Published in final edited form as:

Fungal Genet Biol. 2011 July ; 48(7): 668–676. doi:10.1016/j.fgb.2011.04.001.

Fungal Mating Pheromones: Choreographing the Dating Game

Stephen K. Jones Jr.¹ and Richard J. Bennett^{1,2,*}

¹ Graduate Program in Molecular Biology, Cellular Biology, and Biochemistry, Brown University, Providence, RI 02912

² Department of Molecular Microbiology and Immunology, Brown University, Providence, RI 02912

Abstract

Pheromones are ubiquitous from bacteria to mammals - a testament to their importance in regulating inter-cellular communication. In fungal species, they play a critical role in choreographing interactions between mating partners during the program of sexual reproduction. Here, we describe how fungal pheromones are synthesized, their interactions with G protein-coupled receptors, and the signals propagated by this interaction, using *Saccharomyces cerevisiae* as a reference point. Divergence from this model system is compared amongst the ascomycetes and basidiomycetes, which reveals the wealth of information that has been gleaned from studying pheromone-driven processes across a wide spectrum of the fungal kingdom.

Keywords

GPCR; MAPK kinase; signal transduction; homothallic; heterothallic

1. Introduction

Pheromones are diffusible ‘telesensing’ molecules that may be used for a variety of purposes including quorum sensing, predator detection, and mating regulation. In this review, we examine the intricacies of mating signaling in fungi, concentrating on the roles of pheromones, pheromone receptors, and related factors such as pheromone-degrading proteases, all of which are integral to regulating communication between courting partners. What defines a mating pheromone? At its most basic level, it is a secreted molecule that conveys information from one cell to another, resulting in induction of the corresponding mating response. Signaling can involve unicellular or multicellular organisms; both are present in the fungal kingdom. Historically, the hemiascomycete yeast *Saccharomyces cerevisiae* has provided an excellent model system for exploring the mechanism of pheromone signaling. *S. cerevisiae* mating occurs between two cell types, **a** and **α**, in a manner that is tightly regulated by secreted pheromones. *MATa* and *MATα* cells secrete **a** and **α** pheromones, respectively, that are recognized by sex-specific receptors on the surface of the opposite cell type. We will review how these factors regulate mating in *S. cerevisiae* and discuss how this model compares to pheromone signaling in other fungal species.

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*Correspondence: Richard_Bennett@Brown.edu.

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2. Pheromones Regulate Mating in *S. cerevisiae*, the Model Ascomycete

Mating in *S. cerevisiae* and other ascomycetes is regulated by transcription factors encoded at the *MAT* locus. Haploid cells that contain the *MATa* locus are classified as **a** cells, and these can mate with haploid α cells that express the *MAT α* locus (for reviews see Heitman, 2007 and Lee et al., 2010). *S. cerevisiae* α cells secrete α factor, a mature peptide of thirteen amino acid residues, WHWLGLKPGQPMY, that is the processed product of the *MFa1* and *MFa2* genes. The α factor is initially translated as a prepro-protein of 165 amino acids composed of three regions: a 19 amino acid signal sequence, a 64 amino acid proregion containing several glycosylation sites, and a region containing four (*MFa1*) or two (*MFa2*) repeats of the mature α factor separated by short linker sequences (see Figure 1A). The prepro form is translocated into the endoplasmic reticulum where the signal peptide is cleaved off and extensive glycosylation of the proregion occurs (Kurjan and Herskowitz, 1982; Singh et al., 1983; Waters et al., 1988). Upon transitioning to the Golgi apparatus, these recently added carbohydrate groups are modified and three cleavage reactions occur. First, the Kex2 protease separates the α factor repeats by cleavage after conserved lysine-arginine residues. Second, Kex1 removes these two amino acids via carboxypeptidase activity. Finally, the diamino peptidase Ste13 completes the maturation process by removing excess residues at the N-terminus of the peptide (Figure 1A) (Julius et al., 1984; Dmochowska et al., 1987; Wagner and Wolf, 1987; Caplan et al., 1991). Mature α factor then exits the cell via the classical secretory pathway.

