Genetic Analysis of the MAT-1 Pheromone Gene of Ustilago Hordei and the Study of Morphogenesis During the Mating Response
by CYNTHIA M ANDERSON

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Sciences
Montana State University

Abstract:
Mating in Ustilago hordei, the causal agent of covered smut of barley, is under the control of one locus, MAT, with two alleles, MAT-1 and MAT-2. Genes at this locus encode the cell-cell signaling components responsible for initiating the morphological changes required for formation of the infectious cell type. In this investigation the MAT-1 allele containing the pheromone gene, Uhmfa1, was localized and sequenced. A deletion vector was designed to knock out both the pheromone gene and the previously described pheromone receptor gene (Uhpra1) upon transformation into MAT-1 cells. One non-mating transformant, 14Am-, was isolated. Subsequent replacement of Uhmfa1 and Uhpra1 into 14Am-, and observation of the mating response of each transformant, confirmed the role of these genes as pheromone and pheromone receptor respectively. Northern analysis of the pheromone genes from both mating type alleles (Uhmfa1 and Uhmfa2) revealed upregulation of Uhmfa1 in MAT-1 cells grown in the presence of MAT-2 cells, in the diploid strain, and in 14Am- transformed with Uhmfa1. Conversely, Uhmfa2 appeared to be downregulated in MAT-2 cells grown with MAT-1 cells and in the diploid strain.

A split agar assay was developed that enabled detailed analysis of the morphological transition that occurs during the early stages of mating. The mating response was shown to be asymmetric and occurred at distances up to 400 μm. MAT-1 cells responded first by the formation of conjugation tubes that grew toward MAT-2 cells. MAT-2 cells began conjugation tube formation when MAT-1 conjugation tubes came within 75 μm of them. Conjugation tubes fused tip-to-tip and gave rise to the dikaryotic cell-type.

The role of the following factors in the asymmetric mating response were tested; response time of cells to pheromone, pheromone diffusion rates, and cellular sensitivity to pheromone. MAT-1 cells responded to Uhmfa2 pheromone four times faster than MAT-2 cells to Uhmfa1. Uhmfa2 diffused more than ten times faster than Uhmfa1. MAT-1 cells responded to a much lower concentration of Uhmfa2 (<250 pg) than MAT-2 cells to Uhmfa1 (31 ng).

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APPROVAL

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Cynthia Marie Anderson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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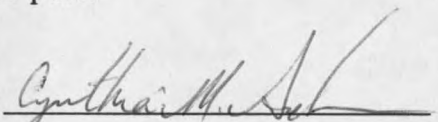
Signature 

Date 1/15/99

This dissertation is dedicated to the memory of my mother, N. Paula (Robbins) Overton, whose death during the course of this research taught me much about life. Her gifts of love and encouragement helped me to persevere even through the most trying of times, and are forever a part of my soul.

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ABSTRACT

Mating in *Ustilago hordei,* the causal agent of covered smut of barley, is under the control of one locus, *MAT*, with two alleles, *MAT-1* and *MAT-2*. Genes at this locus encode the cell-cell signaling components responsible for initiating the morphological changes required for formation of the infectious cell type. In this investigation the *MAT-1* allele containing the pheromone gene, *Uhmfa1*, was localized and sequenced. A deletion vector was designed to knock out both the pheromone gene and the previously described pheromone receptor gene (*Uhpra1*) upon transformation into MAT-1 cells. One non-mating transformant, 14Am-, was isolated. Subsequent replacement of *Uhmfa1* and *Uhpra1* into 14Am-, and observation of the mating response of each transformant, confirmed the role of these genes as pheromone and pheromone receptor respectively. Northern analysis of the pheromone genes from both mating type alleles (*Uhmfa1* and *Uhmfa2*) revealed upregulation of *Uhmfa1* in MAT-1 cells grown in the presence of MAT-2 cells, in the diploid strain, and in 14Am- transformed with *Uhmfa1*. Conversely, *Uhmfa2* appeared to be downregulated in MAT-2 cells grown with MAT-1 cells and in the diploid strain.

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CHAPTER 1

LITERATURE REVIEW

The Barley Covered Smut Pathogen, *Ustilago hordei*

Classification and Description

Ustilago hordei (Pers.) Lagerh. is the fungal agent causing covered smut of barley (*Hordeum vulgare L.)*. This fungus is taxonomically classified in the division *Basidiomycota*, subdivision *Basidiomycotina*. Recent changes in the taxonomy of the rust and smut fungi have grouped all of the smuts, including *U. hordei,* into the class *Ustilaginomycetes* which contains only one order, the *Ustilaginales* (Alexopoulos *et al,* 1996; Carlile and Watkinson, 1994). This order contains two families, the *Ustilaginaceae* and the *Tilletiaceae.* The family to which *U. hordei* belongs, the *Ustilaginaceae*, is differentiated from the *Tilletiaceae* by having a prostrate promycelium which is transversely septate, bearing basidiospores both laterally and terminally. In the *Tilletiaceae*, the promycelium is arial and aseptate or unicellular, bearing only terminal basidiospores (Alexopoulos *et al*, 1996).

The teliospores of *U. hordei* are nonechinulate, globose to subglobose, 5-8 μm in diameter and range in color from light olive brown to brown

(Mathre, 1997; Fischer, 1953). One side of the teliospore wall is a lighter-colored, weaker area from which the promycelium emerges (Fischer and Holton, 1957). Upon germination of the teliospore, the promycelium breaks through the spore wall and the diploid nucleus undergoes the first meiotic division while still within the spore. One nucleus moves to the distal end of the promycelium and a septum is laid down between the two nuclei. The two nuclei undergo the second meiotic division to form a four-celled promycelium in which each cell is uninucleate (Fischer and Holton, 1957). Each promycelial cell produces uninucleate primary sporidia (basidiospores) by lateral budding (Fischer and Holton, 1957; Mathre, 1997). The resulting sporidia are oblong to ovate, hyaline, 9-11 μm long and 4-6 μm wide.

Until 1924, the idea that cereal smut fungi, such as *U. hordei,* could exist in different biological forms had not been considered. James Faris (1924) presented substantial evidence of this phenomenon and identified the first five biological forms based on their differences in pathogenicity on a set of four barley cultivars, Nepal, Hannchen, Texas Winter and Summit. By 1945, thirteen biological forms, now referred to as physiologic races, had been identified by Tapke (1937, 1945) based on pathogenicity on a differential set of eight barley cultivars, Excelsior (C.I. 1248), Hannchen (C.I. 531), Himalaya (C.I. 1312), Lion (C.I. 923), Nepal (C.I. 595), Odessa (C.I. 934), Pannier (C.I. 1330), and Trebi (C.I. 936).

To date, 14 physiologic races of *U. hordei* have been identified. Race 14 resulted during an inbreeding study of race 8 and is recognized as a legitimate race due to its unique pathogenicity pattern. It is virulent on all eight cultivars in the differential set (Pedersen and Kiesling, 1979).

Life/Disease Cycle

Completion of *U. hordei's* life cycle is closely dependent on the relationship with its barley host as an obligate parasite. While there are five early reports of *U. hordei* teliospore formation on artificial media (Fischer and Holton, 1957), such teliospore formation has not been reproducible, and it is widely believed that they are formed exclusively within host tissues (Thomas, 1988).

The teliospores, which serve as the resting spore, are released from smutted barley heads during harvest and threshing, thus contaminating both healthy seeds and the soil in which healthy seed may be sown. In the presence of adequate soil moisture (50%) and favorable temperatures (14-25° C) both the teliospores and the barley seeds germinate. Sporidia of opposite mating-types form conjugation tubes that fuse to form the pathogenic, dikaryotic mycelium. The dikaryotic mycelium penetrates the coleoptile of the germinating barley seedling, invades host tissues and establishes itself in the meristematic tissues of the plant. If the dikaryotic mycelium successfully penetrates the entire length of the coleoptile, tillers of the plant will also become infected. At the time of heading, the mycelium permeates the ovarian

tissues where cells of the dikaryon round up, karyogamy occurs and masses of diploid teliospores are formed in place of the seed (Mathre, 1997; Tapke, 1948). Each mass of teliospores, called a sorus, is 6-10 μm in length, and is covered by a persistent membrane derived from host pericarp (Zundel, 1953; Webster, 1980).

In most cases of infection, barley plants are symptomless, or only slightly stunted, until the time of heading when the sori replacing the seed can be easily seen. The infected heads often emerge from the boot later than uninfected heads, or they may become trapped in the flag leaf sheath and unable to emerge at all. On occasion, depending on environmental conditions, or on aggressiveness of the race, smut sori may form long streaks on leaf blades or near the nodal tissues on the culms (Mathre, 1997; Groth and Person, 1978; Gaudet and Kiesling, 1991).

Control of Covered Smut

Covered smut is readily controlled by the use of chemical seed treatments. The most widely used treatments are the systemic oxathiin fungicides such as carboxin. These have the advantage of providing complete control at low concentrations, and are not phytotoxic except at very high concentrations (Mathre, 1997; Thomas, 1991; Webster, 1980). There are also many cultivars available that have resistance to some of the 14 physiologic races. However, due to the effectiveness of the seed treatments many of the

varieties currently grown are susceptible, but are preferred because of their agronomically desirable traits.

Economic Importance

Ustilago hordei is distributed worldwide. While this organism is most notable for the disease it causes on cultivated barley, it is also capable of causing disease on several species of wild forage grasses as well as on oats and rye (Fischer, 1939; Zundel, 1953; Ainsworth and Sampson, 1950). The effectiveness of protective and systemic fungicidal seed treatments has kept the economic losses due to covered smut in check in areas of the world where these treatments are used consistently. In areas of the Middle East, however, where seed treatments are not regularly used, economic losses are not uncommon (Mathre, 1997). Even in areas where ample control measures are taken to prevent covered smut, neither eradication nor 100% control is seen. Annual cereal smut surveys in the prairie provinces of Canada, Manitoba and Saskatchewan, depict this well. During the years 1989 through 1995 *U. hordei* was found in up to 23 % of the fields surveyed at levels ranging from less than 0.1% up to 7% infected plants (Thomas, 1997; 1995). Since yield reduction is directly proportional to the percentage of infected heads, losses even at these levels of infection can represent a significant loss of income for the farmer. Further losses come in the form of discounted grain prices if the barley is designated as "smutty" according to federal grain standards. This

designation is given when a lot contains more than a small percentage of infected heads (Mathre, 1997)

Genetics of the *U. hordei*-Barley Interaction

Using the flax/flax rust plant-pathogen interaction, Flor (1956) first demonstrated that for each gene that confers resistance in a host plant to a pathogen, a corresponding gene exists that confers virulence to the pathogen. This idea, coined the gene-for-gene concept, has since been shown to operate in many other fungal, bacterial, and viral pathogens as well as in some diseases caused by parasitic plants and nematodes (Agrios, 1988; Flor, 1971). Two virulence genes have been identified in *U. hordei* that conform to the gene-for-gene concept. *Uh v-1* has been shown to control virulence against the resistance gene *UhR1* in cvs Hannchen and Vantage, and *Uh v-2* has been shown to control virulence against *UhR2* in cv Excelsior (Sidhu and Person, 1971; 1972). While these two studies found the virulence genes to be recessive and the corresponding resistance genes to be dominant, another study of the virulence genes in *U. hordei* have found *Uh v-2* to behave as a modified dominant allele under certain environmental conditions (Ebba and Person, 1975).

Further studies of the virulence genes of *U. hordei* have yielded some conflicting, and unusual results. *Uh v-3* controls virulence on cvs Pannier and Nepal, and is linked to *Uh v-2* (Thomas, 1976). In one study, *Uh v-4* and *Uh*

v-5 were found to be duplicate recessive genes controlling virulence on cvs Himalaya and Keystone when present at either one of two genetic loci (Ebba and Person, 1975). However, Thomas (1976) reported that virulence on Himalaya was due to the presence of both *Uh v-1* and *Uh v-2*. *Uh v-6* is an unlinked gene that controls virulence on Lion and Plush, and on Vantage if combined with *Uh v-1* (Thomas, 1976). Virulence on Trebi appeared to be under the control of a single recessive gene (Pedersen and Kiesling, 1979). However, later studies showed that this virulence on Trebi may be capable of a reversal of dominance when one set of experiments yielded results that indicated virulence to be under the control of a single, dominant gene (Person *et al*, 1987). A subsequent set of experiments attributed virulence on Trebi to a recessive gene (Christ and Person, 1987). Thomas (1991) suggested that this reversal of dominance was most likely the result of environmental differences between the experiments as seen with *Uh v-2*, and implicated this as a further complication in understanding the mechanisms of genetic virulence in *U. hordei*.

Ustilago hordei as a Model System

Ustilago hordei is emerging as another model system for the study of smut diseases. In particular, it could prove to be representative of the bipolar smuts that infect small grains. *In vitro* study of *U. hordei* is facilitated by the ability of the sporidia to be easily maintained on artificial media. The sporidia are uninucleate, reproduce in a yeast-like fashion by budding, and are thus

amenable to genetic analysis (Thomas 1988). A genetic transformation system has been developed (Holden *et al.* 1988; Duncan and Pope, 1990) that allows cloned DNA sequences to be introduced into the sporidia. Transformation experiments allow the function of genes to be tested by disruption of cloned sequences using vectors engineered to integrate into the genome homologously, thus disrupting a specific gene or genes. Replacement of the genes can also be achieved in the same way by transformation with vectors that integrate randomly or heterologously into the genome, or by transformation with plasmids containing an autonomously replicating sequence (ARS) (Fincham, 1989). Isolation of an ARS from *U. maydis* (UARS1) (Tsukuda *et al.* 1988) has allowed high efficiency transformation of *U. hordei* with plasmids containing this sequence.

Electrophoretic karyotyping of *U. hordei* is now possible and provides a tool for the study of its cytogenetics (Thomas, 1991). McClusky and Mills (1990) determined the electrophoretic karyotypes for monosporidial strains of each of the 14 races of *U. hordei*. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was used to separate chromosomal DNA of protoplasted sporidia. Each strain displayed a unique karyotype which was conserved among members of individual tetrads and between tetrads of the same race. From this study the haploid complement of chromosomes in *U. hordei* is estimated at 16 chromosomes (McClusky and Mills, 1990).

The ability to easily induce mutations in the haploid sporidia of this organism by using ultraviolet light or chemical mutagens further increases its usefulness as a model. Auxotrophic, fungicide resistant, morphological, and temperature sensitive mutants have been obtained (Hood, 1968; Thomas, 1972; Ben-Yephet *et al.* 1974; Henry *et al.* 1985; Henry *et al.* 1988, Martinez-Espinoza *et al.* 1992). Complementation tests have been performed with many of the auxotrophic mutants, increasing our knowledge of biosynthetic pathways, and allelism (Dinoor and Person, 1969; Henry *et al.* 1988; Martinez-Espinoza *et al.* 1992). Similarly, the genetic control of fungicide resistance has shed light on the dominance or recessiveness, as well as the stability of mutations conferring such resistance (Ben-Yephet *et al.* 1975; Henry et al, 1987).

The primary disadvantage of *U. hordei* as a genetic tool is the inability to complete the sexual phase of the life cycle in culture (Thomas, 1988; 1991). The amount of time required to obtain teliospores from a cross is approximately 3 months, the time needed to raise barley from seed to adult plant.

The Importance of Mating to Pathogenicity in the Smut Fungi

The plant pathogenic smut fungi found in the order *Ustilaginales* consist of nearly 1200 species in more than 50 genera. Approximately 4000 species of flowering plants serve as hosts to these obligate parasites

(Alexopoulos *et al.* 1996). Early in the study of smut diseases it was recognized that host infection and subsequent sporulation was dependent upon the mating of two haploid cells of opposite mating type. Studies conducted in the late 1920's and early 1930's were the first to actually correlate a relationship between mating and pathogenicity in several species of smut fungi. The first, conducted by Stakman and Christensen (1927), showed that, with only a few exceptions, monosporidial lines of *Ustilago maydis* alone were not sufficient to cause gall formation or sporulation on corn. Certain combinations of the monosporidial lines, however, were able to induce gall formation and sporulation, thus supporting the notion that lines of compatible mating type were required for pathogenesis. The exceptions in this study were a few lines that appeared to be monosporidial, but induced galls and were able to sporulate. They suggested that these particular lines were comprised of cells morphologically similar but functionally heterogamous. Further studies with these "solopathogenic" lines revealed that they were actually diploid, since when the spores of the lines germinated, the sporidia from the promycelium segregated for sex (reviewed in Fischer and Holton, 1957).

Smut fungi are only infectious during the dikaryotic phase of their life-cycle. Since mating interactions are necessary to form the dikaryon, and since sexual development in the majority of the smut fungi takes place only within host tissues, the mating-type genes responsible for the regulation of mating

and sexual development are, therefore, considered pathogenicity genes (Kronstad, 1995).

The Genetic Control of Fungal Mating

Fungal Mating Systems

The genetic mechanisms of sexual reproduction in the fungi are diverse and complex. Morphological differentiation of reproductive structures, common in the animal and plant kingdoms, is one of the mechanisms found in the fungal kingdom. This type of differentiation, however, is relatively uncommon. Far more common is the use of incompatibility systems as a means of controlling mating interactions (Raper, 1966). Two types of incompatibility systems exist, each operating in an opposing manner. Heterogenic incompatibility, also referred to as homothallism, restricts mating between strains having different genetic factors, allows self-fertility, and thus favors inbreeding. Homogenic incompatibility, also referred to as heterothallism, prevents mating between strains having the same genetic factors, prevents self- fertility, and thus enhances outbreeding (Raper, 1966; Carlile and Watkinson, 1994).

Structurally complex mating-type loci are responsible for the regulation of mating in heterothallic fungi and in many homothallic fungi. This genetic control of mating-type is classified according to the number of loci and the number of alleles at each locus. The simplest, and most common is the

unifactorial (or bipolar), biallelic system. In this system, the mating of two genetically compatible individuals yields two and only two mating types as the products of meiosis (=bipolar). This is the mating-type system displayed by the Ascomycetes and the Zygomycetes. The more complex mating-type systems are found among the members of the Basidiomycetes. The simplest is the unifactorial, biallelic system described above. Unifactorial systems in the basidiomycetes may, however, contain multiple alleles of the mating-type locus. These systems, termed unifactorial, multiallelic, also yield two and only two products of meiosis. The most complex systems are the bifactorial (or tetrapolar) systems. The bifactorial system, controlled by two genetic loci, may be biallelic or multiallelic at one or both loci. The products of meiosis resulting from a bifactorial cross will consist of four mating types (=tetrapolar), two parental and two nonparental, due to reassortment of the two loci (Alexopoulos *et al.* 1996; Carlile and Watkinson, 1994; Raper, 1966). The use of these systems in heterothallic fungi is easily understood. However, the concept of homothallism becomes somewhat complicated by the fact that many homothallic fungi utilize one of the above genetic systems to control mating. The implication is that, even in its simplest form, the unifactorial, biallelic system, two different mating types are required to form the zygote.

Homothallism is defined as the ability of a single haploid cell to give rise to a diploid cell capable of undergoing meiosis (Herskowitz, 1988). One well studied mechanism of homothallism in fungi that employ one of the above

mating-type control systems involves the ability of a haploid cell to undergo mating-type switching. This mechanism, referred to as pseudohomothallism, is most studied in the ascomycete yeast, *Saccharomyces cerevisiae*. Both heterothallic and homothallic strains of *S. cerevisiae* exist in nature. The mating-type locus of *S. cerevisiae* has two alleles, *MAT***a** and *MAT*α, which gives rise to the two respective mating-types, **a** and α. Two nearby loci, *HML*α and *HMR***a**, contain a copy of *MAT*α or *MAT***a**, respectively. Genes contained at *MAT* are expressed, while those contained at *HML* and *HMR* are not. The only difference between homothallic and heterothallic strains is that homothallic strains contain a functional version of the *HO* (homothallism) gene, whereas heterothallic strains have the defective version, *ho*. The presence of the functional form of this gene provides the ability to change the information at the mating-type locus by a process known as mating-type interconversion. The *HO* gene encodes an endonuclease that specifically produces a double-strand break at *MAT*. Repair of this break leads to the duplicative transposition of information from *HML* or *HMR* to *MAT*. This process results in the ability of a single haploid cell to give rise to progeny of the opposite mating-type thus producing a mixed culture of cells that are interfertile (review by Herskowitz, 1988). A similar model is used to explain mating-type switching in the fission yeast, *Schizosaccharomyces pombe* (Kelley *et al.* 1988; Beach, 1983). Mating-type switching has also been observed in the ascomycetes *Chromocrea spinulosa, Sclerotinia trifoliorum,*

and *Glomerella cingulata* (Glass and Kuldau, 1992), and in the tetrapolar basidiomycete, *Agrocybe aegerita* (Labarère and Noel, 1992). Another mechanism of homothallism, secondary homothallism (sometimes also called psuedohomothallism), is seen in the ascomycetes *Neurospora tetrasperma* and in *Podospora anserina*. In these two fungi a single culture gives the appearance of homothallism because nuclei of opposite mating type are compartmentalized within a single ascospore during first-division segregation or second-division segregation respectively (Webster, 1980).

