COMMUNICATION AND CHOICE IN YEAST MATING

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Em memória de Maria Videira,

que me ensinou que o Saber não ocupa lugar

The single biggest problem in communication is the illusion that it has taken place

George Bernard Shaw

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ABSTRACT

Cell-cell communication is essential for all organisms and a hallmark of multicellularity. In the budding yeast, *Saccharomyces cerevisiae*, mating occurs when two haploid cells of opposite mating types (**a** and α), communicate through secreted pheromones and the corresponding transmembrane receptors, to find each other and fuse. I focused on the mating system of *S. cerevisiae* and used a quantitative approach to ask how yeast cells communicate with each other. I show that α cells advertise their presence strongly and devote about 1% of their protein synthesis to making just enough α-factor pheromone to initiate this communication. The **a** cells can only respond accurately to a small range of secreted α-factor and express a protease, Bar1, to maintain the α-factor concentration within their gradient sensing regime. I argue that this is an efficient way to keep mating economic and robust. I then asked how yeast cells choose a partner and I propose that sexual identity, in yeast, is determined at the cell surface, by which receptors and pheromones each cell expresses. I also report that *S. cerevisiae's* **a** cell receptor, Ste2, is surprisingly promiscuous and can respond to high concentrations of pheromones from distant species. I present evidence that the Ste2 receptors across the Ascomycota are not under positive selection, contrasting with most genes involved in speciation, and this can explain the cross-talk between different receptors and pheromones. I solve this paradox by arguing that most fungi cannot distinguish between self and non-self closely related species and that speciation in fungi is not happening at the receptor/pheromone level. I discuss these findings from a molecular and evolutionary perspective.

SUMÁRIO

A comunicação entre células é essencial para todos os organismos e característica da multi-celularidade. Na levedura unicelular *Saccharomyces cerevisiae*, a reprodução sexual acontece quando duas células de sexos opostos, **a** e α, se fundem. Para se encontrarem, as duas células comunicam através de feromonas e dos correspondentes receptores trans-membranares. Através de uma abordagem quantitativa ao processo de comunicação inter-celular durante a reprodução sexual das leveduras mostro, neste trabalh,o que as células α dedicam cerca de 1% da produção total de proteínas à publicitação da sua presença, através da produção da sua feromona, o factor α. Este forte investimento serve apenas para iniciar o processo de comunicação. No entanto, as células **a** só conseguem detectar com precisão uma pequena gama de concentrações de factor α. Para manter a concentração de feromona no seu regime de detecção de gradientes, as células **a** expressam uma protease, Bar1, que degrada o factor α. Segundo a hipótese que apresento (e discuto), este sistema representa um modo eficiente de tornar o processo de reprodução sexual simultaneamente robusto e económico. Em seguida, estudei como as células de levedura escolhem um possível parceiro e proponho que, nas leveduras, a identidade sexual seja determinada à superficie das células, pelo par feromona-receptores que cada célula expressa. Surpreendentemente, o receptor Ste2, expresso pelas células **a** de *S. cerevisiae*, é promíscuo, conseguindo detectar a presença de concentrações elevadas de feromonas de outras espécies. Apresento evidências de que os receptores do tipo Ste2, do filo Ascomycota, estão sob selecção negativa, ao contrário da maioria dos genes envolvidos em especiação. Resolvo este paradoxo sugerindo que que a maioria dos fungos não consegue distinguir entre a sua própria espécie e espécies evolutivamente próximas e que a especiação dos fungos não acontece ao nível das feromonas e dos correspondentes receptores. Discuto estas descobertas de uma perspectiva molecular e evolutiva.

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This was a long and bumpy ride…

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CHAPTER 1

Introduction

Cell-cell communication is essential for all organisms. In multicellular organisms this communication allows cells to differentiate into multiple cell types and tissues, grow and maintain their appropriate sizes, respond to internal and external signals and preserve homeostasis. Cell-cell signaling is also fundamental for single celled organisms to find food and mating partners. In unicellular as in multicellular organisms, one cell is constantly subjected to a very large number of signals, some of which they can sense and respond to. Appropriate integration and response to these stimuli is fundamental for the organism's survival. For instance, a skin cell at the surface of the human body senses a number of environmental stimuli, like temperature and pressure, while integrating a number of stimuli from their fellow cells, such as signals to divide, stop dividing or even to commit suicide. If the outside temperature rises this information has to reach different parts of the body and, via cell-cell communication, elicit the correct responses, namely increased blood flow and fluid secretion.

There are at least three steps involved in cell-cell communication: 1) detection of an external signal; 2) signal interpretation and 3) response to the signal. Disruption in any of these steps can lead to disease: the loss of cell-cell communication is one of the hallmarks of cancer, while improper communication is one of the bases of auto-immune diseases. As a result, the manner in which cells integrate and respond to different signals is a fundamental and extensively studied problem in biology.

A deep understating, at the molecular and organismal level, of the multiple steps involved in cell-cell communication is not always easy to achieve in higher eukaryotes. Most fungal species use extracellular molecules to signal their presence and detect possible mating partners. Failure to emit or sense these signals leads to sterility. *Saccharomyces cerevisiae* (budding or baker's yeast) is an excellent model to study these processes as it is unicellular, has a short generation time (of approximately 90 minutes), is exceptionally genetically tractable, has both vegetative and sexual life cycles, can live vegetatively either as haploid or diploid cells and shares many of its signaling components with mammalian cells (such as G-Protein Coupled Receptors, Mitogen Activated Protein cascades, etc). When two haploid cells of the opposite mating types are put in contact they can sense each other's presence by exchanging secreted pheromones, which bind to specific receptors at the yeast's surface (Figure 1.1). As the cells grow towards each other they undergo cell wall remodeling and eventually fuse their cytoplasms.

Approximately 15 minutes later, their nuclei fuse, and they re-bud as diploids. This full process can happen in as little as two hours (reviewed in [1]).

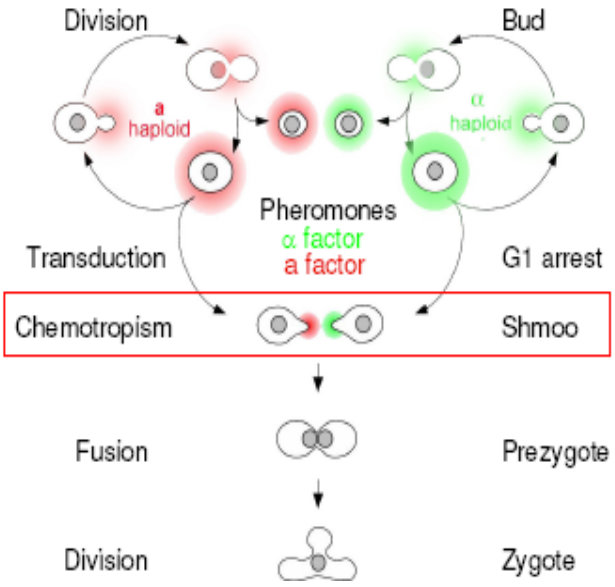

Figure 1.1 – Schematic of the *S. cerevisiae* mating process
Two haploid cells of the opposite mating type can polarize and grow towards each other (chemotropism). These cells form sexual protrusions called shmoos and can fuse their cytoplasms and nuclei to form diploid cells.

For these reasons, mating in budding yeast has been widely studied as an example of inter-cellular communication between eukaryotic cells. The work described in this thesis focuses on the signal sensing step and its implications for gradient detection, self vs. non-self discrimination and partner choice. In Chapter 3 we generate artificial and alternative mating types and ask what defines a possible mating partner by looking at the determinants of sexual identity. In Chapter 2 and Appendix A we look at what happens when one yeast cell is in the presence of several possible partners. We ask how they disentangle the signal gradients formed by large numbers of cells and how they distinguish between close, far away and equally distant partners. In Chapter 4 we extend these studies by looking at the specificity of different fungal pheromone receptors to self and non-self pheromones. Our goal is to look not only at the determinants of specificity but also at the role they might play in speciation.

The purpose of this introduction is to present some background on yeast mating. I start by describing what is common between both mating types, including some of the known molecular details of the signaling pathway and the fusion process. I then introduce the differences between

the mating type loci, present some distinguishing features between the **a**-cells and the α-cells and discuss the role some of these differences might play in gradient shaping and signal detection. Finally, I compare the mating systems of different fungi and give a short overview of how these different species use the mating pathway to communicate

The yeast mating pathway and mating types

As described before, haploid yeast cells exist in two mating types: *MAT***a** and *MAT*α. Both cells express at their surface seven-transmembrane G-protein coupled receptors (GPCRs) that detect the presence of small peptides secreted by the opposite mating type. *MAT***a** cells secrete a-factor and express at their surface the α-factor receptor, Ste2, and *MAT*α cells secrete α-factor and express at their surface the **a**-factor receptor, Ste3. Pheromone binding triggers the dissociation of a trimeric G protein, whose βγ subunits lead to the activation of a Mitogen-Activated Protein (MAP) kinase cascade. Fus3, the MAP kinase at the bottom of the cascade, phosphorylates and activates proteins that induce both cell cycle arrest (via Far1, a cyclin-dependent kinase inhibitor) and the transcription of genes involved in the process of mating triggered by the activation of the transcription factor Ste12. Free $G_{\beta\gamma}$ also recruits Far1 that, in addition to arresting the cell cycle, recruits Cdc24, the guanine nucleotide exchange factor for Cdc42, the small G protein that controls actin polymerization and cell polarization [2] (Figure 1.2 adapted from [3]). This signaling cascade is conserved between the two mating types.

Active vesicle transport to the polarized site allows the deposition and remodeling of the yeast cell wall. The cells then grow, forming the pear-shaped mating protrusions commonly called shmoos. If another cell, of the opposite mating type, is also detecting a gradient and growing towards it, the polarities of the two cells can align and they can fuse their membranes and share their cytoplasm (Figure 1.1). The signal that triggers cell wall dissolution and membrane fusion is not yet known. Upon cell-cell fusion, the nuclei of the two cells migrate towards each other and nuclear fusion, or karyogamy, occurs. The resulting diploid cell can either divide by budding or undergo sporulation, given the right conditions.

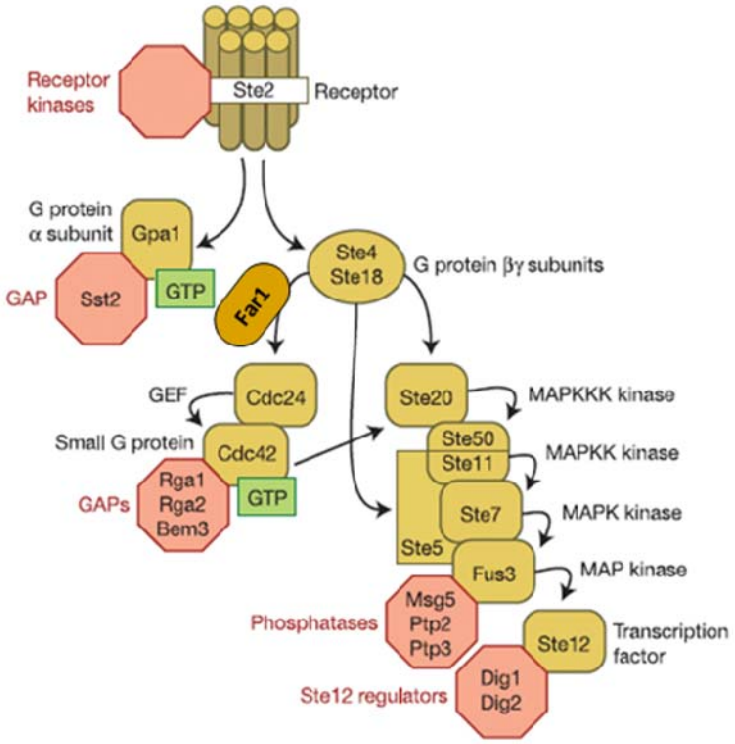

Figure 1.2 - Components of yeast signaling cascade (adapted from [3])
The mating response pathway for a *MAT***a** cell is shown. The α-factor pheromone binds the GPCR, leading to cell cycle arrest and actin polymerization (via Far1). *MAT*α cells express the Ste3 receptor and respond to **a**-factor, but downstream of the receptor the signaling cascade is the same in both cell types.

The differences in gene expression between the two mating types are governed by a single locus (*MAT*) that exists in two alleles: *MAT***a** and *MAT*α. *S. cerevisiae* is a pseudo-homothallic species, meaning that it encodes both mating type alleles in its genome, but each haploid cell expresses only one of them and can only mate with cells that express the opposite allele (for a comparative view on fungi mating systems see [4]) thus, the allele expressed defines the mating type. The *MAT***a** allele is expressed in the **a** cells and encodes two regulators: Mata1 and Mata2. The *MAT*α allele is expressed in α cells and encodes two different regulators: Matα1 and Matα2. Mating type specific proteins include the receptors, the pheromones, the proteins involved in pheromone maturation and secretion and small number of other mating-type specific proteins [5]. Both haploid cells express two transcription factors, Ste12 and Mcm1, that activate (or repress) the transcription of mating-specific genes. In the absence of the regulators, a complex is formed between Ste12 and Mcm1 leading to the expression of **a**-specific genes. In α-cells, Matα2 binds

Mcm1 to repress the **a**-specific genes and Matα1 binds both Mcm1 and Ste12 to activate the transcription of α-specific genes. The Mata1 regulator only plays a role in diploid cells, and at this time there is no function assigned to the Mata2 regulator (reviewed in [6]).

Some classic work in sexual specificity [7] has shown that mutant *MAT*α cells can mate as either **a** or α cells depending on which set of receptors and pheromones are expressed, but this work did not rule out a contribution of the *MAT*α and *MAT***a** loci to the mating process. We asked if the receptors/pheromones are also the major determinants of sexual identity by inducing same sex mating (*MAT***a**/*MAT***a** and *MAT*α/*MAT*α) when the mating pairs express complementary receptors and pheromones.

Yeast cells detect gradients and can mate over a wide range of conditions.

During mating, budding yeast cells need to detect the source of the signal coming from a potential partner. This process has been extensively studied using **a** cells that detect the small peptide pheromone α-factor. The pheromone's presence induces the **a** cells to polarize towards the source of the attractant and grow mating protrusions, or shmoos. **a** cells also secrete a specific aspartyl protease (Bar1) that degrades and inactivates α-factor. **a** cells that lack Bar1 have been described as being supersensitive to α-factor-induced G1 arrest and exhibit mating difficulties [8], [9], but even in the complete absence of the protease, the cells can polarize and grow shmoos.

Detection of chemical gradients is a necessary mechanism for living organisms, and it is often aimed at exploring the environment for nutrient sources, following prey or escaping poisonous environments or predators. *Chemotaxis* (polarized migration) and *chemotropism* (polarized growth) are crucial for various biological events, such as developmental patterning, axon guidance, wound healing.

The yeast *chemotropism* system shares many molecular components with systems requiring gradient detection, such as *chemotaxis*, but the yeast system shows some particularities (reviewed in [10]). First, it is a slow process (polarization in yeast takes approximately one hour, compared to seconds for *chemotaxis*). Second, the typical distance of mating cells is very small suggesting that cells respond to very steep pheromone concentration slopes. It may also involve a temporal aspect, as cells are known to modulate the level of pheromone they produce in response to the incoming signal. Another important difference is that most chemotactic cells quickly adapt

to a homogenous stimulation and return to a basal un-polarized state whereas yeast cells are able to detect gradients at concentrations at which they can spontaneously polarize [11], suggesting that suggesting that this adaptation mechanism might be missing.

Yeast cells can mate both as isolated pairs and in very dense mixtures, meaning that they have to be able to adapt to very different pheromone concentrations and be able to discriminate between many potential partners. A multitude of events in plant, fungal and animal development depends on a similar ability of cells to interact with only one of many possible partners. Examples include the interaction of neuronal growth cones with target cells (reviewed in [12]), myotube or vascular guidance (reviewed in [13-14]), the growth of pollen tubes to reach ovules (reviewed in [15]), and the mating of many fungi, including budding yeast (reviewed in [16-17]).

Bar1 homologues have been identified in different fungi, from *Candida albicans* [18] to *Schizosaccharomyces pombe* [19], always in MAT**a** cells, but these are the only known organisms that have a protease to degrade their own attractant, raising the question: why have yeasts evolved such an unusual and extracellular adaptation mechanism?

We looked at how Bar1 helps yeast cells detect α-factor gradients in controlled micro-fluidic devices, in the presence of single partners and in dense mating mixes.

The two pheromones are very different and this asymmetry is conserved.

There is no known equivalent of Bar1 being expressed by α-cells but this is not the only difference between the communication systems of the two haploid cells. The two pheromones, a-factor and α-factor, are secreted peptides that activate the corresponding receptors at the surface of the opposite cell. The α-factor, secreted by the α-cells, is encoded by two genes, *MFα1* and *MFα2*. The translated peptide is a pro-α-factor that includes several repeats of the pheromone, cleavage sites and a leader sequence. This leader sequence targets the α-factor precursor to the ER where it gets cleaved, at two consensus sequences, by three different proteins Ste13, Kex1 and Kex2. The mature peptides are now 13 amino acids long and get secreted via the normal secretory machinery (Figure 1.3.A). **a**-factor, secreted by the **a** cells, is also encoded by two genes *MFA1* and *MFA2*. The a-factor precursor only includes one copy of the pheromone and does not have a signal sequence for ER translocation and export. The pro-a-factor is processed at the cytoplasm by Axl1 and the cleaved peptide ends with a CAAX box at its C-terminus. This

sequence tags the peptide for a covalent lipid modification. First, two other proteases, Rce1 and Ste24 cleave after the cysteine residue at the CAAX consensus sequence. Then, a complex of two proteins, Ram1 and Ram2, transfer a farnesyl group to the protein. Finally, Ste14 methylates the cysteine residue at the C-terminus. These highly hydrophobic peptides are then transported to the membrane and secreted via an **a** cell-specific transporter Ste6 (Figure 1.3.B, reviewed in [6]).

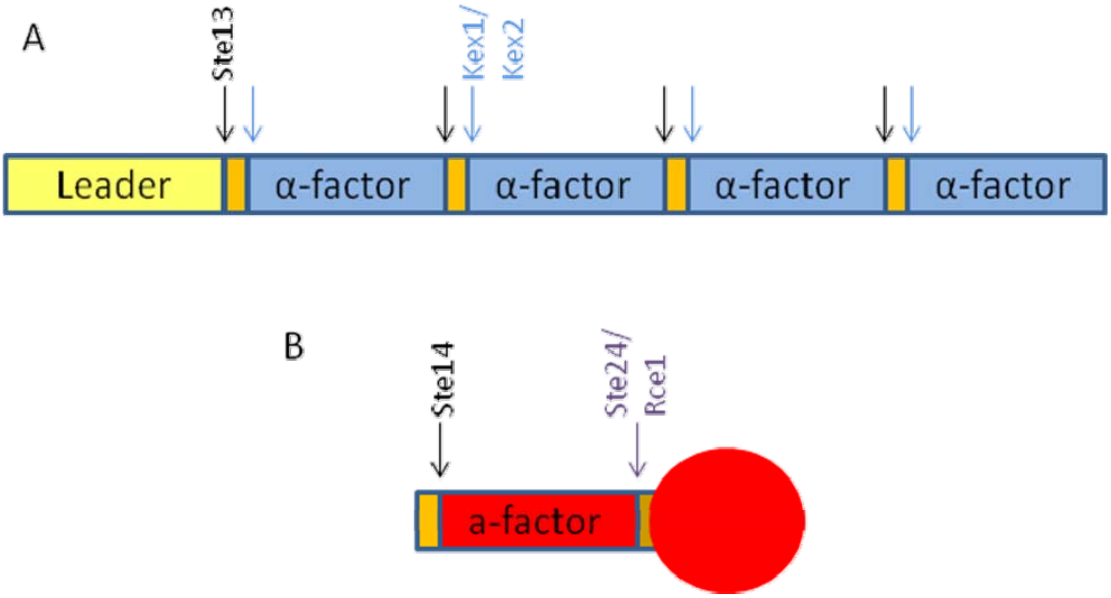

Figure 1.3 – Processing of the *S. cerevisiae* pheromones.
A- Pro-α-factor
B – Pro-a-factor with lipid modification (represented by the large red circle)

Homologues of some of these enzymes exist in higher eukaryotes. Axl1 is related to an insulin maturation protein and Ste6 belongs to a highly conserved family of ABC (for ATP-binding cassette) transporters that includes the cystic fibrosis channel CFTR. The asymmetry in the pheromone's physical-chemical properties is also conserved. In all of the sequenced members of the Ascomycota, the phylum of the Dikarya to which *S. cerevisiae* belongs, it is possible to identify α-like pheromones, existing in one or more repeats, and a-like pheromones, with the CAAX box at their C-terminus. The Basidiomycota, the phylum that includes all mushrooms and smuts, have only lipid-modified pheromones. It is not known why a-factor needs to be so heavily modified, but the farnesyl group is required for signaling and mating. *S. cerevisiae* cells expressing analogs of the pheromone that are non-carboxymethylated and/or non-

farnesylated mate 1000 fold worse [20]. This observation is also true for members of the Basidiomycota, such as the corn smut, Ustilago maydis [21].

Different yeasts share the same pheromones

The pheromone receptors belong to a ubiquitous family of GPCRs that play fundamental roles in cell signaling. *S. cerevisiae* has only three members of this family, but several hundreds have already been identified in humans where GPCRs represent the largest class of cell surface receptors (reviewed in [22]). These receptors are usually involved in transducing extracellular stimuli into intracellular signals and can be activated by light, neurotransmitters, odorants, lipids, hormones, chemokines, sugars, etc (recently reviewed in [23]).

As mentioned before, these receptors are characterized by an extracellular N-terminal domain, seven hydrophobic transmembrane domains and an intracellular C-terminus. Upon agonist binding, the receptor undergoes a conformational change that allows the receptor to associate (or release) its heterotrimeric G proteins, eliciting an intracellular response. These receptors are thought to heterodimerize and be very specific (recently reviewed in [24])

In *S. cerevisiae*, GPCRs are involved in detecting glucose, via Gpr1, and responding to mating stimulus, via the pheromone receptors Ste2 and Ste3. The Ste2 and Ste3 receptors have very little homology, although they are predicted to have the same topology, and signal by binding to the same intracellular machinery. These receptors are conserved and we can find homologs for both Ste2 and Ste3 in very distant fungi (reviewed in [22]).

Their conservation is even more striking in the *Saccharomyces sensu stricto* group. This is a collection of fermenting yeast species that includes *S. cerevisiae*, *S. paradoxus*, *S. cariocanus*, *S. mikatae*, *S. kudriavzevii*, *S. bayanus*, and the hybrid *S. pastorianus* [25], [26]. Genomic analysis of these species has revealed very high synteny and over 80% of their genes show over 80% sequence identity [27]. Additional to the high receptor similarity, *S. cerevisiae*, *S. paradoxus* and *S. mikatae* share the same a and α-factors; *S. cerevisiae* and *S. castelii*, an off-group Saccharomycete, share all but one amino acid in their a-factor pheromone and 10 out of 13 aminoacids in their α-factor-like peptides (this study). This is also known to be true in the Neurospora clade, another family of Ascomycetes, which are believed to share the same α-factor pheromone, although there is some variability between their receptors [28]. Co-existence between *sensu stricto* species has been reported [29], [30]. Natural hybrids between the *sensu stricto*

species can be isolated and lab crosses have shown that there is no significant pre-zygotic isolation (reviewed in [31]) . The fact that the hybrids are sterile (unable to sporulate or giving rise to dead spores) raises the interesting question of why they have not diverged to prevent cross-talk between the pheromones and the receptors. Furthermore, it is not known how specific these receptors are to their own pheromones or how much different species, that may or may not share pheromones, use these GPCRs to communicate.

We generated a synthetic system of receptor expression and tested the receptor's response to pheromones of close and distantly related fungi. We analyze the results from a speciation perspective.

CHAPTER 2

Regulated Cell-Cell Communication Makes Yeast Mating Economic and Robust

Budding yeast cells exist in two mating types, **a** and α, which use peptide pheromones to communicate with each other and induce mating. We used quantitative measurements to understand how yeast cells mate efficiently under a wide range of conditions, ranging from isolated pairs of cells to dense mating mixtures. We show that α cells devote 1% of their protein synthesis to making just enough α-factor to initiate an inter-cellular positive feedback loop that induces cell cycle arrest, polarization, and fusion. Cells make a sharp transition between polarization and budding, despite substantial noise in the strength of signaling. They only respond to pheromone gradients over a narrow range, implying that cells should mate efficiently as isolated pairs but not in dense mating mixtures. We resolve this paradox by showing that **a** cells maintain the pheromone concentration at their surface within this range by regulating the expression of Bar1, the protease that degrades α-factor. We conclude that yeast mate robustly by using the secreted protease to regulate ligand concentration and we discuss this as a general strategy for finding partner cells.

INTRODUCTION

Sex takes time and energy. Mating cells must signal to each other and arrest their cell cycles before they fuse. In nature, the budding yeast, *Saccharomyces cerevisiae*, is mostly found as diploid cells. Haploids exist in two mating types, **a** and α, which can proliferate asexually or mate with each other to form diploids. The **a** cells secrete **a**-factor and bear an α-factor receptor, whereas α cells secrete α-factor and detect **a**-factor. Both express a common signal transduction pathway that is triggered by the binding of mating pheromones to their sex-specific receptors. The signal passes through a MAP kinase cascade and induces gene expression and polarized growth; polarization transforms ovoid cells into pear-shaped shmoos that grow towards each other (chemotropism) and fuse with each other at their tips (see Chapter 1 and Figure 1.1.A for more details, reviewed in [32]).

Mating in budding yeast has been widely studied as an example of inter-cellular communication between eukaryotic cells. The components of the signaling pathway and many of the connections between them are known; Hartwell and his colleagues showed that mating depends on communication between **a** and α cells ("courtship") [33-34], Segal [11] demonstrated that cells polarize up pheromone gradients, the level of noise in the signaling pathway has been measured [35-36]. Bar1 (an α-factor-protease secreted by **a** cells [9]) is essential for efficient

mating and has been proposed to help cells return to the cell cycle if they fail to mate [37], discriminate in favor of pheromone secreted by nearby α cells [37], and modulate the direction of the pheromone gradient when an **a** cell is surrounded by multiple α cells [38]. Despite these studies, we lack an integrated understanding of mating that answers the following questions: do decisions about mating show the same level of noise as signaling? How wide a range of pheromone gradients can cells detect? What is the physiological role of Bar1? How do cells regulate their pheromone response to mate efficiently under a wide range of conditions, varying from a pair of isolated **a** and α cells to very dense mating mixtures, where each cell is surrounded by multiple partners of the opposite mating type?

