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Article

The sulfur metabolism regulator MetR is a global regulator controlling phytochrome-dependent light responses in *Aspergillus nidulans*

Zhenzhong Yu [a,b,*], Jia Gao [a], Olumuyiwa Igbalajobi [a,d], Marek Skoneczny [c], Marzena Sieńko [c], Agnieszka M. Maciejewska [c], Jerzy Brzywczy [c], Reinhard Fischer [a,*]

[a] Department of Microbiology, Institute for Applied Biosciences, Karlsruhe Institute of Technology (KIT)-South Campus, Karlsruhe D-76131, Germany
[b] Key Laboratory of Plant Immunity, Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, National Engineering Research Center for Organic-based Fertilizers, Nanjing Agricultural University, Nanjing 210095, China
[c] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa 02-106, Poland
[d] Michael Smith Laboratories, Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia VGT 1Z4, Canada

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ABSTRACT

Phytochrome-dependent light signaling has been studied in several fungi. In *Aspergillus nidulans* light-stimulated phytochrome activates the high-osmolarity glycerol (HOG) signaling pathway and thereby controls the expression of a large number of genes, many of which are related to stress responses. In a genome-wide expression analysis in *A. nidulans* we found that phytochrome, *fphA*, is under strict expression control of the central regulator of the sulfur-starvation response, MetR. This transcriptional regulator is required for the expression of genes involved in inorganic sulfur assimilation. In the presence of organic sulfur, MetR is probably ubiquitinated and possibly degraded and the transcription of sulfur-assimilation genes, e.g., sulfate permease, is turned off. The expression analysis described here revealed, however, that MetR additionally controls the expression of hundreds of genes, many of which are required for secondary metabolite production. We also show that *metR* mutation phenocopies *fphA* deletion, and five other histidine-hybrid kinases are down-regulated in the *metR1* mutant. Furthermore, we found that light and phytochrome regulate the expression of at least three carbon–sulfur hydrolases. This work is a further step towards understanding the interplay between light sensing and metabolic pathways.

1. Introduction

Most filamentous fungi are able to grow on very poor medium containing salt mixtures as trace elements and inorganic sources for sulfur or nitrogen but are also able to grow on rich media containing ready-to use organic molecules. Thus, metabolic pathways e.g., used for the biosynthesis of amino acids can be turned off if amino acids are provided in the medium. This raises the question about the molecular regulation of the expression of the corresponding genes. In the case of sulfur metabolism, bZIP DNA-binding proteins were identified as central positive regulators for sulfur assimilation in *Saccharomyces cerevisiae* (Met4), *Neurospora crassa* (Cys3), *Aspergillus nidulans* (MetR) and recently in *Cryptococcus neoformans* [1–5]. In the presence of sulfur-containing amino acids, genes required for inorganic sulfur assimilation are turned off, whereas they are switched on if only inorganic sulfur sources are available or under sulfur-starvation conditions [3,6]. Despite the fact that the central positive regulator is conserved in all three fungi, the mechanism of sulfur assimilation control is quite different. Whereas in *S. cerevisiae* and in *N. crassa* transcription of *met4* or *cys3* is repressed in the presence of organic sulfur, the *A. nidulans metR* gene is constitutively expressed and its regulation occurs *via* ubiquitin-mediated inactivation or degradation of the MetR protein [6–9]. In *N. crassa* it was shown that Cys3 binds to specific consensus sequences in the promoters of *cys3*-regulated genes and the basic region-leucine zipper motif is important for that [10]. In *A. nidulans* the C-terminal basic region and the adjacent leucine zipper motif is also essential for activity, because frameshift mutations (*metR1*, *metR2*) in that motif result in methionine auxotrophy [4].

The function of MetR or its orthologues has been related and connected to other cellular pathways. In *Hypocrea jecorina* (*Trichoderma reesei*) a putative E3 ubiquitin ligase (Lim1) was found to be involved in the regulation of cellobiohydrolase [11]. The ligase is an orthologue of the sulfur regulator Scon2 of *N. crassa* and SconB

* Corresponding authors.
E-mail addresses: yuzhenzhong@njau.edu.cn (Z. Yu), reinhard.fischer@kit.edu (R. Fischer).

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in *A. nidulans*. Transcription of *lim1* was regulated by light through the blue-light photoreceptor ENVOY [11].

In a global transcriptional analysis in *A. nidulans* comparing wild type and several mutants affected in the sulfur assimilation pathway (*cysB*, *sconB*, *sconC*), it was found that many genes involved in different stress responses were mis-regulated [12]. This was the first indication for a global effect of the supply with sulfur-containing amino acids on other metabolic pathways. However, because the mutants showed de-repression of the sulfur assimilation pathway, which probably leads to the reduction of the ATP and NADPH levels in the cell, the upregulated stress genes are likely to be a consequence of this general effect on the energy status of the cell. In a recent study, a MetR orthologue has been studied in *Alternaria alternata* (tangerine pathotype) [13]. Several environmental stresses led to an induction of the transcription of *metR*. Deletion of *metR* affected vegetative growth, spore formation and the resistance towards several stresses. Transcriptional profiling revealed severe mis-regulation of the genome, suggesting a more complex role of MetR than only regulating sulfur assimilation.

Mitogen-activated protein kinase (MAPK) signaling pathways play important roles in transducing environmental signals into cellular responses. One example is light sensing in fungi [14,15]. Red light sensing in *A. nidulans* is initiated by the FphA protein called phytochrome [16–18]. Phytochrome signaling occurs through the activation of the HOG MAP-kinase pathway [19].The final step of this kinase cascade pathway is encoded in *A nidulans* by the *hogA* gene, called also *sakA* because of its role in the sensing of other stresses and the latter name will be used further in this article [20].

Here, we show that *A. nidulans* MetR controls a large number of genes and that the phytochrome-dependent light control is under control of MetR. This indicates a novel connection between light sensing and metabolic control.

2. Materials and methods

2.1. Strains, plasmids, and culture conditions

Standard strain construction procedures were used [21]. Media for *A. nidulans* were prepared according to Martinelli [22] and unless otherwise stated, minimal medium was supplemented with 1 mmol/L methionine.

To construct a strain constitutively expressing *fphA*, 1.3 kb of the *gpdA* promoter, the *fphA* ORF and the *Aspergillus fumigatus pyroA* gene were cloned into pJet1.2/blunt vector, yielding plasmid pZY32. The *metR1* mutant was transformed with plasmid pZY32, yielding strain SZY63. To tag SakA with GFP in the *metR1* mutant, a fragment where SakA was C-terminally fused to GFP, was co-transformed with plasmid pZY40 (harboring the *A. fumigatus pyroA* gene). This yielded strain SZY66. To investigate the localization of SakA when *fphA* is constitutively expressed in the *metR1* mutant, plasmid pZY32 and the SakA-GFP fragment, were co-transformed into the *metR1* mutant, yielding strain SZY67. Strains used in this study are listed in Table 1 and primers for strain construction and quantitative real time PCR are listed in Table 2.

2.2. Quantification of conidiophores and cleistothecia

Fresh conidia of wildtype, the *fphA*-deletion strain and the *metR1* mutant, were diluted in supplemented minimal medium with 0.75% agar to a spore density of 5×10^4 cells/mL before the medium solidified. 1 mL medium was spread evenly on each supplemented minimal medium plate (Ø 5.5 cm). The solidified plate was cultured at 37 °C in the dark for 12 h and after 12 h, red light was switched on and the plates illuminated for 4 d. Conidia were collected with water containing 0.1% Tween 20 and quantified. Cleistothecia were quantified under the stereomicroscope.

Table 1
Strains used in this study.

