The Pheromone Cell Signaling Components of the Ustilago *a* Mating-Type Loci Determine Intercompatibility Between Species

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Manuscript received December 14, 1995
Accepted for publication May 1, 1996

ABSTRACT

The *MAT* region of *Ustilago hordei,* a bipolar barley pathogen, harbors distinct mating functions (*a* and *b* loci). Here, we show that the *b* locus is essential for mating and pathogenicity, and can induce pathogenicity when introduced into a strain carrying a *b* locus of opposite specificity. Transformation experiments using components of the *a1* locus and analysis of resulting dual mating phenotypes revealed that this locus harbors a pheromone receptor gene (*Uhpra1*) and a pheromone gene (*Uhmfa1*). These *U. hordei a1* genes, when introduced by transformation, are necessary and sufficient to make *U. maydis,* a tetrapolar corn pathogen, intercompatible with *U. hordei MAT-2,* but not *MAT-1,* strains. *U. hordei* strains transformed with the *U. maydis a1* locus also become intercompatible with *U. maydis a2,* but not *a1,* strains. The interspecies hybrids produced dikaryotic hyphae but were not fully virulent on either corn or barley. Partial, natural intercompatibility was shown to exist between the sugarcane smut *U. scitaminea* and both *U. hordei* and *U. maydis.* These results show that the signal transduction pathway for mating responses is conserved between different smut species. We conclude that, apart from intraspecies compatibility, the Ustilago *a* locus also dictates *inter*compatibility in this group of fungi.

PHYTOPATHOGENIC smut fungi of the genus Ustilago have recently attracted a great deal of interest, and their sexual compatibility systems are being characterized in molecular detail (reviewed in BÖLKER and KAHMANN 1993). The majority of smut species, such as *U. hordei* and *U. scitaminea,* possess a bipolar mating system controlled by one locus with two alleles. Other species, such as *U. maydis* and *U. longissima,* have a tetrapolar mating system controlled by two unlinked loci (FISCHER and HOLTON 1957). *U. maydis* has been studied in detail and it was recently shown that the *a* locus (two alleles in nature) harbors a gene for a small proteinaceous mating pheromone (*Ummfa*) and a gene that is a functional homologue of the *Saccharomyces cerevisiae STE3* gene. This gene probably encodes a pheromone receptor (*Umpra*). Presumably, the a1 pheromone is recognized by the a2 receptor and vice versa (FROELIGER and LEONG 1991; BÖLKER *et al.* 1992; SPELLIG *et al.* 1994). The *b* mating-type locus (with ≥25 natural alleles) contains two divergently transcribed genes that have been called *bW* and *bE* and that are putative transcriptional regulators because each contains a conserved homeobox sequence (KRONSTAD and LEONG 1989, 1990; SCHULZ *et al.* 1990; GILLISSEN *et al.* 1992). It has been shown recently for *U. maydis* that the protein product from a *bW* allele interacts physically with the protein product from a *bE* allele of different specificity, thereby creating a heterodimer (KÄMPER *et al.* 1995). Two haploid partners of opposite *a* specificity put out conjugation tubes that join at the tips to establish cytoplasmic fusion (BANUETT and HERSKOWITZ 1989, 1994; TRUEHEART and HERSKOWITZ 1992; SNETSELAAR 1993). If a compatible bWbE heterodimer is formed due to opposite *b* specificities, a straight-growing, dikaryotic hypha is initiated at the point of fusion. This dikaryon represents the pathogenic cell type that is obligately dependent on the host plant to complete the sexual cycle (SNETSELAAR 1993; BANUETT and HERSKOWITZ 1994).

We have shown that bipolar smut fungi have homologues of the *a* and *b* loci (BAKKEREN *et al.* 1992) and that, in *U. hordei,* these loci are equivalent in structure to the *a* and *b* loci from the tetrapolar smut, *U. maydis* (BAKKEREN and KRONSTAD 1993, 1994). The difference between the bipolar and tetrapolar mating system is due to the genomic organization of the *a* and *b* loci. In the bipolar fungus *U. hordei, a* and *b* are genetically and physically linked to create a large (>150 kb) mating-type region with two natural alleles, *MAT-1* and *MAT-2* (BAKKEREN and KRONSTAD 1994).

Sexual hybridization between different smut species is known to occur (reviewed in FISCHER and HOLTON 1957). It is important to differentiate between simple fusion (KNIEP 1926: cited in FISCHER and HOLTON 1957) or intercompatibility (BOIDIN 1986), and true hybridization or interfertility. In the latter case, the sexual cycle is completed when viable hybrid spores are produced on the host (FISCHER and HOLTON 1957; BOI-

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TABLE 1

***Ustilago* strains**

Strain[a]	Genotype[b]	Source
Uh112	*MAT-1 a1 b1 ade* tester strain	BAKKEREN and KRONSTAD (1993)
Uh100	*MAT-2 a2 b2 ade* tester strain	BAKKEREN and KRONSTAD (1993)
Uh553	*a1 b0 ade hyg*^{r} (=Uh112 *b* deletion *UnbWE1*:: *hyg*)	This work
Uh530	*a2 b0 ade hyg*^{r} (=Uh100 *b* deletion *UnbWE2*:: *hyg*)	This work
Uh551	*a1 b2 ade hyg*^{r} *phleo*^{r} (=Uh553 with *UnbW2* randomly integrated)	This work
Uh549	*a2 b1 ade hyg*^{r} *phleo*^{r} (=Uh530 with *UnbWE1* randomly integrated)	This work
Uh4857-4	*MAT-1 a1 b1* wild-type strain	P. THOMAS; G. BAKKEREN (unpublished data)
Uh4857-5	*MAT-2 a2 b2* wild-type strain	P. THOMAS; G. BAKKEREN (unpublished data)
Um521	*a1 b1*	KRONSTAD and LEONG (1989)
Um518	*a2 b2*	KRONSTAD and LEONG (1989)
UmPBAD8	*a1b0 hyg*^{r} (=Um521 *b* deletion *UnbWE1*:: *hyg*)	KRONSTAD and LEONG (1990)
Us2+, 6+/1−,5−	*MAT-1/MAT-2* wild-type strains	S. SCHENK and H. ALBERT

[a] Uh, *Ustilago hordei*; Um, *U. maydis*; Us, *U. scitaminea*. Note that the strains of *U. scitaminea* were determined to be of either the − or + mating type; we suggest *MAT-2* for the − mating type because of mating with the *U. hordei MAT-1* strain, and *MAT-1* for +.

[b] *ade*, adenine auxotroph; *hyg*^{r}, hygromycin B-resistance; *phleo*^{r}, phleomycin-resistance.

DIN 1986). It is clear that interfertility depends additionally on the availability of a common host for parasites such as the smut fungi. *U. hordei*, a pathogen of barley, and *U. maydis*, which is pathogenic on corn, are not naturally intercompatible. That is, when different strains and mating types are tested, no visible interaction, *i.e.*, formation of conjugation tubes or hyphae, can be observed. However, when we isolated the *b* locus from *U. hordei* and tested its potential function in *U. maydis*, we discovered that the *U. hordei b* locus apparently had allelic specificities different from the resident *U. maydis b* locus and that these *b* genes could interact. This study yielded haploid *U. maydis* transformants that grew with a filamentous morphology and that were pathogenic on corn (BAKKEREN and KRONSTAD 1993). Moreover, when integrated into the genome of a *U. maydis* strain, the *a1* locus from *U. hordei* allowed a mating interaction between this transformant and a *U. hordei MAT-2* strain (BAKKEREN and KRONSTAD 1993).

In this study, we have further analyzed the function of the *U. hordei a* and *b* loci and have addressed the nature of interspecific mating interactions. We are particularly interested in constructing hybrid species to start genetic identification of genes involved in pathogenicity and host range. Specifically, we wanted to investigate the molecular basis for natural intersterility in smut fungi and to identify the components of the mating-type loci involved in interspecific compatibility interactions.

MATERIALS AND METHODS

Strains: Table 1 lists the Ustilago strains employed in this study. The strains were grown and maintained as described previously (BAKKEREN and KRONSTAD 1993).

Terminology: The following abbreviations and gene designations have been used: Uh, *U. hordei;* Um, *U. maydis;* Us, *U. scitaminea; MAT-1* or *-2*, mating-type region of bipolar smut fungi (*e.g., U. hordei*) that harbors the *a* and *b* mating-type loci; *Uhb1, U. hordei b1* gene complex consisting of *bE1* + *bW1; Uhb2, UhbE2* + *UhbW2; UhbE1* or *2, U. hordei b*East allele from *MAT-1* or *-2; UhbW1* or *2, U. hordei b*West allele from *MAT-1* or *-2* (see Figure 1); *Uhpra1*, pheromone receptor gene located on the *U. hordei a1* locus from *MAT-1; Uhmfa1*, mating pheromone gene located at the *U. hordei a1* locus from *MAT-1; Umpra1* and *Ummfa1*, receptor and pheromone genes located at the *U. maydis a1* locus.

