Production of a Mating Inhibitor by *Ustilago hordei*

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ABSTRACT

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Ustilago hordei, the cause of barley covered smut, was found to produce a factor that inhibited its own mating. The mating inhibition factor (MIF) specifically inhibited mating of *U. hordei* and other *Ustilago* spp., but not teliospore germination or sporidial growth. MIF did prevent teliospore germination of *Tilletia caries* and *T. contraversa*. MIF was found at low levels in culture supernatants of either mating type of *U. hordei* grown separately, but at higher levels when both mating types were grown together, in the supernatants of MAT-1 mating type cells transformed with the MAT-1 pheromone gene *mfa1* and of MAT-2 cells transformed with

either *mfa1* or the MAT-1 pheromone receptor gene *pra1*. Diploid cells produced no detectable inhibitor, nor did MAT-1 cells with a disrupted mating type locus that deleted both *mfa1* and *pra1*. MIF production was restored when *mfa1*, but not *pra1*, was added back to the MAT-1Δ cells. MIF activity was altered by protease treatment. Highly purified MIF from MAT-1 cells contained cysteine methyl ester, farnesyl cysteine, farnesyl cysteine methyl ester, and a dodecapeptide with a mass consistent with that of MAT-1 pheromone lacking the terminal cysteine. Since smut fungi must first mate to become pathogenic, mating inhibition has the potential to be an effective method of disease control for these pathogens.

Additional keywords: *Cryptococcus neoformans*, *Ustilago avenae*, *U. maydis*, *U. nuda*.

Fungal plant pathogens are currently controlled by a variety of methods including application of fungicides, host genetic resistance, and cultural practices. No single method works for all pathogens, and for some pathogens, none are effective. In addition, each of these methods has negative features: fungicides can be expensive, may harm beneficial microorganisms, and may pollute the environment; genetic resistance in the host takes years to develop, is often short-lived, and might, in the absence of disease, actually reduce yield; and new cultivation practices are not always accepted by the farmer, and often, while controlling a specific disease, may exacerbate other problems (9,16,31,38). Although metabolic processes have been common targets for antifungal agents, a strategy for the control of plant pests that has not yet been used with fungi is the disruption of the sexual cycle. This strategy has been used with great success with insect pests using sex pheromones (10). Fungal sexual spores are often persistent, allowing long-term survival of the pathogen. These spores then act as the primary inoculum to spread the disease in time and space. Additionally, mating and meiosis increases genetic variability, allowing the introduction and rapid spread of virulence and fungicide resistance genes in a fungal population.

The sexual cycle is particularly important in the disease cycle of the smut-causing fungi such as *Ustilago hordei*. Mating in *U. hordei*, which is controlled by a single bipolar locus, results in the conversion of uninucleate, nonpathogenic, yeastlike sporidia into pathogenic, dikaryotic mycelium (41). It has been previously shown that *U. hordei* controls the initiation of mating with small molecular weight (mw) pheromones (26) that act to signal cells of the opposite mating type. In other higher fungi, pheromones are most often farnesylated peptides ranging in length from 9 to 13 amino acids (1,8,17,29,32,33,39). The early events of mating in *U. hordei* initiated by pheromones include the induction of conjugation tube

synthesis, conjugation tube fusion, and the development of dikaryotic mycelium from the conjugation bridge (26).

While *U. hordei* has a single mating type locus with two alleles, MAT-1 and MAT-2 (commonly designated A and a) (25,42), it has been shown that there are actually two sets of genes required for mating (4). These genes are analogous to the *U. maydis* a and b loci. In *U. hordei*, these sets of genes appear genetically to be a single locus, but are apparently in a region of low recombination, since they are not tightly linked physically (5). As with *U. maydis* (37), the b locus analogue in *U. hordei* is a gene pair designated bE and bW (4), which appear to be regulatory in function. In *U. maydis*, the a locus codes for pheromones and the pheromone receptors (7). The pheromone receptor for *U. hordei* MAT-1 mating type, *pra1*, has been cloned and sequenced (5).

During attempts to purify the pheromones from the two *U. hordei* mating types, a mating inhibiting factor (MIF) was found in the culture supernatants. Similar activities have been reported during the purification of the M factor from *Schizosaccharomyces pombe* (8) and pheromones from *U. maydis* (39). Chemical characteristics of the *U. hordei* MIF, its regulation, and range of activity are reported here.

