Mutations in the *myp1* Gene of *Ustilago maydis* Attenuate Mycelial Growth and Virulence

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ABSTRACT

Mating between haploid, budding cells of the dimorphic fungus *Ustilago maydis* results in the formation of a dikaryotic, filamentous cell type. Mating compatibility is governed by two mating-type loci called *a* and *b*; transformation of genes from these loci (*e.g., a1* and *b1*) into a haploid strain of different mating type (*e.g., a2 b2*) allows filamentous growth and establishes a pathogenic cell type. Several mutants with a nonmycelial colony morphology were isolated after insertional mutagenesis of a filamentous, pathogenic haploid strain. The mutagenized region in one such mutant was recovered by plasmid rescue and employed to isolate a gene involved in conditioning the mycelial phenotype (*myp1*). An 1150 amino acid open reading frame is present at the *myp1* locus; the predicted polypeptide is rich in serine residues and contains short regions with similarity to SH3 domain ligands. Construction of *myp1* disruption and deletion mutants in haploid strains confirmed that this gene plays a role in mycelial growth and virulence.

THE basidiomycete corn pathogen *Ustilago maydis* is capable of switching between a nonpathogenic haploid yeast-like phase and a pathogenic, filamentous phase (dikaryotic cells) as a result of mating interactions (CHRISTENSEN 1963). In addition, environmental factors such as nutrition and exposure to air can influence the switch between budding and filamentous growth (KERNKAMP 1939; GOLD *et al.* 1994). In the laboratory, mycelial colonies of filamentous cells have a white "fuzzy" appearance and can be readily distinguished from colonies of yeast-like cells. This phenotypic difference, and the ease of molecular genetic manipulation of *U. maydis*, provides an opportunity to identify genes involved in dimorphic growth.

The filamentous dikaryon results from the fusion of two compatible haploid yeast-like cells; compatibility is determined by the alleles present at two mating-type loci called *a* and *b* (ROWELL and DEVAY 1954; ROWELL 1955; HOLLIDAY 1961). The *a* mating-type locus, encoding a pheromone and a pheromone receptor, has two alternative forms, *a1* and *a2* (FROELIGER and LEONG 1991; BÖLKER *et al.* 1992; SPELLIG *et al.* 1994). The *a* locus controls cell fusion between strains harboring different *a* specificities (HOLLIDAY 1961). The *b* mating-type locus, with ≥25 different specificities, controls pathogenicity and dimorphism (DAY *et al.* 1971). Once cell fusion has occurred, fusion products that are heterozygous at *b* display filamentous growth and are pathogenic. That heterozygosity at *b* is sufficient for pathogenesis was confirmed by the introduction of a DNA fragment encoding a different *b* specificity into a haploid strain, thus artificially creating heterozygosity at *b*. The transformants grew with a mycelial phenotype and were pathogenic when tested on corn (KRONSTAD and LEONG 1989). Two genes are present at the *b* locus, *bE* and *bW*, each encoding a polypeptide with a homeodomain-like motif (KRONSTAD and LEONG 1990; SCHULZ *et al.* 1990; GILLISSEN *et al.* 1992). The combination of *bE* and *bW* gene products encoded by alleles of different *b* specificities is believed to form a novel transcription factor that maintains the pathogenic, filamentous cell type (GILLISSEN *et al.* 1992). Specificity determinants that play a role in recognition have been identified within the *bE* and *bW* genes (A. R. YEE and J. W. KRONSTAD, unpublished results).

Several fungal pathogens of plants and animals can alternate between yeast-like and filamentous growth (dimorphism). For example, *Ceratocystic ulmi*, the causal agent of dutch elm disease, exhibits dimorphism in response to nutritional conditions (BRUNTON and GADD 1989). Animal pathogenic fungi that exhibit dimorphic growth include *Candida albicans* and *Histoplasma capsulatum* (MARESCA and KOBAYASHI 1989; CUTLER 1991). In most of these fungi, the relationship between virulence and cell morphology is not clear. In *U. maydis*, the fact that pathogenicity and filamentous growth are controlled by the same set of genes (at the *a* and *b* loci) indicates a relationship between the two characteristics; *i.e.*, filamentous growth is required for infectivity. We are interested in dissecting the two phenomena to understand the role of dimorphic growth in pathogenesis

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TABLE 1

***U. maydis* strains**

Strain	Genotype	Source
518	*a2 b2*	KRONSTAD and LEONG (1989)
521	*a1 b1*	KRONSTAD and LEONG (1989)
P6D	*a2 b2* [*a1 bE1 phleo*^{r}]	LAITY *et al.* (1995)
P6D-9	*a2 b2* [*a1 bE1 phleo*^{r}] *myp1*Δ*1* (*hyg*^{r})	This work
NF14	*a2 b2* [*a1 bE1 phleo*^{r}] *myp1*::p11-24 (*hyg*^{r})	This work
NFD8	*a2 b2* [*a1 bE1 phleo*^{r}] *myp1*::p11-24 (*hyg*^{r})	This work
518-6	*a2 b2 myp1*::p11-24 (*hyg*^{r})	This work
521-10	*a1 b1 myp1*::p11-24 (*hyg*^{r})	This work
518-60	*a2 b2 myp1*Δ*1* (*hyg*^{r})	This work
521-32	*a1 b1 myp1*Δ*1* (*hyg*^{r})	This work

for *U. maydis*. Unfortunately, it is difficult to identify recessive mutations in genes required for mycelial growth in *U. maydis* because this phenotype is usually exhibited only by dikaryotic cells and these cells are difficult to culture (DAY and ANAGNOSTAKIS 1971).

Two approaches have been used to isolate *U. maydis* genes involved in mycelial growth. BANUETT (1991) has isolated mutants that fail to give a mycelial reaction when mixed with cells of opposite mating type. This approach could potentially identify genes that play a role in mediating pheromone response or other steps in the fusion process, as well as genes needed for mycelial growth. Using a different approach, BARRETT *et al.* (1993) screened for haploid mutants that displayed a constitutive mycelial phenotype. This strategy allowed the detection of recessive mutations affecting genes involved in the pathway leading to mycelial growth. However, because the *b* mating-type function was not activated in these cells (a single *b* specificity was present), this approach would not necessarily allow the isolation of direct targets of the *b* genes involved in the formation of filamentous cells. This strategy did yield important information on the regulation of dimorphism by factors other than mating. Specifically, the approach of BARRETT *et al.* (1993), and subsequent genetic analysis (GOLD *et al.* 1994), revealed that cAMP levels and protein kinase A play an important role in determining whether *U. maydis* grows with a budding or filamentous morphology.

