Monomorphic Nonpathogenic Mutants of *Ustilago maydis*

Alfredo D. Martínez-Espinoza, Claudia León, Guadalupe Elizarraraz, and José Ruiz-Herrera

Departamento de Ingeniería Genética, Centro de Investigación y Estudios Avanzados del IPN Unidad Irapuato, Km. 9.6 del Libramiento Norte de la Carretera Irapuato León, Apdo. Postal 629, Irapuato 36500, Gto., México.

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ABSTRACT

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We have developed conditions which promote the dimorphic transition of haploid cells of *Ustilago maydis* in vitro by controlling the pH of the media. At low pH (below 5.0) mycelial growth occurs, whereas at neutral pH yeastlike growth takes place. We screened for mutants unable to form mycelium at low pH and obtained 26 mutants. These mutants have been characterized by their cell and colony morphology in different media.

Mutations in 18 strains were found to be recessive when these strains were crossed with the wild type. Other crosses indicated that they were affected in genes other than *a* and *b*. Crosses between mutants suggest that the mutations fall in at least two complementation groups. In addition, mutants were characterized by their pathogenicity to corn seedlings. Mutations which were recessive for pathogenicity were also recessive for morphogenesis in vitro.

Additional keywords: corn smut, dimorphism, pH effect.

The basidiomycete *Ustilago maydis* (DC.) Corda is the worldwide smut pathogen of maize (*Zea mays* L.) and may cause severe yield losses to agriculture when environmental conditions are suitable (8). In the saprophytic phase the fungus is haploid and grows as budding yeasts (sporidia). Mating of compatible sporidia leads to the formation of a dikaryotic mycelium, which invades the plant. The mycelium eventually septates and forms black teliospores, which fill tumors that develop in the plant. Teliospores germinate in the form of a promycelium in which meiosis occurs, giving rise to basidiospores, which multiply by budding, thus completing the life cycle. Normally, the dimorphic transition in *U. maydis* is regulated by the mating-type loci *a* and *b*. The *a* locus is required for cell-to-cell recognition in the mating process (7) and maintenance of filamentous growth (3). The *a* locus has two idiomorphs (*a1* and *a2*). Both idiomorphs have been cloned, and each encodes a pheromone and a receptor for the mating factor synthesized by the compatible partner (7). The *b* locus regulates the steps in sexual development that occur after the fusion of haploid cells. The *b* locus is complex, with at least 25 alleles at each of two genes, *bE* and *bW*. The presence of different alleles is a prerequisite for triggering filamentous growth and tumor induction (11,19).

Recent results from this laboratory (17) demonstrated that dimorphic transition of haploid cells can be obtained in vitro by controlling the pH of the media. Cells grown at neutral or nearly neutral pH display yeastlike growth, whereas below pH 5.0 they display mycelial growth. We subsequently isolated mutants unable to form filaments at acid pH. A protocol for isolating and characterizing the mutants and an analysis of the relationship between mycelial formation and pathogenicity are described.

MATERIALS AND METHODS

Strains, media, and culture conditions. The following haploid strains of *U. maydis* were used in this study: FB1 (*alb1*) and FB2 (*a2b2*) (Flora Banuett, University of California, San Francisco);

RK1725 (*albW1*⁻) and RK1607 (*albE1*⁻) (Regine Kahmann, Institut für Genetik und Mikrobiologie der Universität München); UM031(*alb2*) and UM032 (*a2b1*) (James Kronstad, University of British Columbia, Vancouver, Canada); and the monomorphic mutants obtained in this study. Strains were maintained at -70°C in 50% glycerol. Strains were recovered in HCM liquid medium (13), shaken at 28°C for 2 days, and used as inoculum for subsequent experiments. Cell growth in liquid cultures was measured as described previously (17). To evaluate the "fuzz" reaction (formation of filaments) (2,3), plates of minimal solid medium (13) containing 1% charcoal (9) and adjusted to either pH 3.0 or pH 7.0 were used. Drops of late log cultures of each strain were spotted on the plates, either alone or mixed with drops from cells of the opposite mating type. The fuzz reaction was recorded after 24 to 48 h at 22 to 25°C.