Strain	Genotype	Reference
A. nidulans		
FGSCA4	Wild type	Fungal Genetics Stock Center, Kansas City, Missouri
SJR2	*pyrG89*; *pyroA4*; *nkuA*::*bar*; *veA*$^+$	[23]
SJR3	*argB2*; *pyroA4*, *nkuA*::*bar*; *veA*$^+$	[19]
W1	*yA2*; *pyroA4*; *veA1*	[4]
RM114	*yA2*; *metR1*; *pyroA4*; *veA1*	[4]
RM77	*biA1*; *ΔmetR*; *pyroA4*, *ΔnkuA*::*argB*; *veA1*	[4]
SRJ8	*pyroA4*, *nkuA*::*bar*; *veA*$^+$	Julian Roehrig, Karlsruhe
SJR10	*pyrG89*; *ΔargB*::*trpCΔB*; *pyroA4*, *nkuA*::*bar*; *ΔfphA*::*argB*; *veA*$^+$	[24]
SZY31	*pyrG89*; *ΔsakA*::*AfriboB*; *pyroA4*, *ΔnkuA*::*argB*; *veA*$^+$	[19]
SZY34	*pyrG89*; *sakA*(*p*)::*sakA*::*GFP*::*AfpyrG*; *pyroA4*, *nkuA*::*bar*; *veA*$^+$	[19]
SZY37	*ΔargB*::*trpCΔB*, *sakA*(*p*)::*sakA*::*GFP*::*AfpyrG*; *pyroA4*, *nkuA*::*bar*; *ΔfphA*::*argB*; *veA*$^+$	[19]
SZY61	SJR3 × RM114:*metR1*; *pyroA4*, *nkuA*::*bar*; *veA*$^+$	This study
SZY63	*metR1*; *pyroA4*, *nkuA*::*bar*, *veA*$^+$, transformed with pZY32 (*gpdA*(*p*)::*fphA*, *AfpyroA*)	This study
SZY66	SZY61 transformed with plasmid pZY40 (*AfpyroA in pJet1.2/blunt*) and *SakA C-terminal GFP tagging cassette*	This study
SZY67	SZY61 co-transformed with plasmid pZY32 (*gpdA*(*p*)::*fphA*, *AfpyroA*) and *SakA C-terminal GFP tagging cassette*	This study

2.3. Microscopic localization of SakA upon light exposure

Fresh conidia of wild type (SZY34), the *fphA*-deletion strain (SZY37), the *metR1* mutant (SZY66), and the *metR1* mutant constitutively expressing *fphA* (SZY67), in which SakA is C-terminally tagged with GFP, were inoculated on microscope coverslips with 400 μL supplemented minimal medium. After 8 h incubation in the dark at 37 °C, samples were exposed to white light for 10 min or kept in the dark. Afterwards samples were fixed immediately with 4% formaldehyde for 10 min and washed twice with PBS. Nuclei were stained with DAPI before microscopy.

2.4. Analysis of transcript abundance with quantitative real time PCR

To analyze the transcript abundance of *fphA*, *lreA*, *lreB*, *cryA*, *veA*, and hybrid histidine kinase encoding genes in wild type and the *metR1* mutant, fresh conidia of each strain were inoculated on the surface of liquid supplemented minimal medium in Ø 3.5 cm Petri dishes with an inoculation loop and cultivated in the dark for 18 h. The mycelium was harvested in dim-green light and frozen immediately in liquid nitrogen. To analyze the effect of amino acids on the transcript abundance of *fphA*, wild-type strain was cultured in minimal medium supplemented with different concentrations of arginine, alanine and methionine. To analyze the transcript abundance of *ccgA*, *conJ*, *veA*, and the genes encoding putative carbon–sulfur lyases upon light stimulus, white light was switched on after 18 h incubation in the dark and samples were illuminated for 15 min before harvesting. Fungal RNA extraction kit from Omega was used to isolate RNA. Frozen mycelium was disrupted in a cell homogenizer with an oscillation frequency of 30 hits/min for 5 min. DNA was digested with the TURBO DNA-free kit and purified RNA was diluted to 50 ng/μL with DEPC water.

Table 2
Oligonucleotides used in this study.

Name	Sequence (from 5′ to 3′)
AfpyroA_f	TCACAGCTTGTCTGTAAGCGGAT
AfpyroA_nest_f	GCAGATTGTACTGAGAGTGCACC
Afpyro_r	CTGCTTCCTCAACCTCTCGATTC
gpdA(p)_f	GAATCGAGAGGTTGAGGAAGCAGCAGACGGCGTAA CCAAAAGTCAC
gpdA(p)_r	GAGAGGGGAGCTCGGACATTGTGATGTCTGCTCAAGCGG
fphA(0)_f	ATGTCCGAGCTCCCCTCTC
fphA_nest_r	CGCGCTTGTTTAGGAATGTCGG
fphA(+1500)_r	CATGGCACGGAGATAGGCGT
fphA + 671_f	TGGTTCGCATTGTCAGCGAG
fphA + 4330_notI_r	AAGGAAAAAAGCGGCCGCAGCTGAGTTGAAGGGGA TGAATG
h2b-RT-F	CTGCCGAGAAGAAGCCTAGCAC
h2b-RT-R	GAAGAGTAGGTCTCCTTCCTGGTC
ccgA-RT-F	CGACGCTTCCCTCACTTCTC
ccgA-RT-R	CATCATGGGACTTCTCGTCCTT
ccgB-RT-F	GGAGACTATCAAGGTAAGCATGTACC
ccgB-RT-R	CTTGTCAAAGAGAGCGTCCTTG
conJ-RT-F	CTGAGAAGCAGCGCAACAT
conJ-RT-R	CTCATCGCCAGGCTGGAA
tcsB_rt_f	TATCTCAGCCTCTACCAAGCCAGC
tcsB_rt_r	CGCTGTGCCATTCGGATGATCATAGG
hk2_rt_f	GTGGCCAACAGTTAGCAGACACC
hk2_rt_r	CCGTCAAGTGGCCTGCTGTGA
tcsA_rt_f	CGCGCTCTCTCTCGACGATAACGA
tcsA_rt_r	GCTCAACGAGGGTACGGATGGAC
phkA_rt_f	CTTTGGCCTCCGACATGCTGC
phkA_rt_r	GTCGTGCGTCGGTTCGTGGA
phkB_rt_f	GCATGAGAACACCTCCGCTTCCATC
phkB_rt_r	TCGTGGGCAAAACTGGAGAGTGTC
nikA_rt_f	GGCTTGCGCCGGACGAGAC
nikA_rt_r	GAGTCGACGCCATTCGTGGATTG
hk-8-1-f	GGACGGGATTTGTCATCTCGTCTGC
hk-8-1-r	CGATCATCTTTCGTCAGGTCGGGAAC
hk-8-2-f	TGGGAGCCATGAGACTTGGTGC
hk-8-2-r	ACATCCTAGCCGTAGTTCGTCGTC
hk-8-3-f	GCCTCGCGTGACAATGTCCTCA
hk-8-3-r	GGCCTCTGCAATGACATACTGCTGC
hk-8-4-f	GGCGTCGTCACGCTCGAGAG
hk-8-4-r	ACTCCGAATCGCTTGTCGAATGTGTC
hk-8-5-f	TCCTCACCCCACGGTCGC
hk-8-5-r	AAATCTGCAAGCGTTTGCCACAGC
hk-8-6-f	CAATAAGCGGCTTGCCTCAGATGC
hk-8-6-r	GTGGTGGCCTCGGCAATGAC
hk-8-7-f	CGGTCTCAATGTCGCCCAACTTC
hk-8-7-r	GTGTCCGTCAATGAGCGAGGC
hk-9-f	GCCCGAACCCATGGAATCAGACT
hk-9-r	ACTGCCCAGAGTTTTCAATTTGCGAG
LreA_rt_f	CAACGACTTTGGGGGGTTCG
LreA_rt_r	GATGTATACGTGGCCATGTGTGC
LreB_rt_f	CCAACTAGCTCAGCGCTCTCA
LreB_rt_r	TGGGATTGGAGGAGAGAGTGC
fphA_RTQ_fw	ATGGAGGCGCTGTTGGATACAA
fphA_RTQ_rev	CAGATCTATGCTCATCGTCGGA
CryA_rt_f	CTCTCCAATGGCGCTGATGAG
CryA_rt_r	CGCTGTTGTAAGCTTCGCACC
VeA_rt_f	GCGAGAGCTTGTGGTCAAGG
VeA_rt_r	GTTCAATTCAATGACTGGCGGAGG
AN11039_rt_f	TTAAATACCTTCAACACCCGCTGCTTCC
AN11039_rt_r	GCGGTCCTTGTAACCGGGATCTT
AN03558_rt_f	ATGCCTTCTTACACCGGCTCCTG
AN03558_rt_r	TGACGTCCGCGCGTCGTC
AN03314_rt-f	ATGCTGTATCCCGTGGTCAGGC
AN03314_rt-r	GCGCATCATCGGGCGAATTAAGG
AN7600-qPCR-fw	ACGTTCCTACTCTCCAGACC
AN7600-qPCR-rev	TTCGCAAGGTATAACCGCTC
cysD-qPCR-fw	CTGGTGTTACGGAAGATGCC
cysD-qPCR-rev	GGACAACTTTGAAGGTCGCT
sA-qPCR-fw	CCAGCCAAGATGCATTCCAA
sA-qPCR-rev	ACTCTTCACTTGAGCCACCA
sB-qPCR-fw	GGATATCACCATTGGCCCTG
sB-qPCR-rev	CAAGATGCAATAACGTGCGG
sC-qPCR-fw	AATTGACGACATCTACCGCC
sC-qPCR-rev	TCGACCTTGGTGTTCAGGTA

Quantitative real time PCR was performed with the SensiFAST SYBR & Fluorescein One-Step Kit from Bioline (Luckenwalde, Germany). The *h2b* gene was used for normalization. Each expression level is the mean value of three biological replicates.