Directional signals come in two forms. The first are long range signals that cells move towards by chemotaxis. For instance, starved slime molds emit pulses of cyclic AMP which induce them to crawl towards each other and aggregate using a collective signal that increases with the density of cells. The strength of the signal and its distance from the responding cell can vary over orders of magnitude. Slime molds respond to this challenge by adaptation, adjusting their response so that they are maximally sensitive to small concentration changes around the current concentration of attractant or repellent. The other type of signal is a homing signal, whose purpose is to guide one cell to form an intimate interaction with a single partner. Many cells that home cannot move and must grow or polarize toward their partners. Because a yeast cell can only mate with one partner, it must distinguish the signal from a nearby suitor from the collective signal emitted by more distant cells. Since the signal from distant cells could be stronger than the signal from close neighbors, adaptation of the sort shown by slime molds, bacteria, and neutrophils will not help a yeast cell pick a mate. Instead, cells must find a way of discriminating against the signal from distant cells. Our hypothesis is that proteases like Bar1 discriminate by reducing the half-life of secreted signals and thus keeping signaling local. We took a quantitative approach to the cellular functions that govern mating. We measured the level of pheromone secretion, the range of pheromone concentrations that allow accurate gradient detection, the noise in the decision between mating and proliferation, the induction of the *BAR1* gene, and the role of Bar1 in controlling the mean pheromone level that α cells experience. Although many of these problems have been studied before [11, 35-36, 39] we do not understand fully how yeast cells mate efficiently under a wide range of conditions. In the first half of the paper, we use **a** cells that lack Bar1 so that we can quantify responses to α-factor. We find that 1) pheromone secretion

accounts for about 1% of overall protein synthesis, 2) that this level can initiate courtship but not cell polarization, 3) that cells decide precisely, rather than noisily, whether to proliferate or polarize, and 4) that they only detect gradients over a narrow range of pheromone concentrations. In the second half of the paper, we explore the role of Bar1 in allowing yeast cells to mate efficiently over a wide range of conditions and we find that 5) the regulated secretion of Bar1 makes mating robust by keeping the pheromone concentration at the surface of α cells within the narrow range needed for accurate gradient detection.

RESULTS

Measuring pheromone production and response

Unlike many unicellular eukaryotes, which only make pheromones when they are starved, haploid budding yeast are always ready to mate. This willingness implies a conflict between the benefit of making enough pheromone to signal to potential partners and the energetic cost of pheromone production.

For α cells, this cost is set by the minimum pheromone concentration that **a** cells can detect and the physics of diffusion. At a minimum, α cells must produce enough α-factor to induce a response in **a** cells that lack Bar1, and thus cannot degrade α-factor. This value has been reported to be as little as 0.1 [35, 39] and as much as 15 nM [36]. For simplicity, we assume that a single α cell lies on the surface of an agar plate, very far from any other α cells, that it secretes pheromone uniformly from its surface, and that the pheromone that it secretes diffuses into the agar, which acts as an infinite sink. Under these conditions, continuous pheromone secretion rapidly produces a spatial gradient in pheromone concentration that does not change over time.

A spherical cell producing I molecules per second reaches a steady state concentration of $C_0=I/(4\pi aD)$ at its surface [40], where a is the cell radius (~2.5 μm) and D is the diffusion coefficient (~300 μm^2/sec for α-factor [11]). This simple relationship suggests the upper estimates for the sensitivity to α-factor are unreasonable: if the pheromone concentration at an α cell's surface is 15 nM, it must produce α-factor at 85,000 molecules/sec, about 10 times the estimated rate of total protein synthesis (≈9,000 molecules/sec [41]). This discrepancy is reduced by the fact that it takes only 41 amino acids to encode a molecule of α-factor (as opposed to an

average protein of 450 amino acids [41]), but the pheromone concentration at the surface of an α cell must still be far below 15 nM.

We started by inducing **a** *bar1*Δ cells with different concentrations of synthetic α-factor and scoring their morphology, by light microscopy, after 2h induction. With this information, we generated a calibration curve (Figure 2.1.A) where we make each pheromone concentration correspond to an observed percentage of shmoos. To measure the pheromone secretion rate, we grew α cells exponentially in rich medium, transferred them to fresh medium for up to 2 hours, removed the cells, incubated the conditioned medium with **a** *bar1*Δ cells, and scored the fraction of the cells that had formed shmoos. We then compared the percentage of shmoos observed when the cells had been induced with the conditioned media, to the percentage of shmoos observed when the cells were responding to known concentrations of α-factor. This comparison allowed us to estimate that cells make α-factor at 740 ± 220 molecules/cell/sec (Figure 2.1.B), corresponding to a steady state concentration at the cell surface of ~0.1nM α-factor, far below the pheromone concentrations used in many previous studies, and only enough to occupy 1% of the α-factor receptors (which have a K_d of about 6 nM [42]). Even at this low concentration, we estimate that α-factor synthesis accounts for about 1% of total protein synthesis.

Can **a** cells detect such low levels of α-factor and, if they can, what responses are induced? We began by studying the interactions of one **a** cell with one α cell. Because we wanted to study the behavior of the **a** cell as the number of α cells and the level of α-factor increased, we kept the α cells from mating by using the *far1-H7* mutation; α *far1-H7* cells respond normally to pheromone but have polarization defects that reduce the efficiency of mating [43]. When an **a** *bar1*Δ cell is placed next to an α *far1-H7* cell, it arrests within a few cell divisions and shmoos (Figure 2.1.C). This observation could have two explanations: 1) the basal pheromone secretion of an α cell induces cell cycle arrest and shmooing, or 2) weak stimulation of the **a** cell starts a positive feedback loop. In such a loop, the basal level of α-factor could not induce the **a** cell to shmoo, but could make it secrete more **a** factor, stimulating the α cell and inducing it to secrete enough α-factor to make its partner shmoo.

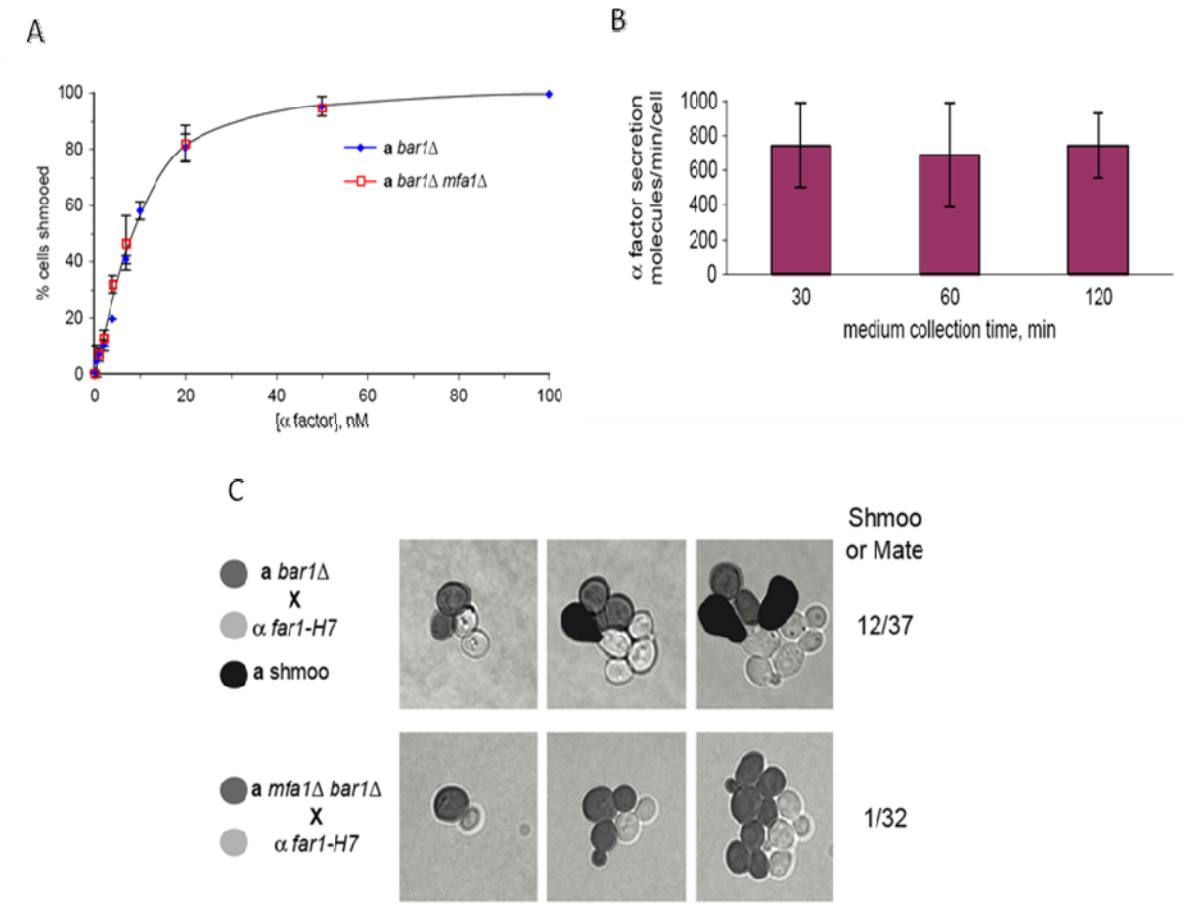

Figure 2.1 Response of a-cells to pheromone
A- Calibrating pheromone response and pheromone secretion. The response of **a** cells to α-factor. Cells were treated with the indicated concentrations of α-factor for 2 hours and the fraction of cells that had formed shmoos was determined by light microscopy. The calibration was performed on two strains, **a** *bar1*Δ (MP384) and **a** *bar1*Δ *mfa1*Δ (YTK277), to check that deletion of the major gene encoding **a**-factor had no effect on the response of cells to α-factor. The assay was performed at least 3 times, at least 200 cells were counted for each time point. B - Bar graphs for the secretion of rates of α-factor. Conditioned medium was collected from α cells (MP634) for the indicated periods and assayed for its ability to induce shmooing in a *bar1*Δ cells (MP384). The assay was performed 4 times, at least 200 cells were counted for each time point. C - The response of individual **a** cells to α cells. Pairs of cells were micromanipulated to touch each other and observed. Bars show standard deviations. See Supplementary Table 2 for statistical tests.

We distinguished these possibilities by comparing the behavior of **a** *bar1*Δ cells that make different amounts of **a** factor; *mfa1*Δ cells lack one of the two structural genes for **a** factor and produce less **a** factor than wild type **a** cells [44]. Cells that make normal levels of **a** factor arrested and formed shmoos when placed next to α cells, whereas the **a** factor-deficient cells

failed to arrest or arrested transiently (Figure 2.1.C), even though both types of **a** cells responded identically to synthetic pheromone (Fig 2-1-A). Thus unstimulated α cells secrete just enough pheromone to make nearby **a** cells secrete more **a** factor and this reciprocation induces the α cell to secrete enough pheromone to induce polarization of the **a** cell.

Do low concentrations of pheromone induce changes in gene expression? We monitored the transcriptional response of the *FUS1* and *BAR1* genes by fusing their promoters to YFP and measuring promoter activity as the rate at which fluorescence changed over time; *FUS1* encodes a protein that plays an essential role in cell fusion and is the most commonly used reporter of pheromone signaling, whereas *BAR1* encodes the α-factor protease. Because we wanted to follow individual cells for long periods and because cells can remove α-factor from the medium by endocytosis [45], we used microfluidics [46] (Figure 2.2.A) to expose **a** *bar1*Δ cells to a wide range of temporally stable concentrations of α-factor. **a** *bar1*Δ cells expressing P_{BAR1}-*YFP*and **a** *bar1*Δ cells expressing P_{FUS1}-*YFP* were distinguished from each other by fluorescent cell wall labeling, mixed together, placed in the same channel and exposed to a pheromone gradient. The *BAR1* promoter responded more strongly and more sensitively than the *FUS1* promoter (Figure 2.2.B): *BAR1* was induced 10 fold by 0.1 nM α-factor, whereas *FUS1* failed to respond at this concentration (Figure 2.2.B, inset). Our results are consistent with the observation that cell fusion needs higher levels of pheromone-induced signaling than cell polarization [47].

Cells respond precisely to pheromone

The induction of *FUS1* by α-factor is noisy: at the same pheromone concentration, different cells transcribe *FUS1* at different levels [35]. We asked if cells showed a similar variation in their fates by examining their morphological response to different pheromone concentrations. We did time-lapse recording of cells, expressing a polarisome marker, Spa2, fused to a fluorescent protein, which allowed us to precisely assess each cell's behavior at each concentration. Cells shmooed at concentrations above 1nM pheromone (Figure 2.2.C) and we saw little cell-to-cell variability: at 0.6 nM α-factor, 96% of cells bud after a delay; at 1 nM, buds and shmoos are equally common; and at 2 nM, 93% of the cells shmoo (Figure 2.2.C). Note that the small fraction of cells that shmoo relative to the results in Figure 2.1.A reflects the short time (≤2 hours from the last round of cytokinesis) allowed for cell polarization in the calibration experiments.

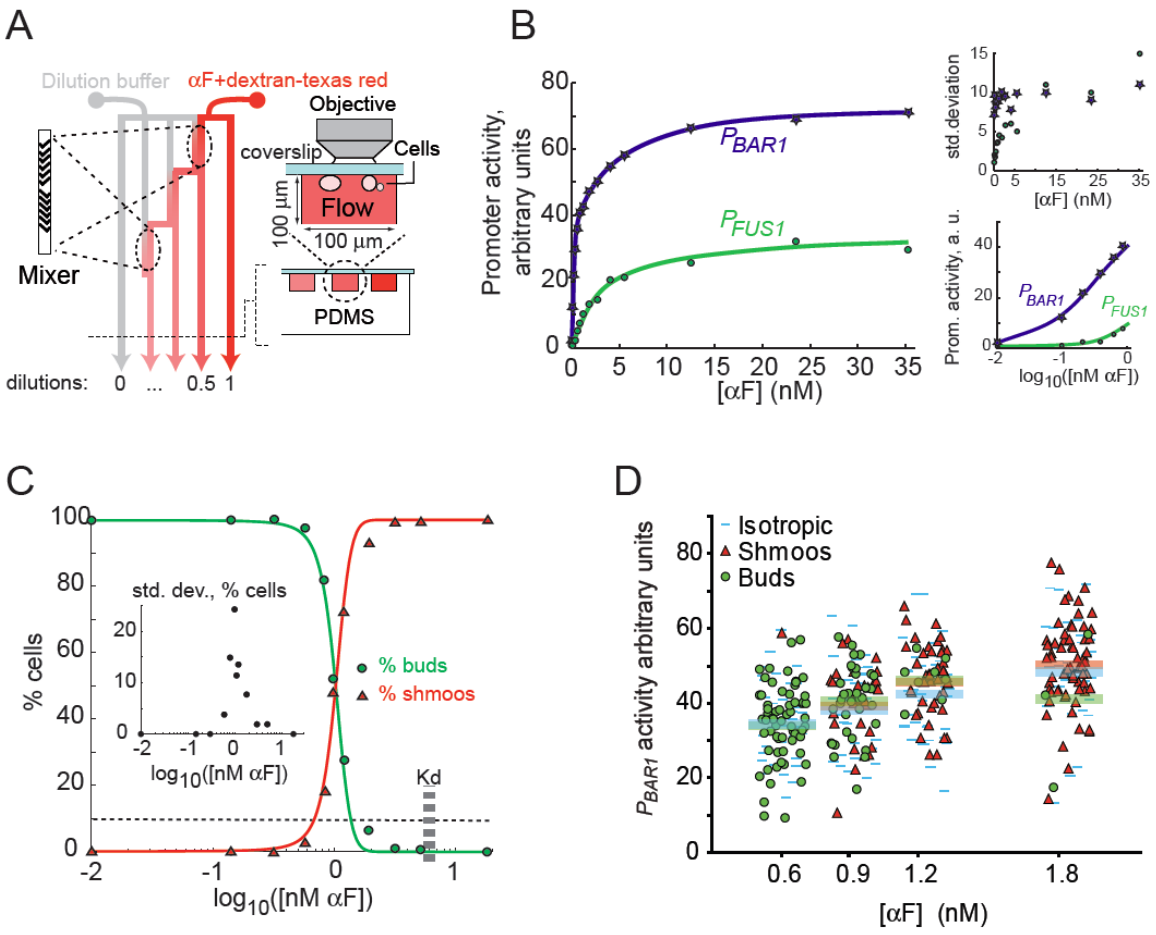

Figure 2.2 - The response of *bar1*Δ cells to homogenous stimulation by α-factor
A) Schematic of a device to produce a range of pheromone concentrations by using chaotic mixers in dilution chambers. The diagram shows a plan view (left) and cross sections at two magnifications (right). B) Induction of the *FUS1* and *BAR1* promoters by α-factor. The *BAR1* promoter is induced by lower levels of α-factor than the *FUS1* promoter. C) Bud/shmoo transition in spatially uniform fields of pheromone. About 4000 cells in seven independent dilution chambers were scored. The inset shows the standard deviation of the fraction of different events between experiments. The measured dissociation constant of α-factor from Ste2 is indicated (K_d) [42]. The half maximal point of the sigmoidal fit is 1.02 +/-0.03 nM and the Hill coefficient for the transition between budding and shmooing is 6.5 +/- 0.6 (95% confidence interval). D) Distribution of *BAR1* promoter activity in cells that budded or shmooed when treated with uniform fields of α-factor with the indicated concentrations. Each point represents a single cell. Cells that budded are shown as green dots, those that shmooed as red triangles, and cells which neither shmooed nor budded in the course of the recording as blue lines. The average promoter activity for each behavior is shown as a thick colored bar.

This precise morphological transition contrasts with the noisy expression from the *BAR1* and *FUS1* promoters, measured as the rate of YFP production. At 0.9 and 1.2 nM α-factor, there were enough cells of each type to perform t tests, which revealed that at each concentration, the expression levels in the three different morphologies were statistically indistinguishable from each other. However, for both cells that shmooed and budded, the response levels were statistically different between 0.9 and 1.2 nM α-factor ($p < 0.025$ for both comparisons) (Figure 2.2.D).

Cells polarize accurately over a narrow range of gradients

We also measured the response of **a** *bar1*Δ cells by exposing them to pheromone gradients in laminar flow chambers (Figure 2.3.A and 2.3.B). These experiments led to four conclusions: i) The transition between budding and shmooing occurs at the same concentration (1 nM) as it does for cells in homogenous pheromone concentrations (compare Figure 2.3.C with Figure 2.2.C), ii) cells in gradients respond faster than those treated with uniform pheromone concentrations (Figure 2.3.D), iii) cells can only detect gradients accurately over a narrow range (mean pheromone concentrations from 0.7 to 2.5 nM) (Figure 2.3.E), and iv) cells detect gradients most precisely at the mean concentration (1 nM), which equals the lowest concentration that induces shmooing in an isotropic field of pheromone (compare Figure 2.2.C and 2.3.E).

As long as we consider the mating of isolated pairs of cells, our results make sense. Single cells signal weakly, are induced to signal more strongly when they encounter a partner of the opposite sex, and cannot overstimulate their partners. But budding yeast also mate efficiently as dense mixtures of **a** and α cells. In this situation, the same positive feedback would be disastrous because the mean pheromone concentration an **a** cell experiences depends on how many α cells are nearby. We start by considering a single **a** cell on an agar surface surrounded by concentric rings of α cells (Figure 2.4.A). The α cells secrete α-factor, which spreads by three-dimensional diffusion through the volume of the agar. If the radius of a ring is r, the contribution an individual α cell makes to the pheromone concentration at the **a** cell falls as $1/r$, but because the number of cells in a ring is proportional to r, each ring of α cells contributes the same amount of pheromone to the location of the **a** cell and thus the pheromone concentration it experiences increases linearly with the number of rings of α cells surrounding it (Figure 2.4.A). If we assume that **a**-factor-induced α cells have 1 nM α-factor at their surface, the α-factor concentration in a dense

film of **a** and α cells would exceed 100 nM, far above the concentration range at which **a** *bar1*Δ cells can detect gradients.

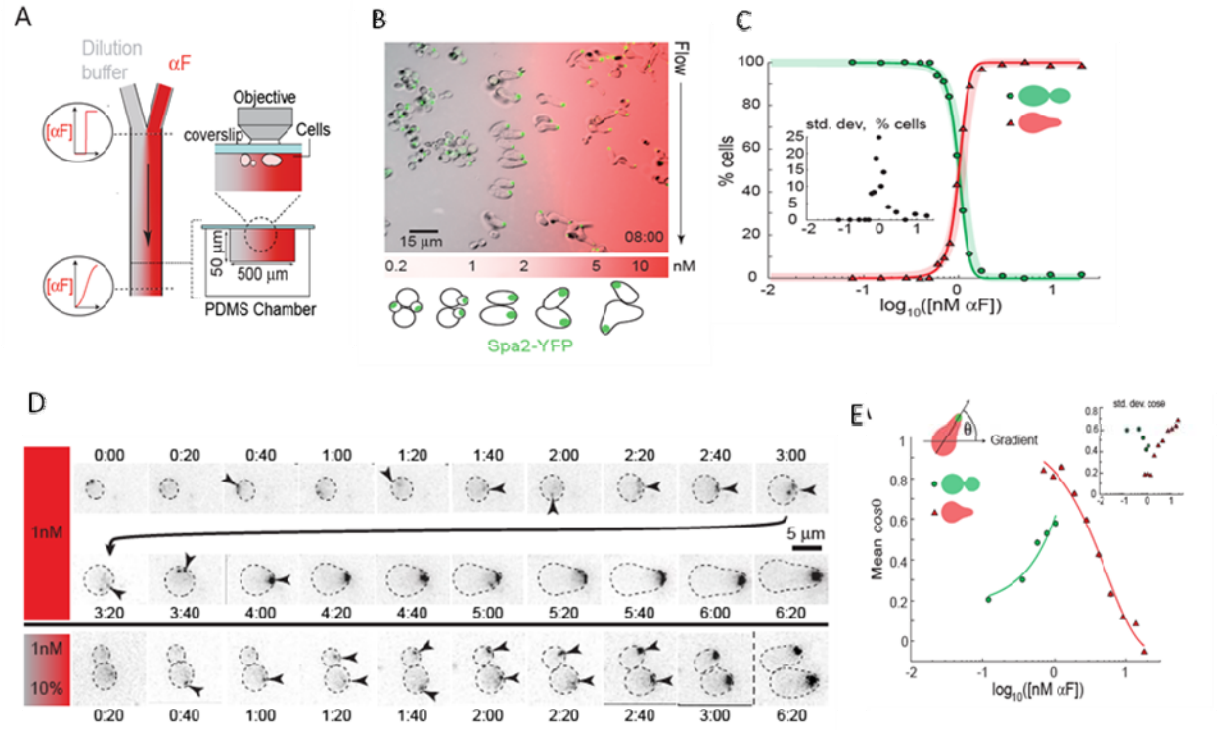

Figure 2.3 - *bar1*Δ cells respond faster to α-factor gradients.
A) Producing pheromone gradients in a laminar flow chamber. Pheromone mixed with a fluorescent dextran and a dilution buffer enter through two ports and diffusion between the two fluid streams creates a temporally stable, exponential gradient (see Materials and Methods for more details). The left hand view is from the top of the apparatus and right hand views are two different magnifications of a cross-section, showing the cells attached to the coverslip that forms the roof of the chamber. B) A field of cells which have been exposed to a gradient of pheromone (red). Concentrations along the gradient are indicated and a schematic representation of stereotypical cell behavior at various concentrations is shown below the micrograph. Cell polarity was recorded by the distribution of Spa2-YFP (shown in green). C) The transition between budding and shmooing, quantified as in Fig. 2.2.C. The lightly shaded, thick curves show the data from spatially uniform pheromone concentrations (Fig. 2.2.C) for comparison. For every cell, the difference in concentration between the two edges of the cell was >5% (expressed relative to the mean concentration the cell experienced). D) A comparison of the timing in individual cells exposed to a uniform field and a gradient of α-factor. Images were taken every 20 minutes and show the fluorescence of Spa2-YFP with the contrast inverted for clarity so that areas of high fluorescence appear black. Note the small unstable Spa2p spots that appeared in the homogenously stimulated cell before a stable polar cap developed. In the gradient, a small Spa2p spot in the direction of the gradient gradually grew stronger. E) The accuracy of gradient detection as a function of pheromone concentration. Accuracy is defined as the mean cosine of the angle between the gradient and Spa2 polar cap.

Bar1 promotes mating by regulating global pheromone concentrations

Regulated secretion of Bar1 is an attractive mechanism for controlling α-factor concentrations. Bar1 limits the lifetime of diffusing α-factor, preventing distant α cells from contributing to the pheromone concentration at the surface of an **a** cell. We argue that the induction of Bar1 has been tuned to keep the α factor concentration at the surface of **a** cells within the narrow range required for accurate gradient detection; the basal expression of Bar1 is low enough to allow **a** cells to detect the low levels of α-factor made by a single cell, but maximal induction of Bar1 allows cells in dense mating mixtures to dramatically reduce what would otherwise be saturating pheromone levels.

To test the role of Bar1 in regulating the α-factor response, we used the *FUS1* promoter to report on the pheromone concentration cells are experiencing. In α-factor-treated cells carrying P_{FUS1}-*YFP,* fluorescence accumulates at an average rate that is constant for at least 12 hours, suggesting that there is little or no adaptation in the pheromone response. Thus we can interpret the transcription of the *FUS1*promoter as a proxy for the pheromone concentration that the **a** cells experience. We compared the induction of P_{FUS1}-*YFP* in three types of **a** cell: *bar1*Δ cells, cells expressing *BAR1* from its own promoter (*BAR1*), and cells expressing Bar1 from the *MET3* promoter (P_{MET}-*BAR1*) in medium lacking methionine (which represses P_{MET3}). At every α-factor concentration, the *bar1*Δ cells gave the strongest induction of P_{FUS1}-*YFP*, but the relationship between *BAR1* and P_{MET}-*BAR1* cells switched as the pheromone concentration rose (Figure 2.4.B). Below 3.5 nM α-factor, cells expressing Bar1 from its own promoter responded more strongly than P_{MET}-*BAR1*, demonstrating that the basal level of Bar1 expression is low. At higher pheromone concentrations, the *BAR1* cells responded more weakly than the P_{MET}-*BAR1* cells. Thus the P_{MET}-*BAR1* cells express too much Bar1 in the absence of pheromone and too little at high pheromone levels, showing that tight regulation of Bar1 production is needed to allow **a** cells to respond appropriately to a wide range of pheromone concentrations.

The induction of Bar1 extends the sensitivity of **a** cells to α-factor. We compared the response of the *FUS1* promoter in a *bar1*Δ and a *BAR1* cells to estimate the pheromone concentration that Bar^+ cells experience. We argue that if the two strains respond equally strongly, their receptors must be exposed to the same concentration of α-factor. The minimum

pheromone concentration required for accurate polarization of *bar1*Δ cells is 0.7 nM and *BAR1* cells require just under 5 nM pheromone before their response reached the same level; but even at 30 nM, *BAR1* cells are still responding more weakly than *bar1*Δ cells respond at 2.5 nM pheromone, their upper limit for accurate gradient detection. These comparisons show that Bar1 induction reduces the pheromone concentration at the cell surface 7- to 12-fold relative to the concentration in bulk solution and that this reduction increases at higher pheromone levels, where BAR1 is more strongly induced.

