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The *a* and *b* Loci of *Ustilago maydis* Hybridize with DNA Sequences from Other Smut Fungi

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The smut fungi are obligately parasitic during the sexual phase of their life cycle, and the mating-type genes of these fungi play key roles in both sexual development and pathogenicity. Among species of smut fungi it is common to find a bipolar mating system in which one locus with two alternate alleles is believed to control cell fusion and establishment of the infectious cell type. Alternatively, several species have a tetrapolar mating system in which two different genetic loci, one of which has multiple alleles, control fusion and subsequent development of the infection hyphae. Cloned sequences from the *a* and *b* mating-type loci of the tetrapolar smut fungus *Ustilago maydis* were used as hybridization probes to DNAs from 23 different fungal strains, including smut fungi with both tetrapolar and bipolar mating systems. In general, all of the smut fungi hybridized with the mating-type genes from *U. maydis,* suggesting conservation of the sequences involved in mating interactions. A selection of DNAs from other ascomycete and basidiomycete fungi failed to hybridize with the *U. maydis* mating-type sequences. Exceptions to this finding include hybridization of DNA from the *a1* idiomorph of *U. maydis* to DNA from one strain of *U. violacea* and hybridization of both *a* idiomorphs to DNA from *Saccharomyces cerevisiae.*

Additional keywords: infectious dikaryon, multiallelic recognition.

The smut fungi are basidiomycete phytopathogens that attack a variety of monocotyledonous and dicotyledonous plants (Fischer 1953; Fischer and Holton 1957). Many of the best-characterized smut fungi cause infections on important crop species, in particular, small grain cereals and corn. One of the main hallmarks of a smut infection is the presence of large masses of sooty black teliospores within the host plant. In small grain cereals, these spores often replace the developing seeds, thereby reducing the yield and quality of the grain (Agrios 1988).

The smuts share many features of their life cycles including, in most species, a yeastlike vegetative cell type and an obligately parasitic, dikaryotic cell type arising from the fusion of compatible haploid cells. The infectious dikaryon, which has a filamentous morphology, grows systemically within host tissue and eventually sporulates—often within galls or floral tissue. The smut fungi differ in some important details of their life cycles, including the range of hosts that can be infected, the influence on host tissue (whether galls are induced), and the location in the host where sporulation occurs (Fischer and Holton 1957). For example, *Ustilago maydis* (DC.) Corda infects seedlings of corn (*Zea mays* L.) and induces galls on any above ground part of the plant; the fungus sporulates primarily within gall tissue. In contrast, *U. hordei* (Pers.) Lagerh. infects seedlings of small grain cereals, in particular barley (*Hordeum vulgare* L.), and sporulates primarily within floral tissue (without inducing galls).

One of the most interesting differences among the smuts is the presence of at least two different mating systems within the group (Holton *et al.* 1968). Many of the species, such as *U. hordei*, possess a bipolar mating system in which a single mating-type locus with two alternate alleles controls dikaryon formation. Other smuts, such as *U. maydis*, display tetrapolar mating in which two loci control dikaryon formation. The tetrapolar smut fungi generally have one locus with two alternate alleles that is thought to control cell fusion and a second locus with multiple alleles that controls the establishment of the dikaryon (Holton *et al.* 1968). If the bunt fungi of the genus *Tilletia* are included in the smut group, then a third mating system (multiple alleles at a single locus) has been reported for *T. controversa* Kühn in Rabenh. (Hoffman and Kendrick 1965).

Besides *U. maydis*, other smut fungi that display a tetrapolar mating system include *Sporisorium* species (smut of maize and sorghum), *U. striiformis* (Westend.) Niessel (smut of several grass species, e.g., *Agropyron subsecundum* (Link) Hitchc.), and *U. longissima* (Sowerby) Meyen (smut of *Glyceria* sp.) (Fischer 1953; Holton *et al.* 1968). Interestingly, there have been reports of interfertility between *U. maydis* and *Sporisorium* species (Rowell and DeVay 1954; A. Budde and S. A. Leong, unpublished results), and coinoculation of corn seedlings with haploid isolates of these fungi can give rise to an infection (Rowell and DeVay, 1954). Smut fungi with bipolar mating systems include those that infect grasses, such as *U. bullata* Berk. and *U. aegilopsidis* Picbauer, and those that infect small grain cereals, such as *U. hordei* and *U. kolleri* Wille (covered smut of barley and oats), *U. nuda* (Jensen) Rostrup and

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U. tritici (Pers.) Rostrup (smut of barley and wheat), and *U. nigra* Tapke and *U. avenae* (Pers.) Rostrup (loose smut of barley and oats) (Holton *et al.* 1968). In many cases, these fungi are interfertile and apparently show only limited genetic differences (Huang and Nielsen 1984). In general, there have been few reports of interfertility between smuts containing a bipolar mating system and those containing a tetrapolar system. One exception is the report of Kniep (Kniep 1926; cited in Fischer and Holton 1957) of the fusion of *U. longissima* with *U. nuda* and with *U. avenae*. An important limitation to the demonstration of fusion or sexual compatibility between different smut species is the dependence on a common host to demonstrate true interfertility. This limitation has been overcome for some of the bipolar species with the discovery that two grasses will serve as common hosts for several smut species (Nielsen 1978).

Given the role of mating in establishing the infectious dikaryon (see Froeliger and Kronstad 1990 for a review), the characterization of the structure and function of the mating-type genes will be important for an understanding of pathogenicity. Molecular analysis of the mating systems in the smut fungi began with the isolation of two alleles of the multiallelic *b* locus of *U. maydis* (Kronstad and Leong 1989). Subsequently, at least five additional alleles have been isolated and characterized (Kronstad and Leong 1990; Schulz *et al.* 1990). The polypeptides predicted to be encoded by the *b* alleles contain a region with similarity to the homeodomain of regulatory proteins from *Drosophila*, and a comparison of different alleles indicated a variable amino terminal portion and a conserved carboxy terminal region (Kronstad and Leong 1990; Schulz *et al.* 1990). The sequences at the *a* locus (*a1* and *a2*) of *U. maydis* have also been isolated and, unlike the *b* alleles, these regions contain large blocks of nonhomologous DNA (Froeliger and Leong 1991; Bolker *et al.* 1992). This organization is similar to that found for the mating-type idiomorphs of *Neurospora crassa* (Glass *et al.* 1988; Metzenberg and Glass 1990).

The availability of cloned DNAs from the *a* and *b* loci of *U. maydis* presented an opportunity to survey additional smut fungi with tetrapolar and bipolar mating systems for the presence of similar sequences. We report here that sequences hybridizing with the DNAs from the *a* and *b* loci of *U. maydis* were found in all of the smut fungi tested. In addition, sequences that hybridized with the *a* DNAs were found in *Saccharomyces cerevisiae* Hansen and, in the case of the *a1* sequence, in *U. violacea* (Pers.) Roussel, but not in several other basidiomycete and ascomycete fungi.

