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Fungi use the SakA (HogA) pathway for phytochrome-dependent light signalling

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Stress-sensing in fungi depends on a signalling cascade comprised of a two-component phosphorylation relay plus a subsequent MAP kinase cascade to trigger gene expression. Besides osmotic or oxidative stress, fungi sense many other environmental factors, one of which is light^{1,2}. Light controls morphogenetic pathways but also the production of secondary metabolites such as penicillin. Here we show that phytochrome-dependent light signalling in *Aspergillus nidulans* involves the stress-sensing and osmosensing signalling pathway. In a screening for 'blind' mutants, the MAP kinase SakA (also known as HogA) was identified by whole-genome sequencing. The phytochrome FphA physically interacted with the histidine-containing phosphotransfer protein YpdA and caused light-dependent phosphorylation of the MAP kinase SakA and its shuttling into nuclei. In the absence of phytochrome, SakA still responded to osmotic stress but not to light. The SakA pathway thus integrates several stress factors and can be considered to be a hub for environmental signals.

Light signalling in *A. nidulans* is of particular interest because this filamentous ascomycete contains at least two classes of photoreceptors. Most fungi contain a system that senses blue light and involves a flavin-containing transcription factor, but many fungi, including *A. nidulans* and *Neurospora crassa*, contain phytochrome in addition^{2,3}. Phytochrome was thought to be confined to plants, but was later discovered in bacteria and fungi^{4–7}. *A. nidulans* was the first fungus where the functionality of phytochrome (FphA) was shown^{8,9}. In order to identify novel components of light signalling, we developed a mutant screening procedure (Supplementary Fig. 1). To this end the *conJ* gene was used, which responds to light very well^{10,11}. The putative promoter (2,000 bp upstream the ATG) was fused to the *N. crassa* nutritional marker gene *pyr4* and introduced into a uracil-auxotrophic *A. nidulans* strain. When cultured in the dark, this strain (SZY17, parent) grew well on agar plates supplemented with uracil and uridine, but showed only residual growth in the dark on minimal medium without the supplements (Fig. 1a). Under light, the strain grew like the wild type on those plates, indicating that the expression of *pyr4* was tightly controlled by light. This strain was used for UV mutagenesis (survival rate 25%) (Fig. 1a). A total of 476 mutant strains were obtained that formed small colonies on minimal medium when grown under light. Of these, only five grew to normal-sized colonies on medium supplemented with uracil and uridine. In addition, three strains grew better in the light than they did in the dark, but more slowly than the other five strains and sporulation was impaired. All eight strains obviously did not grow slowly in light because of other mutations, but probably because the *pyr4* gene was mutated or no longer light-induced. In order to distinguish a mutation in the *conJ* promoter or the inserted *pyr4* gene from a mutation in a gene encoding an upstream regulator of *conJ*, the expression of

the endogenous *conJ* gene and a second light-regulated gene, *cgcA*^{11,12}, was quantified by real time polymerase chain reaction (RT-qPCR). The values were normalized to the constitutive H2B (histone) gene. In seven strains both genes were not light-inducible, whereas in mutant M17-3 light induction was not affected. Thus seven blind mutant strains were isolated. One of the mutants (M10-13) could be complemented with a wild-type copy of *fphA*, showing that mutagenesis of phytochrome was responsible for the phenotype (Supplementary Fig. 2) and proving that the procedure is suitable for isolating regulatory components of light signalling.

Since we aimed at identifying the responsible mutation in one of the mutants by next-generation sequencing of the whole genome, we tried to back-cross all mutants twice with the wild type to reduce the number of background mutations. Mutant M6-9 crossed very well and the desired progeny strains were obtained quickly. From the progeny of the second cross 28 strains with the light-dependent growth phenotype (and thus the expected regulatory mutation) and 28 without that phenotype were chosen for genomic DNA isolation. DNA from each group of strains was pooled for deep sequencing. A single nucleotide deletion was detected in the gene encoding the MAP kinase SakA in the mutant DNA (Supplementary Fig. 3). This suggested a novel function for SakA beyond the function in osmotic and oxidative stress-sensing (Fig. 2a,b). The gene was first described as *sakA* (stress-activated kinase) and later as *hogA*^{12,13}. We will keep the original name *sakA*. In order to verify that the observed phenotype was indeed only caused by the loss of the SakA-function, the mutant was complemented with a wild-type copy of *sakA* (Fig. 2a).

To explore this light-dependent function of SakA, development of the wild-type strain was compared with development of the *sakA*-deletion strain (Supplementary Fig. 4). Light favours asexual and represses sexual development. In blind mutants, development is shifted towards sexual spore formation under light conditions¹⁴. Vegetative growth of the two strains was the same after three days of growth under light, but whereas the wild type produced green conidiospores, the *sakA*-deletion strain produced sexual cleistothecia and only very few conidiospores. In order to further analyse the blind phenotype, the expression of *conJ* and *cgcA* was compared between the *sakA*- and the *fphA*-deletion strains. Both strains showed similar expression patterns (Fig. 2c). After the discovery of the role of SakA in light sensing, we tested whether mutations in other genes of the pathway would also cause blindness. The two-component stress-sensing system in *A. nidulans* consists of a number of proteins, whose activity is controlled by their phosphorylation status (Fig. 2b)^{15,16}. At the end of the cascade SakA is phosphorylated and shuttles into the nucleus to activate the bZIP transcription factor AtfA¹⁷. A *ypdA* mutant is not available, because the gene is essential¹⁸. Induction of *cgcA* or *conJ* by light was not observed in *sskA*-, *sskB*-, *pbsB*- or *atfA*-deletion strains (Fig. 2c). These findings suggest that light and stress signalling share

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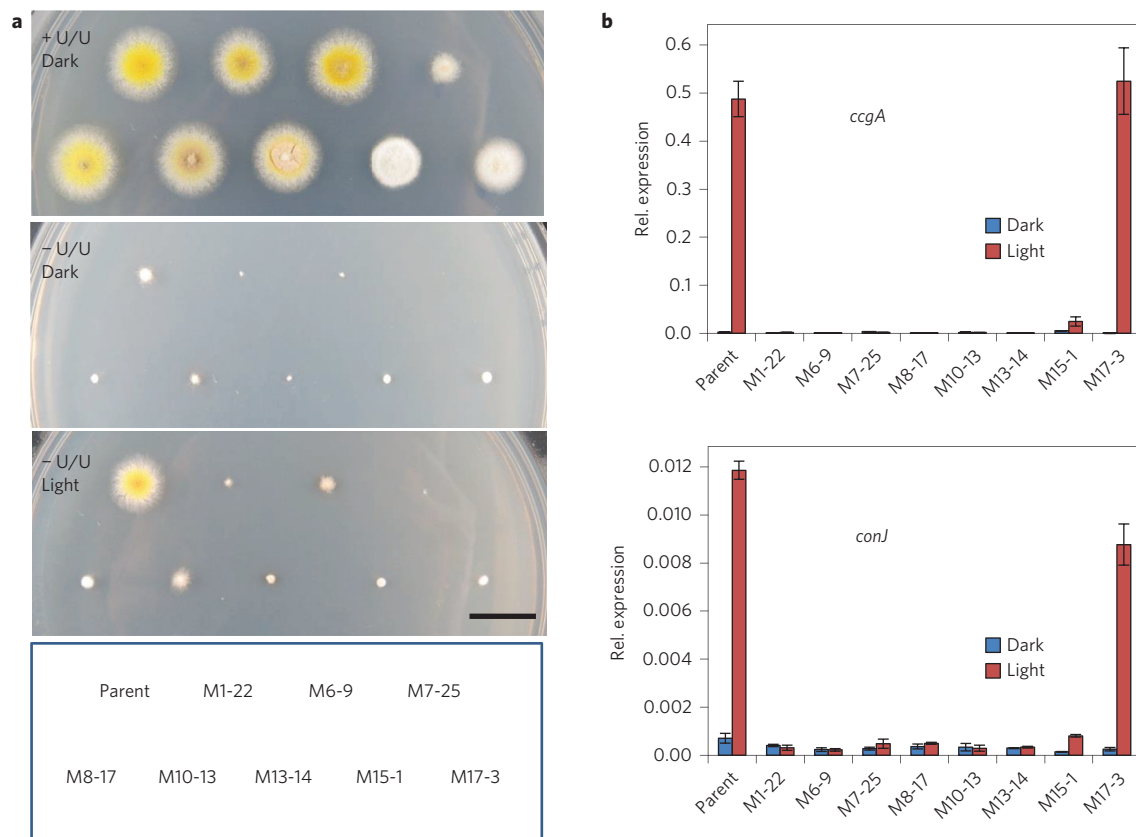


Figure 1 | Isolation of an *A. nidulans* 'blind' mutant. **a, Phenotype of the parent strain (SZY17) used for the mutagenesis approach and the blind mutant M6-9 grown in the presence or absence of uracil and uridine (U/U) in light or in the dark. Colonies were grown on agar plates at 37 °C for 3 days. Scale bar, 1 cm. **b**, Real time RT-qPCR expression analysis of *ccgA* and *conJ* in wild type and the mutants. Fresh conidia were inoculated on the surface of supplemented liquid minimal medium and cultured for 18 h in the dark at 37 °C. The mycelium was harvested under green-light conditions directly or after 30 min illumination with white light and frozen in liquid nitrogen for RNA isolation. The mean values were calculated from three biological replicates and the error bars represent the s.d.**

components of the same signalling cascade. To further test this hypothesis, we analysed whether *ccgA* and *conJ* would also react in response to osmotic stress. Indeed, both genes were induced after addition of 0.5 M NaCl and the parent strain used for mutagenesis grew well on minimal medium in the dark (Supplementary Fig. 5).

In order to test whether any of the other isolated mutants had defects in the SakA-signalling pathway, a growth assay with and without salt in the medium was performed. Whereas the wild type grew equally well under both conditions, the *sakA* mutant only showed residual growth on high-osmolarity medium. Mutant M10-13 was also able to grow, which is consistent with the fact that its phytochrome gene is defective. However, six mutants did not grow. *sakA* itself was not affected, because none of these mutants could be complemented by *sakA*. In order to characterize some of the mutants, we amplified the genes from the *sakA* pathway from two mutants (M1-22 and M15-1) and identified several mutations in *pbsB* (mutant M1-22) that all caused changes in amino acids. In the case of mutant M15-1 an insertion was identified in *sskB*, leading to a frameshift after amino acid 1106 (Supplementary Fig. 6). Thus the protein, if it is produced, lacks most of the kinase domain. Mutant 8-17 was also subjected to genome sequencing. However, we were not yet able to unambiguously identify the causative mutation for the blindness. Two mutants have not been analysed yet (M7-25 and M13-14).

The results of analysis of the mutant strains indicate that the light signal enters the stress-sensing signal transduction cascade at the level of YpdA or SskA. To distinguish between the two possibilities, we tested the two proteins for interaction with phytochrome by

bimolecular fluorescence complementation (BiFC). Fluorescence was detected in the cytoplasm only with the combination of FphA and YpdA (Fig. 3a). The interaction occurred in the dark and was not affected by light. The interaction was confirmed by Co-immunoprecipitation (Fig. 3b).