MATERIALS AND METHODS

Cultures and media. *U. hordei* strains 14A (MAT-1; ATCC #90512), 14a (MAT-2; ATCC #90510) (25), and the diploid strain W-7 (11) were used in this study. Teliospores of *U. hordei* were obtained by inoculating Odessa barley by vacuum infiltration with 14A and 14a sporidia and growing the plants in a glasshouse as described previously (25). Teliospores of *U. nuda*, *U. maydis*, *U. avenae*, *U. kollerii*, *U. bullata*, *Tilletia caries*, and *T. contraversa* were obtained from D. Mathre, Montana State University (MSU), as were cultures of *Fusarium oxysporum*, *F. culmorum*, *Gaeumannomyces graminis*, *Pyrenophora teres*, *Rhizoctonia solani*, *Rhynchosporium secalis*, and *Sclerotinia sclerotiorum*. *Pseudomonas syringae* and *Xanthomonas campestris* cultures were provided by D. Sands, MSU. *Cryptococcus neoformans* strains of a and α mating types were obtained from V. Chaturvedi, University

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of Cincinnati Medical Center. Fungal cultures were routinely maintained on Holliday's complete medium (HCM) (13).

MIF partial purification and bioassay. Sporidial cultures of *U. hordei* were grown in side-arm flasks containing 50 ml of Vogel's minimal medium (VMM) (45) at room temperature on a rotary shaker at 250 rpm to a final density of 450 to 500 Klett units. The cells were removed by centrifugation at $10,000 \times g$ for 10 min. Each 50-ml supernatant was concentrated to 10 ml under vacuum on a rotary evaporator, and 20 ml of methanol was added. Following centrifugation at $15,000 \times g$ for 15 min to remove methanol-insoluble materials, the methanol was removed from the supernatant by rotary evaporation. The solution was dialyzed (size cutoff 6,000 to 8,000 mw; Spectra/Por membrane, Spectrum Medical Industries, Inc., Los Angeles) against distilled H₂O for 4 h at room temperature and dried with a rotary evaporator. The material remaining after drying was dissolved in 1 ml of sterile distilled water, giving a "partially purified" MIF fraction used in some experiments.

To further purify MIF for chemical analysis, *U. hordei* strain 14A cells were grown in 500-ml volumes, the cells removed, and the culture supernatant concentrated fivefold. The concentrated supernatant was extracted with 4× volume chloroform, separated into water and chloroform-soluble fractions, and the water-soluble fraction was precipitated with 4× volume methanol. Following centrifugation at $15,000 \times g$ for 15 min, the supernatant was concentrated by rotary evaporation to a final volume $1/50$ the original culture volume. This concentrated sample was subjected to size-exclusion chromatography using Sephadex G50 (Sigma Chemical Co., St. Louis) and Toyopearl HW55 (Supelco, Bellefonte, PA) with 20% ethanol and 75% methanol as solvents, respectively. Fractions displaying strong mating inhibitory activity were further purified by reverse-phase high-pressure liquid chromatography (HPLC) using 85% methanol on a C18 column.

Peptides were synthesized by the MSU Peptide Facility or Research Genetics Inc. (Huntsville, AL) using solid phase 9-fluorenylmethoxycarbonyl chemistry. Cysteine and cysteine methyl ester (Sigma Chemical Co.) were farnesylated following the protocol of Hrycyna and Clarke (14). Farnesylation was verified by thin-layer chromatography (TLC) on silica 60 (Whatman, Hillsboro, OR) with methanol/chloroform (2:1, vol/vol).

Mass spectrometry was performed at the MSU Mass Spectrometry Facility. α -Cyano-4-hydroxycinnamic acid was used as the matrix for MALDI-TOF spectrometry (PerSeptive Biosystems/Vestec Products, Framingham, MA). Electrospray mass spectrometry was performed with a VG Trio 2 mass spectrometer (VG, Beverly, MA) using MeOH/H₂O/acetic acid (50:50:1) as the solvent.

Mating inhibition was assayed by adding 2 μ l of the partially purified MIF solution to a 1- by 1- by 0.2-cm square of trace elements (TE) agar (26). After the liquid had dried, 2 μ l of a teliospore suspension (1×10^6 spores per ml) or a cell suspension containing an equal mixture of 14A and 14a cells at a final concentration of 1×10^6 cells per ml, was added to the agar square and allowed to dry. The squares were incubated on a microscope slide in a moist chamber at 16°C and observed microscopically after 24 and 48 h.

A novel bioassay was developed to test the activity of unknown samples and synthesized compounds following separation by TLC as described above. The plates were air-dried, examined under short- and long-wavelength UV light, and spots were marked with pencil. Duplicate TLC plates were stained with ninhydrin to confirm the location of the standards and unknowns. After silica between the spots was scratched from the backing to prevent cross-contamination, TE agar squares were placed on the marked spots. Cells were placed on the agar squares as in the standard bioassay. The TLC plate was incubated in a humid chamber for 24 and 48 h, after which the agar squares were removed and the cells examined microscopically.

