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# Conservation of the *b* Mating-Type Gene Complex among Bipolar and Tetrapolar Smut Fungi

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**In the phytopathogenic fungus *Ustilago hordei*, one locus with two alternate alleles, *MAT-1* and *MAT-2*, controls mating and the establishment of the infectious dikaryon (bipolar mating). In contrast, for *U. maydis*, these functions are associated with two different gene complexes, called *a* and *b* (tetrapolar mating); the *a* complex has two alternate specificities, and the *b* gene complex is multiallelic. We have found homologs for the *b* gene complex in *U. hordei* and have cloned one from each mating type using sequences from one *b*East allele of *U. maydis* as a probe. Sequence analysis revealed two divergent open reading frames in each *b* complex, which we called *b*W (*b*West) and *b*E (*b*East) in analogy with the *b* gene complex of *U. maydis*. The predicted *b*W and *b*E gene products from the two different mating types showed ~75% identity when homologous polypeptides were compared. All of the characterized *b*W and *b*E gene products have variable amino-terminal regions, conserved carboxy-terminal regions, and similar homeodomain motifs. Sequence comparisons with the *b*W1 and *b*E1 genes of *U. maydis* showed conservation in organization and structure. Transformation of the *U. hordei b* gene complex into a *U. hordei* strain of opposite mating type showed that the *b* genes from the two mating types are functional alleles. The *U. hordei b* genes, when introduced into *U. maydis*, rendered the haploid transformants weakly pathogenic on maize. These results indicate that structurally and functionally conserved *b* genes are present in *U. hordei*.**

## INTRODUCTION

The genus *Ustilago* (smut fungi), belonging to the Basidiomycetes, is composed of a group of phytopathogens that cause disease on a variety of plants, including monocotyledonous plants such as major cereal crops (Fischer, 1953; Fischer and Holton, 1957). Smut diseases are worldwide in distribution, have the potential to cause major outbreaks, and have major economic impact, especially in developing countries (Agrios, 1988; Gjaerum et al., 1988; Thomas, 1988).

The life cycles of the smut fungi are similar for all species, irrespective of the specific host plant(s) infected, and involve transitions between three cell types. Diploid teliospores are the resting cell type and are disseminated mainly by wind or rain splashes. They germinate by forming a probasidium on which, following meiosis, four basidiospores emerge. The haploid basidiospores represent the second cell type. Those cells grow by budding and can be cultured on defined media. Two basidiospores with compatible mating type will fuse to give rise to the dikaryotic, pathogenic cell type. This third cell type, which exhibits a mycelial growth, proliferates intercellularly in the host plant tissue. Depending on the fungal species, the infectious dikaryon can cause galls on any above-ground parts of the host plant, as is the case for *U. maydis* on maize (for reviews, see Christensen, 1963; Banuett and Herskowitz, 1988; Froeliger and Kronstad, 1990; Banuett, 1992), or, alternatively, can grow asymptomatically with the developing floral meristem and invade the newly formed embryonic tissue, as in the case of *U. hordei* on barley (Thomas, 1988, 1991). Karyogamy takes place in the dikaryotic mycelium and diploid teliospores are formed within the host tissues. This sporulation will give the maturing galls or infected cobs on a maize plant a black, sooty appearance, or will replace all the kernels of the spikes on a barley plant.

One of the most interesting differences within the group of smut fungi is the presence of at least two mating systems (Holton et al., 1968). Many of the species, such as *U. hordei* (covered smut of barley and oats), possess a bipolar mating system in which a single mating-type gene complex with two alternate specificities has been described to control mating and the establishment of the pathogenic dikaryon. The two alternate mating-type specificities have been called "a" and "A." However, because no dominance from one over the other is exerted, they will be called, more appropriately, *MAT-1* and *MAT-2*, following the new genetic nomenclature as proposed by Yoder et al. (1986) and as suggested by Thomas (1991) for

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smut fungi (see Methods). In contrast, species such as *U. maydis* (boil smut of maize), display tetrapolar mating in which two gene complexes control mating and formation of the infectious dikaryon. One gene complex, called *a* in *U. maydis*, has two alternate specificities and controls fusion, and a second gene complex, called *b*, with at least 25 specificities in nature, controls the establishment of the pathogenic dikaryon (Rowell and DeVay, 1954; Holliday, 1961; Puhalla, 1970; Day, 1974; Banuett and Herskowitz, 1989). It is of importance to understand the structure and function of these mating-type gene complexes because they play a key role in the formation and maintenance of the infectious cell type and hence pathogenicity.

In recent years, several of the multiallelic *b* gene complexes of *U. maydis* have been isolated, and it has been shown that this complex consists of two divergently transcribed genes (Kronstad and Leong, 1989, 1990; Schulz et al., 1990; Gillissen et al., 1992). One open reading frame (ORF), called *b*W (for *b*West), encodes a 626–amino acid polypeptide, and the second ORF, called *b*E (for *b*East), encodes a polypeptide of 473 amino acids. *b*W does not share sequence similarity with *b*E, except for a homeodomain region characteristic of regulatory proteins (Scott et al., 1989; Kües and Casselton, 1992), but has a similar structure. That is, alignment of the different alleles of either *b*W or *b*E indicated variable amino-terminal and conserved carboxy-terminal regions. Gillissen et al. (1992) have shown by genetic means that the presence of one *b*E and one *b*W ORF from either compatible parent is required to establish a filamentous, pathogenic dikaryon. It was hypothesized that the *b*W and *b*E gene products interact, and that formation of a bW–bE heterodimer is instrumental in the switch from growth by budding to filamentous, pathogenic growth. Yee and Kronstad (1993) have shown that allelic specificity for two *b*E alleles lies in a hypervariable amino-terminal region extending from amino acid 39 to 87. In *U. maydis*, the *b* gene complex has been shown to be essential for pathogenicity on its host plant, because null mutants having the entire *b* gene complex deleted, although viable, fail to establish a pathogenic dikaryon when mated with a wild-type, compatible strain (Kronstad and Leong, 1989; Schulz et al., 1990; Gillissen et al., 1992).

Recently, the two alleles from the *a* gene complex have been isolated (Froeliger and Leong, 1991; Bölker et al., 1992). The two alleles have large blocks of nonhomologous regions and should therefore be termed idiomorphs (Glass et al., 1988; Metzenberg and Glass, 1990). Each mating-type idiomorph harbors two genes. These genes appear to encode mating-specific pheromones and pheromone receptors, based on comparisons with pheromone and pheromone receptor genes in yeast (Bölker et al., 1992).

We have previously shown, in a survey of 23 different fungal strains (Bakkeren et al., 1992), that hybridization probes from the *U. maydis a* and *b* genes recognize homologs in the genomic DNA of all smut fungi tested. Based on these data, we have concluded that other small grain–infecting smut fungi from the bipolar group (e.g., *U. kolleri*, *U. avenae*, *U. nigra*, and *U. bullata*) possess *b* homologs. Preliminary molecular data suggest that very similar *b* gene complexes are present in this group (G. Bakkeren and J. W. Kronstad, manuscript in preparation). The members of this group are all interfertile and apparently differ primarily in host range and spore morphology. It has been proposed to unite these species into the single species *U. segetum*, with two morphologically different varieties, *U. segetum* var *avenae* and *U. segetum* var *hordei* (Huang and Nielsen, 1984). It appears that *U. hordei* is a good representative of this group because extensive genetic experimentation has been described for this fungus (Thomas, 1988, 1991).

Here, we report the isolation of a *b* gene complex from both mating types, *MAT-1* and *MAT-2*, of *U. hordei*. We have demonstrated that these complexes harbor functional alleles and possess structural and functional conservation compared with the *U. maydis b* gene complex.

## RESULTS

### *U. hordei* Has *b*W and *b*E Homologs

A *U. maydis*–derived DNA probe corresponding to the carboxy-terminal, constant domain of the bE1 protein, was found to hybridize under moderately stringent conditions to genomic DNA of *U. hordei* (Bakkeren et al., 1992). The probe revealed a single restriction fragment, different in size, in (haploid) strains of the opposite mating type: that is, a 1.5-kb BamHI fragment in a *MAT-2* strain (Uh100) and a 2.8-kb BamHI fragment in a *MAT-1* strain (Uh112). Figure 1 shows the respective fragments as inserts of plasmid clones pUhbE2 and pUhbE1. The two *U. hordei* strains can mate successfully by coinoculation on plates containing rich medium supplemented with activated charcoal (Holliday, 1974). These mating reactions result in the appearance of the characteristic mycelial cells, indicative of the dikaryotic, pathogenic cell type. Figures 2L, 2M, and 2N show the corresponding, characteristic phenotypes that can be compared with a typical mating reaction of *U. maydis* in Figures 2A, 2B, and 2C.

We constructed two subgenomic libraries from the *MAT-2* strain (Uh100) and the *MAT-1* strain (Uh112) and, using the *U. maydis* carboxy-terminal *b*E1 probe, identified clones pUhbE2 and pUhbE1, harboring the 1.5- and 2.8-kb BamHI fragments, respectively (Figure 1; see Methods). Subsequently, the nucleotide sequence of these fragments was determined. Small differences were found between these cloned fragments, and each shared ~58% nucleotide homology with the *U. maydis* probe, suggesting that we had isolated parts of two different alleles of a *b*E-like gene from *U. hordei*. The complete *b* homolog–containing regions have been isolated from two strain-specific cosmid libraries made from genomic DNA of a *U. hordei MAT-1* strain (Uh112) and a *MAT-2* strain (Uh100) in the *Ustilago*-specific cosmid vector pJW42 (see Methods).

