THE GENETICS OF DIMORPHISM IN THE SMUT FUNGI

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ABSTRACT. Dimorphic growth in *Ustilago maydis* is regulated by mating-type loci called *a* and *b*. The *b* region encodes two polypeptides (*bE* and *bW*) which control the formation of the infectious dikaryon upon fusion of haploid, yeast-like cells of opposite *a* mating type. Given that there are at least 25 naturally occurring specificities at the *b* locus, and that the locus must be heterozygous to trigger filamentous growth, the molecular basis of self versus non-self recognition is of particular interest. The construction of recombinants between the *b1E* and *b2E* alleles identified a 30 to 48 amino acid region which determines specificity. In addition, hybridization and sequence analyses revealed homologs of the *bE* and *bW* genes in *U. hordei*, a smut thought to have only the *a* mating function. Genes have also been identified that may be regulated by the *b* locus and whose products influence cell morphology. One of these genes, called *rem1*, functions in the switch from yeast-like to mycelial growth.

1. Introduction

The smut fungi are basidiomycete phytopathogens that attack a variety of important crop species including corn, sorghum and small grain cereals (Fischer, 1953; Fischer and Holton, 1957; Christensen, 1963). Smut infections are generally distinguished by the presence of large masses of sooty black teliospores within the host plant. Some species, such as the corn smut fungus *Ustilago maydis*, incite galls on the host and sporulate within gall tissue. Other species, which infect small grain cereals (e.g., *Ustilago hordei*), do not incite galls but produce teliospores preferentially in floral tissue, thereby reducing the yield and quality of the grain (Agrios, 1988).

As a group, the smut fungi have basically similar life cycles in which cells exist in one of three types: diploid, haploid and dikaryotic (Fischer and Holton, 1957). Diploid teliospores are produced in infected tissue and germinate to give the four haploid products of meiosis, which are called basidiospores. Mating interactions between compatible haploid basidiospores yield dikaryotic cells which are capable of infecting the host plant, proliferating within host tissue and eventually sporulating to produce teliospores. Release of teliospores from infected tissue represents a major source of inoculum for subsequent infections.

One of the most interesting biological features of the smut fungi is the presence of at least two different mating systems within the group (Holton *et al.*, 1968). Many of the species, such as *U. hordei*, possess a bipolar mating system in which a single mating-type locus with two alternate forms, *MAT-1* and *MAT-2* (also called *a* and *A*), controls dikaryon formation (Thomas, 1991). Other smuts, such as *U. maydis*, display tetrapolar mating in which two loci control dikaryon formation (reviewed by Froeliger and Kronstad, 1990; Banuett, 1992). The tetrapolar smut fungi generally have one locus with two alternate sequences that is thought to control cell fusion (equivalent to *MAT*), and a second locus with multiple alternative forms that controls the establishment of the infectious dikaryon (Holton *et al.*, 1968). If the bunt fungi of the genus

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Tilletia are included in the smut group, then a third mating system (multiple alleles at a single locus) has been reported for *T. controversa* (Hoffman and Kendrick, 1965)

The tetrapolar mating system of *U. maydis* has been characterized in some detail due to recent efforts to isolate and sequence the mating-type genes. In this fungus, the locus which controls fusion is called *a* (*MAT*), and the locus that establishes the infectious dikaryon is called *b*. Haploid cells can fuse and form the infectious dikaryon only if they carry different sequences at the *a* and *b* loci. The *a* mating type locus has two alternative forms, *a1* and *a2*. These sequences have recently been shown to be idiomorphs (Froeliger and Leong, 1991), and to encode pheromones and pheromone receptors (Bolker *et al.*, 1992). The *b* region has at least 25 different alternative forms and appears to control events after cell fusion necessary for establishment of the filamentous, pathogenic dikaryon. The haploid cell type is not pathogenic. Two genes are present at *b*, *bE* and *bW*. The combination of the *bE* product (473 a.a.) from one version of the *b* locus with the *bW* product (626 a.a.) from another is believed to form a novel regulatory protein that triggers dikaryon formation (Gillissen *et al.*, 1992). The alignment of the predicted amino acid sequences of several alleles of *bE* revealed the presence of a variable N-terminal domain, a central homeodomain-like motif and a conserved C-terminal region (Kronstad and Leong, 1990; Schulz *et al.*, 1990). A similar organization was found for *bW*; however, it should be noted that *bE* and *bW* share sequence similarity only in the homeodomain region (Gillissen *et al.*, 1992).