The involvement of SakA in light sensing led us to hypothesize that SakA is phosphorylated and shuttles into nuclei upon illumination as in the case of oxidative-stress sensing¹⁷. GFP-tagged SakA was observed in the cytoplasm and indeed accumulated in nuclei after 5 min of white-light illumination (Fig. 4a). The same effect was observed with red- but not with blue-light illumination. SakA also shuttled into nuclei in the dark when 0.5 M NaCl was added to the medium. Whereas light-dependent nuclear shuttling was dependent on phytochrome, salt-dependent shuttling was independent (Fig. 4a). Likewise, deletion of components of the blue-light-sensing system, *lreA* or *lreB* did not affect the localization of SakA.

Next, we tested whether SakA would be phosphorylated and thereby activated. Therefore, we analysed the phosphorylation status of SakA with antibodies derived against the phospho-p38 MAP kinase (mammalian homologue of *S. cerevisiae* Hog-1). The total amount of SakA was quantified with a polyclonal antibody against Hog-1. Western blot analysis revealed that after 10 min of illumination the phosphorylation level of SakA increased. Red light was also effective, whereas blue light only sometimes induced a weak increase of the phosphorylation level (Fig. 4b and Supplementary Fig. 7). The phosphorylation increase was not observed in the phytochrome mutant. *LreA* or *LreB* appeared not to be involved in the control of the phosphorylation level (Supplementary Fig. 7). Because the

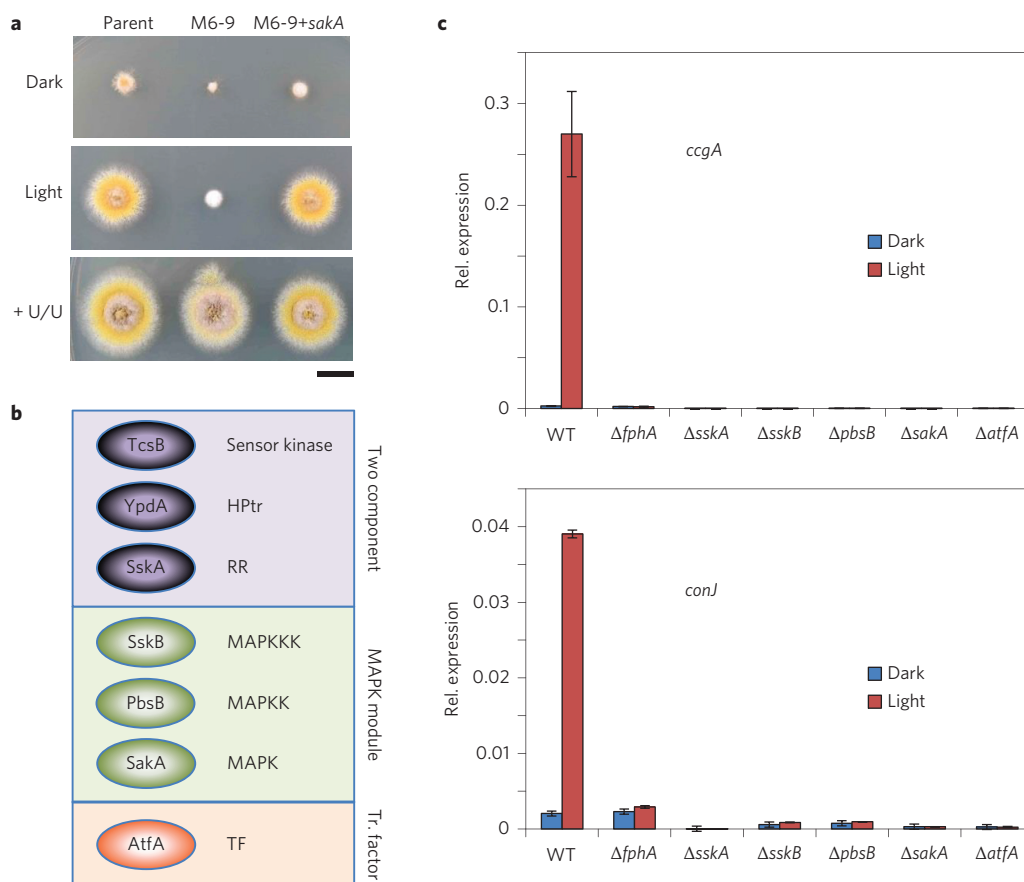


Figure 2 | Impact of the SakA signalling pathway on light sensing. **a**, Colonies of the parent, of the mutant M6-9, and M6-9 complemented with *sakA* (SZY45) grown on minimal medium agar plates for 2.5 days in light, in the dark and on minimal medium supplemented with uracil and uridine. Scale bar, 0.5 cm. **b**, Simplified scheme of the two-component system and MAPK module involved in osmotic and stress sensing in *A. nidulans*. **c**, *ccgA* and *conJ* induction analysed in different mutant strains. Fresh conidia harvested from wild type (SJR2), $\Delta fphA$ (SJP1), $\Delta sskA$ (SZY41), $\Delta sskB$ (A1293), $\Delta pbsB$ (A1289), $\Delta sakA$ (SZY31) and $\Delta atfA$ (SZY42) inoculated on the surface of supplemented liquid minimal medium and cultured for 18 h in the dark at 37 °C. The grown mycelium was exposed to light or kept in the dark for 30 min and frozen in liquid nitrogen for RNA isolation. The expression levels of *ccgA* and *conJ* were measured by real-time PCR. The mean values were calculated from three biological replicates and the error bars represent the s.d.

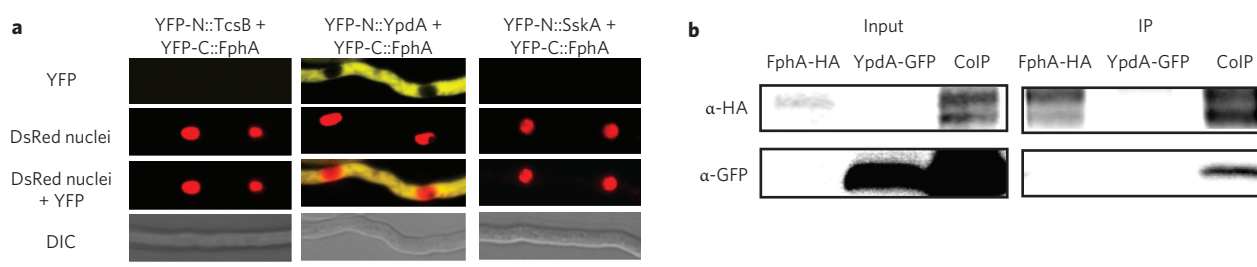


Figure 3 | Phytochrome interacts with the histidine-containing phosphotransfer protein YpdA. **a**, TcsB, YpdA and SskA were each fused to the N-terminal half of YFP and transformed separately together with plasmid pJP5 (*alcA::YFP-CT::fphA*; *pyr4*) into strain SKV103. Strains were cultured in supplemented liquid minimal medium containing 2% threonine and 0.2% glucose. This results in low-level expression of the construct. Scale bar, 10 μ m. **b**, Co-immunoprecipitation of FphA and YpdA. YpdA was fused to GFP and FphA was HA-tagged. The strains were cultured in liquid minimal medium at 37 °C containing 2% threonine and 0.2% glucose for 24 h at 180 rpm. Protein was purified and precipitated with anti-HA agarose.

results obtained with blue light varied from experiment to experiment, we aimed at reducing other stress factors besides light. In our standard experimental setup hyphae were grown on the surface of liquid medium, exposed to air. This guarantees homogeneous light exposure of the mycelium, but hyphae may also suffer from partial desiccation or other stresses. In order to reduce these factors, submerged cultures were analysed (Supplementary Fig. 7e). However, similar results were obtained. Therefore, we established an assay to analyse the level of SakA phosphorylation at the cellular

level (Fig. 4c). Conidia were germinated on cover slips, exposed to light and then processed for immunostaining using the anti-phospho p38 antibody. In the dark control only weak fluorescence was detected in the cytoplasm. After five min white or red light exposure, a strong signal was observed in nuclei. Blue-light illumination resulted also in a nuclear signal, but it was very weak in comparison to the other illumination conditions. This appears to be in contrast to the results obtained with GFP-tagged SakA and the shuttling into nuclei after illumination. This may be a problem resulting

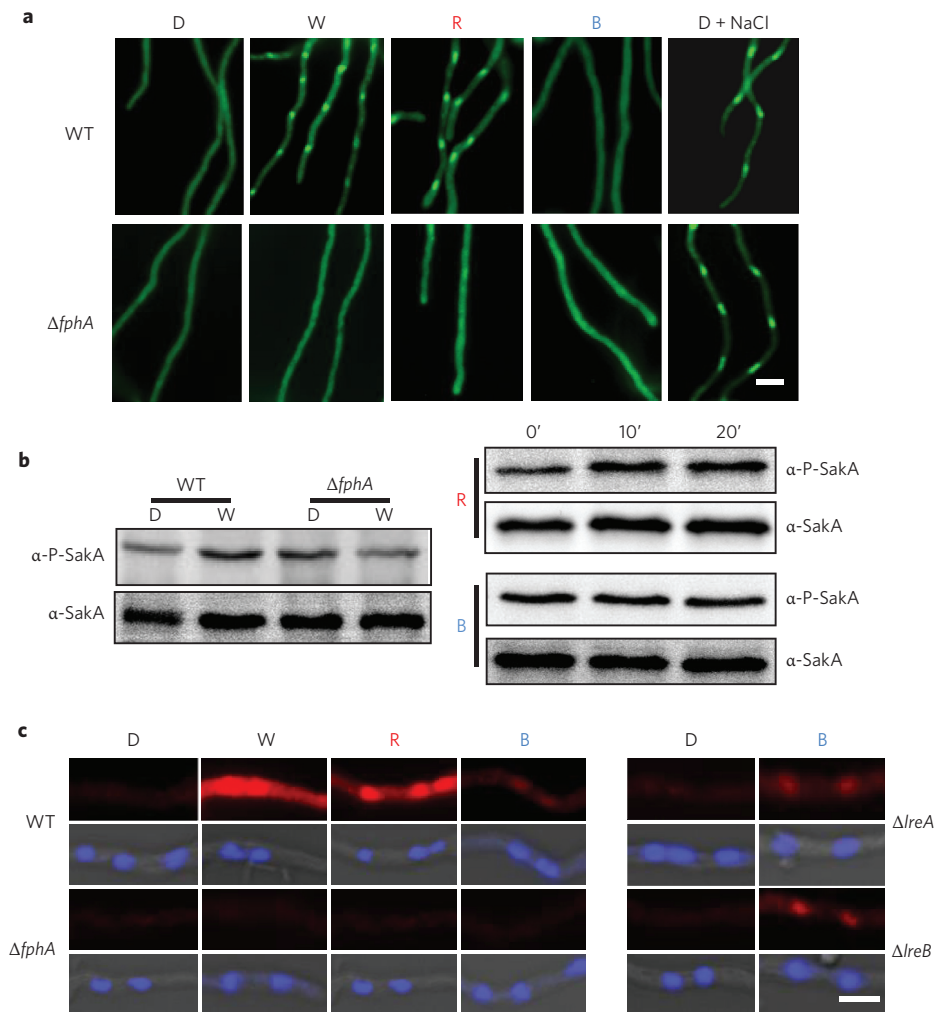


Figure 4 | SakA shuttling and phosphorylation after light exposure. **a**, SZY34 (wild type) and SZY37 ($\Delta fphA$) expressing SakA::GFP under the *sakA* native promoter on supplemented minimal medium in the dark (D) grown for 16 h. The mycelium was fixed in the dark (dim-green light) directly or after 5 min of illumination in white (W), red (R) and blue (B) light. To impose salt stress, minimal medium was replaced by minimal medium with 0.5 M NaCl and incubated for 10 min before fixing. **b**, Phosphorylation of SakA in white (W), red (R) and blue (B) light. Fresh conidia were inoculated on the surface of liquid minimal medium and incubated for 18 h in the dark. The mycelium was harvested directly in the dark or after 15 min illumination with white, or 10 or 20 min illumination with red or blue light and frozen in liquid nitrogen immediately for protein extraction. 35 μ g crude extract of each sample were loaded onto the SDS PAGE and processed for the western blot. **c**, Immunostaining of wild type (SJ2), the *fphA*-deletion strain (SJ10), and the *lreA*- (SSR66) and *lreB*- (SJ70) deletion strains. Conidia were germinated on cover slips at room temperature, then exposed for 5 min to the light as indicated and immediately fixed and processed for immunostaining (upper row of pictures). Nuclei were stained with DAPI (lower row of pictures). Scale bar, 5 μ m.

