Fungal Biology 124 (2020) 447–457

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

On the role of the global regulator RlcA in red-light sensing in *Aspergillus nidulans*

Zhenzhong Yu [a, b], Jennifer Hübner [a], Satur Herrero [a], Victor Gourain [c], Reinhard Fischer [a, *]

[a] Karlsruhe Institute of Technology (KIT) - South Campus Institute for Applied Biosciences Dept. of Microbiology, Fritz-Haber-Weg 4, D-76131, Karlsruhe, Germany
[b] Nanjing Agricultural University, Jiangsu Provincial Key Lab of Organic Solid Waste Utilization, College of Resources and Environmental Sciences, 210095, Nanjing, China
[c] Institute of Toxicology and GeneticsKarlsruhe Institute of Technology Hermann-von-Helmholtz-Platz 1, D-76344, Eggenstein-Leopoldshafen, Germany

ARTICLE INFO

Article history:
Received 28 October 2019
Received in revised form
17 December 2019
Accepted 21 December 2019
Available online 10 January 2020

Corresponding Editor: D.E.N. Rangel

Keywords:
Bye 1
Chromatin remodeling
Light signaling
PHD finger protein
Phytochrome
SPOC domain

ABSTRACT

A large proportion of fungal genomes are under the control of light. Most fungi employ complex light sensing systems, consisting of red-, blue-, and in some cases green-light photoreceptors. Here we studied the light response in *Aspergillus nidulans*. In a genetic screen, followed by whole-genome sequencing we identified a global regulator, which appears to be involved in chromatin structure modification. We therefore named the protein RlcA (regulator of light sensing and chromatin remodeling). The protein comprises a nuclear localization signal, a PHD (plant homeodomain) finger, a TFSII (found in the central region of the transcription elongation factor S-II), and a SPOC domain (Spen paralog and ortholog C-terminal domain). In the mutant, where light-controlled genes were constitutively active, the SPOC domain is missing. RlcA localized to the nucleus and interacted with the phytochrome FphA. The PHD-finger domain probably binds to trimethylated lysine 4 of histone H3, whereas the TFSII domain binds RNA polymerase II. The SPOC domain could mediate interaction with a global repressor protein. In the mutant, repressor recruitment would be hindered, whereas in the wild type repressor release would be induced after light stimulation. Our results add another layer of complexity to light sensing in filamentous fungi.

1. Introduction

Most fungi contain several photosensory proteins to respond and adapt to different environmental conditions (Corrochano, 2011, 2019; Fischer et al., 2016; Rodriguez-Romero et al., 2010; Yu and Fischer, 2019; Schumacher and Gorbushina, 2019; Schumacher, 2017). At the molecular level, the blue-light response has been studied first with ground-breaking work in *Neurospora crassa* (Ballario et al., 1996; Linden and Macino, 1997). It turned out that the two LOV-domain proteins, WC-1 and WC-2 are central components which form the White-Collar Complex (WCC). Both are transcription factors, and WC-1 contains a flavin as chromophore (Froehlich et al., 2002; He et al., 2002). In *N. crassa* almost all light-induced processes respond to blue-light and are likely to be under control of the WC system. The WC light-sensing system is essentially conserved in most filamentous fungi and received additional attention because the WCC is a central component of the circadian clock (Larrondo et al., 2015; Dunlap and Loros, 2017).

In addition to the WC system, many fungi encode one to three phytochromes in their genomes (Rodriguez-Romero et al., 2010; Schumacher and Gorbushina, 2019). Their molecular functions have been studied first in *Aspergillus nidulans* and in *N. crassa* (Blumenstein et al., 2005; Froehlich et al., 2005). In earlier work in *A. nidulans* it was described that the balance between asexual and sexual development was a typical red-light effect, which could be reversed by far-red light exposure (Mooney and Yager, 1990). Not surprisingly, these effects were changed in a phytochrome mutant (Blumenstein et al., 2005). In contrast, deletion of the *N. crassa* phytochromes had only minor effects on the biology (Froehlich et al., 2005; Wang et al., 2016). *A. nidulans* and *N. crassa* are hence two examples where red or blue light appear to play the main role in light sensing. In *Alternaria alternata,* a wide-spread

* Corresponding author. Fax: +49 721 6084 4509.
E-mail addresses: yuzhenzhong@njau.edu.cn (Z. Yu), reinhard.fischer@KIT.edu (R. Fischer).

https://doi.org/10.1016/j.funbio.2019.12.009
1878-6146/

contaminant of food and feed, both light qualities appear to be more interlinked (Igbalajobi et al., 2019).

There is also good evidence that light-dependent gene induction requires chromatin remodeling. In *N. crassa* it was shown that the histone acetyl transferase, NGF-1 is required for WC-dependent gene induction (Grimaldi et al., 2006; Brenna et al., 2012). In *A. nidulans*, it was shown that induction of *ccgA*, a light-induced gene, is accompanied by histone H3 acetylation at lysine 9. The acetylation was dependent on phytochrome, FphA, and the WC-1 protein, LreA. It was also shown that FphA interacts with the acetyl transferase, GcnE (the ortholog of *N. crassa* NGF-1), and LreA with the histone deacetylase HdaA (Hedtke et al., 2015). There is evidence that LreA together with HdaA keep the level of acetylation low in darkness and that FphA together with GcnE cause acetylation in light.

In order to understand the signaling events of the phytochrome response in *A. nidulans*, a mutant screening has been performed (Yu et al., 2016). This genetic screen was based on a light-regulated gene, which was identified previously (Hedtke et al., 2015; Ruger-Herreros et al., 2011). The promoter of one of the light-responsive genes, *conJ*, was fused to the nutritional marker gene *pyr-4*. Because the light-driven *pyr-4* gene was the only gene encoding orotidine 5′-phosphate decarboxylase, the strain was only able to grow on minimal medium supplemented with uracil and uridine. Under light conditions, where the *conJ* promoter was induced, the strain grew well on minimal medium without uracil and uridine. Several mutants were isolated which lost the ability to grow under those conditions. Addition of uracil and uridine to the medium restored growth of some of those mutants, suggesting that the growth defect was only due to mutations in light sensing and hence *conJ* promoter induction and not due to other mutations in the genome. The corresponding mutation of one of the mutants was identified by whole-genome sequencing in the *hogA/sakA* gene (Yu et al., 2016). This result linked light sensing to stress sensing. This link is also conserved in *A. alternata* (Igbalajobi et al., 2019). In this paper we used a different mutagenesis approach to identify mutations which cause constitutive induction of the light-inducible promoter independent of light.

2. Materials and methods

2.1. Strains and culture conditions

Supplemented minimal media for *A. nidulans* were prepared as previously described, and standard strain construction procedures were used (Käfer, 1977). A list of *A. nidulans* strains used in this study is given in Table 1.

2.2. Generation of constitutive mutants with UV-mutagenesis

Fresh spores were washed off from a newly-prepared plate with distilled water and the spore suspension in a 10 ml falcon tube was incubated on ice for 20 min. The supernatant was moved into a new falcon tube. The concentration of conidia was diluted to 1.2×10^4 conidia/ml. 400 μl conidia suspension were added to a Petri dish (14 cm diameter) with 100 ml solid minimal medium supplemented with p-aminobenzoic acid (PABA). Each plate was treated with 0.015 J UV-C light resulting in a survival rate of 25 %. After UV treatment, the plates were incubated in the dark at 37 °C for 3 d to screen for ura-prototrophic (constitutive) mutants.

2.3. Mutant analysis using next generation sequencing (NGS)

The constitutive mutant was back-crossed twice. First, the mutant MD3-8 was crossed to SZY64 and then one progeny (PD-6) with the constitutive phenotype to the parent strain using standard protocols (Todd et al., 2007). The progeny was sorted according to the phenotype. Fifty four bigger colonies (constitutive growth phenotype) and 35 small colonies (like the parent strain) were propagated for genomic DNA isolation. The spores of each progeny were inoculated on the surface of supplemented liquid minimal medium and incubated overnight at 37 °C. The mycelia from all progeny of one class were mixed before grinding in liquid nitrogen. The mixed genomic DNA was subject to whole genome sequencing. Sequencing libraries were prepared from 1 μg of gDNA with the TruSeq Nano

Table 2
Oligonucleotides used in this study. Restriction sites are indicated in small letters.