To determine the effect of MIF on smut fungi other than *U. hordei*, teliospores from *Ustilago* spp. were placed on TE agar with and without serial twofold dilutions of MIF. *Tilletia* spp. telio-

spores were plated on soil extract agar and *C. neoformans* mating was performed on V-8 agar using cell suspensions containing equal concentrations of each mating type. Potential inhibition of growth of nonsmut filamentous fungi and bacteria was determined by adding MIF to paper discs that were placed close to agar plugs of fungi or on lawns of the bacteria. Growth inhibition was determined by a lack of fungal growth near the discs with the fungi or zones of inhibition surrounding the discs with the bacteria.

DNA manipulations. Strains 14A and 14a were transformed with *mfa1* and *pra1*, the pheromone and pheromone receptor genes from MAT-1 mating type cells. These genes were cloned using the *U. maydis mfa1* and *pra1* genes (7), obtained from R. Kahmann, Berlin, as heterologous probes of a genomic library prepared from *U. hordei* MAT-1 mating type DNA in λ 2001 (18). The genes were then cloned into pCM54 (43), which contains a *U. maydis* autonomously replicating sequence, for transformation into *U. hordei*. *mfa1* was also subcloned into pBluescript KS+ (Stratagene Inc., San Diego, CA) for sequencing using the dideoxy chain termination method (35) with Sequenase enzyme (United States Biochemical Corp., Cleveland). pMAT1(-) was designed to knock out the MAT-1 locus including all of *mfa1* and the 3' portion of *pra1*. This vector was constructed by replacing the 5.5-kilobase (kb) *Bam*HI fragment containing *mfa1* and the 3' portion of *pra1* with the 1.8-kb *Bam*HI cassette containing the gene for bleomycin resistance (obtained from J. Kronstad, University of British Columbia). The *Xba*I-*Apa*I fragment was ligated into pBluescript and transformed into MAT-1 cells as described below. Bleomycin-resistant colonies were tested for the ability to mate with MAT-2 cells on charcoal agar (25). DNA from transformants that were unable to mate was analyzed by Southern analysis to confirm that a homologous double crossover event accounted for the inability to mate. pUhp α 1 was made by subcloning the 2.1-kb *Hind*III fragment from the lambda insert into pJS42, which was constructed by removing the *Xba*I fragment from the Hsp70 promoter region of pCM54 (43). pUhm α 1 contained the 2.4-kb *Hind*III-*Eco*RV fragment, including *mfa1*, ligated into pHyg101 (obtained from J. Kronstad). The cloning and characterization of *mfa1* will be described in detail elsewhere.

For transformation of *U. hordei*, strains 14a and 14A were grown in 25 ml of HCM broth to densities of 100 to 250 Klett units and centrifuged at $10,000 \times g$ for 10 min at 4°C. The cells were resuspended in SCS (10 mM sodium citrate and 1.0 M KCl, pH 6.5) and vortexed to remove extracellular polysaccharide. After centrifugation, the pellet was resuspended in 1 ml SCS containing 20 mg of Novozyme 234 (Interspers Products Inc., Foster City, CA) and gently rocked for 1 to 2 h. Cells were incubated until greater than 90% of the cells were converted to spheroplasts, as determined by microscopic observation, and counted with a hemacytometer. Following one wash with SCS and one wash with STC (10 mM Tris-HCl, 50 mM CaCl₂, and 1.0 M KCl, pH 8.0), STC was added to bring the final suspension to 2×10^8 spheroplasts per ml. Fifty microliters of the spheroplasts were added to a microcentrifuge tube with 1 μ l of heparin (15 mg/ml) and 1 to 5 μ l of DNA (1 to 5 μ g/ μ l). After incubating for a minimum of 15 min on ice, 500 μ l of 40% polyethylene glycol 3,500 (Sigma Chemical Co.) in STC was added and mixed well. Following incubation for 5 to 15 min on ice, 500 μ l of SCS was added, and the spheroplasts were pelleted at $3,000 \times g$ for 10 min at 4°C. The pellet was resuspended in 200 μ l of SCS and spheroplasts were spread on HCM agar containing 150 μ g of hygromycin B (Boehringer Mannheim Biochemicals, Indianapolis, IN) per ml or 20 μ g of bleomycin (Sigma Chemical Co.) per ml. The petri plates were incubated in the dark at 25°C until colonies were visible, typically about 1 week.

