Karlsruher Institut für Technologie

# Molecular characterization of the light response in *Alternaria alternata*

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# Erklärung

Ich erkläre, dass ich die Doktorarbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht und die Satzung des Karlsruher Instituts für Technologie (KIT) zur Sicherung guter wissenschaftlicher Praxis in der jeweils gültigen Fassung beachtet habe.

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Olumuyiwa Igbalajobi

# Publikatationliste

Aus dieser Arbeit sind folgende Publikation entstanden:

**Igbalajobi, O., Yu, Z. and Fischer, R.** (**2019**). Red- and blue-light sensing in the plant pathogen *Alternaria alternata* depends on phytochrome and the white-collar protein LreA. *mBio* **10,** e00371-19. I.O. performed 100 % of the experimental work.

**Yu, Z., Ali, A., Streng, C., Igbalajobi, O., Leister, K., Krauß, N., Lamparter, T. and Fischer, R. (2019)**. Two hybrid histidine kinases, TcsB and the phytochrome FphA, are involved in temperature sensing in *Aspergillus nidulans. Mol Microbiol,* in revision. I.O. performed 10 % of the experimental work.

Weitere Publikationen, die nicht direkt mit dem Thema dieser Arbeit verknüpft sind:

**Choi, Y., Lee, M., Igbalajobi, O., Yu, J., and Shin, K. (2019).** Transcriptomic and functional studies of the RGS protein Rax1 in *Aspergillus fumigatus*. *Mol Gen Genet,* in revision. I.O. performed 30 % of the experimental work.

**Igbalajobi, O., Yu, J., and Shin, K. (2017).** Characterization of the rax1 gene encoding a putative regulator of G protein signaling in *Aspergillus fumigatus*. *Biochem Biophys Res Commun* **87**, 426-432. I.O. performed 100 % of the experimental work.

**Igbalajobi, O., Oluyege, A., Oladeji, A. and Babalola, J. (2016).** Antibiotic resistance pattern of *Pseudomonas aeruginosa* isolated from clinical samples in Ekiti State university teaching hospital, Ado-Ekiti, Ekiti State of Nigeria*. British Microbiology Research* **12**, 1-6. I.O. performed 100 % of the experimental work.

**Babalola, J., Oluyege, A., Lawal, O., Akinduro, O. and Igbalajobi, O. (2016).** Antibiotics susceptibility profile of extended spectrum beta lactamase producing gram negative bacteria from widal positive patients in Ekiti State University Teaching Hospital. *British Microbiology Research* **12**, 1-9. I.O. performed 70 % of the experimental work.

**Igbalajobi, O., David, O., Agidigbi, K., Babalola, J. (2015).** Antibiotic resistance pattern of two Indicator bacteria Isolated from cow dungs across ten local government areas of Ekiti State, Nigeria. *Int J Curr Microbiol App Sci* **4**, 8-14. I.O. performed 100 % of the experimental work.

**Odeyemi, A., Ajayi, A. and Igbalajobi, O. (2013).** Plasmid profile of isolated bacteria from Arinta Waterfall in Ipole-Iloro Ekiti. *Journal Microbiol Res* **3**, 32-38. I.O. performed 50 % of the experimental work.

**David, O., Ayeni, S., Akinmoji, O. and Igbalajobi, O. (2012).** Incidence of antibiotic-resistant Enterococci in three edible land snails consumed in Nigeria. *Wayamba Journal of Animal Science* **4,** 270-274. I.O. performed 50 % of the experimental work.

# Abbreviations

| | |
|---|---|
| AOH | Alternariol |
| CRY | cryptochrome |
| cys | cysteine |
| FAD | flavin adenine dinucleotide |
| FRQ | Frequency |
| FphA | Phytochrome |
| LOV | Light-Oxygen-Voltage |
| NLS | Nuclear localization signal |
| PAS | PER, ARNT, SIM repeats |
| Pfr | Far-red-light absorbing form of phytochromes |
| PHR | Photolyase-related domain |
| Phy | Phytochrome |
| PHY | Phytochrome domain |
| Pr | Red-light absorbing form of phytochromes |
| ROS | Reactive oxygen species |
| TLC | Thin layer chromatography |
| TF | Transcription factor |
| WC-1 | White collar 1 |
| WC-2 | White collar 2 |
| WCC | White- Collar- Complex |
| WT | Wild type |
| ZF | GATA type zinc-finger DNA binding domain |

# Contents

# Summary

Light is one of the most crucial environmental factors that impacts on development and metabolic processes of almost all organisms on earth. Filamentous fungi can perceive light using different photoreceptors and transduce the signal into biochemical outputs that modulate cellular and molecular responses within the ambient light environment. The black spore producing fungus *Alternaria alternata* is a common pre/post-harvest plant pathogen capable of producing more than 70 secondary metabolites. *A. alternata* is rarely implicated as human pathogen (allergy). Many processes in *A. alternata* are triggered by light. Whereas red light stimulates sporulation, continuous illumination with blue light completely inhibits sporulation. Interestingly, the inhibitory effect of blue light can be reversed by red light, suggesting cross-talk between the two light-sensing systems.

The *A. alternata* genome encodes a phytochrome (FphA), a white collar 1 (WC-1) orthologue (LreA), an opsin (NopA), and a cryptochrome (CryA) as putative photoreceptors. Apart from the white collar 1 orthologue LreA, which has been studied to some extent, further gene-function analyses have been limited due to difficulties to generate homogenous knock-out strains. However, recently, the CRISPR-Cas9 technology was established in *A. alternata* facilitating rapid and precise editing of genetic sequences and studies of gene function and regulation. Using this technology, we investigated the role of FphA and LreA and the interplay with the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway by creating loss-of-function mutations for *fphA*, *lreA*, and *hogA*.

Deletion of *fphA, lreA* and *hogA* resulted in a reduction of sporulation in the dark, suggesting an activating function of the photoreceptors (FphA and LreA) and HogA. Conidiophore development in *A. nidulans* requires a transcriptional cascade consisting of *brlA, abaA* and *wetA.* We investigated the effect of the deletion of FphA and LreA on *wetA* and *abaA* genes which are part of the central regulatory cascade of conidiation in *Aspergillus*. A *brlA* orthologue was not found in *the A. alternata* genome. After 60 min of white light illumination, the expression of *wetA* and *abaA* was abolished in the *fphA*- and *lreA*- mutant strains confirming the importance of the photoreceptors in the activation of sporulation and asexual development. Germination of conidia was

delayed in red, blue, green, and far-red light. Deletion of phytochrome, but not *lreA*, released the repression under all conditions, suggesting repressing functions of FphA and the presence of additional blue-light photoreceptors, respectively.

Next, we analysed the impact of light on secondary metabolite formation in the absence of FphA, LreA and HogA. The stimulation of alternariol formation was lost in the *hogA*- mutant strain irrespective of light and dark conditions with the upregulation of a yellow compound. Blue- and green-light stimulation of alternariol formation depended on LreA and not FphA. Oxidative stress response to hydrogen peroxide and menadione were enhanced in the *fphA*- *and lreA*- but not *hogA*- deletion strains independent of light due to the upregulation of catalases and superoxide dismutases.

We found that light induction of *ccgA* (clock-controlled gene in *Neurospora crassa* and light-induced in *Aspergillus nidulans*) appears to be strictly dependent on LreA and only to some extent on FphA. In order to further characterize the roles of *fphA, lreA*, and *hogA*, we studied their role in regulating other putative light-regulated genes. The expression of the catalase gene *catA* and short-chain dehydrogenases/reductases gene (*AAT_PT02522*) depended on FphA, LreA, and HogA. Light induction of *ferA* (a putative ferrochelatase gene) and *bliC* (*bli-3,* light regulated, unknown function) required LreA and HogA but not FphA.

Since previous reports in *A. nidulans, N. crassa* and *Trichoderma viride* suggested an interaction between the red and blue light photoreceptors with the HOG MAP kinase cascade, we investigated the expression of osmotic-stress induced genes in the three mutant strains. The transcript levels of *hogA, atfA,* and *ccgA* were reduced in the *fphA*, *lreA*- and hogA- mutant strains. While the expression of *bliC* was dependent on LreA and HogA, the *fphA*- deletion strain exhibited similar expression levels compared to the WT. We further analysed the phosphorylation of HogA by immunostaining under different light conditions. After illumination with red or blue light, fluorescence was detected in the cytoplasm and enriched in nuclei. The stimulation of the signal was not observed in the *ΔfphA* or Δ*lreA* mutant strains suggesting light activation of the HogA pathway depended on FphA and LreA.

The loss of *fphA* enhanced pathogenicity on tomato, whereas the *lreA*- and *hogA*- mutant strains showed reduced virulence as compared to the WT. We also found that phytochrome plays a role in temperature sensing.

# Zusammenfassung

Licht ist ein wichtiger Umweltfaktoren, der die Entwicklungsprozesse und metabolischen Prozesse von fast allen Organismen der Erde beeinflußt. Filamentöse Pilze können Licht mittels verschiedener Photorezeptoren wahrnehmen. Der schwarze Sporen-produzierende Pilz *Alternaria alternata* ist ein weit verbreitetes prä/post-Ernte Pflanzenpathogen, der in der Lage ist mehr als 70 Sekundärmetabolite zu produzieren. *A. alternata* kann auch selten als Humanpathogen Allergien verursachen. Viele Prozesse in *A. alternata* werden durch Licht gesteuert. Rotlicht stimuliert die Sporenproduktion, aber kontinuierliche Beleuchtung mit Blauchlicht inhibiert die Sporenproduktion komplett. Interessanterweise kann der inhibierende Effekt von Blaulicht durch Rotlicht aufgehoben werden, was eine Verknüpfung zwischen den Lichtsensorsystemen suggeriert.

Das *A. alternata* Genom kodiert ein Phytochrom (FphA), ein WC-1 Ortholog (LreA), ein Opsin (NopA) und ein Cryptochrom (CryA) als putative Photorezeptoren. Neben dem White Collar-1 Ortholog LreA, das schon zum Teil untersucht wurde, waren weitere Funktionsanalysen der Gene durch Schwierigkeiten bei der Konstruktion von homologen knock-out Stämmen limitiert. Jedoch wurde die CRISPR/Cas9 Technologie in *A. alternata* etabliert, die schnelle und präzise Veränderung von Gensequenzen und somit Studien von Genfunktion und –regulation zulässt. Mit der Verwendung dieser Technologie, haben wir die Rolle von FphA und LreA sowie das Zusammenspiel mit dem high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) Kinase Signalweg durch loss-of-function Mutationen für *fphA*, *lreA*, and *hogA* untersucht.

Die Deletion von *fphA, lreA* and *hogA* führte zu einer Reduktion der Sporulation im Dunkeln, was eine aktivierende Funktion der Photorezeptoren (FphA und LreA) und HogA suggeriert. Die Entwicklung von Konidiophoren benötigt eine transkriptionelle Kaskade, die aus *brlA*, *abaA* und *wetA* besteht. Nach 60 min Beleuchtung mit Weißlicht wurde die Expression von *abaA* und *wetA* in *fphA* und *lreA* Mutanten aufgehoben, wodurch die wichtige Rolle von Photorezeptoren in der Sporulation und asexuellen Entwicklung bestätigt wurde. Ein BrlA Ortholog wurde nicht gefunden.

Die Auskeimung von Konidien war in Rot-, Blau-, Grün- und Dunkelrotlicht verzögert. Die Deletion von Phytochrom, aber nicht von *lreA*, hob die Repression unter

allen Bedingungen auf, was die hemmende Funktion von FphA und die Präsenz zusätzlicher Blaulicht Rezeptoren bestätigt.

Weitergehend wurde die Auswirkung von Licht und die Rolle von FphA, LreA und HogA auf die Sekundärmetabolitbildung untersucht. Die Alternariolproduktion war sowohl im Licht als auch im Dunkeln strikt von *hogA* abhängig. Stattdessen wurde die Bildung einer gelben Substanz stimuliert. Die Blau- und Grünlicht Stimulation der Alternariolproduktion war nur von LreA abhängig und nicht von FphA. Die Resistenz gegenüber oxidativem Stress durch Wasserstoffperoxid und Mennadion war in dem *fphA* und *lreA*-Deletionsstamm, aber nicht im hogA Deletionsstamm erhöht. Die erhöhte Resistenz war unabhängig von den Lichtbedingungen und kann durch eine Induktion von Katalasen und Superoxiddismutasen erklärt werden.

Zur weiteren molekularen Analyse wurde die Induktion von *ccgA* (clock-controlled gene in *Neurospora crassa* and light-induced in *Aspergillus nidulans*) untersucht. Das Gen wird auch in *A. alternata* durch Licht induziert. Die Induktion war strikt von LreA und nur leicht von FphA abhänig. Für die weitergehende Charakterisierung der Rollen von *fphA, lreA*, and *hogA*, wurde die Regulation weiterer putativer lichtregulierter Gene untersucht. Die Expression des Katalase Gens *catA* and des short-chain dehydrogenases/reductases Gens (*AAT_PT02522*) ist von FphA, LreA and HogA abhängig. Die Lichtinduktion von *ferA* (ein putatives Ferrochelatasegen) and *bliC* (*bli-3,* light regulated, unbekannte Funktion) benötigen LreA und HogA, aber nicht FphA.

Da vorherige Untersuchungen in *A. nidulans, N. crassa* and *Trichoderma viridae* eine Interaktion zwischen den Rot und Blaulicht Photorezeptoren mit der HOG-MAP-Kinase Kaskade suggerieren, wurde die Expression von Genen, die durch osmotischen Stress induziert werden, in den drei Mutanten untersucht. Die Transkriptmenge von *hogA, atfA und ccgA* war in *fphA, lreA und hogA* Mutanten reduziert. Während die Expression von *bliC* von *lreA und hogA* abhängig war, wies der *fphA* Deletionsstamm eine vergleichbare Expression zum Wildtyp auf. Desweiteren wurde die Phosphorylierung von HogA unter verschiedenen Lichtbedingungen durch Immunostaining untersucht. Nach Beleuchtung mit Rot und Blaulicht wurde Fluoreszenz im Zytoplasma und angereichert im Zellkern detektiert. Die Stimulation des Signals wurde nicht im *fphA* oder LreA Mutantenstamm beobachtet, wodurch die Aktivierung des Hog Signalweges durch FphA und LreA suggeriert wird.

Der Verlust von *fphA* verstärkte die Pathogenität auf Tomaten, während *lreA* und *hogA* Deletionsstämme eine abgeschwächte Virulenz im Vergleich zum Wildtyp aufweisen. Zudem fanden wir, dass *fphA* eine Rolle in der Temperaturwahrnehmung spielt.

# 1. Introduction

*Alternaria* is a fungal genus that is ubiquitous in nature. The genus includes saprophytic and pathogenic species. Alternaria species are widely distributed in nature and are commonly found in grains, feeds, sewage, plastic, dead organic matters as well as air conditioning system (Ostry, 2008). *Alternaria* are indispensable in the ecosystem as major degraders of polymeric organic material and their recycling to $CO_2$. They are known to form both synergistic and antagonistic (pathogenic) relationships with, plants, animals and microbes. They are able to produce more than 70 different secondary metabolites, some of which are mycotoxins or antibiotics (Pfeiffer *et al.*, 2007). Some of these compounds impact their virulence and pathogenicity (Ostry, 2008; Tsuge *et al.*, 2013; Wenderoth *et al.*, 2019). Prominent examples among Alternaria species are *A. alternata, A. tenuissima, A. arborescens, A. radicina, A. brassicae, A. brassicicola, and A. infectoria*. The optimum growth temperature ranges between to 25°C and 30°C. They have been implicated as post-harvest plant pathogens in a variety of crops such as brown rot of tangerine, black rot of tomato and apple as well as leaf spot of lemons, grapevine and limes (Magan *et al.*, 1984; Hasan, 1999; Thomma, 2003). In humans, they have been reported as opportunistic pathogens directly linked to cutaneous, subcutaneous infections and asthma (Sanzani *et al.*, 2016). *A. alternata* is a black-spore producing fungus with conidia produced in chains. New individual spores arise directly from the tip of mature parent chains. The spores are melanised. This property protects them against ultraviolet (UV) damage and other environmental stresses. On the basis of their chemical structures, *Alternaria* mycotoxin are divided into five major groups; (1) dibenzopyrone derivatives, which encompass alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT); (2) tetramic acid derivatives, such as tenuazonic acid (TeA), and *iso*-tenuazonic acid (*iso*-TeA); (3) perylene derived, altertoxins I, II, and III (ATX-I, ATX-II, and ATX-III); (4) *A. alternata* f. sp. *lycopersici* TA1, TA2, TB1, and TB2 toxin (AAL TA1, TA2, TB1, and TB2); (5) wide-ranging structures, such as tentoxin (TEN), *iso*-tentoxin (*iso*-TEN), and dihydrotentoxin (DHT), which are cyclic tetrapeptide (Ostry, 2008; Alexander *et al.*, 2011; Lou *et al.*, 2013; Hickert *et al.*, 2017). These mycotoxins are potentially harmful if consumed and

pose a serious risk to human and animal health. Their presence in cereal grains has been suggested to be linked with high levels of human esophageal cancer in China and Africa (Fleck *et al.*, 2016).

Light is a major source of energy for plants and important for many organisms on earth. The wide distribution of photoreceptors that sense, carry and process environmental cues among microorganisms, animals, insects, and plants suggests a more sophisticated role of light beyond their role as source of energy in photosynthesis. The presence or absence of light and direction, intensity, and spectral distribution carry information important for the development and fitness of many microorganisms. The influence of light on biological responses such as sporulation, virulence, secondary metabolite production and the entrainment of the circadian clock in filamentous fungi reveals complex roles of light in photobiology (Blumenstein *et al.*, 2005; Idnurm & Heitman, 2005; Fischer, 2008; Kami *et al.*, 2010; Rodriguez-Romero *et al.*, 2010; Canessa *et al.*, 2013; Röhrig *et al.*, 2013; Dasgupta *et al.*, 2016; Yu *et al.*, 2016; Yu & Fischer, 2019).

## 1.1 Phytochrome enables red- and far-red- light sensing in plants, bacteria and fungi

Living organisms perceive light from the environment using different photoreceptors. The first evidence of red- light sensing in *Alternaria* was reported in *Alternaria solani* (Lukens, 1963). Red light stimulated the formation of conidia from conidiophores. Phytochromes (phys) are a superfamily of red (R) and far-red (FR) photoreceptors which regulate various metabolic processes and development in response to light in plant, fungi and bacteria. They were first discovered over half a century ago in plants (Butler *et al.*, 1959) and afterwards in cyanobacteria, bacteria and fungi such as *A. nidulans* and *N. crassa* (Karniol *et al.*, 2005; Njimona *et al.*, 2014; Lamparter *et al.*, 2004; Lamparter *et al.*, 2003; Lamparter *et al.*, 2002; Lamparter *et al.*, 2017). Phytochromes are composed of two major protein domains; an N- terminal anchoring the tetrapyrrole bilin chromophore, responsible for the photosensory properties and a C-terminal output or regulatory domain, responsible for the transmission of the light signal. The bilin chromophore is covalently attached to a conserved cysteine residue in the phytochrome apoprotein via the C3 sidechain of the bilin A-ring. The core sensory domain typically consists of a PAS, GAF and PHY subdomain with two

additional PAS domains in plant phytochromes (Montgomery & Lagarias, 2002). The C-terminal output end contains a subdomain similar to histidine kinases (HKRD), although a truncation of most of the histidine kinase-related domain does not eliminate the activity of phyB suggesting that this domain is dispensable (Krall & Reed, 2000). In some bacteria and fungi, the histidine kinase domain is followed by a response-regulator domain (RRD) (Pao & Saier, 1997; Szurmant *et al.*, 2007). Plant phytochromes (Phy family) and cyanobacterial phytochromes that bind phytochromobilin and phycocyanobilin respectively have a conserved Cys in the GAF domain (Fry & Mumford, 1971; Rockwell & Lagarias, 2006). Fungal phytochrome is more related to bacterial phytochromes than to plant phytochromes (Lamparter *et al.*, 2004; Lamparter *et al.*, 2003; Lamparter *et al.*, 2002; Blumenstein *et al.*, 2005; Froehlich *et al.*, 2005). Unlike plant phytochromes, the fungal phytochrome harbors a bilin chromophore covalently attached to a cysteine in the PAS domain and lacks the canonical cysteine in the GAF domain (Blumenstein *et al.*, 2005). X-ray crystallographic and *in vitro* studies have established that the PAS and GAF domains of the photosensory core are knotted together and both are crucial for proper incorporation of the chromophore (Wu & Lagarias, 2000; Wagner *et al.*, 2005).

Phytochromes act as photosensors which trigger various biological and metabolic outputs by reversibly interconverting between two conformations, Pr (red light (R)-absorbing) and Pfr (far-red light (FR)-absorbing) via the cis/trans-isomerization of a double bond in the bilin chromophore upon stimulation by red or far-red light (Abe *et al.*, 1985; Siebert *et al.*, 1990; Foerstendorf *et al.*, 2001; Heyne *et al.*, 2002; Bae & Choi, 2008). The Pr form is mostly synthesized in the dark and upon absorption of red light is converted to the Pfr form, which is the active form for many biological responses. The Pfr form after absorption of far-red light or via dark reversion can be converted back to Pr, the biologically inactive form of the photoreceptor (Quail, 1997; Fankhauser, 2001). There are five phytochromes in the plant *Arabidopisis thaliana* designated as *phyA, phyB, phyC, phyD* and *phyE* (Rockwell *et al.*, 2006). Analysis of different mutants of these genes have differential and overlapping roles as biological switch. While *phyA* mediates the control of seedling deetiolation upon continuous far-red illumination, *phyB* mediates the same function upon continuous illumination by red light (Nagatani *et al.*, 1993; Whitelam *et al.*, 1993; Kiss *et al.*, 2003). Unlike in dicotyledons, the phytochrome family in monocotyledons, including

duckweeds, have three different phytochrome genes, homologs of *phyA*, *phyB*, and *phyC* (Mathews & Sharrock, 1997).

Bacteriophytochrome was first characterized in a stem nodulating symbiont of Aeschynomene, *Bradyrhizobium* sp. strain ORS27A (Giraud *et al.*, 2002). Unlike most bacteriophytochromes, *BrBphP* is a bathyphytochrome (phytochrome that undergo dark conversion of Pr to Pfr and thus the ground state is the Pfr form) which lacks an histidine kinase (HK) domain (Giraud *et al.*, 2002; Karniol & Vierstra, 2003; Tasler *et al.*, 2005). It contributes to a far-red light shift in the modulation of various biological responses such as the regulation of photosynthetic signalling and carotenoid metabolism (Beattie *et al.*, 2018). Bathyphytochrome are well distributed among Rhizobia soil bacteria which includes *Rhodopseudomonas palustris*, *Agrobacterium tumefaciens, Agrobacterium vitis* S4, *Rhizobium leguminosarum* 3841, *Rhizobium etli* CIAT652, and *Azorhizobium caulinodans* and *Xanthobacter autotrophicus* (Giraud *et al.*, 2002; Karniol & Vierstra, 2003; Tasler *et al.*, 2005; Rottwinkel *et al.*, 2010). In addition to bathyphytochrome and normal phytochrome in bacteria, there are other phytochromes with unusual spectral properties as observed in the Agp1 and Agp2 from *A. tumefaciens* with an N-terminal chromophore attachment site outside the region where the plant and cyanobacterial phytochrome binds their chromophore (Lamparter *et al.*, 2002).

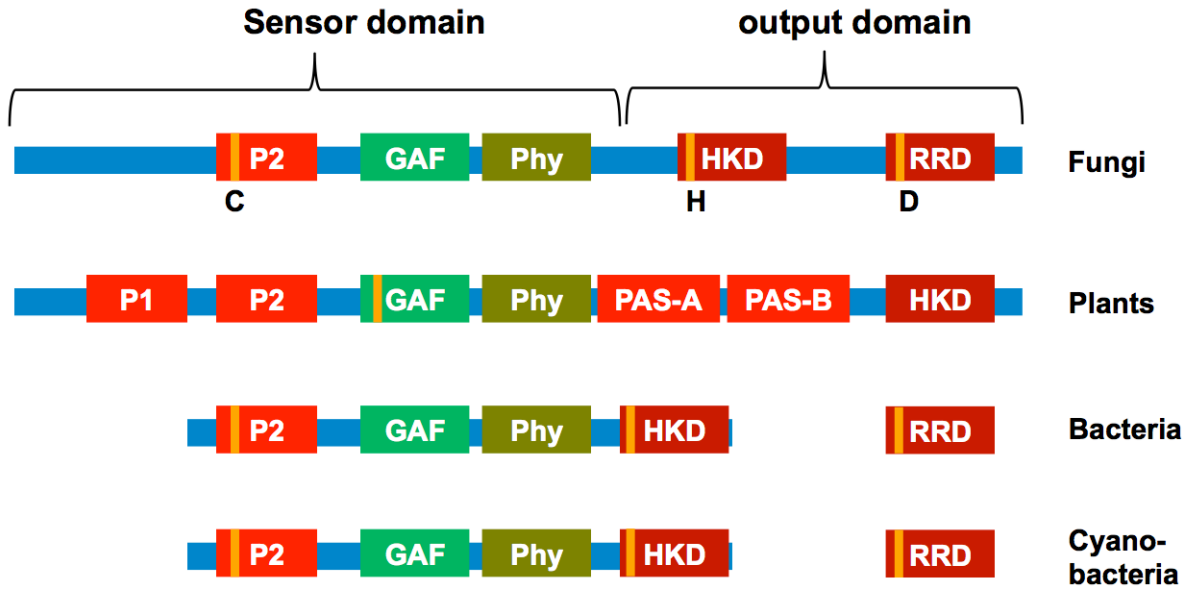


**Fig. 1. Domain organization of plant, cyanobacterial, bacterial and fungal phytochromes**. P1, P2, PAS-A and PAS-B all belong to PAS (Per-ARNT-Sim) domain; GAF, GAF (vertebrate cGMP specific phosphodiesterases, cyanobacterial adenylate cyclases, and

the transcription activator FhlA) domain; PHY, phytochrome domain; HKD, histidine kinase domain; RRD, response regulator domain; C, conserved cysteine for chromophore attachment. H and D represent histidine residue and aspartate residue respectively for autophosphorylation.

Phytochromes have been identified in various filamentous fungi including *N. crassa, A. nidulans, Fusarium graminearum, A. fumigatus*, *Ustilago maydis*, and *Botrytis cinerea* (Blumenstein *et al.*, 2005; Kim *et al.*, 2015; Fuller *et al.*, 2013; Panzer *et al.*, 2019; Hu *et al.*, 2014). *N. crassa* has two phytochrome genes, Phy-1 and Phy-2 respectively. The inactivation of the *phy-1* and *phy-2* had no effect on colony morphology, developmental processes or transcript levels of light-inducible genes (Froehlich *et al.*, 2005; Chen *et al.*, 2009). However, the expression of *con-10* was upregulated in the *phy-2* mutant independent of light suggesting a repressive role (Olmedo *et al.*, 2010). Recently, the role of Phy-2 in light-dependent repression of sexual development has been reported (Wang *et al.*, 2016). In *A. nidulans*, red light promotes asexual development and represses sexual development (Blumenstein *et al.*, 2005; Purschwitz *et al.*, 2008; Röhrig *et al.*, 2013; Mooney & Yager, 1990). Deletion of *fphA* leads to early sexual development, derepression of mycotoxin formation and conidial germination with slight abrogation of asexual developmental processes (Blumenstein *et al.*, 2005; Purschwitz *et al.*, 2008; Röhrig *et al.*, 2013) **(Fig. 2).** Interaction between *A. nidulans* phytochrome (FphA) and histidine phosphotransferase protein (YpdA) in the dark stimulates FphA kinase activity suggesting a role for the phytochrome in the dark (Blumenstein *et al.*, 2005; Brandt *et al.*, 2008; Yu *et al.*, 2016). Light-dependent induction of conidiation genes such as *brlA, wetA, abaA* and fluffy genes were tightly connected to FphA (Ruger-Herreros *et al.*, 2011). Deletion of a phytochrome-like histidine kinase gene in *B. cinerea* resulted in altered and reduced radial growth, sclerotia production, cell wall chitin content and pathogenicity on plants (Hu *et al.*, 2014).

