Pheromone-Related Inhibitors of *Ustilago hordei* Mating and *Tilletia tritici* Teliospore Germination

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ABSTRACT

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Ustilago hordei, the causal agent of barley covered smut, produces mating pheromones that break down to smaller peptide compounds that act as potent inhibitors of mating and germination in several fungi. The pheromones are members of the farnesylated family of proteins. Synthetic peptide analogs of the pheromone derivatives, ranging in size from 4 mers to full length pheromones, were farnesylated, methyl esterified, or both and tested for mating or teliospore germination inhibition with *U. hordei* or *Tilletia tritici*, respectively. *N*-Acetyl-*S*-farnesylcysteine, which inhibits processing of Ras, and other sulfur-containing compounds such

as homocysteine or methionine, were likewise modified and tested. The most potent inhibitors were methionine methyl ester and modified 4-mer peptides from both pheromones. Alanine scanning of the inhibitory 4 mers determined that the native amino acid sequence was specific for a high level of activity. The sulfur amino acids appear to be required for inhibition. Glasshouse studies using selected antagonists of mating and teliospore germination as seed treatments inhibited covered smut of barley and common bunt of wheat, although the level of control was inconsistent. The use of pheromone-related antagonists to mating or teliospore germination is a promising, novel strategy for control of smut and bunt diseases.

Additional keywords: alanine scanning, default mating, farnesyltransferase, methyltransferase, pheromone analogs.

Mating is required for the fungus *Ustilago hordei*, which causes barley covered smut, to become pathogenic. Mating is initiated by sexual pheromones produced by the two mating types of *U. hordei* (MAT-1 and MAT-2). The binding of pheromone to its complementary receptor induces the formation of conjugation tubes. The conjugation tubes of the opposite cell types follow pheromone gradients to locate each other and fuse to form dikaryotic mycelia (27). The dikaryotic mycelium infects the barley seedling.

The pheromones from *U. hordei* are members of the CAAX family of farnesylated peptides (2,34), like those from *Coprinus cinereus* (28), *Schizophyllum commune* (40), *U. maydis* (36), *Schizosaccharomyces pombe* (9,10), *Saccharomyces cerevisiae* (1), *Tremella mesenterica* (32,33), and *Rhodospiridium toruloides* (20). Most of these pheromone peptides are also methyl esterified on the C-terminal cysteine (7,8). Methyl esterification is required for pheromone transport out of the cell by the STE6 transporter in yeast (23). Other important proteins in the CAAX family include the G-proteins Ras (3,30) and transducin (12,15,24), which are involved in the signal transduction pathways of cell development or differentiation and vision, respectively.

The genes for *U. hordei* MAT-1 and MAT-2 pheromones (*Uhmfa1* and *Uhmfa2*) were cloned and sequenced to determine the amino acid sequence of the pheromone propeptides (2). Chemical analysis of culture supernatant performed through various chromatography procedures, matrix assisted laser desorption ionization (MALDI) mass spectrometry, and nuclear magnetic resonance (NMR) elucidated probable pheromone structures. The most active form of *Uhmfa1*, the MAT-1 pheromone, was farnesylated GKSGSGLGYSTC methyl ester, whereas the most active form of *Uhmfa2* was farnesylated GKGEAPYCY methyl ester (21).

Mating inhibitors coeluted with pheromones during purification of the pheromones of *Schizosaccharomyces pombe* (9), *U. maydis* (36), and *U. hordei* (34). The structure of the *U. maydis* inhibitor was not determined but an inhibitory compound extracted from *S. pombe* cultures was identified as an oxidation product of the pheromone (9). A similar compound was found in *Saccharomyces cerevisiae* extracts (5). These oxidation products could be generated chemically by hydrogen peroxide or sodium hydroxide treatment, which remove the farnesyl or methyl ester group respectively, of purified *a*-factor of *S. cerevisiae* (5) or M factor of *Schizosaccharomyces pombe* (10). A truncated *S. pombe* pheromone synthesized as an amide with the C-terminal cysteine removed was also inhibitory to pheromone activity (10). Appressorium formation was prevented in *Magnaporthe grisea* when grown in yeast extract media, possibly due to the presence of a native inhibitor or *Saccharomyces cerevisiae* *a*-factor (4). Cysteine and other peptide compounds derived from the pheromones of *U. hordei* were found in high quantities in mating-inhibiting crude extracts of culture supernatants (21,34).

