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Report

Functional and Physical Interaction of Blue- and Red-Light Sensors in *Aspergillus nidulans*

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Summary

Light sensing is very important for organisms in all biological kingdoms to adapt to changing environmental conditions. It was discovered recently that plant-like phytochrome is involved in light sensing in the filamentous fungus *Aspergillus nidulans* [1]. Here, we show that phytochrome (FphA) is part of a protein complex containing LreA (WC-1) and LreB (WC-2) [2, 3], two central components of the *Neurospora crassa* blue-light-sensing system. We found that FphA represses sexual development and mycotoxin formation, whereas LreA and LreB stimulate both. Surprisingly, FphA interacted with LreB and with VeA, another regulator involved in light sensing and mycotoxin biosynthesis. LreB also interacted with LreA. All protein interactions occurred in the nucleus, despite cytoplasmic subfractions of the proteins. Whereas the FphA-VeA interaction was dependent on the presence of the linear tetrapyrrole in FphA, the interaction between FphA and LreB was chromophore independent. These results suggest that morphological and physiological differentiations in *A. nidulans* are mediated through a network consisting of FphA, LreA, LreB, and VeA acting in a large protein complex in the nucleus, sensing red and blue light.

Results

A. nidulans Senses Red and Blue Light

In this paper, we studied the light response in *A. nidulans* and found a red- and a blue-light photoresponse when the strain was inoculated as a lawn in a topagar layer (Figure 1, and the Supplemental Experimental Procedures available online). Large numbers of conidiospores were only produced in light, but neither red (light-emitting diode [LED] 680 nm) nor blue (LED 450 nm) light alone induced sporulation to the level of white light (Figure 1B). The combination of both light qualities restored full conidiation. In comparison, both, red and blue light inhibited effectively the sexual cycle. Because development is connected to mycotoxin formation, we investigated which effect different light qualities had on sterigmatocystin (ST) production. A representative result is shown in Figures 1C and 1D. Blue light repressed mycotoxin formation to a similar level as white light did. In comparison, ST production was increased under red-light illumination conditions. Light of 700 nm appeared to be more effective than 680 and 740 nm light. This is in agreement with the fact that the absorption maximum of *A. nidulans* phytochrome lies at 705 nm [1]. These results indicate that *A. nidulans* is able to sense red and blue light. Green light (536 nm) was ineffective (Figure S6). Compared to previous results, it was surprising that blue light stimulated asexual development as much as red light did [4].

To understand the blue-light response at the molecular level, we analyzed the role of *N. crassa* White Collar (WC) homologs in *A. nidulans*. White Collar 1 and 2 are interacting transcription factors characterized from the model fungus *N. crassa* and other fungi, with WC-1 acting as a blue-light sensor. Two genes were identified in *A. nidulans* by colony screening with *wc-1* and *wc-2* probes and named *lreA* and *lreB* (light response) before the genome sequence was available [5]. The open reading frames are disrupted by three introns in the case of *lreA* and one intron in *lreB*. The two deduced proteins share between 34% and 37% identity to the *N. crassa* proteins and comprise similar domain organization as WC-1 and WC-2 do (Figure 2A and Figure S1). The 836 amino acid long LreA protein is characterized by a light-, oxygen-, or voltage-sensitive (LOV) and two PER-ARNT-SIM (PAS) domains, a nuclear localization signal (NLS), and a GATA-type zinc-finger DNA binding domain at the C terminus. The LOV domain harbors the flavin adenine dinucleotide cofactor [2, 3, 6]. PAS domains are involved in protein-protein interaction [7]. LreB is only 417 amino acids long and lacks the LOV domain and one PAS domain in comparison to LreA (Figure 2A and Figure S1). To study the role of the two proteins in *A. nidulans*, we deleted the genes from the genome and analyzed the effect on light-dependent regulation of asexual and sexual development (Figure 2B, and Figure S2). In addition to single mutants, we constructed double and triple mutants with the phytochrome *fphA* and included them into the analysis. Conidiospore production was slightly increased in the Δ*lreA* and Δ*lreB* strains, independent of the presence or absence of light. This suggests a repressing function of LreA and LreB. Conidiation was slightly reduced in Δ*fphA* in the dark and in light in comparison to the wild-type. This suggests that FphA activates asexual development. The fact that the *fphA*-deletion mutant still produced 70% of the number of conidia of the wild-type in light points to the presence of other photoreceptors. Interestingly, double mutation of *lreA* or *lreB* with *fphA* or the triple mutation caused a drastic decrease of the number of conidiospores. However, in the dark and in the light, a basal level of conidiation remained in the absence of the blue- and red-light regulatory proteins (Figure 2B).

