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RESEARCH ARTICLE

Light inhibits spore germination through phytochrome in *Aspergillus nidulans*

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Abstract *Aspergillus nidulans* responds to light in several aspects. The balance between sexual and asexual development as well as the amount of secondary metabolites produced is controlled by light. Here, we show that germination is largely delayed by blue (450 nm), red (700 nm), and far-red light (740 nm). The largest effect was observed with far-red light. Whereas 60 % of the conidia produced a germ tube after 20 h in the dark, less than 5 % of the conidia germinated under far-red light conditions. Because swelling of conidia was not affected, light appears to act at the stage of germ-tube formation. In the absence of nutrients, far-red light even inhibited swelling of conidia, whereas in the dark, conidia did swell and germinated after prolonged incubation. The blue-light signaling components, LreA (WC-1) and LreB (WC-2), and also the cryptochrome/photolyase CryA were not required for germination inhibition. However, in the phytochrome mutant, Δ*fphA,* the germination delay was released, but germination was delayed in the dark in comparison to wild type. This suggests a novel function of phytochrome as far-red light sensor and as activator of polarized growth in the dark.

Keywords Aspergillus · Phytochrome · White collar · Light sensing · Germination

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Introduction

Filamentous fungi are extremely versatile organisms, which can grow under very different environmental conditions. They are able to sense various physical parameters and translate them into cellular responses, such as adaptation to osmotic stress or nutrient availability. One major response triggered by environmental conditions is the developmental decision. For instance, the ascomycete *Aspergillus nidulans* grows as hyphae in substrates, develops asexually when it grows at a water–air interface in light and develops complex sexual fruiting bodies when grown in the dark (Adams 1994; Busch and Braus 2007; Rodriguez-Romero et al. 2010). Both types of spores, conidia and ascospores, which are produced in these morphogenetic pathways are well protected against harmful UV light by pigments embedded into the cell wall. Spores are thus well adapted to spreading in the environment. However, once spores land on a nutrient-rich surface, vegetative hyphae are going to be produced. These hyphae are much less well adapted for growth in the presence of UV light and far more susceptible to DNA damage, enzymatic lysis and oxidation (Abad et al. 2010). Therefore, it is not surprising that the spores of some fungi sense light and possibly delay germination until sunset (Bragg 1981; Lucas et al. 1975). Germ tubes are formed and can grow into the substrate where they are further protected. Postponing germination to after sunset hours not only protects the fungus from UV light but also increases the probability for growth under more

humid conditions. An action spectrum of the inhibitory effect suggested the involvement of blue-light receptors in *Pseudoarachniotus marginosporus* and *Aspergillus oryzae* (Bragg 1981; Hatakeyama et al. 2007). However, the mechanism of germination delay and the nature of the involved photosensors remained elusive.

Light sensing, in general, was described in fungi many decades ago, and the molecular basis for light sensing and signal transduction has been unraveled to some extent within the past 20 years (Idnurm et al. 2010; Rodriguez-Romero et al. 2010). The blue-light response in *Neurospora crassa* is one of the best-studied cases and gained extraordinary attention due to the crucial role played by light regulators in the regulation of the circadian rhythm (Baker et al. 2011; Brunner et al. 2008; Heintzen and Liu 2007). In *N. crassa*, blue light controls many photoresponses, among which is the carotinoid biosynthesis. Mutants in the biosynthetic pathway appear white and the corresponding genes were named *albino* (Nelson et al. 1989). Because carotinoid biosynthesis is only light-regulated in hyphae but not in conidiophores, mutants with defects in putative light regulators appear with a white-collar (Degli-Innocenti et al. 1984). Two of the corresponding proteins turned out to be the main regulators for blue-light sensing, called *white collar 1* (*wc-1*) and *wc-2*. They both encode zinc-finger DNA-binding proteins, and WC-1 contains a flavin as chromophore (Ballario et al. 1996; Talora et al. 1999). They are able to form heterodimers and bind to the promoters of light-regulated genes (Froehlich et al. 2002). This blue-light perception and signaling system of *N. crassa* is essentially conserved in many other fungi (Idnurm et al. 2010; Purschwitz et al. 2006). Another player in UV- and blue-light sensing is the functional cryptochrome CryA, which at the same time has photolyase activity and is able to complement the DNA-repair machinery in *Escherichia coli* (Bayram et al. 2008a). Furthermore, there is evidence for red-light sensing in several fungi (Lukens 1965; Mooney and Yager 1990) and has been studied in detail in *A. nidulans*. In this fungus the red- and blue-light sensing systems interplay and regulate light-dependent gene expression (Blumenstein et al. 2005; Purschwitz et al. 2008, 2009). Many fungi contain in addition to blue- and red-light sensors, opsin-like proteins (Brown et al. 2001). However, their potential roles in light sensing in fungi are not well studied yet. Another important player in light-sensing in filamentous fungi is the Velvet protein, VeA, which is not a light sensor itself but is involved in the formation of a light regulator complex (Bayram et al. 2008b). It is involved not only in the control of development but also in the control of the secondary metabolism. Many laboratory strains harbor a mutation (*veA1*), leading to an N-terminally truncated protein in which a bipartite NLS motif is missing. VeA1 strains show strong conidiation even in the dark and a reduced production of secondary metabolites (Bayram and Braus 2012; Calvo 2008; Käfer 1965; Stinnett et al. 2007).