Isolation of monomorphic mutants. Strains FB1 and FB2 were grown separately in HCM liquid medium for 18 h at 28°C to reach a concentration of 1×10^7 to 1×10^8 cells per ml. Cells were centrifuged and washed twice with SCS (sodium citrate, 0.1 M, pH 5.5) and resuspended in 3.6 ml of SCS. Aliquots (0.9 ml) were distributed into four small tubes, and 100 µl of N-methyl-N'-nitro-N-nitrosoguanidine (250 µg/ml) was added. The mixture was incubated at 28°C in total darkness for 40 min to achieve 20% survival (C. Leon, unpublished). The mutagenesis was stopped by adding an equal volume of sodium thiosulfate. Cells were recovered by centrifugation and washed twice with 0.1 M phosphate buffer at pH 7.0. Cells were then resuspended in 1 ml of HCM liquid media and incubated at 28°C for 18 h. Aliquots of 100 µl of treated cells were plated in HMM medium, pH 3.0, supplemented with 1% activated charcoal (17), and the plates were incubated at 25°C. We screened the plates for smooth colonies made of yeastlike cells. Our previous work indicated that under these conditions wild-type strains form mycelial cells—the Fuz⁺ reaction (17). Mutants were screened twice to confirm their colony type. A single mutant from each mutagenic treatment was chosen for further study. A total of 26 mutants were initially isolated.

Phenotypic characterization of monomorphic mutants. Monomorphic mutants were characterized by microscopic observation to determine colony and cell morphology, the latter in both solid and liquid media at pH 7.0 and at pH 3.0. Wild-type cells

Corresponding author: José Ruiz-Herrera; Email: jruiz@mvax1.red.cinvestav.mx

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and mutant strains were grown in liquid media adjusted to pH 3.0 or pH 7.0, fixed with formaldehyde, mounted in a glass slide, and observed under a compound microscope. A camera was adapted to the ocular of the microscope and connected to a computer (Power Macintosh 6100/60av, Apple Computer Inc., Cupertino, CA). Images were captured through the video monitor Quicktime program (Apple Computer). Selected images were then translated to a graphics program (CANVAS, Dereba Software, Miami, FL). Cells were measured with the rulers of the program or manually. In order to compare the data, we calculated the ratio between cell length and width. The number of cells in each hypha and the number of branchings were recorded. These three parameters were related to the wild type, taking the difference between mycelial and yeast cells of wild-type strains as 1.0. At least 20 well-isolated cells of each strain were measured for these calculations.

