Detection of Ustilago hordei in barley leaf tissue by polymerase chain reaction and analysis of the MAT-2 pheromone and pheromone receptor genes
by Deborah Ann Willits

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University

Abstract:
Ustilago hordei, the basidiomycete fungus that causes covered smut of barley, infects barley seedlings but disease symptoms are not visible until heading. The disease is routinely controlled by the application of protective or systemic fungicides to the seed. Resistance genes for U hordei have been described but breeding for resistance to covered smut is time consuming and difficult. We have sequenced the ribosomal internal transcribed spacer (ITS) regions of U hordei and developed a primer pair for polymerase chain reaction (PCR). These primers amplified a 574 bp fragment from DNA of Ustilago spp. but did not amplify DNA from barley or other common barley pathogens. DNA extracted from as few as four U hordei sporidia was detected by this method. U hordei DNA was amplified from leaf tissue at different stages of plant development of inoculated susceptible and resistant plants from a set of barley differential varieties. Detection of the fungus in different leaves of an individual plant was inconsistent. Several highly resistant varieties were shown to contain U hordei DNA in the first leaves but not in the later leaves. Thus, although the fungus can infect some resistant plants, the plants remain symptomless. Detection of U hordei in plant tissue prior to heading should assist efforts for breeding for resistance, aid in analysis of the host/pathogen interaction and allow for the determination of effective resistance genes.

The pathogenic stage of the fungus is controlled by the fusion of sporidia from opposite mating types. This fusion event is determined by the presence of a pheromone and pheromone receptor in each mating type, MAT-1 and MAT-2. We have cloned and sequenced the pheromone receptor, pral, and the pheromone, mfa2, from the MAT-2 locus and determined the length of the DNA region between these genes. Both genes were separately transformed into wild-type cells. MAT-1 cells transformed with pral or mfa2 were much more mycelial than wild-type sporidia and the mycelia was branched. This suggests that the MAT-1 transformed cells were forming conjugation tubes in response to the opposite mating factor now present in the cell. MAT-2 cells transformed with the genes did not differ phenotypically from wild-type. Knockout plasmids were constructed to attempt to disrupt the genes. A predicted active form of the pheromone was determined from the pheromone sequence and a peptide was synthesized. This peptide was carboxyl methyl esterified and famesylated at the cysteine residue before application to MAT-1 cells. Conjugation tube formation of MAT-1 sporidia was initiated by this synthetic peptide.

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Date 4/9/98

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ABSTRACT

Ustilago hordei, the basidiomycete fungus that causes covered smut of barley, infects barley seedlings but disease symptoms are not visible until heading. The disease is routinely controlled by the application of protective or systemic fungicides to the seed. Resistance genes for *U. hordei* have been described but breeding for resistance to covered smut is time consuming and difficult. We have sequenced the ribosomal internal transcribed spacer (ITS) regions of *U. hordei* and developed a primer pair for polymerase chain reaction (PCR). These primers amplified a 574 bp fragment from DNA of *Ustilago* spp. but did not amplify DNA from barley or other common barley pathogens. DNA extracted from as few as four *U. hordei* sporidia was detected by this method. *U. hordei* DNA was amplified from leaf tissue at different stages of plant development of inoculated susceptible and resistant plants from a set of barley differential varieties. Detection of the fungus in different leaves of an individual plant was inconsistent. Several highly resistant varieties were shown to contain *U. hordei* DNA in the first leaves but not in the later leaves. Thus, although the fungus can infect some resistant plants, the plants remain symptomless. Detection of *U. hordei* in plant tissue prior to heading should assist efforts for breeding for resistance, aid in analysis of the host/pathogen interaction and allow for the determination of effective resistance genes.

The pathogenic stage of the fungus is controlled by the fusion of sporidia from opposite mating types. This fusion event is determined by the presence of a pheromone and pheromone receptor in each mating type, MAT-1 and MAT-2. We have cloned and sequenced the pheromone receptor, *pra2*, and the pheromone, *mfa2*, from the MAT-2 locus and determined the length of the DNA region between these genes. Both genes were separately transformed into wild-type cells. MAT-1 cells transformed with *pra2* or *mfa2* were much more mycelial than wild-type sporidia and the mycelia was branched. This suggests that the MAT-1 transformed cells were forming conjugation tubes in response to the opposite mating factor now present in the cell. MAT-2 cells transformed with the genes did not differ phenotypically from wild-type. Knockout plasmids were constructed to attempt to disrupt the genes. A predicted active form of the pheromone was determined from the pheromone sequence and a peptide was synthesized. This peptide was carboxyl methyl esterified and farnesylated at the cysteine residue before application to MAT-1 cells. Conjugation tube formation of MAT-1 sporidia was initiated by this synthetic peptide.

LITERATURE REVIEW

Ustilago hordei

The Host Plant, Barley

The first barley plants were introduced into North America in the 1770's by Spanish missionaries (Tapke, 1937). Today, barley (*Hordeum vulgare* L.) is an important small grain cereal crop that is widely used for animal feed, malting and brewing, and food products. Barley is a reliable crop in temperate areas because of its drought and frost tolerance and ability to grow in alkaline soils. Barley cultivars can have heads that are two-rowed or six-rowed and the seed can be covered or naked (Mathre, 1997). The quality of the barley grain is especially important in the malting and brewing process.

Various bacterial, fungal and viral pathogens of barley can have a major effect on grain quality and yield. Two important fungal diseases are the rusts and smuts. Four smuts cause disease in barley including: *Ustilago hordei* (Pers.) Lagerh., covered smut; *U. nuda* (C. N. Jensen) Rostr., true loose smut; *U. nigra* Tapke, semiloose smut; and *Tilletia controversa* Kuhn, dwarf bunt (Mathre, 1997). Each of these diseases result in the formation of black spore masses in the head of the barley plant replacing the seed. The common name for the *Ustilago* spp. on barley is derived from the sturdiness of the membrane covering the spore mass. In barley covered smut the membrane containing the spores is not easily disrupted as it is in barley semiloose smut and loose smut (Thomas,

1988; Mathre, 1997). The different *Ustilago* spp. differ in their teliospore morphology but *U. hordei* and *U. nigra* can be crossed successfully (Thomas, 1988). Smut diseases not only reduce yield but also affect quality of the seed by contaminating seed lots with smut spores (Mathre, 1997).

Taxonomy

Ustilago hordei is a basidiomycete fungus that causes covered smut of barley (Fischer and Holton, 1957). The group, Ustilaginomycetes, is placed in the phylum, Basidiomycota, of the fungal kingdom. Mushrooms, boletes, puffballs, rusts, smuts and jelly fungi are all in this fungal phylum. The smuts are in the order, Ustilaginales, which is further broken down into two families, Ustilaginaceae and Tilletiaceae, based on the mode of teliospore germination. A main characterization of the smuts is the formation of a thick-walled, binucleate teliospore that undergoes karyogamy to become diploid. This teliospore is the overwintering structure and the place where meiosis occurs. The smuts are important plant pathogens with about 1,200 species that attack over 75 different families of the angiosperms (Alexopoulos et al., 1996).

Life Cycle of *Ustilago hordei*

The infection process of *U. hordei* begins with germination of diploid fungal teliospores on the seed or seedling to form a promycelium and the four products of meiosis referred to as basidiospores or sporidia. These haploid sporidia then reproduce by mitosis and budding and can be cultured on artificial media (Thomas, 1988). *Ustilago*

hordei is heterothallic with a bipolar mating system designated MAT-1 (**A**) and MAT-2 (**a**) (Martinez et al., 1993). Sporidia from opposite mating types form conjugation tubes that fuse to form the infectious dikaryotic mycelium (Fischer and Holton, 1957). This well-developed, septate, dikaryotic mycelium penetrates the coleoptile of barley seedlings and continues to grow intercellularly through the plant behind the growing point until the time of flowering. At this time, diploid teliospores form in place of the barley kernels (Mathre, 1997) (Fig. 1-1). Teliospores of aggressive races can also develop in the leaves and nodes of an infected plant (Gaudet and Kiesling, 1991). The teliospores are covered with a membrane that ruptures during harvest allowing their spread onto healthy seeds. The spores then act as an inoculum source for the germinating seed (Mathre, 1997).

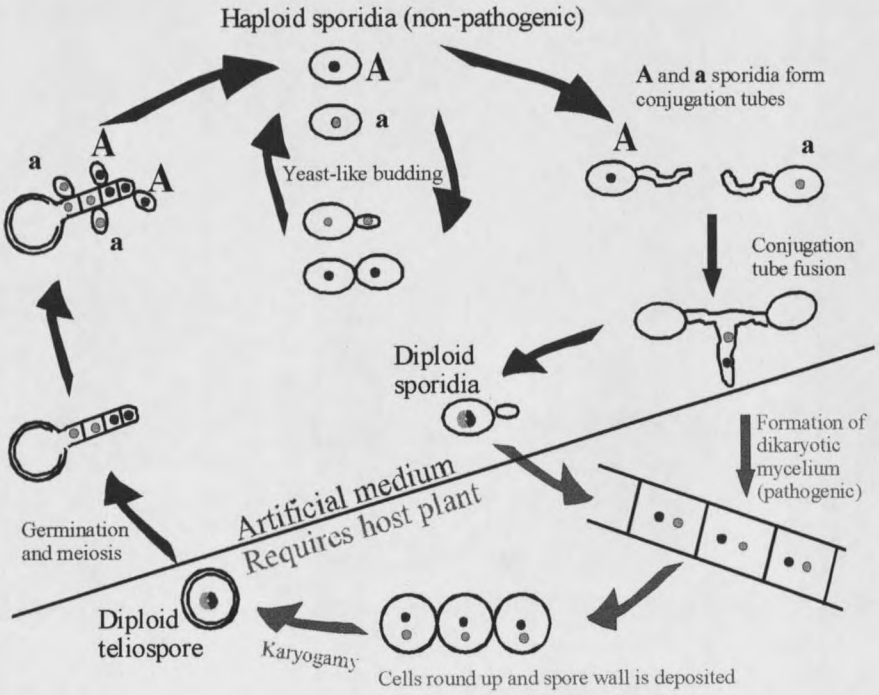

Fig. 1-1. Life Cycle of *Ustilago hordei*.

The yeast-like haploid stage can be manipulated for genetic studies. This sporidial stage can be easily maintained in the lab but the host plant is required for the continuation of the life cycle to form diploid teliospores. Dikaryotic mycelia will form in culture but they are short lived and dissociate into their haploid components (Dinoor and Person, 1969). Ordered tetrad analysis of the products of meiosis of the germinating teliospore is possible by micromanipulation and allows for the mapping of genes to the centromeres (Thomas, 1988).

One advantage of using *U. hordei* for genetic studies is that a transformation system has been developed (Holden et al., 1988). Transformation of fungi involves the formation of protoplasts by breaking down the fungal cell wall in the presence of an osmoticum to maintain cell stability. Calcium ions need to be present along with the protoplasts and transforming DNA for uptake of DNA into the cell to occur. In most systems transforming DNA needs to be integrated into the genome either homologously or randomly for successful transformations to occur. In some systems an autonomously replicating sequence (ARS) has been identified (Fincham, 1989). A 383 bp sequence from *U. maydis* allows autonomous replication of transforming DNA in *U. hordei* as well as *U. maydis*. This ARS functions as an origin of replication and allows extrachromosomal replication of transforming DNA. Thus, transformation frequencies may increase up to 1000-fold when the plasmid is carrying the ARS. About 25 copies of the ARS containing plasmids are maintained in transformed cells of *U. maydis*. One disadvantage of extrachromosomal elements is that they are mitotically unstable and therefore, are lost without constant selection pressure (Tsukuda et al., 1988).

Transformation experiments can be used to analyze gene function including the cloning, disruption and replacement of genes (Fincham, 1989). Transformations carried out with an ARS can be used to determine phenotypic differences due to the presence of a cloned gene.

The haploid sporidial stage of the fungus also allows for mutagenesis and recombination studies (Henry et al., 1988). Ultraviolet light and various chemical mutagens have been used to obtain auxotrophic mutants. Complementation of different auxotrophs of opposite mating type can be observed by the formation of the dikaryotic mycelia when the colonies are combined on a minimal media (Dinoor and Person, 1969). The addition of activated charcoal to the agar has facilitated the observation of dikaryotic mycelia. When this mycelia is present, the colony is distinctively 'fuzzy' on the charcoal containing medium (Martinez et al., 1992).

Disease Incidence and Control

Covered smut has worldwide distribution but can be effectively controlled by sowing resistant cultivars or by the application of systemic or protective fungicides to the seed (Mathre, 1997). Average loss due to covered smut was typically 4 to 5% in a survey in the Punjab but may be as high as 30% (Bedi and Singh, 1974). Similar results were found in two surveys of barley smut infection in Canada in the years 1978-1982 and 1983-1988. The typical loss due to *U. hordei* was 0.5% to 0.7% but some sites were found to have up to 40% infection and the cultivars widely sown at that time displayed varying degrees of susceptibility (Thomas 1984; 1989). Most of the barley growing

regions of the world plant cultivars that are susceptible to covered smut infection and therefore, use fungicides to control the disease (Mathre, 1997; Bedi and Singh, 1974; Thomas, 1984). The systemic oxathiin fungicides including Carboxin, sold as Vitavax®, are widely used as seed treatments to control smut diseases because of their safety and effectiveness even at low quantities (Bedi and Singh, 1974; Agrios, 1988; Thomas, 1991). Vitavax® has been shown to control covered smut completely when used as a seed dressing (Bedi and Singh, 1974; Johnston and Mathre, 1983; Mills, 1975). In regions that routinely plant untreated seed due to economics or unavailability of the chemicals, covered smut can cause economic loss. Economic loss is not only due to loss of yield but also due to contamination of healthy seed with the smut spores at the time of threshing (Mathre, 1997).

Cultivars resistant to *U. hordei* would be of great importance if the pathogen developed a tolerance to these fungicides. This may not be an immediate threat but mutants tolerant to four different fungicides, including carboxin, have been identified in the lab (Henry et al., 1987; Ben-Yephet et al., 1974). Constant fungicide use would create a situation that would select for mutants tolerant to the fungicide. In some mutants, tolerance to carboxin was shown to be polygenically controlled and therefore, may be hard to lose once it becomes established in a population (Ben-Yephet et al., 1975). In France a field example of carboxin resistance has been described for two strains of *U. nuda* on winter barley crops in 1986 (Leroux and Berthier, 1988). These findings indicate the potential for survival of carboxin tolerant strains.

Even though some resistance genes are known, breeding for resistance to covered smut is not routinely done because of the lengthy process involved and the effectiveness of present fungicides (Bedi and Singh, 1974; Mathre, 1997; Thomas, 1988). Plants need to grow two to three months until heading before symptoms of fungal infection become apparent (Groth and Person, 1978). Additionally, infection is inconsistent even in highly susceptible lines, such that a plant without an infected head may not necessarily be resistant. Thus, a population from the next generation has to be tested to see if the parent is truly resistant (Thomas, 1988). The genotype of a partially-smutted plant with both healthy and smutted heads cannot be classified until progeny are tested. These partially smutted plants may be displaying intermediate resistance (Kiesling and Peterson, 1972). If resistant cultivars with good agronomic qualities were available it would benefit regions that plant untreated seed and provide an alternative to fungicides.

Differential Varieties and Resistance Genes

In the gene-for-gene concept as described by Flor (1971), there exists a distinct relationship between resistance genes in the plant and avirulence genes in the fungus. This concept has led to an elicitor-receptor model for the interaction between resistance and avirulence gene products. The resistance gene in the host codes for a receptor that recognizes an elicitor in the pathogen which is the product of the avirulence gene. This elicitor binds to the plant receptor and activates a signal transduction cascade, thereby initiating host defense responses and making the plant resistant to the pathogen. Avirulence genes and resistance genes have now been cloned and sequenced and their

structure supports this hypothesis (De Wit, 1995). Both the resistance gene and corresponding avirulence gene need to be present for the plant to be resistant. This type of resistance is referred to as vertical resistance. Resistance in the host is usually dominant while virulence in the pathogen is usually recessive (Flor, 1971). Each smut race possesses several loci responsible for virulence and avirulence. If virulence is recessive, the three genotypes possible are *vv* which is virulent on the host and *vV* and *VV* which are avirulent (Cherewick, 1958).

The barley differential set described by Tapke (1945) has been used for studying the resistance/avirulence host/pathogen relationship but clear cut resistance genes have not been identified for all of the differential lines. The barley differential set consists of eight varieties that vary in their susceptibility to the different races. Odessa (C.I. 934) is considered universally susceptible to covered smut because no resistance genes have been identified (Tapke, 1945). The differential varieties have not been improved agronomically and are not cultivated varieties. The phenotypes of the barley differential set vary in the type of seed and the growth of the plant. Hannchen (C.I. 531) is the only two-rowed variety in the set. Excelsior (C.I. 1248), Himalaya (C.I. 1312) and Nepal (C.I. 595) have naked seeds. Nepal has a hooded head and Lion (C.I. 923) has black seed. Trebi (C.I. 936) is more agronomically desirable than some of the other differentials. Out of the differential varieties Pannier (C.I. 1330) may be the best choice for use in breeding for resistance to covered smut because it has total resistance to seven of the races and has better agronomic traits (Tapke, 1945). These differences in physical characteristics of the differential set help to distinguish possible seed contaminates.