In this study, we describe a new light response in *A. nidulans*. We found that the spore germination is significantly delayed in the presence of light. Surprisingly, far-red light caused the strongest inhibition and phytochrome was required for this light response.

Materials and methods

Strains and cultivation of *A. nidulans* and determination of the germination rate

Conidia of *A. nidulans* FGSCA4 grown for 2.5 days were harvested from minimal medium plates (Käfer 1977). Ascospores were isolated from cleistothecia from *A. nidulans* FGSCA4 grown on minimal medium containing 6 % glucose for 3 weeks in the dark. Spores were suspended in 0.2 % tween 80 and washed three times with distilled water. Liquid minimal medium with 2 % glycerol, arginine, uracil, uridine and vitamins was inoculated with 0.7×10^6 spores/ml for germination experiments. 500 µl of that suspension was applied to a cover slip and placed in paper-back boxes with adequate light conditions, such as dark, blue light (450 nm), red light (700 nm) and far-red light (740 nm). Light was generated by LED panels and scattered by a diffuser foil. Each panel consists of 24 LEDs (Reuthner Lasertechnik GmbH, Wien, Austria). Light intensities at the bottom of the light boxes reached from 0.51 (700 nm) over 0.56 (450 nm) to 0.9 (740 nm) W/m^2.

To determine the rate of germination, a total of at least 300 spores per sample were examined. A spore was considered as germinated if a germ tube of at least 2 µm was visible. All experiments were repeated minimum three times. For the second germination rate determination, *A. nidulans* was cultivated as described above, but for a total of 40 h. Only the effects of darkness and far red light were examined. Germ tubes of at least 100 spores per sample were counted. The *cryA*-deletion strain (SCK44) was generated in FGSCA1153 (*yA1, pabaA1; argB2; pyroA4; nkuA::bar*). The *cryA* open reading frame was replaced with the dominant marker *ptrA*. Other strains used during this study were the phytochrome-deletion strain SJP1 (*pyrG89*; Δ*argB::trpC*Δ*B*; *pyroA4*; Δ*fphA::argB; veA*+) and the appropriate complementation strain SJP22 [SJP1 transformed with pJP19 (*fphA* with native promoter, *pyr4*)] as well as the *lreA*-deletion strain LAV+(*biA1;*Δ*lreA::argB; veA*+) and the *lreB*-deletion strain LBV+(*biA1*; Δ*lreB::argB; pyroA4; veA*+) (Purschwitz et al. 2008). In addition, the *veA*-deletion strain DVAR1 (*pabaA1, yA2,* Δ*argB::trpC, trpC801,* Δ*veA::argB*) (Kim et al. 2002) and the *veA1* strain GR5 (*pyrG89; wA3; pyroA4; veA1*) (Waring et al. 1989) were used.