Gene Function at the Mating-type Loci

The genes residing at the mating-type locus (*MAT*) in several fungi have been extensively studied and their role in the control of mating are beginning to be understood. A brief overview of the mating-type genes in some of the well-studied pseudohomothallic and heterothallic fungal mating systems will be presented. First however, it is important to understand that although a mating-type locus within a species may have genetically different forms referred to as alleles, this term isn't entirely accurate. Although each mating-type allele within a species maps to a particular part of the genome in compatible mates, the DNA sequence of each mating-type allele is distinct and unrelated (ascomycetes) or distantly related (basidiomycetes). Flanking sequences, on the other hand, are quite homologous among mating-types of the same species. Metzenberg (1990) coined the term idiomorph to describe this type of variation at a genetic locus and to distinguish these forms from

classical alleles. It is also important to understand that a fungal *MAT* locus may contain more than one gene, and that the designation *MAT* refers to a region on a chromosome that has mating-type control function.

As mentioned above, the ascomycete yeast *S. cerevisiae* has a bipolar mating-type system. One of two idiomorphs, *MAT*α or *MAT***a**, resides at the *MAT* locus. Cells carrying *MAT*α are referred to as α mating-type cells, while those carrying *MAT***a** are **a** mating-type cells. *MAT*α contains two genes that code for regulatory proteins, α1 and α2, and *MAT***a** contains one gene that also encodes a regulatory protein, **a**1. α1 is a positive regulator of α-specific genes; expression of this protein is necessary to induce expression of the genes required for α-mating-type function (Sprague *et al.* 1983; Herskowitz, 1989). α2 is a negative regulator of transcription of the **a**-specific genes, in other words it represses the genes that would confer **a**-mating-type function (Wilson and Herskowitz, 1984; Herskowitz, 1989). In **a** mating type cells, it is the lack of α2 expression that allows the constitutive expression of **a**-specific genes. The α-specific genes are not expressed in **a** cells due to the lack of expression of α1. The **a**1 regulatory protein plays no role in the expression of **a**-specific genes, but is necessary in **a**/α diploids where it interacts with α2 to form a unique negative regulatory protein **a**1-α2. This protein is responsible for repressing both **a**- and α-specific genes and the large group of haploid-specific genes expressed in both haploid cell types (Nasmyth *et al.* 1981; Klar *et al.* 1981; Herskowitz, 1989; Kües and Casselton, 1992). **a**-specific genes

include the structural genes for the pheromone, **a**-factor (*MF***a***1* and *MF***a***2*), the α-factor receptor (*STE2*), a protein necessary for **a**-factor secretion (*STE6*), and *BAR1*, an α-factor degrading protease. The α-specific genes include the structural genes for the pheromone, α-factor (*MFα1* and *MFα2*), and the receptor for **a**-factor (*STE3*). Functions of the **a**- and α-specific genes will be discussed in a later section.

The ascomycete fission yeast, *Schizosaccharomyces pombe,* also displays a bipolar mating system. Mating type specificities are only expressed under conditions of nitrogen starvation. The two mating-type idiomorphs, *matM* and *matP,* each contain two divergently transcribed genes. *matMc* and *matPc* are required for conjugation, while *matMm* and *matPm* are required for meiosis (Kelly, 1988). Similar to the products of the *MAT* genes of *S. cerevisiae,* these four genes encode proteins that act as transcriptional regulators of mating-type-specific genes in haploid cells. In diploid cells the gene products interact with one another to repress haploid-specific genes, and induce diploid specific genes (Kües and Casselton, 1992; Kronstad and Staben, 1997).

Functions of the genes at the mating-type locus (*mt*) of the ascomycete *Neurospora crassa* have not yet been fully elucidated, although their roles in the mating process have been genetically assessed by mutational analysis. The two idiomorphs of *N. crassa* are *mt a* and *mt A*. Three genes are present in the *mt A* idiomorph (*mt A-1, mt A-2* and *mt A-3*) and only one gene (*mt a-1*) is

contained within the *mt a* idiomorph. Mating defects occur if there is a mutation within either *mt a-1* or *mt A-1* (Kronstad and Staben, 1997). Directed replacement of the *mt A* locus with *mt a-1* reveals that the presence of the *mt a-1* gene is able confer a mating type switch suggesting that it is the only gene essential to mating in the *mt a* idiomorph (Chang and Staben, 1994). Studies show *mt A-1* to be necessary prior to, but not during ascosporogenesis (Glass and Lee, 1992). The amino acid sequences of the *mt A-1* and the *mt a-1* genes show some homology to the MATα1 protein of *S. cerevisiae* and the *matMc* gene product of *S. pombe*. This suggests that these genes might also encode transcriptional regulators (Bölker and Kahmann, 1993). Recent mutational analysis of *mt A-2* and *mt A-3* revealed that the products of these genes may repress *sdv-1* and *sdv-4* (genes involved in sexual development) in the absence of *mt A-1*. A model has thus been proposed in which MT A-1, MT A-2, and MT A-3 form a complex that regulates the expression of *sdv-1* and *sdv-4* in *mt A* except when crossed with *mt a* (Ferreira *et al.* 1998). It is also possible that interactions between these three polypeptides is important in maintaining mating-type nuclear identity prior to karyogamy (Ferreira *et al.* 1998).

The more complex *MAT* loci of the basidiomycete fungi harbor not only regulatory genes, but also the pheromone and pheromone receptor genes required for cell-cell recognition. The mating-type loci of the tetrapolar fungi *Schizophyllum commune* and *Coprinus cinereus* each contain two unlinked loci

designated *A* and *B*. In *S. commune*, each of these loci contain two subloci designated α and β. *C. cinereus* also has two subloci, α and β, found tightly linked at the *A* locus, but has only one *B* locus. Due to the presence of multiple specificities at each genetic locus in these fungi, literally thousands of mating types are possible. In *S. commune*, $A\alpha$ has 9 specificities, $A\beta$ has 32, and $B\alpha$ and $B\beta$ each have 9 specificities (Vaillancourt *et al.* 1997). As a result, there are over 23,000 possible mating types. Similarly, *C. cinereus* has more than 12,000 possible mating types resulting from approximately 160 specificities at *A* and 79 at *B* (O'Shea *et al.* 1998). The *A* locus of both *S. commune* and *C. cinereus* encode homeodomain proteins. This locus is responsible for the regulation of nuclear pairing, hook cell formation, conjugate division of the nuclei in the tip cell, and hook cell septation. The *B* locus in each of these fungi encode pheromones and pheromone receptors. Nuclear migration and fusion of the hook cell with the subapical cell are the processes regulated by genes located at *B* (Specht, 1995; Wendland *et al.* 1995). Compatible matings occur in both *S. commune* and *C. cinereus* only upon fusion of cells with different alleles at both mating-type loci, e.g., *A1B1* X *A2B2*.

The corn smut fungus, *Ustilago maydis*, is also tetrapolar with two unlinked loci designated *a* and *b*. In this fungus, the *a* locus has only two specificities while the *b* locus has 25. The result is 50 possible mating types. The idiomorphs of the *a* locus (*a1* and *a2*) contain genes that encode

pheromones and pheromone receptors. These genes are not only necessary for cell-cell recognition, but may also play a role in maintenance of the dikaryon (Banuett and Herskowitz, 1989). The *b* locus contains two divergently transcribed genes, *b*E and *b*W that encode homeodomain proteins. These homeodomain proteins are involved in the regulation of genes required for filamentous growth and pathogenesis (Schulz *et al.* 1990). Similar to *S. commune* and *C. cinereus* compatible mating will only occur if both mates contain different alleles at both loci, e.g., *a1b1* X *a2b2*.

U. hordei displays a bipolar mating-type system with only two alternate specificities, *MAT-1* and *MAT-2*, and thus only two mating types are possible. The *MAT* locus of *U. hordei* contains two subloci, *a* and *b* that are related to the corresponding loci of *U. maydis* (Bakkeren *et al.* 1992). *a* contains genes encoding pheromones and pheromone receptors (Chapter 1; Willits, 1998; Bakkeren and Kronstad, 1994) and *b* contains genes encoding homeodomain proteins, Uh*b*W and Uh*b*E (Bakkeren and Kronstad, 1993).

The Role of Pheromones in Fungal Mating

Across the fungal kingdom, chemical mating factors have been implicated, at some level, in the orchestration of mating processes including the induction of gametic structures, chemotaxis or chemotropism. Examples of chemical mating factors from the lower fungi include sirenin produced by *Allomyces* sp., antheridiol and oogoniol produced by *Achlya* sp., and trisporic

acid first isolated from the zygomycete *Mucor mucedo*. The structure of these have all been determined, and are lipoidal substances in the isoprenoid family of secondary metabolites (Webster, 1980). Of primary interest to this study, however, are the peptide pheromones produced by ascomycete and basidiomycete fungi.

Genes encoding peptide pheromones have been cloned from several fungi including the ascomycetes *S. cerevisiae* (Kurjan & Herskowitz, 1982; Brake *et al.* 1985), *S. klyuveri* (Sakamoto, 1986) and *S. pombe* (Davey, 1992), and the basidiomycetes *U. maydis* (Bölker *et al.* 1992), *Rhodosporidium toruloides* (Akada *et al.* 1989), and *Cryptococcus neoformans* (Moore and Edman, 1993). With the exception of *C. neoformans,* at least one of the pheromones from each of the above species has also been isolated and characterized (Andregg *et al.* 1988; Statzler *et al.* 1976; Sakurai *et al.* 1984; Davey, 1992; Spellig *et al.* 1994b; Kamiya *et al.* 1978). In addition, pheromones from *Tremella mesenterica* and *T. brasiliensis* have been isolated and characterized but not cloned (Sakagami *et al.* 1978, 1981; Ishibashi *et al.* 1984). Generally speaking, the fungal pheromones are peptides with mature lengths ranging from 9 to 16 amino acids. With only a few exceptions, the peptides thus far isolated and characterized are reported to be further modified at a C-terminal cysteine by the addition of a farnesyl group and carboxyl methyl esterification (Table 1-1).

Table 1-1. Structures of Fungal Peptide Pheromones

S. cerevisiae	a-factor	Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys(S-farnesyl)-OCH_3
S. cerevisiae	α-factor	Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-OH
S. pombe	M-factor	Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-Cys(S-farnesyl)-OCH_3
S. klyuveri	a-factor(sk1)	X-His-Trp-Leu-Ser-Phe-Ser-Lys-Gly-Glu-Pro-Met(O)-Tyr-OH
S. klyuveri	a-factor(sk2)	Trp-His-Trp-Leu- Ser-Phe-Ser-Lys-Gly-Glu-Pro-Met-Tyr-OH
R. toruloides	Rhodotorucine A	Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys(S-farnesyl)-OH
T. mesenterica	Tremerogen a-13	Glu-Gly-Gly-Gly-Asn-Arg-Gly-Asp-Pro-Ser-GLy-Val-Cys(S-farnesyl)-OH
T. mesenterica	Tremerogen A-10	Glu-His-Asp-Pro-Ser-Ala-Pro-Gly-Asn-Gly-Tyr-Cys(S-farnesyl)-OCH_3
T. brasiliensis	Tremerogen A-1	Asp-Ser-Gly-Ser-Ser-Arg-Asp-Pro-Gly-Ala-Ser-Ser-Gly-Gly-Cys(S-farnesyl)-OCH_3
U. maydis	mfa1	Gly-Arg-Asp-Asn-Gly-Ser-Pro-Ile-Gly-Tyr-Ser-Ser-Cys(S-farnesyl)-OCH_3
U. maydis	mfa2	Asn-Arg-Gly-Gln-Pro-Gly-Tyr-Tyr-Cys Cys(S-farnesyl)-OCH_3

The pheromones and their complementary receptors in each of the species above play a key role in cell-cell recognition leading to fusion of compatible mating type cells. In *S. cerevisiae,* a model representative of the ascomycete yeasts, the pheromone and pheromone receptor genes are regulated by genes residing at the *MAT* loci. **a** cells produce **a**-factor, a 12 amino acid peptide pheromone encoded by *MF***a**, and express the α-factor receptor, encoded by *STE2* (Blumer *et al.* 1988), on their surface. Similarly, α-cells produce a 13 amino acid pheromone peptide encoded by *MF*α, and express the **a**-factor receptor, encoded by *STE3* (Hagen *et al.* 1986), on their surface. The pheromones are secreted across the cell membrane where they form a gradient that is sensed by nearby cells of opposite mating type. Mating interactions occur only among cells in very close proximity to one another. Upon pheromone binding to its receptor, a G-protein mediated signal transduction pathway is triggered that leads to a number of changes in cell physiology allowing the formation of an **a**/α diploid cell. One of the first results of the signal transduction pathway is the arrest of cells at the G1 stage of the cell cycle, prior to DNA replication (Herskowitz, 1988, 1989). Subsequently, morphological changes, known as shmoo formation, occur as the cells stretch toward each other in response to the pheromone gradient (Baba *et al.* 1989; Byers, 1981). Meanwhile, cell surface glycoproteins and agglutinins are synthesized in order to stabilize the association of the mating cells enabling them to fuse (Lipke *et al.* 1989; Herskowitz, 1988; Kües and

Casselton, 1992). Once the a/α diploid is formed, a1 and α2 proteins encoded by the *MAT* loci interact to shut down expression of the **a** and α specific genes. Pheromone and pheromone receptors are, therefore, no longer produced.

Ustilago maydis, representative of the basidiomycetes listed above, utilizes its pheromones and pheromone receptors for cell-cell recognition in the mating response in a manner similar to that of *S. cerevisiae*. Unlike the ascomycete yeasts, however, the pheromone and pheromone receptor genes are actually present at the *MATa* locus. The pheromone genes *Ummfa1* and *Ummfa2* encode pheromone precursors of 40 and 38 amino acids respectively (Bölker *et al.* 1992). Each of the precursors end with a prenylation signal known as a CaaX motif (C = cys, a = an aliphatic amino acid, X = any amino acid) (Bölker *et al.* 1992; Casey, 1995; Schafer and Rine, 1992). Subsequent cleavage and processing results in active pheromones of 13 amino acids (Ummfa1) and 9 amino acids (Ummfa2), both of which are likely to be post-translationally modified by S-farnesylation and carboxyl methyl esterification of the C-terminal cysteine (Spellig *et al.* 1994b). Mature pheromones are secreted by their respective cell types and bind to the pheromone receptors on cells of the opposite mating type. Microscopic *in vitro* experiments to describe the mating process of *U. maydis* have been done primarily in water droplets of small volume with the assumption that these conditions most closely mimic the high humidity and shallow liquid films found in the leaf

whorls of corn where mating and infection occur naturally (Snetselaar and Mims, 1992; Sneteselaar, 1993). In these experiments, conjugation was observed only between cells in very close proximity to each other (one cell length apart or less). This suggests that the pheromones produced by *U. maydis* are not readily diffusible. Experiments in which cells of opposite mating type were coinoculated onto agar solidified minimal media and then covered with mineral oil and a cover slip did result in mating at somewhat greater distances (Snetselaar *et al.* 1996), but this system is considered extremely artificial.

The *U. maydis* pheromone receptor genes, *Umpra1* and *Umpra2* (Bölker *et al.* 1992), encode receptors with similarity to members of the serpentine receptor family. The amino acid sequence of Umpra1 and Umpra2 share significant similarity to the *S. cerevisiae* a-factor receptor, STE3. These three receptors are thought to belong to a subfamily of serpentine receptors specific for the recognition of prenylated peptide mating factors (Bölker *et al.* 1992).

In response to pheromone binding, a series of changes in the physiology of the *U. maydis* cells occurs as seen in the ascomycete yeasts. Cells stop budding, and produce conjugation tubes that grow toward cells/conjugation tubes of the opposite mating type. The conjugation tubes fuse at the tips where formation of the dikaryotic mycelium occurs. Unlike the ascomycete yeasts where pheromone production is shut down after cell fusion, however, *U. maydis* requires pheromone signaling in order to maintain filamentous

growth of the dikaryon (Snetselaar, 1993; Banuett and Herskowitz, 1994; Bölker *et al.* 1992; Kronstad and Staben, 1997).

Mating in *Ustilago hordei* is mediated by readily diffusible pheromones. This was first shown by the demonstration that conjugation tubes could be induced when cells of the opposite mating type were as far away as 10 cell lengths. This same induction of conjugation tubes could also be seen when cells of opposite mating type were separated by a dialysis membrane with a 12,000 MW cutoff (Martinez-Espinoza *et al.* 1993). Bakkeren and Kronstad (1994) cloned the pheromone receptor gene *Uhpra1* and found that it encodes a protein with a predicted amino acid sequence 64% identical and 82% similar to the predicted product of *Umpra1*. Presumably, binding of pheromone to its receptor in this system is also responsible for the morphological changes, conjugation tube formation and growth, and possibly maintenance of the filamentous dikaryon, seen during the mating process.

The role of pheromones and pheromone receptors in mating of the basidiomycetes *S. commune* and *C. cinereus* are very different from those described above. In these fungi anastomosis of haploid vegetative cells is not dependent on pheromone-based recognition. However, recent research has shown that pheromone and pheromone receptor genes are present at the *B* locus in both of these fungi (Vaillancourt *et al.* 1997; O'Shea *et al.* 1998). This implies that the *B*-regulated events of reciprocal nuclear migration and hook cell fusion are controlled by pheromone signaling. The following was

proposed for the series of *B*-regulated events. Upon fusion of compatible monokaryotic mycelia, secreted pheromones encoded by the fertilizing nucleus act as advance signals to activate receptors encoded by the resident nuclei in nearby cells of the mycelium. The pheromone signals prepare the cells for invasion by causing dissolution of the septa so that the fertilizing nucleus can pass from cell to cell until it reaches the tip cell. *A* locus regulation of hook cell formation and conjugate division of the nuclei occurs as the two nuclei of the mating partners are established in the tip cell. Further *B*-regulated signaling then allows fusion of the hook cell to the newly formed cell to form a mature clamp connection (Kronstad and Staben, 1997; Vaillancourt *et al.* 1997).

CHAPTER 2

GENETIC AND MOLECULAR ANALYSIS OF THE *MAT-1* PHEROMONE GENE OF *USTILAGO HORDEI*

Introduction

The basidiomycete smut and bunt fungi are pathogens of more than 1100 species of plants (Fischer and Holton, 1957). To become pathogenic, these fungi must pass through the sexual cycle by fusing two uninucleate sporidia of opposite mating type to form an infectious, dikaryotic mycelium. In *Ustilago hordei*, the causal agent of covered smut of barley (*Hordeum vulgare*) (Mathre, 1997), the mating system is bipolar and controlled by genes at the *MAT* locus (Thomas, 1988). This locus has two alternate specificities, *MAT-1* and *MAT-2*, that correspond to the two mating types traditionally referred to as "**A**" and "**a**" respectively. Genes at this locus are responsible for regulating cell-cell recognition, cell fusion, the morphological change from yeast-like growth to filamentous growth, and maintenance of the infectious dikaryon (Bakkeren and Kronstad, 1996). Evidence for the production of small molecular weight chemical mating factors by the haploid sporidia of *U. hordei* was presented by Martinez-Espinoza *et al.* (1993). Secretion of these mating factors, or pheromones, is the first step in the mating process leading

to the formation of the pathogenic form of this fungus. The genes encoding pheromones and their receptors, therefore, could reasonably be considered pathogenicity genes.

