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The *Ustilago maydis* Regulatory Subunit of a cAMP-Dependent Protein Kinase Is Required for Gall Formation in Maize

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In the plant, filamentous growth is required for pathogenicity of the corn smut pathogen *Ustilago maydis*. Earlier, we identified a role for the cAMP signal transduction pathway in the switch between budding and filamentous growth for this fungus. A gene designated *ubc1* (for *Ustilago* bypass of cyclase) was found to be required for filamentous growth and to encode the regulatory subunit of a cAMP-dependent protein kinase (PKA). Here, we show that *ubc1* is important for the virulence of the pathogen. Specifically, *ubc1* mutants are able to colonize maize plants and, like the wild-type pathogen, cause localized symptoms in association with the presence of hyphae. However, in contrast to plants infected with wild-type cells that often developed galls from initially chlorotic tissue, plants infected with the *ubc1* mutant did not produce galls. These data suggest that PKA regulation is critical for the transition from saprophytic to pathogenic growth and from vegetative to reproductive development. Plate mating assays in which exogenous cAMP was applied suggested that the cAMP and *b* mating-type morphogenetic pathways may be coordinated.

INTRODUCTION

Corn smut of maize is caused by the fungal pathogen *Ustilago maydis* (Christensen, 1963). Disease symptoms include the initial induction of chlorosis and/or anthocyanin pigmentation, followed by the generation of galls consisting of hyperplastic and hypertrophied plant cells and fungal hyphae. These symptoms are an apparent plant response to fungal signals transmitted at the infection foci. The fungus eventually forms a mass of black sexual teliospores within gall tissue, and it is from the sooty appearance of this phase of the life cycle that the smuts derive their name.

Dimorphic growth is a central feature in the life cycle and pathogenesis of *U. maydis*. Haploid strains grow as saprophytic budding cells and can easily be cultured in the laboratory. Compatible haploid mating partners fuse and form a filamentous dikaryon, which is pathogenic and obligately biotrophic. Dikaryotic hyphae colonize plant tissue, ramify locally, and induce galls on all above-ground plant parts. Teliospore formation completes the coincident pathogenic and sexual phases of the life cycle.

Budding haploid strains must possess different alleles at two mating-type loci called *a* and *b* (Rowell, 1955; Holiday, 1961) to form the infectious dikaryon (for recent reviews, see Banuett, 1995; Kahmann et al., 1995). The *a* locus encodes pheromones and pheromone receptors, and each haploid mating partner must contain a different *a* specificity (*a1* or *a2*) for cell fusion to occur (Froeliger and Leong, 1991; Bölker et al., 1992; Trueheart and Herskowitz, 1992). Heterozygosity at the *a* locus also plays a secondary role in the maintenance of the filamentous phenotype and in the transcription of the *b* mating-type genes (Spellig et al., 1994; Hartmann et al., 1996; Urban et al., 1996). Heterozygosity at the *b* locus is required for the production of a stable dikaryon and for filamentous growth (Puhalla, 1970). The DNA at the *b* locus has been cloned and found to encode two homeodomain-containing proteins termed bW and bE (Kronstad and Leong, 1989; Schulz et al., 1990; Gillissen et al., 1992). These proteins have variable N-terminal domains that control dimerization between proteins encoded by different alleles (Kronstad and Leong, 1990; Yee and Kronstad, 1993; Spellig et al., 1994; Kämper et al., 1995).

The *b* gene products are believed to regulate the transcription of a set of target genes that directly or indirectly control morphogenetic transitions and pathogenicity. The *b* locus must be in the heterozygous state for the formation of a pathogenic cell type, with the only described exception to this rule being mutants affected in the *rtf1* (for repressor of tumor formation) gene (Banuett, 1991, 1995). In addition to mating signals, cells of *U. maydis* will switch between budding and filamentous forms in response to environmental conditions, such as nutrient availability (Kernkamp, 1939),

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exposure to air (Gold et al., 1994), and changes in pH (Ruiz-Herrera, 1995).

Several genes have been identified that influence filament formation in *U. maydis*. These include (1) the *uac1* (for *Ustilago* adenylyl cyclase) and *ubc1* (for *Ustilago* bypass of cyclase) genes encoding adenylyl cyclase and the regulatory subunit of the cAMP-dependent protein kinase (PKA), respectively (Gold et al., 1994); (2) the *myp1* gene (for mycelial phenotype), which is required for the filamentous growth and virulence of a haploid strain heterozygous at the *b* locus (Giasson and Kronstad, 1995); and (3) the *fuz7* (*MEK*) gene, a homolog of the *Saccharomyces cerevisiae ste7* gene, which plays a role in filament formation (Banuett and Herskowitz, 1994). Recently, the *gpa3* gene, encoding an α subunit of a heterotrimeric G protein, has also been found to affect filamentous growth and mating competence (Regenfelder et al., 1997). All of these genes appear to be involved in signal transduction processes, but little is known about the downstream genes whose products are responsible for the transition from budding to filamentous growth.