2.5. Microarray analysis

Transcriptomes of two strains, W1 and RM114, both grown in MM-S supplemented with 0.35 mmol/L methionine, were compared in two biological replicates, each in two technical replicates with dye swap. Mycelia were powdered in liquid nitrogen and total RNA was isolated using TRI Reagent (Molecular Research Center) according to the manufacturer's protocol and subsequently precipitated with lithium chloride added to a final concentration of 3.42 mol/L. RNA probes were fluorescently labeled with Cy3 or Cy5 were synthesized using Quick Amp Labeling Kit, two-color (Agilent Technologies) according to the manufacturer's protocol, using 5 μg of total RNA as template. Labeled probes were hybridized to *A. nidulans* custom-designed microarray slides (purchased from Agilent) in an 8 × 15 k format containing oligonucleotides representing all known *A. nidulans* genes identified in the Aspergillus Genome Database (AspGD) version s06-m01-r07. Following hybridization, the microarrays were scanned with an Axon GenePix 4000B microarray scanner (Molecular Devices, LLC). Feature extraction was done with GenePix Pro 6.1. Raw LogRatio results from all biological and technical replicates were Lowess normalized, the resulting data for each gene were averaged and the statistical significance (*P*-values) was calculated with the Acuity 4.0 software. Additional data manipulations were done in Microsoft Excel. The transcriptomic data were submitted to Gene Expression Omnibus and are available under accession number GSE156068. Gene Set Enrichment analysis was performed on the FungiFun server (https://elbe.hki-jena.de/fungifun/; [25]).

2.6. Detection of secondary metabolites

Mycelia and culture media for HPLC analysis were collected from a single Petri dish. Mycelia were ground with glass powder, suspended in 5 mL of water and extracted twice with 5 mL of ethyl acetate for 30 min with agitation. Culture media were homogenized in tissue grinder with 25 mL of water and extracted twice with equal volume of ethyl acetate as above. The combined ethyl acetate layers were evaporated and re-dissolved in 1 mL of MeCN. HPLC analyses were performed using a Knauer dual pump system with a multi-channel UV spectrophotometer based on a diode array technology detector managed by Clarity Chrom controller V2.6.5.517. Waters Nova-Pak® C18, 60 Å, 4 μm, 4.6 mm × 250 mm cartridge column at a flow rate of 1 mL/min with UV detection in the range from 215 to 350 nm was used for analytical separations. The HPLC solvent gradient was 5% MeCN–H_2O (solvent A) and 95% MeCN–H_2O (solvent B) both containing 0.05% formic acid: 0% B from 0 to 5 min, 0 to 100% B from 5 to 35 min, 100% B from 35 to 50 min [26]. Asperthecin, emericellin, shamixanthone and epishamixanthone were identified by comparison of elution time and UV spectra with published data [26,27]. Identity of the xanthones was confirmed by mass spectrometry (not shown). Sterigmatocystin was identified by comparison with a standard (Sigma-Aldrich).

3. Results

3.1. MetR controls much more than sulfur metabolism

The role of *A. nidulans* MetR and its fungal orthologues in *S. cerevisiae* and *N. crassa* in sulfur metabolism is well established. In

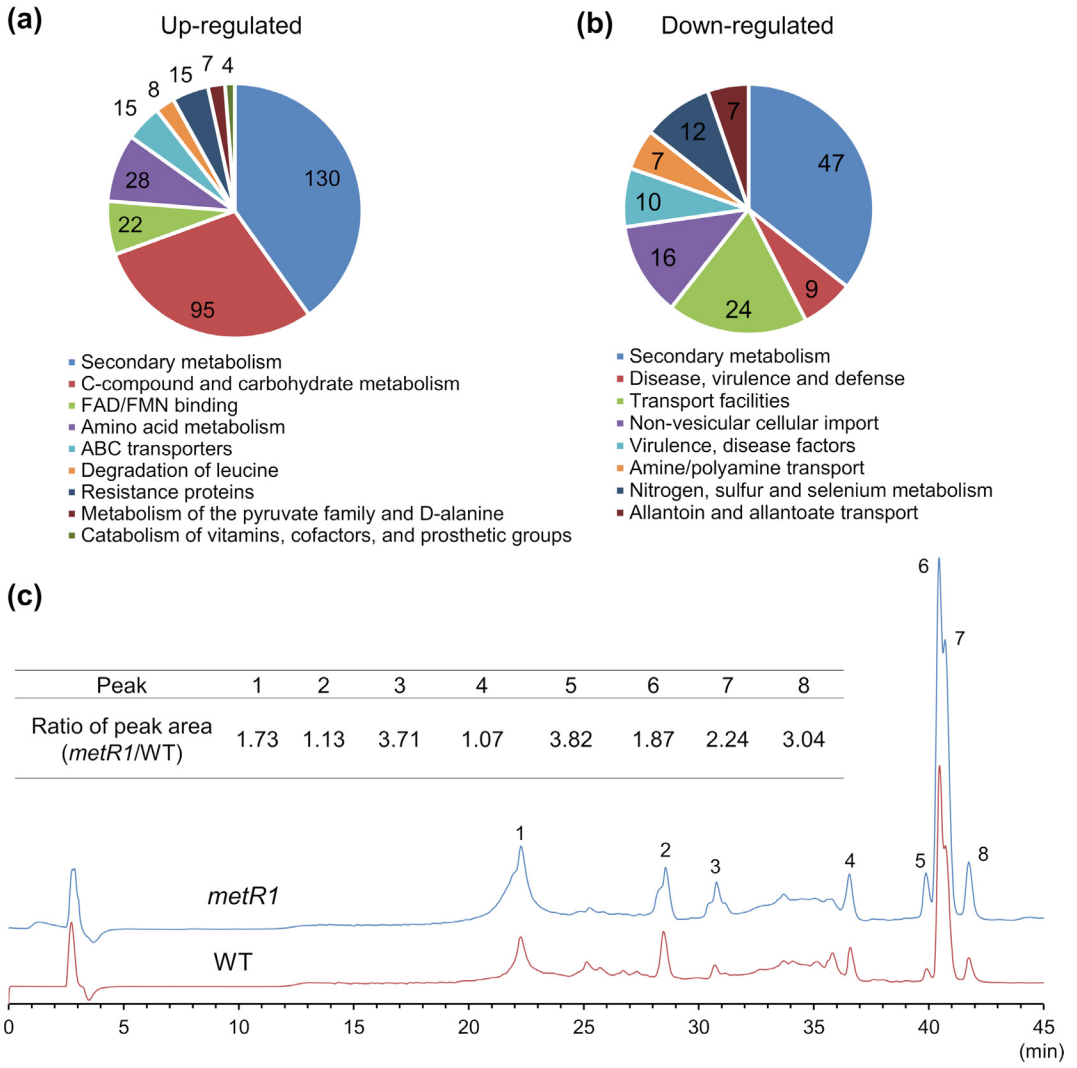

Fig. 1. Enrichment of functional categories among differentially expressed genes in the *metR1* mutant (RM114) in comparison to wild type (W1). (a) Functional categories enriched among 732 upregulated genes. (b) Functional categories enriched among 264 down-regulated genes. The number on each sector in the pie chart represents the number of genes enriched in each category. Pie charts were created with functional categories for adjusted $P < 0.05$. (c) HPLC profiles of the extracts from wild type and the *metR1* strain. The strains were cultured for 6 d and the secondary metabolites were extracted with ethyl acetate from mycelia and the culture media. The inserted Table shows the ratio (*metR1*/WT) of the peak areas for each compound. The identified metabolites are asperthecin (1), sterigmatocystin (2), emericellin (6), shamixanthone (7) and epishamixanthone (8).

