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The *Aspergillus nidulans* Phytochrome FphA Represses Sexual Development in Red Light

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Summary

Phytochrome photoreceptors sense red and far-red light through photointerconversion between two stable conformations, a process mediated by a linear tetrapyrrole chromophore [1]. Originally, phytochromes were thought to be confined to photosynthetic organisms including cyanobacteria, but they have been recently discovered in heterotrophic bacteria and fungi, where little is known about their functions [2, 3]. It was shown previously in the ascomycetous fungus *Aspergillus nidulans* that asexual sporulation is stimulated and sexual development repressed by red light [4]. The effect was reminiscent of a phytochrome response, and indeed phytochrome-like proteins were detected in several fungal genomes [5]. All fungal homologs are more similar to bacterial than plant phytochromes and have multifunctional domains where the phytochrome region and histidine kinase domain are combined in a single protein with a C-terminal response-regulator domain. Here, we show that the *A. nidulans* phytochrome FphA binds a biliverdin chromophore, acts as a red-light sensor, and represses sexual development under red-light conditions. FphA-GFP is cytoplasmic and excluded from the nuclei, suggesting that red-light photoperception occurs in the cytoplasm. This is the first phytochrome experimentally characterized outside the plant and bacterial kingdoms and the second type of fungal protein identified that functions in photoperception.

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Results

A. nidulans FphA Is More Closely Related to Bacterial than to Plant Phytochromes and Covalently Binds Biliverdin

We identified putative phytochrome proteins (*fph* = *f*ungal *ph*ytochrome) in the genomes of several ascomycetous fungi such as *A. nidulans*, *A. fumigatus*, *Gibberella moniliformis*, and the basidiomycetes *Ustilago maydis* and *Cryptococcus neoformans* but not in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, or *Ashbya gossypii*. Fungal phytochromes share several characteristic domains with phytochromes from plants and bacteria (Figure 1A). All phytochromes are composed of a photosensory input domain at their N terminus and a regulatory output domain at the C terminus. The input domain consists of P2, GAF, and PHY subdomains, and the output domains are usually more diverged but contain a histidine kinase domain (HKD) [6]. Some bacterial phytochromes carry an additional response-regulator domain (RRD), a second module known from bacterial two-component systems. Similarly, the output domain of fungal phytochromes accommodates a HKD and a RRD (Figure 1A).

The close relationship between bacterial and fungal phytochromes is further supported by similarities in their putative chromophore binding region (Figure 1B). Plant and cyanobacterial phytochromes harbor within their GAF domain a conserved cysteine residue that covalently attaches phytochromobilin or phycocyanobilin, respectively. *A. nidulans* FphA lacks this cysteine residue but has an isoleucine residue instead (Figure 1B). On the other hand, it has been shown that Agp1 from *A. tumefaciens* and BphP from *P. aeruginosa* covalently attach a biliverdin chromophore at a conserved cysteine residue within the P2 domain. Interestingly, this cysteine is also present in FphA (C195) and is likely to be the site of chromophore attachment (see below) (Figure 1B). These sequence-based results and a phylogenetic analysis of the GAF domain suggest that fungal phytochromes are more closely related to bacterial than to plant phytochromes, and they might have evolved from a bacterial progenitor (Figure 1C) (see also [5]).

To ascertain the role of phytochromes in a filamentous fungus, we have characterized FphA from *A. nidulans*. The open reading frame is interrupted by one 56 bp intron at the 3′-end of the transcript. The deduced protein consists of 1280 amino acids, with a calculated molecular mass of 140 kDa and an isoelectric point of 5.2. To establish whether FphA is able to autoassemble with linear tetrapyrroles, a feature unique to phytochromes, we expressed the gene together with a gene encoding the *Synechocystis* sp. heme oxygenase in *E. coli* [7]. This system enables us to provide biliverdin, which is produced by the heme oxygenase, during the expression. Recombinant FphA autoassembled with biliverdin in *E. coli*, and the resultant holo-FphA revealed a typical red, far-red photoreversible phytochrome signature (Figure 2A). Both forms (Pr and Pfr) are considerably shifted toward longer wavelengths, as are the