To circumvent the potential problems associated with the approaches described above and to identify genes which may play important roles in the infectious dikaryon, we adopted a strategy based on the use of a haploid strain constitutively expressing the mycelial phenotype. This strain was generated by the introduction of *a* and *b* mating-type alleles of different specificity to the resident alleles. The introduced *a* sequences included the *mfa1* gene encoding the *a1*-specific pheromone; the production of pheromones of both *a1* and *a2* specificity in dikaryons is thought to promote filamentous growth via an autocrine response (SPELLIG *et al.* 1994). The introduced *b* sequences encoded the bE1 product, which is believed to interact with the bW2 protein to maintain filamentous growth (GILLISSEN *et al.* 1992). The expression of these genes in this strain leads to a mycelial phenotype and allows the genetic analysis of genes required for activation of filamentous growth. The expression of some of these genes may be regulated by the *a* and *b* loci. For example, response to pheromone or heterozygosity at *b* may result in transcriptional activation of genes needed for filamentous growth.

In this report, we describe the construction and insertional mutagenesis of a haploid strain with a mycelial phenotype. The characterization of one nonmycelial mutant led to the isolation and sequence analysis of a gene (*myp1*) that is required for full expression of the mycelial phenotype that results from heterozygosity at the *b* locus. In addition, we show that disruption or deletion mutations in the *myp1* gene result in strains that have attenuated virulence on corn seedlings.

MATERIALS AND METHODS

Strains and media: *Escherichia coli* strain DH5α [F$^-$, *end*A1, *hsd*R17(r_k^-, m_k^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, f80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R] was used for calcium chloride transformation. *E. coli* strain DH10B [F−, *mcr*A, Δ(*mrr-hsd* RMS-*mcr*BC), f80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara, leu*)7697, *gal*U, *gal*K, *rps*L, *end*A1, *nupG*] was used for electroporation. *E. coli* strains were grown in LB medium, except in electroporation experiments where cells were allowed to recover in SOC (SAMBROOK *et al.* 1989).

The *U. maydis* strains employed in this work are listed in Table 1. *U. maydis* strains were grown in either potato-dextrose medium (PDA and PDB, Difco), YEPS (TSUKUDA *et al.* 1988) or double complete medium (DCM; HOLLIDAY 1974). Formation of aerial mycelium was detected on DCM containing 1% activated charcoal (DAY and ANAGNOSTAKIS 1971; HOLLIDAY 1974). Prototrophy of *U. maydis* strains was tested on minimal medium (HOLLIDAY 1974).

Pathogenicity tests: Seven-day-old "Golden Bantam" (Buckerfield Seed Co., Vancouver, B.C., Canada) corn seedlings were grown in soil, then injected 5 mm above the soil line with 100–200 μl of fungal cell suspensions in dH_2O (10^6 or 10^7 cells per ml depending on the experiment), 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). Pathogenicity tests were also performed on plants grown in the greenhouse.

DNA and RNA procedures: Recombinant DNA techniques

were performed as described by SAMBROOK *et al.* (1989). A cosmid library containing DNA from *U. maydis* strain 518 (BARRETT *et al.* 1993) was employed to isolate the *myp1* gene. Screening of the cosmid library to identify the cosmid pM1-1 (4000 colonies) was done according to SAMBROOK *et al.* (1989) using Magna membrane (Micron Separations). For Southern analysis, DNA samples were transferred onto Zeta-probe membrane (BioRad) as described (SAMBROOK *et al.* 1989). DNA labeling was performed using [α-^{32}P]dCTP and an oligolabeling kit (Pharmacia). Total genomic DNA from *U. maydis* was prepared as previously described (BAKKEREN and KRONSTAD 1993). Polyadenylated RNA was isolated from *U. maydis* and used in Northern blot analyses as previously described (BARRETT *et al.* 1993).

The 3.0- and 4.0-kb *Stu*I genomic DNA fragments from cosmid pM1-1 (carrying the *myp1* gene) were subcloned into vector pUC128 (KEEN *et al.* 1988), giving rise to p3K1 and p4K1, respectively. Nested deletions were generated from p3K1 and p4K1 using the Pharmacia double-stranded nested deletion system. Plasmid DNA templates for sequence analysis were prepared as described by ZAGURSKI *et al.* (1985). The T7 Sequencing Kit (Pharmacia) was used for sequence determination and both strands of the *myp1* gene were sequenced. Three oligonucleotides, MYP2LL (5′-GGAATGGGAACG-GCGGC-3′), MYP3LL (5′-GCAAACGAAGGCCCACC-3′) and MYPLR (5′-GCCGTCGCTGATCCG-3′) were used to determine the *U. maydis* genomic DNA sequence on p11-24. Nucleotide sequence analysis was performed with the Wisconsin Genetics Computer Group software package, version 7.0 (DEVEREUX *et al.* 1984). Homology searches of the GenBank database were conducted with the FASTA program of PEARSON and LIPMAN (1988) and with the BLAST program (ALTSCHUL *et al.* 1990).

Plasmid construction: Plasmid pUBC72 (LAITY *et al.* 1995) was constructed by inserting a 4.2-kb *Eco*RI-*Bam*HI fragment encoding the *bE1* allele (KRONSTAD and LEONG 1990) into plasmid pUC19 (digested with the same enzymes). Then, a 3.6-kb *Eco*RI fragment carrying the *mfa1* gene from the *a1* idiomorph of *U. maydis* strain 521 (FROELIGER and LEONG 1991) was cloned into the unique *Eco*RI site. Finally, a 1.9-kb *Sst*I-*Hin*dIII fragment encoding the phleomycin resistance gene from pUXVble1 (transcribed from the *U. maydis gap* promoter and having the *Saccharomyces cerevisiae trpC* terminator) (G. BAKKEREN and J. KRONSTAD, unpublished data) was blunt-end ligated into the unique *Bam*HI site. For each of these ligations, the vector was first dephosphorylated with calf intestinal phosphatase. Vector pUBC50 was constructed by blunt-end ligation of the 2.0-kb *Xba*I-*Sal*I DNA fragment (encoding the hygromycin resistance cassette from pCM54) (TSUKUDA *et al.* 1989) into the *Eco*RI site of plasmid pBR322. Plasmid pDEL2 (Figure 1D) was obtained by first cloning the 3.0-kb *Stu*I fragment of pM1-1 (Figure 1C) into the *Sma*I site of pUC13. This construct was then digested with *Sal*I, and the vector-containing fragment ligated to the 2.5-kb *Sal*I fragment of pCM54 (encoding the hygromycin resistance marker). Finally, the 2.0-kb *Xho*I/*Stu*I fragment of p4K1 was added by blunt-end ligation into the unique *Hin*dIII site. *U. maydis* cells were transformed using the protocol of WANG *et al.* (1988).

