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Pathogenicity Resulting from Mutation at the *b* Locus of *Ustilago maydis*

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ABSTRACT **This paper explores the genetic basis of the ability of the fungus, *Ustilago maydis*, to induce neoplastic galls in the corn plant (*Zea mays*). Pathogenic mutants of *U. maydis* were produced by ultraviolet irradiation of cultures of nonpathogenic diploids homozygous at the *b* locus. The mutants formed smaller neoplasms, produced fewer teliospores, and showed higher frequencies of meiotic failure and lower rates of basidiospore survival than did the wild-type fungus.**

The corn plant (*Zea mays*) suffers from a neoplastic disease induced by a fungus, *Ustilago maydis*. Two genetic loci (*a* and *b*) in the fungus control sexual compatibility and pathogenicity. The *a* locus has two alleles and controls cell fusion in the pathogen (1). The *b* locus has 18 known alleles, designated by the letters A through R (2), and determines the competence of the dikaryotic fusion product to initiate disease in the host. When introduced through a wound, a competent dikaryon induces the neoplastic growth of meristematic host tissue to form a gall in which is embedded a mass of fungal teliospores (brandspores). Each teliospore is diploid. It germinates to form a promycelium in which meiosis occurs. The haploid meiotic products, or basidiospores, reproduce by budding and form yeastlike cultures. Wound infection by the dikaryon, formed from compatible haploid cells, completes the disease cycle.

We are interested in how the *b* locus controls the induction of the neoplasm; to this end, we have examined the effects of mutation on this control. In this paper we report on the action of some *b* mutants induced by ultraviolet radiation and present evidence that *b* controls the sequence of development from infection to meiosis and basidiospore formation at teliospore germination.

MATERIALS AND METHODS

Media and methods have been described (3, 4). Mating type was determined on double complete medium (CM × 2) in taped dishes after 3-days incubation at 25°C under continuous light (3). The auxotrophic markers *pan-1*, *met-15*, *ad-1* (requirements for pantothenate, methionine, and adenine, respectively) were used to synthesize marked diploids (5). Diploids were also recovered as unreduced products from germinated teliospores. Strains with mutant *b* factors were selected from diploid cells heterozygous for *a* and homozygous for *b* ($a\neq$ $b=$) after treatment with ultraviolet light. The nonmutant $a\neq$ $b=$ diploid cells form yeastlike colonies on CM ×2, whereas solopathogenic diploids heterozygous for both factors ($a\neq$ $b\neq$) form mycelial colonies (3). Artificial mixtures showed that the method was sufficiently sensitive to detect one mutant diploid as a mycelial spot in a background of 5×10^5 cells per plate. Three *b* factors were used: *bG*, *bD*, and *bI*.

Abbreviations: CM, complete medium; CM X2, double-strength CM.

RESULTS

In each selection experiment, mycelial colonies were picked from CM ×2 and inoculated to corn seedlings to test for pathogenicity. The results are shown in Table 1. The two diploids homozygous for *bG* were obtained independently as unreduced products from a natural infection and carried no auxotrophic markers. Six mutants formed galls containing teliospores. The galls were smaller than those produced by normal solopathogenic diploids, took 5–10 days longer to mature, and contained fewer teliospores. Control inoculations of the parental $a\neq$ $b=$ diploids never gave rise to galls.

The teliospores from galls produced by four of the mutants were sown on complete medium (CM), and tetrads were isolated by a method similar to that of Holliday (4). Colonies from single germinated teliospores, with from 20 to 75 cells, were picked to fresh plates of CM, one per plate, and spread. After 2 days of incubation, 16 colonies were selected for testing. All morphological types present were sampled. A sample of 16 does not always include all 4 meiotic products but, for those tetrads in which 3 products are recovered, the fourth may usually be deduced if normal meiosis is assumed.