Tapke (1945) describes which races are virulent on each cultivar except race 14 which is virulent on all of the varieties in the differential set (Pedersen and Kiesling, 1979).

In *Ustilago hordei* two genes have been identified that display the gene-for-gene interaction with virulence being controlled by a single genetic locus. The virulence genes designated *Uh v*-1 (v_1) and *Uh v*-2 (v_2) were effective on Hannchen and Excelsior, respectively (Sidhu and Person, 1971). The corresponding dominant resistance genes are designated R1 for Hannchen and R2 for Excelsior (Sidhu and Person, 1972). Therefore, a pathogen homozygous for v_2 will be virulent on Excelsior but the heterozygous and homozygous dominant genotypes will be recognized by the dominant resistance gene in Excelsior and the host/parasite interaction will be of resistance/avirulence. Two reports have confirmed that virulence on Hannchen was recessive (Fullerton and Nielsen, 1974; Sidhu and Person, 1971) while another found that it was dominant (Cherewick, 1967). To further complicate the issue, it has been found that environmental conditions and the genetic background of the host affect the interactions involving the R2 gene from Excelsior and the heterozygous genotype, V_2v_2, from *U. hordei* (Ebba and Person, 1975).

Studies done on the resistance of the other differentials have not been as straight forward. Virulence on Himalaya was found to be governed by two duplicate recessive genes, v_4 and v_5, at either of two genetic loci (Ebba and Person, 1975). Thomas (1976) had conflicting results that suggest that virulence in Himalaya was determined by a combination of v_1 and v_2. Virulence on Nepal and Pannier seemed to be regulated by a recessive gene, v_3, which is linked to v_2. Three dominant genes were found in Pannier that govern resistance to race 6 of *U. hordei* and one of these genes was found to be

temperature sensitive or incompletely dominant (Kiesling, 1970). Pannier has four independent genes governing resistance to Race 8 (Kiesling, 1971). Virulence on Lion was designated v_6 because it was shown to be different from virulence genes identified thus far (Thomas, 1976). Virulence on Trebi appeared to be dominant in initial studies (Person et al., 1987) but was later found to be recessive (Christ and Person, 1987). The unnamed major allele conferring virulence on Trebi was found to be modified by other polygenes that enhance or depress the major gene effects (Person et al., 1987). The conflicting results of these virulence studies may partly be explained by differences in environmental conditions, seed sources, or strains of the fungus (Thomas, 1988).

Physiologic Races of the Pathogen

Fourteen physiologic races of *U. hordei* have been described (Tapke, 1945; Pedersen and Kiesling, 1979). These races have been identified by their reaction on the barley differential set (Tapke, 1945) except Race 14 which was generated during an inbreeding study of Race 8 (Pedersen and Kiesling, 1979). A karyotype analysis was done for strains from the 14 races using contour-clamped homogeneous electric field (CHEF) pulsed-field gel electrophoresis (PFGE). This method showed the variability in number and size of chromosome length DNA fragments between the different races (McCluskey and Mills, 1990). The races also differ in their aggressiveness. The aggressiveness of the race describes the severity of disease reactions. In some cases this has been defined as the percent infection of inoculated Odessa plants. The aggressiveness components include the extent of smutting in the head, flag leaf and flag

node, the compaction of the peduncle and plant dwarfing. Races 2, 3, 4, and 12 are the most aggressive and 7, 10 and 14 are the least aggressive when all the aggressiveness components are considered for Odessa barley. More aggressive races are capable of producing sori in the leaf tissue (Gaudet and Kiesling, 1991). Therefore, constructing a method to detect the fungus in leaf tissue should be possible and may be more effective for certain races.

The variability of the pathogen in the field creates a problem when assigning a race classification. Since each infective generation of the fungus has gone through a sexual phase, a race will only be stable if it is homozygous. In an experiment to determine natural race variability in Canada, 353 field isolates were analyzed. Only two stable races, 6 and 7, could be identified in this collection. The rest of the isolates were a mixture of two or more races or heterozygous strains that could not be separated into stable entities. Barley cultivars that were resistant to a race may no longer be resistant due to variability of recessive virulence genes (Cherewick, 1958).

PCR Detection of Fungi

Polymerase chain reaction (PCR) is a simple, rapid, and sensitive method to amplify specific DNA fragments and has been used for numerous applications including evolutionary studies, environmental screening and forensics (Foster et al., 1997). Because of the sensitivity of PCR, negative controls should always be included to detect DNA contamination of reagents. PCR has been used to detect a number of fungal

pathogens in plant tissue and many of these examples are based on the internal transcribed spacer regions (ITS) of the ribosomal DNA (rDNA) (Henson and French, 1993). The 18S, 5.8S, and 28S nuclear rDNA genes are highly conserved for different species and are used for phylogenetic studies. Universal primers have been designed from these conserved regions to allow amplification of the ITS regions between the genes. The ITS regions evolve more quickly than the genes themselves and may vary among species within a genus (White et al., 1990). This feature of the ITS regions allows for the identification of sequence variability between related species. Thus, primers that uniquely amplify a given species can be constructed (Henson and French, 1993). This method was sensitive enough to detect differences in the ITS region of three *Verticillium* species; *V. dahliae*, *V. albo-atrum* and *V. tricorpus* (Moukhamedov et al., 1994). It is even possible to detect specific pathotypes of a species. A virulent pathotype of *Leptosphaeria maculans* was distinguishable from an avirulent pathotype using PCR on DNA extracted from infected canola leaves and primers constructed from the ITS region (Xue et al., 1992). High copy number due to 60 to 200 tandemly repeated clusters of the rDNA genes per haploid genome increases sensitivity of this detection strategy allowing the detection of rDNA in a mixed DNA preparation (White et al., 1990; Henson and French, 1993). Fungal DNA can be detected from a DNA extraction of diseased plant tissue when using primers that amplify the rDNA regions. This feature allows detection of pathogens that cannot be cultured (Henson and French, 1993).

Fungal Mating Types

Pathogenesis

Looking back at the life cycle, it is important to note that the pathogenic phase of *U. hordei* occurs when opposite mating types of the haploid sporidia mate to form the dikaryotic mycelium. Until the mating types fuse the fungus is not pathogenic to the plant. Thus, the mating type genes are also pathogenicity genes (Kronstad, 1995). This feature makes the study of mating type genes in *U. hordei* even more important since increased knowledge of these genes could aid in finding a way to prevent the pathogenic phase of the fungus (Sherwood et al., 1998). Each mating type consists of individuals that are morphologically identical but self-incompatible (Kues and Casselton, 1992). Mating types insure that sexual reproduction will only occur with a genetically diverse individual (Kothe, 1996). The four products of meiosis from the germinating teliospore look identical but two will be from one mating type and will not be able to mate with each other and the other two will be from the opposite mating type.

The mating type genes have been studied for a range of fungi including, but not limited to, two ascomycetes; *Saccharomyces cerevisiae* and *Neurospora crassa,* and two basidiomycetes; *Ustilago maydis* and *Schizophyllum commune.* A variety of different mating systems are displayed in these fungi. The general mating system can be either tetrapolar or bipolar. Mating in the bipolar system is controlled by a single genetic locus. In a tetrapolar mating type system, two unlinked mating type loci, commonly designated

a and *b*, control mating. The number of alleles at each locus varies for the different species (review by Kothe, 1996).

Neurospora crassa and *Saccharomyces cerevisiae* both display a bipolar mating type (Kothe, 1996). The brewing yeast, *S. cerevisiae,* which has been well characterized genetically, has one genetic locus with two mating types, MAT**a** and MATα. MAT**a** cells produce the **a**-factor (Mf**a**) which is a secreted peptide that binds to the **a**-factor receptor on the surface of α cells (Anderegg et al., 1988). Similarly, the α-factor (MFα) is produced by MATα cells and binds to the α-factor receptor on the surface of **a** cells (Review by Kues and Casselton, 1992). By analysis of sterile (STE) mutants, the genes that encode the pheromone receptors (STE3 and STE2) have been identified (Hagen et al., 1986; Blumer et al., 1988). Other STE genes isolated were found to be required for processing of the pheromone precursor molecules encoded by MF**a** and MFα (Kues and Casselton, 1992). Systems similar to that of *S. cerevisiae* have been identified in other fungi but a unique feature of *S. cerevisiae* is that it is capable of mating type switching. Both **a** and α contain silent cassettes of each of the mating type genes, therefore mating type switching is possible by a gene conversion event into the active site (Kothe, 1996).

Schizophyllum commune and *Ustilago maydis* both display a tetrapolar mating type system with differing numbers of alleles at each locus. It has been estimated that the worldwide population of *S. commune* consists of ~20,000 mating types because it is multiallelic at **A**α, **A**β, **B**α and **B**β. **A** and **B** are unlinked and considered genetic complexes each consisting of linked α and β loci with multiple alleles. Genes from the

two **A** loci code for transcriptional regulators. Genes from the two **B** loci code for pheromone receptors and multiple pheromones (Vaillancourt et al., 1997). In *S. commune* cell fusion is seen regardless of mating type but events occurring after fusion are distinguished by mating type compatibility (Kothe, 1996).

The mating loci of *U. maydis* are well characterized and offer a model system for the study of tetrapolar basidiomycete mating. *U. maydis* has a less complicated system than *S. commune* with the *a* locus composed of two alleles that regulate fusion of haploid cells (Trueheart and Herskowitz, 1992) and the *b* locus with at least 25 alleles that regulate pathogenesis (Banuett and Herskowitz, 1989; Holliday, 1961). If two haploid cells differ at the *a* locus, they will fuse but completion of the sexual cycle and pathogenicity will only occur if the nuclei differ at the *b* locus (Banuett and Herskowitz, 1994). The *a* locus was cloned by complementation of the linked biochemical marker gene for pantothenic acid, *pan1*-1. It was found that the mating type alleles are ideomorphic, i.e., restriction maps and DNA hybridizations show that they are unrelated but the DNA flanking the gene is similar. There is also only one copy per haploid genome (Froeliger and Leong, 1991). The b locus has also been extensively studied and many of the alleles have been cloned (Kronstad and Leong, 1989) Two regulatory genes exists at the *b* locus and each contain multiple alleles. These genes are designated *bE* and *bW*. Each allele of the b locus codes for a pair of homeodomain proteins that combine to act as transcriptional regulators (Gillissen et al., 1992). Cloned sequences of the mating type genes from *U. maydis* were shown to hybridize to genomic DNA from the other smut fungi. This was true for the *a* and *b* locus in both tetrapolar and bipolar

smuts (Bakkeren et al., 1992). This similarity should allow for the use of the *U. maydis* genes as a probe to isolate and clone the mating type genes from other fungi including *U. hordei.*

Differences have been found between the mating type locus in *U. maydis* and in *U. hordei.* Unlike *U. maydis* a bipolar mating type system is present in *U. hordei.* This bipolar system in *U. hordei* is also composed of *a* and *b* loci (Bakkeren and Kronstad, 1993) but these loci are physically linked. Thus, suppression of recombination in the mating type locus of *U. hordei* results in a large complex MAT locus (Bakkeren and Kronstad, 1994).

Fungal Pheromones and Pheromone Receptors

In *U. maydis* the mechanism of fusion to the opposite mating type has been shown to be controlled by the *a* locus which codes for pheromone and pheromone receptor genes (Bölker et al., 1992). Cells of the MAT-1 mating type have the genes that code for a pheromone, *mfa*1, and pheromone receptor, *pra*1. MAT-2 cells have genes that code for a similar pheromone, *mfa*2, and pheromone receptor, *pra*2. The pheromone receptor of MAT-1 cells, pra1, is located on the cell surface and recognizes the pheromone, mfa2, that is excreted from MAT-2 cells. Similarly pra2 recognizes mfa1.

Mating in *U. hordei* has also been shown to involve a diffusible mating factor or pheromone. The observance of conjugation tube growth of one mating type toward the other when the two types were separated by a dialysis membrane with a molecular weight cutoff of 12,000 was the initial evidence of pheromone production. Conjugation tubes

from opposite mating types grow toward one another and fuse tip to tip (Martinez et al., 1993). This observation and the knowledge that the pheromone and pheromone receptor genes from *U. maydis* hybridize to DNA fragments from *U. hordei* (Bakkeren et al., 1992) led to the conclusion that mating in *U. hordei* is also regulated by pheromones and pheromone receptors.

The pheromone precursor genes from *U. maydis* have been cloned and sequenced. The sequence codes for a short polypeptide, 40 amino acids for *mfa*1 and 38 for *mfa*2 (Bölker et al., 1992). These polypeptides are processed and cleaved to form the active pheromone lipopeptide (Spellig et al., 1994). The amino acid sequence of both genes end with a prenylation signal of C-A-A-X where C is the terminal cysteine, A is any aliphatic amino acid and X is any amino acid (Bölker et al., 1992). The amino acid at the X position determines if it is geranylgeranylated or farnesylated. If the X amino acid is leucine or phenylalanine it is thought that the cysteine is geranylgeranylated. Any other amino acid means that it is farnesylated (Hoffman, 1991; Boyartchuk et al., 1997). The most common prenylation is the attachment of farnesyl. Farnesyl is a isoprenoid lipid molecule containing 15 carbon atoms (Caldwell et al., 1995). This signal has been found in genes that encode precursors for lipopeptide mating factors and thought to be necessary for movement out of the cell (Bölker et al., 1992; Casey, 1995). The active form of the pheromones produced by MAT-1 and MAT-2 cells have also been purified (Spellig et al., 1994). A morphological switch from yeast-like to mycelial growth upon receptor binding was demonstrated in a diploid strain heterozygous for *b* and homozygous for *a*. This strain was used as a biological assay to purify the pheromones.

The secreted pheromones, mfa1 and mfa2, were determined to be 13 amino acids and nine amino acids, respectively, after post-translational modification. Both pheromones were proposed to be farnesylated and carboxyl methyl esterified at the terminal cysteine (Spellig et al., 1994).

The pheromone receptors from *U. maydis* (Bölker et al., 1992) as well as the MAT-1 pheromone receptor from *U. hordei* (Bakkeren and Kronstad, 1994) have been cloned and sequenced. These receptors are similar to each other and to the *S. cerevisiae* **a**-factor and α-factor receptor genes (Bölker et al., 1992; Bakkeren and Kronstad, 1994; Hagen, et al. 1986; Blumer et al., 1988). These pheromone receptors are similar to the G protein-linked serpentine receptors that respond through a phosphorylation signal transduction cascade. These receptors have seven transmembrane domains that define three cytoplasmic loops and three extracellular loops. The transmembrane regions are determined by hydrophobic α helical domains (Blumer et al., 1988). The third cytoplasmic loop may be involved in G-protein coupling. Upon binding of the pheromone to the extracellular loops, the G protein is released and dissociates into an α component and a βγ component (Kothe, 1996). In *U. maydis* and in *U. hordei* the Gα subunit seems to be involved in a signal transduction cascade via kinase phosphorylation (Regenfelder et al., 1997). This cascade initiates cell cycle arrest and the morphological switch from budding sporidia to mycelia formation (Banuett and Herskowitz, 1994).

These studies have increased our knowledge of how fungi distinguish self from non-self as well as improving our understanding of receptor mechanisms. As more is

learned about these systems we may discover unique ways of controlling pathogen development. Such pathogen inhibition has been exhibited by two different systems. The α-factor from *S. cerevisiae* inhibits pathogenicity of the rice blast fungus, *Magnaporthe grisea* (Beckerman et al., 1997). A mating inhibitory factor (MIF) from *U. hordei* inhibits mating or teliospore germination of the different smut species which is required for pathogenicity (Sherwood et al., 1998). Knowledge of the roles of pheromones and pheromone receptors in mating and pathogenesis in different fungal systems is continuously increasing. Although these systems have features in common each seems to have distinguishing characteristics of interest.

PCR DETECTION OF *USTILAGO HORDEI*

Introduction

Ustilago hordei infects barley seedlings but symptoms of the disease, covered smut, are not visible until heading (Groth and Person, 1978). A method to detect the fungus in the plant tissue at various stages of development would allow for the early detection of fungal infection. The presence of fungal mycelia in plant tissue has been examined microscopically for *U. hordei* (Kozar, 1969) and *U. tritici* (Batts and Jeater, 1958). It was found in both cases that the fungus was concentrated in the meristematic regions of the plant. These studies helped to determine the interaction between the host and parasite but microscopic methods tend to destroy plant tissue and lack sensitivity. A detection method based on amplification of fungal DNA would not only be fast but would be less invasive and more sensitive than microscopic techniques. The fungus could be detected in infected leaf tissue using polymerase chain reaction (PCR) with primers that are specific to the fungus (Henson and French, 1993). This method to detect the fungus in plant tissue prior to heading would allow for studies on the effect of resistance genes on the pathogen and may be effectively used to screen cultivars for resistance in a breeding program.