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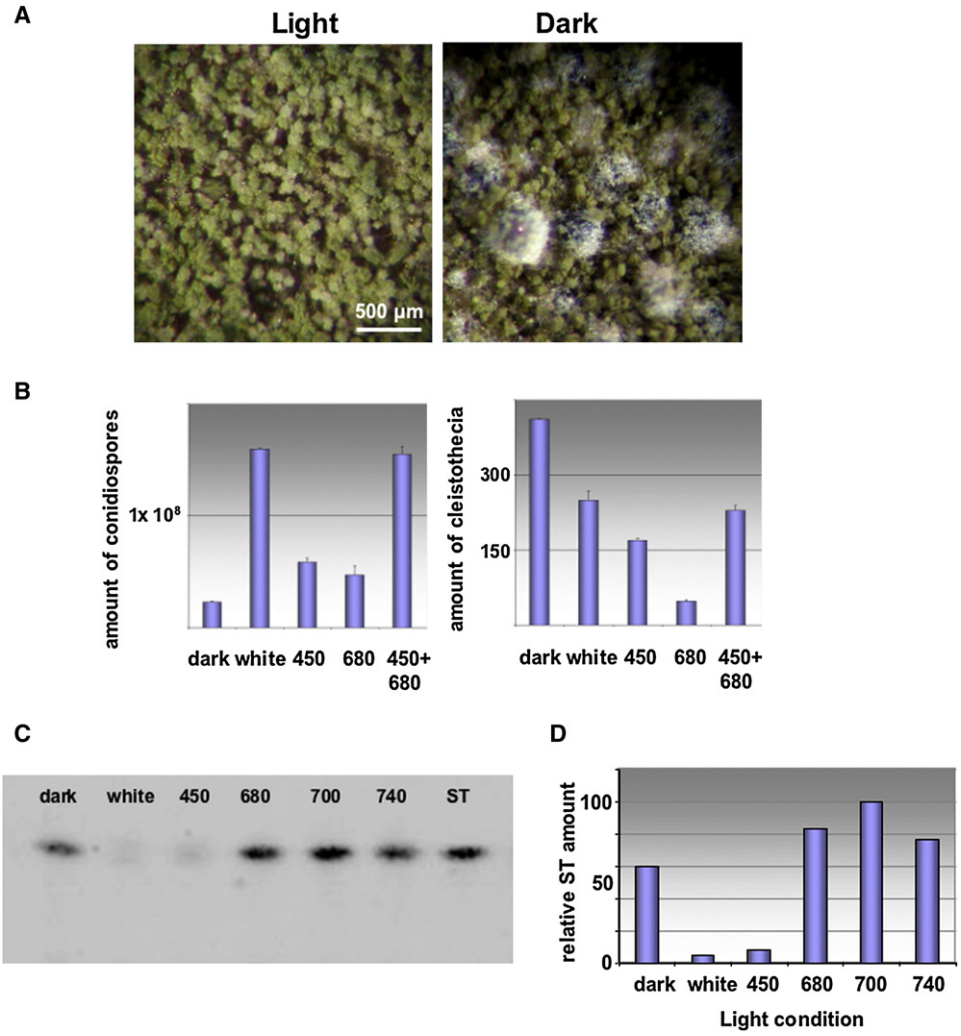


Figure 1. The Light Response in *A. nidulans*

(A) Colonies of FGSCA4 grown under light and dark conditions.

(B) Quantification of conidiospores and cleistothecia formation in the dark, under white, blue (450 nm), red (680 nm), and blue and red light. Conidiospores and cleistothecia were counted as described in the Supplemental Experimental Procedures. The graph shows the average of three independent experiments. Error bars represent the standard deviation.

