



Morphological, genetic and molecular analysis of the mating process in *Ustilago hordei*
by Alfredo Dick Martinez-Espinoza

A thesis submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in
Plant Pathology

Montana State University

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Abstract:

Ustilago hordei is a pathogenic basidiomycete that causes covered smut of barley. Mating in *U. hordei*, which is controlled by a single locus with two alleles, results in the conversion of haploid, non-pathogenic yeast-like sporidia to dikaryotic, pathogenic mycelium. Mating is one of the most important steps in the *U. hordei* life cycle, initiating the sexual phase of this fungus. An improved medium to test the mating reaction and genetic complementation was developed. The addition of activated charcoal to a variety of media enhanced the stability and intensity of the mating reaction. Complementation assays using minimal media containing activated charcoal demonstrated allelism of auxotrophic mutations. The ease and reliability of the mating test allowed for rapid identification of the mating type of unknown isolates and progeny of crosses, and also provided a dependable procedure for performing complementation assays. A comprehensive morphological analysis of the mating process was performed. Evidence of mating-type specific, diffusible, small molecular weight factors was found when sporidia separated by a dialysis membrane were capable of inducing conjugation tube formation by cells of the opposite mating-type. This indicates that these compounds were necessary for recognition of opposite mating-type cells. Genetic control of the mating process was analyzed by the use of mating defective mutants, which were isolated using charcoal-containing media. These mutants were phenotypically characterized and arranged into several classes. These classes indicate the complexity of the genetic control of mating in *U. hordei*. Molecular analysis of the mating process is also described. DNA fragments from *U. maydis* which encode pheromones (*mfa1* and *mfa2*) and pheromone receptor (*pra1* and *pra2*) hybridize with *U. hordei* DNA. *Inf1*, *mfa2*, and *pra2*-like fragments were cloned from a *U. hordei* A (MAT-1) mating-type strain. Mating-type of *U. hordei* is proposed to be determined by specific sequences that encode a pheromone and a pheromone receptor, which serve as mechanisms of cell-to-cell recognition in the initial stages of the mating process.

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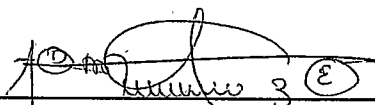
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TO MY WIFE ELVIA
TO MY DAUGHTER ERIKA
TO MY PARENTS LUCITA AND NICANOR

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ABSTRACT

Ustilago hordei is a pathogenic basidiomycete that causes covered smut of barley. Mating in *U. hordei*, which is controlled by a single locus with two alleles, results in the conversion of haploid, non-pathogenic yeast-like sporidia to dikaryotic, pathogenic mycelium. Mating is one of the most important steps in the *U. hordei* life cycle, initiating the sexual phase of this fungus. An improved medium to test the mating reaction and genetic complementation was developed. The addition of activated charcoal to a variety of media enhanced the stability and intensity of the mating reaction. Complementation assays using minimal media containing activated charcoal demonstrated allelism of auxotrophic mutations. The ease and reliability of the mating test allowed for rapid identification of the mating type of unknown isolates and progeny of crosses, and also provided a dependable procedure for performing complementation assays. A comprehensive morphological analysis of the mating process was performed. Evidence of mating-type specific, diffusible, small molecular weight factors was found when sporidia separated by a dialysis membrane were capable of inducing conjugation tube formation by cells of the opposite mating-type. This indicates that these compounds were necessary for recognition of opposite mating-type cells. Genetic control of the mating process was analyzed by the use of mating defective mutants, which were isolated using charcoal-containing media. These mutants were phenotypically characterized and arranged into several classes. These classes indicate the complexity of the genetic control of mating in *U. hordei*. Molecular analysis of the mating process is also described. DNA fragments from *U. maydis* which encode pheromones (*mfa*₁ and *mfa*₂) and pheromone receptor (*pra*₁ and *pra*₂) hybridize with *U. hordei* DNA. *mfa*₁, *mfa*₂, and *pra*₂-like fragments were cloned from a *U. hordei* A (MAT-1) mating-type strain. Mating-type of *U. hordei* is proposed to be determined by specific sequences that encode a pheromone and a pheromone receptor, which serve as mechanisms of cell-to-cell recognition in the initial stages of the mating process.

CHAPTER 1

LITERATURE REVIEW

The PathogenEconomic Importance of the Pathogen

Covered smut of barley, caused by *Ustilago hordei*, is distributed worldwide and seems to be more extensively dispersed than either true loose smut or semi-loose smut (Mathre, 1982). Losses from the disease in areas where seed treatment is not practiced are economically important (Mathre, 1982). Grain quality and yield reduction represent the main monetary losses. Barley with more than a small percentage of heads infected with covered smut is not acceptable according to federal grain standards (Mathre, 1982; Zillinsky, 1983).

In areas of the Middle East and Africa where growers sow untreated seed, covered smut continues to cause economic losses (Mathre, 1982). In the prairie provinces of Canada, including Manitoba, Saskatchewan and Alberta, overall yield losses from barley smuts were calculated to range from 0.7%-1.4% during the years 1978-1982, representing considerable monetary losses. The largest economical loss on the prairies of Canada was \$17.6 million in 1981. Other major losses in those provinces include \$1.1 million in 1980, \$10.9 million in 1978 and \$5.8 million in 1979. The maximum incidence of *U. hordei* was in 1980 at 0.9%. In addition, up to 25% of the plants were infected with

U. hordei in several fields in 1980 and 1982 (Thomas, 1984). In 1983-1988, again in the prairie provinces of Canada, annual losses were between 0.7% to 1.6% representing economic losses of \$8.8 to \$13.2 million. *U. hordei* was the most significant smut in Canada in 1987, having the highest level of infection in that year compared to other smuts. In addition, up to 40% covered smut infection was observed in a field in 1987 in Manitoba and 30% infection in a field in Alberta in 1988 (Thomas, 1989a) being the most important smut in that year in Alberta (Krehm and Beauchamp, 1989). *U. hordei* was present in the provinces of Saskatchewan and Manitoba in 1989 (Weller and Rossnell, 1990) with an average of 70% of the fields infected and an average incidence of infection of 0.8% (Thomas, 1990). It is thought that economic losses occur throughout the world due to smuts, and particularly to covered smut. However, strict surveys like those carried out in Canada have not been widely implemented.

Taxonomy and Description of the Pathogen

The causal organism of covered smut is *Ustilago hordei*, a fungus classified in the division *Eumycota*, and the subdivision *Basidiomycotina*. It belongs in the class *Hemibasidiomycetes* in which the promycelium usually has crosswalls, the order *Ustilaginales* where the promycelium has lateral sporidia at septa, and the genus *Ustilago* (Webster, 1989).

The teliospores or resting structures of the fungus are globose, subspherical to spherical, 5-8 μ m in diameter, brown to dark brown, with a smooth surface and a lighter colored side which is presumably

where germination takes place (Tapke, 1937). The teliospores germinate forming a four-celled basidium called the promycelium from which four oblong to ovate hyaline basidiospores termed sporidia grow. Sporidia are 9-11 μm long and 4-6 μm wide, and represent the haploid phase of the disease (Dinoor and Person, 1969). They bud in a yeast-like fashion to produce numerous secondary sporidia (Fisher and Holton, 1957); fusion of sporidia of opposite mating-type results in the infectious dikaryotic mycelium (Dinoor and Person, 1969; Mathre, 1982).

Teliospores germinate on standard mycological media (Vogel, 1956; Lade, 1967; Holliday, 1974; Hellmann and Christ, 1991), including water agar (Henry et al., 1988, personal observation). Some reports state that peptone plays a role in growth of the fungus (Fisher and Holton, 1957). Maltose, sucrose and glucose seem to be the best carbon sources (Fisher and Holton, 1957), and nitrate is the best nitrogen source. The pH of the medium does not appear to have a strong influence on teliospore germination or growth of the fungus (Fisher and Holton, 1957). Teliospores germinate to produce the promycelium in which meiosis occurs and results in four haploid sporidia which continuously produce haploid, uninucleate sporidia. Although early studies reported teliospore formation on artificial media (Fisher and Holton, 1957), formation of diploid teliospores occurs exclusively in the plant (Thomas, 1988).

Host Range

U. hordei has been reported to occur naturally on rye in Siberia, and on several grasses including *Agropyron cristatum* and *Elymus glaucus*

in the USA (Aisworth and Sampson, 1950). Artificial inoculations using paired sporidial cultures of covered smut from these hosts produced infection on the following grasses: *Agropyron caninum*, *Elymus canadiensis*, *Elymus glaucus*, *Elymus sibiricus*, *Hordeum nodosum* and *Sitanion jabatum* (Fisher, 1939). Other hosts reported include *Agropyron semiconstatum*, *Avena byzantina*, *Avena fatua*, *Avena orientalis*, *Avena sativa*, *Elymus canadiensis*, *Elymus excelsus*, *Elymus glaucus*, *Hordeum bulbosum*, *Hordeum vulgare*, *Hordeum vulgare* var. *trifurcatum*, several *Hordeum* spp. and *Secale cereale* (Zundel, 1953).

Symptoms of Covered Smut

The characteristic symptom of the disease is a purple- black fungal mass of teliospores (sorus) of about 6-10 mm in length. The teliospores are contained within a rather persistent membrane that surrounds the seeds on the entire head of the host (Dickson, 1956). The sorus is enclosed in the floral bracts of the spikelet which frequently appears at maturation of the host (Fisher and Holton, 1957). Smutted heads tend to emerge later than healthy heads or in many cases are trapped in the sheath of the flag leaf. Occasionally, depending on environmental conditions and physiological races, smut teliospores also develop in leaf blades as long streaks or in nodal tissue (Mathre, 1982; Gaudet and Kiesling, 1991).

Disease Cycle

The smutted heads are broken and crushed during harvest, depositing inoculum in the soil and on the surface of healthy seeds. Teliospores are the resting stage and survive in this form between

growing seasons. Teliospores germinate at the same time that seeds germinate (Mathre, 1982). Germination of teliospores in the soil is enhanced by moderate moisture (50%) and cool soil temperatures (10-15°C) (Tapke, 1948). *U. hordei* sporidia were found on hulls of germinating seeds and the infective dikaryotic mycelium colonized the seeds and germinating tissue (Kozar, 1969b). Infection occurred through the coleoptile, and the mycelium advanced through the host tissue and became established behind the growing point where a profuse, thick, branched mycelium was observed. Plants developed more infected tillers when the pathogen penetrated the entire length of the coleoptile (Thomas, 1976; Kozar, 1979b). As the plant grows, the mycelium maintains its position within the growing point until flowering time. Then the fungus permeates the tissue of the ovary and teliospore formation begins by segmentation and rounding of intercellular mycelium that forms the mass of teliospores that replace the seed. The teliospores are contained within a persistent membrane that surrounds the entire head and are released at harvest (Mathre, 1982).

Physiological Specialization

Physiological specialization describes the traits of morphologically similar species that differ from each other in one or more physiologic characters. Specialized races of *U. hordei*, based on pathogenicity, were first indicated in 1924 when 5 different "biological" forms were identified on 4 barley cultivars, Nepal, Hannchen, Texas winter, and Summit (Faris, 1924). Two distinct physiological races were subsequently found in Canada which were

readily distinguished by their reaction on Eureka and Hannchen (Aamodt and Johnston, 1935). From 200 collections of *Ustilago hordei* from 26 states, 8 races were determined based on the differential reaction to 5 barley cultivars. Race 6 was found in 21 of 26 states and in 114 of the 200 collections. Later, in Canada, evidence of 4 new races was reported (Fisher and Holton, 1957). However, three of them proved to be the same when tested on more cultivars.

Thirteen physiological races were identified in 1945 (Tapke, 1945), 5 from a collection of 244 isolates and 8 from previous studies from a total of 444 collections from 33 states. Race 6 was widespread and comprised 61.3% of total collections. The most prevalent races were 1, 5 and 6. In California and Washington, race 6 was the most prevalent. Not infrequently, minor differences in size, teliospore color, spore wall smoothness, compactness of the smutted heads and spore mass, degree of awn destruction and degree of smutted head emergence from the boot have been associated with the different physiological races of *U. hordei*.

Fourteen races are now recognized for *U. hordei* after a new race with a unique pathogenicity pattern was obtained in an inbreeding study of race 8 (Pedersen and Kiesling, 1979).

Control

U. hordei has been adequately controlled by chemical seed treatments. In the early 1900's, formaldehyde was used as a seed disinfectant which gave complete control. However, formaldehyde, besides being a health hazard, causes injury to the seed if not

properly applied (Fisher and Holton, 1957). Later, organic mercuric compounds such as Ceresan (ethyl mercury chloride) were used effectively to control covered smut. However, these compounds were not widely accepted due to health hazards and high cost (Fisher and Holton, 1957). To date, effective control of covered smut can be achieved with several fungicides, including benomyl (Alcock, 1980) and carboxin (Ballinger, 1980). Formulations containing Carboxin and triadimenol also gave complete control (Johnston and Mathre, 1980; Sheridan, 1980).

Because of the success of chemical seed treatment, little effort has been placed on the development of resistant cultivars. However, there are many cultivars that have resistance to some of the 14 physiological races including Excelsior, Hannchen, Himalaya, Lion, Nepal, Pannier, and Trebi (Tapke, 1945). Other resistant cultivars include the six-rowed hulled types Atlas, Sacramento, Velvet, Shaw, Sol and Success, the two-rowed hulled types Spartan, Golden, Pheasant and Horn and the hulless types Himalaya, New Era, Russian and Burbank (Aamodt and Johnston, 1935). Other cultivars resistant to several races of the fungus include BJ28, and C163 (Bedi and Singh, 1972), BHD22, EB582, EB570, EB905, EB 2100, EB 2505, EB 2507, EC24882 and K572/11 all of which seem to be immune to 5 races of *U. hordei* (Shrivastava and Shrivastava, 1976).

Major gene resistance is conditioned by a single dominant gene in some lines, while in others two or three independent factors are known (Fullerton and Nielsen, 1974; Thomas, 1988). Minor genes influencing partial resistance are also known (Emara and Sidhu, 1974). Partial resistance is expressed as partial infection in which not all heads of

the plants become infected (Groth et al., 1976; Mathre, 1982).

However, depth of sowing, soil type and temperature at which inoculated plants are grown can also influence the occurrence of partial smutting (Groth et al., 1976; Mathre, 1982).

Genetic mechanisms of host resistance will be described in detail in the following sections.

Genetics of *Ustilago hordei*

Mutation and Genetic Complementation

The *Ustilago hordei* life cycle is particularly well suited for genetic studies. In the asexual cycle of *U. hordei*, haploid, yeast-like, non-pathogenic cells, which are the result of meiosis from the resting sexual spores (teliospores), are produced. Daughter sporidia are produced by budding. Mutation of *U. hordei* has proven to be performed with relative ease. Mutation rate, lethality and interactive influences affecting mutation were determined by Hood (1968), including the effect of the stage of the cell cycle and repair processes.

Hood (1968) isolated three hundred and twenty three auxotrophic mutants using UV irradiation and selection on minimal media. Three hundred were identified with respect to their special nutritional requirements. Genetic analyses of ninety of these mutants were made using crosses between mutants and wild-type strains. This analysis showed that haploid segregants had the same nutritional requirement as the parents, indicating stability of the mutation (Hood, 1968).

Mutation using UV irradiation combined with inositol starvation seemed to increase the mutation frequency, compared with irradiation

alone (Thomas, 1972). 1.9% of the survivors using UV irradiation followed by inositol starvation were found to carry a nutritional requirement in addition to inositol, whereas 0.11% and 0.17% were recovered using UV irradiation by itself and 0.27% by inositol starvation alone. From these mutants, 118 required an exogenous source of amino acid including serine, leucine, isoleucine and valine, methionine, arginine, lysine, phenylalanine, histidine, and tryptophan. Thirty-six mutants required adenine and 58 required vitamins including niacin, pyridoxin, pantothenic acid and biotin. Interestingly, 1140 mutants required proline in addition to inositol (Thomas, 1972).

Fifty-two auxotrophic, morphological and temperature sensitive mutants were isolated following UV irradiation, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and ethyl methane sulfonate (EMS) (Henry et al., 1988). Auxotrophic strains included 3 adenine (*ade*) mutants and 9 vitamin-requiring strains including nicotinic acid, pantothenic acid, inositol and pyridoxine. Twenty-three amino acid-requiring strains were auxotrophic for arginine, phenylalanine, proline, tryptophane, lysine, histidine, valine, arginine/valine, methionine/lysine, lysine/nicotinic acid, and valine/inositol. In this study 10 morphological (*morph*) mutants were identified by their cauliflower-like appearance rather than the wet yeast-like colony typical of the wild type. These mutants had greatly reduced numbers of sporidia, which were distributed in chains rather than solitary sporidia. Four temperature-sensitive mutants were also obtained. Two mutants were shown to be heat sensitive and two were cold sensitive (Henry et al., 1988).

Fungicide resistant strains have also been obtained. Benomyl, chloroneb, thiabendazole and triadimenol resistant strains were isolated using UV irradiation and MNNG treatments. The inheritance of fungicide resistance was determined (Henry et al., 1985a; Henry et al., 1985b; Henry et al., 1987).

Complementation studies in *U. hordei* have been used to determine allelism of auxotrophic mutations. Genetic complementation tests with 58 arginine requiring mutants were performed (Dinoor and Person, 1969). Dikaryotic hyphae formed when haploid lines of the opposite mating-type containing complementary mutations were mixed on minimal media. Ten different complementation groups were identified, indicating that mutations in 10 different loci which control arginine biosynthesis were obtained (Dinoor and Person, 1969). Henry et al. (1988) performed genetic complementation tests on *U. hordei* auxotrophic mutants, and of twenty one compatible pairs with different biochemical requirements, seven did not complement (i.e., form dikaryotic mycelium). However, some did form mycelium when tested with the standard mating types, indicating some interference with heterokaryon formation between those mutants. However, of the 13 compatible pairs with similar biochemical requirements, four did not complement, indicating allelism (Henry et al, 1988).

Interspecific Hybridization and Echinulation

The fact that several smuts parasitize the same host, e.g. *U. nigra*, *U. hordei* and *U. nuda* on barley and *U. kolleri* and *U. avenae* on oats, and the probability that these fungal species coexist in

nature leads to ample opportunity for cross-hybridization in nature. Interspecific hybridization can result in the development of new physiological (pathogenic) races or species. Intra- and inter-specific hybridization have been repeatedly demonstrated (Holton, 1931; Fisher, 1951).