Table 1. Fungal strains and sources of DNA used in this study

Species	Strain and relevant markers	Source or reference
Ustilago maydis	521 (*a1 b1*)	S. A. Leong
	518 (*a2 b2*)	S. A. Leong
	031 (*a1 b2*)	S. A. Leong
	032 (*a2 b1*)	S. A. Leong
	ATCC 22906 (*a2 bl*)	ATCC
	a1 bx70E b1W::HygB^r	This study
	a2 bx70E b1W::HygB^r	This study
	a1 bE1 b1W::HygB^r	Kronstad and Leong 1989
	a2 bE1 b1W::HygB^r	Kronstad and Leong 1989
Ustilago longissima	*a2 b?*	This study
Saccharomyces cerevisiae	2R26-12C (*MAT*α)	M. Smith[a]
Sporisorium reilianum		R. Frederiksen[b]
U. hordei	a112	C. Person[c]
U. hordei	A100	C. Person[c]
U. kolleri		P. Thomas[d]
U. avenae		P. Thomas[d]
U. aegilopsidis		P. Thomas[d]
U. bullata		P. Thomas[d]
U. nigra		P. Thomas[d]
U. nuda		P. Thomas[d]
U. violacea	001	A. Day[e]
U. violacea	002	A. Day[e]
Puccinia graminis f. sp. *tritici*	SZA 2C-1	L. Szabo[f]
Tilletia controversa	B6-1	D. Mills[g]
T. controversa	H-1	D. Mills[g]
T. caries	T-9	D. Mills[g]
Cryptococcus neoformans		U. Wisconsin Hospital
Neurospora crassa	74A	M. A. Nelson[h]
N. crassa	ORSa	M. A. Nelson[h]
Schizophyllum commune	4 - 40	C. Novotny[i]

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MATERIALS AND METHODS

Sources of strains or DNA. The fungi employed in this work are listed in Table 1. *U. maydis* strains 518, 521, 031, and 032 are part of the collection maintained by S. A. Leong at the University of Wisconsin-Madison. *U. maydis* ATCC 22906 was obtained from the American Type Culture Collection, Rockland, MD. *U. maydis* strains carrying the hygromycin B resistance marker at the *b* locus were constructed as previously described (Kronstad and Leong 1989). It was later determined that the marker disrupted a second open reading frame (ORF) at the *b* locus (designated *bW*; see Results). A haploid isolate of *U. longissima* was obtained from germinated teliospores from the Herbarium in the Department of Plant Pathology, University of Wisconsin-Madison. The spores were isolated from a brown stripe smut infection on rattlesnake mannagrass (*Glyceria canadensis*). *S. cerevisiae* strain 2R26-12C was obtained from M. Smith, Biotechnology Laboratory, University of British Columbia. A haploid isolate of *S. reilianum* (Kühn) Langdon Fullerton was obtained from teliospores provided by R. Frederiksen, Texas A & M University, College Station. *U. hordei* strains a112 and A100 were from the culture collection of the late C. Person, Department of Botany, University of British Columbia. Teliospores of *U. hordei, U. kolleri, U. avenae, U. aegilopsidis, U. bullata,* and *U. nuda* were provided by P. Thomas, Agriculture Canada Research Station, Winnipeg. A strain of *Cryptococcus neoformans* was obtained from the University Hospital, University of Wisconsin-Madison. Purified DNAs from various fungi were provided by the following individuals: A. Day, Department of Plant Science, University of Western Ontario (*U. violacea*); L. J. Szabo, Cereal Rust Laboratory, University of Minnesota (*P. graminis* f. sp. *tritici*); D. Mills, Department of Botany and Plant Pathology, Oregon State University (*T. controversa* Kühn in Rabenh. and *T. caries* Tol & C. Tol); M. A. Nelson, Department of Biology, University of New Mexico (*N. crassa*); and C. Novotny, Department of Microbiology and Molecular Genetics, University of Vermont (*Schizophyllum commune* Fr.:fr.).

Teliospore germination and cell growth. Teliospores of the smut fungi were germinated on potato-dextrose agar (PDA) at 30° C. Single haploid progeny were obtained by dispersing germinated teliospores in sterile water and plating them on PDA for single-colony isolates. Liquid cultures of the smut fungi and of *C. neoformans* were grown at 30° C in complete medium (Holliday 1974). Cells of *U. bullata, U. aegilopsidis, U. kolleri, U. nuda,* and *U. avenae* were grown on PDA at 30° C.

DNA manipulations and hybridization conditions. DNA was isolated from the smut fungi and from *C. neoformans* by vortexing with glass beads (425–600 μm; Sigma Chemical Co.) as described by Elder *et al.* (1983). All DNA samples for hybridization analysis were digested with *Bam*HI. Restriction digestion, agarose gel electrophoresis, and transfer of DNA to membranes were performed as described by Maniatis *et al.* (1982). For most of the experiments, nylon membranes were employed (Zetabind, Cuno Laboratory Products; Nytran, Schleicher & Schuell). (The blots shown in Figs. 5A and 6A below were prepared on nitrocellulose [Schleicher & Schuell]. DNA fragments for use as hybridization probes were separated from vector sequences by electrophoresis on agarose gels (two separations). The fragments were isolated from agarose using the GeneClean Kit (Bio101) and labeled with ^{32}P using an oligolabeling kit (Pharmacia). Hybridizations were performed as follows. Filters with bound DNA were prewashed at 65° C for 16 hr in 0.1× SSC (15 mM NaCl plus 1.5 mM sodium citrate) and 0.5% sodium dodecyl sulfate (SDS). Subsequently, the filters were incubated in prehybridization solution (30% formamide, 5× SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris pH 7.4, 1× Denhardt's solution, 100 μg/ml of calf thymus DNA [Maniatis *et al.* 1982]) for 2 hr at 42° C, the hybridization probe was added, and incubation was continued overnight. The filters were washed in 2× SSC, 0.1% SDS for 30 min at room temperature followed by a 20- to 60-min wash at 45° C in 0.5× SSC, 0.1% SDS. Additional washes at 65° C were performed as indicated in the figure legends. Autoradiography was performed with Kodak X-Omat AR film and one Cronex Lightning intensification screen at −70° C. For some experiments (not shown), the hybridization conditions of Amasino (1986) were employed.

RESULTS

Fungal isolates. The fungi chosen for this study are representative of both basidiomycetes and ascomycetes and include isolates of smut fungi with tetrapolar and bipolar mating systems (Table 1). The smut fungi were chosen to include isolates that infect different monocotyledonous plants. Thus, species such as *S. reilianum* and *U. maydis* infect corn, species such as *U. hordei* and *U. avenae* infect small grain cereals, and species such as *U. bullata, U. longissima,* and *U. aegilopsidis* infect grass species. Two isolates among the smut fungi, *U. violacea* 001 and 002, infect dicotyledonous plants. These isolates have a similar life cycle compared with the smut fungi that infect monocotyledonous plants, but recent evidence (Deml and Oberwinker 1982) indicates that *U. violacea* is not sufficiently related to other *Ustilago* sp. to be in the same genus. *U. violacea* is now placed in the genus *Microbotryum*. *C. neoformans* was chosen because this fungus is an important opportunistic pathogen of humans, and the perfect state of this fungus (genus *Filobasidiella*) is in the same order (Ustilagenales) as the smut and bunt fungi (Kwon-Chung 1975).

