Sex in Fungi: Molecular Determination and Evolutionary Implications
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23 Bipolar and Tetrapolar Mating Systems in the Ustilaginales

INTRODUCTION

Mating, Sexual Development, and Phytopathogenesis

The smut fungi are attractive experimental models to investigate basidiomycete mating systems and to explore the role of mating-type functions in pathogenic development. A fascinating aspect of these fungi is that their ability to cause disease on host plants is dependent on mating interactions between haploid cells leading to formation of an infectious dikaryon. Sex and pathogenesis are thus intimately intertwined because the infectious dikaryon requires a host for proliferation and for the eventual formation of sexual spores (teliospores). *Ustilago maydis*, the corn pathogen, has emerged as the primary model for studying smut fungi and is discussed in other chapters in this book. Here we discuss other species, *Ustilago hordei* in particular, that have provided useful comparative information leading to insights into the genetic basis of bipolar versus tetrapolar mating systems in the smut fungi as a group. We first discuss the importance of smut fungi and the interactions of these pathogens with host plants to provide context for appreciating the role of mating in disease. We then focus on the details of the mating system in *U. hordei*, including the structure and function of the mating-type loci, the genomic organization of these elements, and the sequence of the 527-kb *MAT-1* locus. Comparisons between the tetrapolar mating system in *U. maydis* and the bipolar system of *U. hordei* allowed the development of a detailed view of the genomic basis of mating-system organization. This work sets the stage of a broader examination of the interconnections between genomic organization, mating systems, and pathogenesis in these fungi. In particular, comparisons suggest an evolutionary drive towards larger but genetically less complex mating loci: this results in the genesis of sex chromosomes which promote inbreeding within the species and presumably bestow a selective advantage (chapter 2). The latter might be particularly suited to species occupying specialized niches such as pathogens. However, in several homobasidiomycete mushrooms, such as hymenomycete lineages in the genus *Coprinus*, bipolar mating systems are frequently found and seem to be derived from tetrapolar organizations by losing pheromones and receptors as mating-type determinants;

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in *Coprinellus* (*Coprinus*) *disseminatus* these are not linked to *MAT* (chapter 19). In this context it is rather striking that fusion of *MAT* loci in some phytopathogenic ascomycete lineages, which confers a change from hetero- to homothallism, is persistent over time and might even have been transfered laterally (chapter 6).

Overview of the Smut Fungi

Smut fungi, or smuts for short, have attracted interest for centuries because they form conspicuous fruiting structures on many kinds of plants, and these structures contain black masses of teliospores that give the infected tissue a "sooty" or "smutted" appearance (touched by fire or "Brand" in German and Dutch). In the order Ustilaginales, smuts (family of Ustilaginaceae) are distinguished from related bunt ("bu[r]nt") fungi (family of Tilletiaceae) based on distinct features of the promycelium: septate with lateral and terminal basidiospores (sporidia) in the smuts and a continuous promycelium with terminal basidiospores in the bunts. However, these characteristics proved imprecise and later research proposed only one family for the smuts, the Ustilaginaceae (19). Fischer and Holton (19) consolidated the many described species into 1,162 species belonging to 33 genera, whereas recently Vanky (85) recognized approximately 1,200 species in more than 50 genera. New species are being described continuously (11, 60, 86).

Smuts infect over 4,000 species of angiosperms, including both monocots and dicots in over 75 families (19). However, each smut species generally has a rather narrow host range. Interestingly, a disproportionate share of the host species are in the family Gramineae, and because these plants include the world's most important agricultural crops (cereals and forage grasses), smuts infecting these hosts have been studied extensively for over a century. Some of the earliest official reports of (corn) smut came from France in the mid-18th century (14). Among the best known are *U. maydis* (infecting corn), which is the best characterized at the molecular level and whose complete genome has been sequenced (discussed in chapter 22), *Sporosorium reiliana* (infecting sorghum and corn), and *Ustilago scitaminea* (infecting sugarcane). A group of small grain-infecting smuts have also been studied extensively. These include *U. hordei* (barley and oats), *U. avenae* (oats), *U. kolleri* (oats), *U. nuda* (barley), and *U. tritici* (wheat). In contrast to most species in this group, the last two have a distinctive mode of germination and initiate disease by infecting embryos via flowers rather than young seedlings. *U. hordei* has emerged as the representative for the small-grain-infecting group. Several smuts are also pathogenic on forage grasses (e.g., *Ustilago bullata*), and it is sometimes possible to find grasses that serve as common hosts for several smut species (82). These common hosts have potential utility for testing mating interactions between different smut species. Other fungal pathogens of cereal crops such as the rusts and bunts share similar life cycle features with the smut fungi. The bunts are most similar and include well-known pathogens such as *Tilletia foetida* (syn. *T. laevis*) and *T. caries* (syn. *T. tritici*, causing common bunt or stinking smut on wheat) and *T. controversa* and *T. indica* (causing dwarf and Karnal or partial bunt of wheat, respectively).

As mentioned, the smut and bunt fungi are particularly interesting because of the role of mating in formation of the infectious dikaryon that invades host tissue. The mating systems in these fungi include bipolar and tetrapolar classifications based on genetic experiments, and these fungi provide an opportunity to explore mechanisms of mating in the context of infection and host range. A fascinating additional layer of complexity exists in that infection by these fungi is often governed by so-called avirulence genes in the pathogens and corresponding resistance genes in the host plants. Fungal isolates within a species can carry different complements of avirulence genes constituting many specific so-called "races." Similarly, a host species may harbor different combinations of resistance genes that recognize specific avirulence genes to block infection; this gives rise to many "cultivars" within that species. Genetically, a specific avirulence gene interacts with one specific resistance gene; dominance triggers defense and blocks disease, but a recessive allele in either or both partners is not recognized. This "gene-for-gene" concept actually emerged from research on the rust *Melampsora lini* on flax (20) and the smut *U. tritici* on wheat (57). Over the years, many pathosystems have been shown to follow this concept, including many species in the Ustilaginales with their respective hosts (19). *U. hordei* has emerged as the model for the genetic analysis of avirulence genes, and one avirulence gene has been isolated (25, 50, 70, 79, 80, 81). The molecular basis of recognition and subsequent defense is being worked out and reflects the intimate coevolution of these pathogens with their host plants (13). Finally, some smut and bunt fungi are interesting because they provoke profound physiological and/or morphological changes in their hosts. For example, *U. maydis* incites conspicuous tumors in which massive sporulation occurs, *Tilletia buchloeana* induces a sex change such that pistils develop in an otherwise staminate floret of male buffalo grass (38), and *Microbotryum*

violaceum transforms female flowers of *Silene alba* into male ones, eventually replacing pollen with spores (84).