Processed α factor is recognized by the Ste2 receptor on the surface of **a** cells, which initiates mating signaling. Despite its short length, different regions of α factor have specific roles in Ste2 activation. Thus, the C-terminus of α factor is important for the physical interaction with Ste2, while the N-terminus is more closely associated with downstream signaling events (Naider and Becker, 2004). The central region of the peptide (proline-8 and glycine-9) is thought to adopt a β -turn, which may orient the N-terminal 'signaling' and C-terminal 'binding' domains during the interaction with Ste2 (Shenbagamurthi et al., 1983; Naider and Becker, 2004). While there is tolerance for sequence changes in these regions, specific modifications can either enhance or inhibit signaling (Raths et al., 1993; Zhang et al., 1997, 1998). In either case, the affinity of α factor for its receptor does not correlate with the ability to activate downstream signaling events (Naider and Becker, 2004). In fact, modified pheromones that bind very strongly to Ste2 often perform poorly in signal transduction. These experiments illustrate two important points in pheromone function: (1) Even in short peptide sequences specialized functions are associated with different regions of the peptide. (2) The strength of the receptor-ligand interaction does not predict the strength of the induced biological signal.

The use of unmodified peptides as mating pheromones (e.g. α factor) is characteristic of the ascomycetes, including *Saccharomyces* and *Candida* species. In contrast, pheromones consisting of both peptide and lipid components are found throughout the ascomycetes and basidiomycetes (Olesnicki et al., 1999; Davidson et al., 2000; Fowler et al., 2004; Coppin et al., 2005; Kothe, 2008). *S. cerevisiae* **a** factor is representative of this class and undergoes processing that differs from that of α factor. *MFa1*/*MFa2* genes encode a propheromone of 36 or 38 amino acids, respectively (Fuller et al., 1985; Michaelis and Herskowitz, 1988). Processing of the propheromone occurs in the cytosol, beginning with modification of a cysteine residue in the conserved C-terminal motif CAAX (Anderegg et al., 1988). The cysteine residue is first farnesylated by Ram1 and Ram2 proteins which introduce a lipid moiety, followed by proteolysis of the three most C-terminal residues by Rce1 or Ste24 (Powers et al., 1986; He et al., 1991; Boyartchuk et al., 1997; Chen et al., 1997; Tam et al., 1998; Boyartchuk and Rine, 1998). Further post-translational modifications are performed by Ste14, which appends a carboxymethyl group to the cysteine residue (Hrycyna et al.,

1991; Sapperstein et al., 1994). Subsequently, a two-step proteolysis of the N-terminus occurs: Ste24 cleaves off the first seven residues, then Axl1 cleaves off fourteen additional residues, although Ste24 activity does not appear to be essential (Fujimura-Kamada et al., 1997; Tam et al., 2001; Huyer et al., 2006). The final product is mature **a** factor, a twelve amino acid phospholipid exported by the Ste6 transporter (Figure 1B).

Sex-specific pheromone expression has been demonstrated in other hemiascomycetes such as *Candida albicans*, in the basal ascomycete *Schizosaccharomyces pombe*, and in multiple filamentous ascomycetes including *Neurospora crassa*, *Podospora anserina*, *Magnaporthe grisea* and *Aspergillus nidulans* (see Figure 2) (Zhang et al., 1998; Shen et al., 1999; Bobrowicz et al., 2002; Coppin et al., 2005; Paoletti et al., 2007). Some ascomycete species, however, show significant variations from *S. cerevisiae* pheromone processing. For example, in the filamentous ascomycete *Hypocrea jecorina* (the teleomorph of *Trichoderma reesei*) an unusual precursor form of **a** pheromone has been uncovered. The **a** propheromone exhibits features of both *S. cerevisiae* **a** and α pheromone precursors. In particular, the C-terminal CAAX motif that is associated with farnesylation and carboxymethylation of **a** factor is present, but so are two putative cleavage sites for the α factor protease, Kex2 (Schmoll et al., 2010). The gene encoding this pheromone was demonstrated to be essential for male fertility, but Kex2 processing was apparently dispensable since deletion of putative processing sites did not block mating. The authors found similar 'hybrid' pheromones in several *Fusarium* species, leading them to classify these pheromone precursors as h (hybrid)-type pheromones (Schmoll et al., 2010). Further genetic and biochemical analyses of the h-type pheromones will be required to determine how this class of pheromone precursor is processed. In addition, it will be interesting how this class of pheromone evolved, and whether it involved fusion of **a**- and α -type pheromone precursors. Moreover, this work shows that although **a** and α factors are the prototypical mating pheromones in ascomycetes, sequence, structure, and post-translational processing can vary significantly between species.