Plasmid and strain construction: The *U. hordei b* locus disruption construct was made by replacing a 1.9-kb *Bgl*II fragment from plasmid pUhbWE1 (FIGURE 1 and BAKKEREN and KRONSTAD 1993), thereby removing the amino-terminal regions and promoters from both *UhbE1* and *UhbW1*, with a 3.1-kb Ustilago-specific hygromycin B resistance cassette from plasmid pCM54 (TSUKUDA *et al.* 1988); the 3.1-kb *Hin*dIII fragment was made blunt with the Klenow DNA polymerase fragment and synthetic *Bam*HI linkers were added. The 8-kb *Kpn*I fragment from the resulting pUhbWE1::Hyg construct was used for transformations and resulted in the knock-out of the *b* locus in both *U. hordei MAT-1* (Uh112) and *MAT-2* (Uh100) strains. The high sequence conservation in the carboxy-terminal portions of the genes of the *UhbE1bW1* and *UhbE2bW2* loci (BAKKEREN and KRONSTAD 1993) made it possible to use a single construct for both mating types (Figure 1 and Table 1). Subsequently, *U. hordei b* locus genes of opposite specificity were introduced on an integrative plasmid (pUBle3) carrying a phleomycin-resistant cassette (BAKKEREN and KRONSTAD 1993) to construct the tester strains. This resulted in *U. hordei* strain Uh551 with genotype *a1b2* (upon transformation with plasmid pUhbW2Int; BAKKEREN and KRONSTAD 1993) and in *U. hordei* strain Uh549 with genotype *a2b1* (upon transformation with pUhbWe1Int; BAKKEREN and KRONSTAD 1993; Table 1).

The isolation of the *U. hordei a1* locus on the episomal cosmid paMAT-1 (Figure 1) has been previously described (BAKKEREN and KRONSTAD 1994). DNA fragments (Figure 1) were subcloned in an integrative vector (pUGH1) carrying a hygromycin B-resistant cassette (G. BAKKEREN, unpublished data). Plasmid pUha1-1 was obtained by inserting the 2.6-kb *Hin*dIII fragment from plasmid pUhpra1 into plasmid pUh-

mfa1 (resulting in an unnatural arrangement where the *Uhpra1*-containing fragment is located to the right of the *Uhmfa1*-containing fragment in Figure 1, with *Uhpra1* being transcribed toward *Uhmfa1*).

Mating tests and microscopy: Mating tests were performed on double complete medium (DCM) supplemented with 1% activated charcoal (HOLLIDAY 1974). Colonies were scored as Fuz^+ (+) if they produced the aerial hyphae (fuzzy phenotype) indicative of successful mating and Fuz^- (−) when the colony morphology remained smooth. Mating interactions (formation of conjugation tubes and hyphae) were followed microscopically over time after spreading the transformants and/or mating partners on water-agar (1.5% BBL agar, Becton Dickinson) and incubating at 22° (essentially as described in MARTINEZ-ESPINOZA *et al.* 1992). Staining for nuclei and cell walls was done in 0.1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) for 10 min followed by three washes with water and mounting on microscope slides in 0.7% low melting point agarose with 5 mg/ml Calcofluor white (Sigma Chemical Co.). Microscopy was performed with a Zeiss Axiophot microscope (Carl Zeiss, D-7082 Oberkochen), using UV excitation for fluorescent images.

Pathogenicity tests: Pathogenicity tests on corn were performed as described previously (BAKKEREN and KRONSTAD 1993). Barley inoculations were carried out as follows. Dehulled barley seedlings were germinated for 48 hr at 22° in the dark and were inoculated with paintbrushes at the meristematic region of the ~5 mm-long shoots. The inoculum consisted of a paste of cells, either transformants or mixed mating partners, scraped from potato dextrose agar (PDA). After an additional 48 hr in the dark at 22°, the seedlings were planted in soil and grown in a growth chamber with a 16 hr day/8 hr night regime at 18°, or in the greenhouse with additional illumination as required for a 16 hr day/8 hr night regime.

RESULTS

The *U. hordei b* locus is a pathogenicity factor: The isolation of the *U. hordei b* locus containing *UhbW* and *UhbE* from both *MAT-1* and *MAT-2* mating types (BAKKEREN and KRONSTAD 1993) enabled us to further analyze its role in pathogenicity. Initially, a marker-exchange construct was used to disrupt the resident copy of the *b* locus in both a *MAT-1* and a *MAT-2* strain resulting in strains with genotypes *a1bO* and *a2bO*, respectively (see MATERIALS AND METHODS). In a cross between mating partners with compatible *a* loci, the deletion of a *b* locus in one partner prevented the formation of straight-growing, dikaryotic aerial hyphae (Table 2). However, the formation of conjugation tubes (thinner, meandering mating hyphae) and fusion of these tubes was not affected (not shown). The absence of two compatible *b* loci also prevented pathogenicity on barley (Table 2).

The establishment of heterozygosity at the *b* locus in a haploid cell, by transformation with *b* genes of different allelic specificity, results in colonies with visible aerial hyphae (Fuz^+; Table 2). These hyphae are straight, septated filaments that resemble the dikaryotic hyphae resulting from the mating of wild-type cells (not shown; BAKKEREN and KRONSTAD 1993). Some of the haploids heterozygous at *b* were also found to be solopathogenic in that they produced teliospores on the ears of barley plants, the only visible disease symptoms (Table 2). Position effects of the introduced *b* genes probably affected virulence because not all transformants produce teliospores. Three of the four teliospore populations we obtained were viable and their germination resulted in basidiospores that were identical to the cells used for the inoculum (*i.e.*, Fuz^+ and phleomycin-resistant). We conclude that the *b* genes in *U. hordei* trigger a switch to filamentous growth and pathogenicity in a fashion analogous to *U. maydis* (KRONSTAD and LEONG 1989; SCHULZ *et al.* 1990; GILLISSEN *et al.* 1992; GIASSON and KRONSTAD 1995).

TABLE 2

Mating interactions, colony morphology and pathogenicity of *U. hordei* transformants

	Fuz[a]	Pathogenicity on barley[b]
Cross		
Uh112 (*a1 b1 ade*) × Uh4857-5 (*a2 b2*)	+	7/10
Uh553 (*a1 b0 ade*) × Uh4857-5 (*a2 b2*)	−	0/9
Uh100 (*a2 b2 ade*) × Uh4857-4 (*a1 b1*)	+	4/9
Uh530 (*a2 b0 ade*) × Uh4857-4 (*a1 b1*)	−	0/9
Transformants		
Uh4857-5 (*a2 b2* [pUhbWE1]) 1	+	0/15
Uh4857-5 (*a2 b2* [pUhbWE1]) 2	+	2/19[c]
Uh4857-5 (*a2 b2* [pUhbWE1]) 3	+	2/14[c]
Uh4857-5 (*a2 b2* [pUhbWE1]) 4	+	3/15[c]
Uh4857-5 (*a2 b2* [pUhbWE1]) 5	+	0/18

Two compatible *b* loci are necessary for the production of hyphae and pathogenicity. Note that the adenine auxotrophy in one parent is complemented by the mating partner upon fusion. Five independent transformants were tested for solopathogenicity on the barley cultivar, Odessa.

[a] Fuz^+ or Fuz^- indicating whether or not a cross or haploid transformant produced aerial hyphae on mating medium (see MATERIALS AND METHODS).

[b] Values are the number of plants having at least one smutted ear per total number of inoculated plants.

[c] Small teliospore pockets were formed, sometimes not visible on the outside of an otherwise malformed ear (*e.g.*, having distorted or no awns).

Functional analysis of the *U. hordei a1* locus: The *U. hordei a1* locus on cosmid paMAT1 harbors a pheromone receptor gene, *Uhpra1*, which is 62% identical with the *U. maydis pra1* homologue (Figure 1; BAKKEREN and KRONSTAD 1994). The introduction of DNA fragments carrying active mating-specific components of the *U. hordei a1* locus into a *MAT-2* strain is expected to result in self-stimulation, *i.e.*, formation of conjugation tubes and fusion (BAKKEREN and KRONSTAD 1994). The *Uhpra1* gene (Figure 1) did provoke the production of conjugation tubes upon transformation into a *U. hordei MAT-2* strain (Table 3, first column). In addition, we located another active mating component, having the same effect, on a 2.8-kb *Sal*I/*Hin*dIII fragment (Table 3; Figure 2B, compare with A). Presumably, this fragment contains the pheromone gene, *Uhmfa1;* DNA sequence information from J. E. SHERWOOD (personal communication) supports this conclusion.