Interactions with Host Plants and the Mode of Infection of *U. hordei*

Infection by smut fungi such as *U. hordei* is initiated when seeds are contaminated by dispersed teliospores. Seed and teliospore germination occur together, and the spore produces a basidium with subsequent meiosis to produce four haploid basidiospores (sporidia). In many but not all smuts, the basidiospores grow by budding and can be cultured for molecular genetic manipulations. These cells are not infectious in the absence of compatible mating partners. Most smuts infect their hosts systemically and target meristematic tissues; interestingly, the fungal dikaryon initially grows passively with the plant with subsequent proliferation and sporulation in certain tissues in response to plant developmental changes (e.g., flowering). Teliospores form in sori on stems, leaves, and anthers or in ovaries to replace seeds (particularly in the *Gramineae* [19, 71]). The complete path of infection of *U. hordei* was recently described in a detailed microscopic study (35, 36). The initial mating interaction of haploid cells on the plant surface is a key event, and an interesting observation was that the mating process on the host surface appeared to be more profuse than is seen in vitro. Specifically, many more conjugation tubes (mating hyphae) emerged from basidiospores (often more than two per cell) on the plant surface, and it is possible that stimulatory chemical or physical factors from the plant are sensed to maximize mating partner detection (36). After mating, the dikaryotic filament grows over the leaf surface and can cover considerable distances (on average more than 30 μm) over many epidermal cells without apparent differentiation. The hyphal tip forms a characteristic crook when it grows over a juncture between the long anticlinal walls of epidermal cells. The fungal tip becomes slightly swollen immediately adjacent to this bend, and an appressorium-like structure indicates the site of direct penetration. After penetration, both intra- and intercellular mycelium can be found and the intracellular hyphae do not compromise host cell plasma membranes. Necrotic reactions or other abnormal cell morphologies are not observed, suggesting that *U. hordei* evades and/or actively suppresses host defense mechanisms to establish a biotrophic relationship. Intercellular hyphae can also send several branches into plant cells, some of which seem to terminate within cells. These invading hyphae become encased in an electron-dense interfacial matrix that separates the hyphae and the host plasma membrane. These cells may function as haustoria to acquire nutrients, possibly through induced transporters as occurs with rust fungi (87). Fungal hyphae eventually reach the apical meristem and nodes of the coleoptile, but scant fungal biomass is present, making extensive nutrient uptake and elaborate haustorial structures as seen in rusts unnecessary. However, fungal proliferation occurs when the meristematic region differentiates into a floral meristem and sporulation is initiated. Teliospores are formed inside the spikelets to replace the seeds. Economically, infection results in complete yield loss and massive spore contamination of harvested grain. In contrast to infection of small-grain cereals (as described for *U. hordei*), infection by *U. maydis* occurs on any aboveground part of corn plants and local symptoms (tumors) are induced. The paths of infection for *U. maydis* in corn and for *Sphacelotheca reilianum* in sorghum have been thoroughly studied (72–74, 90). Bunt fungi can also infect locally or systemically (10).

Mating Systems in Basidiomycete Fungi and Smuts

The majority of smut fungi are heterothallic, and many possess a clearly defined sexual stage. Some species, however, produce an ephemeral haploid stage with no separate sporidial cells, making it difficult to establish compatibility relationships and blurring the distinctions between homo- and heterothallism (30). In the species that have been used for extensive experimentation (*U. maydis* and *U. hordei*), the basidiospores produced upon teliospore germination can be isolated and tested for mating specificity on culture medium (28, 29, 67). Successful mating results in the initiation of thin conjugation tubes in response to the exchange of pheromones (encoded by the *a* mating-type gene complex) and can be observed microscopically. These tubes fuse, and a thick, straight-growing, dikaryotic filament is produced if the mating partners have compatible specificity at the *b* mating-type gene complex (encoding a heterodimeric homeodomain transcription factor). When compatible haploid cells are mixed on rich medium supplemented with activated charcoal, a positive mating reaction can be scored as a white, "fuzzy" reaction (Fig. 23.1). Pairings of basidiospores dissected from single basidia (tetrad analysis), or randomly isolated from nature, yield information on the complexity of compatible mating types present in populations and can define whether a bipolar or tetrapolar system is represented (Fig. 23.1).

Among the homobasidiomycetes, approximately 65% of the species regulate sexual compatibility via a

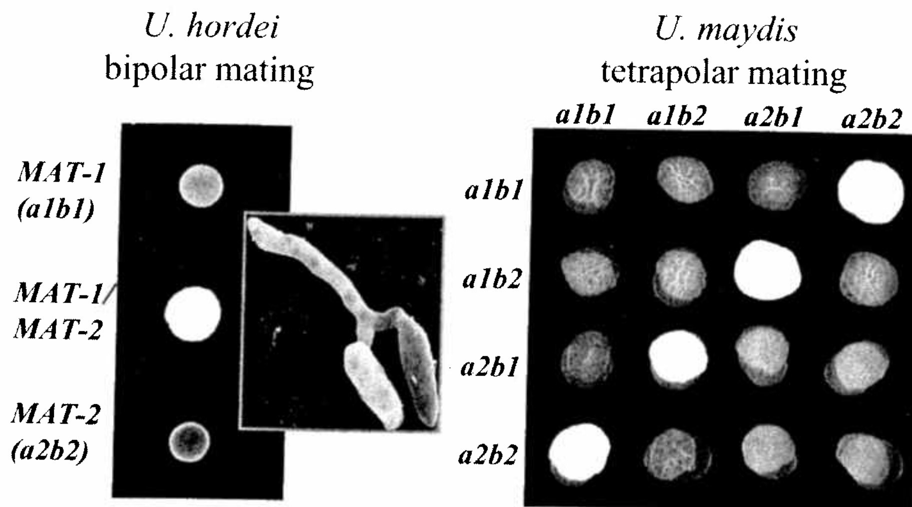

Figure 23.1 Examples of mating interactions on charcoal medium. Aliquots of cultures of haploid basidiospores having the mating genotype indicated are cospotted on complete medium plates supplemented with activated charcoal (28). When both the *a* and the *b* loci are of different allelic specificity, a straight-growing, dikaryotic hypha is produced (inset) and the ensuing colony will have a white "fuzzy" appearance. Note that for *U. hordei* there are only two mating-type alleles (*MAT-1* and *MAT-2*) in nature but that for *U. maydis a* and *b* specificities can assort in all combinations among progeny from genetic crosses.

tetrapolar (bifactorial) mating system. In these fungi, two genetic loci such as the *a* and *b* loci in smut fungi can each possess two or more allelic specificities. For some mushroom fungi such as *Schizophyllum commune* and *Coprinus cinereus* (*Coprinopsis cinerea*), this can result in thousands of mating specificities (chapters 17 and 18). Another 10% of the species are considered to be homothallic (39, 63). Finally, 25% of the species have a bipolar (unifactorial) mating system in which compatibility is governed by a single genetic locus designated *MAT* with alleles *MAT-1* and *MAT-2* (also sometimes called *a* and *A*). This locus can have two or multiple alleles, the latter being common in the homobasidiomycetes and the nonparasitic heterobasidiomycetes. The terms *MAT-1* and *MAT-2* were adopted for *U. hordei* (4). Among the parasitic heterobasidiomycetes, such as the phytopathogenic rust, bunt, and smut fungi, the bipolar mating system is predominant with certain exceptions (19). These exceptions include a multiallelic bipolar system for *T. controversa* (27) and the occurrence of a tetrapolar mating system in several species including *U. maydis*, *Ustilago longissima* (19, 62), and *S. reilianum* (68). Other chapters in this book describe the best-studied tetrapolar system found in *U. maydis*, where the *a* mating-type gene complex has two known specificities and *b* has at least 25 different specificities. Interestingly, recent detailed mating-type analysis of *S. reilianum* revealed at least three specificities for the *a* locus and at least five for *b* (68).

