Identification and Complementation of a Mutation to Constitutive Filamentous Growth in *Ustilago maydis*

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Pathogenicity of the corn smut fungus *Ustilago maydis* involves the formation of a filamentous, infectious dikaryon by fusion of compatible, yeastlike haploid cells. The mating-type loci, *a* and *b*, regulate cell fusion and establishment of the dikaryotic cell type, respectively. On solid medium, compatibility at the mating-type loci, in particular heterozygosity at the *b* locus, is manifested by the formation of aerial hyphae on colonies formed by mating cells. We have employed this "fuzzy" phenotype to identify haploid mutants that constitutively form hyphal filaments and forego cell division by budding. A total of 125 such mutants have been isolated; characterization of one mutant (termed *rem1-1*) revealed that it can participate in infection of the host plant, although it must be paired with a compatible, wild-type mating partner. That is, mutation to the mycelial phenotype is not sufficient to allow a haploid strain to be pathogenic by itself. A cosmid has been isolated that restores the ability of an *rem1-1* mutant to grow with a budding phenotype. Localization of the complementing region on cosmid DNA allowed the construction of an additional mutation by gene disruption. Coinoculation of plants with two compatible strains, each carrying the disruption mutation, gave greatly reduced disease symptoms. The analysis of the *rem1* gene should contribute to an understanding of dimorphic growth and pathogenesis in *U. maydis*.

Additional keywords: filamentous growth, morphological mutants.

The basidiomycete fungus *Ustilago maydis* (DC.) Corda is obligately dependent on infection of corn (*Zea mays* L.) to complete the sexual stage of its life cycle (reviewed by Christensen 1963; Banuett 1992). The infectious cell type of the fungus, which is filamentous and dikaryotic, is formed by fusion of haploid, yeastlike sporidia of compatible mating type. Once the infectious dikaryon has proliferated within host tissue and has induced tumors, sporulation results in the formation of masses of black, diploid teliospores. Vegetative diploids may also be isolated from infected plant tissue (Holiday 1974) or constructed artificially in the laboratory through the fusion of haploid strains carrying complementary auxotrophic mutations (Puhalla 1969).

U. maydis possesses a tetrapolar mating system in which two different genetic loci, *a* and *b*, are involved in establishing the infectious dikaryon. Haploid cells are compatible to fuse and to form the infectious dikaryon only if they carry different genes at both the *a* and *b* loci. Haploid cell fusion is controlled by the a mating-type locus, which has two alternative forms, *a1* and *a2*. The *a1* and *a2* sequences have recently been shown to be idiomorphs (Froeliger and Leong 1991) and sequence analysis suggests that they encode pheromones and pheromone receptors (Bolker *et al.* 1992). The *b* locus has at least 25 different forms and appears to control events necessary for establishment of the filamentous dikaryon after cell fusion. The *b* locus encodes at least two products, *bE* (473 amino acids) and *bW* (626 amino acid); the interaction of the *bE* product from one form of the *b* locus with the *bW* product from another is believed to establish a novel regulatory protein that triggers dikaryon formation (Gillissen *et al.* 1992). The alignment of the predicted amino acid sequences of several alleles of the *bE* and *bW* genes revealed that each contains a variable N-terminal domain, a central homeodomainlike motif and a conserved C-terminal region (Kronstad and Leong 1990; Schulz *et al.* 1990; Gillissen *et al.* 1992). A central question regarding *b* locus activity is how do the different gene products interact to distinguish self from nonself? Recently, a 30–48 amino acid domain has been identified in the variable region of *bE* that determines specificity (Yee and Kronstad 1993). In addition, hybridization studies with the *a* and *b* sequences as probes revealed that these genes are also present in *Ustilago* species that have bipolar mating systems (Bakkeren *et al.* 1992). Apparently, the sequences and/or functions of the *a* and *b* genes have been conserved among this group of phytopathogens.

The finding that *bE* and *bW* contain homeodomainlike regions and the observation that disruption of *b* function blocks formation of the filamentous dikaryon (Kronstad and Leong 1990) 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 the *b* locus are

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grown on the same medium (Kronstad and Leong 1989). This "fuzzy" phenotype 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 the *b* locus and Banuett (1991) used it to identify mutations that block the ability of haploid strains to mate.

To date, the analysis of the functions of the *a* and *b* genes has been hampered by the fact that genes regulated by these loci have not been isolated. The goal of the present work was to identify genes that play a role in the development of the "fuzzy" phenotype, i.e., in formation of the infectious, filamentous dikaryon. Such genes would be candidates for targets of regulation by the *a* or *b* genes. This paper reports the isolation of haploid mutants that constitutively form aerial hyphae on culture medium even though they possess a single version of the *b* locus. Mutant strains with similar characteristics have been described by Stakman *et al.* (1943). These investigators identified the mutants as white sectors on otherwise pigmented colonies and demonstrated that the mutations influenced the ability of strains to form teliospores during infection. In the present work, the influence of a mutation to the mycelial phenotype on pathogenicity has been characterized and a cosmid clone has been isolated that complements the mutation. The gene involved in this phenotype has been designated *rem1* for *r*epressor of *m*ycelial phenotype.

RESULTS

Isolation of mycelial haploid mutants.

It has been observed that haploid strains of *U. maydis* can give rise to variants or mutants displaying a mycelial colony phenotype (Stakman *et al.* 1943). We have isolated 125 of these mycelial mutants from two strains of *U. maydis,* 87 and 272, following mutagenesis with ultraviolet light. In a typical experiment, a suspension of each strain was mutagenized to approximately 10% survival and inoculated onto rich medium containing activated charcoal (Day and Anagnostakis 1971; Holliday 1974). As shown in Figure 1A, a parental strain such as 87 grows to form flat gray colonies on this medium. In contrast, mycelial mutants (Fig. 1B,C) form white colonies which, upon close observation, are composed of filamentous cells; budding cells are observed infrequently in these colonies. Mycelial mutants were obtained at a frequency of approximately 1/1,000 surviving cells and were consistently obtained more readily from strain 272 (81 mutants) than from strain 87 (44 mutants). The mutants displayed a range of phenotypes between weakly mycelial (Fig. 1B) and strongly mycelial (Fig. 1C) and gray, yeastlike sectors frequently appeared on the white colonies. We have designated the mutations to mycelial phenotype *rem* for *r*epressor of *m*ycelial phenotype.