We have accumulated genetic evidence that the cAMP pathway plays a significant role in dimorphism in *U. maydis* (Gold et al., 1994). Specifically, we have isolated the *uac1* gene, which is required for budding growth, that is, strains defective in *uac1* display a filamentous phenotype. These *uac1* mutants are unable to colonize the host plant (Barrett et al., 1993). In addition, we have collected ~150 independent *uac1* suppressor mutants, termed *ubc*, which are suppressed for the filamentous phenotype of *uac1* disruption mutants. Complementation of one of these *ubc* suppressor mutations (*ubc1-1*) led to the isolation of the gene (*ubc1*) encoding the regulatory subunit of PKA that, in plate assays, is required for filamentous growth after mating and for responsiveness to environmental signals (Gold et al., 1994).

The effect of cAMP in eukaryotic cells is primarily transmitted by the action of PKA. It consists of two subunits, the regulatory and the catalytic subunits, that play antagonistic roles (reviewed in Taylor et al., 1990). These proteins generally associate as a heterotetramer of two molecules of each subunit. All enzyme activity is housed in the catalytic subunit of PKA. Each regulatory subunit molecule has two binding sites for cAMP that act with binding kinetics indicative of cooperativity. The holoenzyme tetramer is inactive and thus unable to phosphorylate substrate proteins. Enzyme inactivity is the result of the regulatory subunit binding to the catalytic subunit of PKA and inhibiting catalytic activity. Inhibition is released by the presence of sufficient intracellular cAMP. Upon binding of cAMP, the regulatory subunits are induced to a conformation change that causes the release of two molecules of the free and now active catalytic subunit of PKA. Thus, cAMP indirectly causes the phosphorylation of PKA substrate proteins, which results in their altered activity and, in case of *U. maydis*, leads to morphogenetic change.

In this report, we analyze the interaction of *ubc1* mutants with maize. Because the *ubc1* gene encodes the regulatory subunit of PKA (Gold et al., 1994), *ubc1* mutants are presumed to be derepressed for the phosphorylation of substrates of the catalytic subunit of PKA. Defects in pathogenicity for *ubc1* mutants might therefore indicate a requirement for signal transduction within the fungus, and perhaps from the fungus to the plant, necessary for the induction of galls. Indeed, we found that *ubc1* mutants are defective in the induction of gall formation. In addition, mating experiments with cells exposed to cAMP suggest an interaction of the mating and cAMP signal transduction pathways.

RESULTS

Reduced Symptom Formation by *ubc1* Mutants

Experiments were performed to determine the impact of the *ubc1* mutation on pathogenicity. Pairs of compatible haploid strains differing at the *a* and *b* mating-type loci were coinjected into plants to test the virulence of the dikaryon produced upon fusion. Several strains with different *ubc1* alleles were used to elucidate the role that the regulatory subunit of PKA plays in disease development. Dikaryons were formed by mating compatible strains carrying *ubc1* mutant alleles, including the original *ubc1-1* UV-induced mutation as well as the *ubc1-2* mutation (a disruption mutant; Gold et al., 1994) and the *ubc1-3* allele (a *ubc1* deletion strain; F. Dürrenberger and J.W. Kronstad, manuscript in preparation). All strains used in this study are listed in Table 1.

Inoculations were performed with two additional strains with spontaneous mutations in *ubc1*. These new alleles

Table 1. Strains of *U. maydis* Used in This Study

Strain	Relevant Genotype	Source
1/2	*a1b1* (wild-type strain 521)	Kronstad and Leong (1989)
1/9	*a1b1*, *uac1*::*ble* (COO2P)	Gold et al. (1994)
1/53	*a1b1*, *uac1*::*ble ubc1-1* (COO2P)	Gold et al. (1994)
1/54	*a1b1*, *ubc1-1*	Gold et al. (1994)
1/60	*a2b2*, *ubc1-1*	Gold et al. (1994)
1/68	*a1b1 ubc1-2* (*ubc1*::*hyg*)	Gold et al. (1994)
1/70	*a2b2*, *uac1*::*ble ubc1-1*	Gold et al. (1994)
2/8	*a1b1* (B×11 seventh backcross to 1/2, near isogenic to 1/2)	This study
2/9	*a2b2* (B×22 seventh backcross to 1/2, near isogenic to 1/2)	This study
2/17	*a2b2 ubc1-3*	F. Dürrenberger and J.W. Kronstad (unpublished data)
2/21	*a1a2 b1b2* diploid (d132)	Kronstad and Leong (1989)
4/1	*a1b1 ubc1-4*, small spontaneous deletion in the 1/9 *ubc1* gene	This study
4/4	*a1b1 ubc1-5*, small spontaneous insertion in the 1/9 *ubc1* gene	This study

were identified in a polymerase chain reaction (PCR) screen to identify mobile transposable elements in *U. maydis* (see Methods). The *ubc1-4* and *ubc1-5* alleles were found to have an ~50-bp increase or a decrease in the *ubc1* PCR fragment length, respectively (data not shown). Both mutations are complemented by the *ubc1* gene in transformation experiments (data not shown).

Table 2 shows typical results of plant inoculations with dikaryons produced by the mating of compatible haploid strains containing combinations of wild-type and/or *ubc1* mutated alleles. These inoculations included positive controls of crosses between wild-type strains and between wild-type and mutant strains. We consistently found that dikaryons defective in both *ubc1* alleles were capable of causing symptoms of chlorosis and anthocyanin production. Interestingly, galls were never observed in the several hundred plants inoculated with the various *ubc1* mutant dikaryons.