Sequence from the ribosomal internal transcribed spacer (ITS) regions of *U. hordei* was used to develop a primer pair for PCR. The ITS regions were used because

these sequences can be variable even between closely related species and they occur in high copy number which aids in PCR amplification. This region is easy to clone because of the availability of primers from conserved regions of the ribosomal DNA (rDNA) that can be used to amplify the ITS regions (White et al., 1990). The sensitivity and specificity of this detection method was determined and optimized. The developed primers amplified a 574 bp fragment from DNA of *Ustilago* spp. but did not amplify DNA from barley or other common barley pathogens. DNA extracted from as few as four *U. hordei* sporidia was detected in a sample including barley leaf tissue DNA.

U. hordei DNA was amplified from the leaf tissue of inoculated susceptible and resistant plants at different stages of plant development in a set of barley differential varieties (Tapke, 1945). Detection of the fungus at different stages of growth in an individual plant was inconsistent. A positive PCR product did result more often from plants that had smutted heads. Several highly resistant varieties were shown to contain *U. hordei* DNA in the first leaves but not in the later leaves. Thus, although the fungus can infect some resistant plants, the plants remain symptomless. PCR amplification using these primers allows the detection of fungal DNA from a crude extraction of DNA from an infected plant. This method is sensitive enough to detect the fungus in leaf tissue; therefore, the meristematic regions of the plant do not need to be destroyed. Our PCR detection seems to be specific enough that the primers will only detect DNA from species of *Ustilago* so other pathogens or epiphytes should not cause any problems. This method was used to investigate the distribution of fungal invasion in plant tissue of susceptible and resistant barley varieties.

Materials and Methods

Fungal Strains and Barley Varieties

Teliospores of *U. hordei* and other *Ustilago* spp. were obtained from W. Pedersen, University of Illinois and D. Mills, Oregon State University. Race 8 sporidial cultures were maintained on Holliday's complete media (HCM) agar (Holliday, 1961). *Tilletia controversa* and *T. foetida* teliospores; *Rhynchosporium secalis, Pyrenophora teres, Puccinia striiformis,* and *Erysiphe graminis* infected plant samples; and *Cochliobolus sativus* cultures were provided, respectively, by D. Mathre, M. Johnston, and A. Scharen, Montana State University. The barley differential lines for *U. hordei* as described by Tapke (1945) were provided by W. Pedersen.

Cloning and Sequencing of the PCR Amplified ITS Regions

The ITS regions of *U. hordei* were amplified using PCR and the conserved ITS primers were provided by H. Leung (Washington State University). The rDNA specific primers ITS1, ITS3 and ITS4 were used to amplify the internal transcribed spacer regions using PCR (White et al., 1990). This region was then cloned and sequenced to determine potential primer pairs for the amplification of *U. hordei.*

U. hordei DNA was extracted by boiling 2 mg of teliospores in 1 ml of 10 mM tris pH 8.0 for 20 min and 5 µl of the supernatant was used in a 50 µl PCR reaction

(Henson and French, 1993). PCR reagents and *Taq* DNA polymerase were used according to manufacturers specifications (Perkin-Elmer Cetus, Branchberg, NJ). The final concentration was 1.6 μM for each ITS primer and 5 mM $MgCl_2$. The PCR cycling conditions consisted of denaturation at 94°C for 1.5 min, annealing at 55°C for 2.5 min and extension at 72°C for 3 min for 40 cycles with a final extension at 72°C for 10 min. Ten microliters of the PCR products from ITS 1 and 4, and ITS 3 and 4 were size separated on a 1.5% agarose gel stained with ethidium bromide and viewed with ultraviolet light. The resulting 791bp and 486bp fragments (Figs. 2-1 and 2-2) were then cloned into pCRII and sequenced. One microliter of each PCR product was ligated into pCRII using the TA cloning kit (Invitrogen Corp., San Diego, CA) and transformed into *Escherichia coli* strain DH5α (GIBCO-BRL, Gaithersburg, MD). The clones were screened for the presence of the desired insert and prepared for sequencing using a Qiagen plasmid miniprep kit (Qiagen Inc., Chatsworth, CA). Double stranded DNA was denatured by an alkaline method (Hattori and Sakaki, 1986) and sequenced using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH) and the double stranded dideoxy sequencing method (Sanger et al., 1977) with the M13 universal forward and reverse primers according to manufacturers instructions. Internal primers were also constructed (GIBCO-BRL) to verify the sequence in both directions.

The resulting ITS sequence was entered into the Wisconsin Genetic Computer Group (GCG) sequence database program (Devereux et al., 1984) which identified suitable primer pairs. Primer Uh1, located 42 bases from ITS1, and Uh4, located 137

bases from ITS4 were synthesized (GIBCO-BRL). The T_m value for the primer pair was 58°C. PCR conditions were optimized by adjusting primer concentrations, $MgCl_2$ concentration and annealing temperature. The final concentrations used for PCR with these primers was 1.5 mM $MgCl_2$ and 0.1 μM of each primer in a 25 μl reaction. The program for the thermal cycling was denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min for 40 cycles using three minute denaturation and extension steps for the first three cycles and a final extension for 10 min at 72°C.

DNA Extraction

DNA from fungal cultures, spores or infected leaf tissue was obtained using a modification of a plant tissue extraction (Edwards et al., 1991). Samples were ground in 400 μl extraction buffer, vortexed, and placed at -70°C for 20 min before extracting at room temperature for 40 min. After addition of isopropanol (1:1 v/v) to 300 μl of the supernatant, the samples were placed at -70°C for 5 min or -20°C for greater than 25 min and then centrifuged at 16,000 x g for 20 min. The DNA pellet was dried and suspended in 100 μl of sterile double distilled water for use in subsequent PCR reactions. For each extraction, a negative control was included that contained only extraction buffer to assure that the buffers and reagents were not contaminated.

Determination of Sensitivity and Specificity

The sensitivity of the procedure to detect *U. hordei* was determined by testing a dilution series of sporidia. Sporidia were shaken at 250 rpm overnight in HCM broth. The cells were centrifuged for 5 min at 4500 x g, washed with sterile distilled water, and the concentration determined by counting the cells with a hemocytometer. The cells were adjusted to a concentration of $1x10^8$cells/ml and a serial 10-fold dilution was performed down to $1x10^4$cells/ml. Ten microliters of each dilution was extracted as described above. Thus, an initial concentration of $1x10^8$ cells/ml was equivalent to extracting $1x10^6$ sporidia. DNA from 300 µl, or seventy-five percent of the total amount of DNA, was precipitated with isopropanol (1:1 v/v) and dissolved in 100 µl sterile double distilled water. Therefore, the resulting 100µl of extracted DNA was from the equivalent of 750,000 sporidial cells. Five microliters of the DNA was used per 25 µl PCR reaction so that a PCR reaction of the extraction of $1x10^6$ sporidia would be the equivalent of amplifying the DNA from 37,500 haploid sporidia. A similar extraction of the same dilution series of sporidia was done which included a 1 cm segment of uninfected barley leaf tissue. Control DNA extractions were also done with no sporidia with and without uninfected barley leaf tissue.

The specificity of this reaction was determined by performing PCR amplification using primers Uh1 and Uh4 with DNA from uninoculated barley plant tissue, other common barley leaf pathogens and other members of the Ustilaginales. DNA was extracted from teliospores of *U. avenae, U. bullata, U. kolleri, U. tritici, U. nuda,*

Tilletia controversa, and *T. foetida.* DNA was also extracted from conidia of *Cochliobolus sativus, Pyrenophora teres, Rhynchosporium secalis,* uredia from *Puccinia striiformis* and from leaf samples infected with *Erysiphe graminis.* PCR reactions were performed as stated above with the Uh1 and Uh4 primers.

Inoculation and Greenhouse Experiments

The barley covered smut differential lines described by Tapke (1945) were used for greenhouse experiments. Teliospores of *U. hordei* race 8 were used for plant inoculations. A modification of the vacuum inoculation method was used (Tapke and Bever, 1942). For each barley cultivar, the seeds were dehulled and surface sterilized for 5 min in bleach (5.25% sodium hypochlorite). Since manual dehulling of the seed decreases successful seedling emergence, thirty seeds were initially inoculated to ensure that ten plants could be obtained for PCR analysis (Schafer et al., 1962). Approximately 25 mg of race 8 teliospores were added to the seeds with 0.5 mg of carboxymethylcellulose and 1ml of sterile water containing 0.001% Tween20. The seeds were vacuum infiltrated three times for ten minutes and planted in damp vermiculite at a depth of 3 cm. After the seeds were germinated at 16°C for 7 days, fifteen of the plants were transplanted into 6 inch pots with three plants per pot. A steam sterilized greenhouse mix of soil : sand : peat (1:1:1 v/v) was used for transplanting and the plants were placed in a controlled greenhouse providing 16 hours of light, weekly fertilization and a night/day temperature of 18 °C /21°C.

When the plants were at the three leaf stage, ten plants from each cultivar were tagged with an identification number and leaf tissue was collected from each tagged plant as it was growing. Samples were taken from each leaf after two higher leaves had emerged. For example, the first leaf was collected when the plant had a total of three leaves. The leaf below the flag was collected when the flag leaf had emerged and the flag leaf was collected when the head emerged from the leaf sheath. Preliminary experiments indicated that the distribution of *U. hordei* in an individual barley leaf was not uniform, therefore three samples per leaf were collected to maximize the likelihood for obtaining infected tissue. Each sample consisted of segments from about 2.5 cm below the leaf tip, the center of the leaf and 2.5 cm above the base of the leaf. Uniform tissue size was obtained by using a sterile microcentrifuge tube to punch out the leaf samples. The microcentrifuge tubes containing the leaf samples were stored at -70°C until the DNA was extracted. The presence of smutted heads in each of the tagged plants was also recorded.

Results

Primers and ITS Sequence

DNA extracted from *U. hordei* race 8 teliospores was amplified using ITS 1 and 4 primers and ITS 3 and 4 primers, resulting in 791 bp and 486 bp fragments, respectively. Figure 2-1 shows the resulting bands when 10 µl of each PCR product was size-separated on a 1.5% agarose gel that was stained with ethidium bromide and viewed with ultraviolet light. Each fragment was cloned into pCRII and sequenced.

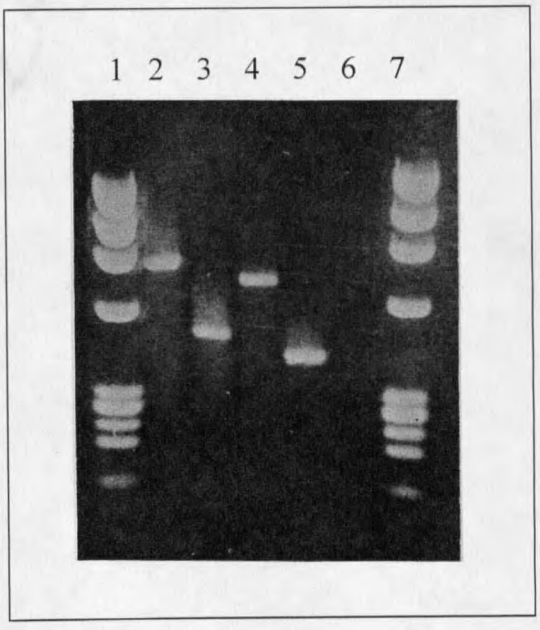

Fig. 2-1. ITS PCR Products. Lanes 1 and 7 were loaded with 1µg of a φx174 *Hae*III digest for a ladder. Lane 2 shows the PCR product which resulted from the use of the ITS primers 1 and 4 on *U. hordei* DNA. Lane 3 shows the PCR product using ITS primers 3 and 4 on the *U. hordei* DNA. Lanes 4 and 5 show the PCR fragments that resulted when *T. controversa* DNA was used for PCR with the ITS 1 and 4 primers and 3 and 4 primers, respectively. There was a slight size difference in the resulting fragments for the two different fungi. Lane 6 was loaded with a control reaction without DNA.

The cloned fragments were used for sequencing and a primer pair was chosen from this sequence. Figure 2-2 diagrams the ITS regions and the location of the ITS primers. The sequence for the primers Uh1 and Uh4 were determined from the ITS sequence, and amplify a 574bp fragment from *U. hordei.*

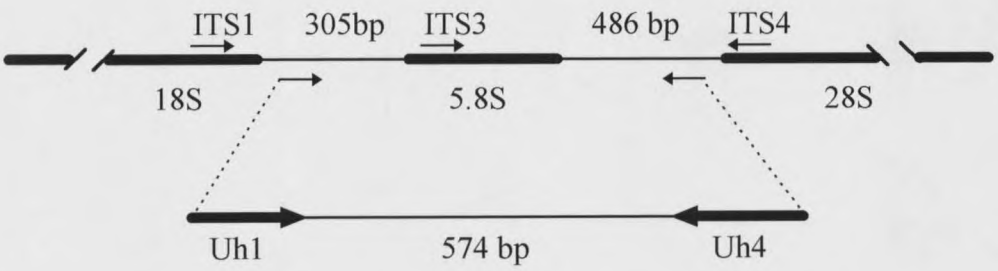

Fig. 2-2. ITS regions of the rDNA of *U. hordei.* The arrowheads indicate the 3' end of the primers. Primers Uh1 and Uh4 were determined from the ITS sequence.

The sequence shows the ITS regions of *U. hordei* spanning the region between the ITS1 and ITS4 universal primer sites including the 5.8S rDNA and primer regions (Fig. 2-3). The sequence was obtained for both directions to improve accuracy of the hard to read regions of the sequence. The 5.8S rDNA region was determined by comparison to this region in *S. cerevisiae* (Bell et al., 1977).

ITS primer 1 ⟶

1 TCCGTAGGTG AACCTGCGGA TGGATCATTT CGATGAAAAA CCTTTTTTCA GAGGTGTGGC

U. hordei primer 1⟶

61 TCGCACCTGT CCAACTAAAC TTGAGCTACC TTTTTCAACA CGGTTGCATC GGTCGGCCTG

121 TCAAACAGTG CGACGCAAGG AGAAAATCCT CGCGTCTGCT GGGCGACGGA CAGACAATTT

181 TATTGAACAC TTTTTGATGA TCTAGGATTT GAAGGAGAAA AAGTCATTTT TACGAATGAA

241 ATCGACTGGT AATGCGGTCG TCTAATTTTA AAAACAACTT TTGGCAACGG ATCTCTTGGT

ITS primer 3 ⟶

301 TCTCCCATCG ATGAAGAACG CAGCGAATTG CGATAAGTAA TGTGAATTGC AGAAGTGAAT

361 CATCGAATCT TTGAACGCAC CTTGCGCTCC CGGCAGATCT AATCTGGGGA GCATGCCTGT

421 TTGAGGGCCG CGAATTGTTT CGAACGACAG CTTTTTTCTT TTGGAAAAGG TTGACGGATC

481 GGTATTGAGG GTTTTTGCCA TTTACCGTGG CTCCCTTGAA ATAGATTAGC GCATCCATTT

541 TATAGGCAAG ACGGACGAAA GCTCGATTTT TGCTCTCTCT TCCCTGCCGG GTTTTGATAC

⟵ *U. hordei* primer 4

601 TATCAGGACT TCGGAGAGGT TGAGATGGGT AGGAGCTCGA CGCAACGGCT TGCTGTTTGG

661 AGTGCTTCTG AAACCCGCCC ATGCCGAGTT TATTTTATTT TATAGAAAGC TAGGAAGGGA

721 TTTATAATAA TTCATCGGCC TCAGATTGGT AGGACTACCC GCTGAACTTA AGCATATCAA

⟵ ITS primer 4

781 TAAGCGGAGG A

Fig. 2-3. Sequence of the ITS regions. The bold regions are the ITS regions (White et al., 1990). The region in the middle of the two ITS regions is the 5.8S rDNA gene (Bell et al., 1977). The primer sequences are underlined. *U. hordei* primers Uh1 and Uh4 anneal to the ITS regions of this sequence and result in the amplification of a 574 bp fragment when used for PCR on *U. hordei* DNA.

Sensitivity of PCR Detection Assay

DNA extracted from a 10-fold serial dilution of sporidia was used for PCR with the Uh1 and Uh4 primers. Figure 2-4 shows the DNA fragments amplified using 5 µl of each extraction per 25 µl PCR reaction. A strong product was still seen when 375 *U. hordei* sporidia were extracted as seen in lane 4. The inclusion of barley leaf tissue in the DNA extraction increased the intensity of the PCR product as seen in lanes 9 to 13. As few as four *U. hordei* sporidia could be detected with these primers in the presence of barley leaf tissue DNA. The control lanes 7 and 14 were negative.