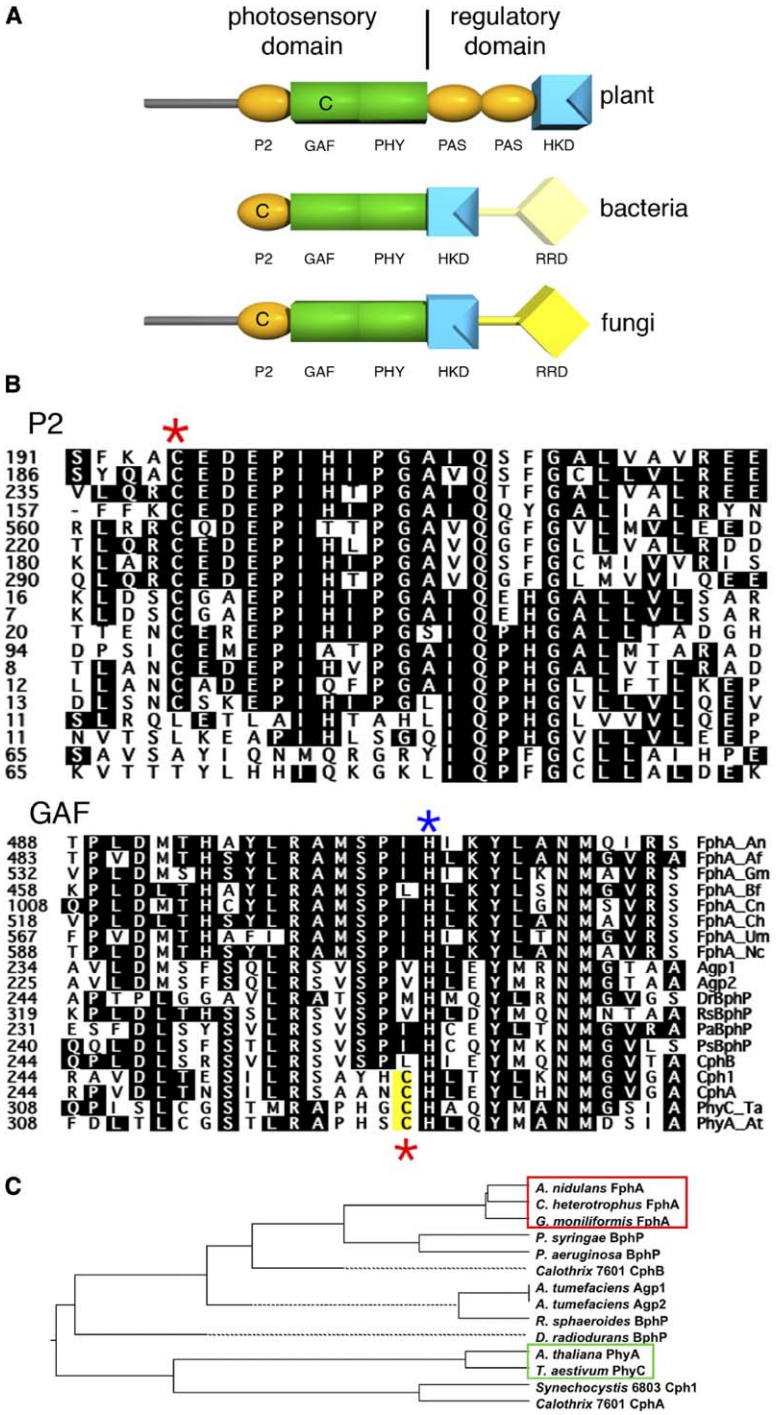

Figure 1. Sequence Analysis of Plant, Bacterial, and Fungal Phytochromes

(A) Domain structure of plant, bacterial, and fungal phytochromes. The following abbreviations are used: P2, a PAS domain; GAF, small ligand binding domain (cGMP-specific phosphodiesterases, cyanobacterial *a*denylate cyclases, and *f*ormiate hydrogen lyase transcription activator FhlA); PHY, phytochrome domain; PAS, *P*ER, *A*RNT, *S*IM repeats; HKD, *h*istidine *k*inase *d*omain; RRD, *r*esponse-*r*egulator *d*omain; and C, conserved cysteine residue involved in covalent chromophore binding.

(B) Amino acid sequence alignment of the two putative chromophore binding regions. On the top, the putative chromophore binding region of bacterial phytochromes, which is located within the P2 domain, is shown. On the bottom, parts of the GAF shown to be the site for chromophore attachment in cyanobacterial and plant phytochromes are shown. Asterisks identify cysteine and histidine residues involved in chromophore binding. Black background denotes identical residues.

(C) Phylogenetic relation of GAF domains from selected phytochromes listed in (B). The red box indicates GAF domains from fungi, and the green box indicates GAF domains from plants. The *A. nidulans fphA* sequence was deposited in the Genbank database under the accession number AJ867583.