Name	Sequence (from 5′ to 3′)
AN1777AscI fw	ggcgcgcc ATG GCT GGT AAG CTC AGT ACT AT
AN1777 PacI rev	ttaattaa CTT CAA GAG TCT TCA GTC GTT C
AN1777full AscI	ATA ggc gcg cc GTA CCA CAC CCT GAA ATG CC
AN1777full PacI	CTA tta att aa GTA GCT AGT AAA TGT CGC ATA GG
h2b-RT-F	CTGCCGAGAAGAAGCCTAGCAC
h2b-RT-R	GAAGAGTAGGTCTCCTTCCTGGTC
ccgA-RT-F	CGACGCTTCCCTCACTTCTC
ccgA-RT-R	CATCATGGGACTTCTCGTCCTT
conJ-RT-F	CTGAGAAGCAGCGCAACAT
conJ-RT-R	CTCATCGCCAGGCTGGAA

Table 1
Strains used in this study.

Strain	Genotype	Reference
A. nidulans		
SKV103	*pyrG89; pyroA4; veA*$^+$	Vienken & Fischer (2006)
SKV104	*yA2, pyrG89, pabaA1; pyroA4, veA*$^+$	Yu et al. (2016)
SJR2	*pyrG89; pyroA4, nkuA::bar; veA*$^+$	Herr & Fischer (2014)
SJR3	*argB2; pyroA4, nkuA::bar; veA*$^+$	Yu et al. (2016)
SZY17	SKV104 transformed with pZY19 (*conJ(p)::pyr4, pyroA*); *veA*$^+$	Yu et al. (2016)
SZY64	*SZY17 crossed to SJR3: argB2; pyroA4, nkuA::bar; veA* + ; pZY19 (*conJ(p)::pyr4, pyroA)*	This study
MD3-8	SZY17 treated with UV light	This study
MD5-1	SZY17 treated with UV light	This study
MD5-15	SZY17 treated with UV light	This study
MD6-5	SZY17 treated with UV light	This study
MD3-8c	*MD3-8 complemented with rlcA*	This study
SJH4	*pyrG89; pyroA4, nkuA::bar; alcA(p)::GFP::rlcA; AfpyrG; veA*$^+$	This study
SJH6	*pJH55 in SJR2 (rlcA::GFP::pyrG)*	This study
SJH41	*pJP5 (alcA(p)::fphA::YFP-C, pyr4) and pJH99 (alcA(p)::rlcA::YFPN, pyroA) in SKV103*	This study
SJH14	*pJP5 (alcA(p)::fphA::YFP-C, pyr4) und pJH71 (alcA(p)::rlcA*$^{1-381}$*::YFPN, pyroA) in SKV103*	
E. coli		
Top 10	F- mcrA cr mrr-hsdRMS-mcrBC) Φ 0lacZac hsd lacX74 recA1 araD139 sd araleu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen, Leek, NL

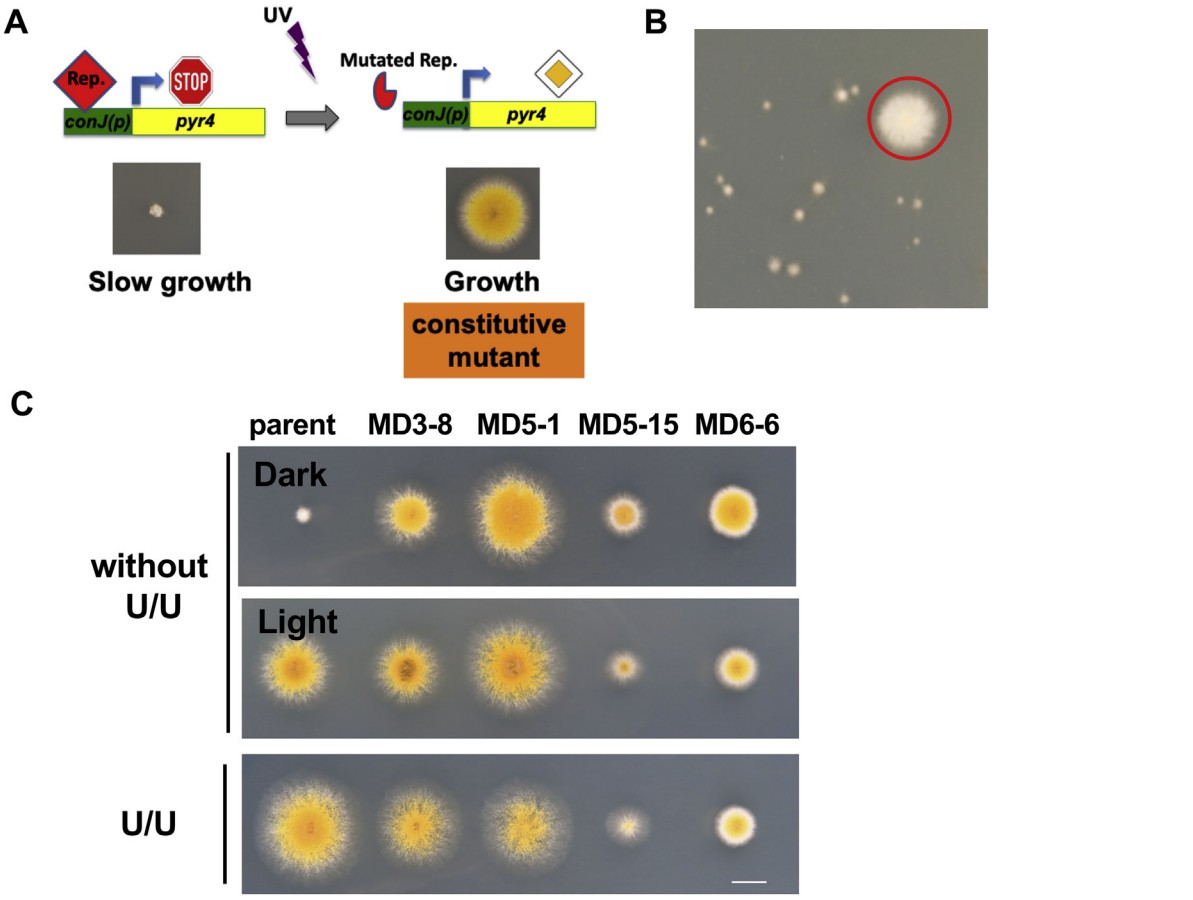

Fig. 1. Screening for constitutive mutants. **(A)** Screening strategy for constitutive mutants by UV-mutagenesis. A hypothetical repressor (Rep.) inhibits transcription of the reporter gene *pyr4*. After mutagenesis of the repressor protein, transcription is possible. **(B)** Phenotype of the mutants on minimal medium agar plates without uracil and uridine after 3 d incubation in the dark. The bigger colony (constitutive mutant) is circled in red. **(C)** The parent strain and the four constitutive mutants MD3-8, MD5-1, MD5-15 and MD6-6 were incubated three d on minimal medium agar plates with or without uracil and uridine (U/U) in the dark or in light.

DNA kit (Illumina), following manufacturer's protocol. Quality and quantity of library products were both assessed with a BioAnalyzer (Agilent) on a high sensitivity DNA chip. Clusters were generated from 10 pM of libraries in one lane of a high throughput flowcell (Illumina). Libraries were then sequenced in paired-end reads, of 50 bp in length, with a HiSeq 1500 (Illumina). The quality of raw sequencing data was assessed with the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were mapped against the reference genome of *A. nidulans* with Bwa (Li and Durbin, 2009). Read mapping was refined with Picard (http://broadinstitute.github.io/picard). Mutation candidates were detected with GATK (McKenna et al., 2010) and visually curated in the IGV genome browser (Robinson et al., 2011). The nucleotide sequence of *rlcA* is available in the Third Party Annotation Section of the DDBJ/ENA/GenBank databases under the accession number TPA: BK011994.

2.4. Plasmid constructions

2.4.1. GFP tagging of RlcA

One kb of the *rlcA* gene was amplified using the oligonucleotides AN1777AscIfw and AN1777PacIrev and cloned as *Asc*I-*Pac*I fragment into vector pMCB17apx. The circular plasmid (pJH54) was used to transform SJR2 for the construction of strain SJH4. After homologous integration into the *rlcA* locus the GFP fusion protein is under control of the *alcA* promoter. For the expression of the RlcA-GFP fusion protein under the control of the endogenous promoter, 1.2 kb of the left border and 1.2 kb of the region before the stop codon of *rlcA* were amplified by PCR. A GFP, pyrG cassette (2.6 kb) was also amplified, fused by PCR and ligated into pJet. The plasmid (pJH55) was verified by digestion and transformed into SJR2 (SJH6).