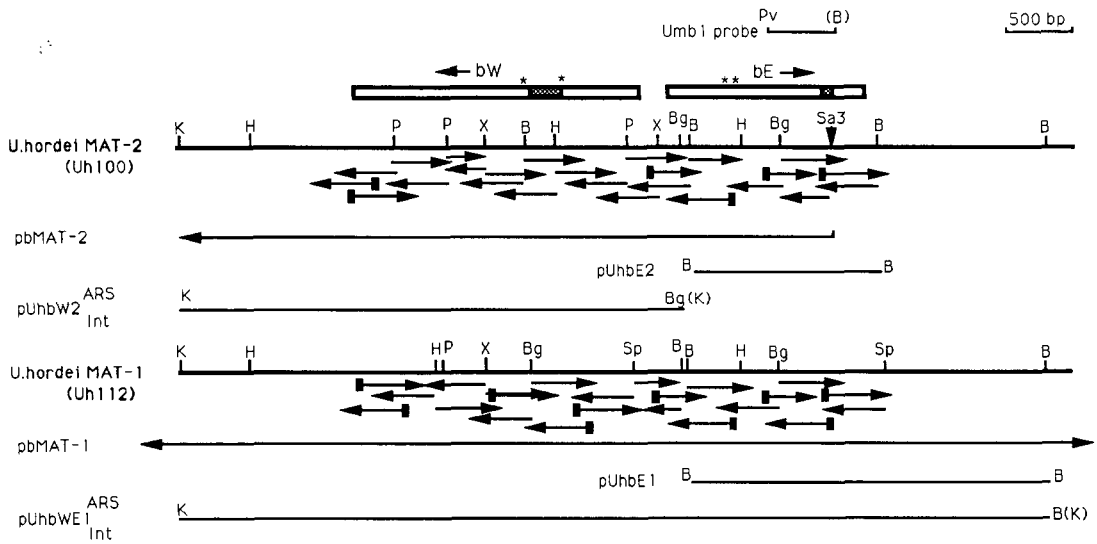


**Figure 1.** Maps and Genomic Organization of the *U. hordei* *b*W and *b*E Genes and Derived Plasmid Constructs.

The bold lines represent the genomic maps of *U. hordei MAT-2* (Uh100) and *MAT-1* (Uh112), respectively, as mapped using cosmid and plasmid clones as well as gel blot hybridization analysis of genomic DNA. Cosmid clone pbMAT-2 extends from the Sau3A site (Sa3) leftward in the direction of the arrow; cosmid clone pbMAT-1 contains the complete region shown (double-sided arrow). Only restriction sites used for cloning are shown: B, BamHI; Bg, BglII; H, HindIII; K, KpnI; P, PstI; Pv, PvuII; Sp, SphI; X, XhoI. The sequencing strategy is given by the small arrows below each genomic map (see Methods). Sites within parentheses are derived from polylinkers during subcloning; the BglII site in pUhbW2 was eliminated during subcloning. ARS and Int indicate that the particular fragment was cloned in an episomal vector or integrative vector, respectively (see Methods). The open bars represent the ORFs designated *b*W and *b*E, respectively, with their respective direction of transcription indicated by the arrows. The stippled boxes indicate the position of the potential introns, and stars mark the homeodomains. The *U. maydis* *b*E1 carboxy-terminal probe (Umb1 probe) used for screening the libraries is indicated.

While mapping these two *U. hordei* cosmid clones, pbMAT-1 and pbMAT-2, with specific *U. maydis* *b*W1 or *b*E1 DNA probes, it became clear that both *b*W and *b*E homologs were present. However, a small 3′ region of the *b*E homolog from clone pbMAT-2 was missing due to a cloning strategy that used genomic DNA partially digested with Sau3A. In analogy to the genes from the *U. maydis b* gene complex (Um*b*W and Um*b*E), we will call the homologs from *U. hordei* Uh*b*W and Uh*b*E. Figure 1 shows a restriction map of the genomic region comprising the *U. hordei b* gene complex for each strain. We completed the nucleotide sequence of the remaining parts of the *b*E homologs and the *b*W homologs from both cosmid clones. Comparison of the nucleotide sequences of the *b* homologs from the two *U. hordei* cosmid clones reveals a remarkable overall homology of 80%. Figures 3 and 4 show the distribution of these homologies between the *b*W and *b*E homologs, respectively. The overall homology between the *U. hordei* homologs of either strain and the *b1* gene complex of *U. maydis*, however, is ~50% (data not shown).

As depicted in Figures 5A and 5B, inspection of the amino acid sequences revealed two divergent ORFs in each cosmid clone as was found for *U. maydis* bW1 and bE1 (Kronstad and Leong, 1990; Schulz et al., 1990; Gillissen et al., 1992; J. W. Kronstad, unpublished data). The predicted bW and bE polypeptides of the *MAT-1* strain (Uh112) and the *MAT-2* strain (Uh100) are very similar in overall homology and organization when each pair of homologs is compared. When aligned with the predicted *U. maydis* b1 polypeptides, it is clear that the organization is very similar, but that there are fewer amino acid identities (Figures 5A and 5B). When we compared the *b* gene complexes from the two *U. hordei* strains, we found that both predicted bW and bE polypeptides have a variable amino terminus and a conserved carboxy terminus. This is also found when different *b* alleles of *U. maydis* are compared (Kronstad and Leong, 1990; Schulz et al., 1990; Gillissen et al., 1992). Table 1 summarizes homologies in amino acid sequences in these variable and constant domains between the different polypeptides. Overall, these results indicate that we have indeed cloned different alleles of the *b* gene complex from two *U. hordei* strains of opposite mating type. We propose to call these alleles Uh*b*W1 and Uh*b*E1 from *U. hordei MAT-1* (Uh112) and Uh*b*W2 and Uh*b*E2 from *U. hordei MAT-2* (Uh100).

When we consider the Uh*b*W alleles (Figure 3) and their presumed protein products (Figure 5A), we have to assume a 262-bp intron for Uh*b*W2 (between positions −620 and −884) and a 285-bp intron for Uh*b*W1, because these introns separate the conserved homeodomain consensus sequence WFqN-R (Scott et al., 1989; Gillissen et al., 1992). The sequence of the 5′ splice junction (GG′GT) as well as the 3′ splice junction (AG′TT) is conserved among the Uh*b*W alleles