The absence of Bar1 dramatically impairs the mating of **a** cells [8]. If the function of secreted Bar1 is to reduce the half life and diffusional range of α-factor in mating mixtures, adding an exogenous protease should rescue the mating of **a** *bar1*Δ cells. We mixed **a** *bar1*Δ and α cells at high density and measured how efficiently the **a** cells mated by following individual cells by videomicroscopy. Because the cells are non-uniformly distributed, the mating efficiency of control *BAR1* cells is only 30%. In the absence of exogenous protease, < 1% of the *bar1*Δ **a** cells mated. As we added increasing amounts of pepsin (the vertebrate protease most homologous to Bar1), the efficiency of mating rose to 30% at 10 μg/ml pepsin and then fell, demonstrating that there is an optimum level of protease activity (Figure 2.4.C) and suggesting that the regulated production of Bar1 is essential for efficient mating. We tested this idea by varying the ratio of α cells to three types of **a** cells: **a** *BAR1*, **a** *bar1*Δ, and **a** *bar1*Δ cells with added pepsin (Figure 2.4.D). The results show that regulated protease production allows cells to mate efficiently under a wide range of conditions. The *BAR1* cells mated efficiently over a 100 fold range of **a**:α ratios, but the **a** *bar1*Δ cells only mated efficiently when **a** cells outnumbered α cells. Adding an exogenous protease reverses this trend: the **a** *bar1*Δ cells with pepsin mated worse as α cells became less frequent. This experiment demonstrates that the absence of protease, or unregulated protease activity make mating fragile for the same reason: only a small range of ratios of **a**:α cells produce pheromone levels in the narrow range needed for gradient detection. This idea is supported by the observation that mixing *BAR1* and *bar1*Δ cells allows cells lacking Bar1 to mate over a wide range of conditions, because the nearby *BAR1* cells regulate their Bar1 production in response to the local rate of α-factor release (MP and JGS, data not shown).

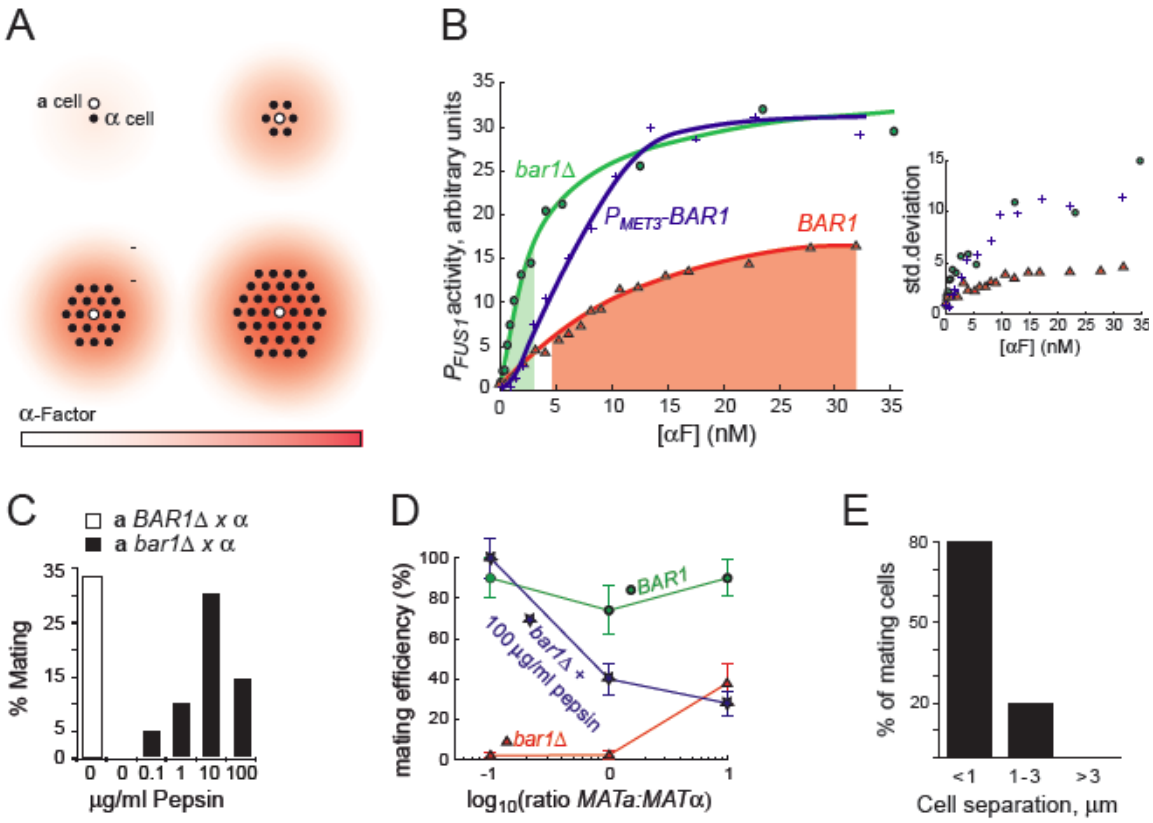

Figure 2.4 - Bar1 controls local pheromone concentrations to ensure efficient mating
A) Each α cell (black dot) produces the same amount of pheromone (red haze), but the contribution from different cells add to each other, increasing the pheromone concentration that an **a** cell (open dot) experiences as the number of α cells rises at a constant cell density. B) *FUS1* promoter activity (estimated from the rate at which the fluorescence of individual cells increased, see Materials and Methods for details) in *bar1Δ (*green), *BAR1* (red), and P_{MET3}-*BAR1* (blue, Bar1 expressed from the *MET3* promoter in the absence of methionine) cells as a function of pheromone concentration. The green shaded area shows the range of mean pheromone concentrations that allowed a *bar1*Δ cells to detect pheromone gradients, and the red area shows the concentration of pheromone where a *BAR1* cells showed a similar level of promoter induction. C) The mating efficiency of **a** *BAR1***a** and of *bar1Δ* cells in mating mixtures containing various amounts of pepsin. The levels of mating at 1 and 100 µg/ml pepsin were significantly different from those at 10 µg/ml ($p < 0.02$, χ^2 test). D) Quantitative matings were performed with various fractions of α cells in the mating mix. The Bar1 status of the **a** cells and the mating efficiency of the minority partner are shown. E) The initial separation between cells that mated. Three independent experiments, totaling 30 fields, each containing about 40 cells (50% **a** and 50% α) were quantified. For each mating event, the shortest distance between the two mating cells was measured just before the first appearance of a Spa2 polar cap in one of the two partners.

If Bar1 is insulating **a** cells from the pheromone secreted by distant α cells, the range of pheromone-based communication should be short. We studied the response of cells as a function of the distance between them. Cells that touch each other arrest and mate, cells that are separated from each other by a cell diameter (5 μm) continue to proliferate, and at intermediate distances, a fraction of the cells arrest and mate (Figure 2.4.E). Because cells bud up pheromone gradients, they can approach a distant partner by budding rather than shmooing towards it, thus postponing the decision between arrest and proliferation.

DISCUSSION

We used quantitative measurements to ask how budding yeast cells can mate efficiently with each other under conditions that range from the equivalent of a desert island (two cells in isolation) to a crowded discotheque (dense mating mixtures). Yeast cells pay to advertise their sexuality by making and secreting pheromones. We show that to minimize this cost, α cells produce the minimum quantity of α-factor needed to induce an **a** cell to increase **a** factor secretion. This induction starts a positive feedback loop that eventually produces enough pheromone to arrest the two cells in G1 and induce them to polarize. Despite noise in their signaling pathway, pheromone-stimulated cells make a precise decision to shmoo or resume budding: **a** cells exposed to less than 1 nM α-factor bud, and those exposed to more shmoo.

Mating partners must polarize so that they grow towards each other, touch, and fuse. Since pheromone gradients direct polarization, cells must be able to detect the gradient produced by the nearest partner despite the presence of other potential partners that are more distant. For **a** cells, this discrimination depends on Bar1, the protease that degrades α-factor. In its absence, we find that cells only respond accurately to a narrow range of gradients, and *bar1*Δ cells only mate efficiently in conditions that keep the overall α-factor concentration low. Figure 2.5.A illustrates the combination of positive feedback and homeostasis involved in mating: **a-** and α-factor induce each other's synthesis allowing the cells to produce the high pheromone levels needed for mating, and α-factor induces Bar1 to prevent **a** cells from being over stimulated.

Pheromone proteolysis by Bar1 has two effects: it reduces the pheromone concentration at the surface of the **a** cell, keeping it within the range needed for accurate polarization, and it reduces the distance that individual pheromone molecules can travel thus emphasizing the contribution from nearby α cells (Figure 2.5.B). The basal level of Bar1 production ensures that **a**

cells continue to proliferate rather than being arrested when they encounter α cells that are too far away to mate with. Figure 2.5.B shows how Bar1's ability to reduce the range of α-factor diffusion helps to separate the contribution of pheromone from different α cells.

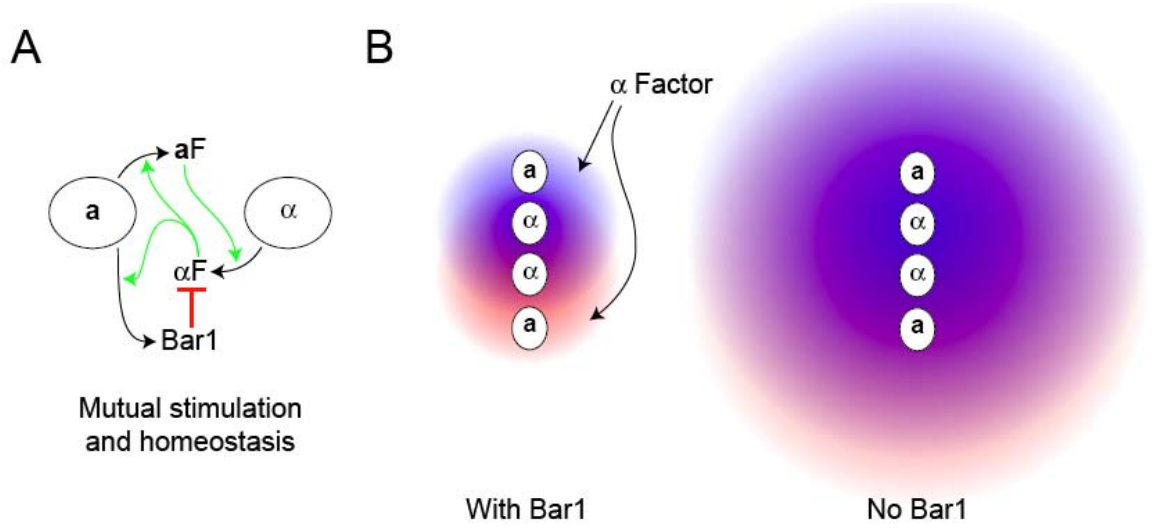

Figure 2.5 – The role of Bar1 in mating
A) Shows the interaction between **a** and α cells during mating. The secretion of α-factor is stimulated by a factor and vice versa and α-factor stimulates the secretion of Bar1 from **a** cells thus preventing the concentration of α-factor at the surface of **a** cells from exceeding the levels needed for accurate gradient detection. B) The effect of Bar1 on insulating **a** cells from α-factor secreted from distant cells. The left panel shows two α cells secreting α-factor, one marked red and the other blue, with the diffusional range of the α-factor restricted by Bar1 secreted from the two **a** cells. In the right panel, the absence of Bar1 increases the diffusional range of the pheromone exposing the **a** cells to higher levels of α-factor and preventing them from distinguishing the pheromone contribution from near and distant cells.

Morphological and transcriptional responses are poorly correlated

We found a profound difference between morphological and transcriptional responses to pheromone. As others have reported [35-36], the transcriptional response to pheromone is noisy; individual cells treated with the same concentration of pheromone show a wide range of promoter activity. In contrast, the morphological response to pheromone is stereotyped, with cells switching from budding to shmooing over a narrow range of pheromone concentrations.

According to our transcriptional reporters, some cells that shmoo are signaling less strongly than those that bud, raising the question of how strongly the morphological responses of cells depends on the activity of the MAP kinase cascade that pheromones activate. Hartwell and his colleagues showed that cells that over express the pheromone-activated transcription factor (Ste12) can polarize towards mating partners in the absence of the MAP kinase cascade that normally transmits information about pheromones to the nucleus [47]. These two observations suggest that other aspects of the pheromone response must determine whether cells bud or shmoo and argue that behaviors under strong natural selection can evolve to be well insulated from the effects of transcriptional noise.

In the absence of Bar1, α-factor-treated cells show two fates: they arrest in G1, fail to polarize, and eventually rebud, or they polarize and shmoo. The cells that shmoo do not adapt to the presence of pheromone. In nature, Bar1 is likely to play a critical role in adaptation by allowing **a** cells to recover quickly from situations that expose them to pheromone without providing them with a mating partner. For example, in an ascus that contains only three viable spores, two **a** and one α, both **a** cells receive α-factor, but only one can mate, and the disappointed suitor should return to the cell cycle as soon as possible.

Ecological and evolutionary aspects of mating

In nature, budding yeast is almost exclusively diploid [48], suggesting that the haploid phase of the life cycle is normally short. Germinating spores and asexually reproducing haploid cells secrete pheromones to advertise themselves to potential mating partners. In the absence of partners, making pheromone slows proliferation [49] and cells should minimize this cost, but in the competition for mates, the cell which makes the most pheromone wins [33-34]. These conflicting needs are resolved by making **a** factor induce α cells to increase the production of α-factor and vice versa. There are two advantages to making the threshold for pheromone induction lower than the one for cell cycle arrest: cells can begin courtship without sacrificing their proliferative potential and they can require that a potential partner demonstrate its fitness by producing the higher amounts of pheromone needed to cause arrest and ultimately mating.

We speculate that pheromone production has been driven upwards by sexual selection. The basal level of pheromone secretion in unstimulated α cells is about 1% of total protein synthesis and the ability of cells to find partners and mate efficiently argues that this level rises at

least 10 fold as **a** and α cells stimulate each other. Hartwell and his colleagues showed that **a** cells efficiently chose partners who made more α-factor than their competitors [33-34]. This behavior will select for increased α-factor production, and because these cells threaten to overcome the ability of Bar1 to keep α-factor concentrations within the range needed for accurate partner location, the spread of "sexier" α cells will select for increased Bar1 expression in **a** cells, selecting for higher α-factor production and so on.

ACKNOWLEDGMENTS

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CHAPTER 3

Sexual Identity in Yeast Mating

The higher fungi can be divided into two phyla: the Ascomycota and the Basidiomycota. Basidiomycetes, have complex mating systems, with one locus encoding regulatory transcription factors and the other encoding a variable number of pheromone and receptor genes. Most ascomycetes have two mating types, each of which expresses one receptor and a single pheromone. In the Ascomycota, both pheromones are small peptides and one of them (**a**-factor) is modified with a farnesyl lipid group that significantly alters its physical-chemical properties. In the Basidiomycota, all pheromones are lipid-modified and this difference is a distinguishing feature between the phyla. We asked whether the conservation in pheromone asymmetry, i.e. the fact that some are lipid modified and some are not, is required in ascomycetes and if the lipid modification of the pheromone plays a role in cell-cell fusion. We cloned receptor and pheromone genes from a homothallic Ascomycete and a heterothallic Basidiomycete and expressed these combinatorially into the yeast *S. cerevisiae* to generate novel, alternative mating pairs. We find that yeast cells can mate even when both mating pairs secrete **a**-like or α-like peptides. Importantly, this is true regardless of whether the cells express the **a**- or α-mating type loci. Thus, we show that, in *Saccharomyces cerevisiae*, the only determinants of mating are the specificity of the receptors and their corresponding pheromones.

INTRODUCTION

Sex costs time and resources and represents a critical moment in an organism's life cycle. Most eukaryotes are obligately sexual and, although some fungi do not have known sexual cycles, most are either homothallic (self-fertile) or heterothallic (self-sterile). The budding yeast, *Saccharomyces cerevisiae,* is considered a pseudo-homothalic species because despite encoding two compatible mating type cassettes and being able to recombinationally switch between them, each haploid yeast cell can only express a single mating locus at a time and can only mate with haploids of the opposite mating type. When expressed, these two compatible mating cassettes define three possible cell types: one diploid (**a**/α cells), unable to undergo sexual fusion but capable of meiosis and sporulation, and two haploid mating types, **a** and α**,** which can fuse with each other to form the diploid, **a**/α cells. Four regulatory proteins, Matα1, Matα2, Mata1 and Mata2, control the expression of cell type specific genes. The presence of Matα1 induces the expression of α specific genes while Matα2 blocks the expression of **a** specific genes. Naturally these regulators are only present in α cells. The **a** mating type is the "default" and it is the one

expressed in the absence of Matα1 and Matα2. In diploid **a**/α cells, the α cell contributes with Matα2, which still represses the *MAT***a** genes, and works with the **a** cell's Mata1 regulator to block the expression of haploid specific genes. There is still no known role for the Mata2 regulator (reviewed in [50]). Haploid specific genes include those involved in all stages of the sexual development. As described in Chapter 1, the two haploid mating types sense each other's presence by reciprocal sets of pheromones and pheromone receptors, with **a** cells secreting **a**-factor and responding to α-factor, and α cells secreting α-factor and responding to **a**-factor.

Beyond the receptors, the signaling pathways are identical in both mating types, although the pheromones for the two mating types are asymmetric with respect to size and physic-chemical properties. While both pheromone are small hydrophobic peptides, **a**-factor is farnesylated and carboxymethylated at a C-terminal CAAX box and requiries a specific transporter for secretion, Ste6, a homolog of multidrug transporters. This asymmetry is conserved across the ascomycetes, but basidiomycetes only express the lipid-modified **a** pheromones (figure 3.1A). Mutations of the CAAX box result in non-farnesylated (or non-carboxymethilated) peptides and lead to significant reductions in mating efficiency, suggesting that the lipid tail is required for recognition and activation of the corresponding **a**-factor receptors [20]. The high hydrophobicity of the **a**-factor pheromone makes it very difficult to work with in a quantitative way and most studies looking at the yeast mating pathway are done with a cells being stimulated with α-factor. Therefore, very little is known about the physical-chemical properties of the **a**-factor pheromone and how they might influence mating efficiency.

A second asymmetry exists between the two mating types, namely that **a** cells and not α cells secrete an aspartyl protease, Bar1, that degrades and inactivates α-factor. **a** cells that lack Bar1 have been described as being supersensitive to α-factor-induced G1 arrest and mate poorly when they have to choose between multiple partners [8]. Other cell-type specific genes include agglutinins and mating type switching enhancers [5].

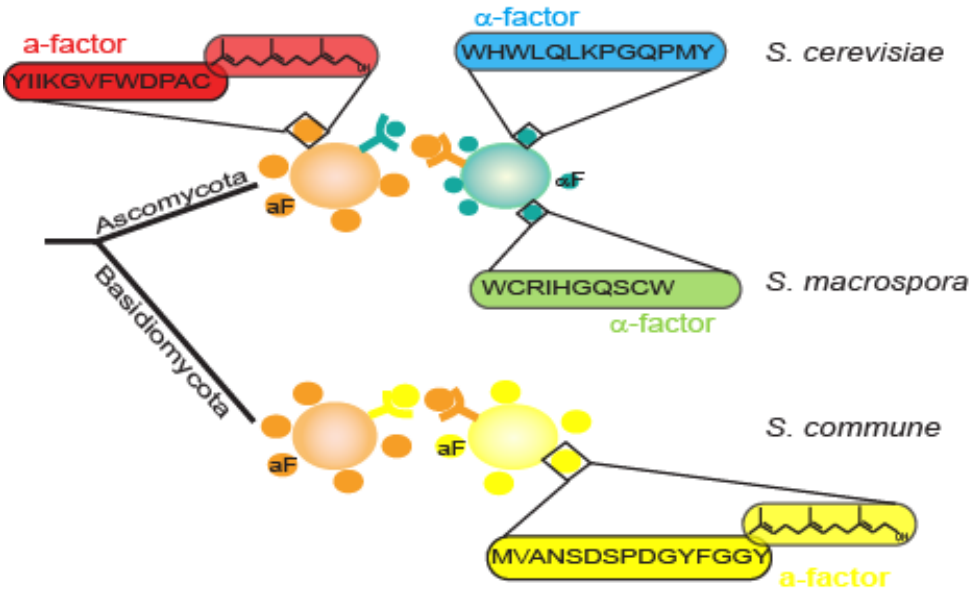


Figure 3.1.A – The Ascomycota and Basidiomycota species communicate via pheromones and GPCRs
The Ascomycota phylum, which includes species like *S. cerevisiae* and *S. macrospora* , communicate via asymmetric pheromones, α-factor like peptides and **a**-factor-like farnesyl modified peptides. The species from the Basidiomycota phylum, like *S. commune*, only use lipid-modified **a**-factor-like pheromones. The large circles represent modified pheromones and the small circles represent the α-factor-like peptides. The sequences for the α-factor like peptides from *S. cerevisiae* and *S. macrospora* are shown in blue and green, respectively. The sequences for the **a**-factor like pheromones for *S. cerevisiae* and *S. commune* are shown in red and yellow, respectively. This color code will be used in all the figures.

In *S. cerevisiae*, sexual specificity is at least partly determined by which set of pheromones and receptors are expressed [51], [7]. However, we wanted to ask if these pheromone-receptor pairs are also sufficient determinants of mating type identity. We hypothesized that given the high level of conservation of **a**-factor (across all dykarya) the farnesyl group might play a role in partner discrimination and/or cell-cell fusion. If this were the case, mating pairs that only communicated via distinct α-factor like peptides, should have significantly impaired mating efficiency. Likewise, if the asymmetry in pheromone properties is the fundamental determinant of specificity, mating pairs that express either α-factor or **a**-factor pheromones only, should also display reduced mating efficiencies. Finally, if mating type identity is defines by additional mating-type specific proteins, than an **a** cell should mate significantly worse with another **a** cell than with an α cell (Figure 3.1.B).

We created several artificial mating types and mating pairs and asked how important these haploid specific asymmetries are in sexual identity.

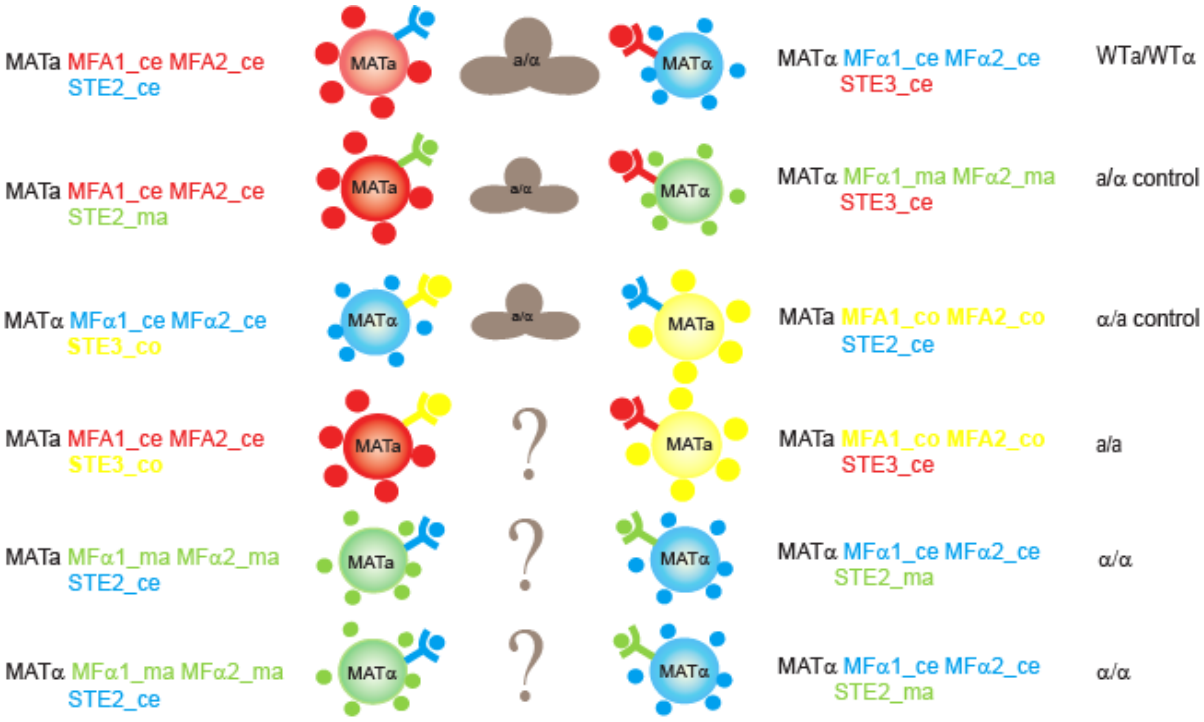


Figure 3.1.B – Artificial mating types
The figure shows six crosses between artificial mating types. The first and second to last columns shows the relevant genotype of the strains. The second and fourth columns show a cartoon of the pheromones, receptors and mating type loci being expressed. The central column represents the expected mating efficiencies. The top cross, between two WT strains, should mate with high efficiency. The two following crosses, between strains expressing heterologous receptors and pheromones but maintaining the a/α asymmetry should also be able to mate. The last three crosses represent combinations of strains that mate using only **a**-factor-like or α-factor-like peptides. As before, the *S. cerevisiae* Ste2 receptor (Ste2_ce) and α-factor pheromone (α_ce) are represented in blue. The *S. cerevisiae* Ste3 receptor (Ste3_ce) and **a**-factor pheromone (**a**_ce) are represented in red. The *S. macrospora* Ste2 receptor (Ste2_ma) and the corresponding α-factor peptide (α_ma) will always be colored green and the clone *S. commune* Ste3 receptor (Ste3_co) together with its **a**-factor pheromone (**a**_co) are depicted in yellow.

RESULTS

S. cerevisiae can mate using heterologous receptor and pheromone pairs.

We wanted to study how disrupting the asymmetry in either pheromone expression or MAT loci would reflect on mating efficiency. To do this we generated multiple artificial mating types by cloning the receptor and pheromone pairs from different fungal species. Since this is a synthetic system, we expected strains carrying the more distant protein homologues to mate worse than wild type *S. cerevisiae* strains. To measure the mating efficiency of these artificial mating types we generated strains that express the heterologous receptor proteins and mated them to strains carrying the matching heterologous pheromone genes. These sets of pairs served as positive controls and were expected to mate as all asymmetries were maintained: an **a** cell always mated with an α cell and they communicated via **a**- and α-like pheromones (Figure 3.2.B and 3.2.C)

To generate this system we chose two fungal species whose receptors had been successfully heterologously expressed in *S. cerevisiae* [52],[53]. *Schizophyllum commune* is a heterothallic Basidiomycete that is predicted to encode at least18 different receptors and more than 75 pheromones, all of which display the farnesylation CAAX motif. Expression of different combinations of the pheromones and receptors define more than 15000 possible mating types (for a review on *S. commune* mating see [4], especially chapter 18). To generate artificial **a-**mating types we cloned one of *S. commune's* Ste3-like receptor (Bbr1) and one of the receptor's matching a-like pheromones (Bbp2(4)), and expressed them in *S. cerevisiae* (Figures 3.1.A and 3.1.B). We will refer to this receptor as Ste3_co, to the pheromone as **a_**co and the pair has been color-coded in yellow in all of the figures and strain table.

Sordaria macrospora is a homothallic filamentous fungus closely related to *Neurospora crassa* (for a review on *S. macrospora* mating see [4], especially chapter 10). To generate artificial α mating types we cloned its Ste2-like receptor (Pre2) and its corresponding α-like pheromone (Ppg1) (Figure 3.1.A and 3.1.B). We will refer to this receptor as Ste2_ma, to the pheromone as α_ma and the pair has been color coded in green in all of the figures and strain table.