2.4.2. Complementation of the rlcA mutant

The *rlcA* gene, including 700 bp upstream and 500 bp downstream of the ORF, was amplified by PCR using oligonucleotides AN1777fullAscI and AN1777fullPacI and cloned into a transformation vector.

2.4.3. Split YFP experiments

The complete *rlcA* ORF (2838 bp) was cloned as *Asc*I and *Pac*I fragment into the destination vector (pMMCB17apx with GFP exchanged to the N-terminal half of YFP). The plasmid (pJH99 or pJH71) was combined with pJP5 (Purschwitz et al., 2008) in SKV103 to obtain SJH41.

2.5. RNA isolation and real-time PCR

Conidia were inoculated with a loop on the surface of supplemented minimal medium in a Petri dish. After 18 h of incubation in constant darkness at 37 °C the mycelial mat was harvested in dim green light. Samples were frozen in liquid nitrogen and stored

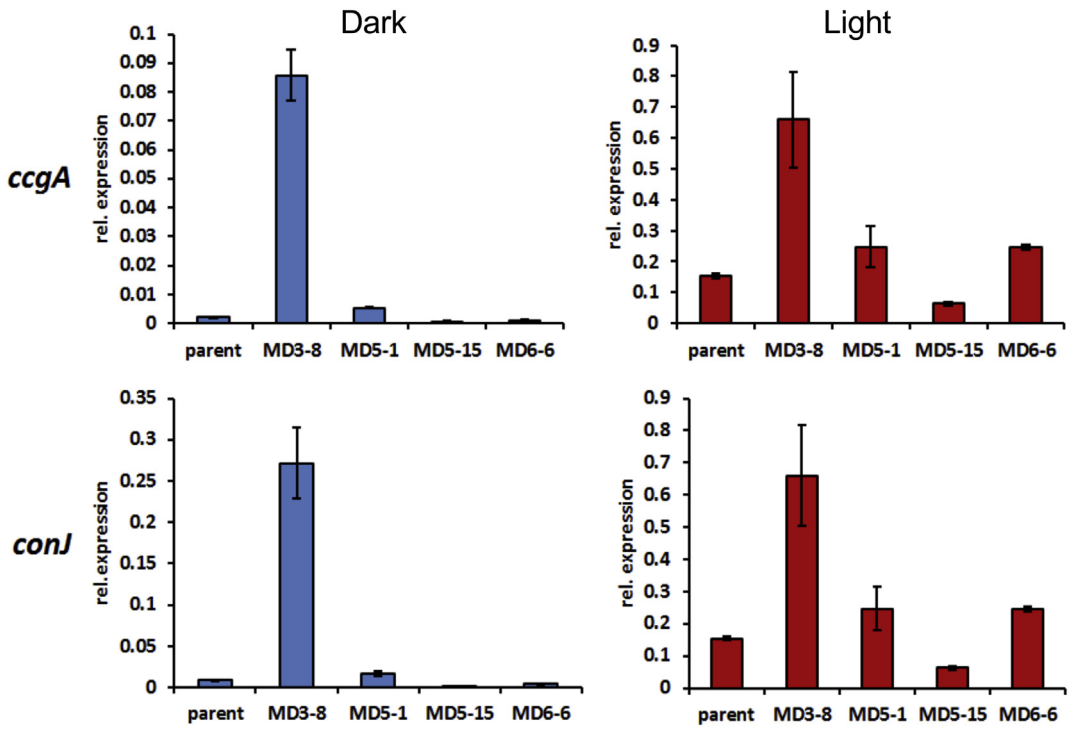

Fig. 2. Determination of the transcript levels of *ccgA* and *conJ* in the parent and the four mutant strains in the dark or in light. Spores of each strain were inoculated on the surface of supplemented minimal medium and incubated for 18 h in the dark at 37 °C. Afterwards, the samples were exposed to light for 30 min or kept constantly in the dark. The mycelia were collected in dim green light immediately and frozen in liquid nitrogen for RNA purification. The expression levels of *ccgA* and *conJ* were normalized to the *h2b* gene. The mean values were calculated based on three biological replicates and the error bars represent standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

at −80 °C until RNA isolation. RNA was isolated with the E. Z. N. A. Fungal RNA Mini Kit (VWR). Cell disruption was achieved by grinding in liquid nitrogen. The isolated RNA was quantified, and an aliquot treated with DNase I. RNA samples were diluted to a final concentration of 50 ng/μl in DEPC water. Quantitative PCR experiments were performed to determine relative mRNA abundance using SensiFAST SYBR & Fluorescein One-Step Kit (Bioline) on an iCycler from Bio-Rad. The expression level of each gene was normalized to the histone H2B gene. The average value was calculated based on three biological replicates. For Oligonucleotides used in this study, see Table 2.

2.6. RNA sequencing and analysis

To prepare the RNA for studying red-light regulated gene expression, the conidia of wildtype SJR2 were inoculated on the surface of supplemented minimal medium and incubated for 18 h at 37 °C in the dark. The samples were exposed to red light for 30 min before collection. To prepare the RNA of parent strain and mutant MD3-8, the strains were incubated on the surface of liquid minimal medium at the same conditions as above in the dark. All the mycelia were collected in dim-green light and frozen immediately in liquid nitrogen. RNA was further purified with the RNeasy plant mini kit (Qiagen). RNA sequencing was performed with BGISEQ-500. Gene expression level was quantified with the software package RESM and differently expressed genes were screened with EBSeq method.

2.7. Microscopy

To determine the localization of RlcA, GFP was fused to the C terminus of RlcA and expressed under the control of the *rlcA* native promoter. For microscopy, fresh conidia were inoculated on coverslips in supplemented liquid medium containing 2 % glucose and incubated overnight at 28 °C. The hyphae were examined under the microscope using the GFP channel. To verify the interaction between FphA and RlcA by biomolecular fluorescence complementation (BiFC), RlcA was tagged with the N-terminal part of YFP. The plasmid was co-transformed with plasmid pJP5 (*alcA(p)::YFP-CT::fphA*) to strain SKV103. To examine the interaction, conidia were grown in supplemented minimal medium with 0.2 % glucose and 2 % threonine before microscopy.

3. Results

3.1. Isolation of constitutive mutants

Previously we used a strain, in which the auxotrophic marker gene *pyr-4* was driven by a light-inducible promoter, in a mutagenesis approach to isolate “blind” mutants (Yu et al., 2016). In those mutants the light-inducible promoter could not be induced anymore. Here, we searched for mutants in which the promoter was constitutively active independent of light. The *A. nidulans* strain SZY17 (parent stain) was used for the UV-mutagenesis (survival rate of 25 %) as previously described (Yu et al., 2016) (Fig. 1 A). More than 100 mutant strains that formed colonies on minimal medium when grown in the dark were obtained, of which four strains, MD3-8, MD5-1, MD5-15 and MD6-6, were chosen for further analysis (Fig. 1C). The mutants grew faster than the parent strain in the dark on supplemented minimal medium without uracil and uridine, although not as good as the parent strain in the presence of uracil and uridine. This growth behavior could be explained by constitutive activation of only the nutritional marker gene, *pyr4*, or by a