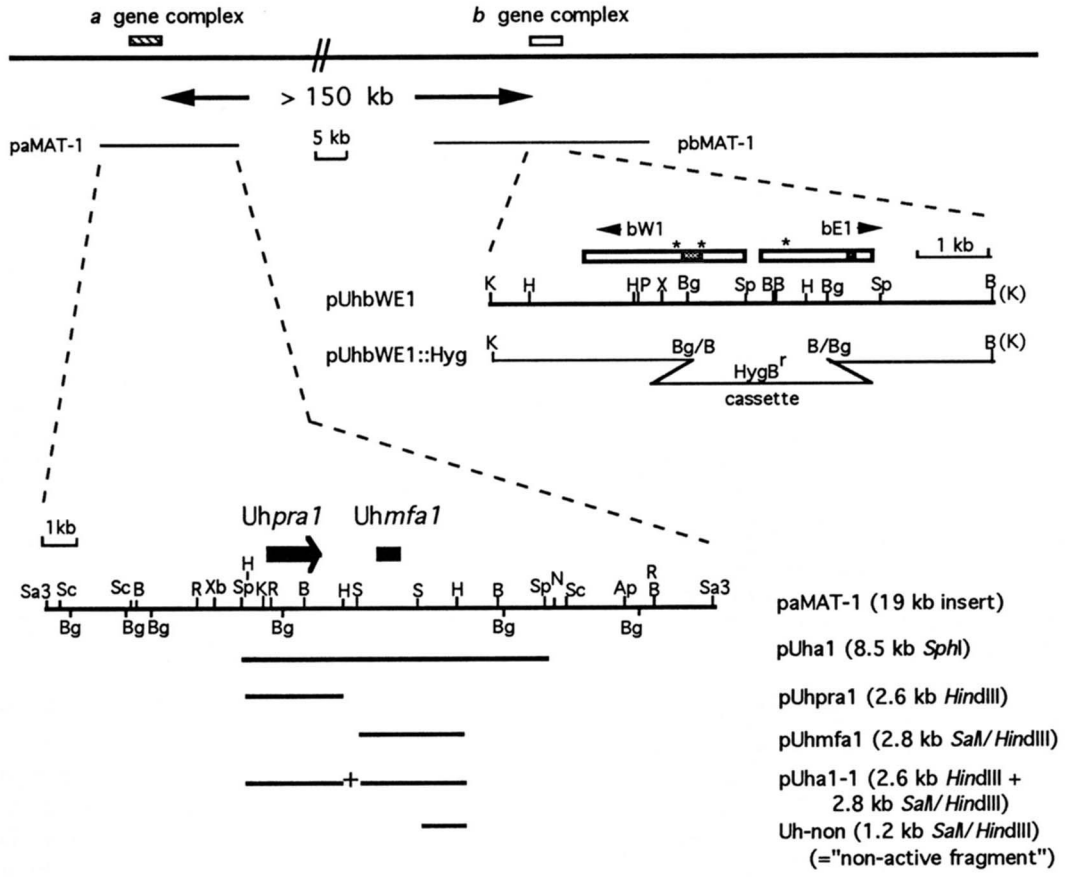

FIGURE 1.—Map of the *U. hordei MAT-1* mating-type locus. The upper line represents the genomic region. □, the position of the *b1* locus consisting of two divergently transcribed open reading frames, *bW1* and *bE1*, isolated on cosmid pbMAT-1 (BAKKEREN and KRONSTAD 1993). The enlarged region shows the genes in detail including the introns (▨) and the homeodomains (*). Cosmid paMAT-1 carries the *a1* locus (▧; BAKKEREN and KRONSTAD 1994). Note that its orientation relative to the *b* locus has not yet been determined. The *U. hordei* pheromone receptor gene, *Uhpra1*, has been described (BAKKEREN and KRONSTAD 1994). The inferred position of the *U. hordei a1* mating pheromone gene, *Uhmfa1*, is indicated by ■ (J. E. SHERWOOD, personal communication). The plasmid names refer to the particular subcloned fragments (see MATERIALS AND METHODS). The arrows indicate the direction of transcription of the genes in question. Restriction enzyme sites are as follows: Ap, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; R, *Eco*RI; S, *Sal*I; Sa3, *Sau*3A; Sc, *Sac*II; Sp, *Sph*I; Xb, *Xba*I.

Active components of the *U. hordei a1* locus (*Uhpra1* and *Uhmfa1*) could also transform a *MAT-2* strain to a dual mating phenotype. Because such transformants already form conjugation tubes due to the presence of a compatible *a* locus, we needed a mating test involving a strain with compatible *a* and *b* loci that would allow the visual scoring of a "fuzzy" colony morphology. Transformants having genotype *a2[a1]b2* should be able to mate with both *MAT-1* (*a1b1*) and *a2b1* strains. The latter tester strain had to be engineered by transformation because recombination between the *a* and *b* loci has not been observed in bipolar smuts (Uh549(*a2b1*) in Tables 1 and 3 and Figure 3A; also, Uh551(*a1b2*) in Tables 1 and 3; MATERIALS AND METHODS). That is, naturally occuring strains of genotype *a1b2* and *a2b1* have not been found (BAKKEREN and KRONSTAD 1994). Table 3 (column two) shows that the *MAT-2* strain transformed with different *a1* locus components mated normally with a *MAT-1* tester strain, indicating that the introduced components did not interfere. Mating tests with the *a2b2* and *a1b2* tester strains showed that, although fusion might occur, filamentous growth was not triggered when the *b* alleles were of the same specificity (columns three and four). The dual mating phenotype became apparent in mating with the *a2b1* strain (last column). The fuz$^+$ phenotype was correlated with the appearance of straight-growing hyphae (Figure 2D, compare B and C). The fact that the *Uhmfa1* gene alone or the *Uhpra1* plus *Uhmfa1* genes together were capable of inducing mating, but the *Uhpra1* gene alone was not, illustrated that both mating partners need to have their pheromone receptors "activated" by the interaction with a compatible pheromone to become mating competent. The results of the transformation experiments with the genes encoding the pheromone signalling components have been summarized in Figure 3A.

The *a* locus specifies intercompatibility between *U. maydis* and *U. hordei*: We have previously shown by genetic means that the *b* loci from *U. hordei* and *U. maydis* are functionally compatible, presumably establishing an

TABLE 3

Functional analysis of the components of the *U. hordei a1* locus in a dual-mating assay

Strain/transformant	Conjugation tubes[a]	Tester strains			
		Uh112 *a1 b1*	Uh100 *a2 b2*	Uh551 *a1 b2*	Uh549 *a2 b1*
Uh4857-5 (*a2 b2*)	−	+[b,c]	−	−	−
Uh4857-5 (*a2 b2* [pUhpra1])	+	+	−	−	−
Uh4857-5 (*a2 b2* [pUhmfa1])	+	+	−	−	+[d]
Uh4857-5 (*a2 b2* [pUhal-1])	+	+	−	−	+
Uh4857-5 (*a2 b2* [Uh-non])	−	+	−	−	−

Results of mating tests with transformants carrying DNA fragments [ ] (see Figure 1) randomly and stably integrated in the genome. Several independent transformants for each construct were tested; ectopically integrated constructs can show "position effects" resulting in strains showing varying degrees of fuzziness when mated. Transformants capable of mating with both tester strains of genotype *a1 b1* and *a2 b1* exhibit a dual-mating phenotype. Note that a cross between engineered strains Uh551 (*a1 b2*) and Uh549 (*a2 b1*) produces a normal Fuz$^+$ colony morphology.

[a] Minus (−) cells are like wild type and grow by budding; Plus (+) cells put out conjugation tubes by themselves in culture (see MATERIALS AND METHODS and Figure 2).

[b] (+) or (−), colony morphologies of mated strains are Fuz$^+$ or Fuz$^-$, respectively.

[c,d] Refer to Figure 2, C and D, respectively, for microscopic views of the cell morphologies from mixtures of mating cells.

active bWbE heterodimer that orchestrates the developmental pathway leading to filamentous growth and pathogenicity (BAKKEREN and KRONSTAD 1993). Cosmid paMAT-1, which carries the *U. hordei a1* locus (Figure 1), allows a *U. maydis* strain (upon transformation) to mate specifically with a *U. hordei MAT-2 (a2b2)* strain (TABLE 4; BAKKEREN and KRONSTAD 1994). We identified the components of the *U. hordei a1* locus responsible for this phenotype by transforming *U. maydis* with various subclones (Figure 1) and by testing transformants for mating with tester strains. Again, the genes introduced by transformation did not seem to have a detrimental effect on the specificity of the mating interactions between *U. maydis* strains (Table 4, first two columns). The results of the interspecific mating interactions are shown in Table 4 (columns three and four). Specificity was maintained according to the compatibility dictated by the introduced mating-type sequence. For example, the *U. hordei a1* locus allowed *U. maydis* to mate with a *U. hordei a2* tester strain, but not with an *a1* strain. It is clear, as illustrated in Figure 3B, that both the *U. hordei*-specific pheromone receptor and the pheromone need to be produced in the transformed *U. maydis* strain for proper recognition to take place. In addition, pheromone receptors of each participating partner need to be activated by the interaction with a compatible pheromone for successful interspecies mating to occur. In a reciprocal experiment, we found that the complete *U. maydis a1* locus could transform a *U. hordei* strain to specifically mate with a *U. maydis* (*a2b2*) strain (bottom row of Table 4).