BphPs in comparison to plant phytochromes. This result is in good agreement with the action spectrum of light-mediated conidiation in *A. nidulans*, where light with a wavelength of 680 nm was maximally effective [4]. Covalent attachment of the biliverdin chromophore was confirmed by zinc-induced red fluorescence (Figure 2B). Red fluorescence is due to the interaction between zinc and covalently attached linear tetrapyrrole molecules. To determine the site of chromophore attachment, we mutagenized the conserved cysteine residue 195. Indeed, this mutation led to a lack of chromophore binding, which in turn resulted in the lack of photoreversibility and zinc-induced red fluorescence (Figures 2A and 2B). Autoassembly was further tested with phycocyanobilin, a chromophore similar to plant phytochrome chromophores. Recombinant FphA autoassembled phycocyanobilin, but the resultant holo-FphA displayed a much weaker photoactivity (data not

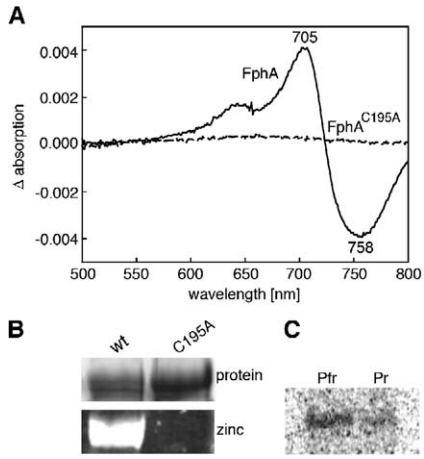

Figure 2. In Vivo Assembly of Recombinant *A. nidulans* FphA by Coexpression of the Apoprotein with a Heme Oxygenase from *Synechocystis* sp. PCC 6803 in *E. coli*

(A) Absorbance-difference spectrum of wild-type (wt) strain and C195A mutant after saturation with red (630 nm) and far-red (750 nm) light.

(B) Zinc-induced red fluorescence (labeled zinc) of equal amounts of wild-type and C195A protein after SDS-PAGE analysis (labeled protein) and electroblotting.

(C) Autoradiogram of FphA Pr and Pfr-forms. Autoradiogram after [γ^{32}P]ATP-labeling, SDS-PAGE, and electroblotting is shown.

shown). These results suggest that BV is likely to be the natural chromophore of FphA. Autophosphorylation of FphA was determined after incubating purified holo-FphA (Pr and Pfr form) with [γ^{32}P]ATP. Both forms of FphA displayed weak autophosphorylation activity (Figure 2C).