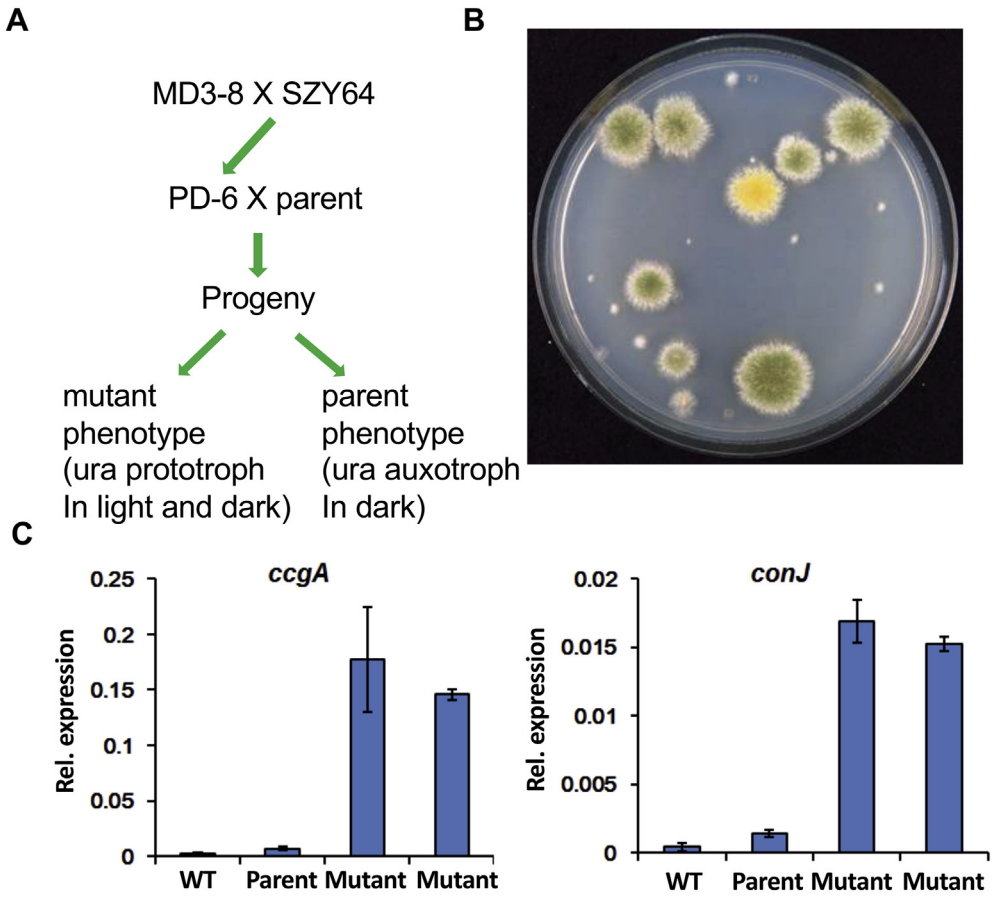

Fig. 3. Reduction of background mutations in MD3-8 by crossings. (A) Scheme of the back-crossing strategy to eliminate background mutations in mutant MD3-8. MD3-8 was crossed to strain SZY64 and the progeny PD-6 was crossed again to the parent strain. Progeny was sorted according to the phenotype. **(B)** Phenotype of the progeny after two rounds of crossings. Ascospores were spread on a supplemented minimal medium agar plate without uracil and uridine and incubated for 3 d in the dark. **(C)** Expression of *ccgA* and *conJ* in wild type, a small and two big colonies from the progeny in **(B)**. The strains were cultured 18 h in the dark on the surface of supplemented minimal medium as above for RNA purification. The mean values were calculated from three biological replicates and the error bars represent standard deviation.

general change of the light response. In order to discriminate between these two possibilities, the transcript levels of the two light inducible genes, *ccgA* and *conJ*, was monitored (Fig. 2). In the dark, the transcript level of both genes was high in MD3-8 in comparison to the parent strain. Even in light, the expression levels of the two genes were higher in the mutant MD3-8 than in the parent strain. Hence, all three genes, *pyr4*, *ccgA* and *conJ* were derepressed in the dark. In contrast the other three mutants showed only very minor or no derepression. Therefore, mutant MD3-8 was used for a detailed analysis.

3.1.1. Identification of the responsible mutation for the constitutive phenotype in the mutant MD3-8

After UV-mutagenesis, many mutations occur in the mutants (Tan et al., 2014; Yu et al., 2016). To identify the responsible mutation for the constitutive phenotype in MD3-8 by next generation sequencing (NGS), we back-crossed mutant MD3-8 twice to reduce the number of background mutations (Fig. 3A). The progeny after two rounds of crossing were sorted according to the phenotype (Fig. 3B). In the small colonies the expression level of *ccgA* and *conJ* were very low in comparison to the expression level in big colonies (Fig. 3C). From this, we concluded that the big colonies should be strains harboring the phenotype-causing mutation, whereas the small colonies should only contain background mutations. Hence, the mycelia of 54 strains forming bigger colonies was collected, genomic DNA isolated and mixed to a single sample. In the same way the mycelia of 35 strains forming only small colonies were processed. The two samples were sent for genome sequencing. By comparing the two genome sequences, two nucleotide substitutions in the gene AN1777 were only observed in the sample derived from the big colonies (MD3-8-2) (Fig. 4A). The codon ATG at position 1312 in AN1777 was changed to a TAG stop codon, which leads to premature termination of the translation (Fig. 4B). To confirm that the constitutive growth phenotype was caused by this premature termination of AN1777, the full-length gene of AN1777 was transformed back into the mutant MD3-8. The plasmid indeed rescued the phenotype of the mutant (Fig. 4C). The complemented strain (MD3-8C) grew very slow in the dark on minimal medium as the parent strain. The gene AN1777 was named *rlcA* (regulator of light sensing and chromatin remodeling)(accession number BK011994). The gene is located on chromosome VII, and the open reading frame of the gene *rlcA* is disrupted by 2 introns, 114 and 54 bp, respectively, in length (RNAseq data). It encodes a protein of 889 amino acids with an N-terminal PHD (plant homeodomain) finger, a TFSII, which is found in the central region of the transcription elongation factor S-II and a C-terminal SPOC (Spen paralog and ortholog C-terminal domain) domain. The premature peptide is 381 amino acids long and only contains the PHD finger domain and

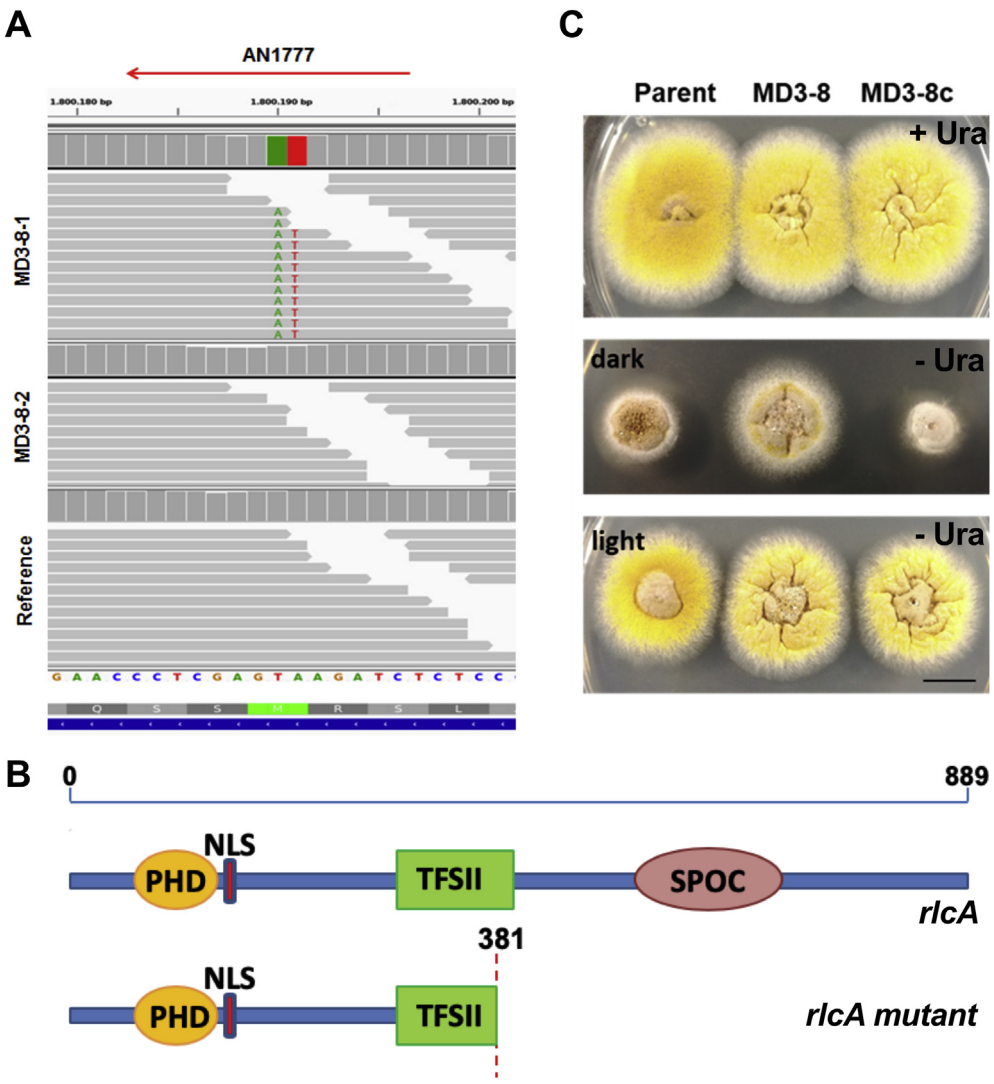

Fig. 4. Identification of the responsible mutation in mutant MD3-8. **(A)** Illustration of two nucleotide substitutions in gene AN1777 (*rlcA*) in MD3-8-1 (colonies with constitutive growth phenotype). The substitution (AT to TA) results in a stop codon, TAG. The mutation was not found in the sequence of MD3-8-2 (parent growth phenotype) or in the reference genome sequence. **(B)** Domain arrangement of RlcA and the premature peptide. RlcA contains an N-terminal nuclear localization signal (NLS), a PHD (plant homeodomain) finger, a TSFII domain, which is found in the central region of transcription elongation factor S-II and a C-terminal SPOC (Spen paralog and ortholog C-terminal domain) domain. The mutant encodes a premature peptide only containing the NLS, the PHD domain and part of the TSFII domain. Domains were identified using HMMER of EnsemblFungi. The NLS was predicted with PSORT II. Phenotype of the complemented constitutive mutant (MD3-8c). The *rlcA* gene including its own promoter was transformed back to the constitutive mutant strain. The parent, the mutant and the complemented mutant were incubated at 37 °C for 3 d at the indicated conditions.