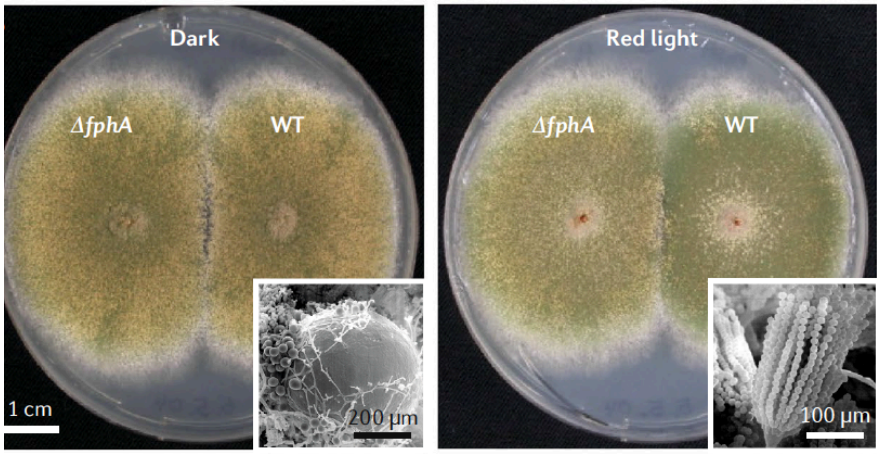


**Fig. 2. Role of *fphA* in *A. nidulans*.** *A. nidulans* strains SAB2 (*ΔfphA*) and FGSC4 (WT) were point-inoculated on agar plates and incubated in the dark (left) or under red-light irradiation (right). The indicated areas were enlarged and displayed as inserts. Deletion of *fphA* enhanced the rate of sexual development under red light illumination (670 nm), while the wildtype produces asexual, green conidiospores (Blumenstein *et al.*, 2005).

Beyond *A. nidulans* and *B. cinerea* phytochromes, light inhibits spore germination in *A. fumigatus* (Fuller *et al.*, 2013). Deletion of the phytochrome lead to release of spores in light and reduced stress tolerance against oxidative and cell wall perturbing agents (Fuller *et al.*, 2013). In *B. bassiana*, red and far-red-light- dependent stimulation of asexual sporulation was reduced in the phytochrome deletion strain with enhanced oxidative stress response against hydrogen peroxide (Qiu *et al.*, 2014). Δ*Bbphy* displayed high sensitivity to osmotic stress, UV and conidial thermal tolerance.

## 1.2 Blue-light-sensing system

### 1.2.1 The white-collar complex (WCC)

The *N.* crassa white-collar complex (WCC) consists of white collar-1(WC-1) and white collar-2 (WC-2) which together form hetero- and homodimers in vitro and function as transcription factor (Ballario *et al.*, 1998). The WC-1 protein contains two PAS(Per-Arnt-Sim) domains, a GATA type zinc finger (ZnF) DNA binding motif and a nuclear localization signal (NLS)(Ballario *et al.*, 1996; Linden & Macino, 1997). The N-terminal PAS domain of WC-1 is classified as a LOV domain (light-oxygen -voltage), noncovalently binding flavin adenine dinucleotide (FAD); a likely chromophore for blue-light-dependent responses. (He *et al.*, 2002; Cheng *et al.*, 2003; Froehlich *et al.*, 2002). WC-2 protein has a zinc-finger and a PAS domain with the exception of a LOV domain

(Linden & Macino, 1997). The orthologues of WC-1 and WC-2 in *A. alternata* are LreA and LreB, respectively. They share similarity in domain orientation with WC-1 and WC-2. The 1025 amino acid long LreA has a LOV domain, two PAS domains, a NLS, and a ZF domain at the C-terminus **(Fig. 4)**. The NLS, however, is not functional (Wang *et al.*, 2015).The 422 amino acid long LreB has a PAS domain and a ZF at the C-terminus (**Fig. 3).**

*N. crassa* WCC has both, light and dark functions. The deletion of the LOV domain of *wc-1* resulted in the loss of light but not dark functions (He *et* al., 2002). The WCC has a role as transcription factor and modulates a range of biological responses in *N. crassa*, including the switch between asexual and sexual development, carotenoid formation, phototropism of perithecial peaks, protein modification and transcriptional regulation of gene expressions, circadian clock and reactive oxygen species(Lakin-Thomas *et al.*, 1990; Linden & Macino, 1997). The deletion of *wc-1 and* wc-2 in *N. crassa* leads to the complete loss of most light-dependent responses (He *et al.*, 2002; Collett *et al.*, 2002; Lee *et al.*, 2003).

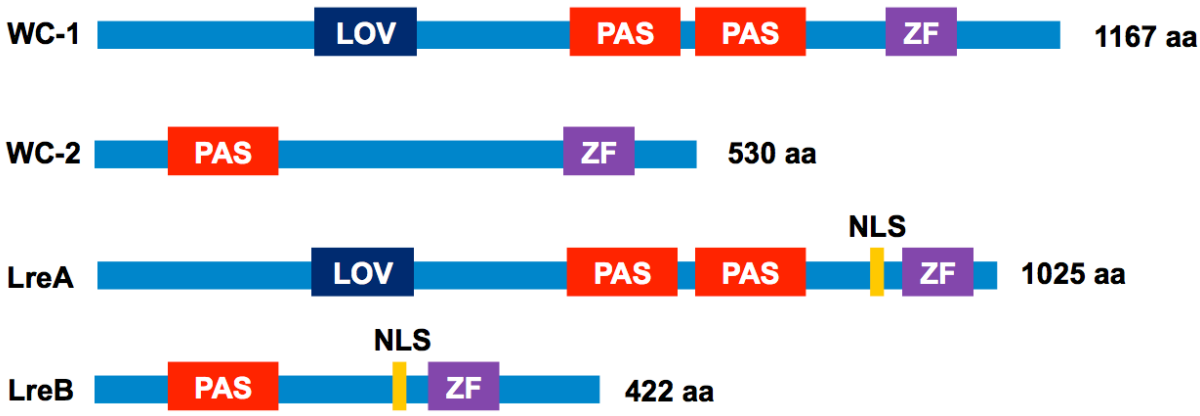


**Fig. 3. Domain organization of *N. crassa* WC-1 and WC-2, and *A. alternata* LreA and LreB.** The blue-light receptors WC-1 and LreA both harbor a LOV (Light-Oxygen-Voltage) for FAD (flavin-adenine dinucleotide) binding, two PAS (Per-ARNT-Sim) domains for protein interactions, and a GATA type zinc-finger DNA binding (ZF) domain. WC-2 and LreB both contain a PAS and a ZF domain

In *A. nidulans*, deletion of the WCC orthologues, LreA and LreB had no effect on light-inducible responses (Purschwitz *et al.*, 2008). Conidiation was enhanced in the *lreA*- and *lreB*-deletion strains irrespective of light and dark. Both deletion strains exhibited reduced mycotoxin synthesis and reduced sexual structures (cleistothecia). This suggests a role for WCC in *A. nidulans* as activators of mycotoxin biosynthesis

and sexual development as well as a repressor of asexual development (Purschwitz *et al.*, 2008). *B. cinerea* WCC modulates light-dependent responses and conidiation in response to stress and environmental conditions (Canessa *et al.*, 2013). Deletion of *bcwcl1* resulted in the release of conidiation independent of light, reduced ability to cope with excessive light and oxidative stress as well as reduced virulence against plant defense mechanisms. Although several transcriptional responses are abolished in the *bcwcl1* deletion strain, the expression of some genes is still induced by light revealing a complex network of blue light sensing systems (Canessa *et al.*, 2013).

In *Trichoderma atroviride*, stimulation of asexual development and light-induced expression of the photolyase gene, *phr-1* is dependent on the orthologues of the WCC Brl1and Brl2 respectively (Casas-Flores *et al.*, 2004). In the dark, deletion of *brl1* resulted in the downregulation of proteins while several proteins were upregulated in the *brl2*- mutant. This suggests independent and complex roles of BLR proteins in the modulation of responses (Sanchez-Arreguin *et al.*, 2012). Mutations of *Cyptococcus neoformans BWC1*, but not *BWC2* rendered the mutant strains insensitive to light (Idnurm & Heitman, 2005). The *bwc1* mutants were highly sensitive to ultraviolet radiation. The inhibitory effect of light on mating and fruiting body formation was released in the *bwc1*- and *bwc2*-deletion strains (Idnurm & Heitman, 2005). Recently, the biological function of the white collar- 1 orthologue, *sfwc1* of the homothallic ascomycete *Sordaria fimicola* in the regulation of phototaxis and fruiting body formation was shown (Krobanan *et al.*, 2019). The *sfwc1* mutant strain exhibited altered expression of light-inducible genes, lacked the fruiting-body zonation and was defective in the phototropism of the perithecial breaks (Krobanan *et al.*, 2019).

### 1.2.2 LOV domain proteins: VIVID and ENVOY

Some fungi contain LOV proteins with an N-terminal cap which regulates photoadaptation in blue light mediated responses. The first member of this group, VIVID, was identified in *N. crassa* with 186-amino acid and binding a flavin (FAD) as a chromophore (Zoltowski *et al.*, 2007; Schwerdtfeger & Linden, 2003) **(Fig. 4).** The expression of VVID is dependent on the WCC. Blue light activation of VIVID enables it to interact with the LOV domain of WC-1, disrupting the WCC complex required for further transcriptional activation of the WCC (Chen *et al.*, 2010a; Hunt *et al.*, 2010;

Malzahn *et al.*, 2010). This negative feedback loop is termed photoadaptation and is dependent on the amount of VVD present in the system.

An orthologue of VVD is the 207-amino acid protein ENVOY identified in *Trichoderma reesei* (*Hypocrea jecorina*) (Schmoll *et al.*, 2005). ENVOY is a functional LOV photoreceptor with a conformational change similar to the light-induced VIVID. Similar to VIVID, ENVOY expression is regulated by the WCC complex. However, in contrast to the negative feedback provided by VIVID, ENVOY appears to provide a positive output on WCC activity (Tisch & Schmoll, 2013). It is involved in light-mediated regulation of various processes including growth, conidiation, sexual development, G-protein signalling, cellulase gene transcription, hydrophobins and sulphur metabolism (Schuster *et al.*, 2007; Schmoll *et al.*, 2009; Seibel *et al.*, 2012). Even though VIVID and ENVOY share similarity in their domain orientation, ENV1 is incapable of complementing the deletion mutant of VIVID suggesting a complex and differential role in signal transduction (Schmoll *et al.*, 2005).


**Fig. 4. Domain organization of *N. crassa* VVD and *T. reesei* ENVOY.** The *N. crassa* and *T. reesei* vivid (VVD) contain light, oxygen and voltage (LOV) domains where FAD is covalently bound to a cysteine upon illumination.

### 1.2.3 Cryptochromes and photolyases

Cryptochromes and photolyases are blue/UV-A photoreceptors containing an N-terminal domain (photolyase-related (PHR) region) that binds noncovalently to the flavin chromophore FAD. In addition to FAD, methenyltetrahydrofolate (MTHF) and deazariboflavin are found in cryptochromes and photolyases as chromophores (Sancar, 2003; Klar *et al.*, 2006; Geisselbrecht *et al.*, 2012; Kiontke *et al.*, 2014). Cryptochromes are distinguished from photolyases by carboxy-terminal extensions of variable length and the absence of DNA repair activity (Kim *et al.*, 2014; Liu *et al.*, 2011; Fischer *et al.*, 2016; Fuller *et al.*, 2015). Cryptochromes are widely distributed among bacteria and eukaryotes and can be classified into three main groups; the animal cryptochromes, plant cryptochromes and CRY-DASH proteins with similarities

to cryptochromes from *Drosophila*, *Arbididoposis*, *Synechocystis* and Human. However, CRY-DASH proteins are absent in humans or *Drosophila*. Cryptochrome is indispensable in regulation of circadian clock in animals.

All species, except plants and animals, encode a member of cryptochrome/photolyases (CPD) protein with dual roles as a photoreceptor and DNA-repair activity. In *N. crassa*, the deletion of the CRY-DASH orthologue affected no light-dependent responses. However, the roles of CRY-DASH cryptochromes in signal transduction has been observed in other filamentous fungi including *A. nidulans, T. atroviride*, *F. fujikuroi and Sclerotinia sclerotiorum* (Bayram *et al.*, 2008a; Garcia-Esquivel *et al.*, 2016; Castrillo *et al.*, 2013; Veluchamy & Rollins, 2008). The deletion of *cyrA* in *A. nidulans* **(Fig. 5)** resulted in derepression of sexual development and genes encoding the regulation of fruiting body formation (Bayram *et al.*, 2008a). Inactivation of the *T. reesei cry1* gene showed decreased repair ability of conidia when exposed to UV light. Light- induction of *cry1* depended on the white-colar-1 orthologue, *brl1* revealing a cross talk with the WCC (Guzman-Moreno *et al.*, 2014). Similarly, the *T. atroviride* cryptochrome/photolyase gene regulates photoreactivation and gene expression in blue and red light. The expression of *blr1*-independent genes in the *cry1* deletion strains were independent of red and blue light (Garcia-Esquivel *et al.*, 2016). In *S. sclerotiorum* , deletion of cryptochrome/photolyases (CPD) resulted in a decrease in sclerotia formation when exposed to UV-A (Veluchamy & Rollins, 2008). In *F. fujikuroi*, *cryD* mutants showed altered morphology in light and increased production of bikaverin (secondary metabolite) under nitrogen starvation. This suggests a negative role for CryD in secondary metabolite biosynthesis and fitness in light.


**Fig. 5. Domain organization of *A. nidulans* cryptochrome.** The cryptochrome in *A. nidulans* harbours a photolyase and FAD binding domain as chromophore.

## 1.3 Green-light sensing with opsin

Opsins belong to the large family of rhodopsins with similarity to the animal type 2 rhodopsins. It consists of seven transmembrane helix motif that binds retinal as a chromophore. The chromophore is covalently linked to a conserved lysine residue through a Schiff base (Ernst *et al.*, 2014). Whereas the retinal conformation upon illumination changes to *13-cis* (Sharma *et al.*, 2006; Spudich, 2006), the orientation is all-*trans* in the dark. The conformation change is important for appropriate biological responses. Opsin genes are evenly distributed among Archaea, fungi and bacteria. Besides the green light sensing rhodopsin, there are also opsin-related proteins (ORPs) and an auxiliary ORP-like rhodopsin called CarO-like rhodopsins in various fungi (Brown & Jung, 2006; Wang *et al.*, 2018). Eventhough opsins have been identified in various fungi including *A. alternata* **(Fig. 6)**, there is still lack of information on their role in signal transduction. The first characterized opsin in ascomycetes was in *N. crassa.* Deletion of *nop-1* in *N. crassa, C. neoformans* and *F. fujikuroi* had no obvious phenotypic defects (Bieszke *et al.*, 1999; Chen *et al.*, 2009; Estrada & Avalos, 2009; Idnurm & Heitman, 2005). Recent study on *Nop-1* in *N. crassa* has shown that a *nop-1* mutant displayed early sexual development and upregulation of genes involved in oxidative stress, catalase and proton transmembrane functions (Wang *et al.*, 2018).

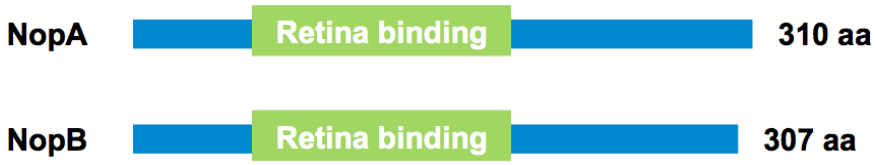


**Fig. 6. Domain organisation of opsin in *A. alternata* (NopA and NopB).** Opsin is a transmembrane protein with retinal as chromophore. Upon absorption of green light, the retinal undergoes a cis–trans isomerization mediating biological responses.

In the necrotrophic phytopathogenic fungus *S. sclerotiorum*, the microbial opsin homolog gene, *sop1* is indispensable in developmental and stress responses (Lyu *et al.*, 2015). *sop1* mutant strain displayed high sensitivity to salt stress, fungicide and osmotic stress. However, they exhibited enhanced tolerance to oxidative stress suggesting a differential role in stress response and adaptability (Lyu *et al.*, 2015). In *F. fujikuroi* and *Phaeosphaeria nodorum* opsin was shown to be an active green-light dependent proton pump (Garcia-Martinez *et al.*, 2015; Fan *et al.*, 2011). Deletion of

*carO* in *F. fujikuroi* resulted in release of spores in light (Garcia-Martinez *et al.*, 2015; Brunk *et al.*, 2018). *CarO* pump activity was highly enhanced upon exposure to glutamate, gluconate, acetate, and indole-3-acetic acid (IAA) (Garcia-Martinez *et al.*, 2015; Adam *et al.*, 2018)

The maize pathogen, *U. maydis* encodes three putative opsins, opsin1, opsin2 and opsin3. Opsin 1 and opsin 2 present in axenic cultures and induced during the entire life cycle are green light-driven proton pumps compared to opsin3 associated with plant infection lacking the proton pump activity (Panzer *et al.*, 2019). Um*ops1* expression is dependent on blue light but not red or far-red light. However, the expression of Um*ops2* is induced by blue, red and far red light. Blue light induction of Um*ops1* but not Um*ops2* was lost in Δ*wco1* strains. This suggests a possible interaction with the white-collar complex and the presence of an additional blue light receptor compensating for the loss of WCO1 activity in the expression of Um*ops2* (Panzer *et al.*, 2019).

## 1.4 Coordination of secondary metabolism in fungi: the velvet family of regulatory proteins

The velvet complex family shares a protein domain that is mostly conserved in filamentous and dimorphic fungi with a distinct role in signal transduction and metabolism (Ni & Yu, 2007; Bayram & Braus, 2012). They interact with one another and non-velvet family to modulate light sensing, interphase between asexual and sexual development, mycotoxin biosynthesis and penicillin production (Bayram *et al.*, 2008b). The biological function of the velvet family has been well studied in *Aspergillus*. *A. nidulans* consists of at least four velvet family members including VeA, VosA, VelB and VelC. They form various protein complexes with themselves as well as with the with the methyltransferase LaeA such as VosA-VelC, VosA-VelB, VelB-VeA-LaeA, and VelB-VelB, controlling asexual and sexual development, mycotoxin biosynthesis as well as viability of spores (Bayram *et al.*, 2008b; Sarikaya Bayram *et al.*, 2010; Park *et al.*, 2012; Park *et al.*, 2014). VosA-VelB protein complex coordinates spore maturation, germination and trehalose production in the conidia.

Although VeA is conserved in most fungi, the function differs in the regulation of developmental processes and secondary metabolite biosynthesis. In the dark, *A. nidulans* VeA and VelB enter the nucleus, VeA dissociates and interacts with the

methyltransferase LaeA, (Bayram *et al.*, 2008b) resulting in a heterotrimeric protein complex which regulates developmental responses and the expression of various secondary metabolite biosynthetic genes in many fungi (Wiemann *et al.*, 2010; Hoff *et al.*, 2010; Chettri *et al.*, 2012; Lind *et al.*, 2015) including *A. fumigatus* (Perrin *et al.*, 2007; Dhingra *et al.*, 2013). Apart from LaeA, there are other methyltransferases affecting velvet-mediated responses via VeA localization in the nuclei or in the cytoplasm. This includes the LaeA-like methyltransferase LlmF, VipC and VapB (Palmer *et al.*, 2013; Sarikaya-Bayram *et al.*, 2014). VipC and VapB can influence the localization of VeA by inhibiting the function of LimF. This results in the regulation of various genes involved in secondary metabolism possibly via chromatin remodelling (Sarikaya-Bayram *et al.*, 2015). VipA is another velvet interacting partner discovered in *A. nidulans*. Interestingly, apart from its interaction with VeA, it also interacts with phytochrome and the white-collar orthologue, LreA in the cytoplasm and nuclei respectively (Röhrig *et al.*, 2017).

Deletion of *veA* from *A. parasiticus* leads to a reduction in sporulation as well as a blockage in both sclerotia formation and biosynthesis of aflatoxin intermediates (Calvo *et al.*, 2004). In *B. cinerea,* the velvet protein complex regulates secondary metabolism, asexual development and virulence (Schumacher *et al.*, 2015). A *veA* mutant in *A. flavus* displayed high sensitivity to oxidative stress and a reduction in the expression levels of oxidative stress-induced genes upon exposure to hydrogen peroxide suggesting a positive role in the modulation of oxidative stress response (Baidya *et al.*, 2014). Similarly, deletion of the *veA* and *laeA* genes in *A. alternata* resulted in reduction in sporulation and altered mycotoxin production (Estiarte *et al.*, 2016). In the fungus *Pestalotiopsis microspore*, while *veA, velB* and *laeA* mutant strains displayed defects in colony growth, sporulation and pigmentation, only the *velB* mutant and not *veA* or *laeA* deletion strains was affected by Congo red (Akhberdi *et al.*, 2018). Moreover, the production of pestalotiollide B polyketide required VelB and LaeA in contrast to the repressing function of VeA in the biosynthesis of the same compound. These suggest a synergistic as well as differential role among the velvet family protein and interacting complexes in modulation of stress responses (Akhberdi *et al.*, 2018).


**Fig. 7. Domain organization of *A. alternata* VeA**. VeA consist of a velvet domain conserved for velvet family proteins, a bipartite NLS, a nuclear export signal (NES) and a PEST domain (rich in proline (P), glutamic acid (E), serine (S) and threonine (T)) motifs.

In contrast to the activating role of velvet family proteins reported in most fungi for developmental processes, the deletion of *vosA, veA* and *velB* in *A. fumigatus* resulted in increase in sporulation and an elevated expression level of *brlA* during asexual development (Park *et al.*, 2012). *vosA* and *velB* mutant strains showed reduction in the amount of trehalose in conidia and increased sensitivity to oxidative and UV stress (Park *et al.*, 2012). Recently, the biological functions of the velvet family proteins and methyltransferase orthologues in developmental responses were characterized in *N. crassa* (Bayram *et al.*, 2019). The localization of VE-1, VE-2, VOS-1, and LAE-1 in the nucleus and cytoplasm were independent of light. Furthermore, VE-1, VE-2, and LAE-1 are involved in the production of protoperithecia during sexual development. *N. crassa* VE-1 and VE-2 successfully complement *A. nidulans veA and velB* mutant strains suggesting a conserved role of the proteins (Bayram *et al.*, 2019).

## 1.5 The MAPK (mitogen-activated protein kinase) cascade

Mitogen-activated protein kinase (MAPK) is a chain of protein kinases which communicate signals from different receptors on the surface of the cell to the DNA in the nucleus. They are important for cell responses from eukaryotes in yeast to human (Qi & Elion, 2005; Raman *et al.*, 2007; Keshet & Seger, 2010). MAPK activity is regulated through a three-tiered cascade composed of a MAPK, a MAPK kinase (MAPKK, MEK) and a MAPK kinase kinase (MAPKK, MEKK) (Qi & Elion, 2005; Raman *et al.*, 2007; Keshet & Seger, 2010). Substrates for the MAPKs include other kinases and transcription factors. These protein kinases can be activated by a wide range of stimuli and stress agents including oxidative, osmotic stressors. These stimuli are detected upon ligand binding to the cell surface by membrane receptors and communicated directly to the MAPKK (Gonzalez-Rubio *et al.*, 2019). Activated MAPKK kinase phosphorylates the MAPK kinase at specific serine/threonine sites which

thereafter results into phosphorylation of a MAP kinase. The activation of the MAPK cascade results in the activation of transcription factors and the expression of specific sets of genes in response to environmental stimuli. Each organism has multiple distinct MAPK cascades involved in the transduction of extracellular signals to cellular responses. In the model organism, *S. cerevisiae*, five MAP kinase pathways have been identified and characterized (Gustin *et al.*, 1998). They play distinct roles in cell wall integrity (protein kinase C, PKC), mating (Fus3), invasive growth (Kss1), and growth under high osmolarity conditions (HOG1) and ascospore formation (Smk1) (Gustin *et al.*, 1998; Breitkreutz & Tyers, 2002). Unlike most filamentous fungi with only three MAP kinase pathways, several *Aspergillus* species, including *A. nidulans*, *A. niger* and *A. fumigatus* have four MAP kinase genes (Reyes *et al.*, 2006; Hamel *et al.*, 2012; Wang *et al.*, 2013). MAP kinases homologous to the yeast Fus3/Kss1 have been identified and characterized in different fungi including *N. crassa, Candida albicans, A. nidulans, A. fumigatus, Cochliobolus heterostrophus*, *Colletotrichum lagenarium*, *C. gloeosporioides*, *Pyrenophora teres* among other pathogenic and non-pathogenic species. In *A. nidulans* homologs of the Fus3 module, SteC (Ste11), MkkB (Ste7), MpkB (Fus3), SteD (Ste50) and AnSte12/SteA (Ste12) have been shown to mediate asexual and sexual development as well as secondary metabolite biosynthesis (Wei *et al.*, 2003; Vallim *et al.*, 2000; Teague *et al.*, 1986; Bayram *et al.*, 2012; Paoletti *et al.*, 2007). In *A. fumigatus,* a Fus3 ortholog, MpkB is crucial for conidiation and dihydroxynaphthalene (DHN)-melanin production (Manfiolli *et al.*, 2019). In *M. grisea*, the MPS1 pathway (homologous to *S. cerevisiae* Slt2 pathway) and the PMK1 pathway (homologous to *S. cerevisiae* Fus3 and Kss1 pathways) have been shown to be involved in penetration peg and appressorium formation respectively (Dean *et al.*, 2005).

*T. atroviride* and *T. reesei* genomes encode three MAPK pathways, Tmk1, Tmk2, and Tmk3, respectively which have been shown to be crucial for asexual sporulation (Medina-Castellanos *et al.*, 2018; Wang *et al.*, 2014). The expression of *tmk3* is light-dependent in *T. reesei* and in *T. atroviride*. Tmk3 regulates asexual development and responses to osmotic and cell wall perturbing agents (Delgado-Jarana *et al.*, 2006; Wang *et al.*, 2013; Esquivel-Naranjo *et al.*, 2016; Wang *et al.*, 2014). Overall, studies have indicated that MAP kinase pathways may play important

roles in regulating growth, differentiation, survival, secondary metabolite biosynthesis as well as pathogenicity.

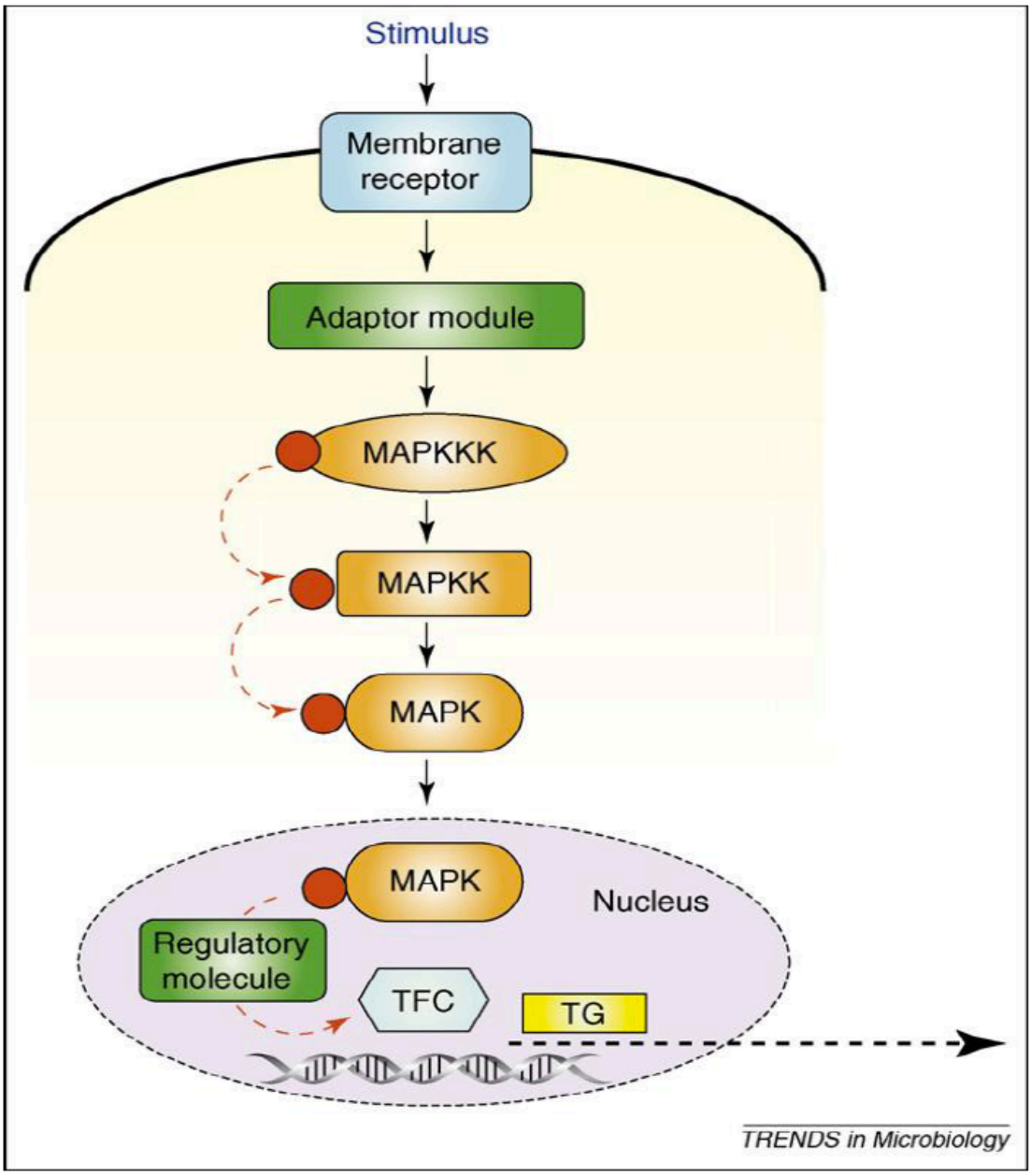


**Fig. 8. General scheme of a MAPK pathway.** The plasma membrane perceives stimulus through specific membrane receptors. The receptor then transfers the perceived signal or stimulus through phosphorylation to the core MAPK cascade which is composed of at least one of either of a MAPK, a MAPK kinase (MAPKK, MEK) and a MAPK kinase kinase (MAPKK, MEKK). This is transmitted via a two-component system or adaptor molecules. Upon phosphorylation, the MAP kinase is translocated to the nucleus where it phosphorylates a transcription factor. The activated transcription factor might be part of a transcription factor complex TFC or a repressor molecule which mediates the expression of the target gene(s) (TG) (Roman *et al.*, 2007).