The progeny of each diploid mutant included two classes: One with the original *b* factor and the other with a mutant *b* factor. Mutant *b* phenotypes within each progeny varied from yeastlike to mycelial on CM ×2. The *a* factor present could be determined in the mating-type test unless the mycelial phenotype was very pronounced and vigorous and obscured the test interactions. The mutants were compatible on CM ×2

TABLE 1. *Mutants isolated from $a\neq$ $b=$ diploids after UV treatment*

Factor	Surviving cells screened × 10^5	Putative mutants tested on corn	Pathogenic mutants	Designation
bG	0.9	1	1	(*bG mut-1*)
bG	1.8	5	2	(*bG mut-2*)*
bD	9.0	12	2	(*bD mut*)*
bI	3.6	3	1	(*bI mut*)

* Only one mutant analyzed.

TABLE 2. *Meiotic products recovered from original b mutants and crosses of related stocks*

Material inoculated	No. of teliospores with 4, 3, 2, 1, more than 4, and abnormal* products					
	4	3	2	1	>4	Abnormal*
a. Original mutants as diploids						
a1 bG / *a2 bGmut-1*	0	4	17	18	0	0
a1 bG / *a2 bGmut-2*	0	0	23	10	0	0
a1 pan bD + met / *a2 + bDmut ad +*	0	1	9	3	1	1
a1 pan bI ad + / *a2 + bImut + met*	0	0	4	3	0	0
b. Haploid mutant recombinants						
a1 bGmut-1	0	0	0	13	0	0
c. Mutant × wild type						
a1 pan bDmut ad × *a2 bI*	2	11	29	11	4	7
a1 pan bDmut ad × *a2 bA*	1	1	1	0	0	3
a2 + bDmut ad × *a1 bA*	12	11	0	1	4	2
a2 + bImut ad × *a1 bA*	9	10	4	0	6	5
d. Mutant × mutant						
a1 pan bDmut ad × *a2 bDmut met*	0	0	6	11	0	0
a1 pan bDmut ad × *a2 bImut met*	6	6	5	0	17	15
e. Wild type × wild type						
a1 pan bD ad met × *a2 + bI + +*	8	10	5	1	5	4

* Tetrads with 3 or 4 products in which there were more than 2 copies of one or more markers.

with their progenitors, with themselves, and with each other, when associated with different *a* factors. A small but variable proportion of the progeny of all 4 mutants showed no mating reaction on CM ×2.

The results of tetrad analysis for the mutants are shown in Table 2*a*. Among a total of 94 tetrads, none with 4 products was found. Five tetrads with 3 products were recovered, all of which could be interpreted as normal tetrads with one product missing. Among the 53 tetrads with 2 products, 12 included all the markers and could have been either ditype or incomplete tetratype tetrads. Of the 34 tetrads in which only one product was recovered, one tetrad was like the original mutant, and thus probably still diploid, while the remainder were of various, presumed haploid, phenotypes. The proportion of normal *b* to mutant *b* segregants among the pooled tetrads of each mutant was close to 1:1.

The frequency with which all 4 meiotic products are recovered tends to be low in *Ustilago*. Holliday (4) found that 10–30% of tetrads gave rise to 3 or 4 products. In our examples (Table 2*a*) the frequencies are even lower, ranging from 0 to 10.2%.

Representative, and presumed haploid, cultures of mutants *bGmut-1*, *bDmut*, and *bImut* were then inoculated into corn, both alone (Table 2*b*) and with different compatible marked haploids carrying wild-type *b* factors different from the mutant progenitor alleles (Table 2*c*). Only prototrophic cultures of *bGmut-1* were available. Inoculated alone they formed galls with teliospores, showing that *bGmut-1* strains are solopathogenic. No parental *bG* factors were recovered among the germination products of these teliospores. Attempts to cross *bGmut-1* strains with compatible haploids, with and without auxotrophic markers, were unsuccessful. Germinated teliospores gave rise only to sporidia like the *b* mutant parent. This appeared to block further analysis of the *bGmut-1*.