Genetics of mutation to the mycelial phenotype.

The original strategy to test the dominance of the *rem* mutants was to isolate diploids by fusion of the mutant strains with either of the parental strains (87 and 272) and to determine the phenotype (yeastlike or filamentous) of these diploids. In addition, diploid formation between mutants of strain 87 and mutants of strain 272, and determination of the diploid phenotype, would theoretically allow complementation tests to be performed. Complementation tests by diploid formation have been described for the analysis of mutations affecting nitrogen utilization in *U. maydis* (Lewis and Fincham 1970). Parental strains 87 and 272 were chosen for mutagenesis because these strains are compatible at the *a* locus and incompatible at *b,* and they contain complementary auxotrophic mutations to allow the selection of diploid fusion products on minimal medium. Previous work showed that these strains readily fuse to yield diploids with the yeastlike phenotype indicative of homozygosity at the *b* locus (e.g., diploid d410, [Kronstad and Leong 1989]).

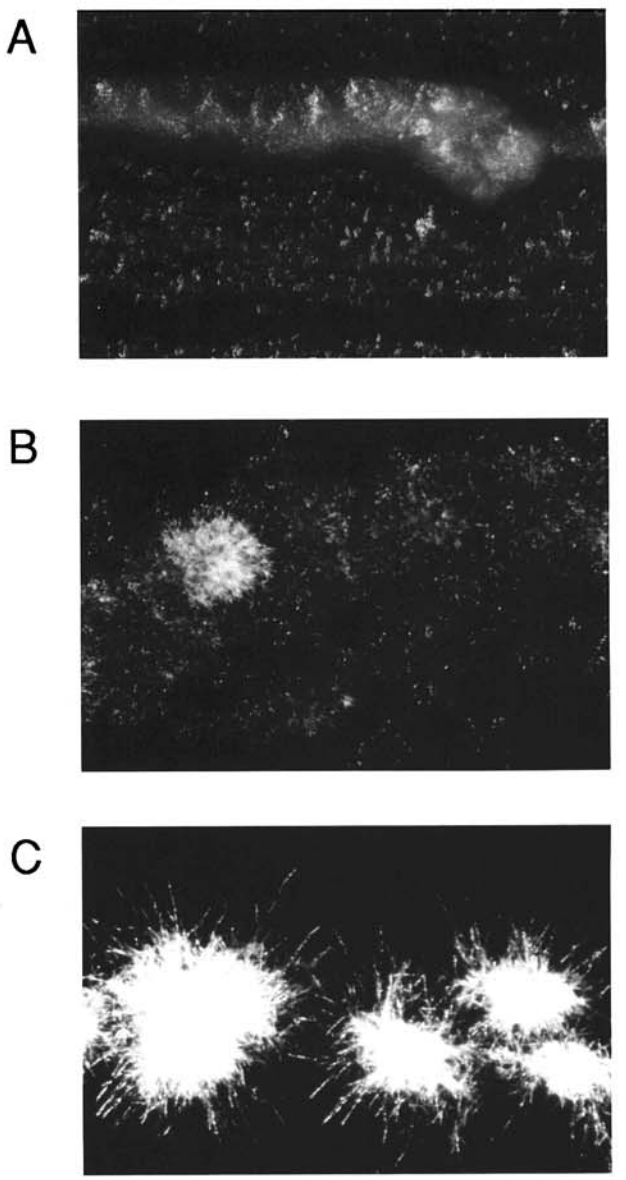

Fig. 1. Colony morphology of parental and mutant haploid strains of *Ustilago maydis.* Photograph of colonies from **A,** the parental strain 87; **B,** the weakly mycelial strain 87-23; and **C,** the strongly mycelial strain 87-18. The colonies were grown for 24 hr on medium containing activated charcoal (Holliday 1974). The magnification is 25× (A) or 50× (B and C).

In general, it proved difficult to isolate diploids when one of the fusion partners carried an *rem* mutation. For example, diploids were obtained with only nine of the mutants from strain 87 (mixed with strain 272) and 18 of the mutants from strain 272 (mixed with strain 87). Presumably, the filamentous growth habit of the mutants interferes with fusion with a budding mating partner. In all cases where diploids were obtained (e.g., mutant strain 87-18 × 272), the resulting fusion product displayed a yeastlike growth habit, suggesting that the *rem* mutation was recessive. That the products of fusion were indeed diploid was demonstrated by mating each strain with *a1 b1* (521) and *a2 b1* (032) tester strains. Positive mating reactions were obtained with both strains, indicating the presence of both *a* idiomorphs in the diploid strains.

Although diploids could be obtained with difficulty in crosses involving one mutant and one parental strain, it proved more difficult to obtain diploid fusion products when two different mutants were mixed. Also, the apparent instability of some of the mutant phenotypes (frequent appearance of yeastlike sectors) raised the concern that revertants rather than mutants might be participating in the fusion events to yield diploids. Overall, the inability to readily generate diploid strains precluded a complementation analysis of the mutations. Because of the difficulty with the genetic analysis of the *rem* mutants as a group, we focused our attention on the characterization of the morphology, and the analysis of the pathogenicity, of one relatively stable mutant with a strongly mycelial phenotype (Fig. 1; strain 87-18). The mutation in this strain was designated *rem1-1*. In addition, a cosmid library was used to complement the mutation in the *rem1-1* strain.

The *rem1-1* mutation blocks budding growth.