It is interesting that the phytopathogenic smut fungi display a dimorphic growth habit similar to that found in some animal pathogens, e.g., *Candida albicans*. *Ustilago* species have the ability to switch between a yeast-like nonpathogenic phase (i.e., the haploid phase) and a filamentous pathogenic phase (i.e., the infectious dikaryon). This dimorphic switch is controlled primarily by the *b* genes. The finding that *bE* and *bW* contain homeodomain-like regions (Schulz *et al.*, 1990; Gillissen *et al.*, 1992), and the observation that disruption of *b* function blocks dikaryon formation (Kronstad and Leong, 1990; Gillissen *et al.*, 1992), suggests that the *b* products are regulatory proteins. The influence of the *b* locus can readily be detected because the mating of compatible haploids on rich medium containing activated charcoal (Puhalla, 1968) results in the formation of white aerial hyphae on the mixed colony. A similar phenotype is seen when diploid or haploid strains carrying two different versions of *b* are grown on the same medium. This "fuzzy" phenotype presumably indicates formation of the infectious, filamentous dikaryon and it provides a convenient assay to detect the activity of the *a* and *b* genes. For example, Day *et al.* (1971) employed this phenotype to isolate mutations at *b*, and Banuett (1991) used it to identify mutations that block the ability of haploid strains to mate; these mutations could conceivably be in genes necessary for fusion, dikaryon formation or both.

The primary goal of our work is to understand the genetics of formation of the infectious dikaryon in the smut fungi. We anticipate that this understanding will provide insight into fungal pathogenesis on plants, into the role of mating-type genes in pathogenesis, and into the regulation and mechanism of dimorphic growth in fungi. Here we describe progress in our analysis of the specificity region of the *bE* gene of *U. maydis* and in the characterization of a *b* locus in the bipolar smut *U. hordei*. In addition, we describe strategies that have proven successful for identifying other genes, besides the *a* and *b* mating-type genes, that play a role in formation of the infectious dikaryon.

2. Materials and Methods

The following strains of *U. maydis* were employed; 518 (*a2 b2*), 521 (*a1 b1*), 87 (*a2 b2 adl-1 leu1-1*), 87-18 (*a2 b2 adl-1 leu1-1 rem1-1*), P6D (*a2 b2 [a1 b1 phl^r]*). The strain P6D is phleomycin resistant (*phl^r*) *U. hordei* strains Uh112 (*MAT-1 adl*) and Uh100 (*MAT-2 adl*) were employed for the isolation of the *b* homologs. Other strains of *U. hordei*, *U. kolleri*, *U. avenae*, *U. bullata* and *U. nigra* were obtained from Dr. P.L. Thomas, Agriculture Canada, Winnipeg, Manitoba. All methods were employed as previously described (Kronstad and Leong, 1989, 1990; Sambrook *et al.*, 1989; Wang *et al.*, 1988).

3. Results and Discussion

3.1. THE *b* GENES OF *U. MAYDIS* AND *U. HORDEI*

3.1.1. *Recombinant alleles define a specificity region in the bE gene of U. maydis.* A series of hybrids between the *b1E* and *b2E* genes was constructed by an *in vivo* recombination strategy diagrammed in Figure 1A. DNA fragments carrying part of the *b1E* gene (truncated at various positions in the variable region) and a selectable marker for resistance to hygromycin B were introduced into a strain carrying a *b2E* allele (518, *a2 b2*). Targeted integration, which occurs at a high frequency in *U. maydis* (Kronstad *et al.*, 1989; Fotheringham and Holloman, 1989), generated recombinants between *b1E* and *b2E*. The transformants resulting from this experiment were screened for their mating specificity in a standard assay on rich medium containing activated charcoal (Puhalla, 1968; Holliday, 1974). The original strain used for transformation mates with *a2 b2* specificity such that it forms white aerial mycelium when mixed with strains carrying the *a1* mating specificity and any other *b* specificity besides *b2*. Three classes of transformants of this strain were found in the mating tests; I) those that mated with the original specificity (*a2 b2*); II) those with a novel *b* specificity (*a2 bx*); and III) those that had switched specificity from *b2* to *b1* (*a2 b1*). These results indicated that *bE* alleles with altered specificity could be generated by *in vivo* recombination. It should be noted that in these experiments, the hygromycin B marker was inserted into the *bW* orf; therefore, in the transformants carrying recombinant *bE* alleles, the *bW* orf is inactive. This allowed the analysis of the *bE* specificity of the transformants without complications arising from the activity of the *bW* gene.