Dominance or recessiveness of the mutations and complementation assays. To determine how the mutations were expressed, the fuzz reaction of monomorphic mutants inoculated with wild-type or mutant strains of the opposite mating type was recorded as described above. A positive fuzz reaction suggests a recessive mutation. The same procedure was performed in order to evaluate if the mutants were affected in the *b* locus, with *bW*⁻ and *bE*⁻ mutants as partners (11). To evaluate if the mutations were due to a single gene or if more than one mutation was involved in the phenotype, crosses were performed in planta between each mutant and a wild-type strain of the opposite mating type, as described in the next section. Galls containing mature teliospores were removed from the plants, washed thoroughly with sterile distilled water, placed in a 70% mixture of commercial bleach and water for 5 min, washed twice with sterile distilled water, and dried. The teliospores were used immediately, or the galls were held at 4°C for later evaluation or further experiments. Galls were mixed with 1 ml of 2% copper sulfate, incubated for 2 h, crushed with sterile needles and scalpel, filtered through cheesecloth, placed in a microcentrifuge tube, and centrifuged at low speed. Sedimented teliospores were washed once with sterile distilled water, and 0.1-ml samples (100 to 150 teliospores per plate) were used to inoculate plates of HCM medium supplemented with carbenicillin (100 µg/ml). The plates were incubated at room temperature for 12 to 18 h. Meiotic segregants were obtained randomly by placing sterile distilled water over the plate and making a homogenate with a glass rod. Sporidia were diluted appropriately and spread over HCM plates to obtain separate colonies. Plates were incubated for 18 to 24 h at 28°C. Colonies were replica-plated on HCM medium, pH 7.0, supplemented with charcoal. Colonies which gave a positive fuzz reaction were discarded, since their phenotype indicated that they were formed by diploid cells. Smooth colonies were selected, transferred to minimal and complete media (pH 3.0, supplemented with charcoal), and incubated as above. A dissecting scope was used to record the colonial phenotype (smooth or fuzzy). In addition, the mating type of the segregants was analyzed. Colonies were replica-plated on four plates of HCM-charcoal medium, pH 7.0. Drops of each one of the mating testers (wild-type strains *alb1*, *alb2*, *a2b1*, and *a2b2*) were placed over the transferred colonies, and the fuzzy phenotype was recorded (see above). After the mating type was corroborated, strains were used for in vitro complementation and pathogenicity assays. To confirm that noncomplementing mutants were not affected in conjugation, we tested the methods described by Snetselaar (20) and Banuett and Herskowitz (5), which, in our hands, did not produce satisfactory results. The procedure was modified to obtain better results: water agar (1%) supplemented with activated charcoal (0.5 mg/ml) was poured in a thin layer, about 2 mm thick, in a petri dish. Small squares were excised and mounted on a glass slide; 5 µl of a particular mutant was placed on an agar square and was air-dried, and 5 µl of a mutant of the opposite mating type was then added at the same spot. The slides containing the crosses were incubated in a moist chamber at 20 to

22°C, and the formation of conjugation tubes and bridges was evaluated microscopically after 4 to 10 h. When photographs were necessary, the charcoal was omitted, though efficiency was reduced.

Pathogenicity assays of monomorphic mutants. Wild-type strains and monomorphic mutants were grown in 3 ml of HCM liquid medium for 24 h. A sample was transferred to fresh HCM liquid medium and grown for 18 h. Cells were centrifuged and washed with sterile distilled water, and the concentration was adjusted to 1×10^8 cells per ml. Suspensions of cells of the opposite mating types were mixed. Aliquots (100 µl) of the mixed suspension were then injected by a small syringe (1) into the stems of 8-day-old seedlings of maize cultivar Cacahuazintle. As controls, plants were inoculated with wild-type strains (FB1 and FB2), either alone or mixed. Other plants were inoculated with the same volume of sterile distilled water. Ten plants were used for each sample, and at least two experiments involving each strain were performed. The plants were maintained in a greenhouse, and symptoms of the disease were recorded as anthocyanin red spots, which are an indicator of infection (8), and tumors or galls characteristic of *U. maydis* infections. In mixtures of wild-type strains or of wild-type strains and recessive mutants, at least four plants in each lot developed tumors. In mixtures of complementing mutants, with few exceptions, tumors were produced in at least two plants in replicated experiments. In reactions scored as negatives, no disease symptoms were observed in repeated experiments. In no case were disease symptoms observed in plants inoculated with a single strain or distilled water.

RESULTS

Isolation and morphological characterization of monomorphic mutants (*myc*⁻). Under the conditions described in Materials and Methods we isolated 26 mutants unable to produce fuzzy colonies when grown in charcoal-containing media at pH 3.0. Colonies of these strains were mostly smooth, and some exhibited a central depression, especially as they became older. Others appeared wrinkled or "spiny." A comparison of colonial morphology of wild-type strains and recessive monomorphic mutants (see below) in media of pH 3.0 is shown in Fig. 1. The smooth phenotype was not due to mutations affecting growth, since all mutants displayed growth rates similar to that of the wild type in liquid minimal medium. These strains were mitotically stable and did not revert after continuous subculturing. These mutants were designated *myc*⁻.