(C) Sterigmatocystin thin-layer chromatography analysis. Conidia of FGSCA4 were top-agar inoculated (10^7 spores per plate) on minimal medium with 1% glucose and cultured under different light conditions. The wavelengths are indicated as numbers above the lanes. Toxin was extracted after 3 days of incubation.

(D) ST densitometry was carried out with the Scion Image Beta 4.03 software. The normalized ST band intensity values were normalized with respect to the highest intensity considered as 100%. Essentially the same results were obtained in two independent experiments.

The observed phenotypes were recomplemented by the corresponding genes expressed under their native promoters (Figure S5). To test whether the conserved cysteine 276 located in the LOV domain is important for function, we generated a mutant in which this cysteine was replaced by alanine. This mutated version did not complement the triple mutant SJP21.3 (Figure S7).

Sexual development in *A. nidulans* wild-type is the preferred developmental pathway in the dark. Under these conditions, deletion of *lreA* caused a 70% reduction of cleistothecia formation, whereas deletion of *lreB* only caused a 30% reduction (Figure 2B). The Δ*fphA* mutant produced the same number of cleistothecia as the wild-type, and the double and triple mutants behaved similar to the *lreB*-deletion strain. In white light, cleistothecium formation was slightly inhibited in the wild-type and nearly completely lost in *lreA* or *lreB* mutants, suggesting an additive effect of light and the absence of the Lre proteins. The sexual cycle was only slightly reduced in the phytochrome mutant in comparison to the incubation in the dark. The complete loss of cleistothecium formation in the Δ*lreA* or Δ*lreB* strains in light was surprisingly largely suppressed by deletion of *fphA*. Double and triple mutants of *lreA*, *lreB*, and *fphA* incubated in the light produced the same number of cleistothecia as in the dark. This suggests that LreA and LreB act as activators of the sexual cycle and their activity is repressed by light through the action of FphA. These results also show that LreA and LreB serve important functions in the dark. In the absence of positive (LreA, B) and negative (FphA) factors, a basal level of gene induction apparently accounts for the formation of sexual structures.