order to unravel if MetR fulfills further functions, we performed genome-wide expression comparisons between wild type and the *metR1* mutant. The *metR1* mutation causes a frameshift in the leucine zipper motif and thereby a replacement of the 65 C-terminal amino acids of the 294 amino acid long protein by a 16 amino acid peptide [4]. *A. nidulans* W1 (containing the *metR* wild type allele) and RM114 (*metR1* mutant) were grown in sulfur-deficient minimal medium, supplemented with 0.35 mmol/L methionine. Transcriptomic analyses revealed that 732 genes were at least two-fold up-regulated whereas 264 genes were more than two-fold down-regulated (Tables S1–S3 online). The analysis of functional categories enriched among differentially expressed genes (Table S4 online) revealed that the regulation of secondary metabolism is apparently disturbed in the *metR1* mutant (Fig. 1a, b). To confirm the effect of the *metR* mutation on secondary metabolite production in *A. nidulans*, HPLC analysis was performed with ethyl acetate extracts of wild type and the *metR1* mutant mycelia. Eight peaks were resolved, six of which were by at least 70% larger in the *metR* mutant than in the wild type (Fig. 1c). The effects on sulfur metabolism are relatively weak and only the sulfate assimilation pathway and homocysteine synthase genes are downregulated in the mutant suggesting that they may be direct targets of this transcriptional activator (Fig. 2 and Table S5 online). On the other hand, cysteine and methionine metabolism genes are upregulated, suggesting that they may be transcriptionally controlled by other regulatory system. However, many other genes were strongly upregulated in the *metR1* mutant (Tables S1, S3 online). The most upregulated gene was *mdpC*, which is a member of the monodictyphenone (mdp) secondary metabolite biosynthesis gene cluster. In the *metR1* mutant, *mdpC* was upregulated 111.6 fold in comparison to wild type (Fig. 2b). Besides *mdpC* all other genes of this cluster were significantly upregulated. The second most upregulated gene was *orsA*, a member of the orsellinic acid and violacerol (F9775) biosynthesis cluster, which was upregulated 75.5 fold in the *metR1* mutant. Likewise, the expression of all other genes in this cluster was upregulated as well. The asperfuranone biosynthesis cluster was also highly expressed in the *metR1* mutant. All genes of the cluster were upregulated and six of them were upregulated more than 15-fold in comparison to wild type. Among the differentially expressed genes, 52 encode putative transcription factors (Table S6 online). In *Neurospora crassa* the palindromic sequence ATGRYRYCAT was characterized as the binding

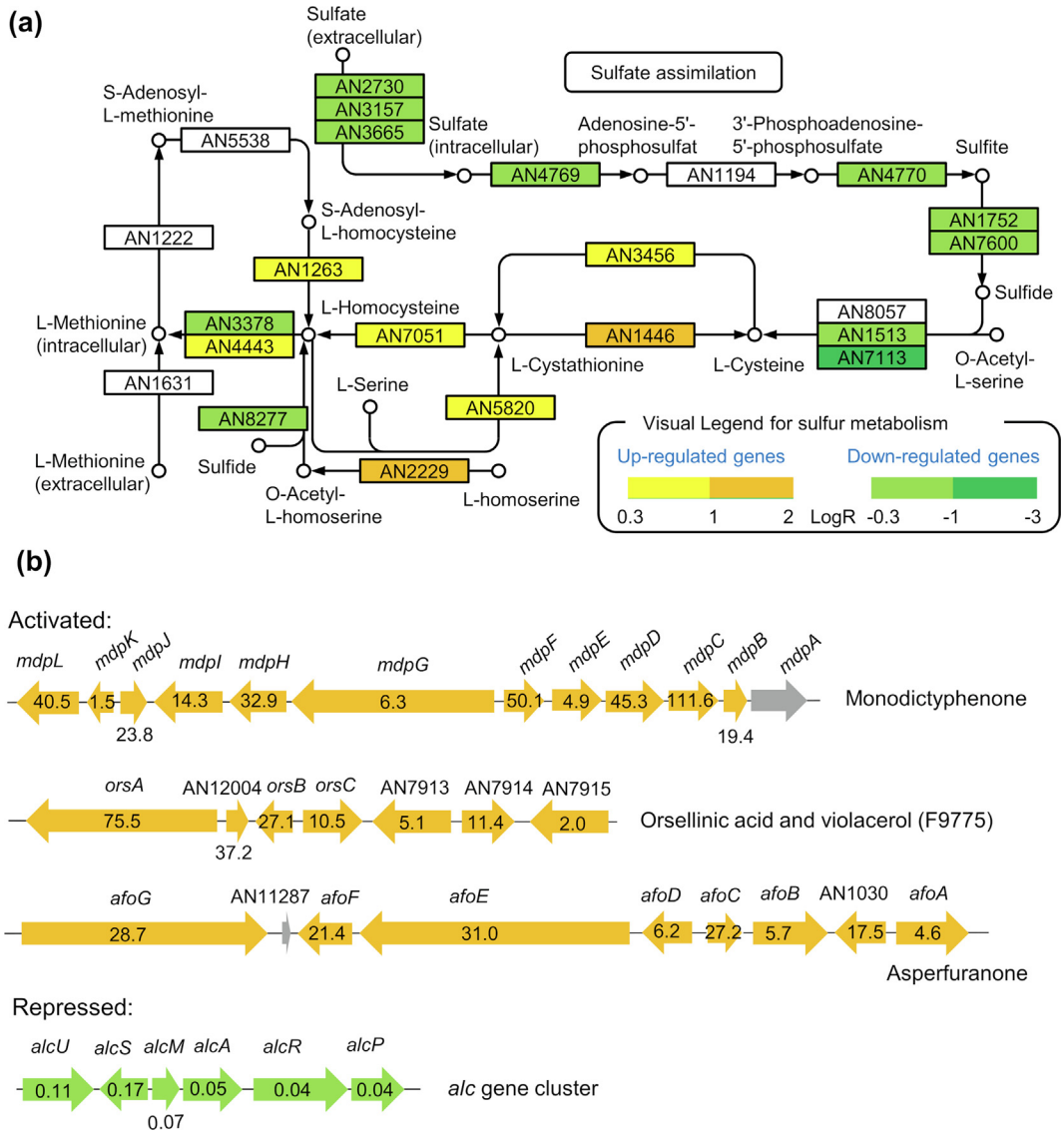

Fig. 2. Transcriptomic effects of the *metR1* mutation. Expression of sulfur metabolism genes (a) and the most significantly up- and down-regulated clusters for secondary metabolite biosynthesis (b) are shown. The numbers on the genes are fold changes of expression level of each gene in the *metR1* mutant in comparison to wild type. Some genes are colored according to regulation in *metR1* as indicated in the figure.

site of CYS3, the homolog of MetR, but this sequence was not detected in the promoters of these putative transcription factor encoding genes. Interestingly, the most down-regulated gene in the *metR1* mutant was AN9008, encoding phytochrome, FphA (Table S2 online). This connection between phytochrome and the sulfur metabolism transcription factor MetR was further investigated in this study.

3.2. MetR strictly controls phytochrome signaling and affects the expression of five other histidine kinases

To validate the expression data obtained in the microarray analysis, we quantified the *fphA*-transcript abundance in wild type, the *metR1* mutant and three Δ*metR*-deletion strains by quantitative real time PCR. In wild type, *fphA* was expressed well, but in all *metR* mutants, *fphA* mRNA was absent (Fig. 3a and Fig. S1 online). In contrast, the expression of other photoreceptors or genes involved in the light response of *A. nidulans* [14], such as *lreA*, *lreB*, *cryA*, and *veA*, was not affected by the absence of MetR (Fig. 3a). Because *metR* is required for inorganic sulfur assimilation and the activity of MetR is reduced in the presence of organic sulfur sources, we tested if *fphA* expression is affected by methionine, cysteine or the non-sulfur containing amino acid alanine. We expected down-regulation of *fphA* in the presence of methionine or cysteine and no effect by alanine. However, *fphA* was upregulated in the presence of all three amino acids (Fig. S2 online). This novel link between the availability of amino acids and phytochrome was not further investigated here.

In the *fphA*-deletion strain, 109 genes were upregulated and 210 downregulated in the dark in comparison to wild type (unpublished RNAseq data). However, only few genes of the differentially expressed genes in the *fphA*-deletion strain overlapped with the differentially expressed genes identified in the *metR1* mutant (Fig. 3b).

Next, we analyzed whether other histidine kinases were also under the control of MetR. The *A. nidulans* genome encodes 14 hybrid histidine kinases, besides phytochrome. Five of them, *hk-8-1*, *hk-8-2*, *hk-8-3*, *hk-8-5*, and *hk-8-7* were down-regulated in the *metR1* mutant in comparison to wild type (Fig. 4).

