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Genetics

Linkage of mating-type loci distinguishes bipolar from tetrapolar mating in basidiomycetous smut fungi

(basidiomycetes/ustilaginales/*Ustilago*/*a* locus/*b* locus)

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ABSTRACT **Sexual compatibility requires self vs. non-self recognition. Genetically, two compatibility or mating-type systems govern recognition in heterothallic basidiomycete fungi such as the edible and woodrotting mushrooms and the economically important rust and smut phytopathogens. A bipolar system is defined by a single genetic locus (*MAT*) that can have two or multiple alleles. A tetrapolar system has two loci, each with two or more specificities. We have employed two species from the genus *Ustilago* (smut fungi) to discover a molecular explanation for the genetic difference in mating systems. *Ustilago maydis*, a tetrapolar species, has two genetically unlinked loci that encode the distinct mating functions of cell fusion (*a* locus) and subsequent sexual development and pathogenicity (*b* locus). We have recently described a *b* locus in a bipolar species, *Ustilago hordei*, wherein the existence of an *a* locus has been suspected, but not demonstrated. We report here the cloning of an allele of the *a* locus (*a1*) from *U. hordei* and the discovery that physical linkage of the *a* and *b* loci in this bipolar fungus accounts for the distinct mating system. Linkage establishes a large complex *MAT* locus in *U. hordei*; this locus appears to be in a region suppressed for recombination.**

A hallmark of the heterothallic basidiomycete fungi is the fusion of cells of different compatibility to form a dikaryotic mycelium in which each cell contains a pair of nuclei. The interaction of two non-self nuclei within one cell triggers a program of sexual development (1–5). Among the homobasidiomycetes, which include the mushrooms such as *Schizophyllum commune* and *Coprinus cinereus* (6, 7), ≈65% of the species regulate sexual compatibility genetically via a tetrapolar (bifactorial) mating system. In these fungi, two genetic loci, each with two or more allelic specificities, control sexual development. Approximately 25% of the species have a bipolar (unifactorial) mating system in which compatibility is governed by a single genetic locus. In the bipolar species, this locus can have two or multiple alleles, the latter being common in the homobasidiomycetes and the non-parasitic heterobasidiomycetes. The remaining 10% consist of homothallic species (6, 8). Among the parasitic heterobasidiomycetes, such as the phytopathogenic rust, bunt, and smut fungi, the diallelic bipolar mating system is predominant with certain exceptions (9, 10). These exceptions include a multiallelic bipolar system for the bunt fungus *Tilletia controversa* (11) and the occurrence of a tetrapolar mating system in several smut species including *Ustilago maydis* and *Ustilago longissima* (10, 12). To our knowledge, the molecular mechanisms underlying the genetic differences between these mating systems have heretofore been unknown.

Mating and dikaryon formation are intricately connected with pathogenicity in the smut fungi, and the mating-type loci in the tetrapolar smut pathogen *U. maydis* have recently been isolated and characterized (10, 13–19). The *a* locus has two specificities (*a1* and *a2*) and consists of a gene complex encoding pheromones and pheromone receptors (14, 15); this locus controls fusion of mating partners (20). The *b* locus has at least 25 naturally occurring allelic specificities and consists of a gene complex encoding homeodomain polypeptides, bW and bE (12, 17–19). Independent assortment of the *a* and *b* loci of *U. maydis* promotes outbreeding because only 25% of the mating interactions in an F_1 population of meiotic products will be successful. In contrast, 50% of the interactions in diallelic bipolar smut fungi, such as the barley pathogen *Ustilago hordei*, will be successful because a single locus (*MAT*) controls mating (8, 21, 22).

The sequences at the *MAT* locus in bipolar smut fungi (*MAT-1* or *MAT-2*; refs. 23 and 24) presumably control fusion events by a mechanism similar to that of the *a* locus of *U. maydis*. In fact, sequences that cross-hybridize with the *U. maydis a* locus and factors that may be diffusible pheromones have been detected in *U. hordei* (25, 26). In addition, bipolar smut fungi do have homologs of the *U. maydis bE* and *bW* genes, and the *b* gene complexes from *U. hordei* are functional when transferred to *U. maydis* (25, 27). These results raise questions such as (*i*) why hasn't a *b* function been identified genetically in bipolar smut fungi and (*ii*) what is the organization of the *b* locus relative to the classically defined *MAT* locus in *U. hordei*? In this report, we present evidence that the *a* and *b* loci are genetically and physically linked in the bipolar species *U. hordei* to establish the *MAT* locus and describe the cloning of an allele (*a1*) of the *a* locus.§

MATERIALS AND METHODS

Strains, Media, and Mating Tests. *U. maydis* strains, 521 (*a1b1*) and 518 (*a2b2*) (16), and *U. hordei* strains, Uh112 (*MAT-1*, *ade*) and Uh100 (*MAT-2*, *ade*) (27), have been described. The bipolar smut strains (25) and all other *U. hordei* strains were obtained from P. Thomas (Agriculture Canada, Winnipeg, MB, Canada). Strains were grown in potato dextrose broth (PDB) or agar (PDA) (Difco) at 22°C. Mating tests were performed with liquid cultures grown in PDB and spotted onto plates containing complete medium with activated charcoal (28). Mixtures of compatible strains give mycelial colonies and incompatible strains form yeast-like colonies (12, 28).