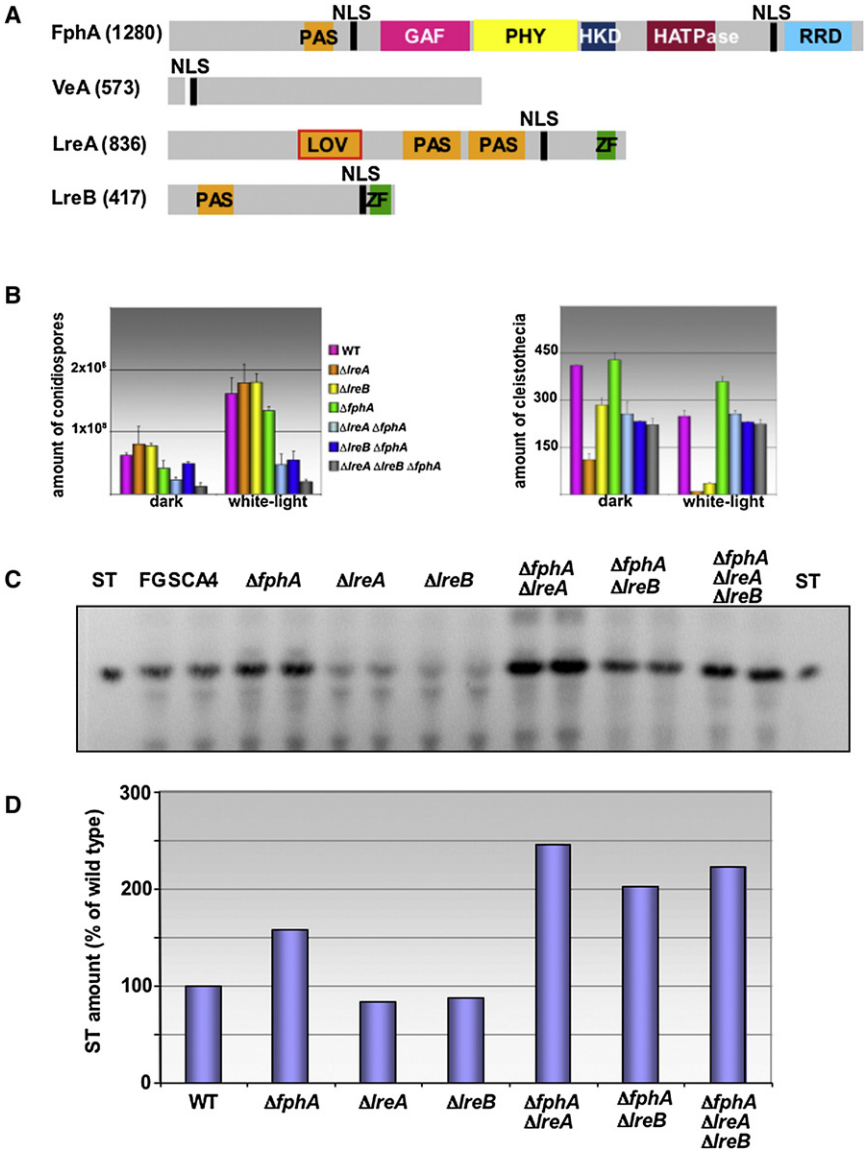


Figure 2. The Role of Phytochrome and White-Collar Proteins in *A. nidulans*

(A) Domain organization of FphA, VeA, LreA, and LreB. Vertical black lines present pat4 nuclear localization sequences, whereas the white vertical line shows a bipartite NLS motif. The following abbreviations are used: PHY, phytochrome domain; GAF, small ligand binding domain; HKD, histidine kinase domain; HATPase, ATPase domain; RRD, response regulator domain; LOV, light, oxygen, voltage domain; PAS, per, arnt, sim domain; ZF, zinc finger.

(B) Effect of deletion of *fphA*, *lreA*, and *lreB* on conidiospore and cleistothecia formation. Quantification was done as described in the Supplemental Experimental Procedures. Error bars represent the standard deviation.

(C and D) Sterigmatocystin formation in light in different mutant strains analyzed by thin layer chromatography (C) and the corresponding quantification (D). So that the low amounts of ST produced in light could be detected, four times more extract was analyzed than in Figure 1C. Essentially the same results were obtained in two independent experiments.

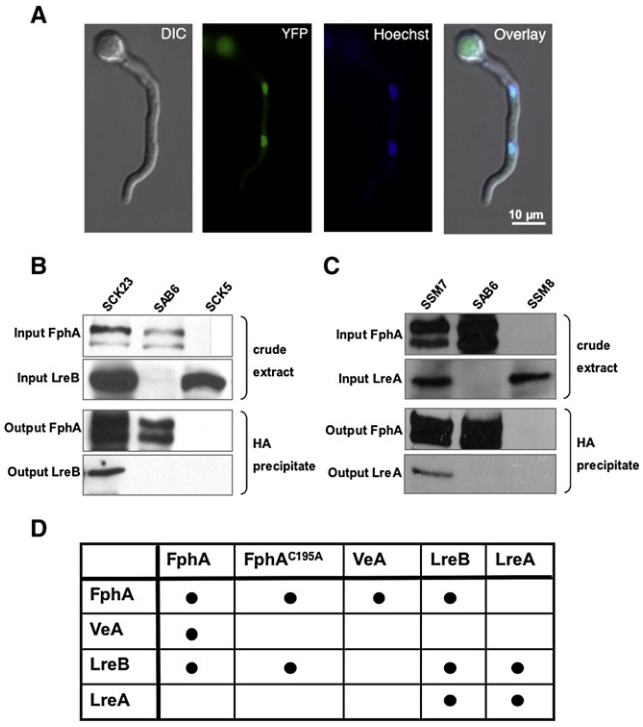
Next, we studied the role of the above characterized light regulators in mycotoxin production. A representative result is shown in Figures 2C and 2D. White and blue light caused a reduction of ST toxin levels compared to those in the dark. The phytochrome mutant strain produced up to 50% more ST and the *lreA* and *lreB* mutants less than the wild-type. The stimulating effect of ST synthesis upon phytochrome deletion was even enhanced in combination with the deletion of the blue-light regulators, LreA and LreB. These results demonstrate a repressing function for phytochrome and an activating function for the WC orthologs and thus a similar regulation as for the formation of cleistothecia.