Treatment of MIF with proteases. To determine if MIF contained a peptide moiety, the partially purified MIF fractions from 14A and 14a cells grown alone and 14a transformed with *mfa1* were treated with pronase E type XXV (20 mg/ml), trypsin type XI (1 mg/ml), or carboxypeptidase Y (0.5 mg/ml). All enzymes were obtained from Sigma Chemical Co. The proteases were mixed

1:1 (vol/vol) with MIF and incubated at 37°C for 24 or 48 h. Samples of each protease without MIF, and MIF without protease, served as control treatments. After incubation, all samples including controls were boiled for 15 min to inactivate the proteases. It was determined in preliminary experiments that boiling had no effect on MIF activity. MIF activity in the samples was determined using the bioassay described above. Slides were examined at 24 and 48 h, and the aerial dikaryotic mycelium per field using the 10× objective was quantified. Protease treatments were repeated three times.

RESULTS

A mating inhibitory factor was found in the supernatants of *U. hordei* cultures. This activity could be partially purified and concentrated by extracting the supernatant with methanol (2:1, vol/vol) and concentrating the methanol-soluble fraction under vacuum. When MIF was added to agar squares prior to inoculation with *U. hordei* teliospores, the spores germinated and the sporidia grew, but mating and subsequent formation of aerial dikaryotic mycelium was completely inhibited (Fig. 1). Interestingly, this was not an all-or-nothing effect. As MIF was diluted, a stepwise return of the mating process was observed. First, cells were able to form

conjugation tubes, but these did not fuse to form bridges. As MIF was further diluted, conjugation bridges formed, but the dikaryotic mycelium did not grow out of the bridge. Eventually, as MIF was further diluted, the mating process could be completed with the formation of dikaryotic mycelium.

The range of biological activity of MIF was determined by exposing other smut fungi to the same concentrations of partially purified MIF used for *U. hordei*. Mating of all *Ustilago* spp. tested was inhibited (Table 1), while teliospore germination and sporidial growth were marginally affected at best. Mating of *C. neoformans*, a human pathogen in the order Ustilaginales (21), was also inhibited in the presence of MIF (Fig. 2, Table 1), although its growth was not affected. MIF inhibited teliospore germination of both *Tilletia* spp. tested, *T. caries* and *T. contraversa* (Fig. 2, Table 1). MIF had no effect on the growth of a number of other fungi and bacteria (Table 1).

The relative quantity of MIF produced by different cell types was examined in order to gain insight into the origin and possible biological role of MIF. The culture supernatants from a diploid culture, W-7, and 14A and 14a cells grown either separately or together in the same flask were examined. Serial twofold dilutions of each partially purified MIF preparation were made, and the last dilution giving 100% inhibition of dikaryotic mycelium formation was noted (Table 2). Diploid strain W-7 produced no detectable MIF, while either mating type culture could be diluted 2:1 before aerial mycelium was observed. When both mating type cells were grown together, MIF yield doubled over when they were grown separately.

As part of a parallel study of *U. hordei* mating, the pheromone (*mfa1*) and pheromone receptor (*pra1*) genes were cloned from MAT-1 cells using the a locus genes *Ummfa1* and *Umpfa1* from *U. maydis* as heterologous probes. *U. hordei mfa1* and *pra1* were separated by about 1.7 kb (Fig. 3A). The amino acid sequence of the *U. hordei mfa1* translation product (Fig. 3B), predicted from the nucleic acid sequence (GenBank accession #AF043940), was similar to other fungal pheromones (29,39). The open reading frame codes for a 42-amino acid precursor, which is presumably processed to a shorter peptide of 13 amino acids (Fig. 3C), as described for *Ummfa1* from *U. maydis* (39). The terminal CAAX motif is typical of farnesylated fungal pheromones, in which the last three amino acids are removed during farnesylation of the cysteine residue (2,14). This terminal cysteine is known to be O-methylated in several fungal pheromones (8,23). The *mfa1* prepeptide showed 55% identity and 68% similarity with the *U. maydis* prepeptide. The sequence for *pra1* has been reported (5). When *mfa1* or *pra1* was transformed into MAT-2 cells, the morphology

TABLE 1. Effect of mating inhibition factor (MIF) on growth, spore germination, and mating of *Ustilago hordei* and related fungi

Pathogen ^a	Disease	Sporidial growth	Teliospore germination	Mating
<i>U. hordei</i>	Barley covered smut	+ ^b	+	–
<i>U. nuda</i>	Barley loose smut	±	+	–
<i>U. avenae</i>	Oat loose smut	+	+	–
<i>U. maydis</i>	Corn smut	±	+	–
<i>Tilletia caries</i>	Wheat common bunt	nt ^c	–	nt
<i>T. contraversa</i>	Wheat dwarf bunt	nt	–	nt
<i>U. bullata</i>	Brome grass smut	+	+	–
<i>U. kolleri</i>	Oat smut	+	+	–
<i>Cryptococcus neoformans</i>	Human pathogen	+	nt	–

^a MIF had no effect on the growth of *Gaeumannomyces graminis* (take-all), *Rhizoctonia solani* (bare patch), *Rhynchosporium secalis* (scald), *Pyrenophora teres* (net blotch), *Fusarium culmorum* (root rot), *F. oxysporum*, *Sclerotinia sclerotiorum*, *Pseudomonas syringae* (kernel, leaf blight), or *Xanthomonas campestris* (bacterial leaf streak).