part of the TSFII domain and hence is likely not fully functional. Despite many attempts, we failed to create a *rlcA*-deletion strain, suggesting that *rlcA* is an essential gene and that the remaining part of the protein in the isolated mutant strain is sufficient to support growth.

RlcA orthologs were mainly identified in filamentous ascomycetes. We performed a blast search using *A. nidulans* RlcA as query and found predicted proteins with 33 %, 32 %, 58 %, 34 %, 32 %, and 23 % identity respectively in *N. crassa*, *Fusarium oxysporum*, *Penicillium brasilianum*, *Metarhizium robertsii*, *Trichoderma guizhouense*, and *Schizosaccharomyces pombe* (suppl. Fig. 1). None of the genes has been characterized so far. A protein with even lower identity with 17 % is found in *Saccharomyces cerevisiae*. It is named Bye 1 and was isolated in a suppressor screening (Kinkelin et al., 2013; Pinskaya et al., 2014; Wu et al., 2003). Single PHD, TFSII, and SPOC domains are found in different proteins with different functions from yeast to human.

3.1.2. RlcA interacts with FphA in the nucleus

The RlcA protein is predicted (82 % probability) to be a nuclear protein (PSORT II). To get further insights into the molecular function of RlcA in *A. nidulans*, we tagged RlcA at the N-terminus and expressed it under the control of the *alcA* promoter (strain SJH4). The plasmid contained only ca. 1 kb of the open reading frame cloned behind GFP. After integration of the circular plasmid into the *rlcA* locus the full-length GFP-*rlcA* gene is under control of the *alcA* promoter. Integration was verified by Southern blot (data not shown). A clear RlcA-GFP signal was observed in the nuclei in the dark (Fig. 5A). We also tagged RlcA at the C-terminus with GFP and expressed it under the control of its own promoter. The fusion

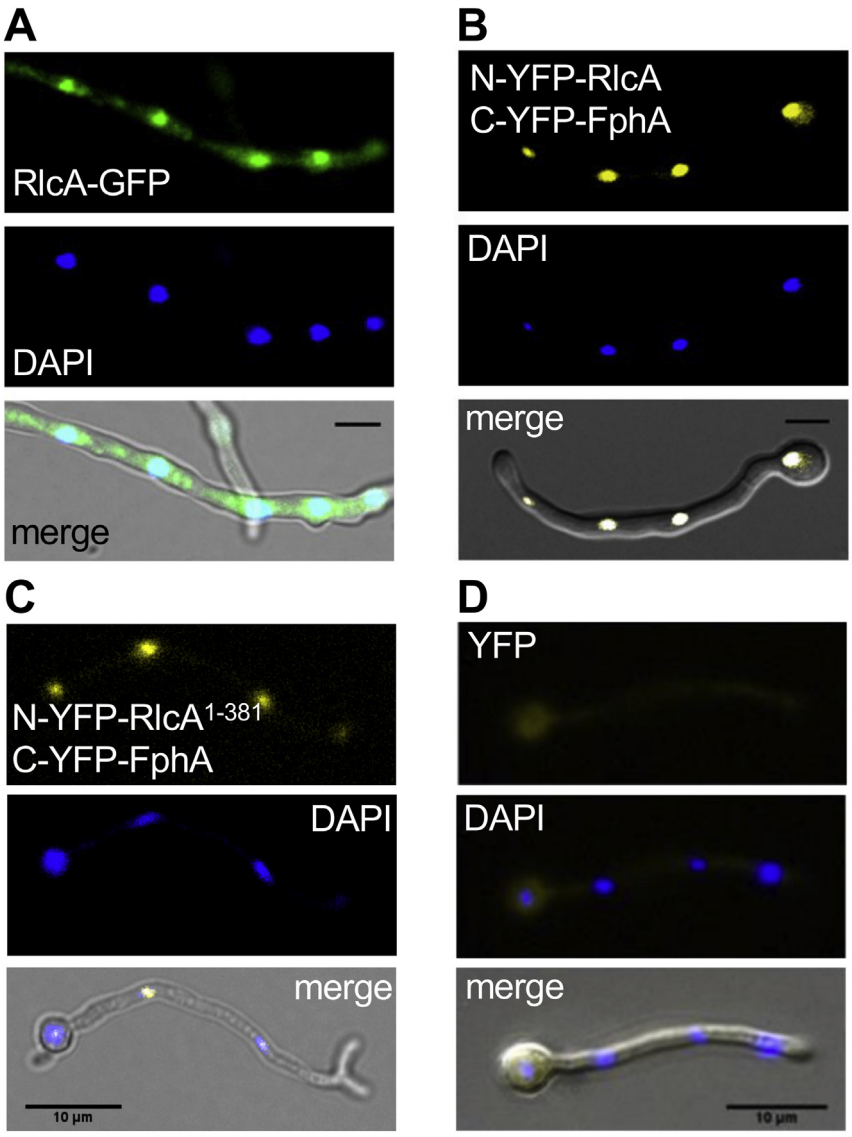

Fig. 5. Localization of RlcA and interaction with phytochrome. **(A)** RlcA localized in nuclei. The spores of strain SJH4 were inoculated on a coverslip with supplemented minimal medium (glycerol as carbon source) and incubated overnight at 28 °C and then observed under the microscope. **(B)** Bimolecular fluorescence complementation (BiFC) to show that RlcA interacts with FphA in the nucleus. Strain SJH41. **(C)** Interaction of the truncated RlcA protein with FphA. Strain SJH14. **(D)** Two control plasmids containing the split YFP halfs fused to two proteins which do not interact were introduced into the same recipient strain as before. **(B–D)** All strains were germinated on a coverslip with minimal medium containing 2 % threonine and 0.2 % glucose.

protein also localized in nuclei (results not shown). We have evidence that the GFP-tagged version was biologically functional, because overexpression of RclA or RclA-GFP both caused a slight induction of conidia formation (data not shown). To analyze whether RlcA interacted with FphA, RlcA was fused to the N-terminus of split YFP. Plasmid pJH71 and pJP5 were co-transformed into the wild-type strain SKV103 and yellow fluorescence was observed in the nuclei (Fig. 6B). We did not see any difference when incubated in light, but it has to be considered that interaction of the split YFP halfs is irreversible and hence light dependency of the interaction of RlcA and FphA cannot be determined in this assay. The RlcA truncated protein, which should be present in the mutant, was also tested for interaction with FphA (Fig. 5C). Indeed, fluorescence was observed in nuclei. This illustrates that the truncated version is expressed and translocated into nuclei. Two control plasmids (one containing FphA and the other one a RlcA version with mutated TFSII domain) introduced into the same recipient strain did not result in YFP fluorescence.