The receptors and pheromone pairs from *S. cerevisiae* are represented by the letters ce. The budding yeast expresses its pheromones from two alleles in the genome called *MFα1* and

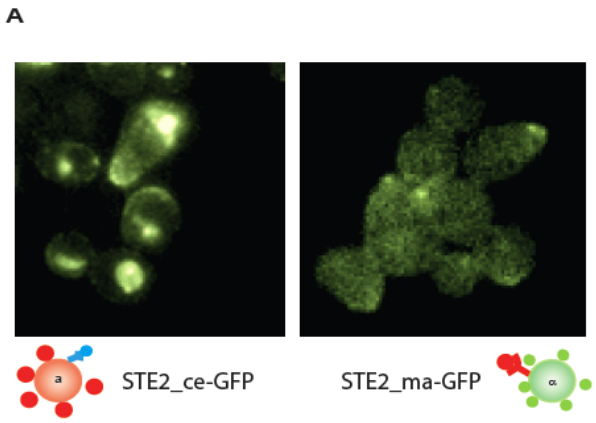
MFα2 for α-factor and *MFA1* and *MFA2* for **a**-factor. Ste2_ce (the α-factor receptor) and the corresponding pheromones, α1_ce and α2_ce have been color coded in blue and Ste3_ce (the **a**-factor receptor), a1_ce and a2_ce have been colored in red.

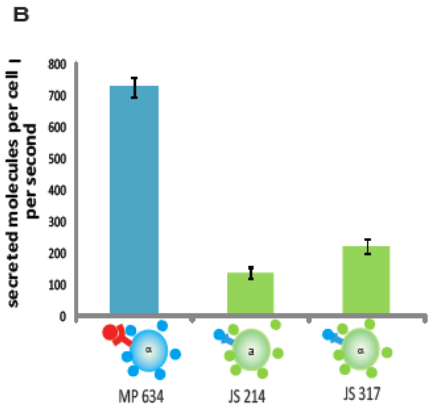
We started by making heterologous receptor green fluorescent protein (GFP) fusions and comparing their level of expression to that of the endogenous receptors in both **a** and α cells (Figure 3.2.A). *STE2_ma* was cloned at the *STE2_ce* locus in an **a** cell and *STE3_co* was cloned at the *STE3_ce* locus in an α-cell. Because these receptors are quite distant from *S. cerevisiae* and showed difficulties communicating with the downstream MAP kinase signaling components, a negative regulator of signaling gene, *SST2*, was deleted). Intrinsically, *sst2Δ* cells show decreased mating efficiency - around 5%, or 10 times worse than wild type cells (Figure 3.2.D and [54]), and this is taken into account when quantifying the alternative matings.

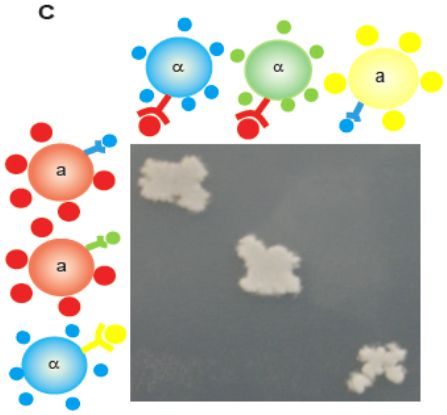
The *MFα_ma* gene was cloned into both α-factor loci and **a**-factor loci and **a**_co into both **a**-factor loci. Both **a** and α-cells were found to express and secrete mature α_ma pheromone, albeit with significantly lower efficiency than α cells secrete their own endogenous α-factor (Figure 3.2.B). We could not make the same type of quantitative measurements for **a**_co because the farnesyl group this pheromone makes it too hydrophobic and unwieldy to work with as it binds unspecifically to most labware surfaces.

We then assayed these strains for mating. The mating pairs constructed and their corresponding genotypes are shown in Figures 3.1.B and 3.2.C. The first mating pair listed served as a positive control of mating between two wild type *S. cerevisiae* strains: *MAT***a** *STE2_ce MFA1/MFA2_ce* x *MATα STE3_ce MFα1/MFα2_ce*. As expected these two strains mate with relatively high frequency, (figure 3.2.C and 3.2.D).

To establish a reference for the extent of mating when using the *S. macrospora* proteins, we mated the following strains: *MAT***a** *STE2_ma MFA1/MFA2_ce* and *MATα STE3_ce MFα1/MFα2_ma*. This pair mates with a reasonable but low efficiency of 1.2% (Figure 3.2.D) and serves as a positive control for the same-sex mating experiments (see below).







D

PAIR	RELEVANT GENOTYPE	% MATING
	(yMP420) MATa WT X (yMP 634) MATα WT	48.3 ± 4.9
	(yMP1255) MATa WT X (yJS212) MATα sst2Δ	4.7 ± 1.5
	(yJS204) MATa STE2_ma sst2Δ X (yJS142) MATα MFα_ma	1.2 ± 0.55
	(yJS351) MATα STE3_co sst2Δ X (yJS319) MATa MFA_co	$5.2x10^{-3} \pm 4.4 x10^{-3}$

Figure 3.2 – Control crosses can mate

A- STE2-GFP fusions were made in strains carrying the receptor from *S. cerevisiae* and *S. macrospora* . Cells were incubated with 10µg/ml of the respective peptide pheromone (α_ce and α_ma) for 2h. Cells were imaged on a Concavilin A coated slide and pseudo-colored. Both receptors are expressed and secreted to the membrane. B – The number of α-factor molecules being secreted per cell per second. The first bar shows the secretion rate for α_se being secreted by an α cell. The two green bars represent the secretion rate for α_ma when expressed in an **a** cell and in an α cell. Error bars represent standard deviations from at least 3 independent trials. C – Non-quantitative mating assay of the control crosses. Cell streaks were replica plated on top of each other and allowed to mate over night in complete media. They were then replica plated on selective media so that only diploids could grow. The figure shows that there is only mating in the diagonals. D – Quantitative mating assay for the control crosses. The shown crosses were allowed to mate on filters for 4 hours (in the case of the wild type) or for 7h (in the case of the *sst2Δ* and heterologous crosses). Filters were then washed and cells plated on selective media to select for diploids. Mating efficiency is calculated as the ratio of the number of zygotes formed divided by the number of available haploids. Errors are standard deviations from at least three independent mating trials. Please note that the **a**_co producing strains (yJS319, last row) were mixed in an excess of 5:1. All the other crosses were done at a 1:1 ratio. See Materials and Methods for detailed descriptions of the protocols.

We then looked at mating efficiency using *S. commune* proteins by mating *MAT***a** *STE2_ce MFA1/MFA2_co* with *MATα STE3_co MFα1/MFα2_ce*. This pair showed almost no mating at all. We hypothesized that the low mating efficiency could be explained by low levels in receptor and/or pheromone expression. Expressing the pheromones and receptors from a multi copy plasmid with a strong promoter showed that pheromone expression was the limiting factor, as increasing the receptor number didn't significantly increase the number of mating events (Figure 3.3.A and data not shown). To correct for this bias, all subsequent experiments were done with the **a**_co expressing strains at a 5:1 ratio to the Ste3_co expressing strains. While mating efficiency was improved, it remained almost two orders of magnitude lower when compared to what was obtained by using the *S. macrospora* proteins. This could be rationalized as reflecting the large phylogenetic distance between *S. commune* and *S. cerevisiae* and a) its genes might not be expressed at the same levels and/or b) the receptor is likely to communicate with the MAP kinase signaling components less effectively.

Although efficiencies varied, all three pairs (expressing either *S. cerevisiae*, *S. macrospora* or *S. commune*s proteins) can mate. We also found that the receptors are quite specific for their pheromones, as no off-diagonal mating can be observed in Figure 3.2.C.

We can recapitulate Basidiomycota matings in an Ascomycete

We then generated a mating pair where both cells express a-like pheromones and STE3-like receptors. In one **a** cell we replaced the endogenous *STE2* receptor with the *S. commune STE3*-like receptor, *STE3_co* (yJS359). In another **a** cell we replaced the endogenous **a**-factor genes with the *S. commune* pheromone **a**_co and the *STE2_ce* receptor with the *STE3_ce* receptor, usually expressed in α cells to make strain yJS360 (this also required the deletion of *ASG7*, an **a**-specific gene that down-regulates Ste3 activity). These strains now express complementary pairs of receptors and pheromones (Figure 3.3.B). While mating efficiency was quite low, at least in the conditions of the experiment, it was actually five times higher than the one obtained when the asymmetry in both pheromones and cell-type background was maintained (Figure 3.3.C).

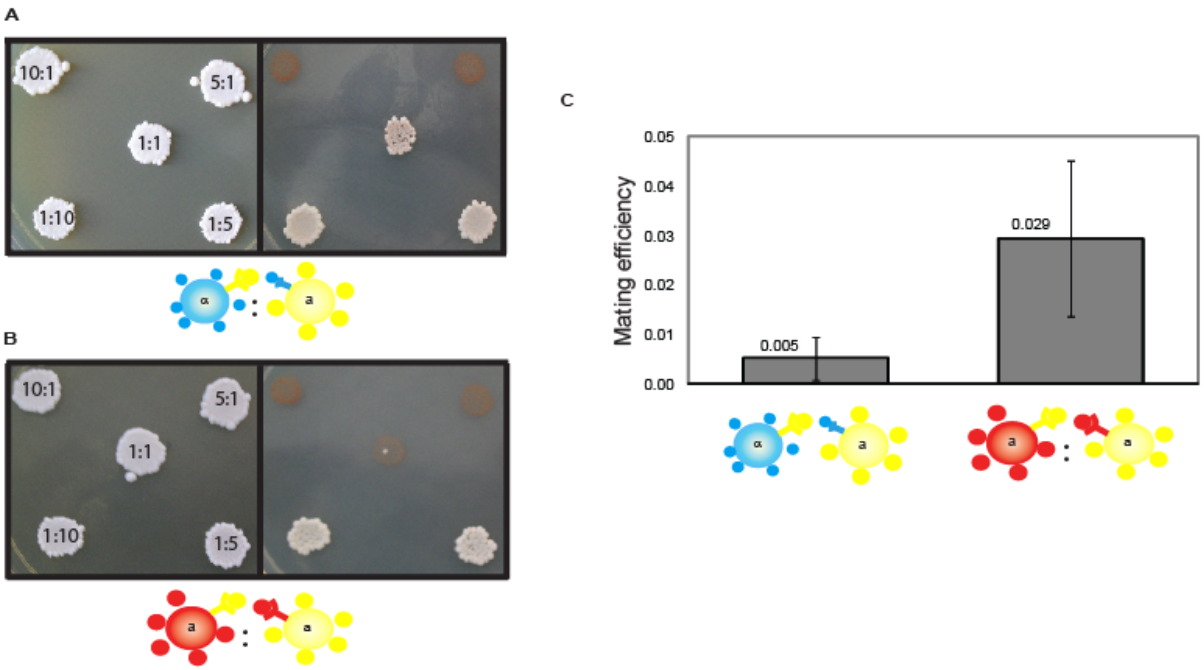


Figure 3.3 – Cells that communicate using only a-factor pheromones can mate
The strains expressing the *S. commune* heterologous receptor and pheromone were mated with either α_ce or **a**_ce producing strains. A and B – the shown strains were mixed at different ratios (10:1, 5:1, 1:1, 1:5 and 1:10), spotted on complete media and allowed to mate over night (left panels). They were then replica plated on selective media that only allows diploid growth (right panels). We saw that consistent mating only happens when the *S. commune* pheromone secreting strains (yellow) were present in excess. This is true whether the mating pairs maintain the a/α asymmetry (A) or express only **a**-factor pheromones and MAT**a** loci. C –Quantitative mating assay. Cells were mixed as described previously and allowed to mate on filters for 7h. Mating efficiency was calculated and error bars represent standard deviations from at least three independent mating trials.

This result was surprising for two main reasons. First, there appears to be no requirement for having asymmetric (farnesylated vs. non-farnesylated pheromones) for Ascomycete mating. This raises the question as to what the functional or evolutionary significance of this asymmetry might be. The second surprising finding follows from the fact that these fused cells are now **a/a** diploids, with no α2 regulator to block the expression of **a**_ce, **a**_co, STE3_ce and STE3_co. These cells should now be expressing both sets of pheromones and receptors and should self-stimulate (or be stimulated by the surrounding non-mated haploids) and progression past G1 should be difficult. I will return to this in the discussion.

The lipid tail is not-required for partner recognition and fusion

The mating pathway in *S. cerevisiae* has been extensively studied but the signal(s) for cell-cell fusion remains to be identified. We have shown that we can make two **a** cells fuse even in the absence of an α-like pheromone. Because this situation appears to mimic mating in the Basidiomycota, we hypothesized that **a**-factor, or some unknown **a**-specific protein, might play a fundamental role in cell-cell fusion. If the farnesyl group of the pheromone is required for membrane fusion, a lipid-modified peptide should be required for mating to occur and one could expect that that two cells of opposite mating types that secrete only α-factor-like peptides to be able to form pre-zygotes but be unable to fuse.

To test this we constructed a mating pair that communicated using only α-like pheromones. Starting from an α-cell we replaced the Ste3 receptor with the Ste2-like receptor from *S. macrospora* , Ste2_ma (yJS220). This strain is now *MATα STE2_ma MFα1/2_ce*, producing S. cerevisiae's α-factor and responding to the *S. macrospora* pheromone α_ma. Starting from an **a** cell, we replaced both **a**-factor producing genes with MFα_ma to make a cell that is *MAT***a** *STE2 MFα_ma* (yJS214). This pair can communicate using only α-like pheromones although the two cell backgrounds remain different, **a** and α (Figure 3.1.B and 3.4.A). These cells can mate with efficiencies comparable to those of the *S. macrospora* controls (the mating of **a** and α cells in which the α-factor and α-factor receptor come from *S. macrospora* and the **a**-factor and **a**-factor receptor come from *S. cerevisiae*) of around 1% (compare second row in Figure 3.4.A with third row in Figure 3.2.D).

This suggests that there is no formal requirement for the lipid-modified pheromone in mating but didn't rule out the possible contribution of the **a** cell to the mating process, for example through a role of **a**-specific genes other than the pheromone in mating. To address a putative role for **a**-specific genes in mating, we started from an α cell and replaced both endogenous pheromone genes with *MFα_ma*, and replaced the naturally expressed *STE3_ce* receptor with *STE2_ce* (yJS317) (Figure 3.4.A).

We now had two mating pairs that could communicate via α-factor like peptides only, but in one pair both cells express only the *MATα* locus. When we compared the mating efficiencies we found that the MATα/MATα pair mated two orders of magnitude worse than the *MAT***a***/MATα* pair (Figure 3.4.A). Several factors could explain this phenotype: a) the α cells might have problems expressing STE2_ce; b) The **a** cells might express different levels of the

pheromone; c) there is some **a**-specific protein that is important for efficient mating; or d) the α/α diploids have difficulties re-budding after fusion, as they could still self-stimulate and be arrested in G1.

A

PAIR	RELEVANT GENOTYPE	% MATING
MATa MATα	yJS204 MATa STE2_ma sst2Δ X yJS142 MATα MFα_ma	1.2 ± 0.55
MATa MATα	yJS214 MATa MFα_ma X yJS220 MATα STE2_ma sst2Δ	0.89 ± 0.45
MATα MATα	yJS317 MATα MFα_ma X yJS220 MATα STE2_ma sst2Δ	1.93 x10^{-3} ± 1.8 x10^{-3}
MATa MATα	yJS385 MATa MFα_ma bar1Δ X yJS220 MATα STE2_ma sst2Δ	0.03 ± 0.021

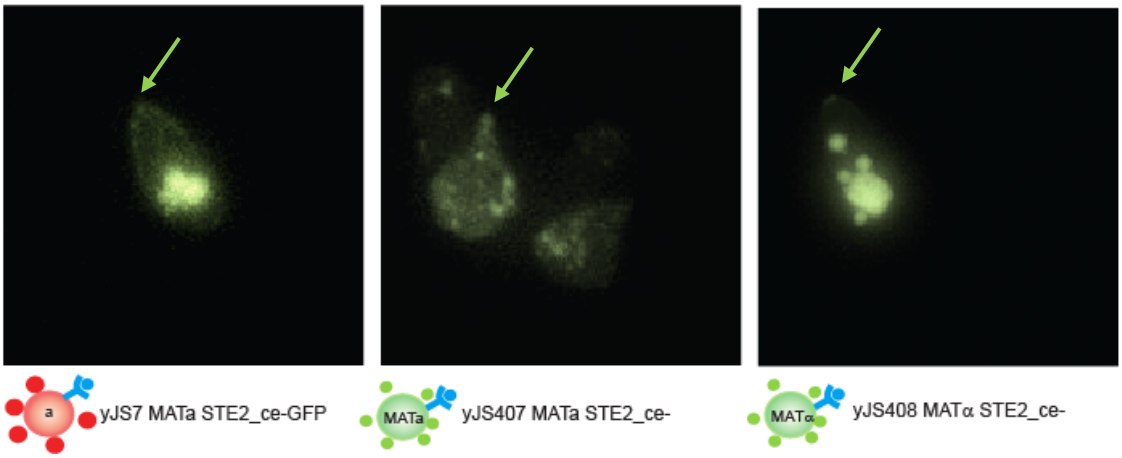


Figure 3.4 - Cells that communicate using only α-factor pheromones mate very poorly
The strains expressing the *S. macrospora* heterologous receptor and pheromone were mated with either α_ce or **a**_ce producing strains, expressing either the *MAT***a** or *MATα* loci. A – Mating efficiency was quantified as described above. Errors represent standard deviations of at least 3 independent trials. Mating pairs where both strains express the *MATα* locus mate 600 times worse than mating pairs that express different mating loci. B – *STE2-GFP* fusions were constructed and cells were induced with the respective pheromones and imaged for receptor localization. There are no significant differences in receptor expression.

The data presented in Figure 3.2.B shows that the α cells actually produce slightly more *S. macrospora* α-factor than **a** cells and it is quite unlikely that this small difference in pheromone secretion could explain such a dramatic effect in the mating efficiencies. We cloned STE_ce-GFP fusions into both the **a** and α mating pairs (yJS214 and yJS317) and induced with

α-factor. The localization and expression of the receptor were analyzed by fluorescent microscopy (Figure 3.4.B). We found no significant difference in the expression of the receptor in the two different cell backgrounds.

A protease is required in the case of α-factor matings

Using the available fluorescent markers to distinguish between different cells (see Supplementary Table 1), we mixed the yJS220 or yJS221 (*MATα MFα_ce STE2_ma*) with its α mating pair yJS317 (*MATα MFα_ma STE2_ce*) and observed these mating mixes, under the microscope (Supplementary Movie 1). STE2_ma expressing cells were found to arrest and induce the mating pathway, as indicated by the activation of the FUS1 promoter, but have difficulty polarizing (as indicated by the movement of the Spa2 dot) and finding a mating partner. The α_ma producing strains also arrest and turn on the FUS1 promoter but are over stimulated and make shmoos. On rare occasions two cells expressing the matching receptor and pheromone pairs find each other, align their polarities, and the fusion process proceeds normally. Although these cells take slightly longer to rebud, their subsequent cell cycles are of normal length indicating that these cells are not being pheromone stimulated.

We asked if the polarity/over-stimulating problems could be responsible for the lowered mating efficiency. A natural **a**-specific gene candidate was the already mentioned protease BAR1. Bar1 is fundamental in shaping the α-factor gradient and helping **a** cells discriminate between possible partners, as argued in chapters 1 and 2. We deleted this protease in yJS214 (the *MAT***a** mating pair, *MFα_ma STE2_ce*) to make yJS385. α_ma secretion is indistinguishable between this strain and its parent, yJS214, (data not shown) but mating efficiencies are now reduced 30 fold, the same reduction observed in wild type *bar1Δ* crosses (Figure 3.4.A), although they still mate 15 times better that the equivalent α/α pairs. Because the secretion levels are the same for both *BAR1* and *bar1Δ* strains, we expect this protease not to cleave α_ma and to act only on *S. cerevisiae*'s α-factor, to prevent the saturation and directionality problems displayed by *MATα MFα_ma STE2_ce* (yJS317) cells, thus improving the mating efficiency.

As described in Chapter 2, adding soluble pepsin to mating mixes could recover the mating efficiency of *bar1Δ* cells. We also observed that in a 1:1 heterologous mating mix, STE2_ma expressing cells seemed to be under-stimulated whereas STE2_ce expressing cells

made long and sometimes multiple shmoos (Supplementray Movie 1). Adding an unspecific soluble protease would presumably help shmooing cells by reducing the overall concentration of α_ce but would have the negative effect of making the *MATα STE2_ma MFα_ce* cells even less responsive. In this case, where both mating cells secrete and communicate via small unmodified peptides, the results would become harder to interpret. We tested the effect of adding different concentrations of both chymotrypsin and pepsin to the two heterologous mating pairs yJS220 (the Ste2_ma α cell) with both the *MAT***a** (deleted for Bar1) and *MATα MFα_ma STE2_ce* strains. Preliminary results indicat that adding soluble protease does increase the number of both **a**/α and α/α diploids formed. We have not been able to raise the number of α/α diploids to the same level, consistent with the idea that these diploids behave like α cells and may have a harder time forming colonies.

Although we saw a decrease in mating efficiency when the two mating cells express the *MAT*α loci (yJS220 × yJS317) this reduction can be mimicked in *MAT***a** *MAT*α matings by deleting Bar1 in the **a** cell (yJS220 × yJS385). The mating efficiency of these pairs was comparable to the one observed when only **a**-factor-like pheromones and MAT**a** loci were expressed.

DISCUSSION

There is no inherent bias for a-a or α-α mating

Our results show that both α cells and **a** cells can mate with themselves if given the appropriate stimuli (summarized in Figure 3.5). Differences in mating efficiency can be explained by difficulties in expressing the heterologous receptors and/or pheromones which lead to polarization problems and divergence in the response between two strains of a mating pair (with some arresting, others cycling and other shmooing). Mating events between these alternative mating types are rare and hard to track by video microscopy, but we have never observed the formation of pre-zygotes or of mating pairs which, after polarizing and finding each other, had problems in the fusion process. This tells us that there is probably no unknown mating type specific protein required for *S. cerevisiae* fusion.

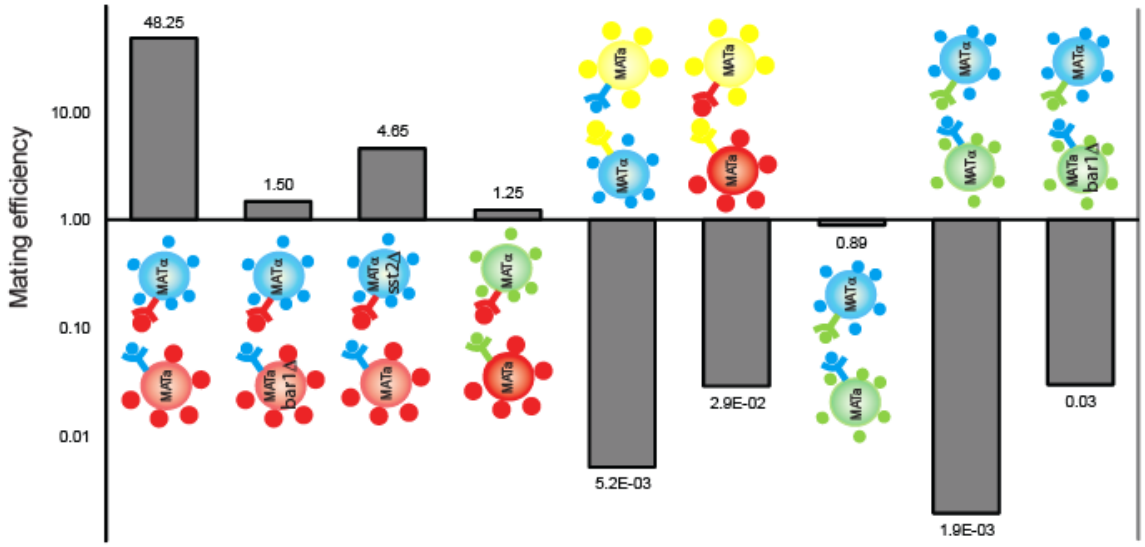


Figure 3.5 – Mating efficiency of all the pairs tested
The mating efficiency of all crosses was quantified as described previously. Cells were allowed to mate for 7h and then plated on selective media to isolate diploids. Please note that the mating efficiencies are plotted in log scale. Bars above the line mean that the mating efficiencies were higher than 1% whereas bars under the line represent mating efficiencies of lower than 1%. It is possible to see that the crosses using the heterologous pheromone/receptor pairs mate worse than the ones expressing the *S. cerevisiae* genes, but there is no significant difference between the mating efficiencies of strains expressing only **a** or only α-like pheromones.

The fact that we can isolate **a/a** and α/α diploids with a certain frequency is not easy to interpret. As discussed above, these cells should self-stimulate, arrest and have difficulties forming colonies. Ideally we would be able to quantify the fusion events by video microscopy and not rely on their colony forming abilities, but because the observed frequency of these a-a and α-α fusions is very rare, a systematic analysis is impossible. .

To test if these diploids can still detect the presence of mating pheromones we tried inducing the α/α pairs with α-factor but observed no response. This told us that these cells might have suffered a mutation in some gene related to the mating pathway or cell cycle progression. A likely candidate is the cyclin Cln3, which plays a role in arresting cells at the G1 phase of the cell cycle. C-terminal truncations of this protein give rise to dominant hyperactive mutants that have shorter G1, smaller size and show no pheromone induced arrest [55], [56]. Cells that acquired this mutation would not self-stimulate and could form colonies. Another explanation could be that the presence of more than one receptor titrates away Gα subunits or other components of the

MAP kinase cascade leading to reduced sensitivity to pheromone. This has been observed when both STE2 and STE3 were simultaneously expressed in **a** cells [57]. We also cannot rule out that an unknown regulator might shut down the pheromone response pathway upon cell-cell fusion, but this is unlikely, as **a**/**a** diploids are known to respond and mate as **a** cells. It is also possible that the cells have adapted physiologically rather than genetically to continual stimulation.

Evolution of the dimorphism

Even homothallic ascomycetes, like *S. macrospora*, express the two types of pheromones (**a** and α-factor like) whereas basidiomycetes only express the farnesylated peptide.

The **a**-factor-like pheromones seem to be conserved over long evolutionary times and the budding yeast's **a**-factor transporter, Ste6, was recently shown to help Drosophila in stem cell migration [58]. Sex is already more costly than asexual reproduction and the production and secretion of a lipid modified peptide is very likely more burdensome than that of a simple one. The fact that this lipid-modified pheromone is so conserved across phyla raises the question of why mating mechanisms that only require α-factor-like pheromones have not evolved. We have no good answer for this question but we speculate that it might have to do with the requirement for Bar1. The diffusion constants of the two peptides are likely to be very different and, as discussed previously, α-cells allocate significant resources into the production of α-factor. Also, as the number of α-cells in a mating mix increase, the number of pheromone molecules increases and receptor saturation can become a problem. To circumvent this problem, the prediction is that a Bar1-like protease was probably "invented" with α-like pheromones. This is not an easy prediction to test as it is relatively easy to identify Bar1 paralogs all across the Ascomycota (it belongs to a conserved family of aspartyl proteases), but not trivial to show which genes encode the true orthologs that work in pheromone inactivation. Functional orthologs have, been identified in evolutionarily close and distant relatives like Candida albicans and Schizosaccharomyces pombe, although they show very little homology to BAR1 [18], [59].