U. hordei seems to hybridize readily with other species including *U. nigra* (Fisher, 1951). Spore cell wall morphology or ornamentation, i.e., smooth, echinulated or wart-like characteristics, has been used as a genetic marker to measure interspecific hybridization. The first hybrids reported were between *U. hordei* and *U. medians* (*U. nigra*) using monosporidial lines of each fungus (Allison, 1935). Head type was intermediate with sori enclosed in a durable but rather loose membrane, and all teliospores were echinulated. Factors for mating-type, pathogenicity, head type and spore cell morphology segregated independently (Allison, 1935).

U. hordei and *U. bullata* which infect both *Agropyron* and *Elymus* spp. also hybridized under experimental conditions (Fisher, 1951). Nine hybrids were recovered; the F_1 spores were echinulated and lighter colored on two sides (*U. hordei* has small, 5-8 μm long smooth teliospores which are lighter colored on one side while *U. bullata* has longer, 8-12 μm , echinulated, dark spores). Teliospore progeny were characterized, and one F_2 hybrid yielded the same spore wall morphology as the F_1 , but several F_2 hybrids yielded diverse spore wall morphology including (1) small echinulated spores which were lightly colored on one side, (2) small smooth spores which were lighter on two sides, (3) small smooth spores which were lightly colored on one side,

(4) large echinulated spores with one side lightly colored, (5) large spores with uniformly colored walls, and (6) large smooth spores with uniformly colored walls. Pathogenicity was the same as in the parents (Fisher, 1951).

Hybrids between *U. hordei*, *U. nigra*, *U. avenae* and *U. kolleri* have been obtained using a common host *Agropyron tsukushiense* var. *transciens* (Huang and Nielsen, 1984). Genetic relationships of these species were based on their ability to cross and the distribution of genes for spore morphology. *U. hordei* strains from 10 collections from 10 countries on 5 continents had an echinulation genotype E_1e_2 resulting in smooth spores. All hybrids between *U. hordei* (smooth spores, E_1e_2) and *U. kolleri* genotype e_1E_2 (smooth spores) had echinulated teliospores. E_1 and E_2 acted as dominant and complementary genes. Teliospore hybrids between *U. nigra* (in which echinulation is conditioned by two dominant, complementary genes E_1 and E_2) and *U. hordei* (E_1e_2), segregated as if echinulation was controlled by a single dominant gene, where E_2 from *U. nigra* was complementing e_2 . Huang and Nielsen, (1984) concluded that a diploid teliospore is echinulated only if it carries both dominant E_1 and E_2 genes. Size of echinulations among hybrids between *U. hordei* and *U. nigra* seems to be affected by a recessive gene(s) found in *U. hordei* that modifies the expression of the major genes (E_1E_2) required to produce echinulation when backcrossed to the smooth-spored parent (*U. hordei*). Echinulation is not affected by the microenvironment. Sorus type seems to be associated with teliospore ornamentation. The least prominent echinulation had a sorus type like that of *U. hordei* while more

prominent echinulation had a sorus type like *U. nigra* (Thomas, 1989b). Recently, hybrids between *U. kolleri* (echinulated spores) and *U. hordei* (smooth spores) were found to possess surface ornamentation which was only 30% of the diameter of normal echinulations and only visible using scanning electron microscopy (Thomas, 1991).

Somatic Recombination

Evidence for parasexual recombination in *U. hordei* has been described (Kozar, 1969a; Megginson and Person, 1974). Compatible combinations of mutant sporidia requiring isoleucine and valine were used in one experiment. Isolation of mononucleated sporidia from excised infected plant tissue grew in minimal media, suggesting that the novel genotypes recovered arose via somatic recombination. Evidence of diploidization was found as tested by DNA content, ability to grow in minimal media and results from the Bauch test (Kozar, 1969a).

Somatic recombination was probably responsible for the genotypes of teliospores after inoculation with a mixture of three different haploid sporidia (Megginson and Person, 1974). The teliospores contained the genetic markers (arg-, leu- and mating type) from all three parents. Somatic recombination was assumed, since inoculations were arranged in a way that the parasitic phase would be completed only if an unusual event had taken place. In addition, parasexual recombination was assumed responsible for the spontaneous appearance of niacin deficiency in sporidial progeny (Megginson and Person, 1974).

Virulence

Genetic analyses of virulence of *U. hordei* on barley supported a gene-for-gene relationship in the *Ustilago-Hordeum* system (Person et al., 1986). Early experiments using tetrad analysis to follow segregation of virulence in *U. hordei* suggested that a single recessive allele for virulence was responsible for a level of infection of 5% in two cultivars of barley. Later it was demonstrated that virulence was due to recessive alleles at different loci for each of the cultivars Excelsior, Lion, Pannier, Trebi, while the gene for virulence on Hannchen was dominant (Thomas, 1988).

Specific genes for virulence of *U. hordei*, *Uhv1* through *Uhv6*, have been identified. *Uhv1* and *Uhv2* are effective against resistance genes in barley on a gene-for-gene basis, *Uhv1* against *Uhr1* in Hannchen and Vantage and *Uhv2* against *Uhr2* in Excelsior. *Uhv3* is a recessive gene, apparently linked to *Uhv2* for virulence on Nepal and Pannier. *Uhv4* and *Uhv5* are duplicate recessive genes at two different loci that control virulence on Lion and Plush (Thomas, 1976).

Despite the polygenic effects that modify avirulence genes (Emara and Sidhu, 1976), environmental influences (Ebba and Person, 1969; Thomas, 1988), and presence of genes influencing virulence (Christ and Person, 1986), the gene-for-gene hypothesis is still the most viable explanation for the basic compatibility or incompatibility of the barley-*U. hordei* interaction (Christ and Person, 1986).

Aggressiveness

The term aggressiveness has been used to describe the variation in degree of infection of susceptible cultivars by the pathogen. The genetic determinants of aggressiveness in *U. hordei* have been and still are the subject of intense study (Thomas, 1988).

Several compatible sporidial lines of *U. hordei* have been found to infect barley cultivars at rates ranging from 1.7% to 41.9% infection (Christ and Person, 1986). This variability was due primarily to additive genetic effects and, to a lesser degree, to genetic interactions such as dominance and epistasis (Christ and Person, 1986). Genetic modification of the expression of *Uhv1* on the cultivar Vantage was studied using two teliospore types. Teliospores homozygous for *Uhv1* were selfed in all possible combinations. Forty-three percent of the infection variability was caused by additive genes and 22% by non-additive genes. Alleles for greater aggressiveness were dominant and heterosis was evident (Emara and Sidhu, 1974). Infection ranging from 15% to 90% on the variety Hannchen due to the effect of *Uhv1* was reported (Thomas, 1988). Twenty-eight percent of this variation was found to be due to *Uhv1*. The rest of the variation was due to the environment (Thomas, 1988). Aggressiveness was also examined on the cultivar Odessa (Thomas, 1988). Race 10 appeared to be homozygous for genes affecting aggressiveness, another dikaryon from unrelated sporidia was heterozygous for polygenic factors affecting aggressiveness. Sixty percent of the genetic effect was additive and 40% was non-additive. One factor affecting aggressiveness appeared to be linked to the mating type locus (Thomas, 1988).

All the studies mentioned above used percentage of infection as the only criterion for aggressiveness. However, variation in aggressiveness among and within races of *U. hordei* was recently reported (Gaudet and Kiesling, 1991) using other components than percent of infection. Aggressiveness was measured according to several components including peduncle compactation, extent of sorus formation in heads, leaves and nodes and plant dwarfing. Aggressiveness should be determined using several criteria besides percentage of infection (Gaudet and Kiesling, 1991).

Molecular Genetics

Two dimensional, isoelectric-focusing/SDS polyacrylamide gel electrophoresis was used to compare polypeptide extracts from teliospores of several *Ustilago* spp, including *U. hordei* (Kim et al., 1984). The same technique was applied to *U. hordei* isolates with known genes for virulence. An initial correlation of a dominant-allele for avirulence with a polypeptide was later modified when tetrads from a heterozygote were backcrossed to the virulent parent. The polypeptide segregated independently from avirulence (Thomas, 1991).

Isozyme variation has also been used to try to define races using starch gel electrophoresis (Hellman and Christ, 1991). Isozymes from different races were obtained and single bands (alleles) were detected for aconitase, adenylate kinase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase and peptidase. Two alleles were found for isocitrate dehydrogenase and malate dehydrogenase and 3 alleles for phosphoglucomutase. However,

racess could not be separated solely by means of this isozyme analysis (Hellmann and Christ, 1991).

One approach toward molecular isolation of fungal genes is to develop a transformation system that allows cloned DNA sequences to be introduced and expressed in fungal cells. A genetic transformation system for *Ustilago hordei* has been reported (Holden et al., 1988). This method consists of the preparation of spheroplasts of *U. hordei* by the use of Novozyme 234 utilizing early log phase cultures, transforming with the plasmid pHL1 linearized at the *Xho*I site. The transformation was confirmed by Southern blots of DNA extracted from the transformants. Transformation frequencies of 10 to 50 resistant colonies per microgram of DNA per 2×10^7 spheroplasts were obtained. The majority of the transformed cells retained resistance to hygromycin B through many mitotic divisions (Holden et al., 1988). An improved transformation procedure has also been described (Duncan and Pope, 1990). Protoplasts were recovered after treatment with KCl buffer solution and Novozyme 234. Stabilized protoplasts were treated with the plasmid pCM54 which contains the hygromycin resistant gene and an autonomously replicating sequence (ARS) from *U. maydis*. The reported transformation rate was 3 to 14 times higher than that previously reported. However, no Southern blot hybridization was done to conclusively demonstrate transformation.

Electrophoretic karyotypes have been determined for monosporidial strains of the 14 races of *Ustilago hordei*. Chromosomal DNA of sporidia was separated by contour-clamped homogeneous electric-field gel electrophoresis (CHEF) (McCluskey and Mills, 1990). A unique

karyotype was observed for each strain. The number of bands for each race varied from a minimum of 15 to a maximum of 19 with approximate length distributions from 170 to 3150 kb. Karyotypes were conserved among members of individual tetrads and between tetrads representing the same race. Southern hybridization was used to map genomic fragments and heterologous conserved genes to chromosomes (McCluskey and Mills, 1990). Electrophoretic karyotyping has also been reported without the need of making protoplasts (McCluskey et al., 1990). Genome plasticity using probes from chromosome-specific DNA libraries was also determined. The demonstration that most single copy probes hybridized with chromosomes of similar size suggests that the variability in karyotype is not generated by translocation (McCluskey and Mills, 1991). Karyotype analysis has led to new approaches to study fungi whose cytogenetics can not be studied by conventional means. In *U. hordei* karyotyping has helped to improve the knowledge of the cytology of this fungus, i.e., number and sizes of chromosomes (Thomas, 1991).

Mating-Type Genetics

Sexuality or mating in fungi has been recognized as one of the most interesting aspects of the biology of this group of organisms. Mating has been found to be very diverse in a wide variety of fungi (Raper, 1966). Different mechanisms have evolved in various groups of fungi to favor cross-mating which enhances outbreeding or promotes inbreeding to preserve the most fit genotypes (Fincham et al., 1979).

Sexual dimorphism is one of these mechanisms of sexuality and it occurs in a number of forms. Most of these cases occur in the *Phycomycetes*. However, this feature is relatively uncommon in fungi as a group (Raper, 1966).

Incompatibility systems are far more prevalent as a means of determining mycelial interactions. There are two basic types of incompatibility among fungi, *heterothallism* (*homogenic incompatibility*) and *homothallism* (*heterogenic incompatibility*). *Heterothallism* prevents self-fertilization, thus restricting inbreeding and promoting outbreeding, while *homothallism* favors inbreeding and self-fertility (Raper, 1966; Fincham et al., 1979).

There are a number of important differences between incompatibility systems. These systems can be distinguished by two major criteria; number of loci and number of alleles at each locus. *Bipolar* and *tetrapolar* are terms that refer to systems with either one or two loci and describe the number of mating types possible among the progeny of a single cross. A bipolar cross yields 2 mating types, while a tetrapolar cross yields 4 mating types. *Biallelic* and *multiallelic* are terms used to distinguish systems with two alleles per locus or those with an large number of alleles.

The types of incompatibility systems that occur in various groups of fungi may be generalized. *Phycomycetes*, especially those belonging to the order *Mucorales*, have a bipolar, biallelic mating system. The *Ascomycete* yeasts have a bipolar biallelic system while the *Euscomycetes* have a similar bipolar, biallelic system but each cell has the capability of acting as either male or female. *Basidiomycetes*

in the *Uredinales* also have a bipolar biallelic system with each cell having the capability of acting as either male or female. In the *Ustilaginales*, some species (i.e., *U. hordei*) have a bipolar, biallelic system while other species (i.e., *U. maydis*) have a tetrapolar system which is biallelic for one locus and multiallelic for the other locus. In either case, mating types are morphologically identical.

Basidiomycetes in the *Hymenomycetes* and *Gasteromycetes* have either a bipolar or tetrapolar multiallelic mating-type system (Raper, 1966).

There are extensive literature reviews on the control of heterothallism or sexuality in fungi (Whitehouse, 1949; Burnett, 1956; Olive, 1958; Raper, 1959; Raper, 1960; Raper, 1966; Nasmyth, 1982) which cover mechanisms of regulation, characteristics of the systems, hypotheses, terminology and biological significance of the mating process. In addition, there are several mating systems that have been well characterized and the molecular mechanisms have been described. A brief review of these systems will be discussed here to give a better understanding of mating. The mating process for several smuts will be also described.

Saccharomyces cerevisiae, an ascogenous yeast, has a bipolar mating system. Mating is determined by the *MAT* locus. Cells with *MAT_a* are referred to as *a* cells while α cells carry the *MAT α* locus. Cells of the opposite mating type can mate while cells of the same mating type cannot. Recognition of cells of the opposite mating type is accomplished by secretion of *a* and α pheromones which bind to receptors on the surface of opposite mating-type cells. Cells fuse to produce an *a*/ α diploid which carries the *MAT_a* and the *MAT α* alleles (Herskowitz,

1988). Switching from one allele to other at the *MAT* locus can occur in haploid cells; thus a colony which begins from a single cell of one mating type can quickly become a mixture of both mating types. This is due to two silent loci designated *HML* α and *HMR* α in which copies of α and *a* reside regardless of the information contained at the *MAT* locus (Hicks et al., 1979; Herskowitz, 1988). Switching is induced by a dominant allele *HO* which encodes an endonuclease that acts on these loci. In the switching process, a copy of *HML* or *HMR* replaces the *MAT* allele which changes the mating-type. The "cassette" model has been suggested for this process in which *MAT* is the active "cassette" or locus carrying the information which identifies the mating-type of the cell. *HML* and *HMR* are silent "cassettes". Switching occurs when the active "cassette" is replaced by information from the opposite mating-type silent "cassette" (Nasmyth, 1982; Herskowitz, 1988).

The structure of the mating-type locus has been elucidated for *S. cerevisiae* (Nasmyth et al., 1981; Draginis, 1990). *MAT* α and *MAT* α contain unique DNA sequences *Y* α and *Y* α , but also contain similar regions common to both mating-types called *W* and *X*. The *Y* region is flanked by a *Z1* and a *Z2* region which are common to both mating types. Unique transcripts from *Y* α and *Y* α , called *a1* and *a2* and $\alpha1$ and $\alpha2$, code for DNA binding proteins that control several target genes (Nasmyth et al., 1981). *a*-specific genes are expressed in *a* cells but are repressed in α cells. *a*-specific genes also include structural genes which are induced by exposure to α factor. α -specific genes, which include the *a*-factor receptor gene, are induced by $\alpha1$ and expressed on α cells (Draginis, 1990). Other molecular mechanisms of regulation of the

"active" and "silent" cassettes have been described, including the *SIR* (Silent Information Regulator) locus, which represses the silent cassette (Draginis, 1990), and regulation of the *HO* gene which initiates the switching. Similarities to the "cassette" model of *S. cereviceae* have been found in the fission yeast *Schizosaccharomyces pombe* (Kelly et al., 1988). Molecular techniques of restriction analysis, Southern blot hybridization and heteroduplex analysis revealed that mating switching occurs by transposition (Beach, 1983). Nucleotide sequences and the mechanism of regulation have been described (Kelly et al., 1988).

The Ascomycete heterothallic fungus, *Neurospora crassa*, has two mating types designated A and a. The mating type alleles A and a control mating in the sexual cycle and also function in heterokaryon incompatibility in the vegetative cycle. The molecular mechanisms of mating type control have been elucidated and the mating-type locus has been cloned. The mating-type clones contain unique DNA segments that are flanked by common DNA sequences (Glass et al., 1988).

In the *Hymenomycetes* and *Gasteromycetes*, which belong in the *Basidiomycetes*, two loci systems are prevalent. Compatibility is controlled by the action of two independent loci A and B (Raper et al., 1958; Day, 1960). In the *Hymenomycetes* each mating-type locus has a large number of alleles. In *Schizophyllum commune* 96 A alleles and 56 B alleles were found in a worldwide survey (Raper et al., 1958). These loci actually consist of four multiallelic genes, $A\alpha$, $A\beta$, $B\alpha$, and $B\beta$ with 9, 32, 9 and 9 alleles respectively estimated to exist worldwide (Raper, 1959; Raper, 1966, Ullrich, 1978). The four mating type genes

are linked in two pairs, $A\alpha B\beta$ and $A\beta B\alpha$, on two separate chromosomes. Complete sexual development occurs only when two strains with allelic differences at $A\alpha$ and $A\beta$ and $B\alpha$ and $B\beta$ mate. A series of distinct developmental events is initiated to convert the two haploid strains into a fertile heterokaryon, with A and B alleles controlling part of the development (Giasson et al, 1989). A alleles regulate nuclear pairing, nuclear conjugation, cellular division, and hook cell formation. B alleles regulate septal dissolution and nuclear migration (Raper, 1966). Two $A\alpha$ mating-type alleles have been cloned ($A\alpha 1$ and $A\alpha 4$) from *Schizophyllum* using chromosome walking with a cosmid library. Transforming DNA is active in the trans configuration, suggesting that $A\alpha$ encodes a diffusible product. Restriction mapping analysis has shown that $A\alpha 1$ and $A\alpha 4$ are located in the same physical region of this chromosome but within a subregion that contains extensive sequence divergence (Giasson et al., 1989).