In contrast, inoculations with dikaryons formed between wild-type partners, or a *ubc1* mutant in combination with a compatible wild-type strain, yielded typical disease symptoms of corn smut. These symptoms included progression from initial expression of chlorosis, and occasional anthocyanin production, to gall formation on leaves and stems. In some cases, plant death was also observed.

Chlorosis was much more common than anthocyanin production on the variety of maize used in these experiments and, in all samples observed, indicated the position of fungal ramification within the host leaves. No symptom development or hyphal ramification was witnessed when plants were injected with water or inoculated with a pair of strains incompatible for mating (data not shown). Figures 1A to 1E show typical disease development from 3 to 14 days postinoculation (dpi) in plants infected with the wild-type dikaryon, the dikaryon heterozygous for *ubc1* (*ubc1/ubc1-1*), and the dikaryon homozygous for *ubc1-1*. In all cases, the wild-type dikaryon or the dikaryons heterozygous at *ubc1* caused similar disease progression, whereas the dikaryon homozygous for *ubc1* mutations was easily distinguishable and never caused gall formation.

Regardless of the mutant *ubc1* allele involved, symptom development in inoculations involving one wild-type strain and any one of the compatible *ubc1* mutant strains was the same and indicated that all *ubc1* mutant alleles are recessive. In addition, the functional equality of the *ubc1* mutant alleles was apparent in the results of pathogenicity experiments. Regardless of the combination of *ubc1* mutant alleles carried by the compatible mating partners, the mutant dikaryon produced symptoms, including chlorosis and anthocyanin production, indistinguishable from those of wild-type dikaryon until ~5 dpi, at which time gall formation began in the plants infected with wild-type cells. In comparison with inoculations of wild-type cells, the frequency of symptomatic plants was generally reduced in inoculations with compatible *ubc1* mutants. Inoculation experiments with dikaryons formed in combinations of the *ubc1-3* deletion strain with either the spontaneous *ubc1-4* or *ubc1-5* mutants also yielded results similar to those with the *ubc1-1* mutants (data not shown).

Table 2. Pathogenicity and Allelism of *ubc1-1* and *ubc1* Disruption/Deletion Mutations

Strains Inoculated[a]	Dikaryon *ubc1/ubc1*	No. of Plants	Disease Rating[b] 0	1	2	3	4	Death 5	Disease Index[c]
1/2 × 2/9	+/+	20	1	3	4	4	7	1	2.80
1/54 × 2/9	+/−	20	1	5	1	5	5	3	2.85
1/60 × 1/2	+/−	20	1	6	3	3	3	4	2.65
1/68 × 2/9	+/Δ[d]	20	2	4	2	3	6	3	2.80
2/17 × 1/2	+/Δ	20	2	3	2	3	7	3	2.95
1/54 × 1/60	−/−	20	4	15				1	1.00
1/60 × 1/68	−/Δ	20	7	13					0.65
1/68 × 2/17	Δ/Δ	20	8	12					0.60

[a] Inoculations were of 10^6 cells per mL for each of the paired strains.
[b] Recorded 10 dpi.
[c] The disease index is calculated as Σ disease ratings divided by the number of plants.
[d] Δ indicates a disruption/deletion mutation.

Microscopic Evidence for Colonization

Fungal ramification was clearly present in symptomatic maize tissue in plants inoculated with either wild-type haploid pairs or *ubc1-1* mutant haploid pairs. Figures 2A to 2E and Figures 2F to 2J present the visualization of wild-type and *ubc1-1* filamentous growth, respectively, within symptomatic tissue from 3 to 14 dpi. In plants inoculated with either the wild type or *ubc1-1* mutants, symptoms were similar during the first 5 days and were accompanied by straight hyphae, which appeared to increase in abundance in symptomatic tissue over time (cf. Figures 2A and 2B and Figures 2F and 2G). The hyphae of the *ubc1-1* mutant were similar to those of wild-type cells, although they occasionally appeared to possess relatively short branches (cf. Figures 2B and 2G). The most obvious distinction between plant symptoms caused by the wild-type and *ubc1-1* infections was the presence or absence, respectively, of gall formation and teliosporogenesis (cf. Figures 2C through 2E and 2H through 2J). Galls first appeared by day 5 (Figure 2B), and teliosporogenesis was most evident within the galls in wild-type infected plants 7 to 14 dpi (Figures 2C to 2E). In inoculation experiments with incompatible strains, sporidial cells were visualized on the surface of fixed leaves but no hyphal ramification was observed (data not shown).