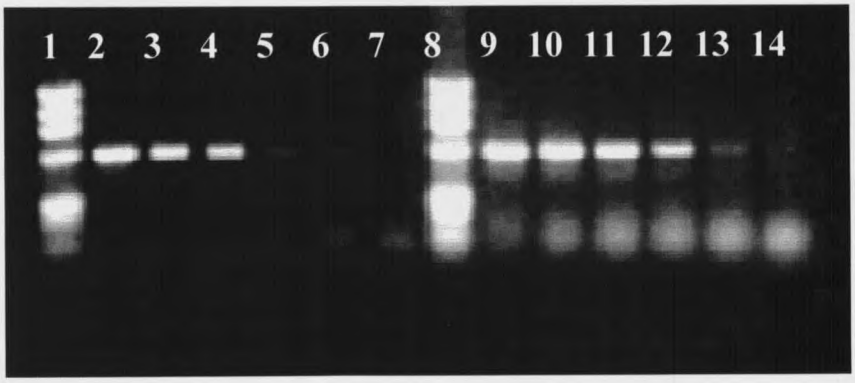

Fig. 2-4. PCR of Sporidia Dilutions. Lanes 1 and 8 were loaded with 1 µg of φx174 *Hae*III digest standard. Lanes 2 through 6 show PCR products of the extraction of 37,500, 3,750, 375, 38 and 4 sporidia, respectively. Lane 7 shows the PCR product of a control extraction with no sporidia. Lanes 9 through 13 are the same as lanes 2 through 6 but barley leaf tissue from an uninoculated plant was added to the sporidia before the DNA extraction. Lane 14 shows an extraction of barley leaf tissue alone.

Specificity of Uh1 and Uh4 Primers

Primers Uh1 and Uh4 specifically amplified DNA from species of *Ustilago*. DNA from uninoculated barley, other potential barley leaf contaminates and from two *Tilletia* spp. did not give a PCR product when amplified with these primers using a 57°C annealing temperature (Table 2-1). The Uh1 and Uh4 primers did amplify a similarly sized product from DNA of all the other *Ustilago* spp. tested with an annealing temperature of 57°C. DNA from *U. bullata* was not amplified when an annealing temperature of 63°C was used.

TABLE 2-1. Polymerase chain reaction assays of barley, barley fungal leaf pathogens and members of the Ustilaginales using Uh1 and Uh4 primers.

Plant pathogen	Common name	Source	PCR product[a]	
			57°C	63°C
Hordeum vulgare	barley	leaf tissue	-	ND
Cochliobolus sativus	barley spot blotch	conidia	-	ND
Pyrenophora teres	barley net blotch	conidia	-	ND
Rhynchosporium secalis	barley scald	conidia	-	ND
Erysiphe graminis	barley powdery mildew	infected tissue	-	ND
Puccinia striiformis	barley stripe rust	uredia	-	ND
Tilletia controversa	dwarf bunt of wheat	teliospores	-	ND
Tilletia foetida	common bunt of wheat	teliospores	-	ND
Ustilago avenae	loose smut of oats	teliospores	+	+
U. bullata	loose smut of crested wheatgrass	teliospores	+	-
U. kolleri	covered smut of oats	teliospores	+	+
U. nuda	loose smut of barley	teliospores	+	+
U. tritici	loose smut of wheat	teliospores	+	+
U. hordei	covered smut of barley	teliospores	+	+

[a] (+) indicates a positive PCR product with Uh1 and Uh4 primers; (-) indicates that no PCR product was present; ND = PCR not done

Analysis of Leaf Tissue from Inoculated Barley Varieties

Leaf tissue from each of the inoculated covered smut differential lines was extracted from each leaf and PCR was done as described above (Table 2-2). The variety Odessa (C.I. 934), which is susceptible to all races of *U. hordei* (Tapke, 1945), produced an unusually high number of leaves when grown under our experimental conditions (Kiesling, 1978). All of the inoculated Odessa plants that survived had smutted heads. Each plant had positive PCR products at multiple collection points. The number of positives varied for each plant and for each leaf. Plant 5 had a strong positive PCR product for each leaf of the plant except the 12^{th} leaf, which was weakly positive. Plant 3, on the other hand, only had strong PCR products for three of the first four leaves, with subsequent leaves negative or weakly positive. Leaves from the uninoculated Odessa control consistently resulted in no PCR product.

Hannchen (C.I. 531) and Pannier (C.I. 1330) have been described as resistant to race 8 of *U. hordei* (Tapke, 1945). In our greenhouse experiment Hannchen and Pannier were not completely resistant (Table 2-2). Five out of the surviving nine Hannchen plants tested had at least one tiller with a smutted head. Therefore, Hannchen is moderately susceptible to race 8 of *U. hordei.* The detection method more consistently resulted in a positive PCR product in the plants that were smutted but the fungus was detected in the early leaves of plants lacking symptoms. Positive PCR products were only found in the later leaves of plants that had infected heads. The variety Pannier had no strong positive PCR products but one plant out of ten was smutted. Pannier was

previously found to be resistant to race 8 of *U. hordei* (Tapke, 1945) but 10% infection would be considered intermediate resistance (Tapke, 1937). The infected plant may have possibly been a seed contaminant of a susceptible variety but this is not likely due to characteristic differences of the barley lines. Nepal (C.I. 595) also displayed intermediate resistance but was not included in the analysis because the uninoculated control plant was smutted.

The other differential lines, Excelsior (C.I. 1248), Himalaya (C.I. 1312), Lion (C.I. 923), and Trebi (C.I. 936) were resistant to race 8 of *Ustilago hordei* (Table 2-2). None of these inoculated plants had smutted heads. However, the fungus was detectable in the early leaves of several plants indicating that the fungus entered the resistant plant but was unable to continue to grow throughout the plant at detectable levels. Evidence of this for Excelsior is shown in Figure 2-5. The pattern of positive PCR products for all the resistant lines was similar to that of Excelsior.

DNA contamination is always a hazard when doing PCR because of the sensitivity of the method. If bands were present in either of the controls, the PCR reaction was repeated with fresh reagents. If contamination occurred during the extraction process the sample could not be used at all for analysis. This accounts for the missing data in the Himalaya table. Weak bands were considered to have potentially resulted from laboratory contamination and were therefore noted but not used for the calculations of percent positive (Fig. 2-5).

Table 2-2. PCR Products obtained from inoculated leaves of the differential set.

Odessa - Susceptible

Plant number	Leaf number 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Infected head
0	○[a]	○	●	●	○	●	○	○	●	◗	●	●	○			+[b]
1	○	◗	●	●	○	○	○	○	died							
2	○	○	○	●	○	◗	○	○	●	◗	◗	○	◗	◗		+
3	●	●	○	●	○	○	◗	○	◗	◗	◗	○	○	○	○	+
4	●	●	○	●	○	○	○	○	●	●	◗	◗	●	◗		+
5	●	●	●	●	●	●	●	●	●	●	●	◗	●	●		+
6	○	●	●	●	○	○	●	●	●	●	●	●	●	●	●	+
7	○	●	●	●	○	○	○	●	●	●	●	●	●	●		+
8	◗	●	●	●	○	○	○	●	○	●	◗	○	◗	●		+
9	○	●	●	●	●	○	●	●	●	○	◗	○	○	●	◗	+
10	○	○	○	●	●	○	●	○	●	●	◗	○	●	●		+
control[c]	○	○	○	○	○	○	○	○	○	○	○	○	○			-
% pos.[d]	36	64	64	100	27	18	36	45	80	60	40	30	50	44	33	100

Hannchen - Moderately Susceptible

Plant number	Leaf number 1	2	3	4	5	6	7	8	9	10	11	12	13	Infected head
0	◗	○	●	died										
1	●	○	●	○	○	○	●	◗	●	●	◗	◗	●	+
2	●	○	●	●	○	○	○	○	○	○	○	○		-
3	●	○	●	○	○	○	○	○	○	○	○	○		-
4	●	○	◗	○	●	●	●	◗	○	●	●	○	○	+
5	●	○	●	○	○	○	●	◗	○	●	●	○	○	+
6	●	○	○	○	○	○	○	○	○	○	○	○		-
7	●	○	●	●	◗	◗	●	○	○	●	◗	●		+
8	○	○	○	○	○	○	○	○	○	○	○	○		-
9	●	○	○	○	○	○	○	○	○	○	○	○	○	-
Control	○	○	○	○	○	○	○	○	○	○	○	○	○	-
% positive	80	0	60	22	11	11	44	0	11	44	22	11	20	44

Pannier - Moderately Susceptible

Plant number	Leaf number 1	2	3	4	5	6	7	8	Infected head
0	○	○	○	○	○	○	○		-
1	◗	○	○	○	○	○	○		+
2	○	○	○	○	○	○	○		-
3	○	○	○	○	○	○	○		-
4	○	○	○	○	○	○	○	○	-
5	○	○	○	○	○	○	○	○	-
6	◗	○	○	○	○	○	○		-
7	○	○	○	○	○	○	○		-
8	○	○	○	○	○	○	○		-
9	◗	○	○	○	○	○	○		-
Control	○	○	○	○	○	○	○		-
% positive	0	0	0	0	0	0	0	0	0

Excelsior - Resistant

Plant number	Leaf number 1	2	3	4	5	6	7	8	9	10	11	12	13	Infected head
0	○	○	○	○	○	○	○	○	○	○	○			-
1	●	○	○	○	○	○	○	○	○	○	○	○		-
2	○	●	○	○	○	○	○	○	○	○	○			-
3	○	○	○	○	○	◗	○	○	○	○	○	○		-
4	○	○	○	○	○	○	○	○	○	○	○	○		-
5	○	○	○	○	○	○	○	○	○	○	○	○		-
6	◗	●	●	○	○	○	○	○	○	○	○	○		-
7	●	●	●	○	○	○	○	○	○	○	◗	○		-
8	●	○	◗	○	○	○	○	○	○	○	○	○		-
9	○	○	○	○	○	○	○	○	○	○	○	○		-
Control	○	○	○	○	○	○	○	○	○	○	○	○	○	-
% positive	30	30	20	0	0	0	0	0	0	0	0	0	0	0

Lion - Resistant

Plant number	Leaf number 1	2	3	4	5	6	7	Infected head
0	○	○	○	○	○	○	○	-
1	○	○	○	○	○	○	○	-
2	○	○	○	○	○	○	○	-
3	◗	●	○	○	○	○	○	-
4	○	●	○	○	○	○	○	-
5	●	○	○	○	○	○	○	-
6	○	○	○	○	○	○	○	-
7	○	○	○	◗	○	○	○	-
8	○	○	○	○	○	○	○	-
9	○	○	○	○	○	○	○	-
Control	○	○	○	○	○	○	○	-
% positive	11	22	0	0	0	0	0	0

Trebi - Resistant

Plant number	Leaf number 1	2	3	4	5	6	7	8	9	10	11	Infected head
0	○	●	●	○	○	○	○	○	○			-
1	○	●	○	○	○	○	○	○	○	○		-
2	●	○	○	○	○	○	○	○	○	○		-
3	○	○	○	○	○	○	○	○	○	○		-
4	○	○	○	○	○	○	○	○	○	○	○	-
5	●	●	○	○	○	○	○	○	○	○		-
6	○	○	○	○	○	○	○	○	○	○	○	-
7	○	●	○	○	○	○	○	○	○	○		-
8	○	○	○	○	○	○	○	○	○			-
9	○	○	○	○	○	○	○	○	○	○	○	-
Control	○	○	○	○	○	○	○	○	○	○		-
% positive	22	44	11	0	0	0	0	0	0	0	0	0

Himalaya - Resistant

Plant number	Leaf number 1	2	3	4	5	6	7	8	9	10	11	12	Infected head
0	○[a]	○			○	○	○	○	○	○	○	○	-[b]
1	○	○			○	○	○	○	○	○	○		-
2	○	○			○	○	○	○	○	○	○		-
3	●	○		○	○	○	○	○	○	○	○		-
4	◗	○		○	○	○	○	○	○	○	○		-
5	●	○		○	○	○	○	○	○	○	○		-
6	●	○		○	○	○	○	○	○	○	○		-
7	○	○		○	○	○	○	○	○	○	○		-
8	○	○		○	○	○	○		○	○	○	○	-
9	◗	○		○	○	○	○	○	○	○	○		-
Control[c]	○	○			○	○	○	○	○	○	○		-
% positive	33	0	?	?	0	0	0	0	0	0	0	0	0

[a] (○) for no product, (●) for a strong product, (◗) for a weak product

[b] (+) indicates a smutted head, (-) indicates the head was not smutted.

[c] Control was a plant of the same variety that was not inoculated with *U. hordei*.

[d] Only the strong positives (●) were used to calculate percent positive for each leaf.

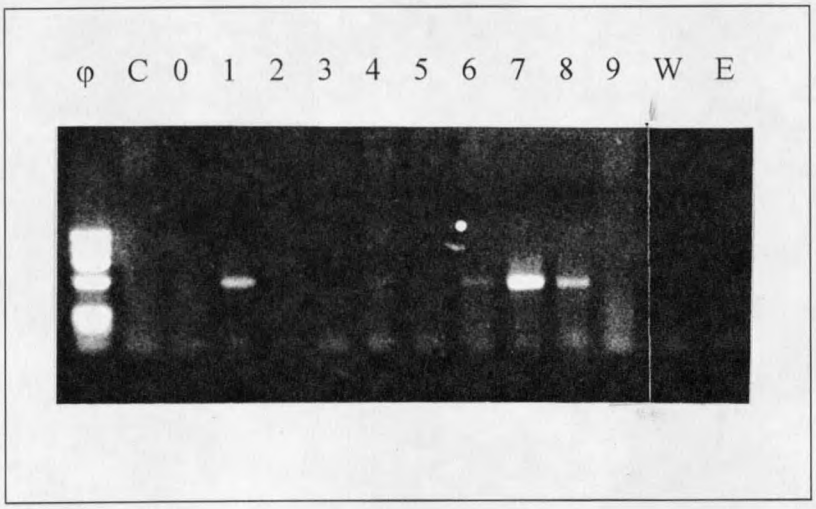

Fig. 2-5. Excelsior PCR Products. PCR products that resulted from the DNA extracted from the second leaf of Excelsior plants. The lane number corresponds to the plant number. The first lane was loaded with the φx174 *Hae*III digest standard. Three control PCR reactions were also included for each set of PCR experiments. The first control was the extraction of leaf tissue from an uninoculated plant from the same variety at the same stage (lane C). The other controls consisted of sterile double distilled water in place of DNA (lane W) and of PCR of a mock extraction performed without tissue (lane E). An example of a strong positive (●), weak positive (◗) and negative (○) PCR products are shown in lanes 7, 6, and 5, respectively.

Discussion

The ability to detect the presence of *Ustilago hordei* in barley tissue would provide a method to study the fungal infection in the host and compare differences in infection for races of *U. hordei* on different barley varieties. The pathway of infection of *U. hordei* in barley tissue had previously been examined microscopically (Kozar, 1969). Tissue preparations and staining were used to identify fungal mycelium and sporidia in the plant. It was found that the fungus was concentrated in the growing point of the plant. A problem with a microscopic evaluation is the lack of sensitivity and the destructive nature of preparing tissue for examination. The nodal regions were destroyed to look for the presence of fungus and therefore, it was not possible to monitor symptom development in the plants that were examined. Fungal mycelium was found to be present in the flag leaf but other leaves were not examined (Kozar, 1969). It was known that teliospores from aggressive races of *U. hordei* can form in the upper leaves of a susceptible plant, but it was assumed that the fungus was not highly concentrated in the leaves (Fischer and Holton, 1957;Gaudet and Kiesling, 1991). In our study leaves were collected when three upper leaves had emerged in order to monitor fungal growth in a less invasive way. This allowed the plant to survive and produce a head so that the PCR reaction in the leaves could be compared to the development of symptoms.

Using PCR to detect fungal DNA in a crude DNA extraction from diseased plant leaf tissue allowed for the detection of small amounts of the fungus. It was found that amplification with primers Uh1 and Uh4 could detect four *U. hordei* sporidia in a DNA

preparation that included barley DNA. This was equivalent to two diploid teliospores or dikaryotic mycelial cells. Thus, PCR with the Uh1 and Uh4 primers is sensitive enough to detect very low amounts of fungal mycelia in leaf tissue of inoculated barley plants. The sensitivity of PCR does require extra precautions to avoid contamination. A previous greenhouse experiment on the resistant variety Excelsior (data not shown) resulted in positive PCR products in the latter leaf samples that seemed to have resulted from contamination from nearby smutted plants. A control of uninoculated plant tissue was always included to monitor the possibility of contamination in the greenhouse, while a control lacking DNA was included to determine and eliminate any reagent contamination that may have occurred.

In a preliminary experiment, the fungus was more easily detected in young leaves. For example, at the four leaf stage the top two leaves were positive and the bottom two were negative. This result might be due to senescence of the older leaves, such that the DNA was harder to extract. This result posed a problem for collecting leaves without interfering with the growth of the plant. We chose to do our greenhouse experiments collecting the leaves at a slightly older stage as opposed to collecting the youngest leaves and interfering with plant growth. Future experiments may more accurately determine the presence of the fungus if the leaves were collected at an earlier stage but fewer leaves taken from the plant.

