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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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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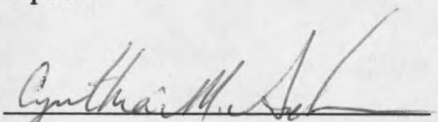
Signature 

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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.