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The *a* Locus Governs Cytoduction in *Ustilago maydis*

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We have developed a cytoduction assay to measure cell fusion quantitatively in the basidiomycete corn smut fungus *Ustilago maydis*. This assay employs a mutation conferring resistance to oligomycin that exhibits non-Mendelian inheritance and presumably affects the mitochondrial genome. After auxotrophic oli^r cells are mixed with prototrophic oli^s cells, prototrophic oli^r cells can be detected at a significant frequency after several hours of incubation, reaching a maximum of 10% of the total prototrophs in the mixture after 18 h. We demonstrate that this cell fusion event occurs only if the mating partners have different alleles of the *a* mating-type locus and is not influenced by the *b* locus. These studies support the view that the *a* locus but not the *b* locus controls establishment of the filamentous, pathogenic state.

The corn smut fungus *Ustilago maydis* undergoes a dramatic change in morphology and growth habit following the conjugation of two haploids to form a dikaryon (for reviews, see references 1 and 6). Whereas the haploid grows by budding and forms yeast-like colonies on solid media, the dikaryon proliferates as an obligately pathogenic filament within its host. Compatibility between haploids is determined by two mating-type loci, *a* and *b*; only when strains which differ at both loci are coinoculated into the plant does a tumorigenic infection occur. This compatibility reaction can be assayed outside the plant by mixing strains together on media containing charcoal: a compatible mating produces a characteristic "fuzz" reaction caused by the filamentous growth of the dikaryon; incompatible reactions yield compact, yeast-like growth. However, since both assays require dissimilarity at *a* and at *b* for the compatible reaction, distinct functions for the two loci cannot be derived from these assays: similarity at one locus leads to an incompatibility indistinguishable from that caused by similarity at both.

Two lines of evidence that argue that the *a* locus governs the ability of two haploids to fuse with one another have been advanced. Rowell (12) reported that only cells which differ at the *a* locus are able to fuse, regardless of their *b* alleles, as assayed by microscopic observation of juxtaposed partners. Another assay for cell fusion involves the formation of diploids on agar medium. In this case, strains with complementing auxotrophies are mixed and subsequently assayed for the ability to form prototrophic colonies, which turn out to be diploids. It has been reported (although quantitative data were not presented) that diploids can be formed between strains that differ at *a* but not between strains that differ only at *b* (10). It should be noted that the normal product of mating between haploid *U. maydis* strains that differ at both *a* and *b* is a dikaryon rather than a diploid. Diploidy occurs later in the life cycle, within plant tumors induced by the fungus.

Cytoduction refers to a mating event in which cellular fusion has been achieved without concomitant nuclear fusion and is detected by the production of haploid exconjugants bearing the nucleus of one parent and the cytoplasmic markers of the other. In organisms that form stable diploids immediately after cell fusion (such as *Saccharomyces cerevisiae*), conjugation can be measured by the production of prototrophs from input parents bearing complementing auxotrophies (9). In *U. maydis*, nuclear fusion and diploid formation do not occur efficiently after the initial cell fusion event and hence cannot be used as a quantitative assay for mating. Cytoduction provides a method for quantitatively measuring conjugation that does not depend on nuclear fusion. The heteroplasmic dikaryon segregates into its haploid components, and these should retain cytoplasmic markers conferred by one of the parents (Fig. 1). Such an assay has been used with *S. cerevisiae* to measure conjugation in strains defective for nuclear fusion (5).

Media and strains. *U. maydis* cells were routinely propagated at 30°C in CM (1% yeast extract, 0.25% Casamino Acids [Difco], 1% glucose, 0.15% ammonium nitrate, and 62.5 ml of a salt solution (described by Holliday [6]) per liter. Minimal medium (MM) contains the same concentration of salts and glucose in addition to 0.3% potassium nitrate. Charcoal plates contain double-strength CM plus 1% activated charcoal. For media containing double-strength CM, the glucose was autoclaved together with the other ingredients. Agar (2%; BBL) was used to solidify all media. Arginine, methionine, and histidine were used at a concentration of 100 μg/ml. Oligomycin (a mixture of oligomycins A, B, and C [Sigma Chemical Co.], stored in ethanol at 10 mg/ml at −20°C) was used at 3 μg/ml in MM plates and at 5 μg/ml in CM plates.