The ability of this method to specifically detect *Ustilago* spp. was important when working with plants inoculated with *U. hordei* in the greenhouse or field. Powdery mildew of barley is a common greenhouse contaminant and the use of fungicides to inhibit mildew may also affect the growth of *U. hordei*. The other leaf pathogens that tested negative with

this method may be present in the field. While contamination by other *Ustilago* spp. is improbable in greenhouse experiments, these primers will not distinguish between *Ustilago* spp. in field grown plants. Comparison of the primer sequences Uh1 and Uh4 to the equivalent sequence in the other *Ustilago* spp. helped explain why these other species were detected with these primers. The ITS region for several *Ustilago* spp. has been sequenced (Abdennadher, 1996). The sequence for Uh1 varied from most of the other species, but Uh4 was very similar. Uh4 has the same sequence for all the species except *U. avenae* where there was one base different. *U. kolleri* and *U. avenae* have the same sequence as Uh1. *U. tritici, U. nuda* and *U. bullata* sequences vary from Uh1 by 4, 7, and 10 bases, respectively. It is not surprising that these primers did not amplify a band for *U. bullata* at higher annealing temperatures because Uh1 is only 50% similar in sequence and the 3' clamp is different. It may be possible to design a different primer pair or vary conditions such that the primers are more specific but the low $MgCl_2$ concentration, low primer concentration and high annealing temperature used here should have optimized specificity with primers Uh1 and Uh4. At an annealing temperature of 65 °C the only positive PCR product was for *U. hordei*, but this band was extremely weak. The ITS sequences of *U. kolleri* and *U. avenae* (Abdennadher, 1996) were so similar to that of *U. hordei* that it may not be possible to differentiate these species.

The ability to detect *U. hordei* in leaf tissue allowed the study of the presence of this pathogen in inoculated differential barley lines. Detection of the fungus at different stages of growth in an individual plant was inconsistent even in plants with smutted heads. The

fungus was detectable throughout the susceptible variety Odessa but the detection was inconsistent. The number of positive reactions for each leaf in a smutted plant ranged from 93% to 14%. This could be a problem with the detection method or growth throughout the plant may be variable. Experiments examining the smutting patterns in barley suggested that the movement of mycelium through tissues is not the same for each seedling (Groth and Person, 1978). Variability in distribution of the pathogen among tillers is known to occur because not all tillers of an individual plant develop symptoms. Mycelium concentration in the leaves of a single tiller could also be variable. Upper leaves of infected plants often resulted in no PCR product. This could be due to the fact that routine failures can occur when too much target DNA is used as a source for extraction (Foster et al., 1997). Extraction from a leaf containing teliospores consistently gave negative results when used at the same quantities for PCR (data not shown).

Groth and Person (1978) concluded that *U. hordei* mycelium is present for at least a short period in resistant plants. Our experiments supported their conclusion. Several highly resistant varieties were shown to contain *U. hordei* DNA in the first leaves but not in the later leaves. Thus, although the fungus could infect the resistant plants, growth was restricted and the plants remained symptomless. It may be that in some host-race combinations, the fungus may be totally excluded from entering beyond the pericarp (Groth and Person, 1978). It would be useful to determine if a symptomless plant has escaped infection, resisted infection, or resisted disease development following a successful infection (Groth et al., 1977). Pannier may be an example of a plant that normally resists infection but if the fungus can infect, disease development is not

inhibited. It was shown that penetrating mycelia were stopped within the first or second cell in the coleoptiles of Pannier (Kiesling, 1971). Our experiments did not detect the fungus even in the early leaves of Pannier suggesting that this was indeed occurring. Kiesling (1971) obtained no smutted Pannier plants out of 115 checks. Having one smutted plant out of ten in our experiment suggests that if infection occurs disease can develop. The negative PCR results in the smutted plant may indicate that in this variety, growth of the fungus is restricted to the growing point and is not present at detectable levels in the leaves. Further experiments on Pannier would have to be performed to verify this. Another possibility could be that the infected plant was not actually Pannier but a seed contaminant. However, each differential has unique morphological characteristics and all of the Pannier plants looked identical. This suggests that the infected plant was truly a Pannier cultivar.

In previous reports, Hannchen was described as resistant to race 8 (Tapke, 1945; Pedersen and Kiesling, 1979). Our result, in which Hannchen proved to be only moderately resistant, may be a result of inbreeding of race 8. A teliospore shown to be virulent only to Odessa was selfed and the progeny were also now virulent to Hannchen (Pedersen and Kiesling, 1979). The increased number of infected Hannchen plants may also be due to the inoculation procedure. Tapke's original experiments classified anything over 36% infection as susceptible, 6 to 35% infection as intermediate and 0 to 5% infection as resistant (Tapke, 1937). Many methods have been attempted to improve the efficiency of the artificial inoculation procedure for covered smut (Shrivastava and Srivastava, 1970). Our inoculation procedure has consistently resulted in 90 to 100%

smutted heads in surviving Odessa plants. Our procedure may also result in increased infection in lines normally resistant to race 8.

Detection of *U. hordei* in plant tissue prior to heading should assist efforts for breeding for resistance and allow for the determination of effective resistance genes. This method may be used to find a plant with exclusive resistance to *U. hordei,* such that, the fungus can not penetrate the plant at all. It may also be used as a screen for resistant plants. Our experiments did not consistently result in a positive PCR product in the leaves of a smutted plant but a modification of this method may reliably detect a susceptible plant. Collecting leaves as they emerge might improve the accuracy, as would collecting leaves at only specific stages. For example, collecting the fifth leaf for PCR analysis would be desirable because it was shown that in resistant varieties the fungus was not present in this leaf. Also, leaf collection right after emergence may increase the likelihood of obtaining a positive PCR product for plants that are smutted because of increased DNA extraction of young leaf tissue. Experiments examining the different races of *U. hordei* on the differential lines or cultivated barley lines would further expand our knowledge of resistance genes available for *U. hordei* and how infection is resisted.

THE MAT-2 PHEROMONE AND PHEROMONE RECEPTOR OF *USTILAGO HORDEI*

Introduction

The mating of sporidia from opposite mating types of *Ustilago hordei* is necessary for the formation of the dikaryotic mycelium and infection of the plant (Thomas, 1988). It has been shown that a diffusible substance is present and responsible for conjugation tube growth toward the opposite mating type (Martinez et al., 1993). Similar substances have been identified in *Ustilago maydis* and *Saccharomyces cerevisiae* mating systems. They have been determined to be short peptides that are presumed to be farnesylated and methyl esterified at the carboxyl terminus of the cysteine residue (Spellig et al., 1994; Anderegg et al., 1988). The peptide, or pheromone, is recognized by the opposite mating type because it binds to a pheromone receptor on the cell surface. The pheromone receptors that have been cloned are thought to be G protein serpentine receptors that respond via a signal transduction cascade (Hagen et al., 1986; Blumer et al., 1988; Bölker et al., 1992)

The identification of the pheromone and pheromone receptor genes from *U. maydis* (Bölker et al., 1992) and the observation that these genes cross hybridize to DNA from *U. hordei* (Bakkeren et al., 1992) allowed for the identification of the pheromone and pheromone receptor genes in *U. hordei*. The pheromone receptor for MAT-1 of *U.*

*hordei, pra*1, was cloned and sequenced previously (Bakkeren and Kronstad, 1994). The MAT-1 pheromone gene, *mfa*1, has also been cloned and sequenced (Sherwood et al., 1998). With this study, the MAT-2 pheromone (*mfa*2) and pheromone receptor (*pra*2) genes have been cloned and sequenced. The sequences have been compared to the genes from *U. maydis.* The distance between the genes has been determined by PCR analysis. Both genes have been transformed into wild-type MAT-1 and MAT-2 sporidia to determine phenotypic expression of the genes. Knockout plasmids were constructed to attempt to disrupt the pheromone and pheromone receptor genes. The amino acid sequence of the pheromone precursor was deduced from the *mfa*2 nucleotide sequence. A synthetic peptide thought to represent the active form of the pheromone was synthesized. This peptide was added to wild-type MAT-1 cells to determine if its presence would initiate conjugation tube formation.

Materials and Methods

Cultures, Media and Plasmids

The two strains of *Ustilago hordei* sporidia,14**A** (MAT-1) and 14**a** (MAT-2), used for transformation and DNA studies were maintained on Holliday's complete media (HCM) (Holliday, 1961). Trace element (TE) agar was used for the assay to determine the effect of a synthetic peptide on wild-type sporidia (Martinez et al., 1993) Three plasmids, pJS42, pKS and pGEM, were used for DNA manipulations. DNA fragments inserted into a variation of pCM54 were used for *U. hordei* transformations (Tsukuda et

al., 1988). This variation, pJS42, had the *Xba*I fragment, consisting of part of the multiple cloning site and the beginning of the hsp70 gene promoter, removed. pJS42 contains hygromycin resistance (hyg), a *U. maydis* autonomously replicating sequence (ARS), a bacterial origin of replication (ori) and ampicillin resistance (amp) (Tsukuda et al., 1988). Thus, it can be selected for in *Escherichia coli* and in *U. hordei* and replicates autonomously in both. DNA fragments were inserted into pBluescript KS+ (Stratagene, San Diego, CA) for sequence analysis. Genes were inserted into pGEM (Promega, Madison, WI) for the gene disruption constructs. pKS and pGEM both contain ampicillin resistance and an *E. coli* origin of replication. All subclones of the original DNA template were carried out in the presence of remelted agarose (Daum et al., 1991).

Screening of 14**a** (MAT-2) Lambda Library

A *U. hordei* genomic library was prepared with race 14 MAT-2 DNA in λ 2001 (Karn et al., 1984). This genomic library was probed with the *U. maydis* pheromone receptor gene from MAT-2, designated *Umpra2,* and the pheromone gene from MAT-2, designated *Ummfa2,* provided by R. Kahmann, Berlin (Bölker et al., 1992). DNA was prepared from phage purified from plaques that hybridized to either of the *U. maydis* probes (Grossberger, 1987). This DNA was restriction enzyme digested with endonucleases that removed the insert DNA (Karn et al., 1984). A Southern blot of this DNA was probed with the *U. maydis* genes that were isolated by elution from an agarose gel by a freeze/thaw method (Qian and Wilkinson, 1991) and random primer labeled using a nonradioactive procedure for colorimetric detection (Boehringer Mannheim,

Indianapolis, IN). A 6kb *Sac*I fragment that hybridized to *Umpra2* and a 5kb *Bam*HI/*Sac*I fragment that hybridized to *Ummfa2* were each excised from an agarose gel and ligated into pJS42 (Daum et al., 1991). These DNA ligation reactions were transformed into *E. coli* strain DH5α (GIBCO-BRL, Gaithersburg, MD). Clones were screened for presence of insert and a restriction map was done for the DNA fragments thought to contain the *pra2* and *mfa2* genes.

U. hordei Transformation

U. hordei races 14**A** and 14**a** were transformed with the *pra2* and *mfa2* genes cloned from *U. hordei*. Plasmids containing these genes were prepared from *E. coli* using a QIAprep spin plasmid kit (Qiagen Inc., Chatsworth, CA) according to manufacturers recommendations. The 6 kb *Sac*I fragment containing *pra2* in pJS42 was used for transformation. The 5 kb *Bam*HI/*Sac*I fragment of *mfa2* cloned into pJS42 was used for transformations, as was a subclone consisting of a 550 bp *Hind*III fragment that contains the complete *mfa2* gene. The transformation procedure was followed as described (Sherwood et al., 1998). Transformants were examined for phenotypic differences from race 14 wild-type strains.

DNA Sequencing

DNA fragments from *U. hordei* that hybridized to the *Umpra2* and *Ummfa2* genes from *U. maydis* were subcloned into pBluescript KS+ (Stratagene). The clones were screened for the presence of the desired insert and prepared for sequencing using a

Qiagen plasmid miniprep kit (Qiagen Inc.). The double stranded DNA was denatured by an alkaline method (Hattori and Sakaki, 1986) for sequencing using the dideoxy chain termination method (Sanger et al., 1977) and Sequenase 2.0, following manufacturer recommendations (United States Biochemical Corp. Cleveland, OH). M13 forward and reverse primers were used to obtain initial sequence data. Primers to continue sequencing further into the genes were then designed from this sequence and synthesized (GIBCO-BRL). The 3.5 kb *Sac*I/*Xba*I fragment from *mfa2* was subcloned and partially sequenced. The 400 bp *Sac*I/*Pst*I fragment containing *mfa2* was subcloned and sequenced in both directions. The sequence from the primer mfa2F was done by the Iowa State sequencing facility and has only been done for one direction.

The 1.7 kb *Sac*I/*Xba*I fragment containing the *pra2* gene was subcloned and the entire fragment was sequenced. This fragment was identified by probing a Southern blot consisting of a restriction digest of the whole λ fragment with the *U. maydis pra2* gene. Initially the M13 forward and reverse primers were used for sequencing. Internal primers were then designed based on sequence within the gene fragment and used to sequence both strands. Sequence data were analyzed and sequence alignments were made using the Wisconsin Genetic Computer Group (GCG) sequence database program (Devereux, et al., 1984).

Distance Between the Genes using PCR

Pra2 and *mfa2* were cloned from two different λ phage from the genomic library, since there was not a single case where both probes hybridized to one λ phage. Therefore, the distance between the *pra2* and the *mfa2* genes was not known. PCR primers were constructed based on sequence from the promoter or coding regions of both genes to attempt to amplify the DNA between the two genes. PCR was performed using these primers with genomic DNA from MAT-2 *U. hordei* sporidia. Primers were constructed for amplification in both directions since the orientation of the genes was not known. Each possible combination was used for PCR using an Elongase kit according to manufacturers instructions (GIBCO-BRL) to allow the amplification of large DNA fragments. One combination of primers amplified a 3.9kb fragment that was ligated into pCRII using the TA cloning kit (Invitrogen Corp., San Diego, CA) and transformed into *E. coli* strain DH5α. The clone containing the desired insert was prepared and sequenced to confirm that the primers were annealing to the desired gene sequence.

Gene Disruption Constructs

A unique *Eco*RV site in the *pra2* gene 350bp from the gene start codon was used to construct a plasmid for gene disruption. The *pra2* *Sac*I fragment was first transferred to the plasmid pGEM, which does not contain an *Eco*RV site. The hygromycin resistance cassette was cut out of pJS42 with *Pvu*II. This blunt ended fragment was inserted into the *Eco*RV site of *pra2* (Fig. 3-10). This construct was designated pPra2ko.

A plasmid to disrupt the *mfa2* gene was also constructed. The whole *Sac*I fragment was inserted into pGEM. No unique restriction sites were present in the *mfa2* gene so site directed mutagenesis was performed to create a *Kpn*I site and a *Eco*RV site. The primers for the site-directed mutagenesis were synthesized (GIBCO-BRL) and the mutagenesis was performed with a QuikChange site-directed mutagenesis kit according to manufacturers recommendations (Stratagene). Primers were constructed such that the restriction sites would be inserted and the terminal cysteine residue of the peptide would no longer be present. The hygromycin cassette was then inserted into the *Eco*RV site as described above (Fig. 3-11). This plasmid construct was designated pMfa2ko.

DNA from both knockout plasmids was prepared for transforming with a Qiagen spin prep kit (Qiagen Inc.). MAT-2 race 14 cells were transformed with pPra2ko both supercoiled and linearized with the restriction endonuclease *Kpn*I and pMfa2ko not linearized. Modifications to the transformation procedure (Sherwood et al., 1998) were made to increase transformation efficiencies when transforming without an ARS. The amount of Novozyme 234 (Interspex Products, Foster City, CA) was decreased to 10mg/ml, the amount of KCl in the SCS and STC was decreased to 0.8M and 1M sorbitol was used in the HCM hygromycin plates instead of 1.2M sorbitol. Transformants that grew on the HCM-sorbitol plates containing 150μg/ml hygromycin were transferred to HCM-hygromycin plates without sorbitol. Transformants that grew were tested for their ability to mate with MAT-1 cells on charcoal agar (Martinez et al., 1992).

Synthetic Pheromone

The mfa2 pheromone amino acid precursor sequence was determined from the nucleotide sequence. A peptide that was considered to be the most likely active pheromone was synthesized by Synpep (Dublin, CA) using solid phase Fmoc chemistry. This peptide that was farnesylated (Hrycyna) and carboxyl methyl esterified at the cysteine residue was a gift from Paula Kosted, Montana State University. One microliter of derivatized peptide (1 μg/μl) was added to a suspension of 14**A** and 14**a** cells on TE agar squares (Martinez 93). All versions of the peptide including the farnesylated form, the methylated form and the form both farnesylated and methylated were added to wild-type sporidia. The peptide that was not modified was also added to both cell types at the same concentration. The squares were incubated on a microscope slide in a moist chamber at 16°C and examined microscopically after 24h for conjugation tube formation. The cells combined with the forms of the peptide were compared to wild-type cells that were grown under the same conditions without peptide.