Isolation of the *U. hordei a1* Locus. A cosmid library containing Uh112 (*MAT-1*) genomic DNA in pJW42 (27) was screened by hybridization with a 3.3-kb *Xho* I–*Bam*HI fragment containing the *U. maydis pan1* gene (14) as described (27). Subclones were constructed in an integrative bleomycin-resistance vector, pUble3 (27). All probes were labeled

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Abbreviations: RFLP, restriction fragment length polymorphism; Mb, megabase pair(s); Uh*pra1*, *U. hordei pra1*.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U07939).

with [α-^{32}P]dCTP (DuPont) by using a random-priming labeling kit (Pharmacia LKB). DNA was transferred to nitrocellulose membranes (Schleicher & Schuell) and blots were hybridized in the presence of 30% (vol/vol) formamide at 45°C as described (25). The filters were washed at 65°C in 0.1 × SSC (15 mM NaCl/1.5 mM sodium citrate, pH 7.0)/0.1% SDS and exposed to film (Kodak XAR5) with an intensifying screen at −70°C.

Pulse-Field Gel Electrophoresis. Chromosome-sized DNA was prepared in 0.5% low-melting-point agarose blocks (*ultra*PURE, GIBCO/BRL) essentially as described (29). To separate the chromosomes, plugs (2×10^8 cells per lane) were cast in an agarose gel in 0.5 × TBE (45 mM Tris-borate/1 mM EDTA) and subjected to countour-clamped homogeneous field pulsed gel electrophoresis at 12°C. For the gel in Fig. 5*A* (1.0% agarose), a Pharmacia LKB 2015 Pulsaphor system was employed with the following conditions: 15 hr at 200 V with a 70-sec switch interval followed by 11 hr at 200 V with a 120-sec switch interval (29). For the gel in Fig. 5*E* (0.7% agarose), a Bio-Rad apparatus was used with the following regime: 48 hr/60-min pulse/50 V, 120 hr/60-min pulse ramped to 15 min/50 V, and 40 hr/30-min pulse ramped to 15 min/70 V.

PCR and Sequence Analysis. The following oligonucleotide primers were used to amplify the *b* sequences from the various bipolar smut fungi: pr1 (5′-GGATCTAGACTGCGTCAGAGTCGTG, positions −518 to −499 of *UhbW1*) and pr2 (5′-AGTCTAGACGCATGTGATATGACGGCA, positions 347 to 366 of *UhbE1*) (27). *Xba*I sites are underlined. PCR conditions were as follows for 30 cycles: 1 min, 94°C; 2 min, 55°C; 3 min, 72°C. Then, there was a final extension for 7 min at 72°C. PCR products were cloned, using the *Xba*I sites, into vector pGEM-3Zf(+) (Promega) and their nucleotide sequence was determined using standard primers. Dideoxynucleotide sequencing (T7 sequencing kit, Pharmacia) was performed on two cloned PCR products for each *b* region (*bW* + *bE*). When base-pair changes were found, the sequence was verified by direct sequencing of the PCR product using a CircumVent thermal cycle sequencing kit (New England Biolabs) and labeled primers, pr1 and pr2.

RESULTS AND DISCUSSION

A *b* Locus Restriction Fragment Length Polymorphism (RFLP) Is Genetically Linked to *MAT* in *U. hordei*. Upon analysis of the *b* gene complex cloned previously from *U. hordei* (27), we discovered a RFLP, detected by a probe specific for the *b* locus (*bE1* probe, Fig. 1) that cosegregated with mating type (*MAT-1* or *MAT-2*) in 86 meiotic progeny from two teliospore populations. The results for 25 of those progeny are shown in Fig. 2*A*. This result indicated that the classically defined *MAT* locus in *U. hordei* might embody a complex region consisting of loci for both the *a* and *b* functions found in *U. maydis*.

Cloning of the *U. hordei a1* Locus. To prove the existence of an *a* locus in *U. hordei*, we drew on the knowledge that in *U. maydis* the *a1* locus is 2.5 centimorgans (2–6 kb) from a pantothenic acid biosynthesis gene (*pan1*; ref. 14). Similarly, a *pan* gene is linked to the *MAT* locus in *U. hordei* (24), and hybridization of the *U. maydis pan1* probe to DNA from 86 progeny of *U. hordei* detected a RFLP that cosegregated (100%) with the *MAT* locus (data not shown). This probe was used to identify a cosmid, paMAT-1, from a genomic library of DNA from a *U. hordei MAT-1* strain. Subsequently, the cosmid paMAT-1 and several subclones (Fig. 1) were tested for mating-type-specific activity by transformation into a *U. hordei MAT-2* strain. Introduction of DNA fragments carrying the *a1* locus should result in transformants that exhibit dual