Impaired Fungus–Plant Signaling in *ubc1* Mutants

The inability of *ubc1* mutants to cause galls could be due to a plant resistance response or to the inability of the fungus

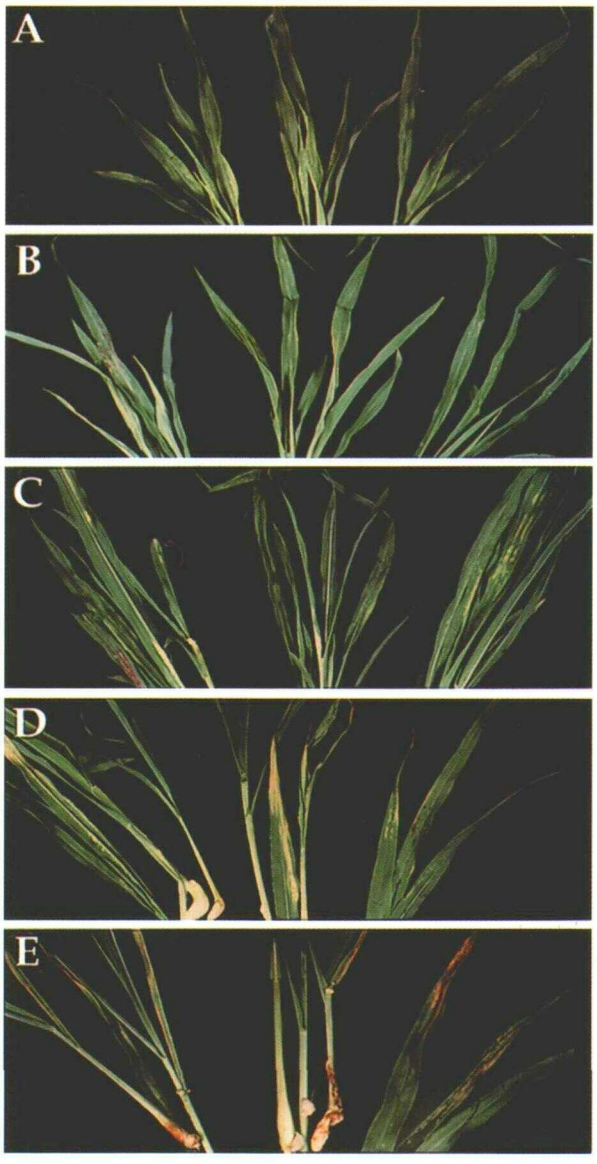

Figure 1. Comparison of Disease Symptoms in Maize Seedlings Caused by Wild-Type or *ubc1-1* Dikaryons.

Infection was with a mixture of wild-type strains (1/2 × 2/9, left), a mix of the *ubc1-1* mutant and a wild-type strain (1/60 × 1/2, center), and two *ubc1-1* mutants (1/60 × 1/54, right). In all cases, the strains were compatible for mating.

(A) Plants 3 dpi.
(B) Plants 5 dpi.
(C) Plants 7 dpi.
(D) Plants 10 dpi.
(E) Plants 14 dpi.

to stimulate gall formation, for example, by generating an inducing signal. To address these possibilities, a competitive inoculation experiment was employed to discern whether primary inoculation with the *ubc1-1/ubc1-1* dikaryon could provide protection from subsequent symptom development after secondary inoculation with the wild-type fungus. This type of protection has been encountered in other systems (Freeman and Rodriguez, 1993; Mahuku et al., 1996). The data presented in Table 3 indicate that there was no protection from wild-type disease progression if wild-type strains were inoculated after primary inoculation with the *ubc1-1* strains. Regardless of the duration (up to 7 days) between initial infection with the *ubc1-1* dikaryon and secondary inoculation with compatible wild-type partners, symptom development was essentially indistinguishable from that in control plants inoculated exclusively with compatible wild-type strains. These results suggest that the inability of the fungus to induce production of galls was likely an inherent defect associated with the *ubc1-1* mutation and not the result of an altered plant resistance response.

Interaction between the cAMP and Mating Pathways

The inability of dikaryons homozygous for defects in *ubc1* to cause gall formation suggests an interaction with the pathogenicity and filamentous growth pathways controlled by the mating-type genes. Therefore, we employed a simple plate assay for aerial hyphae to address the issue of whether regulation of the cAMP signal transduction pathway and mating-type control are independent or coordinated in the induction of filamentous growth. Figure 3 shows the dramatic effect that exogenously added cAMP has on filamentous growth of the *b* heterozygote. The results of mating reactions with compatible haploid strains indicate that cAMP inhibits the production of filaments in the *b* heterozygotic dikaryon (Figure 3A). However, with time, the dikaryon overcomes the initial inhibition and produces filaments. There appeared to be a dose–response relationship because longer exposure to exogenous cAMP caused a greater delay in the production of aerial hyphae.

The 1/9 *uac1* disruption strain was used to control for the filamentous reaction and responsiveness to cAMP (Figure 3). This strain, unlike the others used in the experiment, was always grown first in liquid potato dextrose broth (PDB) with cAMP. This was to achieve budding growth and to control the density and growth stage of cells for plating. However, the resultant colony morphology of 1/9 in response to cAMP on plates is the same if grown first in liquid without cAMP. The dikaryon produced from the cross of 1/2 and 2/9 was able to overcome the presence of cAMP in the plate and begin to produce filaments even after initial growth in liquid containing cAMP; however, 1/9 was not able to overcome the presence of cAMP and produced no filaments.