Plasmid rescue: The *U. maydis* genomic sequence tagged with vector pUBC50 was rescued in *E. coli* by digesting 0.6 μg of total genomic DNA from strain NF14 with restriction enzyme *Xho*I (no *Xho*I sites are present on pUBC50). The enzyme was inactivated by a 15-min incubation at 70°C and the digestion products were treated with T4 DNA ligase (16 hr at 16°C) in a total volume of 100 μl. The ligation mixture was then ethanol precipitated and resuspended in 20 μl of sterile dH_2O. Two microliters of the DNA solution were used for electroporation of *E. coli* DH10B competent cells (BioRad). Approximately 500 tetracycline resistant transformants were obtained and 24 transformants were examined for their plasmid content. The *Xho*I/*Pst*I restriction patterns observed for 21 of the 24 transformants were identical, with fragments totaling 7.4 kb (0.35, 0.45, 2.1 and 4.5 kb; data not shown). One of the clones exhibiting the common restriction pattern, p11–24 (Figure 1B), was selected for further characterization.

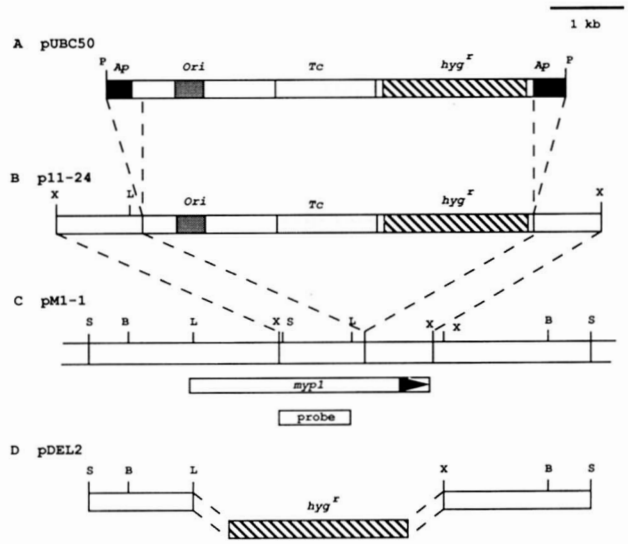

FIGURE 1.—Restriction maps of cloned DNAs employed to characterize the *myp1* gene of *U. maydis*. (A) Map of the plasmid pUBC50 used to mutagenize the mycelial haploid strain P6D. The plasmid is shown linearized at a *Pst*I (P) site and the positions of regions that function in *E. coli* (*Ap, Tc, Ori*) and *U. maydis* (*hyg*^{r}) are indicated above the shaded areas. (B) Map of plasmid p11-24, a derivative of pUBC50 which contains a deletion of part of the *Ap* region and 2.2 kb of flanking genomic DNA from the *myp1* locus. The latter sequences are represented by open boxes at the ends of the linearized plasmid [opened at a single *Xho*I site (X)]. (C) Map of a 7.0-kb segment of the cosmid pM1-1 [two *Stu*I (S) fragments of 3.0 and 4.0 kb] recovered using a 0.9-kb *Xho*I/*Sal*I fragment from p11-24 (B) as a hybridization probe; the position of the probe is indicated below the *myp1* gene. The restriction sites for *Bam*HI (B) and *Sal*I (L) are indicated on the map. Note that not all of the *Xho*I sites are shown. (D) Map of the plasmid pDEL2 employed to delete the *myp1* gene by homologous integration. A 2.5-kb *Sal*I fragment carrying the *hyg*^{r} gene is shown replacing the coding region of the *myp1* gene between *Sal*I and *Xho*I sites; the *Bam*HI sites were employed to generate a linear fragment for transformation of *U. maydis*. Note that the amino-terminal 45 codons for the *myp1* open reading frame are still present on pDEL2.

RESULTS

Construction of the mycelial haploid strain P6D: To identify and isolate genes involved in mycelial growth, we first constructed a haploid strain that mimics the filamentous growth and pathogenicity of the infectious dikaryon. Plasmid pUBC72 (LAITY *et al.* 1995), which carries the *mfa1* pheromone gene of the *a1* idiomorph, the *bE1* allele (from strain 521; *a1 b1*) and a phleomycin resistance marker, was introduced into *U. maydis* strain 518 (*a2 b2*) by integrative transformation. Forty phleomycin-resistant transformants were transferred to medium containing activated charcoal (HOLLIDAY 1974)

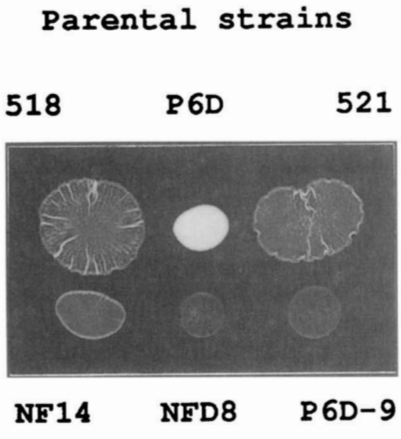

FIGURE 2.—Colony morphology of haploid strains of *U. maydis*. Colonies of wild-type strains 518 (*a2 b2*) and 521 (*a1 b1*) are shown along with colonies of the mycelial haploid strain P6D and the derivatives of this strain, NF14, NFD8 and P6D-9. NF14 is the original nonmycelial mutant of P6D isolated after transformation with pUBC50. NFD8 is a nonmycelial mutant isolated after homologous integration of p11-24 in strain P6D. P6D-9 contains a deletion of the *myp1* gene generated by homologous integration of pDEL2 at the *myp1* locus. DCM medium containing activated charcoal was inoculated with 5–10 μl drops of cultures grown overnight in PDB. The Petri plate was sealed with parafilm and incubated at room temperature for 44 hr.

to detect mycelial growth. All transformants formed colonies with mycelial phenotypes; four of the strains that showed particularly strong mycelial growth were repeatedly transferred to fresh medium to verify the stability and consistency of the phenotype. One of these strains, called P6D (Figure 2), was selected for further analysis. This strain has also been employed in a separate study to assess the influence of heterozygosity at the *a* and *b* mating-type loci on cell fusion (LAITY *et al.* 1995).

The stability of the filamentous phenotype of strain P6D was tested in two ways. First, the strain was grown in liquid medium without phleomycin selection and cells were plated on solid medium containing charcoal (DCM) (HOLLIDAY 1974). No yeast-like, spontaneous mutants were observed among 1337 colonies examined. Second, because our strategy for mutant isolation involved mutagenesis by transformation, we tested the effect of the transformation protocol on the mycelial phenotype of P6D. Cells of P6D were subjected to the transformation protocol (see MATERIALS AND METHODS) except that DNA was not added. Among 1119 colonies arising from the transformation protocol, one showed yeast-like growth. This result suggested the need for caution when identifying morphological mutants after insertional mutagenesis.