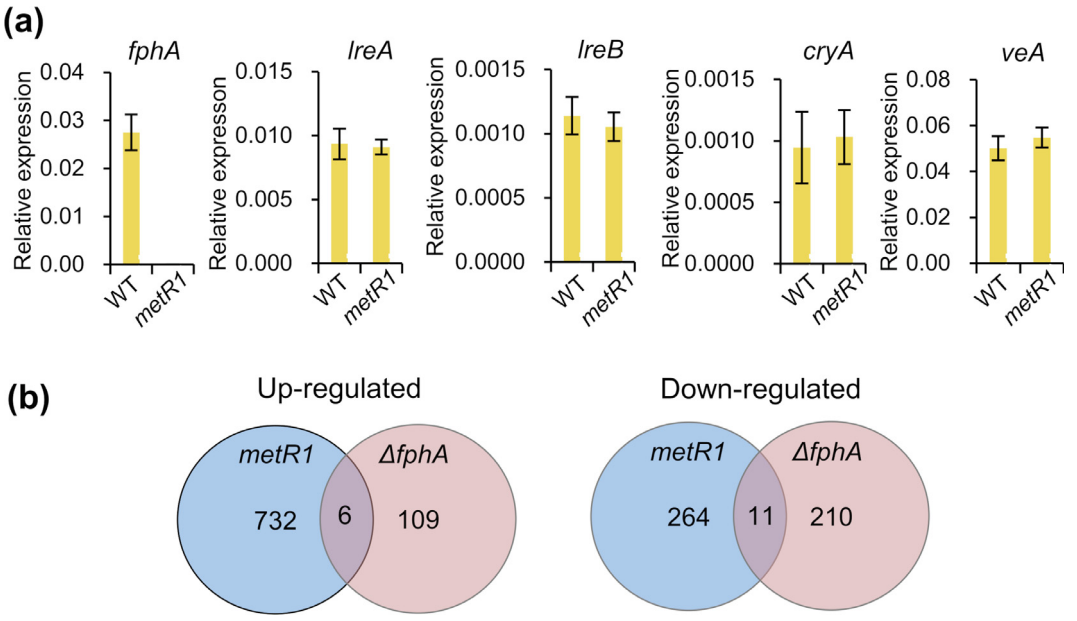

Fig. 3. Expression of some genes related to light signaling in the *metR1* mutant and comparison of differently expressed genes in the *metR1* mutant and the *fphA*-deletion strain. (a) Expression of *fphA*, *lreA*, *lreB*, *cryA*, and *veA* in wild type and the *metR1* mutant. Fresh spores of wild type and the *metR1* mutant were inoculated on the surface of supplemented liquid medium and cultured overnight at 37 °C. The mycelium was collected and frozen immediately in liquid nitrogen for RNA isolation. The expression level of each gene was normalized to the histone *h2b* gene. The error bar represents standard deviation (SD). (b) Venn diagram of differently expressed genes in the *metR1* mutant and the *fphA*-deletion strain.

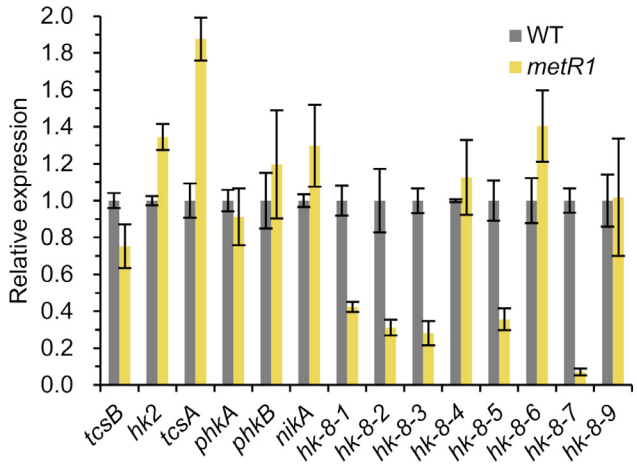

Fig. 4. Expression of genes encoding hybrid histidine kinases in wild type and the *metR1* mutant. Wild type and the *metR1* mutant were cultured on the surface of supplemented liquid medium overnight at 37 °C and the mycelium was collected for RNA isolation as above. The expression level of each gene was first normalized to the histone *h2b* gene and the expression level of each gene in wild type was set as 1.0. The error bar represents standard deviation (SD).

3.3. MetR deficiency phenocopies the fphA-deletion phenotype

One characteristic phenotype of the *fphA*-deletion strain is the effect on the balance between asexual and sexual development [16]. In order to test if *metR* mutation causes similar phenotypes, conidiospores and cleistothecia of *A. nidulans* wild type, the *fphA*-deletion strain and the *metR1* mutant produced in the dark or in light were quantified (Fig. 5). In wild type, light caused a significant increase of the amount of conidiospores in comparison to darkness in all three strains. The *fphA*-deletion and the *metR1*-mutant strain also produced more conidiospores in light but slightly less than wild type. Light inhibits sexual development. Upon illumination, wild type only formed 15% of the amount of cleistothecia produced in the dark. However, light did not inhibit the formation of cleistothecia in the *metR1* mutant or the *fphA*-deletion strain.

Phytochrome signaling occurs through the activation of the HOG MAP-kinase pathway [19]. Because several other stress signals are also transmitted through this pathway, we tested whether the light-dependent MAP kinase SakA/HogA shuttling is abolished in the *metR1*-mutant strain. To this end, SakA was tagged with GFP and expressed under the control of the *sakA*-native promoter in wild type (SZY34), the *fphA*-deletion strain (SZY37), the *metR1* mutant (SZY66), and the *metR1* mutant constitutively expressing *fphA* (SZY67). All strains were inoculated on cover slips with supplemented minimal medium and cultured overnight in the dark before a five minute white light stimulus. SakA-GFP shuttled into nuclei in wild type, whereas SakA-GFP remained in the cytoplasm in the *metR1*-mutant and the *fphA*-deletion strains (Fig. 6a). When the *fphA*-coding sequence was placed under the control of the constitutive *gpdA* promoter, SakA-GFP was again able to shuttle into nuclei. Colonies of the strain overexpressing *fphA* grew well in the dark, but no cleistothecia were formed. Growth was strongly reduced in white light (Fig. 6b). This growth inhibition could be due to hyperactivation of the SakA pathway.

We further analyzed the transcript abundance of two light-inducible genes, *ccgA* and *conJ*, in wild type, the *fphA*-deletion strain and the *metR1* mutant (Fig. 6c). In wild type, both genes were strongly induced after the mycelium was exposed to white light for 15 min. However, in the *fphA*-deletion strain and the *metR1* mutant light induction was lost.

The global regulator of secondary metabolism, VeA, interacts physically with phytochrome [18,28]. Previous genome-wide gene expression analyses have shown that light inhibits *veA* expression