Existing information on the structure and organization of mating-type genes in fungi prompted the inclusion of strains of *N. crassa* and *S. commune*. *N. crassa* has been found to possess idiomorph sequences, i.e., large regions of nonhomologous DNA, at the mating-type locus (Glass *et al.* 1988). As mentioned above, a similar organization has been found for the *a* alleles of *U. maydis* (Froeliger and Leong 1991). *S. commune* is of interest because, unlike *U. maydis*, four different genetic loci are involved in sexual development and multiple alleles exist for all four loci (Raper 1966; Stankis *et al.* 1990).

Hybridization probes for the *a* and *b* loci. Maps depicting the region of the *U. maydis* genome containing the *a* locus are shown in Figure 1A. As described by Froeliger and

Leong (1991) and Bolker *et al.* (1992), the *a* alleles of *U. maydis* are actually idiomorphs and contain large blocks of nonhomologous DNA (approximately 4.0–5.0 kb for the *a1* region and approximately 7.5–8.5 kb for the *a2* region). The sequences within the idiomorphs, which have biological activity specific for each allele, have been identified (Froeliger and Leong 1991; Bolker *et al.* 1992) and are marked on the map. For the *a1* allele, a 3.5-kb *Eco*RI fragment from within the nonhomologous region and including the region with biological activity was employed as a hybridization probe. A smaller fragment of 0.86 kb (*Bam*HI-*Sal*I) from within the region of the *a2* idiomorph showing biological activity was chosen as a hybridization probe.

The *b* locus appears to comprise two multiallelic genes. The first ORF to be identified (Kronstad and Leong 1989, 1990; Schultz *et al.* 1990) encoded a 473-amino acid polypeptide and has been designated *bE* (for *bEast*). A 1.3-kb cloned product of the polymerase chain reaction from within this ORF of the *b7* allele (Kronstad and Leong 1990) was employed as a hybridization probe. The second probe is a 1.3-kb *Bam*HI-*Hin*dIII restriction fragment from a region immediately upstream of the *bE* ORF. This region contains a second ORF, designated *bW* (for *bWest*), believed to encode an approximately 626-amino acid polypeptide with a role in *b* allele function (Gillissen *et al.*, 1992). The *bE* and *bW* nomenclature will be used to designate the two different probes for the *b* locus.

We have confirmed the importance of the *bW* ORF in *b* mating-type function as shown in Figure 2. Strains constructed to contain the hygromycin B gene within the *b1W* ORF (e.g., *a2 bx70E/b1W::HygB*^{r}; see Fig. 1B and Table 1) are capable of mating with wild-type strains (e.g., *a1 b1E/b1W* or *a1 b2E/b2W*) but are incapable of mating with other strains containing insertions of the marker at an identical position (e.g., *a1 b1E/b1W::HygB*^{r}). This result indicates that at least one functional copy of the *bW* region (in either partner) must be present during mating inter-

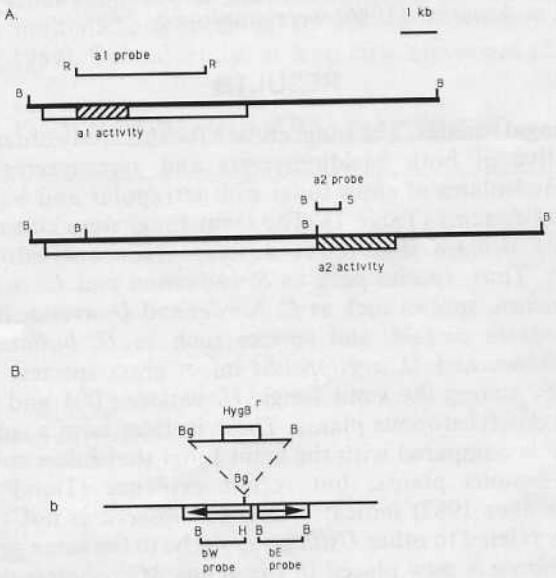

Fig. 1. Regions of the *a* and *b* loci used as hybridization probes. **A,** Maps of the *a1* and *a2* idiomorphs of *Ustilago maydis* are shown with the regions of nonhomology between the sequences depicted as open boxes (Froeliger and Leong 1991). The sequences outside the open boxes show conserved restriction sites (e.g., *Bam*HI) and the regions of the idiomorphs with biological activity are indicated by cross-hatched boxes within the regions of nonhomology (Froeliger and Leong 1991). DNA fragments used as hybridization probes are shown above the maps; a 3.8-kb *Eco*RI fragment was used for the *a1* idiomorph and an 0.86-kb *Bam*HI-*Sal*I fragment was used for the *a2* region. Restriction sites are indicated as follows: B = *Bam*HI, R = *Eco*RI, S = *Sal*I. **B,** Map of the *b* locus of *U. maydis* showing the relative positions and orientations of the two open reading frames labeled *bW* and *bE*. DNA fragments employed as hybridization probes are shown below the map; A 1.3-kb *Bam*HI-*Hin*dIII fragment was used for *bW* and a 1.3-kb *Bam*HI fragment from a polymerase chain reaction product was used for *bE* (Kronstad and Leong 1990). The *Bam*HI sites were incorporated as part of the primer sequences used for amplification. Restriction sites are indicated as follows: Bg = *Bgl*II, H = *Hin*dIII. The insertion of a 3.0-kb *Bam*HI-*Bgl*II fragment carrying the gene for resistance to hygromycin B is shown at the *Bgl*II site (codon 49) in *b1W*. The construction of strains containing this DNA in place of the wild-type *b* locus has been described by Kronstad and Leong (1989).

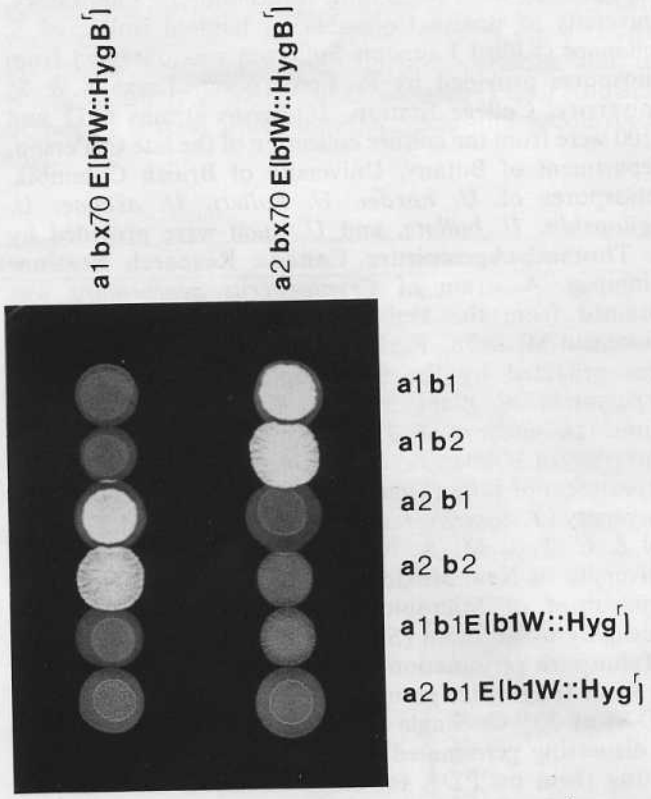

Fig. 2. Disruption of the *b1W* open reading frame in both mating partners blocks mating. A petri dish containing rich medium with 1% activated charcoal (Holliday 1974) was spotted with mixtures of each strain to be tested, and the plates were incubated overnight at 30° C. A positive mating reaction is indicated by the formation of white aerial mycelium on the mixed colonies. Four tester strains with wild-type *b* alleles are shown on the right along with two strains containing a disruption in the *b1W* open reading frame. The *b1* allele has both *b1E* and *b1W*; similarly, *b2* has both *b2E* and *b2W*. Disruption of the *bW* ORF is designated as *b1W::HygB*^{r} and these strains were constructed as described in Kronstad and Leong (1989). As indicated, the spots on the left contain the *b1W* disruption in a strain with the *a1* mating type and the spots on the right contain the same disruption in an *a2* strain. The *b* allele in these strains (*bx70E*) is a recombinant allele with a hybrid *bE* region containing parts of the *b1E* and *b2E* sequences (A. Yee and J. W. Kronstad, unpublished). Specifically, the *bx70E* allele contains codons 1–69 from the *b1E* gene fused to codons 70–410 of the *b2E* gene. This allele allows normal mating as shown by the reactions with the four tester strains.