from the different sensitivities of the two assays. In the shuttling experiment cytoplasmic SakA is also visible, whereas in the phosphorylation assay only phosphorylated SakA will be visualized and thus the cytoplasmic background is very low. Next we tested the role of the photoreceptors and found that only FphA is required, this is also the case for the blue-light-dependent SakA phosphorylation (Fig. 4c). The latter finding was very surprising and may be explained in two ways. It could be that another blue-light receptor (e.g. cryptochrome) senses blue light but somehow requires FphA for signalling. It could also be that FphA itself reacts to blue light. There is some evidence from plants that phytochrome plays a role in blue-light sensing¹⁹.

Our results are indicative of the activation of the SakA pathway upon illumination. In analogy to the events during stress sensing, we anticipate that in the dark the histidine-containing phosphotransfer (HPT) protein YpdA becomes phosphorylated and keeps the response regulator SskA phosphorylated. SskB and the downstream components are inactive. After light exposure, the degree of

phosphorylation of YpdA and SskA decreases and enables interaction of SskA with SskB. This induces the auto-phosphorylation activity of SskB, which in turn initiates the phosphorylation cascade leading to activated AtfA. In agreement with this hypothesis is the fact that the kinase activity of FphA is higher in the dark than in the light²⁰. Therefore, we postulate phosphotransfer from the FphA response regulator domain to the YpdA protein. Phosphotransfer has been demonstrated with heterologously expressed YpdA and FphA, although in this *in vitro* system YpdA transferred the phosphate residue towards the histidine kinase domain of FphA²¹.

Our results clearly show a cytoplasmic function for phytochrome. Phytochrome has been found previously in the cytoplasm⁸, but later it was demonstrated that it interacts with other components of the light signalling machinery within nuclei⁹, where it probably is involved in the derepression of light-induced genes and chromatin modifications¹⁰.

Many microorganisms grow at a water–air interphase, for instance soil-borne organisms at the ground surface, moulds at

the substrate surface, or plant or human pathogens on leaves or on the skin. These habitats are characterized by potentially rather extreme changes of growth conditions and the organisms have to cope with desiccation, or high or low temperatures. One common environmental parameter, which can affect growth at a water–air interphase, is light. Therefore, light is a very reliable source of information that environmental conditions may change drastically. Hence light-sensing is a very effective mechanism for coping with these conditions and adapting correspondingly. In this context, it is not surprising that there is direct cross talk at the gene level between different stress types¹.

It is always considered that photoreceptors sense light and let organisms react correspondingly. However, equally important may be the ‘sensing’ of the absence of light. As mentioned earlier, the kinase activity of phytochrome is high under dark conditions and hence the phosphorylation status of YpdA and the other two-component system players increases, which blocks the MAP kinase components, including the downstream transcription factor AtfA. If AtfA is phosphorylated, spores do not germinate¹⁷ and thus phytochrome-dependent regulation of the MAP kinase pathway appears to be essential for the initiation of hyphal growth. That light indeed delays germination has been shown for *A. nidulans* and its pathogenic relative *A. fumigatus*^{22,23}. Our finding of the involvement of SakA in light-dependent gene regulation adds another level of complexity to light sensing²⁴ and may help to improve the understanding of fungal growth and development and perhaps also have an impact on fungal pathogenicity.

Methods

Strains, plasmids and culture conditions. Supplemented minimal medium for *A. nidulans* was prepared as described previously, and standard strain construction procedures were used²⁵. Standard transformation procedure for *A. nidulans* and *Escherichia coli* were used^{26,27}. Q5 High-Fidelity DNA polymerase for PCR and restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany).

We used SKV103 (*pyrG89; pyrA4; veA*⁺)²⁸, SJ1 (*pyrG89; ΔargB::trpCDB; pyrA4; ΔfphA::argB; veA*⁺)⁹, SJ170 (*pyrG89; ΔlreB::argB; ΔargB::trpCDB; pyrA4; veA*⁺)⁹, SJR2 (*pyrG89; pyrA4; nkuA::bar; veA*⁺)²⁹, SJR10 (*pyrG89; ΔargB::trpCDB; pyrA4; nkuA::bar; ΔfphA::argB; veA*⁺)¹⁰, SSR66 (*yA2; pyrA4; nkuA::bar; ΔlreA::ptrA; pabaA1; veA*⁺)¹⁰, SSR89 (*yA2; ΔlreB::argB; pyrA4; nkuA::bar; ΔlreA::ptrA; pabaA1; veA*⁺)¹⁰, TΔsskA-riboB (*pyrG89; ΔsskA::AfriboB; pyrA4; nkuA::argB; riboB2; veA1*⁺)¹⁸, TFLΔsakA-03 (*pyrG89; ΔsakA::AfriboB; pyrA4; nkuA::argB; riboB2; veA1*)¹⁷, TFLΔatfA-02 (*yA2; pyrG89; ΔatfA::AfpyrG; pabaA1; veA1*)¹⁷, TFL6 (*pyrG89; sakA(p)::sakA::GFP::AfpyrG; pyrA4; nkuA::bar; veA1*)¹⁷, SKV104 (*yA2; pyrG89; pyrA4; pabaA1; veA*⁺), SZY17 (SKV104 transformed with pZY19 (*conJ(p)::pyr4; pyrA4*)). Eight mutants derived from SZY17 after UV treatment, SJR3 (*argB2; pyrA4; nkuA::bar; veA*⁺), SZY31 (TFLΔsakA-03 crossed to SJR3; *pyrG89; ΔsakA::AfriboB; pyrA4; nkuA::argB; veA*⁺), SZY34 (*pyrG89; sakA(p)::sakA::GFP::AfpyrG; pyrA4; nkuA::bar; veA*⁺), SZY37 (*ΔargB::trpCDB, sakA(p)::sakA::GFP::AfpyrG; pyrA4; nkuA::bar; ΔfphA::argB; veA*⁺), SZY38 (*pyrG89; pyrA4; alcA::YFP-C::fphA::pyr4; alcA::YFP-N::ypdA::pyrA; alcA(p)::stuA(NLS)::DsRed; veA*⁺), SZY39 (*pyrG89; pyrA4; alcA::YFP-C::fphA::pyr4; alcA::YFP-N::sskA::pyrA; alcA(p)::stuA(NLS)::DsRed; veA*⁺), SZY41 (TΔsskA-riboB crossed to SJR3; *pyrG89; ΔsskA::AfriboB; pyrA4; nkuA::bar; veA*⁺), SZY42 (TFLΔatfA-02 crossed to SJR3; *pyrG89; ΔatfA::AfpyrG; veA*⁺), SZY44 (*pyrG89; pyrA4; alcA::YFP-C::fphA::pyr4; alcA::YFP-N::TcsB::pyrA; alcA(p)::stuA(NLS)::DsRed; veA*⁺), A1289 (*pyrG89; wA3; argB2; nkuA::argB; pyrA4; pbsB::AfpyrG, se15, nirA14, chaA1, fwA1*), A1293 (*pyrG89; wA3; argB2; nkuA::argB; pyrA4; sskB::AfpyrG, se15, nirA14, chaA1, fwA1*), SZY45 (M6-9 co-transformed with plasmid pCK17 (*pabaA1* gene) and pZY30 (*sakA* gene)), SSM39 (*alcA::fphA::3xHA; pyrG89, veA*⁺)¹⁰, SZY49 (SKV103 transformed with plasmid pZY35), SZY50 (SSM39 transformed with plasmid pZY35).

Construction of the parent strain for mutagenesis. To create a strains that could be used for mutagenesis, the light-inducible gene promoter (*conJ*) was amplified with primers conJ(p)_fwd (5'-ATAATCTAGGCGTTCGCTAGATCTGGCTAAAC-3') and conJ(p)_rev (5'-CTATGCTCGAGTTTGTATGTTATTTAAAGAATTGGTTGTGGTTT-3') and *Neurospora crassa* *pyr4* amplified with primers *pyr4*_fwd (5'-CTTTACTCGATGTCGACCAAGTCAGGAACGCAG-3') and *pyr4*_rev (5'-CAGTGGATCCGATCTTCATCTCGCTTCGCTTCGGG-3'). The 2-kb *conJ* promoter and *pyr4* fragments replaced the *alcA* promoter and *fphA* in plasmid pJP4 (*alcA(p)::YFP-N::fphA*)⁸, yielding the plasmid pZY19 carrying *conJ(p)::pyr4* and *pyrA4* as a selection marker. The plasmid was transformed into SKV104, generating the parent strain SZY17. The growth of this parent strain is controlled by light (Supplementary Fig. 1).

UV treatment. Conidia of the parent strain were harvested freshly-grown from an agar plate with distilled water, and the concentration was adjusted to 1.2×10^4 conidia per ml. 400 µl conidia suspension were added to a 14 cm Petri dish with 100 ml solid minimal medium supplemented with *p*-aminobenzoic acid (PABA). Conidia were distributed evenly using 3 mm glass beads. Each plate was treated with 0.015 UV light in a UV Stratalinker 1800 crosslinker (Stratagene, La Jolla, California) to get a survival rate of 25%. The plates were placed in white light and incubated 3 days at 37 °C.

Mutant analysis. The blind mutant M6-9 was crossed twice to the wild type. Genomic DNA of 28 wild type and 28 mutant progeny were combined respectively and subjected to next-generation sequencing. After alignment of the reads against the reference genome, we checked the depth of coverage along the different chromosomes and could not detect any large insertions or deletions. We thus focused on single nucleotide polymorphisms (SNP). Background mutations present in both the mutant and the wild-type strain were first removed. We found two potential frame-shift mutations in the genes CADANIAG00003997 and CADANIAG00001630 (located at the genomic coordinates II:379061 and VIII:1735159 respectively) as well as one potential missense variant leading to a P/R substitution at position II:303934 in the first exon of the CADANIAT00003967 gene. After visual inspection of these three potential causative mutations, we could validate that the mutation in the gene CADANIAG00001630 encoding SakA leads to a frame shift at amino acid position G111 as a consequence of a one-nucleotide insertion (Supplementary Fig. 3).