U. maydis strains used in this study are listed in Table 1. The oli^r mutation arose spontaneously after approximately 3 × 10^8 cells were plated on five CM-plus-oligomycin plates. To determine whether it is a cytoplasmic mutation, we employed the criterion of the heterokaryon test (7), which examines the ability of a marker to be transferred during a cross in the absence of nuclear fusion and meiosis. The original oli^r isolate, WM9 (*a2 b2*), was mixed with a compatible partner, FB1 (*a1 b1*). After 12 h of incubation at room temperature, when dikaryotic hyphae were just beginning to appear, the mixture was dispersed at the edge of a CM plate and hyphae were micromanipulated to isolated positions. After incubation for 2 days at 30°C, the resultant colonies were restreaked on CM plates and approximately 10 of these colonies were tested for oligomycin resistance and mating

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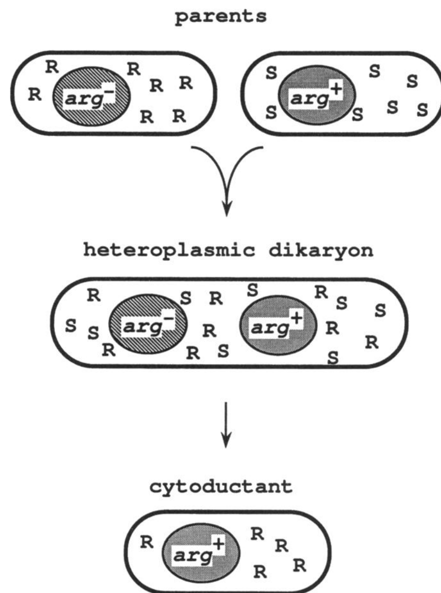

FIG. 1. Cytoduction scheme. Cells are depicted with their nuclear and cytoplasmic (oligomycin-resistant [R] or -sensitive [S]) genotypes. The cytoductant pictured represents only one of the two possible new exconjugants, the other being an *arg*$^-$ *oli*^{s} cell.

genotype. Colonies from eight hyphae were analyzed in this way, and six yielded colonies bearing the *oli*^{r} marker and the nuclear markers (*a1 b1*) of the other parent; one such isolate was WM19. By this assay, the *oli*^{r} mutation displays cytoplasmic inheritance. In addition, whereas *oli*^{s} cells can grow normally on CM plates lacking glucose, *oli*^{r} cells form tiny colonies (<50 cells) after prolonged incubation on such plates, suggesting a defect in respiration. Such a growth impairment might be expected from a cell harboring an altered mitochondrial ATPase, which is the target of the drug in other organisms (8, 14).

TABLE 1. *U. maydis* strains

Strain	Genotype	Source or derivation
FB1	*a1 b1*	F. Banuett
FB2	*a2 b2*	F. Banuett
WM9	*a2 b2 oli*^{r}	FB2 (spontaneous)
WM19	*a1 b1 oli*^{r}	WM9 × FB1[a]
WM21	*a2 b2 arg*$^-$	FB2 (EMS induced)
WM22	*a2 b2 his*$^-$	FB2 (EMS induced)
WM23	*a2 b2 met*$^-$	FB2 (EMS induced)
WM47	*a2 b2 met*$^-$ *oli*^{r}	WM19 × WM23[a]
WM70	*a2 b1*	WM21 × FB1
WM71	*a1 b2 arg*$^-$	WM21 × FB1
WM77	*a1 b1 arg*$^-$	WM21 × FB1
WM79	*a2 b2 arg*$^-$	WM21 × FB1
WM83	*a2 b1 arg*$^-$	WM21 × FB1
WM85	*a1 b1 his*$^-$	WM22 × FB1
WM86	*a1 b1 arg*$^-$ *oli*^{r}	WM47 × WM77[a]
WM97	*a1 b2*	WM22 × FB1
WM101	*a1 b2 arg*$^-$ *oli*^{r}	WM47 × WM71[a]
WM111	*a1 b2 his*$^-$	WM22 × FB1
WM123	*a1 b1 his*$^-$ *oli*^{r}	WM47 × WM85[a]
WM124	*a1 b2 his*$^-$ *oli*^{r}	WM47 × WM111[a]
WM125	*a2 b2 arg*$^-$ *oli*^{r}	WM123 × WM79[a]
WM129	*a2 b1 arg*$^-$ *oli*^{r}	WM124 × WM83[a]

[a] The cross involved only cytoplasmic transfer during mating on charcoal medium. In such crosses, the first strain is the cytoplasmic donor.