On solid medium, mutant strain 87-18 (*rem1-1*) displays a strongly mycelial phenotype (Fig. 1C), compared with the parental strain 87 (Fig. 1A). To look more closely at this phenotype, we compared the cellular morphology of the mutant with that of the parental strain after growth in liquid medium (Fig. 2A,B). Phase-contrast microscopy demonstrated the striking result that the *rem1-1* mutation eliminates the budding pattern of growth seen for haploid strains of *U. maydis* (e.g., strain 87) and converts the fungus almost entirely to a filamentous morphology. Confocal microscopy was employed to determine the distribution of nuclei in the hyphae of strain 87-18 (Fig. 2C,D) compared with the distribution in the parental haploid strain. In general, only one nucleus was detected per cell in the mutant strain 87-18 (*rem1-1*) and in the parental strain. Similar observations revealed that mixtures of compatible haploid strains (carrying different *a* and *b* genes) and diploids heterozygous at *a* and *b* contain both

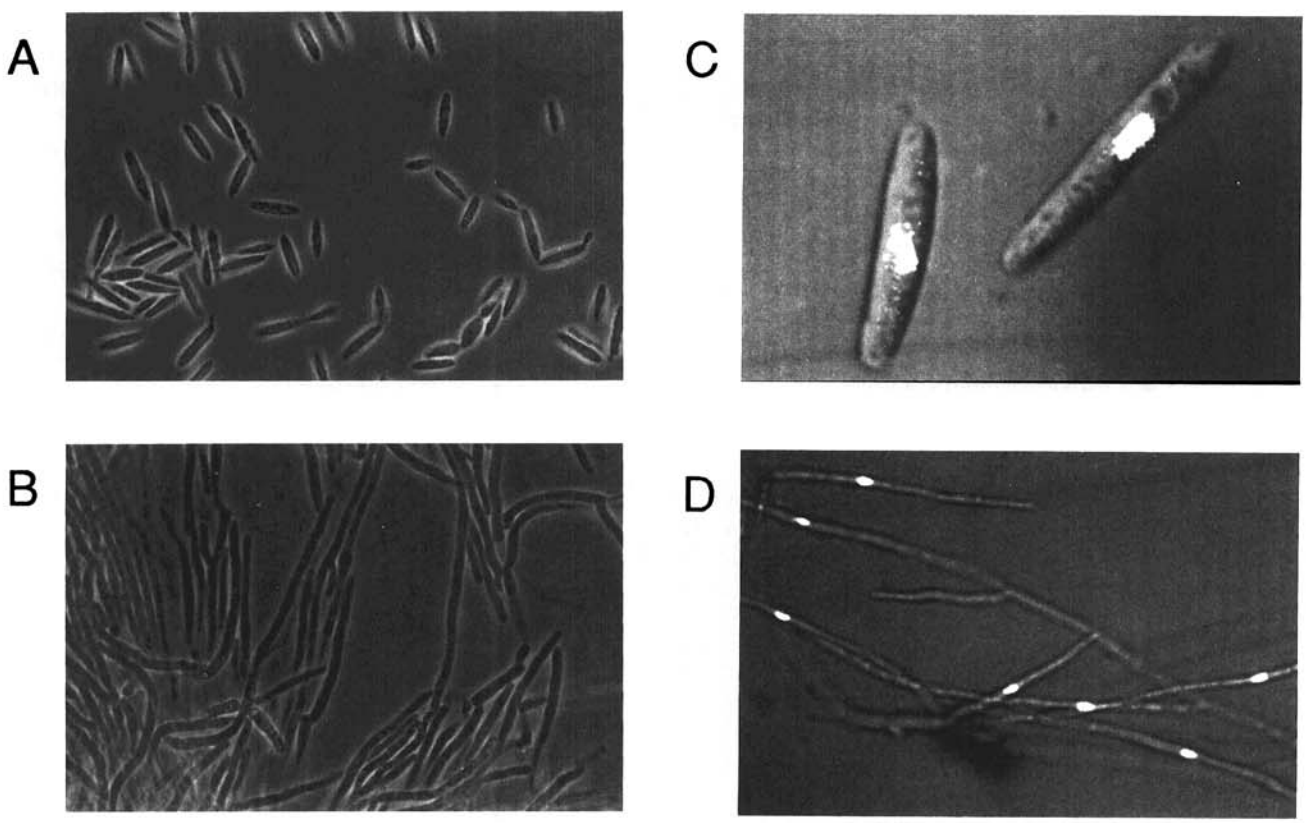

Fig. 2. Cell morphology of parental and mutant strains of *Ustilago maydis*. Phase-contrast micrographs of the cells of **A,** the parental strain 87; **B,** and the *rem1-1* mutant 87-18. The cells were grown for 24 hr in liquid double complete medium (Holliday 1974). The magnification is 1,000×. Confocal microscope images of strains **C,** 87 and **D,** 87-18 are also shown. The photographs were reproduced from a computer image; the estimated magnifications are 4,000× (C) and 1,000× (D). Although not clearly visible in (D), the mycelium of the mutant is septate. Nuclei are generally distributed throughout the filaments, although an occasional cell was observed that did not carry a nucleus.

budding cells and filaments. The latter cells generally appear to be devoid of nuclei and cytoplasm (K. B. and J. W. K., unpublished results). These observations are similar to those reported by Day and Anagnostakis (1971). These investigators found that filaments generated by fusion of haploid cells of opposite mating type were often devoid of cytoplasm and nuclei except in cells at hyphal tips. Similar results have recently been reported by Snetselaar and Mims (1992). While the mutant strain 87-18 superficially appears to mimic the phenotype of the filamentous cell type resulting from mating interactions, microscopic examination suggests that the filaments are different in that the majority of the *rem1-1* cells contained cytoplasm and nuclei.

The *rem1-1* mutation does not confer pathogenicity on haploid strains.

A number of experiments were performed to assess the influence of the *rem* mutation on pathogenicity. The results given in Table 1 reveal that the injection of the original *rem1-1* mutant (strain 87-18) alone into corn plants did not result in disease symptoms. This result was anticipated because the parental strain (87) is auxotrophic (*ad1-1 leu1-1*), and auxotrophs are known to be nonpathogenic (Holliday 1961). For example, Holliday (1961) has demonstrated that mixtures of *ad1-1* auxotrophs, compatible for mating type, fail to give disease symptoms when injected into corn seedlings. It should be noted that the original mutant strain (87-18) was capable of participating in an infection when injected in a mixture with a wild-type, compatible strain (521, *a1 b1*; Table 1).