U. maydis, the causal agent of the disease corn smut, is a close relative of *U. hordei* and has been the subject of extensive study with respect to the genes involved in the mating process. The mating system of *U. maydis* is tetrapolar involving the *a* and *b* loci. The *a* locus contains genes that encode a pheromone precursor (*Ummfa*), and a receptor for the pheromone of the opposite mating-type (*Umpra*) (Bolker, *et al*, 1992; Bakkeren, *et al*, 1994). These genes are primarily responsible for triggering responses necessary for the formation of conjugation tubes and the subsequent fusion of compatible cells. The *b* locus, with more than 33 alleles, encodes two divergently transcribed homeodomain proteins, *b*E (*b*East) and *b*W (*b*West) (Bolker *et al*, 1991; Gillissen *et al*, 1992). Upon fusion of compatible cells, the interaction between *b*E and *b*W polypeptides from different alleles form a heterodimer that allows for sexual development and pathogenicity (Kronstad and Leong, 1989; Schulz *et al*, 1990; Gillissen *et al*, 1992; Spellig *et al*, 1994a; Kamper *et al*, 1995).

U. hordei contains sequences that hybridize to these *U. maydis* genes indicating that similar genes regulate mating and sexual development in both (Bakkeren *et al*, 1992). Recent studies of the structure, organization and function of these mating-type genes in *U. hordei* serve as the beginning of a

model for sexual development in the bipolar smut fungi. Bakkeren and Kronstad (1993) have cloned different alleles of *b*W and *b*E from opposite mating type strains of *U. hordei.* These genes code for polypeptides whose organization and structure are very similar to the *U. maydis* b polypeptides. As in *U. maydis,* the b polypeptides in *U. hordei* appear to control filamentous growth of the dikaryon (Gillissen *et al*, 1992; Bakkeren and Kronstad, 1993; Bakkeren and Kronstad, 1996). Analysis of the organization of the *a1* locus with respect to the *b* locus revealed that these loci were tightly linked genetically. This gives the appearance of a single diallelic *MAT* locus controlling both mating and dikaryon formation. Sequence analysis of part of the *U. hordei a1* locus confirmed that a pheromone receptor-like gene (*Uhpra1)* resided within the *MAT-1* locus (Bakkeren and Kronstad, 1994). Further analysis of the *a1* locus by transformation into a MAT-2 strain indicated the presence of a putative pheromone gene, *Uhmfa1*. Together *Uhpra1* and *Uhmfa1* appear to be responsible for production of the cell signaling components required for early mating responses including conjugation tube formation and fusion (Bakkeren and Kronstad, 1996).

Recently the sequences of the pheromone and pheromone receptor genes from MAT-2 cells were reported (*Uhmfa2* and *Uhpra2, respectively)* (Willits, 1998). The ORF of *Uhmfa2* encodes a 39 amino acid pheromone precursor with 55.3% identity and 76.3% similarity to *Ummfa2*. The predicted precursor contains a C-terminal prenylation signal which appears to be fairly

common to several fungal pheromones isolated to date (see literature review). The sequence of *Uhpra2* was compared to the pheromone receptor gene sequences from *U. maydis* and found to be 64.3% identical to *Umpra2* including the introns. Comparison of the predicted amino acid sequence of Uhpra2 to that of Umpra2 revealed 59.9% identity and 78.6% similarity. Based on the alignment of the predicted protein with the STE3 pheromone receptor of *Saccharomyces cerevisiae* and the Umpra2 receptor of *U. maydis*, seven hydrophobic domains were identified. Based on this information it appears that Uhpra1 and Uhpra2 belong to the G-protein linked, 7-transmembrane segment family of receptors.

Martinez-Espinoza (1993) cloned two contiguous *Bam*HI fragments of 5.0 and 5.5 kb in length from the *MAT-1* allele of *U. hordei*. Transformation of the 5.5 kb fragment into MAT-2 cells caused them to produce conjugation tube-like structures constituitively (Figure 2-1) indicating that the region might contain a pheromone and/or pheromone receptor gene. The purpose of this study was to localize, subclone, and characterize the pheromone gene (*Uhmfa1*) within the *MAT-1* locus, verify the function of both *Uhmfa1* and *Uhpra1* by deletion of those genes at the *MAT-1* locus and subsequent replacement of each gene, and to study the expression of the pheromone and pheromone receptor genes of both MAT-1 and MAT-2 cells.

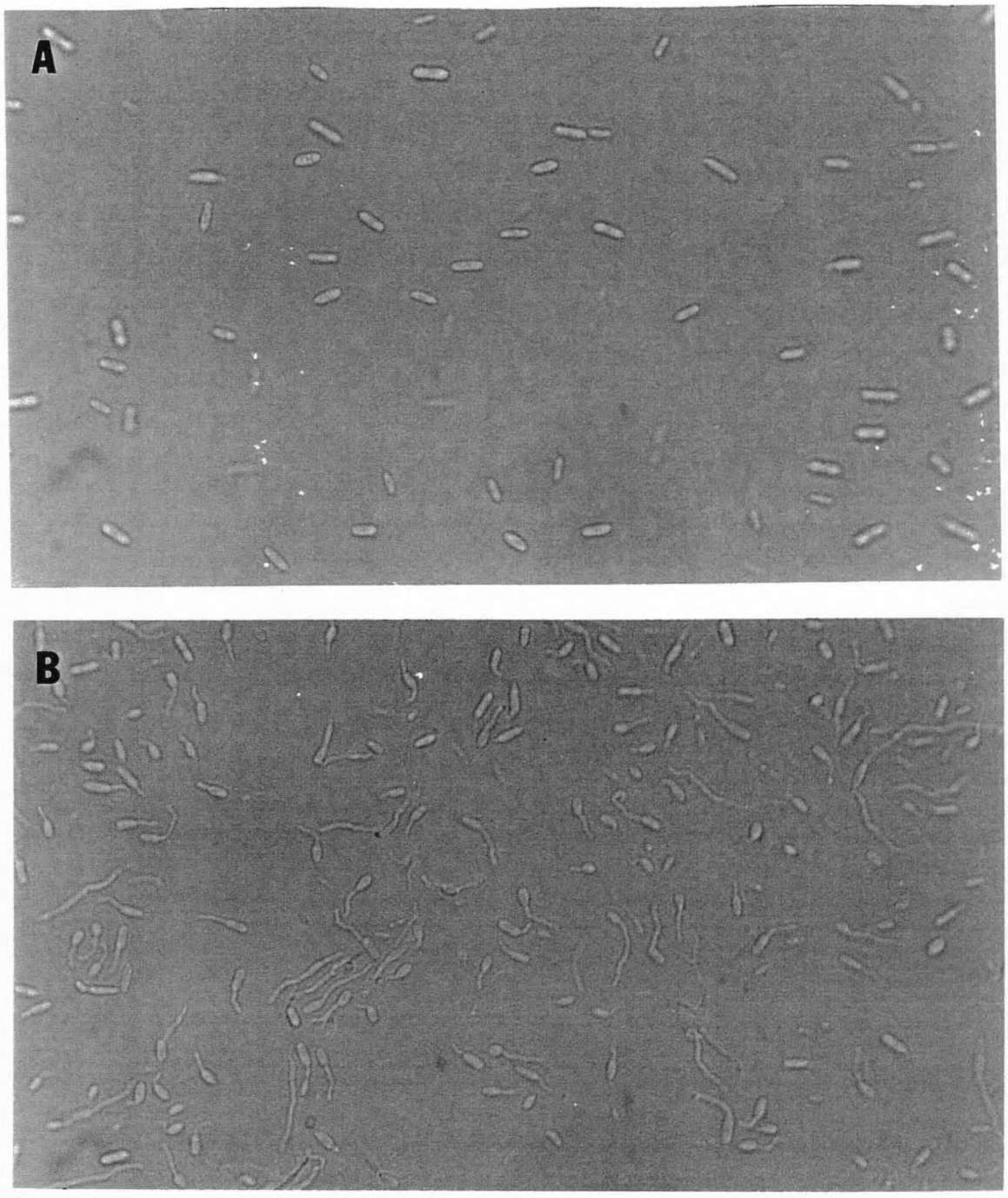

Figure 2-1. *Ustilago hordei* MAT-2 cells transformed with mating-type genes. A) MAT-2 wild-type cells; B) MAT-2 cells transformed with the 5.5 kb *Bam*HI fragment in pHyg101 shows constituitive production of conjugation tubes.

Materials and Methods

Strains and Growth Conditions

U. hordei (Pers.) Lagerh. strain 8A (*MAT-1*; ATCC # 90511) was used to obtain DNA for study of the *MAT-1* locus. Transformations and bioassays used strains 14A (*MAT-1*; ATCC # 90512) and 14a (*MAT-2*; ATCC # 90510). Cultures of *U. hordei* were maintained on Holliday's complete medium (HCM) agar (Holliday, 1974).

Plasmids and Plasmid Constructs

Plasmids pBluescriptK/S+ (Stratagene, La Jolla, CA), pCM54 (Tsukuda, *et al*, 1988), and pHyg101 (obtained from J. Kronstad, University of British Columbia) were used for DNA manipulations. pHyg101 is a derivative of pCM54 contains the *U. maydis* autonomously replicating sequence (ARS) (Tsukuda *et al*, 1988) which allows autonomous replication of the plasmid in *U. hordei*, and the *U. maydis* Hsp70-HygB cassette (Wang *et al*, 1988; Holden *et al*, 1989) for selection in *U. hordei*. It also contains the ColE1 origin of replication and ampicillin resistance gene for replication and selection in *E. coli*. Cloning and maintenance of plasmid DNA was done in *E. coli* strains DH5α and DH10B (GIBCO-BRL, Gaithersburg, MD).

Subcloning and Localization of the Pheromone Gene-Containing Region of *MAT-1*

A restriction map of the contiguous 5.0 and 5.5 kb fragments cloned by Martinez-Espinoza (1993) was constructed. To localize the pheromone gene to the smallest possible region for sequencing, subclones of the 5.5 kb fragment were ligated into pHyg101 and transformed into *U. hordei* MAT-2 cells as described (Sherwood *et al,* 1998). The resulting tranformants were screened microscopically for their ability to constituitively produce conjugation tubes.

Sequencing

Sequencing was done using the dideoxy chain termination method (Sanger, *et al*, 1977) using a Sequenase 2.0 Kit (U.S. Biochemical, Cleveland, OH) and α-^{35}S-dATP. Once the putative pheromone gene was located, internal primers were designed and synthesized (GIBCO-BRL, Gaithersburg, MD) to allow further sequencing and clarification of initial sequence data. All sequences were analyzed using the genetics software, Wisconsin Package (Pearson and Lipman, 1988).

Disruption of *MAT-1* and *MAT-2* Genes

A cosmid clone of the *U. hordei MAT-1* locus, paMAT-1, (a gift from G. Bakkeren and J. Kronstad, University of British Columbia, Vancouver, BC,

Canada) (Bakkeren and Kronstad, 1994) was used to construct a deletion vector in order to knock out the *MAT-1* functions in *U. hordei*. paMAT-1 was digested with *Apa*I and *Xba*I to yield a 12kb fragment containing *Uhpra1* and *Uhmfa1*. This fragment was separated from the vector by gel electrophoresis in 1% low melting temperature agarose. The band containing the 12kb fragment was excised from the gel and ligated (Daum *et al,* 1991) to pBluescript K/S+ This construct was digested with *Bam*HI to remove the 5.5kb fragment containing *Uhmfa1* and the 3' end of the *Uhpra1*. This fragment was replaced with a 1.8kb phleomycin resistance cassette driven by the *U. maydis* heat shock protein 70 (Hsp70) promoter (obtained from G. Bakkeren and J. Kronstad). This new construct, pMAT1(-) was used to transform *U. hordei MAT-1* cells by integration and homologous recombination to create strain 14Amat-. pUhmfa1, containing *Uhmfa1,* and pUhpra1, containing *Uhpra1* (Sherwood *et al,* 1998) were used individually to transform strain 14Amat- to restore pheromone and pheromone receptor functions. In order to restore both functions simultaneously, 14Amat- cells were transformed with the cosmid paMAT-1.

Homologous recombination of pMAT1(-) was confirmed by southern analysis (Southern, 1975) according to Sambook *et al.* (1989). Genomic DNA was isolated from MAT-1 wild-type cells and from 14Am- cells that had been grown in 50 ml of HCM broth to early log phase. Cells were washed with SCS buffer (20mM NaCitrate, pH 5.8; 1.2 M KCl) and protoplasted by incubation

with Novozyme 234 (20 mg/ml in SCS) on a rocker mixer for 1-2 hr. The protoplasts were washed once with SCS and once with 0.1 M EDTA (pH 8.0), 1.2M KCl. Protoplasts were disrupted by suspending them in 0.5 ml 50mM Tris (pH8.0), 0.1 M EDTA , 5µl 10% SDS and 3 µl RNase, followed by incubation in a water bath which decreased in temperature from 65 C to 37 C over the course of the 1 hr incubation time. Cell debris was removed at the end of the incubation time by centrifugation at 14k rpm for 5 min. The supernatant fraction was extracted once with phenol, once with phenol:chloroform (1:1) and once with chloroform. DNA was precipitated by the addition of 0.1 volume 3M NaAcetate (pH 7.5) and 2 volumes of 95% EtOH. DNA was pelleted by centrifugation for 15 min at 14K rpm, washed with 75% EtOH, dried and dissolved in 10 mM Tris buffer.

Genomic DNA for Southern analysis was digested with either *Bam*HI or *Eco*RI. The probes used for Southern hybridization were the 5.5 kb *Bam*HI fragment ligated into pBluescript(K/S) and the deletion vector pMAT1(-). The probes were labeled by nick-translation (Boeringer Mannheim) with ^{32}P-dCTP.

Bioassays

The ability of the *U. hordei* transformants to mate was assessed by a fuz reaction when crossed with opposite mating type cells on charcoal agar (Martinez-Espinoza *et al,* 1993). Mating was observed microscopically by

inoculating 1cm x 1cm x 0.2cm squares of trace element (TE) agar (Martinez-Espinoza *et al,* 1993) with 2 x 10^3 cells each of the transformant being tested and wild-type cells of the opposite mating type. The agar squares were placed on glass microscope slides and incubated in a moist chamber in the dark at 18° to 22° C and observed after 24 h.

RNA Procedures

Total RNA from cells of *U. hordei* was isolated using a modified version of the method described by Kohrer and Domeday (1991). Cells were grown to log phase in HCM broth at RT on a rotary shaker at 250 rpm. The cells were pelleted by centrifugation at 3,500 x g at 4C for 10 min, washed twice with SCS buffer (20 mM sodium citrate pH 5.8, 1.2 M KCl), and resuspended in 1.0 ml AE buffer (50mM sodium acetate, 10 mM EDTA, adjusted to pH 5.0 with acetic acid). Cells were pelleted at 3,500 x g for 5 minutes. The cell pellet was frozen in liquid nitrogen and ground to a fine powder with a precooled mortar and pestle. The cell powder was then added to a microcentrifuge tube containing 0.6 ml phenol equilibrated with AE buffer, 0.5 ml AE buffer and 50 µl 10% SDS, vortexed vigorously for 10 min., then incubated for 30 min at RT, vortexing every 5 to 10 mins. The lysate was centrifuged at 10,000 x g for 10 mins. The aqueous phase was removed and extracted twice with phenol, once or twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). RNA was

ethanol precipitated from the supernatant and incubated at -20C for 30 min or until ready to use. RNA was pelleted by centrifugation at 10,000 x g for 15 min and the pellet was washed with 80% ethanol. The RNA was dissolved in 75 µl RNA buffer (25 mM NaOAc, 2 mM EDTA; pH 5.5). Quality of RNA was assessed by formaldehyde agarose gel electrophoresis. RNA was quantitated using a Gene Quant RNA/DNA Calculator (Pharmacia, Piscataway, NJ). mRNA was isolated from total RNA by oligo(dT)-cellulose (Ambion, Austin, TX) chromatography according to manufacturers recommendations. Northern analysis was done using Hybond- N+ nylon membranes (Amersham, Arlington Heights, IL) using probes labeled by nick-translation (Boeringer Mannheim) with ^{32}P-dCTP. Vectors used for probe synthesis were: pBS494 (pBluescript K/S+::494 bp *Sal*I/*Xho*I fragment containing *Uhmfa1*); pPra1.700 (pBS::700 bp internal *Eco*RV/*Bam*HI fragment of *Uhpra1*); pMfa2.400 (pBS::385bp *Xho*I/*Pst*I fragment containing *Uhmfa2*); pPra2.872 (pBS::872 bp *Pst*I/*Sac*I internal fragment of *Uhpra2*); and pBt680 (pBS::680 bp *Kpn*I/*Ava*I fragment of the *U. maydis* benomyl-resistant β-tubulin gene (*Tub*) from pBen102 [Gold *et al*, 1994]). Expression of *Uhmfa1, Uhmfa2, Uhpra1 and Uhpra2* were quantitated by scanning the northern blots into a Molecular Dynamics PhosphorImager with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). Expression of the β-tubulin gene was calculated for each lane in order to correct for unequal sample loading.

Results

Molecular Analysis of the Pheromone Gene-Containing Region

A restriction map of the 5.0 and 5.5 kb cloned fragments from *U. hordei* MAT-1 cells was constructed (Figure 2-2). Comparison of the restriction map of the 5.5kb and 5.0kb fragments to the *a1* locus and the location of *Uhpra1* (Bakkeren and Kronstad, 1994), indicated that the *Uhpra1* gene spanned the *Bam*HI site between these two fragments (Figure 2-2). The activity of the 5.5kb fragment, therefore, was attributed to the presence of a pheromone gene. Subclones of this 5.5kb fragment were ligated into pHyg101 and transformed into *U. hordei* MAT-2 cells. The ability of the subclones to induce the characteristic phenotype of constitutive conjugation tube production was assessed microscopically in order to localize the pheromone gene-containing region to the smallest possible fragment for sequencing purposes (Figure 2-3). The pheromone gene-containing region of the 5.5kb *Bam*HI fragment was localized to a *Hind*III/*Eco*RV fragment approximately 2.6kb in length. This region was then sequenced from both directions.