Strain P6D was also injected into corn seedlings to determine whether introduction of the *mfa1* and *bE1* mating-type sequences enabled the strain to cause disease. The results (Table 2A) indicated that P6D is weakly virulent because it induced anthocyanin production as well as small leaf and stem galls. This is in contrast to the results obtained with haploid strain 518 (the progenitor strain of P6D), which, as expected, did not induce any disease symptoms. These results demonstrate that P6D is solopathogenic, even though the disease symptoms were less severe than those resulting from the coinoculation of compatible wild-type haploid strains such as 518 (*a2 b2*) and 521 (*a1 b1*). These strains cause severe symptoms ranging from large stem galls to plant death (Table 2). We conclude that the additional mating-type sequences (*i.e.*, the *mfa1* and *bE1* genes) in P6D conditioned establishment of a filamentous, pathogenic cell type.

Isolation of nonmycelial mutants: Insertional mutagenesis via transformation of strain P6D was carried out and transformants were screened for mutants lacking the ability to display a mycelial phenotype. The integrative vector pUBC50 (Figure 1A, specifying hygromycin resistance) was employed for transformation and 30 nonmycelial mutants (nonfuzzy or NF mutants) were recovered from 4079 hygromycin-resistant transformants examined. The mutant strains had colony phenotypes ranging from slightly mycelial to completely yeast like. These mutants presumably arose because of spontaneous or insertional mutation (by pUBC50) of genes involved in filamentous growth. Mating tests were attempted to assess the number of complementation groups present among the 30 NF mutants. Despite repeated attempts, mating reactions (mycelial growth on medium containing charcoal) were not observed between different mutant strains, and genetic analysis of the mutants could not be performed. The inability to obtain mating among the NF mutants has been examined in a separate study (LAITY *et al.* 1995). The results of this work indicated that the block in mating was due to heterozygosity at *b* in the parental strain P6D, a situation which attenuates cell fusion.

One mutant (NF14, Figure 2) was chosen for further characterization and for the isolation of the genomic region carrying the insertion of pUBC50. Plasmid rescue (see MATERIALS AND METHODS) was employed to recover plasmid p11–24; a restriction map of this plasmid is shown in Figure 1B to depict the organization of pUBC50 and genomic sequences.

Disruption of the *myp1* gene attenuates mycelial growth: We next wanted to confirm that the nonmycelial phenotype of NF14 was due to the integration of vector pUBC50 in a gene involved in mycelial growth (called *myp1* for mycelial phenotype) and not to an unlinked mutation. To test this, we attempted to replace the wild-type sequence of *myp1* with a disrupted version by transformation of P6D with plasmid p11–24 (linearized at *Xho*I; Figure 1B). If p11-24 carried part of the *myp1* gene, then homologous integrants should display a nonmycelial phenotype. In contrast, if the nonmycelial phenotype of NF14 was due to a mutation unrelated to the insertional event, the P6D transformants would retain the parental mycelial phenotype.

TABLE 2

Pathogenicity test of *U. maydis* strains on corn plants

Strains	Genotype	Disease symptoms[a]					No. of plants
		A	B	C	E	E	
A.							
518	*a2 b2*	14	0	0	0	0	14
P6D	*a2 b2* [*a1 bE1 phleo*^{r}]	54	63	112	1	0	230
521 × 518	*a1 b1* × *a2 b2*	0	0	18	81	53	152
B.							
NFD8	*a2 b2* [*a1 bE1 phleo*^{r}] *myp1*::p11-24 (*hyg*^{r})	138	10	0	0	0	148
P6D	*a2 b2* [*a1 bE1 phleo*^{r}]	10	12	23	0	0	45
521 × 518	*a1 b1* × *a2 b2*	0	0	0	15	0	15

The results represent the pooled data from four replicates of the inoculation (10^6 cells/ml for the mixtures of strains and 10^7 cells/ml for individual strains).

[a] The rating scheme for the disease symptoms is follows: A, no symptoms; B, presence of anthocyanin; C, galls on leaves; D, galls on stem; E, dead plants. The numbers refer to the number of plants showing the symptoms in each category.

Among 305 transformants obtained with p11-24, 184 were nonmycelial (or very slightly mycelial) and 121 were as mycelial as parental strain P6D. Because 60% of the transformants lost the parental mycelial phenotype, these results suggest that pUBC50 inactivated a gene involved in mycelial growth to generate the mutant NF14. An example of the phenotype of one of the nonmycelial transformants (NFD8) is shown in Figure 2 for comparison with NF14.

Homologous integration of p11-24 at *myp1* in the nonmycelial transformants was confirmed by DNA blot analysis (Figure 3) using the 0.9-kb *Sal*I-*Xho*I genomic DNA fragment from p11-24 as a hybridization probe (Figure 1, B and C). Total genomic DNA from 10 mycelial and 10 nonmycelial transformants (including some that were slightly mycelial) was extracted, digested with *Xho*I, and used for DNA blot analysis. All 10 mycelial transformants displayed a 2.2-kb *Xho*I fragment like the parental strain P6D, indicating that the resident *myp1* sequence was intact (Figure 3B). In contrast, all of the nonmycelial or slightly mycelial transformants tested showed replacement of the 2.2-kb *Xho*I fragment with a 7.4-kb *Xho*I fragment similar in size to p11-24 (Figure 3A). These results indicated that insertion of pUBC50 in *myp1* was the cause of the nonmycelial growth of the original NF14 mutant.

One of the nonmycelial P6D transformants analyzed by hybridization (NFD8) was chosen to test the effect of *myp1* disruption on virulence on corn seedlings (Table 2B). The results showed that the disease symptoms obtained with disruption mutant NFD8 were reduced compared to those observed upon inoculation with strain P6D. A similar experiment with the original NF14 mutant also yielded reduced disease symptoms compared with P6D (data not shown). Overall, these data suggest that disruption of *myp1* results in decreased virulence.

Sequence analysis of the *myp1* gene: The wild-type *myp1* gene was recovered from a cosmid library of *U.*