Protein extraction and immunoblot detection. Fresh conidia were inoculated on the surface of the plates with 25 ml minimal medium containing appropriate supplements and incubated for 20 h in the dark at 37 °C. White, red and blue light were switched on to illuminate the mycelium for 15 min. The mycelia were harvested immediately and frozen in liquid nitrogen for protein extraction. Mycelium was ground in a mortar with liquid nitrogen and afterwards the mycelial powder was collected into Eppendorf tubes. 0.8 ml protein extraction buffer (20 mM Tris-HCl, pH 8, 0.05% Triton-X-100, 150 mM NaCl) containing a protease inhibitor cocktail and 1 mM PMSF was added into each Eppendorf tube and incubated on ice for 20 min. The samples were centrifuged twice at 15,700g at 4 °C. After each centrifugation, the supernatants were moved to new Eppendorf tubes and the pellets were discarded. The protein concentration was measured using the Bradford protein assay and all the samples were adjusted to the same concentration with protein extraction buffer. After denaturing, samples were loaded onto a 10% SDS polyacrylamide gel and blotted to a nitrocellulose membrane. For immunodetection, anti-phospho-p38 MAP kinase (The180/Tyr182) antibodies (#4092; Cell Signalling Technology; Beverly, Massachusetts; dilution 1:1000) against phosphorylated HogA, anti-Hog1p C-terminus antibody (y-215; Santa Cruz Biotechnology; Paso Robles, California; dilution 1:500) against SakA and anti-rabbit IgG (whole molecular)-peroxidase antibody (A0545; Sigma-Aldrich; München, Germany; dilution 1:80,000) were used.

RNA isolation and quantitative real-time PCR. Fresh conidia were inoculated on the surface of liquid supplemented minimal medium in 3.5 cm petri dishes and cultivated in the dark for 18 h. The mycelium was harvested in dim green light directly or after 30 min illumination and frozen immediately in liquid nitrogen. A Fungal RNA Extraction kit from Omega (Norcross, Georgia) was used to isolate RNA and cell disruption was performed with a cell homogenizer at 30 hits per min for 5 min. RNA was treated with TURBO DNA-free kit (Thermo Fisher; Waltham, Massachusetts) and diluted to 50 ng µl⁻¹ with DEPC water. Quantitative real-time PCR was performed with SensiFAST SYBR and a Fluorescein One-Step Kit from Bioline (Luekenwalde, Germany). Each reaction had a volume of 25 µl with 0.2 µM primers and 100 ng RNA. The program started with 10 min of reverse transcription reaction at 45 °C, followed by 2.5 min at 95 °C for the inactivation of reverse transcriptase and 40 cycles of PCR (10 s at 95 °C and then 30 s at 58 °C). To assess the dissociation-characteristics of two-stranded DNA, melting curve analyses were carried out (80 cycles, 95 to 58 °C with 10 s per step). The *h2b* gene was used for normalization. Each expression level is the average of three biological replicates. Primers used for real-time PCR: *h2b*-RT-F (5'-CTGCCGAGAAGAAGCCTAGCAC-3'), *h2b*-RT-R (5'-GAAGAGTAGGTCTCCTTCTGGTC-3'), *ccgA*-RT-F (5'-CGACGCTTCCCTCACTTCTC-3'), *ccgA*-RT-R (5'-CATCATGGGACTTCTCGTCCTT-3'), *conJ*-RT-F (5'-CTGAGAAGCAGCGCAACATC-3') and *conJ*-RT-R (5'-CTCATCGCCAGCGTGAAG-3').

Tagging of proteins with GFP and split YFP. In order to localize SakA in the wild type and the *fphA*-deletion strain under different illumination conditions, we used primers Nest-SakA-F (5'-TTACAGCTATTTCTGCACGGAGC-3') and Nest-SakA-R (5'-CGTGACTGGCAGACTTACCG-3') to amplify a 5-kb *sakA::GFP* transformation cassette from genomic DNA of TFL6. This cassette was transformed into wild type (SJR2), and *ΔfphA* (SJR10) strains. The ORFs of *ypdA*, *sskA* and *tcsB* were amplified with the following primers, *YpdA_YFP_F* (5'-GGGGCGCGCCATGGCGTCAACTACCAACCAAGAC-3'), *YpdA_YFP_R* (5'-CCTTAATTAATTATGCTTTGGCGCTTTTGGATTCTTC-3'), *SskA_YFP_F* (5'-GGGGCGCGCCATGTGCGAAGCAGCTGCTGCC-3'), *SskA_YFP_R* (5'-CCTTAATTAAGTAGAGAGCCCGTCTGACCG-3'), *TcsB_YFP_F*

(5'-GGGGCGCGCCCATGCGGTTCTATTGCCGT-3') and TcsB_YFP_R (5'-CCTTAATTAATCACATCTGCGGTTTCTCCGC-3'). These fragments replaced the *fphA* fragment in plasmid pJP4 (*alcA(p)::YFP-N::fphA*)⁹, yielding plasmids pZY25 (*alcA(p)::YFP-N::ypdA*), pZY24 (*alcA(p)::YFP-N::sskA*) and pZY30 (*alcA(p)::YFP-N::tcsB*). The three plasmids were separately co-transformed with pJP5 (*alcA::YFP-CT::fphA*)⁹ and pJW18 (*alcA(p)::stuA(NLS)::DsRed*)³⁰ into SKV103.

To construct the strains for co-immunoprecipitation, the ORF of *ypdA* was cloned into pMCB17apx (V. Efimov, Piscataway, NJ, USA) with primers YpdA_YFP_F and YpdA_YFP_R, yielding pZY35 (*alcA(p)::GFP::ypdA*). This plasmid was transformed into strains SKV103 and SSM39, generating SZY49 and SZY50.

Co-immunoprecipitation. Fresh conidia were incubated in liquid minimal medium at 37 °C containing 2% threonine and 0.2% glucose for 24 h at 180 rpm, and then the mycelium was harvested by filtration and frozen in liquid nitrogen. Protein extraction was performed as described above and 7 mg raw protein was used for co-immunoprecipitation. 80 µl HA epitope tag antibody (Thermo Fisher) was added to each sample and incubated at 4 °C for 3 h, rotating gently. After the incubation the beads were washed five times with extraction buffer. To release the protein, SDS buffer (4× SDS sample buffer: 240 mM Tris-HCl pH 6.8; 400 mM DTT; 8% SDS; 0.04% bromophenol blue; 30% glycerol) was added to the pelleted beads, which were boiled at 100 °C for 5 min. Western blot analysis was performed as described above. For the detection of GFP, anti-GFP, N-terminal antibody (#G1544; Sigma-Aldrich; München, Germany; dilution 1:1,000) and as secondary antibody anti-rabbit IgG (whole molecular)-peroxidase antibody (A0545; Sigma-Aldrich; dilution 1:80,000) were used. The HA tag was detected with the monoclonal anti-HA antibody (H9658; Sigma-Aldrich; dilution 1:10,000) and anti-mouse IgG (whole molecular)-peroxidase antibody (A9044; Sigma-Aldrich; dilution 1:80,000) as secondary antibody.

Microscopy. For the study of SakA localization in the wild type and the *fphA*-deletion strains under different light conditions, fresh conidia were inoculated on coverslips with supplemented minimal medium and incubated overnight in the dark at 28 °C. In order to avoid the effect of temperature shifts, the samples were placed at room temperature for 2 h before microscopy analysis. For light stimuli, the samples were illuminated in white light for 5 min. For salt stress, the minimal medium on coverslips was replaced by minimal medium containing 0.5 M NaCl and incubated 10 min in the dark. After illumination or salt stimulation, the samples were fixed with minimal medium containing 4% formaldehyde for 15 min and then washed once with PBS. For the analysis of bimolecular fluorescence complementation (BiFC), conidia were incubated on coverslips with supplemented minimal medium containing 2% glycerol and 0.2% glucose overnight in the dark at 28 °C.

Immunofluorescence. Fresh conidia were inoculated onto coverslips with 450 µl supplemented minimal medium and cultivated for 18 h in the dark at room temperature. The samples were exposed to light or kept in the dark in chambers for 5 min and fixed immediately with 3.7% formaldehyde in PBS buffer for 30 min at room temperature. The coverslips were washed three times with PBS buffer and incubated with digestion solution (100 mg driselase, 20 µl 5 U per ml zymolase and 800 mg glucanX diluted in 2.5 ml 50 mM Na citrate (pH 5.8) and mixed with 2.5 ml egg white) for 1 h at room temperature. The coverslips were washed three times with PBS buffer and blocked with 5% BSA in TBS buffer with 0.1% tween 20 (TBST) for 20 min. Afterwards the coverslips were incubated with antiphospho-p38 MAP kinase (Thr180/Tyr182) antibodies (#9211; Cell Signalling Technology; Beverly, MA; dilution 1:400) in TBST buffer with 5% BSA overnight at 4 °C and washed three times with TBST afterwards. Cy3-conjugated anti-rabbit IgG secondary antibody (Jackson Immuno Research; West Grove, PA) was used at 1:200 dilution in 5% BSA in TBST. After 1 h incubation, the coverslips were washed three times with TBST and mounted on microscope slides for observation.

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Author contributions

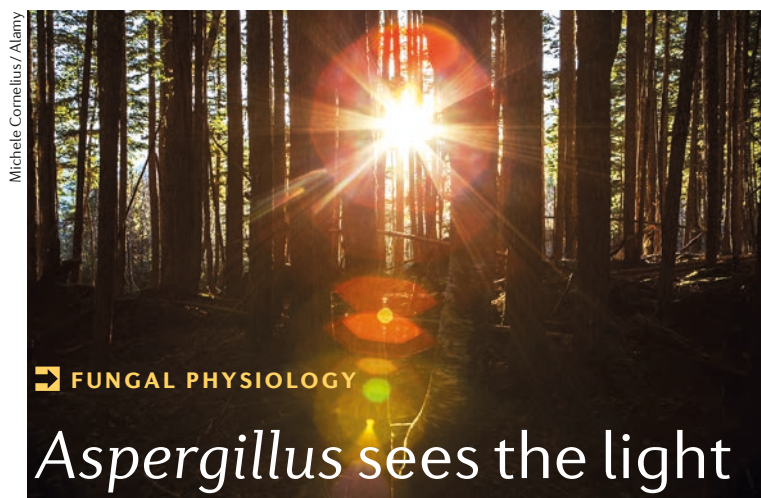
Z.Y. planned and performed the experiments and analysed the data. O.A. was responsible for whole-genome sequencing and analysed the sequencing data. R.F. planned the experiments, analysed the data and wrote the manuscript.

Additional information

Supplementary information is available [online](#). Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to R.F.

Competing interests

The authors declare no competing financial interests.



Michele Cornelius / Alamy

In order to adapt to changing conditions, fungi can sense environmental signals, including light. However, the signalling pathways involved in light sensing are unknown. Now, a new study shows that phytochrome-mediated light sensing in *Aspergillus nidulans* requires a stress-sensing signalling pathway that involves the MAP kinase SakA (also known as HogA).

A. nidulans uses two different systems to sense light: blue light is sensed by a flavin-based photoreceptor, which acts as a transcription factor that regulates gene expression; whereas red light is sensed by the phytochrome FphA, although how this alters transcription is unknown. To elucidate the signalling pathways that regulate gene expression in response to FphA-mediated light sensing, Yu *et al.* used an UV mutagenesis screen to identify

'blind' mutants that are impaired in their ability to sense red light. By sequencing the resulting mutants, the authors identified a single nucleotide insertion in the *sakA* gene, which encodes a MAP kinase that has been previously shown to be a component of signalling pathways sensing osmotic and oxidative stress.