In the *Ustilaginales* incompatibility systems are defined as being either bipolar, one locus with two alleles, or tetrapolar, biallelic for one locus and multiallelic for the other locus.

Ustilago violacea, the anther smut fungus which systemically infects many species of the *Caryophyllaceae* family, has a bipolar mating-type locus termed a_1 and a_2 , which act as a "developmental master switch" controlling the entry into several pathways including vegetative budding, conjugation, dikaryotic hyphae and sporulation (Day and Garber, 1988). Mating-type alleles a_1 and a_2 remain inactive at high temperatures (20-30°C) or in the presence of cations, such that cells continue to bud vegetatively under these conditions (Day, 1979).

When cells containing the two mating-type alleles are brought together, developmental changes occur, going from a saprophytic, yeast-like form to a mycelial form which is characteristic of the parasitic phase (Castle and Day, 1989). Host products can activate or induce mating and mycelium formation (Castle and Day, 1984). One of these products has been isolated and characterized using HPLC, UV absorption spectroscopy and mass spectrometry. The compound has been identified as α -tocopherol (vitamin E). Natural α -tocopherol, synthetic α -tocopherol and other tocopheroles (β , gamma, σ) are very active at low concentrations. It appears that α -tocopherol acts on the mating-type locus affecting gene regulation (Castle and Day, 1984), inducing mycelial formation only in compatible mixtures of haploid sporidia. During conjugation of *U. violacea*, cells of the opposite mating type first pair tightly and then develop a conjugation tube or bridge between them. Recognition of opposite mating-type cells seems to be established by long fine hairs or fimbriae. Fimbriae are not essential for cell pairing but may be involved in later stages of conjugation including development and direction of conjugation tube growth toward opposite mating-type cells. This suggests that fimbriae serve as communication structures between cells or in transporting inducer molecules for the initiation of conjugation tube development (Day and Poon, 1975; Day, 1976; Day and Garber, 1988).

Ustilago maydis or corn smut is a heterothallic fungus with tetrapolar mating-type loci and is another example where the mating-type genetics have been well characterized. Two distinct forms characterize the *U. maydis* life cycle, a unicellular haploid form which

is non-pathogenic and divides as yeast-like cells (sporidia) and the dikaryotic filamentous form which is pathogenic and whose growth is dependent on the living corn plant where it causes tumors on the leaves, tassels and ears (Christensen, 1963). The transitions between forms are governed by two separated loci, the *a* locus and the *b* locus. Formation of the filamentous dikaryon is only possible when haploid cells heterozygous for both the *a* and *b* loci are present. If either identical *a* or identical *b* alleles are present, the filamentous dikaryon form is not developed. The *a* locus has two alleles, a_1 and a_2 , and has been proposed to control the fusion of haploid cells (Rowell and DeVay, 1954; Rowell, 1955; Holliday, 1961). Recently, the two alleles of the *a* locus have been cloned (Froelinger and Leong, 1991; Bolker et al., 1992). a_1 and a_2 mating-type clones contain non-homologous DNA segments which are flanked by similar nucleotide sequences. There is a single copy of either the a_1 or a_2 mating-type sequence within each haploid genome. Each allele is composed of a set of two genes, one encoding the precursor of a polypeptide which appears to be a mating factor (pheromone) and the other which is thought to code for the receptor of the pheromone secreted by cells of the opposite mating-type. The *a* mating-type alleles are proposed to provide the components involved in cell-to-cell signalling (Bolker et al., 1992).

The *b* locus is more complicated, with at least 25 naturally occurring alleles having been described (Rowell and DeVay, 1954; Puhalla, 1968). The *b* locus regulates hyphal growth and pathogenicity of the dikaryon formed by the fusion of two haploid sporidia. The *b*

alleles have to be heterozygous to form hyphae and to induce symptoms in the host.

A variety of molecular mechanisms have been proposed to explain recognition of identical versus non-identical alleles of *Basidiomycetes* incompatibility loci (Kuhn and Parag, 1972; Ullrich, 1978). One hypothesis is that the functional products of the *b* alleles are nucleic acids, either DNA or RNA, and recognition involves information found in the heteroduplex structures (Ullrich, 1978). According to another scheme the *b* locus codes for multimeric proteins (Parag, 1972). Recently four alleles from the *b* locus of *U. maydis* have been cloned (b_1 , b_2 , b_3 and b_4) (Kronstad and Leong, 1990; Schulz et al., 1990). They were found to contain a single open reading frame (ORF) which would encode a protein of 410 amino acids. The protein would have a variable N-terminal region and a highly conserved C-terminal region (60% and 93% identity respectively). The polypeptide appears to be a DNA binding protein containing a motif related to a homeodomain in their constant region (Schulz et al., 1990). It is suggested that *U. maydis* *b* alleles code for polypeptides whose association yields a regulatory protein that governs the developmental program and pathogenicity of this organism (Kronstad and Leong, 1990; Schulz et al., 1990).

In the rest of the *Ustilaginales*, including those that infect small-grain cereals, the mating-type loci are still an enigma in regard to their structure and mechanisms of control. However, it can be generalized that most of the small-grain infecting *Ustilago* species have a bipolar mating-type system (Thomas, 1991). *Ustilago nigra*

(Thomas, 1988), *Ustilago avenae*, *Ustilago kolleri* (Nielsen, 1988), *Ustilago nuda*, *Ustilago tritici*, *Ustilago bullata*, *Ustilago aegilopsis*, *Ustilago phragica* and *Ustilago turcomanica* all have a bipolar mating system, with one locus with two alleles, A and a (Nielsen, 1988; Thomas, 1991). In *Tilletia* spp. a single mating-type locus (bipolar system) has been reported. Multiple alleles apparently occur within *Tilletia controversa* (Hoffmann and Kendrick, 1969) and *Tilletia indica* (Duran and Cromarty, 1977; Royer and Rytter, 1985).

Ustilago hordei has one mating-type locus with two alleles which have been arbitrarily assigned the symbols A and a or "plus" and "minus". Fusion of compatible sporidia can be determined by the "Bauch" test which results in the production of aerial hyphae. Fusion between A and a sporidia results in the production of the dikaryotic hyphae that can infect the host (Fisher and Holton, 1957; Dinoor and Person, 1969; Thomas, 1988).

Recently, a survey of several cereal smut fungi was performed using the cloned a and b mating-type loci from *U. maydis* as probes (Bakkeren et al, 1992). Homology to these loci was found in *U. hordei*, indicating the presence of these loci in this fungus (Bakkeren et al., 1992).

CHAPTER 2

IMPROVED MEDIA FOR TESTING THE MATING REACTION AND
GENETIC COMPLEMENTATION OF *USTILAGO HORDEI*Introduction

Ustilago hordei is a basidiomycete which causes covered smut in barley (*Hordeum vulgare* L.) (Mathre, 1982). *U. hordei* grows asexually as budding, yeast-like sporidia on a variety of rich and minimal media. It is heterothallic, with mating controlled by a single mating-type locus with two alleles designated A and a (Thomas, 1988). When sporidia of opposite mating type are mixed, they are joined by a conjugation bridge. Conjugation initiates a number of developmental changes, including the transition from non-pathogenic, uninucleate sporidia to pathogenic, dikaryotic mycelium. While the asexual cycle can be completed on artificial media, the sexual cycle must be completed on the host. Infection occurs through the coleoptile after which the dikaryotic hyphae advance through the host tissue and become established in the plant meristem (Kozar, 1969b). During flowering, *U. hordei* ramifies throughout the ovarian tissue, forming a fungal mass which differentiates into sexual teliospores in place of the seed (Kozar, 1969b).

While teliospore formation and other late stages of the sexual cycle require growth on the host, the early stages can be monitored

ex planta (Thomas, 1988, and references cited therein). The ability to detect mating of *U. hordei* on artificial media is important for several reasons. First, the mating type of an unknown isolate can be determined without infecting the host and waiting several months until an infected head emerges. Secondly, as previously shown by Dinoor and Person (1969), complementation tests can be performed to determine allelism of mutations in cells of the opposite mating type; non-allelic mutations will complement each other and form dikaryotic mycelium. Finally, as shown with *U. maydis*, the ability to induce and detect dikaryotic mycelium on artificial media can be used to select mating-type mutants (Banuett and Herskowitz, 1988), and for cloning mating-type genes (Kronstad and Leong, 1989; Schulz et al., 1990).

Investigators studying *U. hordei* have long expressed dissatisfaction with the equivocal mating reaction which forms on a variety of agar media (Thomas and Person, 1965; Lade, 1967; Person et al., 1986; Henry et al., 1988; Thomas, 1988). Media containing activated charcoal have been used successfully to enhance the mating reaction of *U. maydis* (Day and Anagnostakis, 1971). I have evaluated the benefit of adding activated charcoal to several complete and minimal media and tested different incubation conditions in an attempt to enhance the *ex planta* mating reaction of *U. hordei*. The suitability of charcoal-supplemented minimal medium for genetic complementation studies is also described.

Materials and Methods

Ustilago hordei Strains

Ustilago hordei (Pers.) Lagerh. strains I4, mating-type A (ATCC #34037) and E3, mating-type *a*, (ATCC #34038) were both obtained independently from Dr. Barbara Christ, Pennsylvania State University and Dr. David Pope, University of Georgia. In keeping with guidelines proposed by Yoder et al. (1988), I am proposing that the mating-type locus of *U. hordei* be designated *MAT1*, and that the A allele be *MAT1-1* and the *a* allele be *MAT1-2*. Since *U. hordei* has only one known mating-type locus (Thomas, 1988), the allele designations can be abbreviated as *MAT-1* for the A allele and *MAT-2* for the *a* allele. Thus, I4 has the *MAT-1* allele and E3 has the *MAT-2* allele. Four sporidial cultures, MSU-1 through MSU-4, were used throughout this study. These were isolated as an unordered tetrad from a teliospore taken from a naturally infected barley head in Montana. In addition, random sporidial isolates of both mating types were isolated from teliospores of races 8 (strains 8A and 8a) and 14 (strains 14A and 14a). Teliospores of races 8 and 14 were kindly supplied by Dr. W. Pedersen, University of Illinois.

Auxotrophic Mutants

Sporidial strains 8A and 14a were grown in liquid Holliday's complete medium (HCM) (Holliday, 1974) to early log phase (Hood, 1968). The sporidia were adjusted to 1×10^3 cells/ml in sterile distilled water and plated on HCM agar. Uncovered petri plates were irradiated with UV light (234 nm wavelength, lamp "A" GE) 30 cm from the UV

source. Irradiation for 40 s (strain 8A) and 35 s (strain 14a) resulted in 90% killing of the cells. Following irradiation, the plates were covered and incubated for 24 h in complete darkness at 20°C, after which they were transferred to a 12 h cycle of light/darkness at 20°C. After 5-6 days of incubation, colonies were replica plated onto Vogel's minimal medium (VMM) (Vogel, 1956) and HCM agar. These plates were incubated at 20°C for two days. Cells that grew on HCM but not on VMM were picked from the master plate, transferred to HCM agar, and incubated as above. This plate was used to replica plate the colonies onto VMM supplemented with amino acids, vitamins, and nucleotides divided into 12 pools as described by Holliday (1956). Putative auxotrophs were purified and retested for auxotrophy and reversion rates.

In order to obtain sporidia of different mating type with a known allelic mutation, strains 8A-1 (*MAT-1 his*) and 14a-2 (*MAT-2 cys*) were crossed. To perform the cross, the sporidia were suspended in sterile distilled water, mixed, and inoculated onto susceptible barley seeds (Odessa, C.I. 934) using the partial-vacuum method described by Tapke and Bever (1942). All *U. hordei* strains and mutants used in this study are described in Table 1.

Media

Several media were tested for suitability in detecting mycelial dikaryons as an indication of successfully mated cultures. These included HCM; Holliday's minimal medium (HMM), double strength Holliday's complete medium (2xHCM) (Holliday, 1974), and VMM.

Table 1. *Ustilago hordei* strains used in this study.

Strain	Genotype	Source
I4	MAT-1 ^a	B. Christ and D. Pope
E3	MAT-2	B. Christ and D. Pope
MSU-1	MAT-1	M. Bjarko ^b
MSU-2	MAT-2	M. Bjarko
MSU-3	MAT-1	M. Bjarko
MSU-4	MAT-2	M. Bjarko
8A	MAT-1	Random isolate from a race 8 teliospore
14a	MAT-2	Random isolate from a race 14 teliospore
8A-1	MAT-1 <i>his</i>	UV-induced His- auxotroph of 8A
14a-2	MAT-2 <i>cys</i>	UV-induced Cys- auxotroph of 14a
814-11	MAT-2 <i>his</i>	Segregant from 8A-1 x 14a-2
8A-6	MAT-1 <i>arg</i>	EMS-induced Arg- auxotroph of 8A; K. Dugan
14a-1	MAT-2 <i>arg</i>	UV-induced Arg- auxotroph of 14a
8A-3	MAT-1 <i>ilv</i>	UV-induced Ilv- auxotroph of 8A
14a-4	MAT-2 <i>ilv</i>	UV-induced Ilv- auxotroph of 14a
8A-2	MAT-1 <i>pro</i>	UV-induced Pro- auxotroph of 8A
14a-10	MAT-2 <i>pro</i>	EMS-induced Pro- auxotroph of 14a; K. Dugan

^a MAT-1 and MAT-2 are the proposed gene designations for mating types A and a, respectively.

^b MSU-1, -2, -3, and -4 were obtained as an unordered tetrad from a single teliospore isolate from Montana.

Activated charcoal (Sigma Chemical Co., St. Louis, MO, USA) was added to each medium at 1% or 5% (w/v). Mating reactions were tested on each medium at 6°C, 16°C, 20°C, and 25°C, using precision incubators (Sherer Dual Jet, Nor-Lake Inc., Hudson, WI, USA) with a fixed light/darkness cycle of 12 h.

The effect of pH on the mating reaction was tested on HMM and HCM with and without 1% activated charcoal. The pH of the media was adjusted to 5.5, 6.5, or 7.5 with 1 M NaOH or 1 M HCl. Duplicate plates were incubated at either 6°C or 20°C. An additional experiment was performed to evaluate the effect of the C:N ratio of the media.

The carbon level of HMM with 1% activated charcoal was adjusted by adding 0.1x, 1x, and 2x the normal concentration of dextrose (1x = 10 g/l). The same was done for the N source, ammonium nitrate (1x = 1.5 g/l). The C:N ratios used were 2xC:2xN, 2xC:1xN, 2xC:0.1xN, 1xC:2xN, 1xC:1xN, 1xC:0.1xN, 0.1xC:2xN, 0.1xC:1xN, 0.1xC:0.1xN. Duplicate plates of each medium were incubated at 6°C and 20°C.

Mating Reaction

Sporidial colonies from fresh cultures grown on HCM agar were suspended in 0.5 ml sterile distilled water at a concentration of 1×10^7 cells/ml. For each "cross" (Thomas and Person, 1965), the mating reaction was initiated by placing 5 μ l of the cell suspension of one strain onto the medium to be tested and then inoculating the same volume of a suspension of the second strain directly onto the first. Mating reactions were evaluated under a Wild M5A dissecting microscope at 18X magnification from day 1 to day 13 after inoculation. Five mating reaction scores, from 0 to 4, were assigned based on the amount of mycelium formed on the sporidial "button". The reactions were 0, with a complete lack of mycelium; 1, indicating a few aerial hyphae; 2, where the colony surface was covered with less than 50% mycelium; 3, where the colony surface was more than 50% mycelium but not completely covered; 4, where the surface was completely covered with mycelium. Mating of auxotrophic strains grown on minimal media containing the appropriate supplement was performed exactly as with the wild type strains. Conjugation bridges were observed by mixing sporidia of opposite mating-type on a flat square of 1.5% water agar

placed on a glass microscope slide. The slide was supported by glass rods in a petri plate containing a moist piece of filter paper and incubated at 16°C for 24 h (Henry et al., 1988).

Fluorescence Microscopy

The number of nuclei in sporidia and hyphae that developed on charcoal agar plates was determined in order to confirm that the hyphal cells were dikaryotic. Cells were placed on a glass microscope slide, fixed with 25% ethanol, and air dried. Five μ l of a 5 μ g/ml DAPI (4,6-diamidino-2-phenylindole) solution were added and allowed to dry. Calcofluor [disodium salt of 4,4'-bis(4-anilobisdiethylamino-5-triazin-2-ylaminol)2,2-stilbenedisulfonic acid] was then added at a concentration of 0.0125 to 0.1 μ g/ml to stain the cell wall. Preparations were examined using a Zeiss fluorescence microscope.

Results and Discussion

Sporidial isolates MSU-1 through MSU-4, I4 and E3 (Table 1) were tested for mating genotype alone and in all possible combinations on each medium at each different temperature. The results of representative matings are shown in Table 2. Crosses of strains having opposite mating type resulted in the formation of white, aerial mycelium (Figure 1). This is distinct from the diffuse mycelium which forms on the agar surface around the periphery of sporidial colonies of some strains (e.g., 14a). However, no aerial mycelium was observed when any of the cultures were inoculated alone or when co-inoculated with a like-mating-type strain (i.e., MSU-1, MSU-2, MSU-3, or MSU-4

Table 2. Effect of medium and incubation temperature on the mating reaction^a observed with *U. hordei* crosses.

Cross	T °C	Media ^b								
		HCM	HCM-1%	HCM-5%	2xHCM	2xHCM-1%	HMM	HMM-1%	VMM	VMM-1%
MSU-1	25	3	4	4	2-3	4	1	4	2-3	3-4
x	20	3	4	4	2-3	4	2	4	2	3-4
MSU-2	16	3	4	4	2-4	4	2-3	4	1-3	3-4
	6	3-4	4	4	3	4	3-4	4	2-3	4
MSU-3	25	3	4	4	2	4	1-2	3-4	2-3	3-4
x	20	3	4	4	2	4	2	4	2-3	3-4
MSU-4	16	2-3	4	3-4	2-4	4	3	4	1-3	3-4
	6	3-4	4	4	2-3	4	3-4	4	2-3	4
E3	25	1	1-3	2-3	0-3	0-3	1	1-3	0-3	1-3
x	20	1	1-3	2-3	0-3	1	1-2	2-3	0-3	1-3
I4	16	0-2	1-3	1-3	1	0	1	1-4	0-2	2-3
	6	0	1-2	1-2	0-1	0-1	1	1-4	0-1	2
I4	25	2-3	3-4	4	ND ^c	ND	ND	ND	ND	ND
x	20	2-3	4	3-4	ND	ND	ND	ND	ND	ND
MSU-2	16	1-2	3-4	3	ND	ND	ND	ND	ND	ND
	6	0-1	3-4	4	ND	ND	ND	ND	ND	ND
E3	25	2	2-4	3-4	ND	ND	ND	ND	ND	ND
x	20	1-2	3	3-4	ND	ND	ND	ND	ND	ND
MSU-1	16	1-2	2-4	2-3	ND	ND	ND	ND	ND	ND
	6	1	3-4	3-4	ND	ND	ND	ND	ND	ND

^a The mating reactions were: 0, with a complete lack of mycelium; 1, indicating a few aerial hyphae; 2, where the colony surface was covered with less than 50% mycelium; 3, where the colony surface was more than 50% mycelium but not completely covered; 4, where the surface was completely covered with mycelium. The values represent the range of mating reactions observed for replicates of a given cross.