The teliospores from an infection of corn seedlings with a mixture of strains 87-18 and 521 were germinated on PDA, and the resulting progeny were tested on medium containing activated charcoal to evaluate the segregation of the phenotype resulting from the *rem1-1* mutation. The progeny analyzed had either a mycelial phenotype or a yeastlike phenotype, but the ratio between the two types varied between experiments. In some cases, very few mycelial progeny were isolated (e.g., 1%) and in other cases up to 47% of the progeny were mycelia. The finding of 47% mycelial progeny indicates that a single locus is segregating; the reduction in the number of mycelial progeny in some experiments could be due to the reduced growth rate of the mycelial progeny compared with the budding progeny. That is, budding cells would have a growth advantage over hyphae and would be present in greater relative numbers upon germination of teliospores and subsequent division of the meiotic progeny.

Prototrophic progeny carrying the *rem1-1* mutation were obtained from the segregation analysis described above and were injected into corn seedlings to assess whether the mutation conferred pathogenicity on a haploid strain. These strains are listed in Table 2 (designated KB), and the determination of the *a* and *b* sequences in some of these strains is shown in Figure 3. None of the prototrophic, mycelial strains resulted in disease syptoms upon injection, indicating that the *rem1-1* mutation was not sufficient to confer pathogenicity. The results for three of these strains (KB2, KB7, and KB14) are shown in Table 1. In contrast, previous work has shown that the presence of an additional *b* gene in a haploid strain (e.g. *a1 b1 +b2*) is sufficient to establish a filamentous cell type and to confer pathogenicity (Kronstad and Leong 1989).

The availability of prototrophic, mycelial haploid strains also allowed a test of pathogenicity when each member

Table 1. Pathogenicity of strains carrying the *rem1-1* mutation or a gene disruption

Strain or cross	Symptoms[a]					Total number of plants
	A	B	C	D	E	
87	20	0	0	0	0	20
87 × 521	2	0	6	5	0	13
87-18	28	0	0	0	0	28
87-18 × 521	9	7	7	12	0	35[b]
87-18 × 518	10	0	0	0	0	10
518 × 521	0	1	4	10	3	18[c]
KB2	10	0	0	0	0	10
KB7	9	0	0	0	0	9
KB14	9	0	0	0	0	9
KB2 × UM031	15	0	0	0	0	15
KB2 × UM032	3	5	4	14	23	49[c]
KB7 × UM031	15	0	0	0	0	15
KB7 × UM032	5	16	15	10	14	60[c]
KB14 × UM031	33	16	4	13	1	67[c]
KB14 × UM032	12	0	0	0	0	12
KB2 × KB14	22	20	8	11	0	61[c]
KB7 × KB14	16	14	6	9	1	46[c]
UM031 × UM032	1	1	5	2	0	9[b]
C002	13	0	0	0	0	13
C001	19	0	0	0	0	19
C001 × 521	19	9	10	3	0	41[c]
C002 × 518	8	4	20	7	0	39[c]
C001 × C002	68	4	0	0	0	72[c]

[a] Disease symptoms are indicated as follows. A = no symptoms; B = anthocyanin; C = galls on leaves; D = galls on stems; E = dead plants. The number of plants in each category is given along with the total number of plants inoculated.

[b] The results represent the pooled data from three replicates of the inoculations.

[c] These data are from two replicates of the inoculations.

Table 2. Strains of *Ustilago maydis*

Strain	Relevant genotype	Source
87	*a2 b2 ad1-1 leu1-1*	S. A. Leong
272	*a1 b2 pan1-1 inos1-3 nar1-1 pyr1-1*	S. A. Leong
518	*a2 b2*	S. A. Leong
521	*a1 b1*	S. A. Leong
d132	*a1/a2 b1/b2*	S. A. Leong
UM032	*a2 b1*	S. A. Leong
UM031	*a1 b2*	S. A. Leong
C001	*a2 b2 C::HygB*	This study
C002	*a1 b1 C::HygB*	This study
KB1	*a2 b2 rem1-1*	This study
KB2	*a1 b2 rem1-1*	This study
KB5	*a2 b?*[a] *rem1-1*	This study
KB6	*a2 b? rem1-1*	This study
KB7	*a1 b2 rem1-1*	This study
KB9	*a1 b2 rem1-1*	This study
KB10	*a2 b2 rem101*	This study
KB14	*a2 b1 rem1-1*	This study
KB16	*a2 b? rem1-1*	This study
KB17	*a1 b? rem1-1*	This study
87-18	*a2 b2 ad1-1 leu1-1 rem1-1*	This study
87-23	*a2 b2 ad1-1 leu1-1 rem*[b]	This study

[a] The *b* sequences were not identified in strains with a question mark (Fig. 3).

[b] The *rem* allele in this strain has not been determined.

of a compatible pair carried the *rem1* mutation. The standard plate assay for determining the mating type of *U. maydis* (Puhalla 1970) could not be employed due to the mycelial phenotype of the progeny. Therefore, the *b* sequences (*b1* or *b2*) present in these strains were determined using a polymerase chain reaction assay and specific primers to distinguish different *b* genes (Fig. 3A,B). The strains carrying *a1* were identified using a hybridization probe specific for the *a1* idiomorph (Bakkeren *et al.* 1992) as shown in Figure 3C. The molecular determination of

