MATING TYPE SPECIFIC ROLES DURING THE SEXUAL CYCLE OF *PHYCOMYCES BLAKESLEEANUS*

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MATING TYPE SPECIFIC ROLES DURING THE SEXUAL CYCLE OF *PHYCOMYCES BLAKESLEEANUS*

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ABSTRACT

Phycomyces blakesleeanus is a filamentous fungus that belongs in the order Mucorales. It can propagate through both sexual and asexual reproduction. The asexual structures of *Phycomyces* called sporangiophores have served as a model for phototropism and many other sensory aspects. The MadA-MadB protein complex (homologs of WC proteins) is essential for phototropism. Light also inhibits sexual reproduction in *P. blakesleeanus* but the mechanism by which inhibition occurs has remained uncharacterized. In this study the role of the MadA-MadB complex was tested. Three genes that are required for pheromone biosynthesis or cell type determination in the *sex* locus are regulated by light, and require MadA and MadB. However, this regulation acts through only one of the two mating types, plus (+), by inhibiting the expression of the *sexP* gene that encodes an HMG-domain transcription factor that confers the (+) mating type properties. This suggests that the inhibitory effect of light on mating can be executed through the plus partner. These results provide an example of convergence in the mechanisms underlying signal transduction for mating in fungi.

This study further investigated the role of mating type genes in the mating related process of mitochondrial inheritance. Due to isogamous fusion of cells in mating in this

fungus, the initial expectation was that *Phycomyces* would demonstrate biparental inheritance but instead uniparental inheritance (UPI) was observed. Analysis of progeny from a series of genetic crosses between wild type strains of *Phycomyces* revealed a correlation between the individual genes in the mating type locus and UPI of mitochondria. Inheritance is from the (+) sex type and is associated with degradation of the mtDNA from the minus (−) parent. These findings suggest that UPI can be directly controlled by genes that determine sex identity, independent of cell size or the complexity of the genetic composition of a sex chromosome. Similar regulation may exist in other eukaryotes or was important at one stage of their evolution in establishing this system of inheritance. This investigation on the genetic regulation of mating and mating-related process of mitochondrial inheritance *P. blakesleeanus* demonstrates a prominent role of the *sexP* gene in both phenomena.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies, have examined a dissertation titled "Mating Type Specific Roles During The Sexual Cycle Of *Phycomyces blakesleeanus*" presented by Viplendra P. S. Shakya, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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LIST OF ABBREVIATIONS

BLR	Blue Light Receptors
BPI	biparental inheritance
CTAB	cetyltrimethylammonium bromide
FAD	Flavin adenine nucleotide
Gsp1	Gamete-specific plus1
HMG	high-mobility group
INM1	inositol monophosphatase
LOV	light, oxygen and voltage
MAT	mating type
mt-	mating type minus
mt+	mating type plus
mtDNA	mitochondrial DNA
MTF	Mating type female
MTM	Mating type male
NRRL	National Regional Research Laboratory
PAS	Per, Arnt, Sim
PDA	potato dextrose agar
RFLP	Prestriction fragment length polymorphism
TDO	Triple drop out

UBC	University of British Columbia
UPI	Uniparental inheritance
wc-1	white collar 1
wc-2	white collar 2
wcoA	white collar one gene A
wcoB	white collar one gene B
YPD	yeast extract-peptone-dextrose

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Dedicated to my Parents

CHAPTER 1

Introduction

Reproduction is fundamental to provide the means of propagating progeny and thus maintains the existence of a species. Most organisms use either an asexual mode or a sexual mode of reproduction; a few are endowed with the choice of using both of those. This flexibility provides an advantage in avoiding the costs attached with sexual reproduction. Most members of the fungal kingdom are capable of both forms of reproduction. Fungi continuously interact with their surrounding environment, which influences aspects of fungal biology including reproduction. Different environmental factors play a role in reproduction including temperature, humidity, nutrient availability, carbon dioxide level, light and pheromones.

Physical factors such as light affect the commitment to a sexual versus an asexual mode of reproduction. For example, in *Aspergillus nidulans,* red or far red light regulates the switch between sexual and asexual modes of reproduction through the photoreceptor FphA (Blumenstein et al., 2005). Red light inhibits sexual growth and promotes the asexual growth in *Aspergillus*. Another example where light influences the mode of reproduction can be seen in *A. nidulans*. This species employs CryA, a photolyase-like protein, which is a blue light and UV-A photoreceptor. Stimulation by blue light inhibits sexual development in this organism. CryA regulates genes required for sexual reproduction and mutations of these genes results in misregulation of sexual reproduction (Bayram et al., 2008). In *Cryptococcus neoformans,* the blue light photoreceptor Bwc1-Bwc2 complex regulates mating by repressing sexual growth under light grown conditions (Idnurm and Heitman, 2005; Lu et al., 2005). The ability to respond to environmental cues contributes to successful adaptation, and

thus may lead to better survival by sensing new niches with better nutrient availability; the aptness to respond to environmental cues also contributes to virulence for many pathogenic fungi.

To determine the environmental and genetic regulation of mating in *Phycomyces blakesleeanus* the effect of the major environmental cue light on the regulation of sexual reproduction is investigated. The natural habitats of *P. blakesleeanus* are currently not fully known or understood well. However, the species is often associated with animal feces, and observations that asexual spore germination requires a break in dormancy from elevated temperatures (such as within an animal gastrointestinal tract) support the hypothesis that the species is often exposed to environmental extremes that would include exposure to light. The second focus of this study is the mechanism of uniparental mitochondrial inheritance and its regulation by the mating type locus. For these studies the filamentous fungus *P. blakesleeanus*, which is the member of subdivision Mucoromycotina, is used as the model system for the several reasons justified later in detail. The three most important reasons for choosing *Phycomyces* are briefly mentioned below. 1) Its highly responsive sensory system for different environmental cues, especially light. 2) Its simple mating type locus compared to other fungi or eukaryotes. 3) Equal size of gametes during sexual reproduction.

Phycomyces blakesleeanus

P. blakesleeanus is a filamentous fungus that belongs in the order Mucorales within the fungal subdivision Mucoromycotina. This is a group that diverged early in evolution and thus is one of the ancient groups of fungi (James et al., 2006). Given its physical appearance of long filaments and a greening color, it is not surprising that it was first characterized as a green alga, named *Ulva nitens* in 1817 in Sweden. When reclassified as a fungus in 1823 its

name became *Phycomyces*, which literally means “algal fungus” (Cerdá Olmedo and Lipson, 1987). *Phycomyce*s is strictly a filamentous fungus unlike some Mucorales (e.g. *Mucor* spp.) that can switch between yeast and filamentous forms depending on the environmental conditions (Orlowski, 1991). *Phycomyces* grows asexually and has a sexual cycle **(Fig. 1)**. The asexual spores are called sporangiospores and sexual spores are called zygospores (Cerda-Olmedo, 2001). The zygospores go through meiosis giving rise to germsporangiospores which are haploid spores resulting from the sexual cycle (Chaudhary et al., 2013; Eslava et al., 1975).

Life Cycle of *Phycomyces*

Asexual Life Cycle

The asexual sporangiospores are multinucleate, ellipsoid in shaped and are surrounded by a thick wall. The spores are dormant in environmentally unsuitable conditions and remain viable for a long time if air-dried or kept refrigerated. Given proper conditions spores germinate by swelling and changing their shape from ellipsoid to globular. The germ tube emerges from the spores after 5-8 hours. Since the spores are dormant, germination is inefficient unless activated. The most common way for activation under laboratory conditions is heat treatment at 48^{o}C for 15 minutes (Cerdá-Olmedo, 1987). When the asexual spores grow they produce one to three germ tubes. These germ tubes grow as hyphae and form a mycelial mat with tube branching during growth (Fig. 1). The hypha is aseptate, and it never fuses to other hyphae under asexual conditions as sometime happens in some other fungi. From these hyphae arise the sporangiophores, which grow directly into the air. Sporangiophores are of two types, the macrophores and microphores. As suggested by the name, the macrophores are long (more than 200 mm) and carry the sporangiophore, which

contains up to 10^5 spores. The microphores are smaller (up to 1 mm) and carry a smaller sporangiophore, which has around 1000 spores. The microphores are suggested to function as a more nutritionally economical way of spore production. Most of the research is done on the macrosporangia and will be referred to simply as sporangia hereafter. The development of sporangiophores is divided into five stages named as I, II, III, IVa and IVb (Cerdá Olmedo and Lipson, 1987) It starts growth as a pointed tube and in the second stage a newly formed sporangiophore swells and develops in to a sporangium, which is bright yellow in color. The third stage is when no expansion in length of the sporangium is observed, but growth is resumed in stage IVa that sees slow growth and a quick change in sporangium color to dark brown or black; at this stage the sporangium also rotates counter clockwise. In the last stage, the sporangiophore reverses its rotation to a clockwise direction. The sporangiophore can reach up to the height of 15-20 cm with the diameter of 100 μm. The sporangiophore contains sporangiospores, which can be dispersed by the rupture of the outer wall of sporangiophore. These spores are then ready to enter a new cycle of asexual growth (Fig. 1) (Cerdá Olmedo and Lipson, 1987).

Sexual Life Cycle

Phycomyces is heterothallic meaning that mating is possible only between opposite sex partners. This heterothallism was first explained by Blakeslee in 1904 in landmark studies on the mating systems in fungi with obligatory-outcrossing species (heterothallism) or species capable of self-mating (homothallism) (Blakeslee, 1904a; Blakeslee, 1904b). *P. blakesleeanus* takes its species epithet from Blakeslee. For heterothallic species like *Phycomyces*, Blakeslee characterized different strains as Plus (+) and Minus (–) arbitrarily as there is no morphological difference. Strains are defined as (+) and (–) based on the mating

reaction with reference to other Plus or Minus strains. When two strains of opposite mating type are in close proximity they go through a process of developmental changes, which culminates in the formation of the sexual structure called the zygospore; provided certain condition are met (Fig. 1). In the initial stages of sexual development when the mycelia of opposite mating types reach within 1 mm of each other, growth pauses. When the mycelia are of opposite mating types the initial structures of mating are formed called zygophores. The zygophores grow towards each other to form progametangia. The progametangia grow to rise above the medium; the gametangia are demarcated by the formation of transverse septa in progametangial cells of both parents. The remaining parts of the progametangia are called suspensor cells. The two gametangial cells then fuse together to form the zygospore which is encircled by black appendages and matures as a blackened and thickened dormant structure. Depending on the parental strains the zygospore dormancy ranges from two to more than 12 months after which they produce the haploid germspores (or meiospores) (Bergman et al., 1969).

Sensory Responses of *Phycomyces*

Phycomyces possesses the capability of responding to many stimuli, the most notable ones are light, gravity, wind and proximity of objects. Most sensory studies in *Phycomyces* have been performed on sporangiophores. Thus, while saying that *Phycomyces* responds to a particular stimulus this means that the sporangiophore responds to that stimulus unless stated otherwise

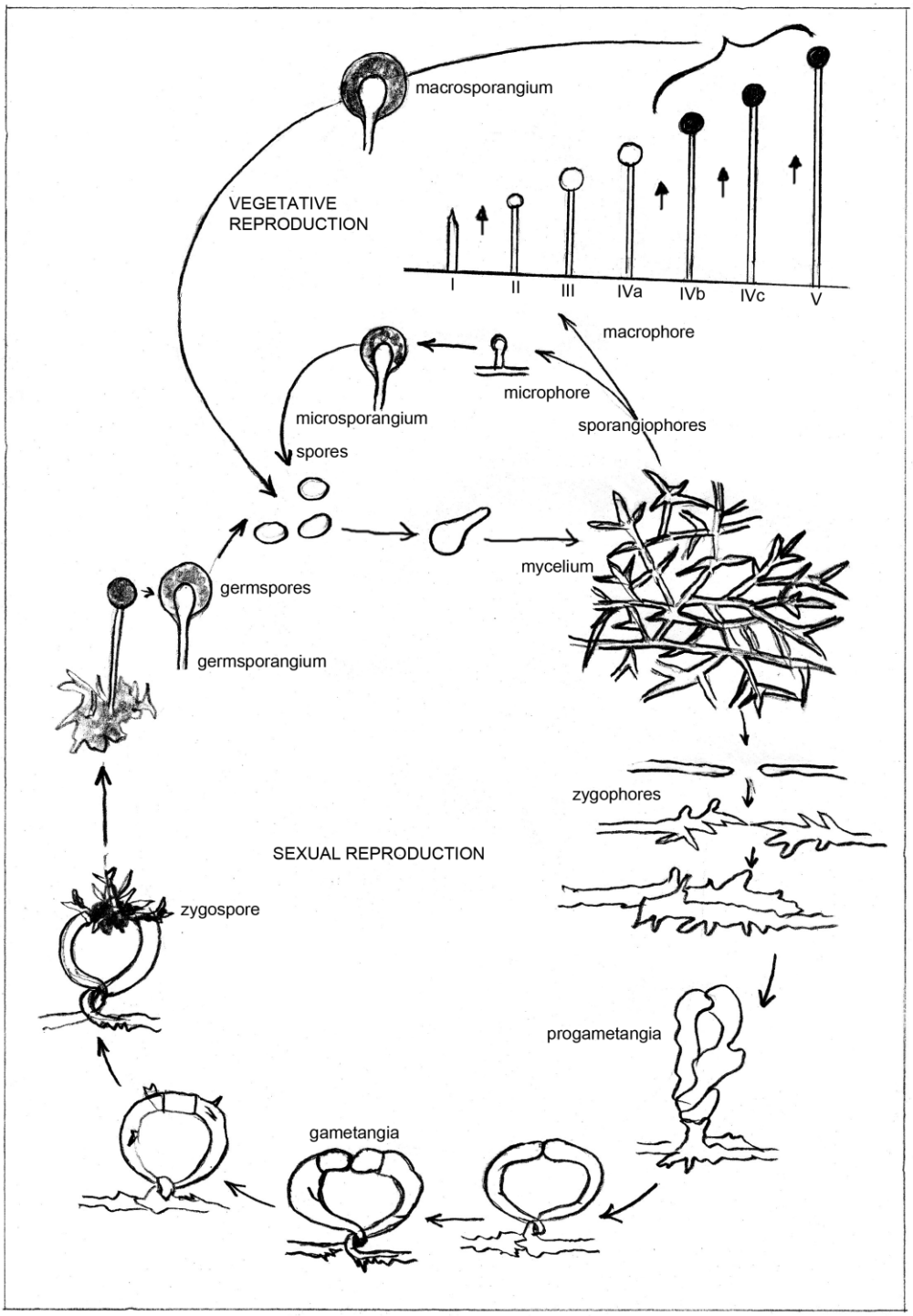

Fig. 1. Life cycle of *Phycomyces*. Vegetative mode of reproduction (asexual) and sexual mode of reproduction. (Redrawn from Cerdá Olmedo and Lipson (1987)

The sporangiophore has a strong gravitotropism response. When stage IV sporangiophores are kept horizontally in the dark they start bending upward, showing negative gravitotropism. This response is so specific that the bending rate is roughly proportional with the extent of deviance from vertical orientation (Dennison, 1964). The experiment of growing *Phycomyces* in a satellite in a microgravity environment demonstrated that the sporangiophore displays loop formation, is twisted, growth is disoriented, though the sexual and asexual reproduction is normal (Parfyonov et al., 1979). The receptor for gravitotropism is yet to be discovered in *Phycomyces* but protein crystals are reported to be required for gravitotropism. Gravitotropic mutants have been isolated, and while these possess these crystals their number is significantly less than the wild type (Eibel et al., 2000).

The *Phycomyces* sporangiophore avoids physical barriers. This is easily seen in petri dish cultures, in which the sporangiophores grow up from the agar medium, around the petri dish sides and out into the open, avoiding contact with the lid. Sporangiophores display this bending when barriers are placed in the vicinity of less than 1 mm of the growing zone. The bending is up to a 40 ^{o} to 50^{o} angle. After moving away from the barrier the vertical growth is resumed and if the physical barrier is removed the bending goes away gradually. Physical barriers sensed by *Phycomyces* are made of glass, plastic, aluminium and Teflon. Besides solid substances, liquid water and concentrated sulfuric acid were also tested by putting the perpendicular sporangiophore just above the surface of liquid, and the avoidance was observed in additional to the negative gravitotropism (Bergman et al., 1969). The sensory perception of *Phycomyces* is not only limited to solid or liquid substances but also the volatile compounds, like methanol or chloroform, are also able to challenge the *Phycomyces*

sensory system (Cohen et al., 1979). Another curious response of sporangiophores is to bend against the slow current of wind: the bending as fast as in two minutes at a rate of around 1° per minute (Cohen et al., 1975). Though the role of most of these sensory responses are not well explained at the molecular level, sensory performance would enable or help *Phycomyces* to disperse its spores in an open area or avoid dangerous locations or toxic compounds, thus increasing the chances of spreading and survival.

Light-sensing in fungi

As with barriers, gravity and chemicals, *Phycomyces* responds to light. *Phycomyces* has served as a model for understanding light sensing, including phototropism and photo-carotenogenesis, and these are described in more detail in the following sections. This fungus attracted researchers for its well-pronounced phototropic response, including Max Delbrück who wanted to use this fungus as a "phage of vision".

Light is one of the most important environmental signals to regulate growth and physiology. In the first place, light is the primary source of energy on earth, harvested by plants via photosynthesis. Even for other organisms, which do not use light as a source of energy, the light sensing system in place would enable the organism to respond to it; hence, it is beneficial to have this capability especially for terrestrial organisms. For example, it could be a signal of an open space and thus a message for an organism for opportunities to disperse progeny in new niches. In fungi, this could be of great importance considering the directional release of spores, as light could serve as the signal for the niches of better accessibility and a sensing system would be a prerequisite for this. In the case of light as a source of a danger signal (e.g. of UV radiation) the organism could initiate the survival response in the form of DNA repair or production of protective pigments like melanin or carotenes. In fungi, light

can affect various traits such as production of asexual or sexual spores, pigmentation, and pathogenicity, which are required for survival in nature. Considering the importance of light signaling, the molecular understanding of these processes is of paramount importance. It is also important from the perspective of understanding of biological aspects which might not be directly related to light (Idnurm et al., 2010). In some organisms, the signaling molecules involved in light sensing may also inform the organism of the absence of light.

Phycomyces is highly sensitive to light, due to the existence of two photosystems that work at different light intensities and over a wide sensitivity range with the threshold of 1 nWm^{-2} and limit of 10 Wm^{-2}. The availability of two photosystems enables *Phycomyces* to elicit both quick and delayed responses. It also allows *Phycomyces* to optimize itself for light induced growth of the sporangiophore. *Phycomyces* shows positive phototropism for blue light and negative phototropism for far UV range wavelengths of light (Corrochano and Garre, 2010; Galland and Lipson, 1987). The phototropic action spectrum of *Phycomyces* shows peaks around 280, 370 and 450 nm; these wavelengths indicate the role of a flavin-like photoreceptor (Curry and Gruen, 1957; Curry and Gruen, 1959; Delbruck and Shropshire, 1960). Besides action spectra data, genetic studies using mutants affected in the synthesis of carotenes or flavins and experiments using riboflavin analogs also suggest the role of flavoproteins as receptor pigment molecules (Otto et al., 1981).