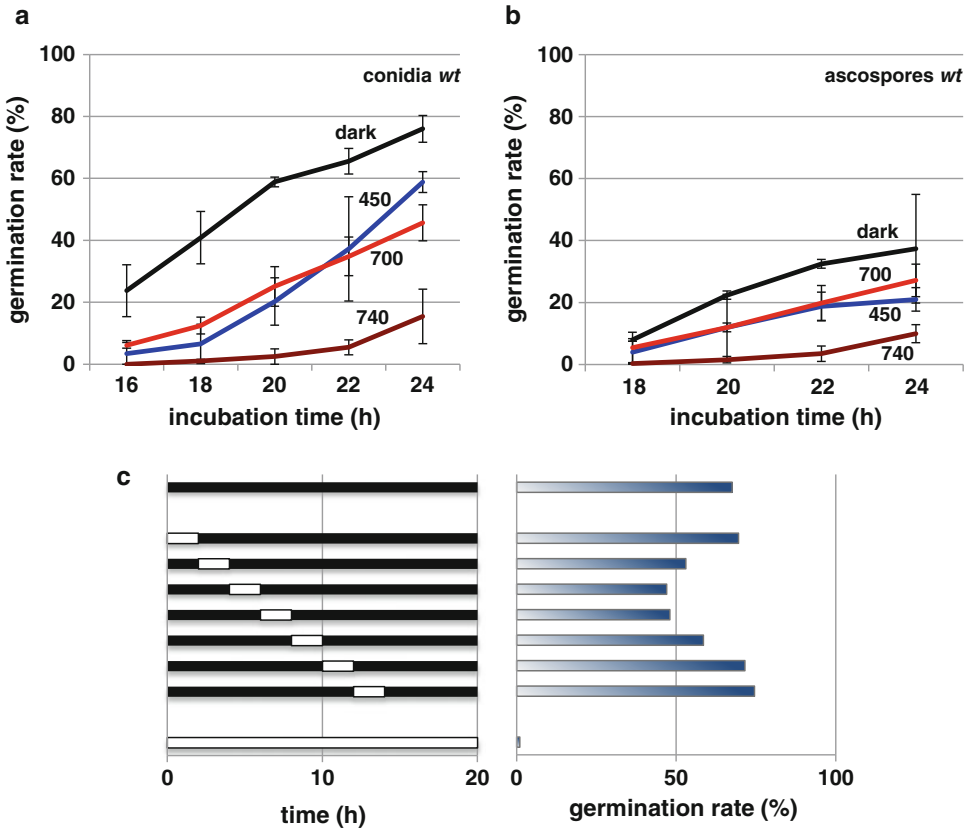

Fig. 1 Analysis of spore germination under different light conditions. **a** Conidia and **b** ascospores from FGSCA4 were incubated in minimal medium with glycerol as carbon source in the dark or under red (700 nm), far-red (740 nm) or blue light (450 nm) for 16–24 h at 23 °C. The germination rate was determined every 2 h. **c** Germination rate determination after 20 h of incubation at 23 °C after incubation in the dark, with 2 h far-red light pulses at different time points (*white bars*) and under constant far red light. About 65 % of conidia were germinated after incubation in the dark (*first row*) and less than 5 % under constant illumination with far-red light (740 nm; *last row*). Error bars represent the standard deviation

For the determination of the number of nuclei in wildtype, *veA*+ strain SCK3 transformed with *gpdA* (*p*)::*dsRed*::*stuA* was used. In parallel the number of nuclei was determined via DAPI staining in strain FGSCA4, SJP1 and SJP22. In each experiment 0.7*10 to the 6 spores/ml were incubated in liquid minimal medium with 2 % glycerol, arginine, uracil, uridin and vitamins for 24 h at 23 °C in the dark or under far-red light (740 nm). Afterwards 7 µl of Triton X-100 were added on each cover slip. Then nuclei were DAPI stained with "Vectashield DAPI" mounting medium. At least 200 spores of each sample were analyzed. The experiment was repeated 4 times.

