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The regulatory network of the White Collar complex during early mushroom development in *Schizophyllum commune*

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ABSTRACT

Blue light is an important signal for fungal development. In the mushroom-forming basidiomycete *Schizophyllum commune*, blue light is detected by the White Collar complex, which consists of WC-1 and WC-2. Most of our knowledge on this complex is derived from the ascomycete *Neurospora crassa*, where both WC-1 and WC-2 contain GATA zinc-finger transcription factor domains. In basidiomycetes, WC-1 is truncated and does not contain a transcription factor domain, but both WC-1 and WC-2 are still important for development. We show that dimerization of WC-1 and WC-2 happens independent of light in *S. commune*, but that induction by light is required for promoter binding by the White Collar complex. Furthermore, the White Collar complex is a promoter of transcription, but binding of the complex alone is not always sufficient to initiate transcription. For its function, the White Collar complex associates directly with the promoters of structural genes involved in mushroom development, like hydrophobins, but also promotes the expression of other transcription factors that play a role in mushroom development.

1. Introduction

Light is an important signal in many filamentous fungi, affecting a large variety of processes, including metabolism, stress and development. While photoreceptors for red, green and blue light have been identified in fungi, the detection of blue light is the most ubiquitous and best-studied light signal in fungi. This is primarily due to the study of photobiology in the model organism *Neurospora crassa* (Chen et al., 2010; Dunlap and Loros, 2005). In this organism, the detection of blue light entrains the circadian clock, which affects many processes, including sexual and asexual development, sporulation and growth rate (Dunlap and Loros, 2017). Blue light is detected by the White Collar complex (WCC) a heterodimer consisting of WC-1 and WC-2 (Ballario et al., 1996; Linden and Macino, 1997). The function of these proteins in light sensing has previously been reviewed in detail (Corrochano, 2019). Both WC-1 and WC-2 contain a GATA zinc-finger transcription factor domain for DNA-binding. In addition, WC-1 contains a light-oxygen-voltage (LOV) domain that can bind a flavin chromophore and is responsible for the structural changes of the WCC upon the detection of blue light (He et al., 2002). Two activated LOV-domains can interact, resulting in a WCC dimer (Malzahn et al., 2010). Monomers of the WCC are able to bind the promoters of light-regulated genes, but dimerization is required for the activation of transcription. The binding of WCC dimers results in the opening of the chromatin by displacement of nucleosomes, and the recruitment of the histone acetyltransferase NGF-1 that acetylates histone H3 on lysine 14 (H3K14ac), promoting transcription (Grimaldi et al., 2006; Sancar et al., 2015). The process is negatively regulated by phosphorylation of the WCC, which prevents it from binding the promoter of *frq*, which encodes the clock-protein FRQ (Schafmeier et al., 2005). Furthermore, the WCC promotes transcription of *vvd*, which encodes the photoreceptor VVD (Heintzen et al., 2001; Schwerdtfeger and Linden, 2003). This protein disrupts dimerization of the WCC by binding to the WC-1 LOV-domain upon activation by light, acting as an inhibitor of the WCC.

Orthologs of WC-1 and WC-2 are found across the fungal kingdom, including mushroom-forming basidiomycetes (Idnurm et al., 2010; Ohm et al., 2013; Todd et al., 2014). This is expected, as blue light is an essential signal for the development of fruiting bodies in many species, including *Coprinopsis cinerea*, *Flammulina filiformis* and *Schizophyllum commune* (Kamada et al., 2010; Li et al., 2023; Ohm et al., 2013). In *S. commune,* deletion of either *wc-1* or *wc-2* prevents mushroom development completely, for which light is an essential signal. In addition, the mycelium becomes more sensitive to UV-light (Ohm et al., 2013). This is presumably due to a lower expression of ferrochelatase and DNA

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photolyase, which are important for preventing light-induced DNA damage. The interaction of WC-1 and WC-2 was shown for the first time in a basidiomycete by a yeast two-hybrid assay in *Pleurotus ostreatus* (Qi et al., 2020). Despite these similarities in function between the WCC in Ascomycota and Basidiomycota, there are also notable differences in the structure of the WCC between these phyla. In basidiomycetes, the WC-1 protein is truncated and lacks a GATA zinc-finger transcription factor domain. Furthermore, no ortholog of VVD has been identified in any basidiomycete (Idnurm et al., 2010). Finally, in the basidiomycete *Cryptococcus neoformans*, the number of light-regulated genes is much lower than in *N. crassa* (Chen et al., 2009; Idnurm and Heitman, 2010). This raises the question whether the mechanism and role of the WCC is conserved between different fungi.

Here we use the model mushroom-forming basidiomycete *S. commune* to study the activity of WC-2 in the presence and absence of light during mushroom development by a combination of transcription factor ChIP-Seq and RNA-Seq. This reveals the regulatory network of the WCC for the first time in a mushroom-forming fungus and shows the similarities and differences in light-sensing between ascomycetes and basidiomycetes.

2. Materials and Methods

2.1. Strains and culture conditions

The *S. cerevisiae* strains Y8800 (*MATa*) and Y8930 (*MATα*) were a gift from Mike Boxem. These strains with the genotype *trp1-901 leu2-3112 ura3-52 his3-200 Δgal4 Δgal80 cyh2R GAL1::HIS3@LYS2 GAL2::ADE2 GAL7::LacZ@met2* were routinely grown at 30 °C in yeast extract peptone dextrose medium (YPD), with or without 20 g L^{-1} agar for static and liquid cultures respectively. For auxotrophic selection, strains were grown on synthetic complete dropout medium (1.3 g L^{-1} medium supplement powder, 1.7 g L^{-1} yeast nitrogen base (Sigma, US), 5 g L^{-1} ammonium sulfate, 20 g L^{-1} glucose, pH adjusted to 5.9 with 6 M HCl (medium supplement powder was equal weight of each alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, uracil, valine)). The medium was supplemented with 1 mM histidine, 1 mM leucine, 0.4 mM tryptophan, 1.68 mM alanine and 0.2 mM 3-amino-1,2,4-triazole depending on the selection conditions.