Interaction of the Blue- and Red-Light Sensing Systems

Because the WC proteins form a complex in *N. crassa*, we asked whether in *A. nidulans* LreA and LreB would interact with each other. First, we studied the localization of LreA and LreB by green fluorescent protein (GFP) fusion. All fusion proteins were shown to be biologically active. LreA and LreB both localized to the nucleus, but LreB also to the cytoplasm (data not shown). To test for physical interaction of LreA and LreB, we used the bimolecular fluorescence complementation (BiFC) assay and cloned the two genes into vectors with the N- or C-terminal yellow fluorescent protein (YFP) halfs, respectively [1]. We transformed the LreA-YFP and LreB-YFP constructs into *A. nidulans* SKV103 and observed fluorescent nuclei (data not shown). The cytoplasm appeared black.

Because the phytochrome and the blue-light-sensing system interacted genetically, we tested whether the proteins would interact physically. By using the BiFC system, we investigated interaction between LreA and FphA and between LreB and FphA. Whereas the first combination did not produce any fluorescent signal in the cell, the second one produced fluorescent nuclei, suggesting LreB-FphA interaction in vivo in the nucleus (Figure 3A). This was surprising because FphA predominantly localized in the cytoplasm, where it also interacts with itself [1]. Our results show that a small fraction of FphA localizes to the nucleus, which was not detected in previous experiments probably because of the high concentration in the cytoplasm [1]. In order to analyze whether light perception of FphA is important for the interaction with LreB, we used a FphA variant in which the chromophore-binding cysteine residue was mutated. Interaction was still detected (data not shown). We also generated FphA derivatives with mutated nuclear localization signals. These mutations did also not affect interaction of FphA with LreB in the nucleus, indicating an alternative nuclear import mechanism for FphA.

The observed interactions of the proteins suggested the existence of a large protein complex, and we wanted to know whether another protein involved in light perception in *A. nidulans*, VeA, would be part of this complex. Therefore, we tested VeA-FphA interaction and the interaction of VeA with LreA or LreB and found that VeA-FphA interaction was positive (data



	FphA	FphA^{C195A}	VeA	LreB	LreA
FphA	●	●	●	●	
VeA	●				
LreB	●	●		●	●
LreA				●	●

Figure 3. Protein-Protein Interaction of Polypeptides Involved in the Photoresponse in *A. nidulans*

(A) Interaction between FphA and LreB in the bimolecular fluorescence complementation assay. FphA was fused N-terminally with the N-terminal half of YFP, LreB was fused N-terminally with the C-terminal half of YFP, and both constructs were transformed into *A. nidulans* SKV103. Hyphae are 3–4 μm in diameter.

(B) Verification of the observed FphA-LreB interaction by coimmunoprecipitation.

(C) Interaction between FphA and LreA shown by coimmunoprecipitation.

(D) Summary of the interactions observed in the BiFC system.

not shown). Interestingly, the interaction was again restricted to the nucleus, despite the presence of both proteins in the cytoplasm [1, 8]. No interaction was detected when the chromophore-binding cysteine in FphA was mutated. In order to confirm the data obtained with the BiFC system in vivo, we did coimmunoprecipitation in vitro by using hemagglutinin (HA)- and GFP-tagged protein versions (Figure 3B, Figure S4A). Although there was no evidence for an interaction between FphA and LreA with the BiFC system, we were able to pull down LreA in coimmunoprecipitation experiments (Figures 3C and 3D). This demonstrates that FphA and LreA reside in the same protein complex without direct protein-protein interaction. As further proof for the existence of a large light-sensing protein complex, we precipitated VeA and detected in the pellet fraction LreA and FphA (Figure S4). Assuming that LreA acts as a blue-light sensor, the last result suggests that red- and blue-light perception occur in the same protein complex. For coimmunoprecipitation experiments, the *alcA* promoter-driven constructs were induced with threonine in the culture medium, resulting in strong expression. To reduce the expression level of these proteins, we used glycerol instead of threonine as carbon source. Under these growth conditions, the proteins were not detectable anymore even in the crude cell extract. Phytochrome, VeA, LreA, and LreB were also not detectable when expressed under their natural promoters.