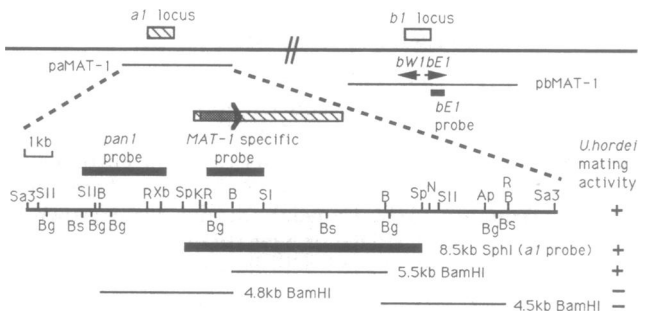

FIG. 1. Map of the *U. hordei MAT-1* mating-type loci. The upper line represents the genomic region. The open box represents the position of the *b1* locus consisting of two open reading frames, *bW1* and *bE1*, isolated on cosmid pbMAT-1 (27). Cosmid paMAT-1 carries the *a1* locus (hatched boxes and enlarged map). Note that the orientation relative to the *b1* locus has not been determined. The stippled arrow within the hatched box indicates the location and direction of transcription of the *U. hordei* pheromone receptor gene, Uh*pra1* (Fig. 4). Subclones (lower four lines) were tested for the presence of the *a1* locus (mating activity) as shown in Fig. 3*B*. Ap, *Apa* I; B, *Bam*HI; Bg, *Bgl* II; Bs, *Bss*H2; K, *Kpn* I; N, *Not* I; R, *Eco*RI; Sa3, *Sau*3A; SI, *Sac* I; SII, *Sac* II; Sp, *Sph* I; Xb, *Xba* I. Solid bars denote the hybridization probes used in this study; the region of hybridization to the *U. maydis pan1* probe is indicated on paMAT-1.

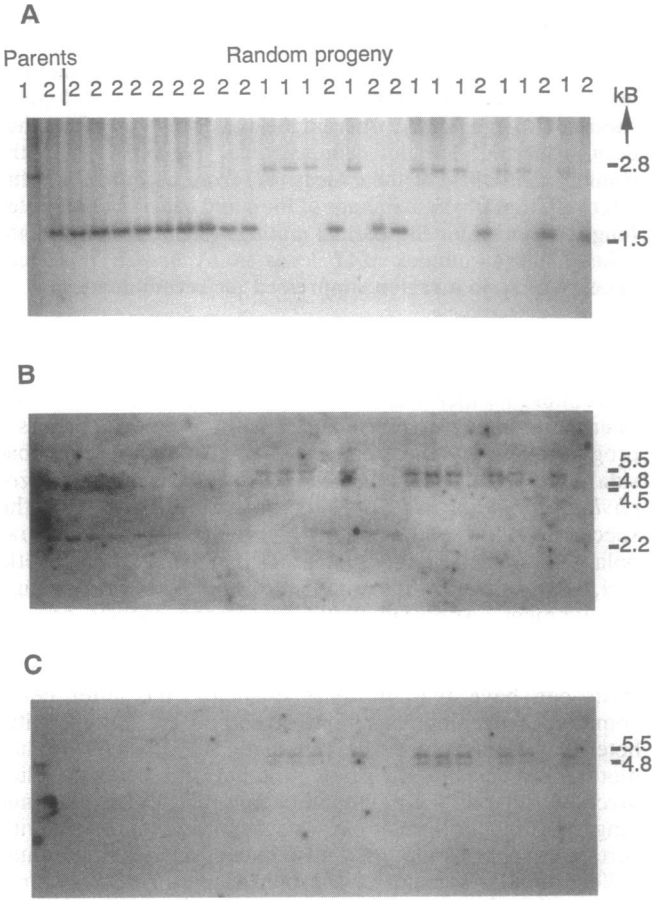

FIG. 2. Segregation of RFLPs within the *U. hordei a* and *b* loci with mating type (*MAT*). Lane labels 1 or 2 refer to mating types *MAT-1* or *MAT-2*, respectively (for a subset of 25 random progeny). The specificity at the *MAT* locus was determined by a mating assay on culture medium. The same DNA blot of total genomic *Bam*HI-digested DNA was hybridized with a *U. hordei b* locus-specific probe, *bE1* (*A*), a *U. hordei a1* locus-specific probe (paMat-1, 8.5-kb *Sph* I fragment) (*B*), or a *U. hordei MAT-1*-specific probe (paMat-1, 2.1-kb *Eco*RI–*Sac* I fragment) (*C*) (see Fig. 1 for locations of probes). The *MAT-1*-specific probe (2.1-kb *Eco*RI–*Sac* I fragment; Fig. 1) hybridizes specifically to genomic DNA from *MAT-1* strains and not to DNA from *MAT-2* strains.