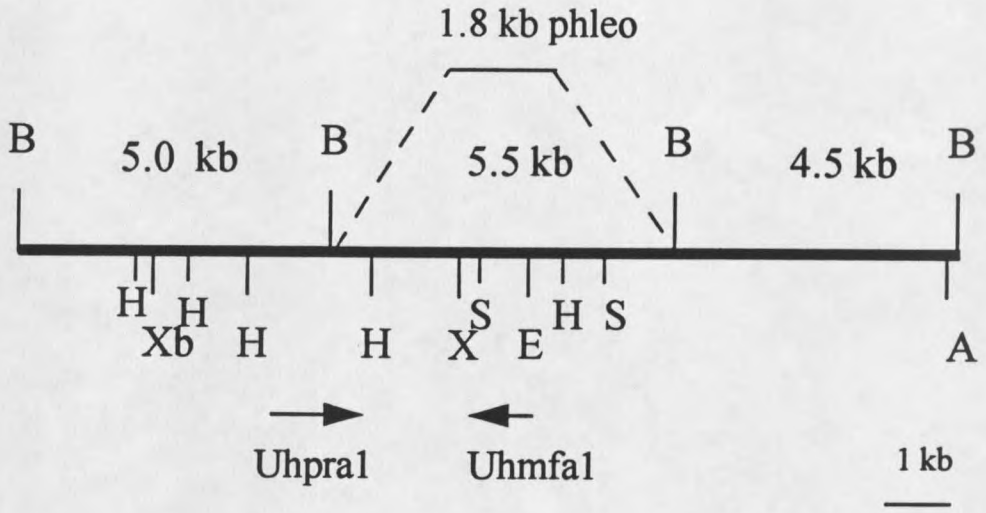

Figure 2-2. The *MAT1* allele of the *MAT* locus of *Ustilago hordei*. Restriction map of the *MAT1* allele. Arrows indicate location and direction of transcription of the pheromone receptor gene, *Uhpra1*, and the pheromone gene, *Uhmfa1*. The deletion vector, pMAT(-), was constructed by removing the 12.0 kb *Xba*I-*Apa*I fragment from paMAT1 and ligating it into pBluescript K/S+. The 5.5 kb *Bam*HI fragment was then replaced with the 1.8 kb phleomycin resistance gene. The two replacement vectors, pUhpra1 and pUhmfa1 were constructed by inserting the 2.1 kb *Hind*III fragment into pJS42 and the 2.4 kb *Hind*III-*Eco*RV fragment into pHyg101 respectively. (A = *Apa*I; B = *Bam*HI; H = *Hind*III; E = *Eco*RV; S = *Sal*I; X = *Xba*I)

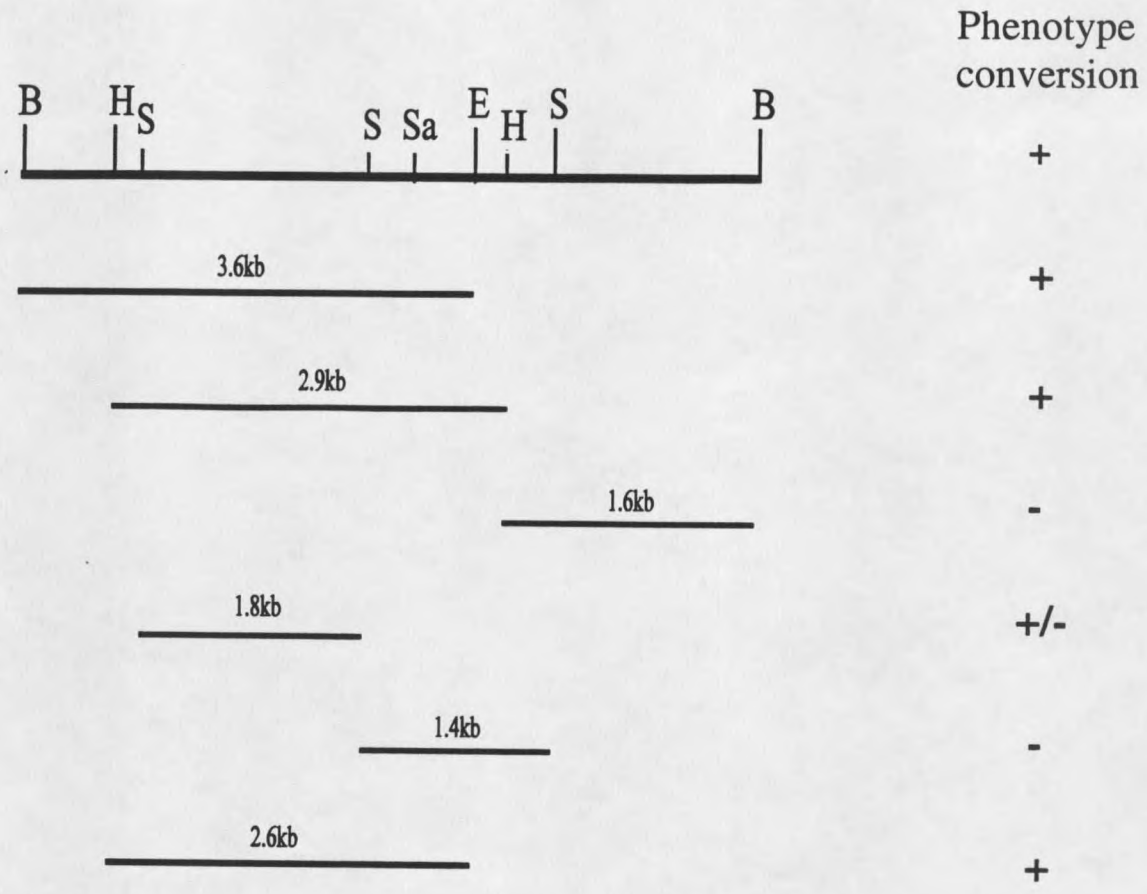

Figure 2-3. Phenotype conversion of *U. hordei* MAT-2 cells. Subclones of the 5.5 kb *Bam*HI fragment from MAT-1 cells were ligated into pHyg101 and transformed into *U. hordei* MAT-2 cells. The ability of each subclone to induce the characteristic phenotype of constituitive conjugation tube production was scored and is shown in the column to the left (+ indicates constitutive production of conjugation tubes was produced by the majority of the cells in any given sample, +/- indicates that production of conjugation tubes occurred, but the phenotype was exhibited by less than a third of the cells in any given sample, - indicates that the cells behaved phenotypically as wild-type). The smallest subclone to yield a phenotype conversion, the 2.4 kb *Hind*III fragment, was used for sequencing *Uhmfa1*. (A = *Apa*I; B = *Bam*HI; H = *Hind*III; E = *Eco*RV; S = *Sal*I; Sa = *Sac*I; X = *Xba*I)

Sequence Analysis of *Uhmfa1*

Analysis of sequence data from the putative *Uhmfa1* gene-containing region of *MAT-1* revealed the presence of a gene with a structure similar to that of the *U. maydis* pheromone gene, *Ummfa1* (Bolker, 1992) (Figure 2-4). Beginning near the *Eco*RV site was a promoter region that contained eight repeated sequence motifs with the consensus sequence ACAAAGGGA. This was the same consensus sequence as the pheromone response element (PRE) described for *U. maydis* (Urban, 1996) and similar to the PRE of *Saccharomyces cerevisiae* (Kronstad, 1987; Dolan, 1989). Other structural features of this gene included a classical transcriptional promoter sequence, the TATAA box. In this promoter the motif TATAA was centered at -53. While the TATAA box typically falls 25 -35 bases upstream from the transcriptional start site in most higher eukaryotes, it is not unusual for the TATAA box of higher fungi to be located much further away (Brown and Lithgow, 1987). In addition to the TATAA box, a CT rich motif was located from -90 to the start codon in lieu of a CAAT box.

Like the *U. maydis* homologues, *Uhmfa1* contained a single, short open reading frame. The ORF of *Uhmfa1* was 126 bp (Figure 2-4) which is comparable to the 120 bp and 114 bp ORFs of *Ummfa1* and *Ummfa2,* respectively. The ORF of *Uhmfa1* encoded a 42 amino acid precursor which is 55% identical and 68% similar to the *Ummfa1* pheromone precursor. The pheromone precursor contains the prenylation signal, CAAX, at the carboxyl

terminus (C = cys, A = an aliphatic amino acid, X = any amino acid) (Casey, 1995; Schafer, 1992).

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-791 GATATCGTAC GATGCATGAC CGCCAACGCA TTGTTCTCTG CTTAATAAAA GGGAAGAAGA
-731 AAGGGACCGT TCAGGTTTCA ACATTCATGT AGGCCAGGGC ACGCTAGAAC AGACACAAAG
-671 GGGAGAATTA CTTGCAAGCA GACCGACGGA CTGTCTCAGA TACTGCACAG CGAATAAATT
-611 TTGATTGTAT GAAAGGGATC TCTCCGGACG GAAAAAGGGC AATTAAACAC GTCCCTTTGT
-551 GTTATGCTCC CACGGTTCTT CTAATTGTTC TCGTGCCAGT CGCAGCGATT TGCGCTTACA
-491 TTGCCGACAC GCCTCTTTGT CTGTCGTTGG GAGTTCAATG CATCGAAACA ATACAGAAAG
-431 AGATCGACAA AGGGACAGAC TATCCATACG AGACGGCGCT AGCCCGAGCT CTCTGGGCGC
-371 AAAAGCCAAA AAAATGAGCG ACCAATTCGA GCTGGAGAAA GGTACGATAA CGGAGATCTG
-311 CACTTGAGTG TTGTACCCTG TCAGGCGTCT CAGGCGTCGT CTGAAGCACC GACAAGCAGC
-251 TTTTATCCTT GACTTGCCTA GTCGAGGTGT TCAGCTATGC GAGTGTGAAC ATGCGCTACT
-191 ACTTTGGCGC TCGTACTCGC CTCATGAAAC ACCCTCTAAG CCGGCATACA CCACTTCGAA
-131 AAGACTTATA AGGAGCACGG CAACCGAGGC TAGTCGACTT TCTCCCCTCA TCCACTCGCC
 -71 CTCACCCACT CTCATCTATA ACACACAGCC TCTTCCGTTC TTTCGAACAA ACACTCTAAA
 -11 CCTCTTTCAC A ATG TTC TCC ATC TTC GCT CAG CCT GCC CAG ACC TCC GTC
                  M   F   S   I   F   A   Q   P   A   Q   T   S   V

  40 TCT GAG ACC CAG GAG TCT CCT GCC AAC CAC GGT GCC AAC CCC GGC AAG
      S   E   T   Q   E   S   P   A   N   H   G   A   N   P   G   K

  88 TCC GGC TCT GGT CTC GGC TAC TCG ACC TGC GTC GTC GCC TAA AGTCTCTGCG
      S   G   S   G   L   G   Y   S   T   C   V   V   A   *