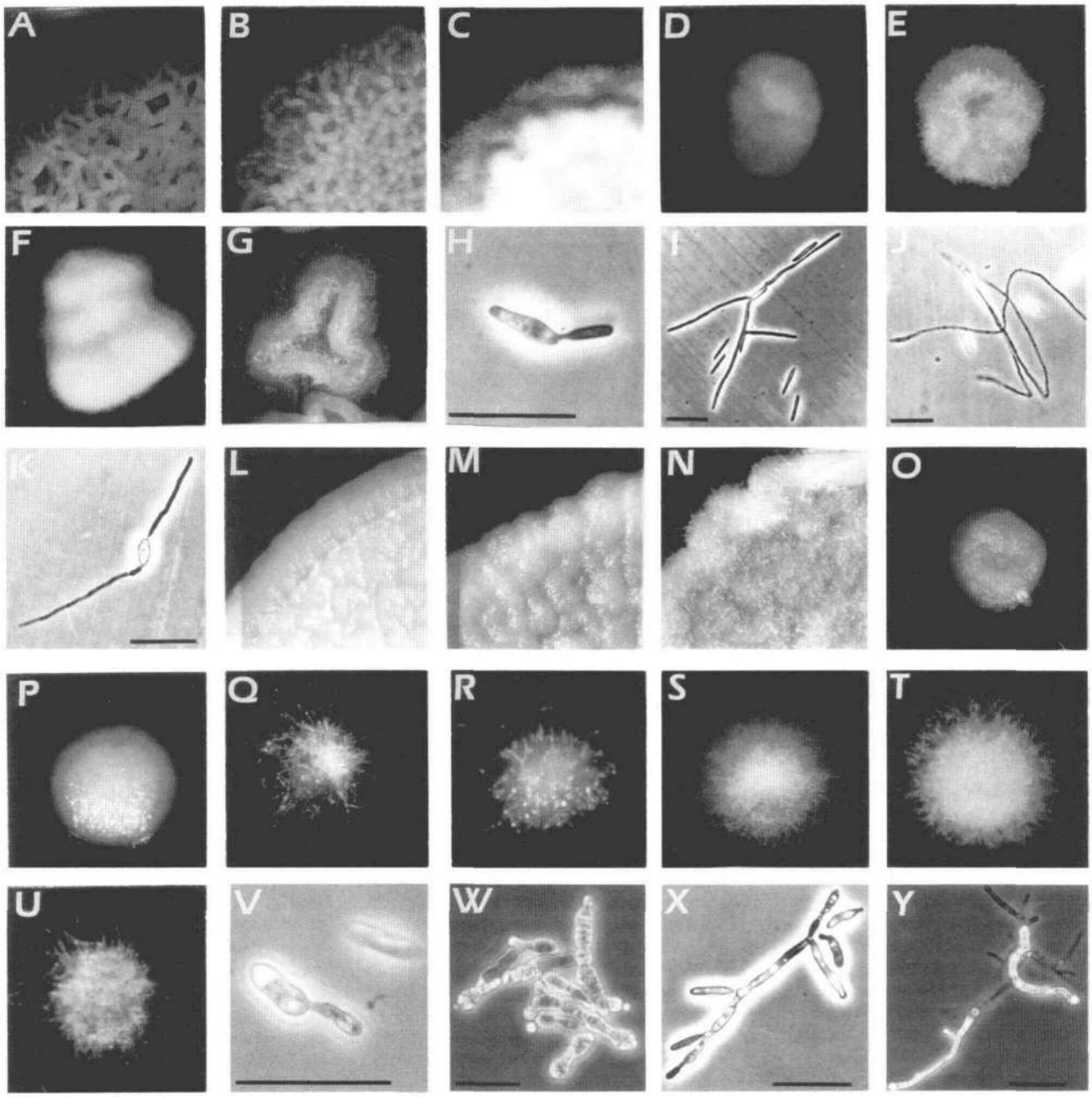


**Figure 2.** Colony and Cell Morphology of Representative Transformants.

The episomal, ARS-containing plasmid constructs introduced into the strains shown are given between parentheses below; brackets indicate stably integrated constructs lacking an ARS (see Figure 1 and Methods). For growth conditions, see Methods. Colonies, 12× magnification. Bars = 25 μm.

**(A)** Sector of a colony of *U. maydis* 518(*a2b2*, pJW42).
**(B)** Sector of a colony of *U. maydis* 521(*a1b1*, pJW42).
**(C)** Sector of a colony from coinoculation of Um518(pJW42) + Um521(pJW42).
**(D)** Colony of transformant Um518(pbMAT-1).
**(E)** Colony of transformant Um518(pUhbWE1).
**(F)** Colony of transformant Um518[pUhbWE1].
**(G)** Colony of transformant Um518[pb1] (See Table 2, footnote f).

and the *U. maydis* *b*W2 allele (Gillissen et al., 1992). Although there could be several alternative start codons, the alignment, as presented in Figure 5A, is optimal in terms of protein length. These alignments indicate that Uh*b*W2 codes for a potential protein product of 647 amino acids, and Uh*b*W1 for a putative 646-amino acid polypeptide.

The Uh*b*E alleles also have a potential intron of 81 bp between positions 1204 and 1286 (Figure 4). Comparison of the predicted polypeptides UhbE2 and UhbE1 with the *U. maydis* *b*E1 gene product, for which the position of the intron has been confirmed (Schulz et al., 1990), reveals a continuous stretch of 27 additional amino acids (Figure 5B). Tentatively, we concluded that the Uh*b*E2 gene codes for a polypeptide of 477 amino acids and the Uh*b*E1 gene encodes a 469-amino acid polypeptide. An unspliced intron, though, would leave the putative protein products in frame and add 27 amino acids. The homeodomain motif WFiN-R is conserved among all *b*E alleles of both *U. hordei* and *U. maydis* (Figure 5B). Peculiar is the finding of a 24-bp direct repeat in the Uh*b*E2 gene (between positions 890 and 915 in Figure 4) resulting in a (repeated) stretch of eight additional amino acids that does not line up with either Uh*b*E1 or *U. maydis* *b*E1 (Figure 5B). Direct sequencing of the cosmid clones pbMAT-2 and pbMAT-1 with a specific oligonucleotide primer gave the same result, excluding the possibility that this repeat is an artifact of subcloning.

Comparison of the intergenic region between the two *U. hordei b* genes from both mating types (Figure 4) and the intergenic region between *b*W1 and *b*E1 of *U. maydis* did not reveal conserved sequences. Because it is likely that this region contains divergent promoters and specific promoter elements, it was anticipated that such homologous elements would be found in the comparisons. Whether this suggests a regulation of the *U. hordei b* genes that is different from *U. maydis* remains to be investigated.

### The *U. hordei b* Homologs Influence Filamentous Growth in a Mating-Type-Specific Manner

We wanted to test whether the cloned *U. hordei* *b*W and *b*E genes exhibited the same functional properties as the homologs from *U. maydis*. Specifically, these genes should induce mycelial growth, resulting in a fuzzy phenotype, when introduced into a haploid strain carrying *b* genes with a different allelic specificity (Kronstad and Leong, 1989).

To manipulate *U. hordei* more easily, we developed an efficient transformation procedure utilizing electrotransformation of partial spheroplasts (see Methods). Figure 2L shows the haploid *U. hordei* strain Uh100 (*MAT-2*) and Figures 2M and 2O show strain Uh112 (*MAT-1*) that were all transformed with the cosmid vector pJW42. The smooth colony phenotype indicates that the vector alone has no effect on colony morphology (or cell morphology; data not shown). When mated (Figure 2N), the characteristic fuzzy phenotype is observed. When cosmid clone pbMAT-1, harboring the Uh*b*1 gene complex, is introduced into the homologous strain Uh112, no effect is seen on either colony morphology (Figure 2P) or cell morphology (Figure 2V). However, cosmid pbMAT-2, carrying the *b*W2 and *b*E2 alleles derived from strain Uh100, when introduced in strain Uh112, induces vigorous mycelial growth (Figure 2Q). Similar results were obtained when cosmid pbMAT-1 was introduced into strain Uh100 (data not shown). It should be noted that cosmid pbMAT-2, which is missing the coding region for the last 70 carboxy-terminal amino acids of the bE2 polypeptide, can still induce mycelial growth when introduced

**Figure 2.** (continued).

**(H)** Budding cell of Um518(pJW42).
**(I)** Filamentous growth of Um518(pbMAT-1).
**(J)** Filamentous growth of Um518(pUhbWE1).
**(K)** Filamentous growth of Um518[pUhbWE1].
**(L)** Sector of a colony of *U. hordei* Uh100(*MAT-2*, pJW42).
**(M)** Sector of a colony of *U. hordei* Uh112(*MAT-1*, pJW42).
**(N)** Sector of a colony from coinoculation of Uh100(pJW42) + Uh112(pJW42).
**(O)** Colony of transformant Uh112(pJW42).
**(P)** Colony of transformant Uh112(pbMAT-1).
**(Q)** Colony of transformant Uh112(pbMAT-2).
**(R)** Colony of transformant Uh112(pUhbW2).
**(S)** Colony of transformant Uh112[pUhbW2].
**(T)** Colony of transformant Uh100[pUhbWE1].
**(U)** Colony of transformant Uh112(pUmbWE1).
**(V)** Budding cell of Uh112(pbMAT-1).
**(W)** Filamentous growth of Uh112(pUhbW2).
**(X)** Filamentous growth of Uh100[pUhbWE1].
**(Y)** Filamentous growth of Uh112[pUhbW2].