The variable regions of the *bE* genes, from representatives of the three classes of transformants, were isolated by polymerase chain reaction and nucleotide sequence analysis was performed. Figure 1B shows a diagram of the recombination points found for transformants in each class. Class I transformants arose from recombination early in the *bE* orf, for example, at codons 28 or 39. Class II transformants had recombination points in the region between codons 40 and 79 and class III transformants had recombination points downstream of codon 87. Overall, these results demonstrate the presence of a region between codons 40 and 79 that determines allelic specificity for the *bE* gene. Recombination upstream of this region did not alter specificity while recombination downstream switched specificity from that of the resident *b2E* allele to that of the incoming *b1E* allele.

Current models for the molecular recognition events mediated by the *b* locus products postulate that the interaction between the *bE* gene product from one haploid parent (e.g. *b1E*) with the *bW* gene product from the other parent (e.g. *b2W*) combine to generate a regulatory activity that directly or indirectly influences the expression of genes required for formation of the infectious dikaryon (Gillissen *et al.*, 1992). It appears that the *bE* and *bW* products from one haploid parent (e.g., *b1E* and *b1W*) are unable to establish the regulatory activity. Given our discovery of a specificity domain in the variable region of the *bE* gene, we postulate that this region of the encoded polypeptide interacts with a corresponding domain in the polypeptide encoded by the *bW* gene (presumably also in the variable portion). The presence of a corresponding region in the *bW* gene is predicted by sequence alignments which reveal two clusters of hypervariable sequences within the variable region (Gillissen *et al.*, 1992; J. W. K., unpublished results). The prediction of our work is that recombinants between *b1W* and *b2W*, within the hypervariable region, should create alleles with specificity different from that of the parental alleles. This would be analogous to the results obtained from the construction of recombinant alleles for *bE*. In addition, it should be possible to find recombinant alleles of *bW* which match recombinant alleles of *bE* in terms of ability to interact and trigger formation of the filamentous cell type. If this turns out to be the case, our model predicts that this approach would lead directly to a molecular description of the amino acid residues that mediate recognition between *bE* and *bW*.