Wild-type *U. maydis* cells grow yeastlike at neutral pH and as mycelium at acid pH in solid or liquid media (17). On the other hand, *myc*⁻ mutants exhibited yeastlike growth in solid media at either pH, whereas in liquid media at pH 3, cell morphology was not strictly yeastlike. The average length of wild-type yeast cells was 11.7 µm, while that of mycelial cells was 27 µm. Their widths, however, were very similar, 1.7 and 1.9 µm for yeast and mycelial cells, respectively. The width of mutant cells remained about the same, with the exception of strains CL1-C, CL2-35, and CL2-39, whose cells were slightly wider. Table 1 compares morphological characteristics of the recessive mutants (see below) and wild-type cells grown in liquid or solid media. Representative strains are shown in Fig. 2. Some strains, such as CL2-21, CL2-35, CL2-39, and CL2-40, displayed a length-width ratio smaller than that of wild-type yeast cells. The extreme case was mutant CL2-11, which appeared as small chains of very short round cells. Hyphae from other mutants contained at most two cells (e.g., CL2-33), and others made only a single branch, which could be distinguished from a bud since it appeared either subapically or in the middle of the mother cell, as in CL1-A and CL1-C.

Genetic analysis of the monomorphic mutants. To determine if the mutations were dominant or recessive, mutant strains were co-spotted with wild-type strains of opposite mating type on charcoal

medium at pH 7.0. A total of eight mutants produced the smooth phenotype. These mutants were not further considered, since the possibility that they had a^- or b^- mutations could not be discounted. The rest of the mutants evaluated formed mycelial colonies when co-spotted with wild-type strains of the opposite mating type, suggesting recessiveness of the mutation. Since the b locus contains two genes (bE and bW) it still was possible that the myc^- mutations were in either bE or bW but would not have been revealed by the type of analysis performed. In order to determine if this was so, $a2b2\ myc^-$ mutants were co-spotted with $a1bW1^-$ and $a1bE1^-$ strains. All $a2b2\ myc^-$ strains gave a positive fuzz reaction with $a1bW1^-$ or $a1bE1^-$ strains. Accordingly $a2b2\ myc^-$ mutants are not affected in either of the b genes. The unavailability of $b2E^-$ or $b2W^-$ tester strains did not allow a similar conclusion to be reached for the $a1b1\ myc^-$ mutants.

Subsequent analyses were performed in order to determine if the myc^- mutants were altered in a single trait or not. For this pur-

pose, 15 mutants were selected. Each mutant was mixed with the opposite wild-type strain (FB1 or FB2), and maize seedlings were inoculated with the mixture. Teliospores were recovered from these crosses, the meiotic segregants were obtained, and their fuzzy phenotype was analyzed at pH 3.0 as described in Materials and Methods. Statistical analysis of the results revealed that mutations segregated in a general 2:2 ratio between the smooth and the fuzzy phenotypes (Table 2), indicating that only one gene was affected in each particular mutant. In only one of the mutants analyzed (CL2-41) was the segregation pattern abnormal (one smooth and nine fuzzy). This strain was not analyzed further. We also determined whether the smooth phenotype was represented in all of the mating types. Segregants of six selected mutants were replica-plated on four plates of neutral media containing activated charcoal and evaluated for mating type with $a1b1$, $a2b2$, $a1b2$, and $a2b1$ tester strains. The data presented in Table 3 and statisti-

Fig. 1. Colonial morphology of wild-type strain FB2 and selected yeast-monomorphic myc^- mutants of *Ustilago maydis* in plates of minimal medium with activated charcoal at pH 7.0 (A) or pH 3.0 (B-F). A and B, FB2; C, CL1-B; D, CL2-39; E, CL2-31; F, CL1-E.