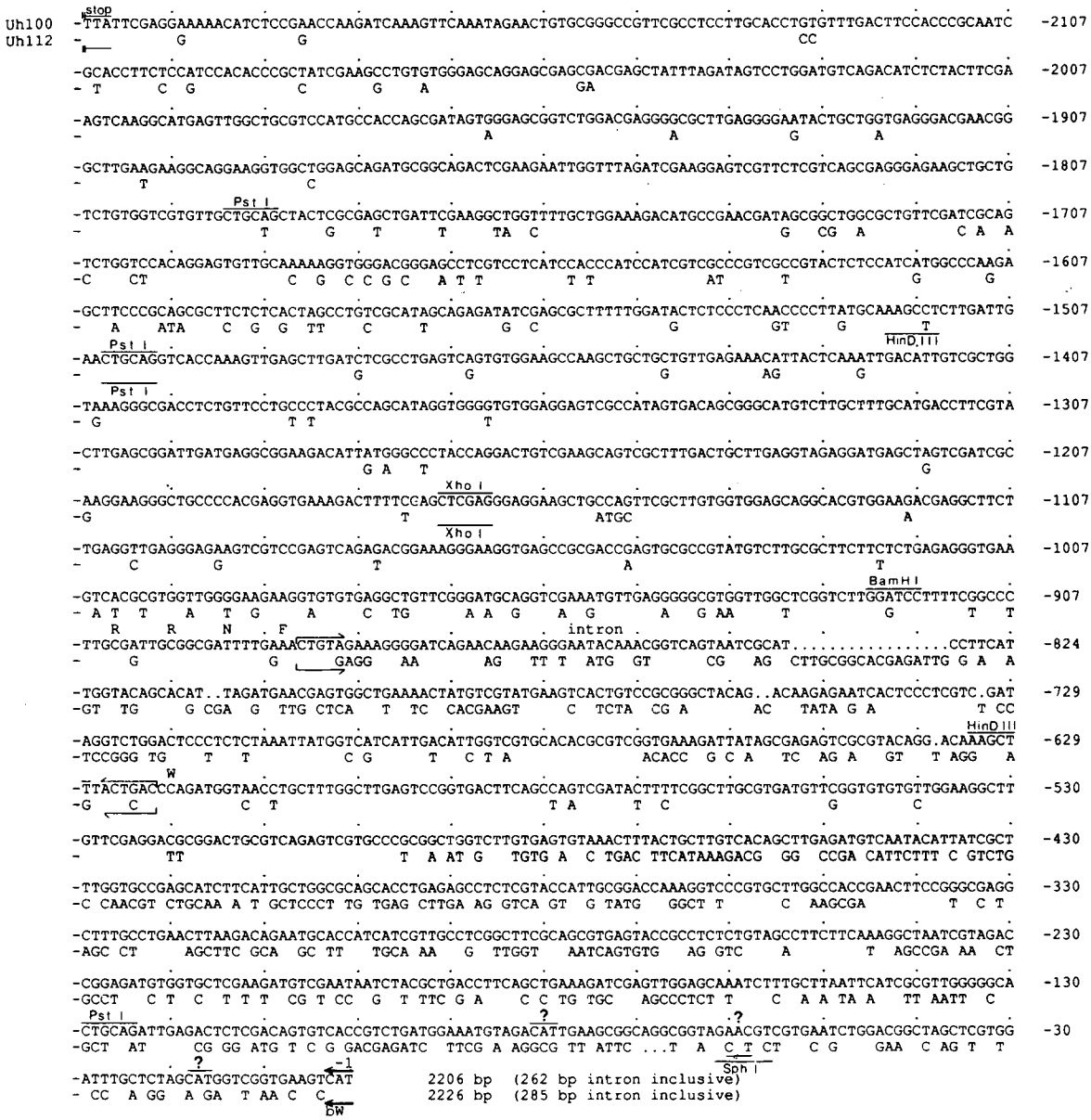


**Figure 3.** Comparison of the Nucleotide Sequences of the Two *b*W Alleles of *U. hordei MAT-2* and *MAT-1*.

The top line represents the nucleotide sequence of the *b*W gene of Uh100 (*MAT-2*) from the stop codon (position −2206) to the start codon (position −1). Note that the noncoding strand is shown to allow continuous alignment with the Uh*b*E2 gene of Figure 4 (compare Figure 1), hence the negative numbering. Other potential start codons are labeled with a bar and a question mark. The sequence is compared with the *b*W allele of Uh112 (*MAT-1*) in the bottom line and only nucleotides that differ are shown. Periods interrupting the sequence do not represent nucleotides but were introduced to allow optimal alignment. The intron position is indicated by the hooked arrows. The letters above the sequence (−619 and −884 to −904) represent the consensus amino acids of the homeodomain (Gillissen et al., 1992). Representative restriction sites are given above (*b*W2) and under (*b*W1) the sequence. The EMBL data base accession numbers for the nucleotide sequences of pbMAT-1 (Uh*b*W1 + Uh*b*E1) and pbMAT-2 (Uh*b*W2 + Uh*b*E2) are Z18532 and Z18531, respectively.

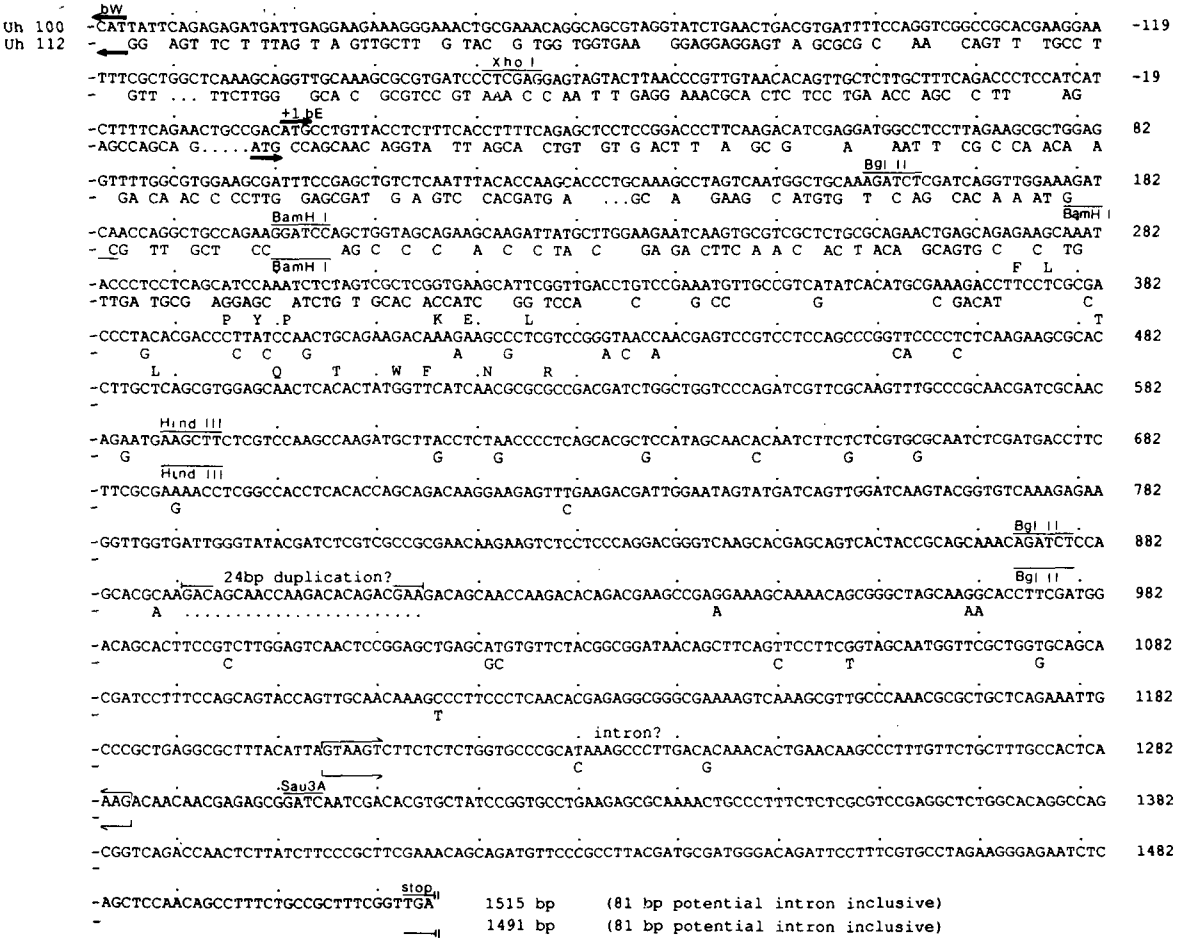


**Figure 4.** Comparison of the Nucleotide Sequences of the Two *b*E Alleles of *U. hordei MAT-2* and *MAT-1.*

The top line represents the nucleotide sequence of the *b*E gene of Uh100 (*MAT-2*) from the start codon (position +1) to the stop codon (position 1515) and is preceded by the intergenic region. The start codon of the Uh*b*W2 gene is at position −215 in the noncoding orientation; this codon is shown at the bottom of Figure 3 at position −1 and serves as the position of overlap between the sequences shown here and in Figure 3. Other symbols are as given in Figure 3. Note the Sau3A site in the Uh*b*E2 gene at position 1301; this is the site at one end of the insert in cosmid pbMAT-2 (Figure 1).

in strain Uh112. Whether or not a truncated UhbE2 polypeptide is involved in the switch from growth by budding to filamentous growth in these transformants, or whether the UhbW2 polypeptide alone is responsible, is not known. It has been noted before that the carboxy-terminal part of the *U. maydis* bE1 or bE2 polypeptides is not absolutely essential for biological activity (Kronstad and Leong, 1990; Schulz et al., 1990).

Because we did not know whether cosmid clones pbMAT-2 and pbMAT-1 contained functions other than those attributed to the *b* alleles, which could have caused the effects we observed, we constructed subclones carrying only the *b*W and *b*E genes of strain Uh112, or only the *b*W gene of strain Uh100. The fragment carrying the *b*W1 + *b*E1 genes was subcloned in either an episomal vector, harboring a *Ustilago*-specific autonomously replicating sequence (ARS), or in an integrative vector lacking an ARS. Similarly, the fragment harboring the *b*W2 gene alone was subcloned in an episomal vector or into an integrative vector (see Figure 1 and Methods). Episomal plasmid pUh*b*W2 provoked a fuzzy colony phenotype when introduced into strain Uh112 (Figure 2R), and induced a cell growth pattern very different from normal budding division, but more reminiscent of "filamentous growth" (Figure 2W; compare to Figure 2V). When we stably transformed Uh112 with