THE MATING-TYPE LOCI OF BIPOLAR SMUT FUNGI

Identification of the *a* and *b* Mating-Type Loci in *U. hordei*

The isolation of the *a* and *b* mating-type gene complexes from *U. maydis* presented an opportunity to search for related sequences in other *Ustilago* species (23, 42). A DNA hybridization survey of a variety of basidiomycete and ascomycete fungi for the presence of sequences with similarity to the *U. maydis a* and *b* genes revealed related sequences in the *Ustilago* species that infect small-grain cereal crops, as well as in *U. longissima* and *S. reilianum* (3). Strongly cross-hybridizing sequences were not found in *Cryptococcus neoformans*, *M. violaceum*, *Puccinia graminis*, *S. commune*, *T. controversa*, *T. caries*, or *Neurospora crassa* (3). Of course, subsequent studies revealed that genes with functions comparable to those of the *a* and *b* gene complexes do exist in other basidiomycetes including *C. neoformans*, *S. commune*, and *C. cinereus*, but these sequences are too divergent to be detected by hybridization (15, 44, 49, 53, 58, 64, 75, 88). One would expect a similar situation for the *Tilletia* and rust species, but these loci remain to be characterized. The hybridization results for the group of *Ustilago* species with bipolar mating systems were interesting because of the similarities observed in the pattern of hybridizing DNA fragments.

Specifically, hybridization with the *bE* gene of *U. maydis* detected a restriction fragment length polymorphism (RFLP) (1.5- or 2.8-kb BamHI fragments) among the strains of *U. hordei*, *U. kolleri*, *U. avenae*, *U. aegilopsidis*, and *U. nigra*, indicating the presence of similar gene complexes in this group. Surprisingly, the RFLP band of 2.8 kb was associated with a positive hybridization result and the 1.5-kb band was associated with a negative result with the *U. maydis a1* sequences. In particular, the two different mating-type strains of *U. hordei* that were tested showed this association (3). These results led to speculation that the *a* and *b* sequences might be genetically linked in *U. hordei* (and the other bipolar *Ustilago* species). As described below, subsequent work revealed that this was the case and provided an explanation for the differences between bipolar and tetrapolar mating systems in *Ustilago*.

FUNCTIONAL ANALYSIS OF THE MATING-TYPE LOCI IN BIPOLAR SMUTS (*U. HORDEI*)

The cloning and analysis of the *b* genes from *U. hordei* revealed that they are very similar in structure to those in *U. maydis*, consisting of a gene complex with divergently transcribed *bE* and *bW* genes (4). Just as for *U. maydis*, the carboxy-terminal ends of the predicted UhbE and UhbW allelic proteins were more conserved (92% identity) than the "variable" amino termini (51% identity); however, comparisons to the *U. maydis* counterparts revealed 64 and 43% similar domains, respectively. The homeodomain, WFxRxR, which is important for function (26), was conserved in all of the proteins. Subsequent work showed that *U. hordei b* genes were able to induce filamentous growth in haploid *U. maydis* strains and these transformants were weakly virulent when inoculated on corn (4). These experiments confirmed cross-species functionality of the *b* mating-type genes after fusion.

The presence of orthologs of *U. maydis a* locus sequences in the bipolar smuts and the demonstration of the involvement of diffusible small-molecular-weight factors (pheromones) in *U. hordei* mating (similar to the process in *U. maydis* [52]) led to attempts to isolate these regions. To isolate the *a* locus, the *pan1* gene from *U. maydis* was used as a probe because it was known to be linked to *a* in *U. maydis* (23). This probe identified a cosmid paMAT-1 in *U. hordei* that was tested for mating-type activity by transformation into a *MAT-2* strain. This clone induced erratic behavior including the formation of long, meandering mating hyphae and cell aggregates in the absence of cells of the opposite mating type (a so-called dual-mater phenotype [5]). Sequence analysis identified the pheromone receptor gene *Uhpra1* on an 8.5-kb SphI fragment which coded for a predicted protein that was 64% identical and 82% similar to Pra1 of *U. maydis*. Similarly, Uhpra2 was found to be 60% identical and 79% similar to Umpra2 and when transformed in a *MAT-1* strain induced the same dual-mater phenotype (1). The *U. hordei* pheromone genes, *Uhmfa1* and *Uhmfa2*, were also cloned and analyzed, and again, their functions were analogous to those of their homologs in *U. maydis* (1, 40). As for *U. maydis*, *Uhmfa1* and *Uhmfa2* are expressed at basal levels but are up-regulated when cells of opposite mating type are encountered. Interestingly, mating inhibition factors (MIFs) have been found in *U. hordei* that are truncated and/or "undecorated" forms of the pheromones, probably oxidative degradation products. Normally, translated Uhmfa is a preprotein which is processed by clipping and becomes farnesylated and carboxy-methylated (41, 69). It was suggested that a gradient of MIFs during teliospore germination might prevent immediate fusion of siblings within tetrads, thereby allowing time for nonsibling interactions (outbreeding). *U. hordei* MIFs also reportedly have an inhibitory effect on the germination of teliospores of several *Tilletia* species, which might confer a competitive advantage during coincident infection of the same host (41).

Smut pheromones are produced and secreted in low quantities by basidiospores, thereby creating a gradient thought to alert and guide potential mates of opposite sexual persuasion. It has been shown that the *U. hordei a* locus genes, *Uhmfa1* and *Uhpra1*, when introduced by transformation, are necessary and sufficient to make *U. maydis* intercompatible with *U. hordei MAT-2*, but not *MAT-1*, strains. In addition, *U. hordei* strains transformed with the *U. maydis a1* locus also became 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 (6). This shows that within these smuts, the machinery and pathways to transmit signals from the receptor once pheromone is bound are conserved. Although the Mfa-Pra interaction is thought to be species specific, many examples of natural intercompatible combinations exist and some are interfertile and infectious on common hosts. The interspecies mating capabilities within the small-grain-infecting smuts were mentioned earlier, but some hybrids do not produce viable or pathogenic progeny (19, 37, 82). Likewise, *U. maydis* and *S. reilianum* can also interbreed and produce teliospores on corn, although no pathogenic progeny have been reported (66). Partial mating capabilities have been observed between *U. scitaminea* and both *U. hordei* and *U. maydis*, indicating that there may be a continuum of pheromone

recognition specificities among the smut fungi (6). A distinction has to be made between fusion of basidiospores, due to sometimes promiscuous responses of pheromone receptors to pheromones, the differentiation to the filamentous cell type brought about by the productive interaction between different *b* alleles from the participating species ("Fuz" reaction), and the subsequent invasion of (common) host tissues resulting ultimately in the production of teliospores that can produce viable, pathogenic offspring. The latter steps point to true hybridization and fertility/fecundity. These issues are intertwined with difficulties in taxonomic placement, which is often based on host range, the species concept based on productive hybridization, and the "biological species" concept which defines a species as organisms that share "morphological" or "taxonomic" characteristics but are, or have become, (partially) intersterile due to geographic isolation.