Real-time PCR experiments

The quantification of the light-receptor encoding transcripts in the spores was done by real-time PCR. Spores were shaken in minimal medium with 2 % glycerol, arginine, uracil, uridine and vitamins in darkness and white-light conditions, respectively (petri dishes). After incubation, spores were harvested by centrifugation and RNA was processed for real-time PCR. For the analysis of *rasA* expression, spores were shaken in minimal medium with glycerol, under dark and far-red light conditions, respectively. cDNA synthesis and RT-PCR were performed using the One-Step RT-PCR Kit from Bioline. *rasA* was amplified with using RasA_RT_for (5′ A ATTGATCCAGAGCCACTTCGTG 3′) and RasA_RT_rev (5′ TGGCAGAGTATTCTTCCTGTCC 3′) as primers. *RasA* real-time was normalized to beta tubulin (*benA*) transcript amplified via PJR3 (5′ CAAGTGTGGTAACCAGGTT GGT 3′) and PJR4 (5′ GGCGTCGAGGCCATGTT 3′). For the amplification of the cDNA of the light receptors, the following primer combinations were used: lreA_RTQ_fw: 5′ GTTCGCTTCCCTGCATTCACA 3′; lreA_RTQ_rev: 5′ CTGAGTTGAAGAGACCATGCC 3′; lreB_RTQ_fw: 5′ ACCTGTCTACTAGAGGCGCAT 3′; lreB_RTQ_rev: 5′ AGGAAGACGCCTTGACAGAGT 3′; fphA_RTQ_fw: 5′ ATGGAGGCGCTGTTGGATACAA 3′; fphA_RTQ_-rev: 5′ CAGATCTATGCTCATCGTCGGA 3′. For normalization of the light receptor transcript levels histone 2B primers H2B-RT fwd (5′ CTGCCGAGAAGAAGCCTAGC AC 3′) and H2B-RT rev (5′ GAAGAGTAGGTCTCCTTC CTGGTC 3′) were used. All experiments were done with three biological and two technical replicates.

Results

Germination of spores is delayed by light

In previous experiments with phytochrome-deletion strains of *A. nidulans*, we noticed that these strains required more