The Fungal Photoreceptor of Blue/UV light

For a molecular understanding of light sensing in fungi, the ascomycete fungus *Neurospora crassa* pioneered the field. White-collar proteins WC-1 and WC-2 were discovered in *N. crassa* as the first fungal photoreceptor complex. These proteins are involved in the blue light induced carotene biosynthesis, induction of sporulation, sexual

development, phototropism of the sexual structure termed the protoperithecia, and also in the regulation of the circadian clock (Corrochano, 2007; Linden et al., 1997). In the 1980's phenotypes of *wc* mutants were described as strains with albino mycelia but normal pigmentation of the conidia, making a white collar like appearance when grown in a test tube. A model for the carotenoid biosynthetic pathway regulated through WC-1 was proposed where WC-1 is a regulator at the top of the carotene biosynthetic pathway, which is impaired for photoinduction processes if *wc-1* or *wc-2* is mutated (Harding and Turner, 1981). Carotene pigment is produced in the dark in conidia, but light is required for its biosynthesis in mycelia.

Before 1984, white-collar mutants were reported, falling into two genetic complementation groups for the loci *wc-1* and *wc-2*. Two of the mutants were mapped to the *wc-1* loci, both on linkage group IV and the third mutant mapped to *wc-2* on linkage group I (Perkins et al., 1982). Later, new mutants for the two genes were isolated and additional phenotypes described. Some of these include the fruiting body protoperithecia formation and bending towards light (Degli-Innocenti and Russo, 1984). More than a decade passed after mutants were isolated to the molecular cloning of the genes (Ballario et al., 1996). The product of the *wc-1* gene is a 125-kDa protein with a zinc finger binding domain and a transcription activation region. A year after the molecular characterization of *wc-1*, the gene encoding the other protein WC-2 was cloned and characterized. WC-2 is also a zinc finger DNA binding protein having transcription activation and PAS dimerization domains, and a model was proposed according to which light induces hetero-dimerization of WC-1 and WC-2 which further results in transcriptional activation of light regulated genes (Linden and Macino, 1997).

Genes of *Phycomyces* Light Sensing

Phototrophic mutants, known as *mad* mutants, in *Phycomyces* were identified first in screens of chemically-induced mutants starting more than 45 years ago in Max Delbrück's laboratory. Since then 10 *mad* genes have been identified in *Phycomyces*, *madA* through *madJ*, with genetic analysis pointing to these all being unlinked genes. Two genes *madA* and *madB* are supposed to encode a photoreceptor, *madC* and *madI* impact photosensor function, and the remaining six *mad* genes have other sensory defects so are most likely the downstream signaling genes or involved in sporangiophore flexibility.

The genes in phototropic mutants of *madA* and *madB* have been identified as homologues of white collar 1 (*wc-1*) and white collar 2 (*wc-2*), respectively, and so are components of a conserved blue-UV light photoreceptor complex (Idnurm et al., 2006; Sanz et al., 2009). However, there are three *wc-1* homologs and four *wc-2* homologs present in the *Phycomyces* genome **(Fig. 2)**. The first *wc-1* like gene is named *madA* (mutant for already known as the *madA* gene), the second *wc-1* like gene is known as *wcoA* (white collar one gene A), and the third *wc-1* like gene is named as *wcoB* (white collar one gene B). Gene structure for the three *wc-1* genes is similar as all have six exons with five introns with the splicing positions identical between them, suggesting a common origin. Phylogenetic analysis further supports that these genes have arisen due to gene duplication events (Idnurm et al., 2010). The three *wc-1* homologs are more closely related to their orthologs from *Mucor circinelloides* in comparison to the paralogs present within the species. WC-1 consists of a zinc finger with the exception of *wcoB* for which the standard zinc finger is absent (this domain is absent from many basidiomycete versions of Wc-1). A LOV (light, oxygen and voltage) domain is a chromophore-binding domain, which binds to flavin (FAD)

such that the protein can absorb UV and blue wavelengths to act as a photoreceptor. In addition, WC-1 has two PAS (Per, Arnt, Sim) domains that are supposed to mediate protein-protein interactions. The *madB* mutant belongs to the family of *wc-2* genes of which in *Phycomyces* there are four *wc-2* homologs named *madB*, *wctB*, *wctC* and *wctD* (for white collar two gene). All have three exons and two introns with shared splicing positions. Phylogenetic studies also suggest that the four *wc-2* genes arose from the sequential duplication events. Gene transcription studies in blue light shows slight repression of *madA* and *madB* in light, whereas induction of expression in light is observed for *wcoA*, *wcoB*, *wctB* and *wctD*. Photoinduction of all these genes is found to be dependent on *madA* and *madB* as in the mutants this induction is lowered or lost, with the exception of *wctB* whose photoinduction is not much affected in the *madB* mutant. The presence of more than one copy of both *wc-1* and *wc-2* genes and their different levels of expression change in response to light suggests one role may be in adjusting to different intensities of light signal. The WC-2 protein contains a zinc finger domain and a PAS domain that is required for dimerization. Yeast two hybrid studies for all WC-1 and WC-2 *Phycomyces* proteins shows an interaction between the MadA and MadB proteins, which is not observed for any of the other WC proteins in any combination (Sanz et al., 2009). Hence it can be hypothesized, as in the other fungi such as *Neurospora*, that MADA and MADB proteins form a photoreceptor complex and regulate light dependent responses and processes. Light affects many prominent aspects of fungal biology such that *madA* and *madB* mutants are defective in photo-morphogenesis, carotene biosynthesis and sporulation. In *Phycomyces,* sexual development is repressed by light, and the fungus has to choose between the asexual and sexual type of growth

(reproduction) with little mating in constant illumination and almost no production of sporangiophores in complete darkness.

The other *mad* genes (*madC* through *madJ*) are being mapped and identified in order to gain further insight in to the components of blue light signaling pathway and the processes it regulates. Availability of the complete *Phycomyces* genome and new genetic map are milestones in this direction, which has already led to genetic mapping of *madC*.

Other fungal homologs of the White collar complex proteins

The overall structure for the two proteins is similar across the kingdom with the exception of the Agaricomycotina and Ustilaginomycotina (Basidiomycota) where the zinc finger DNA binding domain is absent from Wc-1. The Wc-1 and Wc-2 and their homologs are ubiquitous in transcriptional regulation of genes in fungi across the major divisions of the kingdom. Some of the downstream targets regulated through the complex, such as the essential gene *HEM15* are conserved in *Neurospora*, the basidiomycete *Cryptococcus neoformans* and *Phycomyces* (Idnurm and Heitman, 2010). Recently the Bwc1-Bwc2 complex was also reported to regulate the Uve1 endonuclease, which is necessary to protect *C. neoformans* from UV damage and this gene is a direct downstream target of the Bwc1-Bwc2 complex (Verma and Idnurm, 2013). *C. neoformans* and *C. gattii* are human pathogenic fungi; Bwc1-Bwc2 complex mutants show a reduction in their virulence (Idnurm and Heitman, 2005; Zhu et al., 2013).

Light and Mating

Light can have positive or negative effects on mating in fungi. It inhibits mating in representative species of the Ascomycota, Basidiomycota and Mucorales. In *C. neoformans,* mating is regulated through the Bwc1-Bwc2 complex such that in light mating is repressed.

In mutants of *BWC1*, *BWC2* or the double mutant, this repression is lost. *SXI1α*, which encodes a transcription factor, and *MFα1*, encoding a pheromone precursor protein, are two light-regulated genes that are found in the mating type locus of *C. neoformans* and are required for completion of the sexual cycle: the regulation of both genes is altered by loss of the Bwc1-Bwc2 complex. In the ascomycete *Hypocrea jecorina,* light perception is mediated through the Blue Light Receptors (BLR) 1 and 2, the homologs of *WC-1* and *WC-2*. Constant illumination inhibits the sexual development yet at the same time constant darkness is also not conducive for sexual development; this condition slows the formation of stromata. The photoperiod of 12 h light/12 h dark is the most supportive condition for the development of stromata and pigmentation. The inhibition of the sexual development in constant illumination is mediated through BLR1 and BLR2 proteins. In *H. jecorina* 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). Another study in *H. jecorina* revealed that BLR1 and BLR2 negatively regulate the expression of pheromone receptor genes and pheromone precursors in light but not in darkness; interestingly, this regulation appears to be mating type dependent (Seibel et al., 2012).

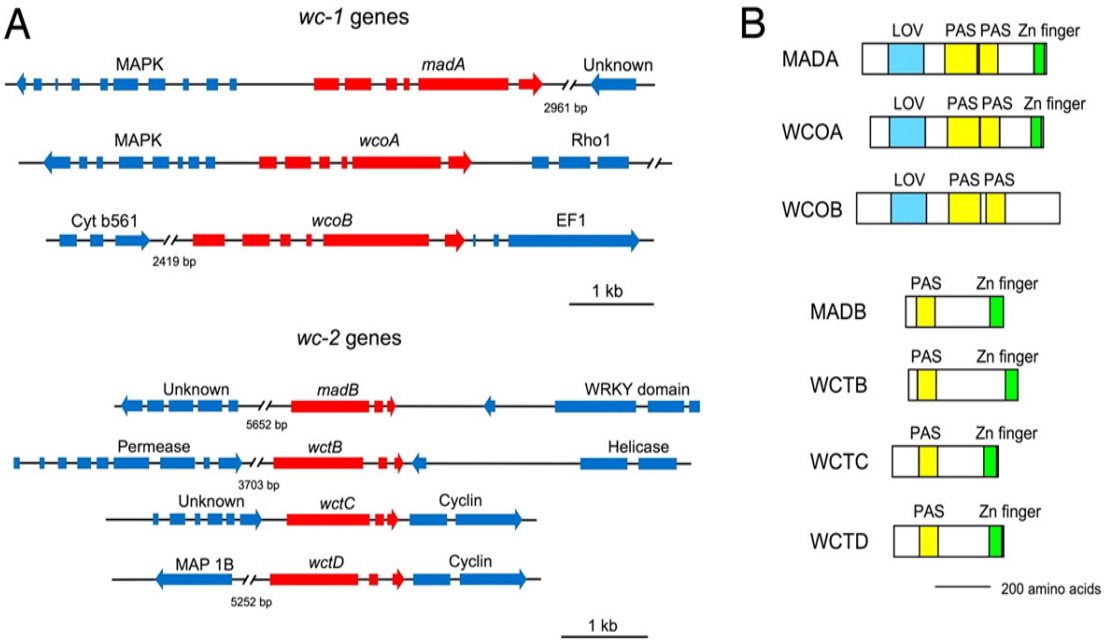

Fig. 2. *Phycomyces wc* genes and proteins. (A) Genomic structure of *Phycomyces wc* genes including flanking genes with their putative identities. Exons, introns, and direction of transcription are indicated. (B) WC proteins in the *Phycomyces* genome and their domains.

Adapted from Sanz, Rodriguez-Romero et al. 2009

Although light regulates processes such as sexual development and reproduction, the exact mode of regulation and the mechanisms through which these processes are regulated by the While Collar complex is not clear.

Carotene and Biosynthesis of Pheromones in *Phycomyces*

The color of wild type changes with the age of the mycelia; the young mycelia most prominently display the yellow color, which is due to the pigment beta-carotene. The mycelia grown in light accumulate more carotene compared to those grown in dark. This is due to light-induced carotene synthesis. Besides light, mating conditions also induce carotene synthesis, as do chemicals like retinol and beta ionone that share physical similarities with parts of the beta-carotene molecule. A conundrum is that light inhibits the process of mating and promotes asexual reproduction, but promotes the biosynthesis of beta-carotene, which is the precursor for the sexual pheromones required for the mating.

Chemically the carotenes are unsaturated hydrocarbons with 40 carbon atoms and a variable number of hydrogen atoms. They are made from four terpenes, which are made of eight isoprenyl groups. The major carotene in *Phycomyces* is β-carotene. One molecule of β-carotene is synthesized from two molecules of geranyl geranyl pyrophosphate. This process involves three enzymes: phytoene synthase, phytoene dehydrogenase and lycopene cyclase. The genetics of biosynthesis involves the two genes *carRA* and *carB*, which encode three enzymes (Fig. 3). The *carRA* mRNA is translated as single polypeptide but encodes two enzymes. The R domain is a lycopene cyclase and the A domain a phytoene synthase (Arrach et al., 2001). The APE motifs (conserved DNA elements for binding of regulatory elements, from *N. crassa* "*al-3* proximal element") located in intervening DNA sequence between the

coding regions of *carRA* and *carB* are suggested to control photoregulation of carotenogenesis, and these motifs are also required for photoregulation in *Mucor* (Ruiz-Hidalgo et al., 1997; Velayos et al., 2000a; Velayos et al., 2000b). Similar regulation is believed to be conserved in the Mucorales (Almeida and Cerda-Olmedo, 2008). Transcription is induced after illumination in all three Mucorales species investigated, i.e. *Phycomyces*, *Blakeslea* and *Mucor*. The two *car* genes for β-carotene biosynthesis in all three of these fungi are also similar in the arrangement of putative regulatory sequence. The regulatory sequences for sexual stimulation of these genes are not yet known.

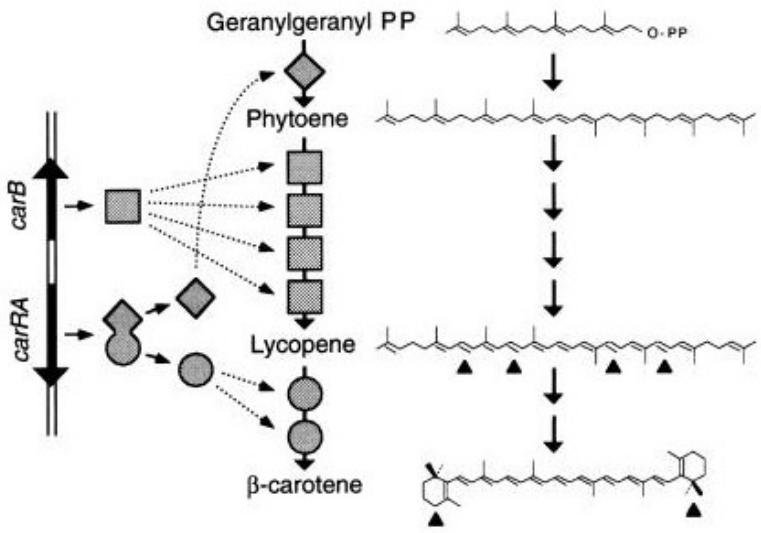

Fig. 3. Genes, enzymes, and chemical reactions for carotene biosynthesis in *Phycomyces*. Two molecules of geranylgeranyl pyrophosphate are converted to one molecule of β-carotene by phytoene synthase (diamonds), four reactions are catalyzed by phytoene dehydrogenase (squares), and two by lycopene cyclase (circles). The scheme on the left represents the genes that code for these enzymes. The chemical changes are marked with arrowheads. (Arrach et al., 2001)

The primary regulators of photoresponses in *Phycomyces* are the *madA* and *madB* gene products, which act together both as photoreceptors and transcription factors (Idnurm et al., 2006; Sanz et al., 2009). The *madA* and *madB* genes belong to Wc family first investigated in *Neurospora*. It is worth noting that the mutants of these genes are linked to the characterization of the genetics of carotene biosynthesis in *Neurospora* (Ballario et al., 1996; Nelson et al., 1989)

Phytoene synthase catalyzes the joining of two molecules of geranyl geranyl pyrophosphate to form phytoene (Arrach et al., 2001). The *carB* gene encodes phytoene dehydrogenase enzyme (Ruiz-Hidalgo et al., 1997). The four double bonds are introduced by four units of phytoene dehydrogenase to form lycopene, and lycopene cyclase (encoded by R domain of *carRA*) catalyzes the formation of the beta rings at the ends of the molecule (Arrach et al., 2001). The mutants of these genes have no or minimal carotene content and thus are visibly different from wild type strains. The *carB* mutants are not able to process phytoene and are white. The mutants of R domain of *carRA* gene are unable to process lycopene and thus accumulate this red carotene and look red. The mutants of the A domain are leaky and look white as the amount of carotene is too low to be visible (Eslava et al., 1974). Blue light upregulates the transcription of *carRA* and *carB* genes, thus explaining how light induces carotene biosynthesis (Ruiz-Hidalgo et al., 1997).

Mating in fungi requires diffusible pheromone-signaling molecules. Hans Burgeff recognized the effect of diffusible pheromones on sexual interactions in Mucor in 1924, a first in any organism (Tagua et al., 2012). In the Mucorales the signaling molecules are

apocarotenoids and are derived from the cleavage of β-carotene. Mutants that cannot either synthesize (i.e. *carB* and *carRA*), those that cannot process (*carS*), and those that are mutants for the genes involved in further processing of apocarotenoids have the similar infertile phenotype. All do not produce the substrate for apocarotenoids, and they can not process the substrate or possibly lack the functional enzymes involved in later stages of pheromone biosynthesis (Sutter et al., 1996). These mutants can respond to the signal from a wild type strain of opposite mating type, but are unable to induce the developmental changes in their respective mating partner: both partners are required to make and sense pheromones to complete the process of mating, and no zygospores are formed (Sutter, 1975).

After β-carotene is synthesized it needs to be metabolized for the biosynthesis of trisporoids pheromones. The mutants that cannot process the β-carotene are sterile. For example, the mutants of the *carS* gene accumulate the β-carotene and cannot stimulate the opposite mating partner. The *carS* gene encodes an oxygenase required for the cleavage of β-carotene ring. This is the first step for the synthesis of apocarotenoids (Medina et al., 2011; Tagua et al., 2012). Apocarotenoids of 18, 15 and 7 carbon atoms have been identified in *Phycomyces* and other Mucorales wild type mating cultures, which shows that the 40 carbon molecule of β-carotene is cleaved into three fragments (Barrero et al., 2011; Polaino et al., 2012; Polaino et al., 2010; Tagua et al., 2012) These apocarotenoids are absent from the mating culture media of *carS* mutants as these mutants lack the enzymatic ability to cleave β-carotene, thus explaining the accumulation of β-carotene in these mutants. Although the accumulation of β-carotene in *carS* mutants could be the result of lack of processing of the metabolite, some studies suggest that the carotenogenesis pathway in *Phycomyces* is regulated by feedback inhibition by one of the apocarotenoid products (Bejarano et al.,

1988). The interruptions by structural analogs such as retinol are considered to block the end-product regulation of carotenogeneis by competing for a binding site in the oxygenase enzyme encoded by the *carS* gene. This binding is possible due to the structural similarity of chemical analogs to the β-carotene ring.

It is interesting to note that light by itself inhibits mating but induces the production of the substrate for pheromone synthesis required for mating, and sexual stimulation has an additive effect on carotene production. This suggests an independent route followed by light and mating to induce carotene biosynthesis. This observation compels us to ask the question of how light inhibits mating when it is indirectly helping to lay the platform for sexual reproduction. This regulation could be through the inhibition of further processing of carotenes to pheromones or light may have more direct control of mating-related genes. In our studies one of the issues we have investigated is the influence of *madA* and *madB* on the mating type locus genes *sexP* and *sexM* in *Phycomyces*. Although the limitation of the lack of genetic manipulation through transformation hinders research on *Phycomyces*, nevertheless the simplicity of *Phycomyces* mating type locus and availability of carotene biosynthesis mutants have enabled us to address the question of how light inhibits mating in *Phycomyces*.