## 1.5.2 The stress-activated MAP kinase (HOG) pathway in fungi

Stress-activated protein kinases or SAPKs are MAP kinases involved in transducing different signals leading to the activation of downstream targets which influences the fitness of the organism (Chen *et al.*, 1996; Franklin *et al.*, 1998). An integral part of the SAPKs is Hog1 and its homologs which in filamentous fungi employ the TGY dual phosphorylation site for signal transduction (Kyriakis & Avruch, 2012). The function of Hog1 in the model yeast *S. cerevisiae* was first limited to osmoregulation (Brewster *et al.*, 1993). However, it was later discovered that Hog1 is important in the adaptation to citric acid, heat and cold stress as well as a role in cell wall integrity, regulation of transcription and translation processes, cell cycle progression and cell membrane ion transport (Lawrence *et al.*, 2004; Winkler *et al.*, 2002; Bilsland *et al.*, 2004; Hayashi & Maeda, 2006; Panadero *et al.*, 2006; Martinez-Montanes *et al.*, 2010; de Nadal & Posas, 2015). In *S. pombe,* phosphorylated Spc1 MAPK in the HOG pathway enters the nucleus to phosphorylate and activate a bZIP TF, *Atf1* which mediates biological functions including conjugation, meiosis as well as osmotic stress response (Shiozaki & Russell, 1996; Wilkinson *et al.*, 1996).

In several filamentous fungi, including *N. crassa*, *A. nidulans, C. lagenarium*, and *M. grisea*, the HOG pathway has been shown to be mostly involved in osmotic and oxidative stress responses, development and virulence (Eaton *et al.*, 2008; Heller *et al.*, 2012; Lamb *et al.*, 2012; Van Nguyen *et al.*, 2013; Nimmanee *et al.*, 2015). *M. grisea* OSM1, was the first HOG1/Spc1/p38 homolog characterized. Deletion of *osm1* resulted in a reduction of asexual sporulation, the inability to cope with hyperosmotic conditions as well as a defect in the accumulation of arabitol in the mycelium (Dixon *et al.*, 1999). Similarly, the OS-2 MAP kinase gene in *N. crassa* plays a role in osmoregulation, fungicide resistance, and response to oxidative stress (Noguchi *et al.*, 2007; Zhang *et al.*, 2002). The *os-2* mutant showed loss of induction of clock-controlled genes, *ccg-1, bli-3* and *con-10* (Watanabe *et al.*, 2007).

In *A. nidulans*, SakA is phosphorylated in response to light, nutrient starvation and hypoxia stress (Lara-Rojas *et al.*, 2011; Yu *et al.*, 2016; Fischer *et al.*, 2016). Deletion of *sakA* resulted in early production of sexual cleistothecia and a defect in asexual sporulation. Light induction of *ccgA* was lost in the *sakA*-deletion strain suggesting a cross talk between the light and osmosensing MAP kinase cascade (Yu *et al.*, 2016). In *A. fumigatus*, Hog1 orthologs SakA and MpkC are crucial for

osmoregulation, oxidative stress response, ability to cope with cell wall perturbing agents, carbon source utilization and virulence (Reyes *et al.*, 2006; Altwasser *et al.*, 2015; Valiante *et al.*, 2015; Bruder Nascimento *et al.*, 2016).

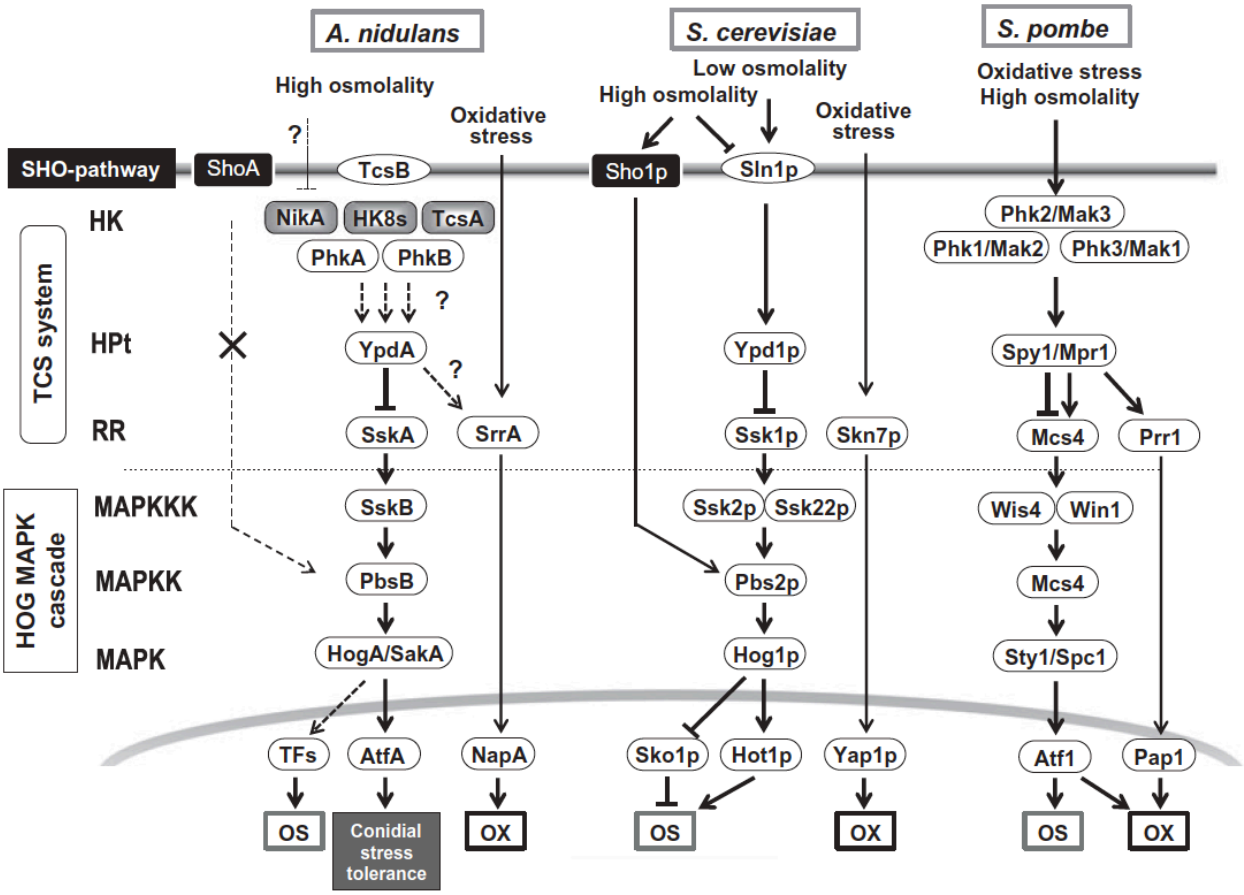


**Fig. 9. Scheme of the HOG pathway signalling cascades in *A. nidulans*, *S.* cerevisiae, and *S. pombe*.** Sho1p (SHO-pathway) activates Pbs2p in *S. cerevisiae*, whereas the homologue in *A. nidulans* has no function. Output responses are represented as OS: osmotic stress response, OX: oxidative stress response (Hagiwara *et al.*, 2016).

The deletion of *Bcsak1* of *B. cinerea* resulted in reduced virulence as well as increased sensitivity to oxidative stress from hydrogen peroxide (Segmuller *et al.*, 2007). However, *Bcsak1* mutant strains exhibited enhanced resistance to the dicarboximide contact fungicide, iprodione (Segmuller *et al.*, 2007). Recently, it was shown that SakA regulates development and Aflatoxin B1 biosynthesis in *A. flavus* (Tumukunde *et al.*, 2019). In the absence of osmotic stress, the *sakA* mutants produced more sclerotia than the wild-type strain. However, this was completely inhibited in medium supplemented with osmotic stress agents. Furthermore, deletion

of *sakA* resulted in the increase of Aflatoxin $B_1$ biosynthesis suggesting a repressing role for SakA in Aflatoxin $B_1$ production in *A. flavus* (Tumukunde *et al.*, 2019*)*.

## 1.6 Light signalling cascades in filamentous fungi

### 1.6.1 Light sensing in *N. crassa*

Blue light regulates many biological functions including asexual and sexual development, carotenoid formation, direction of sexual spore release and a major player in the resetting of the circadian clock in *N. crassa* (Bahn *et al.*, 2007; Ballario & Macino, 1997; Chen *et al.*, 2010b; Corrochano, 2007; Corrochano, 2011; Herrera-Estrella & Horwitz, 2007; Linden & Macino, 1997; Purschwitz *et al.*, 2006). In order to better understand the mechanism of gene regulation in response to light in *N. crassa* , several studies have identified light-dependent genes (Chen *et al.*, 2009; Dong *et al.*, 2008; Lewis *et al.*, 2002; Smith *et al.*, 2010) based on microarray analysis. The estimated light-controlled genes varied from 3% to 14% of the total genome (Chen *et al.*, 2009, Dong *et al.*, 2008, Lewis *et al.*, 2002).

ChIP-seq was used to identify approximately 400 direct targets as well as 27 transcriptional factor genes dependent on the light- activated WCC cascade (Smith *et al.*, 2010). This was in agreement with a previous study which reported the role of the light-activated WCC in the expression of early light-induced genes and few of the TFs genes dependent on WCC signalling cascade (Chen *et al.*, 2009). Furthermore, early light-induced proteins in turn regulate the expression of late light-induced genes. For instance, SUB-1 (*submerged protoperithecia-1*; NCU01154) and CSP-1 (*conidial separation-1*; NCU02713) which are early light-induced TFs play a role in the light-dependent regulation of a large set of late light-induced genes (Chen & Loros, 2009; Sancar *et al.*, 2011). In most of these studies, not all of the genes identified as direct targets of the WCC were light-regulated as some play a role in the dark. The expression of some of the TF genes were repressed upon illumination. This suggests a repressive function for the WCC for specific targets or probably the TFs cross-talk with the WCC cascade in the repression of genes in light (Chen & Loros, 2009; Sancar *et al.*, 2011).

The physical manifestation of the circadian clock through asexual sporulation regulated by the blue light photoreceptor WCC cascade is perhaps the main reason

that *N. crassa* has been extensively studied as the model for circadian clock research in filamentous fungi (Baker *et al.*, 2012). The WCC proteins which act as a transcription factor regulating the expression of the FREQUENCY (FRQ) protein (Crosthwaite *et al.*, 1997; Froehlich *et al.*, 2002). The interaction of FRQ with the WCC complex results in the phosphorylation of the WCC which in turn inactivates it, removing the WCC from the *frq* promoter. This negative loop is further enhanced by the removal WCC from the nucleus (Schafmeier *et al.*, 2005; He *et al.*, 2006; Hong *et al.*, 2008; Schafmeier *et al.*, 2008). The stability of the FRQ protein and inactivation of the WCC is regulated by several kinases including CK1, CK2 and PRD4/ checkpoint kinase 2 and phosphates; PP1 and PP2A (Gorl *et al.*, 2001; Yang *et al.*, 2002; Pregueiro *et al.*, 2006; Heintzen & Liu, 2007). FRQ levels decrease in the course of the subjective night. As negative feedback reaches completion in the mid to late day, FRQ level and rate of FRQ synthesis decreases. The expression level of *frq* peak in the late subjective noon and the FRQ protein level peak around 4-6 h later (Aronson *et al.*, 1994; Garceau *et al.*, 1997; Merrow *et al.*, 2001). The cycle is completed by the delayed release of FRQ-dependent repression which requires the FRQ protein to be phosphorylated for as long as 16-18h (Merrow *et al.*, 1997; Schafmeier *et al.*, 2006).The subsequent phosphorylation of the FRQ makes it attractive to the FWD-1 (F-box/WD-40 repeat-containing protein, an ortholog of the *Drosophila* Slimb protein. Similar to the function in *Drosophila* clock, the FWD-1 is a substrate-recruiting subunit of an SCF-type E3 ubiquitin ligase (Ko *et al.*, 2002; He *et al.*, 2003). Upon interaction of the FRQ with the FWD-1, the FRQ protein is ubiquitylated and targeted to the proteasome where it is degraded facilitating turned over. The cycle can then restart.

### 1.6.2 Light sensing in *A. nidulans*

Analysis of *A. nidulans* genome has revealed orthologues of most photoreceptors found in *N. crassa*. An exception is the absence of VIVID protein and a functional rhodopsin gene (Ruger-Herreros *et al.*, 2011). Genome wide expression analysis identified >400 genes upregulated and less than >100 genes downregulated when competent mycelium was exposed to 30 min of white light (Ruger-Herreros *et al.*, 2011). Some of the upregulated genes were predicted to be involved in asexual development, circadian clock, stress responses, redox reaction and carbon metabolism. A few of these genes are transcription factors predicted to be involved in

the regulation of the light signalling cascade. These TFs regulate several biological functions such as the switch in developmental phases, secondary metabolite biosynthesis as well as different stress responses (Ruger-Herreros *et al.*, 2011). Importantly, the expression of the conidiation master regulator *brlA* and the fluffy genes *flbB* and *flbC* are regulated by light. Deletion of *flbB* and *flbC* disrupted the activation of *brlA* suggesting not only the activation of conidiation by light but also an interaction between the fluffy genes and the central conidiation cascade regulated by *brlA* (Ruger-Herreros *et al.*, 2011).

*ccgB*, a homolog of the *N. crassa ccg-1* (a clock-controlled and glucose-repressed gene of unknown function) (Arpaia *et al.*, 1995; Bell-Pedersen *et al.*, 1996) displayed the highest expression level upon illumination by light. Apart from *N. crassa and A. nidulans, ccgB* is absent in the genome of related species such as *A. niger* and *A. fumigatus* (Ruger-Herreros *et al.*, 2011). While *ccgA* and *ccgB*, are induced by light in a similar manner though with a little difference, other light-induced genes includes *conJ* (homolog of the conidiation and light-dependent gene, *con-10* in *N. crassa*) (Roberts *et al.*, 1988; Olmedo *et al.*, 2010), *cryA*, a UV/blue light photoreceptor involved in sexual development in *A. nidulans* as well as the rhodopsin gene, *nopA*.

Most genes downregulated were predicted to be involved in oxidoreductase responses, nuclear components, transport and nitrogen metabolism (Ruger-Herreros *et al.*, 2011). The most downregulated gene is *veA*. The cross talk between the velvet protein VeA, the WCC and phytochrome was identified at the *ccgA* locus (Hedtke *et al.*, 2015). Whereas red light induced *ccgA* to a greater extent and rapidly, blue light does not in a similar manner. The induction of *ccgA* was completely abrogated in the *fphA*-deletion strain, whereas deletion of *lreA/B* only reduced the expression level. This suggests a crucial role for FphA in the regulation of *ccgA*. In the dark, VeA and LreA bind to the *ccgA* promoter and upon illumination with red or blue light, LreA dissociates from the promoter (Hedtke *et al.*, 2015). Interestingly binding of VeA to the *ccgA* promoter is lost in the *fphA*-deletion strain and binding of LreA is lost in the *veA*-deletion strain suggesting an interaction between the three proteins.

Moreover, the interaction between the FphA and LreA protein has been shown to be involved in histone acetylation and deacetylation resulting in gene regulation. Histone acetylation at the *ccgA* locus upon illumination is lost in both, the *fphA* and *lreA* mutant strains (Hedtke *et al.*, 2015). Therefore, LreA and VeA bind to the

promoters of light-inducible genes in the dark and attracted to histone acetylase GcnE and the histone deacetylase HdaA. LreA represses the activity of GncE and promotes HdaA activity. In light, LreA is released from the promoter and FphA and VeA promote histone acetylation by activating GcnE. In this manner, the expression of light-inducible genes are thus promoted (Yu & Fischer, 2019).

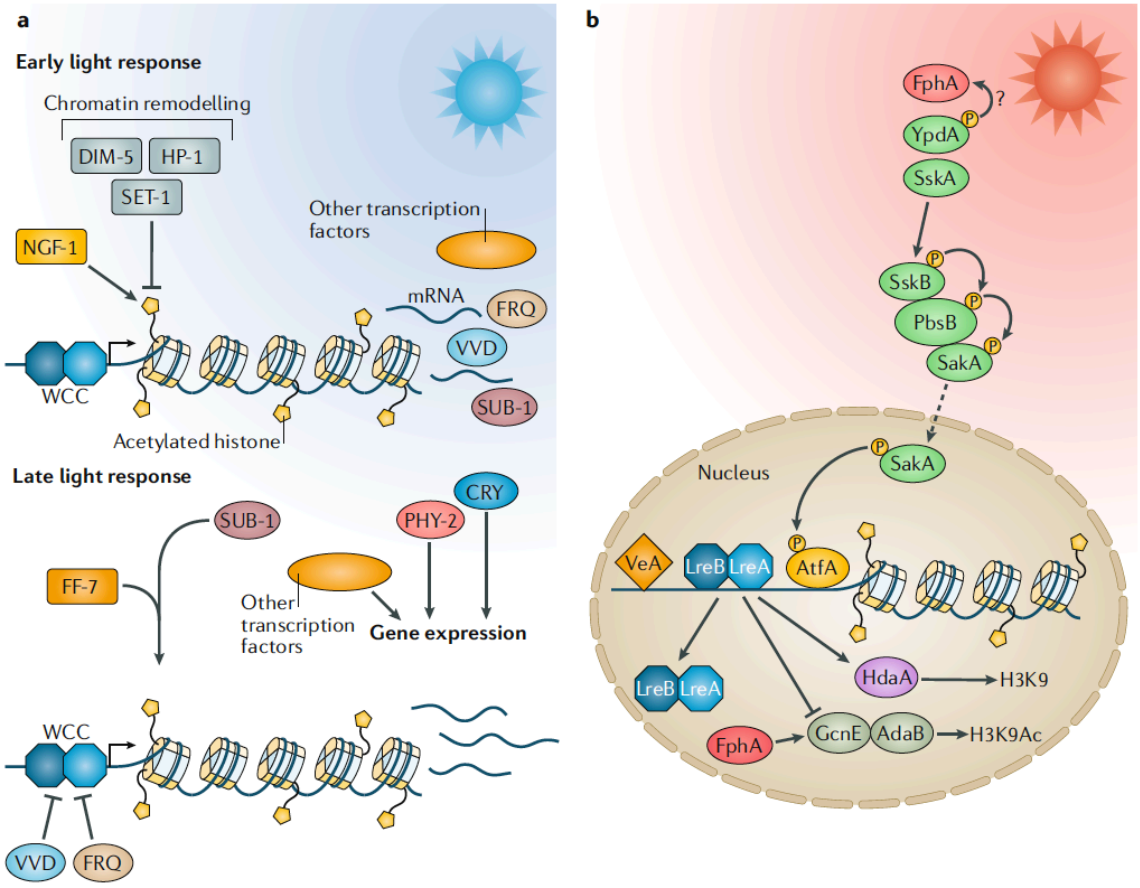


**Fig. 10. Current model of light responses in fungi**. **(a)** In *N. crassa*, upon illumination, the WCC complex binds to the promoter of early light-responsive elements (ELREs) resulting into the induction of several genes and transcription factors including *sub-1*, *frq* and vvd. The FRQ protein (via the circadian clock) regulates the first step in light response while the VVD interacts with WCC leading to photoadaptation. WCC activity and phosphorylation is mediated by several kinases and phosphatases. Chromatin modification regulates light responses. Histone acetylation through NGF-1 is crucial for the induction of several genes, whereas histone methylation through DIM-5, HP1 and SET-1 modulates the repression of light-dependent responses. SUB-1 interacts with FF7 and the WCC complex leading to the induction of late light-responsive genes. Other photoreceptor proteins in *N. crassa* such as PHY-1, PHY-2, NOP-1 and CRY as well VE-1 are indispensable in light responses both at the developmental and molecular level. The expression of TFs induced after first light response regulate several gene expression. **(b)** In *A. nidulans*, red light is perceived by the phytochrome FphA. In the cytoplasm, light-dependent activation of FphA probably leads to dephosphorylation of the phosphotransferase protein YpdA, causing activation of the high osmolarity glycerol (HOG) pathway and, via phosphorelay, activation of the MAP kinase stress-activated kinase (SakA). Phosphorylated SakA shuttles into the nucleus to phosphorylate the transcription factor AtfA,

which activates gene expression. Chromatin remodelling through acetylation of lysine 9 in histone H3 (H3K9Ac) is also involved in light signalling. LreA and VeA bind to the promoters of light-inducible genes in the dark and interacts with acetyltransferase GcnE and deacetylase HdaA. LreA represses GcnE activity and thus light-inducible genes are repressed. Upon illumination, LreA dissociates from the promoter. VeA and FphA promote GcnE activity facilitating gene expression (Yu & Fischer, 2019).

## 1.7 The role of light in plant-fungal interactions

Light is perceived by plants and by microorganisms and is used as cue for interaction. In plants, light is crucial for several biological functions including changes in hormonal levels, secondary metabolite biosynthesis, production and release of volatile compounds which in general influences plant-microbial interactions (Rahman *et al.*, 2003; Wang *et al.*, 2010; Carvalho & Castillo, 2018). Red light regulates the synthesis of cinnamic acid, tryptophan and phenylpropanoid pathways-dependent resistance in plants (Shirasawa *et al.*, 2012; Parada *et al.*, 2014). Red light also represses gray mold incidence (*B. cinerea*) in grapevine, broad bean and tomato plants (Khanam *et al.*, 2005; Ahn *et al.*, 2015; Xu *et al.*, 2017). In fungi, the red and blue light photoreceptors are crucial for secondary metabolite biosynthesis (Bazafkan *et al.*, 2017). Aflatoxin and sterigmatocystin production are repressed by white light (Bayram *et al.*, 2008b) in *Aspergillus* whereas in *A. alternata* the synthesis of alternariol and altertoxin are white and blue light-dependent (Pruss *et al.*, 2014). In the plant pathogen, *C. acutatum*, a notorious fungus capable of infecting several plants including tomatoe, strawberry, apple and mango, light qualities influences melanin production which affects the virulence ability of the fungus (Yu *et al.*, 2013). Light is thus crucial for disease development and defense mechanisms in both plants and fungi.

Furthermore, in most plant fungal pathogens, mutation of the blue light photoreceptor WC homologs attenuates their virulence ability. In the maize leaf pathogen *Cercospora zeae*-maydis, the WC-1 ortholog regulates host stomata tropism and lesion development (Kim *et al.*, 2011a). Similarly, in the rice blast pathogen *M oryzae*, MGWC-1 modulates asexual spore releases which plays a critical role in successful disease development (Kim *et al.*, 2011b).

## 1.8 Objectives of this work

Light is perceived and transduced by most filamentous fungi, where it regulates major fungal behaviour and developmental processes such as morphogenesis, sporulation, growth, stress tolerance, metabolic pathways and virulence. Most fungi including *N. crassa* and *T. atroviride* respond well to blue light and use the white-collar (WC) light sensing system for several biological responses. Although, most fungi contain in addition phytochrome as putative red-light receptor, the role of phytochrome is not clear. In *A. alternata,* blue light inhibits sporulation, but the effect can be reversed by red-light illumination, suggesting the presence and action of blue- and red-light receptors. Furthermore, there is evidence that the HOG MAP kinase pathway serves as a hub for light-dependent responses. The aim of this study is to understand the role of phytochrome (FphA), white collar 1 (LreA) and the interplay with the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway in light sensing and signal transduction in *A. alternata.*

# 2. Results

## 2.1 Evidence for light sensing in *A. alternata*

The first evidence for light sensing in the *Alternaria* genus was reported in *A. solani* (Luken, 1963). Conidiophores produced conidia upon continuous illumination with red but not with blue light. To investigate the effects of light on vegetative growth and asexual sporulation in *A. alternata*, cultures of the wild-type strain were incubated at 28 °C for 12 days in dark and different light conditions. Cultures incubated in dark, red and far-red light exhibited dark brown coloration whereas they appeared pale upon exposure to blue, white and green light **(Fig. 11A)**. Melanisation of spores was independent of light suggesting that the change of the colour of the cultures was dependent on the number of asexual spores produced **(Fig. 11B)**. Whereas sporulation was promoted in cultures exposed to red and far-red light, continuous exposure of cultures to blue, white and green light resulted in sterile aerial hyphae and sporulation was drastically repressed. The effect in green-light was not as strong as the effect of blue and white light **(Fig. 11C)**.

Since a previous study suggested an interplay between the red and blue light system in *A. alternata*, we analysed the effect of red light on the cultures exposed to blue light. Cultures were grown for 48 h in dark and then exposed to 12h of blue light, followed by 12h of incubation in the dark, red or far-red light. Furthermore, we tested another illumination condition where 12h of blue light exposure was followed by 12h of red light, and again 12 h of blue light. All cultures were further incubated for 8 days after the respective treatments **(Fig. 11D)**. The cultures exposed to red or far-red light after blue light exposure produced more spores than cultures incubated in dark after blue-light illumination. The red-light stimulation was nullified by another blue light exposure **(Fig. 11E)**. This suggests an interplay between the red and blue-light photoreceptors as shown in the reversal of blue-light inhibitory ability upon exposure to red and far-red light.

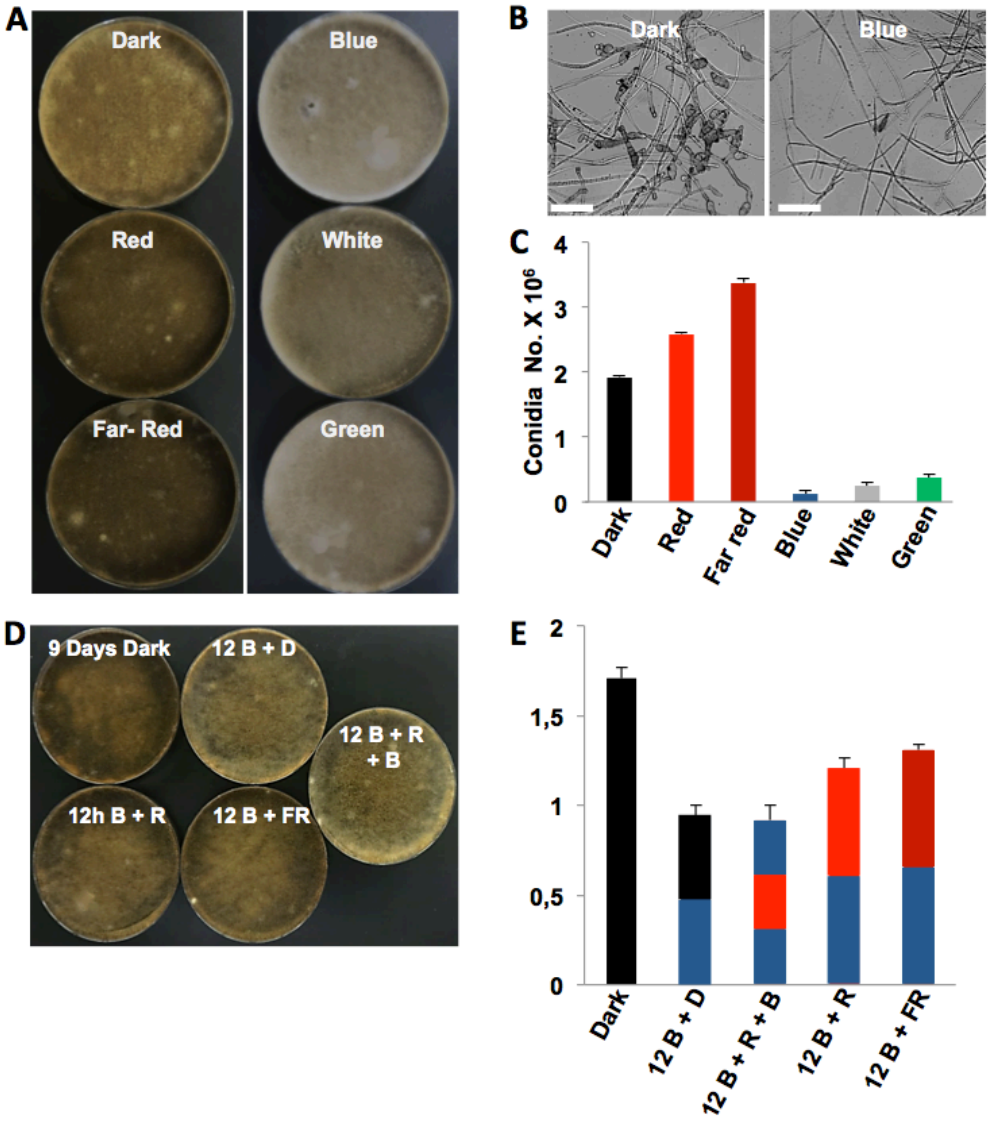


**Fig. 11. Light sensing and photoreversibility of blue-light inhibition of sporulation. (A)** Phenotypic appearance of cultures inoculated with 5 × $10^4$ fresh conidia of the *A. alternata* wild-type evenly spread on mCDB plates and incubated at 28 °C for 12 days in the dark or at different wavelengths as indicate. **(B)** Microscopic pictures of spores in dark and blue-light **(C)** Quantification of the asexual conidia produced on the plates in panel **(A).** Three independent plates of each strain were analyzed, and the mean values for the three samples are displayed. The arrow bar represents the standard deviation. **(D)** Photoreversibility of blue-light inhibition of asexual sporulation by red or far-red light. All cultures first incubated for 48 h in the dark after which they were exposed to 12 h of blue-light followed dark incubation or exposure to 12h of red or far-red light. Control culture was kept in the dark. All the cultures were further incubated in the dark until 12 days. **(E).** Quantification of asexual conidia from the cultures in panel D. Three independent plates of each strain were analyzed, and the mean values for the three samples are displayed. The arrow bar represents the standard deviation.