S. commune was grown on *Schizophyllum commune* minimal medium supplemented with 1.5% agar at 25 °C and 30 °C as dikaryons and monokaryons respectively from small agar inoculum (Peer et al., 2009). When grown at 25 °C all cultures were exposed to a 16/8 hours day/-night cycle unless specified otherwise. Light illumination was supplied as 25.8 μmol m^{-2} s^{-1} in the spectrum of 380 nm to 780 nm. All strains used in this study are derived from *S. commune* H4–8 (*mat*A43*mat*B41; FGSC 9210) (Ohm et al., 2010). For dikaryotic cultures the isogenic compatible strain H4–8b (*mat*A41*matB*43) was used. The *S. commune* Δ*wc-2* strain was published previously (Ohm et al., 2013). For antibiotic selection the medium was supplemented with 15 μg mL^{-1} nourseothricin (Bio-Connect, The Netherlands) or 25 μg mL^{-1} phleomycin (Bio-Connect, The Netherlands).

2.2. Yeast two-hybrid assay

2.2.1. Plasmid construction

Total RNA was isolated with TRIzol (ThermoFisher Scientific, USA) from monokaryotic and dikaryotic H4–8 mycelium grown for 3 days, according to manufacturer's instructions. Next, this was reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen, Germany). To obtain a more complete library, cDNA from monokaryotic and dikaryotic mycelium was combined. The *wc-1* coding sequence was amplified from *S. commune* H4–8 cDNA with primers wc-1-y2h-prey-fw and wc-1-y2h-prey-rv (Table 1) that contain homology arms to pMB29 around the *Not*I digestion site, resulting in a 2616 bp fragment. The *wc-2* coding sequence was amplified from *S. commune* H4–8 cDNA with primers wc-2-y2h-bait-fw and wc-2-y2h-bait-rv (Table 1) that contain homology arms to pMB28 around the *Not*I digestion site, resulting in a 1152 bp fragment. In both forward primers, a guanine was inserted between the homology arm and the gene specific part of the primer, to ensure the gene was inserted in frame. Plasmids pMB29 and pMB28 (a gift from Mike Boxem (Koorman et al., 2016)) were digested with *Not*I and the *wc-1* and *wc-2* fragments were cloned into the respective plasmids with NEB HiFi DNA assembly mastermix (NEB, US). This resulted in the prey plasmid wc-1-pMB29 and bait plasmid wc-2-pMB28. In wc-1-pMB29, the *wc-1* coding sequence is inserted in frame downstream of the GAL4 activating domain, while in wc-2-pMB28 the *wc-2* coding sequence is inserted in frame downstream of the GAL4 binding domain. Both fusion genes are under the control of the ADH1 promoter and terminator. Furthermore, the prey and bait plasmids contain a *TRP1* and *LEU2* gene, respectively, to complement the tryptophan and leucine auxotrophy in the Y2H strains.

Table 1
Primers used in this study. Bold sequences indicate homology arms for Gibson assembly.

Name	Sequence
wc-1-y2h-prey-fw	**GGAGGGCGCGCCAGATCTGCG**ATGCCATTCGAAAAGTACTTCC
wc-1-y2h-prey-rv	**CGACGTCTTACTTACTTAGC**ACCTCCGCCGCTGTCAATA
wc-2-y2h-bait-fw	**GGAGGGCGCGCCAGATCTGCG**ATGTCTACAGTCCACGGCCG
wc-2-y2h-bait-rv	**CGACGTCTTACTTACTTAGC**GGCGTTACTGGGGCCAG
M13R	CAGGAAACAGCTATGAC
prey_fw	GAAGATACCCCAAACC
bait_fw	ATGGCGACATCATCATCGG
wc-2-chip-fw	**GCGTGGCCCCAAGCGTTGGA**TTCTACCTCGGTGGCTATCG
wc-2-chip-rv	**TCAGGGACGTCGTAGGGGTA**GGCGTTACTGGGGCCA

2.2.2. Yeast transformation and selection

S. cerevisiae strains Y8800 and Y8930 were transformed with the plasmids wc-1-pMB29 and wc-2-pMB28, respectively, using the lithium acetate method (Schiestl and Gietz, 1989). Briefly, *S. cerevisiae* was pre-grown overnight at 30 °C at 200 rpm and diluted to an OD_{600} of 0.4 in 50 mL YPD. After growing the culture for an additional 3 hours, the cells were collected by centrifugation at 2500 g for 60 seconds and resuspended in 40 mL 1x TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0). The cells were then centrifuged again at 2500 g for 60 seconds and resuspended in 2 mL LiAc/0.5x TE buffer (100 mM LiAc, 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA pH 8.0). After incubation at room temperature for 10 minutes, 100 μL of the competent cells were mixed with 1 μg plasmid DNA, 100 μg salmon sperm DNA and 700 μL LiAc/PEG-3350/1x TE buffer (100 mM LiAc, 40% w/v PEG-3350, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) and incubated at 30 °C for 30 minutes. Next, 88 μL DMSO was added and cells were heat shocked for 7 minutes at 42 °C. The transformed cells were then collected by brief centrifugation, resuspended in 100 μL 1x TE and plated on synthetic drop-out medium without tryptophan and leucine for strains Y8800 and Y8930, respectively. After incubation at 25 °C for 4 days, transformants were verified with M13R and either prey_fw or bait_fw for preys and baits (Table 1).

2.2.3. Mating and yeast two-hybrid

For the yeast two-hybrid the wc-1-pMB29 strain and wc-2-pMB28 strain were mated. As a negative control, strains with empty pMB28 and pMB29 plasmids in Y8930 and Y8800 were used. Each strain was pre-grown overnight in its respective synthetic drop-out medium. Subsequently 10 μL of the respective baits and preys was mixed with 100 μL -Leu -Trp synthetic drop-out medium and centrifuged for 1 minute at

180 rpm. After incubation for 1 day, 15 μL of each cross was transferred to 150 μL -Leu -Trip synthetic drop-out medium and grown for 2 more days. Finally, 15 μL was spotted to a -Leu -Trp -Ade synthetic drop-out medium agar plate and grown for 2 days. For the Y2H experiment without light, mating was performed in monochromatic red light and subsequently incubated in the dark.