FphA Is a Red-Light Sensor in *A. nidulans*

To analyze the function of *fphA* in *A. nidulans*, we have created an *A. nidulans fphA* null mutant by homologous-gene replacement and compared this strain (SAB2) with a wild-type strain (FGSC4) (Figure 3). It has already been shown that in wild-type strains, sexual development is repressed in the presence of red light, whereas under far-red-light conditions, the block does not occur. If a red-light pulse is followed by a far-red-light pulse, *A. nidulans* develops as under far-red-light conditions. In the dark, the balance between asexual and sexual development is shifted toward meiotic reproduction. It was demonstrated that the mycelium has to acquire developmental competence to make it light sensitive and that the sensitivity is restricted to a window of 6 hr after induction of asexual development [4].

Comparing the *ΔfphA*-strain with the wild-type, we found that the red-light block of sexual development at 670 nm was largely reduced in the *fphA* mutant (Figures 3A and 3B). Incubation of the strains in the dark or in dark-red light (740 nm) led mainly to sexual development, visible as a yellowish color of the colonies. The yellow color is caused by the young cleistothecia, which are embedded into yellow nurse cells, called Hülle cells. In red light, the *fphA* mutant still developed sexually, whereas the wild-type produced green colonies, indicating asexual sporulation. However, the *fphA* mutant produced only about 10% of the number of cleistothecia in comparison to the incubation in the dark. These results suggest that FphA is required for photosensing in *A. nidulans* but indicate that it is not the only sensor for red light (Figure 3D).

FphA Resides in the Cytoplasm

To determine where red-light perception could take place in *A. nidulans*, we tagged FphA C- or N-terminally with GFP. Although the protein was predicted to be nuclear (74% probability, pSortII), we found fluorescence of both constructs in the cytoplasm, and FphA appeared to be excluded from nuclei (Figure 4A). The tagged FphA proteins were proven to be biologically functional by complementation of the Δ*fphA* mutation. The localization pattern did not change in hyphae grown on a coverslip after illumination with red light (results not shown). Cytoplasmic localization of FphA was confirmed by C-terminal HA-tagging and subsequent immunostaining (data not shown).

To analyze the subcellular localization of FphA with a different method, we fractionated crude-cell extracts by centrifugation and detected FphA as HA-fusion protein. We found FphA quantitatively in the pellet fraction after 100,000 × g centrifugation (Figure 4B). The protein could be released into the supernatant after treatment of the fraction with detergent. This result could be explained if FphA is either membrane associated or forms a large protein complex in the cytoplasm. The GFP-localization data support the second alternative.

To analyze whether FphA molecules are able to physically interact, we applied the bimolecular fluorescence complementation (BiFC) system [8]. This system is comparable to the yeast two-hybrid system. Two candidate proteins are each fused with one-half of a fluorescent protein. Interaction of the given proteins restores the full fluorescent protein. We adapted this system for *A. nidulans* and expressed the corresponding plasmids pJP4 and pJP5 alone and in combination in GR5. Fluorescence was obtained in the cytoplasm, when both plasmids were cotransformed (Figure 4C). When either of the FphA fusion proteins was expressed individually, or in combination with other tagged proteins (e.g., ApsB), no fluorescence was detected (not shown).

Discussion

In this study, we demonstrated that FphA is a fungal phytochrome that is able to form a photoactive holoprotein with linear tetrapyrroles, has kinase activity, and is likely to act as a red-light sensor. The discovery of a role for this phytochrome has important implications for the understanding of the photobiology of fungi. Fascinating work has been done in recent years to understand the blue-light response is *N. crassa* and other fungi [9]. Two proteins, WC-1 and WC-2, are central components of this system. One major breakthrough was the discovery of a flavin (FAD) as the chromophore in WC-1 [9, 10]. Because WC-1 is located in the nucleus and acts as a transcription factor, no further signaling cascade appears to be necessary [11]. The analysis of the phytochrome response in this paper is thus the sec-