The observed inhibition of aerial hyphae in the mating reaction could result from interference at the level of conjugation tube formation, cell fusion, or establishment of the

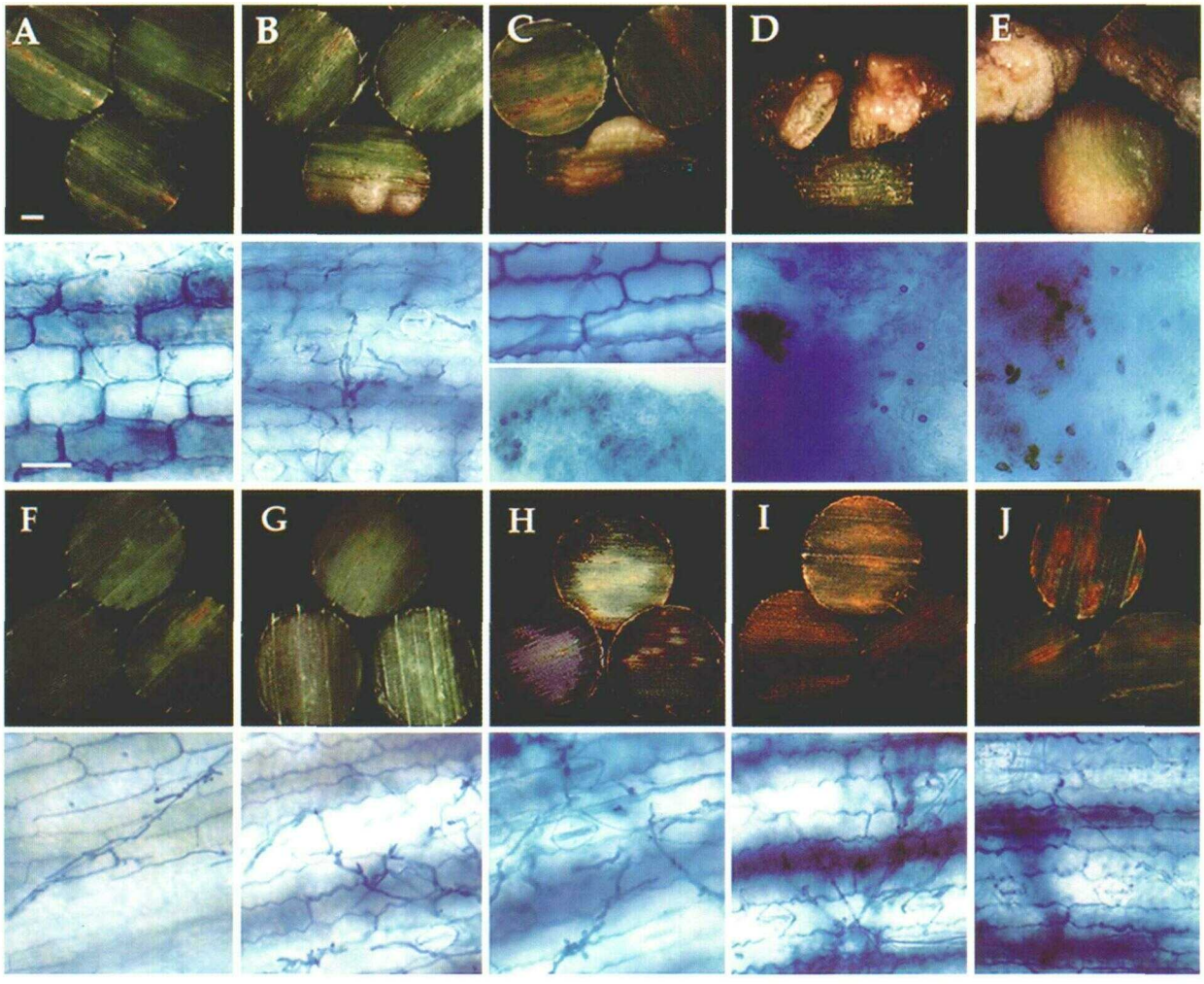

Figure 2. Macroscopic and Microscopic Evidence of Disease Development and Infection in Wild-Type versus *ubc1-1* Infections.

(A) to **(E)** A time course of wild-type dikaryon infection.
(F) to **(J)** A time course of *ubc1-1* dikaryon infection.
The upper halves of **(A)** to **(J)** show the symptoms apparent in 5-mm discs (bar = 1 mm); the lower halves show stained fungal structures, including hyphae and/or teliospores within the symptomatic discs (bar = 50 μm). **(A)** and **(F)** represent 3 dpi; **(B)** and **(G)** represent 5 dpi; **(C)** and **(H)** represent 7 dpi; **(D)** and **(I)** represent 10 dpi; **(E)** and **(J)** represent 14 dpi.

filamentous dikaryon. To distinguish between these three possibilities, we used the diploid strain 2/21, which is heterozygous at the *a* and *b* loci and produces filaments when plated alone on charcoal mating medium or on potato dextrose agar (PDA) amended with India ink (PDA-ink). Filamentous growth of this strain was inhibited if grown in the presence of cAMP when plated on PDA-ink (Figure 3B). The only condition under which the diploid produced significant filamentous growth was when no cAMP had been used in the liquid or on the plates. These results suggest that cAMP and the mating pathway interact after fusion has occurred, although these experiments cannot rule out an additional influence of cAMP on early mating events. A hypothetical interaction between the action of PKA and *b* mating–type control is discussed below.