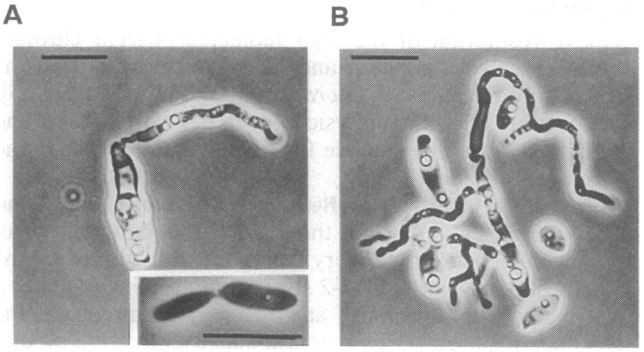

FIG. 3. Conjugation tube formation and cell–cell interactions in *U. hordei*. The conjugation assay (30 hr) was performed as described (30). (*A*) Conjugation tube formation in a compatible mating reaction between a *MAT-1* strain (Uh112) and a *MAT-2* strain (Uh100). (*Inset*) Budding wild-type cell of strain Uh100 (*MAT-2*). (*B*) "Self-mating" behavior (conjugation tube formation and cell aggregation) after introduction of the 8.5-kb *Sph* I fragment carrying the *U. hordei a1* locus (Fig. 1) in a *U. hordei MAT-2* strain (Uh100). (Bars = 10 μm.)

mating behavior, i.e., formation of conjugation tubes and cell aggregation in the absence or presence of cells of opposite mating type. Normally, *U. hordei* responds to mating partners by forming conjugation tubes that mediate fusion (26). A typical mating structure from a mixture of wild-type cells of opposite mating type is shown in Fig. 3*A*. Similar observations have been made for *U. maydis* (30). When tested for mating activity, the episomal cosmid paMAT-1 and 8.5-kb *Sph*I and 5.5-kb *Bam*HI fragments, but not adjacent 4.8-kb and 4.5-kb *Bam*HI fragments (Fig. 1), induced the formation of conjugation tubes and cell aggregates upon transformation into a *MAT-2* strain (Fig. 3*B*). Moreover, when introduced into a *U. maydis* strain, the *U. hordei* 8.5-kb *Sph*I fragment allowed the transformants to interact specifically with a *U. hordei MAT-2* strain but not with a *MAT-1* strain (G.B., unpublished results). Haploid strains of *U. hordei* and *U. maydis* do not normally interact, regardless of mating type.

DNA sequence analysis confirmed the existence of a pheromone receptor gene, *U. hordei* (Uh) *pra1*, having 62% DNA sequence identity with the *pra1* gene of *U. maydis* (15); its predicted amino acid sequence is 64% identical (and 82% similar) to the predicted *pra1* gene product of *U. maydis* (Fig. 4). The Uh*pra1* gene spans the *Bam*HI restriction site be-

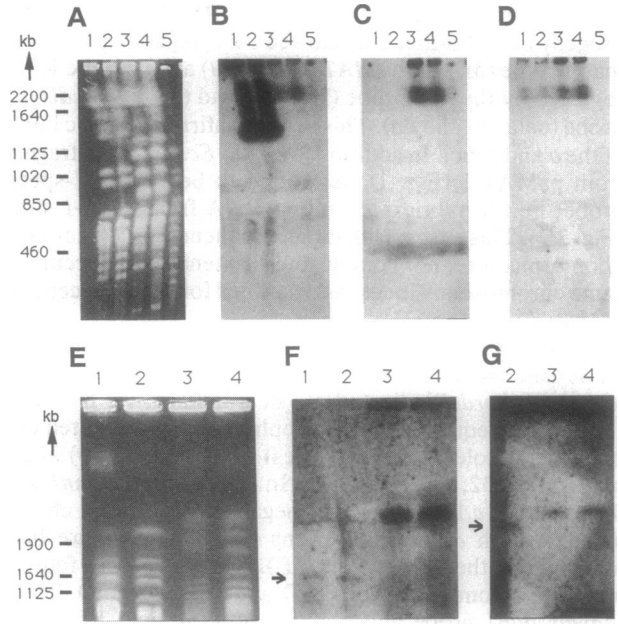

FIG. 5. Localization of the *a* and *b* loci on chromosomes of *U. hordei* and *U. maydis* separated by pulse field electrophoresis. (*A* and *E*) Ethidium bromide-stained agarose gels. (*B–D*) Same DNA blot, made from gel in *A*. (*F* and *G*) Same DNA blot, made from gel in *E*. Blots were hybridized with the following probes (Fig. 1): *B*, *U. maydis pan1*; *C*, paMAT-1 (8.5-kb *Sph* I fragment); *D*, *U. hordei bE1* plus *U. maydis bE5*; *F*, same probe as in *C*; *G*, same probe as in *D*. The *bE5* probe is a *U. maydis b* locus-specific probe and has been described (17). Lanes: 1, *U. maydis* 521 (*a1 b1*); 2, *U. maydis* 518 (*a2 b2*); 3, *U. hordei MAT-1* (Uh112); 4, *U. hordei MAT-2* (Uh100); 5, *Saccharomyces cerevisiae* chromosome-size standard (Bio-Rad).

tween the 4.8- and 5.5-kb *Bam*HI fragments (Fig. 1). The fact that the 5.5-kb *Bam*HI fragment has mating-type activity might indicate that it harbors a gene coding for a *U. hordei* mating factor (Uh*mfa1*). We conclude that the 8.5-kb *Sph* I fragment from paMAT-1 contains the *U. hordei a1* locus. These results allowed us to use sequences from this fragment as *U. hordei a1*-specific hybridization probes for subsequent analysis of the linkage between *a* and *b* loci.