PHYSICAL ASSOCIATION OF THE *a* AND *b* GENE COMPLEXES AT THE *MAT-1* LOCUS OF *U. HORDEI*

The detection of an RFLP at the *b* gene complex in the *Ustilago* species that infect small-grain cereals and that was potentially genetically linked to the *a* locus provided the impetus for further examination of the genetic and physical linkage in *U. hordei*. Initially, a hybridization probe for *UhbE1* was found to detect an RFLP that cosegregated with mating type (*MAT-1* or *MAT-2*) in 86 meiotic progeny from two teliospore populations. No recombinant progeny were obtained, thus indicating tight linkage. The cloning and characterization of the *a* gene complexes from *U. hordei* (described above) provided the specific hybridization probes to also analyze linkage from the perspective of the *a* locus. Both the *a* and *b* probes hybridized to RFLPs that showed 100% cosegregation with mating type in the 86 progeny. Additionally, a 2.1-kb fragment from the paMAT-1 cosmid hybridized specifically to *MAT-1*, indicating that regions of nonhomology or divergence existed between *MAT-1* and *MAT-2*. The probes were also used to demonstrate physical linkage between the *a* and *b* sequences. Electrophoretically separated chromosomes were blotted, and the *a* and *b* probes were found to hybridize to the same chromosome of ~3 Mbp. In contrast, *a* and *b* probes from *U. maydis* detected the *a* locus on a 1.5 Mbp chromosome (later identified as ~1-Mbp chromosome V) and the *b* locus on a 2-Mbp chromosome (later identified as ~2.5-Mbp chromosome I) (Fig. 23.2) (5). Evidence that recombination might be suppressed at *MAT* came from the analysis of 2,182 random progeny from three collections of teliospores. A screen of the progeny for altered mating specificity, e.g., showing a failure to mate with either parental type (possibly due to genotypes *a1b2* and *a2b1*), was unsuccessful, suggesting that the combinations of the mating types *a1b1* and *a2b2* were maintained through meiosis.

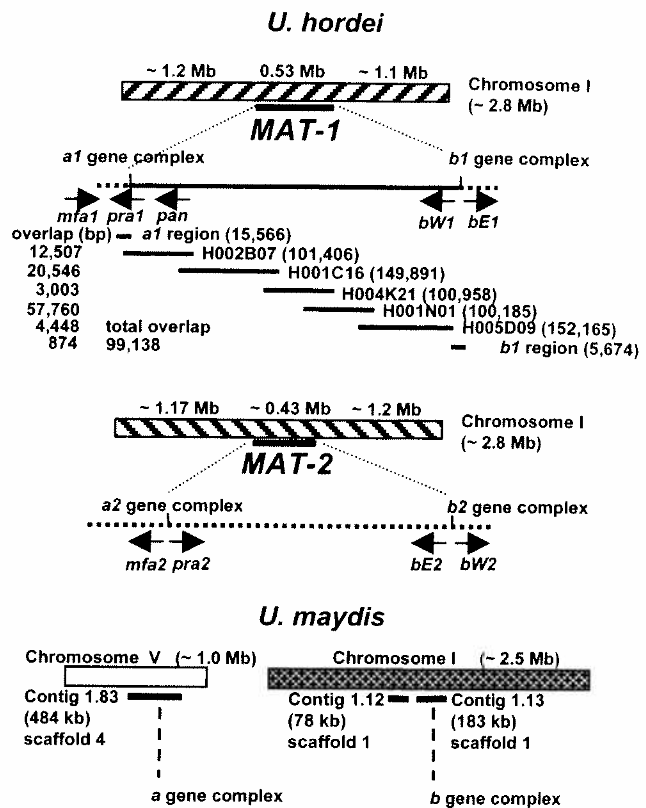

Figure 23.2 Genomic organization of the mating-type loci in *U. hordei* and *U. maydis*. In *U. hordei* the *MAT* locus is defined as the region delimited by the known *a* and *b* mating-type gene complexes, a distance of 526,707 bp for *MAT-1*. The five BAC clones, yielding a total of 625,845 bp of sequence, are indicated (8). In both *MAT-1* and *MAT-2* (430 kb) strains, the locus sits roughly in the middle of the largest chromosome (chromosome I). Note that the *MAT-2* region has not been sequenced but that its length and the orientation of the complexes have been determined (47). In *U. maydis*, the gene complexes are found on two different chromosomes: the *a* locus on contig 1.83 (scaffold 4; Broad Institute http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/) harbored by chromosome V, and the *b* locus on contig 1.13 (scaffold 1) on chromosome I; contig 1.12 is likely linked to contig 1.13 based on the synteny found with *U. hordei* (represented by the solid black lines). Gene names are as follows: *mfa*, mating pheromone gene; *pra*, *a1* pheromone receptor gene; *pan*, pantoate b-alanine ligase gene; *bW1*, bWest1 gene; *bE1*, bEast1 gene (4, 6, 16). Drawing is not to scale. Reprinted from *Fungal Genetics & Biology* (reference 8, Fig. 1) © 2006, with permission from Elsevier.

Unlike the situation in smut fungi with tetrapolar mating systems (such as *U. maydis*), the physical linkage of *a* and *b* in *U. hordei* would ensure that every fusion event

mediated by the *a* genes at *MAT-1* and *MAT-2* during mating would bring together *b* gene complexes of opposite specificity. This result indicates that bipolar smut fungi would require only two *b* specificities, one linked to each *a* specificity. This hypothesis was confirmed by DNA sequence analysis of alleles coding for the variable N-terminal 121 amino acids of bE and 171 amino acids of bW. This analysis revealed only two classes having identical protein sequences among many *U. hordei* isolates from a worldwide collection, each belonging to either *MAT-1* or *MAT-2* (5). That is, only two classes of *b* gene complexes were found among 18 isolates and there was very little variability within each class. Five additional *Ustilago* species similarly had only two classes. No amino acid changes were found when comparing *U. hordei* with *U. nigra* (likely synonymous to *U. hordei*) and *U. aegilopsidis*, while *U. avenae* and *U. kolleri* (82) had the same one conservative base-pair change. *U. bullata*, a more distantly related forage grass pathogen (7), had 3 amino acid changes out of 171 for bW1 and 4 out of 121 for bE1. These results led to the conclusion that most bipolar smut fungi have only two allelic specificities (*MAT-1* with *a1* and *b1* and *MAT-2* with *a2* and *b2*). Moreover, most can probably functionally interact since tests on charcoal mating plates indicated positive interactions in the predicted combinations and several of these have been productively hybridized on a common grass host, *Agropyron tsukushiense* (82).