## 2.2 Generation of *fphA*, *lreA* and *hogA*- mutant strains using the CRISPR Cas 9 gene editing technology

The *A. alternata* genome encodes photoreceptors for blue (LreA), red (FphA), and green (NopA and NopB) light sensing. In addition to these are other components including WC-2 (LreB), the velvet protein family and HogA which have been reported to be involved in light sensing and signal transduction in other fungi. The *A. alternata* LreA consists of 1025 amino acids, and similar to the *N. crassa* WC-1 harbors a LOV domain (with a cysteine residue binding site for the chromophore), a GATA-type zinc finger, two PAS domains, and a predicted NLS. FphA comprises 1,511 amino acids consisting of a PAS domain, a GAF domain and a PHY domain. It encodes a histidine kinase (HK) domain, ATPase domain, and a response regulator (RR) in the C-terminal. The cysteine in the PAS domain and the two nuclear localization signals (NLS) of the phytochrome of *A. nidulans* are conserved in *A. alternata*. An N-terminal extension in front of the photosensory domain is also conserved compared to FphA in *A. nidulans* and *N. crassa*. The *hogA* gene encodes an open reading frame (ORF) of 1,068 bp interrupted by seven introns and giving rise to a polypeptide of 355 amino acids. It has a protein kinase ATP-binding region as well as a MAP kinase site.

In order to assign a role(s) to FphA, LreA, and HogA in light sensing in *A. alternata*, we aimed to inactivate the respective genes using the CRISPR-Cas9 technology. 20 nucleotide protospacer of the respective genes of interest (FphA, LreA, and HogA) adjacent to a 3′ AGG protospacer-adjacent motif (PAM) site close to the start of the ORF were selected and introduced into plasmid pFC332 by PCR and cloning **(Fig. 12A& Fig. S1)**. The resulting plasmid which contains the Cas9 coding sequence from *Streptococcus pyogenes* (codon optimized for *Aspergillus niger*), hygromycin resistance cassette and the single-guide RNA (sgRNA) targeting the genes of interest (FphA, LreA, and HogA) were used for transformation of the wild-type strain (ATCC 66981). Hygromycin resistant transformants were then analysed for possible mutant strains. For *fphA*, 20 hygromycin-resistant transformants were obtained out of which two of the transformants exhibited morphological differences from the rest. In the case of *lreA*, 30 hygromycin- resistant transformants were obtained, with three exhibiting a changed phenotype. Two of the 10 transformants analysed for the loss of HogA function were positive. Presumptive positive

transformants with impaired phenotypes were further analysed via PCR and sequencing of the PCR products in order to ascertain the number of deleted nucleotides. Deletions of 3,398 bp and 535bp were obtained for *lreA* and *hogA* respectively whereas in the case of *fphA*, we detected a deletion and insertion of 528bp and 102bp respectively. There was no difference in the colony growth of the *fphA* and *lreA* mutant with the wildtype strains. In contrast, the loss of the HogA resulted in a defective radial growth.

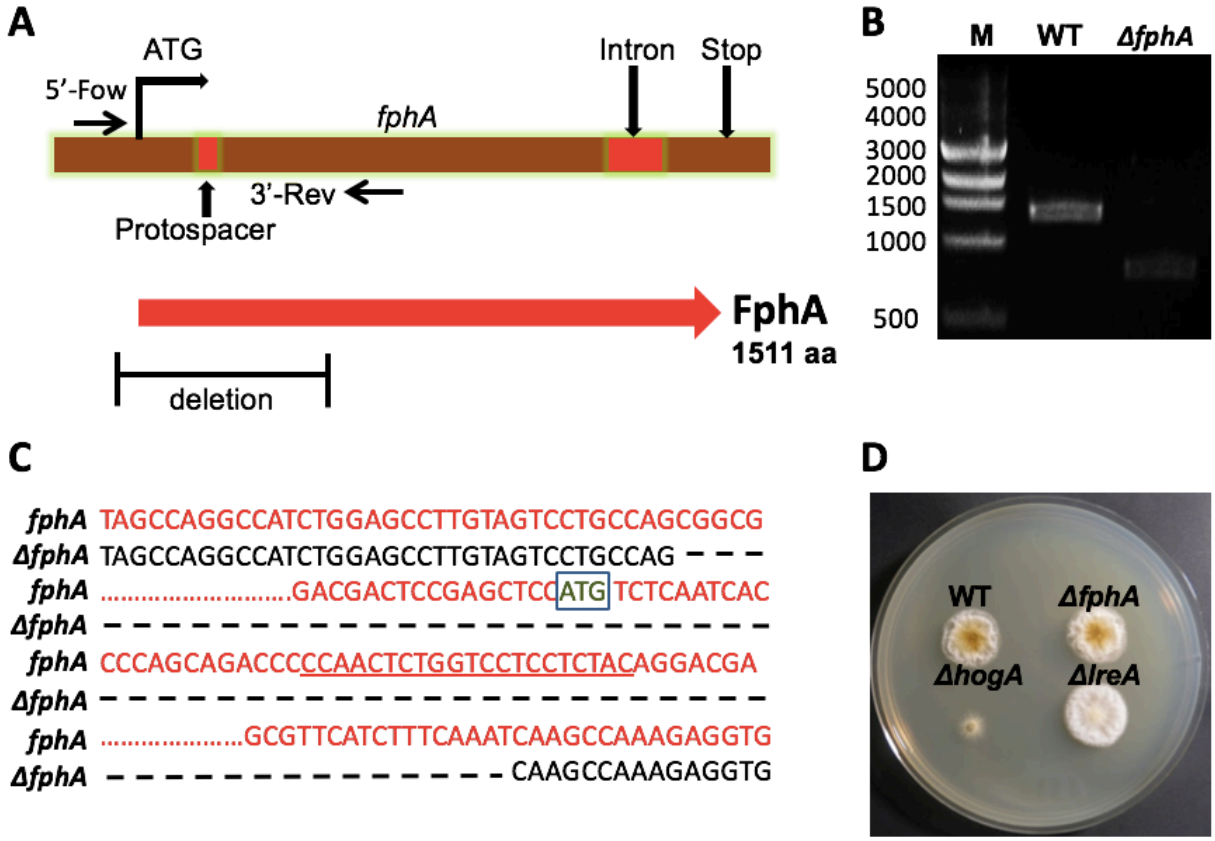


**Fig. 12. Inactivation of the *fphA* gene using CRISPR-Cas9. (A)** Scheme for the inactivation of *fphA*. The primers located upstream and downstream of the protospacer and the protospacer are indicated. The deletion is also shown. aa, amino acids. **(B)** Confirmation of the CRISPR-Cas9-induced inactivation of *fphA* by PCR using the primers indicated in panel **A** and genomic DNA as the template. **(C)** Comparison of the *fphA* mutant sequence (black) with the sequence of the wild type (red) revealed a 528-bp deletion with 467 bp of the *fphA* ORF and 61 bp of the 5′ untranslated region (UTR). The protospacer is underlined. The start codon is boxed. The dashed line shows the deleted nucleotides. The dotted line represents 961 nucleotides which were also missing and not displayed here. **(D)** Pictures of colonies of the WT and the *fphA*, *lreA,* and *hogA* mutant strains incubated at 28°C for 4 days.

## 2.3 Phytochrome and the WC-1 orthologue LreA are involved in sporulation

In order to understand the importance of FphA, LreA, and HogA in sporulation, we investigated the effect of different wavelengths on light regulation of sporulation in the wild-type and the *fphA*, *lreA,* and *hogA* mutant strains **(Fig. 13A, B)**. Continuous illumination with red and far-red light promoted sporulation compared with cultures in dark. Sporulation was repressed in blue, white and green-light. In the dark, conidiation was reduced to 86% in the *fph*A mutant strain. Red and far-red light stimulation of sporulation was abrogated in the mutant. The phenotype change as a result of the loss of function of FphA was rescued after recomplementation with a wild-type copy of FphA **(Fig. 14A, B).** This result suggests that the FphA is a positive regulator of conidiation not only in light but also in the dark. The inactivation of the *lreA* resulted into 51% reduction of conidia in the dark and no difference was observed in blue- light compared to the wild type suggesting the presence of additional blue light receptors. The reduction in sporulation in the *lreA* mutant strain was restored by recomplementation with a wild-type copy of LreA **(Fig. 14A and B).** The *hog*A mutant was drastically affected by continuous illumination as spore numbers were reduced to 48% in dark. However, the culture plates of the *hogA* mutant appeared black compared to other mutants and wild type strains suggesting a negative role for the HogA in melanin production in hyphae **(Fig.15**). Recomplementation of the *hogA* mutant with the wild-type copy was impossible due to inability to isolate viable protoplast from the *hogA* mutant strain.

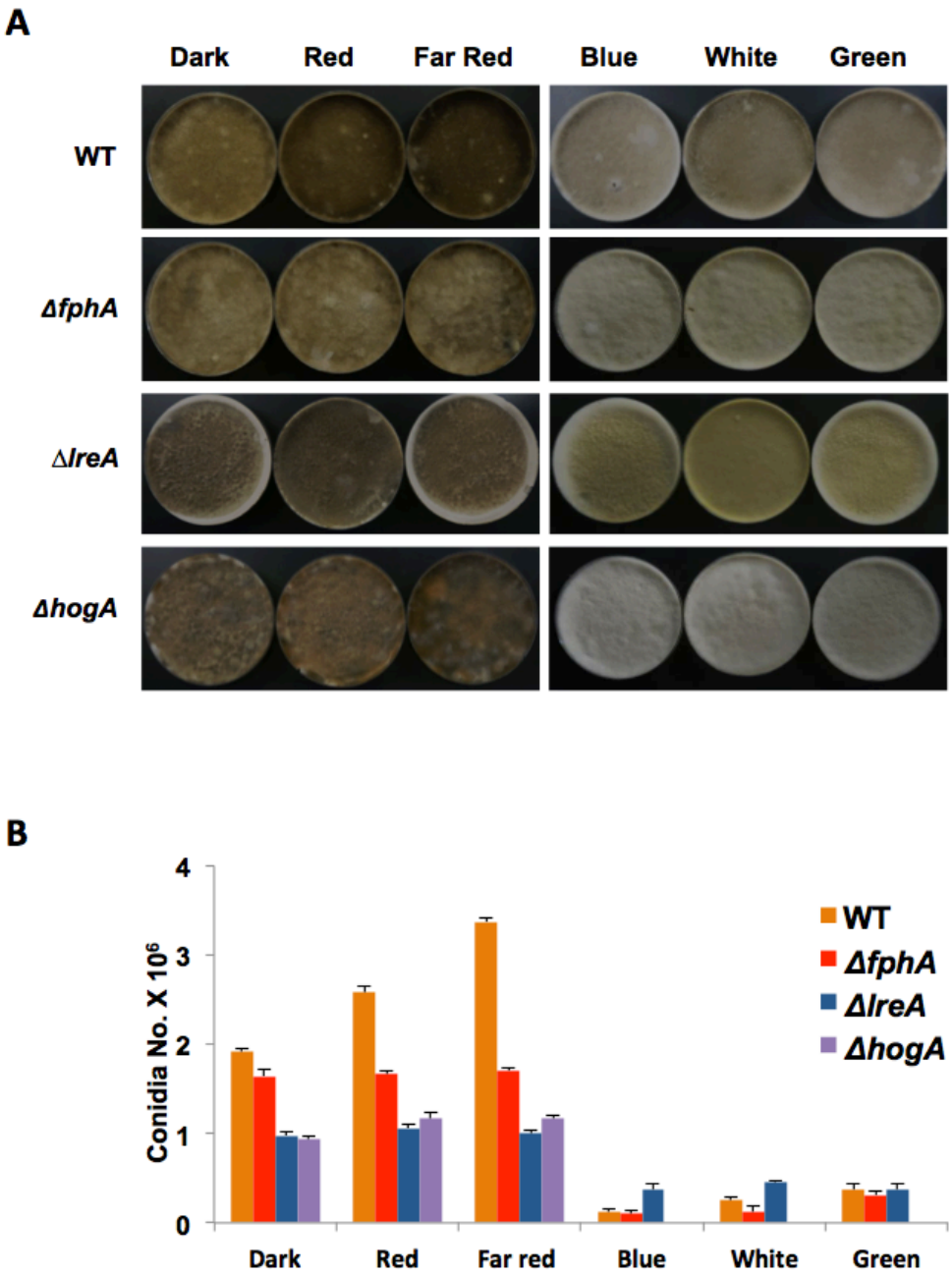


**Fig. 13. Analysis of the number of asexual spores in the *A. alternata* wild type and the *fphA*, *lreA,* and *hogA* mutant strains under different illumination conditions. (A).** Phenotypic appearance of cultures inoculated with 5 × $10^4$ fresh conidia of the *A. alternata* wild-type and corresponding mutant strains evenly spread on mCDB plates and incubated at 28 °C for 12 days in the dark or at different wavelengths as indicated. **(B)** Quantification of the asexual conidia produced on the plates in panel **(A).** Three independent plates of each strain were analyzed, and the mean values for the three samples are displayed. The arrow bar represents the standard deviation.

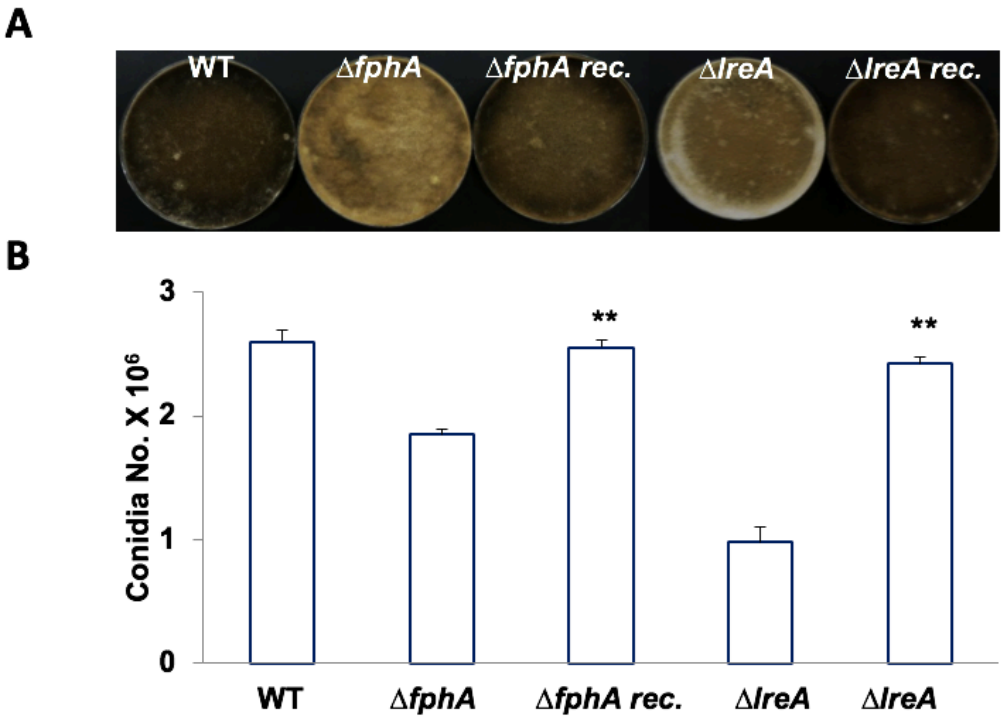


**Fig. 14. Recomplementation of the *ΔfphA* and *ΔlreA* mutant strains**. (**A)** Colony appearance of cultures inoculated with 5 × $10^4$ fresh conidia of the *A. alternata* wild-type, *ΔfphA* and *ΔlreA* and complemented strains evenly spread on mCDB plates and incubated at 28 °C for 12 days in the dark. **(B)** Quantification of the asexual conidia produced on the plates in panel **(A).** Three independent plates of each strain were analyzed, and the mean values for the three samples are displayed. The arrow bar represents the standard deviation. Statistical analysis was performed with Student's t test, **, P ≤ 0.01.

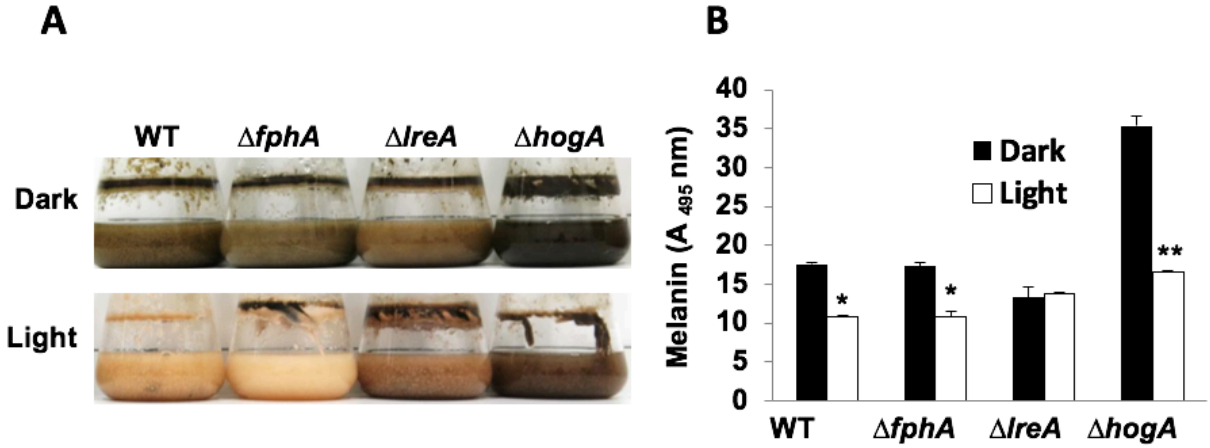


**Fig. 15. HogA negatively regulates the mycelial melanin production. (A)** Pictures of mycelium of the WT, *fphA-, lreA- and hogA*-mutant strain incubated at 28°C (shaking culture) for 7 days in white light and dark conditions. **(B)** Quantitative analysis of melanin. Melanin was purified with 2 % NaOH and the absorbance at 459 nm was measured using the spectrophotometer. All experiments were done in triplicate. The arrow bar represents the standard deviation. Statistical analysis was performed with Student t-test: P ≤ 0.05 (*), P ≤ 0.01 (**).

In *A. nidulans, B. cinerea* and *B. bassiana*, links between light and induction of asexual development genes were shown. Although the *A. nidulans* central regulator BrlA was not identified in the *A. alternata* genome, we found orthologues of many other proteins including the AbaA and WetA . *A. alternata abaA* ORF is composed of 2918 bp with three introns and two introns and is predicted to encode a 850 aa-length protein containing an TEA DNA- binding domain which is conserved in *A. nidulans* and *A. fumigatus*. The *wetA* ORF comprises 1632 bp with no intron and is predicted to encode a 566 aa- length protein with a conserved C-terminal domain. In order to assign a role for FphA and LreA in the regulation of asexual development at the gene level in *A. alternata,* we investigated light induction of five developmental genes (*abaA*, *wetA, csp-1* and *flbC*) **(Fig. 16)**. Mycelia of the WT and the *fphA*, and *lreA* mutant strains were grown in the dark at 28 °C for 36h and exposed to white light for 1 h. After RNA extraction, the amount of mRNA transcript was determined. Expression levels of *abaA* and *wetA* were strongly induced by light in the wild-type but lost in the *fphA* and *lreA* mutant strains. Hence, FphA and LreA appears to be positive regulators for *abaA* and *wetA* light induction. Next we tested the light regulation of the autophagy gene *atg1* and fluffy gene *flbC* whose expression has been shown to be light induced in *B. bassiana* and *A. nidulans* respectively. In *A. alternata,* the induction of *atg1* and *flbC* required the FphA and LreA. At last, we tested an orthologue of *N. crassa csp-1* (conidial separation-1). Light induction of this gene was independent of FphA but strictly dependent on LreA. Taken together the results suggest that FphA and LreA act as activators of asexual reproduction in *A. alternata*, while HogA plays an important role for the general fitness of the organism.

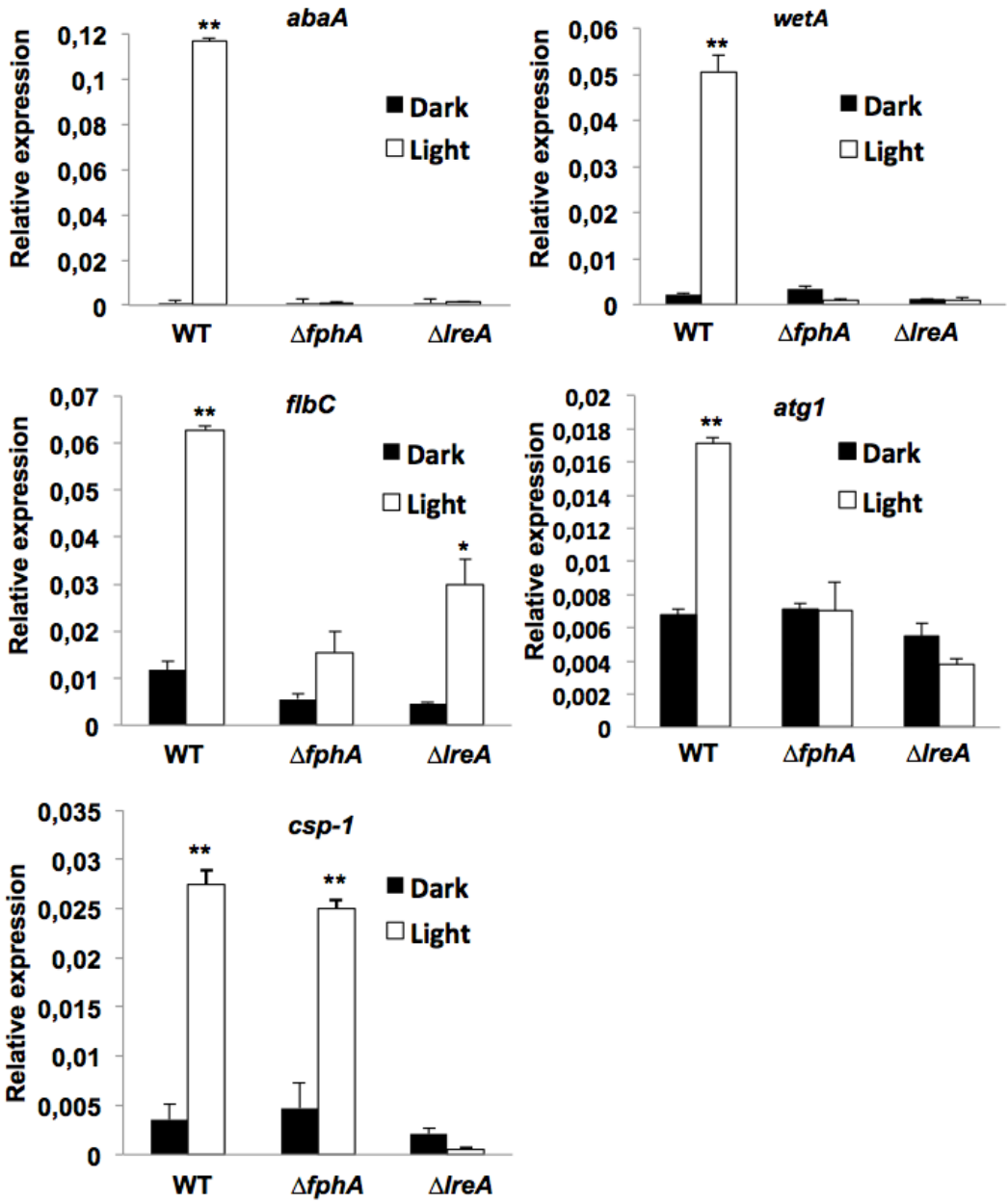


**Fig. 16. Expression analysis of asexual development genes in *A. alternata* wild type and the corresponding mutant strains.** Spores of wild type (WT), *fphA* and *lreA* mutant strains were cultured on the mCDB surface at 28 °C for 36 h in the dark. Mycelium was exposed for 1h to white light. Control samples were collected in the dark. RNA was isolated and the expression levels of the genes were normalized using H2B. The bars present mean values ± SD of three biological replicates. Statistical analysis was performed with Student's t test, *, P ≤ 0.05; **, P ≤ 0.01.

## 2.4 Phytochrome but not LreA is indispensable in light inhibition of germination

In order to test if germination of spores was affected by light, we investigated the effect of different wavelengths of lights on the germination rate. In complete medium at 28°C, germination occurred without delay and there was no difference in the wild type and the corresponding mutants except for the *hog*A mutant strain. However, in minimal medium with glucose substituted with 1% glycerol and incubation at 22°C, we found that after 2 h, ca. 50% of the spores had germinated, and after 3 h, nearly all had produced a germ tube **(Fig. 17A, B).** In red light, 30% of spores germinated after 3 h. Far-red, white, blue and green-light also inhibited germination. Therefore, we hypothesized that FphA or LreA should be involved in light inhibition of germination following their activation by red or blue-light respectively. In the case of *fphA* mutant, the inhibition of germination was released irrespective of light qualities and dark conditions even though germination was faster in dark. This suggests that the FphA negatively regulates spore germination in dark and light conditions. Similar to the *fphA* deletion strain, we hypothesized that the inhibition of germination in blue-light should be released in the *lreA* mutant strain. Contrary to this prediction, blue-light as well as other lights still inhibited the germination of the *lreA* mutant strain. This suggest the presence of additional blue light photoreceptors involved in inhibition of germination by blue-light. Deletion of the *hogA* drastically affected germination in darkness and illumination conditions suggesting an important role for the HogA in light adaptability **(Fig. 17A, B)**.

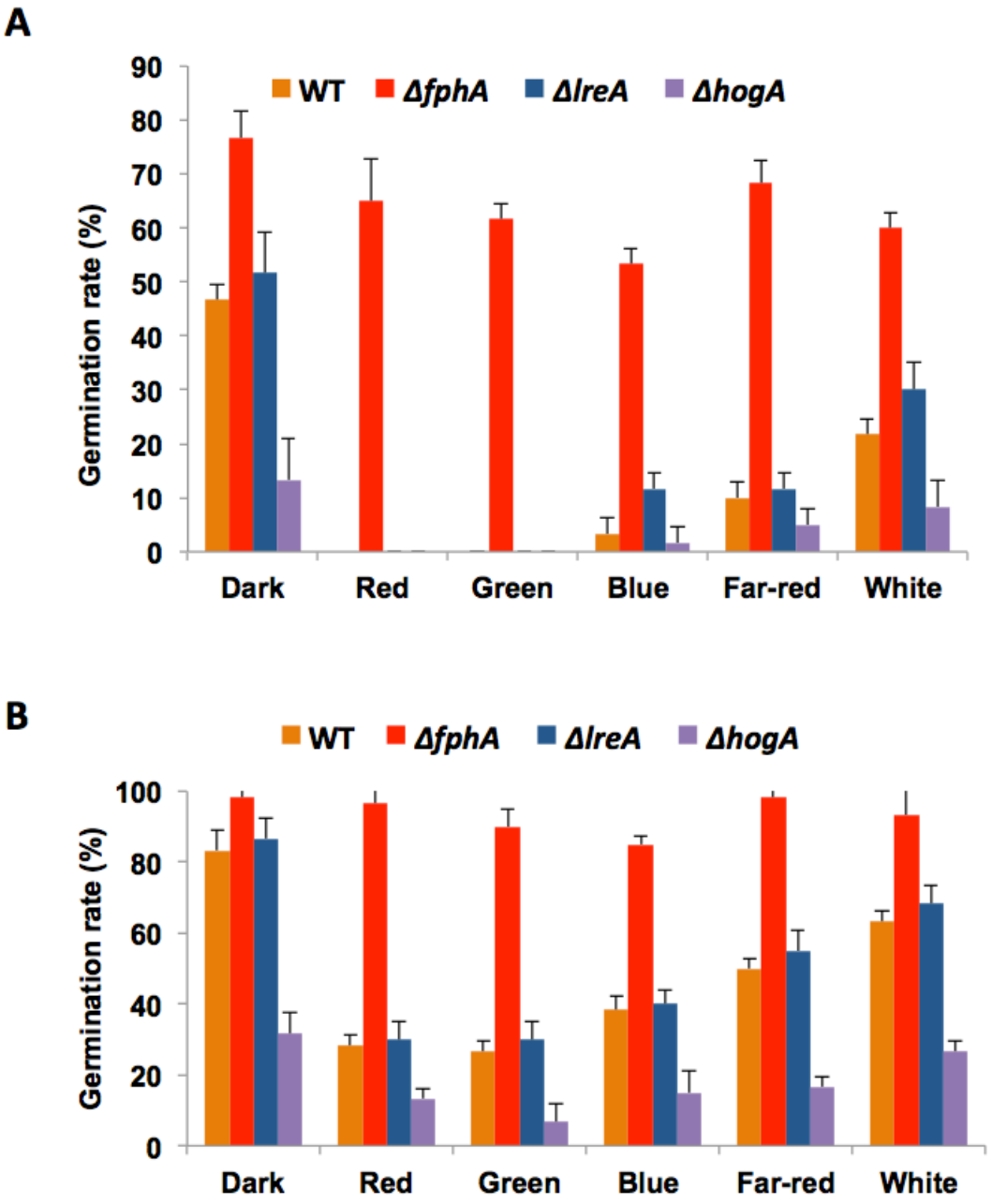


**Fig. 17. Light regulation of conidial germination in *A. alternata*.** Germination rates of conidia from wild-type and different mutant strains in darkness and under constant illumination conditions. A conidial suspension ($10^5$ /ml) was incubated in minimal medium on a coverslip and the percentage of number of germinated conidia determined after 2 h **(A)** and 3 h **(B).** The bars present mean values ± SD of three biological replicates.

