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11 **The inhibition of mating in *Phycomyces blakesleeanus* by light is dependent on**
12 **the MadA-MadB complex that acts in a sex-specific manner**
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ABSTRACT

Light is an environmental signal that influences reproduction in the Mucoromycotina fungi, as it does in many other species of fungi. Mating in *Phycomyces blakesleeanus* is inhibited by light, but the molecular mechanisms for this inhibition are uncharacterized. In this analysis, the role of the light-sensing MadA-MadB complex in mating was tested. The MadA-MadB complex is homologous to the *Neurospora crassa* White Collar complex. Three genes required for cell type determination in the sex locus or pheromone biosynthesis are transcriptionally-regulated by light and are controlled by MadA and MadB. This regulation acts through the plus partner, indicating that the inhibitory effect of light on mating is executed through only one of the two sexes. These results provide an example whereby the mating types of fungi have acquired sex-specific properties, beyond their role in conferring cell-type identity, and provide insight into how sex-determining chromosomal regions can expand the traits they control.

Keywords: blue light sensing; HMG-domain; mating type locus; Mucorales

1. Introduction

Successful outcrossing in organisms requires the recognition of non-self partners and the subsequent initiation of the sexual cycle. In the fungi different mating types are conferred in individual strains by regions of DNA that encode transcription factors, and in addition successful mating often uses small diffusible pheromone molecules to communicate between strains (Lee et al., 2010b). The commitment to undertake the mating process rather than continue with vegetative growth or undergo asexual sporulation integrates multiple environmental signals.

The Mucoromycotina are a branch of the fungi in which most of the key aspects of fungal sex were first established (Ainsworth, 1976). These include the discoveries of sex in fungi, diffusible pheromones, and the reproductive distinction between homothallic and heterothallic species. *Phycomyces blakesleeanus* and the other Mucoromycotina species in the Mucorales characterized to date have a mating type locus that is a region of DNA with minimal similarity between each allele or idiomorph, and each allele encoding a transcription factor of the high-mobility group (HMG) class (Lee and Heitman, 2014). Structurally, this DNA interval is similar to many of the mating type (*MAT*) loci found in the ascomycetes and basidiomycetes. The sex locus of *Phycomyces* contains only one gene that encodes an HMG-domain transcription factor, SexP in plus (+) mating type and SexM in minus (–) mating type. These genes are flanked by two genes encoding a putative RNA helicase and triose phosphate transporter (Idnurm et al., 2008). Other fungal species in the Mucorales have similar

arrangements of mating type transcription factor genes and conserved flanking genes (Camino et al., 2015; Gryganskyi et al., 2010; Idnurm, 2011; Lee et al., 2010a; Li et al., 2011), and thus aspects about the functions of the genes in the *sex/MAT* loci and their regulation may be similar between Mucorales species.

In addition to the requirement of a mating type locus for sexual reproduction, the Mucorales also use sexual pheromones for communication between the two sexes. While ascomycetes and basidiomycetes use peptide-based pheromones, *Phycomyces* and its relatives use derivatives of β -carotene to produce their sexual pheromones (Schimek and Wöstemeyer, 2009; Wöstemeyer and Schimek, 2007). The existing model for the production of trisporoid pheromones requires the presence of both mating partners, as some of the final steps for conversion of precursors to biologically active molecules are carried out in opposite mating types; i.e. the precursors produced by the plus parent would be converted by the minus parent, and vice versa. The trisporoids are derived from the cleavage products of β -carotene and steps up to the synthesis of 4-dihydrotrisporic acid are common in both mating type strains. The processing after this step diverges with the conversion to 4-dihydromethyltrisporic acid in the plus mating type and to trisporic acid in the minus mating type strain. After this, the mutual exchange of the molecules between strains of different mating types has to happen for the further metabolism of trisporic acid.

Light is an important environmental cue that affects many developmental processes across the fungal kingdom (Fuller et al., 2015; Idnurm et al., 2010; Rodriguez-Romero

et al., 2010; Tisch and Schmoll, 2010). The majority of the photobiology research on *Phycomyces* has focused on the properties of the sporangiophores, which are >10 cm long cells that terminate in the production of the sporangiophore containing the asexual reproductive spores called sporangiospores. Research into the responses of these structures to light revealed several features, including a role for shorter wavelengths in the blue and UV parts of the spectrum, a system to sense low and high light intensities, photoadaptation, phototropism towards blue light and the input of at least ten different genes, as defined by mutant isolation and analysis (reviewed by Cerdá-Olmedo, 2001). Two of these ten genes (*madA* and *madB*) have been identified: and they encode homologs of the WC-1 and WC-2 proteins that are the blue light sensors characterized in *Neurospora crassa* (Idnurm et al., 2006; Sanz et al., 2009). WC-1 is a flavin-binding protein presumed to act as the light sensor. It also has a zinc finger DNA-binding domain and together with WC-2 (also a zinc finger DNA-binding protein) this complex can induce transcriptional changes in response to light (Corrochano et al., 2016).