DISCUSSION

ubc1 Mutants Colonize Maize but Do Not Induce Galls

The primary goal of the work described here was to determine the effect of mutations in the *ubc1* gene on the pathogenicity of *U. maydis* on maize. This work is a continuation of

Table 3. Competition of Wild-Type Disease Development by Preinoculation with *ubc1-1* Strains

Day 0 Inoculation[a] (Strains)	Second Inoculation		Dikaryon	No. of	Disease Rating[b]						
	Strains	Day	*ubc1/ubc1*	Plants	0	1	2	3	4	Death 5	Disease Index
1/60 × 1/54	—[c]	—	−/−	19	4	14				1	1.00
1/60 × 1/54	1/2 × 2/9	0	−/−:+/+[d]	19		7		1	6	5	2.95
1/60 × 1/54	1/2 × 2/9	3	−/−:+/+	20		1	3	5	4	7	3.50
1/60 × 1/54	1/2 × 2/9	5	−/−:+/+	19		1	4	3	11		3.26
1/60 × 1/54	1/2 × 2/9	7	−/−:+/+	20	1	1	3	11	2	2	2.90
1/2 × 2/9	—	—	+/+	20		2	4	1	7	6	3.55
—	1/2 × 2/9	3	+/+	20		4		2	11	3	3.45
—	1/2 × 2/9	5	+/+	20		2	1	6	8	3	3.45
—	1/2 × 2/9	7	+/+	20		1	3	9	7		3.30
1/54 × 2/9	—	—	+/−	20		6	4		5	5	3.15
1/60 × 1/2	—	—	+/−	20		7	3		3	7	3.00

[a] Inoculations were of 10^6 cells per mL for each of the paired strains.
[b] The disease ratings were reported at 14 dpi.
[c] Dashes indicate that no inoculation occurred.
[d] Colons separate the two expected dikaryons in cases in which two sets of inoculations were performed.

efforts to identify genes that are not mating-type genes but that are important for the formation of the infectious, filamentous dikaryon (Barrett et al., 1993; Gold et al., 1994; Giasson and Kronstad, 1995). Our analysis of the pathogenicity of dikaryons homozygous for mutations in *ubc1* indicates that the gene is essential for full development of corn smut symptoms. *ubc1* mutants were able to colonize tissue and produce initial disease symptoms, but they were unable to induce gall formation. Fungal development appeared to be arrested before the stage at which sporulation occurs.

Close inspection revealed that the symptoms produced by the *ubc1* dikaryon were generally indistinguishable from those caused by wild-type strains for the first 3 to 5 dpi. At these early times, initial symptoms of chlorosis and anthocyanin production developed, and it was not until ~5 to 7 dpi that the first incipient gall tissue became visible in the wild-type interaction. The initial symptoms were commonly observed in plants infected with *ubc1* mutants, but disease progression was halted before gall formation. The fungus was readily visualized within the host leaf tissue in symptomatic regions, and the abundance of hyphae within the host tissue appeared to be similar between mutant and wild-type infections. In contrast, sporidial cells from incompatible crosses (unable to form the dikaryon) were easily detected at needle wound sites, but no hyphae were observed to colonize plant tissue. Overall, the evidence suggests that the development of the *ubc1* fungus is somehow interrupted in the shift from colonization and ramification to gall formation and sporulation.

Plant inoculations with a *ubc1-1* strain and a compatible *ubc1-2* disruption mutant or a *ubc1-3* deletion mutant produced symptoms very similar to those encountered with the combinations of two compatible *ubc1-1* mutants or combinations of two compatible *ubc1* disruption/deletion (*ubc1-2* × *ubc1-3*) strains. In addition, two spontaneous mutants in *ubc1* (*ubc1-4* and *ubc1-5*) produced similar symptoms in combination with the *ubc1-3* deletion strain. These results confirm that the various *ubc1* mutations are allelic and functionally equivalent because symptom development was identical and no complementation was encountered, regardless of the mutant allele combination employed.

Mutations in the cAMP Pathway Block Different Stages of Infection

The pathogenicity test results reported here were performed with compatible *ubc1* single mutant strains. However, indistinguishable results were observed when *uac1::ble ubc1-1* double mutants were used (data not shown). Thus, the mutation in *ubc1* suppresses the defect in colonization found in *uac1* (previously named *rem1*) disruption strains (Barrett et al., 1993). Combining the pathogenicity data collected for *uac1* (Barrett et al., 1993) and *ubc1* mutants, a view of a pathway to disease development emerges. Mutants in *uac1* (with low cAMP and low PKA activity) are completely nonpathogenic, suggesting that specific substrates of the catalytic subunit of PKA must be phosphorylated to allow colonization. In contrast, mutants in the regulatory subunit of PKA are able to colonize the host. However, gall formation does not occur, suggesting that PKA substrates must favor a lesser degree of phosphorylation for the transmission of fungal signal molecules that induce gall development.