actions. Sequence analysis of the *bW* region from the wild-type *b1* allele also confirmed the presence of a 626-amino acid ORF (J. W. Kronstad, unpublished results).

Hybridization with the *b7E* probe. Initially, a DNA fragment (Fig. 1B) from within the ORF of the *b7E* gene from *U. maydis* was used as a hybridization probe to blots containing DNA from the 23 fungal isolates. As expected from earlier work (Kronstad and Leong 1989, 1990), the probe bound to an 8.5-kb fragment in each of the strains of *U. maydis* tested (lanes a and b, Fig. 3A and B). In fact, the other smut fungi with tetrapolar mating systems (*S. reilianum*, lane d, Fig. 3A and *U. longissima*, lane c, Fig. 3B) also possess an 8.5-kb *Bam*HI fragment carrying sequences homologous to the probe. *U. maydis* is known to give a positive plate mating assay with both *S. reilianum* and *U. longissima* (E. H. Froeliger and S. A. Leong, unpublished results), and coinoculation of corn with *U. maydis* and *S. reilianum* results in infection (Rowell and DeVay, 1954; A. Budde and S. A. Leong, unpublished results). It is interesting to note that the strain of *S. reilianum* tested also showed hybridization to a 1.0-kb DNA (Fig. 3A, lane d) fragment, raising the possibility of a second sequence related to *bE* in this smut fungus.

Hybridization of the *b7E* fragment to DNAs from the smut strains with bipolar mating systems revealed that one of two alternate fragments (2.8 or 1.5 kb) was detected in each isolate (Fig. 3A). The two different strains of *U. hordei* tested were of opposite mating type and contained different size fragments (2.8 kb in a112, lane e and 1.5 kb in A100, lane f). To determine whether the bands represented opposite alleles at a single locus, DNA samples from random haploid progeny of germinated teliospores of *U. hordei* were tested for hybridization. Of the 32 tested, 14 had the 2.8-kb band and 18 had the 1.5-kb band, indicating that the restriction fragment length polymorphism is present at the locus that hybridizes with the *bE* sequence (G. Bakkeren and J. W. Kronstad, unpublished results). Similar results were also obtained upon analysis of random meiotic progeny from teliospores of *U. bullata*, although the progeny containing the 1.5-kb band were underrepresented compared with those containing the 2.8-kb band. These results indicate that, for the *bE* sequence (and *bW*, see below), the smut fungi with bipolar mating systems comprise a group with conserved restriction sites and sequences in the *b* region. Cosmid clones have been isolated that contain the 1.5- and 2.8-kb *Bam*HI fragments from *U. hordei*; preliminary nucleotide sequence analysis indicates that these fragments contain homologs of *bE* and *bW* (G. Bakkeren and J. W. Kronstad, unpublished results).

One additional representative of the bipolar smut fungi, the flower-infecting species *U. nuda*, was tested for hybridization with the *b7E* probe. Because it is difficult to isolate haploid strains of this fungus (Nielsen 1968), DNA was prepared directly from cell material scraped from agar medium containing germinated teliospores. Therefore, the DNA tested was from a mixture of cells representing the progeny of meiosis. As for the other bipolar smut fungi, the *b7E* probe detected the 1.5- and 2.8-kb *Bam*HI fragments (data not shown); thus it appears that these bands are conserved among all of the bipolar smut fungi tested.

As shown in Figure 3A and B, the *b7E* hybridization probe did not detect similar sequences in any of the other nonsmut fungi tested including the ascomycetes *S. cerevisiae* and *N. crassa* and the basidiomycetes, *U. violacea, C. neoformans, S. commune, T. caries,* and *T. controversa.* The lack of hybridization to the *Tilletia* strains is noteworthy because these bunt fungi have very similar