Most fungi are capable of sensing light, and if they do then their genomes invariantly contain a single copy of each of these two homologous genes (Idnurm et al., 2010). However, *Phycomyces* and other Mucorales species have undergone whole genome and/or segmental duplication events such that now they have three or more copies of each gene (Corrochano et al., 2016; Ma et al., 2009; Schwartze et al., 2014). This dramatically expands the options for how light can control different properties in these species, as illustrated by the sub-functionalization observed in *Mucor circinelloides* in the *wc-1* homologs after duplication. In this species, one *wc-1* copy is specialized for

sensing light to trigger carotene synthesis, another copy for phototropism, and the third copy has a function related to carotene biosynthesis (Navarro et al., 2013; Silva et al., 2008; Silva et al., 2006).

In addition to the well-studied phototropism of sporangiophores, responses to light by the vegetative mycelia are also known for *Phycomyces*. These include the induction of formation of the sporangiophores and β -carotene biosynthesis in response to light, both properties also controlled by the MadA and MadB proteins. In addition, light inhibits the mating process when two strains of opposite sex are cultured together. Analysis of action spectra for this inhibition pointed to a blue light and presumably flavin-based effect (Yamazaki et al., 1996). The inhibition of light was more effective on the earlier rather than the later stages of mating (Yamazaki et al., 1996). However, in the last two decades the inhibition of mating by light has not been further investigated, and no genes involved were known.

A somewhat paradoxical situation occurs in *Phycomyces* in that light inhibits mating yet it also induces the production of β -carotene, which is the precursor molecule to the pheromones. To date, one gene that controls the initiation of this pheromone biosynthesis from β -carotene has been identified in *Phycomyces*, that being the *carS* gene that encodes an enzyme to catalyze an asymmetric cleavage of β -carotene to initiate the formation of the pheromones (Sutter, 1975; Tagua et al., 2012). A related carotenoid oxygenase, AcaA is present in *Phycomyces*, and in vitro studies on a recombinant AcaA protein show that it cleaves the product of CarS (Medina et al., 2011).

The *carS* mutants are infertile and accumulate high levels of β -carotene. The *carS* homolog has also been characterized from *Blakeslea trispora* (Burmester et al., 2007; Sahadevan et al., 2013). The chemical steps involved in pheromone production are well-studied in different Mucorales species (Schimek and Wöstemeyer, 2009). Three molecules resulting from an asymmetric cleavage of β -carotene to initiate pheromone synthesis were most recently identified using *Phycomyces* (Polaino et al., 2012; Polaino et al., 2010). Carotenogenesis induced by either light or mating: this effect on carotene biosynthesis is transduced through a complex interplay between light inputs and the presence of a compatible mating partner, the mechanisms of which remain to be elucidated (Almeida and Cerdá-Olmedo, 2008).

The transcript levels of *sexM* and *sexP* genes are higher in cultures of *Phycomyces* with a mix of strains of opposite mating types compared to single strains (Idnurm et al., 2008). Likewise, in *Mucor mucedo* the levels of *sexM* and *sexP* are higher in mating cultures or by treatment of trisporoids (Wetzel et al., 2012). The genes in the mating type/sex locus are obvious candidates for regulation by light. Mutants for the mating type genes *sexM* and *sexP* are not available in *Phycomyces*. However, the availability of the *madA* and *madB* light-sensing mutants, the *carRA*, *carB* and *carS* mutants affected in β -carotene and pheromone biosynthesis, and of isogenic strains, coupled with the amenability to genetic crossing, enables research into how *sexM* and *sexP* expression may be controlled by light and how this affects mating. In this study we investigated the basis of inhibition of mating by light in *Phycomyces*, testing the genes

with a known role in light-sensing or mating that have been defined from mutant or other phenotypes.

2. Material and methods

2.1. Strains, culturing and crossing conditions for Phycomyces

Strains used in this study are listed in table 1. Strains were cultured routinely on potato dextrose agar (PDA) medium. For collection of the asexual spores, strains were grown on PDA medium for 5-7 days. For making the *madA madB* double mutants, first the *madB* mutant strain C109 (–) was crossed to strain A56 (+), and a (+) progeny was obtained carrying the *madB* mutation. This strain was then crossed to two *madA* mutants, with different alleles of the gene, and double mutants in both mating type backgrounds were isolated. For these crosses, the asexual spores from both mating types were incubated on V8 medium plates (5% V8 juice, 0.5 g/L KH₂PO₄, 1 mg/L thiamine, 4% agar; pH 6). Zygosporangia formed after 5-7 days of incubation in the dark. The zygosporangia were harvested and placed on sterile wet filter paper. After dormancy of 2-4 months the zygosporangia germinated to produce the meiospores, which were harvested into sterile water and plated on acidified PDA plates to isolate the single colonies. Various colonies were tested for mating type, to select for the desired mutants in particular mating type background, and mutations in genes were determined either by PCR-RFLP analysis or amplification and sequencing.

To culture mycelia to be used for RNA extraction, the asexual spores were inoculated onto minimal medium agar. The minimal medium has the following components per L: 20 g glucose, 1 g monosodium glutamate, 5 g KH_2PO_4 , 500 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 28 mg CaCl_2 , 1 mg thiamine·HCl, 2 mg citric acid· H_2O , 1.5 mg of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, 1 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 300 μg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 50 μg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 μg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (Cerdá-Olmedo and Lipson, 1987). Heat shock at 45°C for 15 minutes was given to the spores to activate their germination. For single cultures 100,000 spores were plated, for mated cultures 50,000 spores of (+) and (–) mating type strains were mixed and plated. Plates were incubated at room temperature in light (white fluorescent bulbs, 400-800 lux) or dark. To quantify zygosporangium production three random areas of (1 cm^2 each) of the petri plate were selected and the zygosporangia were counted manually from at least two biological replicates.