Mating in Mucoralean Fungi

Mucoralean fungi are historically important when studying sexual reproduction, as they are the species in which the concept of heterothallism and homothallism was explained. Heterothallism occurs in species in the genus *Rhizopus* and *Phycomyces*, and homothallism in species like *Syzygites megalocarpus* (Blakeslee, 1904a; Blakeslee, 1904b). *Phycomyces* is the first fungus outside of the ascomycetes and basidiomycetes in which a mating-type locus was identified (Idnurm et al., 2008). It has a sex-specific or mating-type locus differing

between each of the sexes/mating types, which has been envisaged as the ancestral fungal *MAT* locus (Martin et al., 2010). In *Phycomyces* the *sex* locus (or *MAT* locus) encodes for the HMG domain proteins called SexM and SexP for the minus (–) and plus (+) mating types. Both SexP and SexM are upregulated in the mating cultures. *Phycomyces* is heterothallic with mating possible between opposite sex partners only; however, there are a few strains that behave almost as if homothallic that originate as an outcome of experimental crosses in which the occasional heterokaryons arise carrying both *sexM* and *sexP* in the same mycelium. SexP and SexM proteins of *Phycomyces* are proposed to be divergent homologs such that the two proteins share very low level of amino acid similarity yet they are the closest proteins in tBLASTn searches. For strain UBC21 (+), there is sex-unique region of 5830 bp in comparison to the NRRL1555 (–) strain, which has a shorter sex-unique region of 3,494 bp. The organization of the *sex* locus is such that it consists of an HMG-domain encoding gene flanked by two conserved flanking genes *tptA* (triose phosphate transporter) and *rnhA* (RNA helicase), which is the same for both the sexes. The two genes *sexM* and *sexP* are asymmetrically placed within the sex-unique region and have inverted transcriptional orientation. The (+) sex locus is larger due to the presence of the small repetitive elements in comparison to the (–) sex allele. Phylogeny studies show that SexP is a MATA_HMG domain containing protein and SexM is contains a SOX domain (Martin et al., 2010). These two proteins SexM and SexP are similar to the HMG domain proteins Sry and Sox3 in humans that are believed to be a diverged pair of alleles. The parallels between fungal and animal systems for sex determination have been noted previously (Fraser and Heitman, 2004), and this has been perhaps most dramatically demonstrated by the use of the human Sry protein to complement the mating defects associated with deletion of the *A.*

nidulans matA gene (Czaja et al., 2014) The *sex* loci of two other Mucoralean fungi, *Mucor circinelloides* and *Rhizopus delemar*, are syntenic to that of *P. blakesleeanus*. Promoter elements of *sexP* are conserved, with a common TATA box at 102 bp in *Phycomyces* and transcription factor-binding motif with CCAAT box within 100 bp upstream of the start codon. However no sequence conservation for the promoter region of *sexM* was observed with other Mucorales.

Mitochondrial Inheritance

Mitochondria are double membrane organelles contained within the cell cytoplasm. They are best known as the cell's powerhouse, producing energy currency in form of ATP molecules through the process of oxidative phosphorylation. In humans, mutations in mitochondrial DNA can cause several genetic and metabolic disorders such as type 2 diabetes, neurological diseases such as Alzheimer and Parkinson, Leber's hereditary optic neuropathy, hereditary spastic paraplegia, ischemic stroke, myocardial infarction and cancer (Schon et al., 2012). Most of these diseases are maternally inherited as they are due to mutations in the mitochondrial genome, and in humans during fertilization the mitochondria are contributed by the mother egg cell. The first report of maternally inherited disease due to mutations in the mitochondrial genome (mtDNA) was of Leber's hereditary optic neuropathy in 1988 (Wallace et al., 1988), and since then many more mtDNA mutations are reported implicated in many other diseases. About 1 child in every 5000 to 10000 children born is affected by disease caused by mutation in mitochondrial DNA and can show severe clinical symptoms (Mitalipov and Wolf, 2014), hence understanding of genetics and mechanisms behind mitochondrial inheritance is important. As mitochondria are crucial cell organelles and because they cannot be produced *de novo* they have to be properly inherited from

parents. This inheritance can be biparental (BPI) or uniparental (UPI) depending on the species, and in the biparental organisms recombination between mitochondrial genomes also occurs. Being a cytoplasmic organelle the Mendelian laws of inheritance are not obeyed.

During sexual reproduction the mixing of parental subcellular compartments takes place in the process commonly known as heteroplasmy, so organelles like mitochondria and chloroplasts are mixed and thus their genomes as well. Heteroplasmy is common but maintenance of stable heteroplasmy is rare. Most organisms have developed a method to maintain a homoplasmic state, but how this is achieved is not well explained. Homoplasmy can be achieved either by a process of elimination of mitochondria from one of the fusing cells before gamete formation or by selective degradation after fertilization (Birky, 2001).

In many eukaryotes mitochondrial inheritance is uniparental and this process is genetically regulated. In general, inheritance of mitochondria is expected from the mother cell; e.g. humans get the mitochondria from their mothers. In plants maternal inheritance for mitochondria is predominant, but for plastids this is little more flexible with many examples of paternal or biparental inheritance.

The most trivial and generally provided explanation for maternal inheritance of mitochondria is based on the mammalian scenarios in which the mother cell is big, and so contributes most of the cytoplasmic material, while the sperm is small and loses its mitochondria on fusion with the egg. As reviewed by Mogensen (1996), plants provide many examples counter to the cytoplasmic organelle model of inheritance, as there are examples of paternal inheritance of mitochondria, plastid or both in plants, where the male gametes are also smaller. The conifers can inherit the mitochondria either from egg or pollen cell. Examples of inheritance of both organelles (mitochondria and plastid) from one parent or one

organelle from each parent are available. The Douglas fir has maternal inheritance for mitochondria but paternal inheritance for plastids. The plant *Biota orientalis* inherits both organelles from the paternal cell (Mogensen, 1996). Notably, if the uniparental inheritance of mitochondria is just a consequence of cell size or the amount cytoplasmic material, there would be no need for the various mechanisms existing to ensure the uniparental maternal inheritance in many species.

In fungi, the uniparental inheritance of mitochondria is a common phenomenon but the molecular players implicated for this process are not well defined. In filamentous basidiomycetes UPI is achieved by mutual exclusion of mitochondrial exchange during fusion. In general when anisogamous fusion occurs, e.g. in *A. nidulans* or *N. crassa*, the mitochondria from the larger gamete (protoperithecia) prevails. In isogamous fusions, e.g. in *S. cerevisiae*, BPI is a more regular process (Basse, 2010).

The prominence of uniparental inheritance suggests it must be important. Various mechanisms are reported to regulate UPI. There are pre-fertilization and post-fertilization events that impact mitochondrial inheritance. In brief, different mechanisms are the exclusion of mitochondria from one of the parental gametes prior to fertilization (Moses, 1961), degradation of mitochondria either before or after fertilization by specific nucleases, and by the process of autophagy and ubiquitin-mediated degradation (Sato and Sato, 2013).

The uniparental inheritance provides the homoplasmic state in the progeny, but why is this necessary? For instance, the parental mitochondria were performing their jobs in both original parents, so why are there so many methods to warrant a homoplasmic stage? The model yeast *S. cerevisiae* has biparental inheritance of mitochondria, but again the importance of maintaining a homoplasmic state can be appreciated because only one type of

mitochondria in passed on to the daughter cell (Basse, 2010). Septin-mediated complexes are formed to ensure the differential distribution of mitochondria, which enable the mother cell to ensure a homoplasmic state for daughter cell (Tartakoff et al., 2013).

Various mechanisms to ensure the uniparental inheritance of mitochondria have been discovered, but the principal genetic regulator is still elusive. In microbial models *Chlamydomonas* and *Physarum* mating-type systems direct the process of mitochondrial inheritance (Fedler et al., 2009; Moriyama and Kawano, 2003). Fungi such as *Ustilago maydis* and *C. neoformans* have also suggested the role of mating type locus as a putative master regulator for uniparental mitochondrial inheritance, but the existing models have some drawbacks of complex mating type locus or fusion of unequal size cells. *Phycomyces* has a very simple mating type with only one protein encoded and two similar sized cells fuse to produce the zygospore. Since two similar–sized cells fuse, we initially hypothesized that mitochondrial inheritance in *Phycomyces* would be biparental. However, the genetic evidence we provide in this study proves the inheritance from the (+) plus mating type parent.

CHAPTER 2

MATERIALS AND METHODS

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Strains, culturing and crossing conditions for *Phycomyces*

Strains used in this study are listed in table 1. Strains were routinely cultured on potato dextrose agar (PDA) medium. For collection of the asexual spores, strains were grown on PDA medium for 5-7 days, and spores also saved as permanent stocks at -80°C in 30% glycerol. 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 as then crossed to two *madA* mutants with different alleles of the gene, and double mutants in both mating type backgrounds were isolated. The asexual spores from both mating types were incubated on V8 medium plates (5% V8 juice, 0.5 g/L KH_2PO_4, 1 mg/L thiamine, 4% agar; pH 6). The zygospores are formed after 5-7 days of incubation in dark. The zygospores are harvested and placed on sterile wet filter paper spaced 6-7 cm. After the dormancy for 2-4 months the zygospores 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. Mutations in each gene were determined either by PCR-RFLP analysis or re-sequencing the gene.

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 $MgSO_4.7H_2O$, 28 mg $CaCl_2$, 1 mg

thiamine.HCl, 2 mg citric acid.H_2O, 1.5 mg of $Fe(NO_3)_3.9H_2O$, 1 mg of $ZnSO_4.7H_2O$, 300 µg $MnSO_4.H_2O$, 50 µg $CuSO_4.5H_2O$, 50 µg $Na_2MoO_4.2H_2O$. {Appendix 1 (Cerdá-Olmedo, 1987)}. Heat shock at 45°C for 15 minutes was given to asexual 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 or dark. For zygospore counting three random areas of (1 cm^2 each) of the petri plate were selected and the zygospores were counted manually.

RNA extraction and RT-PCR

Mycelia were collected by scraping them from the plates and immediately frozen in liquid nitrogen and stored in -80°C, until further use. RNA extraction was performed using RNA extraction kit (Qiagen), and 5 µg of total RNA was used for making cDNA by reverse transcription in a 20 µl reaction (Superscript III, Invitrogen). 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.

Fermentas 2X SYBR green mixture was used for amplification of genes from cDNA by quantitative PCR in an ABI7300 machine (Bio-Rad). Primers are listed in Table 2. The ΔΔct method (Livak & Schmittgen, 2001) was used for relative quantification of genes with the actin gene used as the endogenous control as a constitutively expressed gene. The primer concentrations of 0.3 nm were used in qRT-PCR.

Yeast Two Hybrid analysis

The ORFs of *sexM* and *sexP* were cloned previously (Idnurm et al., 2008). For testing the interaction the AD and BD clones in various combination (Fig 9) were co-transformed into the PJ69-4A *S. cerevisiae* strain (James et al., 1996). Double transformants were selected

on the media lacking leucine and tryptophan amino acids (Double drop out, DDO medium). The selected clones were then tested for growth on media without leucine, tryptophan and histidine amino acids (Triple drop out, TDO medium). The TDO media supplemented with 3-amino-1,2,4-triazole (competitive inhibitor for histidine) and media lacking leucine, tryptophan, histidine and adenine (Quadruple drop out, QDO medium) was used to test the strength of interactions.

Cloning of sexM and sexP for localization studies by fluorescence microscopy

The *sexM* and *sexP* were amplified by PCR using Takara Ex Taq from the respective pGAD clones. 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 product 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 DH5alpha strain of *E. coli* and sequenced to identify a clone with no PCR-induced errors.

For localization studies 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 PBS. The imaging was performed using an Olympus confocal microscope (FV300***).***

UV stress test

To test for UV sensitivity, 1,500 spores were plated on acidified PDA media (pH 3.5) and incubated for 24 hours in light. After incubation the UV stress of 100 J/m^2 was given in a

XL-1500 UV cross linker (Spectronics Corporation). After giving this stress the plates were incubated in dark. Results were recorded about 40 hours post incubation. All the experiments were performed on 150 mm X 15 mm petri dishes. While plating the spores a gap of ~15 mm was given from the boundary of the plate to avoid any possible shading effect during the UV stress treatment.

Crosses for the study of mitochondrial inheritance

Strains used as parents in crosses were NRRL1555 sex type (−), NRRL1554 (+), A56 (+), SC10 -1a (+), UBC21 (+), and UBC33 (−). These strains are wild isolates, except for A56. NRRL1555 is the standard laboratory strain, which has been sequenced, and A56 is isogenic to NRRL1555, after 11 backcrosses to introgress the (+) *sex* allele into the NRRL1555 genetic background. KC10B1 (−) and KC10B6 (+) are progeny isolated from a SC10-1a × NRRL1555 cross; they contain the SC10-1a mitochondrial DNA (mtDNA) genotype. KC10F1 (+) and KC10F6 (−) are from an A56 × NRRL1555 cross; they contain the A56 mtDNA genotype. For preparing mycelia for DNA extraction, sporangiospores were inoculated in yeast extract-peptone-dextrose (YPD) medium and incubated for 3 to 5 days. Crosses and progeny isolation were performed as mentioned in section 4.1. Each progeny was isolated from a different zygospore, to ensure that each was the result of an independent meiotic event. The sexes of the progeny were tested by inoculating them adjacent to the standard wild type (+) and (−) strains.

DNA extraction

Mycelium was dried in a freeze-dryer, and genomic DNA was extracted from mycelium broken apart with 2 mm diameter glass beads with a CTAB (cetyltrimethylammonium bromide) extraction buffer following a published protocol (Pitkin

et al., 1996).

Mitochondrial genotyping

Primers ALID0933 and ALID0934 were used to amplify a polymorphic region of the mitochondrial genome, identified by comparing genome sequences of strains NRRL1555 and UBC21 (Joint Genome Institute, US Department of Energy) and suitable for comparing the different mitochondrial genotypes in the other wild type strains. ALID0933 is within the *orf511* gene, and ALID0934 is in the intergenic region between *orf511* and *nad4L*. PCR products were digested with TaqαI restriction enzyme at 65°C and separated by electrophoresis on 2% agarose/1× Tris-acetate-EDTA gels. For PCR from zygospores, a single zygospore was crushed in 10 µl of dilution buffer (Thermo Scientific Direct PCR kit) and incubated for 10 min at room temperature; 2 µl of this was used as the template for the PCRs. Products were either digested with TaqαI and resolved on agarose gels, or sequenced. Another mitochondrial polymorphism between the *atp9* and *trnG* genes was examined in 12 randomly selected progenies from cross 1 and the two parents (NRRL1555 and UBC21). Primers ALID2131 and ALID2132 amplified the region, and the PCR products were gel purified and sequenced, and the sequences were compared for the 14 strains.

Table 1. 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	*madA* (–)	NRRL1555	(Bergman et al., 1973)
A893	*madA* (–)	NRRL1555	(Idnurm et al., 2006)
C109	*madB* (–)	NRRL1555	(Bergman et al., 1973)
CAB26	*madA* (+)	A56 x C47	This study
C5A	*madB* (+)	A56 x C109	This study
AB36	*madA madB* (–)	A56 x A893	This study
AB48	*madA madB* (+)	A56 x A893	This study
CAB13	*madA madB* (–)	A56 x C47	This study
CAB22	*madA madB* (+)	A56 x C47	This study
M1	*carS* (+)	NRRL1554	Sutter 1975
S324	*carS* (–)	NRRL1555	(De la Concha and Murillo, 1984)
C5	*carB* (–)	NRRL1555	(Cerda-Olmedo and Reau, 1970)
S342	*carB* (+)	NRRL1555	Unknown origin
KC10B1	(−)	SC10-1a × NRRL1555	This study
KC10B6	(+)	SC10-1a × NRRL1555	This study
KC10F6	(−)	A56 × NRRL1555	This study
KC10F1	(+)	A56 × NRRL1555	This study

Table 2. Oligonucleotide primers used in this study

Name	Sequence 5´-3´	Purpose
ALID1659	TGTCCTTTCCCTTTACGCC	*Phycomyces* actin for qRT Forward
ALID1660	ACGACCAGCCATATCCAAATG	*Phycomyces* actin for qRT Reverse
ALID1661	CCGTACACCCTAGCATTCTATC	*Phycomyces sexM* for qRT Forward
ALID1662	GTTTCTCTTCATCCGCTTTTCG	*Phycomyces sexM* for qRT Reverse
ALID1671	GATTCTATGCCCAGGACCG	*Phycomyces sexP* for qRT Forward
ALID1672	TCAATGGCGAAGTTCTCTTG	*Phycomyces sexP* for qRT Reverse
ALID1673	CTATCGTCATCTTGGTGCCTG	*Phycomyces carS* for qRT Forward
ALID1674	ACAAGAGTAATACAGTGCCGAC	*Phycomyces carS* for qRT Reverse
ALID1996	CGTTGGACTCTGCCCAGTTTC	*Phycomyces carRA* for qRT Forward
ALID1997	CCAGCGACGAAGCATGTATTC	*Phycomyces carRA* for qRT Reverse
ALID1998	GACCATCTGGAAGCTTATCCG	*Phycomyces carB* for qRT Forward
ALID1999	GGATAACGGTGTTGAAACCAC	*Phycomyces carB* for qRT Reverse
ALID1254	CTATACTTTAACGTCAAGGAGAAA AAACTATAATGGAATCTTTTATGTT TGTTAACG	Cloning of *sexM* for localization in *S. cerevisiae* Forward
ALID1255	CCAGTGAAAAGTTCTTCTCCTTTAC TGTTAATTGAACATAAAGAGAATA TAAGCTC	Cloning of *sexM* for localization in *S. cerevisiae* Reverse
ALID1256	CTATACTTTAACGTCAAGGAGAAA AAACTATAATGAAAGCGAAACAGA CACC	Cloning of *sexP* for localization in *S. cerevisiae* Forward
ALID1257	CCAGTGAAAAGTTCTTCTCCTTTAC TGTTAATAAACCTTGCCCAAAAACT TTCG	Cloning of *sexP* for localization in *S. cerevisiae* Reverse
ALID1287	AATTAATCAGCGAAGCGATG	For sequencing the pYES-GFP vector clones Forward
ALID1272	TGTTGCATCACCTTCACC	For sequencing pYES-GFP
ALID0350	ACTTCAGGGTCAGCTTGC	Internal GFP primer to sequence the gene-GFP fusion
ALID0933	CGAATGACCCGAGAAGCC	*Phycomyces* mitochondrial genotyping
ALID0934	GGAGTGACTCATCTTTCG	*Phycomyces* mitochondrial genotyping
ALID2131	AATTGTTGCTAATCCAGCTC	*Phycomyces* mitochondrial genotyping
ALID2132	AAAAGGTCGTCACCTTCGTC	*Phycomyces* mitochondrial genotyping

Table 3. Crosses and number of progeny analyzed in mitochondrial inheritance study

Cross no.	Parents [(+) × (−)]	No. of progeny analyzed
1	UBC21 × NRRL1555	121
2	SC10-1a × NRRL1555	17
3	SC10-1a × UBC33	26
4	NRRL1554 × NRRL1555	16
5	UBC21 × UBC33	12
6	A56 × NRRL1555	28
7	KC10F1 × KC10B1	22
8	KC10B6 × KC10F6	10

CHAPTER 3

THE MADA-MADB PHOTORECPTOR COMPLEX INHIBITS MATING BY ACTING ON THE PLUS SEX OF *PHYCOMYCES*

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 successful mating often uses small diffusible pheromone molecules to communicate between strains.

The Mucoromycotina are a branch of the fungi in which most of the key aspects of fungal sex were first established. These include the discovery of sex in fungi, diffusible pheromones, and the reproductive distinction between homothallic and heterothallic species. The *sex* or mating type locus was identified in *Phycomyces blakesleeanus* (Idnurm et al., 2008), which paved the way for further investigations in mating in the Mucoromycotina, within the limitation posed that *Phycomyces* is not amenable to transgenic manipulations (Obraztsova et al., 2004). *Phycomyces* and the other Mucoromycotina species characterized to date have a mating type locus that is similar to that found in the ascomycetes and basidiomycetes in terms of a region of DNA with minimal similarity between each allele, and each allele encoding a transcription factor. 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 Mucoromycotina also have similar arrangement of mating type genes and conserved flanking

genes with minor changes (Lee et al., 2010), and thus gene regulation may be similar across species.