2.3. WC-2 ChIP-Seq

2.3.1. Plasmid construction

A plasmid containing the coding sequence of WC-2 fused to an HA-tag under the control of its endogenous promoter was constructed as previously described (Marian et al., 2022). The *wc-2* gene excluding the stop codon and including an 810 bp promoter was amplified from genomic DNA with primers wc-2-chip-fw and wc-2-chip-rv, resulting in a 2165 bp fragment (Table 1). Both primers contained 20 bp homology arms to plasmid pPV009 digested with *Hin*dIII. The *wc-2* fragment was cloned into this plasmid with NEB HiFi DNA assembly mastermix creating plasmid wc-2-HA-pPV009.

2.3.2. Transformation of S. commune

Plasmid wc-2-HA-pPV009 was transformed into *S. commune* Δ*wc-2* protoplasts as previously described (Peer et al., 2009; Vonk and Ohm, 2021). Successful transformants were selected on phleomycin and confirmed by western blot as previously described (Marian et al., 2022).

2.3.3. Chromatin immunoprecipitation

H4–8 and H4–8 Δ*wc-2:: wc-2-HA* dikaryons were grown on porous polycarbonate (PC) membranes (diameter 76 mm; pore size 0.1 μm; Osmonics; GE Water Technologies, US) for 90 hours in either a 16/8 hour day/night cycle or in the dark. At the time of sampling in the light, colonies had been illuminated for 8 hours. Full colonies were collected and ChIP was performed as previously described in triplicate (Marian et al., 2022). Unlike previously described, ChIP was performed on single colonies of each strain. Furthermore, genomic DNA was fragmented with a diagenode bioruptor UCD-200 (diagenode, US) in 300 μL volume on HIGH setting with a 30 second ON, 30 second OFF cycle for 20 minutes. After DNA isolation libraries were prepared with the NEBNext ULTRA II DNA Library Prep Kit for Illumina (NEB, US) according to manufacturer's specification with the NEBNext Multiplex Oligos for Illumina (NEB, US). The resulting libraries were sequenced on the Illumina NovaSeq 6000 platform in SP mode with 2×100 bp output at the Utrecht Sequencing Facility (USEQ, www.useq.nl).

2.3.4. ChIP-Seq read analysis

ChIP-Seq analysis was performed as previously described (Marian et al., 2022; Vonk and Ohm, 2021). Peaks were called with MACS3 (version 3.0.0b1) with default settings for transcription factor peaks (Zhang et al., 2008). Peaks were then filtered for a minimum score of 100 before manual curation where clear artefacts were removed (e.g., repetitive regions). Peaks were correlated with genes if the center of the peak was within 1000 bp of a translation start site. The distribution of peaks was visualized with deeptools pyGenomeTracks (version 3.8) and custom python scripts (Lopez-Delisle et al., 2021; Ramírez et al., 2018).

2.3.5. Motif discovery

Enrichment of motifs was determined with MEME-ChIP from the MEME suite (Bailey et al., 2015). As a positive pool 200 bp fragments around the tip of the ChIP-Seq peaks in WC2-HA in the light were extracted. As a negative control, 10,000 random 200 bp sequences were selected from the genome of *S. commune* H4–8A. The final motif was identified by MEME and the location was determined by Centrimo (both programs are part of MEME suite). The motif was then used in TomTom (part of MEME suite) to search the JASPAR core non-redundant fungal motif database to determine if the motif was previously identified for another transcription factor (Castro-Mondragon et al., 2022).

2.4. RNA sequencing

H4–8 and H4–8 Δ*wc-2* dikaryons were grown in triplicate on porous polycarbonate (PC) membranes (diameter 76 mm; pore size 0.1 μm; Osmonics; GE Water Technologies, US) for 90 hours in either a 16/8 hour day/night cycle or in the dark. At the time of sampling in the light, colonies had been illuminated for 8 hours. RNA was extracted with TRIzol as previously described. After purification, the RNA was cleaned with the GeneJET RNA Purification kit (ThermoFisher Scientific, USA). Libraries were prepared with the Truseq RNA stranded polyA kit (Illumina, USA) according to manufacturer's instructions. The resulting libraries were sequenced on the Illumina NextSeq 2000 platform with a P2 flowcell and 2×50 bp output at the Utrecht Sequencing Facility (USEQ, http://www.useq.nl) Reads were aligned with HISAT2 version 2.2.1 (Kim et al., 2019) and differential expression was determined with Cuffdiff version 2.2.1 (Trapnell et al., 2013). Genes were considered differentially regulated when Cuffdiff considered them significant, and there was a 4-fold change in expression between any conditions, and the minimum expression in either condition was > 10 RPKM.

3. Results

3.1. Yeast two-hybrid shows interaction of WC-1 and WC-2 independent of stimulation by light

To examine if *S. commune* WC-1 and WC-2 form a heterodimer, the coding sequences of *wc-1* and *wc-2* were fused to the *GAL4* activating domain and *GAL4* binding domain, respectively. These plasmids were used to transform the *Saccharomyces cerevisiae* strains Y8800 and Y8930, respectively, to create two compatible fusion strains for yeast two-hybrid (Y2H). Interaction assays revealed that WC-1 and WC-2 indeed interact in a Y2H assay (Fig. 1). To determine the influence of light on this process, the experiment was repeated in the absence of blue light. Interaction still occurred, indicating that the formation of the WCC is independent of blue light.