In *S. cerevisiae*, a systematic study of shorter peptides and those with altered derivations had less activity than native *a*-factor (7, 25). Removal of the farnesyl, methyl ester, or consecutive amino acids from the N terminus of *a*-factor lowered growth arrest and mating restoration activities, but native level activity could be compensated for by increasing the concentration of sample. The levels of pheromone activity from the truncated *S. cerevisiae* *a*-factor analogs resembled those observed with *Uhmfa2* analogs from *U. hordei* (7,21,25). All forms of both *a*-factor and *Uhmfa2* analogs remained active as pheromone. Peptides matching the observed masses from MALDI spectra of active fractions from *U. hordei* culture supernatants were synthesized and farnesylated, methyl esterified, or both to test pheromone properties (21).

Challenging *U. hordei* cells with pheromone analogs and then adding active pheromone showed that some of the analogs competed for pheromone receptor binding (21). In this study, truncated pheromone analogs without and with farnesyl or methyl ester modifications were tested with mixed cells of *U. hordei* to deter-

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mine their ability to block mating. Several of the truncated pheromones were similar to farnesylated cysteine or CAAX mimetics shown to suppress Ras processing by interfering with the farnesyl-transferase or methyltransferase (13,14,22,29,38,39). Each amino acid of the inhibitory 4 mers was replaced with alanine and tested in the same manner to determine if any particular amino acid or acids was required for specific inhibitory activity. Variations of the cysteine compounds, such as homocysteine, *N*-acetyl cysteine, and methionine, also were tested. These compounds were farnesylated and methyl esterified when possible. All compounds also were tested with teliospores of *Tilletia tritici* to determine if they inhibited germination, because the mating inhibitory factor derived from the pheromones of *U. hordei* inhibited germination of teliospores of *T. tritici* and *T. controversa* (34). Greenhouse experiments using inhibitory analogs to treat seed inoculated with *U. hordei* or *T. tritici* were performed to test the hypothesis that inhibition of mating or teliospore germination of these fungi could be a viable strategy to reduce smut and bunt diseases.

The importance of this research has broad implications for all fungal diseases, not just plant pathogens. The relationship of these compounds to those involved in cell differentiation and development, including oncogenes such as Ras, suggests a universal relationship between eukaryotic cell development and the processes controlling them. The compounds studied here relate to development of sexual mating and teliospore germination which, in these fungi, must occur for infection. Similar compounds are being developed and studied for cancer treatments. Related compounds might be designed that specifically inhibit the cellular growth and development for the control of different diseases.

MATERIALS AND METHODS

U. hordei strains 14A (MAT-1; American Type Culture Collection [ATCC] 90512) and 14a (MAT-2; ATCC 90510) (26) were grown and prepared for purification of mating inhibitory factors (MIF) as previously described (34). Standard chromatographic procedures included passage through G-50 Sephadex, Toyopearl HW55, Dowex 1/50, and reverse phase C4, C8, and C18 high performance liquid chromatography (HPLC) columns. Samples were dissolved in water or 1:1 water/methanol. Mass spectrometry was performed on a Vestec/Trio Two instrument with electrospray unit

and Perseptive Biosystems MALDI mass spectrometer at the Montana State University Mass Spec Facility.

Peptides were synthesized by Research Genetics (Huntsville, AL) or SynPep Corporation (Dublin, CA). Peptides were purified by C18 reverse-phase HPLC using a 5 to 65% acetonitrile gradient (all solvents were HPLC Grade; Fisher Scientific, Pittsburgh, PA) with 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis) with a Waters 600E HPLC pump and detector at 254 nm. Purification was monitored using MALDI mass spectrometry with α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich) as the matrix. The peptides were derivatized to determine activity or interference with activity. Peptides were farnesylated by mixing 50 μ l of 50 mM guanidine carbonate (Sigma-Aldrich) to 1 μ mol of peptide dissolved in 200 μ l of dimethyl sulfoxide. Farnesyl bromide (1 μ l) (Sigma-Aldrich) dissolved in 50 μ l of methylene chloride was added to the peptide solution, mixed, allowed to stand for 10 min at room temperature, and vacuum dried (21). The peptides were methyl esterified using thionyl chloride with methanol as the solvent (6). The pH of all samples was adjusted to approximately 5.0 with sodium hydroxide or hydrochloric acid. Samples were dissolved in water or methanol. *N*-Acetyl-L-cysteine (Sigma-Aldrich), homocysteine, and methionine were derivatized by farnesylation or methyl esterification where possible. *N*-Acetyl-S-farnesyl-L-cysteine and *N*-acetyl-S-farnesyl-L-cysteine methyl ester (Sigma-Aldrich) were purchased for comparison. All bioassays were performed a minimum of three times as described below (27) using 1 to 2 μ g of samples.