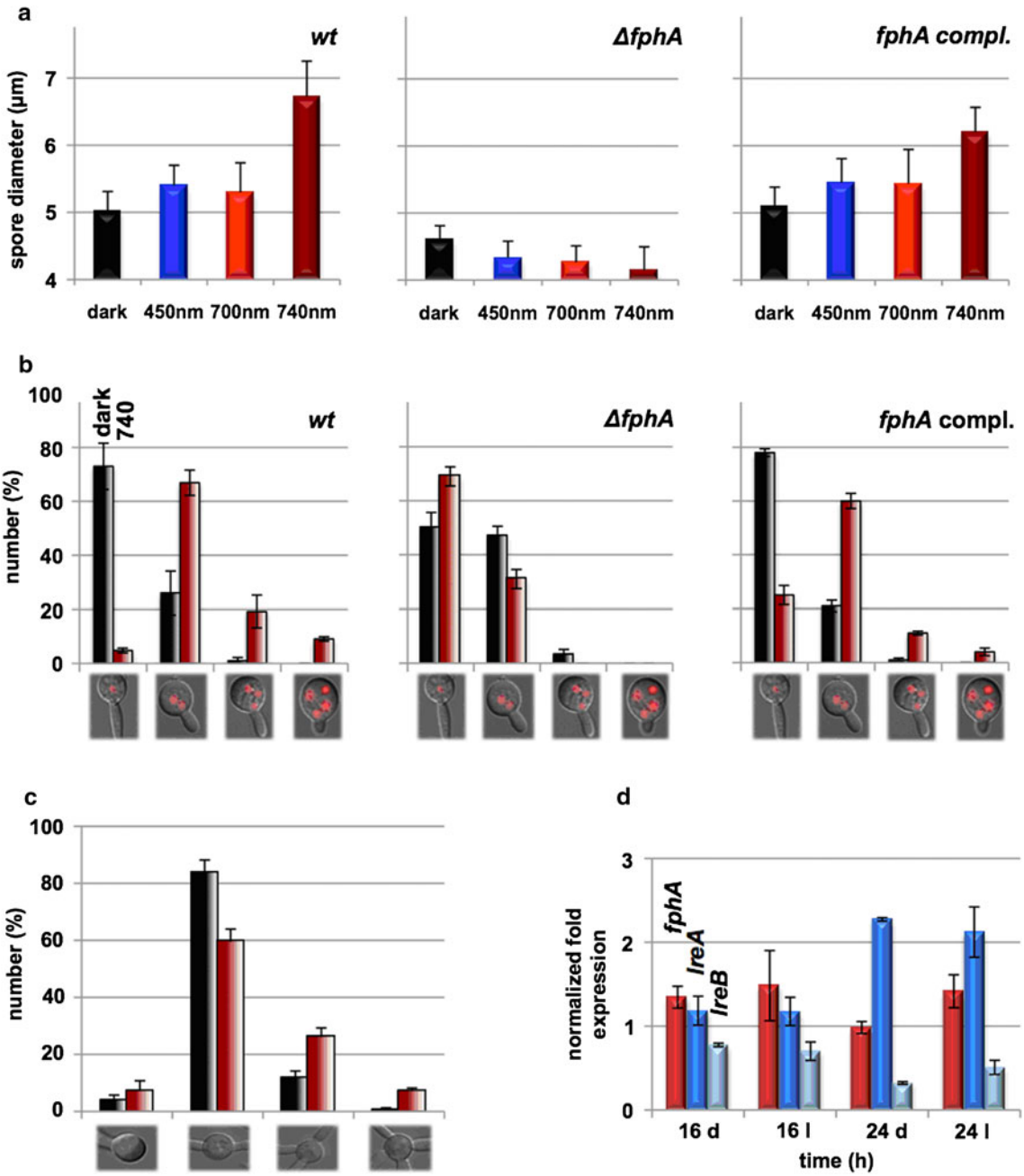

Fig. 2 Analysis of germination inhibition. **a** Conidia were inoculated in minimal medium containing glycerol as sole carbon source, supplemented with uracil, uridine and vitamins. Samples were cultivated for 24 h at 23 °C under different light conditions. The diameter of germinated spores was measured in wild type, a phytochrome-deletion strain (SJP1) and a complemented strain (SJP22). **b** Visualization of nuclei during germination in the dark and under far-red light conditions. DsRed was expressed with the *gpdA* promoter and targeted to nuclei (SCK3). In parallel the number of nuclei was determined via DAPI staining in strain FGSCA4, SJP1 and SJP22. **c** The number of germ tubes of 2-day-old mycelium of FGSCA4 was determined. Far-red illuminated spores form more germ tubes than spores incubated in the dark. **d** Quantification of the transcripts of *fphA*, *lreA* and *lreB* by Real-time PCR analysis. Numbers indicate incubation time in hours. *d* darkness, *l* white light conditions. Error bars represent the standard deviation

time for germination in comparison to Δ*fphA* strains re-transformed with *fphA* constructs. This suggested an activating role for FphA during germination and possibly a novel role for light in this fungus. Therefore, the *A. nidulans* wild-type strain FGSCA4 was analyzed for a light effect on germination. Spores were inoculated in liquid minimal medium with glycerol as carbon source and kept in the dark or illuminated with LEDs of different light qualities during the incubation at 23 °C. Germination was microscopically analyzed. In the absence of light, about