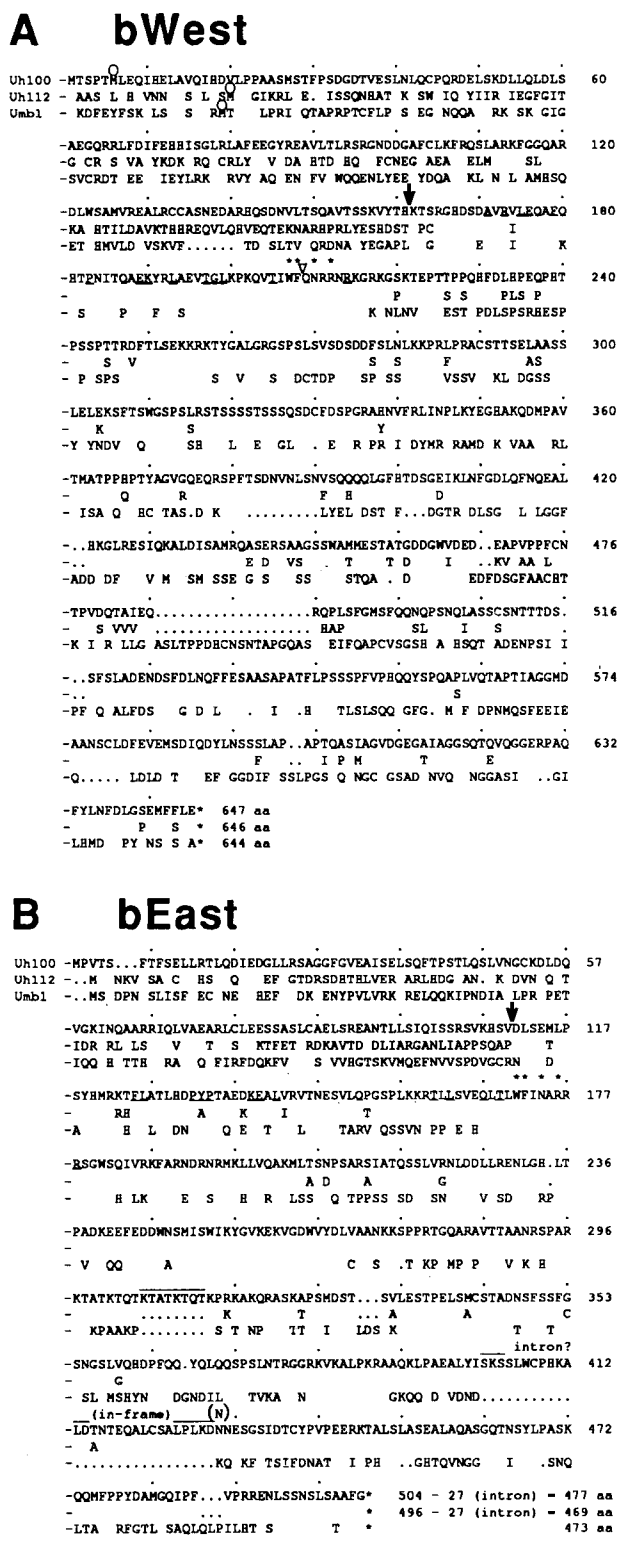


**Figure 5.** Comparison of the Amino Acid Sequences of the Predicted *b* Gene Products of *U. hordei MAT-2*, *U. hordei MAT-1*, and *U. maydis* b1.

construct pUhbW2, the transformant also had a fuzzy phenotype (Figure 2S) and displayed filamentous growth in liquid medium (Figure 2Y).

We conclude that the *b*W2 gene by itself can induce mycelial growth when introduced in strain Uh112, and, moreover, has an allelic specificity different from this strain. As expected, construct pUhbWE1, carrying just the two *b*W1 + *b*E1 genes, induced a fuzzy phenotype in strain Uh100 when stably integrated in the genome (Figure 2T), and also changed the normal growth pattern by budding, resulting in filamentous growth (Figure 2X). Table 2 shows a summary of all the (reciprocal) combinations of *b* alleles and strains tested for colony phenotype. We observed that the haploid *U. hordei* transformants grew much more slowly when harboring different *b* alleles, compared to transformants carrying additional identical *b* alleles or vector sequences alone.

### *U. hordei b* Homologs Trigger Filamentous Growth in *U. maydis*

The similarity between the *b*W and *b*E alleles from *U. hordei* and *U. maydis*, as far as nucleotide and amino acid sequences and organization of the genes are concerned, prompted us to investigate whether this meant functional conservation as well. That is, are the products of the *b* alleles of *U. hordei* capable of interacting with their homologs in *U. maydis* and do they possess an allelic specificity different enough to provoke filamentous growth and possibly to induce pathogenicity? We found that each of the *U. hordei*–derived cosmid clones pbMAT-2 and pbMAT-1 was capable of inducing a mycelial, fuzzy phenotype when introduced into either of two haploid *U. maydis* strains of opposite mating type, Um518 (*a2b2*) and Um521 (*a1b1*). The same *U. hordei b* allele subclones described

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**(A)** bWest. Representation of the predicted amino acid sequence of the Uh*b*W2 gene from *U. hordei* Uh100 (*MAT-2*)(top line), the Uh*b*W1 gene from Uh112 (*MAT-1*)(middle line), and of the *U. maydis b*W1 gene (bottom line). Only residues that differ from Uh*b*W2 are shown. Periods interrupting the sequence do not represent amino acids but were introduced to allow optimal alignment. The numbers refer to the Uh*b*W2 sequence. Stars mark the homeobox motif; the underlined amino acids indicate additional consensus residues found in homeodomain-containing proteins (Gillissen et al., 1992). The position of the intron is given with an open inverted triangle. Open circles above methionine residues indicate alternative start sites.

**(B)** bEast. Representation of the predicted amino acid sequence of the Uh*b*E2 gene (top line), the Uh*b*E1 gene (middle line), and of the *U. maydis b*E1 gene (bottom line). For further explanation see **(A)**. The position of the potential intron in the *b*E genes of Uh100 and Uh112, as compared to *b*E of Umb1, is indicated by "intron ? (in-frame);" note that if the intron is spliced out, these 28 residues will be replaced by amino acid N, resulting in a loss of 27 amino acids (compare with Figure 4).

The vertical arrows indicate the (arbitrary) division of the polypeptides in variable and constant domains; aa, amino acids.

**Table 1.** Conservation of UhbW and UhbE Amino Acid Sequences

| | V Domain | | | | | | C Domain | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % Similarity | | | % Identity | | | % Similarity | | | % Identity | | |
| UhbW2 | 51 | 43 | | 29 | 25 | | 92 | 65 | | 88 | 46 | |
| UhbW1 | 51 | | 48 | 29 | | 28 | 92 | | 64 | 88 | | 46 |
| UmbW1 | | 43 | 48 | | 25 | 28 | | 65 | 64 | | 46 | 46 |
| UhbE2 | 51 | 46 | | 33 | 21 | | 96 | 68 | | 95 | 59 | |
| UhbE1 | 51 | | 48 | 33 | | 27 | 96 | | 68 | 95 | | 57 |
| UmbE1 | | 46 | 48 | | 21 | 27 | | 68 | 68 | | 59 | 57 |

The predicted polypeptides are compared with respect to their variable domains (V domain) considered, arbitrarily, to range for UhbW2 from amino acid 1 to 161 inclusive (refer to the arrow in Figure 5A) and for UhbE2 from amino acid 1 to 110 inclusive (refer to the arrow in Figure 5B), and their constant domains (C domain) considered to be the remaining carboxy-terminal region. The two identical values in each vertical column refer to the two particular polypeptide regions that are compared. The data were generated using the GAP program of the Genetics Computer Group sequence analysis software package (Devereux et al., 1984).

above, that is, pUhbWE1 and pUhbW2 (both episomal and integrative; see Figure 1), were tested in the two *U. maydis* strains. In all cases they induced a mycelial, fuzzy phenotype (Table 2). Figures 2D, 2E, and 2F illustrate representative mycelial colony phenotypes. In liquid cultures, these haploid transformants display a filamentous growth pattern as shown in Figures 2I, 2J, and 2K, representing the corresponding cell morphologies, which can be contrasted with the normal budding division of a haploid strain transformed with the vector alone (compare to Figure 2H). Transformed *U. maydis* strains harboring integrated copies of *U. maydis b* alleles (with different specificity) or *b* genes from *U. hordei* display a fuzzy phenotype (Figure

**Table 2.** Colony Morphology and Pathogenicity of Transformants[a]

| *U. hordei* | | *U. maydis* | | |
|---|---|---|---|---|
| | Fuz | | Fuz | Pathogenicity[b] |
| Uh100(pJW42) | – | Um518(pJW42) | – | 0/15 |
| Uh112(pJW42) | – | Um521(pJW42) | – | 0/13 |
| Uh100(pJW42) × Uh112(pJW42) | + | Um518(pJW42) × Um521(pJW42)[c] | + | 4.6/12[d] |
| Uh100(pbMAT-2) | – | Um518(pbMAT-1) | + | 1.2/13 |
| Uh100(pbMAT-1) | + | Um518(pbMAT-2) | + | 1.1/16 |
| Uh112(pbMAT-1) | – | Um521(pbMAT-1) | + | 2.4/8[d] |
| Uh112(pbMAT-2) | + | Um521(pbMAT-2) | + | 2.8/11[d] |
| Uh100(pUmbWE1)[e] | + | Um518(pUhbWE1) | + | 0.2/9 |
| Uh112(pUmbWE1)[e] | + | Um521(pUhbWE1) | + | 3.0/9 |
| Uh100(pUhbWE1) | + | Um518(pUhbW2) | + | 0/12 |
| Uh112(pUhbW2) | + | Um521(pUhbW2) | + | 0/11 |
| Uh100[pUhbWE1] | + | Um518[pUhbWE1] | + | 1.3/11[d] |
| Uh112[pUhbW2] | + | Um521[pUhbW2] | + | 0/13 |
| | | Um518[pb1][f] | + | 0.9/15 |
| | | Um521[pb2][f] | + | 1.5/8 |

[a] *Ustilago hordei* (Uh) or *U. maydis* (Um) strains were transformed with different *U. hordei b* genes as indicated. pJW42 is the cosmid vector harboring a *Ustilago*-specific ARS. Plasmid designations between parentheses indicate episomal constructs harboring the same ARS; brackets indicate stably integrated plasmid constructs (see Figures 1 and 2). Fuz$^+$, mycelial growth; Fuz$^-$, growth by budding on DCM containing charcoal. Pathogenicity data shown are compiled from different inoculation series and should therefore not be compared with each other. *U. hordei* strains could not be tested for pathogenicity because they have an adenine-requiring mutation.