To confirm the functional role of SakA in light sensing, the authors then generated a *sakA*-deletion strain and compared its development with wild-type *A. nidulans*. Light sensing is known to control fungal morphogenesis – light favours the development of asexual conidiospores, while repressing the development of sexual spores – and indeed, under light conditions, wild-type fungi gave rise to conidiospores, whereas the *sakA*-deletion strain produced mostly sexual spores, confirming that SakA is involved in light signalling in *A. nidulans*.

The SakA stress-sensing pathway is triggered by a two component phosphorelay system that involves a phosphotransferase, YpdA, and a response regulator, SskA, both of which are potential targets of FphA. To discriminate between these two possibilities, the authors tested if FphA could directly interact with YpdA or SskA. By using bimolecular fluorescence complementation and co-immunoprecipitation, they found that the phytochrome directly binds to YpdA but not SskA. Furthermore, by using a GFP-tagged version of SakA and antibodies directed against the phosphorylated form of SakA, the authors found that light sensing induced the phosphorylation and nuclear trafficking of SakA, which was dependent on the expression of the FphA phytochrome.

Collectively, these data demonstrate that the SakA pathway is responsible for phytochrome-mediated light signalling in *A. nidulans* and suggest a model of activation by which light influences the activity of the phytochrome, which then activates YpdA in the two component phosphorelay system; YpdA then regulates the phosphorylation of the MAP kinase SakA and its trafficking to the nucleus, thereby modulating gene expression.

Cláudio Nunes-Alves

ORIGINAL ARTICLE Yu, Z., Armant, O., Fischer, R., Fungi use the SakA (HogA) pathway for phytochrome-dependent light signalling. *Nat. Microbiol.* <http://dx.doi.org/10.1038/nmicrobiol.2016.19> (2016)

FUNGAL PHYSIOLOGY

Red light plugs into MAPK pathway

A classical mutant screen and genetic analyses powered by next-generation sequencing reveal that *Aspergillus nidulans* phytochrome-dependent red light sensing is transmitted via the high-osmolarity-glycerol mitogen-activated protein kinase cascade.

Alexander Idnurm and Yong-Sun Bahn

Most of our understanding of how eukaryotic organisms sense and respond to their environments has been obtained from studying fungi; however, we still do not fully understand how signals are transmitted or the interactions between different stimuli and pathways. In this issue of *Nature Microbiology*¹, Yu *et al.* unexpectedly show that red/far-red light-sensing phytochrome in the model filamentous fungus *A. nidulans* is an upstream component of a stress-sensing mitogen-activated protein kinase (MAPK) pathway.

Light is a major signalling cue that influences fungal physiology, including sexual and asexual differentiation, secondary metabolite synthesis, circadian rhythm and disease outcomes in pathogenic species, processes that are also regulated by many other environmental signals. Responses to blue light, which is sensed using the so-called white collar complex, are the best studied in fungi. Although other wavelengths of light are clearly important for fungal biology, relatively little is known about how signals are transduced and how they relate to other pathways. Phytochromes are a family of proteins that can detect red and far-red wavelengths and signal through a histidine kinase domain. In fungi, phytochrome functions have only been characterized in *Aspergillus* species^{2–4}, in which the phytochrome FphA and the blue-light sensing complex physically interact. How red light signals are translated into different physiological outcomes was unknown. To identify the signalling pathway activated by *A. nidulans* phytochrome, Yu *et al.* devised a genetic screen for 'blind' mutants, that is, ones that grew poorly in the light, and through next-generation sequencing of blind and wild-type progeny identified mutations in the MAPK SakA (also known as HogA), as well as in genes encoding the upstream MAPK kinase PbsB and MAPKK kinase SskB (known as Hog1, Pbs2 and Ssk2, respectively, in *Saccharomyces cerevisiae*).

The high osmolarity glycerol (HOG) pathway is best known as a stress-activated

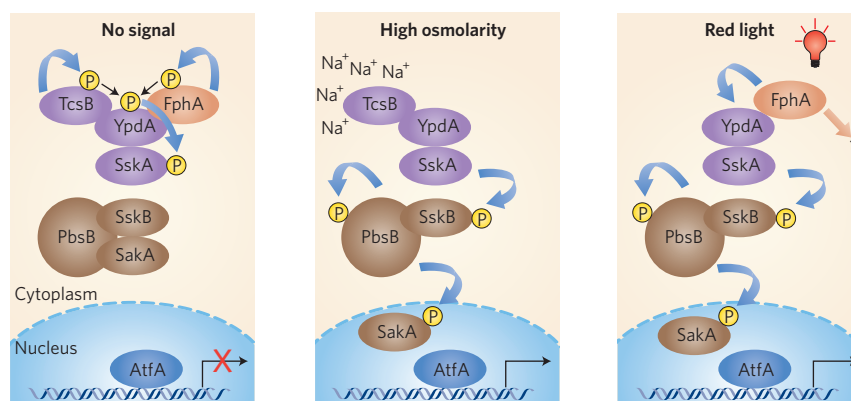


Figure 1 | Revised model of the Hog1 MAPK pathway in *Aspergillus nidulans*. High osmolarity and red light are recognized by histidine kinases TcsB and FphA, respectively (middle and right). Under isotonic or dark conditions (no signal), the histidine kinases are likely to be autophosphorylated and their phosphate groups are relayed to the YpdA phosphotransfer protein and the SskA response regulator. Phosphorylated SskA does not interact with the MAPK kinase kinase SskB. In response to high osmolarity or red light, the phosphorelay system (purple) becomes dephosphorylated to activate a series of kinases (brown), resulting in nuclear translocation of the MAPK SakA and its activation of bZIP-transcription factors, such as AtfA, that regulate target gene transcription. The subcellular localization of these events remains to be fully defined, as FphA is also found in the nucleus under certain conditions. Other targets of FphA are unknown, as indicated by the question mark.

MAPK module (Fig. 1). The human p38 MAPK — which is orthologous to Hog1 — plays important roles in stress and immune responses⁵. In fungi, Hog1 is activated by a multi-component phosphorelay system (consisting of hybrid sensor histidine kinases, phosphotransfer proteins and response regulators), which is responsive to a plethora of environmental stresses, such as osmotic shock, oxidative stress, UV irradiation and high temperature. In addition, the HOG pathway is critical for developmental and differentiation processes in a number of fungal species. Activated Hog1 phosphorylates other downstream kinases and a number of transcription factors. In *S. cerevisiae*, Hog1 can also be recruited to target promoter sites. Given these pleiotropic roles, it is no surprise that the HOG pathway plays pivotal roles in controlling the virulence of most pathogenic fungi⁶.

Through co-immunoprecipitation and bimolecular fluorescence complementation, Yu *et al.* showed that FphA interacts with the YpdA phosphotransfer protein, and phosphorylated SakA translocates to the nucleus in response to light in an FphA-dependent manner, underscoring the role of phytochrome as an input sensor for this pathway (Fig. 1). The HOG pathway has an alternative input sensor, the stress-sensing histidine kinase TcsB, which also acts via YpdA (Fig. 1). Yu *et al.* show that the activities of the two histidine kinases are distinct. For instance, phosphorylated SakA accumulates in the nucleus of wild-type cells in response to either light or a salt stress, and although deletion of *fphA* abolishes SakA nuclear translocation in response to light, SakA is still responsive to salt stress.

The identification of FphA as an upstream activator of the HOG pathway raises new questions. First, how widespread

is this phytochrome-induced signalling pathway in fungi? Phytochrome-encoding genes have been mutated in *Cryptococcus neoformans*, *Neurospora crassa* and *A. fumigatus*, yet phenotypes similar to those of the *A. nidulans* HOG mutants have not been reported in these species^{4,6–8}. However, there is suggestive evidence of a common link between phytochrome, the HOG pathway and an interaction with the blue-light sensing system. For instance, exposing *Trichoderma atroviride* to light increases tolerance to osmotic stress through the HOG pathway. This is not a blue-light dependent effect, yet it does lead to the induction of genes encoding the blue-light sensors⁹. In *N. crassa*, blue light regulates transcription of the *sskB* homologue¹⁰. Uncovering these interconnections will be an exciting direction for future research.

A second question is how the HOG pathway discriminates different incoming signals to activate different sets of downstream genes. Although there are multiple sensor histidine kinases in most fungi, all of their signals appear to be relayed

through a single phosphotransfer protein, converge on an SskA (known as Ssk1 in *S. cerevisiae*)-type response regulator and subsequently activate the Hog1 MAPK module (Fig. 1). If the HOG pathway reads all incoming signals as identical, it should activate the same set of genes regardless of the kind of stress. However, Hog1 is differentially phosphorylated in response to different stresses, and some HOG-dependent genes are regulated by specific stresses. Future transcriptomic analysis after exposure to white, blue or red light could identify light-induced, HOG-dependent genes. Although there may be a certain level of cross-talk between stresses — for example, Yu *et al.* demonstrate that osmotic stress can activate some light-regulated genes — there could be adaptors or scaffold proteins that allow stress-specific cellular responses.

In conclusion, the link between phytochrome and the HOG pathway comes as an exciting find. Yu *et al.* highlight the role of classical mutant screens in making major new discoveries. Their finding of phytochrome signalling via the

HOG pathway paves the way to analyse the extent of this signalling system in fungi and beyond, and the intricacies of light and stress signalling. 

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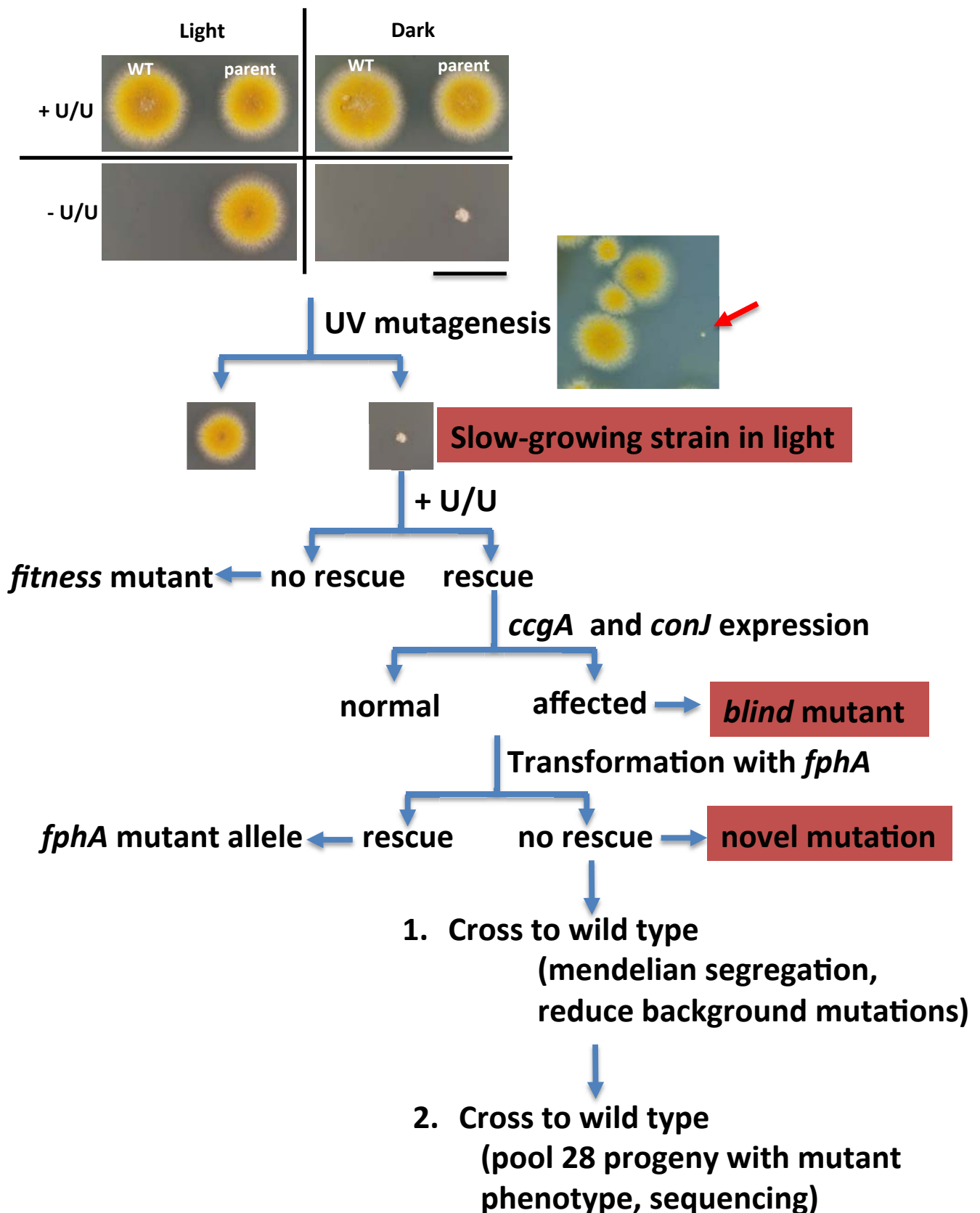
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Fungi use the SakA (HogA) pathway for phytochrome-dependent light signallingZhenzhong Yu¹, Olivier Armant² and Reinhard Fischer^{1*}**Address:**