Auxotrophic strains of *bDmut* and *bImut* (Table 2*c*) were not solopathogenic, presumably because of their adenine requirement (4), but did form galls containing teliospores when inoculated together with compatible, haploid, prototrophic wild-types. Many of these teliospores showed normal segregations of markers from both parents (Table 2*c*). In no case did we recover the original *b* factors from which the mutants were derived. A total of 31 tetrads from these crosses, however, could not be accounted for by normal meiosis. Fourteen had more than 4 products (10 tetrads with 5, 3 with 6, and 1 with 7 products). The remaining tetrads with 3 or 4 products had more than 2 copies of one (13 tetrads), two (3 tetrads), or three (1 tetrad) markers. We do not believe that more than a small fraction of the abnormal tetrads can be due to cross contamination. No tetrad colony was picked for analysis unless it was isolated from other colonies and a single germinated teliospore was visible near its center.

Since it was now clear that mutant strains with auxotrophic markers could be forced to participate in crosses, the two types of crosses *bDmut* × *bDmut* and *bDmut* × *bImut* were made. Both combinations produced galls with teliospores. Only tetrads with one or two products were recovered from the cross *bDmut* × *bDmut* (Table 2*d*). In the class with only one product, 6 were uniformly and vigorously mycelial and prototrophic, 4 were *a2* and prototrophic, and one was mycelial and methionine requiring. In the class with two products all were *a2* and either *ad* or prototrophic and these tetrads are most simply interpreted as incomplete. If the products of the monotypic tetrads are all diploid, those that are *a2* or *met* arising as homozygotes by mitotic recombination, then meiosis failed in $^{11}/_{17}$, or 65%, of the tetrads sampled. In contrast, meiosis in the cross *bDmut* × *bImut* was more nearly normal (Table 2*d*). No apparent diploid strains were recovered and 12 of the 42 tetrads analyzed were normal with 3 or 4 products. However, 17 tetrads had more than 4 products (8 tetrads with 5, 7 with 6, and 2 with 7) and 8 tetrads with 3 or 4 products had more than 2 copies of one (5 tetrads), two (2 tetrads), or three (1 tetrad) markers.

A further sample of 5305 random haploids from a much larger sample of germinated teliospores from the cross *bDmut* × *bImut* was tested for *bD* and *bI*. None was found. We conclude from this result, and our failure to recover *bD* or *bI* from crosses of *bDmut* and *bImut* with *bA*, that *bDmut* and *bImut* are altered at sites which are not only close to each other (upper 5% fiducial limit, 0.03%), but probably part of the region specifying the *b* factor. No recombination between wild-type *b* factors was found in tests with a similar degree of resolution (2).

Holliday (6) reported that $a = b \neq$ diploids are solopathogenic and do not mate in corn with haploid stocks carrying different *a* and *b* factors. Our observation that this was also true of the *bGmut-1* factor prompted a closer look at the ap-

parent equivalence of the haploid mutants and the $a=$ $b\neq$ diploid. Like *bmut* strains, $a=$ $b\neq$ and $a\neq$ $b\neq$ diploids requiring adenine are not solopathogenic. We did not obtain galls in corn inoculated with such diploids mixed with complementary compatible haploids. It is clear from Table 2*c* and *d* that adenine-requiring strains of *bDmut* and *bImut* did participate in crosses. Prototrophic recombinants of *bDmut* and *bImut* were not solopathogenic in preliminary tests. Until induced auxotrophic mutants of the two *bGmut* strains are available, we cannot say whether or not they take part in crosses.

The high incidence of abnormal tetrads in crosses involving *bmut* strains caused us to examine a cross with the same auxotrophic markers, but with wild-type *b* factors. The results are shown in Table 2*e*. Although 18 tetrads with 3 or 4 products had normal segregations for all markers, 5 had more than 4 products (3 with 5 and 2 with 6) and 4 tetrads with 3 or 4 products had more than 2 copies of one marker (1 tetrad), or two markers (3 tetrads). Evidently, abnormal tetrads are not attributable to the effects of mutation at the *b* locus.