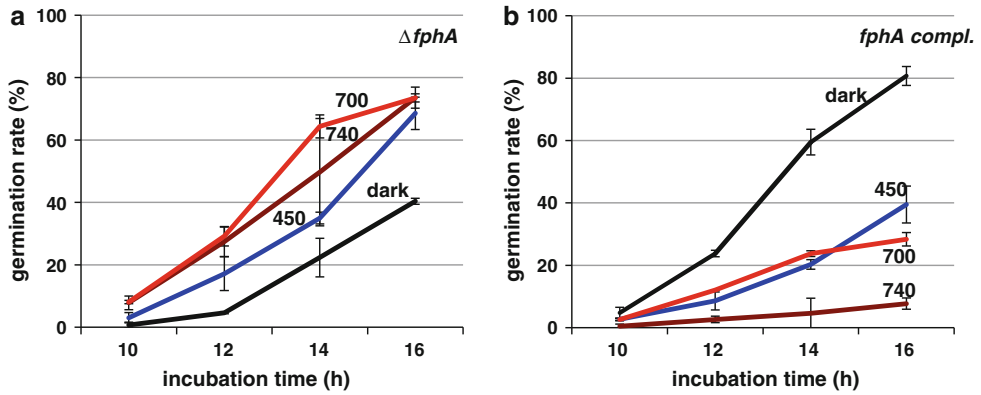

Fig. 3 Analysis of the germination of a Δ*fphA* mutant (**a**) and a re-complemented strain (**b**) under different light conditions. Conidia of a phytochrome-deletion strain (SJP1) (Purschwitz et al. 2008) and a re-complemented strain (SJP22) were cultivated in minimal medium with glycerol as carbon source, supplemented with uracil, uridine and vitamins. Samples were incubated at 23 °C in the dark or illuminated with 450, 700 or 740 nm wavelength, respectively. The rate of germination was determined every 2 h by counting at least 300 spores of each sample

60 % of the conidia germinated after 20 h. Red (700 nm) and blue light (450 nm) suppressed the appearance of the germ tube at about the same rate. Under those conditions, only about 25 % germinated after 20 h (Fig. 1a). Most strikingly, far-red light (740 nm) illumination reduced the germination rate to less than 5 % at that time point. Under these light conditions, it took about 10 h longer to reach the 50 % mark compared to the dark control (results not shown). This effect was essentially the same in conidio- and in ascospores, although the meiotically produced spores germinated slower (Fig. 1b). The inhibitory effect was fully reversible. Once the light was turned off, germination started instantly. After 15 h, nearly 100 % of the spores had germinated.

The experiments described so far were performed with constant illumination. To analyze at which germination stage light is most effective, samples were subjected for 2 h to far-red light illumination at different time points after inoculation. The suppressing effect was the strongest with a 2-h far-red light pulse between 4 and 8 h of growth. Early (0–2 h) and late (after 10 h) illumination did not show an effect (Fig. 1c).

Light leads to prolonged isotropic growth and delays polarity establishment

Spore germination is generally regarded as a three-step process (d'Enfert 1997). First, spores are activated mainly by the presence of water and a suitable carbon source (Osherov and May 2000, 2001). This is followed by a phase of isotropic growth and, finally, the transition to polar growth. We inoculated conidia of FGSCA4 in liquid medium and kept the sample in the dark or illuminated the spores with blue, red or far-red light (Fig. 2a). The diameter of spores with no or only a short germ tube was significantly larger in the samples that were illuminated. The diameter was the largest in the far-red light sample. Thus, the spore diameter correlated with the delay of germination. This result suggests that the light specifically affects the emergence of the germ tube and, hence, the transition from isotropic to polar growth. The same experiment done with spores of an *fphA*-deletion strain showed only slight differences between illuminated and dark-incubated samples. Thus, here spore diameter is the smallest under far-red light conditions, whereas it is the largest in the dark-incubated samples. In general, the diameter in the *fphA*-deletion strain was reduced. A re-complemented strain behaved again like wild type. In agreement with this, we found in young, far-red light treated wild-type germlings up to four nuclei whereas *ΔfphA* germlings illuminated with far-red light behaved similar to wild-type dark-incubated samples (Fig. 2b). More than 70 % of the dark control did not undergo mitosis before germ tube emergence. Only one quarter of the spores had two nuclei. This finding demonstrates that the cell-cycle control is not inhibited by light. Once the large spores germinated, an increased number produced two or more germ tubes from one spore (Fig. 2c).

These results suggested that light delays germination at the transition from isotropic to polar growth. To exclude that the observed effects were due to the absence of light receptors in the early stages of germination, expression analysis of three light regulators was performed (Fig. 2d). Conidia were inoculated and grown at 23 °C and RNA was extracted at different time points. The time span of 24 h comprises the whole germination process at the given conditions. During this period, the expression of the phytochrome *fphA* and *lreB* remained rather constant and *lreA* shows a twofold higher expression after 24 h compared to 16 h of growth.