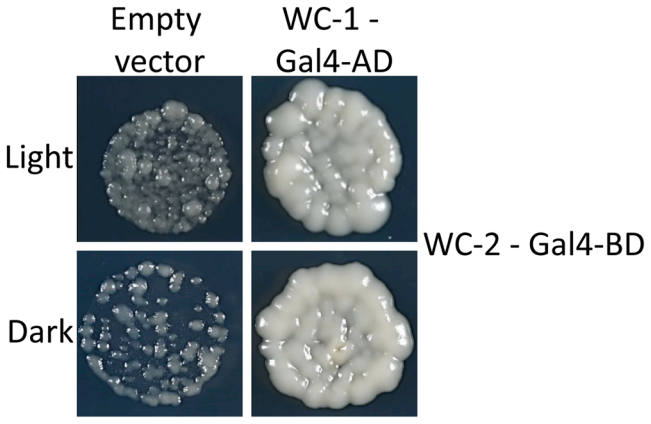

Fig. 1. : WC-1 and WC-2 interact in both light and darkness. Yeast two-hybrid of *wc-1* fused to the Gal4-activating domain (AD) and *wc-2* fused to the Gal4-binding domain (BD) in the light and the dark. The yeast is auxotrophic for histidine due to replacement of the *his3* promoter with the *gal4* promoter. Cultures are grown on yeast drop-out medium lacking adenine. Interaction of WC-1 and WC-2 results in the co-localization of the Gal4 AD and BD, promoting *his3* transcription and complementing the histidine auxotrophy. In both the light and the dark adenine auxotrophy is restored when yeast is transformed with both WC-1 and WC-2 fusion proteins, while an empty vector with only the Gal4-activating domain does not complement the auxotrophy. Additional controls are depicted in Supplementary Figure 3.

3.2. The WCC only associated with the promoters of genes in the presence of light

To study the association of the WCC with the promoters of light-activated genes, we used transcription factor ChIP-Seq of WC-2 in both the light and the dark. Cultures were harvested right before the first signs of light-dependent fruiting became apparent (90 hours after inoculation). First, a Δ*wc-2*Δ*ku80* strain of *S. commune* was transformed with a plasmid encoding the full length *wc-2* gene including a C-terminal HA-tag. After crossing, this resulted in two Δ*wc-2*:: *wc-2-HA* strains with compatible mating types. When crossed, these strains complemented the Δ*wc-2* phenotype and were able to fruit (Fig. 2). The presence of the protein could be detected by western blot (Supplementary Figure 1). The protein was then used for a pulldown of the chromatin of 90 hour old wild type and Δ*wc-2*:: *wc-2-HA* dikaryons with an anti-HA antibody. After purification and sequencing of DNA isolated during the ChIP, the binding sites of WC-2 could be identified. The vast majority of sites where WC-2 is associated with the chromatin were only occupied when the colony was illuminated. In the light a total of 569 binding sites were detected, while in the dark only two binding sites were identified (Supplementary Table 1). This indicates that light is required for the vast majority of DNA-binding activity of WC-2.

The binding sites of WC-2 are enriched in the predicted promoter regions, upstream of the start codon of genes (Fig. 3A). A binding site was associated with a gene if the tip of the peak was within 1000 bp of the translation start site. This resulted in the 569 peaks associated with 549 genes (Supplementary Table 2). The promoters of several previously characterized genes were occupied by WC-2, including the hydrophobins *sc1*, *sc4*, *hyd1* and *hyd7*, and transcription factors *bzt1*, *fst3*, *hom2*, *pri2*, *tea1* and *zfc7*. All of these genes, except *pri2*, have previously been associated with various stages of mushroom development. In total, the promoters of 17 transcription factors were associated with WC-2 binding, indicating that WC-2 has a role in a larger regulatory network (Table 2). It was previously proposed that DNA photolyase *cry1* (protein ID Schco3|2621816) and ferrochelatase *fer1* (protein ID Schco3|2634259) were direct targets of the WCC (Ohm et al., 2013). Indeed, both genes are directly associated with WC-2 binding (Fig. 3B, C).

To identify a putative binding motif of WC-2, the 200 bp around the tip of each peak was used for a motif enrichment analysis and novel motif discovery. The consensus motif CGATSNNNNSC was enriched in the sequences, occurring in 305 of the 565 sequences (Fig. 3D). Moreover, the motif was centered around the tip of the peak associated with a predicted WC-2 binding sites, as is expected for a binding site (Fig. 3E).

3.3. RNA-Seq reveals that WC-2 induces the expression of genes

Light is necessary for the association of WC-2 with the promoter sites of genes in *S. commune*. However, it is unclear if WC-2 promotes or inhibits transcription in *S. commune* and if light is sufficient for this activity. To examine the effect of WC-2 on transcription, we determined the differentially expressed genes (DEGs) between the light and dark in the WT and a Δ*wc-2* dikaryon at the same timepoint as in the ChIP-Seq analysis. A total of 271 genes were differentially expressed in any condition (minimum 4-fold difference in a condition compared to any other condition), the majority of which (195 genes) were differentially regulated in the WT between light and dark conditions (Fig. 4, Table 3, Supplementary Table 3 & 4). In contrast, in the Δ*wc-2* dikaryon, only two genes were differentially regulated between light and dark. This shows that the Δ*wc-2* dikaryon does not respond to light and is effectively blind. Indeed, while there are 218 DEGs between the WT and Δ*wc-2* in the light, only 9 DEGs were identified in the WT in the dark compared to Δ*wc-2* in the light (Table 3).

The majority of DEGs in the WT were upregulated in the light (148 out of 195) (Supplementary Table 3). Therefore, light is an inducer of expression. It would thus be expected that genes regulated by WC-2 are upregulated in the light in the WT, but not in the Δ*wc-2* strain. This is true for all 148 upregulated DEGs in the WT in the light. These genes are enriched in hydrophobins, oxidoreductases, fatty acid desaturases and a family of genes that encode proteins similar to Bacillus haemolytic enterotoxins (Table 4). Only a single transcription factor, *zfc7*, was differentially upregulated in the light. Of the 148 upregulated DEGs in the light, 35 had a putative WC-2 binding site in the promoter (Fig. 4). In order to explore if this low number of putatively directly regulated genes was caused by stringent criteria for DEGs, the overlap of genes with a WC-2 binding site and a minimum upregulation of 2-fold in the light was also determined. This increased the total numer of differentially regulated genes to 352 and the DEGs with a putative WC-2 binding site to 64 (Supplementary Figure 2). Of the 47 DEGs upregulated in the dark a single gene was associated with a WC-2 binding site. This indicates that WC-2 is predominantly a positive regulator of transcription and that light alone is not always sufficient for transcription. Interestingly, genes downregulated during illumination were enriched in the CAZyme classes glycoside hydrolase, carbohydrate esterase and auxiliary activity (Supplementary table 5).