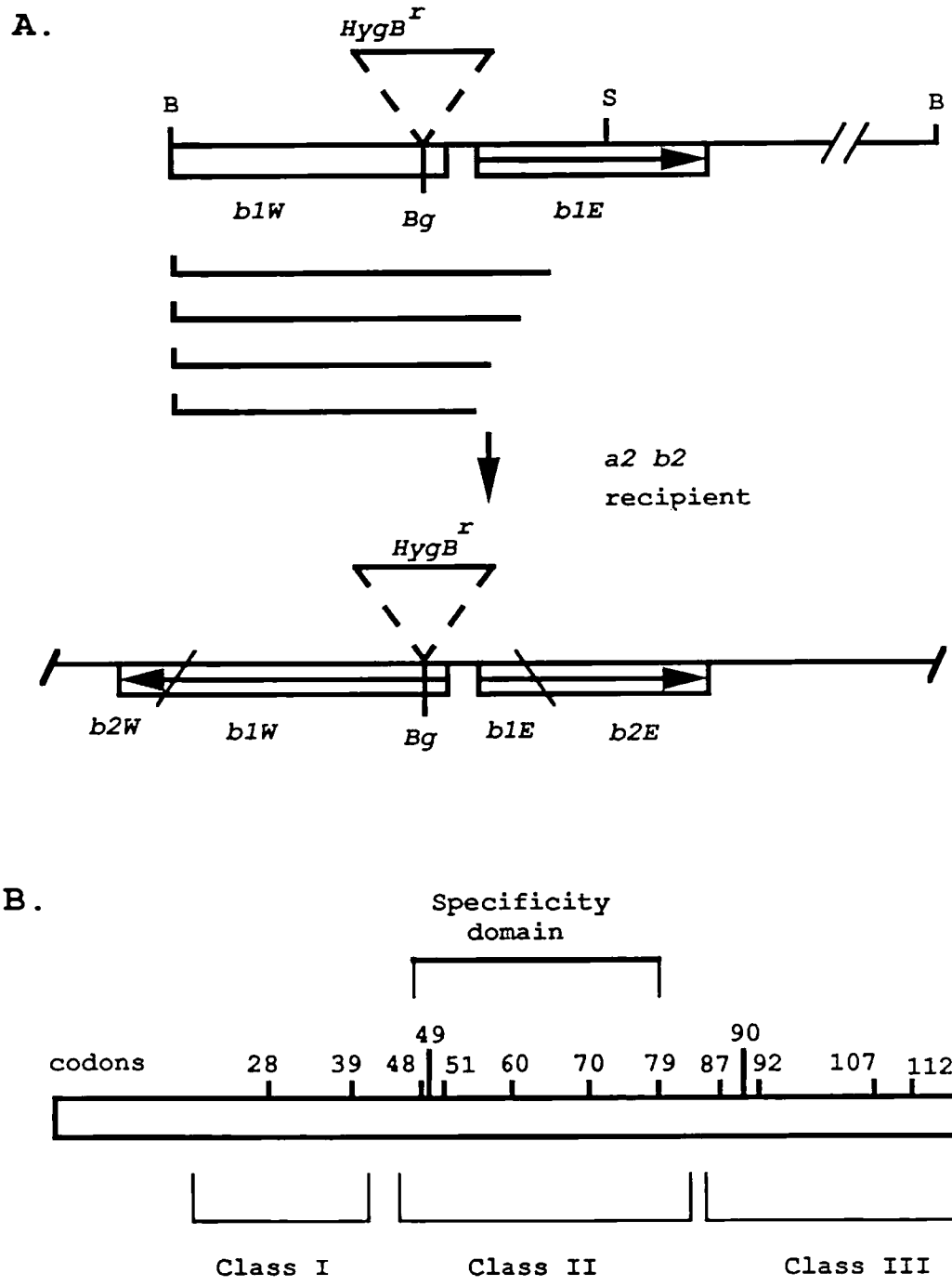

Figure 1. Construction and analysis of recombinant *bE* alleles.
(A) Strategy for the construction of recombinant alleles by targeted integration of truncated versions of *b1E* at the *b2* locus. Note that the hygromycin B resistance gene is inserted into the open reading frame of the *bW* gene. Restriction sites shown are: *Bam*HI, B; *Bgl*II, Bg; and *Sal*I, S.
(B) Recombination map of the *bE* open reading frame. Class I recombinants mate with *b2E* specificity, class II recombinants have specificity different from both *b1E* and *b2E* and class III recombinants have *b1E* specificity.

3.1.2. *The b genes are present in both tetrapolar and bipolar smut fungi.* Hybridization studies with the cloned *a* and *b* DNAs of *U. maydis* revealed that these sequences are present in other smut fungi with the tetrapolar mating system (having both the *a* and the *b* functions), as well as in some smut fungi with the bipolar mating system (Bakkeren *et al.*, in press). Previous genetic analyses suggested that the latter fungi have a single mating-type locus with two alternate forms. Therefore, it was particularly surprising to find that these fungi had sequences that hybridized with the *b* locus of *U. maydis*. We have initiated an analysis of the *b* genes in bipolar smut fungi by isolating cosmid clones that carry the *b* sequences from each mating-type (*MAT-1* and *MAT-2*) of *U. hordei*, a representative of the smuts with the bipolar mating system. The cosmid carrying DNA from the *MAT-1* strain (Uh112) was designated pa112 and the cosmid from the *MAT-2* strain (Uh100) was designated pA100.