In *Mucor circinelloides* the *sexM* homolog is dramatically upregulated by the treatment of pheromone precursor trisporoids, while *sexP* is induced by trisporoids at a lesser level (Wetzel et al., 2012). In mating conditions in *Phycomyces* both *sexM* and *sexP* are upregulated relative to single isolate cultures (Idnurm et al., 2008). If based on the *Mucor* studies, the pheromones seem to be mainly regulating *sexM* but the regulation of *sexP* still needs to be explained. Also the promoter regions of *sexM* and *sexP* are dissimilar, and thus their regulation is suggested to be different (Wetzel et al., 2012).

Another important thing to note is that the pheromone production is induced only when cultures of opposite mating types are present in close vicinity. Thus, some other factor is required to regulate the initiation of mating. Existing data suggest that *sexM* and *sexP* could be regulated by independent factors and a regulator above the level of pheromone biosynthesis, but this factor is yet to be determined and is possibly an environmental cue. 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 (Sutter et al., 1996). The current model suggests that the pheromones are the master regulator for the mating but not the mating type locus genes as happens in Ascomycota. This model raises the question of how the efficient use of resources would be ensured and which partners would initiate the production of pheromone precursors when the pheromone signal from the opposite mating type is not present to communicate the availability of favorable mating conditions. If the

pheromones are major regulators then there needs to be co-ordination with the environment to ensure the favorable conditions for choice of growth mode. This model also misses the link of all these process with environmental variables, such as light, which is known to inhibit mating.

Light is an important environmental cue that affects many developmental processes across the fungal kingdom (Idnurm et al., 2010; Rodriguez-Romero et al., 2010; Tisch and Schmoll, 2010). Considering the prominent light responses in *Phycomyces* it is potentially an excellent system that could be used to delve into how mating-type genes *sexM* and *sexP* in concert with light regulate mating and other developmental aspects of Mucoromycotina. 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 the study of how *sexM* and *sexP* expression may be controlled by light and how this affects mating. An interesting aspect that revolves around light and mating is carotenogenesis. Carotene biosynthesis is the prerequisite for pheromone biosynthesis since carotene cleavage products serve as substrate for pheromone biosynthesis. The chemical steps involved in this process are well studied in *Phycomyces* (Polaino et al., 2012; Polaino et al., 2010; Sutter, 1975)

The repression of mating by light in *Phycomyces* has been known for many years. The wavelength between 350 nm and 490 nm are most effective for the inhibition of zygospore formation. The early stages of mating are inhibited more effectively by wavelengths of 350 nm and 370 nm, while the late stages were inhibited by the wavelength

of 410 nm. The inhibition of light was more effective on the early stages compared to the late stages (Yamazaki, 1996).

However, it is unclear whether or not the repression of mating by light depends on MadA and MadB, or one of the other candidate photosensors that are present in the *Phycomyces* genome (Rodriguez-Romero and Corrochano, 2006; Sanz et al., 2010; Sanz et al., 2009). In *Phycomyces* the role of the MadA-MadB photoreceptor is studied for phototropism (Idnurm et al., 2006; Sanz et al., 2009), in the regulation of heat shock gene *hsp100* (Rodriguez-Romero and Corrochano, 2006), in regulation of the essential gene *hemH* for heme biosynthesis (Idnurm and Heitman, 2005), in regulation of *carA and carRA* genes (Sanz et al., 2010) and a DNA repair gene *UVE1* (Verma and Idnurm, 2013). The conundrum exists for the relationship between light, mating and the process of carotenogenesis in *Phycomyces*, as briefly discussed above. Light inhibits the process of mating and promotes asexual reproduction but at the same time it promotes the biosynthesis of β-carotene, which is a precursor for the pheromones required for mating. Carotenogenesis is light-induced or mating-induced: this effect on carotene biosynthesis is transduced through two separate pathways, as an additive effect is observed when the sexual stimulation is given in the presence of light (Almeida and Cerda-Olmedo, 2008).

For sexual interactions in Mucoralean fungi, the trisporoid pheromone system is the most explained communication system. The existing model for the regulation of pheromone biosynthesis suggests a feedback induction for the synthesis of pheromones (Werner et al., 2012). The trisporoids are derived from the cleavage products of beta-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

plus mating type and to trisporic acid in the minus mating type strain. After this the mutual exchange of the molecules between mating types has to happen for the further metabolism of trisporic acid.

The transcript level of *sexM* and *sexP* genes is increased in *Phycomyces* mating cultures compared to single cultures (Idnurm et al., 2008). In *Mucor* the level of *sexM* and *sexP* is higher in mating cultures or by treatment of trisporoids (Wetzel et al., 2012). The role of mating type genes and the effect of light on their transcript levels is not clear. When strains of opposite mating type are placed near each other, mating happens and pheromones must be produced as the prerequisite of mating. When mating cultures are in dark, mating can progress without inhibition, but it is repressed in light. Thus in such conditions when the pheromones or their precursors are present it is unclear why mating efficiency is decreased considerably. Two possible reasons could be that either the pheromones are degraded or that not enough pheromones are synthesized.

Mating type genes are one of the most obvious candidates for regulators of pheromone biosynthesis, especially as they are putative transcription factors. In this study we have investigated the basis of inhibition of mating by light in *Phycomyces*, using the genes with a role in light-sensing or mating as defined based on mutant phenotypes or linkage analysis.

Results

Genes required for mating are induced during crosses

A series of studies were conducted for examining timing and expression levels of *sexM*, *sexP* and *carS* genes. Figure 4d shows microscope images of crossing cultures at 24 hr, 48 hr, 72 hr and 96 hr to indicate the stages of the sexual cycle at various time points. As

we see at 48 hr, the formation of prozygosporangium was initiated **(Fig 4D).** Harvesting sufficient mycelia at 24 hr was technically challenging for subsequent RNA extractions. At 96 hr the mating process had proceeded as far as zygospore production, yielding plates and samples with a homogenous mix of different stages of mating. For time course studies, four 12 hr intervals between 36 hr and 72 hr were thus selected as an optimal, and subsequent studies after this were on 48 hr incubations.

In a time course of gene expression, strains were inoculated in isolation or as a mix between two mating types, and incubated in the dark for 36, 48, 60 or 72 hr. Mycelia were harvested in the dark, RNA extracted, and the transcript level of three genes measure by q-RT-PCR normalized to the actin gene. The primary observations were that *sexM*, *sexP* and *carS* were induced under mating conditions relative to single culture controls **(Fig. 4A, 4B and 4C).** 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, a consistent observation was that the levels of *sexM*, *sexP* and *carS* were 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. 4A and Fig4B).**

The analysis to calculate the fold change in the expression of genes was performed in two ways. In one way, the single cultures at each time point (36 hr, 48 hr, 60 h and 72 hr) was the base line and this informs about the relative expression of genes in crossed cultures compared to single culture at a particular time point. To look at the expression in a time

course, we also calculated the fold change by taking the 36 hr time point as base line for single culture and cross culture (treating the 36 hr as time zero) and then calculating the fold change in single culture and crossed culture at subsequent time points (48 hr, 60 hr, and 72 hr). This would inform us about the change in the expression in respective culture type (single and crossed) in the time course. This helps to understand the gene expression variation of over time.

We tested the expression of mating type genes *sexM* and *sexP* in mating and single cultures incubated in the dark. One important point is that in mating cultures only half of the material is from either parent. Thus, the representation of expression levels is likely half of what it would be when compared to single culture experiments. As expected the mating cultures have higher expression of both *sexM* and *sexP* when compared to respective single cultures at all the time points tested. We performed the time course experiment with 12 hr intervals to investigate if the expression changes over time. The *sexM* and *sexP* genes are highest at 48 hr and 60 hr time points **(Fig. 4B and 4A)**. When we compared the expression of these genes in single and mating cultures separately taking starting time points of respective cultures as baseline, the expression in single cultures at 48 hr, 60 hr, and 72 hr time points fluctuates or is slightly decreased compared to 36 hr time points. On the other hand in crossed cultures the expression at 48 hr, 60 hr, and 72 hr is either maintained at same level as at 36 hr time point or is up regulated.

Figure 4a shows the expression of the *sexP* gene. The expression of *sexP* is higher in crossed culture compared to single culture at all the time points, though the expression level were changing at different time points. This triggered us to look if the fluctuation in expression is because of change in expression in crossed culture or in single culture or both.

The graph on the right hand side of **Fig. 4A** shows that the expression of *sexP* also changes in single culture. The expression of *sexP* at 60 hrs is lower in single culture. At other time points it is not very different from 36 hrs. But in crossed cultures the fold change was either similar to 36 hrs or higher. Thus, part of the relative fold change in crossed culture is also because of background expression change in the single culture.

The **Fig. 4B** shows the fold change of the *sexM* gene. The left side shows the fold change of *sexM* in crossed culture compared to the single culture, with greatest differences at 48 hrs and 60 hrs. On the right hand side graph, the fold change of *sexM* over time course is plotted. The expression in single cultures goes down at 48 hrs and 60 hrs but come back up at 72 hrs with almost two fold compared to 36 hrs. Again as with *sexP* the crossed cultures show similar expression or the up regulation of *sexM* at different time points.

Figure 4C shows the expression of the *carS* gene. Unlike the mating type genes *sexP* and *sexM*, the *carS* gene is present in both mating type strains, so the fold change for *carS* was calculated compared to both (+) and (-) strains. On the left hand side the upper graph shows the expression of *carS* in crossed culture compared to (-) strain. The *carS* gene is upregulated in crossed cultures at all the time points. The lower graph shows the *carS* expression in crossed culture compared to plus single culture of plus mating type. The levels of *carS* are higher at 60 hr and 72 hrs. From the first look it seems that here the crossed culture shows a slight delay in up regulation. But when we look at the right hand side graph, where the fold change in each culture is plotted in time course manner taking 36 hr as baseline, the NRRL1555 (-) single culture shows the down regulation at 48 hrs and subsequent time points, but in the single culture of NRRL1554 (+) the down regulation of *carS* only starts at 60 hrs time point. The expression in crossed culture is higher at 60 hr and

72 hr time points only. At 48 hr it is not much different from 36 hrs. Thus for *carS* the relative fold change of crossed culture when compared to plus strain seems to be significantly changing only at 60 hr and 72 hr time points.

This analysis is important from the point of view of understanding the role of presence of mating type partner on the expression of *sexM*, *sexP* (the two mating genes) and *carS* (a gene with a role in pheromone biosynthesis). It was important to know if the transcript levels of these genes change during mating although these genes are always expressed at a baseline or if the presence of an opposite mating partner is a cue to increase their expression. This is efficient regulation for utilization of resources to induce sexual reproduction only when it is going to be possible.

Based on these experiments, for the subsequent studies a time point of 48 h after inoculating spores was used. The 48h time point was selected for the following reasons, first at this time point most of the plate is at the same stage and the later time points had a mix of different stages of sexual development, which might have confused the interpretations of results. Secondly, the sexual development progress towards more mature stages at 60 h and the expression of genes should precede the phenotype. Finally, because the 48h time points was most replicative in biological replicates

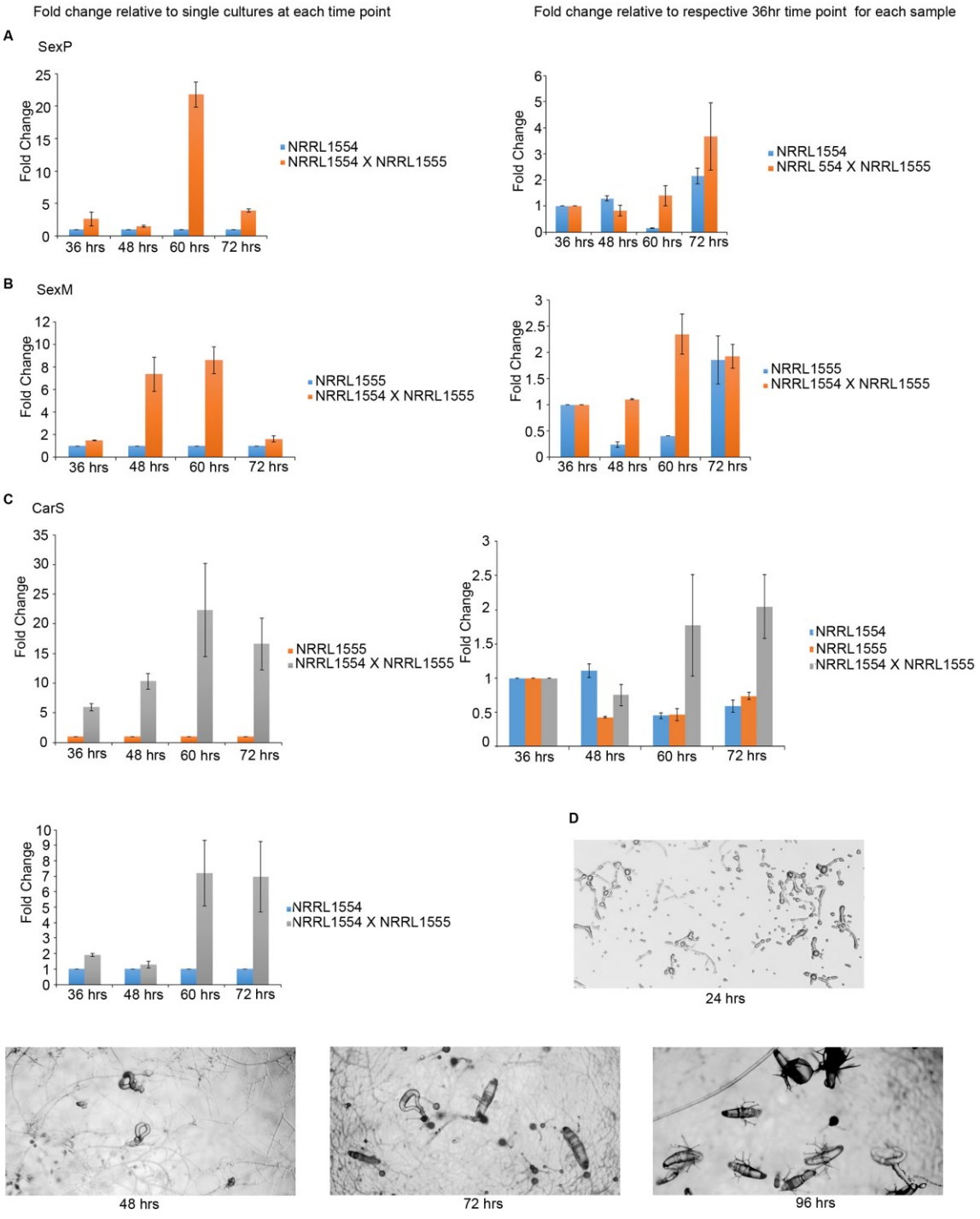

Fold change relative to single cultures at each time point
Fold change relative to respective 36hr time point for each sample
A
SexP
Fold Change
NRRL1554
NRRL1554 X NRRL1555
NRRL 554 X NRRL1555
36 hrs
48 hrs
60 hrs
72 hrs
B
SexM
NRRL1555
C
CarS
D
24 hrs
48 hrs
72 hrs
96 hrs

Fig. 4. Time course of gene expression. The expression profile of *sexP*, *sexM* and *carS* genes in single and crossed cultures of wild type strains. On left hand side the graphs show the fold change in crossed cultures compared to single cultures at different time points mentioned, here the single culture at each time point is the control. The right hand side graphs shows the fold change over time in single and crossed cultures, here the 36 hrs time point is the control and other time points are compared to this. The graphs are as *sexP* (A), *sexM* (B) and *carS* (C). Since the *carS* gene is present in both plus and minus strain, the comparison for single vs crossed culture is performed using both strains. On left hand side, the upper graph for *carS* is when (-) strain (NRRL1555) is control and the lower graph shows the expression profile when (+) strain (NRRL1554) is the control. Panel D shows stages of mating at various time points. The time points are represented on X-axis and the Y-axis represents the fold change.

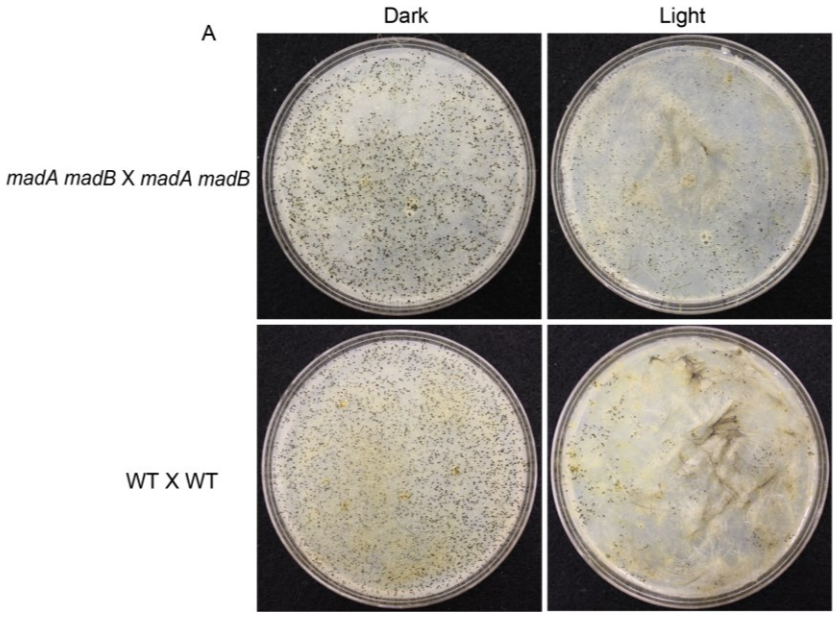
Light inhibits expression of mating genes and loss of the MadA-MadB complex alters the light responses

MadA and MadB are one of three and four members of the White Collar 1 and 2 class of proteins, respectively. 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 (Bergman et al., 1973; Cerda-Olmedo, 2001; Lopez-Diaz and Cerda-Olmedo, 1980). However, the available double mutants were constructed in non-isogenic backgrounds. One challenge in data interpretation is how to compare effects of a mutation in different strain background; 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 related to 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. Thus, we generated two double mutants 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 dark compared to light. To test if the light based inhibition of mating is transduced through the photoreceptor complex MadA-MadB, we made double *madA madB* mutants by two crosses, and then compared the mating in these strains in the light with that of the wild type. In *madA madB* mutants mating was not inhibited by light. We tested the formation of zygospores in light and dark conditions in wild type and mutant strains. The zygospore production in dark was similar in wild type and mutant strains but the mutant strains were able to produce the zygospores in higher number than wild type strains in light **(Fig. 5A)**.

To examine this phenotype at the genetic level we also tested the expression of *sexP*, *sexM* and *carS* genes in light and dark in wild type and mutant strains. In wild type crosses when mating was performed in the light and dark, a noted difference was observed for transcript levels. The wild type crossed cultures incubated in dark have higher expression of *sexP*, *sexM* and *carS* gene compared to incubation in light. The expression of *sexM*, *sexP* and *carS* was examined also in *madA madB* mutant strains. In the double mutant the transcript levels were similar between light and dark and comparable to the wild type dark cultures **(Fig. 5B)**. That is, the expression of *sexM*, *sexP* and *carS* for the double mutants was equal in light and dark. We observed that the effect of 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 geranyl geranyl pyrophosphate were also examined **(Fig. 6)**. Little difference in transcript level was observed for these genes, indicating that the role of light input under these mating conditions is not on the induction of β-carotene biosynthesis. This could also be a result that the mating conditions are inducing enough of the *carRA* and *carB* genes to mask any additional photoinduction.