 140 ATCTACCCCC ATGgtaagtc gaattgcgat tcttttcgtc ttttggcctt tcgtgcaaca
 200 caattctaat gtcggcgact tgttttcttg ggtcaaattt caacctttt cagATCCGTG
 260 ACCCAATCGA CGCGATCGCT GGCTTGATCG AGGCCATCGC CGATGTTTCT CGATGGCATA
 320 GCTTCGTGGA CGCGGCGCCC TCGCTTCCTC ACCTTCGATT TCAAACTTGA TCACATTCAC
 380 TCTCACCCAC CTCTTCAGGC TCGCCTAACA AAGGCTCGAG TCCCACCTTC ATCCGTTCCC
 440 CTTTGTAACT TGTTGACCAT TCCTGAATGA TAGCGCGCTC ATTCAAAAG
```

Figure 2-4. Sequence of *Uhmfa1*, the pheromone gene from the MAT-1 mating type of *U. hordei*. The structural gene for *Uhmfa1* contains eight PRE sequence motifs in the promoter and one 308 bases past the stop codon (bold type). The putative TATAA box is underlined. The pheromone coding regions are shown in bold italic with the amino acid sequence below. An intron is located after the 3' end of the coding region of *Uhmfa1* from bases 153 to 251.

Deletion of the *MAT-1* Locus

To verify the function of both the pheromone and pheromone receptor genes, a deletion vector, pMAT1(-), was used to transform MAT-1 cells. Of 221 transformants tested for the ability to mate with MAT-2 cells on charcoal agar, one strain, 14Amat-, was Fuz-. Southern analysis confirmed homologous recombination at the *MAT-1* locus (Figure 2-5). When wild-type MAT-1 and MAT-2 cells are crossed on agar squares mating occurs resulting in the formation of dikaryotic mycelia (Figure 2-6A). However, microscopic observation on agar squares showed that when 14Amat- was crossed with MAT-2 cells no conjugation tubes were formed by either cell type indicating that the signaling process between the mating types had been successfully disrupted (Figure 2-6B). To show that the deleted genes encoded the pheromone and pheromone receptor and that both were essential to the signaling process, each gene was transformed back into 14Amat- separately and together. pUhmfa1 was transformed into 14Amat- to result in strain 14Am-/mfa1. When this strain was crossed with MAT-2 cells, conjugation tubes formed by the MAT-2 cells grew toward unresponsive 14Am-/mfa1 cells (Figure 2-6C). Similarly, pUhpra1 was transformed into 14Amat- to give rise to strain 14Am-/pra1 and crossed with MAT-2. This time conjugation tubes formed by 14Am-/pra1 grew toward completely unresponsive MAT-2 cells (Figure 2-6D). Finally, when both *Uhmfa1* and *Uhpra1* were added to 14Amat- simultaneously by transformation with paMAT-1, the resulting strain,

14Am-/cos, was able to induce conjugation tube formation by MAT-2 and conjugation tubes were formed by 14Am-/cos that grew toward MAT-2 cells. Conjugation tubes were able to fuse and form dikaryotic mycelia (Figure 2-6E). These observations were consistent with the expected phenotypes and confirmed that *Uhmfa1* and *Uhpra1* were indeed the pheromone and pheromone receptor genes involved in cell-cell signaling and initiation of the early mating process.

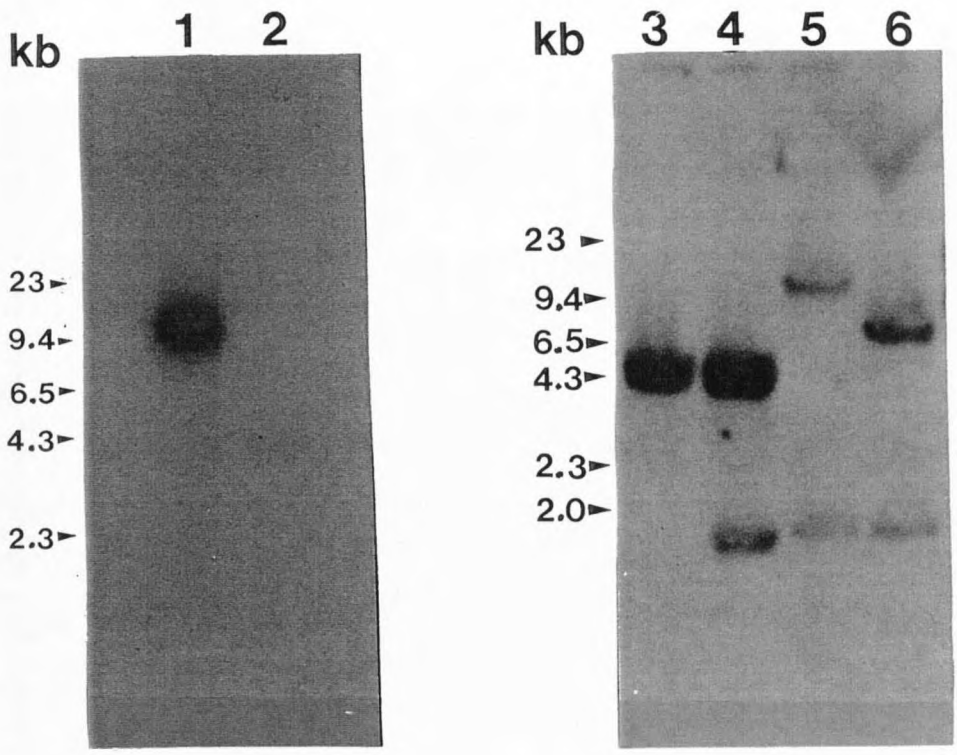

Figure 2-5. Verification of homologous recombination by double crossover between the deletion vector, pMAT1(-) and genomic sequences by Southern blot analysis. Genomic DNA was isolated from wild-type MAT-1 cells and cells of the deletion strain 14Am-. The DNA was digested with *Bam*HI or *Eco*RI and then probed with either the 5.5kb fragment (lanes 1 - 2), or with pMAT1(-) (lanes 3 - 5). Lane 1, DNA from wild-type cells digested with *Eco*RI probed with the 5.5 kb fragment. A single band 10.7kb in length hybridized as predicted. Lane 2, 14Am- digested with *Eco*RI. Lack of signal supports loss of the 5.5kb fragment due to homologous recombination. Lane 3, Wild-type DNA digested with *Bam*HI. Two bands 5.0kb and 4.5kb in length hybridized as predicted. Lane 4, 14Am- DNA digested with *Bam*HI. Three bands of 5.0kb, 4.5kb and 1.8kb hybridized as predicted. The 1.8kb band is representative of the phleomycin cassette that would replace the 5.5 kb *Bam*HI fragment if homologous recombination occurred. Lane 5, Wild-type DNA digested with *Eco*RI. Two bands hybridized as predicted, one 10.7kb and one 1.7kb. Lane 6, 14Am- DNA digested with *Eco*RI. A 7.0kb band and a 1.7kb band hybridized as predicted for homologous recombination.

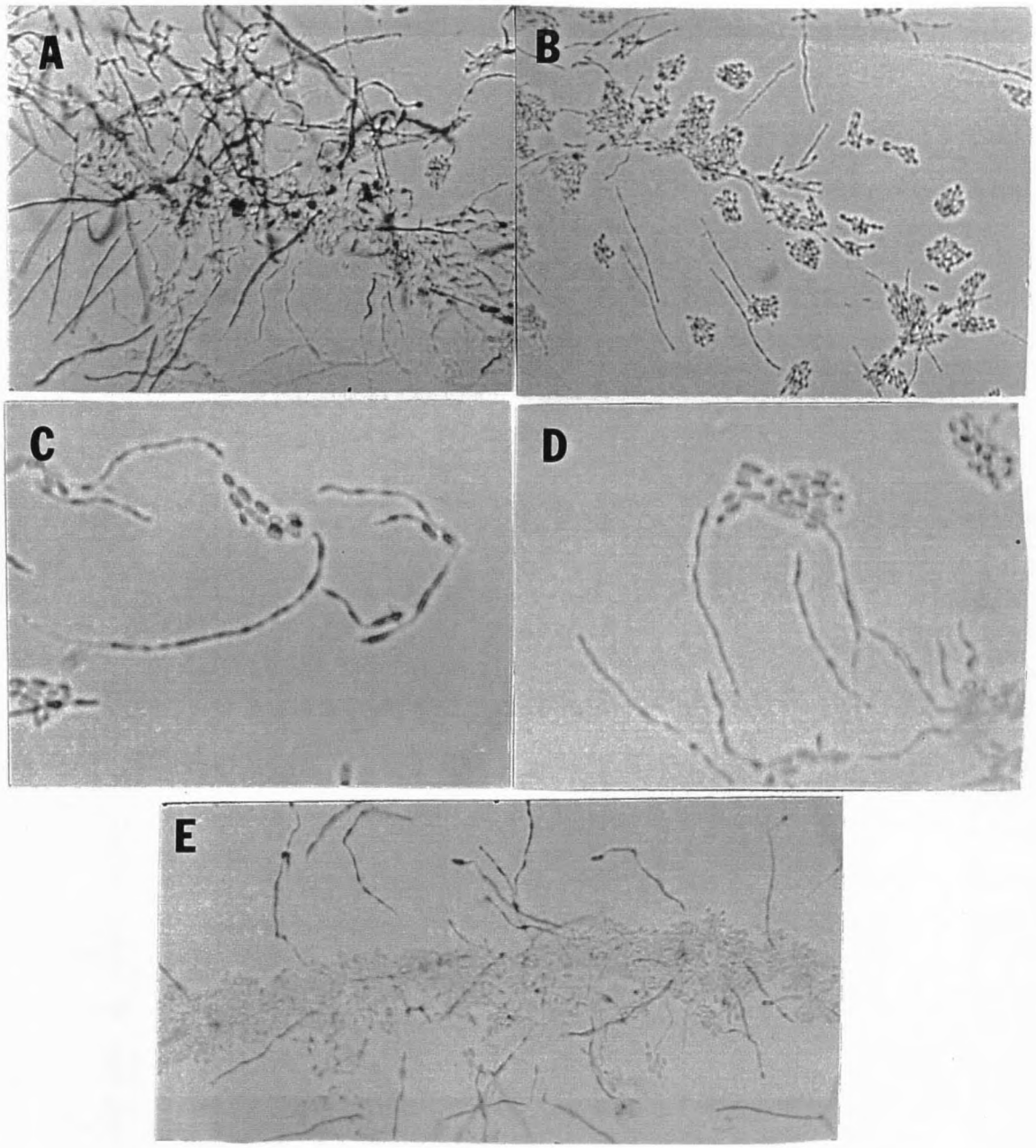

Figure 2-6. Mating reactions of *U. hordei* deletion mutants. A) Wild-type MAT-1 x MAT-2 cells; conjugation tubes are formed, fuse and result in aerial dikaryotic mycelia; B) 14Am- x MAT-2; no conjugation tubes were formed by either mating-type; C) 14Am-/mfa1 x MAT-2; conjugation tubes were formed by only one mating-type, presumably MAT-2; D) 14Am-/pra1 x MAT-2; conjugation tubes were formed by only one mating-type, presumably 14Am-/pra1; E) 14Am-/cos x MAT-2; conjugation tubes were formed by both mating types resulting in mating and the formation of aerial dikaryotic mycelia.

Expression of Mating Type Genes

Northern analysis of *Uhmfa1* and *Uhmfa2* was done in order to examine the relative levels of pheromone gene expression in either mating types alone, when grown together, in diploid strain W7 (Harrison and Sherwood, 1994), and in 14Am-, 14Am-/mfa1, 14Am-/pra1, and 14Am-/cos. In order to compensate for loading errors, each membrane was probed with a highly conserved, 680 bp region of the b-tubulin gene from *U. maydis*. The signal was quantitated using a phosphorimager to standardize the signal when the pheromone genes were used as probes (Table 2-1, Figure 2-7 amd Figure 2-8). These results verified that *Uhmfa1* was not expressed in MAT-2 (14a) cells, or the deletion transformants 14Am- and 14Am-/pra (Figure 2-7). Likewise, *Uhmfa2* was not expressed in MAT-1 (14A) cells (Figure 2-8). *Uhmfa1* appeared to be upregulated by about 5 times when MAT-1 cells were grown in liquid culture co-inoculated with MAT-2 cells, by nearly 20 times in the diploid strain, and by about 84 times in the transformant 14Am-/mfa1 (Figure 2-7). *Uhmfa2*, however, appeared to be downregulated by approximately 3 - 5 times in the diploid and when grown in mixed culture (Figure 2-8). Expression of *Uhpra1* and *Uhpra2* was inconclusive due to the inability to detect enough signal to accurately quantitate.

Table 2-1. Expression of pheromone genes, *Uhmfa1* and *Uhmfa2,* in different strains of *U. hordei.*

U. hordei strain	Probe	Relative *mfa* signal[1]	Relative *Tub* signal[2]
MAT-1	*Uhmfa1*	1.0	1.0
MAT-2	*Uhmfa1*	0	0.84
MAT-1 + MAT-2	*Uhmfa1*	16.1	3.1
W7	*Uhmfa1*	63.8	3.2
14Am-	*Uhmfa1*	0	0.75
14Am-/mfa	*Uhmfa1*	40.3	0.48
14Am-/pra	*Uhmfa1*	0	0.61
14Am-/cos	*Uhmfa1*	0.54	1.3
MAT-1	*Uhmfa2*	0	1.25
MAT-2	*Uhmfa2*	1.0	1
MAT-1 + MAT-2	*Uhmfa2*	0.77	4.4
W7	*Uhmfa2*	1.67	4.7

[1] Counts per minute (cpm) were obtained and compared to the cpm from MAT-1 when probed with *Uhmfa1* , or to the cpm from MAT-2 when probed with *Uhmfa2.*

[2] cpm were obtained and compared to the cpm from MAT-1 when correcting for *Uhmfa1* data or to the cpm from MAT-2 when correcting for Uhmfa2 data.

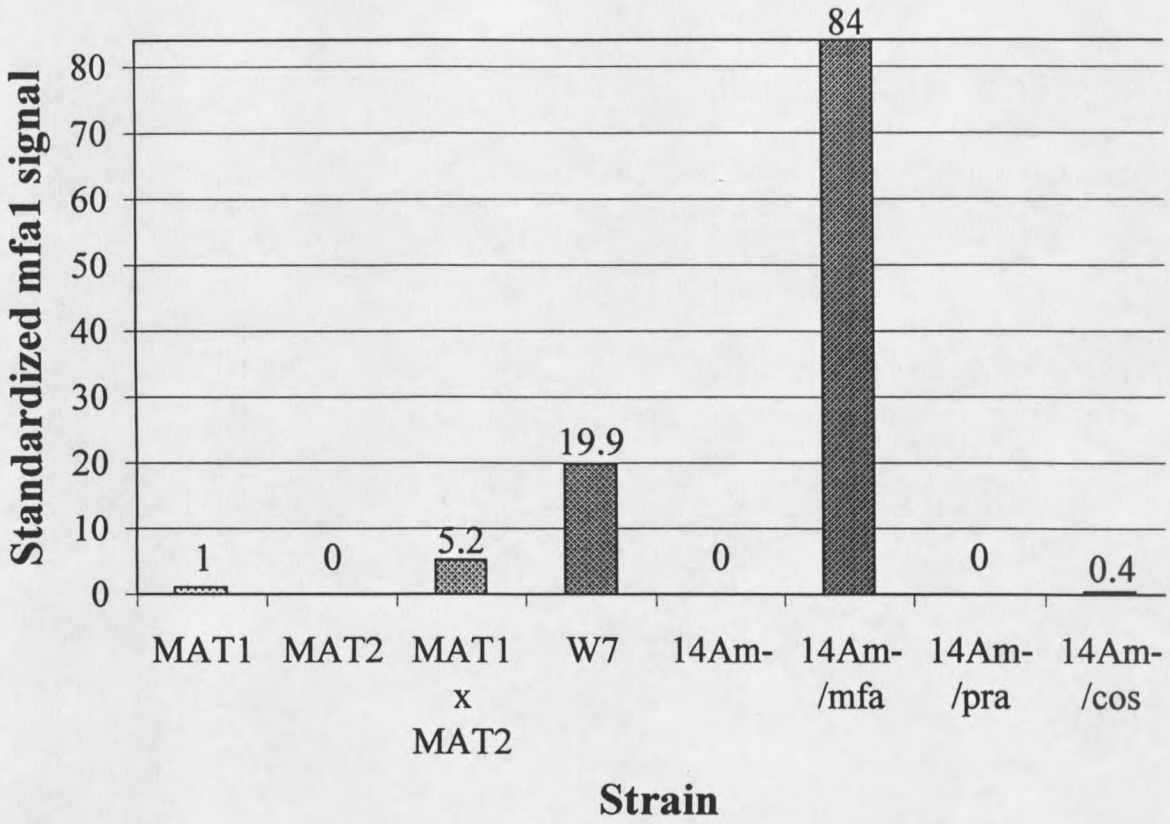

Figure 2-7. Expression of *Uhmfa1* in different strains of *U. hordei*. To correct for uneven loading of mRNA, the signal obtained by probing with *Uhmfa1* was standardized by dividing the relative *mfa* signal by the relative *Tub* signal (Table 2-1).

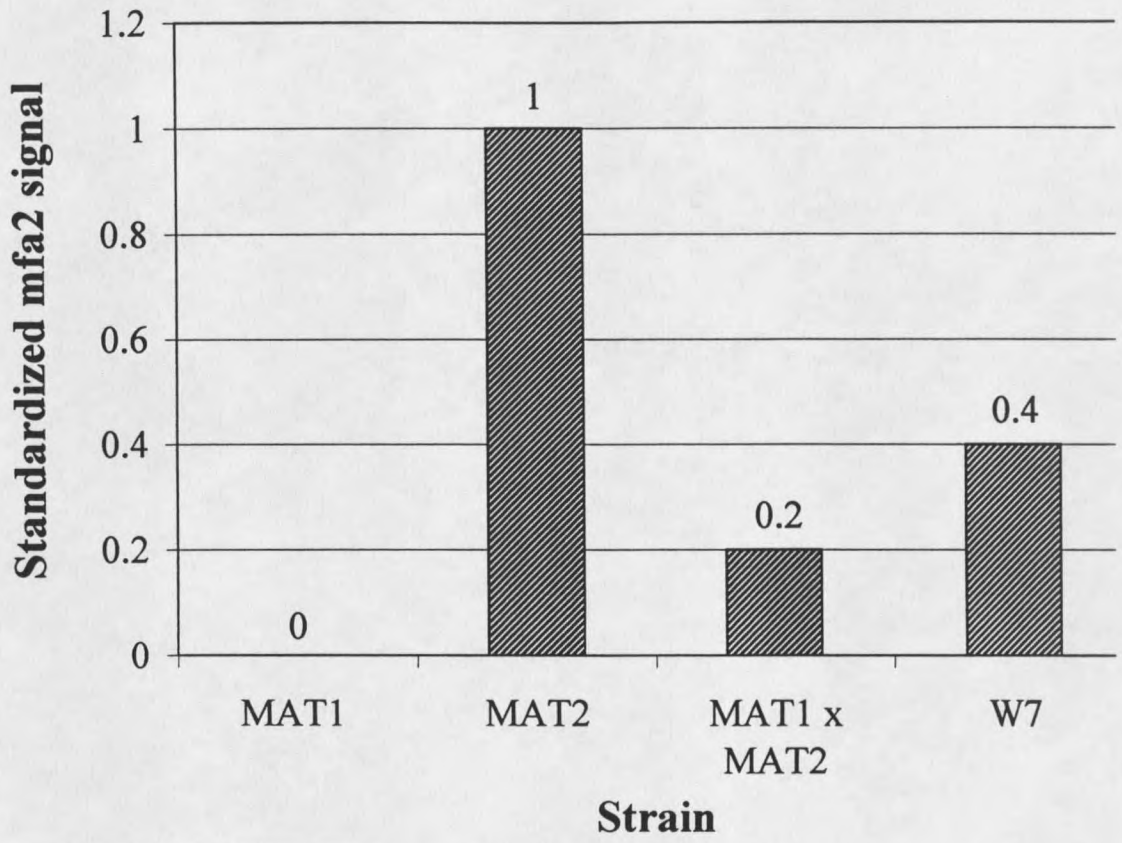

Figure 2-8. Expression of *Uhmfa2* in different strains of *U. hordei*. To correct for uneven loading of mRNA, the signal obtained by probing with *Uhmfa2* was standardized by dividing the relative *mfa* signal by the relative *Tub* signal (Table 2-1).

Discussion

The Structure of *U. hordei* Pheromone Gene *Uhmfa1*

Examination of the sequence of *Uhmfa1* revealed that it shares several structural features with *Uhmfa2* as well as with the genes that encode pheromones in other fungi. The presence of eight DNA elements, conforming to the consensus sequence ACAAAGGGA, in the promoter region of *Uhmfa1* is the first obvious similarity with the pheromone genes of *U. maydis*. In *Uhmfa1* these elements are located 331 bp upstream from the start codon. Another PRE sequence motif is found 190 bp downstream from the 3' splice site of the intron following *Uhmfa1*. Five such sequences also exist in the promoter region of *Uhmfa2* (unpublished results). This 9 bp consensus sequence is also present one time in the promoter of *Uhpra2* (Willits,1998), and a similar sequence is found within the second intron 40 bp upstream from the 3' splice site. In *U. maydis* it has been shown that these elements, known as Pheromone Response Elements (PRE), are present in the promoter regions of *Ummfa1*, *Umpra1*, and *Ummfa2*, within the coding region of *Umpra2* and *bW*, in the intron of *bW* and downstream of *bE*, and are necessary for the induction of pheromone-regulated genes, including upregulation of pheromone itself (Urban *et al*, 1996). Similar pheromone response elements are found in *S. cerevisiae* and *Schizosaccharomyces pombe*. In *S. cerevisiae*, the PRE consists of the 8 bp consensus sequence (A)TGAAACA (Kronstad *et al*, 1987;

Dolan *et al,* 1989) which is similar to, but in opposite orientation of, the PRE of *U. maydis*. The PRE of *S. pombe* consists of a 10 bp consensus sequence TTCTTTGTTY (Sugimoto *et al,* 1990). Each of these PREs have been shown to be recognized by transcriptional regulatory proteins, in *U. maydis* by Prf1 (Hartmann, *et al*, 1996), in *S. cerevisiae* by STE12 (Dolan, 1989) and in *Schizosaccharomyces pombe* by Ste11 (Aono *et al*, 1994). Interestingly, the genes that encode the pheromone of the basidiomycete fungus *Rhodosporidium toruloides* (*RHA*), have several repetitive sequences with the 8 bp consensus CTTTGTTY (Akada *et al*, 1989). The remarkable similarity of this sequence to the *S. pombe* PRE sequence makes it highly probable that it also serves as a pheromone-dependent regulatory sequence. The similarity of PREs among distantly related fungi, and their role in the regulation of pheromone induced genes may be indicative of evolutionary relatedness between classes of fungi that employ a pheromone mediated sexual response.

With respect to the pheromone genes of *U. maydis* and *U. hordei*, four different combinations of core promoter motifs are seen. *Ummfa1* contains the typical TATA and CAAT boxes common to the genes of higher eukaryotes. However, the *Ummfa2* gene contains neither a TATA nor a CAAT box, but instead contains a CT rich region that begins about 90 bases upstream of the start codon as seen in *Ummfa1* (Bolker, *et al*, 1992). *Uhmfa1* contains the typical TATA box and a CT rich motif in lieu of a CAAT box, and *Uhmfa2* contains all three motifs. The combinations found in *U. hordei* are somewhat

unusual for filamentous fungi since a CT rich motif is not usually present in promoters that contain the classical TATA box (Gurr *et al*, 1987). However, the importance of these motifs as core promoter elements in fungal genes has not been entirely determined. Their importance to the regulation of the pheromone genes in both *U. maydis* and in *U. hordei* will require functional study by mutational analysis. It is possible that these sequences are of little importance due to regulation by pheromone and the pheromone response elements (Urban *et al*, 1996). Conversely, these sequences may be important for basal level expression of the pheromone genes with the PREs responsible for pheromone regulation .

A final interesting feature common to both of the *U. hordei* pheromone genes is the presence of an intron located less than 40 bases downstream of the 3' end of the ORF rather than within the ORF. While it is unusual for introns to follow the coding region of eukaryotic genes, it appears to be common in several of the fungal pheromone genes characterized to date. Sequence data published for the *Ustilago maydis* pheromone genes *Ummfa1* and *Ummfa2* (Bolker *et al*, 1992) and for the pheromone genes of *Rhodosporidium toruloides*, *RHA1*, *RHA2* and *RHA3* (Akada *et al*, 1989) each describe introns following the coding regions. Examination of the sequence data for *Cryptococcus neoformans* (Moore and Edman, 1993) also reveals a probable intron of 131 bp with the 5' splice site beginning 26 bp after the ORF, the internal consensus sequence beginning 103 bp from the end of the 5'

splice site consensus, and the 3' splice site located 19 bp from the end of the internal consensus. The M-factor pheromone genes, *MFm1* and *MFm2*, of *Schizosaccharomyces pombe* also each contain an intron, but, rather than the introns beginning shortly past the ORF, the introns are located within the codon for the C-terminal cysteine residue. While this example seems to follow convention in that the introns actually occur within the coding regions, it is interesting to note that these short introns end only 13 bp from the 3' end of the stop codon. Intron position is generally conserved within corresponding genes of different organisms. It would appear that the pheromone genes of filamentous fungi are no exception. Perhaps as more fungal pheromone genes are characterized, their structure would provide clues as to the evolutionary origin of the pheromone mediated sexual response.