[b] Pathogenicity is calculated as an average disease rating per number of injected maize seedlings (inoculum is $10^8$ cells per mL; see Methods).

[c] Inoculum is $10^6$ cells per mL.

[d] Viable teliospores have been recovered from stem galls.

[e] pUmbWE1 contains both *b*W1 and *b*E1 genes of *U. maydis* on an episomal vector (see Methods).

[f] Strains described by Kronstad and Leong (1989) have the respective *b*E1 or *b*E2 gene of *U. maydis* integrated in their genome.

2G versus Figure 2F), suggesting that the *U. hordei* (Uh112) *b* gene complex by itself is recognized by *U. maydis* and is capable of inducing filamentous growth.

The reciprocal *b* gene complex exchange, that is, the *U. maydis* *b*W1 + *b*E1 genes on an episomal vector (pUmbWE1) introduced into either *U. hordei* strain *MAT-1* (Uh112) or *MAT-2* (Uh100), also yielded mycelial colonies (Figure 2U; Table 2). We caution that the degree of "fuzziness" between mycelial colonies should not be used as a measure of compatibility between *b* alleles, because we see some variation in colony morphology even among transformants from a particular transformation experiment. It is not known whether this phenomenon can be caused by a difference in copy number of the transgene.

The cell morphologies of the transformed, filamentous, haploid strains of *U. maydis* and *U. hordei*, heterozygous at the *b* gene complex (Figure 2), differ from the dikaryotic cells recovered from a fuzzy mating reaction between two wild-type strains of opposite mating type (data not shown). Those dikaryotic filaments are devoid of cytoplasm, except for the distal tip where sometimes a binucleated cell can be seen. These filaments cannot be cultured in liquid media, and the binucleated cells immediately fall apart into their haploid constituents reverting to growth by budding. In contrast, our haploid transformants, heterozygous only at the *b* gene complex, are stable in liquid media and do grow, albeit slowly, into multicellular filaments. These filaments are often branched and septate (e.g., see Figures 2J and 2K). Sometimes, after prolonged culturing, parts of the filaments appear empty, thereby clearly showing their septated nature (Figure 2J).

### *U. maydis* Transformants Containing *U. hordei b* Homologs Are Pathogenic

In *Ustilago* species, mycelial growth, seen as fuzzy colony morphology on charcoal plates, is mostly indicative of pathogenicity on the host plant. Haploid *U. maydis* transformants, which have an additional, different *U. maydis* *b*E allele integrated in their genome (e.g., Um518[pb1] and Um521[pb2]), display mycelial growth (Figure 2G) and are weakly pathogenic on maize (Table 2; Kronstad and Leong, 1989). Because our haploid *U. maydis* strains transformed with the various *U. hordei b* alleles displayed a mycelial phenotype, we tested the pathogenicity of these transformants on maize plants. Despite the correlation between mycelial growth and pathogenicity, the appearance of characteristic symptoms upon inoculation of 3-week-old maize seedlings, such as anthocyanin streaking on the leaves and induction of leaf and stem galls, was unexpected. Both *U. maydis* strains transformed with either *U. hordei* cosmid clone or the subclone pUhbWE1 carrying *U. hordei* *b*W1 + *b*E1 genes (either episomal or integrative) were weakly pathogenic with some variability observed between inoculation series. These transformants were comparable in terms of pathogenicity to the *U. maydis* strains Um518[pb1] and Um521[pb2] (Table 2).

The *U. hordei* *b*W + *b*E genes, whether episomal or integrated, apparently are capable of inducing pathogenicity. Moreover, three transformants of the 10 tested were able to induce stem galls and to produce viable teliospores (Table 2, footnote d). To date, we have failed to obtain symptoms using *U. maydis* strains transformed with the Uh*b*W2 gene alone, either when on an episomal vector or when integrated in the genome (Table 2). This could be due to a very weak pathogenicity that rarely gives visible symptoms, or the interaction could simply not be strong enough to establish a pathogenic cell type, even though morphological changes are observed. It is also possible that the Uh*b*W2 gene product cannot functionally interact with the *b*E gene product present in *U. maydis* strains Um518 and Um521. Because the cosmid clone pbMAT-2 does induce pathogenicity when introduced into either of the two *U. maydis* strains, we deduced that the Uh*b*E2 gene product can interact with the *U. maydis* *b*W gene product.

## DISCUSSION

We have described the cloning and characterization of the *b* gene complex from both mating types of *U. hordei.* Sequence analysis revealed two divergent ORFs in each complex, called *b*W and *b*E in analogy with *U. maydis*, with a high degree of conservation in nucleotide and amino acid sequences, and in gene organization, between the two mating types. Transformation of the *U. hordei b* gene complexes into *U. hordei* strains of opposite mating type showed that we have isolated two functional, allelic *b* gene complexes, Uh*b*W1 + Uh*b*E1 from a *U. hordei MAT-1* strain (Uh112) and Uh*b*W2 + Uh*b*E2 from a *MAT-2* strain (Uh100). We demonstrated that the *b* gene complexes of *U. hordei* function in haploid *U. maydis* strains to trigger a switch from growth by budding to mycelial growth and, moreover, to induce weak pathogenicity on maize plants. We have also found that the *U. maydis* *b*W + *b*E genes, when introduced by transformation, trigger filamentous growth in *U. hordei.* We can conclude that despite the fair degree of variability between the predicted b polypeptides of *U. hordei* and *U. maydis* (Table 1), they are obviously capable of interacting, possibly via their polypeptide products in the same manner as is proposed for their species-specific counterparts.

The existence of *b* homologs, involved in dikaryon formation and pathogenicity, has never been genetically demonstrated in the bipolar smut fungi. Our earlier hybridization results showed that homologous sequences were present in smut fungi with bipolar mating systems (Bakkeren et al., 1992). We have expanded on the hybridization results by characterizing *b* sequences from one representative smut fungus with bipolar mating, *U. hordei.* It is interesting that our molecular data reveal a *b* gene complex very similar in structure and function to the *b* complex of *U. maydis*, a fungus with a tetrapolar mating-type system. Thus, *b* functions are present in the bipolar smut fungi, but the genetic requirement for multiple alleles might be absent or refractory to genetic testing. It might be that the *b* functions are constitutive in the bipolar species and that cell fusion is sufficient to trigger formation of the infectious dikaryon.

If this were the case, *b* functions in haploid cells would not be sufficient to produce the filamentous, pathogenic phenotype because haploid strains grow by budding and are nonpathogenic. It is possible that the combination of two different *a* idiomorphs in the dikaryon somehow triggers the activity of the *b* genes. The *U. hordei b* genes by themselves, however, can induce filamentous growth when introduced into a haploid *U. hordei* strain carrying different alleles. We favor the hypothesis that an *a*-like complex and the newly discovered *b* gene complex are tightly linked. Fusion, controlled by the *a* complex, ensures interaction between two *b* complexes with different specificity and hence establishment of the filamentous, pathogenic dikaryon. Consequently, the mating-type (and pathogenicity) functions located on the *a* and *b* gene complexes will segregate as one complex "locus" in genetic tests. We are currently exploring this possibility.

Comparison of the two predicted polypeptides UhbW2 and UhbW1 reveals a lower amount of amino acid identity, especially in the amino-terminal 160–amino acid portion (Figure 5A), than among several *U. maydis* bW polypeptides (Gillissen et al., 1992). The same observation can be made when comparing bE polypeptides (Figure 5B; Kronstad and Leong, 1990; Schulz et al., 1990). It could be that during evolution many more amino acid changes were allowed in the *U. hordei b* alleles to ensure allelic specificity. This would be expected if the presumed *a* gene complex and the newly discovered *b* gene complex were linked.

The length of the bE and, probably, the bW polypeptides is conserved between the two species. Our comparisons suggest that the *U. maydis b*W1 allele codes for a 644–amino acid protein rather than the reported 626–amino acid protein, although alternative in-frame start codons might be used under certain conditions (Gillissen et al., 1992). These authors suggested a polypeptide of 625 amino acids as the product of the related *U. maydis b*W2 allele, because deletions immediately upstream of the preferred start codon (comparable with amino acid position 19 in Figure 5A) still gave a functional product. However, the authors indicated that an in-frame ATG at nucleotide position −58 of Um*b*W2 (comparable with amino acid position 1 in Figure 5A) showed the best fit to the consensus sequence preceding translation initiation in filamentous fungi.