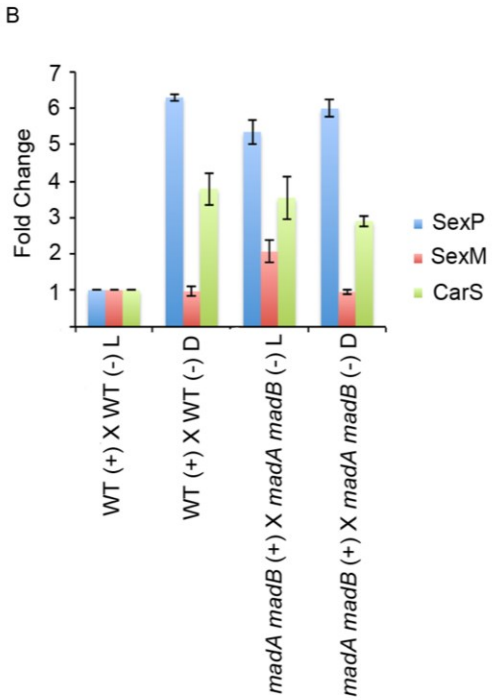


Fig. 5. Mating in wild type and *madA madB* mutants in light and dark. A. The zygospore formation in mutant and wild type crosses in light and dark. B. Expression of *sexP*, *sexM* and

carS in WT and *madA madB* mutants. The expression of *sexP*, *sexM* and *carS* in light and dark in crossed cultures of *madA madB* mutants compared to the wild type strains. The strains used in this experiment are A56 and NRRL1555 for WT (+) and (-), AB48 and AB36 for *madA madB* (+) and (-). The crosses are represented on X-axis and the Y-axis represents the fold change.

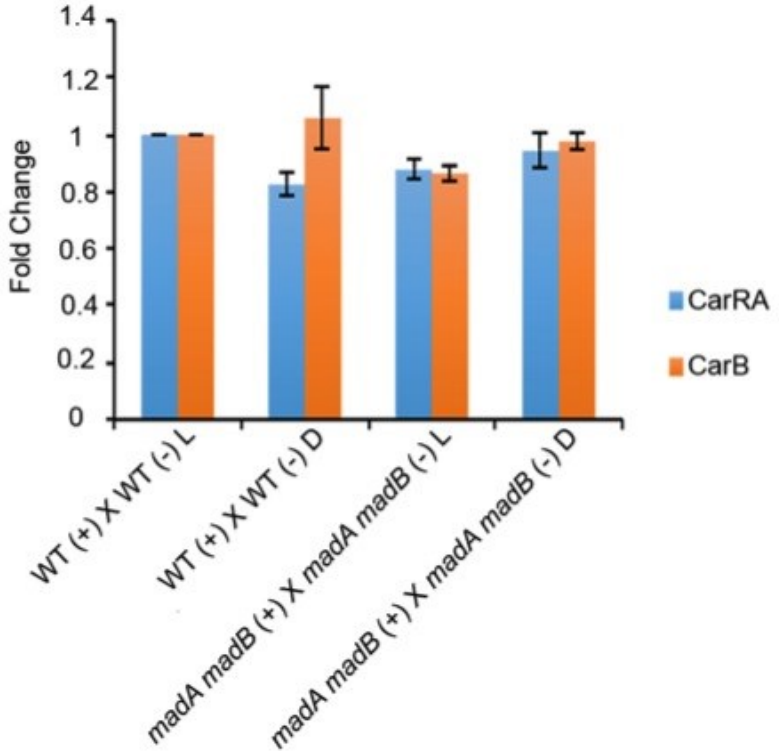


Fig. 6. Expression of *carRA* and *carB* genes in wild type and *madA madB* mutants in light and dark. The strains used in this experiment are A56 and NRRL1555 for WT (+) and (-), AB48 and AB36 for *madA madB* (+) and (-).

Light inhibition is lost in the (+) strains but still present in the (–) 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 mating was reduced approximately four fold. Examination of the single mutants in crosses (*madA* x *madA* or *madB* x *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. Lastly, we compared crosses in which one parent was wild type and the other contained the *madA*, *madB* or double *madA madB* mutants.

We performed crosses in ten combinations, which were incubated in light and dark. Curiously, we observed differences between the reciprocal crosses (switching the mating type of mutant strain). 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 **(Fig7A)**. So, wherever the plus partner is mutant for either *madA*, *madB* or for both genes, the zygospores in both dark and light are comparable in number. But in the reciprocal combination in which the (–) strain has the mutation a significant inhibition by light can be observed. When the (–) strain had the mutation, then light inhibited mating (Fig 7A and 7B). The graph (fig 7B) shows the average number of zygospores counted from three random areas of (1 cm^2 each) of the petri plate. In summary, when the photoreceptor complex is absent from the (+) strain, the light inhibition is bypassed but not when it is absent from the (–) strain. This suggests that the inhibition of mating is mainly caused by the plus strain, possibly by inhibiting transcription of the *sexP* gene.

The basis behind the differences in zygospore numbers observed in the reciprocal crosses was examined at the transcript level. We performed quantitative PCR to investigate the transcript levels of *sexP* and s*exM* genes. We compared all 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 7C).** These results further strengthen a central role of *sexP* targeted by the MadA-MadB photoreceptor complex to inhibit mating in light.

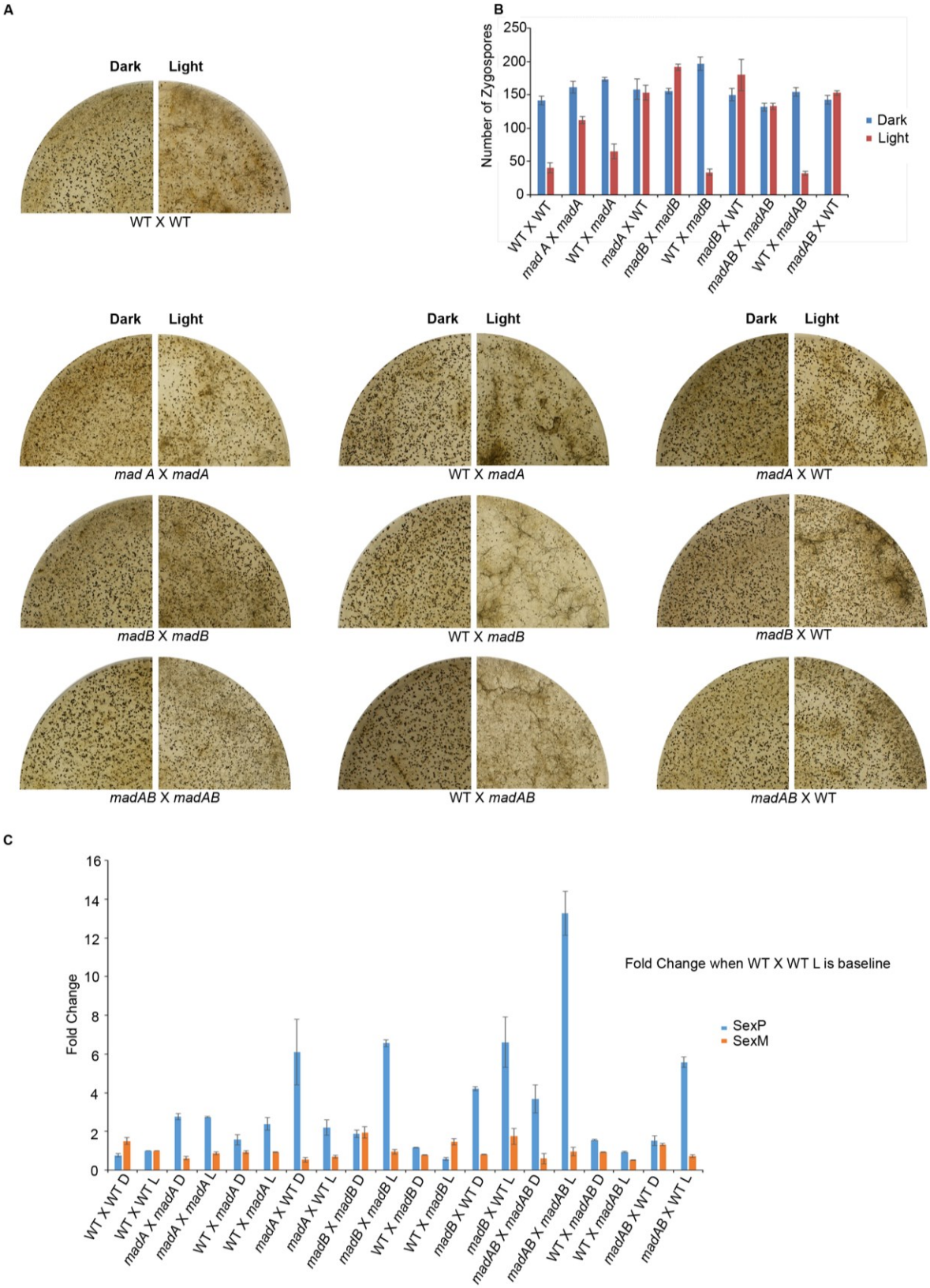

A
Dark
Light
WT X WT
B
Number of Zygospores
250
200
150
100
50
0
Dark
Light
WT X WT
mad A X madA
WT X madA
madA X WT
madB X madB
WT X madB
madB X WT
madAB X madAB
WT X madAB
madAB X WT
Dark
Light
mad A X madA
WT X madA
madA X WT
madB X madB
WT X madB
madB X WT
madAB X madAB
WT X madAB
madAB X WT
C
Fold Change
16
14
12
10
8
6
4
2
0
Fold Change when WT X WT L is baseline
SexP
SexM
WT X WT D
WT X WT L
madA X madA D
madA X madA L
WT X madA D
WT X madA L
madA X WT D
madA X WT L
madB X madB D
madB X madB L
WT X madB D
WT X madB L
madB X WT D
madB X WT L
madAB X madAB D
madAB X madAB L
WT X madAB D
WT X madAB L
madAB X WT D
madAB X WT L

Fig. 7. Mating in light/dark in various crosses. Phenotypes of mating in light/dark in various crosses combination using of wild type, *madA, madB* and *madA madB* mutant strains. The A panel shows the pictures of zygospore formation in crosses incubated in light or dark. The graph in B shows the average number of zygospores formed in various crosses performed in A. The crosses are represented on X-axis and the Y-axis represents the number of zygospores. The graph in C panel shows the expression profile of *sexM* and *sexP* in the crosses in light and dark. The strains used in this experiment are A56 and NRRL1555 for 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 X-axis and the Y-axis represents the fold change.

One level of interaction between light and mating is 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* X *carS* or *carB* X *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 used was NRRL1554, which is not isogenic with NRRL1555, for comparison to *carS* mutants as the plus mutant of *carS* is in the NRRL1554 background. For the *carS* mutant we used four combinations, WT (+) X WT (-), *carS* (+) X *carS* (-), WT (+) X *carS* (-) and *carS* (+) X WT (-) 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 functional cleavage enzyme to process beta carotene.

We compared the expression of *sexP*, *sexM* and *carS* in all four combinations against the cross where both partners are *carS* mutants. In dark *sexP* and *sexM* are upregulated in WT (+) X WT (-) compared to *carS* (+) X *carS* (-) (i.e. M1 x S324), which is acting as baseline for these comparisons **(Fig. 8A)**. In WT (+) X *carS* (-), the *sexP* is 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 thus 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* (+) X WT (-) (i.e. MI X NRRL1555), we expected that *sexP* should be expressed as wild type, as the (-) strain (WT) 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 s*exM*. The *carS* transcript levels were higher in this cross which indicates enough substrate for pheromone production and that the expected pheromone function could be executed.

In the light incubation only *sexM* expression is significantly different in WT (+) X WT (-) and *carS* (+) X WT (-), compared to the control [*carS* (+) x *carS* (-)]. The expression of *sexP* and *carS* are not significantly different from the controls. In WT (+) X WT (-), the result suggests that although the pheromones or other mating cues are available to help in the induction, light inhibits the expression of *sexM* and *sexP* (Fig. 8).

One confounding aspect to interpreting the *carS* crosses is the persistent presence 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. Mutation of this gene blocks the synthesis of β-carotene and renders those strains white (Fig. 3 of pathway in chapter 1). In the *carB* x *carB* crosses, when the samples of dark are compared against light, *sexM* is at slightly lower levels but *sexP* is similar to controls **(Fig. 8B)**.

This 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 more here due to the lack of pheromones, which suggests that pheromone are more important for the expression of *sexM* compared to *sexP*.

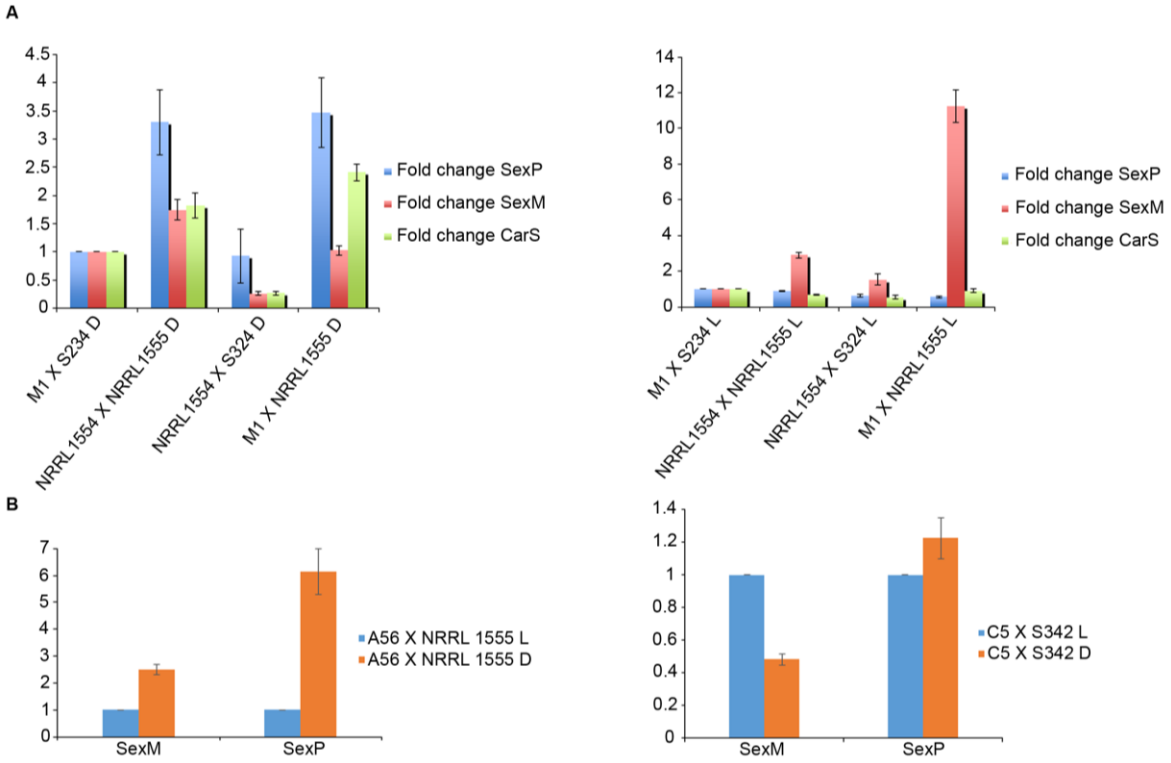


Fig. 8. The expression of *sexP*, *sexM* and *carS* genes in light and dark in crossed cultures of *car* mutants and wild type. A. The expression of *sexP*, *sexM* and *carS* genes in light and dark in crossed cultures of *carS* mutants and wild type. The left side graph shows the expression in dark incubation, the right side graph shows the expression in light incubation. The crosses are represented on X-axis and the Y-axis represents the fold change. B. The expression of *sexP* and *sexM* in crossed cultures of WT and *carB* mutants in light and dark. The genes are represented on X-axis and the Y-axis represents the fold change.

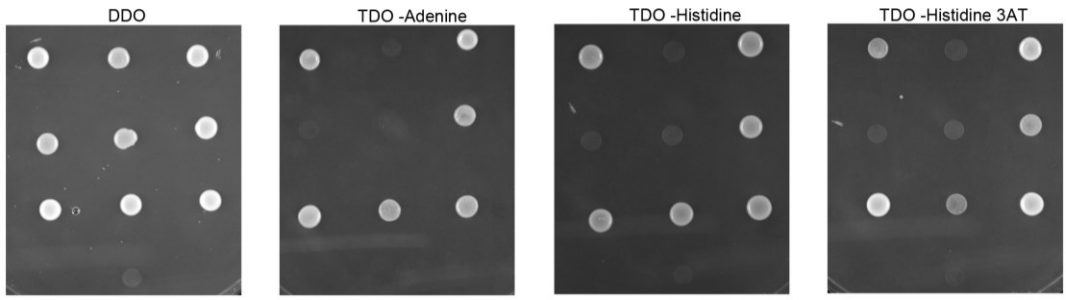
SexM and SexP likely play independent roles in sexual reproduction

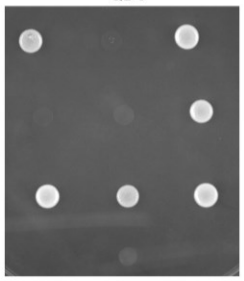
In some ascomycetes and basidiomycetes, the transcription factors within the *MAT* locus play co-ordinated roles during sexual development, including as heterodimeric complexes. Our data thus far on the expression and phenotypic effects suggested that the SexM and SexP proteins would not do this. To 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 physical interactions in the yeast two hybrid system.

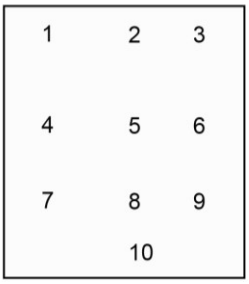
The yeast two-hybrid assays suggest that no physical interaction between SexP and SexM is occurring **(Fig. 9).** When the AD and BD fused clones of SexP and SexM in both combinations (no 6 and 7) were co-transformed into *S. cerevisiae*, growth was observed on selection media (TDO and QDO) but similar growth was also observed when the SexP and SexM clones in BD vector were co transformed with empty AD vector (No 1 and 3). This suggests that the growth on selection media is due to the trans activation by SexP and SexM fused BD vectors. When the SexM and SexP clones in both AD and BD were co transformed (No 8 and 9) they were able to replicate on selection media, but no higher than the background of transactivation. The transactivation is a piece of evidence that SexM and SexP could act as transcription factors, which is 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 heterologous expression in yeast. SexM clearly localized to the nucleus. SexP has a different expression pattern with slight overlap in mitochondrial localization, extra nuclear and extra mitochondrial expression, which also

included formed aggregate structures **(Fig. 10).** This is similar to what was observed when *M. circillenoides* 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 also localize exclusive of each other in a heterologous system.







No.	pGAD	pGBD
1	E. V.	SexM
2	SexM	E. V.
3	E. V.	SexP
4	SexP	E. V.
5	E. V.	E. V.
6	SexP	SexM
7	SexM	SexP
8	SexM	SexM
9	SexP	SexP
10	–	–

Fig. 9. SexM and SexP act in non-cooperative manner. Dissimilar properties of SexM and SexP suggest they act in a non-cooperative manner to control mating. The yeast two-hybrid assays performed in various combinations (1-10) as mention in the list. Yeast two hybrid investigation into SexM and SexP physical interactions, cells were plated onto double dropout medium (DDO, -leucine -tryptophan), triple dropout media (TDO, -leucine -tryptophan, -adenine/-histidine) and for one set 3-amino triazole was added (TDO + 3AT), and on quadruple dropout (QDO, -leucine, -tryptophan, -adenine, -histidine). The details of the strains are provided in the table.