Results

Isolation of *Mfa*2 and *Pra*2 from the Lambda Library

Unique lambda DNAs were identified from the λ 2001 genomic library from 14**a** of *U. hordei* for *mfa*2 and *pra*2. The library was probed with the *Ummfa*2 and *Umpra*2 genes from *U. maydis* but both genes did not cross hybridize to a single λ fragment. The *mfa*2 gene was found on 1λ and *pra*2 on 5λ. The 6 kb *Sac*I fragment from 5 λ and the 5kb *Sac*I/*Bam*HI fragment from 1 λ were each inserted into pJS42. Once these fragments were inserted into the plasmid, restriction digests and southern blots were done to confirm the presence of the genes, construct a restriction map for these regions of DNA and narrow the location of the gene down to a smaller fragment for sequencing (Figs. 3-1 and 3-2).

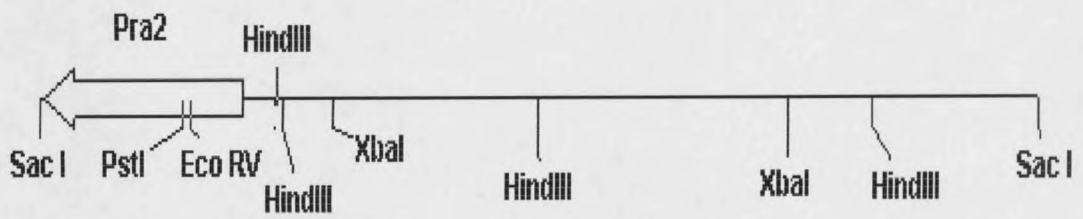

Fig. 3-1. *Pra*2 Restriction Map. Restriction map of the cloned λ fragment containing the *pra*2 gene. The site and orientation of the gene is also shown. This *Sac*I fragment is ~6kb.

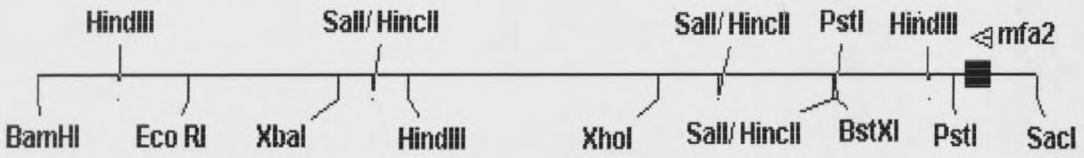

Fig. 3-2. *Mfa*2 Restriction Map. Restriction map of the cloned λ fragment containing the *mfa*2 gene including the site and orientation of the gene. This *Bam*HI/*Sac*I fragment is ~5kb.

Transformation of *U. hordei* Strains
14**A** and 14**a** with *Pra*2 and *Mfa*2 Genes

To show that the cloned genes were active, fragments thought to contain the genes were transformed into wild-type sporidia of both mating types. An ARS was present on the plasmid; therefore, the DNA would not have to integrate to be expressed or replicated. The 14**A** cells transformed with *mfa*2 and *pra*2 differed phenotypically from wild-type 14**A** (Figs. 3-3 and 3-4), while 14**a** transformed cells did not look different from wild-type. The plasmid containing the whole *Sac*I fragment from *pra*2 was used for transformations and transformed 14**A** cells were highly mycelial. The *Bam*HI/*Sac*I fragment of *mfa*2 cloned into pJS42 was first used for transformations. The 550bp *Hind*III fragment thought to contain *mfa*2 was subcloned into pJS42 and also used for transformations. The transformants that contained the *Hind*III fragment had mostly mycelial cells with a lot of branching. The cells transformed with *pra*2 look similar to the cells transformed with the *mfa*2 *Hind*III fragment. The transformants of the plasmid containing the smaller *mfa*2 gene fragment displayed varying degrees of mycelial cells.

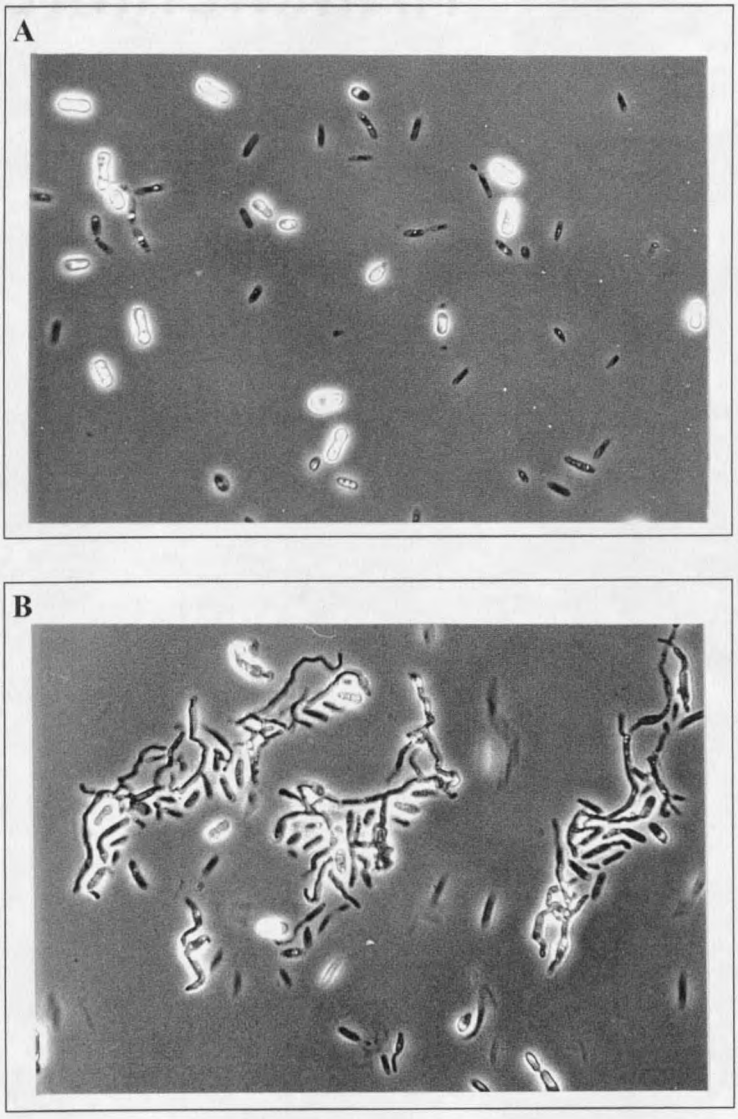

Fig. 3-3. *Pra*2 Transformed Cells. Phase contrast micrographs of *U. hordei* cells transformed with *pra*2 containing *Sac*I fragment in pJS42. **A**) 14**a** transformed with *pra*2. **B**) 14**A** transformed with *pra*2. The 14**a** transformed cells look like wild-type. The 14**A** transformed cells are much more mycelial and the mycelia is branched.
Bar = 20 μm

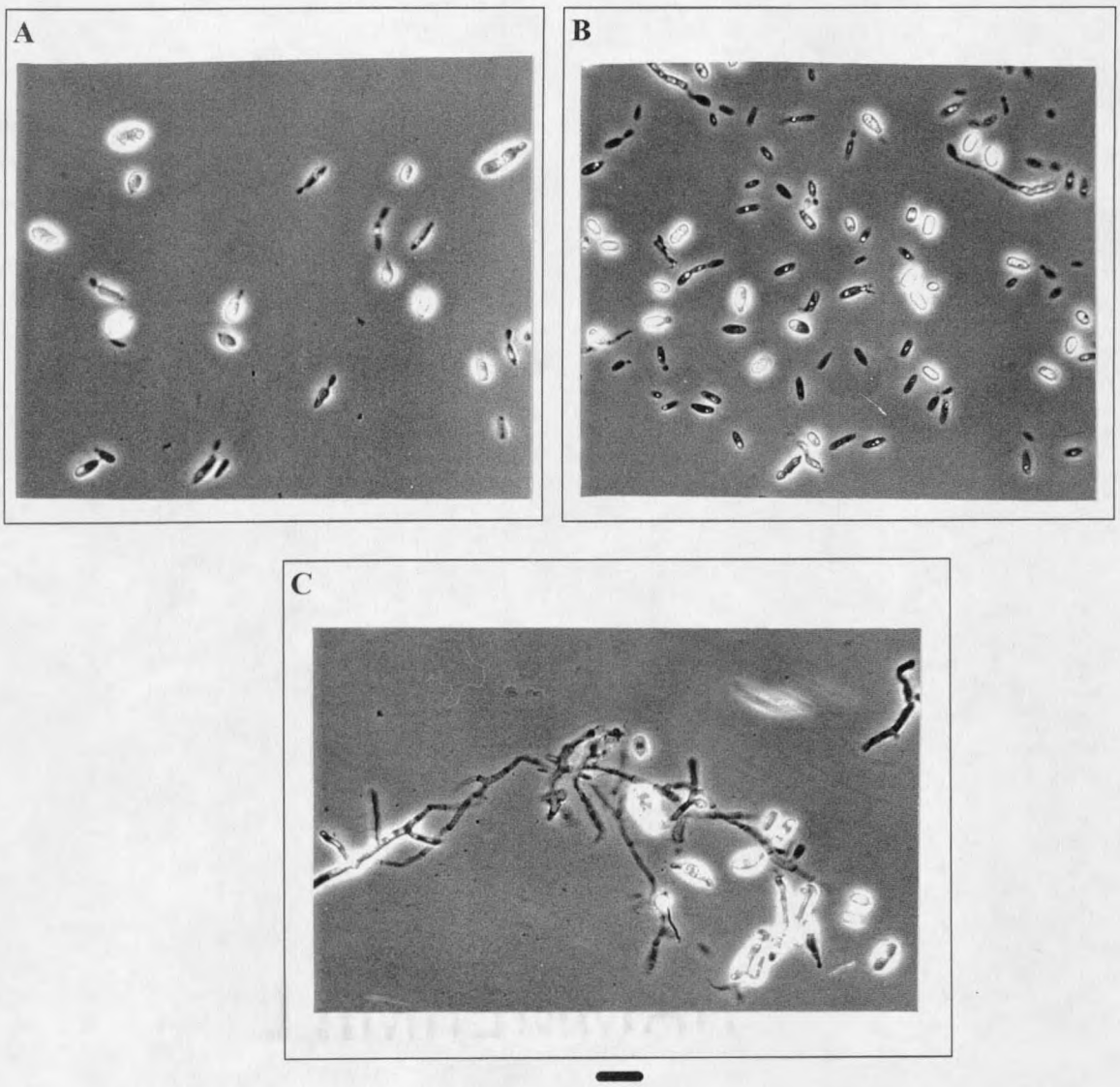

Fig. 3-4. *Mfa*2 Transformed Cells. Phase contrast micrographs of cells transformed with *mfa*2. **A**) 14**a** cells transformed with *mfa*2 *Hind*III fragment. **B**) 14**A** transformed with *mfa*2 *Bam*HI/*Sac*I fragment. **C**) 14**A** transformed with *mfa*2 *Hind*III fragment. The 14**A** cells transformed with *mfa*2 are much more mycelial than 14**a** transformed cells. The 14**A** transformant containing the larger fragment from *mfa*2 shows the typical phenotype from all the transformants identified. A few cells are misshapen and mycelial. The picture shown in **C** is the most mycelial of the transformants identified.
Bar = 20 μm

Sequence of *Pra2* and *Mfa2*

The *pra2* gene was found to be located on the *Sac*I/*Xba*I fragment. This entire fragment was sequenced using the M13 forward and reverse primers and internal primers that were designed by primer walking (Fig. 3-5). The internal primers are indicated on the sequence. The sequences obtained from *U. hordei* were compared to the sequences from *U. maydis* (Bölker et al., 1992). The nucleotide sequence of *pra2*, including the introns, is 64.3% identical between *U. maydis* and *U. hordei*. The nucleotide sequence includes a CAAT motif which is 207 bp upstream of the translation start, ATG. The CAAT motif is the core promoter consensus sequence (Gurr et al., 1987). The second core promoter, a TATAAA motif, is located 28 bases from the CAAT for *U. hordei pra2*. The major transcription initiation point is usually located ~30 bp downstream from this site in higher eukaryotes. The sequence TCTCC is found 25 bp from the TATAAA and is the major transcription initiation site for the *acp*-1 gene from *N. crassa* (Gurr et al., 1987). The putative introns have the typical GTA start and CAG stop. The intron locations were also determined by comparison with the *U. maydis pra2* intron sites and corresponding amino acid sequence. A pheromone response element has been identified in *U. maydis*. In *U. maydis* the short DNA element ACAAAGGGA is present in all genes which are regulated by pheromone (Urban et al., 1996). This sequence was found one time in the promoter region of *pra2*.

Xba I
1 *TCTAGA*GATC AAGGCTTTGG GCCGTCGCCA GCTTTTCCAC AGCAAGCGTG AGTGATGTGG
61 CGACCGTCTA CCGATCATAT TGACGATTTA CTAATCTCGC TGTATCTTTT CATTACATTA
Pra2br→
121 GTCCTGCCTC GCCAGGTAGC CCATTTCTTC ATCAAACTTG CGAAAAGGAC TGAAGGACTG
181 GTGCTGTGAA TGCCCTTAAG CTACAAGCAA TGCAGACAGC GCTTGTCTCT AACATTAATC
Hind III
241 TCTGTCCTGG AATTTCGCCC CACTTCAACG AGCAAAGACT CAACCCTCGT C*AAGCTT*AAA
Hind III
301 **CAAT**AAAACC CATAAAACCT ATAAAAAATT AAA**TATAAA** TTGACAAAAG *CTT*TGGTCGG
Pra2P1→
361 GTTATCTCCC TGCCAAACAC CAAATTTGCG GNGTGTTCCC AGGCTGGTTG **ACAAAGGGA**A
421 GAGTCGATCG ATACCCTGAA AACCGCAGTC ATACATATAT ATAGTGCCTC CTTCGTCCGC
481 CCCAGCTTCG GTTAGTCATG TTCTCACCAA G**ATG**TTCTCT ACAGCCGAAA ACGCCATCTA
541 TGGCGTTCTC TGCCTAATCA CGGCATGTCT TTCCATCTCT TCTCTACCAG TTCACGTCCG
601 GGCGGGGAAC ACAGGGGTCA TTCTCATGAT GTCGTGGTGC TTCGTAGGAC TCTTCAACAA
Pra2rev→ ←
661 GGGTGTCAAC GCTCTCGCGT TCAATCACCG TCTTAACATC TCCTGGAACT TCGGATGCGA
Pra2bf
721 TGTAAGTGCT GTTGTCGAGC GTATCTGGCA GCTCGGCTTG TGCTGTAGCT CCCTTTGCGT
781 TCTACAACGC CTCGAAAGCA TCGCTTCCCT CCGACAGGCG CACTCCACGT ACACGGATCG
*Eco*Rv *Pst* I
841 CCGGCGCCGA GTATGCTTTG ATATCGCAGT CGGTTTGGGT ATACCATTT*C TGCAG*ATCCC
901 TCTCTTCTTC GTGGTTCAGC CATATCGACT CGACGTGGTT GAAGATCTCG GTTGCAGTGC
961 GCCAATCTAC AATTCCGTAC CTGCCTTGTT CGTCTACTAC TTCTGGAGAC TTTTCGTCAG
Pra2for→
1021 CGTTTTGTGC TCTATCTACG CCGgtaagta gtcgtgtgag gggctgcgac tgtataatat
1081 aaactgcata ctcaccaaca gacccatcgc aatacagCTC TTATTCTGAG GTGGTTCATT
Pra2tr→
1141 CTCAGGCGCC TTCAGTTCTC TGCCGCACTC TCGTCGCAAC ACTCCGGTCT ATCGCAGAAA
←Pra2P2
1201 AAATACTTTC GCTTGTTCGC TCTGGCCCTT TGCGAGGCGA CCTTAATCGC CGTTGCCCAG
1261 CTGTATCTTC TCTTTGCGGC ACTTCGGATC ACAGGACTTC TGCCTTACTC CAGCTGGGAA
1341 TACGTGCACT CAGGATTCGC AACGATCAAC TTGGTGCCGA TGGACAGATC AGGTGCCAGC
Pra2P3
1381 TCATCAACAC TGACTTCTTT GGAGGTCTTT AGATGGTTTA CTTTGTCTCC TGGCATTGTG
→
1441 CTTTTTGTCT TCTTCGGTCT CACAGAAGAT GCAAAATCTG CCTATATAGG GTTGTGGCAA
1501 GCTATCAAGA GACTCCGTTT CAAAgtacgg tgagtaatac cgagagcgca cacttcttag
←Pra2tf
1561 ctcagtacgc ctgttgacaa attgtctctc gcttttcaca ggcctagaaa agaatgcgga
1621 ttgaccgaag gacctactgg cttccagGAT AATTCGCTTG ATCTCGACGA TCTCCGCGAG
1681 CAGTCCTATA AAGTTTCGGT CGTAGTTCAC AAAGATGTAA CGGTTCTC**TG** AAAGCTAGTA
Sac I
1721 GTAATCGCGT TGCTGTCTAT CT*GAGCTC*

Fig. 3-5. Sequence of the *Pra2* Gene. The internal primers for sequencing and PCR amplification are labeled above the sequence and underlined and the arrow indicates the direction in which sequence was obtained. The important restriction sites are labeled and italicized. Two putative introns are found in the coding regions and are shown in lowercase letters. The CAAT and TATATAA motifs, the pheromone response element sequence from *U. maydis* and the start and stop codons are in bold.