^b + indicates that the MIF-containing culture behaved similarly to the control lacking MIF, while – indicates that MIF inhibited the indicated function. ± indicates that sporidial growth was slower than the control lacking MIF.

^c nt = not tested.

Fig. 1. Effect of mating inhibition factor (MIF) on mating of *Ustilago hordei*. A, *U. hordei* teliospores germinated on agar lacking MIF. Note that teliospores germinated and sporidia showed some growth before mating, resulting in the formation of the thick aerial mycelium (arrows). B, *U. hordei* teliospores plated on agar with MIF. The teliospores germinated and sporidia grew, but there was no mating as demonstrated by the complete lack of aerial mycelium. Bar = 50 µm.

of the transformants was altered (Fig. 4) such that the cells appeared to constitutively produce conjugation tubelike structures. The amount of MIF produced by these transformants was examined, and they were found to produce even greater amounts of MIF than when strains 14A and 14a were grown together in the same flask (Table 2). Although no cell morphology changes were seen when *mfa1* was transformed into MAT-1 cells (Fig. 4), this transformant produced twice as much MIF as wild-type MAT-1 cells (Table 2).

The MAT-1 locus, including all of *mfa1* and the 3' terminus of *pra1*, was deleted by gene disruption using pMAT1(-) (Fig. 3A). Southern analysis indicated that the vector integrated by a double crossover event (data not shown). Thus, this transformant had no *mfa1* sequence present and only the 5' portion of *pra1*. This mutant was sterile and did not produce conjugation tubes or in-

duce conjugation tubes by MAT-2 cells. This transformant also did not produce any detectable MIF (Table 2).

Because the deletion of the MAT-1 locus included both *mfa1* and *pra1*, we were interested in determining which of these caused the loss of MIF production from this transformant. *mfa1* and *pra1* were transformed individually into the MAT-1(-) strain using vectors with hygromycin resistance as selectable markers. When *pra1* was transformed into the MAT-1(-) strain, the transformant now made conjugation tubes, but did not induce conjugation tubes in MAT-2 cells. When *mfa1* was added back to the MAT-1-deleted mutant, the resulting transformant induced conjugation tubes in MAT-2 cells, but did not make conjugation tubes in the presence of MAT-2 cells. All of these observations are consistent with the presence or lack of functional *pra1* or *mfa1* genes. When tested for MIF synthesis, only the transformant containing *mfa1* produced MIF (Table 2).

Fig. 2. Effect of mating inhibition factor (MIF) on *Cryptococcus neoformans* and *Tilletia caries*. **A**, Both mating type sporidia of *C. neoformans* were plated on V-8 agar lacking MIF. The sporidia mated to form short dark mycelium (arrows). **B**, *C. neoformans* plated on V-8 agar with MIF. The sporidia grew well, but the dark mycelium is completely lacking. **C**, *T. caries* teliospores germinated in the absence of MIF. The spores germinated, formed sporidia, mated, and formed secondary sporidia. **D**, *T. caries* teliospores inoculated on agar with MIF. The teliospores do not germinate even after extended incubation. Bar = 100 μ m.

Partially purified MIF was treated with several different proteases to determine if MIF had a proteinaceous component. Following digestion with pronase E, carboxypeptidase Y, or trypsin for 48 h, mating inhibition was greatly reduced or eliminated when compared with the controls. MIF activity in controls was unaffected by boiling or incubation at 37°C for 48 h. Interestingly, when the partially purified MIF samples were digested with the proteases for 24 h, increases in inhibitory activity were observed.

Attempts to purify MIF to a single compound have been unsuccessful. However, after several rounds of gel permeation and reverse-phase chromatography, the number of detectable compounds was greatly reduced. Before a final separation on a C18 column by HPLC, multiple masses, detected by MALDI-TOF mass spectrometry, ranged from 1,320 to 1,432, consistent with the predicted pheromone, farnesylated PGKSGSGLGYSTC-OMe (mass 1,432), and degradation products of the pheromone including the loss of the *O*-methyl groups, farnesyl groups, or both (Fig. 5A). After passage over a C18 column, the MIF-containing fractions no longer had the larger masses, but primarily contained major masses of 1,110 and 1,133, which are consistent with PGKSGSGLGYST ± Na (Fig. 5B). Mass spectrometry of synthetic PGKSGSGLGYST gave identical masses (Fig. 5C).