2.2. RNA extraction and quantitative reverse transcriptase PCR

Mycelia were collected by scraping them from the plates, immediately frozen in liquid nitrogen, and stored at -80°C until further use. Independent plates were used as replicates in all experiments, with numbers as stated in the figure legends. RNA was purified from mycelia using an RNA extraction kit (Qiagen, Germantown, MD, USA), and 5 μg of total RNA was used for making cDNA by reverse transcription in a 20 μl reaction (Superscript III, Invitrogen, Grand Island, NY, USA). The cDNA was diluted to 60 μl and 4 μl of this was used as template in real time PCR. For both processes the manufacturer protocol was followed.

SYBR green mixture (Fermentas, Vilnius, Lithuania) was used for amplification of genes from cDNA by quantitative PCR in an ABI7300 machine (Bio-Rad, Hercules, CA, USA), with primer concentrations at 0.3 nM. Primers for PCR are listed in Table 2. The $\Delta\Delta Ct$ method (Livak & Schmittgen, 2001) was used for relative quantification of genes with the actin gene used as the constitutively expressed gene.

2.3. Yeast two hybrid analyses

The open reading frames (ORFs) of *sexM* and *sexP* were cloned previously (Idnurm et al., 2008). For testing interactions, the ORFs were fused to the fragments encoding the *Saccharomyces cerevisiae* Gal4 activation domain (AD) and binding domain (BD) in various combination and were co-transformed into the PJ69-4A strain of *S. cerevisiae* (James et al., 1996). Double transformants were selected on the media lacking leucine and tryptophan amino acids (-leu -trp). The selected clones were then tested for growth on media without leucine, tryptophan, adenine or histidine. This medium was also supplemented with 3-amino-1,2,4-triazole (a competitive inhibitor for histidine) and media lacking leucine, tryptophan, histidine and adenine were used to test the strength of potential interactions.

2.4. Heterologous expression of *sexM* and *sexP* for localization studies by fluorescence microscopy

The *sexM* and *sexP* ORFs were amplified by PCR using Takara Ex Taq from plasmids used in the yeast two hybrid experiments. Primers ALID1254 and ALID1255 were used to amplify *sexM* and ALID1256 and ALID1257 were used to amplify the *sexP* gene. The amplicons were purified after ensuring the single band of correct size on the gel. The PCR products along with EcoRI-linearized pYES-GFP vector were transformed in FY834 strain of *S. cerevisiae*. The putative clones were selected on synthetic dextrose (SD) media lacking uracil. Plasmids were rescued into the DH5 α strain of *Escherichia coli* and sequenced to identify clones with no PCR-induced errors.

For localization of proteins the selected clones were transformed back into *S. cerevisiae* and the yeast strains cultured overnight. Cells were stained with MitoTracker Red dye to detect the mitochondria and with Hoechst stain to detect the nucleus for 15 minutes and then washed in phosphate buffered saline. The imaging was performed using an Olympus Fluoview (model FV300) confocal microscope.

3. Results

3.1. Genes required for mating are upregulated during crosses

Microscopy on crossing cultures at 24 h, 48 h, 72 h and 96 h revealed the stages of the sexual cycle at these time points. By 48 h, the formation of prozygosporangium was initiated (Fig. 1A). At 96 h the mating process had proceeded as far as zygosporangium production, but also with a mix of different stages of mating. Harvesting sufficient

mycelia at 24 h was technically challenging for subsequent RNA extractions. Thus for time course studies, four 12 h intervals between 36 h and 72 h were thus selected as an optimal.

Strains were inoculated in isolation or as a mix between two mating types, and incubated in the dark for 36, 48, 60 or 72 h. Mycelia were harvested in the dark under safe red light, RNA was extracted, and the transcript level of the *sexP* and *carS* genes measured by q-RT-PCR and normalized to the actin gene. One important point to note is that in mating cultures only half of the material is from either parent. Thus, the expression levels of the *sexM* and *sexP* genes in mated cultures could also be doubled when making comparisons with single cultures.

The primary observation was that *sexM*, *sexP* and *carS* were induced under mating conditions relative to single culture controls (Fig. 1B and an independent study in Fig. S1). However, a surprising observation was the level of variation in expression in these single culture controls. Thus, direct comparisons between the mated vs. single strain conditions should be viewed with some caution. Nevertheless, the levels of transcripts of *sexM*, *sexP* and *carS* were consistently higher in mating cultures than in single cultures. The second observation was that there was a difference in expression profile between *sexM* and *sexP*. While both genes were induced during mating, there was little evidence that the two genes were co-regulated, as the timing of peak expression levels of these two genes was different (Fig. 1B and Fig. S1).