3. Pheromone Signaling in Basidiomycetes

Mating types in the Basidiomycete phylum often exceed the conservative two-class system typical of ascomycetes (for recent reviews on basidiomycete mating and cell identity see Heitman, 2007; Lee et al., 2010; Raudaskoski and Kothe, 2010; Kruzel and Hull, 2010). In particular, whereas expression of pheromone and pheromone receptor genes in ascomycetes is under the control of the mating locus (e.g., *MAT* in *S. cerevisiae*), mating types in basidiomycetes are themselves defined by interactions between pheromones and their receptors, such as in the smut fungus *Ustilago maydis* and the mushrooms *Schizophyllum commune* and *Coprinus cinereus* (Vaillancourt et al., 1997; Olesnicki et al., 2000).

S. commune has served as a model system for understanding tetrapolar mating, where four different mating types are generated during each meiosis. Two mating loci, A and B, are responsible for regulating mating specificity, and successful mating requires the two parental strains to differ in at least one allele at both the A and B loci (for review see Heitman, 2007; Raudaskoski and Kothe, 2010). The *S. commune* A locus consists of two subloci encoding homeodomain transcription factors that regulate sexual development. The B locus encodes for pheromones and pheromone receptors; two unlinked subloci each expresses a single pheromone receptor and multiple pheromone genes. In total, at least seven receptors and twenty mating pheromones have been identified, with pheromones varying between 10 and 20 amino acids (Vaillancourt et al., 1997; Fowler et al., 2001, 2004; Gola and Kothe, 2003). Notably, pheromones are only able to activate receptors from a different allele in a manner that ensures outbreeding, and the extreme diversity in the pheromone/receptor repertoire effectively results in more than 15,000 *S. commune* mating types.

Basidiomycete species produce only phospholipid pheromones that are structurally similar to *S. cerevisiae* **a** factor, including a conserved CAAX motif, the site for lipid attachment. Studies have examined sequence changes in the *S. commune* pheromone/receptor pair, and show that even moderate amino acid substitutions in either the pheromone or the receptor can alter the specificity of interaction both positively and negatively (Fowler et al., 2001; Gola and Kothe, 2003). Novel pheromone-receptor interactions presumably evolved through gene duplication and subsequent mutation of the duplicated gene. This is not to say that one pheromone only activates one receptor; several receptors recognize multiple pheromones, but receptors cannot be activated by pheromones produced within the same individual (Wendland et al., 1995).

Mating in the corn smut fungus *Ustilago maydis* is also regulated by A and B mating-type loci. In *U. maydis*, the A locus encodes pheromone and pheromone receptor genes, while the B locus encodes homeodomain transcription factors, bE and bW, that regulate filamentation, metabolism, and cell cycle progression (Flor-Parra et al., 2006; Scherer et al., 2006). An important link between mating and pathogenicity exists in *U. maydis*, as only the dikaryotic mating products are infectious and can invade the host plant (Kahmann and Kämper, 2004). While pheromone and pheromone receptors ensure mate compatibility, it is the homeodomain transcription factors that regulate the downstream genes necessary for host invasion (Kothe, 2008). Only compatible bE and bW subunits derived from differing mates can form a functional heterodimer (Kämper et al., 1995). Thus, while the A locus regulates pheromone signaling, formation of conjugation tubes and cell fusion, the B locus determines bE/bW heterodimer specificity and regulates downstream genes involved in pathogenicity (Heimel et al., 2010).

Sexual reproduction has also been extensively studied in the human pathogen *Cryptococcus neoformans*, a basidiomycete that is one of the leading causes of fungal meningitis. This bipolar fungus has two mating types, *MATa* and *MAT α* , that express *Mfa1-3* and *MFa1-3* pheromones, respectively (Kwon-Chung, 1975, 1976; McClelland et al., 2002). The *C. neoformans* *MAT* locus is over 100 kb in size and contains elements of both ancestral *MAT* loci (A and B) found in basidiomycetes like *U. maydis* and *S. commune*. Most likely, this *MAT* locus formed by a series of genomic rearrangements followed by fusion to generate a bipolar mating system from a tetrapolar ancestor (Fraser et al., 2004, 2007; Hsueh et al., 2006). As a direct result of this fusion event, pheromone and pheromone receptor genes are now present at the same locus as the homeodomain transcription factors, *SXI1a* and *SXI2a*, that regulate sexual development (Hull et al., 2005; Hsueh et al., 2008; Kruzel and Hull, 2010). A recent study demonstrated that mating type in *C. neoformans* is still defined by the pheromone-receptor interactions typical of basidiomycetes. Thus, expression of *MFa* pheromone and **a**-pheromone receptor in an α cell (in place of *MFa* pheromone and α -pheromone receptor) resulted in a modified " α " cell that mated as an **a**-type cell (Stanton et al., 2010). Interestingly, the mating products of these crosses did not complete sexual development unless the *SXI2a* gene was also introduced into the α strain background (Stanton et al., 2010). These experiments indicate the importance of pheromones in dictating mating type in *C. neoformans*, but also how the pheromone-sensing pathway is integrated with other regulatory processes necessary to complete the sexual program.