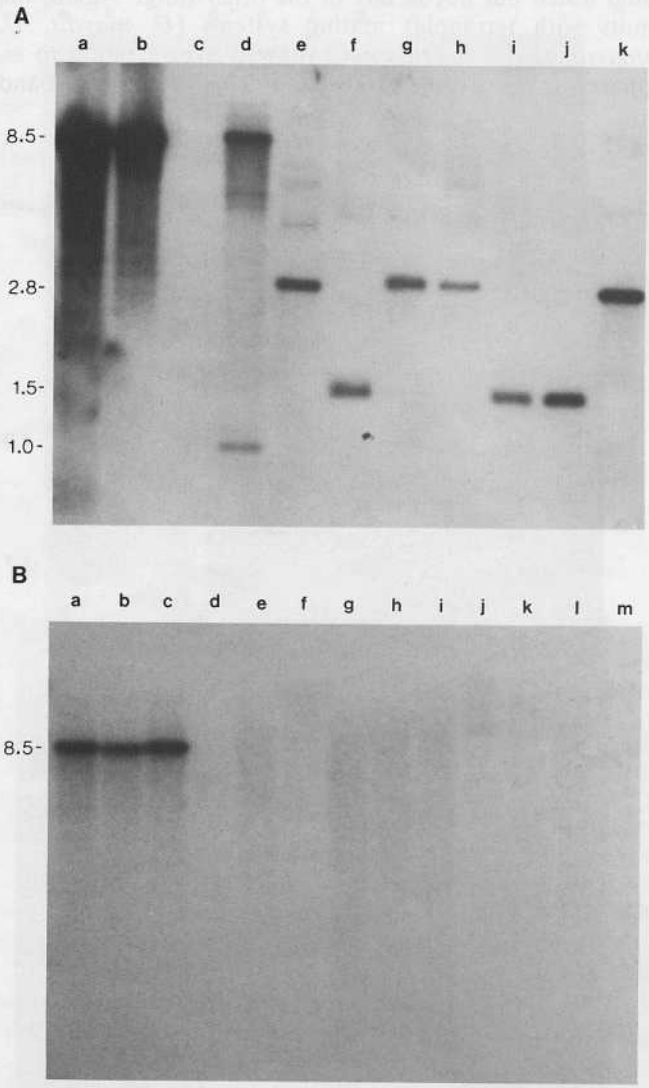

Fig. 3. Hybridization with a DNA fragment from *b7E.* Autoradiograms of Southern blots containing *Bam*HI digested DNAs are shown. The DNAs were electrophoresed through a 0.7% gel, transferred to a membrane and hybridized with a probe from the *b7E* open reading frame (Fig. 1B). The sizes indicated on the left are in kilobases and are based on comparisons with the sizes of *Hin*dIII fragments of lambda DNA run on the same gels. **A,** The lanes contain DNA from the following fungal isolates: a, *U. maydis* 521 (*a1 b1*); b, *U. maydis* ATCC 22906 (*a2 b1*); c, *Saccharomyces cerevisiae* (2R26-12C); d, *Sporisorium reilianum*; e, *U. hordei* a112; f, *U. hordei,* A100; g, *U. kolleri*; h. *U. avenae*; i, *U. aegilopsidis*; j, *U. nigra*; k, *U. bullata.* In addition to the washing conditions described in the text, the filter was also washed in 2× SSC, 0.1% SDS at 65° C for 30 min. The exposure time was 16 hr; shorter exposures indicate a single band is present in lanes a and b. **B,** The lanes contain DNA from the following fungal isolates: a, *U. maydis* 518 (*a2 b2*); b, *U. maydis* 521 (*a1 b1*); c, *U. longissima*; d, *U. violacea* 001; e, *U. violacea* 002; f, *Puccinia graminis*; g, *Tilletia controversa* B6-1; h, *T. controversa* H-1; i, *T. caries* T-9; j, *Cryptococcus neoformans*; k, *Neurospora crassa* 74A; l, *N. crassa* ORSa; m, *Schizophyllum commune* 4-40. The exposure time was 72 hr.

life cycles to smuts and, in some cases, infect the same cereal hosts as the smuts.

Hybridization with the *b1W* probe. Results similar to those obtained with the *b7E* probe were found when a DNA fragment from the region containing the *b1W* ORF was used as a hybridization probe. As shown in Figure 4, cross-hybridizing sequences were found in all of the smut fungi tested but not in any of the other fungi. Again, the smuts with tetrapolar mating systems (*U. maydis, U. longissima,* and *S. reilianum*) showed hybridization to an apparently conserved 8.5-kb band. This is the same band detected with the *b7E* probe in Figure 3A. A polymorphism for *Bam*HI was found among most of the smuts with bipolar mating systems; either an approximately 6.6- or an approximately 1.2-kb band was detected. The exception was *U. bullata*, where an approximately 2.9-kb band was found to hybridize. As mentioned above, preliminary restriction site and nucleotide sequence analyses of cosmid clones from *U. hordei* indicate that the organization of the *bW* and *bE* sequences is similar to that found in *U. maydis* (G. Bakkeren and J. W. Kronstad, unpublished results).

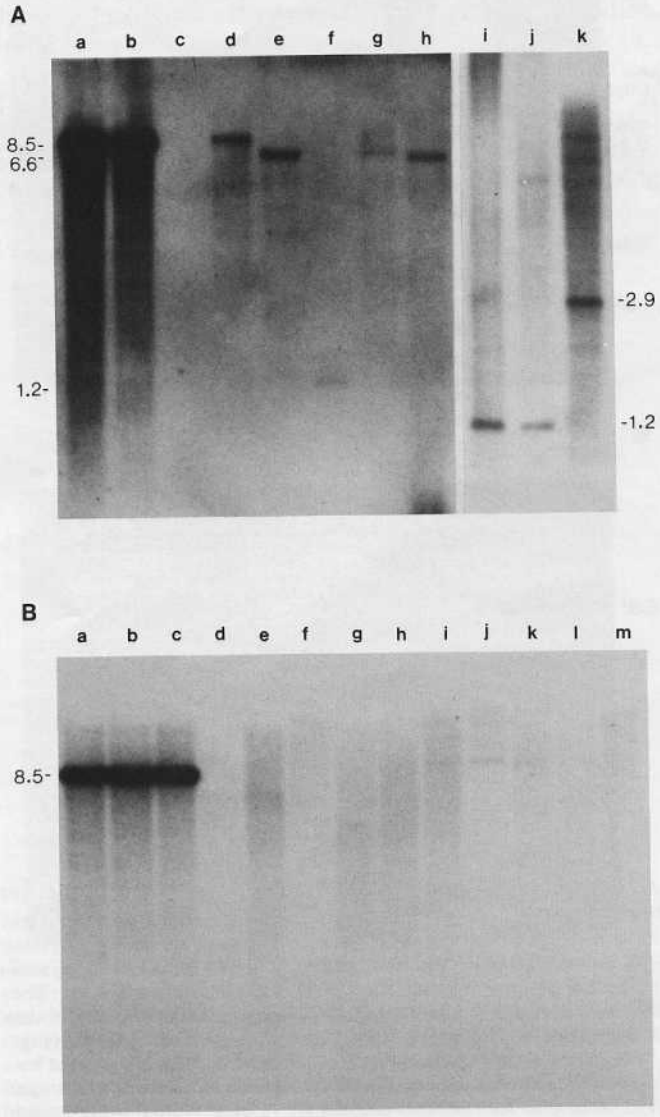

Fig. 4. Hybridization with a DNA fragment from *b1W*. Autoradiograms of Southern blots containing *Bam*HI digested DNA are shown. The DNAs were electrophoresed through a 0.7% gel, transferred to a membrane and hybridized with a DNA fragment from the *b1W* open reading frame (Fig. 1B). The sizes on the left are in kilobases and were determined as in Figure 3. **A,** The lanes are the same as in Figure 3A. Two different blots are shown; lanes a–h on the left were exposed for 10 days and lanes i–k were exposed for 16 hr. Because of problems with background, the blot for lanes a–h received an extra 30-min wash at 65° C in 2× SSC, 0.1% SDS. The blot for lanes i–k was washed as described in the text. **B,** The lanes are the same as in Figure 3B. The filter was washed as described in the text and exposed to film for 16 hr.

Hybridization with the *a1* probe. As mentioned in the introduction, the *a* alleles of *U. maydis* are idiomorphs (Froeliger and Leong 1991; Bolker *et al.* 1992); i.e., large regions of nonhomology were discovered upon comparison of the alleles (Fig. 1A). Therefore, probes taken from within these regions of nonhomology should hybridize only to one of the two mating types of *U. maydis*. An example is shown in Figure 5A, in which the 3.5-kb *Eco*RI fragment probe from the *a1* idiomorph hybridizes only to DNA from *U. maydis a1 b1* (lane a; 10-kb fragment) but not to DNA from *U. maydis a2 b1* (lane b). This probe detects sequences in some of the other smut fungi, e.g., *S. reilianum, U. hordei* a112, *U. kolleri, U. avenae,* and one strain of *U. bullata.* The presence of two bands in the DNAs from these isolates may indicate that the probe overlaps a *Bam*HI site or that two distinct regions hybridize. The lack of hybridization to the remaining smut strains, e.g., *U. aegilopsidis* and *U. nigra*, may be due to a lack of sufficient sequence similarity or to the fact that the strains are of the opposite mating type. The finding of hybridization to only one of the two mating types of *U. hordei* supports the idea that some strains do not show hybridization because they are of the opposite mating type. In addition, only one of two isolates of opposite mating type of *U. bullata* was found to have the bands (compare lanes l and k in Fig. 5A) that hybridize with the *a1* probe. Additional support for the idea that bands detected in Figure 5A are specific to the *a1* mating locus comes from an analysis of random progeny of germinated teliospores of *U. hordei.* The bands that hybridize with *a1* showed approximately a 1:1 segregation ratio, and there was an absolute correlation with mating type, i.e., the isolates with hybridizing sequences were all of the same mating type (G. Bakkeren and J. W. Kronstad, unpublished results). Thus, among the isolates surveyed in Figure 5A, the hybridization results suggest that the strains of *S. reilianum, U. kolleri,* and *U. avenae* tested, along with the a112 strain of *U. hordei* and one of the two strains of *U. bullata*, are of equivalent mating type to *a1* of *U. maydis.*