Fig. 2. Cell morphology of wild-type strain FB2 and representative yeast-monomorphic *myc⁻* mutants of *Ustilago maydis* grown in liquid culture. Cells were grown in minimal medium at pH 3.0 (A and C–H) or pH 7.0 (B) for 18 h. A and B, FB2; C, CL1-C; D, CL2-35; E, CL2-21; F, CL2-33; G, CL2-11; H, CL2-37. Magnification bar, 10 µm.

cal analysis revealed that *myc*⁻ and the mating loci segregated independently.

To determine the number of complementation groups present in the *myc*⁻ mutants, they were reciprocally co-spotted, and the fuzzy phenotype was recorded. Some mutants (e.g., CL2-11, CL2-33, CL2-34, and CL2-39) displayed similar complementation patterns, but in general it was not possible to ascribe the mutants to clear-cut complementation groups (Table 4). One mutant (CL1-C) even failed to complement in vitro with any of the mutants of the opposite mating type, although the mutation was recessive when this strain was crossed with the wild type. To analyze whether problems might arise from the fact that mutants came from distinct genetic backgrounds, we selected a more limited number of mutants for further studies. Crosses were performed in planta between each mutant and a wild-type strain. *Myc*⁻ mutants of opposite mating types were isolated. Reciprocal mating crosses performed in vitro gave exactly the same results as those previously observed (Table 5), suggesting that the aforementioned hypothesis is not a likely explanation. An alternative hypothesis is that some

TABLE 1. Morphological characteristics of yeast-monomorphic *myc*⁻ mutants and wild-type strain FB1 of *Ustilago maydis*^a

Strain	Solid medium cell morphology	Liquid medium		
		L/W ^b	Cells per hyphae	Branches per hyphae
FB1				
pH 3	Hyphae	1.00	1.00	1.00
pH 7	Yeast	0	0	0
CL1-A	Yeast	0.05	0.43	0.25
CL1-B	Yeast	0.22	0.71	0.75
CL1-C	Yeast	0	0	0.25
CL1-E	Yeast	0.22	0.85	0.50
CL1-F	Yeast	0.56	0.14	1.00
CL2-11	Yeast	0	0.57	1.00
CL2-21	Yeast	0	0.28	0.50
CL2-22	Yeast	0.09	0.85	0.50
CL2-31	Yeast	0.20	0.85	0.75
CL2-33	Yeast	0.78	0.28	1.00
CL2-34	Yeast	0	0.85	0.50
CL2-35	Yeast	0	0.57	0.75
CL2-36	Yeast	0	0.14	0.25
CL2-37	Yeast	0.42	0.71	1.00
CL2-38	Yeast	0.44	0.71	1.00
CL2-39	Yeast	0	0.14	0.25
CL2-40	Yeast	0	0.71	0.50
CL2-41	Yeast	0	0	0.25

^a Morphology indexes were determined as described in Materials and Methods. Reported data are values relative to the wild type (1.0).

^b Ratio of length to width.

TABLE 2. Meiotic segregation for colony type in crosses between yeast-monomorphic *myc*⁻ mutants and wild-type strains of *Ustilago maydis*

Cross	Mating type	Smooth : fuzzy ^a	χ^2 Value
CL1-A $\times$ FB2	<i>alb1</i> $\times$ <i>a2b2</i>	142 : 154	0.48
CL1-B $\times$ FB2	<i>alb1</i> $\times$ <i>a2b2</i>	148 : 150	0.01
CL1-C $\times$ FB2	<i>alb1</i> $\times$ <i>a2b2</i>	78 : 84	0.22
CL1-E $\times$ FB2	<i>alb1</i> $\times$ <i>a2b2</i>	85 : 92	0.28
CL1-F $\times$ FB2	<i>alb1</i> $\times$ <i>a2b2</i>	80 : 94	1.12
CL2-11 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	83 : 103	2.15
CL2-21 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	40 : 55	1.36
CL2-31 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	72 : 92	2.43
CL2-33 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	79 : 93	1.13
CL2-34 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	39 : 55	2.72
CL2-35 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	49 : 54	0.25
CL2-38 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	44 : 49	0.15
CL2-39 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	56 : 43	1.73
CL2-40 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	47 : 53	0.36
CL2-41 $\times$ FB1	<i>a2b2</i> $\times$ <i>alb1</i>	8 : 91	70.3 ^b