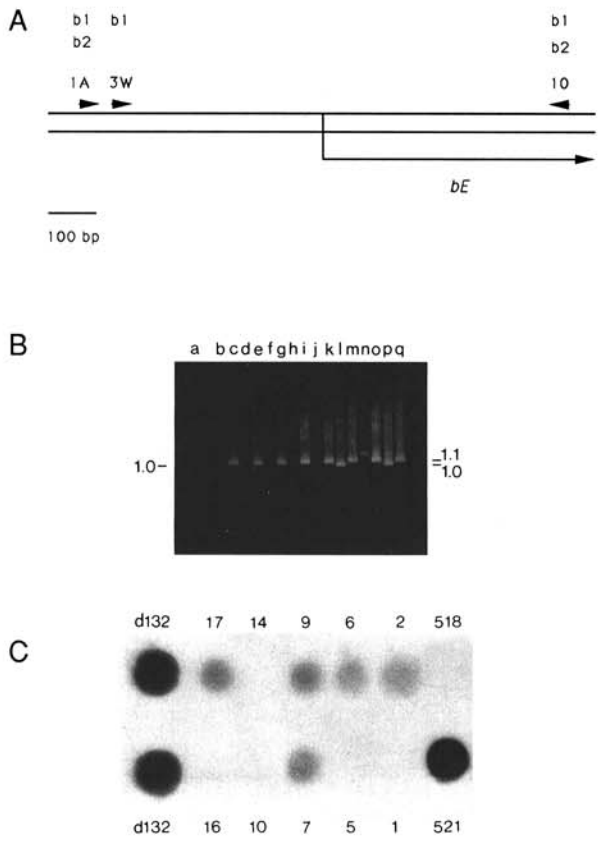

Fig. 3. Determination of the mating types of mycelial strains by hybridization and the polymerase chain reaction. **A,** Diagram of the *b* locus showing the positions of primers for distinguishing the *b1* and *b2* regions. **B,** Agarose gel of the products of the polymerase chain reactions with the primers shown in A. Total genomic DNA was isolated from the mycelial prototrophic progeny of a cross between strain 87-18 (*rem1-1 ade1*) and wild-type strain 521. The lanes contain marker DNAs or amplified products from DNA of various strains as follows: a, 1-kb ladder (BRL, Life Technologies Inc.); b and c, strain 518; d and e, strain KB1; f and g, strain KB2; h and i, strain KB7, j and k, strain KB9; i and m, strain KB14; n and o, strain KB10; p and q, strain 521. For each pair of amplifications, the first lane contains DNA amplified with primers 3W and 10 (1.0-kb product); the second lane contains DNA amplified with primers 1A and 10 (1.1-kb product). The latter pair of primers gives a 1.0-kb amplification product only with genomic DNA from strains carrying the *b1* region (lane l, strain KB14; lane p, strain 521). C, Dot blot hybridization analysis of DNAs from prototrophic progeny of the cross between 87-18 and strain 521. The strain designations are given above and below the dots; note that DNAs from additional progeny are included on the dot blot compared with the polymerase chain reaction experiment. The filter was hybridized with a 3.8-kb *Eco*RI fragment from the *a1* idiomorph of *U. maydis* and exposed to film for 19 hr.

mating type revealed that one of the mycelial progeny was *a2 b1* (strain KB14) and that two others were *a1 b2* (e.g., strains KB2 and KB7). These mating types were subsequently confirmed by inoculations with test strains of various mating-type combinations (Table 1). As shown in Table 1, injection of mixtures of compatible, prototrophic mutants (e.g., KB14 and KB2) gave disease symptoms on corn seedlings comparable to those obtained with some mixtures of wild-type and mutant strains (e.g., KB14 × UM031).

Complementation of the *rem1-1* mutation.

As described earlier, dominance tests performed by the formation of diploids between strain 272 and strain 87-18 suggested that the *rem1-1* mutation was recessive. Therefore, a complementation approach was employed to isolate the corresponding gene. A cosmid library of genomic DNA from wild-type strain 518 was prepared in a *U. maydis* vector (pJW42) carrying an autonomously replicating sequence (Tsukuda *et al.* 1988) and a hygromycin B resistance gene for selection of transformants (Wang *et al.* 1988, 1989; Froeliger and Leong 1991). A pool of DNA from the library was introduced into strain 87-18 (*rem1-1*) and hygromycin B resistant transformants were selected. Subsequently, transformants were transferred to rich medium containing activated charcoal and hygromycin B to assay for the mycelial phenotype. Among 1,859 transformants tested, 209 were found to display a yeastlike phenotype. The high frequency at which these strains arose suggested that reversion had occurred during the transformation procedure. As mentioned earlier, the *rem* mutations tend to be unstable, and growth in liquid medium may actually select for the more rapidly dividing yeastlike revertants.

To distinguish reversion from actual complementation among the yeastlike transformants, each of the 209 transformants was grown to high cell density in liquid, complete medium in the absence of selection with hygromycin B. Two successive transfers were made in liquid medium to allow loss of the cosmid DNA; subsequently, aliquots of the cultures were plated on medium with activated charcoal and the phenotype of individual colonies was assessed. Of the 209 transformants, 206 were found to give hygromycin B-sensitive, yeastlike colonies, indicating that reversion had occurred in these strains. The three remaining transformants displayed the mycelial, mutant phenotype of the original strain 87-18 upon loss of the cosmid (and hygromycin B resistance). The cosmid in each of these transformants presumably carries DNA sequences capable of complementing the *rem1-1* mutation. These sequences may be the *rem1* gene itself or other sequences capable of suppressing the mycelial phenotype of the *rem1-1* mutant.

To confirm complementation, the cosmid DNA present in the three transformants was isolated by *in vitro* packaging of total DNA into phage particles and transfection into *E. coli.* Subsequent isolation of the cosmid DNA and retransformation into strain 87-18 yielded transformants that were yeastlike (100/100 tested) in their phenotype. This result indicated that the cosmid DNA carried a gene(s) that complements the *rem1-1* mutation.

Curing of the cosmid DNA from the transformants again restored the mycelial, mutant phenotype.

Analysis of the DNA from the three complementing cosmids with several restriction enzymes revealed that the inserts in each were highly similar, if not identical (K.B., data not shown). In addition, comparison with the restriction pattern of a cosmid carrying the *b1* allele of *U. maydis* (Kronstad and Leong 1989) indicated that the newly isolated region of genomic DNA was unrelated to the *b* locus. Similarly, the DNA fragments carrying regions of the *a1* and *a2* idiomorphs (Froeliger and Leong 1991; Bakkeren *et al.* 1992) failed to hybridize with the cosmids that complement the *rem1-1* mutation (G. Bakkeren and K. B., data not shown).