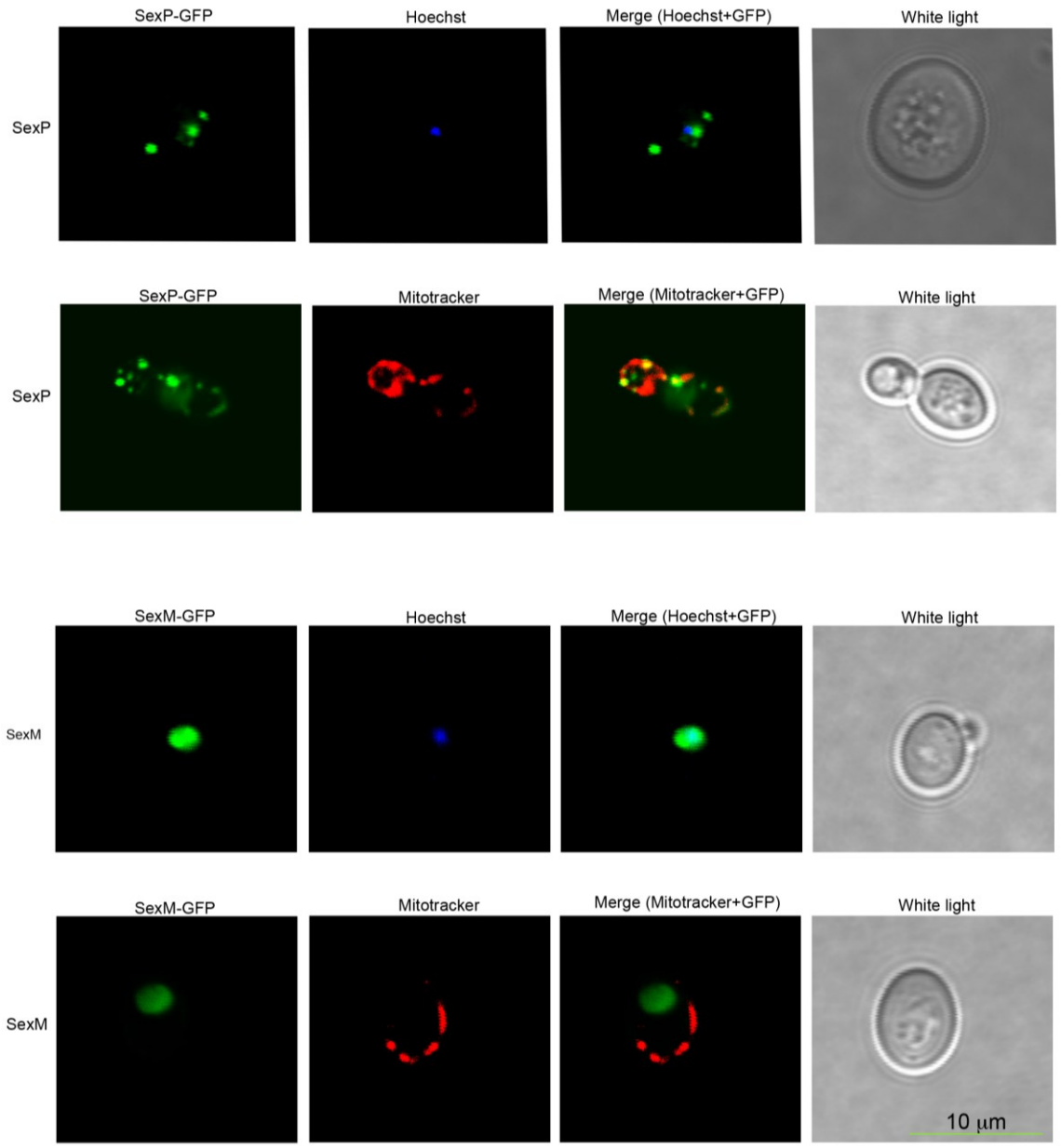


Fig. 10. Subcellular localization of *Phycomyces* SexP and SexM as GFP fusions expressed in *S. cerevisiae*. The top two panels show the localization of SexP and next two panels show the localization of SexM. For both SexP and SexM, the first panel shows the SexP-GFP compared to nucleus (Hoechst stained), the second panel shows the SexP-GFP compared to mitochondria (MitoTracker).

UV resistance is also mediated through the MadA-MadB complex

The White Collar complex has been implicated in protection against UV light. Light induces the expression of the *UVE1* gene that encodes a DNA repair endonuclease in *C. neoformans* (Verma and Idnurm, 2013). In that study, the authors showed by northern blot analysis that increase in the *Phycomyces UVE1* transcript in response to light is regulated through the MadA-MadB complex, as in the mutant background no expression of *UVE1* was seen in the light. Based on this report it was expected that in *Phycomyces* the MadA-MadB complex may also regulate UV sensitive phenotype as observed in *Cryptococcus*. Hence we tested the UV-induced damage phenotype in wild type and *madA*, *madB* and double mutants (*madA madB*) of both plus and minus mating type background **(Fig. 11)**. With a UV dose of 120 J/m^2 the wild type strain recovered after UV stress. The mutant strains showed less recovery but visible difference in recovery was not seen in (+) and (-) strains. These results suggest that the MadA-MadB complex is involved in coping with UV induced stress, which may be through the DNA repair enzyme Uve1.

Regulation of mating by light and pheromones

Based on our findings and previous research a model explaining the effects of light on the mating can be developed **(Fig. 12)**. 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. The darkness removes the inhibitory effect of light on *sexP*. This inhibition is transduced through the MadA-MadB complex thus the *madA*, *madB* or *madA madB* mutants were able to circumvent the inhibitory effect of light on mating. This

bypass is only possible if the (+) mating partner is mutant and suggests that the effect of light is only transduced through the (+) mating partner.

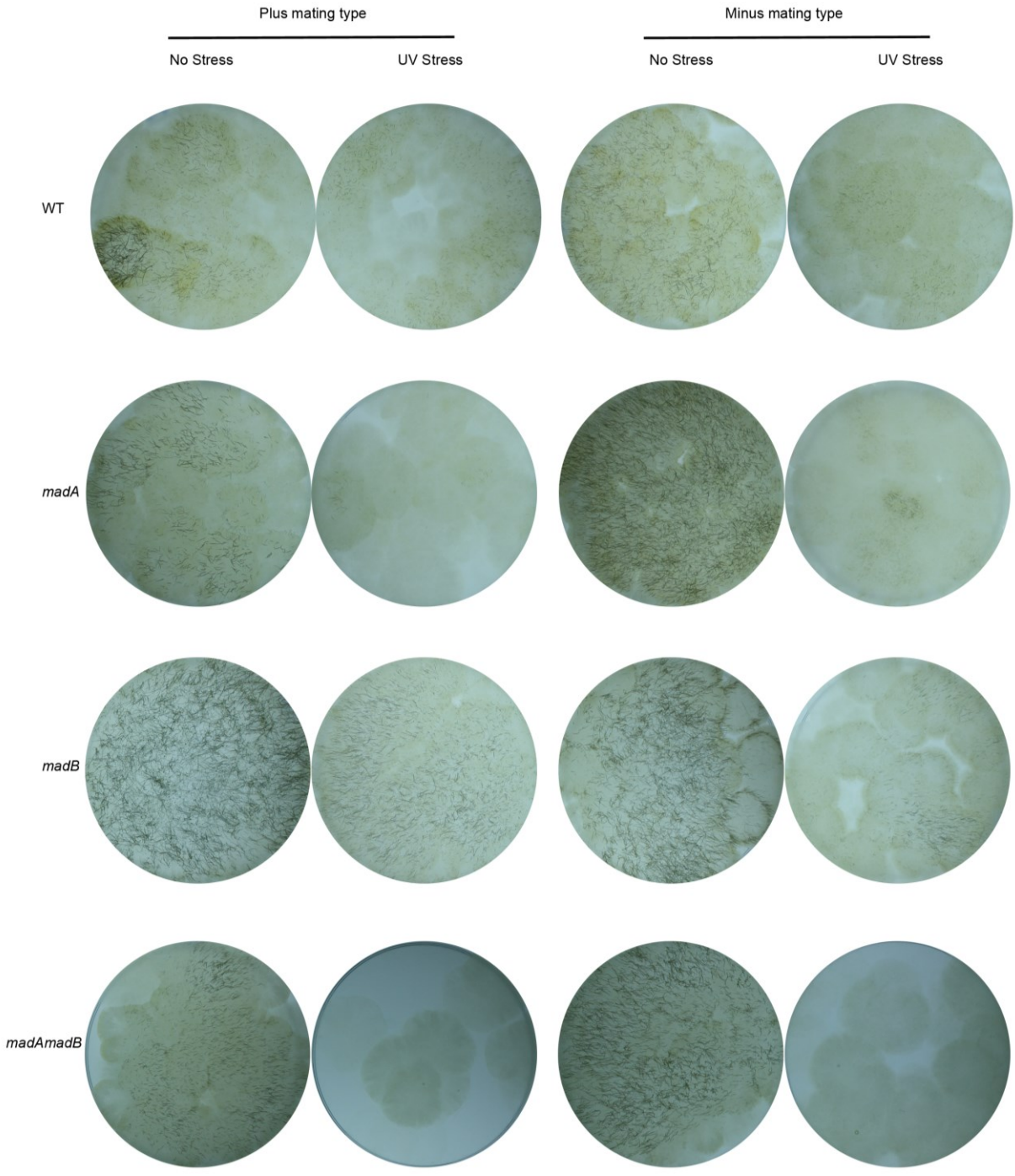

Plus mating type
Minus mating type
No Stress
UV Stress
No Stress
UV Stress
WT
madA
madB
madAmadB

Fig. 11. The UV stress phenotype of wild type and mutants. The left two panes are the strains of (+) mating type. The right two panels are for the strains of (-) mating type. The strains used in this experiment are A56 and NRRL1555 for WT (+) and (-), CAB26 and C47 for *madA* (+) and (-), C5A and C109 for *madB* (+) and (-), CAB13 and CAB22 for *madA madB* (+) and (-).

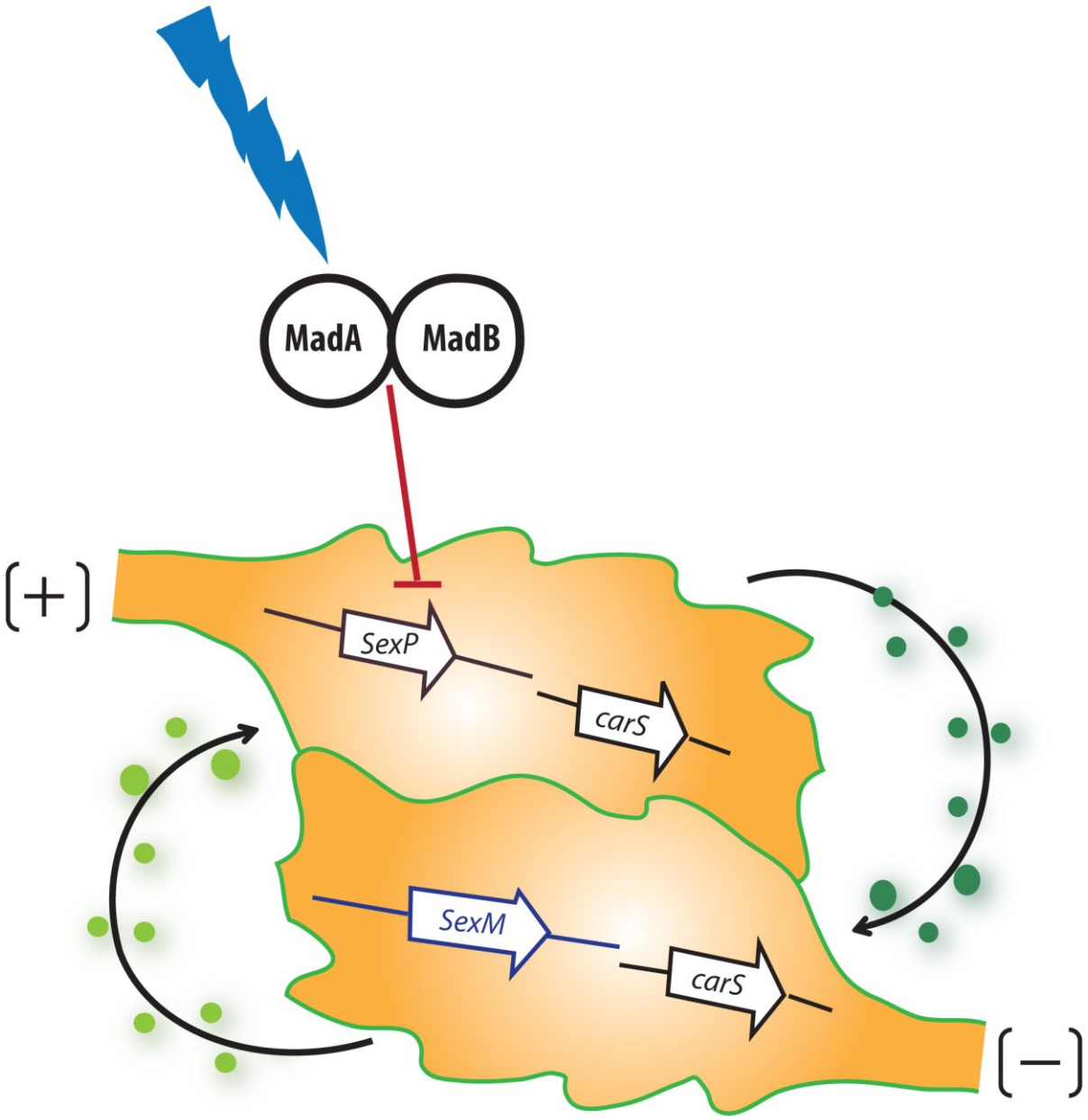


Fig. 12. Model for gene regulation of *sexP* and *sexM* by light and pheromones from mating partners. The light green circles represent the pheromones from (-) partner and dark green circles represent the pheromone molecules form (+) partner. The MadA-MadB photoreceptor complex inhibits the expression of *sexP*.

Discussion

MadA and MadB are known to regulate mycelial and other asexual processes in *Phycomyces* that are affected by light. Although light was reported to inhibit mating (Yamazaki, 1996), the basis for this inhibition was unknown. Our results show that the MadA-MadB complex is also responsible for this inhibition and that both pheromone biosynthesis and mating type locus genes are regulated by MadA and MadB. However, in contrast to the asexual responses, the effects of the light on mating occur via only one of the two sexes.

Identifying the genes required for mating in *Phycomyces* has been a long term, and sometimes heroic effort. In one classic study, Sutter (1975) characterized the *car* mutants affected in β-carotene biosynthesis to reveal two things: first, the *car* genes are important for mating, and second that each mating type synthesizes chemical molecules to induce the formation of zygophores in the opposite mating partner. From finding of point mutation to identification of *carS* required more than 35 years (Sutter, 1975; Tagua et al., 2012). Another study by Sutter et al. (1996) performed chemical mutagenesis and isolated the first mating type specific mutant. The candidate gene, though present in both mating types, is required only in (-) mating type to convert the pheromone from the (+) mating type partner. At present, that mutant still remains unknown. The current model suggests that the two mating types produce different precursors, 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 (-) strain are sent to (+) strain to be converted to active pheromones and the methyl trisporates from (+) striain are processed by (-) to active pheromones, which are trisporic acids in chemical nature (Sutter et al., 1996). The gene *TSP1* encoding for the

enzyme 4-dihrdromethyl trisporate dehydrogenase was identified and cloned and is involved in the biosynthesis of pheromones in *M. mucedo*. Later, the *TSP2* gene was also identified and encodes for another enzyme 4-dihyrdrotrisporin dehydrogenase (Schimek et al., 2005; Wetzel et al., 2009). However, even after identification of the respective genes for these enzymes, it is not confirmed 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 (Ma et al., 2009). The *TSP1* gene is transcribed and also translated in both mating type strains, but the enzymatic activity is only observed in sexually stimulated (-) strain (Schimek et al., 2005). This indicates that at least in *Mucor* that post-translation regulation is also involved in pheromone biosynthesis pathway.

The majority of the research on the photobiology of *Phycomyces* has focused on the properties of the sporangiophores, which are large long cylinders that ultimately terminate in the production of a sphere containing the asexual reproductive spores. Research into these structures revealed several features, including a low and high light sensing system, photoadaptation, and ten different genes defined by mutant isolation and analysis that impacted on the tropism of the structures to blue light (Bergman et al., 1973; Cerda-Olmedo, 2001). Two of these ten genes (*madA* and *madB*) have been identified, and they encode homologs of the WC-1 and WC-2 protein that are the blue light sensors characterized from *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. Most fungi are capable of sensing light, and if

they do then their genomes invariantly contain a single copy of each of these two homologs. However, *Phycomyces* and related Mucoromycotina species have undergone either whole genome or segmental duplication events, such that they have three or more copies of each of these homologs (Fig. 2). 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 *madA* homologs after duplication. One copy is specialized for sensing light to trigger carotene synthesis and the other for phototropism (Idnurm et al., 2010; Silva et al., 2006). The third copy has a unique function related to carotene biosynthesis (Silva et al., 2008)

In addition to the well-studied phototropic response, mycelial responses are also known for *Phycomyces*. These include the induction of formation of the sporangiophores and β-carotene biosynthesis in response to light, both also controlled by the MadA and MadB proteins. Light inhibits the mating process. Analysis of action spectra for this inhibition pointed to a blue light, presumably flavin-based, effect that could well be controlled by one of the multiple WC homologs in the genome (Yamazaki, 1996), However, this observation had not been further investigated, so the genes involved were unknown.

A curious and somewhat paradoxical effect of light on mating is that light inhibits mating yet it also induces the production of β-carotene. While ascomycetes and basidiomycetes use peptide-based pheromones, *Phycomyces* and its relatives use derivatives of β-carotene to produce their sexual pheromones. To date, one gene that controls the initiation of this process has been identified in *Phycomyces*, 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 in the

Phycomyces genome, AcaA, cleaves the product of CarS at least in vitro (Medina et al., 2011) . The *carS* mutant is infertile, and also accumulates high levels of β-carotene. Its homolog has also been characterized from *Blakeslea trispora* (Burmester et al., 2007; Sahadevan et al., 2013).

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 single vs. double mutants is that we tested mating under a single light intensity, and under higher intensities 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 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 7)**. For *Phycomyces* 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.

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; Kamper et al., 1995). In the case of *Phycomyces*, all the evidence presented here, which includes transcript regulation independent of each other (**Fig. 4)**, no evidence for physical interactions **(Fig. 9)**, differences in subcellular localization profiles **(Fig. 10)**, and different photoresponses depending on the strain, suggest that at least in the early stages of mating these genes have cell-type specific functions.

In the basidiomycete *Cryptococcus neoformans* sexual development is inhibited by light as mediated by the Bwc1 and Bwc2 homologs of MadA and MadB photoreceptors (Idnurm and Heitman, 2005). The genes in the *Phycomyces madA* and *madB* phototropic mutants have been identified as homologues of white collar 1 (*wc-1*) and white collar 2 (*wc-2*) genes, respectively, which are components of the conserved blue-UV light photoreceptor complex (Idnurm et al., 2006). Light inhibits mating in *Cryptococcus* and this inhibition can be bypassed by mutation of the *wc-1* gene in either mating partner. In *Cryptococcus*, only one of the mating partners needs to be a *wc-1* mutant to circumvent the light-based repression of the cell fusion stages of mating, as it can be either mating type **a** or α.