The last three columns of Table 4 provide genetic evidence that cytoplasmic fusion occurs to allow interaction between the *b* gene products of the two species. The physical (and genetic) interaction of b products of different specificity is thought to establish filamentous growth (SNETSELAAR 1993; BANUETT and HERSKOWITZ 1994; KÄMPER *et al.* 1995). The experiments summarized in Table 4, column five, demonstrate that, even though the *b* loci have different allelic specificities, cytoplasmic fusion requires compatibility at the *a* locus. The results in Table 4, columns four and six, show that *U. hordei b* alleles of both known specificities, *b1* and *b2*, can interact with the *U. maydis b1* allele. The ultimate genetic test for cytoplasmic fusion is revealed in the last column where mating with a strain lacking the *b* gene complex, *b0*, failed to give a Fuz$^+$ reaction. We deduce from these data that there is interaction between the b products from each contributing mating partner and that, therefore, cytoplasmic fusion must have occurred.

In general, the interspecific mating interactions seem less "vigorous", producing fewer aerial hyphae on the colonies, compared with the situation when compatible partners from the same species were mated. This could be due to different growth rates and/or fewer successful encounters between potential mating partners. The time lag between the subsequent stages of the interactions for the interspecies mating, however, was similar to that of homospecific matings between *U. maydis* cells (SNETSELAAR 1993; BANUETT and HERSKOWITZ 1994). That is, conjugation tubes started to appear after ~3 hr on water agar medium (see MATERIALS AND METHODS). At 6 hr, fused conjugation tubes and the first hyphae could be observed.

Additional genetic experiments aimed at proving cytoplasmic fusion by employing transmission of a mitochondrial oligomycin-resistance marker (TRUEHEART and HERSKOWITZ 1992; LAITY *et al.* 1995) met with no success. One reason why this marker from *U. maydis* failed to function in *U. hordei* might be genetic incom-

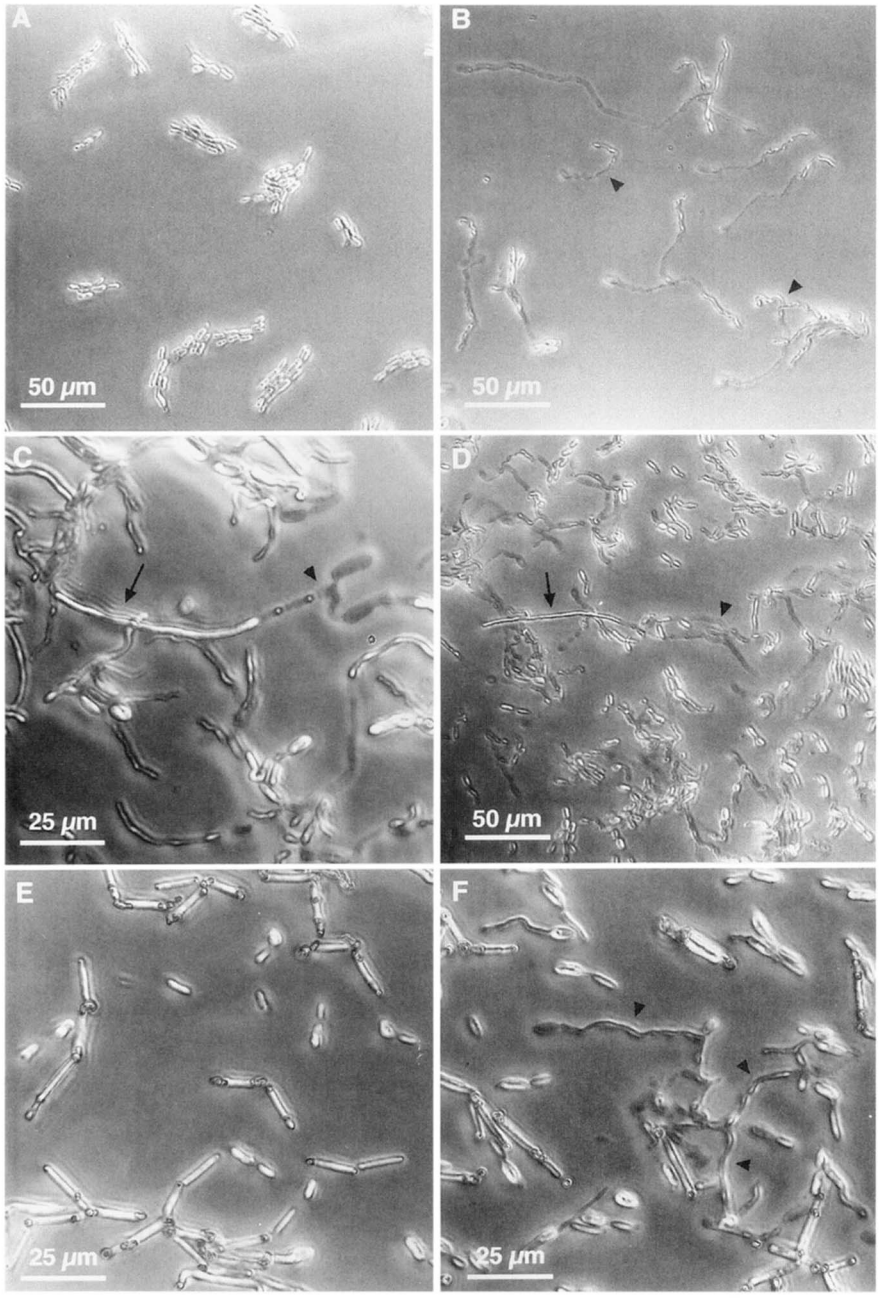

FIGURE 2.—Morphologies of Ustilago transformants and cells involved in mating interactions. The pictures were taken 24 hr after the cells had been spread on water agar and kept at 22°. (A) Normal cell morphology (growth by budding) of a *U. hordei* (Uh4857-5, *MAT-2*) strain transformed with the nonactive plasmid construct, Uh-non (Figure 1). (B) Cell morphology of a *U. hordei MAT-2* (Uh4857-5) strain transformed with plasmid construct pUhmfa1 (Figure 1). Production of the a1 pheromone leads to self-activation of the pra2 receptor resulting in the production of conjugation tubes (arrowheads). (C) Example of a cross between two wild-type *U. hordei* cells, Uh4857-5(*MAT-2, a2b2*) × Uh112(*MAT-1, a1b1*) (Table 3). Note the thick, straight-growing (dikaryotic) hyphae (arrow) that can be easily distinguished from the thinner, meandering conjugation tubes (mating hyphae); compare with 2B. The arrowhead indicates the point of fusion. (D) Example of a cross between a *U. hordei* transformant, Uh4857-5(*a2b2,* [Uhmfa1]), and the engineered tester strain, Uh549(*a2b1*) (Table 3), showing that the acquired dual-mating phenotype results in a filament (arrow) identical to the hypha from a wild-type cross (compare with 2C). (E) Interspecific cross between *U. maydis* transformant, Um521(*a1b1,* [pUha1–1]), and *U. hordei* strain, Uh112(*a1b1*) (Table 4). No interactive response can be observed because *U. maydis* and *U. hordei* do not normally mate. (F) Interspecific cross between *U. maydis* transformant, Um521(*a1b1,* [pUha1–1]), and *U. hordei* strain, Uh530(*a2b0*) (Table 4). Note the formation of conjugation tubes (arrowheads) due to compatible (*U. hordei*) *a* loci; no filaments are produced because of the lack of a compatible *b* locus.

patibility (disharmony) between the *U. maydis* mitochondria and *U. hordei* cells.

Dikaryotic hyphae are formed between *U. hordei* and *U. maydis:* Although a Fuz$^+$ colony phenotype normally correlates microscopically with the occurrence of straight-growing, dikaryotic hyphae, we wanted to confirm this for the unnatural mating interactions. Microscopic observations confirmed the presence of vigorous dikaryotic hyphae between *U. maydis* and *U. hordei* for every cross in Table 4 scored macroscopically as having a Fuz$^+$ colony phenotype. Because the cell shapes and sizes of *U. hordei* and *U. maydis* differ, we could identify the cells of the two different species when they were linked by conjugation tubes. Examples of hyphae in one cross, Um521(*a1b1,*[pUha1-1]) × Uh100(*a2b2*), are shown in Figure 4. Moreover, nuclear staining clearly revealed the dikaryotic nature of the developing filaments and these resembled the filaments resulting from homospecific, wild-type crosses (compare Figure 2C; MARTINEZ-ESPINOZA *et al.* 1992; SNETSELAAR 1993).