The cell morphology and pathogenicity of haploid *U. hordei* and *U. maydis* strains transformed with different *b* alleles differ from those of dikaryotic cells. Normally, dikaryotic cells cannot be cultured in liquid media. Day and Anagnostakis (1971) have developed a way to force the dikaryotic state of *U. maydis* by culturing the mating partners, when each has a different auxotrophic requirement, in minimal medium. Under these conditions, it is possible to obtain slow-growing, branched mycelium, composed mainly of empty cells. Our haploid *U. hordei* and *U. maydis* strains transformed with different *b* alleles resemble such a forced dikaryon, except that more filaments retain their cytoplasm. Wild-type dikaryotic cells may need supporting host plant tissue or plant factors that have a stabilizing and/or mitogenic effect. Having two genetic complements in a single cell, each with (at least) a different *a* and *b* gene complex in the form of two haploid nuclei (often in close proximity), might be responsible for differences in morphology and pathogenicity. However, heterozygosity at both the *a* and *b* gene complexes is not sufficient because a haploid *U. maydis* cell, stably transformed with the opposite *a* gene complex and a (*U. maydis*) *b* gene complex with different allelic specificity, has a morphology and pathogenicity very similar to the haploid *U. maydis* transformants shown in Figures 2I, 2J, and 2K, which are heterozygous only at the *b* gene complex (L. Giasson and J. W. Kronstad, unpublished data). It is possible that plant host factors will have an influence on the stability and/or growth of our haploid transformants.

Dikaryotic cells are more pathogenic than transformed, haploid cells heterozygous at the *b* gene complex, although the latter cell type is able to complete its life cycle and to produce viable teliospores (Table 2; Kronstad and Leong, 1989). All progeny from teliospores derived from stable transformant Um518[pUhbWE1] appeared to have a genotype similar to that of the cells used as inoculum, that is, they all had retained the construct that rendered them phleomycin resistant and filamentous. When germinating teliospores originated from a cross between *U. maydis* strains Um518(pJW42) and Um521(pJW42) (both harboring the episomal cosmid vector pJW42; Table 2), all progeny cells seemed to have lost this plasmid.

Tsukuda et al. (1988) have reported instability of a similar, episomal vector containing the same ARS sequence. Normally present at ~25 copies per cell under selective conditions, the authors reported an 80% loss over 15 generations when cultured in liquid medium lacking selection. Obviously, without selection pressure, the cosmid vector is not retained in the cells that yield the teliospores. Surprisingly, we found that between 3 and 6% of the progeny from teliospores originating from transformants Um521(pbMAT-1) and Um521(pbMAT-2) (both harboring the episomal cosmid vector with different *U. hordei b* gene complexes) had retained these constructs. Cells heterozygous at the *b* gene complex might have a selective advantage for growth in the plant, and the b polypeptide heterodimer, thought to induce genes responsible for filamentous growth and pathogenicity, might also be required for nuclear fusion and spore formation or might at least function until late in spore development.

The discovery, isolation, and subsequent proof of function of the *b* gene complex from two strains representing the two mating types of *U. hordei*, a member of a group of smut fungi displaying bipolar mating, will aid in the development of a pathogen–host system that is representative of a variety of smut diseases. Unfortunately, the transformed *U. hordei* strains used in our study could not be used for pathogenicity tests on barley because they are derived from adenine-deficient laboratory strains, Uh112 and Uh100. However, it should be possible to create pathogenic haploid strains starting from wild-type strains (potentially even of all the other small-grain–infecting smut fungi of the bipolar group); these strains will be invaluable tools for the exploration of the genetics of interaction/resistance responses and pathogenicity.

Because we have previously found by hybridization analysis a homolog of the *a* gene complex of *U. maydis* in members of the bipolar group (Bakkeren et al., 1992) and in this study have proven the presence of *b* genes, this suggests that the distinction between bipolar and tetrapolar mating types may be due to limited genetic experimentation rather than the number of compatibility determinants involved.

## METHODS

### Strains

Strains Um521 (*a1b1*) and Um518 (*a2b2*) of *Ustilago maydis* have been described previously (Kronstad and Leong, 1989). Strains A100 and a112 of *U. hordei* are from the culture collection of the late C. Person, Department of Botany, University of British Columbia. Based on proposed genetic nomenclature for fungi (Yoder et al., 1986) and as described by Thomas (1991), the mating types of smut fungi with bipolar mating systems are designated *MAT-1* (formerly "A") and *MAT-2* (formerly "a"). Note that the "a" mating type of the naturally occurring, proline-requiring strains of *U. nuda*, which would score as "A" mating type against our tester strains (collection of C. Person), is the *MAT-2* standard. Thus, we now designate *U. hordei* a112 as Uh112 (*MAT-1, ade*) and *U. hordei* A100 as Uh100 (*MAT-2, ade*). *U. maydis* strains were grown at 30°C in potato dextrose broth (Difco) or double complete medium (DCM; Holliday, 1974) supplemented with 300 μg/mL hygromycin B (Calbiochem) or 10 μg/mL phleomycin (Sigma) as required. *U. hordei* strains were grown at 22°C in either potato dextrose broth or DCM supplemented with 200 μg/mL hygromycin B or 10 μg/mL phleomycin. Libraries and plasmid constructs were propagated in *Escherichia coli* DH5α (Bethesda Research Laboratories).

### Mating and Pathogenicity Tests

Successful mating between two cells harboring two opposite *a* complexes and two different *b* gene complexes, or the molecular interaction between two different *b* gene complexes within one transformed, haploid cell, gives rise to a mycelial phenotype ($Fuz^+$) when strains are inoculated on DCM plates containing 1% activated charcoal (incubated at room temperature for 1 to 4 days; Holliday, 1974). The colonies of transformants shown in Figure 2 were grown on charcoal plates without selection pressure to enhance expression of the fuzzy phenotype; transformants were grown in liquid DCM with (episomal vectors) or without (integrated vectors) antibiotic selection. Seven- to 10-day-old maize seedlings (cv Golden Bantam) were injected in the internodal region with 50 to 100 μL of fungal cell suspensions ($10^6$ cells per mL for wild-type cells; $10^8$ cells per mL for haploid transformants). Plants were grown in the greenhouse and were scored 3 to 4 weeks after inoculation. The plants were rated for disease symptoms as follows: 0, no symptoms; 1, anthocyanin streaking on the leaves; 2, small leaf galls; 3, galls on the stem; 4, large stem galls; 5, dead plant (with apparent galls) (Kronstad and Leong, 1989). The average disease rating in Table 2 was calculated based on the number of plants inoculated.

### Construction of Libraries

High molecular weight DNA was prepared from *Ustilago* by lysis of protoplasts or vortexing whole cell suspensions for 3 min in the presence of glass beads (as described by Wang et al., 1988; Kronstad and Leong, 1989). A 523-bp PvuII-BamHI fragment (positions 1168 to 1685, including a BamHI site introduced as a polymerase chain reaction primer; Kronstad and Leong, 1990) from the carboxy-terminal region of the *U. maydis b*East1 (*b*E1) gene was employed as a probe to detect *b* homologs in Uh112 (a 2.8-kb BamHI fragment) and in Uh100 (a 1.5-kb BamHI fragment) (Bakkeren et al., 1992; see Figure 1). Note that subsequent sequence analysis indicated that the probe fragment had 58% nucleotide sequence identity to the equivalent region from strain Uh112. Probes were labeled with $\alpha$-$^{32}$P-dCTP (Du Pont) using a random priming labeling kit (Pharmacia LKB Biotechnology Inc.).

Partial libraries were constructed to clone the 2.8- and 1.5-kb BamHI fragments from *U. hordei*. BamHI-digested total genomic DNA was electrophoresed on a 0.7% low melting point agarose (ultraPURE; Bethesda Research Laboratories) gel in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA), and the DNA fragments in the 2.8- and 1.5-kb regions were isolated from the gel. The DNA was recovered by the addition of 2 volumes of TE buffer (10 mM Tris-HCl, 1 mM EDTA), incubation at 65°C, extractions once with phenol, twice with phenol/chloroform (1:1, v/v), and twice with chloroform, and precipitation with 2 volumes 95% EtOH after the addition of 0.1 volume of a 3 M potassium-acetate solution. These fragments were ligated (T4 DNA ligase; Bethesda Research Laboratories) into BamHI cut, dephosphorylated (CIP; Boehringer Mannheim) plasmid pGEM3Z($f^+$) (Promega). The DNA was then dialyzed (TE buffer) and electrotransformed into *E. coli* DH5α cells using a gene pulser (Bio-Rad) linked to a pulse controller as described by Dower et al. (1988).

Approximately 4000 colonies were obtained from the Uh112 sample and 9000 from the Uh100 DNA. These colonies were replica plated on nitrocellulose filters (Schleicher & Schuell) and prepared for colony hybridization analysis as described by Sambrook et al. (1989). Using the *U. maydis* probe for *b*E1 and hybridization conditions as described previously (Bakkeren et al., 1992), we identified clones pUhbE1 and pUhbE2 (Figure 1) after consecutive washes with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 45°C, 0.5 × SSC at 45°C, and 0.5 × SSC at 65°C and exposure to x-ray film (Kodak XAR5) between each wash condition. Subsequently, the insert of pUhbE1 was used to probe cosmid libraries from strain Uh112 and strain Uh100 that contained partial Sau3A genomic fragments of on average 40 kb in length. These libraries were constructed in the *Ustilago*-specific autonomously replicating sequence (ARS)-containing cosmid vector pJW42 (Wang et al., 1988; Froeliger and Leong, 1991), essentially as described by Kronstad and Leong (1989). In a library of 30,000 colonies, representing theoretically 50 genome equivalents of strain Uh112, we identified a single positive clone, cosmid pbMAT-1. Similarly, a single positive clone was obtained from a library comprising 16,000 colonies made from strain Uh100, cosmid pbMAT-2.