Thus, a PKA-controlled switch appears to be important in the pathogenicity of *U. maydis* such that increased PKA activity is required for initial plant infection, whereas reduced

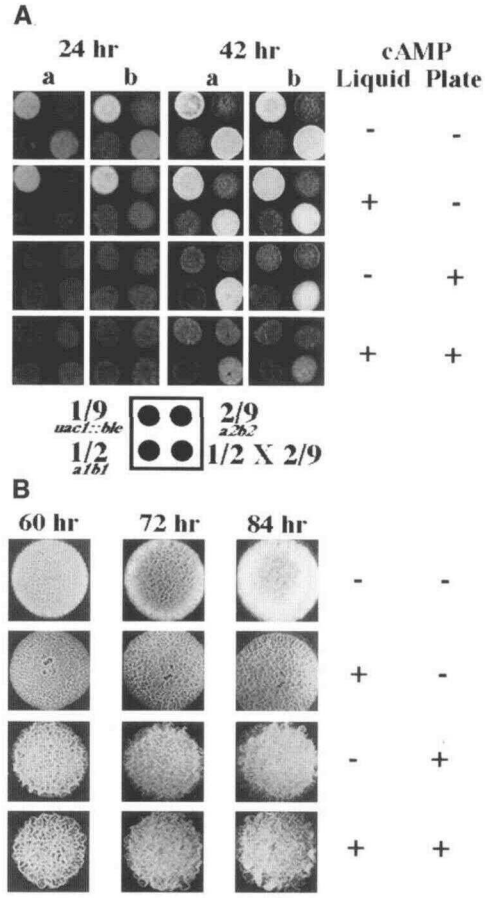

Figure 3. Effect of cAMP on the Production of Filamentous Growth on Plates.

Filamentous growth is visible as white patches. Cells were grown overnight at 30°C in liquid PDB, plated on PDA plates containing India ink, and incubated at 30°C. The (+) and (−) designations at right indicate the presence or absense, respectively, of cAMP in the liquid and/or solid medium. cAMP was added to liquid and solid media at 25 mM where indicated, except that strain 1/9 was always initially grown in liquid in the presence of cAMP to induce budding growth before plating.

(A) The inhibitory effect of cAMP on the mating reaction. The strains in each square are depicted in the diagram. Plates were photographed 24 and 42 hr after plating. Duplicate reactions are shown in a and b at each time point.

(B) A strong inhibitory effect of cAMP on filament formation in a diploid heterozygous for mating type. The diploid strain 2/21 was photographed at 60, 72, and 84 hr after plating. Filamentous growth is visible in the no cAMP treatment as a ring around the outer edge of the colony at 72 hr and covering the colony at 84 hr.

activity is required for a transition to gall formation. It is interesting to speculate that properly regulated PKA is necessary for the production of the signal or signals that *U. maydis* emits to trigger gall formation. Additional switches must be involved in subsequent processes, such as the production of teliospores. These results raise another question, which remains unanswered here, about whether gall formation is necessary for the production of teliospores or whether the defect in *ubc1* influences both processes independently.

The cAMP and Mating Pathways May Interact

Mating assays performed in the presence or absence of cAMP suggest a connection between regulation by the bE and bW heterodimer (homeodomain protein) encoded by the *b* mating–type locus and cAMP signal transduction pathways. Although cAMP did not overcome the ability of the fungus to produce the filamentous dikaryon, it did reduce the rate at which the filaments appeared. Data showing that filamentous growth of a diploid heterozygous at both the *a* and *b* loci is also inhibited by cAMP indicate that this phenomenon is not due simply to a delay in fusion. Therefore, it seems plausible that the bE–bW heterodimer regulates the transcription of genes whose products are the direct substrates of PKA phosphorylation or genes whose expression patterns are regulated by a PKA substrate(s). It is also possible that the *b* heterodimer indirectly leads to increased phosphodiesterase activity and simultaneously decreases *uac1* activity. This would account for the ability of the wild-type dikaryon to produce filaments on plates containing cAMP well before the *uac1* disruption strain, 1/9, which in this experiment was never able to overcome the presence of cAMP in the plates.

An additional finding consistent with a connection between mating and cAMP, reported by Kämper et al. (1995), is that in combination with the wild-type *b2* allele, a mutant *bE2* allele designated E2mut#88 caused symptom development similar to that of the *ubc1* mutants. The E2mut#88 allele, upon introduction into an *a1a2 b2b2* diploid, allowed an apparently very weak (not detectable in the yeast two-hybrid system assay) interaction with the *bW2* allele, leading to filamentous phenotypes on plates. The resultant filamentous transformant was capable of causing anthocyanin production, but no galls were observed. Thus, a weak *b* interaction leads to reduced virulence that is at least superficially similar to that of *ubc1* mutants that are hyperactive for PKA.

A hypothetical interaction between cAMP and *b* mating–type control of morphogenesis that is supported by the data presented in this study is described below. Budding appears to be conditioned by the activity of one or more PKA substrate phosphoproteins that either may act directly in bud formation or may induce the transcription of other genes that perform that function. The *b* heterodimer might counter the effect of cAMP at any of several points to yield filamentous cells. For example, *b* function as a regulator of

transcription could directly or indirectly decrease the transcription of stimulatory genes or, alternatively, increase the transcription of genes inhibitory to budding growth.