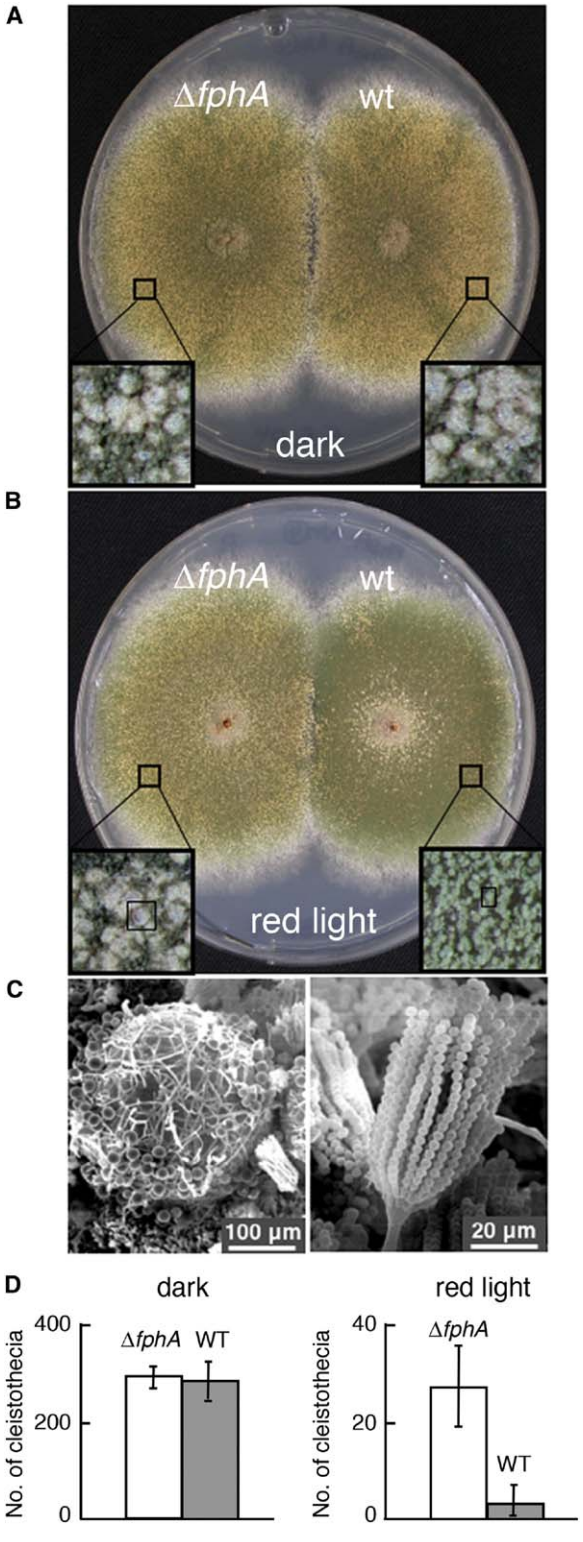

Figure 3. Deletion of *fphA*

(A and B) Strain SAB2 (*ΔfphA*) and FGSC4 (wt) were point-inoculated on agar plates and incubated in the dark (A) or under red-light illumination (670 nm) (B). The indicated areas were enlarged and displayed as inserts.

(C) Scanning electron microscopic pictures showing the developmental structures. On the left, cleistothecium with attached Hülle cells and hyphae is shown. A conidiophore is visible in the right-bottom corner. On the right, enlargement of a conidiophore is shown.

(D) Quantification of the number of cleistothecia. The error bars represent the standard deviation of ten independent countings.

ond example of how fungi sense light and adapt to their environment.