A collection of transposon Tn*5* insertions in one of the cosmids, designated pFuz60, was generated to identify the region responsible for complementation of the *rem1-1* mutation. Specifically, one or more Tn*5* insertions were obtained in each of the *Bam*HI restriction fragments (designated A-N) of the cosmid. As shown in Figure 4A, insertions in *Bam*HI fragments encompassing an approximately 10-kb region (*Bam*HI fragments C, E, and K) blocked the ability of the cosmid to complement the mutation in strain 87-18. A subcloned *Kpn*I fragment spanning this region also complements the *rem1-1* mutation, and Northern blot analysis (Fig. 4C) using the *Bam*HI C fragment as a probe revealed that an approximately 9.0-kb transcript is encoded by this region. Overall, these results indicate that the gene responsible for complementation may be quite large.

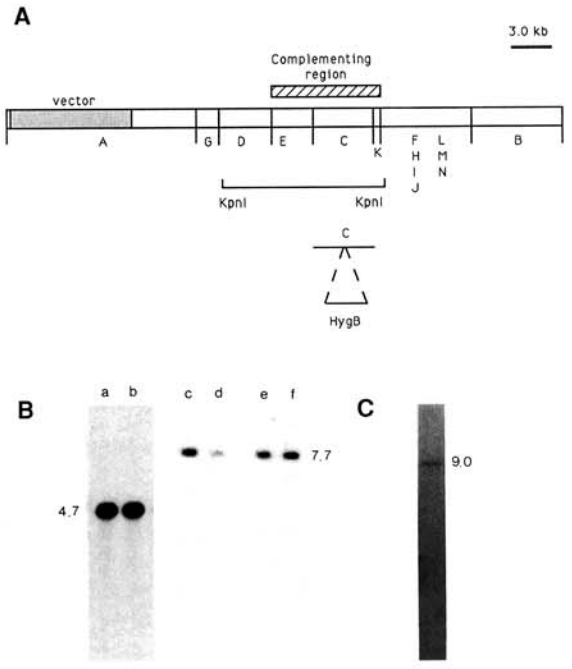

Fig. 4. Localization and disruption of the complementing region on pFuz60. **A,** Diagram of the positions of *Bam*HI fragments of the cosmid pFuz60 and the region in which insertions of transposon Tn*5* block the ability of the cosmid to complement the *rem1-1* mutation. A *Kpn*I fragment that overlaps the complementing region defined by Tn*5* insertion is shown below the map of the cosmid; this fragment was found to complement the *rem1-1* mutation. The position of the hygromycin B resistance marker in the *Bam*HI C fragment is also shown. This construct was employed to generate strains disrupted at the genomic location of the *Bam*HI C fragment. Note that the order of the smaller *Bam*HI fragments (F, H–J, L–N) has not been determined. **B,** Autoradiogram of a Southern blot containing *Bam*HI digested DNAs from the wild-type strains 518 (lane a) and 521 (lane b) and strains containing disruptions of the *Bam*HI C (two isolates of strain C002, lanes c and d; two isolates of strain C001, lanes e and f). The hybridization probe was the *Bam*HI C fragment shown in A and the exposure time was 24 hr. Note that all lanes were taken from the same Southern blot and that the sizes (kb) were determined by comparison with the 1-kb ladder supplied by BRL (Life Technologies Inc.). **C,** Autoradiogram of a Northern blot containing RNA from a mixture of strains 518 and 521. The hybridization probe was the *Bam*HI C fragment and the exposure time was 4 days. The size of the hybridizing band was determined by comparsion to RNA size standards (BRL, Life Technologies, Inc.).

It should be noted that the cosmid pFuz60 also complemented the mutation(s) in five additional, independent mutants (out of five tested). This result suggests that the mutations leading to the mycelial phenotype in these other mutants are in the *rem1* gene or that the sequences on the cosmid can suppress mutations in one or more other genes.

Gene replacement generates haploid strains with the *rem1* phenotype.

The identification of *Bam*HI fragments in which Tn*5* insertions block complementation allowed the construction of additional mycelial mutants by gene replacement. Although the precise position of the complementing sequences on the cosmid is not known, the C fragment from the *Bam*HI digest is completely within the region required for complementation of the *rem1-1* mutation as defined by Tn*5* mutagenesis. Insertion of the gene for hygromycin B resistance into the *Bam*HI C fragment (Fig. 4A) and replacement of the genomic region with this construct resulted in transformants (C002 *a1 b1 C::HygB* and C001 *a2 b2 C::HygB*) with a constitutively mycelial phenotype like that of strain 87-18. That a homologous integration event had occurred at the *Bam*HI C fragment was confirmed by Southern hybridization analysis (Fig. 4B). In general, the strains carrying the disruption were strongly mycelial and displayed a phenotype very similar to that of strain 87-18. These results suggest but do not prove that the pFuz60 cosmid actually encodes the *rem1* gene rather than a gene capable of suppressing the *rem1-1* mutation.

Inoculation of corn seedlings with a mixture of compatible strains, C002 and C001, each containing an insertion of the hygromycin B gene in the genomic region of the *Bam*HI C fragment, did not result in significant disease symptoms (Table 1), although a small proportion (4/72) of the plants showed anthocyanin on leaves. This result suggests that the infection seen in crosses in which both haploids carried the original *rem1* mutation (e.g., KB2 × KB14) may have resulted from leakiness or reversion of the mutation. The strains carrying the disruption mutation were capable of participating in crosses with wild-type strains to cause disease (e.g., C002 × 518; Table 1).

DISCUSSION

The primary goal of the present work was to identify genetic loci, other than *a* and *b*, that are involved in the

formation of the infectious, filamentous dikaryon of *U. maydis*. The strategy was to make use of the ability of haploid, yeastlike strains of *U. maydis* to undergo mutation to a constitutively mycelial phenotype. A number of mutants of this type were generated and characterized phenotypically; however, direct complementation tests were hampered by the low frequency at which mutant strains could participate in diploid formation.

Because genetic analysis proved difficult, attention was focused on the characterization of one mutant (strain 87-18) that displayed a strongly mycelial phenotype. The cellular morphology of this mutant in liquid medium was striking, compared with the parental strain 87, in that the mutant grew with a completely filamentous phenotype, i.e., only hyphae were observed. Clearly, the mutation greatly reduced the ability of the cells to divide by budding. Our microscopic observations (Fig. 2) revealed that strain 87-18 does not contain paired nuclei like those found in the filamentous cell type obtained when haploid cells conjugate (Day and Anagnostakis 1971; Banuett 1992; Snetselaar and Mims 1992). Snetselaar and Mims (1992) have recently published a description of the early events in the formation of the filamentous cell type believed to represent the infectious dikaryon. This description includes a demonstration of the formation of conjugation tubes, the fusion of these structures between mating sporidia and the subsequent outgrowth of the filamentous cell type (containing paired nuclei).