The size of the *MAT* locus in *U. hordei* and the extent of recombination suppression between the *a* and *b* regions were explored in more detail by tagging each gene complex with a different gene encoding a selectable marker and the site for the rare-cutting restriction enzyme I-SceI (Fig. 23.3) (47). Specifically, an *a* region replacement DNA cassette was prepared that contained the gene for phleomycin (phleo) resistance linked to the I-SceI site and a *b* region replacement cassette was prepared with the gene for hygromycin B (hyg) resistance linked to the same I-SceI site. A set of single- and double-tagged strains was then constructed starting with *MAT-1* (*a1b1*) and *MAT-2* (*a2b2*) parents and containing a single phleo cassette at *a1*, a single hyg cassette at *b2*, the cassettes at the *a1* and *b1* gene complexes, and the cassettes at the *a2* and *b2* gene complexes (Fig. 23.3). Chromosome-sized DNA was then prepared from these strains and digested with I-SceI to either cleave the chromosome at *a* or *b* or release the segment of DNA between *a* and *b* at both *MAT-1* and *MAT-2*. The digested DNA was separated by pulsed-field gel electrophoresis, and the DNA segments were identified by Southern hybridization using specific *a* and *b* region clones. These experiments revealed that the distance between *a* and *b* at *MAT-1* is approximately 500 kb and the distance is approximately 430 kb at *MAT-2*. Additionally, the use of the single-tagged strains revealed that the *MAT* locus is close to the center of an ~2.8-kb chromosome. The use of hybridization probes from the *a* or *b* gene complexes and flanking the I-SceI sites also provided evidence that the *MAT-1* and *MAT-2* loci had undergone rearrangements relative to each other. Specifically, the orientation of the *bE-bW* complex relative to the *a* complex differed between *MAT-1* and *MAT-2*. Similarly, the hybridization experiments showed that the organization of the *pra* and *mfa* genes differed between *a1* and *a2* (Fig. 23.3) and revealed that regions of nonhomology existed between these gene complexes. Combined with the size difference between *MAT-1* and *MAT-2*, these experiments indicated that substantial rearrangements existed between *MAT-1* and *MAT-2*.

The single-tagged strains also allowed a direct examination of the frequency of recombination in the interval

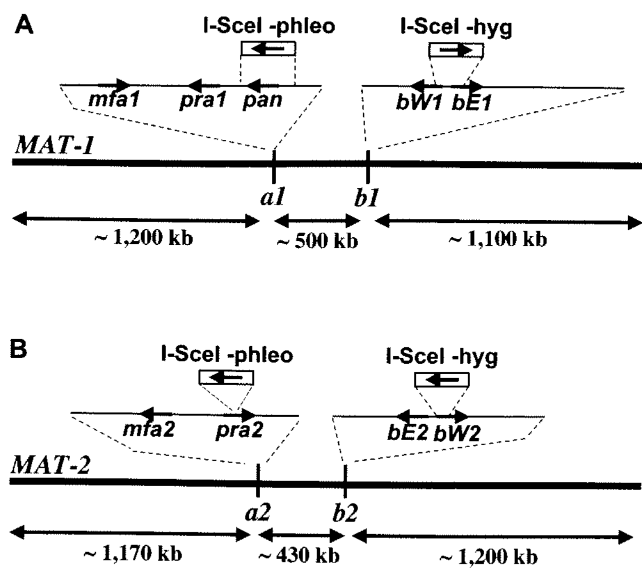

Figure 23.3 Strategy to prove linkage and recombination suppression, and to measure the physical distance between the mating-type gene complexes in *U. hordei*. The two thick lines represent the *MAT* chromosomes with the respective locations of the *a* and *b* mating-type gene complexes for *MAT-1* (A) and *MAT-2* (B). The organization of the mating-type gene complexes is enlarged (compare with Fig. 23.2) and the positions of the integrated constructs used to tag them are indicated (boxes). I-SceI represents the 18-bp recognition sequence for the rare-cutting, intron-homing enzyme from *Saccharomyces cerevisiae* which was linked to the selectable markers for phleomycin (phleo) to tag the *a* complex, and for hygromycin B (hyg) to tag the *b* complex. Digestion with the enzyme I-SceI led to the estimate of the respective distances as indicated by the double-headed arrows (see the text for details) (47). See the legend to Fig. 23.2 for explanation of gene names.

between *a* and *b*. Specifically, two strains tagged with phleo at *a1* were crossed with each of two strains tagged with hyg at *b2* by mixing the strains together on barley seedlings. The strategy was to estimate the frequency of recombination by collecting teliospores from the cross, germinating the spores to obtain meiotic progeny, and selecting double-resistant recombinant progeny on medium containing both hygromycin B and phleomycin. The prediction was that only progeny with the genotype *a1b2* (phleo^r, hyg^r) would be able to form colonies. A sample of 1×10^4 to 2×10^4 random progeny yielded 34 double-resistant progeny (affectionately called drp or "drips"), suggesting that recombination was indeed greatly suppressed in the 400- to 500-kb interval. However, none of the drp strains displayed clear *a1b2* or *a2b1* mating specificity; the strains either failed to give a positive mating reaction with any tester strain or showed mating with more than one specificity. Subsequent hybridization experiments revealed that 13 of the 34 strains had both the *b1* and *b2* mating-type sequences and 32 contained both *a1* and *a2*. These results thus indicated that the drp did not arise from simple recombination events between *a* and *b* but rather from rearrangements at these loci or retention of part or all of the *MAT* chromosome homologs to establish aneuploid or diploid strains. Strains of the expected *a1b2* and *a2b1* mating types have been constructed artificially, so it is known that these combinations are viable and functional (6).

There are many examples of suppression of recombination between orthologous areas in sex-determining regions including the mammalian (human) X and Y chromosomes (46), the mating-type loci in the unicellular green alga *Chlamydomonas reinhardtii* (17), and fungal mating-type loci from species such as the model ascomycete *N. crassa* (24) (chapter 1) and the ascomycetous chestnut blight fungus *Cryphonectria parasitica* (43). In the last species, for example, suppression of recombination occurred in an otherwise highly polymorphic region near the *MAT* locus and it was suggested that this was caused by an ancient inversion, although in this case the *MAT* locus did not seem part of a sex chromosome because recombination between this region and *MAT* was observed.

GENOMIC ANALYSIS OF BIPOLAR AND TETRAPOLAR MATING SYSTEMS IN SMUT FUNGI

The large size of the *U. hordei MAT* region and the suppression of recombination in this area prompted us to propose that the region might function to maintain a set of genes that function together in sexual development and, potentially, in pathogenesis (47). That is, the ~500-kb region might function as a pathogenicity island. We therefore characterized the *MAT* region in more detail with the goal of specifically identifying the genes present at the locus and testing the prediction (8). The genome sequence has not been determined for *U. hordei*, so a physical map was initially constructed for a *MAT-1* strain by using 2,304 large insert bacterial artificial chromosome (BAC) clones (average insert size, 113 kb) to allow characterization of the genome and to isolate the ~500-kb *MAT-1* region. A map containing 38 contigs was constructed by HindIII restriction enzyme fingerprinting of all clones, and an overlapping set of five BAC clones spanning the *MAT-1* locus was identified on a 1.34-Mb contig (Fig. 23.2). These five clones were sequenced, and the assembled sequence was combined with existing sequence from the *a1* and *b1* gene complexes to identify a genomic region of 526,707 bp (GenBank accession no. AM118080) that extended from the *Uhpra1* pheromone receptor gene at the left side of the *a1* locus to the *UhbE1* gene on the right end.