3.1.3. *RlcA as a global regulator*

Since RlcA interacted with FphA, we anticipated that RlcA is involved in red light sensing. In order to explore the regulatory functions of the RlcA protein, a global expression analysis was performed to compare wild type (SJR2), the parent strain (SZY17) and the constitutive mutant (MD3-8). The strains were grown in liquid medium for 18 h at 37 °C in the dark. After the mycelia were exposed to red light for 30 min, or kept in the dark, mycelia were processed for RNAseq experiments using green light as safe light. In total 9459 genes were detected, and 98.4 % of all reads could be mapped to the reference genome. In wild type, 268 genes were up-

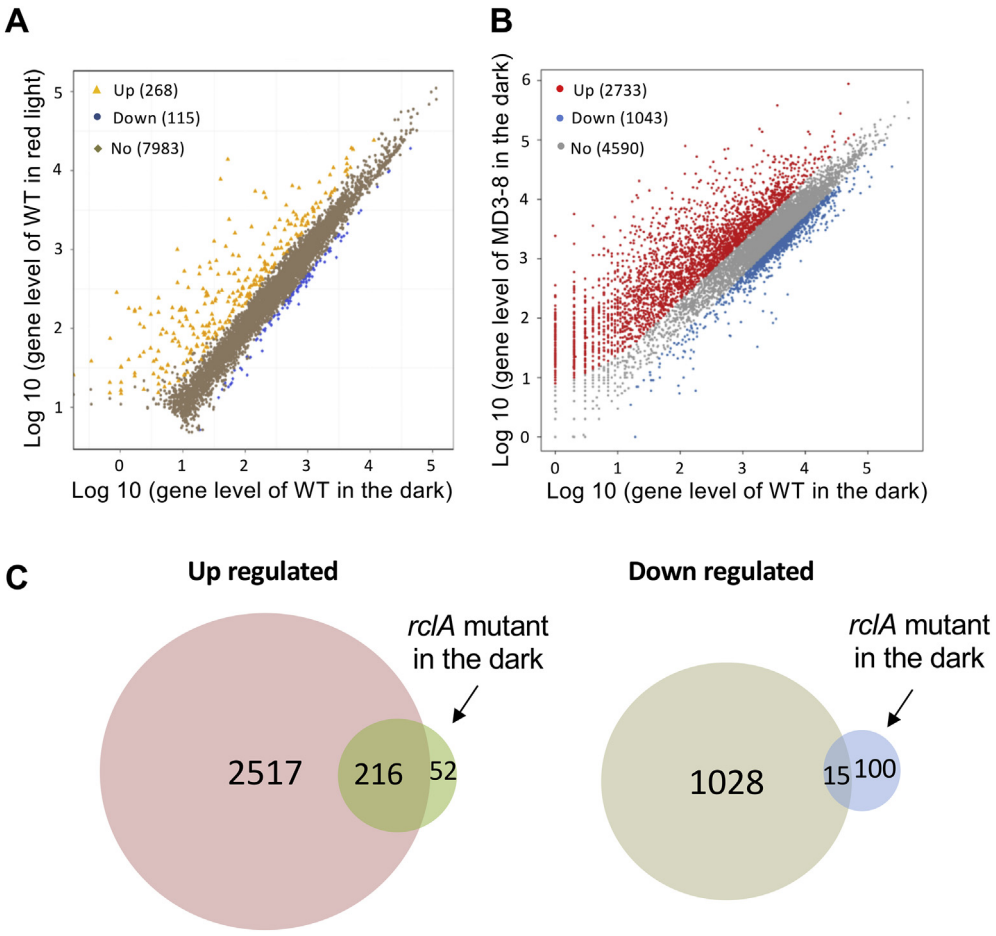

Fig. 6. RNAseq analysis of wild type in the dark and after red-light illumination and comparison of wild type with the MD3-8 mutant. **(A)** Scatter plot of expressed genes of wild type (WT) in light and dark conditions. The differently expressed genes were screened with the EBseq method (PPEE $\leq$ 0.05 and abs (log 2(Y/X))$\geq$1). Up regulated genes (Up), downregulated genes (Down), no significant difference (No). **(B)** Scatter plot of expressed genes in the parent strain compared to the constitutive mutant in the dark. **(C)** Venn diagrams summarizing the overlap between red-light upregulated (left) or downregulated (right) genes and differentially expressed genes in the *rlcA* mutant. 216 genes are upregulated by red light and induced in the *rlcA* mutant. 15 genes are downregulated in red light and repressed in the *rlcA* mutant.

and 115 genes down-regulated after red-light exposure (Fig. 6) (Table 3). The *rlcA* gene nor the mutant allele were regulated by light. In the *rlcA* mutant strain 2733 genes were up- and 1043 genes were down regulated in the dark as compared to the parent strain in the dark. 216 upregulated genes were found in the group of red-light induced genes and upregulated genes in the constitutive mutant (Fig. 6). Hence, more than 80 % of red-light induced genes were de-repressed in the mutant. However, it has to be considered that in strain MD3-8 other mutations besides the RclA truncation occurred and hence some of the mis-regulation could be due to other mutations. Only 15 down-regulated genes were shared between the downregulated genes in the constitutive mutant and the red-light downregulated genes. Among the upregulated genes were several genes characteristic for asexual development, such as the hydrophobin *dewA* (442-fold change) (Stringer and Timberlake, 1994), the hydrophobin *rodA* (107 fold) (Stringer et al., 1991), the transcription factor *brlA* (21 fold) (Prade and Timberlake, 1993), or the G1/S cyclin *pclA* (3 fold) (Schier and Fischer, 2002; Schier et al., 2001). The results suggest repression of those genes through RclA. We tried to address the question if RclA is indeed involved in the regulation of development. However, the *rclA* mutant is impaired in growth (Figs. 1 and 4) and sporulation and has a pleiotropic phenotype. Therefore, we did not analyze sporulation specifically. The fact that more than one third of the *A. nidulans* genome appears to be under the control of the *rlcA* gene, indicates a global role of the protein and may explain an essential function in *A. nidulans.*

4. Discussion

We found that the nuclear protein RlcA is probably a global regulator for gene expression and a new component in red-light sensing in *A. nidulans*. We found that RlcA interacts with phytochrome in the nucleus. A truncated version of the protein leads to the misregulation of several thousand genes, suggesting an important function in gene regulation. The RclA protein harbors an NLS and three additional domains, a PHD finger-, a TFSII-, and a SPOC-domain. All three are well conserved from yeast to human. The only studied orthologue of RlcA is Bye 1 (Bypass of Ess1) in *S. cerevisiae* (Pinskaya et al., 2014; Kinkelin et al., 2013; Wu et al., 2003). Although Bye1 has only 17 % identical amino acids as compared to RclA, it contains all three domains (Suppl. Fig. S1). The protein was identified as a multi-copy suppressor of an *ess1* mutation (Wu et al., 2003).

The first question which arises is why the severe mis-regulation of gene expression in the *rlcA*-mutant strain did not cause a more severe phenotype than the constitutive expression of many red-

Table 3
List of the 15 most up- or downregulated genes (log 2 ratio) found in the RNAseq analysis when wild type was compared to the *rclA* constitutive mutant.

Locus	Gene description	MD/WT	Fold change
upregulated			
AN9218	Putative sterol 14-demethylase (CYP540D1)	9.86	930
AN9310	Transcript induced by light in developmentally competent mycelia	9.81	901
AN7959	hypothetical protein	9.80	892
AN9217	hypothetical protein	9.37	662
AN9277	Proline dehydrogenase activity	9.30	634
AN5243	hypothetical protein	9.22	607
AN8930	DNA binding, RNA polymerase II transcription factor activity	9.19	587
AN8382	hypothetical protein	9.12	559
AN8637	Conidia-specific catalase (catA)	8.98	539
AN3962	hypothetical protein	8.91	506
AN5272	Putative phenylacetate-CoA ligase, role in penicillin biosynthesis	8.79	444
AN8006	Hydrophobin (dewA)	8.78	442
AN9284	hypothetical protein	8.70	424
AN9251	Putative cytochrome P450 (CYP650A1)	8.58	391
AN9164	Predicted oxidoreductase activity	8.49	366
downregulated			
AN7132	hypothetical protein	−9.34	640
AN4659	Putative acyl-CoA synthetase/AMP-binding domain protein	−5.97	62.0
AN7116	hypothetical protein	−5.69	51.8
AN9414	hypothetical protein	−5.21	36.6
AN5956	Predicted DNA binding, transposase activity, role in DNA integration	−5.15	35.1
AN9493	Putative GNAT-type acetyltransferase (*ngn12*)	−4.89	29.3
AN5791	Predicted pheromone precursor (*ppgA*)	−4.57	23.5
AN0611	hypothetical protein	−4.26	19.0
AN5093	hypothetical protein	−4.18	19.0
AN9519	hypothetical protein	−4.15	17.8
AN0785	Putative manganese superoxide dismutase (*sodM*)	−4.02	16.1
AN8788	Predicted DDE1 transposon-related ORF	−3.70	13.3
AN3893	hypothetical protein	−3.54	11.5
AN0223	Predicted DNA binding transcription factor activity	−3.51	11.3
AN3667	hypothetical protein	−3.23	9.25

light regulated genes. In comparison, it is likely that a deletion of the *rlcA* gene is lethal, because we were unable to generate a complete loss-of-function mutant. This suggests that the residual RlcA mutant protein still can fulfil the essential functions. In comparison, *bye 1* deletion in *S. cerevisiae* was not lethal (Wu et al., 2003). Another example that severe mis-regulation of the genome is not necessarily fatal for cell viability is a histone H3 demethylase of *A. nidulans*, KdmB. Lack of *kdmB* led to an upregulation of almost 2000 and the downregulation of a similar number of genes (Gacek-Matthews et al., 2016).