Pathogenicity tests were performed to determine whether the *rem1-1* mutation influenced the ability of *U. maydis* to infect corn seedlings. The results indicate that the *rem1-1* mutation does not allow haploid strains to infect plants by themselves even though the mutation gives the filamentous phenotype expected of the pathogenic cell type. It appears that it is not sufficient for *U. maydis* to grow with a filamentous morphology to be pathogenic on corn seedlings and that additional conditions, not established by the *rem1* mutation, are also required. It is possible that the *rem1-1* mutation influences just part of the process of formation of the filamentous cell type, and that the complete process requires heterozygosity at *b*. That is, it is likely that other genes, which are under *b* control, are required for pathogenicity. Cells carrying the *rem1-1* mutation can still participate in infections when paired with wild-type or mutant strains of compatible mating type. Disease symptoms were virtually eliminated in inoculations with strains carrying a disruption in the region that complements the *rem1-1* mutation (e.g. C001 × C002). Although strains carrying the disruption were capable of fusion with wild-type strains, as demonstrated by pathogenicity tests, it remains to be determined whether these mutant strains can actually fuse with each other. That is, it is possible that the block for pathogenesis, in crosses involving disruption strains, is at the level of cell fusion. It should be reemphasized that these findings could also result from differences between the stability of the original mutation in strain 87-18 and the stability of the mutation created by gene disruption in strains C001 and C002.

Stakman *et al.* (1943) reported that infections initiated with compatible strains, each carrying a "white" trait, resulted in large tumors on plants but failed to yield teliospores. In contrast to the results of Stakman *et al.* (1943), our inoculations with compatible strains, each carrying the original *rem1-1* mutation, gave disease symptoms and, in some cases, teliospores were detected. Thus, the relationship between the mutants described by Stakman *et al.*, which appear to have been filamentous in morphology, and the *rem1* mutants described here is unclear.

It is of interest to speculate about possible roles for the *rem1* gene in *U. maydis*. One possible role might be to encode a repressor that functions to prevent expression of genes involved in establishing filamentous growth. The finding that *rem1-1* is a recessive mutation with a gain-of-function (mycelial growth) phenotype is consistent with this idea. Possible examples of target genes for this putative repressor might include those encoding cell wall biosynthetic enzymes such as chitin synthase or those encoding products involved in selection of sites for bud formation. A useful paradigm for a repressor function for the product of the *rem1* gene is the *RME1* (repressor of meiosis) gene of *Saccharomyces cerevisiae* (Mitchell and Herskowitz 1986). *RME1* encodes a repressor of genes needed for meiosis and sporulation; expression of *RME1* is repressed by the combined products of the MAT*a* and MATα loci, the **a**1/α2 repressor. In *U. maydis*, the heteromeric regulatory protein believed to be established when different alleles of the *b* genes are present in the same cell would play a similar role to that of the *a1/a2* repressor. A prediction of this model would be that the state of the *b* locus (homozygous or heterozygous) would influence expression of the *rem1* gene.

Other possible functions for the *rem1* gene product include a direct role in cell wall biosynthesis, a role in the establishment of the site of deposition for new cell wall material or a role in bud site selection. It may be the case that *rem1-1* cells are unable to establish a site of bud formation and that filamentous growth is the resulting default growth morphology. Given the recent demonstration that *S. cerevisiae* can display pseudohyphal growth (Gimeno *et al.* 1992), it is also possible that the *rem1* gene is involved in sensing the nutritional status of the cell and/or transmitting a signal that triggers a morphological change.

The *rem1* mutation joins previously described mutations that influence cell morphology in fungi (Harold 1990). For example, mutations in *N. crassa*, such as *col-2* (Brody and Tatum 1966) and *crisp-1* (Terenzi *et al.* 1976; Pall *et al.* 1981), influence growth rate, branching, and conidiation. In *A. nidulans*, mutation in the regulatory gene *brlA* influences the switch from apical to budding growth during conidiation (Adams *et al.* 1988). In addition, cell cycle mutants in *S. cerevisiae*, such as *cdc4* and *10* yield arrested cells that have multiple buds and/or elongated shapes (Adams and Pringle 1984; Pringle and Hartwell 1981). These studies make it clear that the *rem1* gene could function in a number of different ways to influence cell morphology.

In summary, a mutation called *rem1* has been identified that causes the dimorphic pathogen, *U. maydis*, to switch from budding to filamentous growth. This mutation does not confer pathogenicity on haploid strains even though the mutant phenotype superficially resembles that of

pathogenic haploid and diploid strains. A cosmid has been isolated that complements the *rem1-1* mutation. Disruption of the complementing region in the *Ustilago* genome yields haploid cells with the *rem* phenotype. In infections where both compatible haploid strains carried a disruption of the complementing region, very few symptoms were found on inoculated plants. We conclude that the disruption mutation influences the growth morphology and the ability of the fungus to establish an infection. The analysis of the sequence and the expression of the region that complements the *rem1-1* mutation will undoubtedly provide insight into dimorphism and pathogenesis in *U. maydis*.

MATERIALS AND METHODS

Fungal and bacterial strains.

The strains of *U. maydis* employed in this study are listed in Table 2. *E. coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) was employed for construction of a cosmid library and for routine transformation experiments. *E. coli* strain DH10B (*F*⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻*rps*L *end*A1 *nup*G) was employed for transformation of cosmid DNA by electroporation. *U. maydis* strains were grown on potato-dextrose media (PDA and PDB, Difco) and on complete or minimal medium as described by Holliday (1974). The formation of aerial mycelium was assayed after growth on double complete medium containing 1% activated charcoal (Day and Anagnostakis 1971; Holliday 1974). *E. coli* strains were grown in LB medium as described (Sambrook 1989).

