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).

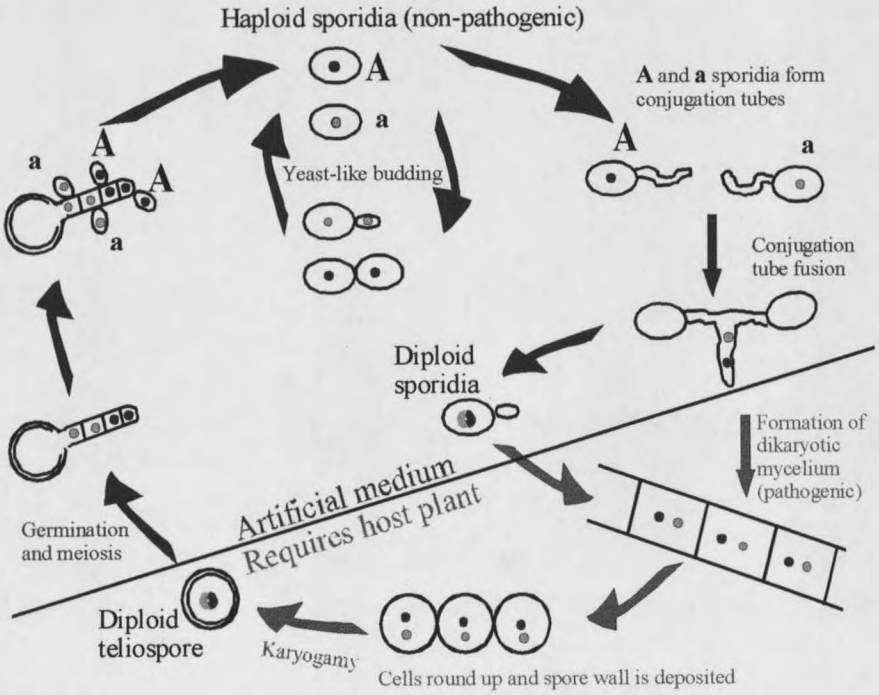

Fig. 1-1. Life Cycle of *Ustilago hordei*.

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.