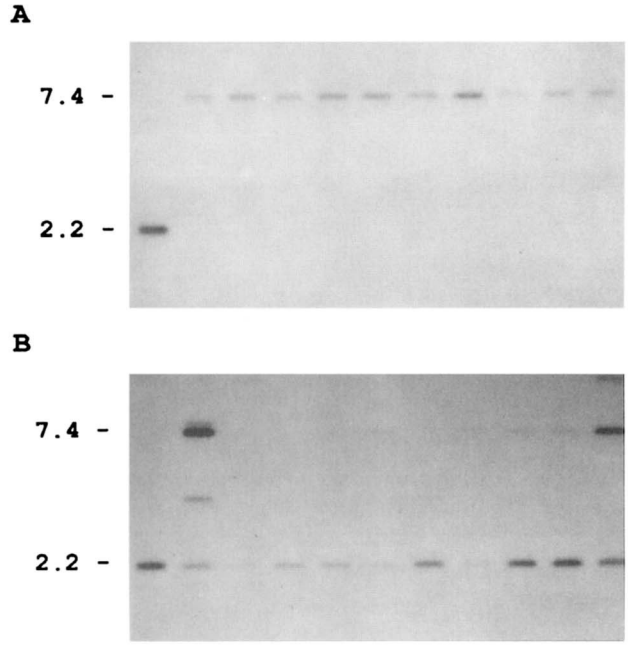

FIGURE 3.—DNA hybridization of a *myp1* gene probe to *Xho*I digests of genomic DNA from nonmycelial and mycelial transformants of strain P6D. (A) DNA from 10 nonmycelial transformants of P6D with p11-24. The left-hand lane contains DNA from the parental strain P6D (hybridization to a 2.2-kb *Xho*I fragment). Note the absence of the 2.2-kb fragment in the transformants and the presence of the 7.4-kb fragment resulting from homologous integration. (B) DNA from 10 mycelial transformants of P6D with p11-24. The left-hand lane contains DNA from P6D. The 2.2-kb *Xho*I band from the wild-type *myp1* gene is present in all of the transformants. In addition, some of the transformants have additional bands that presumably result from ectopic integration of the transforming DNA. Many of the transformants contain a band at 7.4 kb, the size of the transforming plasmid. These bands may result from maintenance of the plasmid in an autonomous state in some of the transformants. The variable intensity of the band at 7.4 kb between various transformants is consistent with this idea. The blots in A and B were both hybridized with an 0.9-kb *Sal*I-*Xho*I *U. maydis* genomic DNA fragment from p11-24 (Figure 1C).

maydis DNA by hybridization with a 0.9-kb *SalI/XhoI* fragment from p11-24 (left end, Figure 1B). The *myp1* gene was initially localized on two contiguous *StuI* fragments (3.0 and 4.0 kb) present on cosmid pM1-1 (Figure 1C). The 0.9-kb *SalI/XhoI* probe was also hybridized to a Northern blot carrying poly(A+) RNA extracted from haploid strain 518 and from a mixture of the compatible strains (518 and 521) that were displaying mycelial growth as a result of mating. A 4.0-kb transcript was detected in both RNA preparations (data not shown). Although this experiment would not reveal subtle regulation of *myp1* transcription between haploids and mating cells, it allowed us to conclude that *myp1* is actively transcribed in both cell types.

The nucleotide sequence of the 3.0- and 4.0-kb *StuI* fragments from pM1-1 was determined and one long open reading frame of 1150 amino acids (nucleotides 302–3754) was identified (Figure 4). The size of the open reading frame is consistent with the size of the transcript (4.0 kb) detected by hybridization analysis. The sequence around the putative initiation codon (GACC**ATG**TC) matches the consensus for the sequence at fungal translation initiation sites in seven of nine positions (BALLANCE 1986). The higher eukaryotic polyadenylation signal AATAAA was not detected, as is often the case for genes from filamentous fungi (BALLANCE 1986). A search for introns by sequence inspection failed to identify candidate splice junctions or sites of lariat formation (BALLANCE 1986) within the coding region.

The 1150 amino acid predicted polypeptide is rich in serine (15.7%), alanine (11.0%) and proline (7.2%) residues. A NCBI BLAST database search (ALTSCHUL *et al.* 1990) with the sequence did not reveal extensive similarity to any known gene, although, as expected, many genes encoding serine-rich proteins gave relatively high scores. Interestingly, stretches of serine and proline-rich regions in the *myp1* sequence also gave matches with the *S. cerevisiae* proline-rich protein verprolin (DONNELLY *et al.* 1993). Upon closer inspection of the three proline-rich sequences in *myp1*, similarity was noted to the proline-rich consensus sequence for SH3 domain ligands (DONNELLY *et al.* 1993; YU *et al.* 1994). The positions of these motifs are underlined in Figure 4. The SH3 ligands are believed to play a role in protein-protein interactions between receptors, signal transduction proteins and cytoskeletal components (REN *et al.* 1993; YU *et al.* 1994). A KYTE-DOOLITTLE (1982) hydrophobicity plot of the predicted *myp1* amino acid sequence (data not shown) also revealed a region between codons 687 and 880 (Figure 4) that is hydrophobic and flanked by clusters of acidic residues. This region could potentially be a membrane spanning domain.

Analysis of the mutation in strain NF14: The position of the original pUBC50 insertion mutation in the non-mycelial mutant NF14 was identified by comparison of sequence information from p11-24 and pUBC50. This analysis revealed that pUBC50 had integrated at codon 856 in the *myp1* open reading frame (Figure 4) in strain NF14. Approximately 1.1 kb of pUBC50 was deleted during the integration process. This is consistent with the finding that the size of the disrupted fragment observed in NFD8 was 7.4 kb (2.2-kb *XhoI* genomic fragment plus 5.2 kb of pUBC50; Figure 3A) instead of the expected 8.5 kb (2.2-kb *XhoI* genomic DNA fragment plus 6.3 kb of pUBC50). These results indicated that a large part of the *myp1* ORF was intact in the disruption mutants (NF14 and NFD8).

Disruption of *myp1* in haploid strains: Given that disruption of the *myp1* gene in P6D (strains NF14 and NFD8) reduced mycelial growth and virulence, it was of interest to ask whether an identical mutation in the *myp1* gene in wild-type haploid cells would result in similar phenotypes after mating. Haploid strains 518 (*a2 b2*) and 521 (*a1 b1*) were transformed with linearized p11-24 (Figure 1B) and the resulting colonies were screened for homologous integration at *myp1* by DNA blot hybridization (data not shown). Two transformants, 518-6 and 521-10, which were shown by hybridization to have the wild-type 2.2-kb *XhoI* fragment replaced by a 7.4-kb *XhoI* fragment of p11-24, were selected for mating and pathogenicity tests. It should be noted that cells of these strains had the same morphology as the wild-type parental strains 518 and 521. However, the mutants did exhibit a slightly slower growth rate in liquid medium when compared with wild-type strains.

In mating tests, these disruption mutants could form mycelial colonies when mixed with wild-type cells or with each other, although the aerial hyphae observed in mixtures of mutant strains were not always as dense as those obtained upon mating of compatible wild-type strains (Figure 5A). Identical results were obtained with several other disruption transformants of strains 518 and 521. It appears from these results that disruption of *myp1* attenuates, but does not eliminate, the ability of compatible haploid cells to form aerial hyphae upon mating.

The results of pathogenicity tests with the disruption mutants 518-6 and 521-10 are shown in Table 3. It is clear that mixtures of the mutant strains, even though they are compatible for mating, failed to produce disease symptoms of the severity seen with mixtures of the wild-type strains. We conclude that the *myp1* gene is required for wild-type levels of virulence.