A Fuz$^-$ colony phenotype would not allow one to distinguish between the absence of an interaction and the formation of only conjugation tubes (*i.e.,* an *a* locus-mediated pheromone response). An example of no interaction is shown in Figure 2E (representative of all crosses in column 3 of Table 4). Figure 2F presents a positive interaction (due to compatible *a* loci) that results in conjugation tubes and fusion but, due to the lack of compatible *b* loci, no filaments and hence a Fuz$^-$ colony morphology.

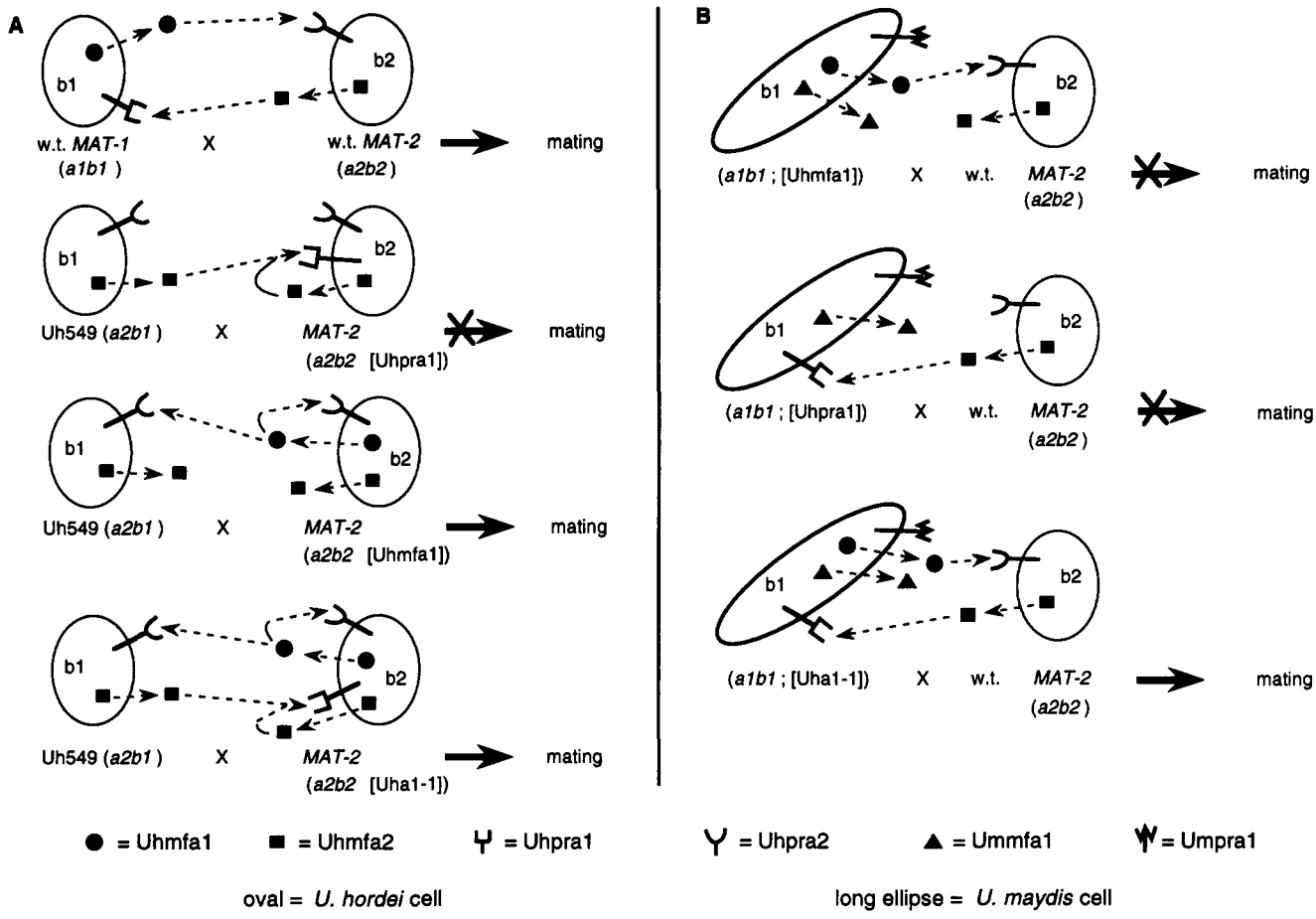

FIGURE 3.—Diagram depicting pheromone signaling during mating interactions between Ustilago cells. The genotypes are given in parentheses. w.t., wild type; Uh549(*a2b1*), constructed tester strain (see Table 1); construct shown between brackets was used to stably transform the strain (compare with Figure 1). The stippled arrows indicate secretion of the pheromones and binding to the cognate receptor. (A) Interactions between *U. hordei* strains. The top panel shows the interaction between wild-type strains, and the lower three panels show the interaction between a tester strain and transformants harboring different components of the *U. hordei a1* locus. (B) Interactions between a wild-type *U. hordei* strain and *U. maydis* transformants harboring different components of the *U. hordei a1* locus. For both the intraspecific (A) and provoked interspecific (B) mating interactions, it appears that the receptors from both partners have to be activated by binding of a compatible pheromone for the initial stages of mating (*i.e.*, fusion via newly formed conjugation tubes) to occur. See text for further explanation.

The provoked interspecific crosses are not pathogenic: The natural, vigorous appearance of the dikaryotic filaments resulting from the interspecific crosses prompted us to test their pathogenicity on corn and barley, the natural hosts for *U. maydis* and *U. hordei*, respectively. Five trials on corn and one trial on barley failed to yield the teliospores expected of a successful infection and completion of the sexual phase of the smut life cycle. At the most, after 1 week, weak symptoms (anthocyanin streaks around the inoculation site) appeared on the first leaf of ~30% of the corn plants inoculated with the crosses that were scored as Fuz$^+$. None of the other crosses showed symptom development. Microscopic analysis of the inoculation sites (at 1-week time intervals) that showed anthocyanin symptoms on the corn leaves did not clearly distinguish between compatible and incompatible interactions. That is, the interspecific dikaryon seemed to be viable and able to ramify, to a certain extent, in the corn seedlings (data not shown). In contrast, no symptoms were observed on barley irrespective of the strains used to inoculate the plants.

Natural intercompatibility between *U. scitaminea* and *U. maydis*: Our conclusion that the Ustilago *a* locus can provoke compatibility between naturally nonmating species prompted us to investigate whether compatibility (reported to occur between several species; ROWELL and DEVAY 1954; FISCHER and HOLTON 1957; HUANG and NIELSEN 1984; DIGBY and WELLS 1989) existed between the species in our collection. During the initial analysis of the mating system in *U. scitaminea*, a smut pathogen of sugarcane (Saccharum spp.; ALEXANDER and SRINIVASAN 1966), we made the surprising observation that the *U. scitaminea* strain of the "minus" mating type (*MAT-2*) could give a positive mating reaction with *U. maydis* strains of *a1b1* and *a1b2* mating types (Table

TABLE 4

Mating interactions between *U. hordei* and *U. maydis* strains transformed with different components from the *U. hordei a1* locus

Strain/transformant	Tester strains						
	Um521 *a1 b1*	Um518 *a2 b2*	Uh112 *a1 b1*	Uh100 *a2 b2*	Uh551 *a1 b2*	Uh549 *a2 b1*	Uh530 *a2 b0*
Um521 (*a1 b1*)	−	+	−	−	NT	NT	NT
Um521 (*a1 b1* paMAT-1)	−	+	−	+	NT	NT	NT
Um521 (*a1 b1* [pUha1])	−	+	−	+	−	+	−
Um521 (*a1 b1* [pUhpra1])	−	+	−	−	−	−	−
Um521 (*a1 b1* [pUhmfa1])	−	+	−	−	−	−	−
Um521 (*a1 b1* [pUhal-1])	−	+	−[a]	+[c]	−	+	−[b]
Uh4857-5 (*a2 b2* [UmpA1])	−	+	+	−	NT	NT	NT

Results of mating tests with transformants containing DNA fragments [ ] (see Figure 1) randomly and stably integrated in the genome. (+) or (−), colony morphologies of mated strains are Fuz^+ or Fuz^-, respectively. Several independent transformants for each construct have been tested; ectopically integrated constructs can show position effects resulting in strains showing varying degrees of fuzziness when mated. The last line shows one reciprocal experiment involving a *U. hordei* strain stably transformed with the *U. maydis a1* locus sequences on plasmid construct pA1. This plasmid carries the *Umpra1* + *Ummfa1* genes on a 10-kb *Bam*HI fragment in plasmid pHL1 (FROELIGER and LEONG 1991). NT, not tested.

[a,b,c] Refer to Figures 2, E and F, and 4, respectively, for microscopic views of cell morphologies.