3.2. *Light inhibits expression of mating genes and loss of the MadA-MadB complex alters the light responses*

Based on the time course experiments for the subsequent studies, on crosses between strains carrying different mutations, 48 h after inoculating spores was used. The 48 h time point was selected for the following reasons. First, at this time most of the plate is at the same stage of mating whereas the later time points had a mix of different stages of sexual development, which might confound the interpretations of expression levels. Second, the sexual development progress towards more mature stages at 60 h and the expression of genes affected by environmental conditions should be influenced at earlier time points (Yamazaki et al., 1996).

MadA and MadB are one of three and four members of the White collar 1 and 2 class of proteins, respectively, in the Mucoromycotina fungi (Corrochano and Garre, 2010). Absence of phototropism or induction of β -carotene synthesis indicates that the double *madA madB* mutants are more severe in phenotype than either a single *madA* or *madB* mutant (Lipson et al., 1980). However, the double mutants were constructed in the 1970s in non-isogenic backgrounds before an isogenic strain became available. There is a caveat in comparing the effects of a mutation in different strain backgrounds: i.e. a difference in expression between two strains may reflect a genuine consequence of the mutation or its mating type under investigation, or it could be a consequence of uncharacterized genetic variation in the strain backgrounds. In order to avoid this issue, wherever possible we used or aimed to compare strains that were isogenic other than

their mating types. To achieve this we therefore generated double *madA madB* mutant strains of each sex with two different *madA* alleles and the same *madB* mutation (only one *madB* allele is known) in an isogenic background.

Mating is more efficient in the dark compared to light. To test if the inhibition of mating by light is transduced through the MadA-MadB photoreceptor complex, the mating in *madA madB* strains in the light and dark was compared with that of wild type strains. The zygospore production in the dark was similar in wild type and mutant strains. In *madA madB* mutants mating was not inhibited by light. In the light the mutant strains produced the zygospores in higher number than wild type strains (Fig. 2A).

To examine the basis of this trait at the gene level, we tested the expression of *sexP*, *sexM* and *carS* genes in light and dark in wild type and mutant strains. In wild type crosses a noted difference was observed for transcript levels when mating was performed in the light and dark. The crosses using wild type strains incubated in darkness have higher expression of the *sexP* and *carS* genes compared to incubation in light. The transcript levels of the three genes were examined also in strains with mutations in *madA* and *madB*. In the double mutant the transcript levels were similar between light and dark and comparable to the wild type dark cultures (Fig. 2B). That is, the expression of *sexM*, *sexP* and *carS* for the double mutants was similar in light and dark. We observed that the effect of the absence of light is more prominent in affecting the transcript levels of *sexP* compared to *sexM*.

For comparison, the expression levels of the two genes, *carRA* and *carB*, required for synthesis of β -carotene from the precursor molecule geranyl geranyl pyrophosphate were also examined. Both genes are regulated by light in single strains (Ruiz-Hidalgo et al., 1997; Sanz et al., 2010). However, negligible differences in transcript levels were observed for these two genes in mating cultures (Fig. S2), indicating that the role of light input under these mating conditions is not on the induction of the genes for β -carotene biosynthesis. This could also reflect that mating conditions are a sufficiently strong signal to alter transcript levels of the *carRA* and *carB* genes that masks any addition from photoinduction.

3.3. Light inhibition is lost in the (+) *mad* mutant strains but still occurs in the (–) *mad* mutant strains

Concurrently with the transcript analysis, the phenotypes of the *madA madB* mutants were compared to wild type. These mutants produced almost equal numbers of zygospores in the light and in the dark, in contrast to the wild type in which under light conditions zygospore production was reduced approximately four fold. Examination of the single mutants in crosses (*madA* $\times$ *madA* or *madB* $\times$ *madB*) also indicates loss of light sensing. There was no additive effect of having both *madA* and *madB* genes mutated in the same strain compared to the single mutants (Fig. 3B).

Reciprocal crosses in which one parent was wild type and the other contained the *madA*, *madB* or double *madA madB* mutations were tested. Crosses were performed in ten

combinations under light or dark conditions. Curiously, we observed differences between the reciprocal crosses. In those crosses in which the strains of the (+) sex carried the photosensor mutation then mating in light was as efficient as if it occurred in the dark; that is the (+) parent was blind (Fig. 3A). In the reciprocal combination in which the (-) strain has the mutation, light still inhibited zygosporangium production (Fig 3A and 3B). To reiterate, wherever the plus partner is mutant for either *madA*, *madB* or for both genes, the production of zygosporangia in both dark and light are comparable in number. This indicates that the inhibition of mating is through the light-sensing properties of the plus strain.

The basis behind the differences in zygosporangium numbers observed in the reciprocal crosses was examined at the transcript level. One hypothesis is that light could inhibit transcription of the *sexP* gene. Quantitative PCR was used to investigate the transcript levels of *sexP* and *sexM* genes. We compared the crosses against the wild type cross incubated in the light. As expected, the expression of the *sexP* gene was higher in the crosses where the plus mating partner is mutant for either *madA*, *madB* or for both genes. The expression of *sexM* is not appreciably different in any of the crosses (Fig. 3C, Fig. S3). These results further strengthen a central role of *sexP* targeted by the MadA-MadB photoreceptor complex to inhibit mating.