Mating bioassays with *U. hordei* were performed by adding the pheromone analogs to agar squares. After the sample had absorbed into the agar, a mixture of both mating-type cells was placed on the square. Following incubation, the number of dikaryotic mycelia formed were counted and compared with the control level with no sample added (34). *T. tritici* teliospore germination bioassays also were performed on agar squares prepared in the same manner, and percent germination determined over 5 to 7 days.

Extracted mating-stimulating fractions from strain 14A culture supernatants were treated with 1 M sodium hydroxide or 30% hydrogen peroxide (5,10) to remove the methyl ester or farnesyl group, respectively. Receptor binding competition studies using cysteine methyl ester, methionine, and its methyl ester were performed as described previously (34).

TABLE 1. Percent inhibition of mating of *Ustilago hordei* by pheromone analogs^a

Peptide ^b	Underivatized peptide	Peptide methyl ester	S-Farnesyl peptide	S-Farnesyl methyl ester
MAT-1 (Uhmfa1)				
PGKSGSGLGYSTC (13 mer)	50	20	40	40 ⁺⁺
GKSGSGLGYSTC (12 mer)	50	20	0	40 ⁺⁺
GLSGSGLGYSTC	20	90	90	>90 ⁺⁺
KSGSGLGYSTC (11 mer)	15	15	36	100 ⁺
PGK	36	...	...	...
SGSGLGYSTC (10 mer)	0	60	75	50 ⁻
GSGLGYSTC (9 mer)	0	0	36	75 ⁻
LGYSTC (6 mer)	60	0	0	0 ⁻
YSTC (4 mer)	45	80	100	100 ⁻
YSTM	60	85	...	...
MAT-2 (Uhmfa2)				
EGKGEPAPYC (10 mer)	0	40	100	100 ⁺⁺
GKGEPAPYC (9 mer)	20	65	100	100 ⁺⁺
GKGEPAPYM	36	36 ⁺	...	...
KGEPAPYC (8 mer)	0	0	75	5 ⁺
GEPAPYC (7 mer)	0	36	95	100 ⁺
APYC (4 mer)	65	50	100	95 [±]
Methionine	95	100	...	...
Cysteine	0	100	100	100
Homocysteine	80	40	95	90
<i>N</i> -Acetyl cysteine	50	50	100	60

^a For each assay, 1.0 μ g of each pheromone analog was used. Percent inhibition was determined by comparing the number of dikaryotic mycelia formed with and without treatment of analog. Standard deviations ranged from 0 to 35%, with percent inhibition levels of 0 and 100% having 0.0 standard deviation. Superscripts indicate pheromone activity of the compound, from strong (++) to no (-) conjugation tube-inducing ability, in opposite cell type (21).

^b Underlined letters indicate an amino acid substitution.

To test the ability of the analogs to inhibit disease, 30 seeds were placed in a test tube with 1 ml (barley cv. Odessa) or 0.5 ml (wheat cv. Butte) of carboxy methylcellulose (0.0001 g/ml), Tween 20 (0.001%) and the test compound at 66 or 330 µg per seed in sterile distilled H₂O. The seed were vacuum infiltrated for 30 min and air dried. Teliospores (15.0 to 20.0 mg) were added and the tubes were gently vortexed or inverted until the seed were evenly coated. Seed were planted in the field or in moistened vermiculite in styrofoam cups that were placed in boxes covered with clear plastic and incubated with a cycle of 16°C, 12-h day and 18°C, 12-h night. After noting percent seed germination for each treatment, the seedlings were potted in greenhouse potting soil when 1 to 4 inches tall and placed in the glasshouse with illumination for 18 h/day and temperatures of 24°C day and 18°C night. Symptoms were examined at plant maturity.