5, column 1) but not with *U. maydis* strains of *a2b2* or *a2b1* mating type (Table 5, column 3). That is, a clear Fuz^+ colony morphology appeared suggesting compatible *a* loci and functional *U. scitaminea b* loci of a specificity different from the *b* loci of *U. maydis*. Furthermore, genetic evidence for the involvement and interaction of the *b* allele products, necessarily preceded by cytoplasmic fusion, was obtained from matings between *U. scitaminea* minus (*MAT-2*) strains and a *U. maydis* tester strain with the same *a1* locus but lacking a functional *b* locus (Table 5, column 5). No Fuz^+ phenotype was observed in these reactions indicating that *b* allele interactions were required. Interestingly, the *U. scitaminea* plus (*MAT-1*) mating type did not produce a Fuz^+ colony morphology when combined with *U. maydis* strains of any genotype.

Microscopic analysis of the mating interactions (Table 5) revealed a correlation between a Fuz^+ colony phenotype and the occurrence of straight-growing hyphae. The hypha produced from a compatible *U. scitaminea* wild-type cross (Figure 5A, arrowhead) was very similar to a hypha emerging from a "intercompatible" interaction between a *U. maydis* and a *U. scitaminea* cell (Figure 5C, arrowhead). Nuclear staining with DAPI (see MATERIAL AND METHODS) confirmed that hyphae similar to those shown in Figure 5 were dikaryotic in

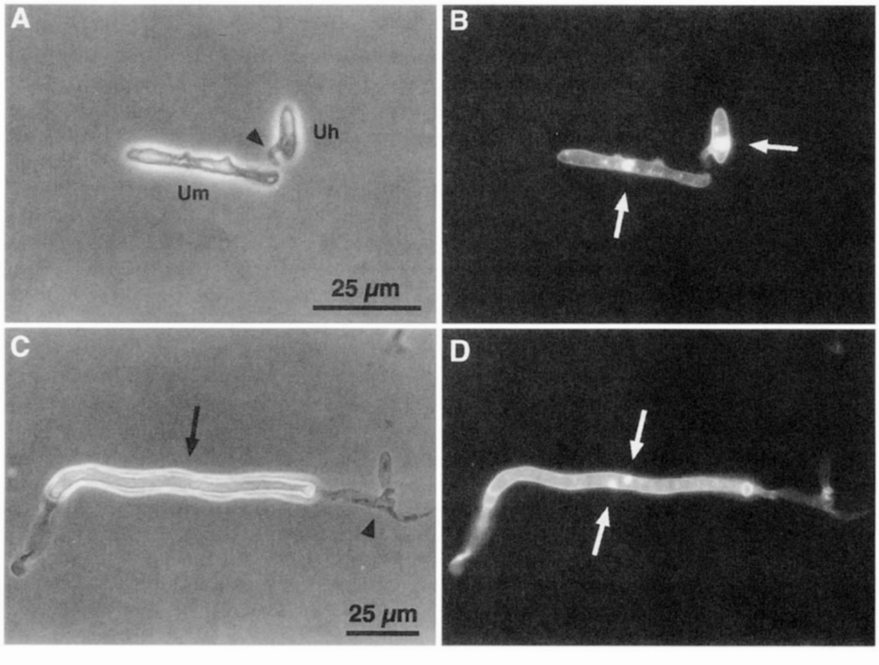

FIGURE 4.—Mating interactions between *U. hordei* and transformed *U. maydis* cells. After 24 hr, the cells were scraped from water-agar plates and stained with DAPI and calcofluor (see MATERIALS AND METHODS). A and C are bright field views; B and D show the same cells under fluorescent conditions. (A and B) *U. hordei* Uh100(*MAT-2, a2b2*) [Uh] and *U. maydis* Um521(*a1b1,* [pUha1–1]) [um] cells are fused via a conjugation tube (arrowhead in A). No filament has yet been formed, and each cell still contains its cytoplasm and haploid nucleus (arrows in B). (C and D) A mating interaction from the same cross at a further stage producing a filament (hypha) in which the cytoplasms have converged (arrow in C). The filament emerged from the fused conjugation tubes (arrowhead in C), leaving behind empty cell bodies. This filament is dikaryotic; the arrows in D indicate the two nuclei in a single, nonseptated cell.

TABLE 5

Mating interactions between *U. scitaminea*, *U. hordei* and *U. maydis*

Strain	Tester strains							
	Um521 *a1 b1*	Path[b]	Um518 *a2 b2*	Path[d]	UmPBAD8 *a1 b0*	Uh112[f] *a1 b1*	Uh100[g] *a2 b2*	Uh533 *a1 b0*
Us 2+ or 6+ (*MAT-1*)	−[a]	0	−[c]	0	−	−	+/−[h]	−
Us 1− or 5− (*MAT-2*)	+[a]	1 (67)	−[c]	0	−[e]	+	−	+[i]
Uh4857-4 (*MAT-1*)	−	NT	−	NT	NT	−	+	−
Uh4857-5 (*MAT-2*)	−	NT	−	NT	NT	+	−	−
Um521 (*a1b1*)	−	0	+	5 (64)	−	−	−	NT
Um518 (*a2b2*)	+	5 (64)	−	0	−	−	−	NT

(+) or (−), colony morphologies of mated strains are Fuz$^+$ or Fuz$^-$, respectively. The combinations may show varying degrees of fuzziness when mated. NT, not tested.

[a] Fuz reaction was identical when a *U. maydis* tester strain with genotype *a1 b2* was employed.

[b,d] Shown is the maximum pathogenicity of the crosses from the first and third column, respectively, as assayed on corn. The disease rating is as follows: 0, no symptoms; 0.5, some chlorosis around the inoculation site; 1, anthocyanin production (streaks) around the inoculation site; 2, small leaf galls; 3, small stem gall; 4, big stem gall; 5, dead plant. The number in parentheses indicates the percentage of plants having that maximum disease rating. Note that the four compatible combinations of *U. scitaminea*, 2+ and 6+ (*MAT-1*) with 1− and 5− (*MAT-2*), gave a very fuzzy colony morphology that correlated with a disease rating of 1 on ~50% of the corn plants. In contrast, *U. scitaminea* crosses of like-mating types were Fuz$^-$ and not pathogenic. When tested individually on corn, none of the strains produced symptoms.

[c] Fuz reaction was identical when a *U. maydis* tester strain with genotype *a2 b1* was employed.

[e] Cells do interact; *cf.* Figure 5D.

[f] Fuz reaction was identical when another, wild-type, *U. hordei MAT-1* tester strain, Uh4857-4, was employed.

[g] Fuz reaction was identical when another, wild-type, *U. hordei MAT-2* tester strain, Uh4857-5, was employed.

[h] Some interaction is seen, ~1% of the cells seem to produce short protrusions that might be conjugation tubes (see Figure 6D).

[i] The colony consists of cells interacting and producing only conjugation tubes; no hyphae were observed at the microscopic level (see Figure 6C).

nature (data not shown). The Fuz$^-$ reaction between *U. scitaminea* minus (*MAT-2*) and the *U. maydis* strain deleted for the *b* locus (Table 5, column 5), showed, upon microscopic analysis, that the partners were still able to interact. That is, conjugation tubes and cell fusion were observed (Figure 5D). No interaction whatsoever was seen between the *U. scitaminea* minus (*MAT-2*) mating type and a *U. maydis* strain of genotype *a2b2* (Figure 5B).

The dikaryotic filaments resulting from the interspecific crosses prompted us to test whether these interactions would cause disease when inoculated into corn seedlings, the normal host plant for *U. maydis*. The results of the plant inoculations are summarized in Table