Since the detected peptide appeared to be the pheromone without the cysteine, TLC and electrospray mass spectrometry were used to detect smaller compounds that might be expected if the pheromone was breaking down between the threonine and cysteine residues. Cysteine methyl ester, farnesyl cysteine, and farnesyl cysteine methyl ester (Fig. 3D) were detected and their presence confirmed by comparison to synthetic standards (data not shown). When the compounds separated by TLC were bioassayed by placing inoculated agar squares directly on the TLC plate, the synthetic and natural compounds showed similar activities; all were inhibitory to mating to different degrees. When different concentrations of the synthetic compounds were assayed for their effect on mating, cysteine alone had little effect when compared with the control (Fig. 6A and B), while farnesyl cysteine or farnesyl cysteine methyl ester both inhibited mating (Fig. 6C and D). A similar result was observed when the peptide PGKSGSGLGYST was assayed (Fig. 6E). In all of these cases, however, mating inhibition was not complete unless the concentration was increased to a level that also inhibited cell growth. The compound that had an activity most similar to MIF was cysteine methyl ester, with which mating was inhibited but the cells were still able to grow into colonies (Fig. 6F). Cysteine methyl ester also inhibited the germination of *T. caries* teliospores (data not shown).

DISCUSSION

During attempts to purify the *U. hordei* mating pheromones (26), a mating inhibitor was found in the culture supernatants. This activity was capable of totally masking pheromone activity, since no expected pheromone activity (i.e., induction of conjuga-

TABLE 2. Relative quantity of mating inhibition factor (MIF) produced by different cell types of *Ustilago hordei*

Cell Type	Relative MIF production ^a
Diploid	0
MAT-1	2
MAT-2	2
MAT-1 + MAT-2	4
MAT-1 (<i>mfa1</i>)	4
MAT-2 (<i>pra1</i>)	8
MAT-2 (<i>mfa1</i>)	8
MAT-1 (<i>MAT-1Δ</i>)	0
MAT-1 (<i>MAT-1Δ, mfa1</i>)	1
MAT-1 (<i>MAT-1Δ, pra1</i>)	0

^a Determined by serial twofold dilution of the crude extract concentrate. End point was the first dilution that did not completely prevent conjugation tubes.

tion tubes in cells of the opposite mating type) was observed in these supernatants. Interestingly, when the mating assay was performed on agar containing activated charcoal, MIF no longer inhibited mating. Therefore, the neutralization of MIF, presumably by adsorption to the charcoal, could explain the increased mating response of *U. hordei* that has been observed in the presence of activated charcoal (25).

Mating inhibitors have been previously found in fungal culture supernatants in the course of pheromone purification. While isolating M factor from *Schizosaccharomyces pombe*, Davey (8) found an inhibitor that was attributed to an oxidized form of the pheromone. Spellig et al. (39) noted a similar inhibiting activity during the purification of the mating factors from *U. maydis*. In both cases, the inhibitor and pheromone copurified through several purification steps, indicating that the pheromones and inhibitors were chemically related.

Several early observations led to the suspicion that the *U. hordei* mating inhibitor was either physically related to, or coordinately expressed with, pheromones. For example, the quantity of MIF found in the culture supernatants mirrored the expected quantity of pheromone. In both *Saccharomyces cerevisiae* and *U. maydis*, pheromones are produced constitutively at low levels until exposed to pheromone of the opposite mating type, at which time they are up-regulated (12,20,44). In *Saccharomyces cerevisiae*, pheromone synthesis is repressed in diploid cells (12,20). Similarly, *U. hordei* sporidia of either mating type produce low levels of MIF, but diploids made no detectable MIF. MIF production increased when MAT-1 and MAT-2 cells were grown together or when MAT-2 cells were transformed with the MAT-1 pheromone or pheromone receptor. In these transformants, the cell synthesizes both a pheromone and the receptor that recognizes it. In fact, these transformants have an altered morphology that suggests that they are constitutively producing conjugation tubes (Fig. 4) (5), and are

Fig. 3. The MAT-1 mating type locus. **A**, A restriction map of the MAT-1 locus showing the location of the pheromone receptor gene *pra1* and pheromone gene *mfa1*. pMAT-1(–) was constructed by replacing the central *Bam*HI fragment with a 1.8-kilobase (kb) *Bam*HI cassette containing the BleoR gene and ligating the resulting 8.3-kb *Xba*I-*Apa*I fragment into pBluescript. pUhp_{pra1} was constructed by ligating the 2.1-kb *Hind*III fragment into pJS42, and pUhm_{mfa1} was made by inserting the 2.4-kb *Hind*III-*Eco*RV fragment into pHyg101. **B**, The amino acid sequence of the MAT-1 pheromone precursor deduced from the nucleic acid sequence of *mfa1*. **C**, The predicted structure of the MAT-1 pheromone after processing. **D**, Structures of *mfa1*-related compounds consistent with those detected in MIF. A = *Apa*I, B = *Bam*HI, E = *Eco*RV, H = *Hind*III, and X = *Xba*I.