RESULTS

Having previously determined the pheromone and receptor competition activities of pheromone analogs (21), the goal of this study was to see if these compounds, particularly those without pheromone activity, could inhibit mating or teliospore germination of fungi as observed with naturally occurring pheromone breakdown products (34). Oxidation of purified pheromones from *S. cerevisiae* (5) and *Schizosaccharomyces pombe* (10) have been shown to produce mating inhibitors. Partially purified *U. hordei* pheromones were treated with sodium hydroxide and hydrogen peroxide to chemically remove the methyl ester or farnesyl group. Removal of the farnesyl by hydrogen peroxide treatment only reduced pheromone activity, but treatment with sodium hydroxide resulted in a potent mating inhibitor. Although exposure to sodium hydroxide was reported to remove the methyl ester (5), the pheromone appeared to break down with this treatment to the same smaller peptides seen in mating inhibitory fractions (21,34) from culture supernatant, including farnesylated cysteine methyl ester, as indicated by the mass spectra of this sample (data not shown). To determine if peptides that matched these masses were inhibitors to mating or teliospore germination, synthesized peptide analogs of both *Uhmfa1* and *Uhmfa2* were derivatized and tested using the mating bioassay. All variations of the peptides were tested, including unmodified, farnesylated, methyl esterified, and both (Table 1).

Peptides made as farnesylated or farnesylated methyl esters generally showed the most inhibition of mating (Table 1). Of the fully derivatized MAT-1 peptides, the 12- or 13-mer peptides, which have pheromone activity, inhibited mating the least, while the 4-mer peptide, which has no pheromone activity, inhibited mating completely when farnesylated or fully derivatized. The small peptide that would represent a *Uhmfa1* trypsin cleavage product from the N terminus, PGK, prevented mating 35%. The underivatized 9- and 10-mer *Uhmfa1* peptides were the only *Uhmfa1* analogs that did not inhibit mating to some degree. The two longest derivatized MAT-2 peptides, which were extremely active as pheromones as measured by their ability to induce conjugation tubes on MAT-1 cells at very low concentrations (21), inhibited mating completely (Table 1). The MAT-2 4 and 7 mers (Fig. 1), which had only slight pheromone activity, nearly completely inhibited mating as farnesylated ± methyl esters. The effects of various analogs on the mating inhibition of *U. hordei* are shown in Figure 1.

Mass spectra of the mating inhibitory factor indicated the presence of a mass consistent with a geranylated 13-mer *Uhmfa1* (21). The geranylated MAT-1 13-mer peptide was synthesized and induced conjugation tubes better and faster than the farnesylated 13-mer but did not inhibit mating. However, the geranylated 13-mer methyl ester did reduce mating by 50%. The geranylated 12-mer *Uhmfa1* and its methyl ester inhibited mating 40%. The geranylated 9-mer of *Uhmfa2* inhibited dikaryon formation by 80% but its methyl ester had no effect. In a mating bioassay, when MAT-2 cells were preincubated with the geranylated *Uhmfa1* analog before mixing with MAT-1 cells and 12- or 13-mer MAT-1 pheromone was added to the agar, formation of dikaryotic mycelia was inhibited more than 80%.

To examine the importance of different structural moieties of the pheromone on mating inhibition, peptides of each mating-type pheromone were made with altered sequences. The lysine, which is recognized by trypsin, was replaced with a leucine in the *Uhmfa1* 12-mer and the cysteine was replaced with a methionine in the MAT-1 4-mer and the 9-mer *Uhmfa2*. The derivatized form of the 12-mer *Uhmfa1* with the lysine to leucine replacement inhibited at least two times more than any derivatized form of the native 12- or 13-mer. The replacement of cysteine with methionine in the MAT-1 4-mer increased the inhibitory effect of the peptide by 15% and the methyl ester was very similar to the native 4-mer methyl ester. The methionine substitution in the 9-mer

Fig. 1. *Ustilago hordei* mating bioassays with different pheromone analogs added to a mixture of MAT-1 and MAT-2 cells. **A**, Control with no treatment, **B**, MAT-1 10-mer farnesylated methyl ester, **C**, farnesylated MAT-2 7 mer, **D**, methionine, **E**, farnesylated N-acetylcysteine, and **F**, farnesylated MAT-2 9-mer methyl ester. **F**, Notice that this analog does not inhibit formation of conjugation tubes (arrows).