The *a* and *b* Loci of *U. hordei* Are Genetically Linked and Together Define the Classical *MAT* Locus. Hybridization with

A

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   1 ATGCTCGACCACGTTACGCCATTCTTTGCA CTTTTTGCCTGCATCCTGGTTCTTTTCGCA CTTGGCTGGCACATTAGAAGTCGAAATGTC GGTACCATCACTCTCTCGCTCTATCTTTTC
 121 TTCGGCAATCTCGACAACTTTGTGAATTCG GTGGCATGGTGGAGCACCGCTGAAGATAAG GCTCCAGGCTTTTGTGAAGTCAgtaagtat gaagttcaacaaaaagattccgtgagaccg
 241 tcgtcactaatcataataccaatgtcgcca tgtactcgcagGCATCAGGCTTCGACATGC CCTGTATATTGCAATCCCTGCCTCAAACCT CGTAATTGCACGCAAATTGGAGAGTATCGC
 361 CTCAACAAGACAAGTCAGGGCCAGTGCATC GGAACACAAAAAATCGATTATTATAGATCT GCTCATCTCTGTGGGACTGCCAGTCCTGTA CGTTTCCTTGATGATCGTCAACCAGACAAA
 481 TCGTTACGGAATCATCGAGCAAGTTGGCTG CTGGCCTTTTCTCTCACTATCCTGGGTCTG GGTTCTGCTGGTAGCTGCGCCGGTTCTCAT AGTGTCCTTCGCTTCTGCAGTCTACAGTGg
 601 taagtgatcacaagtttttcgaacacgtat cctcctcgaaacgttgctgacacttcatcc ttgcgacagTCCTAGCCTTCCGATGGTTCT GGATTCGTCGACGCCAATTCCAGGCAGTGC
 721 TGGCCAGCAGCGCATCGACACTCAACAAAG CAAGATACATTCGGCTTCTAGTGCTTACCG CCATCGACATGCTGCTTTTCTTCCCAATCT ACGTTGGATCCGTTTCTGATACCATCAGAG
 841 GAGCTATCACAACGTCCTATGTGTCCTGGT CGTACGTACACACTGGGTTCAGTTACATCC CTCAATTCTCTGCAGAAGTAATGGAGATGC AGCCTTCCTTCAAGGCGAGGCTGATTCTTT
 961 CTCGTCTGGTATGTCCGATCTCCGCCTACA TCTTCTTCGCGATGTTTGGCCTGGGCCAAG AGGCGCGTCAAGGCTATAAGCACGCAGTTC TTAAGGCTCTGGTCTTCTGCAAATTGCGCA
1081 AGGAACGCCAAAAGCCCATACAAAAgtaag ttcgtgcagagcgttggcctacctattctt tcaatcagctgacacgccagggtttccctc gtttcgttgcagCCATATCGTTGCAAACAT
1201 AGAGGTGGTAACTTTCCAGTCCAGGGAGAC TTCCGGTGGGATCGACGGAAGCCCGCACTC GGAGAAATTCAGCATCAATACTCCAACAAA GTACGAAGAAGCATGA
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B

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                                                                  intron1
                                                                     V
Uh  MLDHVTPFFALFACILVLFALGWHIRSRNV GTITLSLYLFFGNLDNFVNSVAWWSTAEDK APGFCEVSIRLRHALYIAIPASNLVIARKL ESIASTRQVRASASEHKKSIIIDLLISVGL 120
Um       I      V FF    MPFA   K K    L M  IW ML          MV  K TA L   AY  L V    L F         A                  GPGD RRAV      CL I
                                                  intron2
                                                     V
Uh  PVLYVSLMIVNQTNRYGIIEQVGCWPFLSL SWVWVLLVAAPVLIVSFASAVYSVLAFRWF WIRRRQFQAVLASSASTLNKARYIRLLVLT AIDMLLFFPIYVGSVSDTIRGAITTSYVSW 240
Um    II T       S     L EA    MMVF   L          IV  LC     A         V                 I RSH V   L                  TIAAQ KSS SIP G
                                                                                 intron3
                                                                                    V
Uh  SYVHTGFSYIPQFSAEVMEMQPSFKARLIL SRLVCPISAYIFFAMFGLGQEARQGYKHAV LKALVFCKLRKERQKPIQNHIVANIEVVTF QSRETSGGIDGSPHSEKFSINTPTKYEEA* 359
Um    S      NQ    YP SLVL ENT QRN    A     L            L V     E F HR  L   R    PKASALQ V  D       R HD FDA.NT TK   SD DMRGS. A *
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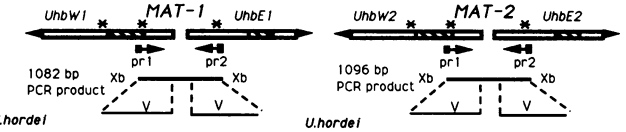
FIG. 4. Molecular analysis of the potential pheromone receptor gene, Uh*pra1*, at the *U. hordei a1* locus. (*A*) DNA sequence of the Uh*pra1* gene. Sequence comparison with the *U. maydis pra1* gene (15) indicated three potential introns (lowercase type). The following features are depicted in order in boldface type: start codon; *Kpn* I, *Eco*RI, *Bgl* II, and *Bam*HI-restriction sites (compare Fig. 1); and stop codon. (*B*) Predicted product of 359 amino acids translated from the DNA sequence depicted in *A*, excluding the three potential introns to maintain a proper open reading frame (*U. hordei*, Uh). This polypeptide has been compared with the 357-amino acid predicted product of the *U. maydis pra1* gene (*U. maydis*, Um). Only amino acids that differ are shown; dots indicate a gap. (Sequence alignment was generated using the GAP program of the GCG sequence analysis software package.)