Light inhibition of spore germination depends on phytochrome but is independent of VeA, CryA and the WC proteins

We showed above that the phytochrome plays a role in the swelling of conidia. Next, we analyzed the role of several light receptors in the germination delay. Conidia of corresponding mutant strains and the complemented strains were inoculated and analyzed as described above. Both, a *veA*-deletion as well as a *veA1*-mutant strain showed the same light-induced delay as the wild type (results not shown). The same was true for the white-collar mutants *lreA* and *lreB* and for *cryA*. Neither mutant showed any differences to wild type (results not shown). However, as observed before, phytochrome appeared to be the main player in mediating the germination inhibition. Far-red and also red-light inhibition was released in the *fphA* mutant (Fig. 3). The *fphA* re-complemented strain behaved again like wild type. The timing of germination was slightly shifted in comparison to the previous experiments with wild type (Fig. 1). This is due to different strain backgrounds. The pattern, however, is comparable. Unexpectedly, germination was delayed in the dark in the *fphA* mutant. After 14-h incubation, only about 20 % of the spores had germinated in comparison to 60 % in the complemented strain. The germination delay in the absence of phytochrome in the dark was surprising and suggests a role of FphA independent of light.

Starvation and illumination with far-red light have additive effects on germination delay

In the previous experiments, light delayed the polarization of conidiospores and ascospores. Because germination is also delayed in the absence of nutrients, we asked whether a combination of the two parameters would cause an additive effect. Conidia were washed with distilled water three times and then incubated in water at 30 °C. Whereas conidia produced a germ tube after 8–10 h in the presence of nutrients, it took much longer under starvation conditions. However, after 30 h more than 95 % of the conidia produced a germ tube when incubated in the dark (Fig. 4a, b). Far-red light reduced the number of germinated conidia after 30 h below 1 %. Conidia did not even undergo swelling. This was different from the light effect when conidia were grown in nutrient-containing media. Here, the block occurred at the polarization stage and, thus, after swelling of the conidia (Fig. 2).

These findings led to the question how the delay of germination could be explained at the molecular level. The RasA-protein has been described as an important signaling component to link nutrient sensing and germination (Osherov and May 2000). A dominant active *rasA*-mutant germinates also in the absence of nutrients. To test, whether the germination block of far-red light is