A complementary way to look at the "invention" of Bar1 would be through the light of the handicap principle, as defined by Amotz Zahavi in the 1970's. This theory predicts that a sexual signal, to be honest, should be costly. A common example is the peacock tail, an extravagant trait with very high cost, which is believed to be under sexual selection as peahens choose mating partners by the size and beauty of their tails. The "honest signal' would be α-

factor as it has been shown that **a** cells prefer to mate with the highest pheromone producers [33] and that α cells spend considerable resources on its production (Chapter 2). Bar1 could have evolved as a test to the quality of the signal. If **a** cells were able to detect it even after very strong degradation, the signal was guaranteed to be strong and, consequently, coming from a healthy partner.

The evolutionary barrier between different mating systems might not be very high

Close relatives can have completely different mating systems. A well studied example is the *Sordariaceae* family, which includes the already described homothallic fungi *S. macrospora* , heterothallic species like the model organism *Neurospora crassa* and pseudo-homothallic ones, like *Neurospora tetrasperma*. Interestingly, all these species share the same α-factor-like peptide and their specificity is likely to occur via events downstream of the receptors and differences in their life cycles.

S. cerevisiae can only mate when stimulated by cells of the opposite mating type. Some wild species encode an endonuclease, HO, which cleaves the DNA at the mating loci. These cells undergo mating-type switching (reviewed in [60]) and can then mate with their daughters. Some others have suffered a mutation and are no longer capable of switching and these cells are believed to mate preferentially with their sisters right after sporulation (see chapter 1 and Appendix A for more details). Even with homothallic strains most mating is likely to be with sister spores, since this option is available immediately on germination, whereas spores cannot switch mating type until they have already divided once. This pseudo-homothallism contrasts with the better defined life cycles of obligatory homothallic or heterothallic species. However, recent studies [61] ,[62], [63] have challenged this view by reporting autocrine stimulation and same sex mating in species previously thought to be heterothallic. This might be relatively common in fungal pathogens that are usually isolated with only one mating type, from the wild.

Similarly, in the budding yeast, whole genome transcription studies [64], have shown that **a** cells transcribe from the *MFα1*, *MFα2* and *STE3* loci upon α-factor induction, genes previously thought to be expressed in α cells only.

Our report that *S. cerevisiae* can be made to mate as **a/a** or α/α pairs, regardless of which set of pheromones or mating loci they are expressing indicates that these mating system's distinctions are not very likely to represent strict evolutionary barriers and that

pheromone/receptor activation, more than mating type loci or mating system, are the primary determinants of sexual identity.

ACKNOWLEDGMENTS

I would like to thank Dr. Stefanie Pöggeler for sharing plasmids with *Sordaria macrospora*'s PRE2 and PPG1, Dr. Thomas Fowler for sharing plasmids with BBR1, BBR2, BBP2(4) and BBP1(1) genes from *Szhyzophylum commune*, and members of the Murray Lab for discussions. This work was supported by grants from NIH (GM 68763 and GM 43987 to A. M), as well a fellowship to.J.G-S. from the Fundação para Ciência e Tecnologia (SFRH/BD/15220/2004).

CHAPTER 4

Evolution and Specificity of the Ste2 Receptor

In *Saccharomyces cerevisiae* mating culminates when two haploid cells of opposite mating types fuse. The two cells communicate through secreted pheromones and the corresponding transmembrane receptors. This communication mechanism is conserved across the species and we asked whether receptor specificity could be playing a role in reproductive isolation. We looked at the evolution of the specificity of the peptide receptor Ste2 in the phylum Ascomycota. We have identified around sixty STE2 and α-factor homologs in the Ascomycota phyla. We synthesized 23 of these pheromones and cloned 17 of the corresponding receptors in *S. cerevisiae*. Using a fluorescent reporter for pheromone induced gene expression and a FACS-based assay we tested the response of the receptors to the presence of the 23 pheromones. Ten out of the fifteen heterologously expressed receptors successfully respond to self-pheromone and we have identified response clusters, defined as groups of receptors that respond to the same pheromone. We report that *S. cerevisiae* STE2 is surprisingly promiscuous and can respond to high concentrations of pheromones from species as distant as *Debaryomyces hansenii* and this promiscuity is not restricted to the *S. cerevisiae* receptor. We present evidence that the receptors seem to be under negative selection, contrasting with most genes involved in speciation, and this can explain the cross-talk between different receptors and pheromones.

INTRODUCTION

In the previous chapter we reported that the pheromone/receptors pairs are the main determinants of sexual specificity and identity in *S. cerevisiae*, so the first step toward hybridization between two species, S_1 and S_2 requires that the Ste2 homologue of one species is able to interact with the α-factor pheromone produced by the other species (and likewise for Ste3 and **a**-factor). In Chapter 1 we discussed the observation that some closely related species share the same pheromones despite clear post-zygotic barriers to genetic exchange between them. *S. cerevisiae* and *S. paradoxus*, for example, have very high genome synteny and homology, share ecological niches, express the same pheromones and natural hybrids between them have been isolated, but the hybrids are sterile, giving rise to dead spores (reviewed in [65]). Evolution of pre-mating isolation, caused by divergent mating signals and preferences, has been suggested as an important component of speciation in many taxa [66-67]. Therefore, speciation, defined as the process through which organisms become incapable of interbreeding, could occur via changes in specificity between the receptors and its corresponding pheromones as a first step towards pre-

zygotic isolation. We asked how specific are the STE2 like receptors and how much of a role they might play in fungal speciation.

RESULTS

Identification of receptor and pheromone sequences

The Ste2 proteins are seven transmembrane GPCRs and we have identified around 70 homologs of the *S. cerevisiae* STE2 receptor from the Ascomycota phyla by both BLASTP and BLASTN homology. The protein sequences of annotated species were used as bait and we confirmed the receptor topology using a TMHMM v2.0 predictor [68]. The list of receptors is tabulated in Supplementary Table 3. We used this large data set of identified receptor sequences and aligned their amino acid translations using a Hidden Markov Model (HMM) downloaded from the PFAM website [69] (Supplementary Tables 5 and 6). The predicted membrane topology of each protein was checked against this alignment to ensure that those residues predicted to be inside the membrane were aligned with each other. An alignment of a subset of 48 representative nucleotide sequences was used to generate a phylogenetic tree using a maximum parsimony method from the MEGA4 program (Figure 4.1 and Supplementary Table 6).

We then identified the peptide pheromones of 65 ascomycete species by homology using BLAST together with literature reports of α-factor sequences from different species. As described in Chapter 1, the sequences of α-factor like pheromones usually code for a leader sequence and several peptide repeats, flanked by a conserved cleavage sequence. The immature peptide is cleaved by the Kex2 protease, which is known to cleave the sequences 'KR' and 'RR' specifically [70]. The list of the predicted pheromone peptides can be found in Supplementary Table 4. The peptides have aligned with the corresponding receptors and this concatenation is shown in Supplementary Table 5.

STE2Sc is quite promiscuous and responds to the pheromones of distant relatives

From our large dataset we chose and synthesized the α-factor-like pheromones from 24 different Ascomycota species, distributed across the phylum, from close and distant *S. cerevisiae* relatives (Figure 4.1).

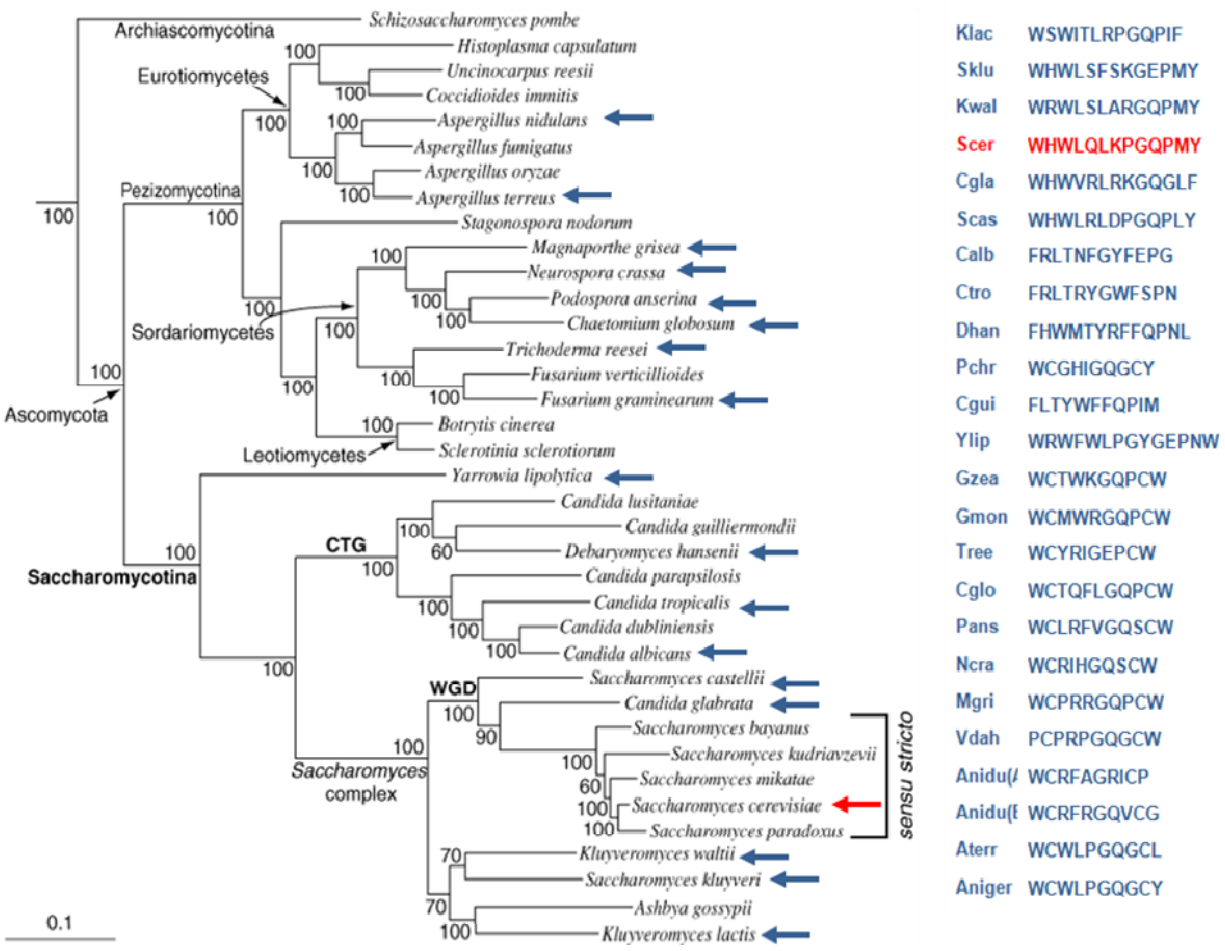


Figure 4.1 – Phylogenetic tree and synthesized pheromones
Phylogentic tree was adapted from [71]. Arrows represent some of the species for which we synthesized pheromones, all across the Ascomycota phylum. The table on the left shows all synthesized peptides. See Materials and Methods for more details.

*MAT***a** *S. cerevisiae* cells respond to α-factor by arresting the cell cycle in G1, expressing mating specific genes and polarizing towards the attractant. Highly stimulated cells grow a protrusion commonly known as a shmoo. We induced a *MAT***a** *bar1Δ S. cerevisiae* strain with two different concentrations of these pheromones. We have shown that *bar1Δ* **a**-cells can accurately polarize over to a small range of pheromone concentration, at around 1nM (see Chapter 2). As expected, when these cells were induced with 100nM of the synthetic pheromones they responded very strongly to the presence of the *S. cerevisiae* α-factor, arrested and shmooed (Figure 4.2). In these conditions STE2_ce showed high specificity and only shmooed in response

to the pheromones of the very close relative *S. castellii* and to a lower extent, to the pheromone of *C. glabrata* (Figure 4.2.A).

When we looked at the induction levels of these cells by using FACS to follow the pheromone-induced expression of P_{FUS1}-*YFP*, we could see that the pheromones from *S. kluyverii*, *K. lactis* and *K. waltii*, all close relatives of *S. cerevisiae*, also induced the MAP kinase pathway and, in the case of *S. kluyverii*, in a very strong way. This discrepancy between the FACS and the microscopy results was not particularly surprising as we already knew (see Chapter 2, and [35-36]) that the *FUS1* promoter (a mating specific promoter) is very noisy and cells can show the same levels of pFUS1 induction and still display very different morphologies.

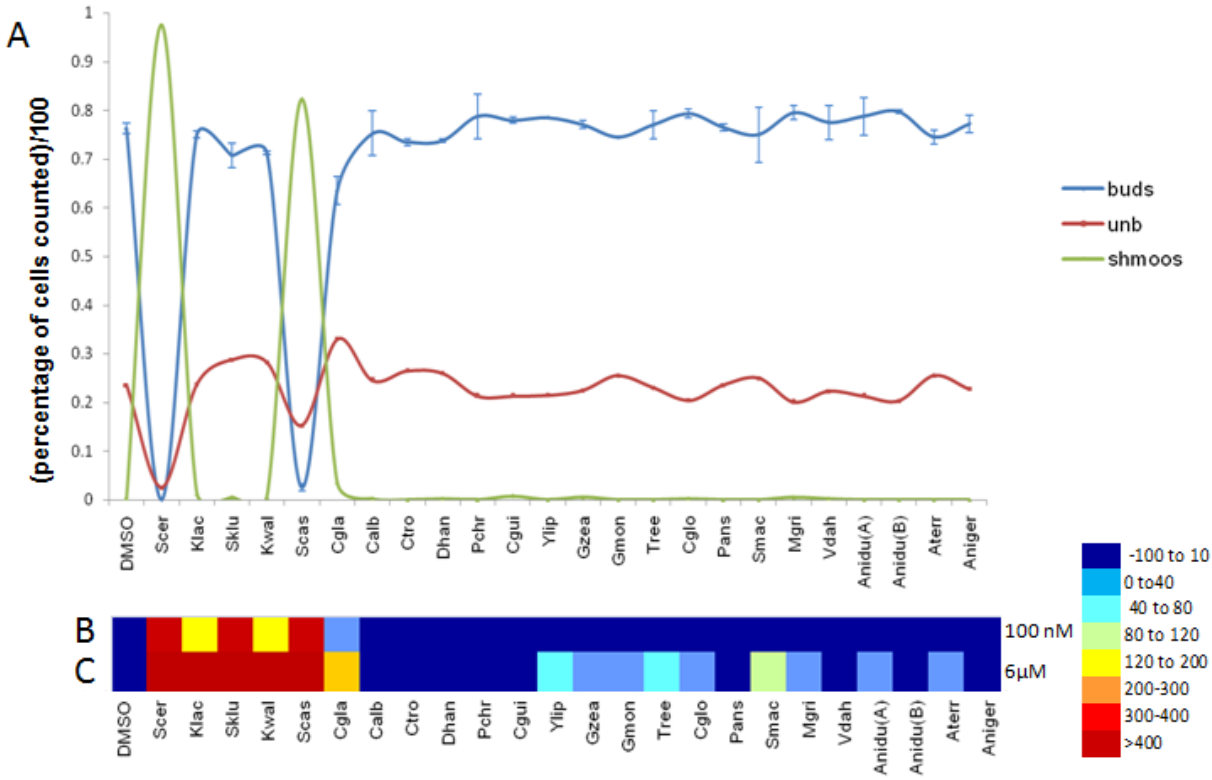


Figure 4.2 – Response of MATa *bar1Δ* cells to different α-factor peptides
Exponentially growing cells, expressing yellow fluorescent protein (YFP) under the *FUS1* promoter, were incubated with the 24 different pheromones or with DMSO for 2h. A) Cells were incubated with 100nM of each pheromone, sonicated and fixed with 60% cold EtOH for 20. The plot shows the normalized number of cells that shmoo, bud or remain unbudded. At least 200 cells were scored per condition and error bars are standard deviations from three independent trials. B) and C) Cells were incubated either with 100nM (B) or with 6µM (C) of the 24 pheromones and their YFP levels analyzed by FACS. Fluorescence levels are in arbitrary units and were normalized using the non-induced values. Because there is significant variation between trials we set the threshold for activation at 120 arbitrary fluorescent units (hot colors on plots).

We then increased the pheromone's concentration 60-fold to 6 μM and tested the cells for pFUS1 induction. The STE2_Sc receptor could now detect the presence of the pheromone of species as distant as *K. lactis*, even in the presence of the Bar1 protease (Figure 4.2.C). Saturating concentrations of all of the other pheromones yielded no response at all. Given the higher sensitivity of the FACS assay when compared to the morphological analysis, the subsequent experiments were done by FACS only.

Receptors from distant fungi can be expressed in S. cerevisiae and activate the mating pathway.

To ask if this promiscuity was specific to STE2_ce we chose 15 receptors, corresponding to a subset of the tested pheromones, plus STE2_ce and STE3_ce as positive and negative controls, respectively (Figure 4.3). Seven of the more distant receptors had STOP codons interrupting their coding sequences and we used the annotated proteins of other sequenced species to manually "splice" these sites and synthesize their DNA (see Materials and Methods for a detailed description and Supplementary Table 7 for the predicted splicing sites). A total of 17 receptors were cloned into the endogenous STE2 locus in *S. cerevisiae* (by homologous recombination, deleting the wild type STE2 receptor). All strains have the afore mentioned promoter fusion with pFUS1 driving YFP. To assure that a non responsive phenotype was due to no pheromone binding and not to poor communication between the heterologous receptor and the budding yeast's G_α subunit (Gpa1) we deleted the G_α subunit's negative regulator, SST2 [72], to yield a total of 34 strains (17 "WT" and 17 *sst2Δ*, see strain table, Supplementary Table 1.C)

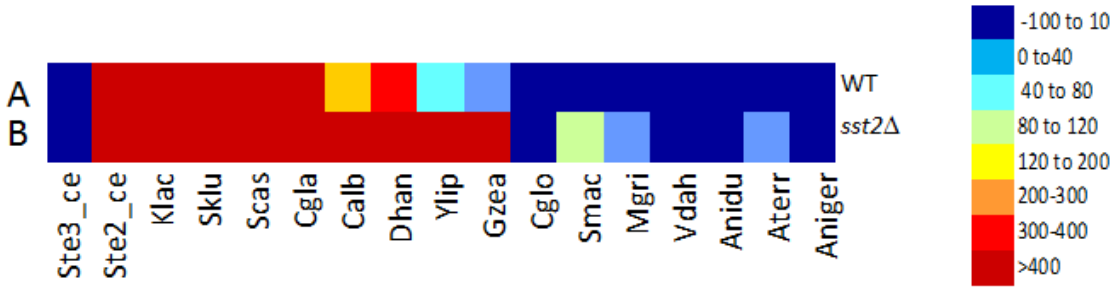


Figure 4.3 Response of the cloned receptors to the corresponding pheromone
Each strain was treated with DMSO or 6μM of the self α-factor for two hours and fluorescence levels were analyzed by FACS. The Ste3 receptor from *S. cerevisiae* (Ste3_ce) was induced with the *S. cerevisiae*'s α-factor as a negative control. The fluorescence values are in arbitrary units and normalized using the non-induced values. A- WT strains, B- *sst2*Δ strains.

We tested the response of the receptors to the self pheromone by measuring the levels of the pFUS1 driven fluorescent protein (Figure 4.4.A).

S. cerevisiae's signaling machinery can recognize the receptors of relatives as distant as *Debaryomyces hansenii*. After this point the receptors become too divergent and communication only happens in the absence of Sst2. Even in the absence of the regulator, receptors of fungi more distant than *Sordaria macrospora* show no response to self pheromone (Figure 4.3.B). This can be because the receptors fail to communicate to the signaling pathway or because they fail get efficiently expressed at the plasma membrane.

Heterologously expressed receptors can respond to pheromones from other species.

We then treated each of the strains with saturating concentrations of all of the 24 synthetic pheromones and used the FACS based assay to look at receptor response. The results are presented as heat matrices in Figures 4.4.A and 4.4.B or as normalized data in Supplementary Figures 8 and 9.

The Ste2 receptor from *S. cerevisiae* is clearly the most promiscuous responding to 10 out of the 24 pheromones tested, even from distant species. We believe that is probably because Ste2_ce has the advantage of being expressed in its natural host and not because it is intrinsically less specific. The receptors from the species closest to *S. cerevisiae* are also not specific and all respond to each other's pheromones. The receptors from *S. castelii* and *C. glabrata* are the ones that discriminate the better but this difference fades out once we delete sst2, indicating that this distinction is probably due to problems signaling to the MAP kinase cascade and doesn't necessarily reveal higher specificity. In fact, once we deleted Sst2, the Ste2 receptors from *S. castelii* and *S. kluyveri* could respond to a family of very distant pheromones, including those of *G. moniliformis*, *T. reesei* and *Y. lipolytica*. This last pheromone strongly activated 5 out of the 16 receptors in the *sst2Δ* background, even from far-away species, but its receptor is remarkably specific and only responded to the self pheromone. Interestingly, one of the pheromones from *A. nidulans* and the one from *A. terreus* could elicit a response when the cells expressed the *K. lactis* receptor and, in the absence of Sst2, even the *S. cerevisiae* STE2 could detect their presence. The receptors from and *S. kluyveri* and *D. hansenii* were activated by a large number of pheromones (9 and 6, respectively) and, after Ste2_ce, are the least specific. The fact that they don't just respond to any pheromone reveals that there is some specificity, but it is not very high.

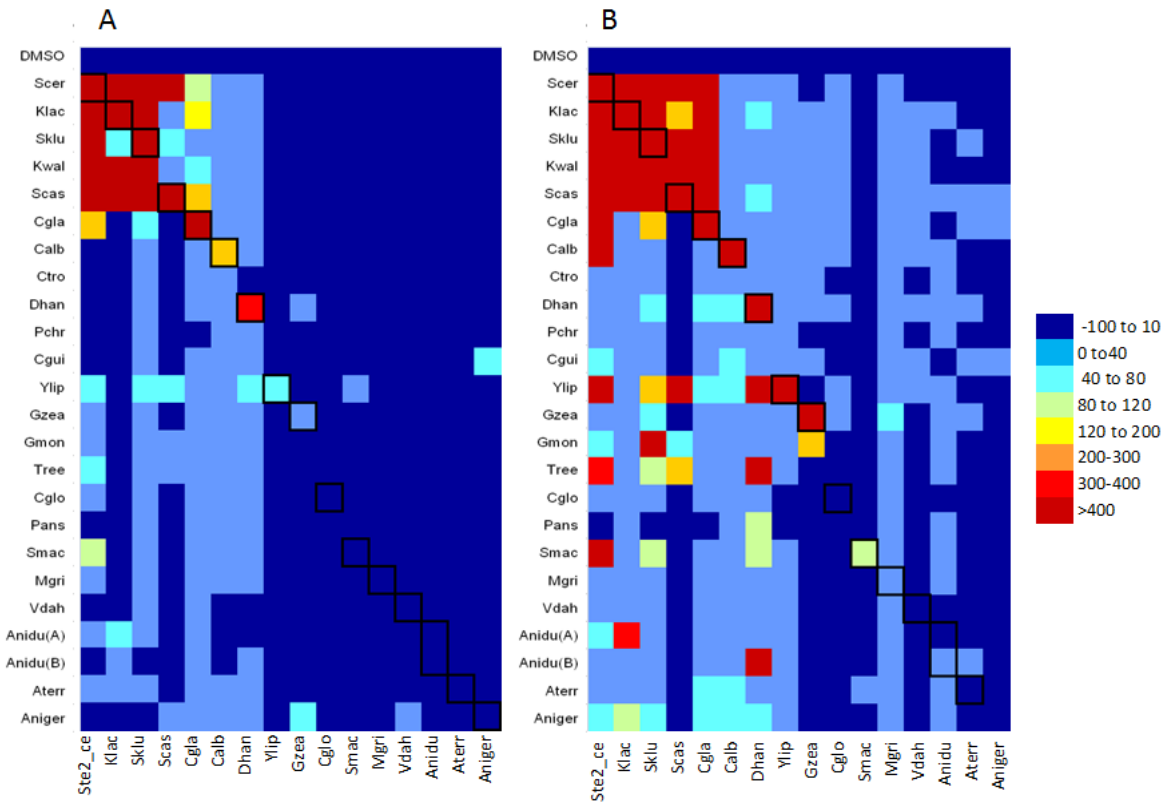


Figure 4.4 – Response of the cloned receptors to all pheromones
Each strain was treated with DMSO or 6μM of the 24 α-factor-like peptides for two hours and fluorescence levels were analyzed by FACS. The fluorescence values are in arbitrary units and normalized using the non-induced values. The vertical axis shows all the pheromones tested and the horizontal axis the receptor being expressed. Because we have more pheromones than receptors the matrices are not square and black boxes are used to show the response of the receptors to the self-pheromone, forming an imperfect diagonal. A- WT strains, B- *sst2*Δ strains.

On the other hand, it is also interesting to note that some pheromones could not activate any of the receptors. This was the case for the *C. tropicalis*' pheromone, even though this peptide is quite similar to the *C. albicans*' one and the receptor from *C. albicans* could respond, even if weakly, to the more divergent pheromone of *D. hansenii*. This is also the case for the pheromone of *C. globosum* (that doesn't even activate its own receptor) and *P. chrysogenum*, a peptide with only 4 aminoacid changes when compared to the pheromone of *S. macrospora*.

We wanted to test whether this promiscuity was caused by the saturating concentrations of α-factor so we chose a smaller group of receptors, that had shown cross-talk or activation before, and we analyzed their response to a lower concentration of some selected peptides. As expected (Figures 4.5.A and 4.5.B), the receptors now show more specificity (compare for

instance the response of the *C. glabrata* Ste2) and some pheromones lose the ability to activate some of the receptors (compare for instance the induction pattern of the α-factor's of *S. kluyveri* or *Y. lipolytica*), but they remain quite unspecific, particularly the ones evolutionarily closer to *S. cerevisiae*.

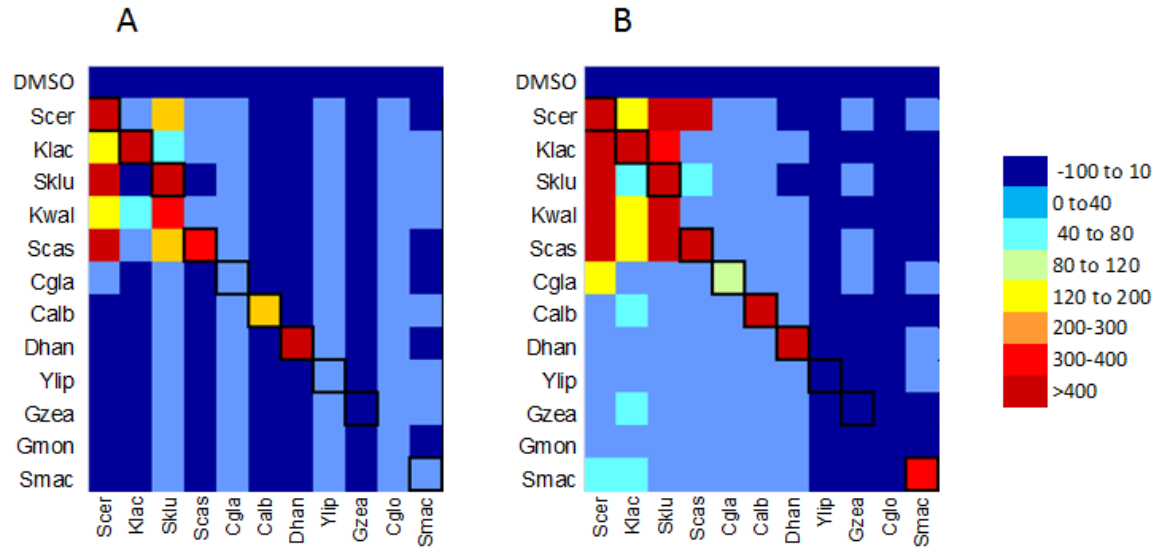


Figure 4.5 - Response of the cloned receptors to a subset of the pheromones
Each strain was treated with DMSO or 100nM with a subset of 12 α-factor-like peptides for two hours and fluorescence levels were analyzed by FACS. The fluorescence values are in arbitrary units and normalized using the non-induced values. The vertical axis shows the pheromones tested and the horizontal axis the receptor being expressed. Because we have more pheromones than receptors the matrices are not square and black boxes are used to show the response of the receptors to the self-pheromone, forming an imperfect diagonal. A- WT strains, B- *sst2*Δ strains.