The examination and annotation of the 527-kb *MAT-1* sequence revealed the presence of 47 candidate protein-coding genes, of which 20 were designated hypothetical proteins. The remaining 27 genes encoded proteins with similarities to proteins of known function. Contrary to our prediction (47) and unlike the findings with the *MAT* locus of *C. neoformans* (49), the majority of the functions were not obviously related to mating or pathogenicity, at least at the level of sequence inspection. These included functions for metabolism (trehalose phosphatase, a-mannosidase, and ferric reductase), gene expression (ribosomal proteins and TATA-binding protein), and signaling (GTPases and glycogen synthase kinase). The region of the *a1* gene complex contained the known *Uhpra1* and *Uhmfa1* genes along with an ortholog of the *rba2* gene found at the *a* locus in *U. maydis*. Genes adjacent to the *a1* locus included the *pan1* gene for pantothenic acid biosynthesis (known to be linked to the *a* locus in *U. hordei* and *U. maydis*), as well as genes predicted to encode a ribosomal protein, a DAHP synthase, and an oligopeptide transporter. In addition to the mating functions, there are hints of functions that are potentially conserved in their association with the *a* locus. For example, a gene related to pantothenic acid biosynthesis is also associated with the *MAT* locus in *C. neoformans* (21) and genes for oligopeptide transporters are found near *MAT* in *U. hordei* and *U. maydis* or are mating regulated in *S. commune* (48). The *b1* gene complex of *UhbW1* and *UhbE1* also has an associated gene predicted to encode an N-terminal acetyltransferase.

The characterization of the *MAT-1* sequence and the annotation of the genes in the region revealed a remarkable accumulation of repeated DNA elements such that ~50% of the total sequence was repetitive (Color Plate 2). This remarkable feature of the locus resulted in a pattern of islands of one to four genes separated by extensive stretches of repeated sequences. The *a1* region had the largest collection of contiguous gene sequences with seven genes, and the largest stretch of repeats spanned approximately 64 kb. The repetitive sequences fell into four classes: (i) gypsy-type retrotransposons, (ii) copia-type retrotransposons, (iii) partial and intact copies of long terminal repeats (LTRs) with and without associated retrotransposons, and (iv) putative DNA transposons. In total, there were ~100 copies of retrotransposons in the region, although only 12 of these appeared to be intact, and the predicted coding regions of the gag-pol proteins in these contained numerous stop codons. Therefore, it was not clear whether any of these elements had retained function. There was sequence evidence that the repetitive elements had been mobile in the past. For example, LTR elements were present in the coding regions of two genes encoding a predicted isocitrate dehydrogenase and a vacuolar protein-sorting function. Additionally, there were numerous examples of LTRs interrupting transposons, retrotransposons, or other LTR elements. One particularly striking example involved a transposon designated Tho1 of 4,132 bp in length that was apparently interrupted five times by LTR1 (the most commonly found LTR), once by LTR10, once by a retroelement called Tuh5, and once by a protein-coding gene with similarity to a gene in *U. maydis* (Color Plate 2) (see reference 8 for more details).

Given the repetitive nature of *MAT-1*, it was of interest to determine whether the rest of the *U. hordei* genome showed a similar repetitive character. The BAC clones and physical map provided an opportunity to attempt to answer this question. First, the ends of the BAC clones from the tiling set of the contig carrying *MAT-1* were sequenced and tested for similarity to the repeats in *MAT-1*. The tiling path included 3 clones from the *b1* gene complex side of *MAT-1*, the 5 clones within the region (Fig. 23.2), and 14 clones on the *a1* locus side. This analysis revealed that the LTR, transposon, and retrotransposon sequences were distributed across the approximately 1.3-Mb contig, although it was not possible to determine their density. To examine the whole genome, seven types of repetitive sequences from *MAT-1* were hybridized to filters containing all 2,304 BAC clones from the map construction. Three patterns of hybridization were detected: (i) widespread distribution such that the majority of the BAC clones hybridized, (ii) intermediate distribution (e.g., one probe detected 249 BAC clones), and (iii) hybridization at a higher frequency to BAC clones that contained the *MAT-1* sequence (8). Overall, these results indicated that the *U. hordei* genome is likely to be highly repetitive but that some types of repeats have accumulated preferentially at *MAT-1*. The highly repetitive nature of the *MAT-1* sequence was consistent with the difficulties in completing and assembling the sequence; in this regard, the physical map and the end sequences of the mapped BAC clones proved invaluable in determining the orientation and positions of sequence contigs. However, these findings also indicate that it might be challenging to complete the sequence of the entire *U. hordei* genome. Overall, the repetitive nature of the *MAT-1* sequence might provide clues with regard to the lack of recombination in the region. That is, a scrambling of repetitive elements between *MAT-1* and *MAT-2* may interfere with pairing during meiosis and reduce opportunities for recombination. It is also possible that large inversions may be present given the orientation differences we observed for the mating-type gene complexes (47).

The availability of the *MAT-1* sequence and the regions around the *a1* and *b1* complexes allowed a comparison to be made with the corresponding regions in the recently sequenced genome of *U. maydis*. The opportunity for this analysis was suggested during the process of annotating the *U. hordei* genes when it became apparent that for many, the closest orthologs were *U. maydis* genes, and that the genes in the same island in *MAT-1* often matched consecutive genes in the *U. maydis* genome (Color Plate 2). Initially, the *U. maydis* sequence contigs carrying the *a* and *b* gene complexes were identified by BLAST: the *a* complex was present on contig 1.83 on chromosome V, and the *b* complex was on contig 1.13 on chromosome I. Synteny seemed to be maintained in the unique regions of the *MAT-1* locus when contig 1.13 was linked to contig 1.12 (Color Plate 2). The alignments of the *MAT-1* sequence with contigs 1.83, 1.12, and 1.13 of *U. maydis* dramatically illustrated the tremendous accumulation of repetitive elements at the *MAT-1* locus and the substantial rearrangements (deletions, insertions, inversions, and translocations) that have occurred since the two species diverged from a common ancestor (Color Plate 2) (8). For example, the distribution of the single-copy regions across the 527-kb sequence of *MAT-1* corresponds to an ~80-kb region around the *a* genes and an ~150-kb region around the *b* genes in *U. maydis*. Remarkably, the order of genes around the *a* gene complex in *U. maydis* was generally conserved in the *MAT-1* sequence of *U. hordei*. Similarly, there is evidence of conservation of synteny for the

region containing the *b* genes between *U. maydis* and *U. hordei*. However, a pattern of interdigitation of *a*-associated and *b*-associated genes exists such that the *MAT-1* locus contains interspersed genes that have locations on different chromosomes in *U. maydis*. One interpretation of this pattern is that a tetrapolar arrangement of *a* and *b* gene complexes was present in the common ancestor of these fungi and that an interchromosomal rearrangement fused the regions together to establish a progenitor *MAT* locus for the bipolar pattern. Subsequently, the *MAT* locus accumulated repetitive elements and these contributed to a large number of inversion and transposition events to generate the current arrangement of genes at *MAT-1*. There was also evidence of rearrangements that might have involved other regions of the *U. maydis* genome. That is, 4 of the 47 genes identified in *MAT-1* were not found to be associated with the contigs carrying the *a* and *b* gene complexes in *U. maydis* (8).