^b Abbreviations for media are: HCM, Holliday's complete medium; HCM-1%, HCM supplemented with 1% activated charcoal; HCM-5%, HCM supplemented with 5% activated charcoal; 2xHCM, double-strength HCM; 2xHCM-1%, 2xHCM supplemented with 1% activated charcoal; HMM, Holliday's minimal medium; HMM-1%, HMM supplemented with 1% activated charcoal; VMM, Vogel's minimal medium; VMM-1%, VMM supplemented with 1% activated charcoal.

^c ND = not determined.

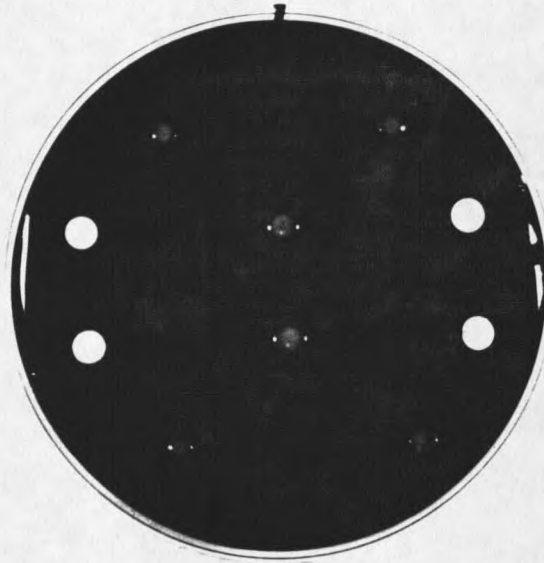


Figure 1. Mating reactions of sporidia isolated from a single teliospore on Holliday's complete medium supplemented with 1% activated charcoal. Top row from left to right: MSU-1 alone, MSU-2 alone; second row: MSU-1 x MSU-2, MSU-1 x MSU-3, MSU-1 x MSU-4; third row: MSU-2 x MSU-3, MSU-2 x MSU-4, MSU-3 x MSU-4; bottom row: MSU-3 alone and MSU-4 alone. Strains of opposite mating type show abundant, white, aerial mycelium, while strains inoculated alone or crossed with a like-mating-type strain have a yeast-like appearance.

alone, or MSU-1 x MSU-3 or MSU-2 x MSU-4) (Figure 1). These colonies retained a yeast-like appearance.

The addition of activated charcoal to any of the media tested resulted in an enhanced mating reaction when compared to the same media without charcoal (Table 2). The addition of 5%, instead of 1%, activated charcoal to HCM did not affect the mating reaction at any temperature. Compared to the reaction of newly isolated sporidia (MSU isolates 1-4), a weak mating reaction was observed when either of the independently obtained isolates of I4 and E3 were crossed with each other. The I4 x E3 cross displayed a weak mating reaction even when activated charcoal was added to the media, although charcoal usually strengthened the reaction to some degree. However, the mating reaction was improved when either E3 or I4 was crossed to a recently isolated strain (e.g., MSU-1 or MSU-2) (Table 2). Since crosses using any of the strains recently isolated from teliospores (including the MSU strains, strain 8A, and strain 14a) gave strong mating reactions, it is assumed that strains I4 and E3 display reduced fertility due to their long history of being maintained as sporidial cultures.

While the addition of activated charcoal enhanced the intensity of the mating reaction of *U. hordei*, incubation temperature also exerted a strong effect (Table 3). At higher temperatures (25°C and 20°C), the mating reaction was strongest at an earlier time, usually 2-3 days after inoculation. After that time the sporidia overgrew the mycelium, eventually resulting in a yeast-like colony. At lower temperatures (6°C), the strongest mating reaction was delayed until 6-10 days after inoculation, but the reaction remained stable indefinitely. Therefore,

Table 3. Effect of temperature and medium on the duration of the maximum mating reaction.

Media	Temperature			
	6°C	16°C	20°C	25°C
HCM ^a	7-13 ^b	3-6	2-4	2-4
HCM-1%	6-13	2-9	1-6	1-4
HCM-5%	6-13	2-7	1-5	1-4
2xHCM	8-11	4-8	1-6	1-5
2xHCM-1%	6-11	3-11	3-5	1-4
HMM	8-13	3-9	1-6	1-4
HMM-1%	4-13	3-13	2-8	1-3
VMM	6-11	3-8	1-6	1-3
VMM-1%	6-12	2-12	1-9	1-6

^a Abbreviations for media are the same as in Table 2.

^b Days of incubation during which the strongest mating reaction was observed. Each value represents the range of the initial and final day of incubation, from 12 different crosses, during which the maximum reaction was observed.

if high temperatures are used, the plates must be observed during the 1-2 day window during which the mycelium is most apparent. Generally, the addition of activated charcoal to a medium resulted in a shorter incubation time before the maximum mating reaction was observed and increased the number of days that the mating reaction could be observed, regardless of incubation temperature. Thus, activated charcoal increased both the rate and stability of the mating reaction.

The aerial mycelium which developed on charcoal-containing media did not extend beyond the inoculation point. Mycelium transferred to a new plate containing a charcoal-containing medium remained stable, but

did not spread. However, if the mycelium was transferred to a complete or minimal medium without activated charcoal, it was soon overgrown by sporidia, resulting in a yeast-like colony. Therefore, the use of activated charcoal-containing media allows formation of stable dikaryotic mycelium but does not support growth of the mycelium.

The pH of the media had little or no effect on mating or stability of the reaction on any of the media (data not shown). Furthermore, the carbon:nitrogen ratio of the media had no effect on the mating reaction (data not shown).

The transition from sporidial to mycelial growth should result in a change in nuclear number from mononucleate to dikaryotic (Thomas, 1988). The number of nuclei in sporidial and hyphal cells obtained from the surface of charcoal-containing agar medium were examined by fluorescence microscopy (Figure 2). All sporidia showed one nucleus per cell (Figure 2A), while the hyphae were found to be dikaryotic (Figure 2B) as expected. Although some mycelial cells appeared to contain only one nucleus, the presence of 2 nuclei in most cells confirmed that the mycelium was dikaryotic and, therefore, resulted from mating.

In addition to being useful in identification of the mating type of unknown isolates, the ability to reliably form dikaryotic mycelium on an agar medium has utility as a method for assigning mutants to complementation groups (Dinoor and Person, 1969; Henry et al., 1988). When cells of opposite mating type with non-allelic mutations are crossed, the mutations should complement and permit mycelium formation. However, allelic mutations will not complement and mycelium will not

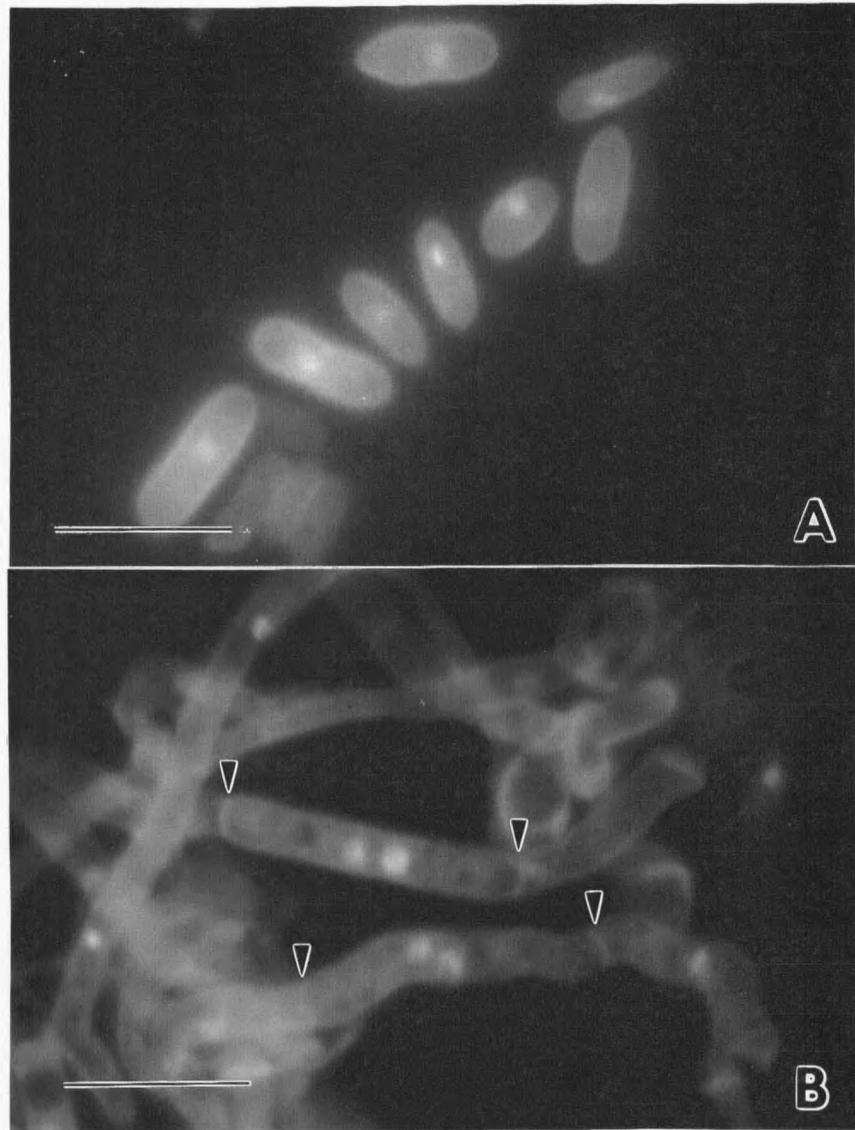


Figure 2. Fluorescence micrographs of DAPI-stained *U. hordei* cells grown on HCM with 1% charcoal. (A) Sporidia from MSU-1, showing a single nucleus per cell, and (B) hyphae from a MSU-1 x MSU-2 cross showing two nuclei per cell. Single arrows indicate stained septa. Bar = 10 μm .

form. In order to analyze the use of minimal medium supplemented with charcoal for complementation tests, auxotrophic mutants of 8A and 14a were induced by UV irradiation or EMS treatment (Table 1). Mutants with similar nutritional requirements derived from strains of opposite mating type were tested alone and together using VMM + 1% activated charcoal as shown in Figure 3. As expected, the auxotrophic mutants did not grow when inoculated separately. However, abundant mycelium was observed when complementing auxotrophic strains were co-inoculated, such as 8A-1 (His-) and 14a-1 (Arg-), or two separately isolated proline auxotrophs, 8A-2 and 14a-10 (Figure 3). In contrast, two independently obtained *Ilv*- mutants with opposite mating type (8A-3 and 14a-4) and two auxotrophs of opposite mating type having the same histidine requirement (8A-1 x 814-11) did not form mycelium, indicating that these mutations were allelic. Since 814-11 was a segregant from a cross in which 8A-1 was a parent (Table 1), these strains were known to contain an allelic mutation and, thus, this cross served as a non-complementing control. Since all of the mutant strains were capable of forming mycelium with the appropriate wild-type strains (Figure 3), it was evident that the lack of mycelium in the crosses involving allelic mutations was not due to an inability of these strains to mate. This conclusion was confirmed by microscopic examination of the crosses which did not form mycelium. In all cases, conjugation bridges were evident (not shown). As pointed out by Henry et al. (1988), it would be prudent to include these controls when performing complementation tests to rule out possible secondary mutations which might affect mating, such as a defective mating-type gene. Dinoor and Person (1969)

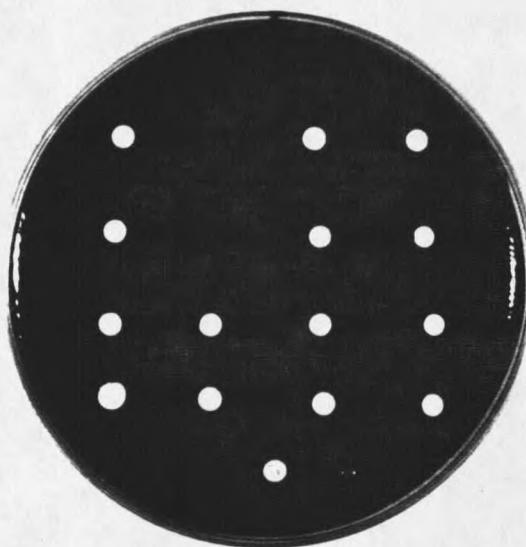


Figure 3. Complementation tests using auxotrophic mutants on Vogel's minimal medium plus 1% charcoal. Crossing mutants with complementing mutations results in abundant white, aerial mycelium, while crosses of cells with allelic mutations do not grow or form mycelium on the minimal medium.

Top row, left to right: 8A-1 (His-) x 14a-4 (Ilv-), 8A-1 (His-) x 814-11 (His-), 8A-1 (His-) x 14a-1 (Arg-), 8A-1 (His-) x 14a.

Second row: 8A-1 (His-) x 14a-4 (Ilv-), 8A-3 (Ilv-) x 14a-4 (Ilv-), 8A-3 (Ilv-) x 14a-1 (Arg-), 8A-3 (Ilv-) x 14a.

Third row: 14a-1 (Arg-) x 8A-1 (His-), 14a-1 (Arg-) x 8A-6 (Arg-), 14a-1 (Arg-) x 8A-3 (Ilv-), 14a-1 (Arg-) x 8A.

Fourth row: 8A-2 (Pro-) x 14a-1 (Arg-), 8A-2 (Pro-) x 14a-10 (Pro-), 8A-2 (Pro-) x 14a-4 (Ilv-), 8A-2 (Pro-) x 14a.

Bottom row: 8A alone, 8A x 14a, 14a alone as controls showing the wild type mating reactions.

have previously shown that dikaryotic mycelium could form when complementing *U. hordei* auxotrophic mutants of opposite mating type were "crossed" on minimal media. My findings demonstrate that the addition of activated charcoal to minimal medium enhances dikaryotic mycelium formation without affecting genetic complementation.

It is obvious from my results that the addition of activated charcoal to a medium does more than simply provide increased contrast between the white mycelium and black background. The addition of activated charcoal to defined media enhances the mating reaction in *U. hordei* (Table 2) while decreasing the time of incubation required for a maximal mating reaction (Table 3). The mycelial form also lasts longer in the presence of charcoal (Table 3). In addition, diploid strains of *U. hordei*, which grow as sporidia on media lacking activated charcoal, form mycelium on charcoal-containing media (R. Harrison and J.E.S., personal observation). These observations support the hypothesis (Day and Anagnostakis, 1971; Banuett and Herskowitz, 1988) that activated charcoal removes an inhibitor of mycelium formation and/or stability from the media. In the case of *U. maydis*, filtration of minimal medium through activated charcoal did not allow dikaryon formation, suggesting that the inhibitor is of fungal origin, as opposed to being a contaminant in the medium (Day and Anagnostakis, 1971). It is also possible that the charcoal is immobilizing chemical mating signals (pheromones), which might otherwise become diluted by diffusion into the agar.

Complete or minimal media containing charcoal offers several advantages over media used historically for examining mating of

U. hordei (Fisher and Holton, 1957; Lade, 1967; Dinoor and Person, 1969; Henry et al., 1988). First, the mating vs. non-mating reactions are easily discerned, eliminating the need for inexact morphological characterization, such as "dull-rough" for compatible strains versus "glossy-smooth" for incompatible pairings (Lade, 1967). Thus, these media can be used for a quick and accurate demonstration of the mating type of a strain. This is particularly useful when examining large numbers of isolates or segregants from teliospores during genetic studies of the *U. hordei*-barley interaction (Thomas and Person, 1965; Pedersen et al., 1977; Person et al., 1986). In addition, this plate assay conclusively demonstrates complementation of auxotrophic mutants. When the proper control crosses are included, additional microscopic observation for "long" vs "short" hyphae and conjugation bridge formation (Henry et al., 1988) are unnecessary. As has been previously demonstrated with *U. maydis* (Banuett and Herskowitz, 1988; Kronstad and Leong, 1989; Schultz et al. 1990), the ability to easily distinguish mating should also allow for the selection of mating-type mutants and cloning of mating-type genes from *U. hordei*.

CHAPTER 3

MORPHOLOGICAL AND MUTATIONAL ANALYSIS OF
MATING IN *USTILAGO HORDEI*Introduction

Ustilago hordei is a basidiomycete which causes covered smut of barley (*Hordeum vulgare* L.) (Mathre, 1982). Infection occurs through the coleoptile, advances through the host tissue and becomes established at the plant meristem. During flowering, *U. hordei* ramifies throughout the ovarian tissues, forming a fungal mass that differentiates into sexual teliospores replacing the seed (Kózar, 1969). *U. hordei* grows asexually as budding, yeast-like sporidia on a variety of rich and minimal media. The fungus is heterothallic with mating controlled by a single mating-type locus with two alleles designated "MAT1-1" and "MAT1-2", (equivalent to the traditional mating type alleles A and a, respectively) (Thomas, 1991).

The pathogenic form of *U. hordei* is a dikaryotic mycelium that arises following conjugation of two opposite mating-type sporidia. The mechanisms by which fungi in the *Ustilaginales* conjugate is very diverse. In *Tilletia* species (e.g. *Tilletia caries*), complementary, haploid sporidia conjugate by means of short, lateral conjugation tubes forming an H structure. A secondary binucleate spore forms from this structure and a pathogenic hypha emerges from this spore (Webster,

1989). In many *Ustilago* species, sporidial conjugation takes place by the production of tubes which form a bridge, out of which pathogenic, dikaryotic mycelium develops. Conjugation tubes have been observed in several species including *U. avenae*, *U. nigra*, *U. kolleri*, *U. longissima* (Webster, 1989), and *U. violacea* (Day, 1976). Only recently have conjugating sporidia been observed in *U. maydis* (Snetselaar and Mims, 1992).