DISCUSSION

Selection for pathogenicity in *Ustilago maydis* can be made independently of the host. The mutants recovered from $a\neq$ $b=$ diploids form smaller galls containing fewer teliospores than $a\neq$ $b\neq$ diploids. The sequence of development at teliospore germination is also defective. Meiotic reduction occurs and the majority of germination products are haploid, but their survival is drastically reduced and few tetrads with more than two products are recovered. The incidence of meiotic failure is greatly increased when *bDmut* is homozygous. All of these defects are overcome in matings of the form *bDmut* $\times$ *bA* and *bDmut* $\times$ *bImut*. The latter mating also shows that *bDmut* and *bImut* are distinguishable. Each retains part of its original progenitor's specificity. Analogous results have been found in *Schizophyllum commune* (7) for mutants of the *B* mating-type factor, which is made up of two loci. Here specificity resided in the nonmutant locus. As yet we have no evidence for subunits of *b* in *U. maydis* (2). Our evidence suggests that the *bGmut-1* factor is also different from *bDmut* and *bImut*. The two latter mutants are clearly different from the $a\neq$ $b\neq$ and $a=$ $b\neq$ diploids in their ability to mate in corn with a compatible strain. Diploids may not be pathogenic in combination with haploids if the resulting dikaryon is unstable due to the difference in ploidy. Such dikaryons in *Coprinus* are unstable (8). This seems unlikely in *Ustilago* since $a\neq$ $b=$ diploids produce galls when paired with compatible haploids (6). While failure of cell fusion appears to be ruled out by the ability of $a=$ $b\neq$ diploids to mate on CM $\times 2$, we have as yet no evidence that the hyphae formed in corn can fuse with haploid cells.

The *b* factor clearly exercises control of morphogenesis after the fusion of cells with different *a* factors. If the cells carry identical and normal *b* factors nothing happens. But, if they carry different *b* factors, infection hyphae are formed. The test for mating type on CM $\times 2$ is based on this interaction.

Mutants that mimic the sequence that occurs in $b\neq$ diploids show defects not only in gall development but in meiosis and basidiospore formation. Basidiospore survival is reduced in diploids like *bD/bDmut*. Thus, the control exercised by *b* continues to meiosis and basidiospore formation.

Tetrads from the crosses described in Table 2 showed abnormalities not associated with mutant *b* factors. A simple explanation is that some teliospores had two nuclei, both of which underwent meiosis to form sets of eight products. With at least three markers 2, 4, 6, or 8 genotypes may be distinguished in a set of 8. The number of genotypes and their relative frequencies will depend on the second-division segregation frequency for each marker. These determine the frequencies of tetratypes expected for each pair of markers. For example, if all pairs of markers are ditype, only 2 or 4 genotypes are distinguishable. Some incomplete sets, with only 3 or 4 out of 6 or 8 genotypes, may be expected because of the sampling error inherent in the method of analysis. Such sets with more than two copies of one or more markers will simulate gene conversion. Some 36 tetrads of this kind were found among the 246 summarized in Table 2*b–e*.

Kernkamp and Petty (10) described variation in germination patterns in *U. maydis* teliospores from various crosses and from nature. Some showed high frequencies of a pattern with two promycelia produced on opposite sides of the spore. Such a pattern could be associated with twin meiosis. Twin meiosis was found by Gutz (9) in certain diploid strains of the yeast *Schizosaccharomyces pombe*. If copulation was not followed by karyogamy, both diploid nuclei underwent meiosis separately to form eight-spored asci. We have observed two-promycelia germination in teliospores from the crosses shown in Table 2 and are presently attempting to correlate them with twin meiosis. Since more than one pair of markers is required to detect twin meiosis, one would normally not expect to detect it in material from nature where *a* and *b* are the only readily available markers.

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