Pheromone Structure

Like the structure of the pheromone gene *Uhmfa1*, the *U. hordei* pheromone precursor, Uhmfa1, (Figure 2-4) shares several similarities with other fungal pheromone precursors. As seen in *S. cerevisiae, S. pombe, R. torruloides* and *U. maydis* (Brake, *et al,* 1985; Davey, 1992; Akada, *et al,* 1989; Bolker, 1993) the precursor peptide is considerably longer than the actual pheromone peptide with lengths from 36 to 62 amino acids. The ORF of *Uhmfa1* encodes a 42 amino acid precursor which is well within this range. The C-terminus of the peptide precursor contains a sequence motif, CAAX, which indicates that the mature pheromone might be post-translationally

modified by the addition of a prenyl group to the cysteine via thioether linkage and subsequent cleavage of the terminal three amino acids. Alanine, the C-terminal amino acid of the Uhmfa1 precursor molecule, most often signals prenylation with a farnesyl moiety (Clarke, 1992). It is also possible that the free carboxyl group of the cysteine becomes methylated (Casey, 1995). It has been shown that N-terminal proteolysis occurs after an asparagine (N) residue in the cases of the *S. cerevisiae* **a** factor (Marcus *et al,* 1990) and both **M** factor precursors of *S. pombe* (Davey, 1992). Based on mature length of known peptide pheromones and this cleavage point, it is surmised that cleavage might occur after the second N residue of the *Uhmfa1* precursor to yield a mature peptide pheromone of 13 amino acids (PGKSGSGLGYSTC(S-farnesyl)-OCH_3).

The above described post-translational modifications have been verified to occur in the *S. cerevisiae* **a**-factor, both of the *S. pombe* pheromones (Davey, 1992), the *R. toruloides* pheromone, rhodotorucine-A (Kamiya *et al,* 1979) and tremerogen A-10 pheromone of *Tremella mesenterica* (Sakagami *et al,* 1981). The role of the prenyl group on fungal lipopeptide pheromones is not entirely certain. One hypothesis proposes that the prenyl group is required for secretion. Since the lipopeptide pheromones lack a traditional signal sequence required for targeting to the endoplasmic reticulum for entry into the secretory pathway, it is possible that the farnesyl group is involved in a novel secretory pathway requiring interaction between the farnesyl moiety and a

membrane-bound transport protein. Evidence in support of this hypothesis is seen in the role that the farnesyl group of the *S. cerevisiae* **a**-factor plays in the STE6-dependent secretion of this pheromone. STE6 is a transport protein with similarities to the mammalian multidrug resistance protein (Mdr) involved in the ATP-dependent excretion from cells (McGrath and Varshavsky, 1989). It has been shown that prenylation is required for secretion of **a**-factor by STE6 (Caldwell *et al*, 1994). Likewise, there is evidence to suggest that a similar mechanism is involved in the secretion of rhodotorucine A and tremerogen A-10 (Tsuchiya *et al*, 1978).

Deletion of the MAT-1 locus

Deletion of the pheromone and pheromone receptor genes located in the *MAT-1* locus of MAT-1 mating-type cells resulted in complete loss of their ability to mate with MAT-2 cells. Subsequent replacement of the pheromone and pheromone receptor genes individually and then together by transformation with autonomously replicating vectors containing the necessary sequences, provided strong evidence that these genes are functionally active, and necessary for progress through the sexual cycle. An interesting observation made during long term microscopic observation of mating assays of crosses 14Am-/mfa1 x MAT-2 and 14Am-/pra1 x MAT-2 was the ability of a few responsive cells to fuse with completely nonresponsive cells. Such fusion resulted in dikaryon formation (data not shown). Although such fusions were extremely rare, their occurrence implies that only one set of

pheromone/receptor interactions is absolutely necessary for mating to occur. Further analysis of the resulting dikaryons of such fusion events could be done to determine viability and pathogenicity. Such analysis might provide clues to the roles played by pheromones and their receptors in pathogenicity.

Expression of Mating-Type Genes

Expression of *Uhmfa1* appeared to occur at a low constitutive level in MAT-1 cells. This basal level of expression increased by approximately five-fold when exposed to MAT-2 cells, and an increase of nearly twenty-fold was seen in the diploid strain. These results suggest that exposure of MAT-1 cells to Uhmfa2 stimulates an increase in the production of Uhmfa1. This might be necessary in order to ensure that MAT-2 cells are able to sense the presence of, and form conjugation tubes toward MAT-1 cells during the mating response. The increase in expression by eighty-five times in 14Am-/mfa1 was most likely due to the presence of several copies of the transforming plasmid in this strain. While it is not known how many copies of the plasmid are maintained in this *U. hordei* strain, it has been estimated that plasmids containing the *U. maydis* ARS may be maintained at approximately 25 copies per *U. maydis* cell (Tsukuda *et al*, 1988). Expression of *Uhmfa1* in strain 14Am-/cos was less than half that seen in the wild-type strain, 14A. The low level of *Uhmfa1* expression in this strain was, perhaps, the reason that this strain mated somewhat less efficiently than the wild-type.

Expression of *Uhmfa2* followed a pattern opposite that of *Uhmfa1*. *Uhmfa2* appeared to be downregulated in MAT-2 cells exposed to MAT-1 cells and expression levels in the diploid strain were decreased to 35% of that of MAT-2 alone. It is more difficult to explain a decrease in pheromone gene expression when exposed to cells of the opposite mating-type. Morphological changes of cells of opposite mating-types occurs in an asymmetric fashion, that is, conjugation tubes are formed by MAT-1 cells before they are seen in MAT-2 cells. It may be that expression of the pheromone genes and production of each of the pheromones occurs in such a way as to cause the cells to respond sequentially rather than simultaneously.

The analysis of mating functions in plant pathogenic fungi can offer a wealth of information on pathogenesis and avirulence. This is especially true in the smut fungi where sexual development takes place only within host tissues. In many of the smut diseases, the keys to the transition from nonpathogenic sporidia to pathogenic dikaryotic mycelia seem to lie in the earliest stages of the sexual cycle; pheromone production, pheromone reception and the subsequent morphogenesis to form the infectious cell type. Without the occurrence of these processes the pathogen is unable to infect the host. Careful study of the genetics and cellular biology of the *U. hordei* mating process will lead to a better understanding of pathogenicity, as well as cell-cell signaling, cellular differentiation of mating and other structures, gene

regulation, the role of sexual dimorphism in pathogenesis, and signal transduction pathways.

CHAPTER 3

MORPHOGENESIS OF *USTILAGO HORDEI* DURING THE EARLY STAGES OF THE SEXUAL CYCLE

Introduction

Ustilago hordei (Pers.) Lagerh. is a basidiomycete smut fungus that is obligately parasitic on barley (*Hordeum vulgare* L.). The life cycle of *U. hordei* begins as the teliospores germinate to produce a promycelium from which haploid, yeast-like sporidia (basidiospores) arise. Sexually compatible sporidia then fuse via a conjugation bridge and produce a dikaryotic mycelium. The dikaryotic mycelium penetrates the coleoptile of the germinating seedling and establishes itself in the meristematic tissue of the plant. At the time of flowering the dikaryotic mycelium ramifies throughout the ovarian tissue and forms masses of teliospores that replace the seed (Kozar, 1969). Pathogenesis in *U. hordei*, and in the plant pathogenic smut fungi as a whole, is absolutely dependent upon progression into the sexual cycle.

Mating in *U. hordei* is controlled by one locus, *MAT*, with two alleles, *MAT-1* and *MAT-2* (Thomas, 1988). The *MAT* locus is responsible for regulating cell-cell recognition, cell fusion, the morphological change from yeast-like to filamentous growth, and maintenance of the infectious dikaryon

(Bakkeren and Kronstad, 1996). Each of these alleles contain genes that encode the pheromones (*Uhmfa1* and *Uhmfa2*) (Chapter 2; Willits, 1998) and pheromone receptors (*Uhpra1* and *Uhpra2*) (Bakkeren and Kronstad, 1994; Willits, 1998) responsible for cell-cell signaling during the earliest stages of the mating process.

A preliminary description of the morphological changes that take place during mating has been described (Martinez-Espinoza *et al.* 1993). By using a simple assay in which cells of opposite mating type were coinoculated onto small squares of agar and observed microscopically, it was shown that the pheromones were diffusible, since cells of opposite mating type were able to form conjugation tubes and fuse even when located up to 10 cell lengths (100μm) from each other. The use of this method to observe mating, however did not allow for the distinction between MAT-1 and MAT-2 cells. In an attempt to solve this problem, the "sandwich method" was used in which cells of opposite mating type were placed on small agar squares separated by a piece of dialysis membrane (Martinez-Espinoza *et al.* 1993). While this method allowed for the distinction of mating types during mating, it did not allow for observation of the fusion process and dikaryon formation. It did, however, provide evidence that the pheromones were of small molecular weight and capable of diffusion away from the secreting cell. The limitations imposed by these methods prevented further detailed analysis of the morphogenesis that

occurs during the transition from a nonpathogenic haploid sporidium to an infectious dikaryotic mycelium.

This study reports the development of a mating assay, the split agar assay, that allows individual mating types to be distinguished and, at the same time, allows long term observation of the mating process from signaling to dikaryon formation. Using this assay it was possible to elucidate differences in the time it takes each mating type to respond to pheromone signaling and made it possible to determine the limits of the ability of *U. hordei* to mate over long distances. Additionally, diffusion rates of synthetic pheromones could be calculated with this assay. Synthetic pheromones were also used to assess cellular response time and sensitivity to pheromone.

Materials and Methods

Cultures

U. hordei strains 8A (*MAT-1;* ATCC # 90511) and 14a (*MAT-2;* ATCC # 90510) were used. Cultures were maintained on Holliday's complete medium (HCM) (Holliday, 1974).

Split Agar Assay

In order to make detailed observations of the morphological changes that occur in each mating type during the course of mating in *U. hordei,* the split agar assay was developed (Figure 3-1). TE agar (Martinez-Espinoza *et al.* 1993) squares (1 cm x 1 cm x 0.2 cm) were placed onto microscope slides,

cut in half and the halves separated. Two microliters of a 1 x 10^6 cell suspension of MAT-1 cells were placed near the lower edge of the upper half of the square while an equal amount of MAT-2 cells were placed on the upper edge of the other half. After the cell suspension droplets dried, the two halves were pushed together. The closest distance between the two mating types within this procedure was generally between 75 and 125 μm. The slides were placed in a moist chamber and incubated for up to 72 hours. Microscopic observations were made at various times over the course of the incubation period. To determine the maximum distance over which mating could occur, cell suspension droplets were inoculated at increasing distances from each other. The distance between mating cells was determined using an ocular micrometer after successful conjugation tube fusion and dikaryon formation.

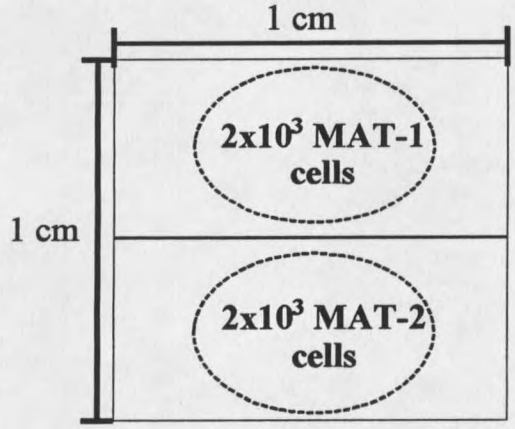

Figure 3-1. Diagram of the Split Agar Assay

Synthetic Pheromones

Based on *U. hordei* gene sequences of *Uhmfa1* and *Uhmfa2* (Chapter 2; Willits, 1998), predicted pheromone peptides were synthesized (Research Genetics, Inc., Huntsville, AL). Uhmfa1 was a 13mer with the amino acid sequence, PGKSGSGLGYSTC, while Uhmfa2 was a 9mer with the amino acid sequence GKGEPAPYC. The C-terminal cysteine of each was farnesylated ($Uhmfa1_f$ and $Uhmfa2_f$) (Hrycyna and Clarke, 1992) or farnesylated and methylated ($Uhmfa1_{fme}$ and $Uhmfa2_{fme}$) (Brenner and Huber, 1953). These synthetic pheromones were used to assess response time, diffusion rates and cellular sensitivity.

Diffusion Assay