Nucleotide sequence analysis of the *b*-like regions on the cosmids pa112 and pA100 revealed open reading frames analogous to the *bE* and *bW* genes of *U. maydis*. When the predicted amino acid sequences were aligned, the two *bE* alleles (one from each mating type) showed the same organization as was found for *U. maydis bE* (Kronstad and Leong 1990; Schulz *et al.*, 1990), i.e., variable amino termini, constant carboxy termini and a homeodomain-like motif. At the amino acid level, the two *bE* alleles from *U. hordei* were 81% identical to each other, and each gene was approximately 50% identical to the *b1E* allele of *U. maydis*. The *bW* alleles of *U. hordei* also possessed variable amino terminal regions, constant carboxy terminal regions and homeodomain-like motifs. These alleles were 74% identical to each other at the amino acid level and approximately 40% identical to the *b1W* allele of *U. maydis* . Overall, the sequencing results indicated that the *b* genes are structurally conserved between smut fungi with bipolar and tetrapolar mating systems.

Given the structural conservation, it was of interest to determine whether the sequences were functionally equivalent. Two tests of *b* allele function, based on colony morphology and pathogenicity (Kronstad and Leong, 1989), were performed. First, *U. hordei b* alleles were introduced into *U. maydis* haploid strains, e.g., *bE* and *bW* from *U. hordei* into *U. maydis*, and the resulting transformants were tested for their morphology on medium containing activated charcoal. The presence of two different *b* specificities in a single strain, either on an episomal vector or integrated, results in the formation of mycelial colonies; in contrast, haploid strains normally form yeast-like colonies. Placing either of the cosmids, pa112 or pA100, from *U. hordei* into *U. maydis* yielded transformants showing mycelial growth. Similarly, placing the cloned *b1E* and *b1W* alleles from *U. maydis* into strains of either mating-type of *U. hordei* also gave mycelial transformants. The transformants of *U. maydis*, which contained *U. hordei b* genes, were also injected into corn seedlings to determine whether the *U. hordei* and *U. maydis b* gene products would productively interact to yield a pathogenic cell type. These *U. maydis* transformants did give weak disease symptoms on corn seedlings indicating that the products of the *b* genes from *U. hordei* and *U. maydis* are capable of interaction.

Sequence analysis to date on the *bE* and *bW* variable regions from other bipolar smut fungi indicates that there may only be two alleles for each gene among this group of fungi. That is, the predicted amino acid sequences of the variable regions of *bE* and *bW* from 4 other bipolar smut fungi, *U. bullata*, *U. nigra*, *U. avenae* and *U. kolleri*, fall into two related groups. In one group, the sequences are almost identical to the genes from the *MAT-1* strain of *U. hordei*; in the other, they are almost identical to the sequences from the *MAT-2* strain. In contrast, a similar analysis of several *bE* genes from *U. maydis* revealed a large number of amino acid differences in the variable region (Kronstad and Leong, 1990; Schulz *et al.*, 1990). A more extensive survey of *bE* and *bW* variable regions from several isolates of *U. hordei* is currently underway to explore the extent of variability and to further establish the idea that only two alleles exist for *bE* and *bW* in the bipolar smut fungi.

3.2. OTHER GENES INVOLVED IN FORMATION OF THE INFECTIOUS DIKARYON.

3.2.1. *Isolation of constitutively mycelial mutants in U. maydis.* The ability of strains of *U. maydis* to form mycelial colonies on medium containing activated charcoal has proven to be a useful assay for the state of the *a* and *b* mating-type sequences (homozygous or heterozygous) and for the pathogenicity of a particular strain. In fact, it was this mycelial or "fuzzy" phenotype that originally provided the assay to clone the *b* locus (Kronstad and Leong, 1989). With this in mind, we set out to search for mutations that would cause normally yeast-like haploid strains of *U. maydis* to constitutively form mycelial colonies independent of the state of the *a* or *b* loci. To date, over 100 mutants have been isolated, following mutagenesis with ultraviolet light, which display a completely mycelial growth habit. This phenotype is in contrast to haploid or diploid strains carrying two different forms of the *b* locus. The latter strains generally grow with a yeast-like morphology in liquid and form yeast-like colonies which become covered with white aerial mycelium on solid medium. The mutation to a mycelial phenotype has been designated *rem1* for repressor of mycelial phenotype.