Deletion of *myp1* in haploid strains: The plasmid pDEL2 (Figure 1D) was employed to generate strains carrying a null allele of *myp1*. In pDEL2, the coding sequence of *myp1* was replaced by a marker specifying hygromycin resistance to ensure that the gene was completely inactive. To achieve gene replacement, pDEL2 was linearized with *Bam*HI (Figure 1D) and transformed into strains 518, 521 and P6D. Homologous integration events leading to gene replacement in the transformants were identified by DNA blot hybridiza-

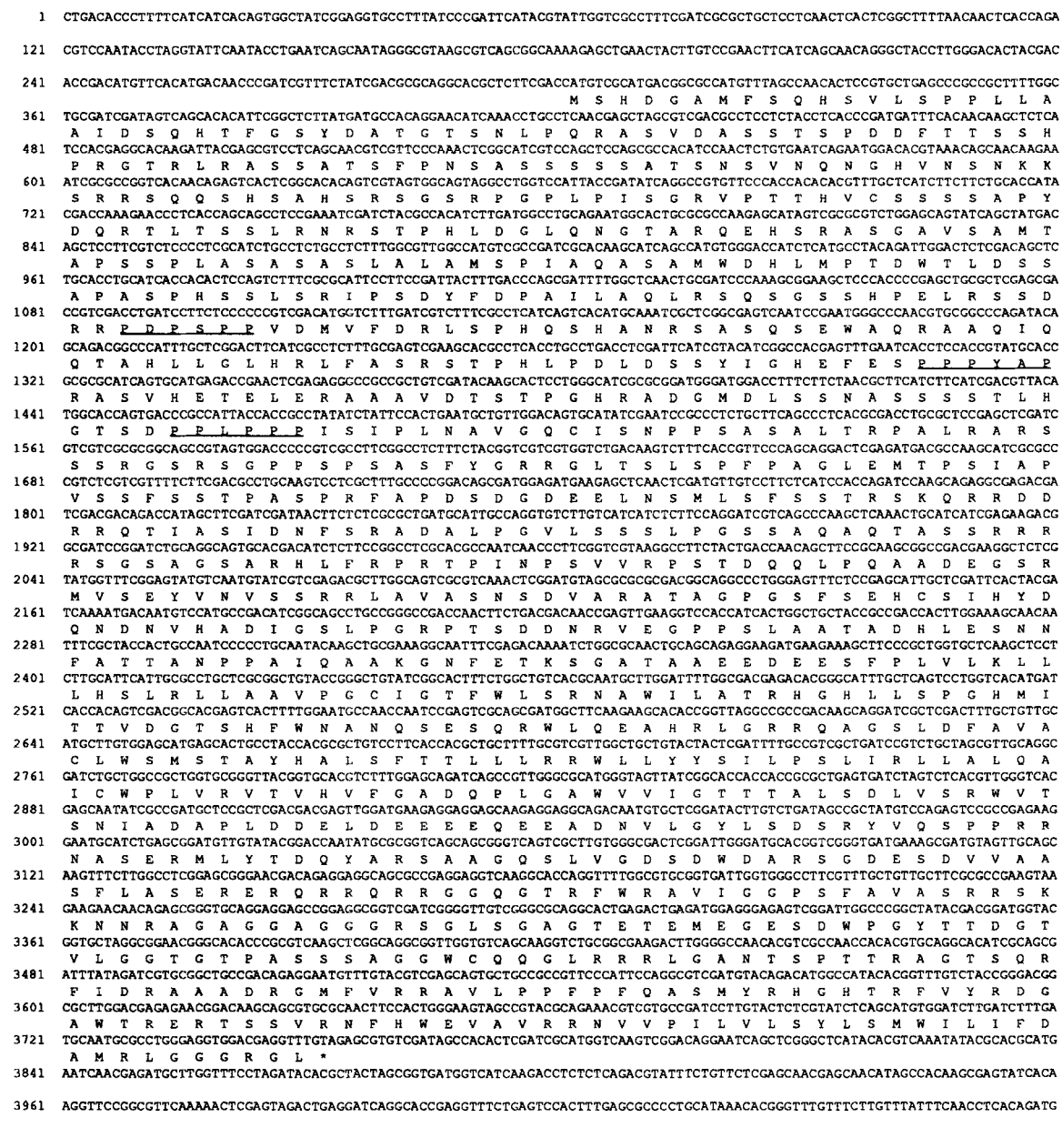

FIGURE 4.—Nucleotide sequence and open reading frame of the *myp1* gene. The numbers at the left refer to the nucleotide sequence and the numbers on the right indicate the amino acid sequence (standard one letter code). The stop codon is marked with an asterisk. The positions of proline-rich, putative SH3 ligand motifs are underlined. The accession number for the sequence is L33919.

tion (data not shown) and deletion mutants were identified for each strain. All five P6D deletion mutants obtained in this experiment were nonmycelial (or slightly mycelial) on DCM with charcoal (Figure 2). Transformants of 518 and 521 carrying the *myp1* deletion retained wild-type cellular and colony morphology. As with the *myp1* disruption mutants, these deletion strains exhibited a slightly slower growth rate in liquid medium when compared with wild-type strains (Figure 5B).

Representative deletion mutants 518-60 and 521-32 were selected for mating tests to determine whether the mutation influenced filamentous growth (Figure 5B). The compatible strains 518 and 521 formed a colony that was covered with the dense aerial hyphae indicative of a positive mating reaction. Similarly, coinoculation of deletion mutant 518–60 (*a2b2 myp1Δ1*) with compatible wild-type strain 521 (*a1b1*), or deletion mutant 521–32 (*a1b1 myp1Δ1*) with compatible wild-type strain 518 (*a2b2*), resulted in a mycelial reaction. The mixture of both deletion mutants, 518-60 and 521-32, also formed weakly mycelial colonies indicating that deletion of the *myp1* gene in both mating partners attenuates but does not block formation of aerial hyphae.

The pathogenicity of deletion mutants 518-60, 521-32 and P6D-9 was also tested by inoculation into corn seedlings. As can be seen in Table 4, there was a slight decrease in virulence when the deletion mutant P6D-9 was used as the inoculum compared with inoculation