Uhmfa2 peptide increased the inhibitory properties by 15% compared with the native peptide, with the methyl ester form contributing no additional inhibitory activity.

Cysteine compounds previously identified in *U. hordei* culture supernatants were very inhibitory to mating (34); therefore, methionine, as the only other sulfur-bearing amino acid, was tested for similar activity. Methionine and its methyl ester were effective mating inhibitors (Table 1). Methionine cannot be farnesylated due to the thiol methyl group. Cysteine methyl ester, methionine, and its methyl ester, in competition studies with any form of MAT-1 or MAT-2 pheromones, had no effect on the induction of conjugation tubes (data not shown), suggesting their inhibitory effect was not due to competition for pheromone receptor sites. Other forms of cysteine varied in their ability to inhibit mating. The farnesylated forms of homocysteine and *N*-acetyl cysteine

were very inhibitory to *U. hordei* mating (Table 1). *N*-Acetyl cysteine, which lacks a positive charge on the N terminus of the molecule, had lower inhibitory activity, 50 to 60%, in other forms. Homocysteine alone inhibited 80% of mating.

Mating of *U. hordei* sporidia was strongly inhibited by the 4-mers APYC and YSTC; therefore, the amino acids in these peptides were altered to determine which were required for activity. Changes made to the YSTC sequence to determine the necessity of the sulfur amino acid cysteine included YSTE, YSTF, and YSTL. None of the substitutions for cysteine in the 4-mer were inhibitory to mating even when methyl esterified (data not shown). The specificity of the MAT-1 and MAT-2 4-mer sequences were examined by alanine scanning. Each amino acid other than the final cysteine was individually replaced with alanine, producing AAYC and APAC for APYC and ASTC, YATC, and YSAC for

TABLE 2. Percent inhibition of *Ustilago hordei* mating with alanine-scanned peptide 4 mers^a

Peptide ^b	Underivatized peptide	Peptide methyl ester	S-Farnesyl peptide	S-Farnesyl methyl ester
APYC	63.8 ± 27.8	53.3 ± 20.8	100.0 ± 0.0	95.0 ± 5.0
AAYC	45.0 ± 8.7	48.3 ± 2.9	80.0 ± 21.8	61.7 ± 5.8
APAC	31.7 ± 10.4	48.3 ± 2.9	91.7 ± 7.6	53.3 ± 10.4
AAAC	38.3 ± 2.90	56.7 ± 20.8	81.7 ± 15.3	100.0 ± 0.0
YSTC	48.3 ± 17.6	72.5 ± 6.5	100.0 ± 0.0	100.0 ± 0.0
ASTC	53.3 ± 5.8	63.3 ± 2.9	93.3 ± 5.8	66.7 ± 17.6
YATC	50.0 ± 8.7	43.3 ± 20.2	56.7 ± 28.9	50.0 ± 15.0
YSAC	36.7 ± 10.4	51.7 ± 12.6	58.3 ± 14.4	55.0 ± 18.0

^a Data are the mean and standard deviation of three experiments.

^b For each assay, 1.0 µg of each peptide was used. Percent inhibition was determined by comparing the number of dikaryotic mycelia formed with and without treatment with the peptide.

TABLE 3. Effect of *Ustilago hordei* pheromone analogs on teliospore germination and life cycle progression of *Tilletia tritici*

Treatment (1 µg/100 µl of agar) ^a	Inhibition of teliospore germination (%) ^b	Postgermination effects
SGSGLGYSTC ± FME	75	...
GSGLGYSTC + F	75	...
GSGLGYSTC + ME	100	...
YSTC ± FME	0	100% inhibition of primary sporidia
EGKGEPAPYC + F, ME, or FME	0	Probasidia lyse, no primary sporidia
Farnesylated cysteine	75	...
Cysteine methyl ester	100	...
Homocysteine ± F, ME, or FME	0	100% inhibition of primary sporidia
Methionine ± ME	100	...

^a Derivation of compound: F = farnesylated, ME = methyl ester, and FME = farnesylated methyl ester.

^b Standard deviation ranged from 0 to 33%; all values of 0 or 100% had 0.0% standard deviation.