If our hypothesis is right, that the truncated RlcA protein is partially functional it has to be considered that the protein still contains the NLS and the PHD finger domain and most part of the TSFII domain. PHD finger domains were first identified in plants and contain a Cys_4-His-Cys_4 domain (Schindler et al., 1993). Many of the proteins, in which this domain is found, are involved in chromatin-mediated gene regulation (Aasland et al., 1995; Shi et al., 2006; Champagne and Kutateladze, 2009). The Bye 1 protein of *S. cerevisiae* was shown to bind to H3K4met3 (Kinkelin et al., 2013), and the human PHD finger protein ING recruits acetyltransferase and deacetylase to the promoters (Champagne and Kutateladze, 2009). The tri-methylated lysin 4 stage of histone H3 (H3K4met3) is characteristic for actively transcribed genes. Because the PHD finger is unaffected in the *rclA* mutant, binding of the truncated RlcA protein to DNA is still likely. The second domain, which is to a large extent remained in the truncated RlcA protein is the TFSII domain. This domain was found in the general transcription elongation factor TFIIS (Morin et al., 1996). This factor stimulates RNA polymerase II to read through DNA regions which promote the formation of stalled ternary complexes. The third domain in RlcA is the SPOC domain (Zhang et al., 2016). It could interact with a repressor or a repressor complex such as Tup 1 (Smith and Johnson, 2000).

Although we did not test the interaction of RclA with RNA polymerase II or the Tup 1 repressor complex, the well-characterized domains allow us to propose a model, which explains the observed effects (Fig. 7). We speculate that the PHD finger domain interacts with lysine 4 in histone H3, and the TFIIS domain interacts with RNA polymerase II. RclA may recruit other proteins required for chromatin modifications, such as histone acetyl transferase (GcnE) or the histone deacetylase, HdaA. They were previously described to be involved in the regulation of light-regulated genes (Hedtke et al., 2015). SPOC domains are found in regulatory proteins such as the Arabidopsis flowering regulator FPA or the human co-repressor SHARP (Zhang et al., 2016; Mikami et al., 2013). It probably recruits a repressor, or repressor complex to the DNA, which inhibits transcription. A fungal candidate for such a repressor is the Tup1 protein (Smith and Johnson, 2000). Upon certain signals, such as an activation of phytochrome in the case of light-regulated genes, the repressor could be released, and transcription can occur. In the mutant strain, the repressor would be absent because of the lack of the SPOC domain and thus RNA polymerase II could transcribe the gene at a basal level without external stimuli. The transcription can be further stimulated by red light, because phytochrome induces the HOG pathway and thus activates probably the AtfA transcription factor (Yu et al., 2016) (Fig. 2). This situation in the mutant allows us to speculate about the wild type situation. Here, the release of the repressor would be initiated by

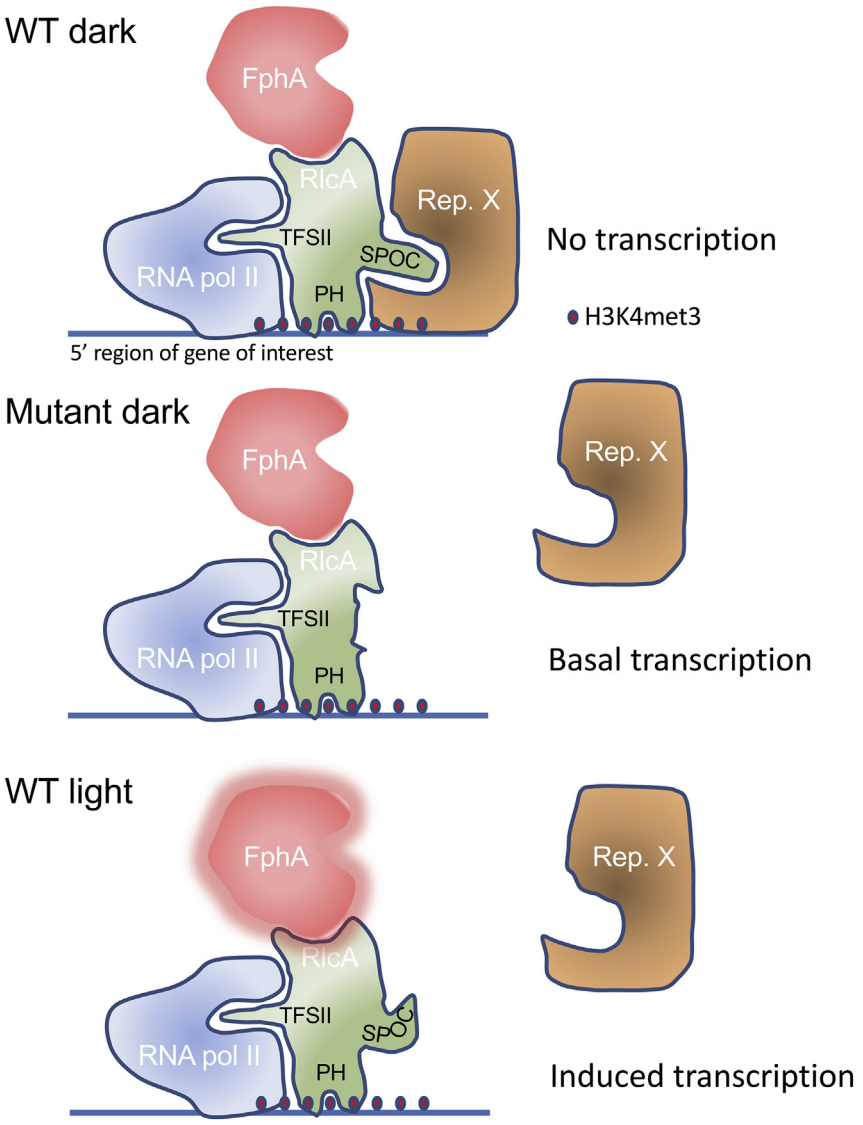

Fig. 7. Proposed model for *rlcA*-dependent gene activation of red-light induced genes. For details see the Discussion.

illumination of the phytochrome. This model proposes thus an important function of RclA as a hub which may integrate different signals at different promoters.

Acknowledgement

This work was supported by the German Science Foundation (DFG, Fi459/19-1). This manuscript is part of the special issue Fungal Adaptation to Hostile Challenges intended for the third International Symposium on Fungal Stress - ISFUS-2019, which was supported by FAPESP 2018/20571-6 and CAPES 88881.289327/2018-01.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.12.009.

References

Aasland, R., Gibson, T.J., Stewart, A.F., 1995. The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem. Sci. 20, 56–59.
Ballario, P., Vittorioso, P., Margrelli, A., Toalora, C., Cabibo, A., Macino, G., 1996. White collar-1, a central regulator of blue light responses in *Neurospora crassa*, is a zinc finger protein. EMBO J. 15, 1650–1657.
Blumenstein, A., Vienken, K., Tasler, R., Purschwitz, J., Veith, D., Frankenberg-Dinkel, N., Fischer, R., 2005. The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. Curr. Biol. 15, 1833–1838.
Brenna, A., Grimaldi, B., Filetici, P., Ballario, P., 2012. Physical association of the WC-1 photoreceptor and the histone acetyltransferase NGF-1 is required for blue light signal transduction in *Neurospora crassa*. Mol. Biol. Cell 23, 3863–3872.
Champagne, K.S., Kutateladze, T.G., 2009. Structural insight into histone recognition by the ING PHD fingers. Curr. Drug Targets 10, 432–441.
Corrochano, L.M., 2011. Fungal photobiology: a synopsis. IMA Fungus 2, 25–28.
Corrochano, L.M., 2019. Light in the fungal world. Annu. Rev. Genet. in press.
Dunlap, J.C., Loros, J.J., 2017. Making time: conservation of biological clocks from fungi to animals. Microbiol. Spectr. 5. FUNK-0039-2016.
Fischer, R., Aguirre, J., Herrera-Estrella, A., Corrochano, L.M., 2016. The complexity of fungal vision. Microbiol. Spectr. 4. FUNK-0020-2016.
Froehlich, A.C., Liu, Y., Loros, J.J., Dunlap, J.C., 2002. White collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. Science 297, 815–819.