We showed that FphA expressed in *E. coli* assembles well with biliverdin, whereas the attachment of phycocyanobilin was less effective. From these results and the fact that the chromophore attachment site is similar to the ones found in biliverdin binding BphPs, we hypothesize that biliverdin is the natural chromophore of FphA. However, it has to be noted that the peaks in the difference spectrum (Figure 2) were slightly different from the peaks of the action spectrum [4]. The discrepancy may be due to the fact that the natural chromophore in *A. nidulans* is different from biliverdin and thus has different spectroscopic properties. To solve this question, FphA needs to be purified from *A. nidulans* and the structure of the chromophore determined. The question remains how *A. nidulans* produces linear tetrapyrroles. Although several fungi contain heme oxygenases [12], we did not find any similar sequences in *A. nidulans*. This could be due to low homologies between heme oxygenases [13], or the reaction could be performed by a novel enzyme, different from the ones identified to date. A similar scenario has been discovered recently in *Staphylococcus aureus* [14].

The detection of FphA autophosphorylation supports the hypothesis that signal transduction of the light response occurs through phosphorylation. Most likely, autophosphorylation occurs at a conserved histidine residue, followed by a transphosphorylation to an aspartate residue in the C-terminally located response-regulator domain. This dynamic phosphorylation might be the reason for the observed weak kinase activity in vitro.

Deletion of *fphA* from the *A. nidulans* genome led to partial derepression of sexual development under red-light conditions (670 nm). This phenotype was only detectable in *veA* wild-type strains (data not shown). The *veA* gene has long been related to the light response in *A. nidulans* [15]. *veA* mutant strains predominantly develop asexually, independent of the light conditions. Deletion of the gene caused completely asexual strains [16]. Because overexpression of *veA* results in an activation of the sexual cycle and even an induction of sexual development in liquid culture, it appears to be an activator. At the same time, it seems to repress asexual development. Sequence analysis, however, did not reveal any evidence for a light-sensing role or DNA binding capacity. Therefore, it could well be that the gene is not directly involved in light sensing but acts as a regulator of sexual development. If the *fphA* mutation was analyzed in a *veA1* mutant background, no derepression of sexual development was observed (data not shown). This could be due to the largely reduced sexual cycle in the *veA1* background and suggests that VeA acts downstream of FphA or that VeA and FphA operate in parallel developmental pathways.

Another question is where light perception occurs. In

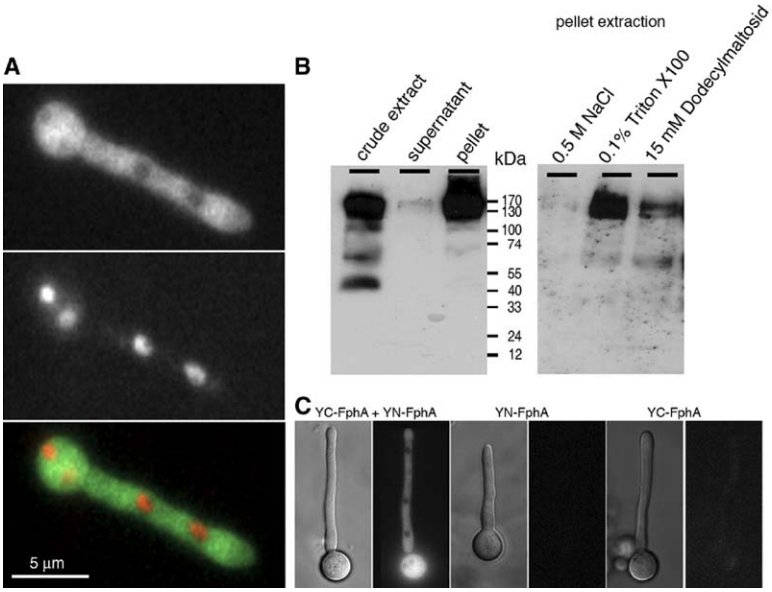

Figure 4. Localization of FphA

(A) The *fphA* open reading frame was fused to GFP and coexpressed with nuclear-targeted DsRedT4 in germlings of *A. nidulans*. The following are shown: on top, GFP fluorescence; at middle, DsRed fluorescence; and on bottom, overlay and colorization of the picture.