It is interesting to note that one of the two mating strains of *U. violacea* (002) also shows weak hybridization to the *a1* probe (Fig. 5B, lane e). This result was unexpected given the recent classification of *U. violacea* into a different genus. As mentioned below, no hybridization was detected to either of the *U. violacea* strains with the *a2* probe. Hybridization was also detected between the *a1* probe and the DNA from a *MAT*α strain of *S. cerevisiae* (Fig. 5A, lane c), perhaps indicating a conservation of sequences with a role in mating (discussed below). In general, the other ascomycete or basidiomycete fungi (Fig. 5B) did not show hybridization to the *a1* probe.

Hybridization with the *a2* probe. As predicted from the analysis of the *a* idiomorphs of *U. maydis*, the *a2* probe showed strong hybridization only to a fragment of approximately 6.0 kb (shown at 6.3 kb in Fig. 6) from the *a2* strains of *U. maydis* (lane b, Fig. 6A and lane a, Fig. 6B). In addition, a band of the same size was detected in *U. longissima* (Fig. 6B, lane c). This result was anticipated because this strain of *U. longissima* gave a positive mating reaction when mixed on agar plates with strains of *U. maydis* carrying the *a1* idiomorph (E. H. Froeliger and S. A. Leong, unpublished results). The same mating tests revealed that the *b* gene present in this strain appears to

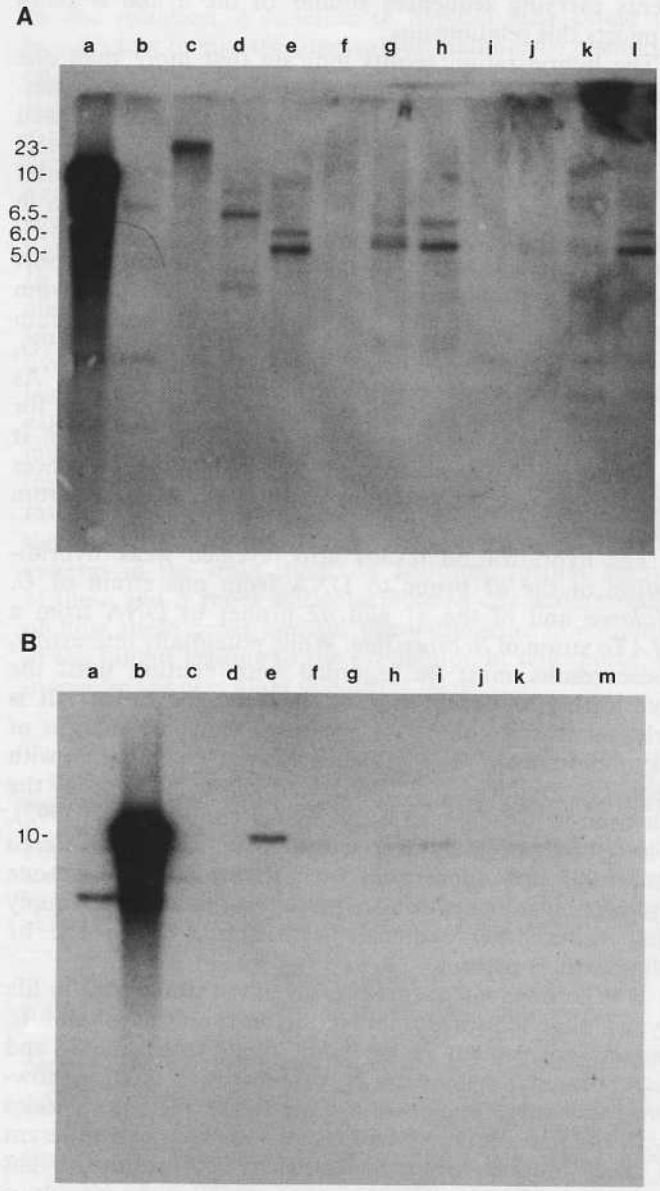

Fig. 5. Hybridization with a DNA fragment from the *a1* idiomorph. Autoradiograms of Southern blots containing *Bam*HI digested DNAs are shown. The DNAs were electrophoresed through a 0.7% gel, transferred to a membrane, and hybridized with a DNA fragment from the *a1* idiomorph (Fig. 1A). **A,** The lanes are the same as in Figure 3A except that an additional lane containing DNA from a haploid isolate of *U. bullata* is in lane 1. This isolate is of opposite mating type to the isolate analyzed in lane k (B. Gibbard and J. W. Kronstad, data not shown). The filter was exposed to film for 40 hr. **B,** The lanes are the same as in Figure 3B. The filter received an additional 20-min wash at 65° C in 1× SSC, 0.1% SDS and was exposed to film for 72 hr. The bands below 10 kb in lanes a and b probably represent nonspecific hybridization since they are in both mating types. Similar bands are seen in lanes a and b in A.