Fig. 2. *Tilletia tritici* teliospores exposed to different pheromone analogs. Teliospores were treated with A, control, with no treatment, B, farnesylated MAT-2 10-mer methyl ester, and C, methionine methyl ester.

YSTC. These peptides, derivatized as the other analogs, generally had less mating inhibitory activity compared with the native 4 mers (Table 2). The peptide AAAC, made as a control, had inhibitory properties similar to the native 4 mers.

The compounds listed in Table 1 also were tested for their effects on *T. tritici* teliospore germination. Only those analogs that had any effect on the germination or life cycle progression are listed in Table 3. The methyl esterified MAT-1 9 mer completely inhibited the germination of *T. tritici*. Farnesylated cysteine, farnesylated MAT-1 9-mer, and farnesylated MAT-1 10-mer methyl ester inhibited germination 75%. The MAT-1 4 mer ($\pm$ farnesyl methyl ester) did not inhibit germination but did inhibit the formation of primary sporidia. All forms of the MAT-2 10 mer affected *T. tritici* life cycle progression where germination was not affected, but the probasidia lysed after formation with this form of Uhmfa2 (Table 3; Fig. 2B).

The derivatized forms of homocysteine did not affect germination of *T. tritici* but did inhibit formation of primary sporidia. Methionine, however, as well as its methyl ester, inhibited teliospore germination completely in *T. tritici* (Table 3; Fig. 2C). The acetylated cysteine had no effect on germination or life cycle progression of *T. tritici* in any form.

Glasshouse experiments performed using analogs to coat seed of barley and wheat before being inoculated with *U. hordei* and *T. tritici*, respectively, gave promising, but inconsistent, results (Tables 4 and 5). The individual compounds were selected because of their in vitro activity. In three experiments using 66 μ g per seed, cysteine, methionine, their methyl esters, and YSTC reduced barley covered smut by 15 to 60% (Table 4) and, in two experiments also using 66 μ g per seed, reduced common bunt of wheat by 0 to 90% (Table 5). Efficacy was not noticeably improved when the test compounds were increased to 330 μ g per seed (Tables 4 and 5). None of the compounds showed consistent detrimental effects to the plants as measured by percent viability. One interesting effect was an increase in plant height when certain sulfur-containing analogs were used (data not shown). Obtaining consistent disease control remains a major goal.

DISCUSSION

The pheromones of *U. hordei* appear to be degraded through time in an oxidative environment, such as when cells are grown in broth culture. Although some of the compounds identified from *U. hordei* culture supernatant retained pheromone activity (21), others inhibited mating, as found with pheromone oxidation products of *Saccharomyces cerevisiae* (5) and *Schizosaccharomyces pombe* (9). The mixture of compounds with inhibitory activity, originally referred to as MIF (34), also was an effective teliospore germination inhibitor in some *Tilletia* spp., including *T. tritici*. Sodium hydroxide treatment of the stimulatory fraction from culture supernatant, which contained pheromone, generated apparent breakdown products with mass values consistent with the MIF compounds. When these compounds were synthesized and tested individually, they were less inhibitory to mating except for a few forms, most notably the C-terminal 4 mers of either pheromone, which resemble CAAX mimetics in reverse. Only two of the *U. hordei* pheromone-derived compounds were effective at inhibition of *T. tritici* teliospore germination.

Several mechanisms have been elucidated in other systems that might explain the mating inhibitory activities observed in this study (Fig. 3). These include competition for receptor binding (41), loss of pheromone gradient (11), competition with farnesyl- or methyltransferases (22,29,38,39), transport inhibition of the pheromone (41), and direct interactions with caveolin affecting signal transduction (19,35,42). We have shown previously that some of the identified compounds bind to the pheromone receptor in a competitive fashion (21). However, teliospore germination in *T. tritici* was not triggered or did not progress in the presence of these compounds. No explanation for the inhibition of teliospore germination has been addressed as to mechanism, especially because the peptide compounds are not known to be specific to the organism.