3.4. Interaction between light and the pheromone pathway

Light is a known regulator of the two genes that are required to synthesize the precursor of pheromones, β -carotene, so the effect of light could be mediated directly on *sexM* or *sexP*, or on one of these components that then act indirectly on *sexM* and *sexP*. To test this, crosses were set up with either *carS* $\times$ *carS* or *carB* $\times$ *carB* strains and compared to their wild type parents. In this case, due to the technical challenges of generating progeny from sterile strains, the wild type (+) parent used was NRRL1554, which is not isogenic with NRRL1555, for comparison to *carS* mutants because the mutant of *carS* in the (+) sex was isolated in the NRRL1554 background (Sutter, 1975). For the *carS* mutant we used four combinations: wild type (+) $\times$ wild type (-), *carS* (+) $\times$ *carS* (-), wild type (+) $\times$ *carS* (-) and *carS* (+) $\times$ wild type (-), and these crosses were incubated in light and dark. Darkness being the optimal condition for mating should provide the best availability of pheromones in wild type strains, and the *carS* mutant cross should be devoid of pheromones, as the strains lack the functional cleavage enzyme to process β -carotene.

The expression of *sexP*, *sexM* and *carS* was compared in four crossing scenarios using the cross where both partners are *carS* mutants as the baseline. In dark *sexP* and *sexM* are upregulated in wild type (+) $\times$ wild type (-) compared to *carS* (+) $\times$ *carS* (-) (i.e. M1 $\times$ S324), whose transcript levels are acting as baselines for these comparisons (Fig. 4A). In wild type (+) $\times$ *carS* (-), the *sexP* transcript levels are not significantly different from controls but *sexM* is almost four-fold down compared to controls. The *carS* gene is expressed at lower levels in this cross; thus, there may be insufficient pheromone levels to induce transcription of *sexM* and/or there is auto-regulation through the pheromone

precursors. The mutant strains could have less efficient feedback regulation by the *carS* product(s), and the possibility of the less stringent regulation of mating involved genes should not be ruled out in *carS* mutants. The *carS* gene of the plus partner, though functional, was not induced to produce substrate for pheromones. In *carS* (+) × wild type (-) (i.e. MI × NRRL1555), we expected that *sexP* should be expressed as wild type, as the (-) strain (wild type) has functional *carS*, so could produce pheromones to induce *sexP*. This can be the case if the pheromones are playing any role in promoting the expression of *sexP*. In this cross we observed the expected trend of expression of *sexP* and *sexM*. The *carS* transcript levels were higher in this cross, which indicates that there is enough substrate for pheromone production and that the expected pheromone function could be executed.

In the presence of light only *sexM* transcript levels are significantly different in wild type (+) × wild type (-) and *carS* (+) × wild type (-), compared to the control [*carS* (+) × *carS* (-)]. The expression of *sexP* and *carS* are not significantly different from the controls. In wild type (+) × wild type (-), the result suggests that although the pheromones or other mating cues are available to help in the induction, light inhibits the expression of *sexP* (Fig. 4B).

One confounding aspect to interpreting the *carS* crosses is the presence of high levels of β -carotene, which may itself impact gene regulation. Hence, we examined crosses in strains carrying mutations in the *carB* gene, which encodes phytoene dehydrogenase (Ruiz-Hidalgo et al., 1997). Mutation of this gene blocks the synthesis of β -carotene

and renders those strains white. In the *carB* × *carB* crosses, when the samples cultured in the dark were compared against those exposed to light, *sexM* was at slightly lower levels but *sexP* is similar to controls (Fig. S4).

The experiments using the *carB* and *carS* mutants suggests that although the light plays an important role for the regulation of *sexP*, pheromones are also required for the induction because loss of either *carB* or *carS* impacts the ability of light to alter transcript levels. The *sexM* gene is affected by the lack of pheromones, which suggests that pheromones are more important for the expression of *sexM* compared to *sexP*.

3.5. *SexM* and *SexP* play independent roles in sexual reproduction

In some ascomycetes and basidiomycetes, the transcription factors within the *MAT* locus play coordinated roles during sexual development, including as heterodimeric complexes. Our data thus far that observed differences in expression and phenotypic effects suggested that the SexM and SexP proteins would not do this. To explore further the possibility of a SexM-SexP physical interaction, we fused the ORFs of both proteins to the DNA binding or activation domains of yeast Gal4, and tested for interactions in the yeast two hybrid system.

The yeast two-hybrid assays suggest that either no physical interaction occurs between SexP and SexM, or that it is very weak (Fig. 5A). When the AD and BD fused clones of SexP and SexM in both combinations were co-transformed into *S. cerevisiae*, growth

was observed on media used to detect interactions (without adenine or histidine) but similar growth was also observed when the SexP and SexM clones in the BD vector were co-transformed with the empty AD vector. This indicates that the growth is due to the transactivation by SexP and SexM fused BD vectors. When the SexM and SexP clones in both AD and BD were co-transformed they were able to replicate on the test media, but no higher than the background of transactivation. The transactivation is one piece of evidence that SexM and SexP could act as transcription factors, predicted based on the presence of high mobility group (HMG) domains within them.