Froehlich, A.C., Noh, B., Vierstra, R.D., Loros, J., Dunlap, J.C., 2005. Genetic and molecular analysis of phytochromes from the filamentous fungus *Neurospora crassa*. Eukaryot. Cell 4, 2140–2152.
Gacek-Matthews, A., Berger, H., Sasaki, T., Wittstein, K., Gruber, C., Lewis, Z.A., Strauss, J., 2016. KdmB, a Jumonji histone H3 demethylase, regulates genome-wide h3k4 trimethylation and is required for normal induction of secondary metabolism in *Aspergillus nidulans*. PLoS Genet. 12, e1006222.
Grimaldi, B., Coiro, P., Filetici, P., Berge, E., Dobosy, J.R., Freitag, M., Selker, E.U., Ballario, P., 2006. The *Neurospora crassa* White Collar-1 dependent bluit light response requires acetylation of histone H3 lysine 14 by NGF-1. Mol. Biol. Cell 17, 4576–4583.
He, Q., Cheng, P., Yang, Y., Wang, L., Gardner, K.H., Liu, Y., 2002. White collar-1, a DNA binding transcription factor and a light sensor. Science 297, 840–843.
Hedtke, M., Rauscher, S., Röhrig, J., Rodriguez-Romvero, J., Yu, Z., Fischer, R., 2015. Light-dependent gene activation in *Aspergillus nidulans* is strictly dependent on phytochrome and involves the interplay of phytochrome and white collar-regulated histone H3 acetylation. Mol. Microbiol. 97, 733–745.
Herr, A., Fischer, R., 2014. Improvement of *Aspergillus nidulans* penicillin production by targeting AcvA to peroxisomes. Metab. Eng. 25, 131–139.
Igbalajobi, O., Yu, Z., 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 e00371-00319.
Käfer, E., 1977. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv. Genet. 19, 33–131.
Kinkelin, K., Wozniak, G.G., Rothbart, S.B., Lidschreiber, M., Strahl, B.D., Cramer, P., 2013. Structures of RNA polymerase II complexes with Bye 1, a chromatin-binding PHF3/DIDO homologue. Proc. Natl. Acad. Sci. U. S. A. 110, 15277–15282.
Larrondo, L.F., Olivares-Yanez, C., Baker, C.L., Loros, J.J., Dunlap, J.C., 2015. Circadian rhythms. Decoupling circadian clock protein turnover from circadian period determination. Science 347, 1257277.
Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760.
Linden, H., Macino, G., 1997. White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. EMBO J. 16, 98–109.
McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M.A., 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303.
Mikami, S., Kanaba, T., Ito, Y., Mishima, M., 2013. NMR assignments of SPOC domain of the human transcriptional corepressor SHARP in complex with a C-terminal SMRT peptide. Biomol. NMR Assignments 7, 267–270.
Mooney, J.L., Yager, L.N., 1990. Light is required for conidiation in *Aspergillus nidulans*. Genes Dev. 4, 1473–1482.
Morin, P.E., Awrey, D.E., Edwards, A.M., Arrowsmith, C.H., 1996. Elongation factor TFIIS contains three structural domains: solution structure of domain II. Proc. Natl. Acad. Sci. U. S. A. 93, 10604–10608.
Pinskaya, M., Ghavi-Helm, Y., Mariotte-Labarre, S., Morillon, A., Soutourina, J., Werner, M., 2014. PHD and TFIIS-Like domains of the Bye 1 transcription factor determine its multivalent genomic distribution. PLoS One 9, e102464.
Prade, R., Timberlake, W.E., 1993. The *Aspergillus nidulans brlA* regulatory locus consists of two overlapping transcription units that are individually required for conidiophore development. EMBO J. 12, 2439–2447.
Purschwitz, J., Müller, S., Kastner, C., Schöser, M., Haas, H., Espeso, E.A., Atoui, A., Calvo, A.M., Fischer, R., 2008. Functional and physical interaction of blue and red-light sensors in *Aspergillus nidulans*. Curr. Biol. 18, 255–259.
Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., Mesirov, J.P., 2011. Integrative genomics viewer. Nat. Biotechnol. 29, 24–26.
Rodriguez-Romero, J., Hedtke, M., Kastner, C., Müller, S., Fischer, R., 2010. Fungi, hidden in soil or up in the air: light makes a difference. Annu. Rev. Microbiol. 64, 585–610.
Ruger-Herreros, C., Rodriguez-Romero, J., Fernandez-Barranco, R., Olmedo, M., Fischer, R., Corrochano, L.M., Canovas, D., 2011. Regulation of conidiation by light in *Aspergillus nidulans*. Genetics 188, 809–822.
Schier, N., Fischer, R., 2002. The *Aspergillus nidulans* cyclin PclA accumulates in the nucleus and interacts with the central cell cycle regulator NimX(Cdc 2). FEBS Lett. 523, 143–146.
Schier, N., Liese, R., Fischer, R., 2001. A pcl-like cyclin of *Aspergillus nidulans* is transcriptionally activated by developmental regulators and is involved in sporulation. Mol. Cell. Biol. 21, 4075–4088.
Schindler, U., Beckmann, H., Cashmore, A.R., 1993. HAT3.1, a novel Arabidopsis homeodomain protein containing a conserved cysteine-rich region. Plant J. 4, 137–150.
Schumacher, J., 2017. How light affects the life of Botrytis. Fungal Genet. Biol. 106, 26–41.
Schumacher, J., Gorbushina, A., 2019. Light sensing in plant- and rock-associated black fungi. Fungal Biol. in press.
Shi, X., Hong, T., Walter, K.L., Ewalt, M., Michishita, E., Hung, T., Carney, D., Pena, P., Lan, F., Kaadige, M.R., Lacoste, N., Cayrou, C., Davrazou, F., Saha, A., Cairns, B.R., Ayer, D.E., Kutateladze, T.G., Shi, Y., Cote, J., Chua, K.F., Gozani, O., 2006. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature 442, 96–99.
Smith, R.L., Johnson, A.D., 2000. Turning genes off by Ssn6-Tup 1: a conserved system of transcriptional repression in eukaryotes. Trends Biochem. Sci. 25, 325–330.
Stringer, M.A., Dean, R.A., Sewall, T.C., Timberlake, W.E., 1991. *Rodletless*, a new *Aspergillus* developmental mutant induced by directed gene inactivation. Genes Dev. 5, 1161–1171.
Stringer, M.A., Timberlake, W.E., 1994. *dewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. Mol. Microbiol. 16, 33–44.
Tan, K., Roberts, A.J., Chonofsky, M., Egan, M.J., Reck-Peterson, S.L., 2014. A microscopy-based screen employing multiplex genome sequencing identifies cargo-specific requirements for dynein velocity. Mol. Biol. Cell 25, 669–678.
Todd, R.B., Davis, M.A., Hynes, M.J., 2007. Genetic manipulation of *Aspergillus nidulans*: meiotic progeny for genetic analysis and strain construction. Nat. Protoc. 2, 811–821.
Vienken, K., Fischer, R., 2006. The $Zn(II)_2Cys_6$ putative transcription factor NosA controls fruiting body formation in *Aspergillus nidulans*. Mol. Microbiol. 61, 544–554.
Wang, Z., Li, N., Li, J., Dunlap, J.C., Trail, F., Townsend, J.P., 2016. The fast-evolving *phy-2* gene modulates sexual development in response to light in the model fungus *Neurospora crassa*. mBio 7, e02148.
Wu, X., Rossettini, A., Hanes, S.D., 2003. The ESS1 prolyl isomerase and its suppressor BYE1 interact with RNA pol II to inhibit transcription elongation in Saccharomyces cerevisiae. Genetics 165, 1687–1702.
Yu, Z., Armant, O., Fischer, R., 2016. Fungi use the SakA (HogA) pathway for phytochrome-dependent light signaling. Nat. Microbiol. 1, 16019.
Yu, Z., Fischer, R., 2019. Light sensing and responses in fungi. Nat. Rev. Microbiol. 17, 25–36.
Zhang, Y., Rataj, K., Simpson, G.G., Tong, L., 2016. Crystal structure of the SPOC domain of the Arabidopsis flowering regulator FPA. PLoS One 11, e0160694.