^a Expected ratio of 2 : 2.

^b Significant χ^2 value.

noncomplementing mutants synthesize abnormal polypeptide products that interfere with the organization of normal protein aggregates. At least two complementation groups were determined in this set of selected mutants: group I, containing CL1-B, CL1-E, and CL2-21; and group II, containing CL2-33. Strains CL2-31 and CL2-35 did not complement with any other mutant. It remains to be determined whether mutants CL2-31 and CL2-35 contain mutations that identify different complementation groups.

Mixtures of mutants of opposite mating types were able to fuse and form conjugation tubes, although some mutant combinations formed a reduced number of conjugation tubes under the assay conditions (Table 6). These results demonstrate that noncomplementing *myc*⁻ mutants were nevertheless competent in conjuga-

TABLE 3. Segregation of mating type and fuzzy phenotype in progeny of crosses between yeast-monomorphic *myc*⁻ mutants and wild-type strains of *Ustilago maydis*

Cross	Ratio of smooth to fuzzy phenotype in mating type: ^a				χ^2 Value ^b
	<i>alb1</i>	<i>a2b2</i>	<i>alb2</i>	<i>a2b1</i>	
CL1-B $\times$ FB2	8/9	8/8	14/9	14/7	5.5
CL2-11 $\times$ FB1	11/18	5/10	6/7	8/10	12.8
CL2-21 $\times$ FB1	8/5	4/5	2/7	3/5	5.4
CL2-31 $\times$ FB1	10/6	11/13	7/10	17/11	7.4
CL2-33 $\times$ FB1	10/15	11/6	10/9	11/11	4.5
CL2-34 $\times$ FB1	4/7	11/12	15/10	3/5	16.1

^a Expected ratio 1/8 : 1/8.

^b Not significantly different from the expected ratio at $P > 0.01$.

TABLE 4. Complementation assays in vitro and in planta among yeast-monomorphic *myc*⁻ mutants of *Ustilago maydis*

<i>a2b2</i>	<i>alb1</i>									
	CL1-A		CL1-B		CL1-C		CL1-E		CL1-F	
	IV ^a	IP ^b	IV	IP	IV	IP	IV	IP	IV	IP
CL2-11	+	+	+	+	-	-	+	+	+	+
CL2-21	-	+	-	-	-	-	-	-	+	+
CL2-31	-	-	-	-	-	+	-	-	+	+
CL2-33	+	+	+	+	-	-	+	+	+	+
CL2-34	+	+	+	+	-	-	+	+	+	+
CL2-35	-	-	-	-	-	-	-	-	+	+
CL2-36	-	-	-	+	-	-	-	-	-	-
CL2-37	+	+	-	-	-	-	-	-	-	-
CL2-38	+	+	+	+	-	+	-	-	-	+
CL2-39	+	+	+	+	-	-	+	+	+	+
CL2-40	+	+	+	+	-	+	+	+	+	+

^a For in vitro (IV) assays, each mutant was crossed with mutants of the opposite mating type; + = complementation, indicated by the formation of filaments (the fuzz reaction); - = noncomplementing reaction, recorded when a yeastlike colony was observed.

^b For in planta (IP) assays, maize seedlings were inoculated with a mixture of mutant cells and cells from a mutant of the opposite mating type; + = production of anthocyanin pigments and formation of tumor galls; - = lack of symptoms.