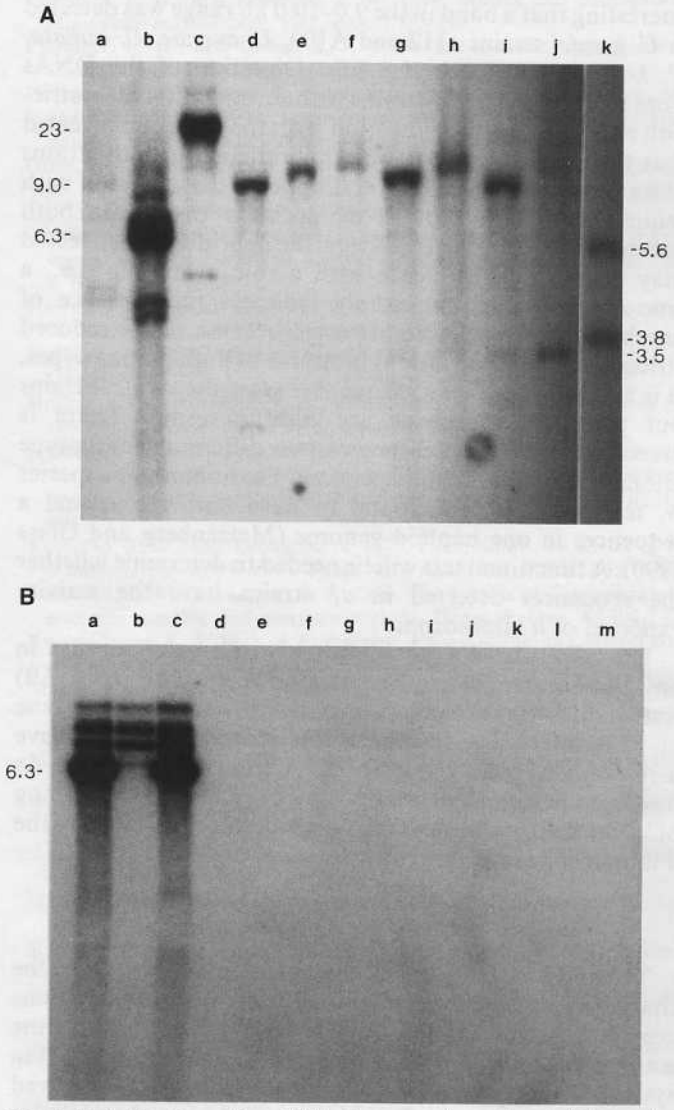

Fig. 6. Hybridization with a DNA fragment from the *a2* idiomorph. Autoradiograms of Southern blots containing *Bam*HI digested DNAs are shown. The DNAs were electrophoresed through a 0.7% gel, transferred to a membrane and hybridized with a DNA fragment from the *a2* idiomorph (Fig. 1B). **A,** The lanes contain DNA from the following fungal isolates: a, *U. maydis* 521 (*a1 b1*); *U. maydis* ATCC 22906 (*a2 b1*); c, *Saccharomyces cerevisiae* (2R26-12C); d, *U. hordei* a112; e, *U. hordei* A100; f, *U. kolleri*; g, *U. avenae*; h, *U. aegilopsidis*; i, *U. bullata*; j, *U. nigra*; k, *Sporisorium reilianum*. All lanes are from the same gel; distortion of the DNA samples in lanes e, f, and h occurred during electrophoresis. Subsequent analysis has shown that the bands in lanes d and e are of the same size. Several non-mating-type specific bands are present in lanes a and b, but restriction mapping (Froeliger and Leong, 1991) of the *a2* idiomorphs indicates that, as shown, the 6.0-6.3 kb *Bam*HI fragment is expected to hybridize. The exposure was 63 hr. **B,** The lanes are the same as in Figure 3B. The filter was exposed to film for 72 hr. Non-mating-type specific bands are present in lanes a, b and c; as in A the 6.0–6.3 kb band is expected to hybridize in strains with the *a2* allele. Some of the nonspecific bands are similar in size to those seen in A.

have a different specificity than the *b1* or *b2* alleles of *U. maydis*. The *U. maydis* and *U. longissima* DNA samples gave the strongest signals with the *a2* probe. Hybridization signals of lesser intensity were detected for bands of approximately 23 kb in *S. cerevisiae*, approximately 3.5 in *U. nigra* and bands of 5.6 and 3.8 kb in *S. reilianum*. It is interesting that a band in the 9.0–10.0 kb range was detected in *U. hordei* strains a112 and A100, *U. avenae, U. bullata, U. kolleri,* and *U. aegilopsidis*. Digestion of the DNAs from the two *U. hordei* strains with several different restriction enzymes and hybridization with the *a2* probe revealed that the same fragments were hybridizing in both strains (data not shown). Therefore, it appears that a region with sequence similarity to the *a2* probe is present in both mating-type genes. Alternatively, the hybridization detected may not be to a sequence with a role in mating (i.e., a true *a2* homolog) but simply indicates the presence of another related sequence (detected because of the reduced stringency of hybridization) in strains of both mating-types. It is also possible that *a2* sequences are present in *a1* strains but that the sequences are nonfunctional. There is precedence for the presence of two different mating-type idiomorphs in one haploid genome. The homothallic species *N. terricola* has been found to have both the **A** and **a** sequences in one haploid genome (Metzenberg and Glass 1990). A functional test will be needed to determine whether the sequences detected in *a1* strains have the activity expected of *a* idiomorphs.

The *a2* probe did not detect hybridizing sequences in any of the ascomycete or basidiomycete fungi (Fig. 6B) tested, with the exception of *S. cerevisiae* (Fig. 6A, lane c). It is interesting that both the *a1* and *a2* probes gave a reasonably good signal to DNA from *S. cerevisiae*. As discussed below, these results may be related to the finding of a homolog of the *STE3* gene of *S. cerevisiae* in the *a* idiomorphs of *U. maydis* (Bolker *et al.* 1992).

DISCUSSION

The most striking result obtained in this study is the finding that the smut fungi with bipolar mating systems contain sequences that hybridize with the DNAs from the *a* and *b* loci of *U. maydis*, a fungus with a tetrapolar mating system. These results suggest that the sequences involved in mating are conserved among the bipolar and tetrapolar smut fungi. Future work must focus on determining whether the sequences detected by hybridization actually play a role in mating in the bipolar species. Specifically, the sequences must be isolated and tested for activity. We are pursuing these goals with *U. hordei* as a representative of the smut fungi with bipolar mating systems. As mentioned earlier, cosmids containing the regions that cross-hybridize with *bE* and *bW* have been identified (G. Bakkeren and J. W. Kronstad, unpublished results); preliminary analysis of the nucleotide sequences of the hybridizing regions suggests significant similarity to the *b* region of *U. maydis*. For the *a* locus, our results raise the possibility that some strains of the bipolar smuts may have both idiomorphs.

The four species that infect seedlings of small grains, *U. hordei, U. kolleri, U. avenae,* and *U. nigra,* are all interfertile and apparently differ primarily in host range and spore morphology. Given these biological observations, it has been proposed that the four species be united into the single species, *U. segetum,* with two morphologically different varieties, *U. segetum* var. *avenae* (Perss) Brun. and *U. segetum* var. *hordei* (Pers.) Rbh (Lindeberg and Nannfeldt 1959; cited in Huang and Nielsen 1984). Our finding that these fungi have conserved restriction fragments carrying sequences similar to the *a* and *b* genes supports this relationship.

The hybridization results indicate that more than one region with *b* sequences may be present in some species. The clearest example is *S. reilianum*, which showed hybridization of the *bE* fragment to two different *Bam*HI fragments. Although this result could indicate a *Bam*HI site within the *S. reilianum* sequence, the conservation in size of one of the bands (8.5 kb) compared with *U. maydis* strains suggests that this is not the case. Additional support for the presence of more than one *b* sequence comes from the finding that attempts to clone the *b* sequences from one strain of *U. hordei* yielded two different cosmids (G. Bakkeren and J. W. Kronstad, unpublished results). As discussed below, Groth (1975) has presented evidence for two genes conditioning mycelial growth in *U. hordei*. It is conceivable that these genes are the *b*-like sequences detected during the isolation of the cosmid clones from *U. hordei*.

The hybridization results also revealed weak hybridization of the *a1* probe to DNA from one strain of *U. violacea* and of the *a1* and *a2* probes to DNA from a *MAT*α strain of *S. cerevisiae*. While potentially interesting, these results must be regarded with caution until the hybridizing sequences can be characterized further. It is relevant in this context to note that sequence analysis of the *a* idiomorphs of *U. maydis* has identified a region with sequence similarity to *STE3* (encoding the receptor for the pheromone **a**-factor) of *S. cerevisiae* (Bolker *et al.* 1992). The hybridization of the *a1* probe to DNA from *U. violacea* strain 002 may suggest that genes related to pheromone production or perception are present in this strain or simply that some other sequence with similarity to the *a1* idiomorph is present.