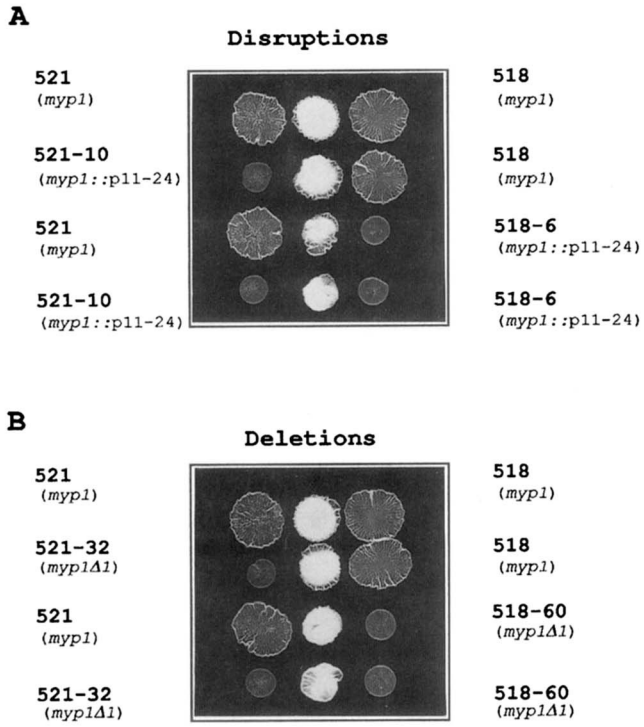

FIGURE 5.—Mating reactions of haploid strains carrying mutations in the *myp1* gene. The colonies on the left and right are individual strains inoculated onto DCM medium containing activated charcoal. The colonies in the center are the mating reactions (formation of aerial hyphae) arising from mixtures of the strains indicated on the left and right. The reaction between strains 518 and 521 at the top of each panel indicates the amount of aerial hyphae produced by wild-type mating interactions. Identical results were obtained in four repetitions of the mating reactions with the strains shown in both panels. (A) Mating reactions of strains 518-6 (*a2 b2 myp1* p11-24) and 521-10 (*a1 b1 myp1* p11-24), carrying a disruption mutation in the *myp1* gene, with wild-type or mutant strains. (B) Mating reactions of strains 518-60 (*a2 b2 myp1Δ1*) and 521-32 (*a1 b1 myp1Δ1*), carrying a deletion mutation in the *myp1* gene, with wild-type or mutant strains. Five to ten microliter drops of overnight PDB cultures were spotted on the plates and allowed to dry. The plates were sealed with parafilm and incubated at room temperature for 44 hr.

with the parental strain P6D. The severity of disease symptoms was more dramatically reduced when a mixture of two deletion strains (518-60 and 521-32) was used as the inoculum, compared with a mixture of compatible wild-type strains (518 and 521). These data indicate that deletion of *myp1* results in a reduction in virulence.

DISCUSSION

Construction and mutagenesis of a pathogenic haploid strain: To identify recessive mutations in genes involved in filamentous growth in *U. maydis*, we constructed a haploid strain (P6D) that would mimic properties of the infectious dikaryon that are normally conditioned by heterozygosity at the *a* and *b* mating-type loci, *i.e.*, filamentous growth and pathogenicity. Plasmid insertion mutagenesis of this strain proved to be an effective method of generating mutants with a nonmycelial colony morphology. In this work, a circular plasmid (pUBC50) was employed for mutagenesis, and insertion of this plasmid led to the isolation of the *myp1* gene. In other fungal systems (LU *et al.* 1994), restriction-enzyme mediated integration (REMI; KUSPA and LOOMIS 1992) has proven effective for generating mutations and this technique could potentially be applied to isolate additional nonmycelial mutants in *U. maydis*. Genetic analysis of the mutations in our collection of nonmycelial mutants (*e.g.*, to establish complementation groups) was not possible because the strains were defective for mating due to the presence of two different *b* specificities (LAITY *et al.* 1995).

Phenotypes of *myp1* disruption and deletion mutants: The *myp1* gene was identified following transformation of plasmid pUBC50 into strain P6D and subsequent characterization of the nonmycelial mutant NF14. This mutant formed yeast-like colonies with a markedly different appearance compared with colonies of the mycelial haploid P6D. The same insertion mutation, when present in haploid strains 518 (*a1 b1*) and 521 (*a2 b2*), caused a less marked reduction in the mycelial growth (indicative of infection hyphae) that normally results from a mating reaction. Similar results were obtained with derivatives of haploid strains 518 and 521 that carried a deletion of the *myp1* locus. That is, these strains also showed a slight reduction in myce-

TABLE 3

Pathogenicity of *myp1* disruption mutants on corn plants

Strains	Genotype	Disease symptoms					No. of plants
		A	B	C	D	E	
518-6	*a2b2 myp1*::p11-24 (*hyg*^{r})	26	0	0	0	0	26
521-10	*a1b1 myp1*::p11-24 (*hyg*^{r})	27	0	0	0	0	27
518-6 × 521-10	*a2b2 myp1*::p11-24 (*hyg*^{r}) × *a1b1 myp1*::p11-24 (*hyg*^{r})	71	10	0	0	0	81
518 × 521-10	*a2 b2* × *a1b1 myp1*::p11-24 (*hyg*^{r})	3	1	0	53	16	75
521 × 518-6	*a1b1* × *a2b2 myp1*::p11-24 (*hyg*^{r})	5	1	4	47	16	73
521 × 518	*a1b1* × *a2b2*	4	5	1	59	9	78

The results were obtained from plants inoculated in two replicates of the experiment. The rating scheme for the disease symptoms is described in Table 2. The plants were inoculated with cells at a density of 10^6 cells/ml.

TABLE 4

Pathogenicity of *myp1* deletion mutants on corn plants

Strains	Genotype	Disease symptoms					No. of plants
		A	B	C	D	E	
P6D	*a2 b2 [a1 bE1 phleo^r]*	15	60	24	3	0	102
P6D-9	*a2 b2 [a1 bE1 phleo^r] myp1Δ1 (hyg^r)*	18	75	9	0	0	102
518-60	*a2b2 myp1Δ1 (hyg^r)*	11	0	0	0	0	11
521-32	*a1b1 myp1Δ1 (hyg^r)*	11	0	0	0	0	11
521 × 518-60	*a1b1 × a2b2 myp1Δ1 (hyg^r)*	0	2	1	15	5	23
518 × 521-32	*a2b2 × a1b1 myp1Δ1 (hyg^r)*	2	2	3	10	4	21
518-60 × 521-32	*a2b2 myp1Δ1 (hyg^r) × a1b1 myp1Δ1 (hyg^r)*	39	51	2	0	0	92
521 × 518	*a1b1 × a2b2*	0	0	6	60	30	96

The results were obtained from plants inoculated in two replicates of the experiment. The rating scheme for the disease symptoms is described in Table 2. The plants were inoculated with cells at a density of 10^6 cells/ml for all mixtures except P6D and P6D-9, which were at 10^7 cells/ml.

lial growth upon mating that was similar to that found with strains carrying the disruption mutation. As with disruption of the *myp1* gene in strain NF14, deletion of the gene in a mycelial haploid background (P6D-9) resulted in a drastic reduction in mycelial growth.

The more pronounced phenotypes observed upon disruption or deletion of *myp1* in P6D compared with mutation of the gene in haploid mating partners (during mating reactions) can be viewed in the context of the relative vigor of the filamentous growth and pathogenicity of haploid, diploid and dikaryotic cells. For example, mixtures of compatible haploid strains form dikaryons with distinctive mycelial growth on culture medium; these mixtures cause severe disease symptoms upon injection into plants. Diploids that are heterozygous for the *a* and *b* mating-type loci also form mycelial colonies, but the disease symptoms caused by these strains are reduced compared with the haploid mixtures (HOLLIDAY 1961; KRONSTAD and LEONG 1989, 1990). The engineered haploid strain P6D displays a relatively weak mycelial phenotype, and a reduced ability to cause disease symptoms, compared with mating mixtures and diploids. In this context, it is not surprising that disruption or deletion of the *myp1* gene might cause a more dramatic phenotype in a weakly pathogenic haploid strain like P6D. In addition, allelic differences at other loci could contribute to the vigor of mycelial growth and pathogenicity in haploid mating partners and might compensate for a defect in *myp1*; such differences would not be present in the haploid strain P6D. There is precedent for the influence of the specific genetic background on the penetrance of mutations affecting morphology in fungi. For example, BLACKETER *et al.* (1993) recently reported variation in the severity of phenotypes of elongated morphology mutants in *S. cerevisiae* (*ELM1–3* genes) depending on genetic background.