One question concerns the position of the original fusion event that joined the *a* and *b* gene complexes together. From Color Plate 2 it is apparent that *a*-associated genes from *U. maydis* are distributed in the *MAT-1* locus from positions 1 to ~332 kb. Thus, one could use the 332-kb coordinate as the right boundary of the putative fusion event. The left boundary is more difficult to establish because of the interdigitation of *a*- and *b*-associated genes described earlier that extends from ~90 to 332 kb. Inspection of this interval did not reveal obvious features that may have participated in the rearrangements, although among the five types of retroelements and four types of transposons, the eight sequences related to the Tho4 transposon are found only in this interval and are roughly distributed with the gene islands (G. Bakkeren and J. Kronstad, unpublished observations). In addition, if one focuses on the clusters of genes around the 258- and 332-kb positions in *MAT-1*, it appears that these genes (in an inverted orientation) represent the ends of the *U. maydis* 1.12 and 1.83 contigs that appear at *MAT-1* (Color Plate 2). Interestingly, these regions are flanked by exceptionally large clusters (10 to 20 kb) of the LTR1 element (Bakkeren and Kronstad, unpublished). These interpretations are therefore suggestive of the action of transposable elements in the translocation events that might have taken place in a common ancestor. It is also possible that the comparison has revealed rearrangement events that occurred in the *U. maydis* sequences after separation. Finally, given the highly repetitive nature of the *MAT-1* sequence, it is possible that some of the observed differences in gene order might be due to assembly errors.

An analysis of the sequence contigs carrying the mating-type regions of *U. maydis* was also performed, and it was found that these regions contained very few repetitive elements. Initially, the sequences of each of the different types of repetitive elements from *MAT-1* (LTRs, retroelements, and transposons) were used to search the *U. maydis* contigs and the complete genome sequence. Short sequences with weak similarity were found that were related to 2 of the 12 LTR elements from *MAT-1* and one of the transposon sequences. In addition, sequences related to each of the five retrotransposons from *MAT-1* were also present in the *U. maydis* genome. Interestingly, none of the sequences related to the elements from *MAT-1* were present on contigs 1.12, 1.13, and 1.83, which are associated with the *a* and *b* loci in *U. maydis*. A closer examination of these contigs identified very few repetitive sequences, and these were short (i.e., <100 bp in length). Taken together, these results indicate that the mating-type regions of *U. hordei* and *U. maydis* are dramatically different with a paucity of repetitive elements in *U. maydis* and approximately 50% representation of these sequences in the *MAT-1* region of *U. hordei*.

IMPLICATIONS OF BIPOLAR AND TETRAPOLAR MATING FOR PATHOGENIC FUNGI

The analysis of the genomic organization of the *a* and *b* gene complexes provides a simple explanation for the observed differences in the mating systems of smut fungi (5). However, this analysis also revealed the highly repetitive nature of the *MAT-1* sequence with repeated sequences spread across the whole genome of *U. hordei* (although the overall proportion is not known [8]). The accumulation of repetitive sequences in sex-determining regions of a variety of organisms is well documented, and there are clear examples in the fungi *Microbotryum violaceum* and *Cryptococcus neoformans*, which exhibit bipolar mating. Indeed, Hood (31, 33) has shown that the chromosomes carrying the mating-type locus in *M. violaceum* are dimorphic and rich in repetitive sequences. Specifically, sequence analysis of random genomic fragments revealed that DNA from the sex chromosomes was twice as likely to contain transposable elements than DNA from autosomal chromosomes. Overall, the genome of *M. violaceum* appears to contain 15% or more repetitive DNA (33). A similar situation exists with the bipolar *MAT* loci in *Cryptococcus* species, but the level of repetitive sequences appears to be lower at 13.2% for *MATa* and 17.3% for *MAT*α in *C. neoformans* var. *grubii* (21). This level can be compared with a

5% overall genome content of transposons, although this estimate comes from the *C. neoformans* var. *neoformans* genome (51). The shared features raise the possibility that a bipolar mating system might have contributed to the accumulation of repetitive elements at the mating-type locus and throughout the genome in these fungi. It has been postulated that the lack of "purifying recombination" in sex-determining regions leads to the accumulation of transposable elements and repeats (12), offering an explanation for the increased abundance of such elements compared to the rest of the genome.

The examination of the mating-type regions for the tetrapolar system of *U. maydis* revealed a striking departure from the paradigm of repeat accumulation (8). If *U. maydis* and *U. hordei* are truly representative, the results suggest that the *MAT* loci (sex chromosomes) in these species have evolved by quite different paths. Several possible contributing factors come to mind. For example, it is possible that the extant features are the result of differences in the potential for inbreeding versus outbreeding. For inbreeding species, a lack of different parental stretches of DNA for "purifying recombination" might sustain transposable element loads. Inbreeding may have an influence similar to that of asexuality with regard to the accumulation of transposable elements for *U. hordei*. Arkhipova and Meselson (2) hypothesized that, relative to asexual organisms, sexual activity may limit the proliferation of transposable elements within a genome even though new elements may be introduced through sex. Tetrapolar mating is generally thought to promote outbreeding because teliospore germination generates progeny that only have a one-in-four chance of being compatible. In *U. hordei*, one-half of the progeny from a teliospore would be compatible, thus potentially favoring inbreeding. Other possibilities for the observed differences between *U. hordei* and *U. maydis* could involve the interconnections between mating and the need for host infection to complete sexual development. For example, the length of the saprobic phase on the host surface before mating and initiation of infection might influence the window of opportunity for different smuts to find a compatible nonsibling partner. Thus, outbreeding potential could be influenced by inoculation density and the extent of mixing of gametes on the plant surface. This is further complicated by whether the smut species cause local or systemic disease after infection of seedlings, flowers, or older plants and whether infection occurs underground or aboveground. Thus, the differences in the pathogenic lifestyles of these fungi could have a major influence on mate detection. Additional features of importance include the role of the host genetic background in allowing completion of the sexual cycle after mating. Different smuts that infect small-grain cereals and grasses have the potential for interactions with compatible isolates of different species (e.g., *U. hordei* and *U. bullata*) on plants that serve as common hosts (18). However, even if successful cell fusion and dikaryon formation occur, the contributions of avirulence genes in the backgrounds of the mating partners may preclude successful sexual development because of a host defense response. A clear gene-for-gene system has not been described for *U. maydis*, while *U. hordei* and other smuts that infect small-grain cereals are genetically well characterized with regard to their avirulence genes. Finally, another consideration is that the different smut species may be more or less likely to bypass mating altogether because it is known that teliospore germination can directly lead to an infectious dikaryon (e.g., due to "partial or delayed [meiotic] reduction as described for *U. maydis* [14]).