4. Discussion

Light is an important developmental signal in fungi (Corrochano,

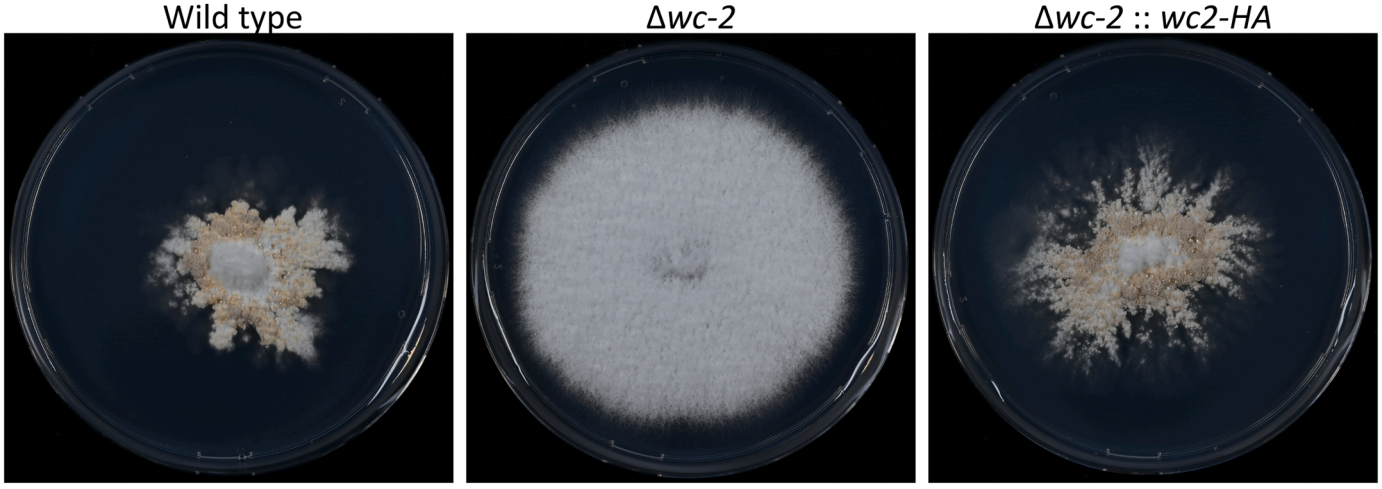

Fig. 2. : *wc-2-HA* fully complements the Δ*wc-2* phenotype. Cultures of wild type, Δ*wc-2* and Δ*wc-2*:: *wc-2-HA* dikaryons were grown for 7 days in the light. While a Δ*wc-2* dikaryon does not show any mushroom development for the wild type and Δ*wc-2*:: *wc-2-HA* dikaryon develop mature mushrooms.