The mating process, and more specifically the process of sporidial conjugation, has only been studied in detail in *U. violacea*, in which physical contact of cells is generally required (Poon et al., 1974). Pairing of sporidia of opposite mating type in *U. violacea* is aided by fimbriae, which extend from the sporidial walls (Day and Poon, 1975; Poon and Day, 1975). It has been suggested that fimbriae act as conduits for a reciprocal exchange of information between the two mating-type cells before conjugation tubes form (Day and Poon, 1975).

Genetically, mating is controlled by the *MAT* locus which is assumed to act as a master switch regulating cell-to-cell recognition, cell fusion, regulation of the yeast-to-mycelium morphological transition, control and maintenance of nuclear number and ploidy, control of pathogenicity and indirectly meiosis and teliospore formation (Day and Garber, 1988; Thomas, 1988; Banuett, 1989). One approach to study these processes and their genetic control is the isolation of haploid mutants that can no longer mate (MacKay and Manney, 1974a; MacKay and Manney, 1974b; Banuett and Herskowitz, 1988). In several systems, the isolation of mating defective mutants has allowed a complete description of the different steps involved in

mating as well as the isolation and characterization of genes required for mating which are distinct from the mating-type locus (*MAT*) (MacKay and Manney, 1974a; Banuett, 1991). In *Saccharomyces cerevisiae*, these genes (*STE* genes) are expressed in a cell-type specific manner (e.g., in *a* or α haploid cells but not in *a*/ α diploid cells) and are the targets of regulatory proteins encoded by the *MAT* locus (Herskowitz, 1988). Other genes code for components of the signal transduction system or are involved in the initiation of meiosis and sporulation (Herskowitz, 1989). In *U. maydis*, 80 mating-defective (*Fuz*⁻) mutants were isolated (Banuett, 1991). The *Fuz*⁻ phenotype ranged from a complete lack of mycelium production, to the formation of very few hyphae, to morphologically altered filaments. Three genes, *Fuz-1*, *Fuz-2* and *rft1*, which are necessary for the formation of hyphae, have been described in detail (Banuett, 1991).

While a general knowledge of the mating process in *U. hordei* has been described (Fisher and Holton, 1957; Mathre, 1982; Thomas, 1988), there has been no detailed description of the early stages of mating or the optimal conditions at which mating occurs. In this chapter I present a comprehensive description of the morphological changes that occur during mating in *U. hordei* and evidence for the involvement of diffusible mating factors. Furthermore, to begin the characterization of the mating process at the genetic level, I describe the isolation and phenotypic characterization of *Fuz*⁻ mutants.

Materials and Methods

Ustilago hordei Strains

Ustilago hordei (Pers.) Lagerh. wild-type strains 8A (Race 8, MAT-1) and 8a (MAT-2) (see Chapter 2) were used to study the mating process. To isolate mating-defective mutants, wild-type strains 8A and 14a (race 14, MAT-2) and auxotrophic strains 8A-6 (race 8, MAT-1, *arg*-) and 14a-2 (race 14, MAT-2, *cys*-) (see Chapter 2) were used.

Analysis of Mating

To study the *U. hordei* mating process in detail, early to mid-log phase wild-type cells grown in Holliday's Complete Medium (HCM) broth (Holliday, 1974) were adjusted to 1×10^6 cells/ml in distilled water, mixed, plated on water agar and incubated at 21°C. An alternative technique referred to as the "sandwich method" was developed to distinguish the individual cell types during the mating process (Bandoni, 1965; Abe et al., 1975). Early to mid-log phase wild-type cells were grown in HCM broth and adjusted to 1×10^6 cells/ml in distilled water. TE agar, which contains the trace elements from Vogel's Minimal Media (VMM) (Vogel, 1956) with final concentrations of 0.005% citric acid, 0.005% ZnSO_4 , 0.0017% $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.00005% H_3BO_3 , 0.00005% $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.00005% MnSO_4 (w/v), was poured as a thin (2mm) layer in a petri plate. One cm squares were excised and mounted on a microscope slide. Five μl of a cell suspension was spread onto the squares and air dried. A prepared dialysis membrane (Sambrook et al., 1989) with a 12,000-14,000 mw cut-off pore size was placed on top of the agar square, after which an agar square containing air dried

cells of the opposite mating type was placed on the dialysis membrane. The microscope slide containing the "sandwiches" was supported by glass rods in a petri plate containing a moist piece of filter paper and incubated at 16 °C for 48-72 hrs. Optimal conditions for conjugation tube induction with the sandwich method were use of TE medium, pH 5.6, incubated at 16°C in the dark (S. Gerhardt, personal communication). This combination permitted the evaluation of the production of mating factors and the induction of conjugation tubes on opposite mating-type cells while minimizing the formation of mycelium which was unrelated to mating.

Fluorescence microscopy was performed as described in Chapter 2.

Isolation of Mating-Defective (Fuz-) Mutants

Sporidial strains 8A-6 and 14a-2 were grown in liquid HCM to early log phase (Hood, 1968). The sporidia were adjusted to 1×10^3 cells/ml in sterile distilled water and plated on HCM agar. Uncovered petri plates were irradiated with UV light (234 nm wavelength lamp "A" GE) 30 cm from the UV source. Irradiation for 40 s (strain 8A-6) and 35 s (strain 14a-2) resulted in a 10% survival rate. Following irradiation, the plates were covered and incubated for 24 h in complete darkness at 21°C, after which they were transferred to a 12 h cycle of light/darkness at 21°C. Discrete colonies had formed after 5-6 days of incubation at which time the colonies were replica plated onto HCM agar supplemented with 1% activated charcoal (HCM-C) (see Chapter 2). After incubation at 21°C for 24 hrs, the agar surface of each plate was sprayed with sporidia of the opposite mating type suspended in sterile

distilled water at 5×10^6 cells/ml using a sterilized atomizer until uniform coverage was obtained. The mating reaction was evaluated after incubation for 24-48 h at 21°C, under a Wild M5 dissecting microscope. Putative mating (*Fuz*-) mutants which did not form white aerial mycelium as occurs in wild type mating (see Chapter 2) were selected from the HCM agar master plate. After purification, these mutants were tested for the proper auxotrophic marker on VMM supplemented with arginine or cysteine. To confirm the *Fuz*- mutation, putative mutants were re-tested 2-3 times by placing 5 μ l of a 5×10^6 /ml cell suspension on separate HCM-C agar plates, incubating at 21°C for 24 h and spraying with opposite and like mating-type cells. The mutants were also checked by spotting a cell suspension of an opposite mating-type strain directly onto the mutant suspension. All mating reactions were incubated at 21°C and observed daily for 3 days.

Phenotypic Characterization of *Fuz*- Mutants

The phenotypes of the *Fuz*- mutants were characterized for colony morphology using a dissecting microscope (Wild M5A) and for cell morphology using a Zeiss compound stereo microscope. The stage at which mating was defective was determined by examining mixed cells on agar or by the "sandwich method". In the former, a cell suspension of each *Fuz*- mutant was placed alone or mixed with the wild-type strain of the opposite mating type on a thin water-agar square mounted on a microscope slide. The cells were incubated at 16°C and examined daily for 3 days. For the "sandwich" method, suspensions containing 1×10^6 cells/ml of each *Fuz*- strain and appropriate wild-type culture were

used as inocula. The sandwiches were incubated at 16°C and evaluated daily for 4 days.

Results and Discussion

The *U. hordei* Mating Process

Historically, mating in *U. hordei* has been described as fusion, anastomosis or conjugation of cells of the opposite mating type resulting in the production of dikaryotic infective hyphae (Fisher and Holton, 1957; Dinooor and Person, 1969; Mathre, 1984; Thomas, 1988). However, no detailed description of events that occur during the early stages of *U. hordei* mating have been reported.

The formation of conjugation tubes by *U. hordei* sporidia was the first observable step of mating when cells of opposite mating types were mixed together on a suitable medium such as water agar. First, polar conjugation tubes emerged from the cells (Figure 4A). The tubes elongated and fused with conjugation tubes from sporidia of the opposite mating-type to form conjugation bridges (Figures 4B and 5A, 5B, 5C). When cells were in close proximity, conjugation could occur within two hours after mixing both mating types on water agar when incubated at 21°C. A filamentous structure then developed out of the conjugation bridge. This mycelium has never been observed to emerge from one of the mated sporidia which sometimes occurs with *U. maydis* (Snetselaar and Mims, 1992). After conjugation, septa developed either at the base of the hypha or at the base of each sporidium (Figure 5C and 5D). The mycelium was stained with DAPI and found to be dikaryotic (Figure 5C). This dikaryotic mycelium represents the infection form of *U. hordei*.

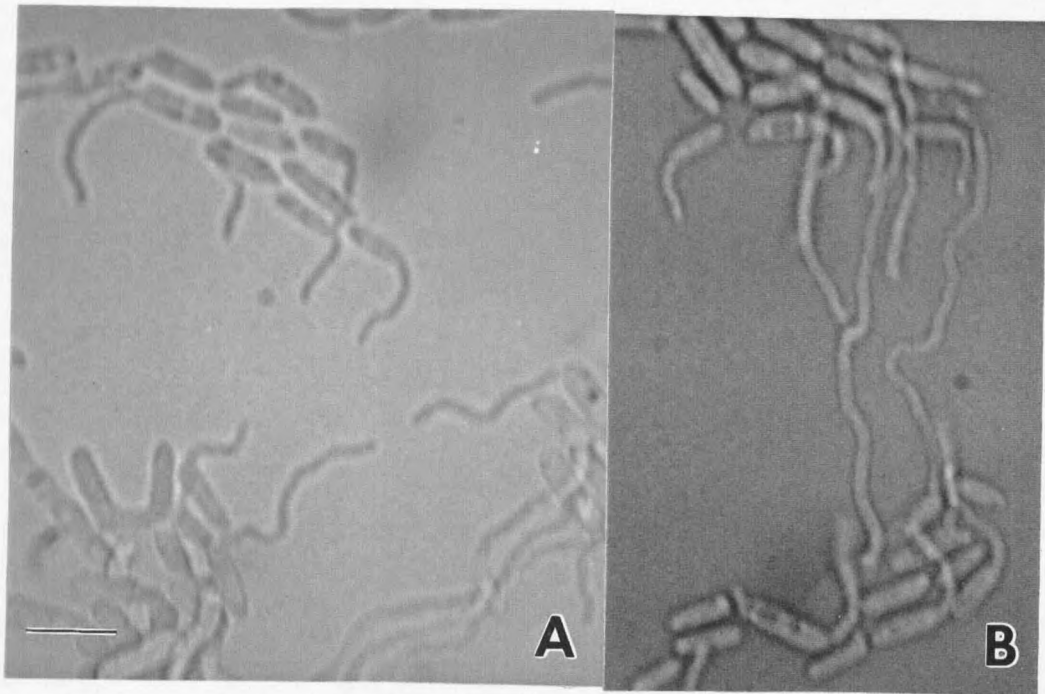


Figure 4. Conjugation tubes of *U. hordei*. (A) Sporidia of opposite mating type were mixed and spread on an agar surface. Polar conjugation tubes emerge from cells and grow toward sporidia or conjugation tubes of opposite mating type. (B) Long distance induction and tip-to-tip fusion of conjugation tubes to form conjugation bridges. Bar = 10 μm

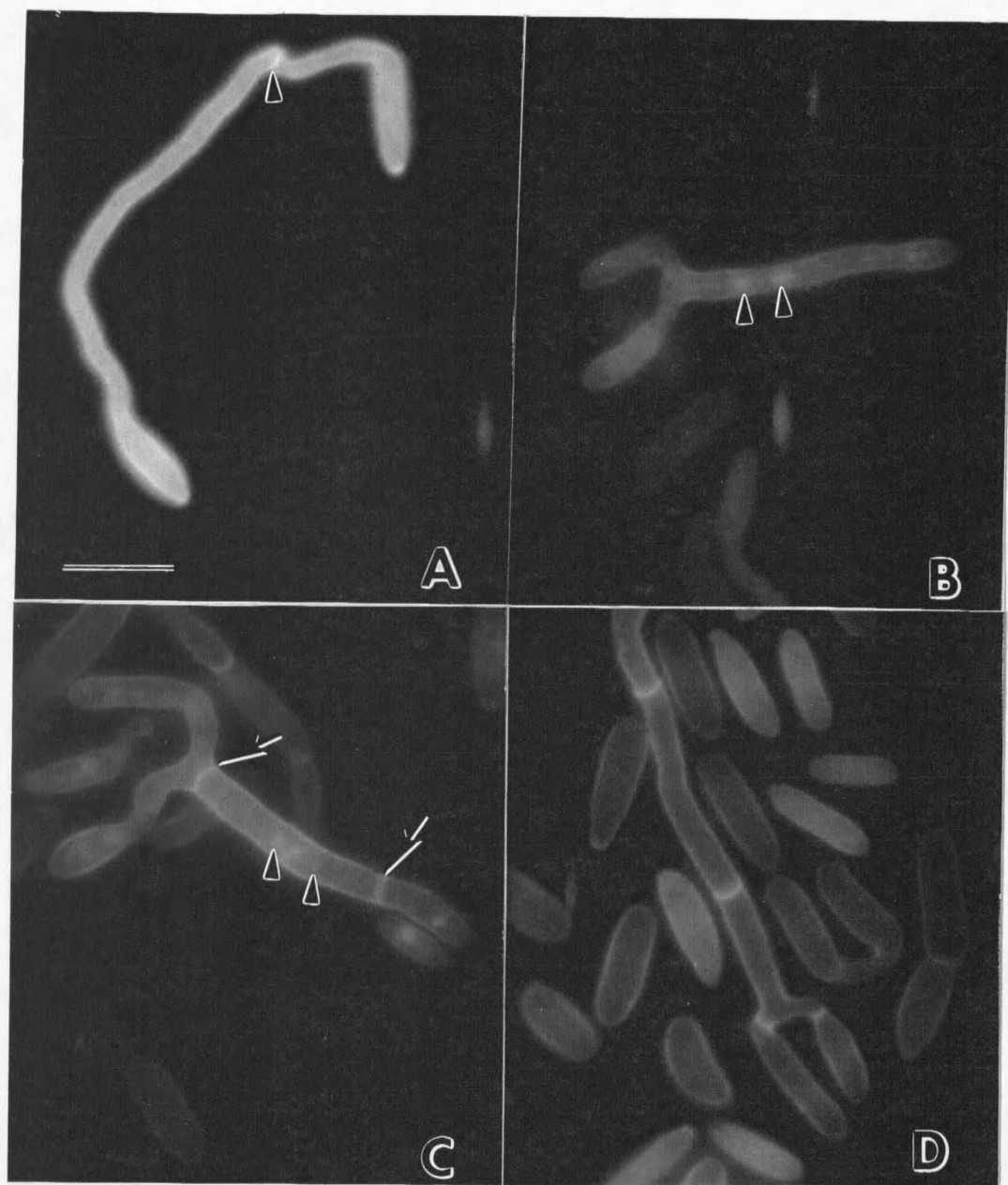


Figure 5. (A) Calcofluor-stained cell walls of mating sporidia forming a long conjugation bridge. The arrowhead indicates point of fusion. (B) Calcofluor and DAPI stained dikaryotic mycelium developing from conjugated sporidia. Arrowheads indicate the two nuclei. (C) Elongation of dikaryotic mycelium. Arrowheads indicate nuclei double arrows indicate septa. Cells were stained with Calcofluor and DAPI. (D) Conjugated sporidia and dikaryotic mycelium stained with calcofluor. All pictures were taken after mixing opposite mating-type sporidia on agar squares. Bar = 10 μ m.

Conjugation of *U. hordei* did not require that the cells have physical contact or even be in close proximity as reported for *U. violacea* (Day, 1976), *U. longissima*, or *U. avenae* (Webster, 1989). In *U. hordei*, cells of opposite mating type were able to fuse when located at a considerable distance from each other (Figure 4B and 5A), as much as 8-10 times the length of a sporidium (70-100 μ m). Conjugation tubes were usually meandering with sharp curvatures. There was usually a single polar conjugation tube produced per cell, although there were occasionally 2 polar tubes, but never more than two. Fusion of conjugation tubes was always tip-to-tip. Conjugation occurred over a wide range of temperatures, from 6°C to 25°C, although it took longer at lower temperatures. Sporidial conjugation was always between two cells; there were no incidence of multiple conjugation as described with *U. violacea* (Poon et al., 1974).

Evidence for Mating Factors

From the observations described above, there were numerous indications that sporidia were able to communicate with each other, even at a considerable distance. The induction of conjugation tubes by cells of the opposite mating type as far as ten cell lengths away, the observation that fusion of conjugation tubes was always tip-to-tip even though the tubes take a meandering path before fusing, and the directed growth of conjugation tubes toward opposite mating-type cells or colonies were indications that diffusible compounds were acting as signals for mating. The use of the "sandwich method", which was developed to discern the individual cell types during the early stages

of mating, provided clear proof of the presence of small molecular weight, diffusible mating factors, or pheromones. When cells of opposite mating type were placed on agar squares with dialysis membrane between them, the cells formed conjugation tubes (Figure 6C and 6D). However, conjugation tubes were not induced if cells of the same mating-type were used (Figure 6A and 6B). The results indicated that mating-type specific compounds capable of diffusing through dialysis membranes with a 12,000 m.w. cutoff were involved in conjugation tube induction. The size of the membrane pores eliminated the possibility that fimbriae were directly involved in intercellular communication, as described with *U. violacea* (Day and Poon, 1975), since the fimbrial subunit mass of *U. violacea*, and presumably similar with *U. hordei*, was reported to be 74,000 daltons (Gardiner and Day, 1985).

The presence of diffusible mating factors and the fusion of hyphal tips or conjugation tubes has been described previously for other fungi (Burnett, 1956). Tip-to-tip fusion of conjugation tubes suggested that fusion resulted from a single species-specific substance which created a concentration gradient in the vicinity of each tip. This would result in a slight but critical concentration gradient between the two tubes when they lie a short distance from each other (Raper, 1952; Raper, 1960). Diffusible mating factors have been shown to be involved in mating of *Rhodospiridium toruloides* (Abe et al., 1975) and *Tremella mesenterica* (Bandoni, 1965), basidiomycetes which reproduce asexually as budding yeast. Pheromones from *R. toruloides* and *T. mesenterica* (Bandoni, 1965), have been purified and found to be short peptides of 11 amino acids (Rhodotorucin A) and 12 amino acids (Tremmerogen A).