The evidence to date with *U. maydis* suggests the hypothesis that species with tetrapolar mating have less repetitive DNA. An interesting analysis would be to trace repeat content in the genomes and at the mating-type loci in a set of smut species with different levels of evolutionary separation. Recent molecular phylogeny studies using ribosomal sequences and whole-genome scans for DNA length polymorphism profiles have started to resolve issues regarding taxonomic placement within the smuts (7, 54, 61, 76, 77). For example, *M. violaceum* resided previously in the genus *Ustilago* (*U. violacea*), *U. maydis* has been placed in the genus *Sporisorium*, and *U. hordei* is considered a representative of the "true" *Ustilago* species. These relationships and the emerging molecular view of smut phylogeny will be a valuable guide to choose species for further sequence analysis.

We have attempted to assess the evolutionary distance between *U. hordei* and *U. maydis*. A rough estimate comes from calculating mutation rates between several genes present on the recently sequenced *U. hordei MAT-1* locus (8) and homologs in the *U. maydis* database (MIPS: Munich Information Center for Protein Sequences, http://mips.gsf.de/genre/proj/ustilago). No precise clock exists for the smuts, but substitution rates at fourfold degenerate sites were estimated at 2.2 $\times 10^{-6}$ mutations per year per kb for mammals (45) and at 11 $\times 10^{-6}$ mutations per year per kb for *Drosophila* (78). In general, no good estimate exists for fungi, but some researchers have used 1 $\times 10^{-6}$ mutations per year per kb for the internal transcribed spacer

region, which might not be as precise (9). Assuming a "clock" of between 2.2×10^{-6} and 11×10^{-6} mutations per year per kb, our limited set of data would suggest the divergence between *U. maydis* and *U. hordei* to be between 21 and 27 million years (Bakkeren and Kronstad, unpublished).

As mentioned, there may be special evolutionary considerations for fungi that spend their lives intimately associated with their plant hosts and whose evolution was coincident with the evolution, domestication, and widespread monoculture cultivation of cereal crops. Interestingly, the timing of divergence of bipolar and tetrapolar mating systems might be coincident with the divergence of host plants. The cereal plants such as barley, corn, rice, and wheat are thought to have diverged from a common ancestor 50 to 70 million years ago. In thinking about parallel evolution of smuts and their hosts, one curious feature of the *MAT-1* locus is the discovery of gene islands in a sea of repetitive sequences. This organization is remarkably similar to the pattern found in the highly repetitive genomes of cereal crops (59, 65, 89). Overall, these observations reinforce the unique aspects of mating-type evolution in smut fungi in the context of interactions with host plants.

UNANSWERED QUESTIONS AND FUTURE WORK

Is *MAT* Carried on Sex Chromosomes in *U. hordei*?

It is clear that recombination is suppressed within the *U. hordei MAT* locus, and an open question is whether this suppression extends past *MAT* to include part or all of the chromosome arms. That is, the situation may be similar to that of *M. violaceum*, where a length polymorphism for the chromosomes harboring the two opposite mating-type loci, that is, dimorphic sex chromosomes, has been described (31). The absence of recombination has also been reported for almost the entire chromosome carrying the mating-type locus in *Neurospora tetrasperma* (24, 55).

Does the Genome of *U. hordei* Contain Fewer Genes than That of *U. maydis*?

The abundance of repetitive sequences identified at the *MAT-1* locus potentially extends throughout the genome of *U. hordei* (8). The genome sizes for *U. hordei* (~19.6 Mb) and *U. maydis* (~20.5 Mb) are similar (at least by the inaccurate method of electrophoretic karyotyping), so it would be interesting to compare the overall level of repetitive DNA, particularly in the context of potential deleterious effects of transposable elements. There is clear evidence for gene disruption by elements within *MAT-1* (8), and it would be interesting to determine whether *U. hordei* has fewer genes overall or fewer active genes. The prediction from the analysis of the *U. hordei* genome is that other small-grain smut fungi will also have repetitive genomes.

What Is the Evolutionary History and Level of Activity of the Transposable Elements at the *MAT-1* Locus?

The abundance of repeats at the *MAT-1* locus provides a rich opportunity to examine the biology of transposable elements in fungi. In particular, there is an opportunity to use the elements to trace patterns of activity. For example, some elements may be preferentially inserted into others in *MAT-1* and such a pattern has been described for transposable elements in *Magnaporthe grisea* where Maggy and MGL (*M. grisea* LINE retrotransposon) are found inserted into *Pot2*, but the reciprocal arrangement is not observed (83). More detailed analysis of the variation in the *MAT-1* LTR sequences, both solo and associated with retrotransposons, may provide insight into the evolutionary history of the elements and of different parts of the *MAT* locus. In particular, the examination of intraelement LTR sequences may shed light on whether certain elements may have transposed more recently than others. Finally, Hood et al. (34) presented evidence for ripping in *M. violaceum*. This process could contribute to the inactivation and control of transposable elements, and the occurrence of ripping at *MAT-1* needs to be examined in *U. hordei*.

How Do the *MAT-1* and *MAT-2* Regions Differ in *U. hordei*?

The *MAT-2* locus is estimated to be 430 kb in length, and this region remains to be sequenced for comparison with *MAT-1*. This type of comparative analysis has been quite informative for the *MATa* and *MATα* loci of *Cryptococcus neoformans* (22). Hood et al. (32) also presented evidence that the A1 and A2 sex chromosomes in *M. violaceum* contain different densities of functional genes. One could imagine that *MAT-2* contains the same genes as *MAT-1* or that rearrangements have resulted in the complement of genes identified at *MAT-1* being distributed elsewhere in the genome with some retained at *MAT-2*. Related questions include how many of the genes at *MAT-1* and *MAT-2* are essential, how many are actually involved in mating or virulence, and how many are pseudogenes? The evidence for insertion of LTRs and transposable elements into some of the genes in *MAT-1* raises the question of whether functional copies exist

elsewhere in the genome or whether the strains have lost specific metabolic functions. The local movement of transposable elements might explain the linkage between proline auxotrophy and mating type in some smut fungi such as *U. nuda* (56).

The reasons for suppression of recombination for the *MAT-1* and *MAT-2* intervening sequences also need to be explored. One initial experiment would be to use a *MAT-1* strain and swap *a2* for *a1* and *b2* for *b1* to create a strain that would potentially mate with a *MAT-1* (*a1 b1*) strain but that would have the same intervening region. The frequency of recombination could then be tested, and one could assess whether heterozygosity for genes contained with *MAT-1* and *MAT-2* is important for sexual development and virulence.

What Is the Evolutionary History of Bipolar and Tetrapolar Mating Systems in Smut Fungi?

The sequence comparisons for *U. hordei* and *U. maydis* suggest that the tetrapolar system is ancestral and gave rise to the bipolar system in the Ustilaginales. One wonders then whether the bipolar system arose once or several times and whether the history of changes in mating systems can be traced in the phylogeny of the smuts. The approach to answer some of these questions would be to link a detailed phylogenetic analysis (e.g., by multilocus sequence typing) with the selective analysis and sequencing of mating-type regions. In particular, the phylogeny of the unique genes at *MAT* would be interesting to compare among different smuts.

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