## 2.5 LreA is involved in blue- and green-light-dependent biosynthesis of alternariol

*A. alternata* is considered as the most important toxin producing species of the *Alternaria* genus. Since they are known as producer of a large spectrum of secondary metabolites among which are the mutagenic alternariol (AOH) and altertoxin (ATX) and are both stimulated by light, we analysed the effect of the inactivation of *fphA*, *lreA* and *hog*A on secondary metabolites biosynthesis under different illumination conditions. 5000 spores of the wild-type and corresponding mutant strains were evenly spread on modified Czapek Dox broth (mCDB) agar plates and incubated for 7 days at 28°C . Alternariol was extracted in the agar medium using ethyl acetate and analysed with thin-layer chromatography (TLC). In the case of the WT, whereas we observed a slight increase in the amount of AOH produced in cultures exposed to red-lights compared to dark, there was no difference in other bands in red-light and dark **(Fig. 18A)**. The continuous exposure of cultures to far-red and blue-lights resulted in the reduction of the amount of most bands with the exception of AOH. While there was no difference in the amount of bands produced in cultures exposed to white-light in comparison to dark, green-light promoted the amount of other bands with the exception of AOH. In dark, we observed no difference in the secondary metabolite profiling of the WT and corresponding mutant strains, though the amount of AOH produced in *lreA* mutant strain appeared reduced. The deletion of *hogA* resulted in the complete loss of AOH production irrespective of light or dark conditions. In contrast there was a large increase in the amount of a yellow band (probably altertoxin (ATX) produced under similar condition except in green-light in the *hogA* deletion strain. The inactivation of *fphA* and *lreA* resulted in reduced production of AOH under constant illumination with red, far-red and even dark conditions.

Furthermore, while the amount of AOH produced was drastically reduced in the *lreA* mutant strain in green-light, illumination with blue and white-light resulted in no AOH production in the *lreA* mutant suggesting an activating role for alternariol biosynthesis in green, blue and white-light with the exception of far-red light **(Fig. 18A)**. In order to further analyze the effect of the inactivation of *fphA*, *lreA* and *hog*A on secondary metabolites biosynthesis at the molecular level , we studied the expression of the polyketide synthase (PKS) gene responsible for alternariol formation, *pksI* **(Fig.**

**18B)**.The expression was stimulated by red and far-red light and to a lower extent by blue light. Red-light-dependent induction of *pksI* was lost in the Δ*fphA, ΔlreA,* and Δ*hogA* mutants. Contrary to our prediction, the induction of *pksI* was still observed in the Δ*fphA* mutant in far-red light suggesting additional far-red light effect which might be light independent **(Fig. 18B)**.

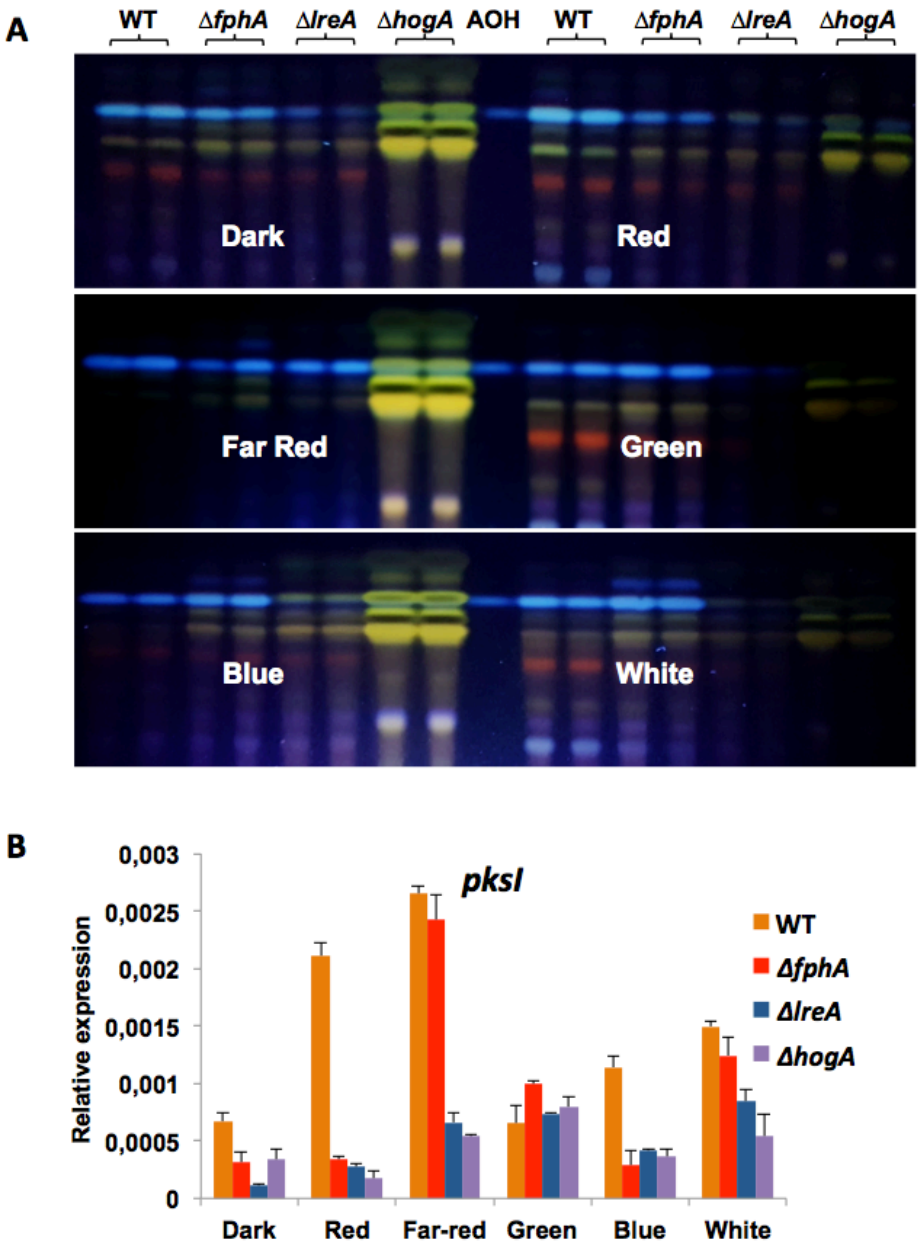


**Fig. 18. Analysis of secondary metabolites in the WT and mutant strains by thin-layer chromatography. (A)** Extracts from the WT and mutant strains grown for 7 days on mCDB agar plates at 28°C in darkness or under different illumination conditions. Fresh conidia of the WT and *fphA, lreA* and *hogA* mutant were spread evenly on the agar surface. An AOH standard was applied on the TLC for comparison. **(B)** Expression analysis of the *pksI* gene involved in AOH biosynthesis. Liquid mCDB medium was inoculated with conidia and grown without shaking for 7 days. Mycelia were harvested, frozen in liquid nitrogen and processed for RNA extraction and real-time PCR analysis. The mean of the results from three biological replicates

and three technical replicates is shown. Expression was normalized to the expression of the H2B gene.

## 2.6 Phytochrome, LreA (WC-1), and HogA regulate gene expression

The red- and blue-light receptors in *A. alternata* appear to play similar but also unique roles compared to those in *N. crassa*, *A. nidulans*, *B. cinerea* and *C. neoformans* Therefore, the next question was about their roles at the gene level. To this end, we investigated light induction of five genes. The first candidate was *ccgA*. Light induction of *ccgA* or *ccg-1* requires the FphA and *hogA* in *A. nidulans* and HogA and the WCC in *N. crassa*. Mycelia of the WT and the *fphA-, lreA-,* and *hogA*- deletion strains were grown in the dark at 28°C for 36 h and then exposed to white light for 30 min. After RNA extraction, the expression levels of tested genes were measured by real-time PCR and normalized to the H2B gene. Indeed, *ccgA* was induced to about 13-fold in the light compared to the dark (**Fig. 19)**. In the *fphA*-deletion strain, light induction of *ccgA* was reduced to 20% and to even less in the *lreA* and *hogA* mutants. Hence, light induction of *ccgA* required the FphA, HogA, and LreA. The next candidate was the catalase gene *catA*, whose expression depended on the MAP kinase cascade in *N. crassa*. Similar to the regulation of *ccgA*, the inactivation of *fphA*, *hogA* and *lreA* resulted in a decrease in mRNA expression of *catA.* The same regulatory behaviour was true for a gene whose translational product shares similarity to short-chain dehydrogenases/reductases (*AAT_PT02522*). The gene was identified in *A. nidulans* in RNA sequencing (RNA-seq) approaches to isolate light-regulated genes Next, we tested the light regulation of *ferA*. This gene shares similarity to the *fer* gene of *N. crassa*, *B. cinerea* and *C. neoformans*; it encodes a ferrochelatase, an enzyme that catalyzes the terminal step of heme biosynthesis. The gene is light-regulated in a white collar-dependent manner In *N. crassa*, *B. cinerea* and *C. neoformans.* In *A. alternata*, it was strongly induced by white light. The light induction was independent of FphA but strictly dependent on LreA. In the *hogA*- deletion strain, light induction was still observed, although the mRNA expression was reduced in comparison to that in the WT. At last, we tested an orthologue of *N. crassa bli-3* (blue light induced-3; unknown function). Light induction of this gene also required the LreA and HogA but not the FphA. The results indicate a complex regulatory network for light-regulated

genes in *A. alternata*, with similarities to *N. crassa*, *A. nidulans*, *B. cinerea* and *C. neoformans*.

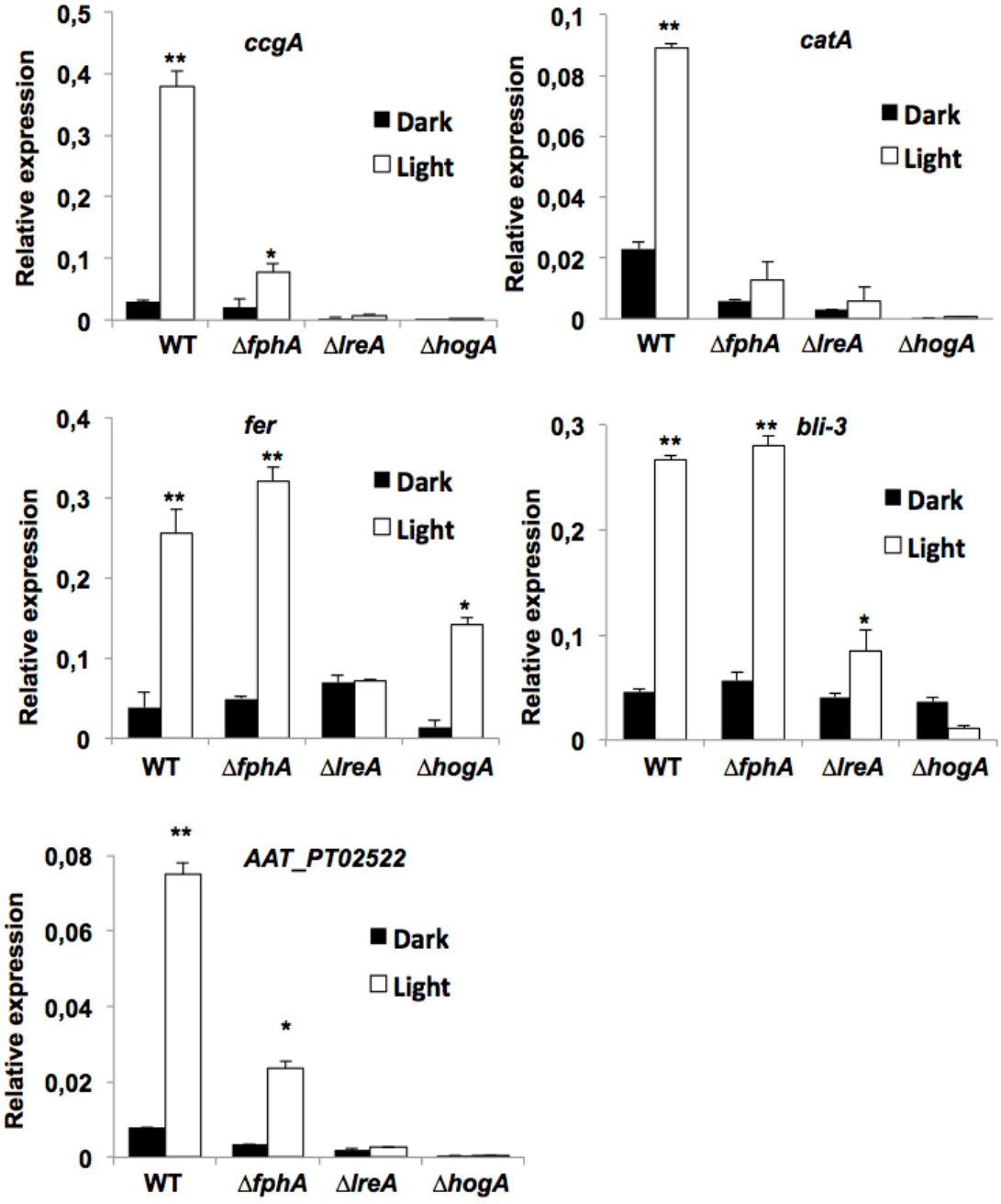


**Fig. 19. Expression analysis of light-induced genes in the WT, *ΔfphA, ΔlreA* and *ΔhogA* strains.** Spores of wild type (WT), *fphA, lreA* and hogA mutant strains were cultured on the mCDB surface at 28 °C for 36 h in the dark. Mycelium was exposed for 30 min to white light. Control samples were collected in the dark. RNA was isolated and the transcript the expression levels of the selected genes were normalized using H2B. The bars present mean values ± SD of three biological replicates. Statistical analysis was performed with Student's t test, *, $P \leq 0.05$; **, $P \leq 0.01$.

## 2.7 Light sensing and stress responses

### 2.7.1 Multistress responses in *A. alternata* are dependent on FphA, LreA, and HogA

The cross talk between light sensing and stress signalling cascade has been reported in several filamentous fungi including *N. crassa, B. cinerea, A. nidulans, A. fumigatus*, *T. atroviride* and *B. bassiana*. In order to assign a role for FphA, LreA, and HogA in the regulation of stress responses in *A. alternata*, we investigated the effect of osmotic, oxidative, and cell wall-degrading agents on the spores and mycelial plugs of wild-type and the mutant strains on medium supplemented with these chemical agents. Plates were incubated at 28°C for 4 days in the dark **(Fig. 20A)**. There was no difference in the radial diameter of the *fphA* and *lreA* mutant to osmotic stress with NaCl and KCl or the cell wall stress compounds, Congo red and SDS compared to WT. The *hogA* mutant strain was highly sensitive not only to KCl and NaCl but also to other chemical agents tested. The inactivation of the *fphA* and *lreA* resulted in enhanced resistance of their spores to $H_2O_2$ and menadione compared to the WT strain **(Fig. 20A, B)**. We observed no significant differences between samples grown on medium supplemented with tested chemical agents under illumination conditions and darkness (data not shown). Unexpectedly, mycelia of all mutant strains were as resistant to oxidative stress as the WT strain **(Fig. 21A, B)**. The results indicate that different structures (mycelium and conidium) of *A. alternata* have different sensitivity to oxidative stress.

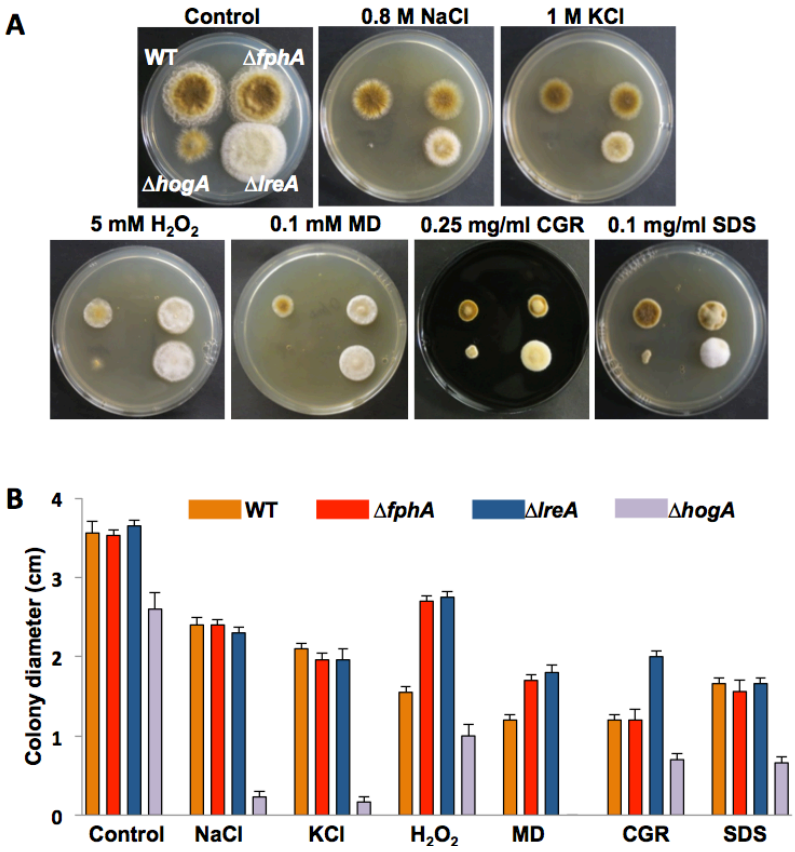


**Fig. 20. FphA, LreA and HogA mediate stress response in conidia. (A)** Colony growth of WT and the *fphA*, *lreA* and *hog*A mutant strains under different conditions (mCDB plates supplemented with 0.8 M NaCl, 1 M KCl, 5 mM $H_2O_2$, 1 mM menadione (MND), 0.25 mg/ml Congo red (CGR), or 0.1 mg/ml SDS) incubated for 4 days at 28°C in the dark. **(B)** Quantification of the colony diameter from the colonies in panel **(A).** The experiments were repeated three times and the arrow bar represents the standard deviation.

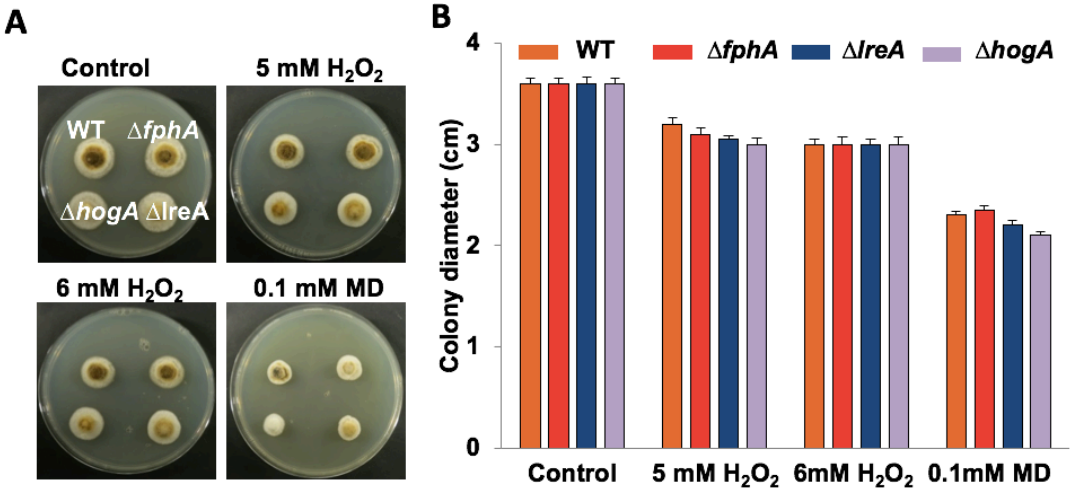


**Fig. 21. FphA, LreA and HogA are dispensable in oxidative stress response in growing mycelia. (A)** Mycelial plugs from the growing edge of 2-day colonies from the WT and mutant strains were used to inoculate mCDB plates supplemented with 4 mM $H_2O_2$, 6mM $H_2O_2$ and 1 mM menadione (MND) and then incubated at 28°C for 3days**. (B)** Quantification of the colony

diameter from the colonies in panel **(A).** The experiments were repeated three times and the arrow bar represents the standard deviation.

In order to better understand the tolerance of oxidative stress in the conidia of the *fphA* and *lreA* mutant strains, we analysed the transcript levels of four superoxide dismutases and two catalases (CAT) genes in the presence of 4 mM $H_2O_2$ **(Fig. 22)**. The transcript level of all tested genes was upregulated in the *fphA* and *lreA* mutant compared to the WT. Taken together, our results suggest a negative role for the FphA and LreA in the oxidative stress response that is independent of light.

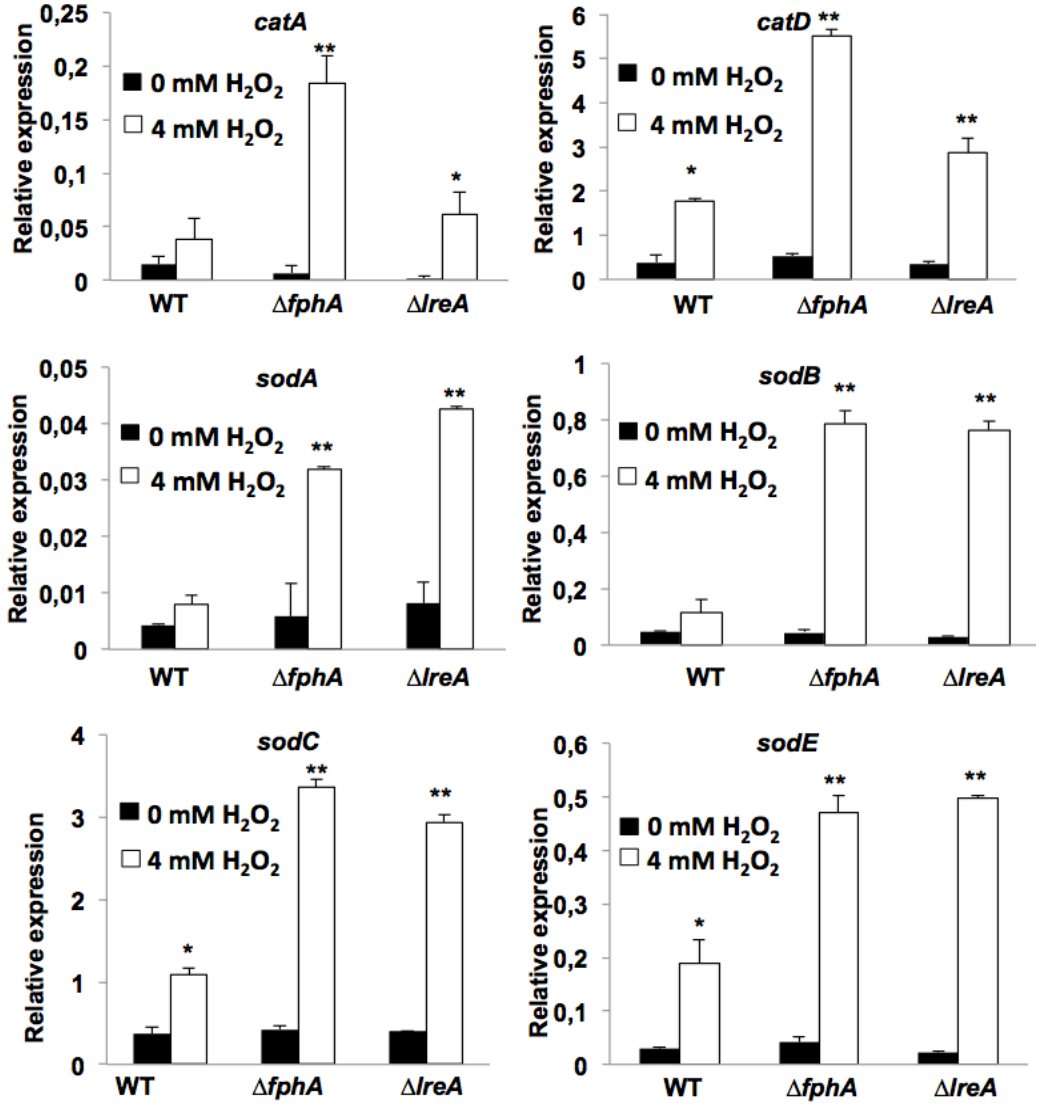


**Fig. 22. Transcriptional profiling of light and/ or oxidative stress regulated genes and the role of FphA, LreA and HogA.** Strains were grown on mCDB liquid medium for 18h (shaking) at 28˚C and then supplemented with 4 mM $H_2O_2$. and further incubated for 30 minutes. RNA was isolated as previously described and mRNA transcript expression levels of the selected genes were normalized using H2B. The bars present mean values ± SD of three

biological replicates. Statistical analysis was performed with Student's t test, *, P ≤ 0.05; **, P ≤ 0.01.

Several reports in filamentous fungi including *N.* crassa, *A. nidulans*, *T. atroviride* and *B. bassiana* suggested a cross talk between the phytochrome, white-collar complex and the HOG signalling pathway. Therefore, we analysed the expression of selected genes (*hogA, atfA, ccgA* and *bliC*) in the WT and mutant strains in medium supplemented with/ without 0.8 M NaCl **(Fig. 23)**. The inactivation of *fphA* and *lreA* resulted in reduced transcript levels of *hogA, atfA* and *ccgA*. LreA but not *fphA* is required for the induction of *bliC.* Deletion of *hogA* lead to complete abrogation of the induction of *ccgA* and *bliC.*

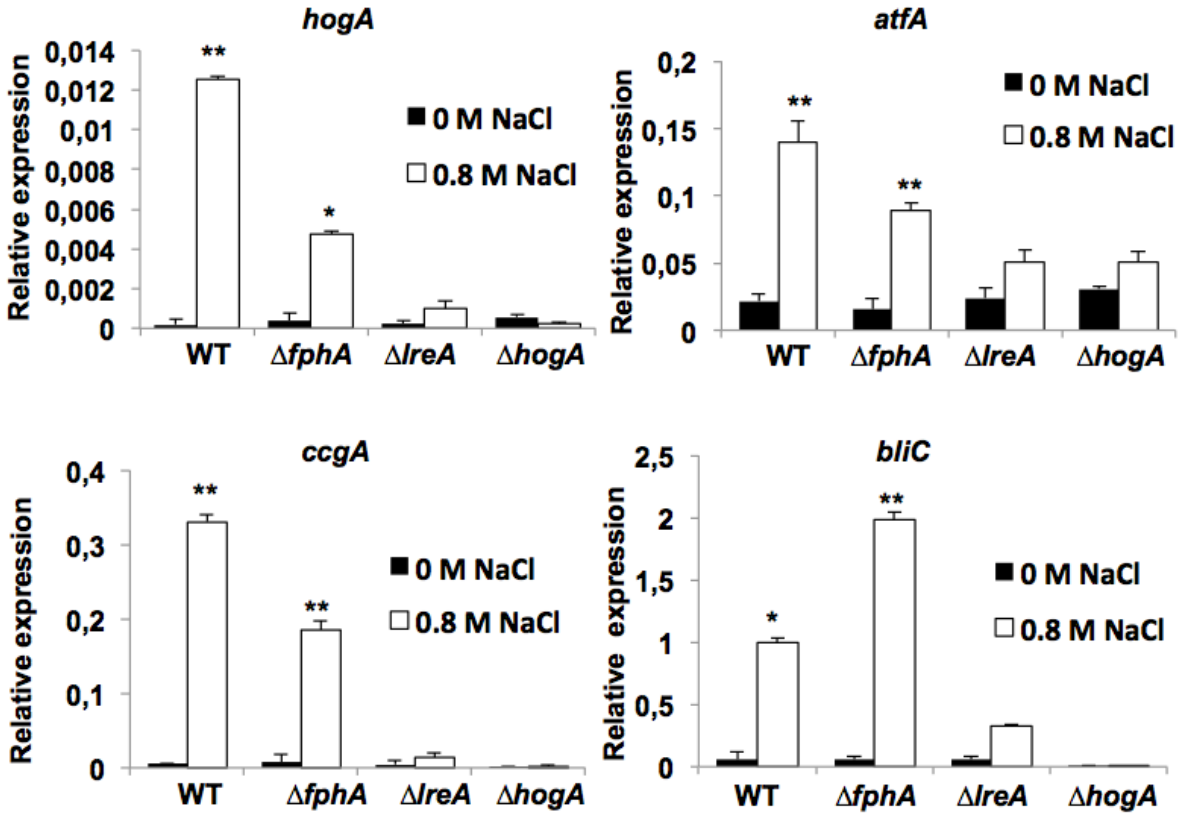


**Fig. 23. Transcriptional profiling of light and/ or osmotic stress regulated genes and the role of FphA, LreA and HogA.** Strains were grown on mCDB liquid medium for 18h (shaking) at 28°C and then supplemented with 0.8 M NaCl and further incubated for 30 minutes. RNA was isolated as previously described and mRNA transcript expression levels of the selected genes were normalized using H2B. The bars present mean values ± SD of three biological replicates. Statistical analysis was performed with Student's t test, *, P ≤ 0.05; **, P ≤ 0.01.