thus responding to a compatible pheromone-receptor interaction. MAT-1 cells transformed with *mfa1* also produced increased levels of MIF. The only difference between this transformant and wild-type MAT-1 cells was an increased copy number of *mfa1*. Indeed, unlike cells transformed with pheromone or pheromone receptor genes of the opposite mating type, these cells have a normal cell morphology. Last, and most critically, when the mating type locus was deleted from MAT-1 cells, this mutant no longer produced MIF. MIF production was restored by adding back the pheromone gene, but not the pheromone receptor gene.

An additional indication that MIF was directly related to mating, and perhaps to pheromones, was its specificity. MIF completely inhibited mating of *Ustilago* spp., but had little effect on teliospore germination or sporidial growth. The loss of MIF activity following protease treatment demonstrated that, like the pheromones, some moiety of MIF was protein. The high enzyme concentrations and long incubation times needed to eliminate MIF activity is not unusual for peptides that are farnesylated or *O*-methylated at the carboxyl terminus (3), which would be expected if MIF was related to the pheromone. The finding that a shorter protease treatment actually increased inhibitory activity may indicate that a protease substrate present in the crude MIF preparation was partially digested to yield MIF. Further purification and analysis by MALDI-TOF, electrospray mass spectrometry, and TLC were consistent with the interpretation that MIF contained various moieties of the MAT-1 pheromone including the peptide lacking the terminal cysteine, farnesyl cysteine, cysteine methyl ester, and farnesyl cysteine methyl ester (Fig. 3D). Bioassays performed with these purified compounds (Fig. 6) indicated that cysteine methyl ester had a mating inhibiting activity most similar to MIF, but a combination of these compounds at critical concentrations might also give MIF-like activity.

How might the MAT-1 pheromone give rise to MIF? One possibility is that imprecise processing of the pheromone results in the excretion of imperfect pheromone molecules. For example, the carboxypeptidase that processes the farnesylated yeast a factor normally removes the last three amino acids from the CAAX motif, but stops at the cysteine because it quickly becomes methylated due to the farnesyl signal (14). However, the carboxypeptidase will continue removing amino acids in cases in which the cysteine does not become methylated (14). This type of processing error could account for the presence of the peptide observed in the MIF fraction that lacks the terminal cysteine.

It is also possible that *U. hordei* possesses an enzymatic activity that removes the terminal cysteine, with its decorations, from the

pheromone. Since individual mating type cells produce MIF, the peptidase would need to be able to have activity on its own pheromone. Pheromone-cleaving enzymes have been described in *Saccharomyces cerevisiae* (24,40) and *Rhodospiridium toruloides* (28),

Fig. 5. MALDI-TOF mass spectrographs of **A**, partially purified MAT-1 mating inhibition factor showing masses at 1,433, corresponding to the predicted pheromone (farnesylated PGKSGSGLGYSTC methylester), and 1,111 (PGKSGSGLGYST). Intermediate peaks correspond to various breakdown products of the pheromone. **B**, Inhibitory fraction as in **A** after passing over C18 reverse-phase high-pressure liquid chromatography. Masses at 1,111 and 1,134 correspond to PGKSGSGLGYST $\pm$ Na. **C**, Synthetic PGKSGSGLGYST (compare with **B**).

Fig. 4. *Ustilago hordei* transformed with the pheromone gene from MAT-1 cells. **A**, *U. hordei* strain 14a (MAT-2) transformed with the 5.5-kilobase *Bam*HI DNA fragment containing *mfa1*. Note the distorted cell morphology and conjugation tubelike projections. **B**, *U. hordei* strain 14A (MAT-1) transformed with *mfa1*. These cells have normal cell morphology, but produce twice the mating inhibition factor produced by wild-type 14A cultures. Bar = 20 μ m.

although in neither case does the enzyme have the specificity observed here, cleaving between a threonine and cysteine, nor have the products been described to inhibit mating.