As a second experimental direction, we examined the subcellular localization of SexM and SexP by fusing these proteins to GFP and expressing those constructs heterologously in *S. cerevisiae*. The expectation was that both proteins would be targeted to the nucleus. SexM clearly localized to a single large organelle, and with colocalization with a nuclear stain (Fig. 5C). SexP has a different expression pattern, with potential mitochondrial localization but also included aggregate structures in the cytoplasm, and no evidence for nuclear localization (Fig. 5B). This subcellular localization for the *Phycomyces* Sex proteins expressed in *S. cerevisiae* is similar to what was observed when *M. circillenioides* SexM was expressed in yeast as a GFP fusion, whereas the SexP protein could not be visualized (Wetzel et al., 2012). Hence these two proteins localize exclusive of each other in a heterologous system.

4. Discussion

Light can be a potent environmental signal that affects fungal biology. MadA and MadB, homologs of the *Neurospora crassa* WC-1 and WC-2 proteins, are known to regulate the mycelial and other asexual processes in *Phycomyces* that are affected by light. Light was also reported to inhibit the mating of *Phycomyces* (Yamazaki et al., 1996); however, the basis of this inhibition was unknown and the possible role of MadA-MadB has remained untested. Our results show that the MadA-MadB complex is also responsible for inhibiting mating, and that both pheromone biosynthesis and mating type locus genes are regulated by MadA and MadB. However, in contrast to the vegetative responses to light, the effects of the light on mating occur via only one of the two sexes.

Identifying the genes required for mating in *Phycomyces* and the other Mucorales has been a long-term process, and to date only a handful of genes have been identified. For example, from isolating a mutant to the identification of the mutation in the *carS* gene took more than 35 years (Sutter, 1975; Tagua et al., 2012), and other genes still remain to be identified (Sutter et al., 1996). Hence, understanding the molecules and genes required for pheromone production has often involved a suite of different Mucoromycotina species.

The two mating types in the Mucorales produce different precursors for pheromones, which can only be processed further by the opposite mating type to the active form of pheromones (Sutter, 1975; Sutter et al., 1996). The methyl trisporins from the (–) strain are sent to the (+) strain to be converted to active pheromones and the methyl trisporates from the (+) strain are processed by the (–) strain to active pheromones,

which are trisporic acids (Sutter et al., 1996). The gene *TSP1*, encoding the enzyme 4-dihydromethyl trisporate dehydrogenase, was identified and cloned and is involved in the biosynthesis of pheromones in *Mucor mucedo*. Later, the *TSP2* gene was also identified and encodes for the enzyme 4-dihydrotrisporin dehydrogenase (Schimek et al., 2005; Wetzel et al., 2009). However, even after identification of the respective genes for these enzymes, it has not been possible to confirm that these genes are required for mating. Gene disruption is limited to a few Mucorales species, and this is an important proof-of-function especially given the high level of genome or large scale gene duplication seen in this lineage (Corrochano et al., 2016; Ma et al., 2009; Schwartze et al., 2014). The *TSP1* gene is transcribed and also translated in both mating type strains, but the enzymatic activity is only observed in a sexually-stimulated (–) strain (Schimek et al., 2005). This indicates that post-translational regulation is also involved in the pheromone biosynthesis pathway, at least in *M. mucedo*.

The first discovery from this research is that the inhibition of mating by light is dependent on the MadA-MadB complex. Mutation of either gene, or in combination, abolishes the repression by light. Under the light intensities used there was no additional effect of mutation of both the *madA* and *madB* genes in the same strain, contrasting to the situation for phototropism or induction of carotene synthesis by light. One caveat to interpreting the impact of single vs. double mutants is that we tested mating under a single light intensity, and it is possible that under a range of intensities that an effect could be observed. Thus, the other two homologs of *wc-1* and other three

homologs of *wc-2* do not appear to be involved, and instead MadA-MadB must integrate light into these different processes.

The second main discovery from this research is that the photoinhibition of mating is dependent on the sex (or mating type) of the strain. Loss of the light-sensing function from the *sexM* strain still enabled repression of mating by light, whereas in the reciprocal situation loss in the *sexP* strain caused mating to occur as efficiently as in the dark (Fig. 3). For *Phycomyces* strains to overcome the light-based inhibition, the (+) mating type partner has to be a *madA* or *madB* mutant. These features of *Phycomyces* suggest that light-based regulation of mating is transduced primarily through the (+) partner, and the (+) partner initiates signaling via pheromones that are received by the (–) partner, which then responds to complete the mating process. Although the two mating types of *Phycomyces* are identical in appearance and produce equally-sized gametangia, they have specific roles at the molecular level. In addition to the experiments reported in this study, the mating type genes control the uniparental inheritance of the mitochondrial genome (Shakya and Idnurm, 2014).

In some of the best-studied models of fungal sexual reproduction, the components encoded in each allele of the mating type locus physically interact to co-regulate gene expression (Dranginis, 1990; Kämper et al., 1995). In the case of *Phycomyces* the experiments presented here, which includes transcript regulation independent of each other (Fig. 1), a lack of evidence for physical interactions (Fig. 5A), differences in subcellular localization profiles of the proteins (Fig. 5B and 5C), and different

photoresponses depending on the strain, suggest that at least in the early stages of mating these genes have cell-type specific functions.