### Sequence Analysis

Subclones from pUhbE1 and pUhbE2 and cosmid clones pbMAT-1 and pbMAT-2 were constructed in pGEM3Z($f^+$) using the restriction sites shown in Figure 1, and sequenced according to the strategy outlined by the arrows. Plasmid inserts or whole cosmids were sequenced directly (double-stranded templates), essentially as described by Zagurski et al. (1985), using the standard forward and reverse M13

primers, or oligonucleotide primers (indicated by the arrows preceded by a black box in Figure 1), $\alpha$-$^{35}$S-dATP (Du Pont), and a T7 sequencing kit (Pharmacia LKB Biotechnology Inc.). Sequencing data were analyzed by use of the software package of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

### Plasmid Constructions

#### *Uh100-Derived Plasmids*

The *U. hordei* *b*West2 (*b*W2) gene was obtained from a longer 5.8-kb BglII subclone in pGEM3Z (BamHI site) as a 4-kb KpnI fragment (pUhbW2; Figure 1). This KpnI fragment was cloned in a newly constructed, episomal *Ustilago* vector, pCM60 (essentially pCM54 [Tsukuda et al., 1988] except with pUC18 backbone; S. Gold and G. Bakkeren, unpublished data), to yield pUhbW2ARS, or in a newly designed, integrative vector pUble3 to yield pUhbW2Int. pUble3 contains a phleomycin/bleomycin coding region (Gatignol et al., 1990) inserted into the *Ustilago*-specific expression cassette of pDWH10 (D. W. Holden and S. A. Leong, University of Wisconsin, Madison), which was then cloned in pGEM3Z (G. Bakkeren, unpublished data).

#### *Uh112-Derived Plasmids*

The Uh112 *b* gene complex was reconstructed by ligating a 7.0-kb BamHI fragment containing the Uh*b*W1 gene into partially BamHI-digested, dephosphorylated pUhbE1. A 6.8-kb KpnI fragment containing both the Uh*b*W1 and Uh*b*E1 genes was then cloned in plasmid pCM60 and pUble3. Replacement of the 1.9-kb BglII fragment in both constructs reintroduced the 21-bp BamHI fragment (deleted from earlier plasmid constructs due to subcloning) and yielded pUhbWE1ARS and pUhbWE1Int, respectively. Plasmid pUmbWE1 containing both the *U. maydis* *b*W1 and *b*E1 genes on an episomal vector was constructed by cloning of a 6-kb PstI fragment (Kronstad and Leong, 1990) in the PstI site of plasmid pCM54.

### Transformation of *U. maydis* and *U. hordei*

Transformation of spheroplasts of *U. maydis* using PEG was performed essentially as described by Wang et al. (1988). *U. hordei* cells, only partially converted into spheroplasts (to maximize regeneration frequency), were obtained as described for *U. maydis*. After three consecutive washes with ice-cold 1 M sorbitol, the cells were resuspended to a density of $2 \times 10^9$ cells per mL and 20% PEG was added to a final concentration of 1%. One to 2 $\mu$g of ethidium bromide-CsCl gradient-purified DNA in 5 $\mu$L of TE buffer (10mM Tris-HCl, pH 7.6, 1mM EDTA) was mixed with 1 $\mu$L of a 15 $\mu$g/$\mu$L solution of heparin on ice. After the addition of 40 $\mu$L of cells and a 1-min incubation on ice, the suspension was transferred into an ice-cold 0.2-cm electrode gap gene pulser cuvette (No. 165-2086; Bio-Rad). A gene pulser (Bio-Rad) linked to a pulse controller set at 2.5 kV/cm, 200 $\Omega$ resistance in parallel with a 25 $\mu$F capacitor resulted routinely in a discharge with time constant of 4.4 to 4.6. Approximately 25% of the cells survived this treatment. With 10% spheroplast regeneration, we were able to obtain a transformation frequency of $\sim$5000 colonies per $\mu$g using a derivative of episomal plasmid pCM54. The plasmid DNAs used for transformation were purified by CsCl gradient centrifugation according to Sambrook et al. (1989). The integrative vectors were linearized with a restriction enzyme prior to transformation.

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## REFERENCES

**Agrios, G.N.** (1988). Plant Pathology, 3rd ed. (San Diego: Academic Press), pp. 474–486.

**Bakkeren, G., Gibbard, B., Yee, A., Froeliger, E., Leong, S., and Kronstad, J.** (1992). The *a* and *b* loci of *U. maydis* hybridize to DNAs from other smut fungi. Mol. Plant-Microbe Interact. **5,** 347–355.

**Banuett, F.** (1992). *Ustilago maydis*, the delightful blight. Trends Genet. **8,** 174–180.

**Banuett, F., and Herskowitz, I.** (1988). *Ustilago maydis*, smut of maize. Adv. Plant Pathol. **6,** 427–453.

**Banuett, F., and Herskowitz, I.** (1989). Different *a* alleles of *Ustilago maydis* are necessary for maintenance of filamentous growth but not for meiosis. Proc. Natl. Acad. Sci. USA **86,** 5878–5882.

**Bölker, M., Urban, M., and Kahmann, R.** (1992). The *a* mating type locus of *U. maydis* specifies cell signalling components. Cell **68,** 441–450.

**Christensen, J.J.** (1963). Corn smut caused by *Ustilago maydis*. In American Phytopathology Society Monograph, No. 2. (St. Paul, MN: American Phytopathological Society), pp. 1–41.

**Day, P.R.** (1974). Genetics of pathogenicity. In Genetics of Host-Parasite Interactions, A. Kelman and L. Sequeira, eds (San Francisco: W.H. Freeman and Company), pp. 36–91.

**Day, P.R., and Anagnostakis, S.L.** (1971). Corn smut dikaryon in culture. Nature New Biol. **231,** 19–20.

**Devereux, J., Haeberli, P., and Smithies, O.** (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. **12,** 387–395.

**Dower, W.J., Miller, J.F., and Ragsdale, C.W.** (1988). High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acids Res. **16,** 6127–6145.

**Fischer, G.W.** (1953). Manual of North American Smut Fungi. (New York: Ronald Press).

**Fischer, G.W., and Holton, C.S.** (1957). Biology and Control of the Smut Fungi. (New York: Ronald Press).

**Froeliger, E.H., and Kronstad, J.W.** (1990). Mating and pathogenesis in *Ustilago maydis*. Semin. 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.

**Gatignol, A., Dassain, M., and Tiraby, G.** (1990). Cloning of *Saccharomyces cerevisiae* promoters using a probe vector based on phleomycin resistance. Gene **91,** 35–41.

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

**Gjaerum, H.B., Tjamos, E.C., and Viranyi, F.** (1988). Basidiomycetes I, ustilaginales. In European Handbook of Plant Diseases, I.M. Smith, J. Dunez, D.H. Phillips, R.A. Lelliott, and S.A. Archer, eds (Oxford: Blackwell Scientific), pp. 462–472.

**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.

**Holliday, R.** (1961). The genetics of *Ustilago maydis*. Genet. Res., Camb. **2,** 204–239.

**Holliday, R.** (1974). *Ustilago maydis*. In Handbook of Genetics, Vol. 1, R.C. King, ed (New York: Plenum), pp. 575–595.

**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. USA **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.

**Kües, U., and Casselton, L.A.** (1992). Homeodomains and regulation of sexual development in basidiomycetes. Trends Genet. **8,** 154–155.

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

**Puhalla, J.E.** (1970). Genetic studies on the *b* incompatibility locus of *Ustilago maydis*. Genet. Res., Camb. **16,** 229–232.

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

**Sambrook, J., Fritsch, E.F., and Maniatis, T.** (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

**Schulz, 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.

**Scott, M.P., Tamkun, J.W., and Hartzell, G.W., III.** (1989). The structure and function of the homeodomain. Biochim. Biophys. Acta **989,** 25–48.

**Thomas, P.L.** (1988). *Ustilago hordei*, covered smut of barley and *Ustilago nigra*, false loose smut of barley. Adv. Plant Pathol. **6,** 415–425.

**Thomas, P.L.** (1991). Genetics of small-grain smuts. Annu. Rev. Phytopathol. **29,** 137–148.

**Tsukuda, T., Carleton, S., Fotheringham, S., and Holloman, W.K.** (1988). Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. Mol. Cell. Biol. **8,** 3703–3709.

**Wang, J., Holden, D.W., and Leong, S.A.** (1988). Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. Proc. Natl. Acad. Sci. USA **85,** 865–869.

**Yee, A.R., and Kronstad, J.W.** (1993). Construction of chimeric alleles with altered specificity at the *b* incompatibility locus of *Ustilago maydis*. Proc. Natl. Acad. Sci. USA. (in press).

**Yoder, O.C., Valent, B., and Chumley, F.** (1986). Genetic nomenclature and practice for plant pathogenic fungi. Phytopathology **76,** 383–385.

**Zagurski, R.J., Baumeister, K., Lomax, N., and Berman, M.L.** (1985). Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gen. Anal. Tech. **2,** 89–94.