The 3.5kb *Sac*I/*Xba*I fragment that hybridized to *U. maydis mfa*2 was cloned into pKS for sequencing. Sequencing with the M13 reverse primer identified the promoter sequence and *mfa*2 gene of *U. hordei*. The *Sac*I/*Pst*I fragment was subcloned and sequenced in both directions using the forward and reverse primers. The primer mfa2f was constructed to sequence more of the 3' end of the sequence (Fig. 3-6).

Only 250 bases of the promoter region were available on this piece of DNA from the λ library. The *mfa*2 nucleotide sequence for *U. hordei* also contained a CAAT motif located 161 bp upstream of the translation start site. Thirty base pairs downstream from the CAAT was a TATATAA. The region between the core promoters and the translation start is also very pyrimidine rich. These CT-rich sequences are present in highly expressed genes and may be promoter elements (Gurr et al., 1987). The sequence TCTCC was also found in the promoter region of *mfa*2 located 77 bp from the TATATAA. The transcriptional start site is not known for either of these genes because no mRNA analysis has been done. There appears to be an intron located 18 bases downstream from the translational stop of *mfa*2. The GTG start and TAG stop sequence of introns was present and the resulting intron region is 54 bp. An intron sequence was also found following the stop codon in the *mfa*1 and *mfa*2 genes for *U. maydis* (Bölker et al., 1992).

```
                                        Sac I
   1 GGGGGATCCA CTAGAGCGGC CGCCACCGGT GGAGCTCGAG GATCTCCCTC TGCACTCTAC

  61 TTGCTTCTCA TCAGCGTCGA AAAAAGCAAT CATCACAGCG CTCAAGGACT CATCTAAAGG

 121 TATATAACGG ACGTGTCAAA CTCCTCATCA CCGACATTTT CCCATCAGTC CACATCTCTT
     Primer mfa2R ⟶
 181 CCACACTCTC CACTACACAC TCTCTCTCCT TTCTCACTCT TCGTCTTTCA GACTTTCAAA
                   M  F  S   L  F  E   T  V  A  A   A  V  K   V  V  S
 241 CACCCTTCAC AATGTTCTCT CTTTTCGAAA CTGTTGCTGC CGCCGTCAAG GTTGTCTCTG
     A  A  E  P   E  H  A   P  T  N   E  G  K  G   E  P  A   P  Y  C
 301 CCGCCGAGCC CGAGCACGCC CCTACCAACG AAGGCAAGGG CGAGCCCGCT CCTTACTGCA
     I  I  A  *
 361 TCATCGCCTA AATGGTCCCT ACCGCCATTg tgcacagacc tctaccgtga gtactttctc
               Pst I                 ⟵ primer mfa2R1
 421 ttctcgcttc tgcagtcaaa tagGGGGCGG TGCTGACAAT AGATTCGCTT CTTATTGTGA
                                                              Primer
 481 CGTGGATATA GATGGGCAGA TGCGTCTCAG TGCCGGGATA CACCAGTGCC GGGGATGAGG
      mfa2F ⟶ Hind III
 541 TGAAGAATGG AAGCTTCGAG TAGGTCGAGT TCGAATAGCT CCCGCTTTCA GCCCATGTCC

 601 CTTCCTACTT CTTCTCGGTT ATCGGTAGTC ACGTCCTACC TCTTTTGTAC TTCTTTGTTA

 661 CTTCCTTTAC TCTCCAACGT CGGCCGACGG AATTTCATCG ACATTCCCCC CTTATCTCTA

 721 TCGCTGTGTT GAGTTCTGTG TGATCGTTTG ATATGAGATC GAGTCTCCTT TACCACACGG

 781 ATCAGTTTCG TTGCACTTTC CCAGTTATTC CTTTTGAACG CTTCTTCTTA ATATATGGCA

 841 TTTTCGCGAA AATCGAGCTT TAATTGCCTT TCGTGAATTT GTCATTGGTG CGAGGGCGAA

 901 GACTTAGGCG GAGGGCTTCT GTCGAGTGAG ACCAAGAGGA TCGTCCATCG AGAGCTGCGA

 961 TCCATTTTTG ATAAGGCTTG GAGAAGACTT CGACCCACAT CGTTGGAGGC GCCTGCAGGT

1021 CGACTGGATC GGCTGTGGTG TCTTCGCCGG CAATCTCAGA GTCCTTGAGC TGTTTGGGAC

1081 GGAAGGACCG CAGCTTGACC TTGTGTTCGA CCTTGAGCGG GCCTGGAGAT TCTTCCATTT

1141 TTCGGGACGA TAATCTTCAT CCTCGTCTTC ATTACCGTGC TTGCCACATC ATCGACATTG

1201 ATGCTTCGGA CTGCCTTGCC TTCCCCTTTG CGCGTGTCGA AGGGCTGGCG TTGGCTGTTT

1261 GCCCGGCCTT GTCTGAGCAT TGGGACAGAT CGACTGGAGA
```

Fig. 3-6. Sequence of the *Mfa2* Gene. The relevant restriction sites and amino acid translation are written above the corresponding sequence. The asterisk indicates the stop codon. A potential intron located after the stop codon is indicated by lowercase letters. Primer sequences used for primer walking and PCR amplification of the fragment between *mfa2* and *pra2* are underlined and the arrow indicates the direction of extension. The CAAT and TATATAA box of the promoter are in bold as are the start and stop codons of the exon and intron. The sequence from the *Sac*I site to the *Pst*I site has been sequenced in both directions.

The amino acids were found to be 59.9% identical and 78.6% similar for the *pra*2 genes from *U. hordei* and *U. maydis* (Fig. 3-12). The gene from *U. maydis* had been previously aligned with the STE pheromone receptor from *S. cerevisiae* (Bölker). The hydrophobic domains that correspond to the transmembrane regions were identified for *S. cerevisiae* (Hagen et al., 1986) and are similar for *U. maydis* and *U. hordei.*

```
U. hordei    1 MFSTAENAIYGVLCLITACLSISSLPVHVRAGNTGVILMMSWCFVGLFNK
               |||. ||. :||||||:.:|:|.||  :|:.| |.||:||| |||.||.||
U. maydis    1 MFSGKENVSFGVLCLLAGCISTSSCLIHLQAKNIGVLLMMFWCFTGLVNK

U. hordei   51 GVNALAFNHRLNISWNFGCDVSAVVERIWQLGLCCSSLCVLQRLESIASL
               |:|||||:.|.:.|.:|||:||::||.||:|||||.||||||||:||||
U. maydis   51 GINALAFNNSLRLAWTLGCDLSAIIERTWQFGLCCSALCVLQRLEGIASL

U. hordei  101 RQAHSTYTDRRRRVCFDIAVGLGIPFLQIPLFFVVQPYRLDVVEDLGCSA
               |||||| ||:||: :|::||||:| ||||:||:|||||:|:|::||||
U. maydis  101 RQAHSTVWDRKRRLLIDFGVGLGLPALQIPMFFIVQPYRLNVIENIGCSA

U. hordei  151 PIYNSVPALFVYYFWRLFVSVLCSIYAALILRWFILRRLQFSAALSSQHS
               |:|.||||||:|.:|||:||::|.:||.|:||||:||| ||.||||||||
U. maydis  151 PLYASVPALFIYHLWRLLVSLVCAVYAVLVLRWFMLRRRQFTAALSSQHS

U. hordei  201 GLSQKKYFRLFALALCEATLIAVAQLYLLFAALRITGLLPYSSWEYVHSG
               |||||||||||||||:|| .|:..:|:|::::..|.|.|||||.||. ||..
U. maydis  201 GLSQKKYFRLFALAICERVLVSAGQFYVIIQSLQIGGLLPYTSWAEVHTN

U. hordei  251 FATINLVPMDRSGASSSTLTSLEVFRWFTLSPGIVLFVFFGLTEDAKSAY
               |. | :||:|  : ||  | ||.::|||.|.|::.|||||||||:|.|.|
U. maydis  251 FNRILFVPVDTIAHSS..LLSLSILRWFSLTPAMALFVFFGLTEEAQSVY

U. hordei  301 IGLWQAIKRLRFKD.........NSLDLDDLREQSYKVSVVVHKDVTV
                : |.|: .|   ..         :||||:.: .:: |.||:|::|..:
U. maydis  299 KARWKALINLCSSKGKKQTDGRESLDLEAFESHGSKFSVLVQRDTVI
```

Fig. 3-7. Amino Acid Sequence Comparison Between the *Pra*2 Gene from *U. hordei* and *U. maydis*. A line connecting two amino acids indicates that they are identical and a dot indicates that they are similar. The bold regions are the amino acids that correspond to hydrophobic domains in the STE3 protein from *S. cereviseae.*

The amino acid sequence was also compared for the *mfa2* genes from *U. hordei* and *U. maydis* and were 55.3% identical and 76.3% similar (Fig. 3-8). The amino acid sequence is the predicted pheromone precursor. The precursor would then be processed to obtain the active pheromone. The pheromone from *U. maydis* was determined by amino acid sequencing of an active fraction purified from MAT-2 cells. The cysteine was not identifiable by amino acid sequencing and was deduced to be modified by farnesylation as expected due to the CAAX signal (Spellig et al., 1994).

A)

```
U. hordei     1 MFSLFETVAAAVKVVSAAEPEHAPTNEGKGEPAPYCIIA 39
                |:|:|||||||. .|..||.::|..||.:|:|: ||:||
U. maydis     1 MLSIFETVAAAA.PVTVAETQQASNNENRGQPGYYCLIA 38
```

B)

S-farnesyl
/
Asn-Arg-Gly-Gln-Pro-Gly-Tyr-Tyr-Cys-OCH_3

C)

S-farnesyl
/
Glu-Gly-Lys-Gly-Glu-Pro-Ala-Pro-Tyr-Cys-OCH_3

Fig. 3-8. Amino Acid Sequence Comparison Between the *Mfa2* Gene from *U. hordei* and *U. maydis*. **A**) Comparison of the amino acid sequence from the coding region of *mfa2* for *U. hordei* and *U. maydis*. A line connecting two amino acids indicates that they are identical and a dot indicates that they are similar. **B**) The structure of the MAT-2 pheromone from *U. maydis* after predicted processing (Spellig et al., 1994). **C**) The predicted structure of the MAT-2 pheromone from *U hordei* after processing.

DNA Region Separating *Pra2* and *Mfa2*

Since both of the genes were not found on a single λ fragment, another method had to be used to determine the distance between the *mfa2*and *pra2* genes. Primers from both of the genes were synthesized and used for PCR. The primers pra2bf and pra2br were used from the *pra2* gene (Fig. 3-5). The primers used from the *mfa2* gene were mfa2R and mfa2R1 (Fig. 3-6). The only fragment amplified from all the possible combinations of these primers was a 3.9 kb fragment amplified using the PCR primers pra2bf and mfa2R1. The pra2bf primer was located 200bp from the start codon of *pra2*. It amplifies in the direction of the gene promoter. The mfa2R1 primer was located past the stop codon of the pheromone gene and would amplify in the direction of the promoter. The PCR product was cloned into pCRII and sequenced with the forward and reverse primers. The sequence identified was exactly the same as the pheromone receptor and pheromone gene sequence from the primer sites. This region was mapped and the distance between the genes determined to be 3.6 kb.

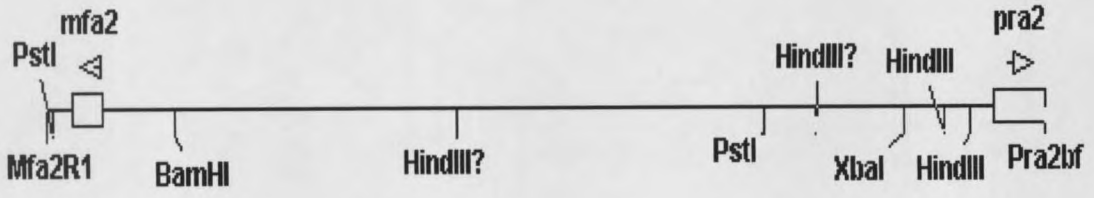

Fig. 3-9. Restriction Map of the DNA Fragment Between *Mfa2* and *Pra2*. Mfa2R1 was the primer used from the *mfa2* gene and Pra2bf was the primer used from the *pra2* gene. The genes are labeled by the open block and orientation of the genes is indicated by the arrow above.

Gene Disruption Experiments

Wild-type 14**a** cells were transformed with the plasmid constructs for disruption of the *pra*2 and *mfa*2 genes (Figs. 3-10 and 3-11). The transformation method was adjusted because the first transformation only resulted in 1-3 transformants with the knockout DNA. The transformation procedure with lower KCl and Novozyme concentrations increased the number of transformants up to 25 to over 100 per reaction. The transformation procedure was repeated numerous times and individual transformants were transferred to HCM containing 150μg/ml hygromycin and then mated on HCM agar containing charcoal. Single colonies of sporidia that were hygromycin resistant were obtained from transforming 14**a** with the interrupted *pra*2 gene. Out of the colonies transferred, 285 grew successfully and were tested for the ability to mate with 14**A** cells. All of the pra2ko transformants tested mated with 14**A** which was easily observed by the appearance of 'fuz' on charcoal agar. Wild-type 14**a** cells were also transformed with pMfa2ko. Over 225 transformants that grew on hygromycin were obtained and combined with 14**A** cells on charcoal agar. All the cells transformed with pMfa2ko mated with 14**A** cells.

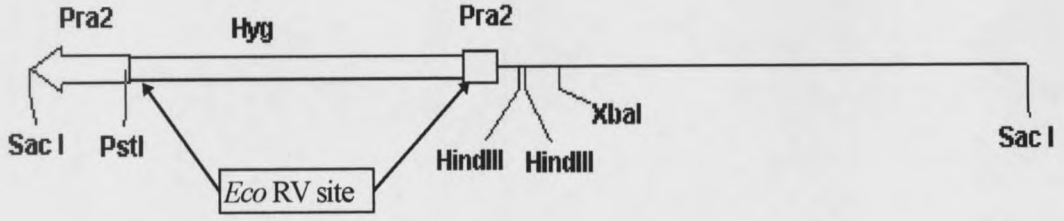

Fig. 3-10. Restriction Map of pPra2ko. Insertion of the hygromycin cassette into the *Eco*RV restriction site of *pra*2. pGEM contains this *Sac*I fragment and the resulting size is 12kb.

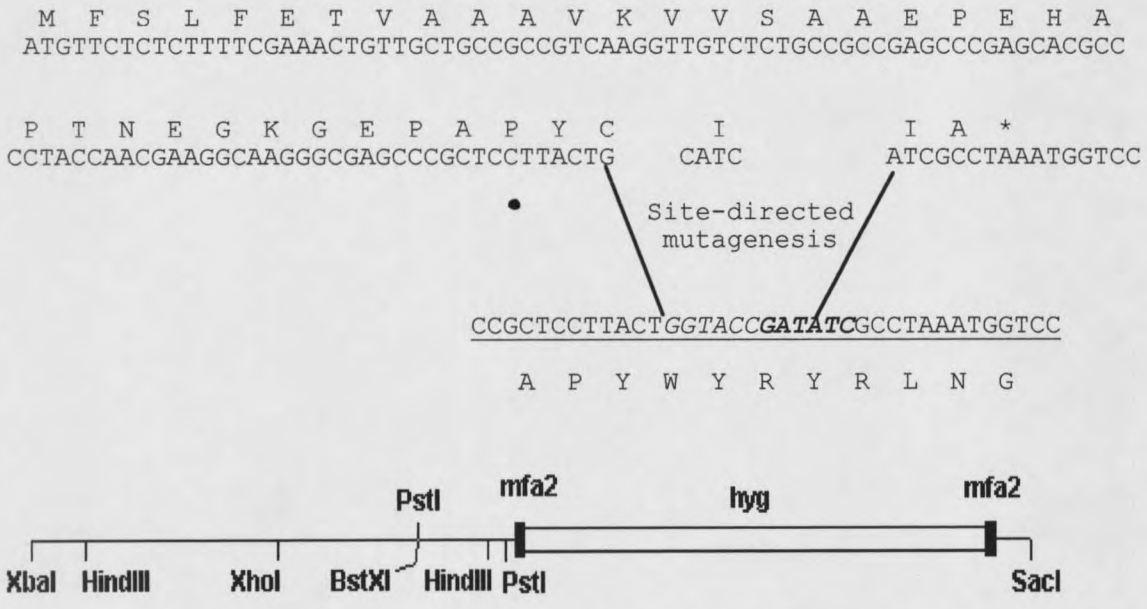

Fig. 3-11. Site-Directed Mutagenesis and Restriction Map of pMfa2ko. The site directed mutagenesis performed with the *mfa*2 gene added a *Kpn*I and an *Eco*RV restriction site at the cysteine residue. The *Eco*RV site is in bold and was the site where the hygromycin cassette was inserted. The amino acid that corresponds to the original nucleotide sequence is positioned above the second nucleotide of the codon. The primer used for the site directed mutagenesis is underlined and the new amino acid sequence which resulted after the mutagenesis is positioned below the primer. The plasmid containing the disrupted gene is 9.5 kb.