4. Transduction of the Pheromone Signal

Pheromones are recognized by G protein-coupled receptors (GPCRs), which reside on the surface of recipient cells (Xue et al., 2008). GPCRs represent the largest family of receptors and are currently the most targeted proteins in pharmacological design (Filmore, 2004). The N-terminus of these receptors is extracellular and is followed by seven membrane-spanning regions, while the C-terminus is intracellular and can play a direct role in downstream

signaling (Dosil et al., 2000). GPCRs typically interact with large ligands via their extracellular domains whereas small molecules target GPCR transmembrane domains (Eglen and Reisine, 2009). Both modes of binding are important for peptide ligands, resulting in conformational changes that propagate the signal to cytoplasmic G proteins (Naider and Becker, 2004; Umanah et al., 2010).

The best-studied fungal GPCR is Ste2, the *S. cerevisiae* receptor for α factor (Eilers et al., 2005). The N-terminus of α factor contacts multiple regions of Ste2, including transmembrane (TM) domains 5 and 6, and extracellular domains 2 and 3 (see Figure 3)(Lee et al., 2001; Lin et al., 2003; Naider and Becker, 2004). The interaction of α factor with TM5 and TM6 is particularly interesting, since these TMs flank the third intracellular loop (IC3) critical for G protein activation (Stefan and Blumer, 1994; Schandel and Jenness, 1994). In addition, the C-terminus of α factor (Gln¹⁰ and Tyr¹³) interacts with residues in TM1 (Lee et al., 2001; Son et al., 2004). Although a high-resolution structure of the ligand-receptor interaction is not available, proposed models reflect the flexibility of the beta turn in the central region of α pheromone (Lin et al., 2003; Umanah et al., 2010). There is also support for oligomerization of Ste2, with recent work showing that oligomer interfaces include TM1, TM4, and TM7, potentially allowing for large complexes to form (Wang and Konopka, 2009; Kim et al., 2009). In *C. neoformans*, a constitutively active GPCR involved in mating was recently identified. *Cryptococcus* pheromone receptor 2 (Cpr2) was shown to activate G protein signaling in the absence of any pheromone ligand (Hsueh et al., 2009). Cpr2 is related to the canonical *C. neoformans* pheromone receptors, Ste3a and Ste3 α , and is able to form homodimers with itself or heterodimers with Ste3a/Ste3 α . Cpr2 utilizes the same G protein-coupled cascade as these receptors and promotes both heterothallic (α - α) and homothallic (α - α) mating. Constitutive activity of the receptor is due to the presence of a leucine residue at position 222 in TM domain 6. In most ligand-dependent GPCRs, a proline residue is found at the corresponding position, and this rigid amino acid mediates conformational changes between active and inactive states (Baldwin, 1993). Although Cpr2 cannot substitute for conventional Ste3a/Ste3 α receptor activity, it is required for normal cell fusion and clamp cell formation, and competes with Ste3a/Ste3 α receptors for access to downstream signaling components (Hsueh et al., 2009). Constitutively active GPCRs may similarly function to regulate mating in other fungi. For example, the role of pheromone/pheromone receptors in determining cell mating type has been lost from two mushroom species, so that cell identity is now solely determined by the homeodomain *MAT* loci (i.e., mating is bipolar) (Aimi et al., 2005; James et al., 2006). One possible explanation is that pheromone receptors became constitutively active in these species, alleviating the need for activation by pheromones from a different allele (Hsueh et al., 2009). Genome sequences have also revealed the presence of multiple receptor-like genes in *S. commune* and *C. cinereus*, although the function of these genes is unknown (Raudaskoski and Kothe, 2010). While constitutively active GPCRs have been found in various mammalian systems, the discovery of Cpr2 in *C. neoformans* suggests that ligand-independent receptors, or at least receptors with diverged functions, may be more common in fungi than previously envisaged.