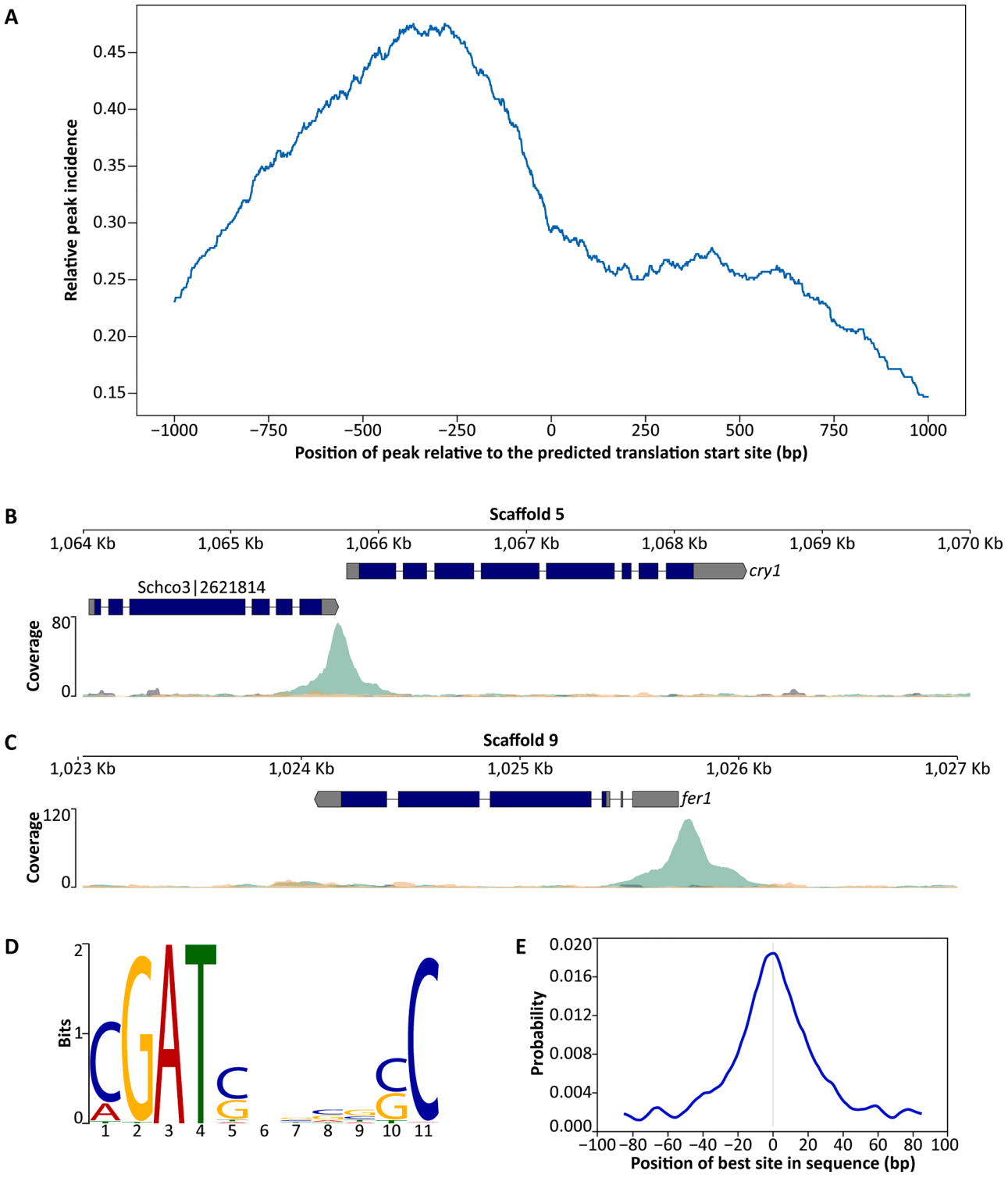

Fig. 3. : WC-2 activates transcription by binding the promoter of light-regulated genes that contain the motif CGATSNNNNSC. A: The relative occurrence of binding sites in the 1 kb upstream to 1 kb downstream of the translation start site (TSS). WC-2 preferentially binds upstream of the TSS of genes in the promoter region. B: ChIP-Seq peak of WC-2 in the promoter of the DNA photolyase *cry1* (protein ID Schco3|2621816). WC-2 binds upstream of the *cry1* TSS. C: ChIP-Seq peak of WC-2 in the promoter of the ferrochelatase *fer1* (protein ID Schco3|2634259). WC-2 binds upstream of the *fer1* TSS. D: The predicted consensus motif recognized by WC-2. E: The predicted motif is enriched in the center of WC-2 peaks, indicating that the motif is important in the interaction of WC-2 and chromatin.

2019). In many mushroom-forming fungi, blue light is a key signal for the initiation of fruiting-body development (Morimoto and Oda, 1973; Perkins, 1969; Perkins and Gordon, 1969; Tsusué, 1969). Blue light is detected by the WCC and its downstream gene regulation is essential for the development of mushrooms in *S. commune* (Ohm et al., 2013). Here we describe for the first time the direct targets of the WCC in a mushroom-forming basidiomycete, by a combination of functional genomics, gene expression analysis and transcription factor ChIP-Seq. This shows that the heterodimerization of WC-1 and WC-2 to form the WCC occurs independently of light. Furthermore, we show that light is

Table 2
Transcription factors that are associated with a WC-2 binding site in the light. The predicted DNA-binding domain of each transcription factor is listed. Four of the transcription factors associated with a WC-2 binding site have previously been characterized: Fst3, Hom2, Pri2 and Zfc7 (De Jong et al., 2010; Ohm et al., 2011, 2010; Vonk and Ohm, 2021).

Protein ID	Transcription factor type	Name
Schco3|1107091	Transcription factor TFIIS	n.a.
Schco3|2208139	SRF-type	n.a.
Schco3|2481568	TEA/ATTS	n.a.
Schco3|2485027	Fungal Specific TF	n.a.
Schco3|2519514	TEA/ATTS	Tea1
Schco3|2520843	bZIP	n.a.
Schco3|2525437	Fungal Specific TF	Pri2
Schco3|2533051	Fungal Specific TF	n.a.
Schco3|2543273	Fungal Specific TF	n.a.
Schco3|257987	Homeodomain	Hom2
Schco3|2601101	C2H2 zinc finger	Zfc7
Schco3|2619061	Helix-loop-helix	n.a.
Schco3|2623333	HMG	n.a.
Schco3|2624398	bZIP	n.a.
Schco3|2629275	Fungal Specific TF	Fst3
Schco3|2631700	Fungal Specific TF	n.a.
Schco3|2645246	bZIP	n.a.

necessary for the WCC to associate with the promoters of genes, but that this interaction is not always sufficient to initiate transcription.

As previously described in multiple ascomycetes and the basidiomycete *P. ostreatus*, WC-1 and WC-2 heterodimerize to form the WCC (Corrochano, 2019; Qi et al., 2020). In *S. commune* this action is independent of light, as yeast two-hybrid assays not exposed to blue light still show interaction of WC-1 and WC-2. This is similar to the previously described heterodimerization of WC-1 and WC-2 in *N. crassa* (Linden and Macino, 1997). In contrast to the *N. crassa* WCC, the complex requires light for interaction with genomic DNA (Sancar et al., 2015). It is tempting to speculate that this is caused by the lack of a zinc-finger DNA-binding domain in the basidiomycete WC-1 ortholog. The presence of two GATA zinc-fingers is often required for the function of these transcription factors (Chen et al., 2012; Hasegawa and Shimizu, 2017). Indeed, many GATA zinc-finger transcription factors contain both an N-terminal and C-terminal domain. For example, in *S. commune* five of the twelve predicted GATA zinc-finger transcription factors contain two GATA zinc-finger domains (Marian et al., 2022). Therefore, a single WCC in ascomycetes may be able to interact with the chromatin independently of light by the dual activity of the WC-1 and WC-2 GATA zinc-finger domains, while light-dependent homodimerization of two WCCs may be required in basidiomycetes lacking the WC-1 GATA zinc-finger domain.

A total of 569 WC-2 binding sites, associated with 549 genes were identified for WC-2 during early dikaryotic development. This would indicate that 3.4% of all genes are directly regulated by WC-2, similar to the number of light-regulated genes in ascomycetes (Chen et al., 2009; Idnurm and Heitman, 2010). Except for genes related to mushroom development and protection against UV damage, no pathways were enriched in the ChIP data. The consensus motif CGATSNNNNSC was enriched in the center of WC-2 binding sites. This motif is very similar to the motif identified for WC-1 that also contains a central CGAT as the primary component of the binding site (Froehlich et al., 2002). Therefore, it appears that the loss of the WC-1 GATA zinc-finger in basidiomycetes does not change the specificity of the WCC. However, unlike the motif identified in *N. crassa*, no palindromic repeat could be identified in the genome (Guo et al., 2009). Furthermore, the motif was not found in all WC-2 binding sites. It is unclear if other proteins are required for protein interaction, or if a less conserved motif may allow for weaker DNA interaction.