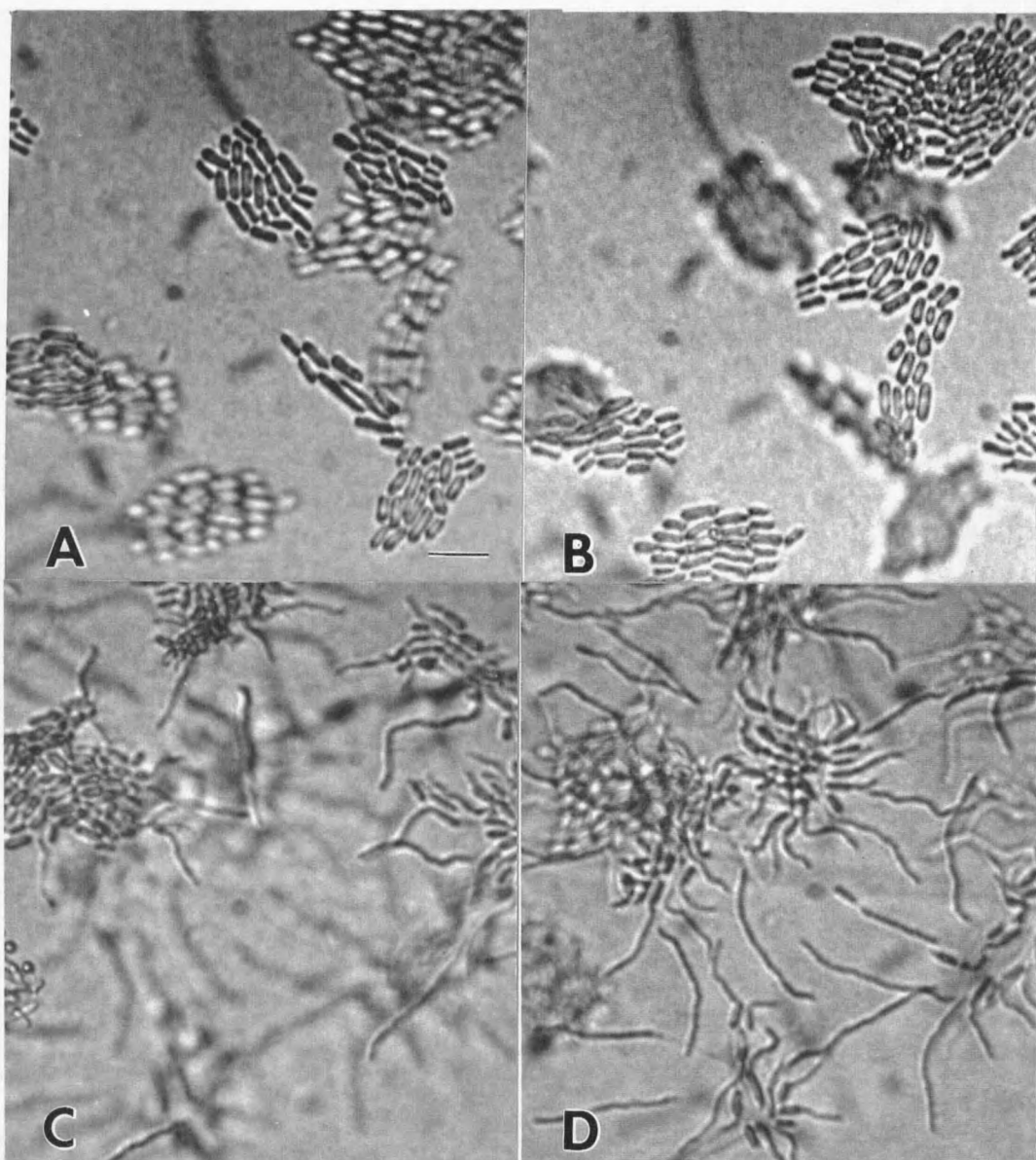


Figure 6. Induction of conjugation tubes by cells of opposite mating type through a dialysis membrane. (A) and (B) Identical microscope fields of cells of same mating type placed on opposite sides of a dialysis membrane showing no induction of conjugation tubes. Microcolonies which are in focus in (A) are unfocused in (B) and vice versa. (C) and (D) Identical microscope fields of cells of opposite mating type placed on opposite sides of a dialysis membrane. Conjugation tubes are evident on both cell types. Microcolonies in focus in (C) are unfocused in (D) and vice versa. Bar = 20 μm .

Both contain an S-isoprenyl cysteine at the C-terminus (Tsuchiya and Fukui, 1978; Akada, 1989). *Saccharomyces cerevisiae* a and α cells, each secrete a mating factor that acts specifically on cells of the opposite mating-type. The α factor, secreted by α cells, is a 13 amino acid peptide. This is the only known non-farnesylated pheromone from a higher fungus. The a factor is found in two slightly modified forms, a_2 and a_3 , both of which have a S-farnesyl cysteine as a structural component (Anderegg et al., 1988). It has been proposed that *S. cerevisiae* cells locate a mate by sensing a gradient of the pheromone from the opposite mating type and elongate, or "schmoo", in the direction of that cell (Duntze et al., 1970; Mackay and Manney, 1974; Herskowitz, 1988). *U. hordei* conjugation tubes may also create a gradient of the mating factor compound, allowing tubes from opposite mating-type to "sense" each other. For this to be true, the pheromone must be excreted from the conjugation tube tip itself, since the tubes grow toward the tips of tubes arising from cells of the opposite mating type before they finally fuse end to end. It is not known at this time whether the *U. hordei* pheromones are synthesized constitutively. While this is the case for *S. cerevisiae* mating factors (Duntze et al., 1970; Strazidis and Mackay, 1982; Herskowitz, 1988), only "A" cells of *R. turoloides* produce Rhodotorucine A constitutively, but production of Rhodotorucine a is induced only after exposure of "a" cells to Rhodotorucine A (Abe et al., 1975).

Isolation and Characterization
of Fuz- Mutants

Charcoal-containing media have been shown to be suitable to screen for mating-defective mutants (*Fuz-*) mutants with *U. maydis* (Banuett, 1991). Using a similar rationale, *U. hordei* *Fuz-* mutants were screened for and detected by the lack of white, mycelial colonies which form when wild-type strains of *U. hordei* are crossed (see Chapter 2). The mycelial *Fuz+* reaction of the wild-type strains was easily distinguished from mating-defective mutants, which remain yeast-like or have a drastic decrease in mycelium formation. Twenty-six individually isolated mating mutants of *U. hordei* have been obtained (Table 4).

Table 4. *Fuz-* variants of *Ustilago hordei*.

Variant Class	Conjugation Tube Synthesis	Pheromone Synthesis ^a	Cell Type ^b
8A-6 <i>MAT-1</i>			
I (4) ^c	-	-	S
II (5)	-	-	M
III (2)	-	+	S
IV (2)	+	+	S
14a-2 <i>MAT-2</i>			
V (3)	-	-	S
VI (2)	-	-	S(round)
VII (1)	-	-	S/M
VIII (2)	-	-	M
IX (2)	-	+	S
X (3)	-	+	M

^a Determined by ability to induce conjugation tube formation in opposite mating type cells separated by dialysis tubing.

^b S=sporidia; M=mycelial; S/M=Spordia and short mycelium.

^c Number in parentheses indicates the number of variants within each class.

Thirteen were derived from mating-type A cells (*MAT-1*) and thirteen were derived from mating-type a cells (*MAT-2*). Some of these mating-defective mutants resemble those obtained in *U. maydis* (Banuett, 1991) where *Fuz-* mutants had varying degrees of mycelium production. Some mutants produced no hyphal filaments, while others produced sparse or morphologically altered mycelium. All of the mutations in *U. hordei* appear to be mitotically stable since they have not reverted after continuous culturing. All mutants are *Fuz-* over a range of temperatures (6°C, 16°C, 21°C and 25°C), indicating that none are temperature-sensitive.

Each mutant was characterized according to colony and cell morphology. A wide variety of colony and cell morphologies were found (Figures 7 and 8). Some mutants had a yeast-like, shiny and smooth colony morphology. Cells of these mutants had the size and shape of normal wild-type sporidia or sporidia that were round and smaller than wild-type cells or that produced numerous small daughter sporidia (Figure 8B). Other mutants had white to yellow-opaque colonies with numerous narrow and shallow ridges giving a cauliflower appearance. These mutants had deformed sporidia resembling short pieces of mycelium (Figure 8C). Also found were mutants with mycelial, dry, opaque, rough colonies with deep ridges giving a mycelial pinwheel-like appearance. These mutants had long, thick mycelial filaments (Figure 8D) which were sometimes mixed with distorted small pieces of mycelium. Other colonies of the cauliflower or pinwheel type had a shiny and smooth appearance to different degrees. These mutants usually had a combination of normal looking sporidia with deformed short pieces of

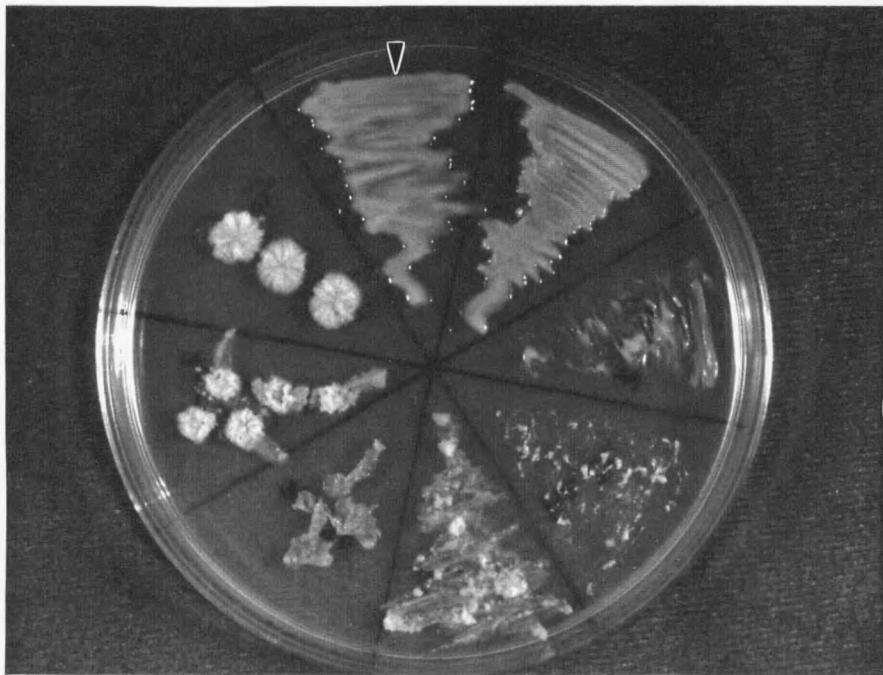


Figure 7. Colony morphology of several mating-defective mutants. The arrowhead points to wild-type strain 8A. Clockwise from 8A are the wild-type 14a and examples of increasingly mycelial *Fuz-* mutants.

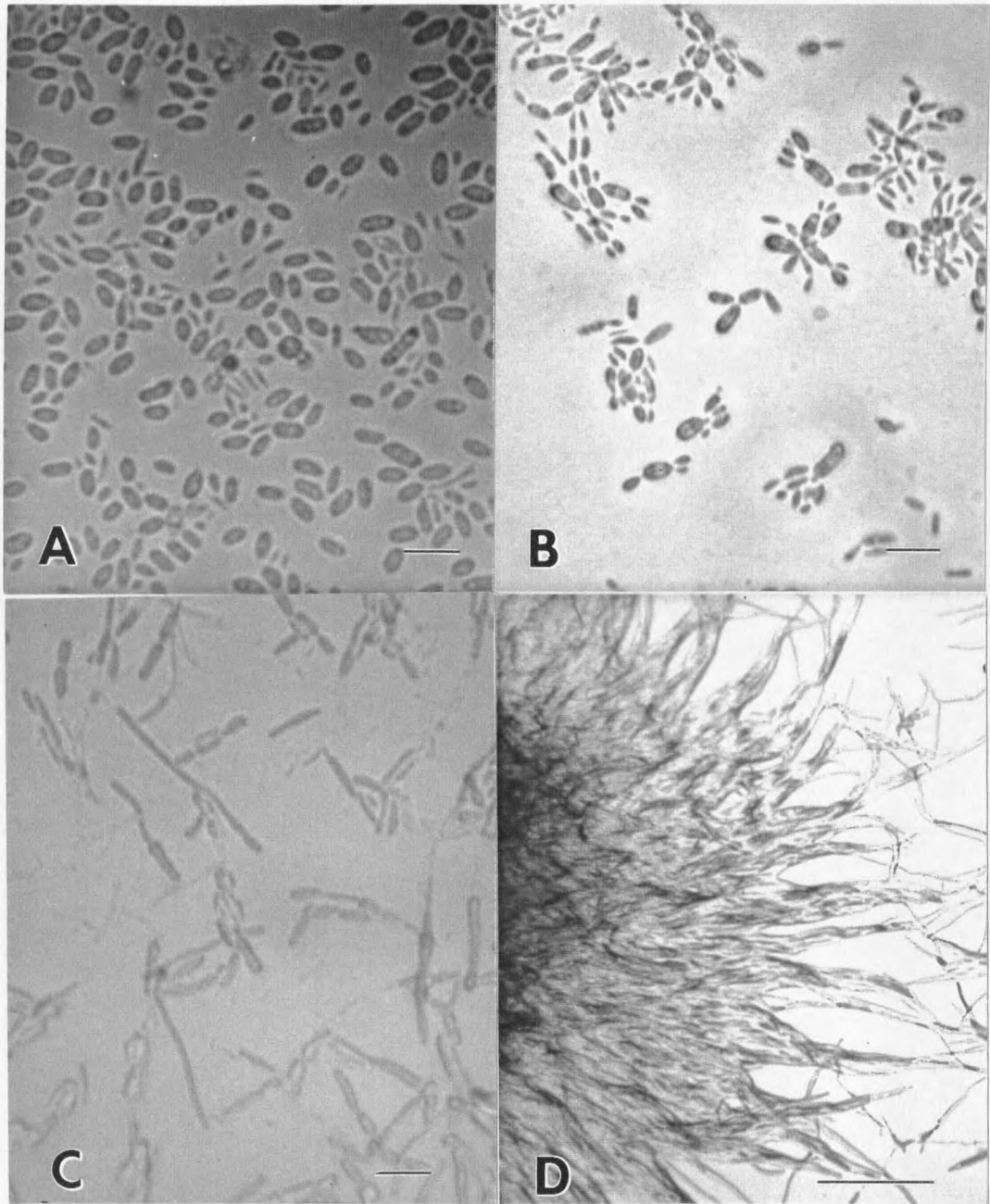


Figure 8. Cell morphology of several mating mutants. (A) Wild-type sporidia. (B) An example of a class III producing numerous small daughter sporidia. (C) A class VII mutant with deformed sporidia resembling short pieces of mycelium. (D) An example of a class II mutant with long mycelial filaments. Bars on A, B, and C = 20 μm ; bar on D = 100 μm .

mycelium-or thick filaments. Some of these colony and cell morphologies resembled those described as pseudohyphal (*ph*), cauliflower (*cf*), modified pseudohyphal (*mph*) or dull (*dl*) mutants in *U. violacea* by Zielinski and Garber (1982).

The *Fuz*- mutants were selected on the basis of their inability to form mycelium when mixed with cells of the opposite mating-type. However, there are several steps prior to mycelium formation which can be delineated with *U. hordei*. Thus, the mutants were examined for the ability to form conjugation tubes, to induce conjugation tube formation, tube fusion, formation of dikaryotic mycelium, and stability of the mycelium. To determine the specific lesion for each mutant, the mutants were examined after inoculation with the wild-type strain of the opposite mating type on TE agar and also examined using the "sandwich" method. The latter method allows the reaction of each cell to be observed independently. Thus, it was possible to determine whether a *Fuz*- mutant was incapable of producing a conjugation tube, or of inducing conjugation tube synthesis in the wild type cells of the opposite mating type. Unfortunately, it was not possible to perform complementation tests or other genetic analysis of the *Fuz*- mutants, since these require that cells be able to mate (Thomas, 1991; see Chapter 2).

Using these two methods, the *Fuz*- mutants were placed into groups (Table 4). (1) Mutants that were morphologically similar to wild-type sporidia, did not produce conjugation tubes but still induced conjugation tubes on cells of the opposite mating type (classes III and IX). This indicated that they still produced pheromone, but were

unable to detect or process the signal produced by the opposite mating type. Such mutants were characterized in *Saccharomyces cerevisiae* (Mackay and Manney, 1974a) which are defective in the signal transduction pathway (Herskowitz, 1988). It is also possible that a mutation affects genes essential for conjugation tube synthesis.

(2) This group included mutants that grew as sporidia, produced conjugation tubes at a reduced level, and induced conjugation tubes in cells of the opposite mating type (class IV). This might indicate that the pheromone receptor was altered or diminished in number and thus had a reduced ability to detect pheromone. Apparently pheromone biosynthesis was not affected. (3) This group of mutants grew as sporidia but were not able to detect the pheromone from the opposite mating type since they did not make conjugation tubes. They also did not produce an active mating factor, since they did not induce conjugation tubes on cells of the opposite mating type (classes I, V and VI). This group apparently included mutations which affected both pheromone synthesis and detection. This suggests that the mutation was in a regulatory gene which governs both pheromone production and response to pheromone. (4) This group included mutants that grew as mycelia and did not induce conjugation tubes in opposite mating-type cells (classes II and VIII). These classes apparently were unable to produce active mating factor. In addition, control of development of mycelia was altered. This could be the result of single or multiple mutations; it is possible that some genes controlling mycelial growth also repress pheromone synthesis. (5) This group grew as mycelia and induced conjugation tubes on cells of the opposite mating type at a

very reduced frequency (class X). This class may be similar to the previous group but with a reduced production of active pheromone. Mutants with reduced or altered mating factor production have been isolated in *S. cerevisiae* (Mackay and Manney, 1974a). (6) These mutants had an altered cell morphology, with sporidia that appeared to be germinating or converting to mycelium. They also did not produce mating factor (Class VII). These might result from mutations in a gene(s) similar to those in the fourth group, but with a different cell morphology.