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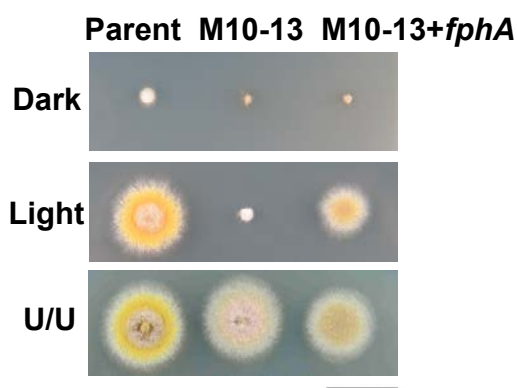
Figure S1



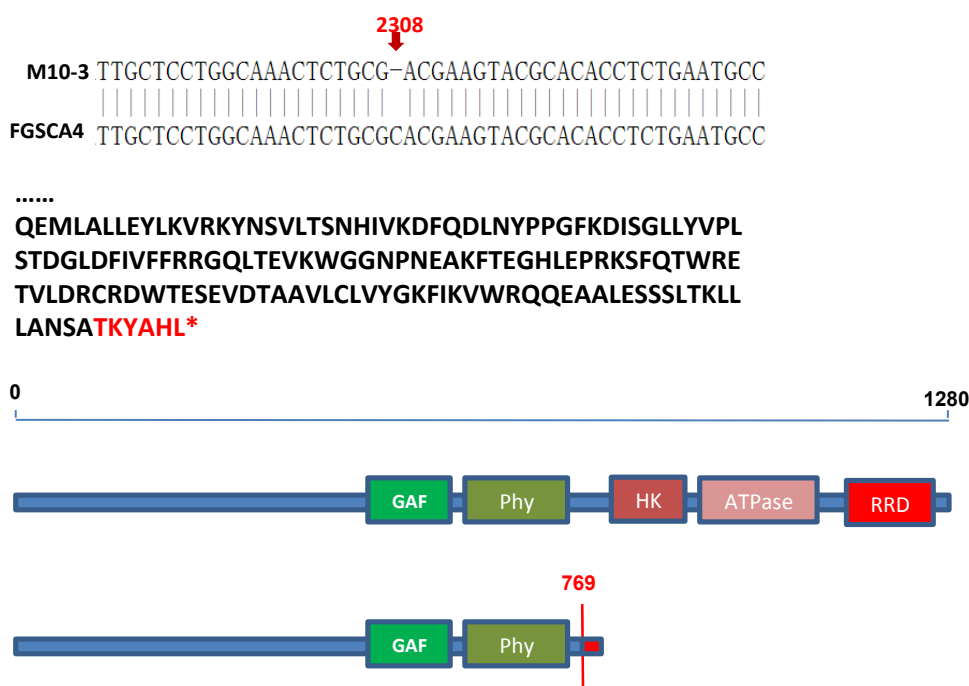
Suppl. Figure S1: Scheme of whole-genome sequencing based screening for novel components involved in light signaling. We constructed a strain (parent strain) in which the nutritional marker gene *pyr4* was placed under the control of a light-controlled promoter (*conJ*). This strain grows like wild type in light on minimal medium without uracil and uridine, but grows very slowly in the dark. After UV treatment, the putative *blind* mutants showed a slow-growing phenotype on minimal medium without uracil and uridine in light. These slow-growing mutants were transferred to minimal medium with uracil and uridine to eliminate fitness mutants. The mutants, whose phenotype could not be rescued, were further confirmed by checking light inducible genes *ccgA* and *conJ* expression in the dark and light with real-time RT-PCR. The *blind* mutants were the ones in which *ccgA* and *conJ* could not be induced by light. These *blind* mutants were complemented with *fphA* to eliminate *fphA* mutagenesis. In order to reduce background mutations, all *blind* mutants were crossed to wild type. After two subsequent crosses, progeny were sorted by phenotype. 28 progeny with a slow-growing phenotype and 28 progeny with normal growth were used for multiplex whole-genome sequencing. Scale bar, 1 cm.