Mixtures of compatible haploid strains each carrying the disruption or the deletion mutation showed a reduction in virulence upon injection into corn seedlings. This phenotype is consistent with an attenuated ability of these strains to form infection hyphae upon mating. The *myp1* gene apparently plays a role in establishing or maintaining the filamentous cell type that normally results from mating. This dikaryotic cell type is required for proliferation of the fungus in the plant.

It should also be noted that a slower growth rate was apparent for the haploid strains carrying the disruption or the deletion mutation when compared with wild-type cells. It was not the case, however, that the observed phenotypes of the mutants were due to auxotrophy because these strains were capable of growth on minimal medium. Overall, the characterization of the disruption and deletion mutants indicates a role for the *myp1* product in filamentous growth and virulence, as well as a role in the growth of haploid cells.

Characterization of the *myp1* gene: Nucleotide sequence analysis of the *myp1* gene revealed a long open reading frame that could potentially encode a polypeptide of 1150 amino acids. The amino terminal half of the inferred product is rich in serine and alanine residues and contains several proline-rich motifs. The carboxy terminal portion of the predicted product contains a region of hydrophobic amino acids flanked by stretches of acidic residues; this region may specify a membrane spanning domain. A database search did not reveal extensive similarity between the *myp1* sequence and any known gene.

The proline-rich motifs of the *myp1* gene are similar in sequence to ligand motifs recognized by SH3 domain containing proteins (YU *et al.* 1994). These sequences were detected because of their similarity to regions in the proline-rich protein, verprolin, of *S. cerevisiae* (DONNELLY *et al.* 1993). In yeast, defects in verprolin (*vrp1*) result in cells that are larger than wild-type cells and that have a distorted morphology. It has been proposed that verprolin contains SH3 ligand motifs and that the protein interacts with cytoskeleton-associated proteins. Interestingly, the *BEM1* gene of *S. cerevisiae* contains SH3 domain motifs and plays a role in the polarized

growth that occurs during budding and during mating response (CHENEVERT *et al.* 1992). Defects in Bem1p lead to general cell enlargement rather than polarized growth in response to pheromone. These observations indicate an important role for proteins containing SH3 domains and SH3-ligand motifs in fungal morphogenesis. If the putative SH3 ligand motifs in the predicted *myp1* product of *U. maydis* are authentic, then this polypeptide may participate in morphogenetic processes similar to those involving verprolin and Bem1p.

Involvement of the *myp1* gene in morphogenesis: The sequence organization of the *myp1* gene product and the phenotypes of mutants defective in *myp1* suggest a role for the polypeptide in hyphal elongation in *U. maydis*. We speculate that the *myp1* product may participate in the organization of the cytoskeleton or other factors necessary for directed growth at hyphal tips in filaments or at the tips of budding cells. Haploid *U. maydis* cells generally have an elongated morphology during budding growth and the elongation of a bud resembles hyphal growth. The slower growth of budding cells carrying *myp1* mutations may simply result from a reduced rate of cell wall deposition during bud elongation. For filamentous growth, the loss of *myp1* function could potentially impair the hyphal elongation necessary to initiate mating (*i.e.*, formation of conjugation tubes) and/or the growth of the filamentous dikaryon that results from mating. That the defects in these processes are not complete, even in strains deleted for *myp1*, suggests that there may be structural or functional homologs of the *myp1* product. The apparent redundancy of *myp1* function is also consistent with the fact that compatible haploid strains carrying the *myp1* deletion partially retained the ability to form aerial hyphae upon mating. In terms of pathogenesis, the *myp1* defect greatly reduces the ability of the fungus to cause disease symptoms in the host plant. Again, this phenotype is consistent with a role for the *myp1* product in hyphal elongation, a trait that is likely to be necessary for tissue invasion.

Genes required for elongation of hyphal or yeast-like cells have been identified in other fungi. For example, a large number of genes have been described that play a role in pseudohyphal growth in *S. cerevisiae*, a growth pattern typified by elongated cells. These genes include the *STE7, 11, 12* and *20* genes, which encode components of the pheromone response pathway (LIU *et al.* 1993), the *RAS2* gene in the cAMP pathway (GIMENO *et al.* 1992), the *PHD* genes whose overexpression enhances pseudohyphal growth (GIMENO and FINK 1994), as well as a set of genes designated *ELM* for elongated morphology (BLACKETER *et al.* 1993). Mutations in the *ELM* genes result in constitutively pseudohyphal growth (BLACKETER *et al.* 1993). In *Candida albicans*, the *PHR1* gene has been identified a playing a role in apical cell growth and morphogenesis (SAPORITO-IRWIN *et al.* 1995). The *PHR1* gene is regulated by pH and appears to encode a cell surface protein with a glycosylphosphatidylinositol membrane anchor. A mutant defective for *PHR1* was unable to carry out apical growth of either yeast or hyphal forms.

In many fungi, protein phosphorylation plays an important role in morphogenesis. For example, the *ELM1* gene of *S. cerevisiae* encodes a serine/threonine protein kinase believed to regulate differentiation into the pseudohyphal growth pattern (BLACKETER *et al.* 1993). Other fungal genes encoding protein kinases and playing a role in morphogenesis include the *cot1* gene of *Neurospora crassa* (YARDEN *et al.* 1992), the *orb5* gene of *Schizosaccharomyces pombe* (SNELL and NURSE 1994) and the *YCK1* and *YCK2* genes of *S. cerevisiae* (ROBINSON *et al.* 1993). In *U. maydis*, recent evidence has implicated the cAMP dependent protein kinase (PKA) in the switch between budding and filamentous growth (GOLD *et al.* 1994). That is, low PKA activity is correlated with filamentous growth in *U. maydis*. It is possible that the *myp1* gene product and PKA are components of the same morphogenetic pathway in *U. maydis*.

In summary, we have isolated and characterized the *myp1* gene that plays a role in morphogenesis. This gene joins a growing list of *U. maydis* genes involved in morphogenesis and virulence. These genes include the mating-type genes at the *a* and *b* loci, components of the cAMP pathway such as the *uac1* and *ubc1* genes (GOLD *et al.* 1994), and *fuz7*, which encodes a MEK/MAPKK kinase involved in pheromone response (BANUETT and HERSKOWITZ 1994).

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