Excretion of farnesyl cysteine methyl ester might occur due to degradation of internal pheromone or other prenylated peptides. In *Saccharomyces cerevisiae*, prenylated proteins are potentially degraded intracellularly (47) and the prenylated cysteine transported out of the cell by STE6, which is normally involved in a factor excretion (19). Methylation is essential for export by STE6 (34). Farnesyl cysteine methyl ester and cysteine methyl ester might be directly excreted from *U. hordei* in an analogous fashion. Interestingly, farnesyl cysteine and analogs such as *N*-acetyl-farnesyl cysteine (AFC) have been the subject of a number of studies because of their ability to interfere with numerous processes in mammalian cells that involve prenylated peptides similar to the *U. hordei* pheromones, including Ras-related GTP-binding proteins (46), mouse peritoneal macrophage chemotaxis (46), human platelet aggregation (15), and superoxide formation in human neutrophils (30). AFC appears to act as a competitive inhibitor for substrates of the C-terminal methyl transferase (46) or by directly interfering with binding of the farnesylated signal to the receptor (22,36). Thus, the farnesyl cysteine and its methyl ester found in MIF may act

either by preventing the synthesis of active pheromone by *U. hordei* by interfering with methylation or by competitively binding to the pheromone receptor.

Why would *U. hordei* maintain a MIF-producing activity or produce a pheromone that degraded into a mating inhibitor? One possible reason is that this would inactivate pheromone when there were no potential mates in the vicinity. Pheromone accumulation would destroy the pheromone gradient necessary for conjugation tubes to locate each other (26). Another consequence of MIF being present immediately following teliospore germination might be the delay of conjugation of sister sporidia. This is consistent with the observation that, at least under laboratory conditions, *U. hordei* sporidia do not immediately fuse following teliospore germination, but double numerous times before mating occurs (Fig. 1). This is contrary to the life cycle of several other smut fungi, including *U. nuda* and *Tilletia* spp., in which sporidia fuse immediately after germination. This would result in an increased chance for *U. hordei* sporidia to mate with sporidia arising from nearby teliospores. Indeed, conjugation tubes can be observed growing between the colonies resulting from the germination of teliospores on artificial medium (data not shown). The end result would be an increase in the outbreeding potential for this fungus.

Fig. 6. Effect of synthetic compounds found in mating inhibition factor on mating of *Ustilago hordei*. Each compound was added to *U. hordei* teliospores using the standard bioassay. **A**, Control; **B**, cysteine (8 nM); **C**, farnesyl cysteine methyl ester (300 pM); **D**, farnesyl cysteine (300 pM); **E**, synthetic peptide PGKSGSLGYST (225 pM); and **F**, cysteine methyl ester (30 nM). Bar = 50 μ m.

One apparent inconsistency with MIF being a pheromone derivative is that pheromones, by nature, must be very specific, while MIF inhibits mating or teliospore germination in a number of smut and bunt fungi. However, while the pheromones from *U. hordei* and *U. maydis*, for example, have regions that are dissimilar, they also show extensive homology; the pheromone amino acid sequences are identical at 6 of the 13 residues (48%), while the prepheromones are 55% identical and 68% similar at the amino acid level. It is conceivable that the homologous regions are involved in the nonspecific MIF activity, while the dissimilar regions are involved in specificity. A more likely explanation, however, is that the cysteine derivatives, which are common moieties in fungal pheromones, are involved in the relative nonspecific nature of MIF.

While there are effective systemic fungicides available for control of many cereal smuts including covered smut, others are not as easily controlled, such as loose smut and dwarf bunt. Even covered smut causes significant yield losses in developing countries where the use of pesticides is not widespread for economic reasons (27). All fungicides are under increased regulatory scrutiny for environmental and public health reasons. It is important, therefore, that novel strategies for the control of plant diseases continue to be developed. In many fungi, and the cereal smuts in particular, the sexual cycle is intimately involved in the disease cycle. The sexual cycle often results in the formation of dormant spores that are capable of surviving cold, heat, or drought. These spores may then act as the primary inoculum for the disease when a new crop is planted. The sexual cycle also increases genetic variability through meiosis and the incorporation of new genes through outbreeding. In the smut fungi, the sexual cycle is a critical point in the disease cycle because mating is necessary for the conversion of nonpathogenic sporidia to pathogenic dikaryotic mycelium. Thus, it is possible that disruption of the sexual cycle of fungal plant pathogens could be an effective new strategy for controlling fungal diseases. This might be accomplished by directly applying a chemical inhibitor to the plant. Alternatively, and depending on the genetic complexity of the biosynthetic pathway, an inhibitor might also be delivered by a microbial MIF producer, or the plant itself might be genetically engineered to produce the inhibitor. Interestingly, it has been shown recently that the yeast α factor and culture supernatant from a MAT1-1 strain specifically inhibit infection of MAT1-2, but not MAT1-1 mating types of *Magnaporthe grisea* by preventing appressorium production (6). The fact that these compounds and *U. hordei* MIF are naturally occurring fungal products with very specific activities should increase their attractiveness as potential disease control agents because of limited detrimental effects on the environment and nontarget organisms.

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