Figure S2

a

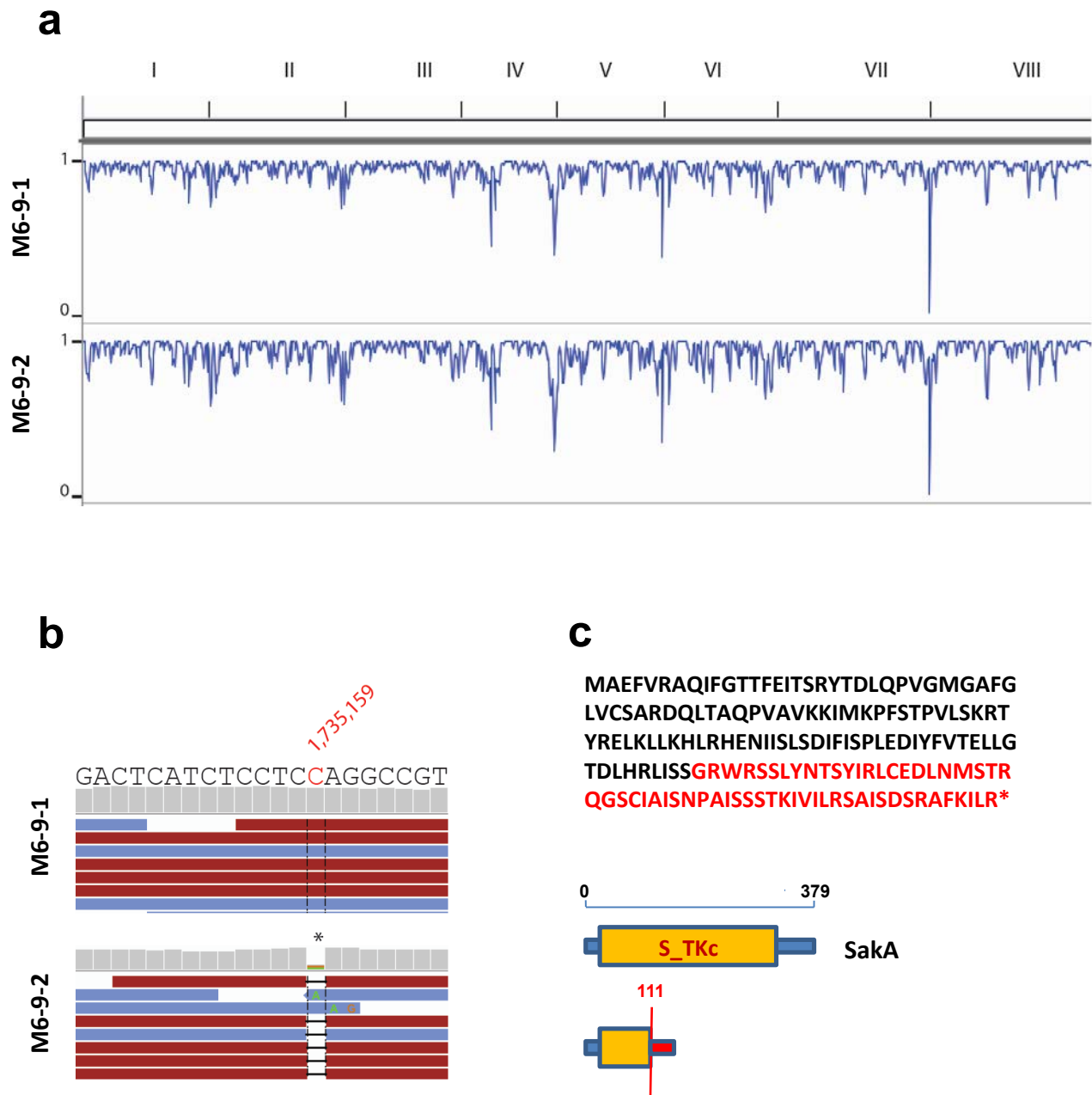


b



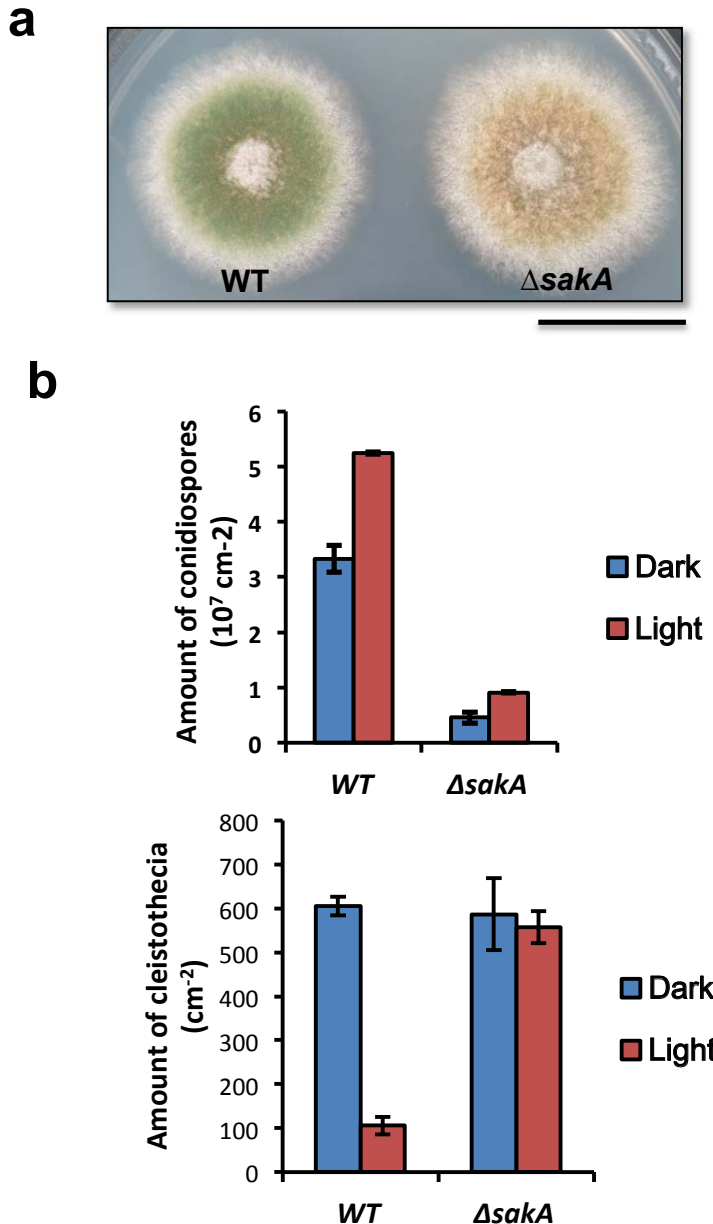
Suppl. Figure S2: Phytochrome is mutated in mutant M10-13. (a) The growth delay phenotype of M10-13 when grown in light is rescued by re-complementation of the strain with the *fphA* gene. M10-13 was co-transformed with the plasmid pJR17 (pCR2.1-TOPO containing the entire *fphA* gene) and pCK17 (pCR2.1-TOPO containing the *pabaA* gene). Colonies were cultured on supplemented minimal medium for 2.5 days. Scale bar, 1 cm. (b) Illustration of the frame-shift mutation leading to premature translation termination of *fphA*. The truncated protein lacks the histidine kinase (HK), ATPase domain and the response regulator domain (RRD).

Figure S3



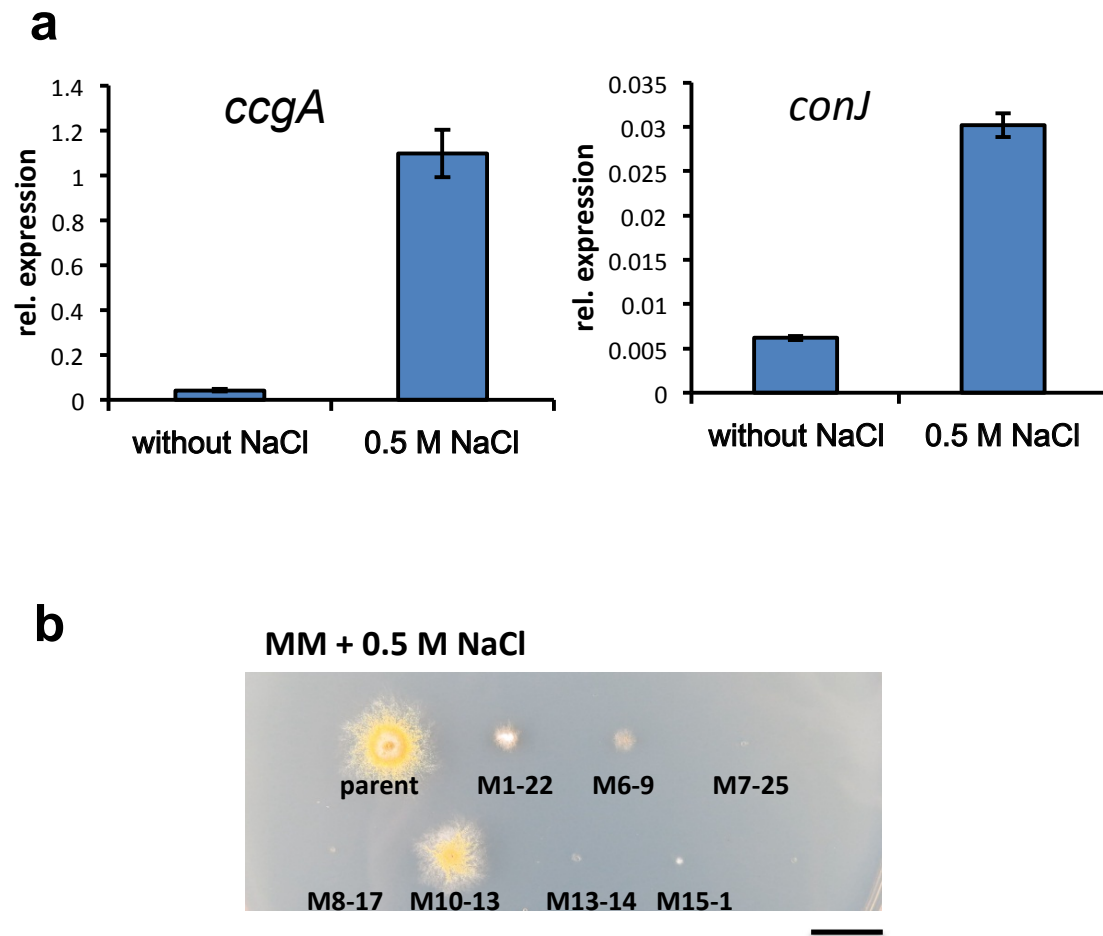
Suppl. Figure S3: Identification of the mutation in the mutant M6-9 by whole genome sequencing. (a) Coverage analysis shows that the genomes of M6-9-1 (only background mutations) and M6-9-2 (putative phenotype-causing mutation and background mutations) are identical. No big deletions or insertions were observed. **(b)** A 1 nt deletion was detected in the *sakA* gene. **(c)** The translated protein of the mutated *sakA* gene lacks the kinase domain.

Figure S4



Suppl. Figure S4: The balance between asexual and sexual development is shifted towards sexual development in the *sakA*-deletion strain. (a) The *sakA* deletion strain produces less green conidia as compared to wild type. The colonies grew 2.5 days on supplemented minimal medium. Scale bar, 1 cm. (b) Quantification of the conidiospore production of wild type and the *sakA*-deletion strain in dark and in light. Fresh conidia were added to supplemented minimal medium containing 7.5% agar to a final concentration of 1×10^6 spores/ml and 2 ml of the medium were poured on minimal medium agar plates. All plates were incubated at 37 °C in the dark for 12 hours and then half of them were exposed to light. After 5 days of cultivation, 3 pieces of agar were cut out with a hole puncher of Ø 8 mm from each plate. To wash off the conidia, each agar was put into a 2 ml Eppendorf tube with 1ml water containing 1% Tween and shaken at 200 rpm for 1 hour at 37 °C. Conidia were then counted in a Neubauer chamber. Cleistothecia were counted under the stereo microscope. The mean values were calculated from three biological replicates and the error bars represent the standard deviation.

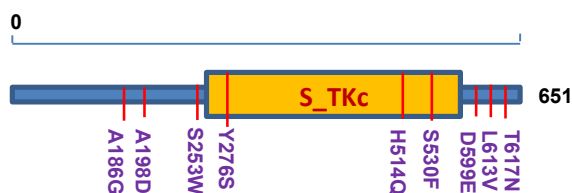
Figure S5



Suppl. Figure S5: *ccgA* and *conJ* can be induced by osmotic stress. (a) *ccgA* and *conJ* expression levels were up-regulated under salt stress. Conidiospores (1×10^6 spores/ml) were cultured in 300 ml flasks with 60 ml of liquid supplemented minimal medium at 37 °C shaken at 180 rpm. After 18 hours, 8.6 ml pre-warmed minimal medium with 4 M NaCl, or without NaCl for the control, were added to each flask. The mean values were calculated from three biological replicates and the error bars represent the standard deviation. (b) Parent strain and mutant M6-9 grew normally in the dark at 37 °C on supplemented minimal medium containing 0.5 M NaCl without uracil and uridine. The colonies were incubated 2.5 days. Scale bar, 1 cm.

Figure S6

a M1-22

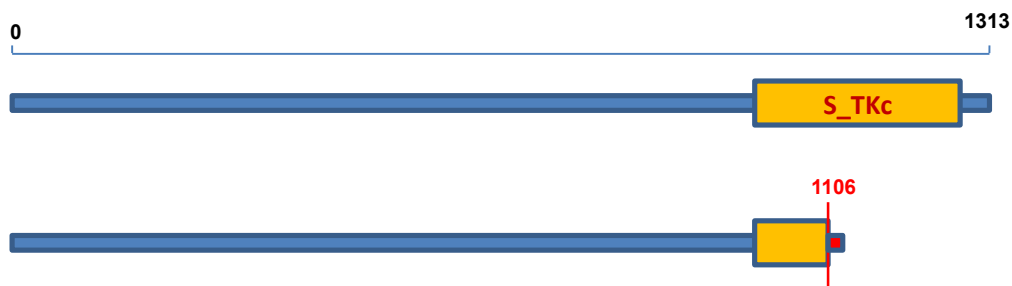


b M15-1

3571
↓

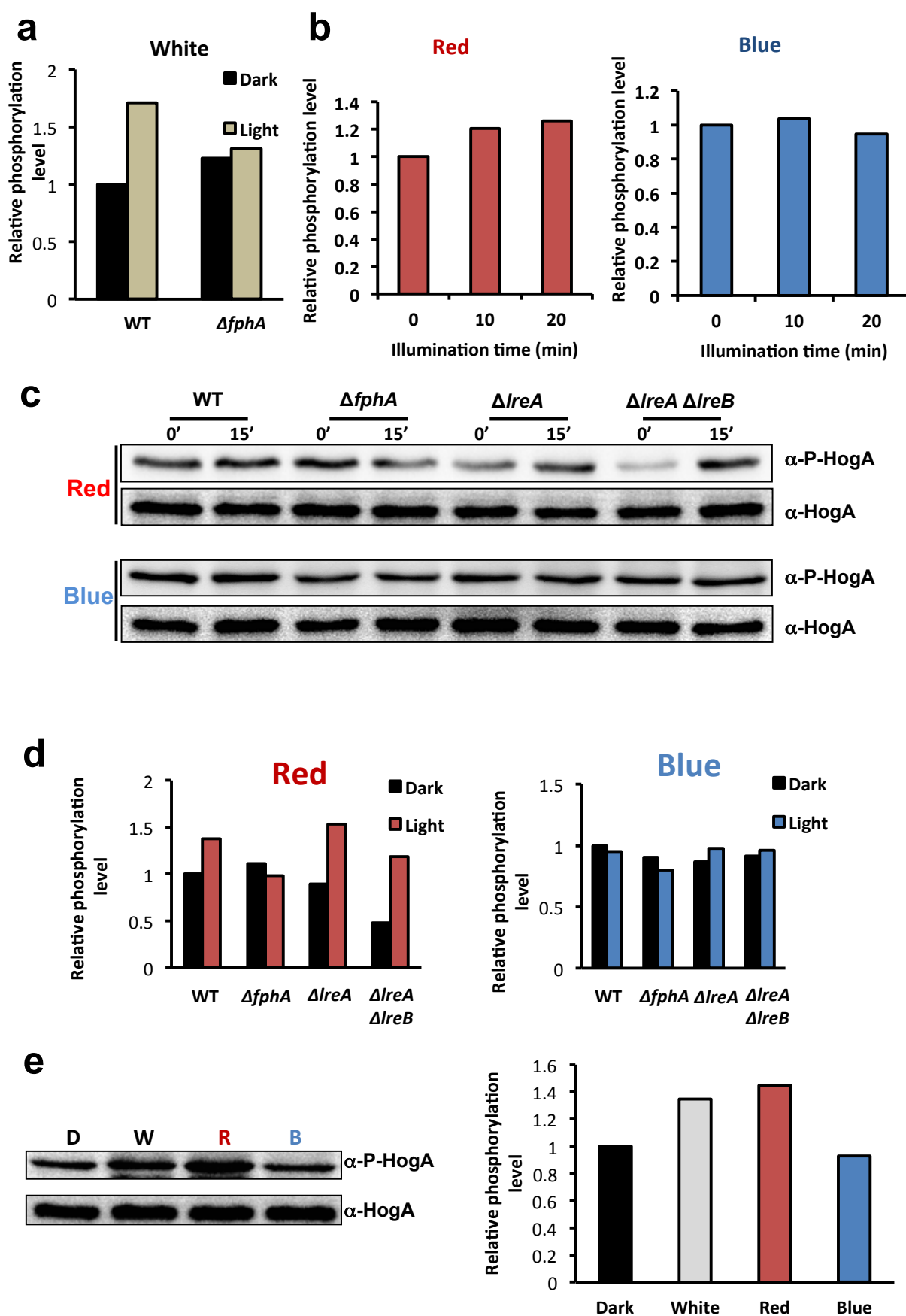
M15-1 TCCTTGAGCATGGCCGTGTCGAGGGACGAGACGGTTATCATGGTTTAT
 |||||
 FGSCA4 TCCTTGAGCATGGCCGTGTCGA-GGACGAGACGGTTATCATGGTTTAT