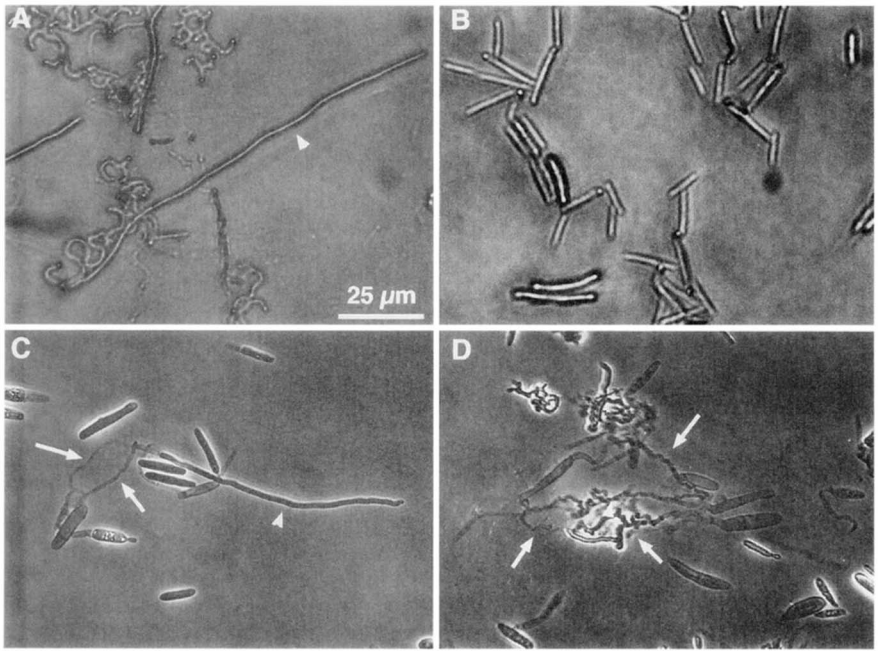

FIGURE 5.—Microscopic analysis of the interaction between *U. scitaminea* and *U. maydis*. Note the thin, meandering conjugation tubes (arrows) and the thicker, straight-growing hyphae (arrowheads) emerging from fused conjugation tubes. (A) Mating between *U. scitaminea* wild-type strains 5- (*MAT-2*) and 2+ (*MAT-1*). (B) Mating between *U. scitaminea* strain 5- (*MAT-2*) and *U. maydis* strain 518 (*a2b2*); no interaction was observed. (C) Mating between *U. scitaminea* strain 5- (*MAT-2*) and *U. maydis* strain 521 (*a1b1*); note that two different cell types are linked by the conjugation tubes and result in the production of a single hypha. (D) Mating between *U. scitaminea* strain 5- (*MAT-2*) and *U. maydis* strain PBAD8 (*a1b0*); interaction was observed, but no straight-growing hyphae could be detected.

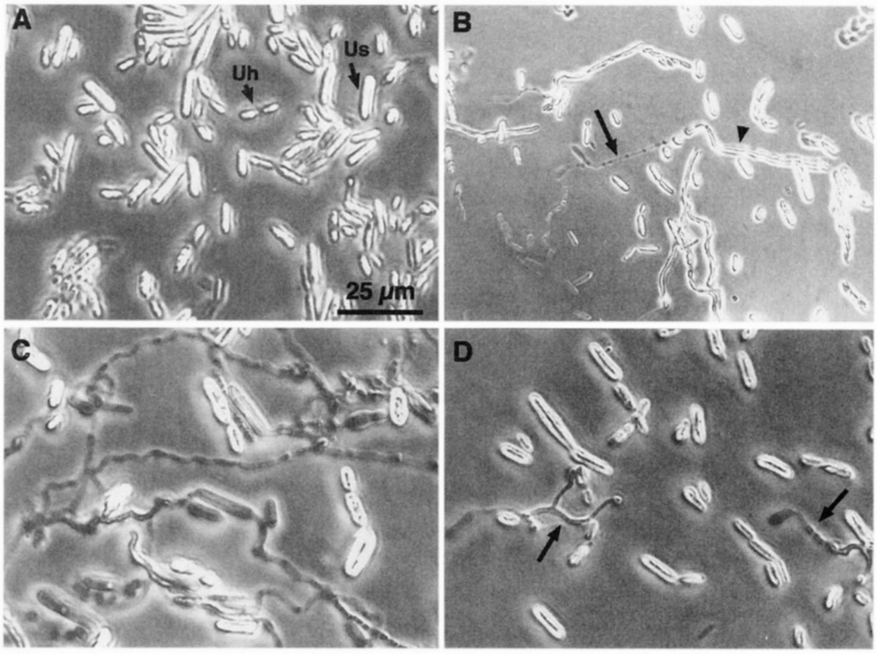

FIGURE 6.—Microscopic analysis of the interaction between *U. scitaminea* and *U. hordei*. (A) Mating between *U. scitaminea* strain 5- (*MAT-2*) [Us] and *U. hordei* strain Uh100 (*MAT-2*) [Uh]; no interaction was observed between the distinct cell types. (B) Mating between *U. scitaminea* strain 5- (*MAT-2*) and *U. hordei* strain Uh112 (*MAT-1*); two different cell types are fused via conjugation tubes (arrow) from which a thicker hypha emerges (arrowhead). The combined cytoplasms are dikaryotic (data not shown) and are found in the tip leaving behind empty cell bodies and a septated filament. (C) Mating between *U. scitaminea* strain 5- (*MAT-2*) and *U. hordei* strain Uh533 (*a1b0*); the distinct cell types interact via many (fused) conjugation tubes but fail to produce straight-growing hyphae. (D) Mating between *U. scitaminea* strain 2+ (*MAT-1*) and *U. hordei* strain Uh100 (*MAT-2*); a small fraction of the cells respond by producing conjugation tube-like protrusions (arrows).

5 (columns 2 and 4). No teliospores were produced from any of the inoculations, but there was a correlation between the crosses scored as Fuz^+ and weak symptom formation. At the most, after ~1 week, chlorosis or anthocyanin streaks around the inoculation site appeared on the first leaf of the inoculated plants; these symptoms were reminiscent of the symptoms seen from the corn inoculations with the forced intercompatible crosses. None of the other crosses (Fuz^-) developed symptoms.

Natural intercompatibility between *U. scitaminea* and *U. hordei*: Natural intercompatibility was subsequently found between *U. scitaminea* and another bipolar species, *U. hordei*. Table 5 (column 6) shows that *U. scitaminea* minus (*MAT-2*) mating types interacted with *U. hordei MAT-1* strains but not with *U. hordei MAT-2* strains (column 7). Microscopically, the Fuz^+ reaction correlated with the appearance of hyphae (compare Figure 6, A and B) that were dikaryotic as revealed by staining with DAPI (data not shown). Mating with a *U. hordei MAT-1* strain lacking a *b* gene complex (*a1b0;* Table 1) proved that the Fuz^+ reaction was dependent upon the productive interaction of *b* gene products following fusion because no straight hyphae were observed, although the cells interacted profusely via conjugation tubes (Figure 6C). Unexpectedly, this interaction resulted in a weak Fuz^+ colony morphology (Table 5, column 8). The *U. scitaminea* plus (*MAT-1*) mating type did not interact with *U. hordei MAT-1* cells (Table 5, column 6) but seemed to interact with *U. hordei MAT-2* cells (column 7). This particular interaction was too weak to give a Fuz^+ colony morphology, but microscopic examination revealed that a small fraction (~1%) of the cells responded by putting out protrusions that resembled conjugation tubes (Figure 6D). These cells appeared to be *U. hordei* based on their size and shape. The *U. scitaminea* and *U. hordei* crosses were not tested on barley.

While it is difficult to quantitate the strength of the natural interspecies interaction, the timing of the formation of conjugation tubes and dikaryotic hyphae was similar to those of homospecific matings (SNETSELAAR 1993; BANUETT and HERSKOWITZ 1994) and the forced interspecies matings described above.

DISCUSSION

We have shown previously that the *U. hordei* mating-type locus, *MAT,* harbors physically linked loci, called *a* and *b,* that are homologous to the *a* and *b* loci in *U. maydis* (BAKKEREN *et al.* 1992). Furthermore, we showed that the *U. hordei b* loci were structurally and functionally similar to the *b* loci in *U. maydis* (BAKKEREN and KRONSTAD 1993). In this study, we demonstrate that in *U. hordei,* the *b* locus is essential for orchestrating the morphological switch from budding to filamentous growth and for the pathogenicity of mated partners. The bEbW heterodimer, formed when the two molecular species encoded at the *b* locus have opposite specificities, can set off this developmental program in both a dikaryon, *i.e.,* the product of a mating between compatible partners, and in a haploid transformant. The solopathogenic haploid *U. hordei* strains constructed in this study should prove invaluable in mutational analyses to identify other genes, as has been demonstrated for a solopathogenic haploid strain of *U. maydis* (GIASSON and KRONSTAD 1995).

We have also shown before that the *U. hordei a1* locus bears a pheromone receptor gene (*Uhpra1*) with homology to the *U. maydis* gene, *Umpra1* (BAKKEREN and

KRONSTAD 1994). Here we demonstrate that the *U. hordei a1* locus harbors at least one additional gene, the pheromone gene *Uhmfa1,* and we show that this gene and the *Uhpra1* gene code for cell signaling components that initiate the formation of conjugation tubes and cell fusion. Our transformation experiments indicate that the proposed pheromone and receptor combination dictates mating-type specificity in *U. hordei.*

The dual mating phenotypes, obtained after transformation of a *U. hordei MAT-2* strain with various components of the *U. hordei a1* locus, and the forced interspecific mating interactions between *U. maydis* cells, transformed with the same components and *U. hordei* tester strains, demonstrated that both mating partners need to be activated by a receptor and its compatible pheromone for mating to occur. BÖLKER *et al.* (1992) reached similar conclusions while analyzing the mating responses between *U. maydis* cells. The pheromone-receptor combination operates at the very beginning of a signaling pathway that is otherwise conserved between mating types and, as we have shown here, between species. The enzymatic machinery responsible for expression of the mating-type genes and the proper maturation of the gene products must be conserved as well. We have not ruled out the possibility that small open reading frames, such as those present at the *a* loci of *U. maydis* (SPELLIG *et al.* 1994), might also be located at the *U. hordei a* locus and might contribute to the interspecies mating capability. This possibility seems unlikely given the well-established roles of the identified pheromone and receptor genes.