Based on our findings and previous research a model explaining the effects of light on mating can be developed (Fig. 6). We propose the MadA-MadB photoreceptor complex inhibits the expression of *sexP* gene in light. The pheromones from opposite mating partners are also required for the expression of *sexM* and *sexP* in mating conditions, the pheromones induce the expression of these genes. Darkness removes the inhibitory effect of light on *sexP*. This inhibition is transduced through the MadA-MadB complex and so the *madA*, *madB* or *madA madB* mutants were able to circumvent the inhibitory effect of light on mating.

Mating compatibility in fungi is determined by the identity of the mating-type locus, with only opposite mating-types undergoing successful outcrossing. Traditionally, the mating type in fungi is considered to have a relatively minimal role, just in regulating the cell's mating type identity, rather than as a major regulator driving distinct developmental differences between strains as would be considered for the equivalent sex chromosomes in animals (Shiu and Glass, 2000). This explains why "mating type" is used to define individual strains in place of "sex" or "gender" (Billiard et al., 2011). Despite the common use of "mating type" to describe fungi, at the molecular level mating types are different. Analysis of gene expression profiles by microarrays, RNA-sequencing or chromatin immunoprecipitation (ChIP)-sequencing in Ascomycota species reveals in many that there is differential gene expression between mating type

or that genes not considered part of mating are regulated by the transcription factors within *MAT* loci. However, defining a mating type dependent phenotype is not common.

One interesting parallel with the research here on *Phycomyces* is found in the ascomycete *Fusarium verticillioides*, where the light-dependent induction of the synthesis of β -carotene requires the *MAT1-2-1* gene (Ádám et al., 2011). This indicates that there is a central role for this mating type factor between light input, possibly via the WC-1 protein, and the carotenoid pathway. A second parallel is in another ascomycete, *Trichoderma reesei*, where the orthologs of WC-1 and WC-2 control the light-based inhibition of the production of sexual spores. The mutants of *BLR1* and *BLR2* can produce sexual spores in constant illumination, unlike wild type strains (Chen et al., 2012). *BLR1* and *BLR2* negatively regulate the expression of pheromone receptor genes and pheromone precursors in light but not in darkness; interestingly, this regulation is mating-type dependent (Bazafkan et al., 2015; Seibel et al., 2012).

In summary, these findings demonstrate that in *Phycomyces* the (+) strain is responsible for sensing light and integrating that signal in terms of the commitment towards sexual or asexual reproduction. The *madA* and *madB* genes are required for sensing light to inhibit mating. The experiments addressing where in the mating process this inhibition occurs point towards a central role of the production of the trisporic acid pheromones, both at the transcript level for the regulation of *carS* after β -carotene is synthesized and because induction of *sexM* and *sexP* is impaired in mutations that block pheromone production.

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766 **Table 1**

767 *P. blakesleeanus* strains used in this study.

Name	Genotype	Parent(s)	Reference
NRRL1555	Wild type (-)		(Bergman et al., 1973)
A56	Wild type (+)	Isogenic with NRRL1555	(Alvarez and Eslava, 1983)
NRRL1554	Wild type (+)		Unknown origin
C47	<i>madA</i> (-)	NRRL1555	(Bergman et al., 1973)
A893	<i>madA</i> (-)	NRRL1555	(Idnurm et al., 2006)
C109	<i>madB</i> (-)	NRRL1555	(Bergman et al., 1973)
CAB26	<i>madA</i> (+)	A56 × C47	This study
C5A	<i>madB</i> (+)	A56 × C109	This study
AB36	<i>madA madB</i> (-)	C5A × A893	This study
AB48	<i>madA madB</i> (+)	C5A × A893	This study
CAB13	<i>madA madB</i> (-)	C5A × C47	This study
CAB22	<i>madA madB</i> (+)	C5A × C47	This study
M1	<i>carS</i> (+)	NRRL1554	(Sutter, 1975)
S324	<i>carS</i> (-)	NRRL1555	(De la Concha and Murillo, 1984)
C5	<i>carB</i> (-)	NRRL1555	(Cerdá-Olmedo and Reau, 1970)
S342	<i>carB nicA</i> (+)	Crosses	(Alcalde et al., 2016)