It was shown recently that VeA is largely localized in nuclei when grown in the dark, but VeA presence in the nucleus is decreased when grown in the light [8]. Because VeA itself probably is not able to sense light, we asked whether VeA subcellular localization was dependent on the presence of the phytochrome and found that indeed the decrease of VeA levels in the nucleus was partly dependent on FphA (Figure S3). The fact that nuclear concentration of VeA is prevented by blue light [8] reflects that both red- and blue-light-sensing systems are required for effective nuclear localization.

Discussion

Light sensing is very important for organisms in all kingdoms to adapt to changing environmental conditions and is mediated only by a few photoreceptor molecules, two of which are phytochromes, for red light, and flavoproteins, for blue light. Phytochromes were thought to be confined to photosynthetic organisms including cyanobacteria [9–11] but have been recently discovered in heterotrophic eubacteria and in fungi [1, 12, 13]. Phytochrome as well as blue-light sensors are absent from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Phytochrome, FphA, and a fungal-specific protein, VeA, have been described as important components of the light response in *A. nidulans* [1, 4]. Certain mutant strains of *A. nidulans* were reported to react on blue light (436 nm), in addition to red light, suggesting the presence of a blue-light-sensing system, as well [14]. In addition to the morphogenetic decisions, light reduces the formation of the *A. nidulans* mycotoxin ST. This regulation involves also the *veA* gene, suggesting common regulatory pathways [15].

The blue-light response is best studied in *Neurospora crassa* but has been recently studied also in other fungi [2, 3, 13, 16–18]. Two main players are WC-1 and WC-2, which are both transcription factors. WC-1 contains flavin as the light-receptor molecule [2, 3]. Both proteins are nuclear localized, but a fraction of WC-2 was also detected in the cytoplasm, and both undergo light-dependent phosphorylation. Neither light nor phosphorylation had an effect on the localization [19]. Besides the well-studied blue-light response in *N. crassa*, a red-light response has been described several decades ago as potentiation of X-ray-induced genetic damage by far-red light. This effect was reversible when the culture was illuminated with red light after far-red-light exposure and thus resembled a phytochrome response [20]. Indeed, two phytochrome homologs were identified recently in the genome, but deletion of the phytochromes did not cause any obvious developmental phenotype [16]. Genetic damage was not analyzed in this publication.

We show in this paper that morphological and physiological differentiation in *A. nidulans* is regulated through an interplay between two light-sensing systems, which involves direct protein-protein interaction (Figure 3). This raises the question of how common such an interaction would be, given that in many fungal genomes, photoreceptors for several light qualities were found. In *N. crassa*, the white-collar protein complex was purified, and WC-1 and WC-2 were identified in a ratio of 1:1 [2]. This depicts that in *N. crassa*, which contains two phytochromes and also a VeA ortholog, only blue-light perception is mediated through the protein complex. Similar to the results obtained for *N. crassa*, deletion of the phytochrome gene in *Cryptococcus neoformans* did not exhibit a phenotype [18]. On the other hand, the function of the WC proteins as blue-light receptors is well conserved during fungal evolution [17, 18].

There are examples that fungi respond to blue and to red light [21], but a functional and physical interplay between the two light-sensing systems has not been reported yet in any other fungus.

After the discovery of the interplay between the red- and the blue-light response in *A. nidulans*, it will be the challenge for future research to identify pathway-specific transcription factors, which convert the light response into differential gene expression. These transcription factors are likely to be interaction partners of the response regulator of FphA (Figure 2A). Whether the changes of the activities of VeA, LreA, and/or LreB are due to modulations of the protein activities or due to changes of the protein levels is another important question to be solved for better understanding photosensory responses. Our results suggest that photosensing in fungi appears not only to share crucial proteins such as phytochrome with higher plants, but also that integration of different light qualities is an ancient process conserved from bacteria [22] to fungi to plants [23].

Supplemental Data

Experimental Procedures, seven figures, and two tables are available at http://www.current-biology.com/cgi/content/full/18/4/255/DC1/.

Acknowledgments

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