the *U. hordei a1* probe detected a RFLP, among the 86 progeny of *U. hordei*, that showed cosegregation (100%) with mating type (*MAT-1* or *MAT-2*; Fig. 2*B*) and with the RFLPs revealed by the *bE1* probe (Fig. 2*A*) and the *U. maydis pan1* probe (data not shown). This result confirmed genetic linkage of the *a* and *b* loci. In addition, a 2.1-kb *Eco*RI–*Sac* I fragment from paMAT-1 (Fig. 1) was found to be a *MAT-1*-specific probe; i.e., it hybridized only to DNA from *MAT-1* strains (Fig. 2*C*). This suggests that the *a* sequences of *U. hordei* are idiomorphs, i.e., nonhomologous sequences that occupy the same chromosomal locus, as described for the *a* sequences of *U. maydis* (14, 15, 31).

The *a* and *b* Loci Are Located on the Same Chromosome in *U. hordei*. To test whether the cosegregation of the *a* and *b* loci in *U. hordei* indicated physical linkage, we hybridized the *a* and *b* sequences to electrophoretically separated chromosomes (molecular karyotypes) of *U. hordei* (29) and *U. maydis* (ref. 32; Fig. 5 *A* and *E*). In *U. maydis*, the *pan1* probe detected the *a* locus on a 1.5-megabase pair (Mbp) chromosome in both *a1* and *a2* strains (Fig. 5*B*; compare bands indicated by the arrow in Fig. 5*F*). The *b* locus was found on a 2-Mbp chromosome (Fig. 5 *D* and *G*; see arrow). The *U. maydis pan1* probe and the *U. hordei a1* and *bE1* probes hybridized to a 2- to 3-Mbp chromosomal DNA band in *U. hordei* (Fig. 5 *B–D*). The use of electrophoretic conditions to achieve resolution of the high molecular weight chromosomes revealed that the *a* and *b* loci were present on the largest chromosome in *U. hordei* [≈3 Mbp (29); Fig. 5 *E–G*]. These results provide physical evidence for linkage and confirm the genetic evidence for cosegregation of the *a* and *b* loci in *U. hordei* (Fig. 2).



U. hordei		
Uh112 [a] (Winnipeg)	511 bp 170 aa	365 bp 121 aa
Uh4857-4 (Winnipeg)	0/511	0/365
Uh4632-3 [b] (Ethiopia)	0/511	0/365
Uh4634-1 (Azarbadjan)	0/511	0/365
Uh4635-3 (Canary Islands)	0/511	0/365
Uh4639-1 (Spain)	0/511	0/365
Uh4640-3 [b] (Kenya)	0/511	0/365
Uh4641-1 (Australia)	N.D.	0/365
Uh4642-1 (Tunesia)	0/511	0/365
U. avenae (USA)	0/511	1/365 [c,d]
U. kolleri (Quebec)	0/511	1/365 [c,d]

U. hordei		
Uh100 [a] (Winnipeg)	514 bp 171 aa	365 bp 121 aa
Uh4857-5 (Winnipeg)	0/514	0/365
Uh4632-1 [b] (Ethiopia)	1/514 [c]	0/365
Uh4634-4 (Azarbaijan)	0/514	0/365
Uh4635-1 (Canary Islands)	0/514	0/365
Uh4639-4 (Spain)	0/514	0/365
Uh4640-1 [b] (Kenya)	0/514	0/365
Uh4641-4 (Australia)	0/514	0/365
Uh4642-3 (Tunesia)	0/514	0/365
U. nigra (Winnipeg)	0/514	0/365
U. bullata (Western Canada)	5/514 bp (3/171 aa)	7/365 bp [e] (4/121 aa)
U. aegilopsidis (Turkey)	0/514	0/365

FIG. 6. Variation among alleles of the *b* genes from bipolar smut fungi. The geographic origins of the collections are given in parentheses. Specificity at the *MAT* locus was determined by a mating assay on culture medium; all the bipolar species used are interfertile. Open boxes with arrowheads represent the *U. hordei* open reading frames, Uh*bW1* and *-2* and Uh*bE1* and *-2* (see Fig. 1). Hatched boxes represent introns, asterisks denote homeodomains (27), and V indicates the variable N-terminal regions. In the latter regions, Uh*bW1* differs by 275 bp of 511 bp from Uh*bW2* and Uh*bE1* differs by 193 bp of 365 bp from Uh*bE2* (27). Solid squares indicate the positions of the oligonucleotide primers, pr1 and pr2 [including *Xba* I cloning sites (Xb)], used to amplify the indicated region by the PCR. For example, PCR amplification in the *MAT-1* strain resulted in a product of 1082 bp consisting of, respectively, the N-terminal 511 bp (170 amino acids) of Uh*bW1*, a 206-bp intergenic region, and the N-terminal 365 bp (121 amino acids) of Uh*bE1*. Subsequently, the sequence of the PCR products was determined. For example, 0/511 means that no base-pair change was found in the 511-bp N-terminal region of Uh*bW1*. ND, not determined; *U.*, *Ustilago*.