TABLE 5. Reciprocal complementation of allelic yeast-monomorphic *myc*⁻ mutants of *Ustilago maydis* in vitro^a

<i>a2b2</i>	<i>alb1</i>					
	CL1-B	CL1-E	CL2-21	CL2-31	CL2-33	CL2-35
CL1-B	-	-	-	-	+	-
CL1-E	-	-	-	-	+	-
CL2-21	-	-	-	-	+	-
CL2-31	-	-	-	-	-	-
CL2-33	+	+	+	-	-	-
CL2-35	-	-	-	-	-	-

^a See Table 4 for a description of the complementation assay.

tion. We also carried out allelic tests to determine whether complementation among some mutants could be intragenic. For this purpose teliospores that came from several crosses were harvested and germinated. Sporidia were recovered, and their phenotype was analyzed as described in Materials and Methods. At least 200 colonies were evaluated. We observed the presence of both smooth and fuzzy colonies in the following ratios: CL2-33 *a2 b2*/CL2-21 *a1 b1*, 82% smooth and 18% fuzzy; CL2-33 *a2 b2*/CL1-B *a1 b1*, 67% smooth and 33% fuzzy. These results indicate that CL2-33 is not allelic with CL2-21 or CL1-B.

Pathogenicity of *myc*⁻ mutants. Inoculation of maize seedlings with *myc*⁻ mutants together with wild-type strains of the opposite mating type showed that their recessivity was also expressed in pathogenicity. In all cases, inoculations with such mixtures induced disease symptoms similar to those caused by wild-type strains (not shown). In a further experiment, corn seedlings were inoculated with mixtures of *myc*⁻ mutants of opposite mating types. In some inoculations, disease symptoms were milder than those observed in plants inoculated with wild-type strains. Thus, plants inoculated with CL1-A/CL2-11 and CL1-A/CL2-21 crosses did not produce galls, but clear production of anthocyanins was observed in more than three plants; and plants inoculated with crosses CL1-A/CL2-34, CL1-B/CL2-40, CL1-C/CL2-31, CL1-C/CL2-38, and CL1-F/CL2-38 developed small tumors, but only two plants developed large tumors. All complementing pairs of mutants were pathogenic (Table 4). Highly significant was the observation that most mutant pairs which did not complement in vitro were nonpathogenic. Mating pairs carrying allelic mutations, representing the putative complementation groups (see above) CL1-B/CL1-B, CL2-33/CL2-33, CL2-31/CL2-31, and CL2-35/CL2-35, were nonpathogenic. These results are evidence that genes affected in *myc*⁻ mutants are involved in filamentous growth and pathogenicity.

DISCUSSION

Previously we demonstrated that pH regulates dimorphism in the plant-pathogenic fungus *U. maydis* (17). The only other fungus whose morphogenesis is also regulated by pH is the human pathogen *Candida albicans* (21). In the present study we used the colony morphology in media of acid pH to isolate yeastlike mutants of *U. maydis*. Crosses between allelic recessive mutants gave rise to the smooth phenotype, providing evidence that mycelial growth at acid pH is related to fuzzy growth displayed in the mating reaction. Our data demonstrate that these mutants were not affected in *a* or *b* alleles or in their ability to mate with strains of the opposite mating type. The following evidence supports these conclusions: (i) mutants co-spotted on agar plates with wild-type strains of the opposite mating type produced conjugation tubes and bridges and, in later stages, mycelial growth, and these fusion events led to the characteristic macroscopic fuzzy appearance of the colonies; (ii) *a2b2 myc*⁻ mutants mated with *a1bW1*⁻ and *a1bE1*⁻ mutants; (iii) mutants and wild-type mating pairs were as

virulent to corn seedlings as wild-type mating pairs, and all produced fertile teliospores in the tumors.