Mutagenesis.

Actively growing cultures of *U. maydis* strains 87 and 272 (10^8 cells per milliliter) were exposed to ultraviolet light for sufficient time to kill 80–90% of the cells (generally 2–3 min) and dilutions were plated directly on rich medium containing activated charcoal (Day and Anagnostakis 1971; Holliday 1974). Petri plates were sealed with Parafilm and incubated at 30° C for 2–3 days in the dark. Tn*5* mutagenesis was performed as described by de Bruijn and Lupski (1984).

DNA manipulations, library construction, and transformation.

Recombinant DNA techniques were performed essentially as described (Sambrook *et al.* 1989; Kronstad and Leong 1989). Restriction and DNA modifying enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim (Indianapolis, IN), and Pharmacia (Uppsala, Sweden). A cosmid library was constructed in the *U. maydis* vector pJW42 as described previously (Wang *et al.* 1988; Wang *et al.* 1989; Froeliger and Leong 1991). Total genomic DNA was isolated from *U. maydis* strain 518 and partially digested with *Sau3A*1 to yield fragments of 30–40 kb. These fragments were ligated into the *Bam*HI site of pJW42; the *Bam*HI ends of the linearized vector were dephosphorylated with calf intestinal phosphatase as recommended by the supplier (Boehringer Mannheim). Ligated DNA was packaged *in vitro* using the Gigapack packaging extract of Stratagene (La Jolla, CA) and transfected into *E. coli* strain DH5α. Approximately 10^6 individual transfectants were pooled from transfection plates (LB containing 50 μg/ml ampicillin), grown for 6 hr in LB containing ampicillin, and DNA was prepared as described (Ish-Horowicz and Burke 1981). *U. maydis* strains were transformed using the protocol of Wang *et al.* (1988). Cosmid DNA was recovered from putative transformants by packaging total DNA from *U. maydis* with Gigapack packaging extract (Stratagene) and transfection into *E. coli* DH5α. Cosmid DNA was also recovered by electroporation into *E. coli* DH10B, followed by selection on LB agar plates containing ampicillin.

The polymerase chain reaction was employed to identify the *b* genes present in mycelial strains. A Perkin-Elmer Cetus thermal cycler (Norwalk, CT) was employed with an initial 7-min incubation at 94° C followed by 25 cycles of 1 min at 94° C, 1 min at 55° C, and 2 min at 72° C. Three oligonucleotide primers were employed to distinguish between the *b1* and *b2* sequences. Primer 1A (5′ AACGGATCCTCATAAGCCTCCTCGTAT 3′) anneals to a sequence within the open reading frame of both *b1W* and *b2W*; this sequence is 413 bp upstream of the initiation codon for the *bE* gene. Primer 10 (5′ AAGGATCCATAGCGTGAGCTGATGA 3′) anneals to a region 497 bp downstream of the intiation codons (within the open reading frame) of both *b1E* and *b2E*. Primer 3W (5′ ATGGATCCTCATACACTCGTCGTAG 3′) anneals only to a sequence 431 bp upstream of the *b1E* initiation codon. This primer was designed to not anneal to *b2* DNA because of several mismatched positions. To distinguish the *a* idiomorphs present in mycelial mutants, a DNA fragment from the *a1* idiomorph (Froeliger and Leong 1991; Bakkeren *et al.* 1992) was labeled using the Oligolabeling Kit of Pharmacia and hybridized to total nucleic acid (2–5 μg spotted on a nitrocellulose filter) as described (Sambrook *et al.* 1989). Total nucleic acid was isolated from *U. maydis* using a method developed for *S. cerevisiae* (Elder *et al.* 1983). Southern hybridization analyses were performed as described (Bakkeren *et al.* 1992).

The disruption strains C001 and C002 were constructed by transformation with a version of the *Bam*HI C fragment (cosmid pFuz60) containing the hygromycin B resistance marker. The resistance marker was obtained as a 3.0-kb *Hin*dIII restriction fragment from the plasmid pHL1 (Wang *et al.* 1988) and inserted at a unique *Hin*dIII site near the center of the 4.7-kb *Bam*HI C fragment.

RNA analysis.

A mixture of mating cells of strains 518 and 521 was prepared by inoculating 300 μl of an overnight culture of each strain (in PDB) onto petri dishes containing double complete medium containing 1% activated charcoal (Day and Anagnostakis 1971; Holliday 1974). The plates were sealed with Parafilm and incubated at 30° C for 30 hr. This procedure resulted in a lawn of strong filamentous growth on the petri plates. These cells were collected with a glass rod, frozen in liquid nitrogen, and stored at −70° C. Total RNA was extracted from 40 g of cells (wet weight)

by grinding the material to a fine powder with a blender in the presence of liquid nitrogen and extracting the RNA with guanidine thiocyanate (Chirgwin *et al.* 1979). Polyadenylated RNA was purified on oligo(dT) cellulose as described (Sambrook *et al.* 1989). Northern blot analysis was performed using formaldehyde-agarose gels (Ausubel *et al.* 1987). RNA (0.85 μg) was transferred to Zetabind membrane and hybridized to a 4.7-kb *Bam*HI fragment (C fragment) labeled with ^{32}P using the Pharmacia Oligo-labeling Kit. A 0.24- to 9.5-kb RNA ladder (BRL) was used to estimate RNA size.

Inoculation of plants.

Seven-day-old seedlings ("Golden Bantam," Buckerfield Seeds, Vancouver, BC) were grown in Sunshine Mix and injected at the soil line with 50–100 μl of *U. maydis* cell suspensions (10^6–10^7 per milliliter) using a 1-ml syringe and a 26 gauge needle. Plants were maintained in a Conviron model E15 growth chamber with cycles of 14 hr of illumination (26° C) and 10 hr of darkness (21° C).

Microscopy.

Confocal microscopy was performed using a Bio-Rad MRC 500 microscope (Richmond, CA). Nuclei of *U. maydis* were stained with mithramycin essentially as described (Slater 1976). *U. maydis* cells were grown for 24–48 hr on rich medium containing activated charcoal (Day and Anagnostakis 1971; Holliday 1974) prior to examination.

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