[a]Strains and DNA sequences as described (27).

[b]Two independent sets of basidiospores were analyzed.

[c]Conservative change.

[d]Single base-pair change found at the same position in *Ustilago avenae* and *Ustilago kolleri*.

[e]Loss of a *Bam*HI restriction site with consequent loss of a RFLP (see figure 3A, lane K, in ref. 25).

Limited Variability Exists Between *b* Alleles of Bipolar Smut Species. Physical linkage of the *a* and *b* loci in bipolar smut fungi would ensure that every fusion event mediated by the *a* locus of *MAT-1* and *MAT-2* strains would bring together *b* loci of opposite specificity and result in formation of the infectious dikaryon. This scenario suggests that the bipolar smut fungi require only two *b* specificities, one linked to each *a* specificity. To test this hypothesis, we analyzed the variability found in the predicted amino acid sequences of the N-terminal regions of the *bW* and *bE* genes from a world-wide collection of *U. hordei* strains. The N-terminal region is variable among different alleles of the *bW* and *bE* genes in *U. maydis* (17–19) and *U. hordei* (27) and harbors specificity determinants (33). The sequence analysis (Fig. 6) revealed that there are only two classes of *b* gene complexes in *U. hordei* and that there is very little variability within each class. As expected, one class of *bW* and *bE* genes is associated with the *MAT-1* mating type and the other is associated with *MAT-2*. The *bW* and *bE* variable regions from *MAT-1* and *MAT-2* isolates of five additional *Ustilago* species with bipolar mating systems also showed similar classes when compared with *U. hordei* (Fig. 6). Overall, the finding of only two classes of *b* loci supports the hypothesis that most bipolar smut fungi have only two allelic specificities.

Recombination Between the *a* and *b* Loci Is Suppressed. Given the linkage of the *a* and *b* loci in *U. hordei*, meiotic recombination could occur between the loci to generate progeny with genotypes *a1 b2* or *a2 b1* that are unable to mate with either parental strain (*a1 b1* or *a2 b2*). However, a search among 2182 random progeny, obtained from three collections of teliospores, failed to yield candidate recombinant strains with altered mating specificity. Although genetic and physical distances have not been correlated in *U. hordei*, it is known that the *pan1* gene and the *a1* locus in *U. maydis* are separated by 2.5 centimorgans (2–6 kb; ref. 14). Recombination must be suppressed in the *MAT* region containing the *a* and *b* mating-type loci in *U. hordei* because preliminary physical mapping with rare-cutting restriction enzymes and pulse field gel analysis suggested that the *a* and *b* loci are >150 kb apart (data not shown). Recombination in this region might be suppressed due to genetic control or because of differences in the genomic organization of the *MAT* region in the two mating types. In *Chlamydomonas reinhardtii*, the mating-type region contains many genes, is recombinational suppressed, and displays intrachromosomal translocations, inversions, and large deletions (spanning at least 190 kb) between the two mating types (34).

The linkage of sex determinants (i.e., *a* and *b* sequences) to establish the bipolar mating system may reflect the need to keep functionally related genes together. Such a situation would make biological sense given the requirement for two distinct mating types to establish the infectious dikaryon. Certainly, the clustering of genes for sex determination has been proposed as the reason for mosaic sex chromosomes in higher eukaryotes (35). In this context, it is conceivable that additional genes for sexual development (and pathogenicity) are also clustered within the *MAT* region of *U. hordei*.

The finding that the genome organization of conserved mating-type loci in smut fungi accounts for apparent major differences in mating strategies suggests that the mechanisms for establishing the infectious dikaryon are also conserved in this group of fungi. Because smut fungi are obligately depen-

dent on infection to produce the diploid teliospores necessary for meiosis, sexual development is dependent not only on compatibility but also on the host species or cultivar preferences of the mating partners. In this regard, it is possible that host range differences contributed to the evolution of the two mating systems in smut fungi. Although it is not known whether one type of mating system evolved from the other (for opposing viewpoints, see refs. 5 and 6), it is likely that tetrapolar mating evolved after the divergence of basidiomycete from ascomycete fungi because tetrapolar mating systems have not been found in the latter group (1).

Based on relative complexity and on the suggestion that basidiomycetes evolved from ascomycetes (5), one might imagine that the tetrapolar system (two loci) evolved from the bipolar system (one locus). Initially, translocation events in a diallelic bipolar species could have separated the *a* and *b* loci. Subsequent mutation of *b* genes would be then required to generate multiple allelic specificities. It is also possible that the loci might have been separated after multiallelic factors evolved. Generally, it is argued that tetrapolar mating systems give the selective advantage of increased outbreeding capacity and, therefore, increased adaptability (8). However, even though 65% of the basidiomycetes are tetrapolar, increased outbreeding capacity is not necessarily the driving force in evolution. For example, an inbreeding or nonbreeding fungal species may be highly specialized and very successful in its claimed niche. Certainly, bipolar smut fungi are quite specialized and successful in their ability to parasitize grass and cereal hosts. *Ustilago nuda* is an example of one such bipolar smut species. This species is an embryo-infecting smut that can establish a dikaryon by mating between the four meiotic progeny immediately after germination of a teliospore on floral tissue of barley and wheat plants. In this species, the opportunity for outbreeding is minimal because of the short haploid stage in the life cycle.