The auxotrophs WM21, WM22, and WM23 were derived by ethyl methanesulfonate (EMS) (Sigma Chemical Co.) mutagenesis (60 min; 3% EMS in 100 mM potassium phosphate, pH 8.0) of FB2 (55% survival, 3,000 colonies screened). The reversion frequency of these mutations ranges from 5×10^{-6} (*met*$^-$) to less than 10^{-7} (*arg*$^-$ and *his*$^-$). Standard genetic methods (6) were used to generate the other *oli*^{s} strains in Table 1. In order to transfer oligomycin resistance readily to many different strains, a *met*$^-$ *oli*^{r} derivative (WM47) was obtained by mating WM19 to WM23, micromanipulating hyphae as described above, and screening the cells in the resultant colonies for the Met$^-$ Oli^r phenotype. Subsequent transfer of the *oli*^{r} determinant, for example, to the *arg*$^-$ strain WM77, was performed selectively, by mating WM47 to WM77 and selecting for cytoductants on MM containing oligomycin and arginine but lacking methionine. For two strains, WM125 and WM129, the *oli*^{r} mutation arrived via a *his*$^-$ intermediate.

Cytoduction assay. Figure 1 schematically depicts the rationale for the assay. When an auxotrophic *oli*^{r} strain is mixed with a compatible prototrophic *oli*^{s} strain, the resultant dikaryotic cells contain each of the input nuclei in a cytoplasm that is presumably mixed (heteroplasmic), containing mitochondria derived from each parent. Since the growth of *U. maydis* dikaryons is unstable outside their maize host (4, 13), subsequent segregation of the input nuclei and mitochondria should result in haploid cells (cytoductants) containing cytoplasm derived from one parent and a nucleus derived from the other. Formation of cytoductants can be readily assayed by plating a mating mixture on the appropriate selective medium, on which neither parent can grow. To this end, we used the *oli*^{r} cytoplasmic mutation described above and utilized auxotrophic mutations and the incompatibility loci as nuclear markers. A mating between an *arg*$^+$ *oli*^{s} strain and an *arg*$^-$ *oli*^{r} strain should yield *arg*$^+$ *oli*^{r} cytoductants, which, unlike either parent, can grow on MM plus oligomycin. Although an *arg*$^+$/*arg*$^-$ *oli*^{r} diploid would be expected to grow on this medium, such exconjugants do not arise at a detectable rate because the frequency of nuclear fusion by *U. maydis* outside the plant is very low.

To perform quantitative mating assays, strains were grown to saturation overnight in CM medium, and 0.2 A_{600} unit each of both parents was spotted together on an agar plate in an area of ~3 cm^2. The plate was sealed with Parafilm and incubated faceup at room temperature. The cells were then scraped from the plate with a wooden applicator stick and dispersed in distilled water. Since crosses between strains differing at *a* tend to yield clumped aggregates, dispersal in these cases required rubbing the cells against the side of the tube in a small quantity of water with the applicator stick. Dilutions were made in distilled water, and cells were plated on MM to determine the titer of the total prototrophic cells and on MM plus oligomycin to measure cytoductants. Colonies were counted after 2 to 3 or 4 to 5 days of growth at 30°C on MM or MM-plus-oligomycin plates, respectively. Mating tests were used to confirm that the cytoductant colonies possessed the nuclear genotype of the *arg*$^+$ parent and were not diploids or dikaryons.

In preliminary tests, it was found that cytoductants appeared at a frequency of 5 to 10% after incubation overnight of compatible, appropriately marked strains (WM47 [*a2 b2*] × FB1 [*a1 b1*]). To determine the minimum time required to achieve cell fusion, the proportion of cytoductants was

TABLE 2. Time course of cytoduction[a]

Coincubation period (h)	Cytoduction frequency[b]
0	$<6 \times 10^{-7}$
0.5	$<1.7 \times 10^{-6}$
2	8.0×10^{-4}
3	6.3×10^{-3}
6	2.1×10^{-2}

[a] WM125 (*a2 b2 arg⁻ oli*ʳ) and FB1 (*a1 b1*) cells were mixed together and incubated on charcoal plates for the indicated time in the dark (see Materials and Methods for details).