One of the mycelial haploid mutants, strain 87-18 (carrying *rem1-1*) has been characterized with respect to the distribution of nuclei in the hyphae and pathogenesis. Previous descriptions of the filaments that are thought to represent the infectious, filamentous dikaryon of *U. maydis* revealed that, in general, the cells are devoid of cytoplasm and nuclei except for those at the hyphal tips (Day and Anagnostakis, 1971). The mutant strain 87-18 differs in that cytoplasm and nuclei are present throughout the hyphae and each cell appears to have only one nucleus. Pathogenicity tests, performed by injecting mutant strains (carrying *rem1-1*), alone or with compatible haploid strains, into corn seedling revealed that the mutation did not allow haploid strains to be pathogenic by themselves. This is in contrast to weakly pathogenic haploid strains constructed by introducing a different set of *bE* and *bW* genes (e.g., *b1E*, *b1W*) into a haploid strain (e.g., *b2E*, *b2W*) (Kronstad and Leong, 1989). It is clear that mutation in the *rem1* gene (conferring the mycelial phenotype) is not sufficient to confer pathogenicity on a haploid strain. The haploid strains carrying the *rem1-1* mutation were capable of giving high levels of infection when paired with wild-type strains of compatible mating-type. However, mixtures of compatible strains, each carrying the *rem1-1* mutation, gave reduced levels of infection compared with infections caused by wild-type strains. Thus, the *rem1-1* mutation appears to reduce the ability of *U. maydis* to cause disease symptoms.

Stakman *et al.* (1943) reported the occurrence of "white" mutants of *U. maydis*, which have a similar phenotype to the *rem1* mutants described here. Interestingly, Stakman *et al.* (1943) reported that all of the white mutants were capable of causing disease symptoms but they were unable to produce teliospores in infected tissue.

The *rem1-1* mutation in strain 87-18 has been complemented by the introduction of a cosmid library and screening for transformants with a yeast-like morphology. Three transformants, all of which appear to contain the same cosmid, were obtained from a screen of approximately 2000 transformants. These cosmids, when introduced into strain 87-18, give transformants with a yeast-like morphology. Loss of the cosmid, due to growth in medium lacking hygromycin B, resulted in cells with the mycelial phenotype of the original mutant strain 87-18.

The region of the cosmid complementing the *rem1-1* mutation has been identified by Tn5 insertion mutagenesis. Tn5 insertions in an approximately 9 kb region block complementation upon transformation into strain 87-18. Insertion of the hygromycin B marker into this region and replacement of the genomic sequence with the disrupted sequence in haploid strains results in cells with a mycelial phenotype. This result suggests that the cosmid DNA does indeed carry the *rem1* gene, rather than a suppressor of the *rem1-1* mutation.

3.2.2. *Isolation of mutants blocked in formation of the infectious dikaryon.* One of the main problems in attempting to genetically dissect pathogenesis in the smut fungi is the requirement that two haploid partners must mate to form the infectious, dikaryotic cell type. Specifically, it is difficult to identify recessive mutations that block the ability of the cells to switch from budding to

filamentous growth. To overcome this problem, we have constructed a haploid strain of *U. maydis* (P6D) that contains each of the *a* locus sequences and two different *b* locus sequences. This strain mimics diploid strains heterozygous at both *a* and *b* in that it forms mycelial colonies on charcoal medium and it is pathogenic (albeit weakly) upon injection into corn seedlings. With this strain, we have undertaken a screen for mutations that block the ability of the strain to form mycelial colonies on charcoal medium. Mutations have been generated by random insertion of an integrative plasmid vector and by treatment with ultraviolet light. A large number of mutants have been isolated (designated nf for nonfuzzy), and examples of the phenotypes of several of the mutants are shown in Figure 2. The phenotypes vary from almost as mycelial as the parental strain to completely yeast-like. In addition, one class of mutants has a phenotype of slow growth and abnormal cell shape. These mutants may have defects in cell wall biosynthetic functions that are involved in filamentous growth. Attempts are currently being made to complement two of the mutations that block the mycelial phenotype of P6D.