It is perhaps not surprising, given the similarities in life cycles, that sequences similar to the *a* idiomorphs of *U. maydis* are present in the smut fungi with bipolar and tetrapolar mating systems. It is especially interesting, however, that the *b* sequences are present in the smut species with bipolar mating systems. In *U. maydis*, two different *b* alleles must be brought together by cell fusion (thought to be controlled by the *a* locus) to establish the infectious dikaryon. In addition, at least 25 different alleles are known for the *b* locus and any combination of two alleles will establish the infectious dikaryon. Despite many years of genetic analysis (Fischer and Holton 1957; Thomas 1991), a multiallelic locus with a role in dikaryon formation or pathogenicity has not been described for the bipolar smut fungi. A single genetic locus (the *a* locus) with two alternate alleles is thought to control both fusion and formation of the infectious dikaryon.

Although a *b* locus has not been genetically defined in the smut fungi that show bipolar mating, two genes that

condition mycelial growth in haploid isolates of *U. hordei* have been described by Groth (1975). It is conceivable that these genes have functions like *b* and that these are the genes that we detect by hybridization with the *b* probes. Thus, a *b* function may be present in the bipolar smut fungi but the genetic requirement for multiple alleles may be absent. It might be the case that the *b* function is constitutive in the bipolar species and that cell fusion is sufficient to trigger formation of the infectious dikaryon. In this situation, *b* function in haploid cells would not be sufficient to give the filamentous, pathogenic phenotype because haploid strains are yeastlike and nonpathogenic. It is possible, however, that the combination of two different *a* idiomorphs in the fusion product somehow triggers the activity of the *b* genes. An alternative explanation for the apparent absence of a multiallelic *b* locus in the bipolar species would be to postulate that the *a* and *b* loci are tightly linked to create a single complex mating-type locus. In this case, it would be sufficient to have two alternate alleles at the *b* locus, each linked to different *a* alleles. Fusion events between cells with opposite *a* idiomorphs would automatically bring together different *b* alleles and lead to establishment of the infectious dikaryon. Genetically, the *a* locus would be the only locus recognized in mating tests. The detailed analysis of the organization of the *a* and *b* sequences in progress for *U. hordei* should resolve these issues and provide considerable insight into the differences between bipolar and tetrapolar mating systems in the smut fungi. In addition, further analysis of the *a* and *b* genes will contribute to our understanding of how these genes control the pathogenic development of the fungus leading to the production of teliospores, the inoculum for new plant infections.

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LITERATURE CITED

Agrios, G. N. 1988. The smuts. Pages 474-486 in: Plant Pathology. 3rd ed. Academic Press, Inc., San Diego.

Amasino, R. 1986. Acceleration of nucleic acid hybridization rate by polyethylene glycol. Anal. Biochem. 152:304-307.

Bolker, M., Urban, M., and Kahmann, R. 1992. The *a* mating type locus of *U. maydis* specifies cell signalling components. Cell 68:441-450.

Deml, G., and Oberwinker, F. 1982. Studies in Heterobasidiomycetes, Part 24. On *Ustilago violacea* (Pers.) Rouss. from *Saponaria officinalis* L. Phytopathol. Z. 104:345-356.

Elder, R. T., Loh, E. Y., and Davis, R. W. 1983. RNA from the yeast transposable element Ty1 has both ends in the direct repeat, a structure similar to retrovirus RNA. Proc. Natl. Acad. Sci. U.S.A. 80:2432-2436.

Fischer, G. W. 1953. Manual of North American Smut Fungi. Ronald Press, New York. 343 pp.

Fischer, G. W., and Holton, C. S. 1957. Biology and Control of the Smut Fungi. Ronald Press, New York. 622 pp.

Froeliger, E. H., and Kronstad, J. W. 1990. Mating and pathogenesis in *Ustilago maydis*. Sem. Dev. Biol. 1:185-193.

Froeliger, E. H., and Leong, S. A. 1991. The *a* mating-type genes of *Ustilago maydis* are idiomorphs. Gene 100:113-122.

Gillissen, B., Bergemann, J., Sandmann, C., Schroeer, B., Bolker, M., and Kahmann, R. 1992. A two-component regulatory system for self/non-self recognition in *Ustilago maydis*. Cell 68:647-657.

Glass, N. L., Vollmer, S. J., Staben, C., Grotelueschen, J., Metzenberg, R. L., and Yanofsky, C. 1988. DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. Science 241:570-573.

Groth, J. V. 1975. Two additive, independent genes for mycelial growth versus sporidial growth of haploid cultures of *Ustilago hordei*. Can. J. Bot. 53:2233-2239.

Hoffman, J. A., and Kendrick, E. L. 1965. Compatibility relationships in *Tilletia controversa*. Phytopathology 55:1061-1062.

Holliday, R. 1974. *Ustilago maydis*. Pages 575-595 in: Handbook of Genetics, Vol 1. R. C. King, ed. Plenum, New York.

Holton, C. S., Hoffman, J. A., and Duran, R. 1968. Variation in the smut fungi. Annu. Rev. Phytopathol. 6:213-242.

Huang, H.-Q., and Nielsen, J. 1984. Hybridization of the seedling-infecting *Ustilago* spp. pathogenic on barley and oats, and a study of the genotypes conditioning the morphology of their spore walls. Can. J. Bot. 62:603-608.

Kronstad, J. W., and Leong, S. A. 1989. Isolation of two alleles of the *b* locus of *Ustilago maydis*. Proc. Natl. Acad. Sci. U.S.A. 86:978-982.

Kronstad, J. W., and Leong, S. A. 1990. The *b* locus of *Ustilago maydis* contains variable and constant regions. Genes & Dev. 4:1384-1395.

Kwon-Chung, K. J. 1975. A new genus, *Filobasidiella,* the perfect state of *Cryptococcus neoformans*. Mycologia 67:1197-1200.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Metzenberg, R. L., and Glass, N. L. 1990. Mating-type and mating strategies in *Neurospora*. BioEssays 12:53-59.

Nielsen, J. 1968. Isolation and culture of monokaryotic haplonts of *Ustilago nuda*, the role of proline in their metabolism, and the inoculation of barley with resynthesized dikaryons. Can. J. Bot. 46:1193-1200.

Nielsen, J. 1978. Two grasses as common hosts for all species of *Ustilago* parasitic on wheat, barley and oats. Can. J. Bot. 56:2712-2716.

Raper, J. R. 1966. Genetics of Sexuality of Higher Fungi. Ronald Press, New York.

Rowell, J. B., and DeVay, J. E. 1954. Genetics of *Ustilago zeae* in relation to basic problems of its pathogenicity. Phytopathology 44:356-362.

Schultz, B., Banuett, F., Dahl, M., Schlessinger, R., Schafer, W., Martin, T., Herskowitz, I., and Kahmann, R. 1990. The *b* alleles of *U. maydis*, whose combinations program pathogenic development, code for polypeptides containing a homeodomain-related motif. Cell 60:295-306.

Stankis, M. M., Specht, C. A., and Giasson, L. 1990. Sexual incompatibility in *Schizophyllum commune*: From classical genetics to a molecular view. Sem. Dev. Biol. 1:195-206.

Thomas, P. L. 1991. Genetics of small-grain smuts. Annu. Rev. Phytopathol. 29:137-148.