### 2.7.2 Light induces an early transient phosphorylation of HogA through phytochrome and LreA

Given that the MAP kinase cascade regulates several downstream targets once activated by phosphorylation and light has been reported to have stimulated this process in *A. nidulans* and *T. atroviride*, we then analysed if that also occurs in responses regulated by light through the phytochrome and LreA (WC-1) in *A. alternata.* Conidia were germinated for 3 h on coverslips in the dark, exposed for 5 min to red or blue light, and processed for immunostaining using the antiphospho-p38 MAP kinase antibody, which has been used to detect *A. nidulans* phosphorylated SakA **(Fig. 24A)**. After illumination with red or blue light, HogA was rapidly phosphorylated within 5 minutes and the fluorescence was detected in the cytoplasm and enriched in the nuclei. Phosphorylation decreased to basal level 15 minutes after the stimulus suggesting that HogA phosphorylation is an early light response subjected to a photoadaptation mechanism. Phosphorylation of the HogA was not detected in the *fphA* and *lreA* mutant strains. The results are in agreement with reduced expression of *ccgA* in the *fphA* and *lreA* mutant strains after illumination with red or blue-light **(Fig. 24B)**.

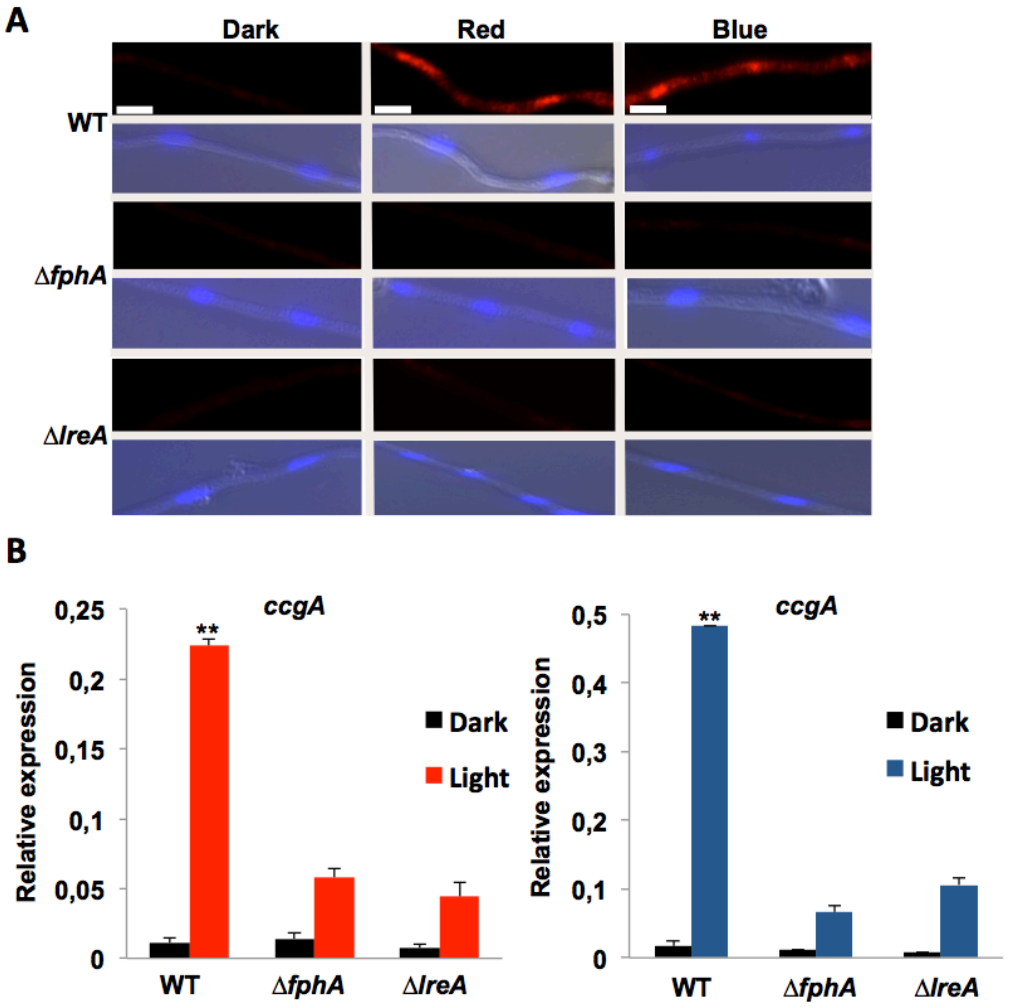


**Fig. 24. Transient phospho-activation of HogA by red and blue-light and the role of FphA and LreA. (A)** Microscopic pictures of germlings of WT and mutant strains incubated in the dark or under red or blue-light. Conidia were germinated for 3 h at room temperature and processed for immunostaining. Scale bar = 5 µm. **(B)** Expression analysis of *ccgA* in the dark and under red- or blue-light conditions. Hyphae were grown for 36 h at 28°C and then exposed for 30 min to red or blue light. RNA was extracted and *ccgA* expression quantified by real-time PCR using H2B for normalization. The mean of the results from three biological and three technical replicates is shown. Statistical analysis was performed with Student's t test, **, $P \leq 0.01$.

## 2.8 Phytochrome is involved in temperature sensing in *A. alternata*

### 2.8.1 Phytochrome modulates vegetative growth and asexual sporulation at high temperature

There is evidence that the phytochrome is involved in temperature sensing in bacteria and plants (Njimona & Lamparter, 2011; Njimona *et al.*, 2014; Legris *et al.*, 2016; Jung *et al.*, 2016; Burgie *et al.*, 2017; Qiu *et al.*, 2019). Most phytochromes have a C-terminal histidine kinase or a histidine kinase related region, which could act as temperature sensor. Temperature effects on the kinase activity and the absorption spectra of the sensory core (PAS-GAF-PHY) in vivo has been described for the bacterial phytochrome (Njimona & Lamparter, 2011; Njimona *et al.*, 2014). Therefore, the next question was about the role of the FphA in temperature sensing in *A. alternata.* To this end, we investigated the effect of the inactivation of *fphA* on vegetative growth and asexual sporulation. We found that temperature shifts did not affect the sporulation of the WT strain in the dark whereas in the *fphA*-deletion strain, less conidia were produced at 33 °C than at 28 °C **(Fig. 25 A, B)**. Light stimulation of sporulation in red light at 28 °C was lost in the WT after a temperature shift to 33 °C. Spore numbers were reduced to 72%. The loss of FphA resulted in reduced colony growth at 33 °C compared to the WT strain **(Fig. 25 C, D).** These results suggest a role for the FphA in adaptability to temperature changes.

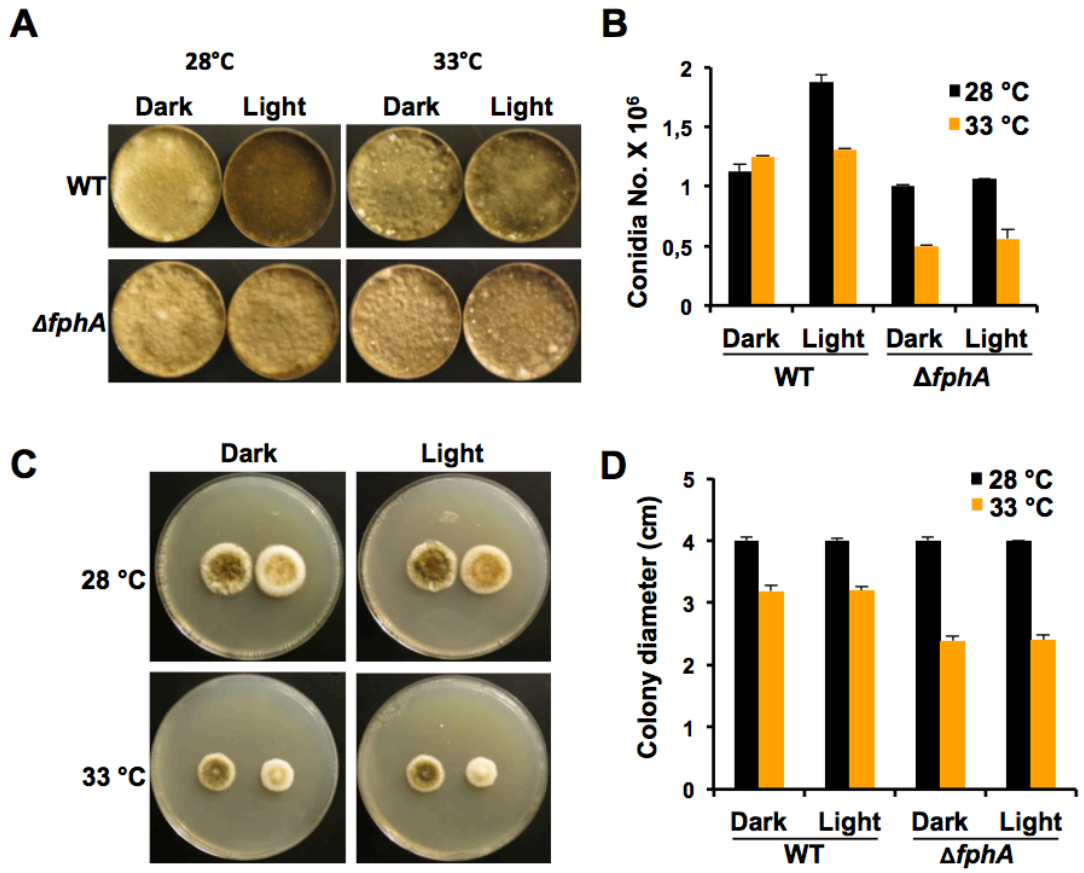


**Fig. 25. Effect of a temperature shift on sporulation and colony growth in *A. alternata* and the role of FphA (A)** Colony appearance on mCDB plates inoculated with 5,000 conidia evenly spread on the agar surface and incubated at 28 °C and 33 °C for 12 days in the dark or light conditions. **(B)** Quantification of the conidia produced on the plates in panel **(A).** Three independent plates of each strain were analyzed, and the mean values for the three samples are displayed. The arrow bar represents the standard deviation **(C)** Pictures of colonies and radial of the WT and *fphA* mutant strains incubated at 28 °C and 33 °C for 5 days **(D)** Quantification of the colony diameter from the colonies in **(C).** The experiments were repeated three times and the arrow bar represents the standard deviation

## 2.8.2 FphA regulates gene expression in response to a temperature shift

Since FphA regulates phenotypic response at high temperature, next we asked if *A. alternata* FphA could act as thermometer at the gene level. Hence, we analysed the transcript levels of four light induced gene (*ccgA, catA, catB* and *catD*) after the stains were subjected to a temperature shift. Mycelia of the WT and the *fphA*-mutant strains were grown in the dark at 28 °C for 40 h. Thereafter, they were shifted to 28°C or 33°C respectively and further incubated for 15 minutes. Indeed, transcript levels of *ccgA* and the catalase genes (*catA*, *catB* and *catD*), were upregulated in the WT strain but reduced in *fphA*-deletion strain after a temperature shift from 28 °C to 33 °C in the dark

**(Fig. 26)**. Therefore, the FphA is required for the induction of light-inducible genes at high temperature.

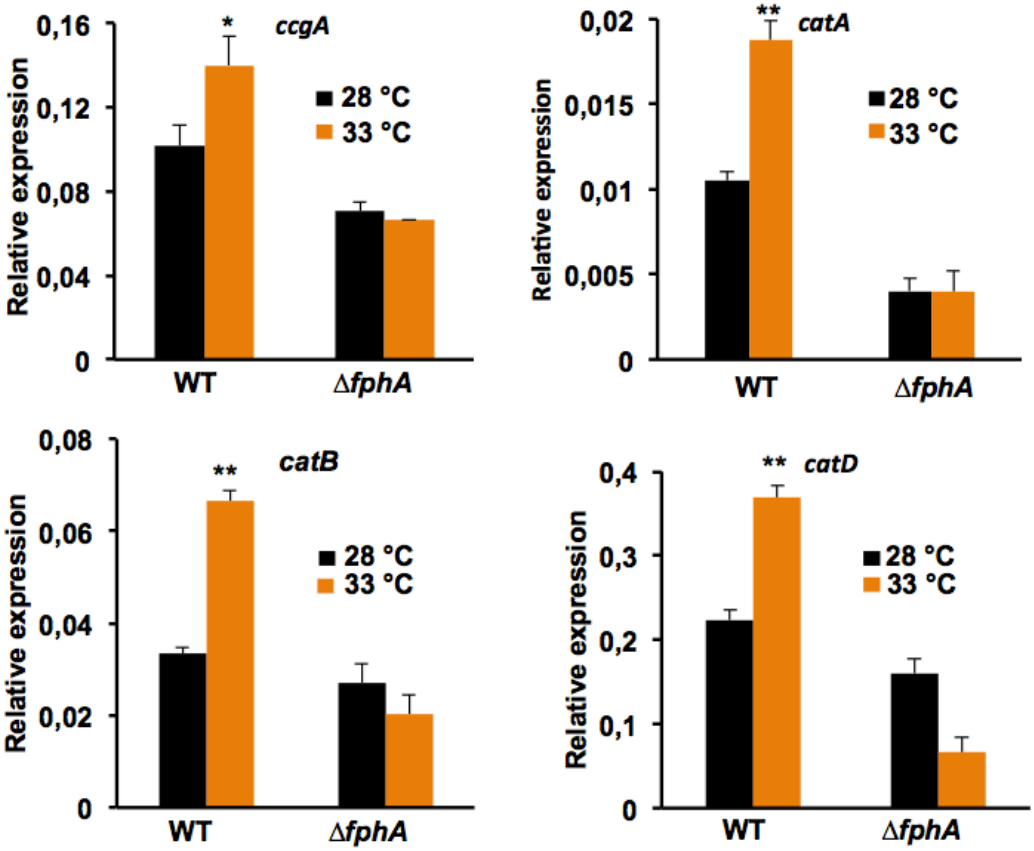


**Fig. 26. Expression analysis of light-responsive genes at different temperatures in *A. alternata* wild type (WT) and the Δ*fphA* strains.** Spores of wild type (WT) and the *fphA* mutant strain were cultured on the surface of mCDB at 28 °C for 40 h in the dark. The mycelia were then transferred onto the surface of fresh mCDB pre-warmed to 28°C or 33 °C and incubated for 15 minutes in the dark. Then, the mycelia were frozen immediately in liquid nitrogen and RNA isolation was done as previously described and mRNA transcript expression levels of the selected genes were normalized using H2B. The bars present mean values ± SD of three biological replicates. Statistical analysis was performed with Student's t test, *, $P \leq 0.05$; **, $P \leq 0.01$.

## 2.9 Role of FphA, LreA and HogA in virulence of *A. alternata*

Since *A. alternata* is a common food contaminant, we assayed the colonization potential of the WT, *fphA-, lreA-, and hogA*-mutant strains on tomato using conidia and mycelial plus of corresponding strains. Tomatoes were immersed in 70% ethanol for 5 min, rinsed with sterile water, and air-dried in a clean bench. The air-dried tomatoes were wounded (approximately 1cm in depth) at the equator with a sterile scalpel prior to inoculation with 10 µl of 1 x $10^5$ fresh conidia of the WT and mutant strains **(Fig.**

**27A)**. In the case of mycelial plug assay, tomato was wounded with a cork borer 5 mm in diameter. Wounds were inoculated with mycelia plugs from 36 h actively growing plates of the WT and mutant strains **(Fig. 27C)**. All samples were incubated at 20°C for 7 days in white-light and darkness for lesion development. In the case of tomato inoculated with conidia, we observed that necrotic lesions were slightly reduced on tomatoes infected with the *lreA*-mutant strain incubated in the dark and light as compared to WT **(Fig 27A, B)**. The *hogA*-mutant strain did not cause any necrotic lesions. This could be due to the reduced fitness of the strain rather than a specific effect on pathogenicity. The *fphA*-mutant strain displayed enhanced pathogenicity irrespective of light and dark conditions compared to WT and the *lreA*-mutant strains. In contrast, all the strains with the exemption of the *fphA* mutant exhibited same pattern of aggressiveness on tomato inoculated with mycelia plugs **(Fig. 27C, D)**.

In order to further understand why the asexual conidia is more important in successful colonization compared to mycelia, we analysed the transcript levels of genes involved in reactive oxygen species (ROS) resistance in growing conidia supplemented with 4 mM $H_2O$ **(Fig. 28).** The transcript levels of all tested genes were upregulated in the *fphA* mutant with the exception of *catB*, in comparison to the WT. Deletion of *lreA* resulted in reduced transcript levels of all tested genes with the exception of the *sodB* and *sodC* genes. HogA is important for the induction of all the genes **(Fig. 28)**. Taken together, our results suggest a negative role for FphA in virulence.

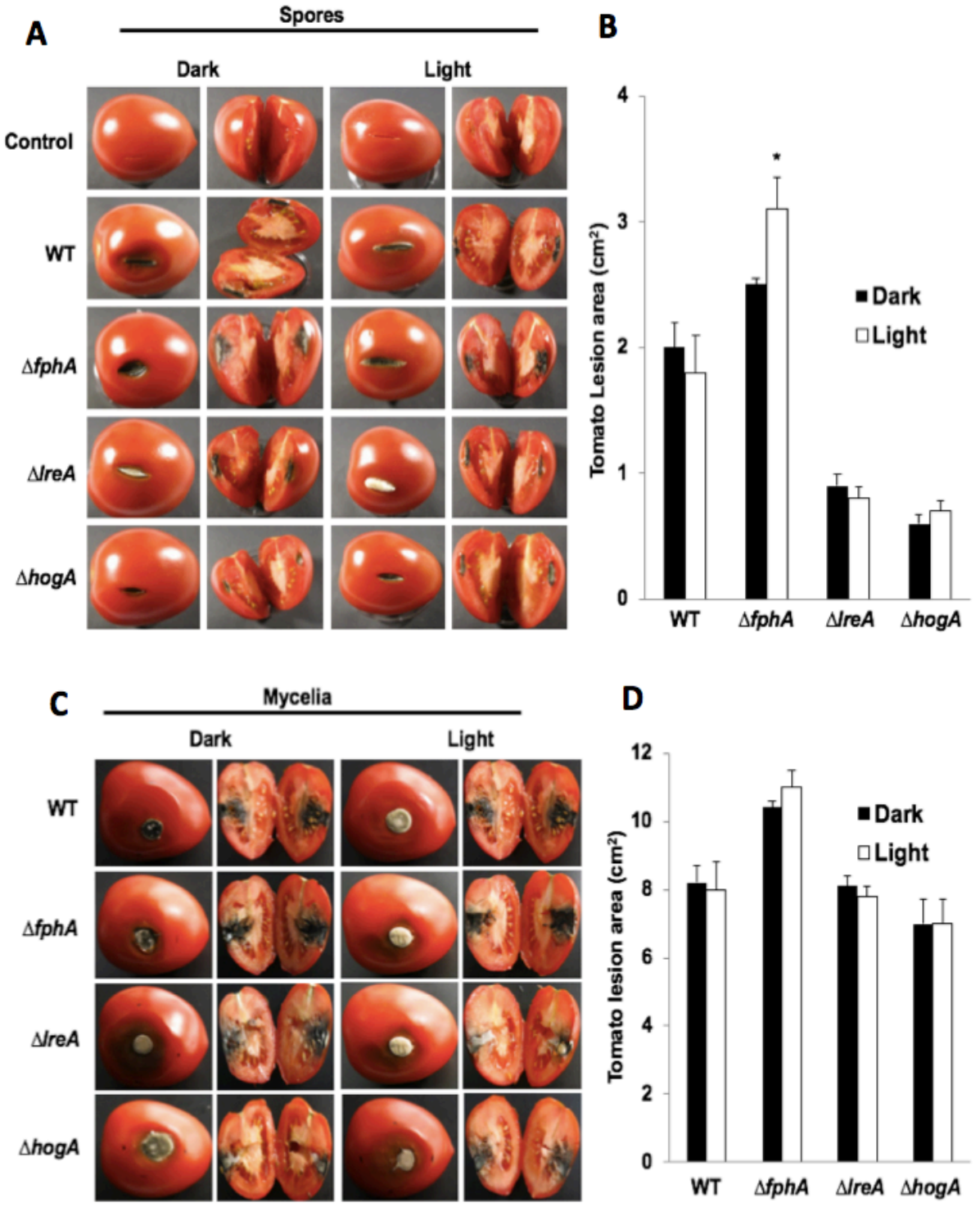


**Fig. 27. Virulence assay on tomatoes. (A)** Fresh conidia of the WT and the Δ*fphA, ΔlreA and the ΔhogA* mutant strains were inoculated on tomato fruits and incubated at 20 °C for 7 days in the dark or under white light. To the control tomatoes water was added instead of a spore suspension. (**B)** Quantification of the lesion areas in panel A. **(C)** Mycelia plugs from 36 h actively growing plates of the WT and mutant strains were inoculated in a 0.5 cm diameter hole and incubated as described above. (**D)** Quantification of the lesion areas in panel **(C).** The mean value was calculated from three biological replicates. The experiment was repeated three times. The arrow bar represents the standard deviation. Statistical analysis was performed with Student's t test, *, P ≤ 0.05.

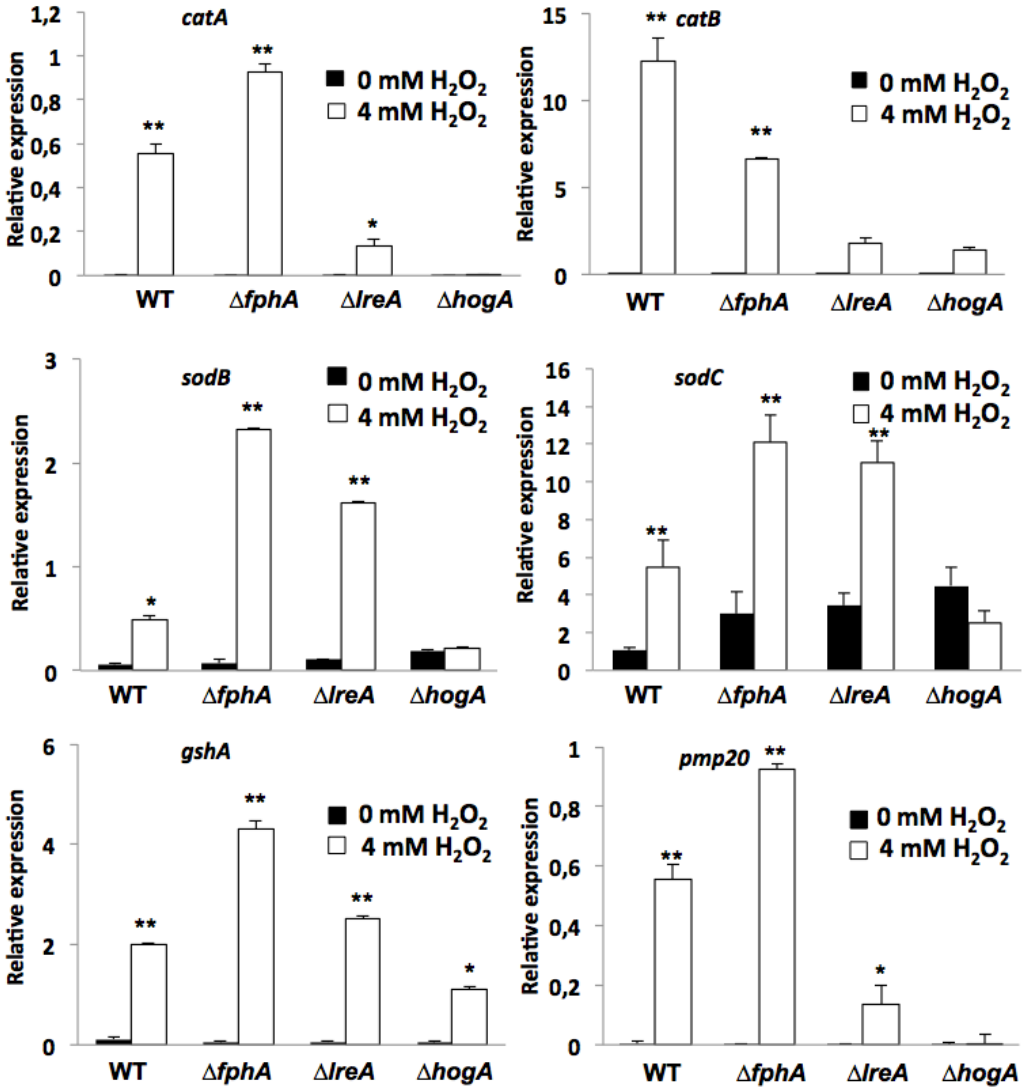


**Fig. 28. Expression analysis of genes involved in reactive oxygen species (ROS) resistance in the growing conidia of WT, *ΔfphA, ΔlreA* and *ΔhogA* strains.** Fresh spores of wild type (WT) and the mutant strains were scrapped from surface of mCDB plates, filtered and incubated at 28 °C for 2 h (shaking) in the dark and then supplemented with 4 mM $H_2O_2$.The samples were further incubated for 1 h. Thereafter, the spores were centrifuged, supernatant discarded, and the spore pellets were frozen immediately in liquid nitrogen and RNA isolation was done as previously described. mRNA transcript expression levels of the selected genes were normalized using H2B. The bars present mean values ± SD of three biological replicates. Statistical analysis was performed with Student's t test, *, $P \leq 0.05$; **, $P \leq 0.01$.

# 3. Discussion

## 3.1 *A. alternata* as a bridge between *N. crassa* and *A. nidulans* light sensing signalling cascade

Light is an environmental signal that plays an important role for most organisms, either as a tool for information or source of energy. The fungal kingdom is estimated to contain over a million species, and it is estimated that a majority of them respond to light which influences several physiological and biological responses including vegetative growth, development, secondary metabolism and virulence. *N. crassa* photobiology is regulated by blue light in a WCC complex-dependent *crassa* (Bahn *et al.*, 2007; Ballario & Macino, 1997; Chen *et al.*, 2010b; Corrochano, 2007; Corrochano, 2011; Herrera-Estrella & Horwitz, 2007; Linden & Macino, 1997; Purschwitz *et al.*, 2006). In contrast, early studies on the expression, regulation and *in vitro* photochemistry of the two red light photoreceptors PHY-1 and PHY-2 suggest a photobiological role which was rather unclear since strains containing deletions of *phy-1* and *phy-2* were not compromised in any known photoresponses (Froehlich *et al.*, 2005). However, few years later it was discovered that the loss of *phy-2* or *phy-1* affected light- dependent expression of asexual development and early development genes. *phy-1* and *phy-2* mutants also displayed defective sexual development. Protoperithecia formation commenced earlier in the Δ*phy-2* mutant upon exposure to red light suggesting that phytochromes regulates the switch between asexual and sexual reproduction (Wang *et al.*, 2016).