The fact that a large number of receptors is not specific was quite surprising. In the budding yeast, pheromone recognition leads to cell cycle arrest preventing asexual reproduction, so the cost of inducing the mating pathway and not mating is probably very high. However, 100nM is still a quite high pheromone concentration and we should distinguish between biochemical and biological specificity. It might be true that, as we see, the receptors can detect the presence of distant pheromones, but only at concentrations that the cells don't usually express in nature. To know whether the response that we observe has significance in terms of mating or cell cycle arrest, we should generate dose response curves for the different receptors and pheromones. This will require large scale data acquisition which, although feasible, is not trivial.

Another reason why this was surprising is because sex genes are generally assumed to diverge faster than the rest of the genome (recently reviewed in [73]), so we didn't expect cross-

talk between the receptors and pheromones from very distant species. This led us to question the assumption that the mating recognition genes are under positive selection.

The STE2 receptor genes are not under positive selection

Mutations in a nucleotide can leave the aminoacid sequence unaltered (synonymous substitutions) or give rise to a different aminoacid (nonsynonymous substitutions). Starting from the already described receptor nucleotide alignment (Supplementary Table 4) we estimated the rate of evolution ω as the ratio of the rates of nonsynonymous (*dN*) and synonymous (*dS*) substitutions for each codon in the alignment. Positively selected codons retain a higher proportion of mutations that change the corresponding amino acid compared to those that do not result in an amino acid change, so codons under positive selection should have $dN/dS>1$, whereas codons under purifying (or negative) selection should have $dN/dS<1$.

We used two different approaches to estimate ω, REL, for Random Effects Likelihood and FEL, for Fixed Effects Likelihood (see Materials and Methods for a more detailed description of these methods), from the Datamonkey website [74]. REL assumes a distribution of rates across sites and infers the rate at which individual sites evolve given this distribution. FEL estimates the ratio of *dN* and *dS* substitutions on a site-by-site basis. The *dN/dS* values and p-values for each codon are reported in Supplementary Table 10 A Figure 4.6.A shows that there was some diversity in the values of *dN/dS* found for each codon, when *dN/dS* was plotted for those codons with p-value less than 0.1 (p-value is derived for the test $dN \neq dS$ using a likelihood ratio test). However, Figure 4.6.A does not support the hypothesis that Ste2 evolves quickly. In fact, both methods find that while a large number of sites are under purifying selection, no sites are found to be under positive selection at either the 0.05 or the 0.1 significance level.

It is important to note that the identification of residues under positive selection is not trivial. This is especially hard with long sequences and sequences that cover a long evolutionary period, as is the case with our receptors. To overcome this problem we repeated the analysis, limiting our data set to the 17 Saccharomycete's sequences. The data is presented in Figure 4.6.B using the same scale as in Figure 4.6.A. As expected, when we reduce the size of the data set we also reduce the number of significant codons and the plot looks sparser, but it is also clear that, if anything, the codons are under even stronger purifying selection (Supplementary Table 10 B).

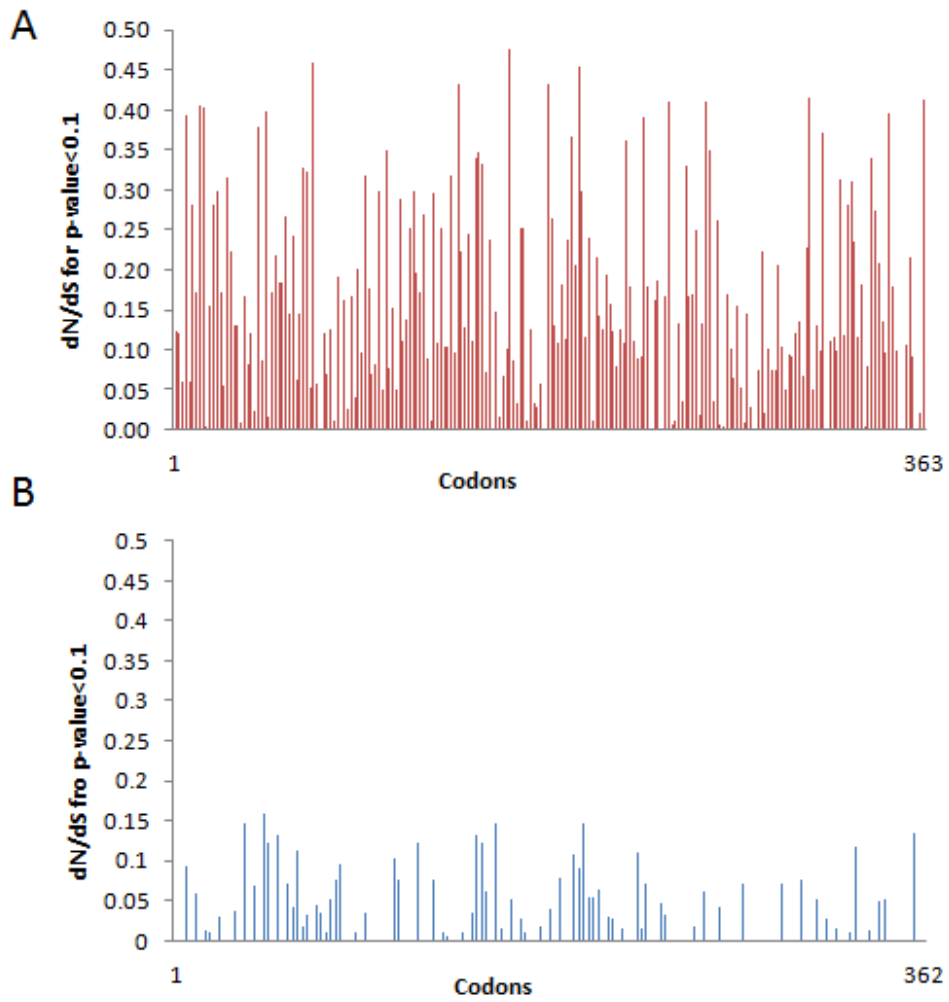


Figure 4.6- dN/dS across the Ste2 receptor alignment
Values of dN/dS were calculated using an FEL approach as described in the text and in the Materials and Methods, for an alignment of the 41 nucleotide sequences encoding Ste2-like proteins from the different yeast species annotated at the GeneBank website. The mean dN/dS value at this significance level was 0.162, and the variance was 0.014.

As was mentioned before, the Ste2 receptors are membrane proteins and have a conserved topology of seven membrane spanning hydrophobic domains. We wanted to see where the aminoacids predicted to be under purifying selection would fall on the receptor structure. We used a topology prediction program (via the webserver ConPred II **[75]**) to generate a snake-plot of the *S. cerevisiae*'s Ste2 receptor. While the REL analysis suggested that all sites are under purifying selection, the FEL analysis found just 220 of the 363 codons analyzed at significance

level p = 0.1. Figure 4.7 shows those amino acids corresponding to codons found to be under purifying selection circled in dark blue. These amino acids are distributed amongst transmembrane domains, intracellular and extracellular loops.

However, we note that there is less evidence of negative selection for those codons predicted to fall in the extracellular loops and this is one of the regions, together with residues at the extracellular face of various transmembrane domains, where the pheromone has been suggested to bind.

We have shown that the *S. cerevisiae* receptor is promiscuous and can detect and respond to the presence of pheromones of distant species and this response is stronger as the concentration of pheromone increases. This lack of specificity is not unique to the *S. cerevisiae* receptor and Ste2 receptors from several species respond to pheromones other than self. By using computational methods we show that the Ste2 receptor is not under positive selection and this might explain its lack of specificity.

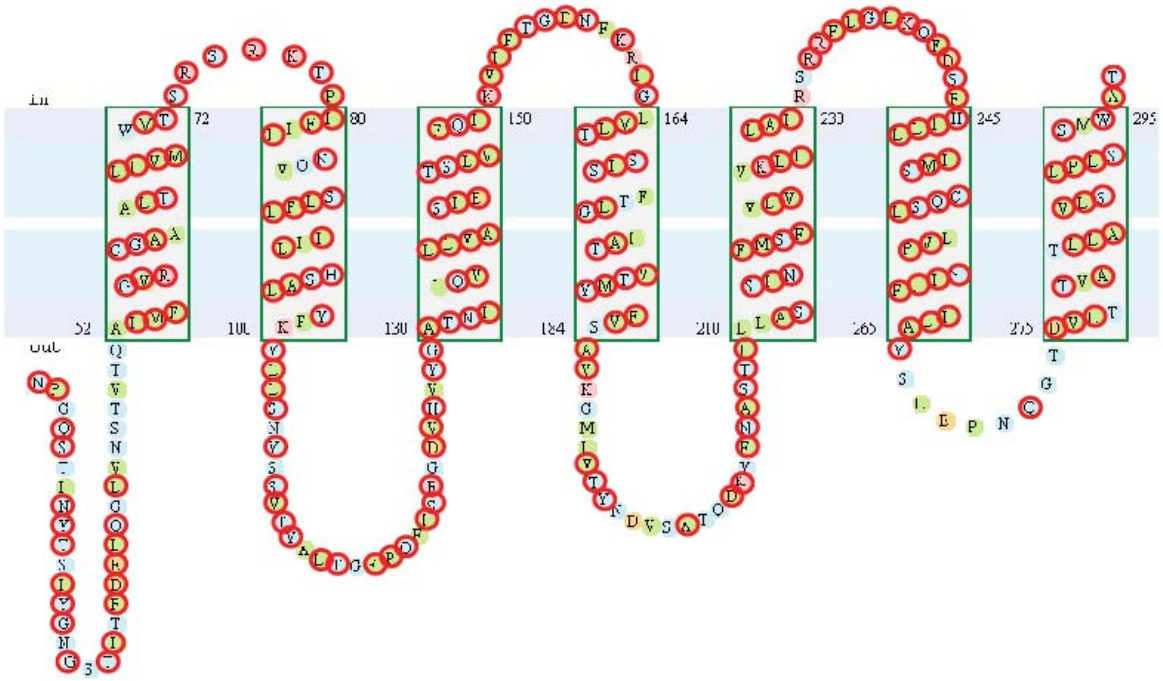


Figure 4.7 - The predicted transmembrane topology of the Ste2p protein from *S.cerevisiae.* The snake plot was generated by the ConPred II webserver [75]. Hydrophobic residues are in green, hydrophillic in blue, positive and negative charges in pink and orange respectively. Those residues found to be under negative selection at the 0.1 significance level (218/363), for the full alignment, were subsequently marked with red circles.

DISCUSSION

Sex genes are commonly believed to be under positive selection

Speciation occurs when two populations can no longer interbreed and give rise to fertile progeny but the mechanisms by which it happens remains an interesting question in Biology. Species boundaries are known to be maintained by the sterility of hybrids, for example due to the presence of incompatible genes [76-77] and, in general, comparative sequencing studies show that reproductive genes evolve more rapidly than other genes and, in many cases, have been shown to be under positive selection (recently reviewed in [73]). This divergence is believed to be important in the establishment of reproductive barriers between species [78]. The observation that the Ste2 receptors do not seem to be under positive selection together with the fact that the receptors are quite promiscuous, suggests that speciation is not happening via the receptor/pheromone pairs.

The *sensu stricto* yeasts can mate at high frequency but have speciated.

Saccharomyces cerevisiae can divide clonally both as a diploid or a haploid cell through asexual reproduction but when two haploid cells, of the opposite mating type, are put in each other's presence they mate with very high frequency (see Chapters 2 and 3). Not all fungi are as prone to mate as the budding yeast; although sexual cycles have been found to exist in all fungal phyla and the number of fungi with no known sexual reproduction has been decreasing as genomic and molecular techniques improve: for species such as *Candida glabrata* and *Coccidioides immitis,* population studies have provided evidence for genetic recombination, however a sexual cycle has yet to be observed in the laboratory [79-80].

As described in Chapter 1, the species of the *Saccharomyces sensu stricto*, are known to have high synteny and over 80% sequence identity [27]. Their receptors and pheromones did not evolve faster than the rest of the genome and some of them share the peptide and the lipid-modified pheromones. These species have also been mated in the lab and recently, McClean and Grieg [81] have shown that although *S. cerevisiae* and *S. paradoxus* can interbreed with high frequency, when given a choice (in competitive matings) they prefer to mate within their species. They explain this finding with mating timings, as *S. paradoxus* has a longer developmental period. This gives support to previous work that has also shown that selection for reproductive isolated leads to differences in mating timing rather than changes in the receptor/pheromone

specificity [82]. More recently, a comparative study of transcription regulation of the mating pathway between different yeast species has shown that the Ste12 binding sites have diverged significantly faster than the rest of the genome and this can be a source of speciation [83].

The *sensu stricto* yeasts often share the same ecological niches and have been isolated together in several field studies [29-30]. Natural hybrids between the *sensu stricto* species are also common even though they give rise to dead spores (reviewed in [65]). Some of these hybrids have been characterized and are believed to have some fitness advantage when compared to their parental strains. *S. pastorianus*, a natural hybrid between *S. cerevisiae* and *S. bayanus* seems to have gotten the best of both worlds, being able to metabolize some unusual sugars and ferment at a wide range of temperatures [84]. This might indicate that pre-zygotic isolation has not been strongly selected for, raising the interesting question of why speciation occurred.

Unfortunately not much is known about the ecology of most fungal species and it is difficult to speculate about possible natural barriers to mating. We mentioned the fact that different species might have different mating timings and this could become an effective natural barrier. It is also known that closely related species can prefer to grow at different temperatures and might have significantly different cell cycle lengths and life cycles in the wild. Finally, because most mating genes are expressed under the same regulatory network, changes in the regulatory regions of mating genes (in cis or trans) might have a larger target size and can facilitate faster evolution than a system where both genes would have to mutate at the same time for speciation to occur. Our results suggest that the receptor/pheromone system does not significantly contribute to pre-zygotic isolation.

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CHAPTER 5

Discussion

When haploid cultures of opposite mating types are mixed(...), the cells may put out hyphal ('copulatory') processes; and when these meet, fusion may occur and a dumb-bell-shaped zygote be formed. (...) Experiments in which a plus cell was placed close to a minus cell and allowed growth (...) showed that the mating reaction is probably hormone- controlled, since mating response developed without contact between cells of opposite mating type; but it was found that only the minus cells put out copulatory processes.

J. D. Levi, 1956

In 1956, in a four paragraph, one figure, *Nature* paper, J.D. Levi reported that two yeast cells, of opposite mating types, could respond to each other at a distance [85] and predicted the existence of pheromones. This communication process between two yeast cells has been extensively studied in the past decades. These cell types are now called MAT**a** and MATα, instead of plus and minus, and the predicted hormones have been identified as **a**- and α-factor. We know many of the molecular details involved in how these signals induce the "mating reaction" in the cells and how the shmoos, or "hyphal processes" are put out. However, and among many phenomena, why only the MATα (or the minus) cells change their morphology in response to the opposite mating type, remains unknown. We focused on this system of communication and asked: how do cells find each other? How do they choose a partner? And how to they distinguish between self and non-self? In this Chapter, I will try to present an overview of what I believe to be our contributions to answering these questions and I discuss the use of yeast as an experimental model to the study of cell-cell communication in more general terms.

Yeast as a model organism for the study of chemotropism

In the first part of this thesis we focused on the mechanisms used for detecting the position of a mating partner, more specifically on the role played by the Bar1 protease. Since yeast cells do not move, the two (**a** and α) cells reach their partners by growing toward each other, in a process called shmooing. A pre-requisite for successful mating is thus the correct recognition of the direction of the mating partner, in order to be able to properly orient the mating

projection. This process requires two different mechanisms: distinguishing between close and distant cells and fine tuning the direction of the shmoo to meet the one from the mating partner.

The secreted form of Bar1 plays a role in the first mechanism, by degrading the pheromone produced by the α cells. By providing an exogenous protease to the mating mix, it is possible to significantly recover mating in *bar1*Δ cells (Chapter 2).

We then looked at the role of localized degradation of α-factor, either through endocytosis and degradation or localized inactivation of the pheromone by Bar1 retained at the cell wall (Appendix A). Although endocytosis is commonly implicated in down-regulation of the response and recovery from induction, we believe that it may reveal an additional role in facilitating gradient detection and shaping the gradient itself. A similar mechanism may also be applicable to *chemotaxis* of eukaryotic cells; it is known that eukaryotic cells, unlike prokaryotes, usually assess receptor occupancy across their membrane before initiating directional movement (recently reviewed in [86]).

We had already argued that yeast and other eukaryotes use very different mechanisms for chemotropism and we suggest two reasons for this difference: the first is that a haploid yeast cell has a different mission from a chemotactic bacterium or slime mold. The yeast cell is looking for an individual cell at close range, whose general properties (size, pheromone production rate) it has evolved to match, whereas the chemotactic cells are looking for an object of unknown size at an unknown distance. Were chemotactic cells to release ligand-degrading molecules, they would be unable to detect distant sources of attractant. Were yeast cells to adjust the coupling between pheromone concentration and intracellular signal strength, they would detect the sum of the pheromone delivered from nearby and distant cells. Using a secreted interceptor reduces the half life of free pheromone and thus privileges the signal from nearby cells over that from distant cells. The second reason for preferring an interceptor comes from the biology of mating. After meiosis, the four yeast spores (two **a** and two α) are tightly packaged in the ascus, a specialized cell wall. The germinating spores can commit to mating before emerging from the ascus. If the ascus wall provides a barrier to α–factor diffusion, continued pheromone secretion could eventually overwhelm receptor adaptation. Through regulated Bar1 production, α-factor levels can be kept in the range needed for efficient mating.

Is regulating ligand concentration, rather than ligand-signal coupling, unique to budding yeast? We suspect not. In animal development, responding cells must often pick one of several

identical targets and, in several cases, the target cell produces a diffusible signal and the seeker makes a signal-destroying protease ([87-88]. Recent studies have shown that fibroblasts [89-90] and axonal growth cones [91-92] can only detect gradients of their ligands in a very narrow range of concentrations. Yeast chemotropism might thus rely on a general mechanism for gradient sensing that is widely used by cells trying to find and interact with specific targets. It would be interesting to examine whether a similar mechanism functions in other systems which rely on signaling gradients for directing cell fusion, as is the case, of the wiring of axons in the developing neural system.

Yeast as model organism for the study of hydrophobic signals.

In wild type mating mixes, it is uncommon to see shmoos, as cells "prefer" to bud rather than to shmoo towards a partner (Figure 2.4.E). This change in morphology seems to be specific to cells detecting high concentrations of pheromone. Nevertheless, and as J.D. Levi observed over 50 years ago, when shmoos appear, they always originate in the **a** cells. The fact that six repeats of the α-factor pheromone exist in the *S. cerevisiae* genome while there are only two of the **a**-factor (potentially leading to differential expression), should not matter in mating mixes where an excess of **a** to α cells exist, or at long time scales, with secretion not being balanced by degradation. Also, our data shows that there is no intrinsic reason for this difference in morphology, as *MAT*α cells expressing the Ste2 receptor and exposed to α-factor can shmoo (see for instance Figure 3.4.B). Why then, do we never observe mating protrusions in α cells?

One hypothesis is that some **a**-factor protease secreted by α cells, equivalent to Bar1, remains to be identified. This is unlikely, as several genetic screens looking at mating sensitivity have been made (and failed to identify such enzyme), and few genes remain to be annotated in the yeast genome.

Another hypothesis is that there is something different about how the Ste3 receptor communicates with the MAP kinase cascade, either by being less sensitive to the presence of **a**-factor or by being more efficient at depleting the pheromone from the medium. Deletion of Sst2, the negative regulator of the Gα subunit, can in fact induce prolonged arrest in MATα cells, in the presence of **a** cells, but this is the same phenotype as observed in **a** *sst2Δ* cells. Faster turnover of the Ste3 receptor could potentially lead to efficient **a**-factor depletion from the medium. However, this would require the endocytosis rate of receptor to respond to the extracellular

concentration of the pheromone, or shmoos should be observed in the presence of a large excess of **a** cells.

Finally, shmoos have been observed in α cells treated with high concentrations of synthetic **a**-factor. This pheromone is very hydrophobic and, during these induction experiments, several chemical tricks have to be used to keep the **a**-factor in solution and to prevent non-specific binding to most polymers (meaning most lab surfaces). This leads us to suggest a third hypothesis: possibly, the physical-chemical properties of the pheromone help in keeping its effective concentration below the threshold necessary for shmoo formation, by removing them from the effective pheromone pool. Under normal mating conditions, the pheromone's fatty group on one **a**-pheromone could react with the farnesyl groups of neighboring peptides, forming aggregates, or micelles, preventing recognition by the receptor.

Three lines of evidence support this view: 1) the **a-**factor pheromone has very high membrane partitioning, indicating that its diffusion properties are probably very different from those of the α-factor pheromone; 2) it has been purified as a high molecular weight complex [93], suggesting that large aggregates might form; 3) when we generated our mating pair strains, using only MAT**a** cells expressing only **a**-factor-like pheromones, no shmooing was ever observed, indicating that there is nothing specific to the α cells preventing these changes in morphology.

It would be very interesting to study this in more detail, as **a**-factor-like pheromones are ubiquitous in the fungal kingdom and hydrophobic signals are commonly used in higher eukaryotes as signaling molecules (see, for instance, a recent review on Wnt processing [94]). It is known that preventing mating or mutating components of fungal MAP kinase cascades can reduce virulence of several human and plant fungal pathogens (for reviews see [95-96], [97]), and recent reports have shown adding enzymes with lipolytic activity to the surface of plant leaves can prevent fungal invasions [98]. We suggest that the physical-chemical properties of these signals might play important roles in receptor recognition and cell-cell communication. Yeast, could be used as an experimental model to further study these processes. Simple experiments, such as changing the hydrophobicity of the medium during mating, or making **a** cells secrete different concentrations of **a**-factor, could potentially give very useful results. If the lipid tails interact with each other at physiological concentrations, we predict that increasing the number of **a**-factor molecules would not necessarily make a big difference, but changing that the critical micelle concentration should.

Yeast as a model system for the study of sexual-selection

In a section of Chapter 3, we discussed 1) how a protease seems to be required in the case of mating pairs that communicate by secretion of α-factor-like peptides only, 2) how we observed the formation of multiple shmoos (a sign of over-stimulation) when no protease was present and 3) how Bar1 might have evolved in parallel to the appearance of α-factor-like peptides. In Appendix A we present evidence for the requirement of Bar1 in intra-ascal mating and discuss the prevalence of these events in the "wild". It has been known that yeast cells pay a cost to advertising their presence to possible mating partners, and that cells prefer to mate with the partners that advertise the most [33]. We have shown that the α cells produce significant amounts of their pheromone, even when they are not being stimulated (Figure 2.1.B) and we estimate this to account for approximately 1% of the total protein synthesis. Moreover, in response to the presence of a mating partner, the α cells are most likely expressing at least 10 times more pheromone (Figures 2.1.C and 2.2.C). These efforts seem to be wasted in the presence of Bar1 and, in Chapter 2, we proposed that this is a case of runaway sexual selection, with **a** cells choosing to mate with the α cells that express the most α-factor, and having to secrete Bar,1 to keep the pheromone concentration from saturating its receptors. Here we would like to extend this view and see Bar1 in the light of the handicap principle. This was already briefly mentioned in the discussion of Chapter 3, where we present a complementary way to look at the "invention" of Bar1.

The handicap principle was first proposed in 1975 [99] and this theory predicts that a honest sexual signal should be costly. In Zahavi's own words: "*the marker of quality should evolve to handicap the selected sex in a character which is important to the selecting sex, since the selecting sex tests, through the handicap, the quality of its potential mate in characters which are of importance*". This could be used to explain traits as the peacock tail or the size of the deer's antlers. Peahens choose mating partners by the size and beauty of their tails, as female deers select on the size of the male's antlers. These extravagant displays have a high cost to the bearers. Peacocks are poor flyers and the brightness of their tails makes them more attractive not only to females but also to predators; however, from the female perspective, the fact that males can carry around such unpractical tails might be a sign of fitness. Similarly, the large branched antlers of moose can get tangled in trees and reduce their likelihood of escaping predators, but they also help them fighting other males and can be a sign of a healthy carrier.

For the past few decades, many theories have built on the handicap principle, or tried to disprove it, but lack of good experimental models has left most of the discussion limited to theoretical arguments. I would like to argue that the yeast mating system might provide a good framework to study sexual selection in the lab. The "honest signal' would be α-factor given the **a** cell's preference for high pheromone secreters [33] and that α cells spend considerable resources on its production. Bar1 might have evolved as a test to the quality of the signal. If **a** cells are able to detect it even after very strong degradation, the signal is guaranteed to originate from a fit partner. In yeast, it would be possible to measure the fitness cost of producing variable levels of pheromone and relate that cost to their mating efficiency. For example, **a** *bar1Δ* cells should mate more efficiently with α cells expressing less pheromone (*mfα1Δ MFα2*, for example) and preliminary results indicate that this is true (JGS, data not shown). It would thus be interesting to compare the long term fitness of diploids formed from crosses between **a** *bar1Δ* and α *mfα1Δ* to the efficiency of diploids that had been selected on the basis of the handicap principle (**a** *BAR1* mating with α *MFα1 MFα2)*.

Yeast as a model system for the study of self/non-self recognition.

One of the best studied examples of adaptive evolution is that of the immune system. Immune system cells have to be able to distinguish between self and non-self and respond only to the latter. T-cells have to detect the presence of pathogens, by recognizing small peptides presented by the Major Histocompatibility Complex (MHC). The MHC has to able to present a very large number of peptide sequences and its binding pocket is known to be under positive selection (reviewed in [100]). Parasites represent the major selective force for variability at the MHC, as failure to recognize their epitopes can lead to infection and misrecognition is one the causes of auto-immune diseases. The receptors at the surface of the T-cells must thus be very specific, and respond *only* to non-self signals, and, simultaneously, very variable, to respond to *all* possible non-self signals.

GPCRs are also known to be quite specific and able to discriminate between a wide range of stimuli [101]. One extensively studied example is the collection of GPCRs that function as odor receptors, representing the biggest family of GPCRs in mammals. These olfactory receptors are able to distinguish between a still undetermined number of signals, although it is known that each receptor is usually capable of detecting more than one odor (reviewed in [102]). These

receptors have been shown to be under positive selection and it is believed that the selective force for the variability is also likely to be related with prey detection and predator evasion ([102], [103]).