In a variation of the split agar assay, thin TE agar was cut into 1cm x 1cm squares and placed onto microscope slides. A 0.2 cm strip was cut from the lower portion of the square and inoculated with 1 µl of synthetic pheromone (1 µg/µl in water). Two µl of a 1 x 10^6 cell/ml suspension of MAT-1 or MAT-2 cells were placed on the remaining portion of the square. After the pheromone solution and cell suspension droplets dried, the two agar pieces were carefully pushed together. To determine the amount of time it took for cells in direct contact with pheromone to begin conjugation tube formation, 1 µl of synthetic pheromone solution (125 pg/µl Uhmfa2, or 25ng/µl Uhmfa1) was placed on an agar square, dried, and inoculated with

2µl of a 1 x 10^6 cell/ml suspension of MAT-1 or MAT-2 cells (Figure 3-2). The agar squares were incubated for up to 72 hours on microscope slides in a moist chamber and observed microscopically at various times. The rate of diffusion was determined by the formula $d / (t-c)$, where d = the distance between the seam formed by the junction of the two portions of the cut agar square and the cells furthest from the seam that formed conjugation tubes, t = the time required to traverse the distance (d), and c = the amount of time for conjugation tubes to be formed by cells in direct contact with pheromone.

All experiments were conducted three times with three replicates per time interval. SAS statistical analysis software was used to calculate the means, standard deviation and standard errors of diffusion rates per time interval and to perform a t test to determine the significance of differences in diffusion rates over time. The same statistical tests were done to compare diffusion rates of the farnesylated vs the farnesyl methyl ester forms of each pheromone, as well as to compare the diffusion rates of Uhmfa1_{f} vs Uhmfa2_{f} and $\text{Uhmfa1}_{\text{fme}}$ vs $\text{Uhmfa2}_{\text{fme}}$.

Sensitivity Assay

Dilutions of synthetic pheromone solutions were added to agar squares, dried, and inoculated with 2µl of a 1 x 10^6 cell/ml suspension of opposite mating type. The ability of cells to form conjugation tubes in response to the pheromone dilution was determined.

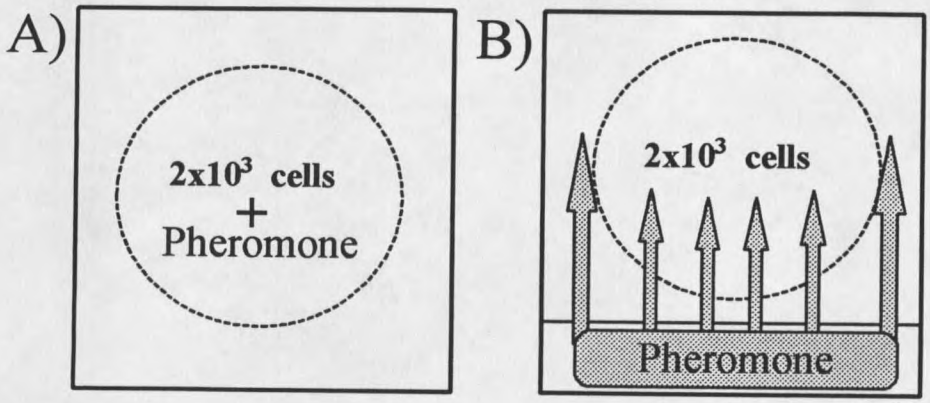

Figure 3-2. Diagram depicting the diffusion assay. Response time for cells to their respective pheromone was determined for each experiment. One microliter of synthetic pheromone solution (125 pg/µl Uhmfa2, or 25ng/µl Uhmfa1) was placed on an agar square, dried, and inoculated with 2µl of a 1 x 10^6 cell/ml suspension of MAT-1 or MAT-2 cells (A); A 0.2 cm strip was cut from the lower portion of the square and inoculated with 1 µl of synthetic pheromone (1 µg/µl in water). Two microliters of a 1 x 10^6 cell/ml suspension of MAT-1 or MAT-2 cells were placed on the remaining portion of the square. After the pheromone solution and cell suspension droplets dried, the two agar pieces were carefully pushed together (B).

Results

The Early Mating Response of *U. hordei* is Asymmetric

The split agar assay was used to observe the morphological changes that occur as a result of the cell-cell signaling events that trigger the mating response in *U. hordei*. The first notable event was the apparent arrest of cell division in MAT-1 cells that occurred between 10 and 16 hours after inoculation. This was immediately followed by the formation of a thin protrusion from one of the ends of the cell that, at first, appeared mycelial in nature. Given a few more hours the protrusion elongated and it become apparent that the structure was a conjugation tube and not a vegetative mycelium (Figure 3-3A). Conjugation tubes were distinguished from vegetative mycelia by being narrower, thinner walled, and curvilinear in growth habit. They were also obviously directional in their growth toward cells of the opposite mating type. Mycelia, on the other hand, appeared wider, thicker walled, were primarily straight in their growth habit, and grew in random directions with respect to opposite mating-type cells.

With further incubation some of the MAT-2 cells, which had continued to divide, arrested and began to form conjugation tubes 6 to 8 hrs later. Interestingly, it appeared that the formation of conjugation tubes by MAT-2 cells did not occur until MAT-1 conjugation tubes were within 75 μm of MAT-2 cells, or unless they were located across from areas of profuse MAT-1

conjugation tube production (Figure 3-3B). Conjugation tubes of both mating types grew toward the opposite mating type until tip-to-tip fusion occurred (Figure 3-3C). Before the fusion event occurred, conjugation tubes were often observed to be growing in a spiral fashion as the tips of the conjugation tubes coiled toward one another. Finally, at the point of fusion, an aerial dikaryotic mycelium emerged (Figure 3-3D). The above observations led to the conclusion that the mating process was asymmetric, that is, specialized mating structures were not formed simultaneously by both mating types, but underwent a distinct, mating-type specific, sequence of events.

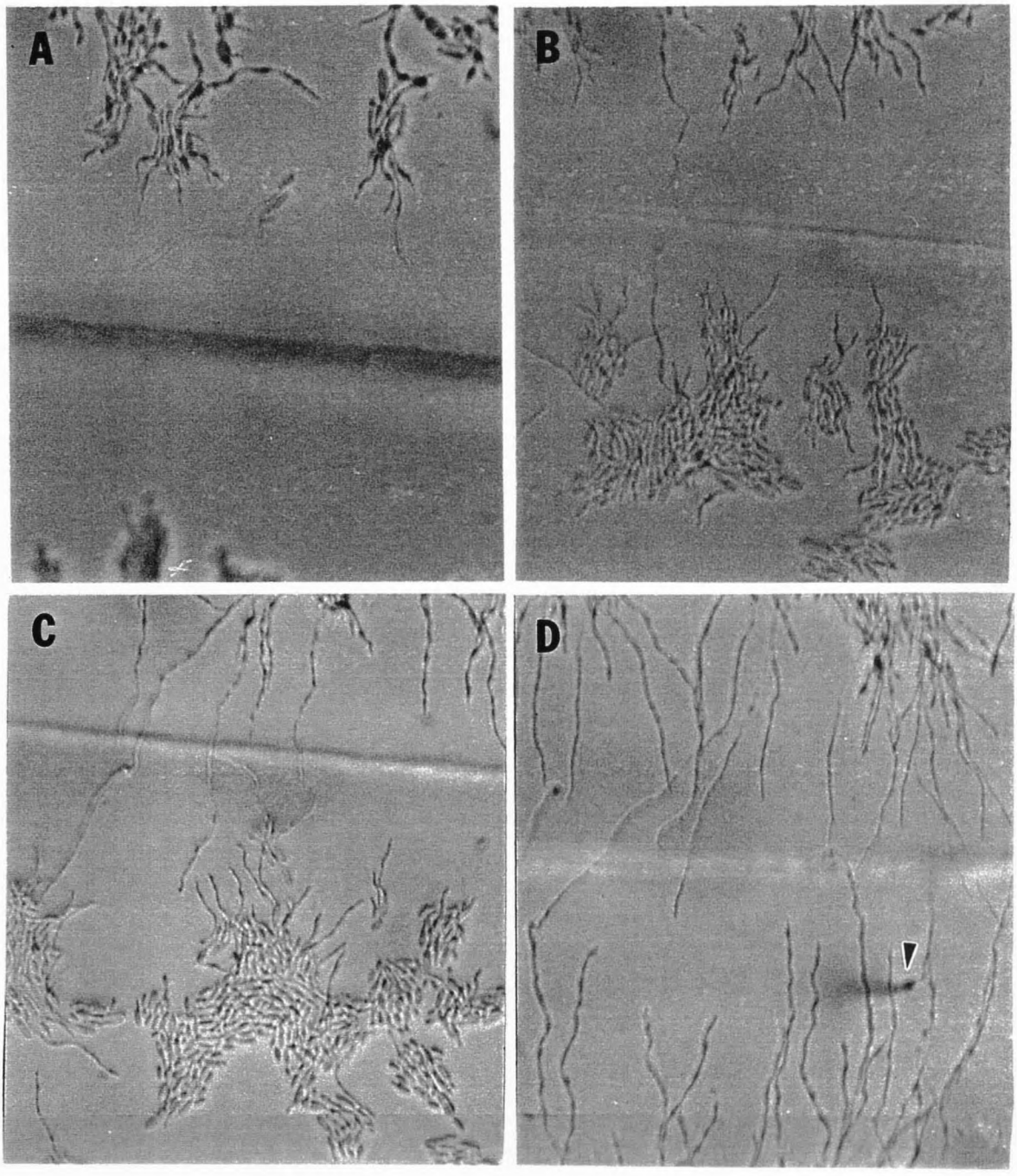

Figure 3-3. Asymmetry of the early mating response of *U. hordei*. A) 14 h after inoculation, MAT-1 cells begin conjugation tube formation while MAT-2 cells continue to divide. B) 18 h after inoculation, MAT-2 cells are now forming conjugation tubes. C) 20 h after inoculation, arrow indicates tip-to-tip fusion of conjugation tubes. D) 24 h after inoculation, dikaryotic mycelium (arrow) being formed at the point of conjugation tube fusion.

Use of the split agar assay also revealed that mating in *U. hordei* occurred readily between cells located much greater than 125 μm from each other. Therefore, the limits of this long-distance mating ability were tested. It was found that successful induction of conjugation tubes and their subsequent fusion occurred between cells separated by approximately 450 μm after 60 - 72 hours of incubation (Figure 3-4). Longer incubation periods resulted primarily in the formation of vegetative mycelia that was unable to mate during this period of observation.

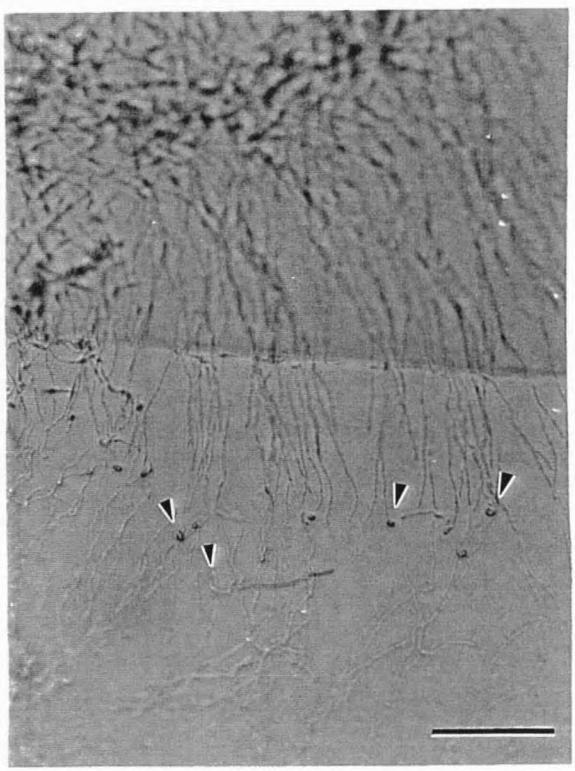

Figure 3-4. Long distance mating between cells located approximately 400 μm to 500 μm apart. Arrows indicates points of conjugation tube fusion and formation of dikaryotic mycelia. (Bar equals 100 μm)

Factors Influencing Asymmetry of the Mating Response in *U. hordei*

U. hordei is unique among the pheromone producing yeast and yeast-like fungi that have been studied in that mating can occur over distances greater than 400 μm (over 40 times a cell length). This led to the presumption that *U. hordei's* pheromones were readily diffusible. However, this did not explain the asymmetry of the mating response, but instead raised questions about differences in diffusibility of the pheromones and about the sensitivity of cells to pheromone produced by the opposite mating type. To determine whether or not the pheromones differed in diffusibility, the diffusion rates of synthetic peptides representative of the predicted mature pheromones were calculated. Initial experiments with the synthetic pheromones $Uhmfa1_{fme}$ and $Uhmfa2_{fme}$ revealed a lag time between inoculation of cells onto squares containing pheromone and conjugation tube formation (Figure 3-5). This lag time was very different for the two mating types; 3 to 6 hrs for MAT-1 cells to respond to $Uhmfa2_{fme}$ and 12 to 16 hours for MAT-2 cells to respond to $Uhmfa1_{fme}$. Similar results were seen with $Uhmfa1_f$ and $Uhmfa2_f$. Since the assay used to measure pheromone diffusion rates was based on the phenotypic change from normally dividing cells to cells producing conjugation tubes, this lag time was taken into consideration during each experiment by running controls in which the lag time for each mating type was determined.

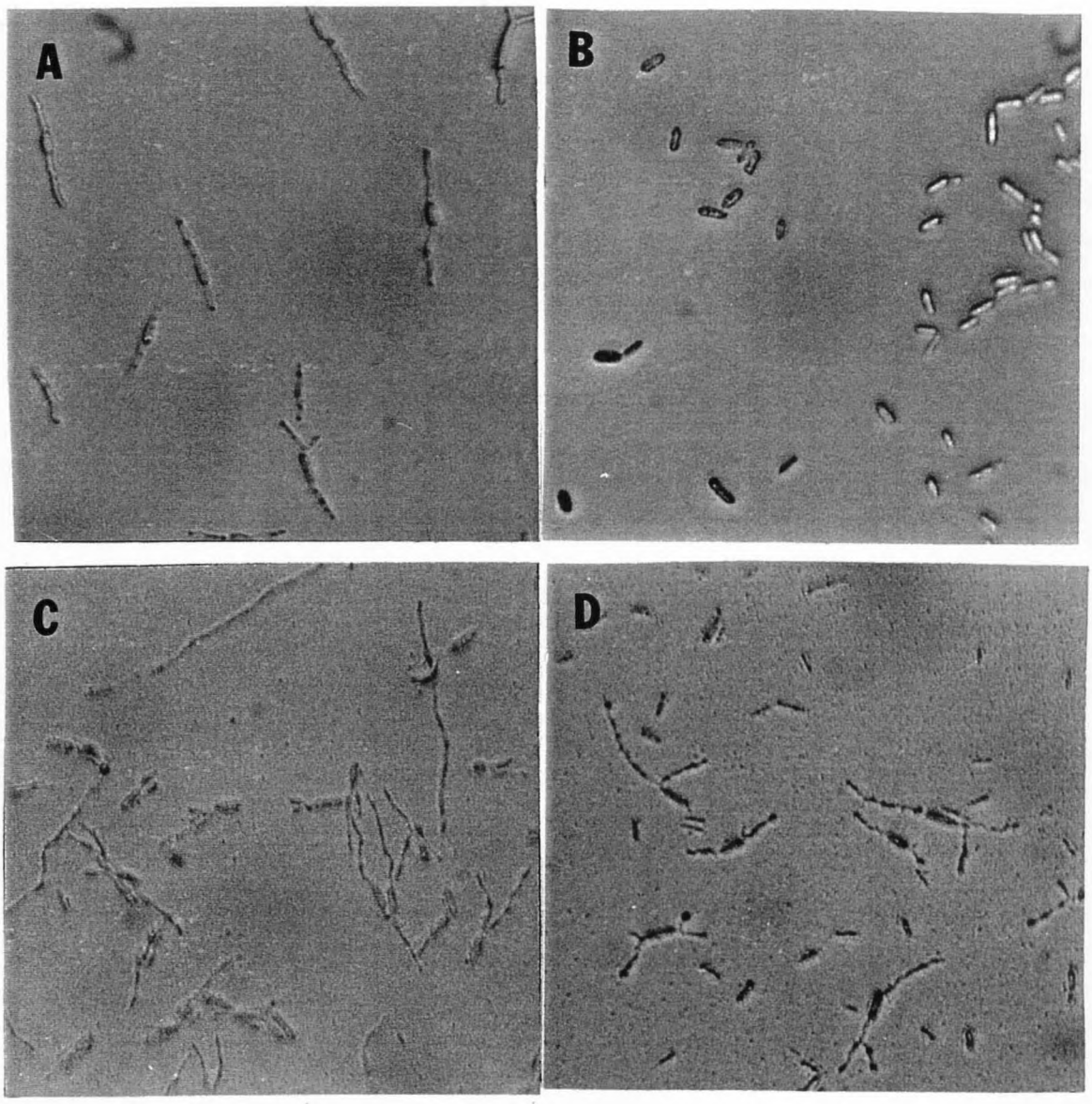

Figure 3-5. Timing of conjugation tube formation in response to direct application of synthetic pheromone. Synthetic pheromones Uhmfa1$_{fme}$ and Uhmfa2$_{fme}$ were inoculated onto TE agar squares with cells of the opposite mating type to determine the amount of time required for each to induce conjugation tubes. A) After 3 h Uhmfa2$_{fme}$ has induced conjugation tubes in MAT-1 cells, but B) no conjugation tubes were produced by MAT-2 cells in response to Uhmfa1$_{fme}$. C) After 12 h, MAT-1 cells responded vigorously to Uhmfa2$_{fme}$. D) After 12 h MAT-2 cells began to form conjugation tubes in response to Uhmfa1$_{fme}$.

Diffusion rates for both derivatives of each pheromone were determined at three time points to determine if the rates changed over time, as might occur if the peptides were unstable during the course of the experiments. There was no significant difference in the rates of diffusion over time for any of the pheromones tested. There were, however, significant differences between the diffusion rates for the farnesyl and farnesyl methyl-ester derivatives of both Uhmfa1 and Uhmfa2 (Figures 3-4 and 3-5). The farnesylated peptides diffused more slowly than their farnesyl methyl-ester counter parts. This was most likely due to differences in hydrophobicity between the two modifications.

Of primary interest was a comparison of the diffusion rates between the pheromones of the two mating types since a difference might account for the asymmetric mating response that was observed. Indeed, the diffusion rates of both derivatives of Uhmfa2 were found to be far greater than the rates for both derivatives of Uhmfa1 (Figure 3-8). At 189 and 203 μm/h for $Uhmfa2_f$ and $Uhmfa2_{fme}$, these diffusion rates were more than ten times greater than the rates of $Uhmfa1_f$ and $Uhmfa1_{fme}$ at 11.5 and 18 μm/h, respectively.

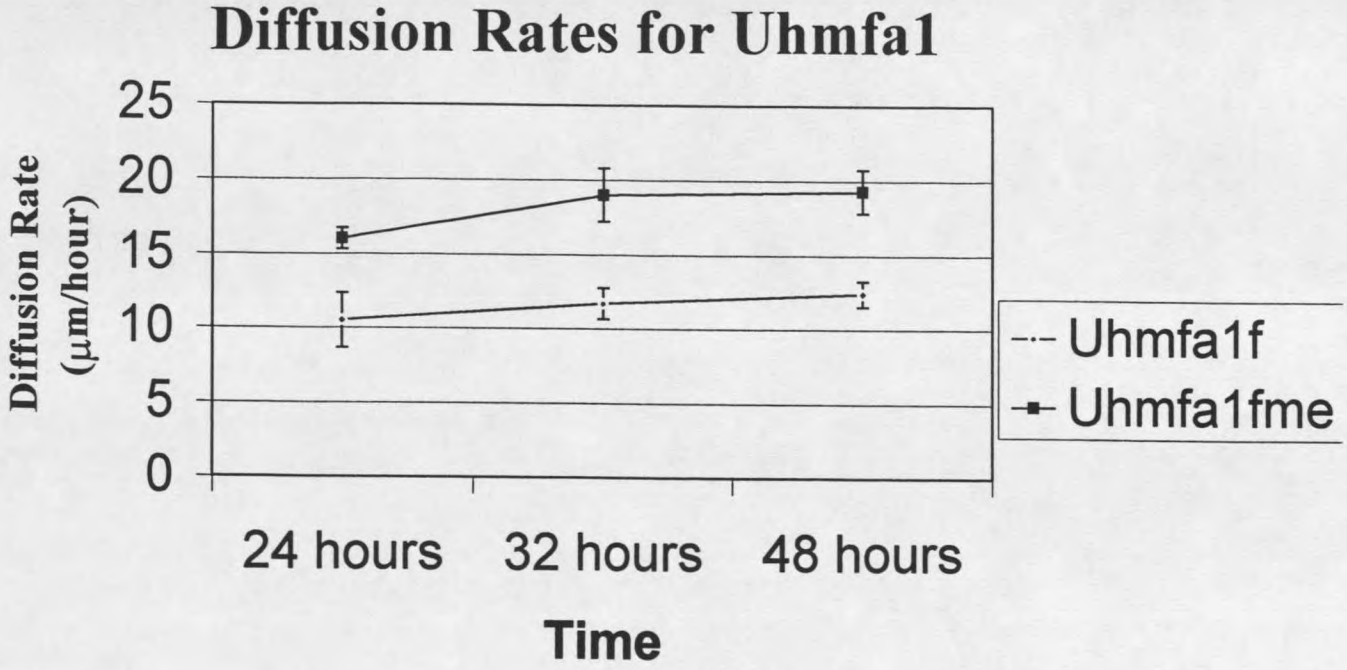

Figure 3-6. The diffusion rates for Uhmfa1$_{fme}$ and Uhmfa1$_{f}$ at 24 h, 32 h and 48 h. There was no significant difference in the rate of diffusion over time for Uhmfa1$_{fme}$ or Uhmfa1$_{f}$ at $P < 0.1$. ($P=0.19$ for Uhmfa1$_{fme}$ and $P=0.58$ for Uhmfa1$_{f}$). The error bars show one standard error above and below the mean. There was, however, a significant difference between the diffusion rates of the farnesyl and the farnesyl methyl ester derivatives at $P<0.05$. ($P=0.0055$).

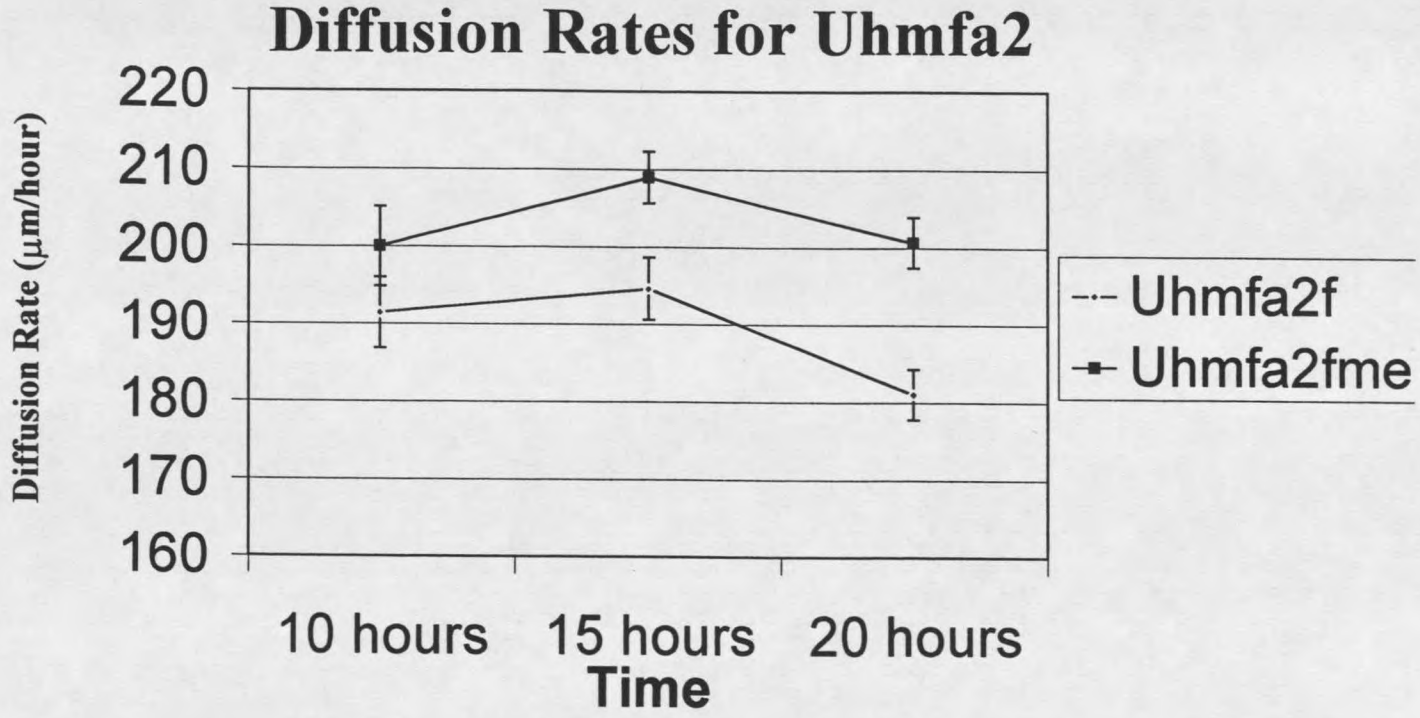

Figure 3-7. The diffusion rates for Uhmfa2$_{fme}$ and Uhmfa2$_{f}$ at 10 h, 15 h and 20 h. There was no significant difference in the rate of diffusion over time for Uhmfa2$_{fme}$ or Uhmfa2$_{f}$ at $P < 0.1$. ($P=0.39$ for Uhmfa2$_{fme}$ and $P=0.15$ for Uhmfa2$_{f}$). The error bars show one standard error above and below the mean. There was, however, a significant difference between the diffusion rates of the farnesyl and the farnesyl methyl ester derivatives at $P<0.05$. ($P=0.0471$).

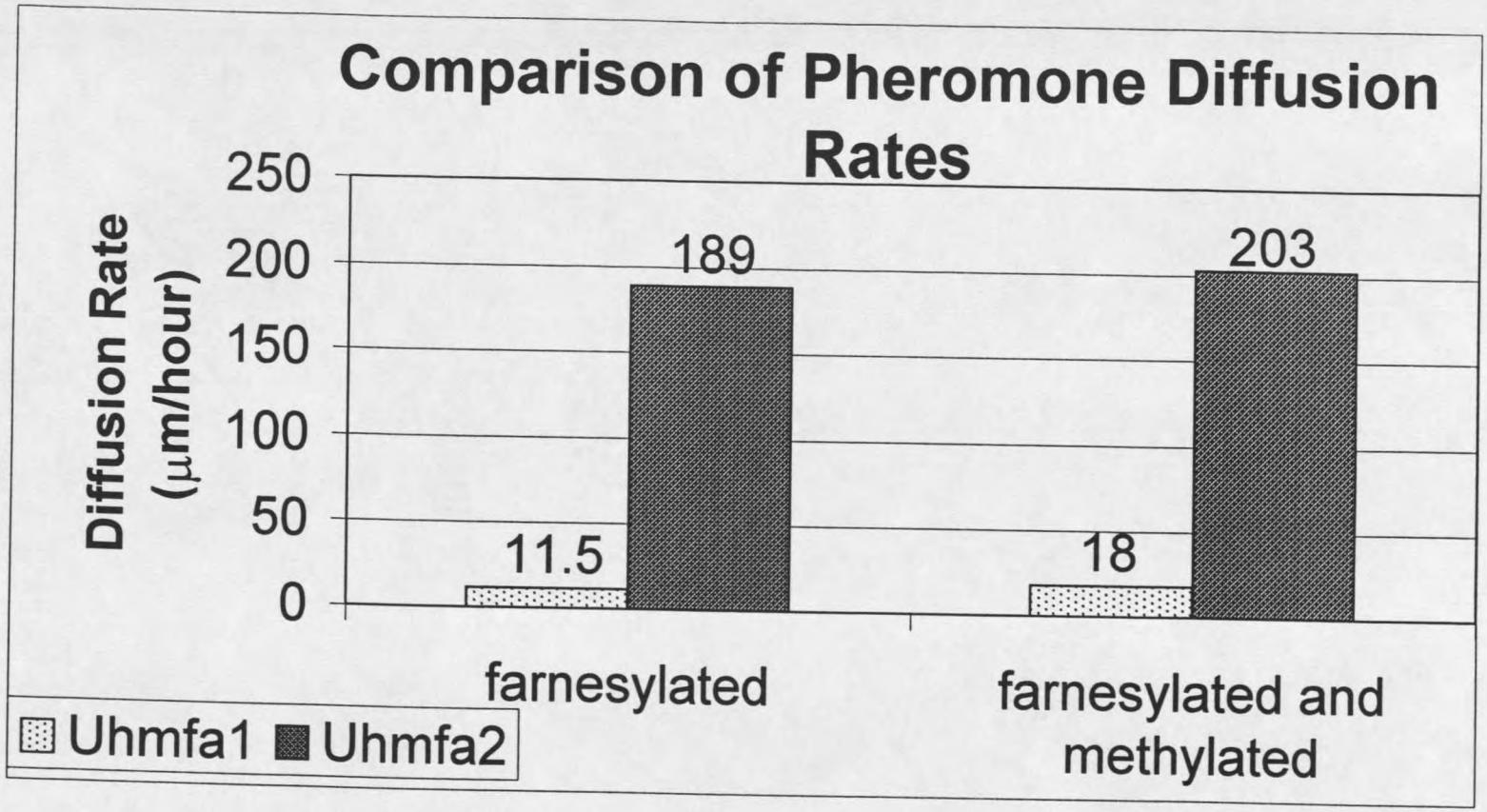

Figure 3-8. A comparison of the diffusion rates of $Uhmfa1_f$ vs $Uhmfa2_f$ and $Uhmfa1_{fme}$ vs $Uhmfa2_{fme}$.

Another factor that might play a role in causing the mating response of *U. hordei* to be asymmetric would be a difference in the sensitivity of each mating type to its conjugate pheromone. As seen in Figure 3-9, MAT-1 cells responded very well to less than 250 picograms of Uhmfa2$_{fme}$. This was significantly less than the 15.5 nanograms of Uhmfa1$_{fme\$16}$ required to induce conjugation tubes in merely a few MAT-2 cells (Figure 3-10).

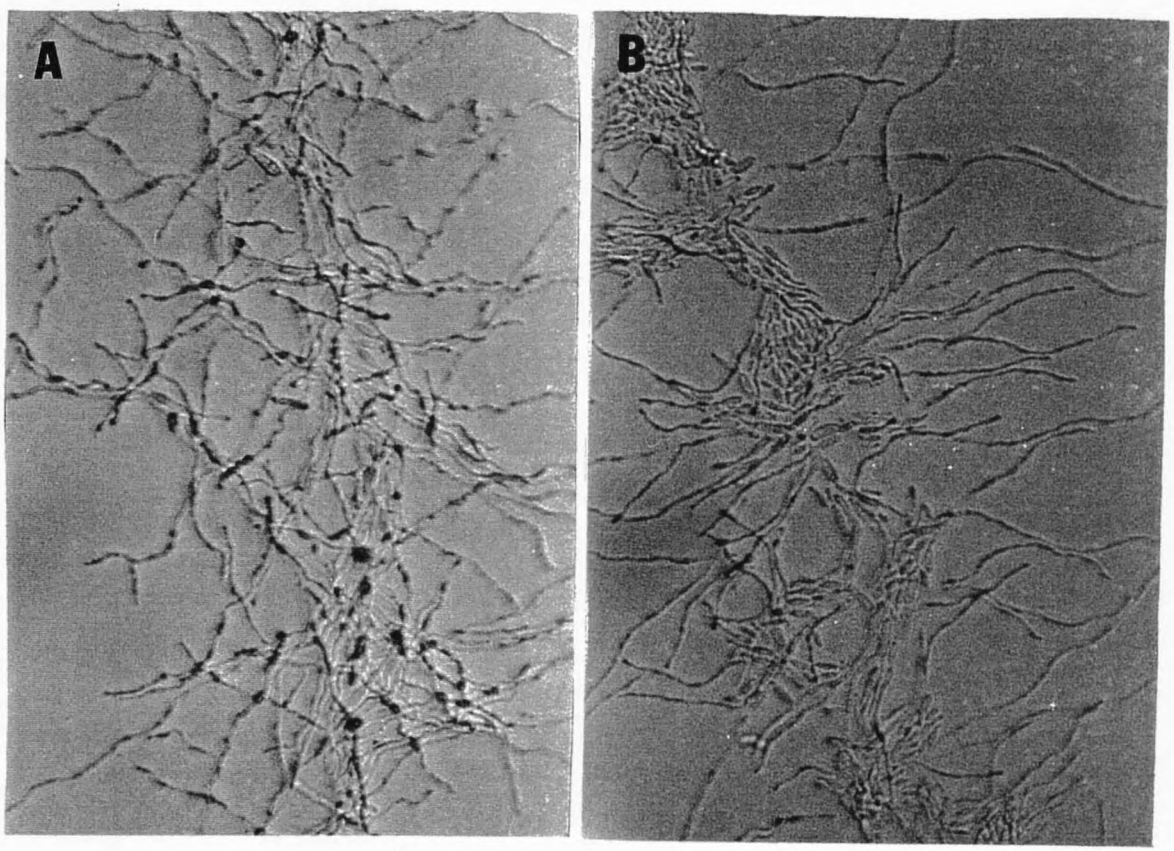

Figure 3-9. The sensitivity of MAT-1 cells to Uhmfa2$_{fme}$ was tested by applying 1µl of a ten-fold serial dilution of the pheromone to cells and assessing their ability to form conjugation tubes in response. A) Undiluted pheromone (1 µg) was able to induce conjugation tube formation in all cells. B) At a 1/4000 dilution (250 pg), many of the cells were still able to form conjugation tubes, while many were dividing normally.

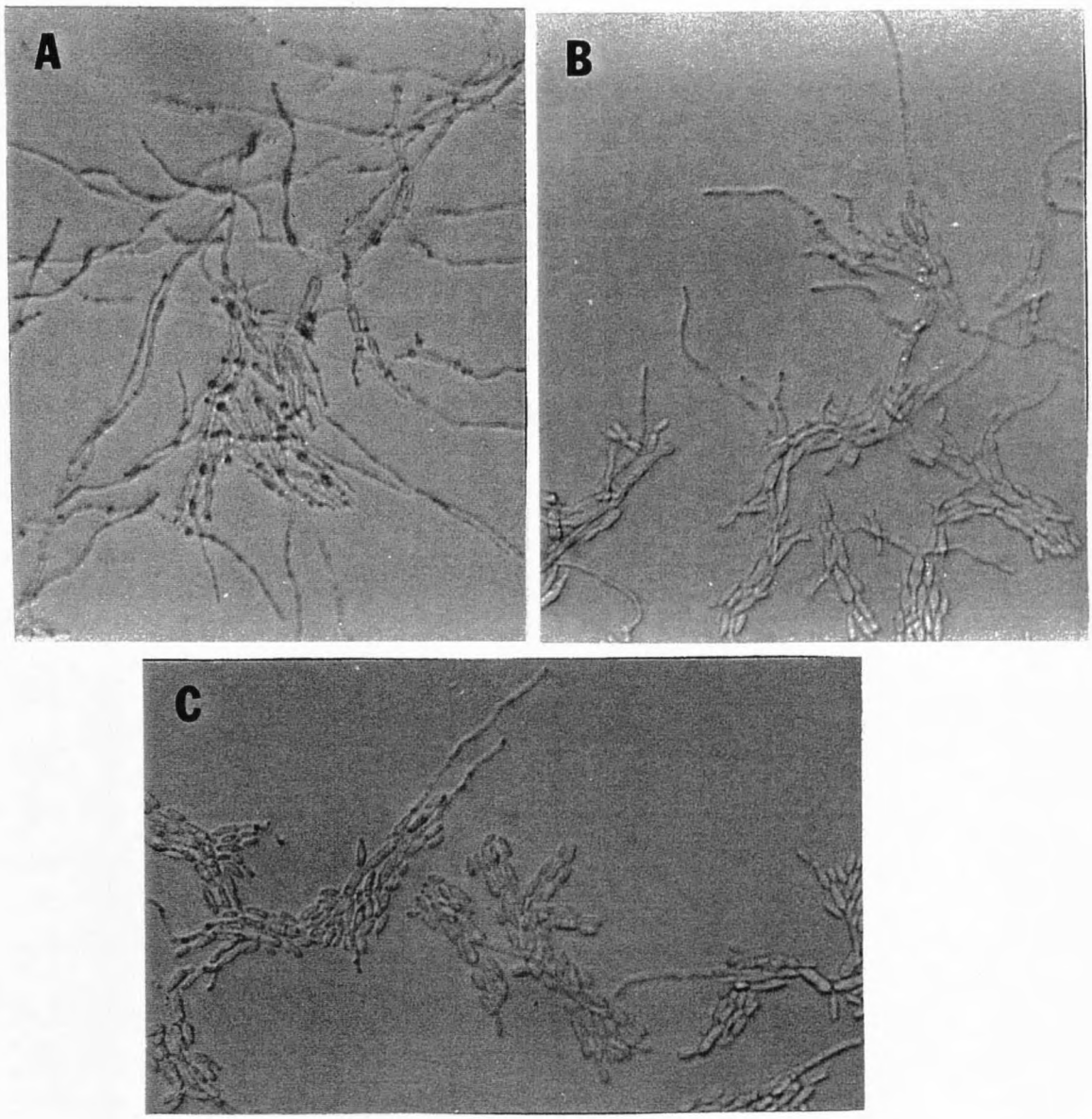

Figure 3-10. The sensitivity of MAT-2 cells to Uhmfa1$_{fme}$ was tested by applying 1µl of a two-fold serial dilution of the pheromone to cells and assessing their ability to form conjugation tubes in response. A) In response to undiluted pheromone, all cells formed conjugation tubes when exposed to 1 µg of Uhmfa1$_{fme}$; B) Many cells still formed conjugation tubes in response to 31 ng of Uhmfa1$_{fme}$, but they were shorter and several cells divided normally; C) Only a few cells formed conjugation tubes when 15.5 ng of Uhmfa1$_{fme}$ was added, and most divided normally.

Discussion

During the course of mating in *U. hordei* a number of events occur that result in a carefully orchestrated morphological transition in which nonpathogenic sporidia of opposite mating type fuse to form the infectious dikaryon. Careful, long term analysis of these events using the split agar assay revealed that the mating response was asymmetric, like that seen in *Rhodosporidium torruloides* (Abe *et al.* 1975). In an asymmetric response one mating type undergoes morphological changes first, followed by changes in the opposite mating type. This is unlike the symmetric response seen in *Saccharomyces cerevisiae* in which both mating types begin to form the characteristic schmoo at the same time (Jackson and Hartwell, 1990). Snetselaar *et al.* (1996) reported that *U. maydis* strains *a1* and *a2* also respond in a slightly asymmetric manner when placed 100 μm apart under paraffin oil droplets. They reported that *a2* cells produced conjugation tubes that elongated more quickly than those produced by *a1* cells, but that both mating types formed conjugation tubes of similar length within 5 hrs after inoculation, and fusion occurred by 10 hrs. While this might have been considered an asymmetric response, it is certainly not as drastic as that seen in *R. toruloides* and *U. hordei.*