In *U. maydis*, two mating-type loci (*a* and *b*), govern the life cycle transitions of this fungus. The *a* locus has two alleles a_1 and a_2 (Froelinger and Leong, 1991) that controls cell fusion (Puhalla, 1969; Bolker et al. 1992). The *b* mating-type locus has 25 alleles and controls pathogenicity and is necessary for filament formation (Banuett, 1989; Kronstad and Leong, 1989). Recently, homology to the *b* locus of *U. maydis* (*bE* and *bW*) was found in *U. hordei* DNA indicating the presence of a *b*-like locus in this fungus (Bakkeren et al., 1992). The importance of these sequences in *U. hordei* mating is not known.

The *MAT* locus controls sporidial conjugation between cells of the opposite mating type. The initial morphological changes, i.e., induction of conjugation tubes by cells of the opposite mating type, are induced by small molecular weight diffusible substances that are, by definition, pheromones. Pheromones induce tube synthesis and presumably direct tube growth toward tubes of the opposite mating type, which results in the tip-to-tip fusion of these tubes. Thus, the early stages of the mating process include a reciprocal information

exchange-between opposite mating type cells with pheromones acting as the signals. With the use of mating defective mutants, it has been possible to infer some of the general features of the mating type regulatory system. Several of the *Fuz-* mutants isolated from either mating-type have lost the capacity to produce active pheromone and no longer induce wild type cells to form conjugation tubes. Other mutants have apparently lost their ability to detect or process the sex factor from the opposite mating type suggesting mutations in the pheromone receptor or signal transduction machinery required to activate conjugation tube biosynthesis. Other alleles that control the developmental change from sporidia to mycelium are also involved in conjugation tube formation and pheromone synthesis.

The structure of the mating-type locus (*MAT*) of *Ustilago hordei* is yet unknown. Recent results describing the *U. maydis* *a* locus indicates that pheromone structural genes and the genes for pheromone receptors are clustered at this single locus (Bolker et al., 1992). These results with *U. hordei* indicate a similarly complex, multigenic process under the control of the *MAT* locus.

CHAPTER 4

MOLECULAR ANALYSIS OF THE MATING-TYPE

LOCUS OF *U. hordei*Introduction

Mating in *Ustilago hordei* is controlled by the *MAT* locus, which has two alleles (*MAT-1* and *MAT-2*) (Thomas, 1988) giving rise to two mating-type cells. In other fungi the *MAT* locus has been proposed to be a developmental master locus regulating major transitions in the life cycles of these organisms (Day and Garber, 1988; Herskowitz, 1988). In *U. hordei*, *MAT* is proposed to control recognition between cells, cell fusion, the morphological change from the yeast form to the mycelial form and pathogenicity (Thomas, 1988).

The first step of the mating process in *U. hordei* is the recognition of cells of the opposite mating type. After recognition, conjugation tubes develop from the sporidia, elongate and fuse with conjugation tubes from the opposite mating type. Out of the recently formed conjugation bridge, the dikaryotic, pathogenic mycelium develops (see Chapter 3). Recognition of cells of opposite mating type is carried out by diffusible, low molecular weight mating factors, i.e., pheromones. These mating factors were able to pass through a dialysis membrane and induce conjugation tube formation by cells of the opposite mating type (see Chapter 3).

The life cycle of *U. hordei* is completed with the infection of barley plants by the dikaryotic mycelium and by the eventual production of diploid teliospores that replace the developing barley grains. Teliospores germinate on barley seed or artificial media to produce a short four-celled promycelium from which four haploid sporidia develop. These haploid cells can be propagated on artificial media where they multiply vegetatively by budding. When compatible haploid lines are mixed they produce a characteristic aerial mycelium on solid complete medium containing activated charcoal (see Chapter 2).

In *U. maydis*, the corn smut disease-causing organism, the *a* mating-type locus has two alleles, a_1 and a_2 , and has been proposed to control the fusion of the haploid sporidia (Rowell and Devay, 1954; Rowell, 1955; Puhalla, 1969). Recently, these alleles have been cloned and molecular analysis has revealed that the two alleles consist of regions of non-homologous DNA (Froelinger and Leong, 1991). The a_1 and the a_2 alleles are defined by unique regions of 4.5 Kb for a_1 and 8 Kb for a_2 . Each allele is composed of a set of two genes; one encodes a precursor for a peptide mating factor (pheromone) and the other codes for the receptor of the pheromone secreted by cells of the opposite mating type. The mating-type alleles thus provide the components involved in cell-to-cell signalling (Bolker et al., 1992). In a recent molecular survey among smuts using the *a* and *b* loci of *U. maydis* as probes, the a_1 allele was found to hybridize to DNA from one mating-type of *U. hordei* (MAT-2) and the a_2 allele hybridized to DNA from both mating-types, suggesting that these sequences are conserved among the smut fungi (Bakkeren et al., 1992).

Since previous results (see Chapter 3) indicated the presence of diffusible, low molecular weight mating factors in *U. hordei*, one objective of this investigation was to see if genes similar to the mating factor genes (*mfa*) and mating factor receptor genes (*pra*) of *U. maydis* are present in *U. hordei*. Another objective of this investigation was to clone the DNA fragments of *U. hordei* containing the *mfa* and *pra* equivalents and to determine if they play a role in mating of *U. hordei*.

Materials and Methods

Strains, Plasmids and Culture Conditions

Ustilago hordei (Pers) Lagerh. wild-type strains 14a (race 14, MAT-2) and 14A (MAT-1) (see Chapter 2), were used to obtain DNA and to perform DNA-mediated transformations. Fresh sporidial cultures were started every 5-7 days. *Escherichia coli* strains LE392 (*hsdR514* [rk-,mk+] *supE44*, *supF58*, *lacY1* [lacIZY] *kz*, *galT22*, *metB1*, *trpR55*); and P2392 (P2 lysogen of LE392) were used as hosts for lambda phage. Plasmid pCM54 (6.3 Kb) a derivative of pHL1 (Holden et al., 1988) which contains an autonomous replicating sequence (ARS) from *U. maydis* was used to subclone the fragments of interest and to perform *U. hordei* transformations.

Wild-type strains were grown on Holliday's complete medium (HCM) (Holliday, 1974) at 21°C or 25°C. *U. hordei* transformants were grown on HCM containing 150 µg/ml of hygromycin at 21°C or 25°C.

DNA Procedures

Isolation of *U. hordei* DNA was performed by preparing protoplasts as described by Holden et al. (1988). Phage DNA was isolated as described by Grossberger (1987). Plasmid DNA was isolated using the boiling miniprep method described by Sambrook et al. (1989). Radioactive labeling was done by nick translation (Boehringer Mannheim). Restriction digests were performed according to the supplier's instructions (New England Biolabs, Beverly, MA).

Plasmids containing the entire *U. maydis* *a1* allele (*mfa*₁ and *pra*₁) and the *mfa*₂ gene from *a*₂ allele were obtained from Dr. S. Leong (USDA, Madison, WI). Complete *a*₁ and *a*₂ alleles from *U. maydis* were obtained from Dr. R. Kahmann (Institut für Genbiologische Forschung, Berlin GmbH). Vectors containing the *a1* allele were digested with *Xho*I yielding a 2.3 kb DNA fragment which contained *mfa*₁ (mating factor encoded by *a*₁) and a 3.8 kb DNA restriction fragment which contained *pra*₁ (pheromone receptor encoded by *a*₁). The plasmid containing *mfa*₂ (mating factor encoded by *a*₂) was digested with *Bam*HI and *Eco*RI to yield a 2.1 kb DNA fragment which contained *mfa*₂. *pra*₂ (pheromone receptor encoded by *a*₂) was isolated by digestion of the appropriate vector with *Hind*III. All probes were separated from vector sequences by electrophoresis on 1% agarose and purified using Gelase according to manufacturer's instructions (Epicentre Technologies, Madison, WI).

Genomic Library from *Ustilago hordei*

A phage library from *U. hordei* MAT-1 DNA was constructed using lambda phage EMBL3 (Stratagene Cloning Systems, La Jolla, CA).

U. hordei genomic DNA was partially digested with *Sau3AI* and appropriately sized DNA (10-20 kb) was obtained from agarose gels using DEAE-strips (Schleicher & Schuell Inc., Keene, NH) or a Gel-X extractor (Genex Corporation, MD, USA). *Bam*HI-digested EMBL3 was ligated to the sized *U. hordei* genomic DNA with T4 DNA ligase. Recombinant lambda phage was packaged using Gigapack Gold packing extract (Stratagene, La Jolla, CA). Recombinants were selected using the Spi- selection, in which only recombinant phage will grow on P2 lysogenic *E. coli* strains. The library was then amplified and stored at 4°C over chloroform.

Cloning *U. hordei* DNA Homologous
to *U. maydis mfa* and *pra*

The lambda library was titered and screened to identify and isolate the desired recombinants. The recombinant plaques were transferred to nitrocellulose filters according to Sambrook et al. (1989). Two replica filters of each plate were made. One set of filters was hybridized to a mixture of ³²P-labelled *mfa*₁ and *pra*₁ DNA sequences of *U. maydis*. The other set was hybridized to a mixture of *mfa*₂ and *pra*₂. Hybridization was performed as described by Sambrook et al. (1989). Washed filters were then exposed to x-ray films. Positive plaques were picked and stored. Purification of positive plaques was done by isolating the phage and repeating the hybridization procedure until all plaques gave positive signals.

Characterization of the phage inserts was performed by restriction endonuclease analysis and gel electrophoresis. Lambda DNA from positive clones was obtained using the miniprep method described by Grossberger (1987). The DNA was digested using *Bam*HI, *Eco*RI, *Sal*I,

SacI and *XhoI*, separated by gel electrophoresis and transferred to a Zeta probe nylon membrane (Bio-Rad, Richmond, CA). The membrane was then hybridized independently to ^{32}P -labelled *mfa*₁, *mfa*₂, *pra*₁ and *pra*₂ DNA. The same conditions were used for all hybridizations. Plasmid vectors containing *U. maydis mfa*₁, *mfa*₂, *pra*₁ or *pra*₂ were digested with the proper enzymes (see DNA procedures), transferred to a nylon membrane and used as positive controls to ascertain that the hybridization procedures were done properly. The membrane was then hybridized to the respective probe using the same conditions as used to evaluate the recombinant phage. DNA restriction fragments that hybridized to the *U. maydis* mating factor and/or pheromone receptor genes were excised from the gel and ligated to pCM54 (Sambrook et al., 1989). The ligation mixture was then transformed into competent *E. coli* strain DH5 α cells (GIBCO BRL Inc.) and plated on LB medium containing 50 $\mu\text{g/ml}$ ampicillin. Ampicillin-resistant colonies were picked and miniprep DNA was screened for insertion of the restriction fragment of interest. As no visual selectable marker was present, selection was made by testing a large number of isolates. Plasmids containing appropriately sized DNA were selected and increased. Plasmids were digested with *BamHI* to confirm the size of the DNA fragment that was subcloned.

Transformation of *U. hordei*

Protoplasts and DNA transformations were performed as described by Holden et al. (1988). Plasmids containing the DNA fragments which hybridized to *U. maydis mfa* and or *pra* genes were transformed

separately into *U. hordei* (MAT-1 and MAT-2). For protoplast regeneration, half of each transformation mixture was plated directly onto HCM plates containing 1.2 M sorbitol and 150 $\mu\text{g/ml}$ hygromycin and incubated at 21°C for 7-8 days under dark conditions. The remaining half of the protoplasts were incubated at 4°C overnight and then plated as indicated above. Transformants were isolated and grown on HCM agar containing hygromycin B and analyzed on HCM charcoal agar co-inoculated with race 14 MAT-1 and 14 MAT-2 wild-type sporidia. The transformants were also analyzed using the "sandwich method" (see Chapter 3). In addition, the transformed cells were analyzed microscopically on water agar squares for cell phenotype.

Results and Discussion

Cloning *U. hordei* DNA Homologous to *U. maydis* *mfa* and *pra*

Froelinger and Leong (1991) and Bolker et al. (1992) described the genomic organization of the *a* locus on *U. maydis* as containing blocks of non-homologous DNA of 4.5 kb for *a*₁ and 8 kb for *a*₂. The non-homologous sequences are flanked by identical sequences. Each allele, *a*₁ and *a*₂, is determined by a set of two genes, *mfa* (mating factor) and *pra* (pheromone receptor). The *a*₁ and *a*₂ alleles from *U. maydis* have been shown to hybridize to DNA from *U. hordei* (Bakkeren et al., 1992). *a*₁ hybridized to *U. hordei* MAT-2 and *a*₂ showed hybridization to both MAT-1 and MAT-2 (Bakkeren et al., 1992). I used DNA sequences from *U. maydis* that encode *mfa*₁, *mfa*₂, *pra*₁ and *pra*₂ as probes to determine if similar sequences and organization could be found in *U. hordei*.

An amplified phage library was screened with a_1 (a mixture of mfa_1 and pra_1) and with a_2 (a mixture of mfa_2 and pra_2). Seventy-nine out of 25,000 plaques hybridized to both a_1 and a_2 . One plaque hybridized faintly to only a_1 and another hybridized faintly to only a_2 . Ten plaques that hybridized strongly to both probes were selected and increased. Six of those plaques showed strong hybridization after purification and re-probing. These phage were purified 3 times to achieve homogeneity. The selected phage were then increased as minipreps (Grossberger, 1987) and DNA was isolated from each phage. The DNA was digested with *Sma*I, *Bam*HI, *Hind*III, *Sac*I and *Xba*I, transferred to a nylon membrane and probed individually with mfa_1 , mfa_2 , pra_1 and pra_2 . After preliminary results, the selected phage were digested with *Bam*HI and *Eco*RI, separated by gel electrophoresis, blotted to a nylon membrane and probed again with each of the probes singly. Two bands from *Bam*HI-digested phage DNA hybridized with the probes; mfa_1 hybridized to a 5.0 kb fragment, mfa_2 hybridized to a 4.5 kb fragment, pra_1 hybridized weakly to both fragments and no hybridization to pra_2 was found (Figure 9). Thus, hybridization of the *U. maydis* a alleles to DNA from *U. hordei* indicated that *mfa*-like and *pra*-like DNA sequences are present in two *Bam*HI restriction fragments from *U. hordei* MAT-1 DNA. One fragment, the 5.0 kb *Bam*HI fragment, contains mfa_1 -like sequences and possibly pra_1 -like sequences. The 4.5 kb fragment, contained a mfa_2 -like and possibly pra_1 sequences. It is possible that the hybridization of *U. maydis* mfa_1 , mfa_2 and pra_1 genes to *U. hordei* DNA may simply indicate that some other sequences with similarity to these probes are present in *U. hordei*. However, previous

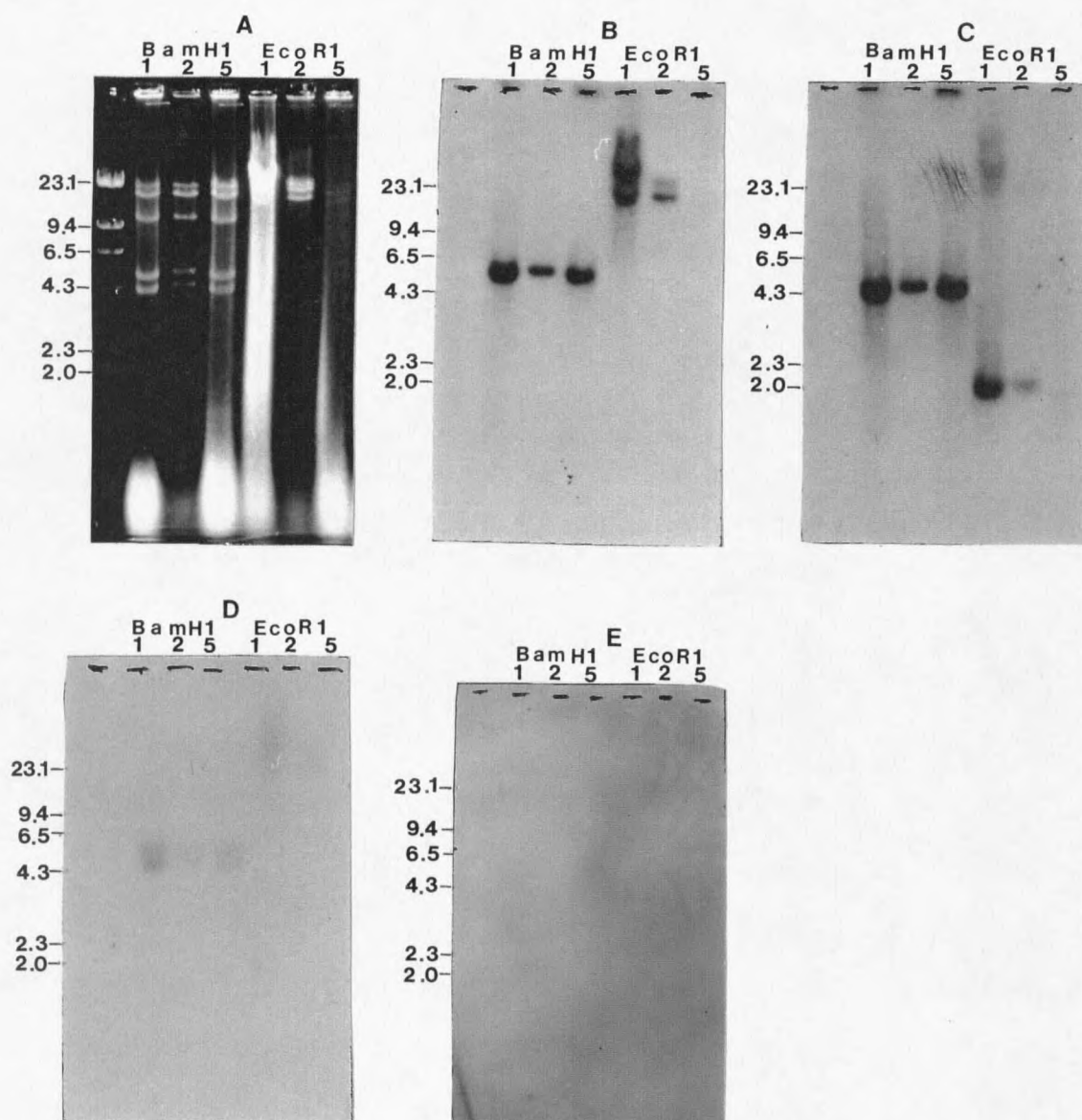


Figure 9. Autoradiograms of southern blots containing the recombinant phage DNA probed with *U. maydis* *a* locus genes. Panel (A) shows the ethidium bromide-stained gel from which the southern blots were made. Phage 1, 2 and 5 were digested with *Bam*HI and *Eco*RI. Sizes indicated to the left are in kb and based on the sizes of *Hind*III fragments of lambda DNA. (B) Southern blot probed with *mfa*₁. (C) Southern blot probed with *mfa*₂. (D) Southern blot probed with *pra*₁. (E) Southern blot probed with *pra*₂. No hybridization was observed.

evidence for the production of small, diffusible mating factors by *U. hordei* (Chapter 3) coupled with the hybridization of *U. hordei* DNA to mating factor and pheromone receptor genes of *U. maydis* strongly suggests that the *U. hordei* sequences encode pheromones and their receptors.