As was mentioned before, most mating genes are believed to be under positive selection (reviewed in [73]). In yeast, the selective pressure would not be to defend themselves against external threats, but to generate barriers against the formation of sterile hybrids. When we started this project we expected the fungi mating system to behave in a similar way to that of the immune system: when presented with a large number of different peptides, the yeast cells would be able to discriminate between their pheromone and pheromones of different species and this recognition should happen at the receptor binding site. This is not what we saw. When we induced the receptors of different fungal species, we observed that a high number of the tested receptors showed poor specificity and responded to the pheromones of distant relatives (Chapter 4). Although we presented the cells with very high concentrations of pheromones it is not unlikely that they might experience around 100nM pheromone levels in the wild. As we discussed earlier (Chapter 2), yeast cells are likely to secrete 1nM pheromone when stimulated. If the contribution of one single stimulated cell can be of around 7000 molecules per second, a colony of cells is likely to experience extremely high concentrations of pheromone. It would be interesting to generate a dose-response curve for some of these receptors, responding to different pheromones, like the one in figure 2.1.A.

Another common notion in sexual selection is reinforcement (recently reviewed in [104]). Reinforcement, or the Wallace effect, is a way to increase reproductive isolation. During the process of speciation, the formation of less fit or even infertile hybrids, between two newly isolated species, is possible. These hybrids will be selected against by natural selection and, over evolutionary times, barriers can appear to prevent cross mating between the isolated species and to favor within species mating only (assortative mating). In sympatric species (species that share the same geographical niche), the formation of hybrid zones, or regions where it is possible to isolate hybrids, is relatively common. A well studied example is that of two *Drosophila* species (*pseudoobscura* and *persimilis*) which have different wing colors, share geographical niches, and can mate to form sterile hybrids (first described by [105]. Males court females from both species, but females prefer to mate within their own species; however, this preference is stronger in regions of overlap between the species, when compared to the preference of populations that

don't often get a chance to reproduce with other species [106]. The interpretation is that populations that rarely form hybrids (geographically separated) are not under strong selection to deepen their speciation, whereas sympatric populations that generate high numbers of sterile hybrids, have an advantage in preventing inter-species mating. The sterility of the hybrids would reinforce their speciation.

Again, this is not what we see in yeast. As mentioned before, the species from the *sensu stricto* group can mate with each other and give rise to sterile hybrids, but there are "naturally" isolated hybrids, between species that physically coexist in wineries and breweries. It is important to notice that at least one of the isolated hybrids, *S. pastorianus*, might have an advantage when compared with the parental strains (*S. cerevisiae* and *S. bayanus*) as it can ferment very efficiently and at low temperatures [84]. This means that, although infertile, these naturally occurring hybrids might not be selected against as vegetatively growing diploids. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* are commonly isolated together in the wild ([29],[30]), and it would be interesting to test for the presence of hybrids between these two species, in their natural environment. On the other hand, recent studies with *Neurospora* species that can fuse in the lab, have shown that the reproductive barrier is stronger in species that geographically co-exist than in species isolated from very distant regions [107], suggesting that these species might be evolving to generate higher reproductive barriers. However, it is worth noting, that the differences in mating preference, were post pheromone/receptor recognition, again supporting our observation that speciation in fungi is not happening at this recognition level.

The fact that closely related fungi have difficulties discriminating between self and non-self, taken together with our observation that the receptors and pheromones are the major determinants of mating identity and specificity (Chapter 3), raises some interesting points. If sexual specificity is determined by which receptors and pheromones are being expressed, and if the receptors are promiscuous and the pheromones are unfaithful, the notion of identity becomes blurry. Also, the idea that fungi might be able to communicate intra and inter-species is an attractive one and I would like to conclude with the suggestion that yeast might also be a good model for the study of multicellularity.

APPENDIX

Local Pheromone Degradation

The evidence presented in Chapter 2 shows that **a** cells use a protease, Bar1, to keep α-factor concentration at their surface within the narrow range needed for accurate gradient detection. We have also shown that the protease's activity can be mimicked by adding a soluble protease to the mating mixes, supporting a previously published model where soluble protease could work by increasing the steepness of the gradient and shielding the a-cell against possible far away partners [38]. These models can explain problems in gradient sensing and what happens in closely packed mating mixes, as it occurs frequently in the wild, but they cannot explain how yeast cells distinguish between two equally attractive partners. This is likely to be the case in intra-ascal mating. After meiosis, the four yeast spores (two **a** and two α) are tightly packaged in the ascus, a specialized cell wall. The germinating spores can form to mating pairs and each potential partner is at the same distance, at the same stage of the cell cycle and presumably secreting comparable amounts of pheromone. It is believed that self-fertilization plays an important role in the sexual reproduction of wild yeast species [108] and the occurrence of intra-ascal mating has been reported [109] (and see Chapter 3).

We wanted to study how yeast cells can mate efficiently over a wide range of conditions, from when there is only one partner available, to dense mating mixes or in the situation of equally attractive partners.

Starting from the ascus situation, we developed a mathematical model, where two **a** cells and two α cells attract each other. If we assume that these cells do not perturb the gradient this is a situation analogous to an electric dipole (Figure A1.1.B). In this case, the field lines (pheromone flux) at the **a** cell position will be concentrated at the location facing the mid-point between the two secreting cells. Consequently, the cell is expected to polarize towards the mid-point between the cells. Now, if the **a** cells can degrade the pheromone at their surface, acting as a perfect sink, this situation is very analogous to that of an electric quadropole, with the **a** cells becoming "negative charges" (Figure A1.1.A). The flux lines would no longer point to the midpoint between the secreting α cells, but to each of them (Figure A1.1.C,E). Such degradation would, thus, help the cells discriminate between the two equally attractive partners. This model is, naturally, a very simplified version of what I probably happening in nature. It relies on the need for the **a** cells to act as a perfect sink with the effective concentration of α-factor at the membrane equal to zero; it also depends on the cells not touching, so that the gradient formed by

the protease is of a smaller scale than the cell-cell distance, but it provides an attractive model to explain how one cell could disentangle similar and equally distant signals.

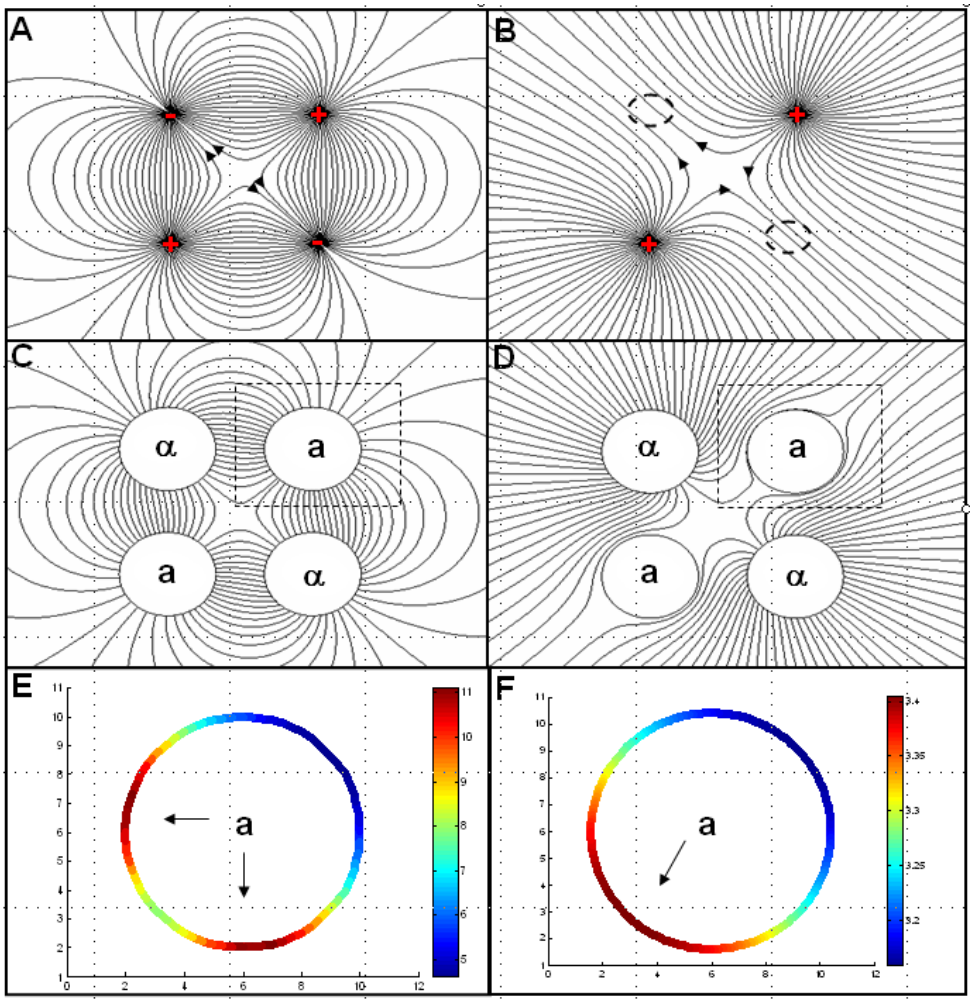

Figure A1.1 - α-factor gradient in germinating spores.
A - Field lines of a quadrupole: two positive charges and two negative charges. B - Electric field lines due to two positive stationary charges of equal magnitude in a dipole arrangement. C - Diffusive field lines for localized degradation model. We assumed that local degradation on the a-cell is strong enough, so that the cells can be viewed as perfect sinks with zero concentration on the boundary. D - Diffusive field lines for simple diffusion model. **A** cells can not degrade and are considered reflective. E - The α-factor concentration along the perimeter of an **a** cell in the presence of localized degradation. Note the two peaks, corresponding to equivalent maxima pointing towards the two alternative partners. F - The α-factor concentration in the absence of local degradation. Note that only a single peak is present, pointing midway between the two secreting cells.

Based on this model we hypothesized that localized degradation of the pheromone should help in partner discrimination and we proposed three possible mechanisms that could help in disentangling gradients generated by equivalent signaling centers: localized degradation at the cell wall, regulated receptor endocytosis and polarized secretion of the protease.

We began testing these hypothesis by looking at the distribution of the α-factor receptor, Ste2, in wild type **a** cells. For this we generated a fusion protein with a GFP C-terminus tag. As expected expression of the receptor is low during asexual life cycle (present uniformly across the membrane) and is up-regulated upon pheromone stimulation, is recruited to the shmoo tip and reaches its maximum right before fusion (Figure A1.2).

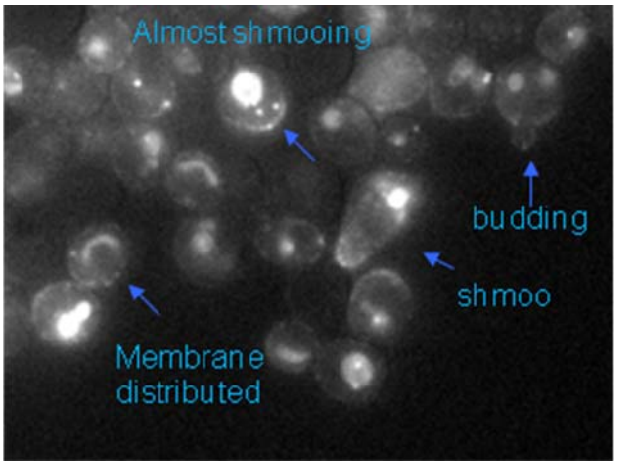

Figure A1.2 – Ste2-GFP localization
A strain expressing a STE2-GFP fusion was constructed and cells were treated with 1μg/ml of α-factor for 1h. After this period cells were imaged and the localization of the protein was analyzed at different stages of the cell cycle.

As described before, α-factor binds the **a**-cell G-coupled receptor Ste2 and, upon pheromone binding, the receptor is internalized (together with the α-factor that gets degraded) and signals downstream. This mechanism depletes α-factor from the extracellular space and assures that signaling is maintained only for as long as there is external stimulus. We searched the literature for mutants that could check the prediction that this local depletion is important for mating, and we chose 8 point mutants that were known to play a role in receptor endocytosis or pheromone binding affinity (Table A1.1).

Receptor mutant	Reference	Described Phenotype				
		pFus1 induction		α-F binding	Endocytosis	Arrest
		with Ste2 wt	without Ste2 wt			
F55V	[110]	80%	NT	30× less	NT	lower
F204S	[111]	50%	80%	almost none	none	$>10^{-8}$ M
S219P	[110]	60%	NT	8×less	NT	lower
S259P	[110]	112%	NT	none	NT	lower
Y266C	[111]	30% to 50%	80% to 55%	3×less	less	$>10^{-8}$ M
S184R	[112]	40%	77%	NT	NT	NT
N205K	[112]	45%	90%	NT	NT	$>10^{-8}$ M
N205D	[112]	41%	65%	NT	NT	NT

Table A1.1 Receptor mutants expressed
Eight receptor mutations were chosen from the published literature. All but F55V, S259P and S219P had been described as dominant negative mutants. F55V was chosen for its low binding affinity to the pheromone, F204S for its strong reduction in endocytosis, and Y266C for its intermediate phenotype. All receptors were expressed in the presence and absence of the wt Ste2 receptor and under 3 different pFus1 promoters [113] (either from plasmids or from the genome) to generate a total of 32 strains.

These mutants were expressed under different promoters in the presence and absence of the WT receptor and the cells response tested under varying α-factor concentrations in a flow-chamber. We could clearly identify combinations where the cells were less responsive to the presence of the pheromone, but we could never mimic a pFUS1 response curve like the one in Figure 2.4.B. This wasn't particularly surprising as most receptor mutations are likely to have pleiotropic effects and not play a role in endocytosis or binding affinity, only. Also, altering one of these receptor roles might change its function in unexpected ways. For instance assuming that the Ste2 level on the cell surface is set by the balance between secretion and internalization, blocking or limiting the endocytosis of the receptor probably increases the level of Ste2 at the

membrane and might lead to increased MAP kinase pathway activation and this could add up to the expected effect of less pheromone depletion.

We then wanted to look at the distribution of the protease upon pheromone induction and during mating. As before, we generated a BAR1-GFP fusion protein. Figure A1-3B shows Bar1-GFP localized to the shmoo tip in cells uniformly stimulated by 5µg/ml α-factor and in Figure A1.3A the localization of the same fusion protein is shown in a normal mating situation.

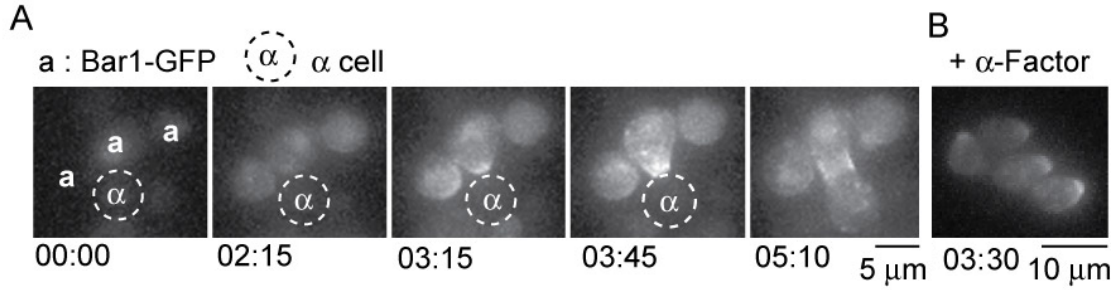

Figure A1.3 BAR1-GFP localization
A- BAR1-GFP *MATa* cells mating with a *MATα* cell and BAR1 polarization to the fusion site.
B) BAR1-GFP *MATa* after 3:30h of uniform induction with 0.5µg/ml α-factor

The BAR1-GFP signal is very strong at the surface of the cell and we wondered if there could be local activity form the cell wall. Full secretion of the protease into the surrounding medium was broadly assumed until Moukadiri et al. [114]. reported extraction of Bar1 from the cell wall in significant amounts. There is also evidence that Bar1 is active from early compartments of the secretory pathway [115] and that the exocyst localizes to the tip of the shmoo. To investigate the localization of the protease, we started by looking at Bar1 induction upon pheromone stimulation. Using a BAR1-TAP tagged strain we stimulated cells with different concentrations of α-factor and compared the levels of the protease in both the supernatant and the cell pellet at several time points (Fig. A1.4.A,B).

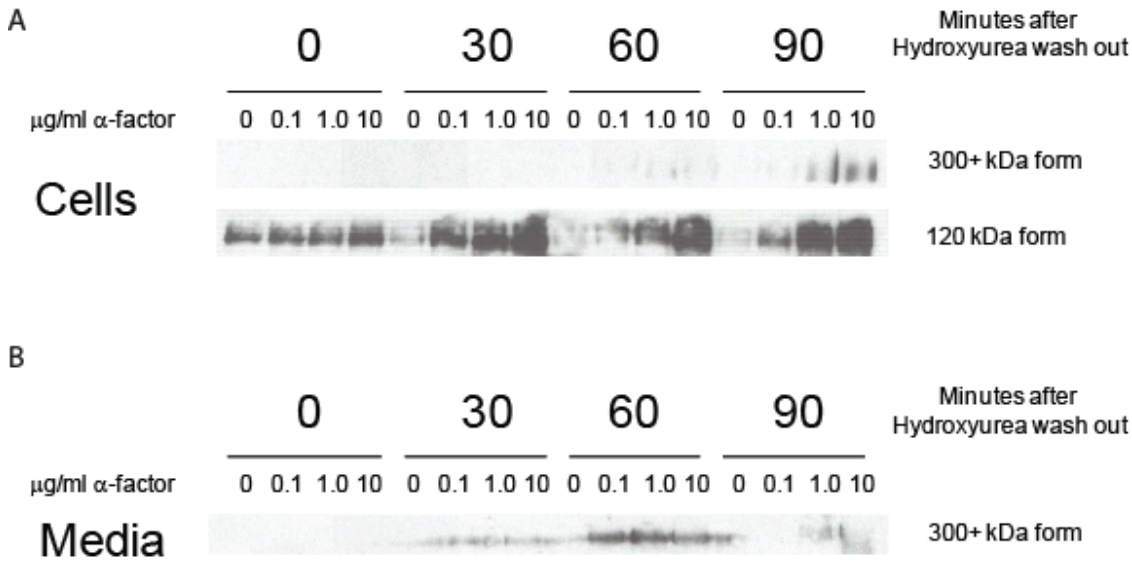

Figure A1.4 - SDS-PAGE and Western-blot with anti-TAP antibody for BAR1-TAP
Bar1-TAP cells were grown in YPD at 30º C and arrest with 10 mg/ml Hydroxyurea for 2 hours, or until 95%-100% of the cells were large-budded. Cells were released from the arrest by hydroxyurea wash out and split into 4 cultures. These were induced with 4 different α-factor concentrations 0, 0.1, 1.0 or 10.0 mg/ml. 1.2mL samples were removed at 0, 30, 60 and 90 minutes and spun down. A- Cells were lysed using NaOH/b-mercaptoethanol. B- The supernatant was EtOH precipitated. Both cell lysate and precipitated media were incubated with an anti-TAP antibody for 2h, washed and imaged.

Bar1p exists in two different forms. One of very high molecular weight (over 300kDa) that is mainly secreted and another one of around 120kDa which is only found associated with the cells. It is known that Bar1p is heavily glycosylated [116] and this could account for this difference in protein size. This data also suggested that there might exist a cell wall bound form of Bar1, working from the tip of the shmoo.

The previous results seem to indicate that effective mating can only happen when BAR1 acts both as a secreted and a bound molecule. The secreted form lowers the overall concentration of pheromone and prevents saturation of the receptor. The cell-wall bound form locally changes the shape of the pheromone gradient and allows proper partner discrimination. In this case, MATa *bar1Δ* cells expressing a protease at their surface, should, in principle, be able to discriminate between partners. To test this hypothesis we developed a protocol to specifically bind a protease to the cell wall of yeast cells.

We took advantage of the high affinity of biotin to avidin to mimic a situation of localized degradation by a cell-wall bound protease (for detailed experimental procedures see the Materials and Methods). We reacted the cell wall of MAT**a** *bar1Δ* yeast cells with a malemide modified avidin To measure the extent of binding we added fluorescently labeled biotin to treated and untreated cells Figure A1.5.A,B.

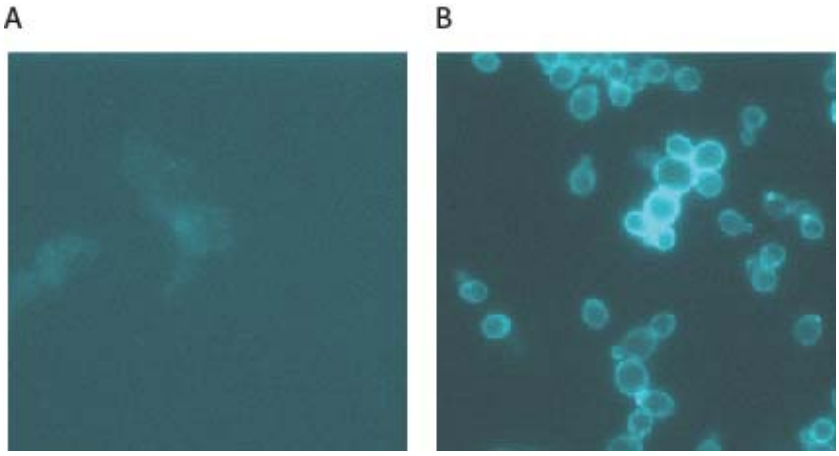

Figure A1.5 – Biotin binding
Cells were treated with a maleimide modified avidin, washed and incubated in the presence of Biotin-FITC. The cells had been previously treated with PBS (A) or a 1mM DTT in PBS solution (B), showing that there is no non-specific binding of the biotin in the absence of the avidin.

We then conjugated an NHS-modified biotin to chymotrypsin by making it react to the protease's amino-groups. This compound was subsequently incubated with avidin bound cells. Figure A1.6 shows the protease conjugated *bar1Δ* cell stimulated by the presence of α-factor. It is possible to see that the conjugated cell is cycling and buds at least 5 times. The non-conjugated daughters, on the other hand, detect the presence of the pheromone and shmoo.

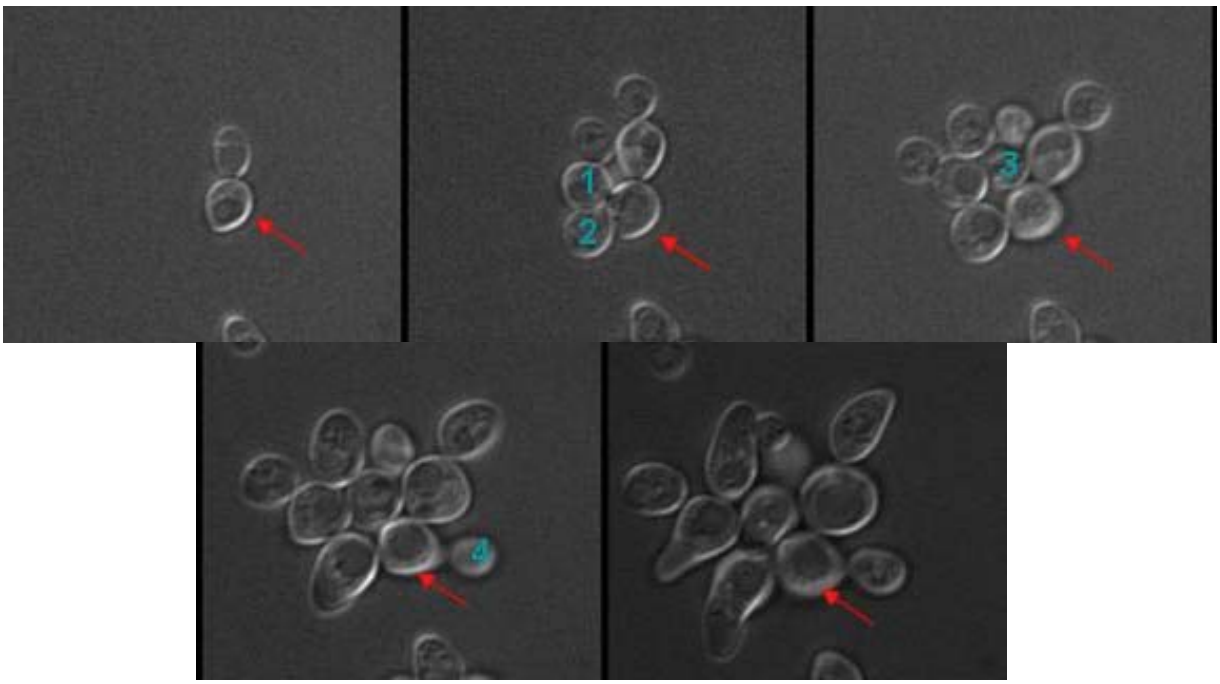

Figure A1.6 – Cell wall bound chymotrypsin
Cells were treated with DTT, incubated with maleimide-avidin washed out and incubated with a chymotrypsin bound biotin. Cells were washed and imaged for 8h in the presence 0.1µg/ml of α-factor. Red arrow identifies the chymotrypsin treated mother cell and numbers track the appearance of the daughter cells.

We have reported previously (Chapter2) that adding an external, soluble protease to a mating mix of wild type α and *bar1*Δ **a** cells can recover the mating efficiency to wild type levels. Matthieu Piel had also observed, during these experiments, that a small number of **a** cells still displayed difficulties finding a mating partner. This happened when the **a** *bar1*Δ cell was surrounded by two equally attractive α cells (Figure A1.7). Because polarization is a slow process, the α cells manage to reorient and match their shmoo tip to that of the off-gradient **a** cells, thus allowing for efficient mating.

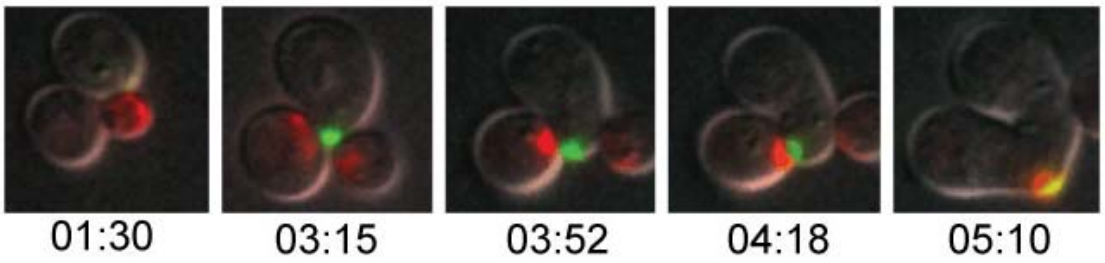

Figure A1.7 - *bar1*Δ *MAT*a cells show alignment problems even in the presence of soluble protease.
A *bar1Δ* MAT**a** cell (SPA2-YFP, green dot) was faced with two nearby MATα cells (SPA2-RFP, red dot) and it polarized to a point located in between them. Despite this confusion, most of these cells usually mated because one MATα cell redirected its polar cap with the mis-directed shmoo tip of the *bar1Δ* MAT**a** cell

To quantify the mating recovery of cell-wall bound protease treated *bar1Δ* **a** cells we needed to prevent the α-cells from finding and tracking the putative partners. To do this we used α *far1h7* mutants cells. This mutant was first described in [43] and shows normal cell-cycle arrest in response to α-factor but it polarizes randomly (or with a bias for the place of the bud scar at very high pheromone concentrations).

We made three predictions:

- If the soluble protease doesn't significantly help in discriminating between partners, even in the presence of an exogenous protease, the mating efficiency between *bar1Δ* and the *far1h7* mutants should not show any improvement;
- If the cell wall bound protease helps in this polarization process, the presence of cell-wall bound chymotrypsin should improve the mating efficiency, even when the mating pairs were *far1h7;*
- Presence of both soluble and cell wall bound protease should significantly improve the mating efficiency of *far1h7* cells.