.....
 KRQALGRVLEGTNEADKSLAVLSSSATNVTLRWQQGQYIGGGTFGSVYA
 AINLDSNYLMAYKEIRLQDPQLIPKISQQIRDEMGVLEVLDPNIVSYHGIE
 VHRDKVYIFMEYCSGSLATLLEHGRVEGRDGYHGLCAPTSRRFSVSPSIR
 NRAPRHQARKYPA *



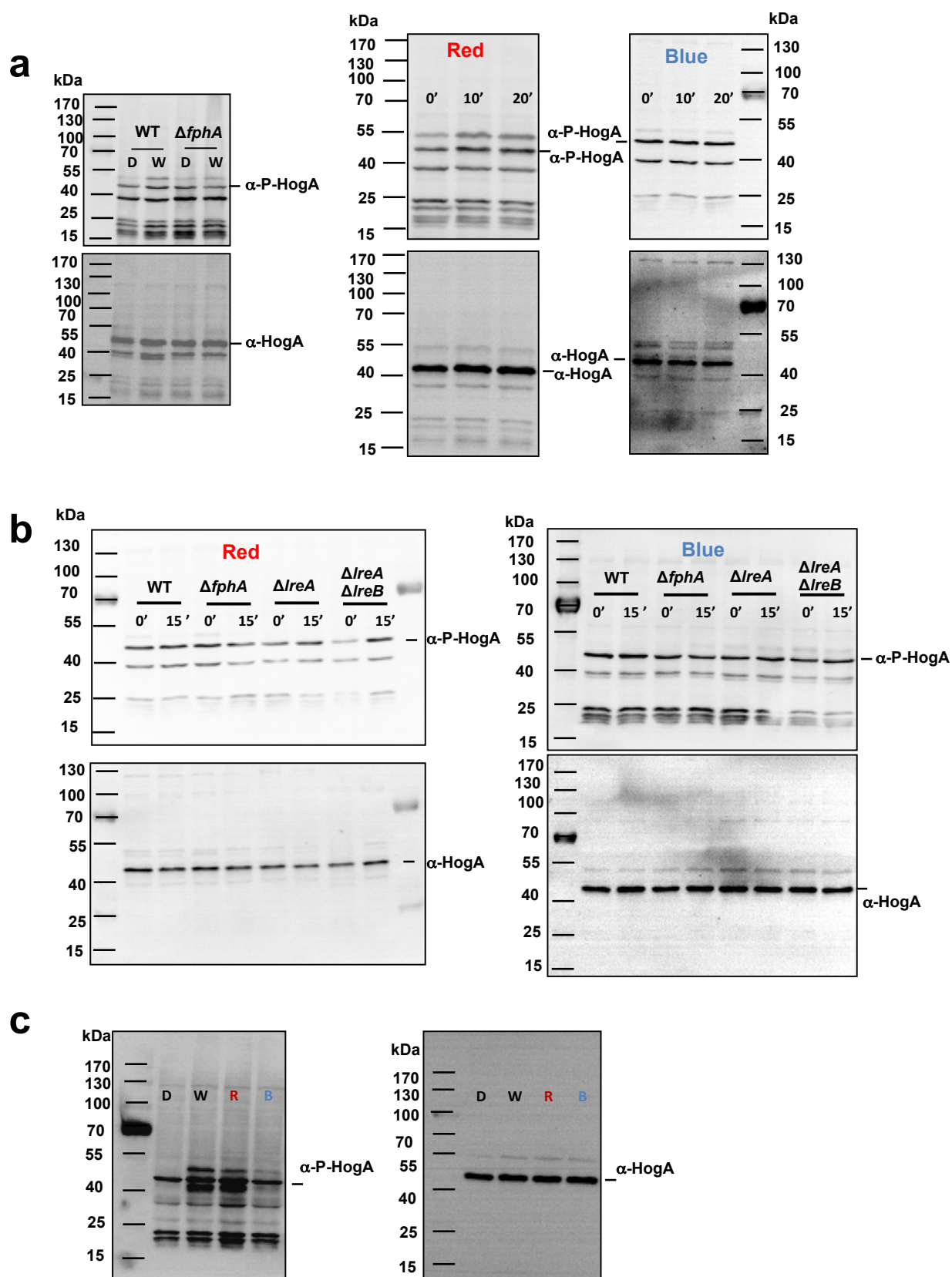
Suppl. Figure S6: Variations found in *pbsB* and *sskB* in M1-22 and M15-1 respectively. (a) Several single-base substitutions were detected in the *pbsB* gene in M1-22. (b) A one-nucleotide insertion was found in the *sskB* gene in M15-1. This results in the loss of the kinase domain.

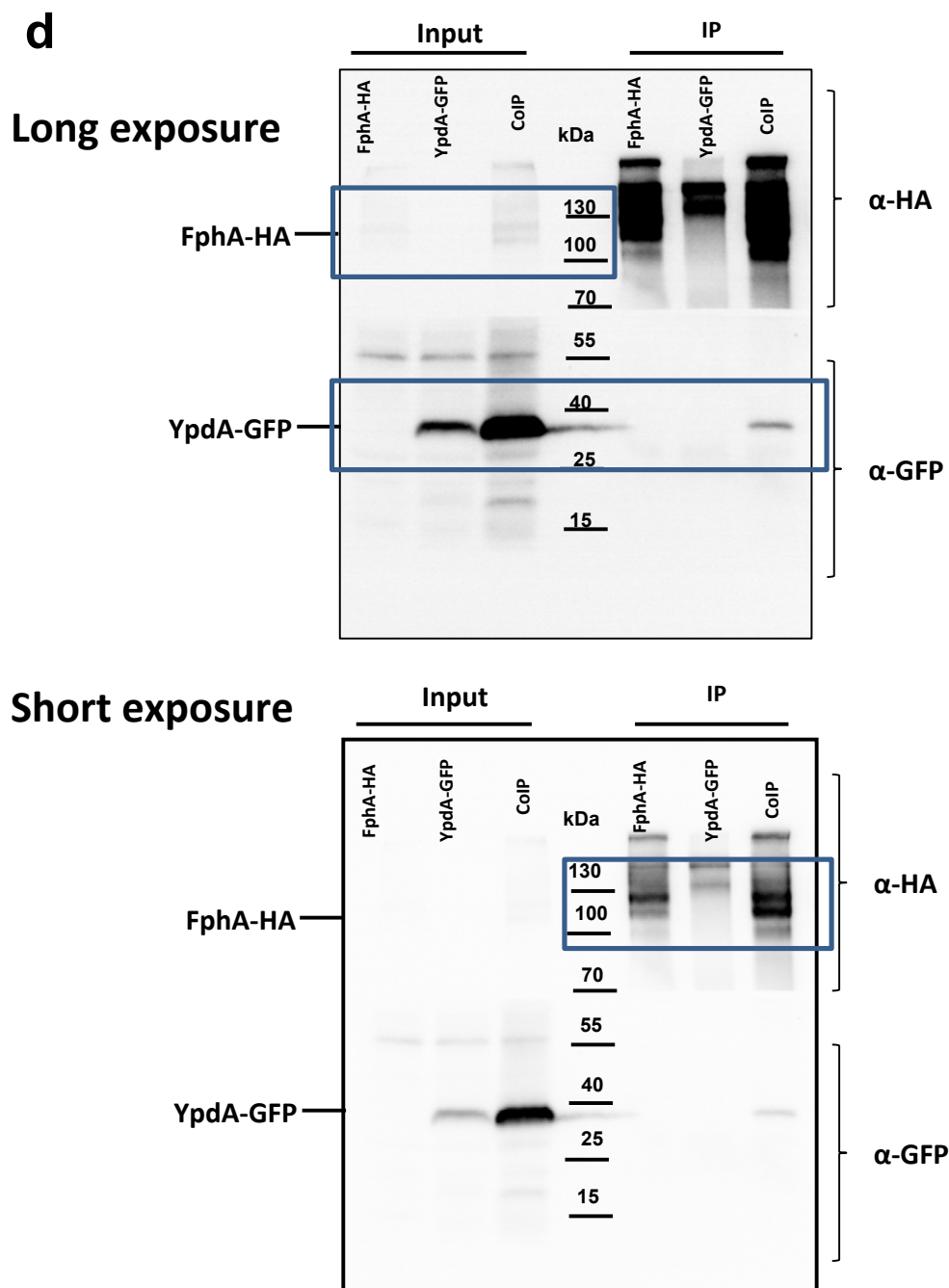
Figure S7



Suppl. Figure S7: Analysis of the phosphorylation signal by Western blotting. (a) and (b) show the relative phosphorylation of SakA corresponding to Figure 4 b. Western blot signals were quantified using ImageJ. (c) Analysis of the phosphorylation level in wild type (SJR2), the *fphA*-deletion strain (SJR10), the *lreA*-deletion (SRS66) and the *lreA/lreB*-double-deletion strain (SSR91). (d) Quantification of the bands displayed in (c). (e) Conidiospores (1×10^6 spores/ml) were inoculated into a 2 l flask with 400 ml of liquid supplemented minimal medium, incubated at 37 °C and shaken at 180 rpm. Each 50 ml culture was transferred into a 300 ml flask, which had been pre-warmed at 37 °C in light chambers. Three flasks were placed into white, red and blue light chambers separately and one was put into a dark chamber. After 15 min illumination, the mycelium was filtrated and frozen immediately in liquid nitrogen for protein extraction and Western blotting. The graph illustrates the relative phosphorylation levels.

Figure S8





Suppl. Figure S8: Original western blot images. (a) Original western blot images of Fig. 4b (b) Original western blot images of Suppl. Fig. 7c. (c) Original western blot images of Suppl. Fig. 7e. (d) Original western blot images of Fig. 3b.