A commitment of resources is required for pheromone biosynthesis and therefore it is important to ensure the necessary conditions are available for mating. Moreover, sexual reproduction is costly compared to asexual reproduction. Further, because of the pheromones produced during mating are sensed by related Mucorales, one risk is a futile commitment to mating and a worse risk is parasitism by the Mucorales species that attack using a mating-based strategy (Schultze et al., 2005). Thus, when the choice is available the organism will opt for sexual growth only when required and the conditions are less favorable for asexual

growth. We propose that SexM and SexP do not physically interact and the possibility of having a common regulatory target is unlikely. However, this supposition is based in part of interpretation of interactions assessed by experiments performed in yeast *S. cerevisiae*. In the interaction studies, both SexM and SexP induced a transactivation response when bound to the Gal4 DNA binding domain. No additional effect was observed in the presence of the opposite Sex protein. If the interaction were weak, then it would be difficult to detect above the background of transactivation.

We also observed that the asexual growth of *madA madB* mutants and the wild type strain is similar. Moreover in the previous literature no difference sex/mating type has been in mentioned. For example, while measuring the phototropism of mutants and wild type if there was a difference in growth, then the comparison for the bending of mycelia towards light would not be comparable.

In summary, these findings demonstrate that the (+) strain is responsible for sensing light and integrating that signal in terms of the commitment towards sexual or asexual reproduction in *Phycomyces*. The investigation of which genes are regulated by light to inhibit mating provides clear evidence that the *madA* and *madB* genes are involved in this process. 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

CHAPTER 4

Uniparental Mitochondrial Inheritance in *Phycomyces*

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Introduction

The uniparental inheritance (UPI) of mitochondria is common among eukaryotes as an outcome in progeny after sexual reproduction. For instance, with very rare exceptions, all people have the same mitochondrial genotype as their mother. The underlying basis by which the sexes of the parents control this form of inheritance, that is non-Mendelian in pattern, is not understood. Two major factors have complicated the understanding of the role of sex-specific genes in the UPI phenomenon: in many cases (i) fusion occurs between cells of unequal size or (ii) mating requires a large region of the genome or chromosome that includes genes unrelated to sex determination. *Phycomyces* has a simple mating type locus encoding only one high-mobility group (HMG) domain protein, and mating occurs by fusion of isogamous cells, thus providing a model system to investigate the links between sex-determination and UPI without the two limitations mentioned above.

The processes accounting for this pattern of inheritance include the selective degradation of organelles of one genotype and the disproportionate contributions of the cytoplasmic contents, together with an underlying selective benefit of homoplasmy, whereby cells house a single mitochondrial genotype (Birky, 2001; Sato and Sato, 2013). In *Saccharomyces cerevisiae* mitochondrial inheritance is affected by the position of the bud on the zygote (Strausberg and Perlman, 1978), which recently was associated with septin distribution in the zygote (Tartakoff et al., 2013). These findings explain why some progeny

exhibit UPI for mitochondria, but the regulation for inheritance of only one type of mitochondria to daughter cell from the mother cell is yet to be studied. Plants provide many examples that contradict the hypothesis that cell size is the sole governing factor for uniparental inheritance. *Biota orientalis* inherits both chloroplasts and mitochondria from the sperm cell, which is much smaller than egg cell. In this plant the sperm cell enters the archegonium with its nucleus tightly surrounded by its cytoplasm, where it is able to make the sperm cell cytoplasm exclusively enclosing the zygote nucleus. The further process of development leads to the formation of cell wall surrounding the pro-embryo nuclei enclosing paternal cytoplasm with its organelles. The female cytoplasm is eventually degenerated during the developmental process (Mogensen, 1996).

There are other hypotheses to explain uniparental inheritance of the mitochondria. One is a dilution hypothesis, according to which the paternal mitochondria exists in such a low concentration that it is dilute in comparison to the maternal mitochondrial load and cytoplasmic contents explanations have strong support. However, a more widely accepted hypothesis is the selective elimination of the paternal mtDNA, which leaves only maternal mtDNA. There are several other mechanisms that are proposed for the selective elimination of paternal mitochondria.

The first mechanism is ubiquitin-mediated selective degradation of paternal mitochondrial. This mechanism is more common in mammals. In some of the mammalian species either in the fertilized egg cell or in the sperm itself the paternal mitochondria is ubiquitinated which in turn is subjected to proteasome-mediated degradation (Sutovsky et al., 1999).

The second mechanism for selective degradation is based on the phenomenon of

autophagy. In *C. elegans* the paternal mitochondria are engulfed by autophagosomes, targeting them to lysosomes where they are destroyed as part of the overall process of autophagy, or to be more precise mitophagy (Sato and Sato, 2011). Al Rawi et al (2012) have shown in *C. elegans* that the autophagosome markers LGG1 and LGG2 start staining for autophagosome formation immediately post-fertilization. These autophagosomes colocalize with the sperm mitochondrial structure post-fertilization. At the end of first division cycles these mitochondria disappear suggesting a degradation mechanism. Using RNAi knockdown experiments for LGG1 and LGG2, which interferes with autophagosome formation, they have also shown enhanced stabilization of the cytoplasmic components including paternal mitochondria (Sato and Sato, 2011). Autophagy-dependent mitochondrial degradation mechanisms are also suggested for mice (Al Rawi et al., 2012).

Other mechanisms for UPI are based on the destruction of mtDNA. For example in *Drosophila*, the paternal mitochondrial DNA within the sperm cell is degraded by two mechanisms. One is nuclease-dependent where the responsible nuclease is called EndoG. The remaining mtDNA is further eliminated by a special mechanism wherein sperm investment cones, which are actin containing structures, sweep the mtDNA in to a waste bag structure which is further eliminated (DeLuca and O'Farrell, 2012). A nuclease-dependent mtDNA mechanism is also suggested for *Physarum polycephalum*, which has equal size gametes like *Phycomyces* (Moriyama and Kawano, 2003). Hence, a number of mechanisms contribute to the uniparental inheritance of mitochondria.

In most cases, the maternal parent contributes the mitochondria into the zygote. However, whether or not the genes on sex chromosomes or in mating-type loci that specifically determine the sex of the parent are directly required for UPI is untested. This is

because it has not been possible to uncouple the effects of differing cell sizes (anisogamy) or the numerous genes present on sex chromosomes from their potential contribution to UPI. To answer this fundamental question requires an analysis in an organism with equally sized gametes (isogamy) and a simple single-gene sex-determining system.

Members of the fungal kingdom may undergo a sexual cycle that involves the fusion of equally-sized cells and whose sex types are determined by a small region of the genome known as the mating type (*MAT*) locus. However, many of these species, like the model ascomycete yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* (Dujon et al., 1974; Seitz-Mayr et al., 1978; Strausberg and Perlman, 1978; Wolf et al., 1978), inherit the mitochondrial genotypes from either parent. Similarly, some basidiomycete mushroom species initiate their sexual cycle through the fusion of equally-sized cells, and depending on the species they can exhibit the inheritance of the mitochondrial genome from just one or either parent. In species with uniparental inheritance, the complex genetic makeup of their mating type systems have prevented implicating sex-determining genes directly, with some exceptions that are discussed below (Basse, 2010; Griffiths, 1996). *Phycomyces*, as a member of the subphylum Mucoromycotina, is a distant relative of the ascomycete and basidiomycete species. Mating involves the fusion of two identically sized gametangium cells, hence the terms "(+)" (plus) and "(−)" (minus) are used to designate the sex or mating type of strains. Sex determination is governed by a small genetic locus, which in *Phycomyces* contains a single gene for each allele, either *sexM* or *sexP* (Idnurm et al., 2008). The alleles share no DNA sequence similarity, although both SexM and SexP proteins contain a high-mobility group (HMG) domain found in transcription factors. This scenario resembles the *SOX3*/*SRY* HMG-domain proteins encoded by the X and Y chromosomes of humans and other mammals but entails a

small (<6-kb) locus encoding one protein rather than a sex chromosome.

The expectation was that *Phycomyces* would exhibit biparental inheritance of its mitochondrial genome, as seen in other fungi that undergo fusion of isogamete-like cells.

RESULTS

A genetic map of *Phycomyces* was recently created using nuclear molecular markers in progeny after crossing two wild type parents (Chaudhary et al., 2013). The mitochondrial genome was not included in that study because the genome sequencing strategy of the Joint Genome Institute had removed the mtDNA sequence from the genome assembly and browser. Revised genome sequences with the missing sequences became available. Oligonucleotide primers were designed to amplify a part of the mtDNA that is variable between strains (Fig. 13A and B) and used on the 121 progeny from the cross used to make the genetic map. The progeny were derived from independent meiotic reduction events, because each zygospore forms independently of the others, and a single strain was isolated from the zygospores germinated. The difference in PCR-restriction fragment length polymorphism (RFLP) patterns between the strains was used to track the source of the mitochondrial genome in the progeny. Rather than exhibiting biparental inheritance, all 121 progeny had inherited their mitochondrial genome from one parent (Fig. 13C). To ensure that this result was not a peculiarity of the molecular marker employed, an additional polymorphic site in the mitochondrial genome was identified. This region was amplified and sequenced from 12 randomly selected progeny: all had inherited the allele from the (+) parent (Fig.14). This result suggests that there is transmission of the full mitochondrial genome from the (+) parent into the progeny.

Segregation of the mitochondrial genome in progeny from a series of crosses shows

that UPI is consistently observed in *Phycomyces* (Fig. 13C and Table 3). In all crosses UPI is from the (+) parent, including in crosses between a selection of different wild type isolates. These wild type isolates are all unique and North American in origin (from Canada for UBC21 and UBC33 and from the United States for the others), and their isolation dates span over a century, from Albert Blakeslee's collection from the turn of the 20th century to an isolate from South Carolina collected in 2010.

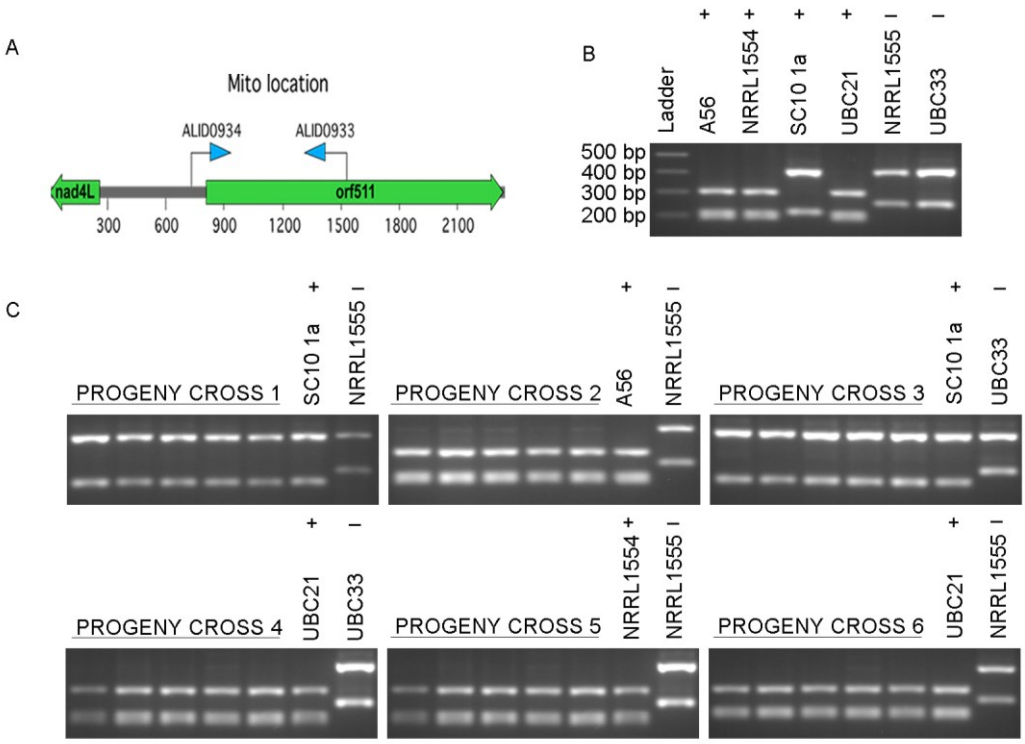

Fig. 13 UPI of mitochondria occurs in *Phycomyces*. (A) Positions of the oligonucleotide primers used to amplify a variable part of the mitochondrial genome. (B) Agarose gel showing the sizes of the PCR products of an mtDNA fragment cut with TaqαI restriction enzyme from the parental strains. (C) Gels of the PCR products of the mtDNA fragment cut with TaqαI, from parents and five representative progeny from eight crosses (Table 3).

Adapted from (Shakya and Idnurm, 2014)

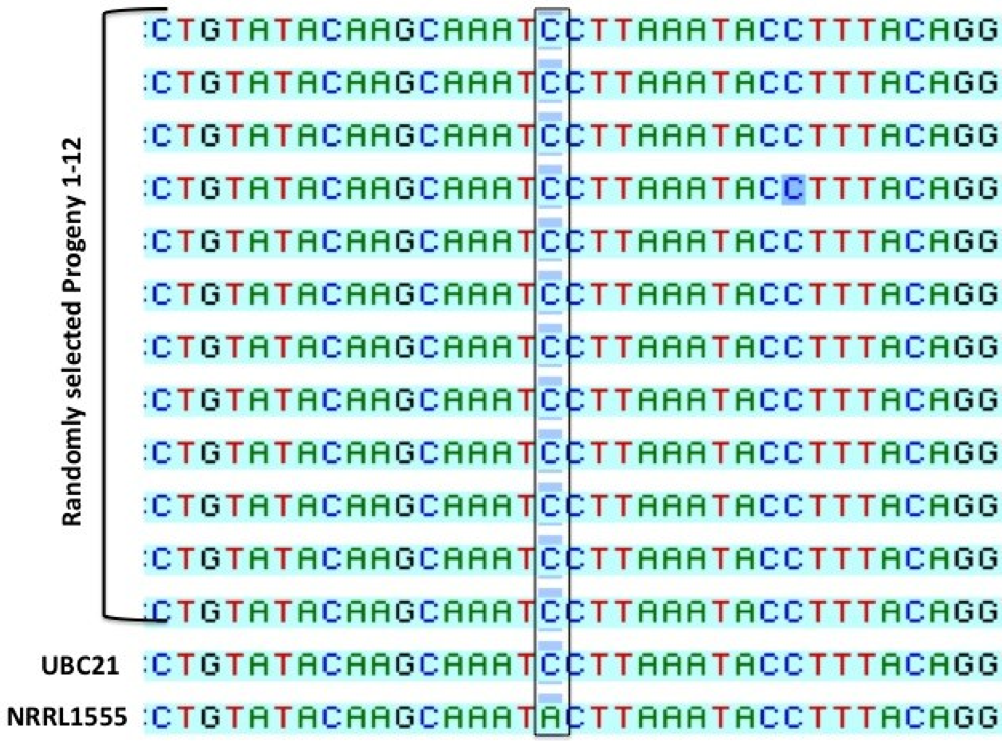

Fig. 14. Second marker for mitochondrial genotyping show UPI in *Phycomyces*. Two parent strains and randomly selected 12 progeny were genotyped for mitochondrial polymorphism between the *atp9* and *trnG* genes. Alignment of the sequencing traces for the 12 progeny from a cross between UBC21 x NRRL1555, and the two parents, with the polymorphic nucleotide boxed.

One model for this pattern of recombination is that the mitochondrial genomes carry a "drive" element that enforces their inheritance in the next generation. To test this, reciprocal crosses were carried out between strains in which the mtDNA was switched into the opposite mating type background, as outlined in Fig. 15A. UPI of the mtDNA from the (+) parent was still observed in these crosses independently of the mitochondrial genotype, which establishes that the mitochondrial genome is not contributing to UPI (Fig. 15B, crosses 7 and 8 of Table 3).

Strain A56 is isogenic with strain NRRL1555 except for its *sex* allele, having been made from 11 backcrosses to introgress the sex locus from strain UBC21 into the NRRL1555 genetic background (Alvarez and Eslava, 1983). That is, strains A56 and NRRL1555 are considered identical at the DNA level with the exception of the *sex* locus (and mitochondrial genotype, which is derived from UBC21; Fig. 13B). The cross between A56 and NRRL1555 also produced progeny in which all carried the mtDNA from the (+) parent A56 **(number 6, Table 3, Fig 13C)**. Hence, because crosses 7 and 8 **(Table 3, Fig. 15B)** eliminate the possibility of a "drive" element in the mitochondria and that the difference between strains A56 and NRRL1555 is solely the *sex* locus, this indicates that the control of UPI lies with the *sex* locus genes to the exclusion of other elements in the genome.

The meiotic reduction process in *Phycomyces* is not always perfect, with the emergence of heterokaryons that can be easily identified by their morphology if they carry the two alleles of the *sex* locus. Three such strains were examined in the progeny sets (two from UBC21 x NRRL1555 and one from NRRL1554 x NRRL1555), and all had inherited the (+) parent mitochondria. This indicates that the selection process occurs with fidelity even if nuclear fusion or chromosome disjunction does not and that UPI is initiated early in

the mating process. Since both mating types were present in these heterokaryons, this also indicates that selection is most likely ensured by the degradation of (−) mitochondria rather than the protection and preferential replication of (+) mitochondria.

Electron microscopy of the developing zygospores of *Phycomyces* has illustrated that mitochondria lie adjacent on either side of the cell walls as the walls dissolve between the two gametangia, suggesting that the events leading to UPI must occur post-fusion (O'Donnell KL, 1976).

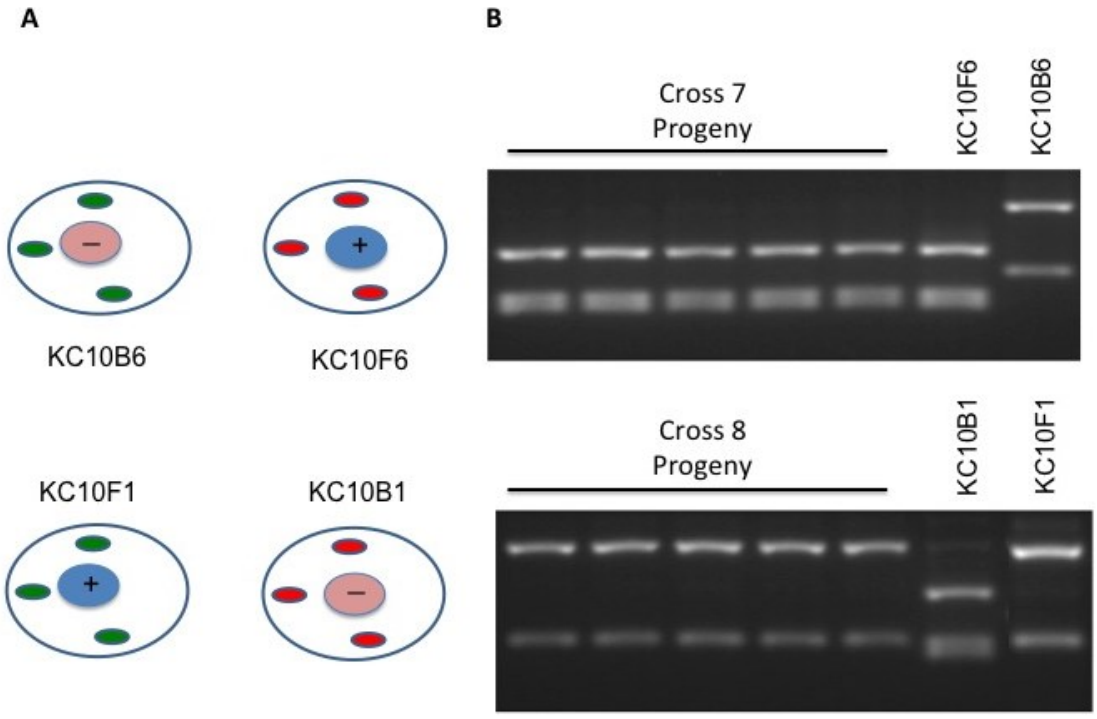

Fig. 15. The mitochondria are inherited from the (+) parent, regardless of the origin of the mitochondria. A Cartoon demonstrating how the mitochondria switching was performed using the reciprocal crosses. B. The mitochondria are inherited from the (+) parent regardless of the origin of the mitochondria. Agarose gels show the mtDNA fragment cut with TaqαI from the strains in which the mitochondrial genome was switched into the other sex background

The timing of mitochondrial degradation was assessed by PCR on zygospores. Zygospores are the single cells that result from the sexual fusion of (+) and (−) gametangia and also act as resting structures that are dormant for 2 months to over a year. The PCR was performed using a DNA extract from single zygospores with the marker used in the previous experiments (Fig. 13A), and the PCR products were sequenced. Analysis of the mitochondrial genotypes demonstrates that degradation of mtDNA from the (−) parent occurs early during the process of zygospore maturation, since this is observed within a week after the formation of the zygospore (Fig. 16).