To test the first prediction we mixed the α *far1h7* cells with untreated *bar1Δ*, in the presence of different concentrations of chymotrypsin. Contrary to our expectations we saw that soluble protease does help *bar1Δ* □ *far1h7* cells mate and, apparently, the improvement is independent of the ability of MATα partner to polarize (purple on plots of figure A1.8), although the mating efficiency remained quite low.

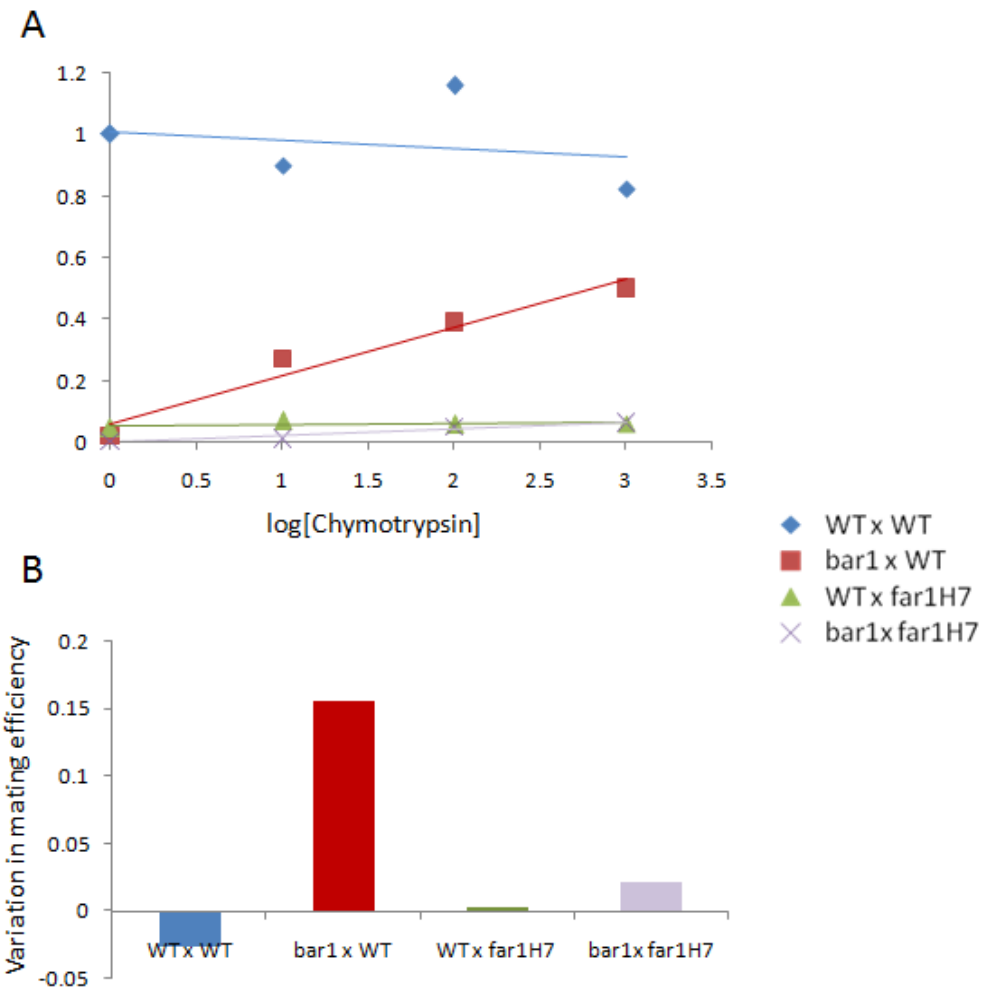

Figure A1.8 – Soluble protease helps cells polarize
The shown mixes of cells were let mate for 4h in the presence of different concentrations of Chymotrypsin. A- Their mating efficiency was normalized to that of wild type BAR1 cells in the absence of protease and ploted. Lines represent linear fits. The slopes of these lines are plotted in B. Mating between *bar1Δ* and f*ar1H7* improves in the presence of soluble chymotrypsin (right most, purple bar).

To test the second prediction we wanted to compare the mating efficiencies of these α *far1h7* cells when mixed with wild type, *bar1Δ* and *bar1Δ* cells that had been previously treated to have cell-wall bound chymotrypsin. Unfortunately we were never able to make this comparison in a quantitative manner. We believe this is for two reasons: first, because, and as was pointed out before, the *far1h* mutants have very low mating efficiency even when mated with wt cells. Adding soluble protease did increase that efficiency and adding cell-wall bound protease

had the same effect, but we never managed to increase the mating efficiency above a reasonable noise level that would allow us to make precise comparisons. The second problem is related to the extent of yeast conjugation, as it was quite difficult to control. Adding too much protease would leave the cells irresponsive to pheromone and unable to mate, adding too little would make them too sensitive and unable to properly detect gradients.

Although there is an attractive theoretical argument for the role of localized pheromone depletion for proper partner discrimination we could not show experimentally that is a fundamental requirement. First, adding soluble protease to *bar1Δ* mixes can recover the mating efficiency to wild type levels. Second, even when we prevent the α-cells from finding and tracking their *bar1Δ* mutant partners (by expressing the *far1-h7* mutant) adding soluble protease improves the mating efficiency, suggesting that lowering the overall α-factor concentration is more important than actually fine tuning the polarities, as long as the cells are given enough time to find each other. Finally, although we cannot rule out a contribution of receptor internalization for local pheromone removal, experiments with endocytosis mutants change the receptor level at the same time they change endocytosis rate and thus can't be used as a test of the theory.

The requirement for the local depletion model relied on the existence of two equally attractive partners, secreting similar amounts of pheromone, at an equal distance from the detecting cell. It is possible to imagine that this is not a very likely situation, as small differences in cell cycle of pheromone production levels could be enough to help one cell distinguish and commit to one partner or to another. We thus lack a firm a conclusion on whether anything beyond the soluble protease is really required for accurate partner orientation.

ACKNOWLEDGMENTS

I would like to thank Dr. Matthieu Piel for sharing the data in Figure A1.7, Scott Shuyler for the shown Western blots, Naama Barkai and Noa Rappaport for the theoretical model and Dr. Thomas Kramps for discussions. I'm particularly grateful to Dr. Soni Lacefield and Dr. Dawn Thompson for giving me the support I needed to let this project go. This work was supported by grants from NIH (GM 68763 and GM 43987 to A. M), as well a fellowship to.J.G-S. from the Fundação para Ciência e Tecnologia (SFRH/BD/15220/2004).

MATERIALS AND METHODS

Strain construction and manipulation.

Standard yeast manipulation methods were used. Strains used in this study are listed in Supplementary Table 1. All the strains constructed in this study are derivatives from JYL03 and JYL04 (W303 background). Strains with total deletions, gene replacements or partial C-terminal deletions of genes, or with C-terminal insertion of fluorescent proteins, were produced according to standard methods. [117]. All fluorescent protein cassettes come from plasmids produced by K. Thorn [118]. Strains with fluorescent reporters were constructed by inserting a plasmid containing YFP under the control of the *FUS1* promoter at the *LEU2* locus. Cassettes were amplified by PCR from plasmids made from the pFA6a backbone with a pair of primers that included 40 to 70 bp upstream and downstream of the targeted genomic region and integrated into the genome by homologous recombination. The heterologous receptor/pheromone strains were cloned into the endogenous genomic locus of the corresponding genes in *S. cerevisiae* (see Supplementary Table 1). Galactose inducible strains were constructed by replacing the promoter of GPA1 with pGAL1, at the endogenous locus. These strains were always grown with galactose as the only carbon source unless otherwise noted.

Cell-wall staining

TexasRed succinimidyl ester and Pacific Blue succinimidyl ester were obtained from Molecular Probes. They were diluted to a final concentration of 10mg/ml in fresh DMSO (from Sigma).Aliquots of 10µL were kept at -20˚C and protected from direct light. Exponentially growing cell cultures were harvested and washed twice with 1% PBS. They were diluted in 1% PBS (with 0.1M $NaHCO_3$) to a final volume of 990 µL. One aliquot of the dye was added to the cells (final concentration of 0.1 mg/ml) and vigorously vortexed until no cell clumps remained. Cells were incubated for 5 minutes at room temperatures with occasional vortexing. 500µL YPD were added to block the remaining die and cells were washed twice with ddH_20.

Choice of proteases

We tested commercial proteases (from Sigma) that could mimic the barrier activity. For this we performed a halo assay in which we coat complete media agar plates with *bar1*Δ cells. We then place in the center of the Petri dish a disk of paper embedded in α-factor. We mixed the proteases

is different concentrations with the *bar1*Δ cells and choose the one that recovered the cells from the arrest the better.

Preparation of agar plates for mating assays

YPD agar media was prepared according to standard yeast methods. Before cooling down PBS buffered to pH6.8 was added at a ratio of 1:10 and pH was checked with measuring strips and corrected as needed with NaH_2P0_4 or Na_2HPO_4. Temperature of the media was monitored and BSA was added as soon as media reached 60˚C to a final concentration of 0.1%. This media was then split into different flasks which already contained different volumes of chymotrypsin to reach the desired final protease concentrations. BSA was obtained from Sigma as a lyophilized powder, diluted to a concentration of 10% in ddH_20 and kept at 4˚C before using. α-Chymotrypsin from bovine pancreas was obtained from Sigma and diluted into a solution of 11.5% glycerol in PBS (pH 6.8). Aliquots were frozen and kept at -80˚C until needed and after thawed were never re-used.

Non-Quantitative Mating assays

Fresh colonies were streaked into selective media and allowed to grow overnight at 30˚C. Mating pairs were replica plated on top of each other into the required agar plates and allowed to mate for approximately 24h. They were then replica plated on diploid selective media and grown for another 24h before screening.

Quantitative mating assays

Cell cultures were grown overnight to saturation and then allowed to resume exponential growth by diluting them 50-fold into fresh growth medium, and incubating them for 4-5 hours at 30˚C before the experiment. Cultures were harvested, washed 2 times with ddH_20 and ressuspended in water. A sample of the cultures was then sonicated for 1-2 minutes in an ice-water bath in a 2" cup horn of a Branson Sonifier 250 and counted using a Beckman Coulter Counter. Cultures were diluted into 500ml of ddH_20 for a final concentration of $1X10^7$cells/ml. They were then mixed 1:1 with the corresponding mating pair (unless otherwise noted) for a final volume of 1ml and sonicated for 1 minute.

The mixes were then sucked into filters (0.22µm, 25 mm, Durapore, Millipore), using a 12-sample vacuum filtration manifold (Millipore). Filters were placed on agar plates and allowed to mate for 4h or 7h at 30˚C. Filters were then washed in plastic tubes with 1.5 ml of ddH_20 and diluted. Approximately 200 cells were plated on single drop-outs (haploid controls) and the corresponding double-drop-out media plates (to select for diploids). Mating efficiency was calculated as $ME = \frac{\#zygotes}{\min\{\#haploidA, \#haploidB\}}$

Microscopy

Time-lapse movies were acquired using a Nikon upright microscope, a motorized XYZ stage (Prior), a piezoelectric device from Princeton Instruments placed between the objective turret and a 60X (1.40, WD 0.21) or a 40X (1.30, WD 0.2) insert oil objectives, a CCD camera (CoolsnapHQ, Roper), a motorized excitation and emission filter wheels (Ludl). The whole setup was controlled by Metamorph (Molecular Devices) software. A typical time-lapse recording would acquire data from 3 different positions per minute of time-lapse. We used about 40 positions for mating movies, corresponding to a time-lapse of 15 to 20 minutes. At each position, one DIC picture was acquired, followed by a fast Z-series acquired using the streaming mode and the piezo-electric device to move the objective, to record the localization of a fluorescently labeled protein (e.g. Spa2-YFP) throughout the cell volume with minimum exposure time. At each time-point, the stage was first centered on a 1µm diameter fluorescent bead using a home-made macro. This corrects for potential drift along the X-, Y-, or Z-axes during long recordings.

Mating movies

To record cells behavior during mating, chambers were assembled that allowed long recordings of several mating mixes in parallel. A 1 mm layer of PDMS was produced between two glass slides separated by 1 mm thick spacers. One slide was removed and the other was kept to form the bottom of the chamber. Holes were cut in the slab of PDMS, forming independent chambers. The slide was placed on a hot plate (around 50C). 4x complete synthetic growth medium was placed in a plastic tube on the hot plate and mixed 1:1 with a molten solution of 3% w/v agarose in water. An excess of this mix was then placed in the holes and they were covered with a clean coverslip. The chamber was then placed at 4˚C for a few minutes to let the agarose

gel. The cells and mating mixes were prepared, resuspended in water at a concentration of about 10^7 cells/ml and sonicated. Agarose type IX-A (Sigma, A2576), which has a gelling point of 17˚C, was melted in water at a concentration of 3% and cooled to room temperature. The coverslip covering the hard agar pad in the chamber was removed and all extra agar lying on the PDMS walls of the chamber was removed. A drop of 1 µl of the mating mix was placed on the middle of the agar pad and left to dry. It was then covered with a drop of the melted room temperature low gelling-point agar (about 10 µl for a $1cm^2$ agar pad) and all the chambers were covered with a single large coverslip. The low gelling agar was squeezed between the hard agar pad and the coverslip, in order to form a single layer of cells on top of the hard agar pad. The whole chamber was placed at 4˚C for a few minutes to allow the top layer of agar to form a gel. The chamber was allowed to warm up and checked for stability under a low magnification objective (no freely moving cells should be observed). It was then placed on the microscope stage and positions were chosen for time-lapse recording.

Microfluidic chambers

The design and fabrication of the chambers was based on regular soft lithography techniques (reviewed in[46]). Cells were bound to the chambers by cross-linking with Concanavalin A, a lectin which strongly binds to the yeast cell wall. The chambers and tubing were pre-coated with BSA. When several strains were to be studied in parallel in a given chamber, they were differentially stained prior to binding to the chamber by covalently labeling their cell walls with fluorescent dyes (see the cell wall labeling protocol). The two inlets to the chamber were coupled to two reservoirs: one contained containing complete synthetic growth medium and the other containing the same growth medium plus the desired concentration of α-factor and 200 µg/ml of Texas Red conjugated dextran (MW 3000, Molecular Probes). Using the fluorescent dextran in the pheromone flow, the flows were adjusted to be equal at the point where they first encountered each other in the chamber. We recorded the fluorescent dextran profile (as well as cellular behavior), both to calculate the local pheromone concentration and to check the temporal and spatial stability of the α-factor concentration during the experiment.

Image processing and analysis

Cell behavior was analyzed semi-automatically. Interactive scripts facilitated the analysis and recording of various aspects of the behavior of each cell. For promoter activity, we measured the total intensity in the YFP image from each cell. The total YFP intensity in cells treated with a constant level of pheromone would increase linearly for hours and the slope was measured to obtain the promoter activity for individual cells. Images of the fluorescent dextran were used to estimate the α-factor concentration.

Single cell interactions

We monitored the interaction of individual **a** and α cells by micromanipulating the cells into contact with each other on the surface of YPD plates. The α cells carried the *far1-H7* mutation which prevents accurate polarization and thus blocks mating without affecting cell cycle arrest or pheromone production. The response of cells of a *bar1*Δ (MP384) and a *bar1*Δ *mfa1*Δ (TK277) to an individual α-cell was scored as 1 if after 6,5h hours the cells shmooed or formed a zygote and as 0 if they kept on budding. χ^2 statistics were used to test whether the responses were different, p = 0.0002. The response of *a bar1*Δ *mfa1*Δ (TK277) cells to synthetic α-factor was compared to that of *a bar1*Δ (MP384) cells and both calibration curves could not be distinguished under Kolmogorov-Smirnov statistics (3 independent measures, for n=7 and α=0.05 D_{exp} = 0.123467 and $D_{p=0.05}$ = 0.48342).

Ste2 receptor identification and alignment

We started from the annotated receptor homologs (from the NCBI database) and used the amino acid translations of this set of sequences to identify putative homologs in the genomes of other species through pBLASTn search. Some of these translations displayed STOP codons interrupting the coding sequence and we predicted their spliced sites based on annotated sequences (Supplementary Table 7 shows the predicted nucleotide and protein consensus sequence) and this characterization was used to manually splice those genomic sequences found using BLAST. The membrane topology of the translations of these predicted STE2 homologs was predicted using TMHMM v2.0 [68]. The amino acid translations were aligned using a Hidden Markov Model downloaded from the PFAM website (authors Mian N, Bateman A), [69].

The predicted membrane topology of each protein was checked against this alignment to ensure that those residues predicted to be inside the membrane were aligned with each other.

Pheromone identification and alignment

The sequences of alpha-factor like pheromones usually code for a leader sequence and several peptide repeats, flanked by a conserved cleavage sequence. The immature peptide is cleaved by the Kex2 proteinase, which is known to cleave the sequences 'KR' and 'RR' specifically [70] . We identified the peptide pheromones of 65 Ascomycete species by homology using BLAST together with literature reports of PPG1 sequences from different species. In some cases the brevity of the PPGA locus and lack of peptide repeats meant it was necessary to use synteny of closely related species to identify genomic regions of interest. Species in which this analysis was carried out include: *Coccidioides immitis, Uncinocarpus reesii, Botrytis cinerea, Sclerotinia sclerotiorum*, and *Stagnospora nodorum* available from the Broad Institute. The mature pheromone peptide sequences were initially aligned using MAFFT. The alignment was then manually adjusted taking into account the conserved W at the start of the peptide, and the largely conserved GQ residue pair towards the end of the pheromone (Supplementary Table 5, pheromones have been concatenated with the corresponding receptors and start at residue 285).

Peptide synthesis

23 representative peptides (distributed across the phylogenetic tree) were synthesized by Biomatik Corporation and HPLC purified to >95% purity (Figure 5.1). Upon receipt, the peptides were diluted in DMSO to a final concentration of 3mM, aliquoted and kept at -20˚C.

Receptor and pheromone cloning

Gene replacements were made as described in [117]. The STE2, STE3, MF(α)1, MF(α)2, MFA1 and MFA2 genes from S. cerevisiae were amplified by PCR from *Sacharomyces cerevisae* W303 genomic DNA. The STE2-like receptors from *Kluyveromyces lactis*, *Saccharomyces kluyveri*, *Saccharomyces castellii*, *Candida glabrata*, *Candida albicans*, *Yarrowia lipolytica* and *Debaryomyces hansenii* were amplified from genomic DNA (gift from Dawn Thompson, Broad Institute). PRE2 from *Sordaria macrospora* and from *Penicillium chrysogenum* and PPG1 from *Sordaria macrospora*, gifts from Dr. Stefanie Poggeler, were amplified from plasmids using the

primers described in [52] and from an unpublished plasmid. PRE2 from *Neurospora crassa* was amplified from an unpublished plasmid, gift from Dr. Louise Glass. BBR1, BBR2, BBP2(4) and BBP1(1) genes from *Szhyzophylum commune* were amplified from plasmids, gifts from Dr. Thomas Fowler [53]. The sequence for the PRE2 receptor from *Podospora anserina* has one intron and was amplified by RT-PCR and cloned by Dr. Robert Debuchy. The sequences for receptors *Aspergillus nidulans*, *Aspergillus niger, Aspergillus terreus*, *Chaetomium globosum*, *Gibberella zeae*, *Magnaporthe grisea* and *Verticillium dahliae* all contain one or more introns and were spliced manually (Supplementary Table 7) and DNA was synthesized by DNA2.0 Inc.
All receptors and pheromones were cloned into the pFA6 background using the Pac1 and Asc1 sites. Receptors for the receptor specificity project (Chapter 4) were transformed by homologous recombination, using the Nourseothricin genes as marker, into the endogenous STE2 locus of two yeast strains (JS135 and JS240), which had the STE2 receptor deleted with the URA3 gene from *K. lactis*. All strains were confirmed by sequencing of the STE2 locus.

Response to pheromone

The response of the receptors to the different pheromones was done by flow cytometry and by light microscopy. The tested strains carried a copy of a mating specific promoter (pFUS1) driving a yellow fluorescent protein (P_{FUS1}-*yEVenus*) as a reporter. Exponentially growing cells were harvested, washed twice with ddH_20, sonicated for 2 minutes in an ice-water bath in a 2" cup horn of a Branson Sonifier 250. Cells were counted using a Beckman Coulter Counter and diluted into low-fluorescent synthetic complete media diluted to a final density of 5×10^6 cells/ml. The cells were incubated in 96-well plates with the indicated concentrations of synthetic pheromone for 2h, at 30˚C, with strong and constant shaking to prevent the formation of cell clumps.
Levels of YFP induction were measured using a BD LSRFortessa analyzer, with 488 nm excitation at 100mW, using a reflection from a 505 nm dichroic long pass mirror and transmission through a 530 / 30 band-pass filter. Detection was done using the FACSaria Software and analysis using FlowJo and Matlab. Gating was done using FlowJo. Shmooing cells grow larger and form protrusions altering the forward and side-scatter profiles. Cells were gated manually from the non-induced samples and gates were transferred to the induced samples and we confirmed that the gated samples included approximately 80% of the full sample. Different statistical measures were acquired and we use the mean of each fluorescence distribution as a

mesure of induction. Mean fluorescence levels were normalized by subtracting the mean fluorescent levels of the corresponding uninduced strains. 4uL samples of selected conditions were imaged under the microscope and screened for changes in morphology and the presence of shmoos.

Pheromone secretion measurements:

Cell cultures were grown overnight to saturation and then allowed to resume exponential growth by diluting them 50-fold into fresh growth medium, and incubating them for 3 hours at 30˚C before the experiment. We measured the pheromone secretion by harvesting medium that had contained α-factor expressing cells and comparing its activity to synthetic pheromones. 1.5ml plastic tubes, 12 ml glass tubes and 1ml plastic syringes were filled with a solution of 2% BSA in PBS and incubated overnight at 4˚C to prevent non-specific binding of α-factor. Conditioned medium was collected from α-factor producing cells (MP634 for *S. cerevisiae* pheromone, JS214, JS385 and JS317 for *S. macrospora* pheromone producing cells). Cells were grown in YPD to an OD_{600} of 0.4-1.2, centrifuged, washed twice with water, diluted to 1 x 10^7 cells/ml in pre-warmed YPD with 2% BSA and transferred to the BSA coated glass tubes. Cells were grown in a roller drum at 30˚C with for 30 min, 1 hr and 2 hr. The conditioned media was removed with the BSA coated syringe and filtered using a 0.45μm sterile filter (pre-coated by passing the BSA solution through it). The responding a *bar1*Δ cells ((MP384 and JS204 expressing *S. cerevisiae* and *S. macrospora* receptors, respectively) cells were grown overnight on YPD to an OD_{600} of 0.4-1.2 and ressuspended to 2X10^5cells/ml in pre-warmed YPD with 2% BSA and transferred to the BSA coated tubes. The cells were then incubated either with different concentrations of synthetic α-factor (from 0 to 100nM) to generate a calibration curve, or with different dilutions of the previously described conditioned media (no dilution, or diluted 2-fold or 10-fold), to estimate the secretion rate, and grown at 30˚C for 2h. The fraction of cells that had formed shmoos was determined by light microscopy at least 4 independent times and at least 200 cells were counted, per condition. *S. cerevisiae* and *S. macrospora*'s α-factor like pheromone peptides were synthesized by Biomatik Corporation and HPLC purified to >95% purity. The response curve of MP384, JS204, JS385 and JS317 to the synthetic α-factors (% of shmoos vs. α-factor concentration) was fit to $A\frac{x^n}{k^n + x^n}$ using IgorPro 4.07 (Wave Metrics, Inc, Lake Oswego, OR,

USA). The fit was used to estimate the concentration of α-factor the **a**-cells in conditioned media were sensing. The goodness of the fit was tested with Kolmogorov-Smirnov statistics for α=0.05 and n=7 or n=8 [119]. The fit parameters for the 2 curves (with *S. cerevisiae* and *S. macrospora*'s α-factor) and the statistical significance tests can be found in Supplementary Table 2. The pheromone secretion rate, η, was estimated using $\eta = \frac{0.6 \times 10^5 \times C}{r \times N_0 \left(\exp\left(\frac{T}{K} \right) - 1 \right)}$, where r is the average replication time in seconds (5400), N_0 is the initial number of cells (200000, 100000 or 20000) and T is incubation time in seconds (1800, 3600 or 7200).

Rate of evolution ω

We calculated ω using two different methods [74] from the Datamonkey website The first (Random Effects Likelihood, REL) assumes a distribution of rates across sites and infers the rate at which individual sites evolve given this distribution. Parameters for discretized distributions of *dN* and *dS* are estimated from the entire alignment, using three synonymous and three non-synonymous rate categories. The ratio of the posterior and prior odds of having $dN/dS > 1$ (or $dN/dS < 1$) at a given site is used to determine whether a site is under positive (or negative) selection. In contrast the second (Fixed Effects Likelihood, FEL) estimates the ratio of *dN* and *dS* substitutions on a site-by-site basis. In this case a codon-substitution model is used to estimate *dN* and *dS* and a likelihood ratio test is used to calculate a p-value for the test $dN \neq dS$. The *dN/dS* values and p-values for each codon are reported in Supplementary Table 10.

Snake plot

The topology of the Ste2 receptor was predicted using the webserver ConPred II, which combines the results of several proposed transmembrane topology prediction methods [75].

Protein conjugation

We used the protein biotinylation and recovery protocol described in: http://www.sigmaaldrich.com/sigma/bulletin/BK101bul.pdf. The biotin is modified with an amidohexanoic acid linker and a NHS (N-hydroxysuccinimide ester) conjugate. This reacts with the amino-groups in the chymotrypsin. Extent of the labeling reaction is quantified by an HABA displacement assay [120]. The preparation was eluted with 1x PBS ate pH 6.8, aliquoted with

11.5% glycerol and frozen at -80˚C until needed. An average sample contained around 0.5mg/ml of protein with 5 to 10 molecules of biotin per protein molecule. The conjugated proteased was tested for activity by measuring recovery form α-factor induced arrest in both halo assays and serial dilutions after one cycle of freezing. 50μg/ml of labeled protease are enough to mimic the behavior of the WT Bar1 cells. The protease is still fully active after at least one cycle of freezing and thawing and after 5 minutes at 65ºC (trying to mimic what happens when the protease is mixed with the warm agar). Streptavidin−Maleimide from Sigma (S9415) was then added to the biotinylated chymotrypsin at a 1:4 ratio, in PBS, at pH 6.8. The reaction was left at room temperature for 10 min. Extent of binding was quantified by using a fluorescently labeled biotin.

Yeast conjugation

Exponentially growing yeast cells were washed twice with 1x PBS and sonicated for 2 minutes in an ice-water bath in a 2" cup horn of a Branson Sonifier 250. Cells were counted using a Beckman Coulter Counter and diluted to a final density of 2×10^5 cells/ml. Cells are the incubated in a solution of pH 6.8 PBS with 0.1M DTT as a reducing agent, for 30 minutes, at room temperature. Cells are washed several times with ddH_2O and mixed either with the chymotrypsin-biotin-avidin-maleimide reagent or with FITC-biotin-avidin-maleimide. The maleimide reacts with the exposed sulfur groups on the yeast cell wall. Conjugated cells are washed twice with pH 6.8 PBS and re-suspended in the appropriate media. Extent of binding is inferred from the level of fluorescent signal at the yeast cell wall (Figure A1.5).

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SUPPLEMENTARY TABLES