Pheromone Peptide Synthesis

A peptide that was considered to be the most likely active pheromone (Fig. 3-8C) was synthesized and added to 14**A** and 14**a** wild-type sporidia. Other pheromones that have been purified were believed to be processed by farnesylation and methyl esterification of the cysteine, cleavage of the peptide after the cysteine and cleavage of the peptide after an asparagine in the amino acid sequence (Spellig et al., 1994; Moore

and Edman, 1993). Cleavage after the asparagine and after the terminal cysteine of the mfa2 precursor for *U. hordei* resulted in a 10 amino acid peptide as determined by translation of the coding region of the nucleotide sequence. The synthesized peptide, EGKGEPAPYC, was farnesylated and carboxyl methyl esterified at the cysteine residue (obtained as a gift from Paula Kosted). All of the forms of the peptide were added to 14**A** cells including peptide alone, methylated peptide, farnesylated peptide and the peptide that was both methylated and farnesylated. The most active form of the peptide was farnesylated and methylated (Fig. 3-11). The peptide alone added to 14**A** cells did not affect the cells. The peptide did not affect 14**a** cells in any of the forms (Fig. 3-11).

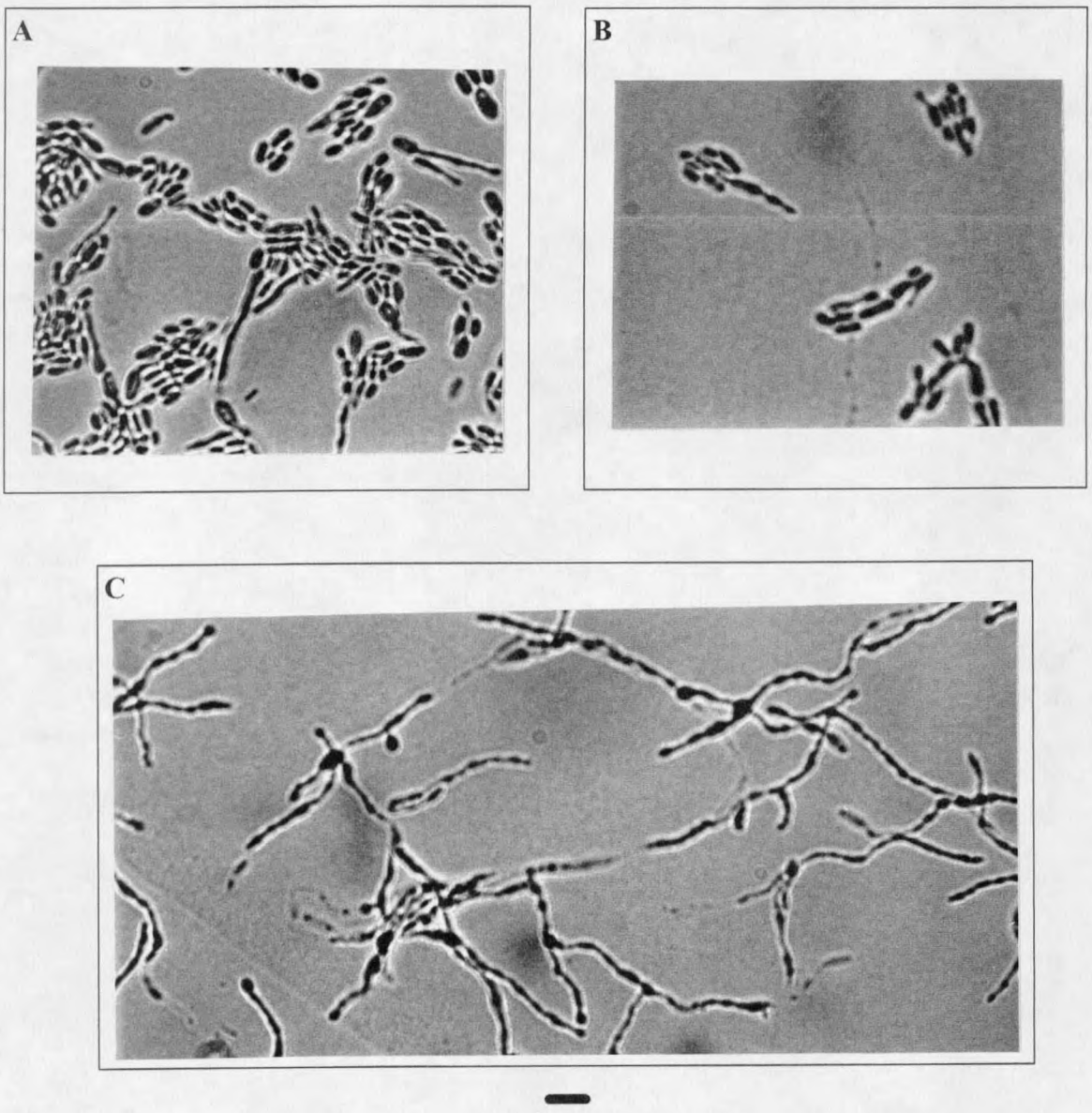

Fig. 3-12. The Effect of Synthetic Mfa2 Pheromone on 14A and 14a Wild-Type Sporidia. **A**) 14**a** + EGKGEPAPYC-farnesyl-OCH3. **B**) 14**A** + EGKGEPAPYC **C**) 14**A** + EGKGEPAPYC-farnesyl-OCH3. The 14**A** cells with just the peptide look like wild-type as do 14**a** cells with the farnyslated and carboxy methylated peptide. The 14**A** cells with the derivatized peptide were very mycelial which indicates conjugation tube formation. Conjugation tube formation is initiated in 14**A** cells with the peptide farnesylated, carboxy methylated or both. Bar = 40μm

Discussion

*Pra*2 and *Mfa*2 Transformants

The expected phenotype was observed for 14**A** cells transformed with the *mfa*2 and *pra*2 genes. If these genes are functional in 14**A** then the cells transformed with *pra*2 would have both the *pra*1 and *pra*2 receptors expressed on the surface of the cell. The *pra*2 receptor on the surface of 14**A** cells would respond to the pheromone produced by these cells. This would result in conjugation tube formation but the growth of the conjugation tubes could not be directional since the pheromone is emitted from itself. This would lead to a mycelial cell which may be branched because it is looking for a conjugation tube from the opposite mating type that is not present.

The *mfa*2 gene in 14**A** would have a similar phenotype. The 14**A** cell has the receptor to mfa2 on its surface which will respond to the pheromone it is now producing itself. Many of the transformants did not display a phenotype significantly different from wild-type 14**A**. This may be due to the fact that only 250bp of the promoter region are available on the DNA fragment used for transforming. This may be enough of the promoter to allow expression of the gene but not at high levels. The *mfa*2 *Hind*III transformant obtained that had an increase in mycelial production may have been inserted into the genome of the fungus in such a way that the expression of pheromone was enhanced. The smaller fragment may have been integrated into the genome more

efficiently than the larger fragment. Thus, transformation with the smaller fragment gave a transformant that was highly mycelial.

Distance Between *Mfa2* and *Pra2*

PCR analysis was the only way found to determine the distance of the DNA fragment between the *pra2* and *mfa2* genes. Sequence analysis confirmed that the primers had annealed to the DNA regions in the genes themselves. The genes were 3.6kb from start to start and were in opposite orientations on the chromosome. Our determination of the orientation of the genes was such that the *mfa2* gene should have been present on the opposite end of the λ fragment containing the *pra2* gene. Sequencing and restriction analysis of both fragments indicated that the *mfa2* gene was not present on the λ fragment even though the distance between suggests that it should be. Therefore, a rearrangement of DNA fragments may have occurred in the construction of the λ library. This is a possibility since the genomic DNA is partially digested to create the library.

Gene Disruption

The plasmid constructs should have interrupted the respective genes if transformation and double cross over occurred in the homologous region of the genome. The interrupted gene should no longer have been functional and, therefore, mating would not occur. The pheromone receptor gene would have been disrupted by the hygromycin

cassette near the middle of the coding frame and would not be functional if inserted with double cross-over at the site of the gene. If the pheromone receptor was not present on the transformed cells, conjugation tubes would not form in the presence of the MAT-1 pheromone and mating would not occur. None of transformants lacked the ability to mate. These transformants must be either the products of single cross-over at the homologous gene, random cross-over events due to nonhomologous recombination, or the plasmid DNA was maintained extrachromosomally.. Transformation of *U. maydis* with circular plasmid DNA primarily resulted in nonhomologous recombination integrative events. When the plasmid DNA was linearized prior to transformation of *U. maydis*, homologous integrative events occurred most often. It was also observed that cells transformed with linearized plasmid DNA could be maintained as concatamers of this DNA in an extrachromosomal state. This was a novel form of replication since the plasmid did not contain autonomous replicating sequences (Fotheringham and Holloman, 1990). For our experiments, transformation efficiencies were increased when linearized plasmid DNA was used. The transformants were only screened for the lack of mating so it is not known whether or not the extrachromosomal replication may have been occurring in *U. hordei*. Double cross-over events may not have occurred in any of our transformations or the loss of function of the pheromone receptor may have been lethal to the cell. The lack of recombination in the mating type locus of *U. hordei* suggests that double cross-over events may be extremely rare in this area and therefore, no knockouts were identified (Bakkeren and Kronstad, 1994).

A similar situation exists for the transformation of a 14**a** cell with the plasmid construct with the pheromone gene interrupted. All of the transformed cells could successfully mate with the opposite mating type. If double cross over had occurred at the homologous region in the genome, the gene would have been intact except for the terminal cysteine and the C A A X motif which is required for farnesylation of the pheromone gene. It has been found in other systems that the pheromone is not active when it is not farnesylated (Spellig et al. 1994; Anderegg et al., 1988). Experiments with the synthetic peptide thought to be the active pheromone, substantiate this observation. The peptide alone did not initiate conjugation tube formation in 14**A** cells but the farnesylated, methylated forms of the peptide did. Thus, the mating assay should have been substantial to determine the loss of pheromone activity in the transformants.

One problem with this pMfa2ko construct may have been the lack of homologous DNA in the promoter region. Only 250bp of homologous DNA were present on the promoter side of the gene which would limit the chance for cross-over in this region. A construct with more of the promoter may improve the chances of getting a double cross-over transformant. It may be that double cross-over did occur but the lack of pheromone farnesylation was lethal to the cell. Thus, any knockout transformants would not divide.

Pra2 and *Mfa2* from *U. hordei*

It was previously shown that the *a* and *b* loci from *U. maydis* hybridized to DNA sequences from *U. hordei* (Bakkeren et al., 1992). This fact allowed the cloning of the MAT-2 *U. hordei* pheromone and pheromone receptor genes by hybridization to the *U.*

maydis Ummfa2 and *Umpra2* genes. Comparison of *pra2* from *U. hordei* with *U. maydis Umpra2* indicated that the nucleotide sequence of the genes are 64.3% identical. Fungal pheromone receptors sequenced thus far seem to be G protein receptors with seven transmembrane regions (Blumer et al., 1988; Bölker et al., 1992, Hagen et al., 1986).

The pheromone genes between *U. hordei* and *U. maydis* were also very similar with the amino acid sequence 55.3% identical. The amino acid sequence of *U. hordei mfa2* was found to have a C A A X motif for prenylation of the pheromone. This CAAX motif is found in other fungal pheromones including *U. maydis* mfa1 and mfa2 and *S. cerevisiae* **a**-factor. The structure of the *S. cerevisiae* **a**-factor pheromone was shown by mass spectrometry to be methyl esterified and farnesylated at the terminal cysteine (Anderegg et al., 1988). Farnesyl is a isoprenoid lipid molecule containing 15 carbon atoms (Caldwell et al., 1995). The enzyme that attaches the farnesyl to the peptide has been identified and called farnesyl transferase (Boyartchuk et al., 1997). It is thought that the attachment of this isoprenoid lipid to the peptide is necessary to direct the protein to the membrane so that it can be excreted from the cell (Caldwell et al., 1995). A signal is necessary for movement to the membrane because the peptide is too small to contain the transmembrane sequence of about 25 lipid soluble amino acids that normally occurs in proteins targeted for membranes. The pheromone and pheromone receptor from *U. hordei* can now be further analyzed to determine if they are processed as expected from homology to other pheromones and receptors.

SUMMARY

Ustilago hordei is an important barley pathogen that can cause a reduction in yield and contamination of seed with smut spores (Mathre, 1997). Fungicidal seed treatments effectively control the disease (Johnston and Mathre, 1983) but it is necessary to have alternatives in case isolates resistant to the fungicide become established in the population or if fungicides are unavailable (Ben-yephet et al., 1974). One alternative to fungicide use is to plant resistant varieties. Several resistance genes are known but breeding for resistance to *U. hordei* is not widely done. One hindering factor is the confusion surrounding the knowledge of available resistance genes. Many discrepancies are found in the literature concerning resistance to *U. hordei* (Thomas, 1988). The ability to detect the fungus in the leaf tissue of inoculated plants may help clear up the confusion by more quickly determining if a plant is resistant. The use of PCR as described here would help determine if the fungus is excluded from entering the plant or if its growth is inhibited once it enters. The PCR primers may also be useful in breeding programs to determine at an earlier stage of growth if a plant is susceptible or resistant to *U. hordei*. This method is less invasive and the meristems of the plant are not destroyed.

PCR primers Uh1 and Uh4 were designed from the ITS regions to take advantage of the variability of these regions between related species and the high copy number of the ribosomal genes (White et al., 1990). Thus, the primers uniquely amplify DNA from *Ustilago* spp. and can detect *U. hordei* DNA from a mixed preparation. Experiments

with PCR amplification of DNA from leaf tissue of a barley differential set demonstrated the presence of the fungus in the first leaves of resistant plants. The fungus was not detectable in the later leaves of symptomless plants. Therefore, if the fifth or sixth leaves of a plant give a positive PCR product, these experiments determined that the plant may be considered susceptible to *U. hordei*. This PCR detection method is highly sensitive and can theoretically detect as few as two teliospores. This result has both positive and negative features. The method is definitely sensitive enough to detect fungal mycelia in leaf tissue but false positives due to contamination are also possible. The material to be detected would have to be isolated from any sources of contamination such as smutted plants. If the necessary precautions are taken and controls are included with the DNA extractions and PCR reactions, this method should be very useful in early detection of *U. hordei* in barley leaf tissue.

Another possible alternative to the use of fungicides is to eliminate the pathogenic stage of the fungus. Since the fungus has to mate to be pathogenic it may be possible to block mating and thus block pathogenicity (Kronstad, 1995). The analysis of the pheromone and pheromone receptors from *U. hordei* may help determine a way to control mating. A mating inhibitory factor has been found that inhibits conjugation tube formation in *U. hordei* (Sherwood et al., 1998). Further knowledge of pheromone binding and the signal transduction cascade that initiates conjugation tube formation may expose other methods to control pathogenicity by blocking mating. The sequence of the *pra2* gene from *U. hordei* is closely related to other fungal pheromone receptors that respond via a G protein phosphorylation cascade (Bölker et al., 1992; Hagen et al., 1986;

Blumer et al., 1988). Many steps in this pathway may be inhibited and lead to a loss of mating and pathogenicity.

The sequence of the *mfa2* and *pra2* genes from *U. hordei* can now be analyzed along with the other fungal pheromones and pheromone receptors. A problem encountered in this analysis of the MAT-2 mating factors of *U. hordei* was the inability to obtain transformants that did not mate. Over 500 cells transformed with either the interrupted *pra2* or *mfa2* genes mated successfully with MAT-1 cells. This result could be due to the lack of recombination in the MAT locus of *U. hordei.* The lack of recombination results in a bipolar mating type instead of the tetrapolar mating type found in *U. maydis* (Bakkeren and Kronstad, 1994) The lack of a knockout mutation in the large number of transformants tested may also be due to a lethal mutation that occurs when the mating type genes are disrupted. Further analysis of the mating type genes from *U. hordei* may answer some of these questions.

The cloning of *pra2* and *mfa2* will allow for experiments to analyze expression of the genes. It is not known if the pheromones from both mating types are constituitively expressed or if the expression of one is induced upon receptor binding to the other pheromone. Experiments have shown that MAT-1 cells form conjugation tubes before MAT-2 cells when opposite cell types are placed on adjacent agar squares. The pheromone produced by MAT-2 also seems to be much more diffusible than the MAT-1 pheromone (Cynthia Anderson, Montana State University, personal communication). Messenger RNA expression experiments would also determine the expression of the genes in the cells transformed with *pra2* and *mfa2*.

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