It has been suggested that the reason for asymmetry in the mating response of *R. toruloides* is the constitutive production of rhodotorucin *A* by *A* cells. Upon reaching an ***a*** cell, rhodotorucin *A* induced conjugation tube

formation and the *de novo* production of a second pheromone, *a*-factor (Abe *et al.* 1975). Recent studies of the expression of the *U. hordei* pheromone genes, *Uhmfa1* and *Uhmfa2* (Chapter 1) demonstrated that both mating types express constitutive levels of pheromone. This suggests that *U. hordei* may differ from *R. toruloides* in the cause of mating asymmetry.

The asymmetry seen in the early mating response of *U. hordei* might be explained by differences in the sensitivity of the two mating types to their respective pheromones and by differences in the diffusibility of those pheromones. This study showed that MAT-1 cells, which are the first to respond, were able to respond to nearly 100 times less pheromone than MAT-2 cells. Furthermore, once MAT-1 cells were exposed to pheromone, conjugation tube formation was observed in only 3 to 6 hours, while it took 12 to 16 hours for MAT-2 cells to respond to pheromone. In addition, the fact that Uhmfa2 diffused over 10 times faster means that MAT-1 cells could, in theory, receive the mating signal much sooner than MAT-2 cells. The combination of increased sensitivity and faster response time in MAT-1 cells, and the possibility of "early reception" of Uhmfa2 by MAT-1 cells, provides strong evidence that these factors contribute to the asymmetry of the early mating response.

Perhaps the most surprising result that came from observing the mating process using the split agar assay was the ability of *U. hordei* to mate over extremely long distances with respect to cell size. Cells were able to form

conjugation tubes that grew directionally toward the opposite mating type, were able to fuse, and form aerial dikaryotic mycelia when located more than 400 μm apart (over 40 times a cell length). The mating process of most pheromone-producing fungi such as *S. cereviseae, R. torruloides,* and *U. maydis* requires that the cells be in fairly close proximity to one another (one to four cell lengths) in order for the fusion of specialized mating structures to occur. *In vitro* experiments to describe the mating process of *U. maydis* have been done in water droplets of small volume with the assumption that these conditions most closely mimic the high humidity and shallow liquid films found in the leaf whorls of corn where mating and infection occur naturally (Snetselaar and Mims, 1992; Snetselaar, 1993). In these experiments conjugation was observed only between cells in very close proximity to each other (one cell length apart or less) suggesting that the pheromones produced by *U. maydis* are not readily diffusible in an aqueous environment. However, experiments in which cells of opposite mating type were coinoculated onto agar solidified minimal media then covered with paraffin oil and a cover slip did result in mating at somewhat greater distances (approximately 100μm) (Snetselaar *et al.* 1996). It is likely that these conditions created an artificial environment where the hydrophobic pheromones could diffuse more easily. Since no other pheromone-producing fungus studied thus far has been reported to be capable of mating at distances greater than one to a few cell lengths, *U. hordei* appears to be unique in its ability to successfully mate at such long

distances. It is likely that this phenomenon is due both to the readily diffusible nature of the pheromones in an aqueous environment, especially Uhmfa2, and to the ability of *U. hordei's* conjugation tubes to grow extremely long and still maintain the ability to function as gametic structures.

It is difficult to speculate as to the reason *U. maydis* and *U. hordei* pheromones appear to behave so differently with respect to diffusibility, especially when they seem so similar chemically. Hydrophobicity plots (data not shown) reveal that Ummfa1 and Uhmfa1 have similar profiles as do Ummfa2 and Uhmfa2. They do, however, differ in their possible secondary structures (data not shown). It is possible that differences in the secondary structure of *U. maydis* and *U. hordei* pheromones are responsible for conferring a hydrophobic nature to the pheromones of *U. maydis* and a hydrophilic nature to those of *U. hordei*. This might explain why the pheromones differ in their apparent diffusibility.

If the described diffusibility of *U. hordei's* pheromones, and the ability to mate across long distances hold true in nature, then this may be a means by which out-crossing potential could be increased. The proximity of opposite mating-type sporidia to each other as they bud off the promycelium would tend to favor inbreeding, as seen in many species of the smut fungi including *Tilletia* spp. and *U. nuda,* unless a mechanism were present to discourage it. In the case of *U. hordei*, the sporidia of germinating teliospores do not necessarily fuse immediately, but are capable of dividing numerous times

before entering the sexual cycle (data not shown). In fact, mating between sporidia of different teliospores has been observed in *U. hordei,* at least *in vitro.* The production of a pheromone-related mating inhibitory factor (MIF) by *U. hordei* has been reported (Sherwood *et al.* 1998) and may be responsible for such a delay in mating between sister sporidia. A possible out crossing mechanism might include MIF, a readily diffusible set of pheromones, and the ability to form very long conjugation tubes.

Since mating is essential to pathogenicity in the smut fungi, each factor involved in the regulation of the mating response could be considered a pathogenicity factor and merits further study. It will be interesting to determine the factors involved in the increased sensitivity of MAT-1 to Uhmfa2. It may simply be that more receptors are available on the surface of MAT-1 cells to bind pheromone, thus causing amplification of the signal cascade at the earliest point. Or, it may be much more complicated with the factors involved further down the signal transduction pathway. As we gain insight to the intricate workings of mating in the smut fungi, we may find innovative ways to prevent the formation of the infectious cell type. This could lead to more effective, specific and environmentally innocuous means of controlling the diseases caused by this group of organisms.

CHAPTER 4

SUMMARY

Covered smut of barley, caused by the basidiomycete fungus *Ustilago hordei*, is an economically important disease affecting barley primarily in areas of the world where the use of protective and systemic seed treatments are not consistently used. Progression into the sexual cycle is essential for the conversion of the non-pathogenic, haploid sporidia into pathogenic, dikaryotic mycelia. Mating in *U. hordei* is under the control of one locus, *MAT*, with two alleles, *MAT-1* and *MAT-2*. Genes at this locus encode the cell-cell signaling components responsible for initiating the morphological changes required for formation of the infectious cell type. The subclone of a *MAT-1* genomic DNA fragment thought to contain a pheromone gene was sequenced. The sequence revealed the presence of the pheromone gene, *Uhmfa1*, with structural similarities to previously described fungal pheromone genes. *Uhmfa1* encoded a pheromone precursor of 42 amino acids. A CAAX motif, located at the carboxyl terminus of the precursor, was present and likely signals farnesylation of the mature pheromone.

In order to confirm the function of this pheromone gene and the previously descibed *MAT-1* pheromone receptor gene, *Uhpra1*, a deletion

vector was designed to knock out both of these genes simultaneously by double crossover homologous recombination upon transformation into MAT-1 cells. One transformant, 14Am-, was isolated that completely lacked the ability to mate. *Uhmfa1* and *Uhpra1* were transformed back into 14Am- separately and together. Observation of the mating response of the resulting transformants provided strong evidence that the genes were functionally active and necessary for progress through the sexual cycle.

Northern analysis was used to study the expression of the pheromone genes from both mating type alleles in haploid, mated and diploid cultures, as well as in deletion and replacement transformants of MAT-1 cells. Upregulation of *Uhmfa1* was seen in MAT-1 cells grown in the presence of MAT-2 cells, in the diploid strain, and in 14Am- transformed with *Uhmfa1*. Conversely, *Uhmfa2* appeared to be downregulated in MAT-2 cells grown with MAT-1 and in the diploid strain.

To increase understanding of the mating process, the split agar assay was developed that enabled detailed analysis of the morphological transition during the early stages of the mating process. It was revealed that the mating response in *U. hordei* is asymmetric and can occur at distances up to 400 μm. In assays where cells were separated by 75 μm to 125 μm, MAT-1 cells responded first by the formation of conjugation tubes that grew toward MAT-2 cells. MAT-2 cells began conjugation tube formation as MAT-1 conjugation

tubes came within 75 μm of them. Conjugation tubes elongated toward each other, fused tip-to-tip and gave rise to the dikaryotic cell-type.

It was hypothesized that asymmetry in the mating response of *U. hordei* could be explained by differences in sensitivity of the two mating types to their respective pheromones and by differences in the diffusibility of those pheromones. Synthetic pheromones representative of *Uhmfa1* and the *MAT-2* pheromone, *Uhmfa2*, were made in order to test this hypothesis. It was revealed that there was a significant lag between the response time of MAT-1 cells to pheromone compared to the response time of MAT-2 cells. Once MAT-1 cells were exposed to pheromone, conjugation tube formation began to take place in only three hours, whereas it took nearly 12 hours for MAT-2 cells to respond to pheromone. In addition, this study showed that MAT-1 cells were able to detect and respond to 250 pg of pheromone whereas MAT-2 cells needed between 15.5 ng and 31 ng, nearly a ten-fold difference in sensitivity. Furthermore, at approximately 200 μm/hr, Uhmfa2 diffused over 10 times faster than the approximately 15 μm/hr of Uhmfa1. These results provide strong evidence that differences in response time in addition to differences in cellular sensitivity to pheromone, and differences in pheromone diffusion rates contribute to the asymmetry of the mating response seen in *U. hordei*.

The apparent decrease of *Uhmfa2* expression and the apparent increase in expression of *Uhmfa1* may also play a role in asymmetry of the mating

response. An increase in the production of Uhmfa1 would allow the relative concentraion of that pheromone to increase in the area of the MAT-1 cell. This, in effect, would provide the less sensitive MAT-2 cells with a higher concentration of pheromone and increase the liklihood of pheromone binding to Uhpra2. The decrease in production of Uhmfa2 might simply be a way for MAT-2 cells to conserve energy, allowing for the production of conjugation tubes in response to MAT-1 cells. Since MAT-1 cells are much more sensitive to pheromone, a much lower concentration is required to direct growth of the conjugation tube toward the compatible MAT-2 cell. The following model depicts the role that each of the above factors may play in causing the mating response of *U. hordei* to be asymmetric (Figure 4-1).

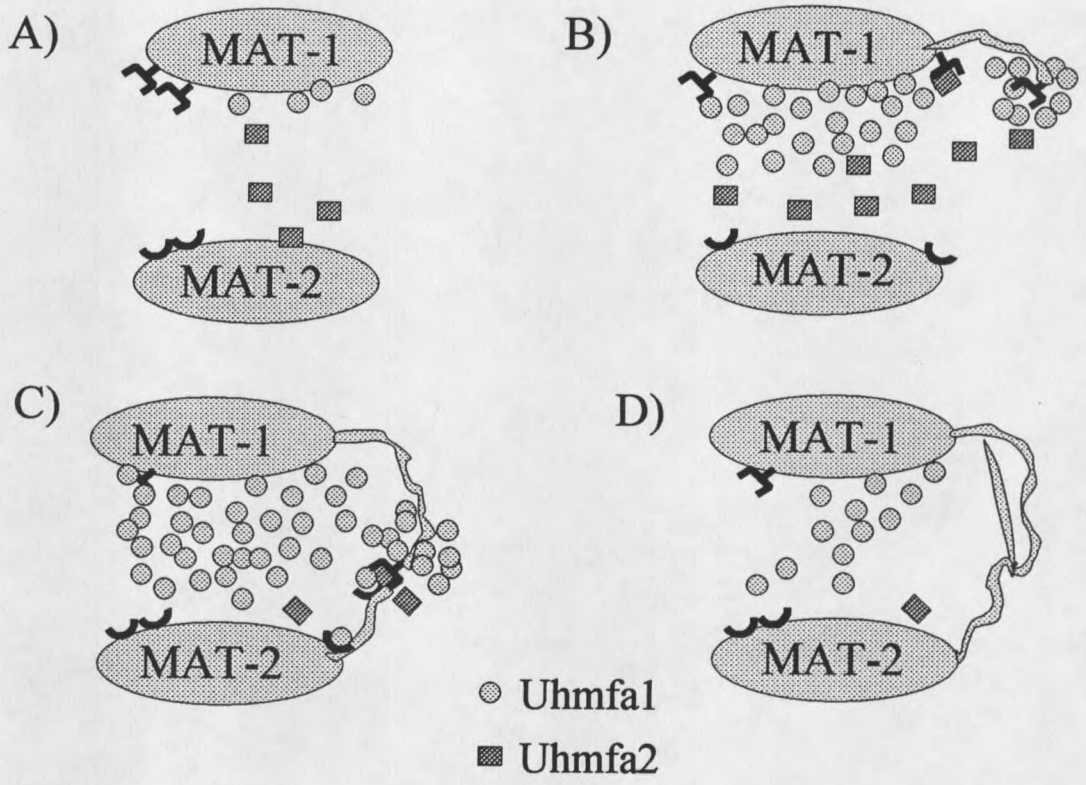

Figure 4.1. Asymmetric mating in *U. hordei*. Pheromones of each mating type are expressed constituitively. Uhmfa2 diffuses faster and is, therefore, likely to bind the MAT-1 pheromone receptor (Uhpra1) sooner (A). When Uhpra1 is bound, cell cycle arrest of the MAT-1 cell occurs followed immediately by the formation of a conjugation tube that grows toward the MAT-2 cell, and expression of *Uhmfa1* is upregulated (B). As the MAT-1 conjugation tube grows toward the MAT-2 cell, and as more Uhmfa1 is produced, the liklihood of Uhmfa1 binding to the MAT-2 pheromone receptor (Uhpra2) increases. When Uhpra2 is bound, the MAT-2 cell enters cell cycle arrest, forms a conjugation tube that grows toward the MAT-1 cell, and expression of *Uhmfa2* is decreased (C). The conjugation tubes grow toward one another until they fuse at the tips resulting in the formation of an aerial dikaryotic mycelium. Expression of Uhmfa1 remains upregulated in the dikaryon due to its role in maintenance of filamentous growth (D).

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