In *A. nidulans*, red and blue light induces conidiation and represses sexual sporulation (Blumenstein *et al.*, 2005; Brandt *et al.*, 2008; Purschwitz *et al.*, 2008); in contrast to *A. fumigatus* where red or blue light does not have an effect on asexual development (Fuller *et al.*, 2013). Unlike in *N. crassa*, the *A. nidulans* LreA/LreB complex represses asexual development and promotes sexual development, whereas FphA represses sexual development and promotes asexual development (Bayram *et al.*, 2010; Purschwitz *et al.*, 2008). At the molecular level, the master regulator of conidiation *brlA* rapidly responds to light and the expression is dependent on the photoreceptor complex composed of a phytochrome FphA and white collar complex LreA and LreB as well as the fluffy genes (Ruger-Herreros *et al.*, 2011). Similarly, blue

light regulates sporulation in *B. cinerea* and *T. atroviride* respectively. In *B. cinerea*, *bcwcl1* mutant exhibited early and persistent conidiation under all light conditions with the loss of sclerotia development in comparison to the wild type. Overexpression of WCC prevents asexual development suggesting that the BcWCL1/BcWCL2 complex represses conidiation through the proliferation of aerial hyphae in response to blue-light. In several species of the genus *Trichoderma*, a brief pulse of light triggers conidiation (Canessa *et al.*, 2013). Blue-light-induced conidiation in *T. atroviride* is mediated by the blue-light regulators BLR-1 and BLR-2 (Casas-Flores *et al.*, 2004; Castellanos *et al.*, 2010). Deletion of either *blr-1* or *blr-2* in *T. atroviride* resulted in the loss of conidiation upon exposure to light (Casas-Flores *et al.*, 2004; Castellanos *et al.*, 2010). Similarly, the WCC orthologues; FgWc-1 and FgWc-2 negatively regulate sexual development in *F. graminearum* (Kim *et al.*, 2015). However, deletion of the phytochrome (*FgFph*) exhibited no morphological defects in comparison to the wild type consistent with the observations in *N. crassa* and *C. neoformans* (Idnurm & Heitman, 2005; Froehlich *et al.*, 2005). Phytochrome is crucial for conidiation in response to red/far-red light in *B. bassiana* (Qiu *et al.*, 2014).

In comparison to light-dependent asexual development in several filamentous fungi including *A. nidulans* and *N. crassa*, light also modulates asexual sporulation in *A. alternata*. Red and far-red promotes sporulation in *A. alternata* unlike in *A. nidulans* where far-red light reverses the red-light effect. In addition to this, blue, white and green-light inhibits sporulation. This is different from *A. nidulans* where blue and white light stimulate sporulation with the absence of a green-light effect. This suggests a more sophisticated photobiological response in *A. alternata*. In related fungi, it was reported that photosporogenesis consists of two phases; inductive and terminal phase. Whereas light stimulates conidiophore formation in the inductive phase, the formation of conidia in the terminal phase is promoted in darkness. A brief exposure of conidia at the terminal phase to light impairs sporulation (Leach, 1967; Witsch & Wagner, 1955). Another hypothesis in the regulation of sporulation by different light qualities in *Alternaria* is the fact that a mycochrome pigment system producing an unknown red substance is crucial for sporulation (Kumagai, 1986). Continuous exposure of culture plates to short wavelength in the blue light spectrum photooxidize the red substance resulting into inhibition of sporulation. However long-term exposure to red and far-red light (longer wavelengths) enhanced the production of this unknown red pigment

(Kumagai, 1986). Our findings show that FphA and LreA positively regulates asexual sporulation in *A. alternata.* The loss of function of *lreA* greatly impaired sporulation, whereas the FphA only regulates high level of spore production. Similarly, the dark role of the two photoreceptors in sporulation is in agreement to what has been reported in *A. nidulans* and *N. crassa.* At the molecular level, both photoreceptors, phytochrome and the white-collar orthologue LreA, have activating functions with respect to the induction of asexual development genes. Another interesting observation was the fact that far-red light had the same effect as red-red light evident in the photoreversibility of blue light effect. Apparently, if red light causes photoconversion of FphA into the far-red form (Pfr) and far-red light its reversion back to the red-light form (Pr), one would expect that FphA in the Pfr form is inactive and the strain should respond as in the dark. This apparent contradiction has been observed before in *A. nidulans, Beauveria bassiana* and *B. cinerea* (Röhrig *et al.*, 2013; Qiu *et al.*, 2014; Schumacher, 2017). The contradiction might be as a result of the possibility of a far-red sensing system with a maximal absorption in red light which do not affect photophysical properties but rather influences its molecular interactions. In plant, photoconversion and nuclear trafficking cycles influences phyA response to far-red light (Rausenberger *et al.*, 2011). In fungi, the mechanism for far-red light responses has yet to be investigated.

Light may pose as a direct source of stress affecting radial growth and conidial germination. Similar to *A. nidulans* and *A. fumigatus,* red and blue light inhibited spore germination in *A alternata*. In contrast to A. *nidulans* and *A. fumigatus*, we observed a repressing role for green light in spore germination. Deletion of the *fphA* but not *lreA* resulted in the release of spores in light supporting the presence of additional blue light receptors. Interestingly, germination was already enhanced in the dark in the *fphA* deletion different from *A. nidulans* and *A. fumigatus* where there were no significant differences in the germination rates of the phytochrome mutants in the dark compared to the wild-type strains in the light. This suggests heterogeneity in phytochrome-mediated germination kinetics of spores across fungi.

At the gene level, *A. alternata* responds to white, blue, red, far-red and green-light producing an increase in expression of light-induced genes. This includes the *ccgA* which is unique compared to *N. crassa* and *A. nidulans.* The expression of *ccgA, catA* and *AAT*_PT02522 genes are regulated by both the FphA and LreA in *A. alternata.* However, the light responsiveness of the genes is more impaired in the *lreA*

mutant compared to the *fphA* mutant strain. In addition, the *ferA* and *bliC* genes expression are strictly dependent on the LreA and not the FphA. It has been shown in in *B. cinerea, C. neoformans*, *N. crassa* and *P. blakesleeanus* that the transcription of *fer* and *bliC* is induced in light in a White Collar complex (WCC)-dependent manner (Canessa *et al.*, 2013; Idnurm & Heitman, 2010). In the case of *ccgA*, our result is different from *N. crassa*, where the expression of *ccgA* is strictly WC-1 dependent and *A. nidulans* where deletion of *fphA* but not *lreA* completely abrogate the induction of *ccgA* upon white light exposure. The importance of FphA and LreA in the induction of *ccgA, catA* and *AAT*_PT02522 might be that the LreA together with the white-collar orthologue LreB and the FphA acts a switch for light-dependent chromatin remodelling and gene expression. In the case *fer* and *bliC*, it could be that their expression is regulated by other modifies such as transcriptional co-regulators which interact with the chromatin structure.

## 3.2 The MAPK HogA cascade as a hub for FphA and LreA- mediated responses

The *A. alternata* HogA signalling pathway is similar to the stress activated protein kinase (SAPK) which have been extensively studied in *S. cerevisiae, A. nidulans, A.* fumigatus and other filamentous fungi (Brewster *et al.*, 1993; Han & Prade, 2002; Kawasaki *et al.*, 2002; Lara-Rojas *et al.*, 2011; Fischer *et al.*, 2016; Yu *et al.*, 2016; Garrido-Bazan *et al.*, 2018; Rispail *et al.*, 2009; Manfiolli *et al.*, 2019). SakA/Hog1p orthologs have been shown to have roles in osmotic and oxidative stress responses as well as responses to cell wall agents, injury, asexual and sexual development, fungicides and pathogenicity (Eaton *et al.*, 2008; Heller *et al.*, 2012; Lamb *et al.*, 2012; Van Nguyen *et al.*, 2013; Nimmanee *et al.*, 2015). The SAPK pathways are activated via the phosphorylation of the conserved threonine and tyrosine residues in the TGY motif located in the catalytic domain which in turn activates the phosphorylation of various substrates resulting into the regulation of biological responses (Ferrigno *et al.*, 1998; Day & Quinn, 2019). Light acts as a stress cue regulating DNA repair, ROS production, osmoadaptation transcription of genes encoding chaperones, secondary metabolites (Rosales-Saavedra *et al.*, 2006; Schuster *et al.*, 2007).

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Consistent with the role of Hog1 in osmoadaptation and various stress responses in *S. cerevisiae, N. crassa* and other filamentous fungi, deletion of *hogA* impaired virtually all physiological and metabolic processes in *A. alternata.* It became apparent that the MAPK HogA is a central regulator of all physiological and metabolic responses and important for fitness in *A. alternata*. One interesting observation was the fact that the conidia but not mycelia of the *fphA* and *lreA* mutants displayed enhanced resistance to hydrogen peroxide and menadione. These observations suggest that either *A. alternata* uses different mechanisms of protection against oxidative stress in conidia and mycelia, or that germinating conidia are much more sensitive to stress, perhaps due to differences in in cell wall structure and composition, increased DNA replications and huge transcriptional shift required for germination to occur (Rosen *et al.*, 1974; Sephton-Clark *et al.*, 2018). The increased tolerance of the conidia of the *fphA*- and *lreA* mutants to oxidative stress is in agreement with the upregulated transcripts of all five SOD genes and four CAT genes in comparison to the WT strain. In *B. bassiana*, Δ*Bbphy* showed increased antioxidant capability and upregulation of catalases and superoxide dismutases genes under oxidative stress (Qiu *et al.*, 2014). Similarly, analysis of 15 histidine kinases mutants including the *fphA* in *A. nidulans* resulted into production of reactive oxygen species (ROS). However, there was no link between ROS production and oxidative stress response in the HK mutant strains (Hayashi *et al.*, 2014). Our results suggest a repressing function of FphA and LreA in tolerance to oxidative stress in conidia of *A. alternata*.

Light activates the MAPKs from *A. nidulans* (SakA) and *T. atroviride* (TMK3) (Esquivel-Naranjo *et al.*, 2016; Yu *et al.*, 2016). Induction of light-inducible genes is impaired in both Δ*sakA* and Δ*tmk3* mutants. In *A. nidulans,* the accumulation of SakA in the nuclei is visible after few minutes of illumination with red or white light, but not with blue light. Light phosphorylate the MAPK SakA in an FphA-dependent manner (Esquivel-Naranjo *et al.*, 2016; Yu *et al.*, 2016). Deletion of *lreA or lreA/lreB* did not affect the SakA phosphorylation in red light/ blue light (Yu *et al.*, 2016). In *N. crassa* and *T. atroviride*, blue light activates the MAPK cascade in a WCC- dependent fashion. In *N. crassa*, dark and light responsiveness of the WCC complex modulates the expression of *os-4* (MAPKKK) by binding to the promoter locus. Deletion of the WCC binding sites in the *os-4* promoter affected *os-4* expression and OS-2 phosphorylation rhythms (Lamb *et al.*, 2011). Rhythmic expression of the histidyl-phosphotransferase

gene, *hpt-1*, which peaks in the evening was also indirectly regulated in a WCC-dependent manner (Lamb *et al.*, 2011). In the case of *T. atroviride,* the MAPK Tmk3 was phosphorylated rapidly upon exposure to blue light in a Brl1-dependent manner. In *A. alternata*, high-osmolarity induction of the transcription of *hogA* and the transcription factor gene *atfA* depended on *lreA* and partially on *fphA*. The salt induction of *ccgA* and *bliC* showed a similar regulatory pattern with samples illuminated with white light. This is different from *A. nidulans*, where the MAP kinase SakA was still able to shuttle into the nuclei upon salt stress even in the absence of the FphA. We also observed a transient phosphorylation of the MAPK HogA upon exposure to red and blue light. Similar to red light stimulus, blue light could not cause HogA phosphorylation in the *fphA* and *lreA* mutant strains. Most importantly, *A. alternata* can distinguish between salt, oxidative and light signals. This suggests a more sophisticated and interactive dialogue between the red and blue light receptors in *A. alternata.*

## 3.3 Phytochrome as a thermometer for seasonal cues

Bacteria respond to environmental stimuli via the two-component regulatory system consisting of His kinase sensors and response regulators (Gao & Stock, 2009). The ability to colonize and infect plant in *Agrobacterium fabrum* by Ti DNA transformation procedure has been shown to be dependent on the *vir* genes, which are under the control of the VirA-VirG two component regulatory cascade (Jin *et al.*, 1993). At elevated temperature, the expression of *vir* genes are down-regulated due to the autokinase activity of *virA* which reduces upon temperature shift from 28°C to over 32–37°C (Jin *et al.*, 1993). Dark reversion of bacterial phytochrome Agp1 and histidine kinase activity of Agp1 and cyanobacterial phytochrome Cph1 has been shown to be temperature-dependent (Njimona *et al.*, 2014; Njimona & Lamparter, 2011). Most importantly, the phosphorylation activity of the holo protein increases with a temperature shift from 5 °C to 25 °C and decreases with a temperature shift from 25 °C to 40 °C. In addition, the spectral properties of Agp1 are influenced by temperature and these temperature effects are regulated via the His-Kinase domain. However, the effect of temperature sensing mediated by Agp1 AND Cph1 in vivo is yet to be established. The impact of Cph1 on gene expression is rather low and insufficient to be linked to a temperature effect (Hubschmann *et al.*, 2005).

In plants, light and temperature regulates plant growth, development and metabolism. The effect of temperature on phytochrome-mediated germination has been investigated in *Arabidopsis* (Heschel *et al.*, 2007). It was shown that phyA promotes germination at warmer temperature, whereas phyB was crucial for germination to occur across a range of temperatures. *phyE* deletion mutant was impaired in germination at colder temperature (Heschel *et al.*, 2007). This suggests the possibility that different phytochromes regulates germination at different temperatures indicating a differential and specialize role of individual phytochromes which are temperature-dependent. In agreement with this finding, spectroscopic analysis of purified phyB revealed that warmer temperatures drastically induced the reversion of active phyB to its inactive form (Song *et al.*, 2017; Jung *et al.*, 2016; Legris *et al.*, 2016).

In bacterial phytochrome, temperature induced decrease of kinase activity in darkness and in the light as well as for the apophytochrome. *P. aeruginosa* phytochrome has been shown to regulate stress response, quorum sensing and heat tolerance. In filamentous fungi, the effect of phytochrome- mediated heat tolerance has been characterized. The human pathogenic fungus, *A. fumigatus* can grow at 50°C and even survives up to 75°C via a unique mechanism which regulates its adaptability to temperature changes in the environment and most importantly in the lungs (Hartmann *et al.*, 2011). In addition, the MAPK SakA/HOGA pathway regulates adaptability to temperature shifts in both *A. nidulans* and *A. fumigatus* (Ji *et al.*, 2012). In *A. nidulans,* upon heat shock at 42°C, the induction of two light-inducible genes *ccgA* and *ccgB* was drastically but not completely reduced in the *fphA* mutant. This suggests an additional role for the FphA in temperature sensing. In *Aspergillus Kawachii*, the transcript level of gene of *ypdA*, *atfa* and *hogA* of the HOG pathway is significantly downregulated upon a temperature shift from 40°C to 30°C (Futagami *et al.*, 2015). Remarkably, common to fungal and bacterial phytochrome is the change of spectral properties at elevated temperatures. These recent findings are indicative for an early evolution of role of the phytochrome as a thermosensor prior to their role as a photoreceptor.

In *A. alternata*, we provided evidence that phytochrome mediated vegetative growth, asexual development and gene expression in response to temperature shifts from 28°C to 33°C. Colony growth and sporulation of the *fphA*-deletion strain was

affected with an increase in temperature. Additionally, the stimulation of sporulation by red light at 28°C in the wildtype was lost after a temperature shift to 33°C. Consistent with that in *A.* nidulans, our results show that FphA regulates the expression of light-inducible genes (*ccgA*, *catA, catB* and *catD*) upon temperature shifts. The loss of *fphA* resulted into a decrease in the expression levels of all tested genes compared to the WT. In agreement with our result, it has recently been shown that the *Alternaria* FphA Pr form absorption decreased by 10% upon a temperature shift from 15°C to 50°C, whereas a decrease from 50°C to 15°C promoted the full recovery of the spectra (Yu *et al*., submitted). This suggests that the observed *in vivo* phenotype upon temperature shifts may be attributed to the altered spectral properties of the His-kinase module of *A. alternata* phytochrome. Thus, the negative relationship between temperature and kinase activity of *A. alternata* FphA points to the biological role of FphA as a thermosensor of different seasonal cues crucial for stability and fitness of the organism.

## 3. 4 Light perception and virulence in *A. alternata*

Light is an important regulator of fungal pathogenesis and has been reported as a relevant variable with the potential to affect the outcome of the plant-pathogen interaction by modulating either plant defense responses, virulence of the pathogen or both. Supporting this concept, upon colonization or pathogen infection, light induced the synthesis of several compounds in plants. This includes low molecular weight compounds with antimicrobial properties such as phenolics, tannins, terpenoids and glucosinolates (Miranda *et al.*, 2007; Mellway *et al.*, 2009; War *et al.*, 2012). A direct effect of light on fungi virulence ability is consistent with several findings on the role of light in the regulation of conidiation, spore germination, cell wall integrity, osmotic and oxidative stress responses and plant invasion by many fungi. Molecular studies have allowed the identification and characterization of different photoreceptors with roles in light-mediated plant invasion, in species such as *Aspergillus*, *Botrytis*, *Neurospora*, *Sordaria*, *Candida*, and *Fusarium* (Carvalho & Castillo, 2018*)*. These light-responsive gadgets and photoreceptors have specific functions in stimulating asexual conidia and sclerotia formation, reactive oxygen species (ROS) homeostasis and secondary metabolism upon exposure to light or darkness (Schumacher, 2017; Yu & Fischer, 2019). Increasing day lengths from 18 to 20–24 h with white florescent lamps suppress severity of powdery mildew (*Podosphaera pannosa*) in rose due to a reduction in the

quantity of spores produced and light inhibition spore germination (Suthaparan *et al.*, 2010). Thus, photoreceptors are involved in colonization potential of fungi. Analysis of the role of light or the WCC 1 orthologs, revealed a contribution to virulence though their precise function differs among fungi-host interactions. The involvement of WC-1 orthologs in virulence have been shown for the causal agent of rice blast disease, *M. oryzea* as well as *F. oxysporum, B. cinerea* and the human pathogen *C. neoformans.* In *M. oryzea* and *B. cinerea,* light-dependent disease suppression is mediated by the blue- photoreceptors WC- 1 (Kim *et al*., 2011b; Canessa *et al*., 2013)*;*in contrast, the contribution of the WCC complex to virulence in *C. neoformans* is independent of light (Idnurm & Heitman, 2005). The role of the phytochrome in fungal virulence is not clear.

In *A. alternata,* the link between photoreceptors, light regulation and stress adaptation may explain the observed effects of the inactivation of *fphA* and *lreA* on virulence. The release of spores irrespective of light and darkness and the upregulation of the intracellular concentrations of catalases and superoxide dismutases in the *fphA* mutant could directly lead to higher stress tolerance and better growth in tomato. If the increased tolerance of the *fphA* mutant is responsible for the increase in virulence, the question arises why the *lreA* mutant appeared less pathogenic. The key for the answer to this question could be that spore germination is inhibited by light in an FphA-dependent manner. Fungal spores provide protective cover against environmental assaults. Another reason could be the fact that LreA has a strong impact in the regulation of major development processes including secondary metabolite production and ability to cope with excessive light. In the absence of LreA, and under white light, almost no AOH was produced. In agreement with this hypothesis it was recently shown that AOH is a colonization factor for virulence in *A. alternata*. Moreover, it was shown that the polyketide gene from the melanin biosynthesis gene cluster, *pksA,* was downregulated in the *lreA*-deletion strain (Saha *et al.*, 2012). Melanin can act as a scavenger for reactive oxygen species and thus a reduction of the melanin content should lead to higher sensitivity towards the defense reactions of the tomatoes (Hamilton & Holdom, 1999; Heinekamp *et al.*, 2012). As expected, deletion of *hogA* resulted in compromised lesion development. We observed no significant difference in the virulence pattern of the strains when tomato was inoculated with mycelia plugs with the exception of the *fphA* mutant strain. One possible scenario is that isotopic growth/ spore germination is essential for the activation of transcriptional network

involved in cell wall biogenesis, protein synthesis and protein modification. Importantly, these network can serve as a regulatory unit that governs protective mechanism against stress and environmental stimuli (Rosen *et al.*, 1974; Sephton-Clark *et al.*, 2018). Thus, the conidia are potential target for chemical inhibitors and antioxidants produced by the plant defense system.

Based on this study, we proposed a model of light signalling in *A. alternata* **(Fig.30).** Upon illumination, the WCC complex and phytochrome activates the expression of light-inducible genes via chromatin modification. The red and blue-light plugs into the HOG MAP kinase pathway activating downstream targets and responses. Several developmental processes in *A. alternata* are WCC and phytochrome-dependent though with distinct and overlapping roles.

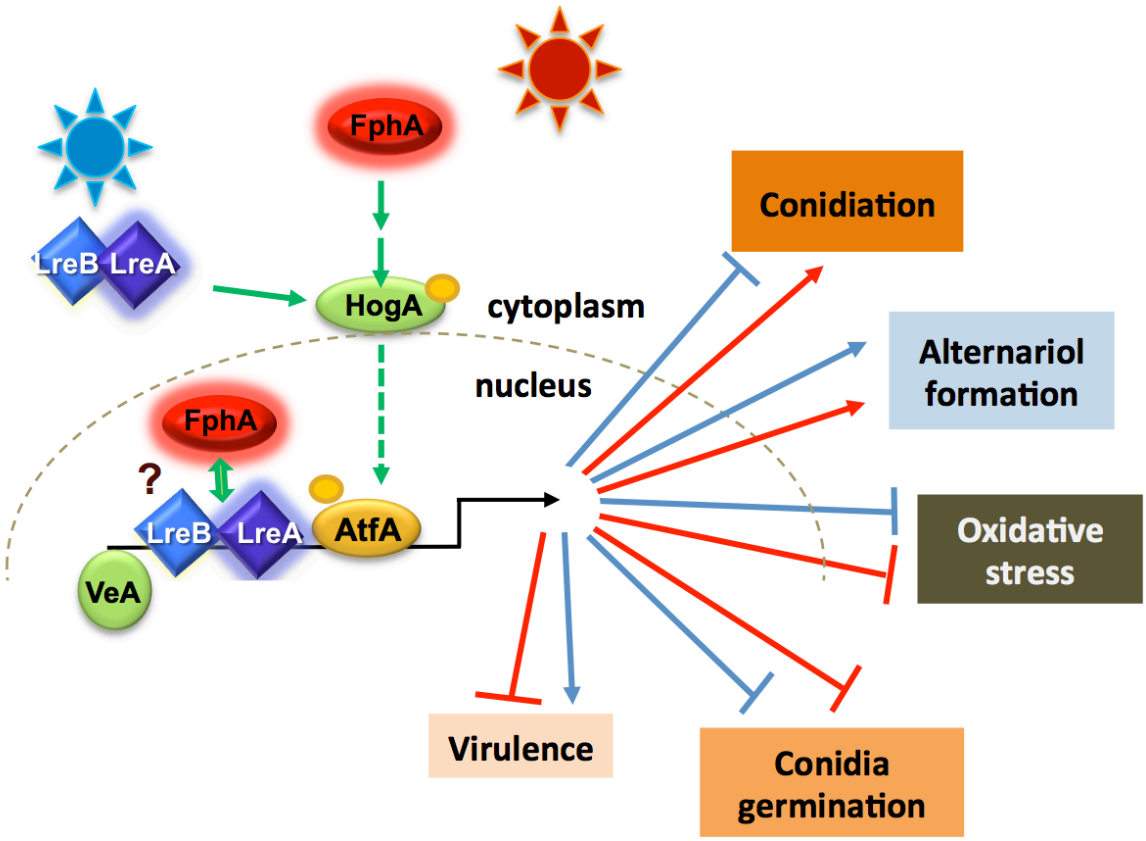


**Fig. 29. Proposed model of light signalling cascades in *A. alternata***. Red and blue light plugs into the HOG MAP kinase cascade activating downstream targets. Phosphorylation of HogA is stimulated by red and blue light and depends on phytochrome and the WC complex. The phytochrome and the WC complex along with VeA play probably regulates the expression of light-inducible genes via chromatin modification. Binding of the WC complex and of VeA to the promoter of light-regulated genes has been shown in *A. nidulans* but not yet in *A. alternata*. Many morphological and physiological processes are controlled in different ways by blue or red light.

# 4. Materials and Methods

## 4.1 Chemicals and equipment used in this study

Chemicals used in this study were purchased from Roth (Karlsruhe), Roche (Mannheim), Sigma Aldrich (Seelze), Sigma (Taufkirchen), Invitrogen (Karlsruhe), Applichem (Darmstadt), IBA (Goettingen) and Serva Feinbiochemica (Heidelberg). DNA polymerases for PCR, restriction endonucleases, makers for DNA and SensiFAST SYBR & No-ROX One-Step Kit for mRNA quantification were manufactured by New England Biolabs (Frankfurt), Fermentas (St-Leon-Rot) and Bioline (Luckenwalde). Other chemicals are indicated in the text. Equipment and kits used in this study are listed in table 1

**Table 1. Equipment used in this study.**

| Equipment | Type | Manufacturer |
|---|---|---|
| **Microscopy** | Axio Imager. Z1<br><br>Nikon Eclipse E200 | Carl Zeiss Microimaging GmbH, Germany<br>Nikon Instruments Europe BV, Amsterdam, Netherlands |
| **Autoclave** | Biomedis | Biomedis Laborservice GmbH, Germany. |
| **Real-Time PCR** | MyiQ™ Single Color Real-Time PCR Detection System | Bio-Rad, U.S. A |
| **Dry oven** | Model 30-1060 | Memmert GmbH, Germany |
| **Thermocycler** | Labcycler & Gradient | SensoQuest GmbH, Germany |
| **Heating block** | Thermo mixer 5436 | Eppendorf, Germany |
| **Digital camera** | Canon PowerShot G15 | Canon, Japan |

| | | |
|---|---|---|
| **Magnetic stirrer** | Heidolph MR 3000 | Heidolph, Germany |
| **Weighing instrument** | Kern 440-47N<br>Satorius R200D | Sartorius, Gottingen<br>Kern, Germany |
| **pH meter** | Hanna HI 208 | Hanna, Romania |
| **Spectrophotometer** | ND- 1000<br>JASCOV-550 | NanoDrop, USA<br>JASCO GmbH, Germany |
| **Centrifuge**<br><br><br><br>**Shaker/incubator** | Centrifuge 5415 R<br>Centrifuge 5415 D<br>Universal 320R<br>Heraeus-Incubators series 6000<br>HT Infors<br>Kelinschutller KM-2 | Eppendorf, Hamburg<br>Eppendorf, Hamburg<br>Hettich, Tuttlingen<br>Kendro, Langenselbold<br>Infors AG, Switzerland<br>Edmund Buhler GmbH, Tubingen |
| **Gel image** | FastGene® FAS-V Imaging System | NEPON Genetics EUROPE GmbH |
| **Power supply apparatus** | Power Pac 3000 | Bio-Rad, Munich |

## 4.2 Microbiological methods

### 4.2.1 *Escherichia coli* transformation and identification of positive clones

Frozen aliquots of competent cells were first thawed on ice for 5- 8minutes, mixed with plasmid DNA or ligation mixture and incubated on ice for 20-30 min. The cells were heat-shocked at 42 °C for 45-60s and afterwards transferred on ice for 5 min. The cells were incubated at 37 °C at 180 rpm for 1 hr in 250 µl of SOC medium. After incubation, culture was pour-plated on agar medium containing ampicillin (100 µg/ml) for selection and incubated overnight at 37 °C. Colonies were picked and cultured in test tubes. Positive clones were determined by digesting the isolated plasmids with enzymes that excise the insert, followed by agarose gel electrophoresis.

**Table 2. *E. coli* growth media.**

| Medium or stock | Composition |
|---|---|
| **SOC** | 2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 2.5 mM KCl, 10 mM $MgSO_4$ and 20 mM glucose. |
| **LB** | 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract and 1 % (w/v) NaCl to pH 7.5 with NaOH. |
| **Antibiotic (1000x)** | 100 µg/ml ampicillin. |

### 4.2.2 *A. alternata* transformation

Fungal spores were harvested from an mCDB culture plate and inoculated into 200 ml liquid mCDB medium for overnight cultivation at 28°C and 180 rpm. The mycelium was harvested by filtering, washed with 0.7 M NaCl, and digested in a Kitalase suspension (150 mg in 15 ml of 0.7 M NaCl) for 1 h with gentle shaking at 120 rpm at 30°C. Protoplast quality and quantity were checked microscopically. Protoplasts were separated from cell fragments by filtering through two layers of Miracloth and precipitated at 2,430 rpm for 10 minutes at room temperature. The Kitalase solution was discarded, and protoplasts were washed once with 10ml of ice-cold 0.7 M NaCl, centrifuged at 2,430 rpm for 10 minutes. The supernatant was discarded, and protoplast resuspended in 400 µl STC solution. Ten micrograms of plasmid DNA was added to the protoplasts, followed by a 10 minutes incubation on ice. DNA uptake was induced with a heat shock at 42°C for 5 min, and after a 5 minutes incubation step on ice. The mixture was transferred to a 50 ml falcon tube and 1 ml of PEG solution were added to the protoplasts, followed by 15 minutes of incubation at room temperature.

The suspension was mixed with 50 ml warm regeneration medium and split into two petri dishes. After overnight incubation at 28°C, the transformation plates were overlaid with 15 ml warm regeneration medium containing hygromycin (80 µg/ml).