5. The MAP Kinase Signaling Cascade

Pheromone activation of the GPCR initiates a signal transduction cascade that includes a mitogen-activated protein kinase (MAPK) pathway (see Figure 4) (for reviews see Bardwell, 2004; Hoffman, 2005; Chen and Thorner, 2007). In *S. cerevisiae*, activation of Ste2 or Ste3 receptors targets a G protein complex composed of three subunits, α (Gpa1), β (Ste4), and γ (Ste18). During signaling, the α subunit exchanges GDP for GTP, and dissociates from the $\beta\gamma$ complex. The $\beta\gamma$ dimer subsequently interacts with three proteins important for the response to pheromone: Ste20, a p21-activated protein kinase, Ste5, a scaffold protein that

coordinates the MAPK pathway, and Far1, a protein involved in cell cycle control. Signal transduction requires Ste20-mediated phosphorylation and activation of Ste11, the first kinase in the MAPK pathway, which then drives a cascade of phosphorylation reactions involving Ste7 and Fus3 (Zhou et al., 1993; Choi et al., 1994). At the culmination of the pathway, phosphorylated Fus3 accumulates in the nucleus and activates Ste12, the transcription factor required for mating gene expression. Fus3 also phosphorylates (and inactivates) Dig1 and Dig2, two negative regulators of Ste12 activity (Elion et al., 1993; Tedford et al., 1997; Breitskreutz et al., 2001). While the role of the G $\beta\gamma$ subunits in transducing the pheromone signal is well established, the G α subunit may also promote signaling via the RNA binding protein Scp160, although the mechanism is unknown (Guo et al., 2003). In comparison, studies in *C. albicans* and *K. lactis* show that the G α and G β subunits function in a coordinated manner, since both subunits are important for activation of the mating program (Coria et al., 2006; Dignard et al., 2008). Conversely, the response to pheromone in *S. pombe* does not involve G $\beta\gamma$ subunits, and the G α subunit is solely responsible for signal transduction (Obara et al., 1991; Ladds and Davey, 2000). Thus, even between this subset of ascomycetes, alternative G protein-coupled mechanisms have evolved to transmit the pheromone signal.

The role of the scaffold protein, Ste5, has been of particular interest during pheromone signaling, as it tethers the Ste11, Ste7 and Fus3 kinases, and delivers these proteins to the plasma membrane via its association with the G-protein β subunit (Marcus et al., 1994; Choi et al., 1994; Printen and Sprague, 1994; Whiteway et al., 1995). More recently, Ste5 plasma-membrane recruitment was shown to involve a PH (pleckstrin-homology) domain that preferentially binds to phosphoinositides concentrated in mating projections (Garrenton et al., 2006, 2010). Docking of Ste5 with the membrane promotes activation of Ste11 by Ste20, which is also localized to the membrane via interactions with G $\beta\gamma$ and the small GTPase, Cdc42 (Drogen et al., 2000; Lamson et al., 2002).

Ste5 also limits cross-talk between alternative MAPK signaling pathways. During the response to pheromone Ste7 preferentially targets the Fus3 kinase, while during filamentous growth (e.g., upon nitrogen starvation) Ste7 activates the Kss1 kinase. Recent experiments show that Ste5 increases the affinity of Ste7 for Fus3 over Kss1, thereby promoting activation of the pheromone-signaling pathway over the program of filamentous growth (Good et al., 2009). A further mechanism preventing MAPK cross-talk involves Fus3-dependent degradation of Tec1, the transcription factor targeted by Kss1 during the filamentation pathway (Bao et al., 2004; Chou et al., 2004; Saito, 2010).

The output from pheromone signaling in *S. cerevisiae* can be separated into three steps: (1) Ste12-mediated transcription of genes involved in mating, (2) formation of polarized mating projections (or shmoo) that promote growth towards the prospective mating partner, and (3) G1 cell cycle arrest that allows fusion of unreplicated nuclei during karyogamy. The latter two processes require Far1, a protein distantly related to Ste5 that can also act as a protein scaffold (Peter et al., 1993; Butty et al., 1998). Activation of Far1 by Fus3-mediated phosphorylation leads to cell cycle arrest due to Far1 inhibition of Cln2-Cdc28 kinase activity (Chang and Herskowitz, 1990; Peter et al., 1993; Peter and Herskowitz, 1994). During mating projection formation, a Far1-Cdc24 complex also shuttles from the nucleus to the cytoplasm in response to pheromone and promotes interaction of G $\beta\gamma$ with a Cdc24/Cdc42/Bem1 protein complex (Butty et al., 1998; Shimada et al., 2000; Wiget et al., 2004). This complex catalyses actin assembly, resulting in polarized growth towards the highest concentration of pheromone. Recent experiments suggest that Fus3 can also polarize the cell via a Far1-independent mechanism. While the Cln/Cdc28 kinase normally prevents the polarized recruitment of Ste5 and other polarity proteins to the plasma membrane, inhibition

of Cln/Cdc28 activity by Fus3 promotes polarized growth in response to pheromone (Yu et al., 2008).