Differential expression analysis of wild type and Δ*wc-2* dikaryons performed under the same conditions as the ChIP, revealed that a Δ*wc-2* strain has almost no change in expression when grown in the light or the dark. This is in contrast to reports in *N. crassa* where transcriptional changes could still be observed in a deletion background of the WCC (Ma et al., 2022). In the genome of *S. commune* no orthologs of the photoreceptors *vvd* (a truncated homolog of *wc-1*) and *nop-1* (an opsin

Table 3
Upregulated genes between wild type and Δ*wc-2* dikaryons grown in the light and dark for 90 hours. Values indicate the number of upregulated genes in the horizontal condition compared to the vertical condition.

Strain		WT		Δ*wc-2*	
	Condition	Light	Dark	Light	Dark
WT	Light	-	148	155	146
	Dark	47	-	7	6
Δ*wc-2*	Light	63	2	-	0
	Dark	73	4	2	-

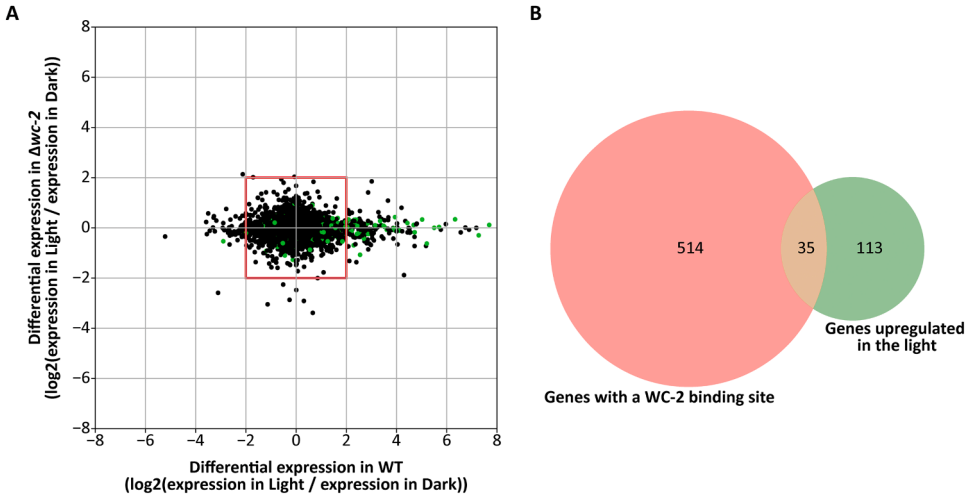

Fig. 4. : Expression analysis of *S. commune* wild type and Δ*wc-2* dikaryons in the dark and the light with a 4-fold change in expression cut-off. A: Log2 fold-change of all genes in the light compared to the dark in the wild type (x-axis) and Δ*wc-2* dikaryon (y-axis). Genes inside the red box change less than 4-fold in expression and are not considered differentially expressed. Genes highlighted in green are associated with a WC-2 binding site. The majority of genes are only differentially regulated in the WT between light and dark conditions. B: Venn diagram of genes associated with a WC-2 binding site (red) and upregulated DEGs in the WT in the light compared to the dark (green). Many genes presumably regulated by WC-2 are not differentially expressed in the conditions we assayed.

Table 4
Enrichment analysis of differentially upregulated genes in the light in the wild type. Determined by Fisher's exact test. P-values are corrected for false discovery rate.

PFAM	Annotation term	Description	DEGs with annotation term	Corrected p-value	Protein IDs of enriched DEGs
PF01185	**Hydrophobin**	Fungal hydrophobin	7	< 0.000	Schco3|1374227; Schco3|2526471; Schco3|2547619; Schco3|2604198; Schco3|2624306; Schco3|2634970; Schco3|73533
PF05791	**Bacillus_HBL**	Bacillus haemolytic enterotoxin (HBL)	4	0.001	Schco3|2626545; Schco3|2633454; Schco3|2634267; Schco3|2634274
PF00732	**GMC_oxred_N**	GMC oxidoreductase	4	0.022	Schco3|2638753; Schco3|2643062; Schco3|2663917; Schco3|2693570
PF05199	**GMC_oxred_C**	GMC oxidoreductase	4	0.020	Schco3|2605412; Schco3|2638753; Schco3|2643062; Schco3|2693570
PF00487	**FA_desaturase**	Fatty acid desaturase	3	0.042	Schco3|2511002; Schco3|2630033; Schco3|2691273

responsive to green light) have been found, but a phytochrome ortholog is present (Corrochano, 2019; Marian et al., 2022; Ohm et al., 2013). A function for this phytochrome has not been established, but it is known that red light does not affect mushroom development and that *S. commune* is primarily responsive to blue light (Perkins and Gordon, 1969). Despite the large number of putative WC-2 binding sites, only a small subset of 35 (64 with a 2-fold cut-off for DEGs) of these genes was differentially regulated in the same conditions, indicating that association of WC-2 when exposed to light is not always sufficient for the activation of transcription. This is not surprising, as light is a ubiquitous signal that is known to affect many different processes in fungi. Therefore, other regulatory mechanism may modulate the response to light in addition to WCC. Indeed, in many fungi the activity of the WCC is transient and diminishes during extended illumination. However, the primary regulator of the WCC in *N. crassa*, VVD, is not found in basidiomycetes (Idnurm et al., 2010; Marian et al., 2022). Nor is there an evident entrainment of a circadian rhythm in *S. commune*. The overlap in DEGs and genes associated with a WC-2 binding site, indicates that WC-2 has a role in the formation of primordia at the stage we assayed. Multiple hydrophobins that are associated with the structure of primordia, such as *sc1* and *sc4*, are upregulated and associated with WC-2 binding. Moreover, the transcription factor gene *zfc7* was identified in both datasets. This C2H2 zinc-finger was previously shown to have a role in the progression of primordia development (Vonk and Ohm, 2021). This indicates that this process is dependent on light-activation of the WCC, thus promoting the expression of *zfc7*. Therefore, we propose that WC-2 in *S. commune* is a regulator of multiple processes, including early primordia development, but that the role of WC-2 can vary based on other developmental cues and environmental conditions. Previously, two transcription factors with a similar developmental phenotypes as a Δ*wc-2* dikaryon, Hom2 and Tea1, have been identified (Ohm et al., 2011; Pelkmans et al., 2017). WC-2 is associated with the promoters of both of these genes. It is tempting to speculate that these transcription factors work in a cooperative fashion to promote mushroom-development and that this ensures their co-regulation during the initiation of fruiting. In order to develop a more complete overview of the effect of the WCC on transcription, differential expression analysis with shorter illumination times should be performed.