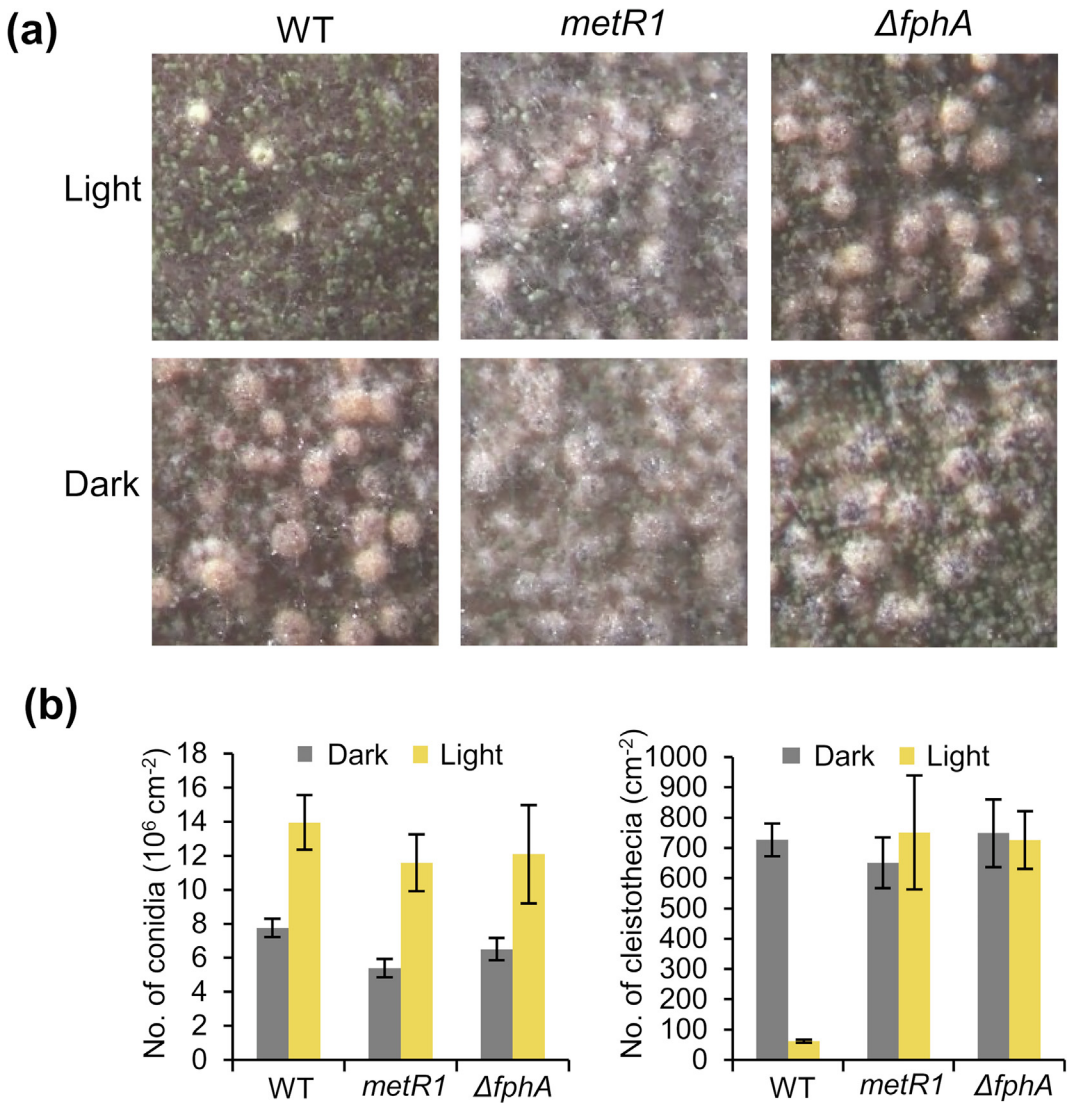

Fig. 5. Quantification of conidiophores and cleistotheicia of wild type, the *metR1* mutant and the *fphA*-deletion strain. (a) Images of the colony surfaces. Same amounts of fresh spores of each strain were evenly spread on the agar plates of supplemented minimal medium and incubated in the dark at 37 °C for 12 h and afterwards half of them were shifted to white light and further incubated for 4 d. Images of the colony surfaces were taken with stereo microscope. The scale bar is 100 μm. (b) Cleistothecia and conidiospores were quantified.

after the mycelium of *A. nidulans* is exposed to white light for 30 min [29]. Because here we found dramatic mis-regulation of a large proportion of genes involved in secondary metabolism in the absence of functional MetR, we compared the expression of *veA* in wild type and the *metR1* mutant. Whereas *veA* was repressed in light in wild type, no significant difference was observed between light and dark conditions in the *fphA*-deletion strain or the *metR1* mutant (Fig. 6d). This effect was not observed in the genome-wide expression analysis (Fig. 1), because the analysis was performed in *veA1* strains.

3.4. Growth under sulfur-starvation conditions

Because MetR was described as the central regulator for sulfur assimilation, and because MetR appears to be connected to phytochrome, we asked if *fphA* contributes to sulfur assimilation. One possibility is that MetR regulates sulfur assimilation genes through FphA. In order to circumvent *fphA*-expression regulation through MetR, we placed *fphA* under the control of the *gpdA* promoter. Growth of different strains was tested on minimal or complete medium with or without methionine (Fig. 7a). In contrast to wild type (with the *veA1* mutation or with the *veA* wild-type allele), *metR1* mutants (RM114 and SZY61) did not grow on medium without methionine. Growth of strain SZY63, in which *fphA* was constitutively expressed, also strictly depended on the presence of methionine (Fig. 7a).

Light represses sexual development and promotes asexual development in *A. nidulans*. To investigate if inorganic sulfur assimilation affects the developmental processes of *A. nidulans* in light, the phenotype of wild type (FGSCA4) on sulfur-starvation minimal medium in the dark and in light was analyzed (Fig. 7b, c). Sexual development of *A. nidulans* was repressed on standard minimal medium in light in comparison to dark conditions but small amounts of cleistothecia were still formed on the surface of the colony. However, when the concentration of inorganic sulfur (SO_4^{2-}) in minimal medium was reduced from 2 to 0.1 mmol/L, light com-

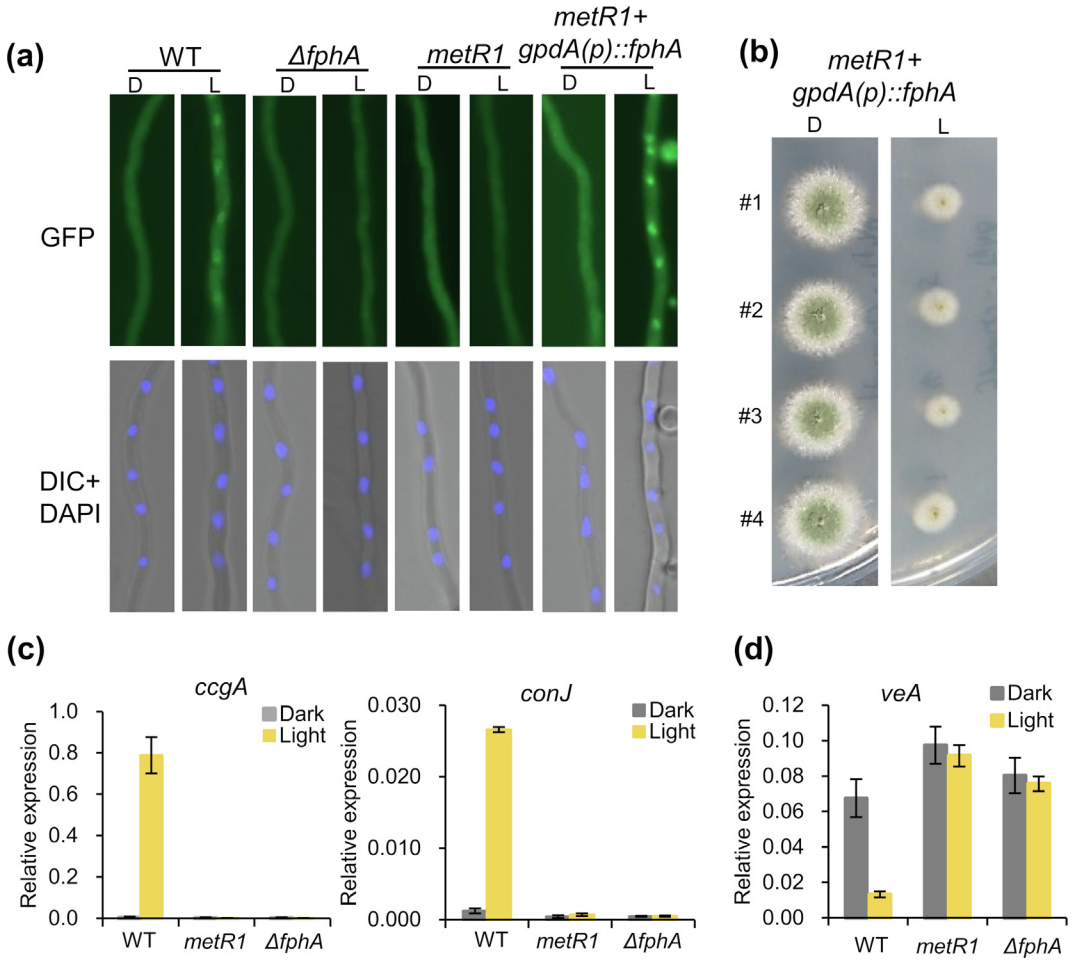

Fig. 6. Localization of SakA and expression of light-regulated genes upon light stimulus. (a) SakA-GFP shuttling upon light. Fresh spores from each strain were inoculated on the microscope coverslips with 400 μL supplemented liquid minimal medium. The samples placed in a Petri dish were incubated at 37 °C for 12 h in the dark and then exposed to light or kept in the dark for 5 min before formaldehyde fixation. (b) Four independent colonies were grown for 2 d in the dark (left) or in white light (right). (c) Expression of light-inducible genes, *ccgA* and *conJ*, in different strains strains upon light stimulus. Wild type (SJR2), the *metR1* mutant (SJR10), and the *fphA*-deletion strain (SZY31) were cultured on the surface of supplemented liquid medium at 37 °C for 18 h and then exposed to white light for 15 min or kept in the dark. The mycelium was collected in dim green light and subjected to RNA isolation. The expression level of each gene was normalized to the histone *h2b* gene. The error bar represents standard deviation (SD). (d) Expression of the light-repressed gene, *veA*, in different strains upon light stimulus. The experiment was performed as described above.

pletely inhibited sexual development. Conidia formation was increased with increased sulfur concentrations with higher numbers of conidia in light.

We further measured the expression of the crucial genes involved in sulfur assimilation, which encode sulfate permease (*sB*), ATP sulfurylase (*sC*), adenosine 3′-phosphate 5′-phosphosulfate (PAPS) reductase (*sA*), β subunit of sulfite reductase (AN7600) and cysteine synthase (*cysB*) (Fig. 8). On the standard minimal medium, the expression of these genes in wild type and the *fphA*-deletion strain was not affected significantly by light, but in the *sakA*-deletion strain their expression was increased especially in the dark in comparison to wild type. After the concentration of inorganic sulfur (SO_4^{2-}) was reduced from 2 to 0.2 mmol/L, the expression of these genes in wild type was repressed in light, which was dependent on FphA (Fig. 8).