(B) Cell fractionation and detection of FphA::HA by western blotting. Crude extract was centrifuged at 100,000 × g for 1 hr, and the pellet was resuspended in buffer (left blot) or in buffer containing 0.5 M NaCl, 0.1% Triton X100, or 15 mM Dodecylmaltosid. Twenty micrograms of protein were loaded into each lane.

(C) BiFC analysis of FphA. FphA was N-terminally fused with the C-terminal or the N-terminal half of YFP and transformed individually (middle and right panels) or together into *A. nidulans*. Phase contrast and fluorescence pictures are shown.

the case of WC-1 in *N. crassa*, the light receptor is a nuclear flavo protein [9–11]. We detected FphA as a GFP-fusion protein in *A. nidulans* in the cytoplasm—excluded from nuclei—and have yet no evidence for a shuttling between the cytoplasm and the nucleus (data not shown). This result seems to contrast the cell fractionation experiments, where FphA was pelleted at 100,000 × g. However, this could be explained if FphA forms a large protein complex. Likewise, it was shown in plants that phytochrome associates with electron-dense particles [17]. This was also the case for plant phytochrome expressed in yeast [18]. Considering these data, we hypothesize a signaling cascade transmitting the primary light-perception signal from the cytoplasm to the nucleus, where the phytochrome response causes a repression of sexual-development-specific genes.

Genome analysis of *N. crassa* and *A. nidulans* revealed that both fungi possess components of several light-sensing systems. The *N. crassa* genome even encodes two phytochrome-like (Phy-1, Phy-2) and one bacteriorhodopsin-like protein (Nop-1) in addition to the proteins for blue-light perception [19, 20]. However, no red-light response has been described in this fungus yet, and deletion of the two phytochromes or the *nop-1* gene did not cause any phenotypic alteration [20] (J. Dunlap, personal communication). Similarly, deletion of a phytochrome did not change light sensitivity of *Cryptococcus neoformans*, but deletion of the WC-homologs did [21]. Likewise, *A. nidulans* contains orthologs of WC-1 and WC-2, although development is regulated by red light. A blue-light response (436 nm) was only reported in a certain *A. nidulans* mutant strain (*bliA1*) [22, 23]. Our results suggest another red-light sensor system in addition to phytochrome because derepression of sexual development in the *fphA* mutant did not result in the same number of cleistothecia as in the dark. The nature of this system remains to be discovered because—unlike the situation in *N. crassa*—*A. nidulans* only contains one phytochrome gene in the genome. The presence of a variety of different light-sensing systems in several, perhaps all, fungi suggests a robust nature of this adaptive environmental response. To detect the functions and cellular responses of the different sensor systems is obviously not always easy under laboratory conditions. It remains to be determined how the different light-sensing systems process the light signal into developmental and physiological decisions to adapt optimally to changing environmental conditions and how they interact. The fact that deletion of *fphA* causes an easily visible developmental phenotype and the amenability of this organism to genetic, molecular, and cell biological methods open a door for detailed structure-function analyses and a further understanding of the signaling cascades related to phytochromes. This may also help to get further insights into the function of phytochromes in plants.

Conclusions

We conclude that the *A. nidulans* phytochrome FphA acts as a potential red-light sensor and represses sexual development under red-light conditions. Red-light perception probably occurs in the cytoplasm.

Supplemental Data

Supplemental Data include Experimental Procedures and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/20/1833/DC1/.

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Accession Numbers

The Genbank accession number for the *A. nidulans fphA* sequence reported in this paper is AJ867583.

Note Added in Proof

The data referred to as "J. Dunlap, personal communication" are currently in press (Froehlich, A.C., Noh, B., Vierstra, R., Loros, J.J., and Dunlap, J.C. (2005). Genetic and molecular analysis of phytochromes from the filamentous fungus *Neurospora crassa*. Eukaryotic Cell, in press).