Several of the mechanisms mentioned above seem to be at work in the mating-inhibitory activity of the pheromone analogs used in this study. Analogs that have pheromone activity, whether fully

TABLE 4. Effect of seed treatment with pheromone analogs on covered smut of barley (cv. Odessa)^a

Effect	Uninoculated control	Inoculated control	Cysteine	Cysteine methyl ester	Methionine	Methionine methyl ester	YSTC
66 μ g/seed ^b							
% Plant viability	86.7 $\pm$ 11.6	89.0 $\pm$ 9.5	90.0 $\pm$ 8.9	84.7 $\pm$ 6.8	85.3 $\pm$ 10.8	84.7 $\pm$ 13.7	82.3 $\pm$ 22.0
% Infected plants	5.3 $\pm$ 9.2	56.0 $\pm$ 14.4	40.0 $\pm$ 24.5	34.7 $\pm$ 15.5	40.3 $\pm$ 20.1	36.7 $\pm$ 8.2	34.3 $\pm$ 17.7
% Disease reduction	...	...	32.3 $\pm$ 28.1	34.0 $\pm$ 29.7	31.3 $\pm$ 20.2	32.3 $\pm$ 16.2	41.7 $\pm$ 20.3
330 μ g/seed ^c							
% Plant viability	90	100	90	93	97	80	23
% Infected plants	0	57	41	36	45	42	0
% Disease reduction	...	...	28	27	21	26	100

^a Mean $\pm$ standard deviation.

^b Experiment was repeated three times; each experiment used 30 seeds per treatment.

^c Experiment performed once.

TABLE 5. Effect of seed treatment with pheromone analogs on common bunt of wheat (cv. Butte)^a

Effect	Uninoculated control	Inoculated control	Cysteine	Cysteine methyl ester	Methionine	Methionine methyl ester	YSTC
66 μ g/seed ^b							
% Plant viability	80 $\pm$ 9.9	76.5 $\pm$ 4.9	75 $\pm$ 2.8	73.5 $\pm$ 19.1	70 $\pm$ 14.1	80 $\pm$ 4.2	...
% Infected plants	4.5 $\pm$ 6.4	82.5 $\pm$ 13.4	54.5 $\pm$ 52	42 $\pm$ 28.3	16 $\pm$ 7.1	22 $\pm$ 25.5	...
% Disease reduction	...	...	38 $\pm$ 52.3	51.5 $\pm$ 26.2	81 $\pm$ 5.7	76 $\pm$ 26.9	...
330 μ g/seed ^c							
% Plant viability	100	70	90	90	77	63	80
% Infected plants	0	90	93	37	74	37	29
% Disease reduction	...	...	0	59	18	59	68

^a Mean $\pm$ standard deviation.

^b Experiment repeated twice; each experiment used 30 seeds per treatment.

^c Experiment performed once.

derivatized or not, appeared to inhibit by destroying native pheromone gradients causing default mating (Fig. 3E) (11). Fully derivatized analogs that lack pheromone activity can compete for receptor binding with the native pheromones (Fig. 3D) (21). Analogs that were farnesylated but not methyl esterified might act as competitive inhibitors for the methyltransferase enzyme in pheromone processing or the Ras signal transduction system (Fig. 3B). The inhibition by cysteine methyl ester, methionine (and methyl ester), and 4-mer peptides (and methyl esters) might be due to the sulfur coordinating with the farnesyltransferase molecule similar to the CAAX mimetics with Ras (Fig. 3A) (16,31). Farnesyltransferase is involved with the modifications of numerous proteins; therefore, inhibition of this enzyme might be expected to have pleiotropic effects. These secondary effects may include the disruption of signal transduction, mediated by Ras proteins, required for teliospore germination in *T. tritici*.

The underivatized MAT-1 peptide analogs had a pattern of decreasing inhibitory activity, which paralleled pheromone activity, as the N-terminal amino acids were sequentially removed. The tripeptide, PGK, which would represent a tryptic digest product of Uhmfa1, appeared to contain most of the inhibitory activity of the entire peptide. The 10 mer that would be the other product of a trypsin digest of the 13 mer had no inhibitory activity. This supports the conclusion that amino acids at the N terminus of the MAT-1 peptide are responsible for peptide specificity. The MAT-1 6- and 4-mer C-terminal peptides also inhibited mating, suggesting an alternative interaction or specificity for that portion of the peptide. Amino acid substitutions, such as lysine to leucine and cysteine to methionine, increased both the hydrophobicity and inhibitory activity of these molecules compared with similarly derivatized native compounds (15,24). However, the alanine-scanned 4 mers showed a trend of less inhibitory activity against mating in *U. hordei*, which implies some sequence specificity, because the native 4-mer sequences are more active at causing inhibition.