In *U. maydis* it is known that important aspects of the cell cycle are regulated by the *a* and *b* loci: mating, mycelial formation, and pathogenicity. Accordingly, the *myc*⁻ mutants isolated in this work must be defective in genes normally involved in mycelial development and under the control of those regulatory loci. Previous studies from several laboratories have provided evidence for the involvement of a number of genes in the mycelial growth of *U. maydis*. Mutants affected in filamentous growth and pathogenicity have been isolated as deficient in filament formation by Banuett (1). These strains contained recessive mutations that defined three genes, *fuz1*, *fuz2*, and *rtf*. Whether these mutants are different from the *myc*⁻ strains isolated in the present work remains to be determined. A gene (*fuz7*) involved in mycelial growth, conjugation, and pathogenicity was identified by Banuett and Herskowitz (4). This gene codes for a member of the MAP kinase kinase (MEK/MAPKK) family. In contrast to the behavior of *myc*⁻ mutants, *fuz7*⁻ strains were affected in the formation of conjugation tubes. Another gene involved in *U. maydis* filamentous growth, has been found to encode adenylate cyclase (9). UV light (14) or insertional mutagenesis (10) of partial diploids with the genotype *a2b2* (*a1bE1*) were used to isolate nonmycelial mutants of *U. maydis*. The authors cloned the gene affected in one of the mutants (*myp1*). Disruption of the gene reduced, but did not abolish, filament growth and virulence (10). Nonpathogenic mutants of *U. maydis* have also been isolated by direct testing on plants of strains mutagenized by restriction enzyme-mediated integration (6).

In other fungi, genes involved in morphogenesis have also been identified. Liu et al. (15) cloned a gene of *C. albicans* (*CPH1*) whose deletion blocked mycelial growth of the fungus in a solid medium. Like the *U. maydis myc*⁻ mutants, the *CPH1* disruptants were not affected in their mycelial growth in a liquid medium. The product of this gene is a homolog of *STE12* from *Saccharomyces cerevisiae*, which is a target of the MAP kinase involved in the pheromone response (16). A pH-regulated gene involved in *C. albicans* morphogenesis is *PHR1* (18). *PHR1* disruption in both alleles led to gross morphological alterations of the fungus, reducing apical growth in both yeast and hyphal cells at neutral and alkaline pH.

It is a common observation that human fungal pathogens display alternate morphologies in their saprophytic and pathogenic stages (reviewed in 22). A similar phenomenon was previously indicated for *U. maydis* (1,4,7,11,12,19) and confirmed by the observation that nonfilamentous mutants are affected in pathogenesis (see above). Our results led us to conclude that some of the genes affected in *myc*⁻ mutants are involved in both dimorphism and pathogenicity. This assertion is supported by the observation that most mutant pairs not complementing in vitro were nonpathogenic. More important is the observation that mating pairs carrying allelic mutations were nonpathogenic. The observation that some mutant mixtures were unable to complement in vitro but induced disease symptoms in plants can be explained in two ways. The fuzz reaction is a macro-observation, and if a limited number of fusion events occur they may escape detection. However, these few fusion events may be sufficient to induce symptoms in the plant. The plant may also provide factors not existing under in vitro conditions, which may help fusion and mycelial development (4).

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TABLE 6. Formation of conjugation and fusion tubes by allelic yeast-monomorphic *myc*⁻ mutants of *Ustilago maydis*^a

<i>a2b2</i>	<i>a1b1</i>						
	CL1-B	CL1-C	CL1-E	CL2-21	CL2-31	CL2-33	CL2-35
CL1-B	+						
CL1-C	+	+					
CL1-E	+	+	+				
CL2-21	+	+	+	±			
CL2-31	±	+	±	±	+		
CL2-33	+	+	+	+	+	+	
CL2-35	±	+	±	±	+	±	+

^a + = Production of conjugation tubes and fusion events. ± = Formation of conjugation tubes, but fewer fusion events than in the wild type.

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