To analyze if light signaling may indeed be involved in sulfur metabolism, we analyzed differentially expressed genes in RNAseq data performed with wild type under light and dark conditions (unpublished data) and searched for genes connected to sulfur metabolism. Genes directly involved in sulfur assimilation, such as sulfate permease, ATP sulfurase, cysteine synthase, and homocysteine synthase were not light-regulated. However, three genes (AN1103, AN3558 and AN3314) encoding putative carbon–sulfur lyases were upregulated in light (Fig. 9). Carbon-sulfur lyases catalyze the cleavage of carbon–sulfur bonds and mobilize sulfur from organic molecules. To confirm whether the induction of these genes is phytochrome-dependent, their transcript abundance was measured in wild type and the *fphA*-deletion strain (Fig. 9). In wild type, they were all induced by light and the induction depended on phytochrome.

4. Discussion and conclusion

Genome-wide expression analyses of several fungal species revealed that besides morphogenetic pathways a large proportion of the metabolism appears to be under the control of light regulation [29–31]. Here, we show a novel link between sulfur-metabolism regulation and light responses in *A. nidulans*. We found

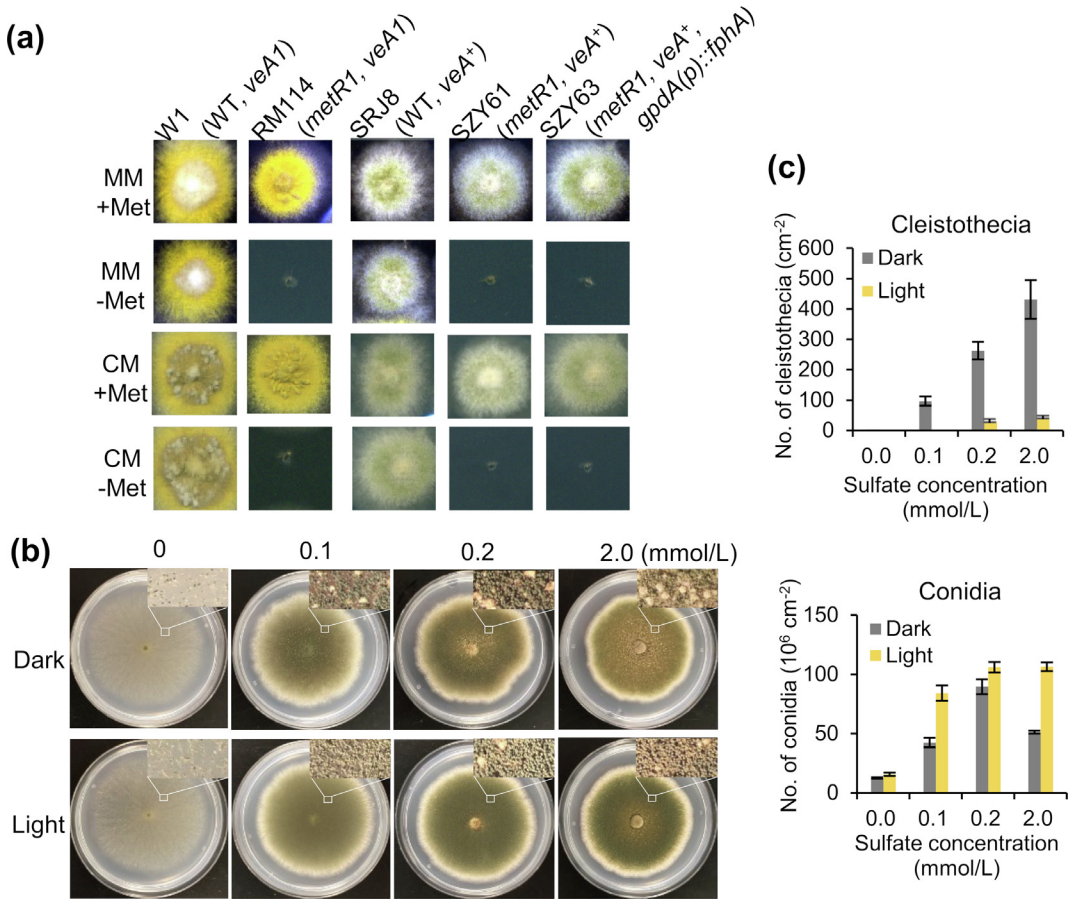

Fig. 7. Growth of *metR1* strains compared to wild type under different conditions. (a) Wild type strains and *metR1* mutants were grown at 37 °C for 2 d on minimal (MM) or complete (CM) medium supplemented with 1 mmol/L methionine (+Meth) or without methionine (−Meth). (b) Phenotype of the wild type strain under different concentrations of inorganic sulfur. Wild type FGSCA4 was cultured at 37 °C for 6 d in light or in the dark on minimal medium with different concentrations of inorganic sulfur as indicated. (c) Cleistothecia and conidiospores were quantified. The error bar represents standard deviation (SD).

that mutation of the sulfur-starvation response regulator MetR causes mis-regulation of hundreds of genes, one of which was the phytochrome *fphA*. The finding is in agreement with recent findings in *A. alternata*, although in that study *fphA* did not appear on the list of downregulated genes [13]. These results suggest that MetR is not only a regulator of sulfur metabolism but appears to control expression of about 10% of the genome. However, it cannot be decided yet if MetR controls all those genes directly or indirectly. It was striking that MetR apparently affects the expression of 51 putative transcription factors. Given that MetR appears to be involved in the expression control of many genes, the question arises why vegetative growth was only affected under sulfur-starvation conditions. Obviously, all other mis-regulated gene functions were somehow compensated or not necessary for hyphal growth, like the *fphA* gene. Similarly, FphA controls hundreds of genes, but vegetative growth is also not affected in the absence of FphA [16,29]. This phenomenon was also reported for another global regulator of *A. nidulans*, RlcA [32].

Moreover, we found that especially several secondary metabolite gene clusters are up- or downregulated in the absence of MetR. Those clusters are under the control of VeA and LaeA. VeA was unaffected in the *metR1* mutant and *laeA* was upregulated by 50% (calculated from the microarray data). The absence of LaeA did not affect *metR* expression significantly (data not shown), although in *A. fumigatus metR* was downregulated in a Δ*laeA* strain [33]. Our results suggest that the mis-regulation of the secondary metabolite clusters is not due to mis-regulation of VeA or LaeA.

The most striking observation of this study was the fact that MetR is apparently strictly required for phytochrome production. This raises the question if light perception and the status of sulfur supply are somehow connected and if so, what is the molecular mechanism. Because *metR* is required for inorganic sulfur assimilation and the MetR protein inactivated or degraded in the presence of cysteine, we tested if *fphA* expression is affected in the presence of methionine, cysteine or the non-sulfur containing amino acid alanine. However, *fphA* was even upregulated in the presence of all three amino acids tested, even in the presence of alanine. This could mean that in the wild-type strain, MetR activates *fphA* through the binding sites in the promoter. This is supported by the fact that *metR* deletion caused a drastic decrease of the *fphA* transcript level. In the mutant strain MetR1 might repress transcription, because it still could bind to the promoter. If the concentration of organic sulfur increases, the MetR1 protein could be degraded and therefore activation of *fphA* transcription would occur. This could explain effects of cysteine and methionine, however, the effect in the presence of alanine cannot be easily explained. But it points to a novel link between amino acid availability and phytochrome.