The farnesylated 4 mers were more inhibitory than any other analog. Not all of the inhibitory activity can be accounted for by competition with the pheromone receptors. Farnesylated 4-mer peptides corresponding to the C-terminal amino acids remaining after the -AAX cleavage can act as a substrate and competitively inhibit the methyl esterification also required for processing of Ras (Fig. 3B) (39). Methyltransferase requires a farnesylated peptide as substrate and utilizes *S*-adenosyl-methionine as the methyl donor. Lack of methylation of the native pheromones would result in incomplete processing and an inability to be transported out of the cell. In this study, it was the derivatized C-terminal four amino acids without the -AAX motif that obstructed mating.

Although derivatized Uhmfa1 and Uhmfa2 4 mers compete for receptor binding, the 4-mer peptides alone do not (21). It has been shown that CAAX peptides act as competitive and nonsubstrate inhibitors to farnesyltransferase (Fig. 3B) (16,31) by coordinating the cysteine sulfur with the zinc ion after farnesyl diphosphate has bound (18). Mating was not inhibited if the cysteine in YSTC was replaced with glutamate, phenylalanine, or leucine, even if methyl esterified. Therefore, the cysteine was required for inhibition.

Farnesyltransferase is somewhat nonspecific to peptide or protein substrate and can be utilized by other molecules that require farnesylation (17). Ras is one of these molecules that requires farnesylation for activity. This G-protein, involved in signal transduction for cell development and differentiation, is ubiquitous to all eukaryotes (3). The peptide 4 mers may be recognized by the farnesyltransferase as a CAAX mimic, even though reversed, and bind to it as nonsubstrate inhibitor. This would interfere with other cellular processes that required farnesylated proteins, such as Ras, which in turn would affect its functions in cell differentiation and development, including processes involved in mating and teliospore germination.

Cysteine compounds are very inhibitory to mating of *U. hordei* (21,34). Similarly derivatized cysteine compounds, known to

compete with methyltransferase, also inhibited Ras activity (39). Methionine and farnesylated homocysteine derivatives were very inhibitory to *U. hordei* mating and *T. tritici* germination. Only the farnesylated version of *N*-acetyl cysteine was highly inhibitory to *U. hordei* mating, but no effect was seen with germination of *T. tritici*. Methyl-esterified compounds appeared to best inhibit *T. tritici* germination, again suggesting an alternate mechanism.

Following germination of *T. tritici* teliospores, a basidium emerges from which primary sporidia form. These quickly fuse, resulting in the formation of secondary dikaryotic sporidia, which are the infective form of this plant pathogen. Previously, lipid components extracted from *T. controversa* teliospores were shown to be inhibitory to germination and sporidial formation of *T. tritici* (37). Here, different analogs affected different steps of this process, including teliospore germination, integrity of the basidium, and formation of primary sporidia. The interesting observation that methionine and cysteine methyl esters inhibit germination of *T. tritici* teliospores suggests these compounds may be non-specific. Ras-related processes that control cell development or a signal related to germination might be affected by these sulfur-containing amino acids or peptides. The addition of methionine or cysteine compounds may also affect regulation of the sulfur biosynthetic pathway, interfering with the cycling of *S*-adenosyl methionine, *S*-adenosyl homocysteine, or cysteine and, ultimately, reactions dependent on them, including pheromone methylation. Further analyses of the metabolites from the sulfur biosynthetic pathway and mechanistic studies with the inhibitory compounds will need to be performed to better understand their activity.

The use of pheromone antagonists to mating or teliospore germination is a promising, novel strategy for control of smut and bunt diseases. Obtaining consistent results in controlling disease is currently a major goal. Clearly, presentation of these compounds

Fig. 3. Steps in pheromone processing leading to mating in *Ustilago hordei*. Pheromone analogs listed on the right of arrows are proposed to inhibit farnesyltransferase (a), methyltransferase (b), endoprotease (c), receptor binding (d), and pheromone gradient (e).

to the fungus at the right time and place is critical. Disease was reduced to varying degrees with all of the antagonists tested. Interestingly, analogs that worked best for covered smut, such the peptide YSTC, did not work as well for common bunt, for which cysteine and methionine methyl esters showed the greatest and most consistent activity. However, results between tests were inconsistent, the cause of which is not currently understood.

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