[b] Fraction of total prototrophic colonies that are resistant to 3 mg of oligomycin per ml, as determined by differential plating.

monitored over shorter periods. Table 2 shows the result of such an experiment. After only 3 h of coincubation, nearly 1% of the prototrophic partners have received a drug-resistant cytoplasm, and by 6 h, the level of cytoduction has reached 20% of the maximum level. Therefore, the assay measures the frequency of an event that begins soon after the mating partners come into contact and provides evidence that mating in *U. maydis* occurs with high efficiency.

The *a* locus governs cytoduction. We next determined whether the *a* and *b* loci control cytoduction. To this end, we constructed strains of four different mating types—*a1 b1*, *a1 b2*, *a2 b1*, and *a2 b2*—that were either prototrophic and oli^s or arg^- and oli^r. This set of strains was mated in all 16 possible combinations of arg^+ oli^s and arg^- oli^r, and the frequency of cytoduction was measured in each case (Table 3). Only in cases in which the *a* loci of the two parents differ is a measurable number of cytoductants produced. If one compares reciprocal matings, in which the mating genotypes of the partners are invariant but the cytoplasmic donor and recipient are switched, it is apparent that mating genotype does not affect the directionality of cytoplasmic transfer.

Our results demonstrate unequivocally that dissimilarity at *a* is required for the formation of cytoductants by conjugating cells. The most likely possibility is that the failure of this process in mixtures of cells similar at *a* reflects the inability of the partners to achieve cell fusion. However, it could also reflect the inability to mix cytoplasmic contents after plasma membrane fusion. One can state this proposition as the failure to achieve nuclear migration into the recipient cytoplasm. In other basidiomycetes, notably *Schizophyllum commune*, in which cell fusion is believed to be promiscuous, a concerted migration of the nucleus of one partner to the hyphal tip of the other constitutes the first step in the formation of the dikaryon and fails to occur in certain incompatible crosses (11). The failure to observe cytoductants in such a system might be interpreted as a failure in nuclear migration rather than in cellular fusion. Alternatively, the absence of cytoduction in a given cross could imply that the heteroplasmic dikaryon is unable to segregate its mitochondrial DNA to create a homoplasmic cell. This explanation obtains only if oligomycin sensitivity is dominant.

TABLE 3. Influence of the *a* and *b* loci on cytoduction[a]

Cytoplasmic donor (arg^- oli^r) mating type	Cytoduction frequency[b] for nuclear donor (arg^+ oli^s) mating type			
	a1 b1 (FB1)	*a1 b2* (WM97)	*a2 b1* (WM70)	*a2 b2* (FB2)
a1 b1 (WM86)	$<5 \times 10^{-7}$	$<4 \times 10^{-7}$	0.016	0.024
a1 b2 (WM101)	$<4 \times 10^{-7}$	$<5 \times 10^{-7}$	0.02	0.025
a2 b1 (WM129)	0.021	0.056	$<4 \times 10^{-7}$	$<3 \times 10^{-7}$
a2 b2 (WM125)	0.089	0.031	$<6 \times 10^{-7}$	$<3 \times 10^{-7}$

[a] Cells were mixed together as described in Materials and Methods and incubated for 17 to 20 h on charcoal plates in the dark.

[b] Fraction of total prototrophic colonies that are resistant to 3 mg of oligomycin per ml, as measured by differential plating. Fractions greater than 0.01 represent averages of two experiments.

The studies described here have made it possible to make further distinctions between the roles of *a* and *b* in the life cycle of *U. maydis*. Here we have shown that at *a*, heteroallelism is required for cytoduction whereas at *b*, it is not. Other studies have shown that *b* is required for pathogenicity whereas *a* is not (2). Thus, the *b* locus is not important for early steps in the formation of the filamentous, pathogenic form. In contrast, the *a* locus is required for the establishment of the dikaryotic state and, on the basis of its behavior in diploid strains, is argued to play a role in the maintenance of the dikaryotic state (2). Recent characterization of the *a* locus has revealed that it encodes a mating pheromone and a membrane receptor for the pheromone from the opposite mating type (3).

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