Summary. The *a* and *b* mating-type sequences of *U. hordei* and *U. maydis* have been employed as hybridization probes to find a molecular explanation for the genetic difference between bipolar and tetrapolar mating systems. We determined that the difference between these mating systems is due to distinct genome organizations of the *a* and *b* loci. These loci are linked together in a large *MAT* complex in the bipolar system and are unlinked in the tetrapolar system. The results for the smut fungi raise the possibility that the organization of mating functions might distinguish bipolar from tetrapolar mating systems in other basidiomycetes.

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1. Alexopoulos, C. J. (1962) *Introductory Mycology* (Wiley, New York), 2nd Ed.
2. Raper, J. R. (1966) *Genetics of Sexuality in Higher Fungi* (Ronald Press, New York).
3. Casselton, L. A. (1978) in *The Filamentous Fungi*, eds. Smith, J. E. & Berry, D. R. (Arnold, London), Vol. 3, pp. 275–297.
4. Raper, C. A. (1983) in *Secondary Metabolism and Differentiation in Fungi*, eds. Benett, J. W. & Ciegler, A (Dekker, New York), pp. 195–238.
5. Whitehouse, H. L. K. (1949) *Biol. Rev.* **24**, 411–447.
6. Raper, J. R. & Flexer, A. S. (1971) in *Evolution in the Higher Basidiomycetes*, ed. Petersen, R. H. (Univ. of Tennessee Press, Knoxville), pp. 149–167.
7. Kües, U. & Casselton, L. A. (1993) *J. Cell Sci.* **104**, 227–230.
8. Koltin, Y., Stamberg, J. & Lemke, P. A. (1972) *Bacteriol. Rev.* **36**, 156–171.
9. Halisky, P. M. (1965) *Botan. Rev.* **31**, 114–150.
10. Fischer, G. W. & Holton, C. S. (1957) *Biology and Control of the Smut Fungi* (Ronald Press, New York), p. 622.
11. Hoffmann, J. A. & Kendrick, E. L. (1968) *Phytopathology* **59**, 79–83.
12. Puhalla, J. E. (1970) *Genet. Res. Camb.* **16**, 229–232.
13. Banuett, F. (1992) *Trends Genet.* **8**, 174–180.
14. Froeliger, E. H. & Leong, S. A. (1991) *Gene* **100**, 113–122.
15. Bölker, M., Urban, M. & Kahmann, R. (1992) *Cell* **68**, 441–450.
16. Kronstad, J. W. & Leong, S. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 978–982.
17. Kronstad, J. W. & Leong, S. A. (1990) *Genes Dev.* **4**, 1384–1395.
18. Schulz, B., Banuett, F., Dahl, M., Schlessinger, R., Schafer, W., Martin, T., Herskowitz, I. & Kahmann, R. (1990) *Cell* **60**, 295–306.
19. Gillissen, B., Bergemann, J., Sandmann, C., Schroeer, B., Bölker, M. & Kahmann, R. (1992) *Cell* **68**, 647–657.
20. Trueheart, J. & Herskowitz, I. (1992) *J. Bacteriol.* **174**, 7831–7833.
21. Mather, K. (1942) *Nature (London)* **149**, 54–56.
22. Kirby, G. C. (1984) *Heredity* **52**, 35–41.
23. Yoder, O. C., Valent, B. & Chumley, F. (1986) *Phytopathology* **76**, 383–385.
24. Thomas, P. L. (1991) *Annu. Rev. Phytopathol.* **29**, 137–148.
25. Bakkeren, G., Gibbard, B., Yee, A., Froeliger, E., Leong, S. & Kronstad, J. W. (1992) *Mol. Plant Microbe Interact.* **5**, 347–355.
26. Martinez-Espinoza, A. D., Gerhardt, S. A. & Sherwood, J. E. (1993) *Exp. Mycol.* **17**, 200–217.
27. Bakkeren, G. & Kronstad, J. W. (1993) *Plant Cell* **5**, 123–136.
28. Holliday, R. (1974) in *Handbook of Genetics*, ed. King, R. C. (Plenum, New York), Vol. 1, pp. 575–595.
29. McCluskey, K. & Mills, D. (1990) *Mol. Plant Microbe Interact.* **3**, 366–373.
30. Snetselaar, K. M. & Mims, C. W. (1992) *Mycologia* **84**, 193–203.
31. Metzenberg, R. L. & Glass, N. L. (1990) *BioEssays* **12**, 53–59.
32. Kinscherf, T. G. & Leong, S. A. (1988) *Chromosoma* **96**, 427–433.
33. Yee, A. R. & Kronstad, J. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 664–668.
34. Ferris, P. J. & Goodenough, U. W. (1994) *Cell* **76**, 1135–1145.
35. Egel, R. (1992) *Nature (London)* **357**, 23–24.