Among smut species, natural intercompatible combinations are common and some are interfertile and infectious on common hosts (FISCHER and HOLTON 1957; HUANG and NIELSEN 1984). For example, ROWELL and DEVAY (1954) described crosses between *U. zeae* (= *U. maydis*) and *Sphacelotheca reiliana* (= *Sporisorium reilianum;* a smut pathogen of sorghum and corn) that produced teliospores on corn. However, no pathogenic progeny were obtained and therefore no evidence of hybridization was found. More recent work has identified positive, mating-type specific interactions between isolates of *U. scitaminea* and *S. reilianum.* Similar to our experiments, no teliospores were formed and only a "flecking reaction" was observed after inoculation on corn and sorghum (K. E. DAMANN, personal communication). Assuming that *b* gene products of different species can productively interact, which we have demonstrated to occur during the natural interaction between *U. scitaminea* and both *U. hordei* and *U. maydis,* the *a* loci assume likely roles as determinants of intercompatibility. It follows that, in certain cases, sexual incompatibility (intersterility) could be considered a lack of proper pheromone signaling.

The natural interspecies matings described in this paper indicate that *U. scitaminea* has a (partially) promiscuous cell signaling system. That is, the pheromones from *U. scitaminea* are recognized by its own receptors, but additionally by those of other species and vice versa; the receptors of both partners have to be activated by a compatible pheromone for fusion to occur. Although there still seemed to be mating-type specificity, because only a limited response was seen in a mating interaction between *U. scitaminea* plus (*MAT-1*) and *U. hordei MAT-2* strains (Table 5, column 7), and no response at all was found with *U. maydis a2* strains (Table 5, column 3). It is possible that the pheromone of only one of the mating partners interacts with the receptor from the other partner and that the other pheromone-receptor interaction fails to trigger a response. Alternatively, one or both of the pheromones may be relatively poor ligands for the "cognate" receptors and only few cells might be stimulated above a certain threshold level needed to trigger conjugation tube formation. In this respect, it should be mentioned that the pheromones from the ascomycetous yeast species, *S. cerevisiae* and *S. kluyveri,* exhibit some physiological cross-reaction (MCCULLOUCH and HERSKOWITZ 1979). For example, the *S. cerevisiae* α-factor receptor, STE2p, has a 3400-fold higher affinity for the *S. cerevisiae* α-factor pheromone than for the *S. kluyveri* α-factor pheromone (SEN and MARSH 1994). However, even though the α-factor receptors of these two species are 50% identical and do bind each others pheromone to transduce a signal that induces G1-arrest, this interspecies interaction does not lead to actual mating. Indeed, the *S. kluyveri* receptor gene is poorly regulated and expressed on the cell surface when introduced into an *S. cerevisiae* mating type **a** strain (SEN and MARSH 1994), suggesting less functional conservation of this signal transduction pathway between these species than between the Ustilago species studied in this paper.

So-called "biological species" are groups of organisms that belong to a single species because they share "morphological" or "taxonomic" characteristics, but that are, or have become, (partially) intersterile due to geographic isolation (ALEXOPOULOS 1962; BURNETT 1983; RAYNER 1991; ESSER and BLAICH 1994). Biological species are common in the fungal world, but only two convincing examples have been described in the smut fungi. The species *U. cynodontis* and *U. striaeformis* each encompass several (partially) intersterile groups among many world-wide collections (FISCHER and HOLTON 1957; DIGBY and WELLS 1989). Upon genetic analysis of the progeny, it might be possible to determine whether the intersterility (intercompatibility) factors reside on these species' *a* loci and are in fact the pheromone signaling components. Alternatively, intersterility genes that determine the limits of interbreeding populations within a species could be distinct from the mating-type genes. Such genes have been described for the root-rotting homobasidiomycetous fungus, *Heterobasidion annosum* (CHASE and ULLRICH 1990). In any case, natural sexual intercompatibility could indicate the ex-

istence of intermediate species actually undergoing speciation. It is tempting to propose that, at least within the genus Ustilago, mutations in the pheromone-receptor signaling components play a role in speciation. However, other factors (genes) are likely involved because the *a* locus determines only sexual intercompatibility and not fecundity. Equally plausible would be a scenario wherein the acquisition (or loss) of host determinants leads to a limited host range and niche-partitioning that then in turn would allow for the drift of the pheromone-receptor signaling system. In this regard, it is interesting to note that in the homobasidiomycetous fungus, *Schizophyllum commune,* mating-type loci are found that encode a multispecific system of several pheromone and pheromone receptor genes (WENDLAND *et al.* 1995). Without the need for specialization on a particular host, this saprotrophic fungus could have evolved by generating multiple factors for self/nonself recognition to increase its outbreeding potential.

The taxonomic classification of smut species is often based on host range and spore morphology. As stated before (BURNETT 1983; BOIDIN 1986; ESSER and BLAICH 1994), comprehensive genetic data, including compatibility tests, should be considered when reviewing taxonomic data. The natural (partial) intercompatibility between *U. scitaminea* and the two other species and the forced interspecific matings illustrate the close relationship between some Ustilago species. Given a common host, these combinations might be interfertile and therefore con-specific (BOIDIN 1986). However, a comparison of the 5S ribosomal RNA genes between *U. hordei* and *U. maydis* allowed BLANZ and GOTTSCHALK (1984) to put these species into separate but related groups. Indeed, parasites with specific host ranges complicate a comprehensive species concept. It seems, nevertheless, that with the emerging data on mating-type genes, with compatibility tests, and with the availability of techniques such as DNA sequence comparison of polymorphic regions (BLANZ and GOTTSCHALK 1984) and the analysis of cell wall carbohydrates (PRILLINGER *et al.* 1993), the species concept in the smut fungi, particularly in the bipolar group of the genus Ustilago, should be reexamined.

Our results suggest that the straight-growing, dikaryotic, filamentous hyphae that we obtained from forced interspecific matings can be recognized by the host and can trigger a response. Chlorosis and anthocyanin production in corn leaves, the first visual symptoms resulting from inoculation with pathogenic *U. maydis* strains, might indicate the induction of defense-related genes (*e.g.,* genes of the phenylpropanoid pathway). There might be several reasons why the dikaryons from forced interspecific crosses fail to display full virulence. First, the basic defense response of the host might be sufficient to arrest the slow-growing, less vigorous "hybrid-species" filaments. Second, the *U. hordei* and *U. maydis* cytoplasms and/or nuclei could be incompatible (genetic disharmony) preventing stabilization and further development of the dikaryotic hyphae (RAYNER 1991). However, we can see (extensive) filaments in the corn plant for several weeks after inoculation suggesting that the filaments are capable of slow growth within the plant. A third possibility is that there might be an "inhibitory effect" from the "alien" fungal partner. In case of the corn inoculations, the alien partner would be *U. hordei* because this species is not pathogenic on corn. This inhibitory effect could be due to a species-specific avirulence gene(s) that is recognized by a host resistance gene(s). Species-specific avirulence genes, in this case, would be scored genetically as host-range genes. It is conceivable that other genes, besides avirulence genes, might have evolved to fix the acquired differences that resulted in the distinction of the species, *U. hordei* and *U. maydis.* It might be possible to find a host or cultivar that would allow the proliferation of the forced interspecific crosses between *U. hordei* and *U. maydis.* Teliospore formation might then occur, but differences in chromosome numbers could cause problems during germination and subsequent meiosis or result in aneuploidization.

The *a* locus of the smut fungi seems to be sufficient, when transferred into a different species, to trigger interspecific mating responses between the transformed species and the "donor" species. We have shown this for two species, *U. hordei* and *U. maydis,* but it might be possible to use this method to force mating (or at least fusion) between other, naturally nonmating species of the genus Ustilago or even between genera of heterobasidiomycete fungi. If the Ustilago *a* loci prove to be general determinants of intercompatibility between smut species, the strategy described in this study could open many avenues to genetically identify genes involved in pathogenicity and host range.

The authors wish to thank Drs. FRANZ DÜRRENBERGER, LOUISE GLASS, CARDY RAPER, MYRON SMITH and GILLIAN TURGEON for critical comments on the manuscript and valuable discussions, Dr. JOHN E. SHERWOOD, Montana State University, Bozeman, Montana, for communicating results (confirmation of the location of the *Uhmfa1* gene) before publication, and Drs. SUSAN SCHENK and HENRIK ALBERT, Hawaiian Sugar Planters' Association, Aiea, Hawaii, for the *U. scitaminea* strains. This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada to J.W.K.

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Communicating editor: M. E. ZOLAN