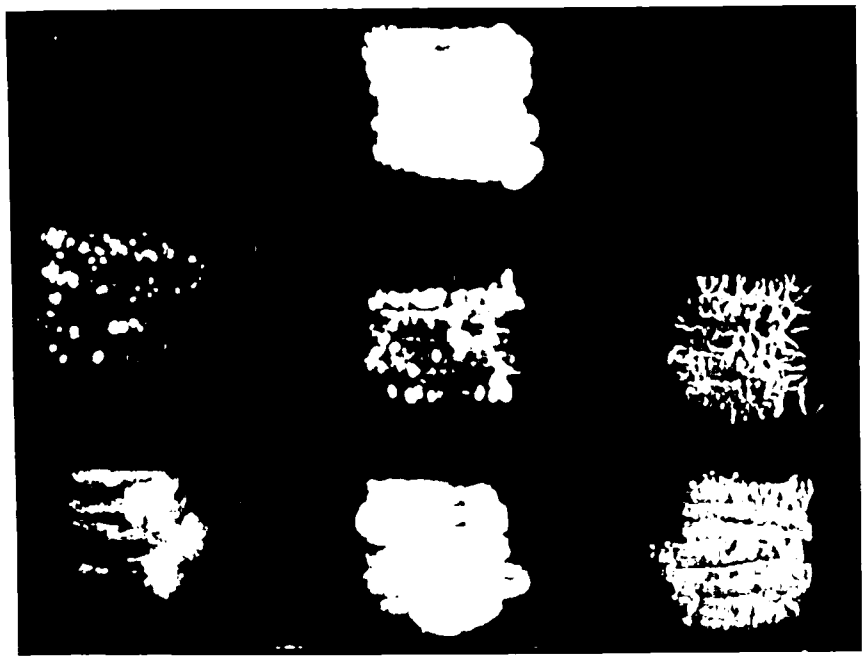

Figure 2. Colony morphology of the mycelial haploid strain P6D and nonfuzzy mutant derivatives. The colonies were grown on rich medium containing activated charcoal (Holliday, 1974) for 48 hours at 30°C.

4. Summary.

The analysis of the mating-type genes of *U. maydis* and *U. hordei* has provided an entry point to begin to understand the formation of the infectious, filamentous dikaryon from nonpathogenic yeast-like haploid strains. We have focused on the key step that triggers dikaryon formation; i.e., the recognition of the presence of products of different alleles of the *b* genes (*bE* and *bW*) in the same cell. Specifically, we have identified an approximately 40 amino acid region in the *bE* gene that we believe determines the specificity of the interaction of *bE* product with *bW*. What is needed now is a complementary analysis of *bW* to identify the corresponding region of specificity in this gene. This analysis will allow a detailed investigation of the specific amino acid residues that interact to mediate recognition.

Our work on *U. hordei* has revealed the surprising finding that smut fungi with bipolar mating systems, that is, without a genetically defined *b* locus, do indeed have *b* genes. Sequence analysis

of these genes revealed that they have the same basic organization as the genes in the tetrapolar smut *U. maydis*. In addition, transformation experiments indicate that the sequences are functionally conserved when moved between species. The problem now is to explain why a *b* locus has not been identified genetically in smut species with bipolar mating systems. Our working model is that the *b* sequences are physically linked to the genetically defined *MAT* locus such that only a single mating-type function with two alternate forms, *MAT-1* and *MAT-2*, appears to be present.

A complete understanding of the regulatory control exerted by the *b* locus will require the identification and characterization of other genes whose expression may be regulated by *b* and whose products play additional regulatory or structural roles in dikaryon formation. We have identified a gene, *rem1*, which appears to play a central role in the switch between budding and filamentous growth. We envision that the product of the *rem1* gene establishes a site near one apex of the yeast-like haploid cell and that bud formation occurs at this site. Loss of the *rem1* function results in loss of the ability to bud; the fact that the resulting phenotype is completely filamentous suggests that this is the default growth morphology for *U. maydis*.

In contrast to the *rem1-1* mutation, which causes haploid cells to display a filamentous phenotype, other mutations have been identified which prevent expression of the mycelial phenotype induced by heterozygosity at the *a* and *b* loci. The characterization of these mutations and the corresponding genes will provide a more complete picture of the regulatory pathway controlled by mating-type and of the biochemical changes needed to change fungal cell morphology.

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