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770 **Table 2**

771 Oligonucleotide primers used in this study.

Name	Sequence 5'-3'	Purpose
ALID1659	TGTCCTTTCCCTTTACGCC	<i>Phycomyces</i> actin for qRT Forward
ALID1660	ACGACCAGCCATATCCAAATG	<i>Phycomyces</i> actin for qRT Reverse
ALID1661	CCGTACACCCTAGCATTCTATC	<i>Phycomyces sexM</i> for qRT Forward
ALID1662	GTTTCTCTTCATCCGCTTTTCG	<i>Phycomyces sexM</i> for qRT Reverse
ALID1671	GATTCTATGCCCAGGACCG	<i>Phycomyces sexP</i> for qRT Forward
ALID1672	TCAATGGCGAAGTTCTCTTG	<i>Phycomyces sexP</i> for qRT Reverse
ALID1673	CTATCGTCATCTTGGTGCCTG	<i>Phycomyces carS</i> for qRT Forward
ALID1674	ACAAGAGTAATACAGTGCCGAC	<i>Phycomyces carS</i> for qRT Reverse
ALID1996	CGTTGGACTCTGCCCAGTTTC	<i>Phycomyces carRA</i> for qRT Forward
ALID1997	CCAGCGACGAAGCATGTATTC	<i>Phycomyces carRA</i> for qRT Reverse
ALID1998	GACCATCTGGAAGCTTATCCG	<i>Phycomyces carB</i> for qRT Forward
ALID1999	GGATAACGGTGTTGAAACCAC	<i>Phycomyces carB</i> for qRT Reverse
ALID1254	CTATACTTTAACGTCAAGGAGA AAAACTATAATGGAATCTTTTA TGTTTGTTAACG	Cloning of <i>sexM</i> for localization in <i>S. cerevisiae</i> Forward
ALID1255	CCAGTGAAAAGTTCTTCTCCTTT ACTGTTAATTGAACATAAAGAGA ATATAAGCTC	Cloning of <i>sexM</i> for localization in <i>S. cerevisiae</i> Reverse
ALID1256	CTATACTTTAACGTCAAGGAGA AAAACTATAATGAAAGCGAAA CAGACACC	Cloning of <i>sexP</i> for localization in <i>S. cerevisiae</i> Forward
ALID1257	CCAGTGAAAAGTTCTTCTCCTTT ACTGTTAATAAACCTTGCCCAA AACTTTTCG	Cloning of <i>sexP</i> for localization in <i>S. cerevisiae</i> Reverse
ALID1287	AATTAATCAGCGAAGCGATG	For sequencing the pYES-GFP vector clones
ALID1272	TGTTGCATCACCTTCACC	For sequencing the pYES-GFP vector clones
ALID0350	ACTTCAGGGTCAGCTTGC	Internal GFP primer to sequence the gene-GFP fusion

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Figure legends

Fig. 1. Time course of mating and transcript levels of three genes involved in mating. (A) Strains were crossed and kept in the dark, with stages of mating photographed at four time points. (B) The transcript levels of *sexP*, *sexM* and *carS* genes in single and mated cultures of wild type strains (NRRL1554 and NRRL1555), showing the induction of these genes during crosses. The error bar indicates the standard error between two replicates.

Fig. 2. Mating and gene expression in wild type and *madA madB* mutants in light and dark. (A) Examples of petri dish cultures of crosses between *madA madB* or wild type strains in the light or dark. The black structures are zygospores. (B) The expression of *sexP*, *sexM* and *carS* in light and dark in crossed cultures of *madA madB* mutants compared to the wild type (WT) strains. The strains used in the experiment are A56 and NRRL1555 for wild type (+) and (-), AB48 and AB36 for *madA madB* (+) and (-). The crosses are represented on the X-axis and the Y-axis represents the fold change relative to wild type $\times$ wild type in the light. The error bar indicates the standard error between three replicates.

Fig. 3. Phenotypes of mating and gene expression in light vs. dark in various cross combinations using wild type (WT), *madA*, *madB* or *madA madB* mutant strains. Strains used in crosses are written in the order of the (+) parent $\times$ (-) parent. (A) Zygospore formation in crosses incubated in light or dark. (B) The average number of zygospores

formed in various crosses performed in (A). The crosses are represented on the X-axis and the Y-axis represents the number of zygospores. The error bar indicates the standard error between three areas counted from two different plates. (C) Transcript levels of *sexM* and *sexP* in the crosses in light (L) or darkness (D). The strains used in this experiment are A56 and NRRL1555 for wild type [WT; (+) and (-)], CAB26 and C47 for *madA* (+) and (-), C5A and C109 for *madB* (+) and (-), CAB13 and CAB22 for *madA* *madB* (+) and (-). The crosses are represented on the X-axis and the Y-axis represents the fold change relative to the wild type x wild type cross in the light. The error bar indicates the standard error between two replicates.

Fig. 4. The expression of *sexP*, *sexM* and *carS* genes in light and dark in crossed cultures of *carS* mutants and wild type. All crosses are written in the order of the (+) parent x (-) parent. (A) The left graph shows the expression in dark (D) incubation. (B) The right side graph shows the expression in light (L) incubation. The *carS* mutants are M1 (+) and S324 (-), and wild type strains are NRRL1554 and NRRL1555. The crosses are represented on the X-axis and the Y-axis represents the fold change relative to the *carS* x *carS* mutants. The error bar indicates the standard error between three replicates.

Fig. 5. Dissimilar properties of SexM and SexP suggest they act in a non-cooperative manner to control mating. (A) Yeast two hybrid investigation into SexM and SexP physical interactions, in which cells were plated onto different media. Growth in the absence of adenine (-ade) or histidine (-his) with SexM or SexP fused to the Gal4 DNA

binding domain indicates transactivation by the two *Phycomyces* proteins. (B) and (C) Subcellular localization of *Phycomyces* SexP and SexM as GFP fusions expressed in *S. cerevisiae*. For both SexP and SexM, the top panels show the Sex-GFP compared to nucleus (Hoechst stained), the bottom panels show the SexP-GFP compared to mitochondria (MitoTracker).

Fig. 6. A model for gene regulation of *sexP* and *sexM* by light and pheromones from the two mating partners. The structures represent the zygophores produced early in the mating interaction. The green circles represent the pheromones from the (–) partner and light brown circles represent the pheromone molecules from the (+) partner. In response to light, the MadA-MadB photoreceptor complex inhibits the expression of *sexP*, causing mating to be inhibited.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

Figure 6