Another puzzling result is a very little overlap between genes differentially expressed in the *metR1* and *ΔfphA* mutants. Since

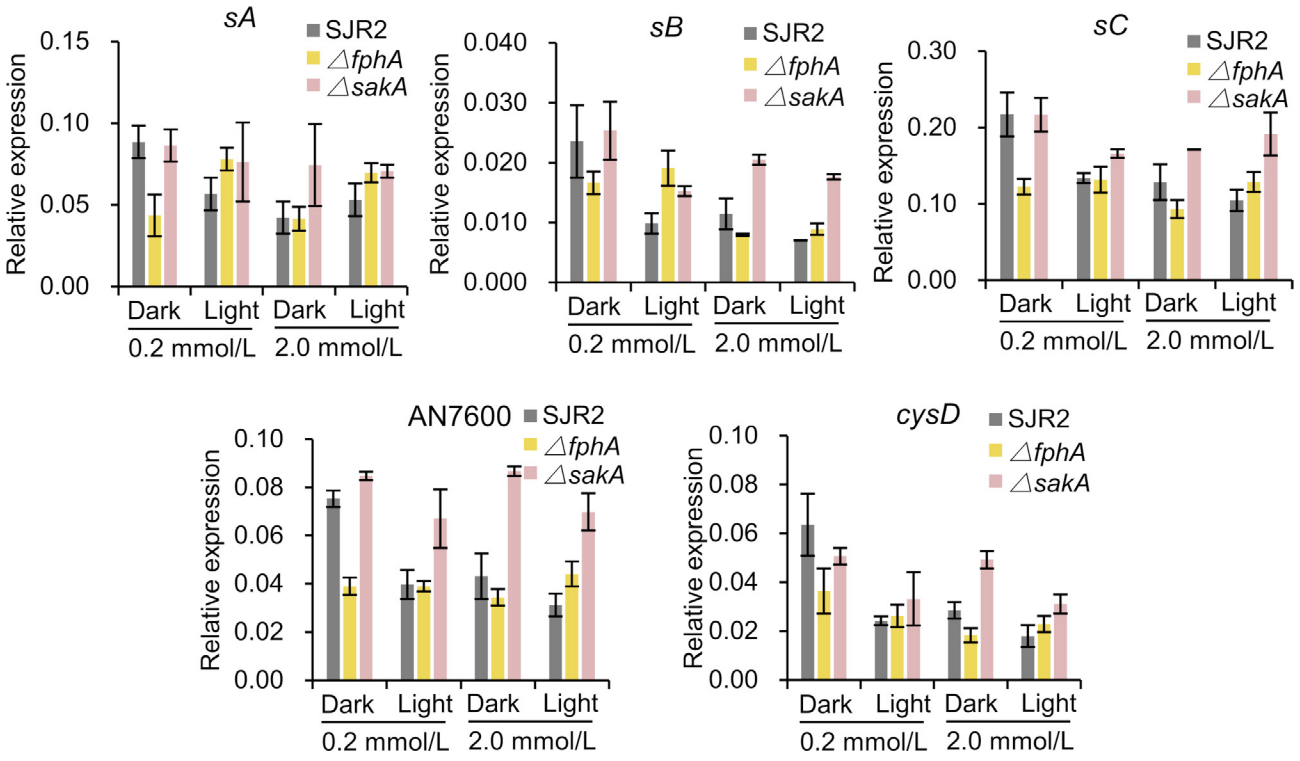

Fig. 8. Expression of key genes involved in sulfur assimilation in light and dark at different inorganic sulfur concentrations. The wild type strain (SJR2), the *fphA*-deletion strain (SJR10) and the *sakA*-deletion strain (SZY31) were cultured on supplemented medium with different concentration of inorganic sulfur at 37 °C for 18 h in the dark and then exposed to white light for 15 min or kept in the dark. The mycelium was collected in dim green light and subjected to RNA isolation. The expression level of each gene was normalized to the histone *h2b* gene. The error bar represents standard deviation (SD).

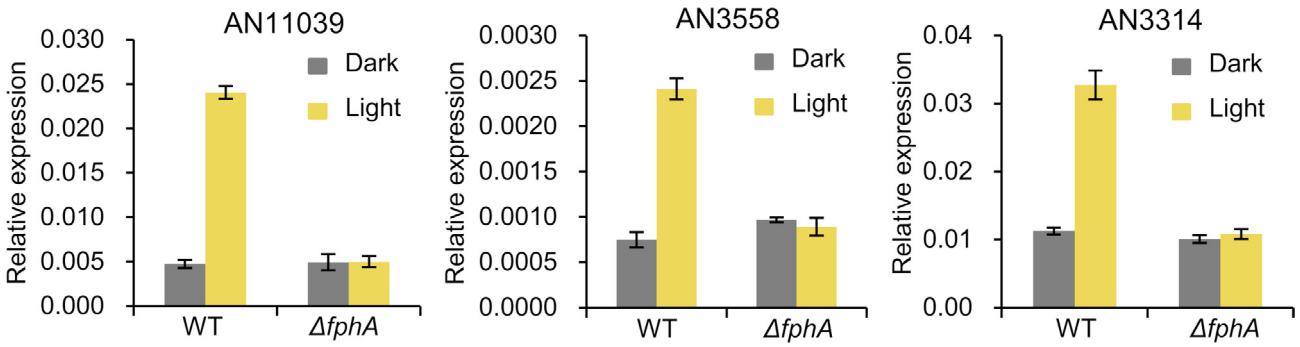

Fig. 9. Expression of carbon–sulfur lyases encoding genes upon light stimulus in wild type and the *fphA*-deletion strain. The light experiment was performed with wild type and the *fphA*-deletion strain as above. The expression level of each gene was normalized to the histone *h2b* gene. The error bar represents standard deviation (SD).

activity of MetR is controlled by SCF^{SconB} ubiquitin ligase we compared sets of genes differentially expressed in the *metR1* mutant to those differentially expressed in the *sconB* mutant (Table S3 online) [12]. However, the two sets of genes also showed only little overlap (Fig. 10a). Since we found that expression of 52 transcription factors is significantly changed in the *metR1* mutant it suggests that MetR may take part in controlling other still unidentified processes (Fig. 10b).

In summary, we found a novel linkage between light sensing and metabolism control. The *metR* gene, originally associated with sulfur metabolism, appears to be a more general transcription factor controlling directly expression of *fphA* and indirectly light responses and secondary metabolism in *A. nidulans*.

Conflict of interest

This authors declare that they have no conflict of interest.

Acknowledgments

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Author contributions

Zhenzhong Yu designed and performed most of the experiments and wrote a draft of the paper. Jia Gao and Olumuyiwa Igbalajobi performed also some of the experiments. Marek Skoneczny and Marzena Sienko did the microarray analyses. Agnieszka M. Maciejewska did the HPLC analyses. Jerzy Brzywczy designed the expression analyses experiments and edited the manuscript. Reinhard Fischer supervised the students in the laboratory in Germany, designed the experiments, and edited the manuscript. All of the authors discussed and commented on the manuscript.

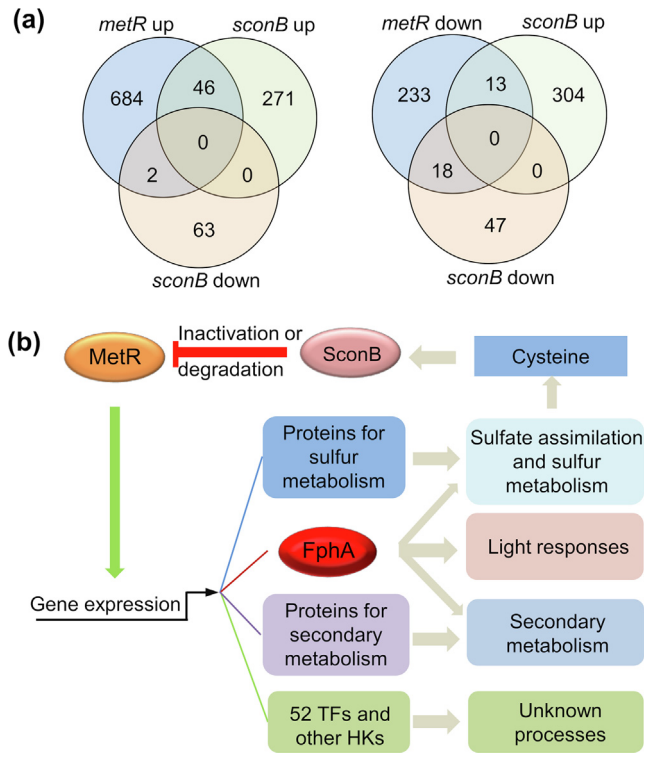

Fig. 10. Regulatory features of *metR*. Sets of genes differentially expressed in the *metR* and *sconB* mutants are compared by Venn diagrams (a) and biological processes controlled by MetR are summarized in (b).

Appendix A. Supplementary materials

Supplementary materials to this article can be found online at https://doi.org/10.1016/j.scib.2020.11.001.

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Zhenzhong Yu obtained his Ph.D. degree in Microbiology at the Karlsruhe Institute of Technology in Germany, where he continued his postdoctoral research to investigate the light signaling pathway in fungi. In 2019, he became a professor at Nanjing Agricultural University. His current research focuses on elucidating how enviromental cues regulate the development of *Aspergillus nidulans* and *Trichoderma guizhouense*.

Reinhard Fischer studied Biology in Marburg, did a postdoc in Athens, GA, USA and returned to Germany in 1993. Since 2004 he has been a professor for Microbiology in Karlsruhe. His work is focused on cell biology and genetics of filamentous fungi. The laboratory studies light sensing, polar growth, the formation of secondary metabolites, and the biology of nematode-trapping fungi.