In *C. albicans*, *FAR1* plays an even more central role in the pheromone response than its homolog in *S. cerevisiae*. *C. albicans far1* mutants are defective in all three aspects of the pheromone response; they lack the transcriptional response to pheromone as well as the morphological response and cell cycle arrest (Côte and Whiteway, 2008). A *STE5* homolog, *CST5*, also exists in *C. albicans*, and loss of this gene prevents signaling in response to pheromone, similar to *S. cerevisiae* (Côte et al., 2011; Yi et al., 2011). Mating in *S. pombe* utilizes a related MAPK pathway, except mating is also controlled via a nutrient-sensing, cAMP-regulated protein kinase A (PKA) pathway (Mochizuki and Yamamoto, 1992; Lengeler et al., 2000; Hoffman, 2005). Dual regulation of mating has also been demonstrated in the basidiomycetes *U. maydis* and *C. neoformans*, where MAPK and cAMP pathways act coordinately to regulate mating processes (Wang et al., 2000; Lengeler et al., 2000; Lee et al., 2003). Curiously, *U. maydis* cells undergo cell cycle arrest while responding to pheromone but do so while in G2 of the cell cycle, and this may promote increased polarized growth of the mating cell (García-Muse et al., 2003).

6. Pheromone Signaling, Phenotypic Switching, and Biofilm Formation

While *S. cerevisiae* readily undergoes mating, other fungi often require environmental cues to direct when mating occurs (Lengeler et al., 2000; Li et al., 2007; Xue et al., 2008). In the case of *C. albicans*, the regulation of mating is even more dramatic, as it is controlled by a unique phenotypic switch. Referred to as the ‘white-opaque’ switch, **a** and α mating types can undergo a heritable change from a rounded, white colony morphology to a flatter, opaque colony morphology (for recent reviews see Soll, 2009; Lohse and Johnson, 2009; Morschhäuser, 2010; Alby and Bennett, 2010). This switch only occurs in **a** or α cells, since the master regulator of the white-opaque switch, Wor1, is repressed by an **a1**/ α 2 protein complex expressed in **a**/ α heterozygotes (Miller and Johnson, 2002; Huang et al., 2006; Zordan et al., 2006; Srikantha et al., 2006). Cells in the opaque state, but not in the white state, respond to pheromones by forming polarized mating projections and subsequently undergoing mating (Miller and Johnson, 2002).

While opaque cells undergo shmooing and fusion, white cells can mount their own specific response to pheromone. Work in the Soll lab established that white cells exposed to pheromone upregulate genes involved in adhesion, thereby stimulating biofilm formation on inert surfaces (Daniels et al., 2006; Sahni et al., 2009). These biofilms may provide a favorable environment for opaque cells to mate, but may also enhance the ability of cells to gain a foothold for infection of the host. Notably, although both the white and opaque responses utilize the same MAPK cascade and receptors, the mating response in opaque cells is mediated by the transcription factor Cph1/Ste12, while the response in white cells is dependent on the Tec1 transcription factor, a factor important for filamentation in both *C. albicans* and *S. cerevisiae* (Madhani and Fink, 1997; Schweizer et al., 2000; Yi et al., 2008; Sahni et al., 2010).

How does the same pheromone elicit two different responses in white and opaque cells? One possibility is that different domains of the pheromone receptor are utilized in the two cell types. Removal of the intracellular IC3 region from Ste2 prevents signal transduction in both cell types, while removal of the IC1 region prevents pheromone signaling only in white cells (Yi et al., 2009; Sahni et al., 2010). Since white and opaque cells show differential expression of around 450 genes (Lan et al., 2002; Tsong et al., 2003) it remains to be seen if other phase-specific factors regulate this duality in pheromone signaling.

7. Pheromone Antagonists and Homothallic Mating

Antagonists of pheromone activity also regulate signaling in fungi, including the conserved extracellular protease, Bar1. Bar1 was initially identified in *S. cerevisiae* **a** cells by its ability to inhibit the response to α factor (Hicks and Herskowitz, 1976). The Bar1 protein is a highly glycosylated aspartyl protease that is secreted from **a** cells in response to α factor (Manney, 1983; MacKay et al., 1991). The *S. cerevisiae* protease is active over a wide range of pH and temperature, and cleaves α pheromone between leucine-6 and lysine-7 residues, inactivating it (MacKay et al., 1991). In both *S. cerevisiae* and *C. albicans*, *BAR1* deletion mutants exhibit hypersensitivity to α pheromone (MacKay et al., 1988; Schaefer et al., 2007). A related activity has been discovered in *S. pombe* that degrades P-factor, the diffusible pheromone secreted by P cells that induces mating in M cells (Imai and Yamamoto, 1994; Ladds and Davey, 2000). Surprisingly, the *S. pombe* activity is a carboxypeptidase rather than an aspartyl protease, suggesting that pheromone-degrading activities have independently evolved multiple times.