A small group of genes (47) was downregulated when grown in the light compared to the dark. These genes included 17 CAZymes of various classes (glycoside hydrolase, carbohydrate esterase and auxiliary activity). During mushroom development the cell wall undergoes

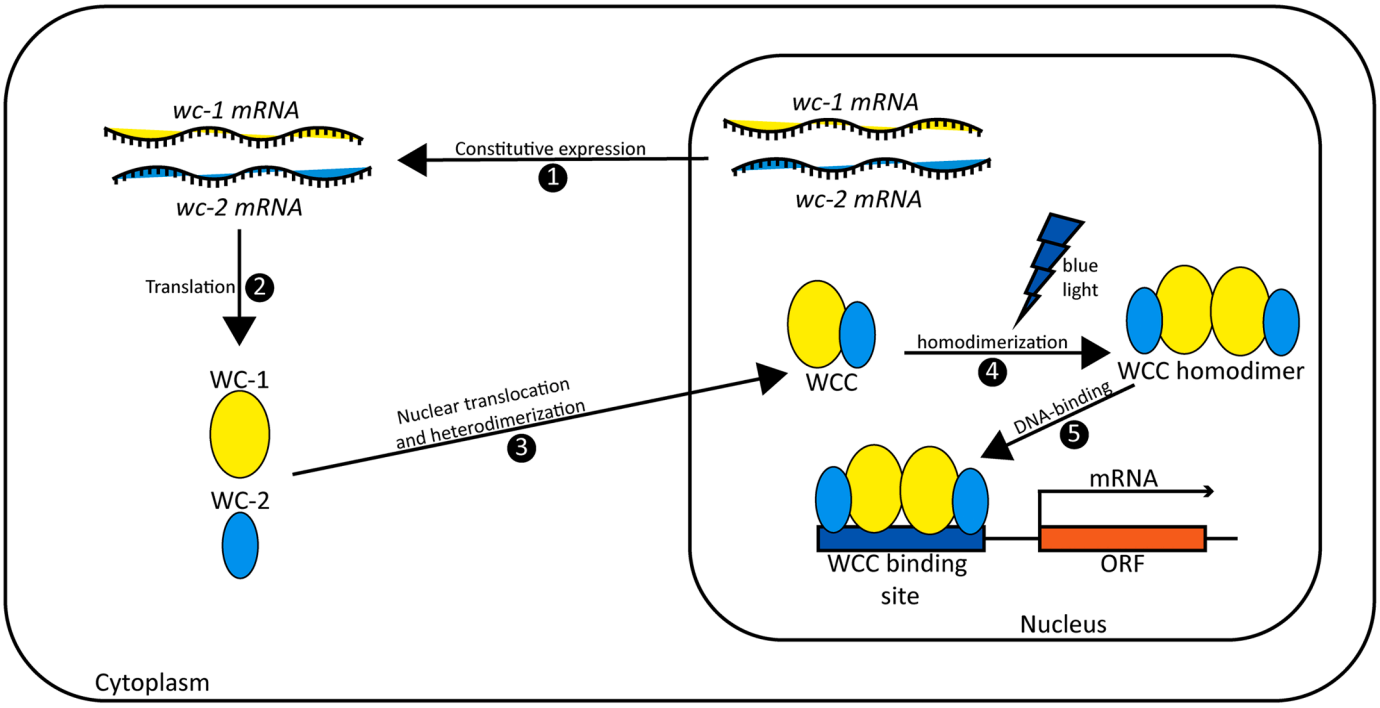

Fig. 5. : Model for the regulation of transcription by the White Collar complex (WCC) in *S. commune*. 1: Both *wc-1* and *wc-2* are constitutively expressed. 2: mRNA of *wc-1* and *wc-2* is translated into the proteins WC-1 and WC-2, respectively. 3: WC-1 and WC-2 dimerize to form the WCC and are translocated to the nucleus due to the presence of a nuclear localization signal on both proteins. 4: In the presence of blue light the WCC may dimerize by the interaction of two WC-1 proteins. 5: After dimerization of the WCC, it may become active to promote transcription of light-regulated genes. Co-factors that influence the activity of the WCC have not yet been identified in *S. commune*.

extensive remodeling (Nagy et al., 2023) and this may explain the differential expression of these genes. Furthermore, in *Pleurotus eryngii*, the expression of several groups of CAZymes is affected by blue light during mushroom development (Xie et al., 2018).

Based on these results and the orthology of the complex between *N. crassa* and *S. commune*, we propose the following mechanism of light detection and subsequent gene regulation in *S. commune* (Fig. 5). Both *wc-1* and *wc-2* are constitutively expressed and the translated proteins dimerize to form the WCC. The WCC is not able to associate with chromatin due to the lack of a GATA zinc-finger dimer and is in an inactive state. When light is detected, two WCC homodimerize by the interaction of the LOV domains of WC-1. This homodimerization brings together two GATA zinc-finger domains that bind to the promoters of light-regulated genes to promote transcription. For some genes, this association is sufficient for the initiation of transcription (i.e. photolyase and ferrochelatase), while for others, the recruitment of additional regulators is required to initiate transcription. The nature of these regulators is not yet known, but candidates include additional transcription factors like Hom2 and Tea1. Studies on different time-points of gene regulation are required to identify the full extent of the regulatory network downstream of the detection of light. Furthermore, protein-interaction studies may reveal putative co-factors of the WCC during different environmental and developmental conditions.

CRediT authorship contribution statement

Robin A. Ohm: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Marieke J. P. Van der Poel:** Investigation. **Peter Jan Vonk:** Writing – original draft, Visualization, Validation, Supervision, Investigation, Formal analysis, Data curation. **Zoé E. Niemeijer:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The RNA sequencing and ChIP sequencing reads have been deposited in the NCBI Sequence Read Archive and can be accessed under BioProject ID PRJNA1073722.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127736.

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