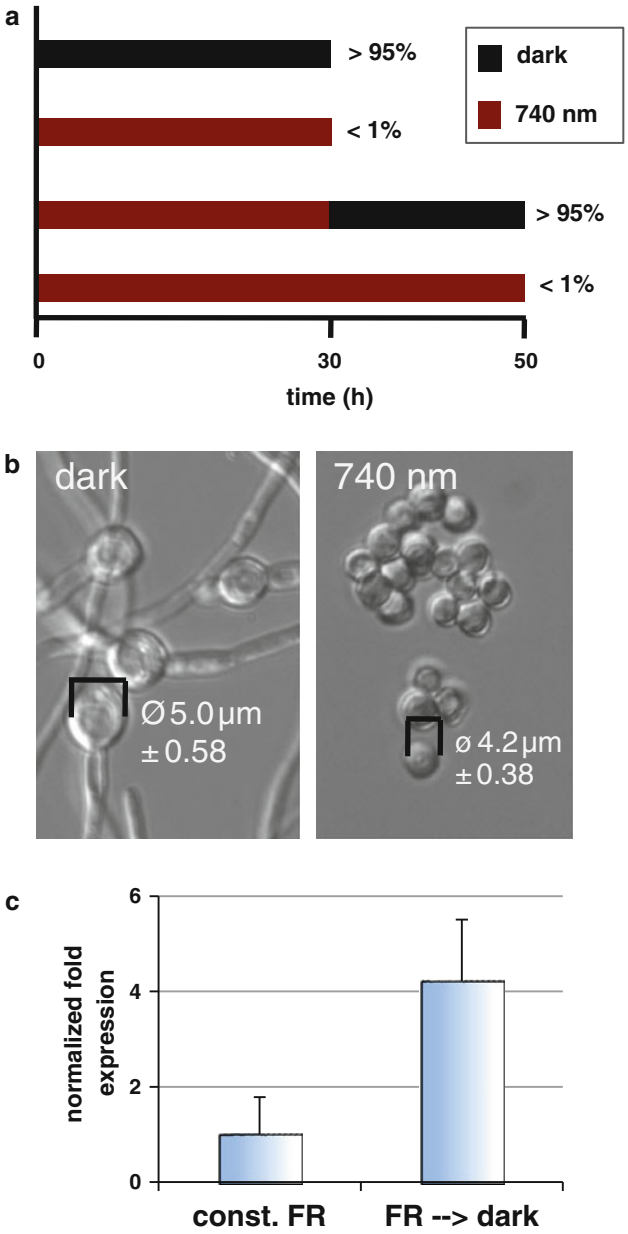

Fig. 4 Effect of light on germination in the absence of nutrients. **a** Conidia of wild type FGSCA4 were thoroughly washed and cultivated in ultra pure water (18 mΩ*cm) at 30 °C in the dark or under far-red light conditions. The germination rate was determined after 30 and 50 h. After 30 h a far red light incubated sample was transferred to the dark. In the absence of nutrients far red light completely blocked germination whereas in the dark, almost all spores produced a germ tube. **b** After 30-h incubation, spore diameters were measured. In far-red light, the spore diameter was only about 4.2 µm and, therefore, comparable to fresh, uncultivated conidia. **c** Conidia were shaken at 30 °C in distilled water for 40 h under far-red light conditions. One sample was transferred to the dark for the last 10 h. After incubation RNA was isolated and the transcript levels of *rasA* determined by real-time PCR

related to changes in the expression of the *rasA* gene, we quantified the *rasA* RNA level in conidia after far-red light illumination and after transfer to the dark. Whereas the mRNA level was very low when germination was blocked, the level increased fourfold after release of the block (Fig. 4c).

Discussion

In this paper, we found that the germination of conidia and ascospores in *A. nidulans* is largely delayed by light. In the absence of nutrients, the first stage of germination, namely isotropic growth, is affected. With nutrients available, light retarded the second phase, the polarization of the cell. Although blue light inhibited germination to the same extend than red light, the blue-light receptor, LreA and the WC-2 homologue LreB appeared not to be required for this response. Likewise, *cryA* deletion did not release the blue-light block, although a sensory function has been described for this protein (Bayram et al. 2008a).

Another interesting outcome of this paper is that far-red light had the most severe effect in the germination delay. Phytochrome is an interconvertible chromoprotein with absorption maxima at 707 and 754 nm depending on whether it was illuminated before with the one or the other wavelength (Blumenstein et al. 2005; Tasler et al. 2005). Our result shows that 740 nm caused the strongest germination inhibition indicates that FphA is converted into the Pr form. This is in contrast to other red-light responses in *A. nidulans*, where the Pfr form appears to be the effective species (Blumenstein et al. 2005; Brandt et al. 2008). FphA is a histidine kinase containing protein and comprises a C-terminal domain with similarity to bacterial response regulator domains. It was shown that FphA is able to autophosphorylate and transphosphorylate interacting FphA molecules at the response-regulator domain (Brandt et al. 2008). The transphosphorylation activity was higher in the Pr form. This suggests a transphosphorylation event in the signal cascade causing the germination delay under far-red light conditions. In disagreement with this hypothesis is the finding that the FphA occurs apparently also in the Pr form under dark conditions (Brandt et al. 2008). However, these results rely on in vitro experiments with FphA expressed and assembled in *E. coli*. Further in vivo experiments are required to determine the phosphorylation status of FphA under different light conditions and the corresponding transphosphorylation activity.

Another intriguing hypothesis is that the Pr phytochrome is the inactive form of FphA and cannot stimulate growth, whereas the Pfr form does. This is further supported by the fact that the complete absence of FphA causes a severe germination delay similar to the delay after illumination with far-red light in wild type. This strongly suggests a role of phytochrome in the dark. Considering the response-regulator domain at the FphA C terminus it could be that this signaling domain takes part in other signaling pathways besides light signaling. One candidate could be the RasA protein as suggested by the induction of the expression after release of the far-red light block (Fig. 4) (Osherov and May 2000). A RasA GTPase activating protein, GapA, has recently been shown to be crucial for the transition from isotropic to polar growth (Harispe et al. 2008), indicating that not only the expression level but also the GTP-state of RasA might be controlled by light. It will be the challenge for future analyses to examine the RasA activity in response to light as well as interactions between these regulators and the light receptors.

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