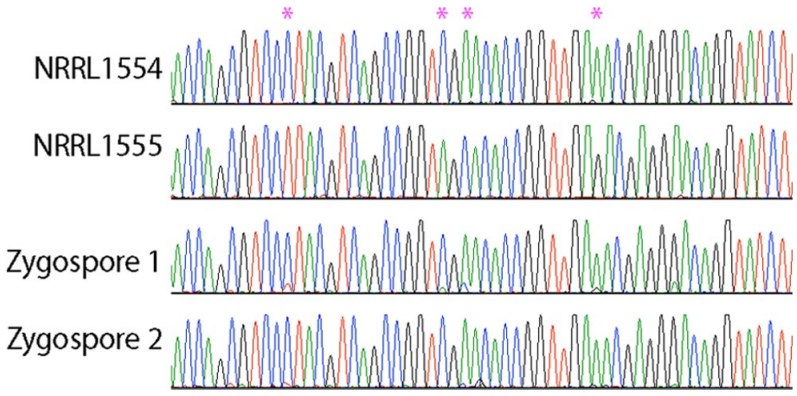

Fig. 16 The mtDNA from the (−) parent is degraded in the zygospore. DNA sequencing chromatograms of a fragment of the mitochondrial genome amplified from the parents NRRL1554 (+) and NRRL1555 (−) and two single zygospores (one week old). The asterisks show the nucleotide sequences that differ between the parents. A trace of the mtDNA from the (−) parent is still amplified from zygospore 1.

Adapted from (Shakya and Idnurm, 2014)

DISCUSSION

Two other species of basidiomycete fungi with UPI linked to their mating type alleles are worth comparing with *Phycomyces* (Basse, 2010). In the first, *U. maydis*, the pheromone *MAT* locus controls uniparental inheritance; however, this is mediated by two genes within the locus, neither of which is required for pheromone biosynthesis or in determining the cell type (Fedler et al., 2009; Mahlert et al., 2009). In *U. maydis* α2 mating type locus genes *lga2* and *rga2* regulate UPI of mitochondria. Lga2 and Rga2 are mitochondrial proteins involved in regulating uniparental inheritance by facilitating the loss of mating type *a1* strain associated mitochondrial DNA. The mitochondria from only one mating type are not inherited in all the crosses but the inheritance is affected by strain background in some cases, rather than the mating type background (Fedler et al., 2009). The second species is *Cryptococcus neoformans*, in which mitochondrial inheritance is governed at the pre- and post-zygotic stages of the sexual cycle (Gyawali and Lin, 2011; Gyawali and Lin, 2013). The post-zygotic control is in part conferred by the Sxi1α and Sxi2**a** homeodomain proteins, which also specify the mating types of the parents. A similar situation likely occurs in the related species *Cryptococcus gattii*. Crosses between congenic strains of *C. gattii* differing only in mating type exhibit UPI, while in crosses using strains with different genetic background both parents can contribute their mitochondrial DNA to the progeny (Voelz et al., 2013; Zhai et al., 2013). In addition, in *C. neoformans* the environmental and genetic conditions alter the proportion of uniparental versus biparental inheritance (Gyawali and Lin, 2013; Toffaletti et al., 2004; Yan et al., 2004; Yan et al., 2007), and cytological analysis indicates that the mating type α cell delivers its nucleus to the **a** cell (McClelland et al., 2004). Thus, neither *U. maydis* nor the *Cryptococcus* species rely on sex-determining genes

for the sole determinant of UPI, as is the case in *Phycomyces*.

The mechanism whereby SexM or SexP, encoded by the *sex* locus of *Phycomyces*, contributes to UPI remains to be elucidated. For instance, it may be that the role of SexM is to trigger degradation of the mitochondria from that strain or that the role of SexP is to protect the mitochondria from being degraded, or SexP can trigger the degradation of mitochondria from the minus strain, or a combination of systems. An alternative hypothesis is that one mitochondrial type is amplified during the sexual cycle, although this seems less likely in the context of evidence from other eukaryotes of degradation of the organelles from one parent. The absence of a stable transformation system for this species prevents gene manipulation approaches to explore this trait (Obraztsova et al., 2004). Nevertheless, finding this correlation between mating type and UPI in *Phycomyces* provides research directions in more tractable eukaryotes, to test if the genes required for sex determination also control UPI more widely.

We hypothesize that the equivalent genes that control the sex of an organism also control UPI or may have done so in the past. In some organisms the original roles of the sex-determining factor in UPI may have been superseded by other genes. In *U. maydis*, the *lga2* and *rga2* genes in the *a* mating type locus regulate UPI of mitochondria (Fedler et al., 2009). These two genes have been incorporated into a locus that controls the sex of the cell but are not involved in determining sex of the mating partner. A similar scenario may have occurred in the green alga *Chlamydomonas reinhardtii*, which has a large mating type (*MT*) locus. In this organism, isogametes fuse, and in the progeny the mitochondrial genotype is from the *minus* parent and, conversely, the chloroplast genotype from the *plus* parent (Armbrust et al., 1993; Ferris et al., 2002; Goodenough et al., 2007). The *ezy-1* and *ezy-2* genes that are

located within *MT* have been implicated in chloroplast inheritance (Armbrust et al., 1993; Ferris et al., 2002; Goodenough et al., 2007). If so, this would represent another example in which the control of UPI is through genetic factors that have been recruited into the region of the genome that includes the sex-determining genes. Even more similar to *Phycomyces* is evidence that the homeodomain protein Gamete-specific plus1 (Gsp1) is also involved in regulation UPI of mitochondria in *C. reinhardtii*. *GSP1* is one of the elements controlling zygote formation in *Chlamydomonas* that was originally identified as a mating type plus (mt+) specific expressing gene. The ectopic expression of *GSP1* in mating type minus (mt-) gametes resulted in the transcription of zygote-specific genes (Zhao et al., 2001). The co-deletion of this gene and the adjacent homolog of an inositol monophosphatase (*INM1*) impairs UPI. Besides the impaired UPI, this mutant also shows defective zygote maturation and the fusion of gametes only results in the formation of vegetative diploids, as shown by the presence of both mt+ and mt- markers in the diploids. Both *INM1* and *GSP1* are required to restart all the functional defects of the mutant (Nishimura et al., 2012). This study also emphasizes that UPI is part of sexual development and is regulated by a transcription factor protein with a role in mating, as SexP does in *Phycomyces*.

A recent study in *Volvox carteri* suggests that another transcription factor encoded in the mating type locus is the global regulator to establish the gender specificity to the cell in the green algae (Geng et al., 2014). *V. carteri* is a relative of *C. reinhardtii* (both in the Volvocine) but the mating involves oogamy. In *V. carteri* the gametes are defined as sperm cells produced from mating type male (MTM) and egg cells produced by mating type female (MTF). The *MID* gene in *C. reinhardtii* is present only in MT- and encodes a transcription factor of the RWP-RK family (Goodenough et al., 2007). In *V. carteri* the MID homolog

VcMID is expressed in male cells (MTM). The ectopic expression of this protein in MTF cells induces them to produce sperm packets instead of producing egg. When the VcMID gene is silenced in male cells (MTM) they develop like egg cells. Authors of this study indicate that the significance of their findings is that a gene originally involved in sex determination in an isogamous organism like *C. reinhardtii* can evolve additional functions, in their case differentiation in cell sizes in *V. carteri* (Geng et al., 2014). Examining UPI in the strains with altered expression of MID in the green algae will likely yield additional insights into the role of the transcription factors in the mating type locus and patterns of organelle inheritance.

In summary, the present study in *Phycomyces* provides an example in which genes located within the sex-determining region of the genome likely have two functions. One is to determine the mating type or sex of the cells, and the other is to direct uniparental inheritance during mating. Given that those two processes are intimately linked in eukaryotes, we propose that this represents an ancestral state in eukaryotes to coordinate the distribution of both nuclear and mitochondrial genetic material during sexual reproduction.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Reproduction can be asexual or sexual, and fungi like *Phycomyces blakesleeanus* have the ability to use both ways to propagate in the environment. The fungi need sensory mechanisms to perceive which mode of reproduction to select depending on environmental cues like nutrient availability or the availability of mating partners. The research in this dissertation originally aimed to be an investigation into the effects of light as a signal in inhibiting mating in *Phycomyces*. However, perhaps the finding of wider significance from this work, which is described in chapter three, when coupled to the evidence of uniparental inheritance described in chapter four is that the concept that the current distinction between mating type and sexes is artificial. After outlining the discoveries from the two chapters, I will expand on the evidence they provide that although the two mating types of *Phycomyces* are identical in appearance, they have unique and fundamental roles in the cell, and that the use of the term "*sex*" locus to describe what others in the fungal genetics community refer to as a "*MAT*" locus was a serendipitous choice when made by Richard Sutter and other workers in the late 1980s.

Chapter three explores the factors required for light to inhibit mating in *Phycomyces*. Sexual reproduction is inhibited in the presence of light and favored in the absence when other required conditions are met. On the one hand light promotes the production of β-carotene, which serves as the substrate for the pheromone production, and at the same time light acts as an inhibitory factor for mating. The MadA-MadB photoreceptor complex has been characterized as the light sensing receptor to transduce various mycelial and asexual responses such as phototropism and synthesis of β-carotene (Idnurm et al., 2006; Sanz et al.,

2009). The inhibitory role of light on mating in *Phycomyces* had not been explained at the molecular level. In fungi mating is regulated through the region in the genome known as the mating type locus. *Phycomyces* has a simple mating type locus with just one gene designated as *sexP* in the plus partner and *sexM* in the minus partner. We discovered that light has different influences on the expression of *sexM* and *sexP* genes. Using the *madA*, *madB* single and double mutants, these studies revealed *sexP* as the prominent target of light acting through the MadA-MadB complex. The mating inhibition is circumvented if the plus partner does not posses a functional MadA-MadB photoreceptor complex.

This complex is conserved in the fungal kingdom; e.g. the *Phycomyces madA* and *madB* genes are homlogs of *wc-1* and *wc-2*, which were first discovered and characterized in *Neurospora* (Ballario et al., 1996). Our studies explain the role of a conserved photoreceptor complex in regulating mating in a sex-dependent manner. Previous studies have suggested the role of Wc homologs in light-based inhibition of mating in fungi. For example in the basidiomycete fungus *C. neoformans*, the mating is inhibited by light but the mutants for Wc homologs in any mating type (**a** or α) can circumvent the inhibition (Idnurm and Heitman, 2005). The finding of our studies assigns a new role for the conserved photoreceptor complex in *Phycomyces* to inhibit the mating through a single mating partner.

The *Phycomyces* mating type locus encodes only one high-mobility group (HMG) domain protein, *sexM* in minus and *sexP* in plus mating type. Though the involvement of two mating partners of opposite sex is essential for the completion of sexual reproduction, both can have different roles in perceiving environmental factors. The *sexM* and *sexP* genes are upregulated in mating conditions in the dark (Idnurm et al., 2008), and recent studies indicate that the pheromones upregulate transcript levels of the *sexM* and *sexP* homologs in *Mucor*

but the regulation is more prominent for *sexM* (Wetzel et al., 2012). Looking at our and the *Mucor* studies together suggests a system in which the expression of *sexP* is influenced by light while *sexM* regulation is more likely a secondary consequence solely through pheromone signaling. The present study does not exclude the influence of pheromones on *sexP*: this is also one layer of regulation and presumably both light and pheromones affect the expression of *sexP*. Probably pheromones are required for basal transcript levels and the absence of light acts as signal for the upregulation of *sexP* or relieves the repression of *sexP* expression caused by light. The differential regulation of *sexM* and *sexP* can provide the flexibility of one strain acting as the primer or initiator to start the process of mating as a "proposing partner". To perceive the environmental cue and conveying the message to the genes involved in mating enables the fungus to make the most efficient use of the resources. The finding of a mating-dependent influence of light helps us to appreciate the dynamics of the conserved machinery in regulating diverse functions.

At present genetic manipulations through transformation are not possible in *Phycomyces*, which limits many investigations. Ideally in future studies the role of MadA-MadB ought to be tested for a possible direct regulation of the transcription of *sexP* by the photoreceptor complex. The *madA madB* mutants (with two *madA* alleles) developed during this study can serve as resources for future studies, like deciphering the role of the MadA-MadB complex in asexual vs. sexual reproduction. Alternatively, *Mucor circinelloides* is a species in which the mating types have also been characterized and gene disruption is feasible. For instance, the *sexM* gene was disrupted in this species, yielding a sterile strain (Lee et al., 2010). However, there are also major limitations in this species as well, including the lack of selectable markers that would work for transformation in a (+) parent. Moreover

the wild type strains currently being studied for mating though produce zygospores, but those zygospores do not germinate.

Chapter four describes the surprising discovery that *Phycomyces* has uniparental inheritance of its mitochondrial genome during mating. The key finding is the contribution of the mating type locus to that process. The uniparental inheritance (UPI) of mitochondria during sexual reproduction is prevalent in eukaryotes; e.g. we inherit out mitochondria from our mothers. The causal reasons for UPI through the sexes of the parents are still not well defined. Two major reasons that make understanding of UPI a daunting task are that sex determination at the DNA level involves large regions of genome and fusion of unequal sized cells. *Phycomyces* provided the model where both of those problems are avoided as it has simple mating type and two equal size cells from opposite mating partners, which fuse together to form the zygospore. Using crosses between diverse wild type isolates, the results of chapter four revealed that the genes in the mating type locus regulate the UPI of mitochondria. The mitochondria are always inherited from the plus partner in *Phycomyces*. The combination of these crosses and the cross between the isogenic strains shows that the *sexP* gene is the regulator for UPI of the mitochondria. The series of crosses also included the switching of mitochondria to make a set of four strains with the same mitochondrial genome but different mating type. Progeny derived from crosses with those strains disprove the possibility of regulation of mitochondrial inheritance by mitochondria itself. Future experiments of RNA sequencing can be performed to investigate for differences in the transcript profile of mitochondria or the strain. The expression profile could be searched for putative genes encoding the enzymes that might be involved in the degradation of the

mitochondrial genome from the minus mating partner and/or those protecting the mitochondria from the plus mating partner.

The findings from *Phycomyces* are worth comparing with recent studies in *Chlamydomonas* and *Volvox* (Geng et al., 2014; Nishimura et al., 2012) extending the evolutionary aspect of the regulatory role of the mating type genes in various cell functions into the green algae. UPI of mitochondria (and chloroplasts) is also present in this early plant lineage. Any function conferred by a gene in a mating locus, other than cell identity, has two possible explanations: either the gene involved in the regulation is recruited to the mating locus or the gene is the master regulator of cell identity but gained the new function during evolution. The *sexP* gene may have always had a role as a driver of UPI or may have acquired this new role in UPI after its primary role of establishing cell identity. More importantly this finding will also help to explore in other organisms how the key transcription factors of cell determination can acquire new roles during evolution, to regulate functions other that cell identity.

The uniparental inheritance of mitochondria is ensured by various mechanisms but the master genetic regulator has not yet discovered. The SexM and SexP proteins are equivalent to HMG domain proteins Sry and Sox3 in humans (Idnurm et al., 2008). The discovery of regulation of UPI of mitochondria by *sexP* in *Phycomyces* provides a strong impetus to consider exploring the role of SRY and SOX3 in the regulation of mitochondrial inheritance in mammals.

Mating compatibility in fungi is determined by the identity of the mating-type locus, with only opposite mating-types undergoing successful out crossing. 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. This explains why "mating type" is used to define individual strains in place of "sex" or "gender" (Billiard et al., 2011). Gamete differentiation is governed by each sex in most animals and plants; e.g. the female sex would produce the egg (large size gamete) and male sex will produce the sperm (small size gamete), so that the sex and anisogamy for gametes are linked. But in fungi the difference in size of cells involved in mating is not linked to the mating type. Many filamentous fungi of Ascomycetes produce small (spermatia or microconidia) and large (ascogonia or protoperithecia) gamets, but haploid strains of each mating type (*A* and *a*) producing are capable of both large and small gametes (Billiard et al., 2011).

In fungi mating-type determination is regulated in the haploid stage unlike other eukaryotes where sex differentiation is mostly related to the diploid stage. Thus the discoveries of mitochondrial inheritance in fungi regulated by mating type locus genes should be attributed to the mating identity of the strain, rather than the differentiated cell function identity. The work presented in chapters 3 and 4 shows that a species with strains of different mating type, though seemingly identical in appearance, still can have fundamental differences in their properties. Indeed, by Hurst and Hamilton's (Hurst and Hamilton, 1992) definitions for mating type vs. sex, *Phycomyces* should be considered as having two sexes, (+) and (–).

Despite the common use of "mating type" to describe fungi, at the molecular level we already are well aware that both mating types are different. The textbook examples are the roles of the **a** and α proteins in regulating cell-types of *Saccharomyces cerevisiae* (Madhani,

2006). The Sordariomycetes (Ascomycota) also provide several examples of differential expression between mating types. For instance, this is observed in *Neurospora crassa* and *N. tetrasperma* (Samils et al., 2013; Wang et al., 2012). One interesting parallel with the research here on *Phycomyces* is found in *Fusarium verticillioides*, where the light dependent induction of the synthesis of β-carotene requires the *MAT1-2-1* gene (Adam 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 *Hypocrea jecorina* 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 (Seibel et al., 2012).

To summarize, even with the transformation system in *Phycomyces* not yet developed, its simple mating type locus, availability of mutants, and the ability to perform sexual crosses makes *Phycomyces* an effective species for investigating certain fundamental areas of biology. This thesis research has provided novel findings on the role of *sexP* in executing the light-mediated inhibitory role on mating by MadA-MadB and in regulating mitochondrial inheritance, opening new avenues of future research to understand the link of environmental cues and mating and mitochondrial inheritance in more tractable model systems. The findings from *Phycomyces* add another set of evidence that fungal mating types are the same as sexes in plants and animals, and illustrates the contribution of microbial organisms in understanding basic cell features which may be common across eukaryotic lineages.

APPENDIX

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1. Proceedings of National Academy of Sciences

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   Arrach, N., R. Fernandez-Martin, E. Cerda-Olmedo, and J. Avalos. 2001. A single gene for lycopene cyclase, phytoene synthase, and regulation of carotene biosynthesis in Phycomyces. *Proceedings of the National Academy of Sciences of the United States of America*. 98:1687-1692.

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