One important function for pheromone-degradation is aiding recovery from cell cycle arrest. Thus, destruction of α pheromone by Bar1 can help cells that do not undergo mating in reentering the cell cycle (Hicks and Herskowitz, 1976). Bar1 degradation of α pheromone by **a** cells may also sharpen pheromone gradients, allowing potential mating partners to be identified more effectively (Barkai et al., 1998). Surprisingly, loss of Bar1 activity in *C. albicans* allowed a novel program of homothallic mating to occur. *MTLa* cells of *C. albicans* were shown to secrete both **a** and α pheromones; Bar1 degradation of α pheromone prevented auto-activation of mating, but *bar1* mutants underwent self-mating even in the absence of α cells (Alby et al., 2009). A similar mechanism of autocrine pheromone signaling may drive homothallism in the related hemiascomycete, *Lodderomyces elongisporus*. *L. elongisporus* is thought to form sexual spores via a homothallic mechanism, yet the genome sequence shows that while it contains homologs of α pheromone and α pheromone receptor, it does not contain **a** pheromone or **a** pheromone receptor (Butler et al., 2009). Given the mechanism of self-mating in *C. albicans*, it is plausible that autocrine signaling via α pheromone and its cognate receptor could be responsible for self-fertilization in *L. elongisporus*.

Homothallism is also a direct consequence of pheromone signaling in other fungal systems. For example, in the filamentous ascomycete *Aspergillus nidulans*, self-mating depends on expression of both an α -box domain protein and a HMG (high mobility group) protein. In several species, these *MAT* proteins are found in opposite mating types and yet self-fertility in *A. nidulans* requires expression of both factors in a single cell type (Paoletti et al., 2007). It was further demonstrated that multiple pheromone-signaling genes are upregulated during homothallic mating, and that mutants lacking homologs of both *STE2* and *STE3* receptors were unable to self-mate (Seo et al., 2004; Paoletti et al., 2007). Pheromone signaling is therefore necessary for homothallic mating in *A. nidulans*, although expression of pheromone-signaling genes was curiously independent of the α -box and HMG transcriptional regulators (Paoletti et al., 2007; Kronstad, 2007).

Similar regulation of homothallic mating may exist in the filamentous ascomycete *Sordaria macrospora*, where two pheromone genes (analogous to *MFa* and *MFa*) and two pheromone receptor genes (analogous to *STE2* and *STE3*) are expressed (Pöggeler, 2000; Pöggeler and Kück, 2001). Single pheromone/pheromone receptor mutants did not show mating defects, whereas the corresponding double mutants were significantly compromised for mating (Mayrhofer et al., 2006). Loss of one pheromone-receptor pair therefore does not block mating, but the loss of both pairs results in infertility. In contrast, recent studies in *Gibberella zeae* suggest that this homothallic ascomycete can self-mate in the absence of

both pheromone genes or both pheromone receptor genes (Lee et al., 2008; Kim et al., 2008). This example illustrates that homothallic mating can occur independently of pheromone signaling, yet still remain dependent on the MAPK pathway. It is possible that constitutive signaling downstream of the pheromone receptors occurs and is responsible for mating in *G. zeae* (Lee et al., 2008).

Homothallic mating is not restricted to ascomycetes and has been described in the basidiomycete *C. neoformans*, where the phenomenon of haploid (or monokaryotic) fruiting in α cells was shown to represent a novel α - α mating cycle (Lin et al., 2005, 2007, 2010). Haploid fruiting refers to the ability of *C. neoformans* α strains to undergo filamentation and sporulation (Wickes et al., 1996). Originally thought to involve asexual reproduction, the Heitman group demonstrated that fruiting is due to fusion between α strains and results in meiotic products (Lin et al., 2005). Significantly, loss of the three MF α pheromones, the Ste3 α pheromone receptor, or a G protein involved in pheromone signaling, all lead to a reduction in haploid fruiting and diploidization (Wang et al., 2000; Shen et al., 2002; Lin et al., 2005). These results establish that pheromone signaling contributes to same-sex mating in *C. neoformans*, and an autocrine feedback loop may operate similar to that in *C. albicans*.