Transformation of *U. hordei* with
4.5 kb and 5.0 kb *Bam*HI Fragments

In order to see if the cloned fragments from *U. hordei* with homology to the *U. maydis* *a* locus have a role in the mating process, the lambda inserts were subcloned and transformed into *U. hordei*. DNA from a phage containing both the 4.5 kb and the 5.0 kb fragment was digested with *Bam*HI, purified by gel electrophoresis and ligated into pCM54. Ligation mixtures were transformed into *E. coli* and plasmids from transformants resistant to ampicillin were analyzed. Two transformants out of 18 contained the 4.5 kb *Bam*HI fragment and one out of 90 contained the 5.0 kb *Bam*HI fragment. pCM54 containing the 4.5 kb fragment or the 5.0 kb fragment were phenol extracted and RNase treated. Each plasmid was transformed into *U. hordei* strain 14 MAT-1 and 14 MAT-2. The introduction of the 4.5 kb *Bam*HI fragment cloned from MAT-1 did not produce any change in the phenotype of the MAT-1 or MAT-2 cells. The transformants mated normally with wild-type cells of the opposite mating type on medium containing activated charcoal, but did not mate with cells of the same mating type and were not Fuz+ when spotted alone on charcoal agar.

Transformation of one mating type of *U. maydis* with the *a* allele of the opposite mating-type caused the cells to behave as double

maters, *i.e.*, they produced a Fuz+ reaction when co-spotted with similar or opposite mating type cells on medium containing activated charcoal (Bolker et al., 1992). However, *U. hordei* transformants containing the 4.5 kb *Bam*HI fragment did not show this behavior.

When evaluated by the "sandwich technique", these transformants induced conjugation tubes on cells of the opposite mating-type but not on cells of the same mating-type. The transformants produced conjugation tubes when induced by cells of the opposite mating type, thus behaving phenotypically as wild-type cells.

When the 5.0 kb *Bam*HI fragment was introduced into *MAT*-2 cells, it produced a change in the phenotype (Figure 10). The colonies of *MAT*-2 transformants had narrow, shallow ridges resembling the morphology of certain *Fuz*- mutants described in Chapter 3, while *MAT*-1 transformants showed no change. Transformed *MAT*-2 cells produced conjugation tube-like structures which arose either from one end or from both ends of the cell (Figure 10). These tubes sometimes branched. A short aerial mycelium was frequently produced from these tubes. This mycelium was produced in the absence of fusion of conjugation tubes to each other or to opposite mating type cells. The tubes and/or aerial mycelia were uninucleate with a low incidence of binucleate filaments.

MAT-1 and *MAT*-2 transformants containing the 5.0 kb *Bam*HI fragment mated readily with opposite mating-type cells on charcoal-containing medium, but did not mate with same mating-type cells. Both transformants induced conjugation tubes on opposite mating-type cells but did not induce conjugation tubes on cells of similar mating type.

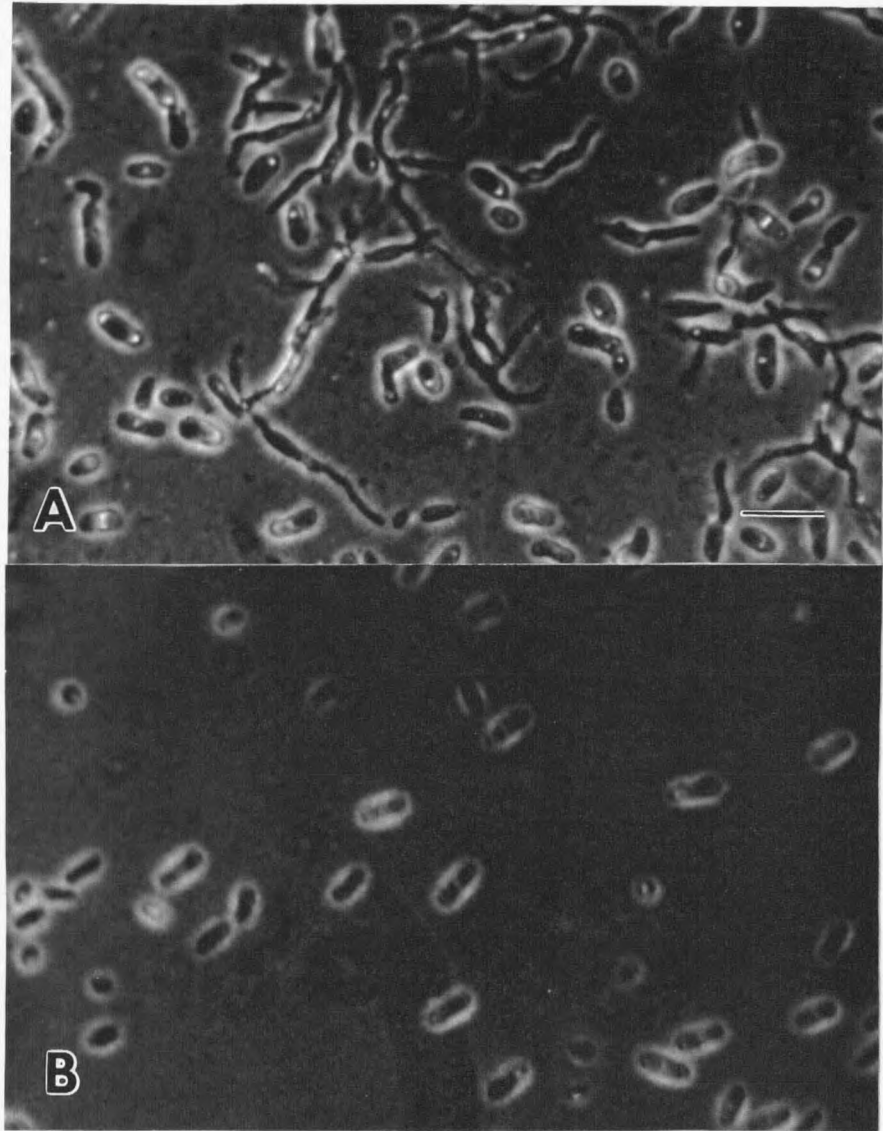


Figure 10. Phenotype of *MAT-2 U. hordei* cells transformed with 5.0 kb *Bam*HI fragment *MAT-1* DNA. (A) Conjugation tube-like are produced constitutively. Tubes developed from either one end or both ends of the sporidium. Conjugation tubes produced by transformed cells branch readily and produce an incipient aerial mycelium. (B) *MAT-2* cells transformed with 4.5 kb *Bam*HI fragment *MAT-1* DNA. Bar = 20 μ m.

These results indicate that the cloned 5.0 kb *Bam*HI fragment may code for a signal which activates the production of conjugation tubes. This 5.0 kb fragment may contain the pheromone receptor gene, so that transformed cells would have both the resident and the introduced pheromone receptors as well as the resident mating factor gene. The introduced pheromone receptor would be activated by the resident mating factor and the cell would therefore produce conjugation tubes constitutively. If this were true, it could be inferred that both pheromone receptors have a similar transduction pathway since both are able to produce conjugation tubes in the same cell. It is also possible that the 5.0 kb *Bam*HI fragment codes for the *MAT-1* mating factor which would activate the resident receptor of the *MAT-2* cell since the transformants did not induce conjugation tubes on cells of the opposite mating type, it is possible that the *MAT-1* mating factor is not secreted from the transformed cells or is not properly processed.

Despite the production of conjugation tubes by transformed *MAT-2* cells, no fusion was observed when these cells were mixed with wild-type cells of the same mating type and no induction of conjugation tubes was observed when the transformants and cells of the same mating type were evaluated by the sandwich technique. However, when transformants were challenged against wild-type cells of the opposite mating type, they readily induced conjugation tubes and fusion occurred.

Based on these results, it can only be speculated as to the possible genes encoded on the cloned fragments. What is certain is

that the 5.0 *Bam*H1 kb fragment contains DNA sequences that induce conjugation tube-like structures on recipient cells of the opposite mating type.

These preliminary molecular results and the strong evidence of mating factors (see Chapter 3) indicate that there are specific pheromones (mating-factors) and receptors encoded by the *MAT* locus on *U. hordei*. It is proposed that each *U. hordei* mating type is determined by specific sequences that encode a pheromone and a pheromone receptor. The pheromones would act as the signal which activate the pheromone receptor on cells of the opposite mating type. Thus each mating type will have a mating-type specific pheromone and a mating-type specific pheromone receptor (Figure 11).

Based on the predicted amino acid sequence, the *a* locus of *U. maydis* appears to encode specific pheromones and receptors which provide the components involved in the cell-to-cell recognition (Bolker et al., 1992). The structural organization of the *a* locus on *U. maydis* is similar to that of the mating-type locus on *Neurospora crassa* (Glass et al., 1990; Staben and Yanofsky, 1990), containing non-homologous DNA which gives mating type activity. These non-homologous DNA fragments are flanked by homologous DNA fragments. Mating pheromones and their receptors have been described in *Saccharomyces cerevisiae* (Michaelis and Herskowitz, 1988; Herskowitz, 1988), and pheromones have been described in *Rhodospiridium turoloides* (Akada et al., 1989), *Tremella mesenterica* (Sakagami et al., 1979) and *Tremella brasiliensis* (Ishibashi et al., 1984). Pheromones and their receptors also seem to be present in *U. hordei* as key elements in the mating process.

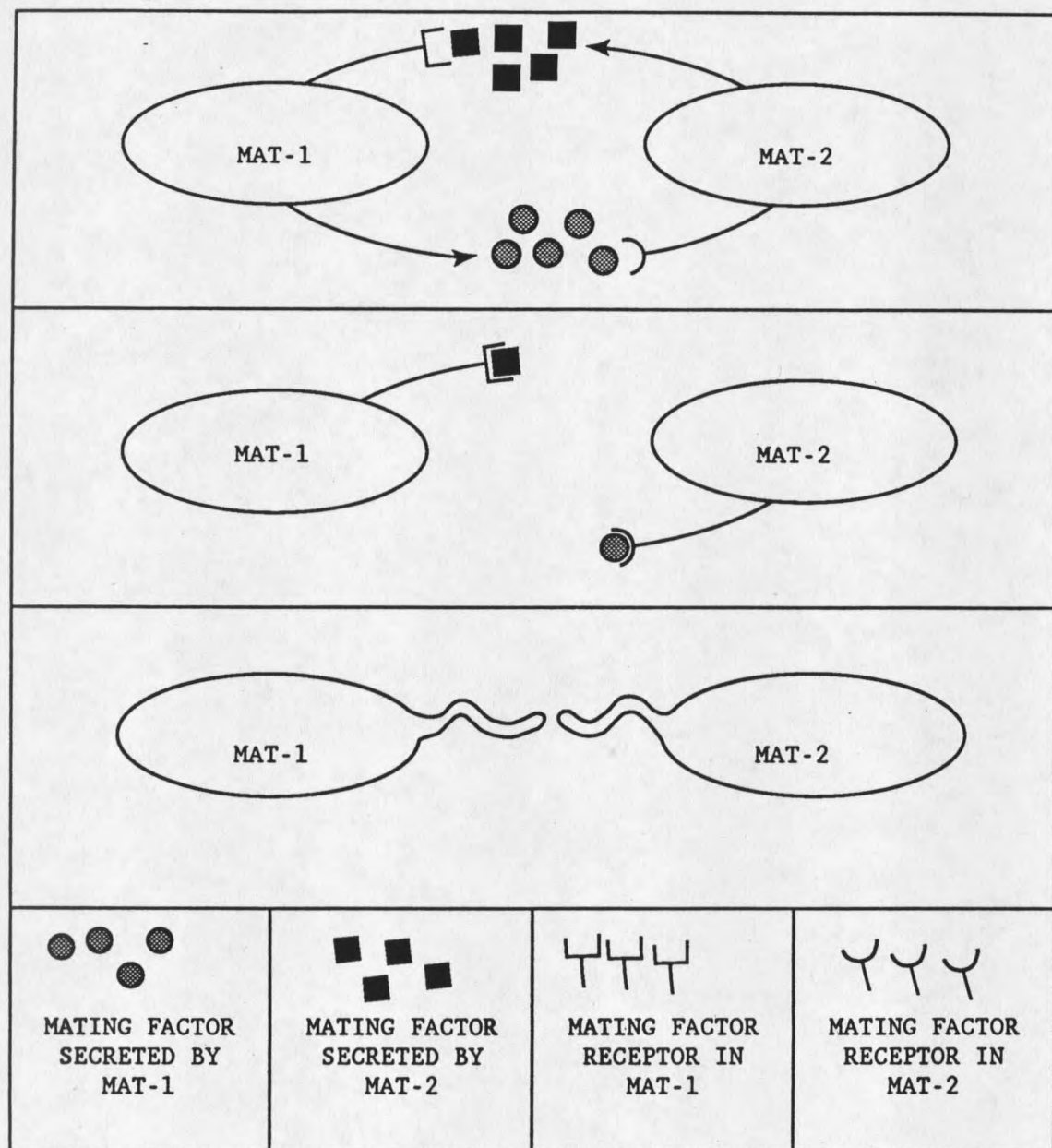


Figure 11. Cell-to-cell recognition on *U. hordei*. Elongated cells represent the sporidia with their respective mating-type. Pheromones and the receptors are shown schematically as indicated. Arrows indicate the production of mating factors. Pheromone binding to the specific receptors leads to the recognition of cells of the opposite mating-type and to the production of conjugation tubes.

Mating mechanisms are extremely variable among the diverse groups of fungi. All of the strategies developed by these organisms have the fundamental role of governing the sexual behavior to ensure the important biological event of outbreeding as means of generating genetic diversity and, consequently, a better opportunity for survival.

CHAPTER 5

SUMMARY

Mating in *U. hordei* is essential for completion of its life cycle. Mating controls the entrance into the sexual phase, resulting in the conversion of haploid, non-pathogenic yeast-like sporidia (asexual phase) to dikaryotic, pathogenic mycelia which represent the sexual phase. Mating in *U. hordei* is controlled by a single locus with two alleles (bipolar mating-type) giving rise to two mating types, *MAT-1* and *MAT-2*.

In this investigation, a comprehensive morphological, genetic and molecular analysis of the mating process in *U. hordei* is described.

An improved medium to test the mating reaction was developed. The addition of activated charcoal to a variety of media enhanced the mating reaction as measured by mycelium formation. The incubation time during which the strongest mating reaction occurred was also reduced. Complementation assays using minimal medium containing activated charcoal demonstrated allelism of mutations in auxotrophic sporidial strains of opposite mating type. The ease and reliability of this mating test allowed for a rapid identification of the mating type of unknown isolates and progeny of crosses. This medium also proved to be dependable for performing complementation tests, eliminating the need for inexact morphological characterization and microscopic observation needed with other historically-used media.

In the morphological analysis, I found that when sporidia of the opposite mating type were mixed and placed on water agar, both cells begin to produce conjugation tubes within two hours at 21°C. The growth of the conjugation tubes was directed toward the tip of tubes arising from cell of the opposite mating type. These tubes fused and dikaryotic, pathogenic mycelium emerged from this conjugation bridge. For the first time in *U. hordei*, evidence of diffusible small molecular weight mating factors was found when sporidia separated by a dialysis membrane were still capable of inducing conjugation tube formation by cells of the opposite mating type. We can infer from these results that these mating factors, or pheromones, are involved in cell-to-cell signalling and in the recognition of opposite mating-type cells. This cell-to-cell communication initiates the production and fusion of conjugation tubes which is the first step in the mating process.

Charcoal-containing medium was shown to be suitable for screening mating-defective (*Fuz*-) mutants of *U. hordei*. The mutants were detected by the lack of white, aerial colonies which formed when wild-type strains were crossed. Twenty six mutants were arranged into several classes based on the ability to form conjugation tubes, the ability to induce conjugation tube formation by opposite mating type cells and cell morphology. The classes are: (1) those that still produce conjugation tubes and induce conjugation tube formation by wild-type sporidia of opposite mating type, (2) those that do not form conjugation tubes but still induce conjugation tube formation, and (3) those that no longer produce or induce conjugation tubes. Sporidial and mycelial mutants were found in each class. These mutants

provide an indication of the genetic complexity involved in this critical-phase of the *U. hordei* life cycle.

Molecular analysis of the *U. hordei* MAT locus revealed that *mfa*₁ (mating factor encoded by *a*₁), *mfa*₂ (mating factor encoded by *a*₂), and *pra*₂ (pheromone receptor encoded by *a*₂) DNA sequences from *U. maydis* hybridized to DNA from *U. hordei*. The homologous DNA fragments were cloned from a *U. hordei* MAT-1 strain using a phage library. *mfa*₂ hybridized to a 4.5 kb *Bam*HI restriction fragment, *mfa*₁ hybridized to a 5.0 kb *Bam*HI fragment and *pra*₁ hybridized to both of the restriction fragments. Upon transformation with the 5.0 kb *Bam*HI fragment from a MAT-1 strain, MAT-2 *U. hordei* transformants, produced conjugation tube-like structures constitutively, indicating that the cloned fragment may act as a signal that triggers conjugation tube formation. Based on these results and the strong evidence for small molecular weight mating factors, it is proposed that the *U. hordei* MAT locus contain sequences that encode mating factors (pheromones) and pheromone receptors, and that each mating-type will have a specific pheromone and a specific pheromone receptor. Mating type is thus determined by components involved in cell-to-cell recognition, which is the first stage in the mating process in *U. hordei*.

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