**Table 3. Solutions for *A. alternata* transformation.**

| Buffer or Medium | Composition (1 litre) |
|---|---|
| **0.7 M NaCl** | 40.9 g 1M |
| **STC** | 182.2 g sorbitol, 10 ml TrisHCl 1 M (pH 7.5), 10 ml $CaCl_2$1M |
| **PEG in STC** | 40% polyethylene glycol 4000 in STC |
| **Regeneration** | 342.3 g saccharose, 5 g hefeextrakt, 5 g casein-hydrolysat, 7.5 g agar. |
| **Antibiotic (1000x)** | 80 ug/ml hygromycin |

**Table 4. Media and stock solutions for *A. alternata* cultivation.**

| Medium or Stock | Ingredients (1 liter) |
|---|---|
| **mCDB** | 50 ml 20x Salt stock solution, 40 g glucose/10 g glycerol, pH 5.5 |
| **20x Salt stock solution** | 20 g $NaNO_3$, 5 g $NH_4Cl$, 20 g $KH_2PO_4$, 5 g KCl, 5 g NaCl, 10 g $MgSO_4$. $7H_2O$, 0.2 g $FeSO_4$. $7H_2O$, 0.2 g ZnSO4. $7H_2O$ |

**Table 5. Strains used in this study.**

| Strain | Genotype | Source |
|---|---|---|
| **ATCC 66981** | Wild type | Christopher Lawrence (Blacksburgh, VA, USA) |
| **SOI1** | Δ*fphA528* | This study |
| **SOI3** | Δ*lreA3398* | This study |
| **SOI4** | Δ*hogA538* | This study |
| **SOI5** | Δ*fphA528* + *Alt. fphA* | This study |
| **SOI6** | Δ*lreA3398* + *Alt. fphA* | This study |

### 4.2.3 Culture conditions and quantification of conidiophores

*A. alternata* ATC66981 cultures were grown on modified Czapek Dox broth (mCDB) and incubated for 12 days at 28°C. For white-light experiments, a 10-W energy-saving lamp (Flair energy) was used; for red-, far-red-, blue- and green-light conditions, light-proof ventilated boxes with wavelength (680, 740, 450 and 550 nm respectively)-specific LEDs were used. All plates were inoculated with 5x $10^4$ conidial suspension quantification. Conidia were washed and harvested in 1% Tween 20, filtered for separation from the mycelium and diluted. The number of conidia was counted in a Neubauer counting chamber.

### 4.2.4.Assays for cellular stress

Fresh conidia of different strains were collected from cultured grown on mCDB plates incubated at 28°C for 12 days. Drops of conidial suspension containing 5000 of the WT or mutant strains of *fphA*, *lreA* and *hogA* were inoculated on mCDB supplemented with NaCl (0.8 M), KCl (1 M) for salt stress and with $H_2O_2$ (5 mM), menadione (1 mM) for oxidative stress. To assay tolerance to cell-wall degrading agents, Congo Red (0.25 mg/ml) and SDS (0.1 mg/ml) were added to the medium. All cultures were incubated at 28°C for 4 days. The experiments were carried out in triplicate.

### 4.2.5 Germination assay

Fresh conidia containing 1 × $10^5$ fresh conidia of the WT, *fphA lreA* and *hogA* mutant strains were inoculated into liquid minimal medium containing 1% glycerol. Four hundred microliters of the suspension was applied to a sterile coverslip, placed in a 10-mm petri dish, and incubated at 22°C for 2 and 3 h in the dark or under light conditions (blue light [450 nm], green light [550 nm], red light [700 nm], far-red light [740 nm], or white light). To determine the rate of germination, a total of at least 100 spores per sample were examined microscopically. All experiments were carried out in triplicate.

### 4.2.6 Melanin assay

The melanin composition of the WT, *fphA*, *lreA,* and *hogA* mutant strains was analyzed on mCDB liquid medium after incubation at 28°C (shaking culture) for 7 days. Mycelia of the respective strains were filtered and frozen in liquid nitrogen. The frozen mycelia were ground into powder, suspended in NaOH solution, and boiled at 100°C for 2 h. The solution was acidified to pH 2.0 with 5 M HCl and centrifuged at 10,000 × g for 20 min. The resulting melanin solution was dissolved in 2% NaOH, and the absorbance at 459 nm was measured using a spectrophotometer.

### 4.2.7 Extraction and analysis of secondary metabolites by thin-layer chromatography

The secondary metabolites composition of the WT, *fphA*, *lreA,* and *hogA* mutant strains was analysed on mCDB plates grown for 7days at 28°C in darkness or under red, far-red, green, blue or white. Three disks from each plate were excised into 2ml Eppendorf tubes with the back of a blue pipette and the secondary metabolite component extracted by shaking with 1 ml ethyl at room temperature for 1h. The samples were centrifuged at 13000 rmp for 5 minutes and the solvent transferred to new 1.5 ml Eppendorf tubes. The solvent was thereafter vaporized in a speed vac and the pellet resolved in 100 µl ethyl acetate. 20 µl of the crude extract of the WT and mutant strains were applied on TLC plates coated with 0.25 mm silica gel as stationary phase (Pre-coated TLC plates SIL G-25, Macherey- Nagel, Düren, Germany). The

mobile phase composed of toluol, ethylacetate and formic acid (5:4:1). As a standard, TLC-prepared AOH was used. The mycotoxins were visualized using UV light at 254 nm.

### 4.2.8 Immunofluorescence

Fresh conidia were inoculated onto coverslips (placed in Petri dish) with 400 µl mCDB medium and cultivated for 3 h in the dark at room temperature. The samples were exposed to light or kept in the dark for 5 minutes before fixation. Fixation of samples was done in complete darkness with 3.7% formaldehyde in phosphate-buffered saline (PBS) buffer and incubated for 30 min at room temperature. The coverslips were washed three times for 10 minutes with 1x PBS buffer and thereafter transferred to new Petri dishes. The coverslips were incubated with 200 µl digestion solution for 1 h at room temperature. The coverslips were then washed three times for 10 minutes with 1x PBS buffer. The coverslips were incubated for 10 minutes at -20°C with precooled methanol. The methanol was removed, and the coverslips were washed two times for 5 minutes with 1x PBS. 200 µl blocking solution were added to the coverslips and incubated for 15 minutes. The coverslips were transferred to new Petri dishes and incubated with antiphospho-p38 MAP kinase (Thr180/Tyr182) antibodies (no. 9211, 1:400 dilution; Cell Signaling Technology, Beverly, MA) in TBST buffer with 5% BSA overnight at 4°C and washed three times for 10 minutes with TBST afterwards. Cy3-conjugated anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) was used at a 1:200 dilution in 5% BSA in TBST. After 1 h of incubation, the coverslips were washed three times with TBST and mounted on microscope slides for observation.

### 4.2.9 Virulence assay

Virulence assay were conducted on pre-wounded tomato inoculated with conidial suspension as described previously with little modification (Zhang et al., 2014). Prior to inoculation, tomato was surface disinfected with 70% ethanol and carved by a sterile scalpel in a length of 1cm deep. 10 µl of fresh conidial suspension containing $10^5$ of WT and mutant strains were inoculated on pre-wounded tomato. In the case of mycelial plug test, tomato was wounded with a cork borer 5 mm in diameter. Wounds were inoculated with mycelium plugs from 36h actively growing plates of the WT and mutant

strains. All samples were incubated at 20°C for 7 days for lesion development. Lesion on the surface of the tomato were measured manually while the extent of colonization was recorded using the Image J software.

## 4.3 Molecular biological methods

### 4.3.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed according to the manufacturer protocol with Q5 polymerase from New England BioLabs (Germany). Oligonucleotides were synthesized by Eurofins Genomics (Germany). dNTPs were purchased from Roth (Karlsruhe). The PCR program was modified based on the length of the amplicon and Tm of the primers. The program included a denaturation, initial step for 3 min at 98°C; all following denaturation steps for 20 s at 98°C; annealing, 5 cycles at 65°C for 20 s, 5 cycles at 63°C for 20 s, 25 cycles at 58°C for 20 s; and elongation, 10 s at 72°C. Standard reaction mixture volumes were 50 µl, including 1 U Q5 reaction buffer, 200 µM dinucleoside triphosphates (dNTPs), 0.5 µM primers, 1 U Q5, and 100 ng of plasmid DNA. The primers used in this study are listed in **Table 6.**

### 4.3.2 CRISPR-Cas9 plasmid construction

The CRISPR-Cas9 vectors with specific sgRNA genes, containing the respective protospacer sequences as well as a 6-bp inverted repeat of the end of the protospacer to complete the hammerhead cleavage site, were generated in a single cloning step. New protospacer sequences were inserted into the linearized pFC332 vector by combining two PCR fragments amplified from plasmid pFC334 and the pFC332 vector in a NEBuilder reaction (New England BioLabs, Frankfurt, Germany). The primers, which contain the variable regions, used to generate the sgRNA gene fragments were obtained from MWG Eurofins and listed in **Table 6**. The amplified fragments were flanked by 30-bp complementary sequences to each other and the linearized vector in order to generate the functional vectors in a single NEBuilder reaction. The fragments were amplified from pFC334 with proofreading polymerase Q5 (NEB) by a touchdown PCR program (denaturation, initial step for 3 min at 98°C; all following denaturation steps for 20 s at 98°C; annealing, 5 cycles at 67°C for 20 s, 5 cycles at 65°C for 20 s, 25 cycles at 63°C for 20 s; and elongation, 10 s at 72°C). Standard reaction mixture

volumes were 50 µl, including 1 U Q5 reaction buffer, 200 µM dinucleoside triphosphates (dNTPs), 0.5 µM primers, 1 U Q5, and 100 ng of plasmid DNA. Plasmid pFC332 was linearized using PacI and assembled with the PCR fragments, following the NEBuilder protocol.

**Table 6. Oligonucleotides used in this study.** The red letters indicate the protospacer sequences.

| Oligonucleotide | Sequence 5' - 3' |
|---|---|
| **Crispy 2.0_fwd** | GGTCATAGCTGTTTCCGCTGA |
| **Crispy 2.0_rv** | TGATTCTGCTGTCTCGGCTG |
| **Proto_fphA_fwd** | GTCCGTGAGGACGAAACGAGTAAGCTCGTCCAACTCTGGTCCTCCTCTACGTTTTAGAGCTAGAAATAGCAAGTTAAA |
| **Hh_fphA_rv** | GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCACAACTCCGGTGATGTCTGCTCAAGCG |
| **Proto_lreA_fwd** | GTCCGTGAGGACGAAACGAGTAAGCTCGTCAATAGTCGCGCAGAACGACAGTTTTAGAGCTAGAAATAGCAAGTTAAA |
| **Hh_lreA_rv** | GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAAATAGTCGGTGATGTCTGCTCAAGCG |
| **Proto_hogA_fwd** | GTCCGTGAGGACGAAACGAGTAAGCTCGTCCAAGGACCAGCTTACTAGCCGTTTTAGAGCTAGAAATAGCAAGTTAAA |
| **Hh_hogA _rv** | GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCACAAGGACGGTGATGTCTGCTCAAGCG |
| **fphA_test_fw** | CGTACTCTCGTCACGAGCAAG |
| **fphA_test_rv** | CTTGACGTCTAGTGCTTGGCT |
| **lreA_test_fw** | TCTGCTTGGCTGGGACATG |
| **lreA_test_rv** | GTGGCGGGATGAAGCCTT |

| | |
|---|---|
| **hogA_test_fw** | ATCGCATTTGGTGCCTGCC |
| **hogA_test_rv** | AGCATGATCTCAGGGGCTC |
| **lreA_comp_fw** | GACCGGTGTTTATCGTCTCAG |
| **lreA_comp_rv** | GAAGCGAAGCAAGGCAAGAC |
| **fphA_comp_fw** | TACTCTCGTCACGAGCAAGTG |
| **fphA_comp_rv** | TGACGTCTAGTGCTTGGCTTG |
| **ccgA_RT_fw** | GTCAACTCTGTCAAGAACGC |
| **ccgA_RT_rv** | TTGATCTTGTCACCAGCAGC |
| **h2B_RT_fw** | ACAAGAAGAAGCGCACCAAG |
| **h2B_RT_rv** | CGTTGACGAAAGAGTTGAGAA |
| **fer_RT_fw** | TGGACCCTATATTGCACGGAG |
| **fer_RT_rv** | GGTGTTCGGACCATTTCCTGA |
| **bliC_RT_fw** | GACCCCTACACGCAGAAGAA |
| **bliC_RT_rv** | GGTCAACAGGCAGAACTTGGT |
| **AAT_PTO2522_RT_fw** | GTCAACGGTGCTAAGGTGTAC |
| **AAT_PTO2522_RT_rv** | CCTGCAATGTTCTGACCATGC |
| **hogA_RT_fw** | CCTGAAATACGTCCACTCCG |
| **hogA_RT_rv** | GAGACCGAAGTCGCAAATCTG |
| **atfA_RT_fw** | CACCGTACACAACCCATTCTC |
| **atfA_RT_rv** | CTCCAGGTGTCTGCAAGTTTC |
| **catA_RT_fw** | GGCATTCTTACCGACACATCG |
| **catA_RT_rv** | TGTGTAGAACTTGACGGCGAAA |
| **catD_RT_fw** | CAACGTCTCCCTCGACAAG |
| **catD_RT_rv** | CAGTGAGAAGCATCAAGTCGG |

| | |
|---|---|
| **sodA_RT_fw** | ACACCACCATCTCATGGAACAT |
| **sodA_RT_rv** | CGTGTGTCTTGTTGTGGGGTT |
| **sodB_RT_fw** | GAGGCCAAGCAAAAGGAAGAC |
| **sodB_RT_rv** | GAAGAGGCTGTGGTTGATGTG |
| **sodC_RT_fw** | CATCAACCACTCGCTCTTCTG |
| **sodC_RT_rv** | GAACTTGTCCTCATCACCCC |
| **sodE_RT_fw** | GAGGCCAAGCAAAAGGAAGAC |
| **sodE_RT_rv** | GAAGAGGCTGTGGTTGATGTG |
| **pksI_RT_fw** | GGAAAACGTCACTTGGTGGA |
| **pksI_RT_rv** | TGTGCCTCTCGCAATTAGGA |
| **abaA_RT_fw** | CAAAAGTCACAACGTCACGGC |
| **abaA_RT_rv** | GTAGGTTCCTGTAGTGACGCA |
| **wetA_ RT_fw** | TACAATCAACTGAGGCACCGT |
| **wetA_ RT_rv** | AGCTGAAGGTTGGTGTTGAG |
| **flbC_ RT_fw** | CAATGGCAGTCGATAACGT |
| **flbC_ RT_rv** | GTGTGGTTGTTGTTGAGGGTA |
| **flbD_ RT_fw** | TCTGCATTGTTTTGGTGACCG |
| **flbD_ RT_rv** | GTCACAGCACGAGGATCAG |
| **csp-1_RT_fw** | GGTCGCAGAGTGTCATTGC |
| **csp-1_RT_rv** | CTGGAGGTCCTAGACTGGA |
| **atg1_RT_fw** | AGACCATGAGGAAAACGCCG |
| **atg1_RT_rv** | AATGCCTGGGACTCACGG |

### 4.3.3 Purification of genomic DNA

Prior to DNA purification, samples were loaded on agarose gel after PCR to check for purity and identify DNA fragments. 2 % Midori Green Advance DNA Stain (NIPPON Genetics EUROPE GmbH) which emits green fluorescence when bound to DNA were added to the gel. 1kb DNA ladder was loaded on the gel as a DNA size standard marker. Gels were run at 100 V – 135 V for about 20-30 minutes until and DNA was examined using the FastGene® FAS-V Imaging System (NIPPON Genetics EUROPE GmbH). DNA purification of fragments were done according to the manufacture's protocol using the Zymoclean Gel Recovery kit (Zymo Research, USA). The concentration of the purified DNA fragments was determined by NanoDrop 1000 (NanoDrop, USA).

**Table 7. Solutions for DNA agarose gel electrophoresis.**

| Solution | Composition |
|---|---|
| **50x TAE buffer** | 40 mM Tris-acetate, 1 mM EDTA, pH 8.0 |
| **6x Loading buffer** | 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol |

### 4.3.4 DNA digestion, cloning and sequencing

Plasmids or DNA fragments were digested with appropriate restriction enzymes according to manufacturer's protocol. Digestions was performed in 50 µl total volume and incubated at 37°C for 1 h. Fragments of DNA to be used for ligation was extracted from the gel as described in 4.3.5. Cloning of fragments was performed using NEBuilder HiFi DNA Assembly kit. The molar ratio of vector to insert was 1:1 in a volume of 20 µl incubated at RT for 30 minutes. For blunt end fragments, pJET1.2/blunt cloning vector was used. After the ligation, the sample was transformed to competent cells. Plasmid or genomic DNA fragments used for sequencing were amplified with Q5 polymerase. Premixed DNA fragments (6ng/ul) in total volume of 15 µl with sequencing

primers were used for sequencing. DNA sequencing was done by commercial sequencing (MWG Biotech, Ebersberg).

### 4.3.5 Plasmid DNA extraction from *E. coli*

Plasmids used in this study were isolated from *E. coli* cells using a NucleoSpin Plasmid EasyPure kit (MACHENEREY-NAGEL, Düren). For quick and efficient way to extract *E. coli* plasmid DNA without commercial kits, the akali-lysis method was used (Sambrook *et al*., 1989). *E. coli* was grown overnight in LB medium with appropriate antibiotics at 37°C. Cells were harvested in 1.5 ml Eppendorf tube by centrifugation at 13000 rmp for 1 minute. The pellet was suspended in 200 µl Tris-EDTA buffer, mixed by pipetting or vortex. 200 µl of alkali buffer were added and mixed gently. 200 µl neutralization buffer were added to the mixture, inverted 4-6 times. The samples were incubated on ice and afterwards centrifuged at 13000 rmp for 10 minutes. 800 µl of the supernatant were transferred to a new 1.5 ml Eppendorf tube with 800 µl isopropanol. Samples were gently inverted and placed on ice for 30 minutes. Samples were centrifuged at 13000 rmp for 10 minutes at 4 °C. Supernatants were discarded and pellets were washed with 500 µl of 70% ethanol. Samples were centrifuged at 13000 rmp for 5 minutes at 4 °C. Eppendorf were left open at room temperature to dry the pellet. The pellets were eluted in 20 µl TE buffer.

**Table 8. Solutions used for Plasmid DNA extraction (Alkali-lysis method).**

| Solution | Composition |
|---|---|
| **Tris-EDTA buffer** | 2 ml 0.5 M EDTA (pH 8.0), 5 ml Tris-HCl (pH 7.5), 10 mg RNase in 100 ml |
| **Alkali-lysis buffer** | 0.2 M NaOH, 1% SDS |
| **Neutralization buffer** | 1.5 M Potassium acetate, pH 4.8 |
| **TE Buffer** | 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 |

**Table 9. Plasmids used in this study.**

| Name | Genotype | Source |
|---|---|---|
| **POI1** | *fphA* Cas 9 protospacer in pFC 332 vector | This study |
| **POI2** | *lreA* Cas 9 protospacer in pFC 332 vector | This study |
| **POI3** | *hogA* Cas 9 protospacer in pFC 332 vector | This study |
| **PO14** | *fphA* gene in pJet 1.2 vector | This study |
| **PO15** | *lreA* gene in pJet 1.2 vector | This study |
| **PO16** | *hogA* gene in pJet 1.2 vector | This study |

### 4.3.6 Isolation of genomic DNA from *A. alternata*

To a 1.5-mL Eppendorf tube containing 700 µl of lysis buffer, a small lump of mycelia from young culture is added by using a sterile toothpick, with which the lump of mycelia is disrupted or grind into powder using liquid nitrogen. The samples were incubated at 68°C in a thermos mixer (gently shaking) for 30 minutes. After adding 375 µl of 8 m potassium acetate, samples were kept on ice for about 25-30 minutes and thereafter centrifuged at 13, 000 rpm for 20 minutes. 700 µl of supernatant were transferred to another 1.5-mL Eppendorf tube with an equal volume of isopropyl alcohol added. The tubes were mixed by inversion briefly and samples were incubated for 15 minutes at room temperature. The samples were centrifuged at 13000 rmp for 10 minutes at 4 °C. The supernatants were discarded, and the resultant DNA pellets were washed in 200 µl of 70% ethanol. After the pellets was centrifuged at 13000 rmp for 5 minutes, the supernatants were discarded. The DNA pellets were air dried and dissolved in 50 µl of deionized H2O or TE buffer. 1 µl of the DNA was used in 25 to 50 µl of PCR mixture.

**Table 10. Solutions used for genomic DNA extraction**

| Solution | Composition |
|---|---|
| **Lysis buffer** | 50 mM EDTA, 50 mM pH 7.5 Tris-HCl |
| **8 M Potassium acetate** | 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of distilled water |

### 4.3.7 RNA isolation and quantitative real-time PCR

Fresh conidia were inoculated on the surface of liquid mCDB medium in a petri dish and incubated for 36 h in darkness at 28°C. Young mycelial mat was harvested in complete darkness or after 30 minutes of illumination (with white-light, red or blue LED lamps depending on the experiment) and frozen immediately in liquid nitrogen and stored at -80°C until RNA isolation. Frozen mycelia were ground into powder, and total RNA isolated using the E.Z.N.A. fungal RNA minikit (VWR). The isolated RNA was quantified, and an aliquot purified with RNA TURBO DNA- free KIT. RNA samples were diluted to a final concentration of 50 ng/ µl. Quantitative real-time PCR reactions were carried out using gene- specific primers **(Table 8),** with SensiFAST SYBR & No-ROX One-Step Kit from Bioline (Luckenwalde, Germany). Each reaction mixture is 25 µl with 0.2 µm primers and 100ng RNA. RT-qPCR was performed as follows : incubation for 10 min at 50 °C for the reverse transcription reaction, then 5 min at 95 °C for the inactivation of reverse transcriptase , followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 58 °C for 30 s and extension at 72 °C for 15 s, followed by a melting curve analysis in order to check the specificity of fragment amplification. After each PCR, we performed melting curve analyses to show the specific amplification of single DNA segments and the absence of nonspecifically amplified DNA. Transcript levels of the target genes were normalized against with histone *H2B* gene expression. All of the measurements were repeated twice, each with three biological replicates.

### 4.3.8 Transcriptional profiling of osmotic and oxidative stress-associated genes

In order to investigate osmotic and oxidative stress responses, transcripts of genes induced by osmotic and oxidative agents were analysed. Prior to the addition of the stress agents, fresh conidia were inoculated in a 50-ml flask liquid mCDB medium and incubated for overnight at 28°C and 180 rpm. The culture was then supplemented with 0.7 M NaCl or 4 mM $H_2O_2$ and further shaken for 30 min. Total RNA isolation and real-time PCR with respective primers for the genes of interest were performed as described above.

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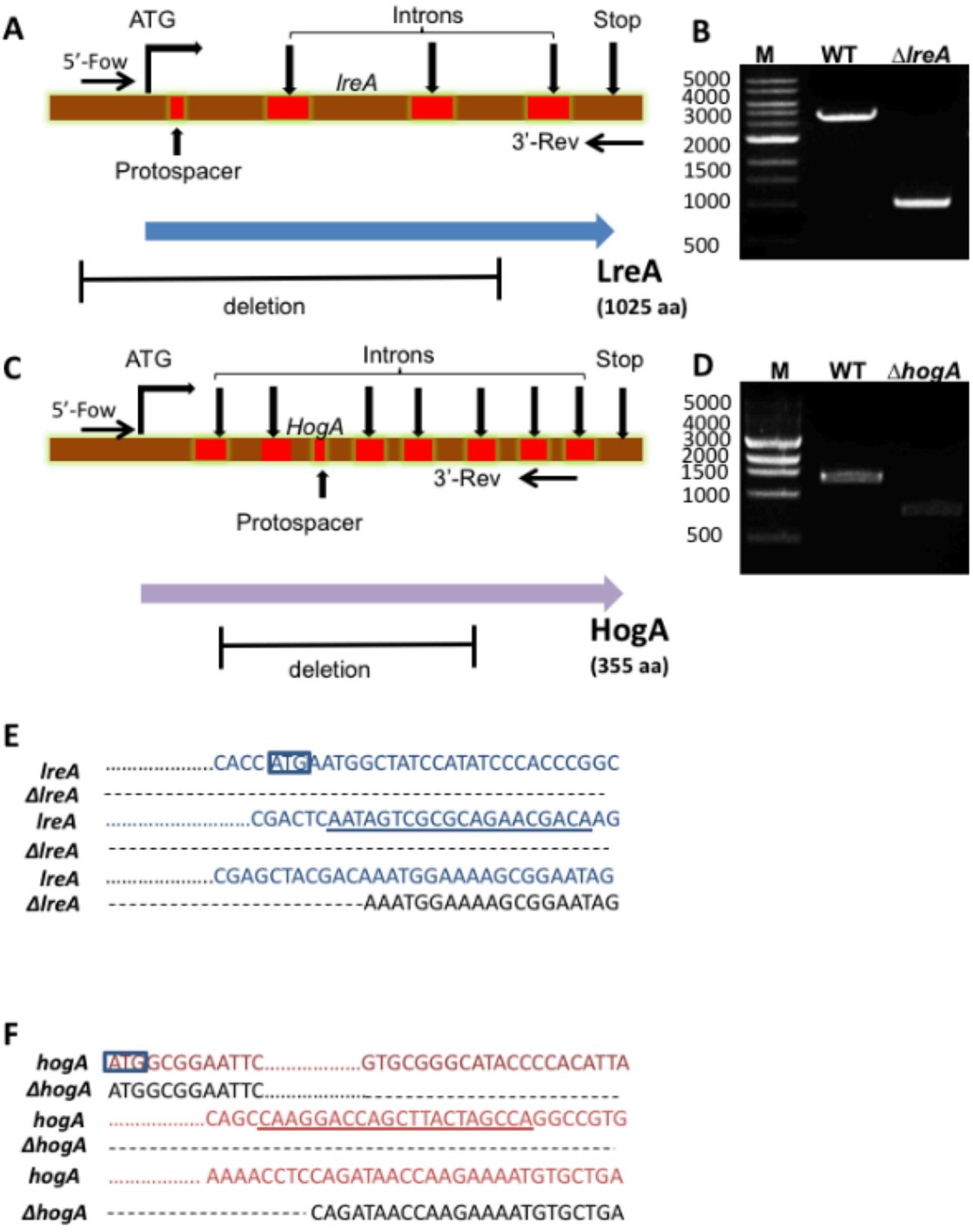
# Appendix




**Suppl. Fig. S1: Inactivation *of lreA* and *hogA* using the CRISPR/Cas9 technology. (A)** Scheme of the *lreA* locus with the protospacer used for inactivation indicated. The shown primers were used to amplify the region in WT and the Δ*lreA* mutant **(B)**. **(C)** Scheme of the hogA locus with the protospacer used for inactivation indicated. The shown primers were used to amplify the region in WT and the Δ*hogA* mutant **(D). (E, F)** Sequence comparison of the WT

loci and the corresponding mutants. The dashed line shows the missing nucleotides (4,410 bp for *IreA* and 1,108 bp for *hogA)*. The dotted line represents 949 nucleotides, which were not displayed here.

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RESEARCH ARTICLE
Applied and Environmental Science

# Red- and Blue-Light Sensing in the Plant Pathogen *Alternaria alternata* Depends on Phytochrome and the White-Collar Protein LreA

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**ABSTRACT** The filamentous fungus *Alternaria alternata* is a common postharvest contaminant of food and feed, and some strains are plant pathogens. Many processes in *A. alternata* are triggered by light. Interestingly, blue light inhibits sporulation, and red light reverses the effect, suggesting interactions between light-sensing systems. The genome encodes a phytochrome (FphA), a white collar 1 (WC-1) orthologue (LreA), an opsin (NopA), and a cryptochrome (CryA) as putative photoreceptors. Here, we investigated the role of FphA and LreA and the interplay with the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway. We created loss-of function mutations for *fphA*, *lreA*, and *hogA* using CRISPR-Cas9 technology. Sporulation was reduced in all three mutant strains already in the dark, suggesting functions of the photoreceptors FphA and LreA independent of light perception. Germination of conidia was delayed in red, blue, green, and far-red light. We found that light induction of *ccgA* (clock-controlled gene in *Neurospora crassa* and light-induced gene in *Aspergillus nidulans*) and the catalase gene *catA* depended on FphA, LreA, and HogA. Light induction of *ferA* (a putative ferrochelatase gene) and *bliC* (*bli-3,* light regulated, unknown function) required LreA and HogA but not FphA. Blue- and green-light stimulation of alternariol formation depended on LreA. A lack of FphA or LreA led to enhanced resistance toward oxidative stress due to the upregulation of catalases and superoxide dismutases. Light activation of FphA resulted in increased phosphorylation and nuclear accumulation of HogA. Our results show that germination, sporulation, and secondary metabolism are light regulated in *A. alternata* with distinct and overlapping roles of blue- and red-light photosensors.

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