8. Post-Cell Fusion Roles For Mating Pheromones

In diverse fungal species such as *S. cerevisiae*, *C. neoformans* and *U. maydis*, pheromones are essential for the events leading to cellular fusion. In *S. cerevisiae*, mating pheromones have also been shown to play a critical role in the steps following fusion. In studies using spheroplasts, cells could be induced to fuse at high frequency, but efficient nuclear fusion required the further addition of α factor to spheroplasts (Rose et al., 1986). The mechanism by which α factor stimulates nuclear fusion in *S. cerevisiae* is unknown. In other fungal systems, it is clear that pheromones are completely dispensable for cell fusion but are critical for downstream events in the mating program. In mushrooms, for example, cell fusion can occur indiscriminately between multiple mating types, but pheromone-receptor interactions subsequently define mating compatibility (Vaillancourt et al., 1997; O'Shea et al., 1998; Brown and Casselton, 2001). In these cases, pheromone signaling is required both for directed nuclear migration and formation of specialized clamp cells (Raudaskoski and Kothe, 2010). Finally, in both *S. cerevisiae* and *C. albicans*, nuclear karyogamy requires the activity of a pheromone-induced, minus-end directed microtubule motor, Kar3 (Meluh and Rose, 1990; Sherwood and Bennett, 2008), and similar microtubule motors are now implicated in mediating nuclear migration in basidiomycetes (Raudaskoski and Kothe, 2010). The precise role of pheromone signaling in directing many of these processes has yet to be determined, however.

8. Conclusions

Studies on fungal species have been at the cutting edge of multiple fields in biology, including the elucidation of mechanisms involved in GPCR-coupled signal transduction. With the advent of new molecular tools and genome sequences for diverse fungal species, there is now a renaissance into characterizing sexual programs in these systems. It is already apparent that while some aspects of mating are highly conserved (e.g. the role of lipid-modified pheromones and the MAPK cascade), there is also a considerable plasticity in pheromone signaling mechanisms. Further understanding of these differences will reveal how signaling pathways can be re-programmed during evolution, and particularly how species can switch between homothallic and heterothallic modes of mating. In addition, it will be exciting to determine how pheromone signaling has been adapted for other cellular processes, such as biofilm formation, that can further aid the fungal lifestyle.

Acknowledgments

We thank members of the Bennett lab for comments on the manuscript. This work is supported by NIH training grant T32GM007601 to SJ and by the NIH (AI081560 and AI081704) and NSF (MCB1021120) to RJB. RJB holds an investigator in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.

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Figure 1. Processing of *S. cerevisiae* **a and α mating pheromones**

A). Processing of α factor: The secretion signal (blue) is cleaved to produce the α propheromone, which is glycosylated in the endoplasmic reticulum. Upon transport to the Golgi apparatus, three proteolytic steps remove the proregion (green) and connecting regions (yellow) that separate copies of mature α factor (pink). **B)** Processing of **a** factor: In the cytosol, **a** propheromone is farnesylated at a conserved cysteine in the C-terminal CAAX motif (C = cysteine, A = aliphatic, X = any). The amino acids (yellow) preceding the cysteine are then removed and replaced with a carboxymethyl group. Finally, two proteolytic events remove N-terminus amino acids (green) to produce the mature **a** factor (pink).

Figure 2. Dikarya phylogenetic tree

The dikarya sub-kingdom includes the Ascomycota and Basidiomycota phyla. Only species discussed in the text are included here; genera *Sordaria* → *Magnaporthe* are within the Sordariomycete class. Tree is not drawn to scale.

Figure 3. The *S. cerevisiae* Ste2 pheromone receptor

In *S. cerevisiae*, the G-protein coupled receptor Ste2 recognizes α factor via extracellular (EC) loops 2 and 3 and transmembrane domains 1, 5 and 6 (shown in pink). Ste2 associates with G-proteins and activates downstream signaling primarily via the C-terminal tail and intracellular (IC) loop 3. The IC2 region is not shown.

Figure 4. The mating MAPK signaling cascade

Following activation of the pheromone receptor in *S. cerevisiae*, the associated G-protein α subunit exchanges GDP with GTP, releasing β and γ subunits. The $\beta\gamma$ complex then interacts with both the Ste5 scaffold and Ste20, which causes sequential activation of kinases Ste11, Ste7 and Fus3. Finally, Fus3 enters the nucleus where it regulates the transcription factor Ste12 to regulate expression of genes important for mating. The Kss1 kinase is primarily involved in filamentation signaling but can also substitute for Fus3 in mating signaling.