The most straightforward example of a possible mechanism for the promotion of filamentous growth by *b* might be the repression of the transcription of a gene encoding a direct substrate of PKA that is required for budding either directly or as a positive factor for the transcription of downstream genes required for the process. Alternatively, *b* might indirectly inhibit PKA activity needed to phosphorylate and thus activate the protein(s) required for budding. The *b* heterodimer could accomplish this by, for example, causing an increase in the abundance of phosphodiesterase and/or a reduction in adenylyl cyclase activity. It seems unlikely, however, that the *b* heterodimer directly impacts PKA activity because dikaryons heterozygous for *b* and mutant at *ubc1* have a dramatic phenotype.

It is conceivable that addition of cAMP to mating reactions interferes with the efficiency of cell fusion and thus dikaryon production. If this is the case, it is possible that mating filaments are reduced in abundance due to regulation of adenylyl cyclase activity via a heterotrimeric G protein in the pheromone response pathway. Recently, a mutant disrupted in one of the four *U. maydis* G protein α subunit genes, *gpa3*, has been described (Regenfelder et al., 1997). *gpa3* was shown to be involved in pheromone response, but the null mutant also was quite similar to a *uac1* mutant in phenotype. If a feature of the pheromone response pathway is to reduce *uac1* activity, it may be that reduced cAMP levels are important for the formation of fusion hyphae. In this context, we have employed cytoduction assays to demonstrate that exogenous cAMP does not reduce the frequency of fusion events (C. Laity and J.W. Kronstad, unpublished results; S.E. Gold, unpublished results). Thus, pheromones seem unlikely to induce mating hyphae simply by reducing intracellular cAMP content through inactivation of *uac1*.

It is clear that high exogenous cAMP interferes with the production of filaments in the diploid strain 2/21 that does not require fusion for the formation of aerial hyphae (Figure 3B). Qualitatively, the ability of the diploid strain to form aerial hyphae appeared to be more sensitive to cAMP than that of the mating mixture. These findings are consistent with an interaction between the cAMP pathway and the function of the b protein heterodimer after fusion. Further support for this idea comes from the construction of a diploid strain that is homozygous for a defect in *ubc1* and that is strongly attenuated for the formation of aerial hyphae and pathogenicity (F. Dürrenberger and J.W. Kronstad, manuscript in preparation).

cAMP in Other Phytopathogenic Fungi

A role for the cAMP signal transduction pathway in various processes of plant pathogenic fungi is now emerging (reviewed in Kronstad, 1997). Changes in cAMP levels have been associated with dimorphism in the Dutch elm disease pathogen *Ophiostoma ulmi* (Brunton and Gadd, 1989), in appressorium production and host penetration in the rice blast fungus *Magnaporthe grisea* (Lee and Dean, 1993; Beckerman and Ebbole, 1996), and in the induction of germination of *Fusarium solani* spores in response to flavonoid compounds typically found in root exudates in the rhizosphere (Ruan et al., 1995). Interestingly, the cAMP pathway also plays a role in the virally induced hypovirulence of *Cryphonectria parasitica* (Choi et al., 1995; Wang and Nuss, 1995), the causal agent of chestnut blight. Thus, the cAMP signal transduction pathway appears to consistently play an important role in fungal plant pathogenesis, albeit in somewhat disparate capacities.

METHODS

Fungal and Bacterial Strains and Culture Conditions

Strains of *Ustilago maydis* employed in this study are listed in Table 1. *U. maydis* strains were grown in liquid potato dextrose broth (PDB) or on solid potato dextrose agar (PDA). Where noted, 1% India ink was added to PDA (PDA-ink) for enhanced visualization of filament production. *Escherichia coli* was grown in or on Luria-Bertani medium with appropriate antibiotics. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersberg, MD) or XL1-Blue (Stratagene, La Jolla, CA) was used for all DNA manipulations.

DNA Manipulations

Polymerase chain reaction (PCR) amplification and cloning steps were performed by standard methods (Ausubel et al., 1987). The deletion in strain 2/17 (*ubc1-3*) was made by standard methods in which amino acids 40 to 460 of the *ubc1* gene were replaced with a *ble* cassette (kindly provided by F. Dürrenburger, University of British Columbia, Vancouver).

Additional Alleles of the *ubc1* Gene

As part of a project to identify transposable elements in *U. maydis*, we used the *ubc1* gene as a potential transposon trap. One hundred independent spontaneous *ubc* class mutants, half of which were defective in *ubc1* (determined by complementation), were assayed for alterations in *ubc1* PCR product size. The mutants were isolated by inoculating each PDB culture tube with a different filamentous colony of the filamentous *uac1* disruption mutant. Due to the rapid budding growth of strains possessing suppressor mutations, compared with the filamentous parent, it was possible to isolate a large number of *ubc* class mutants. That is, within a period of 2 weeks, ~75% of the culture tubes contained a turbid suspension typical of budding growth. A single mutant of increased (*ubc1-4*) and a single mutant of decreased (*ubc1-5*) fragment size in the *ubc1* gene were identified by a PCR-based screen for size alterations in these spontaneous mutants. The alteration in fragment size of these two mutants was an ~50-bp increase or decrease for *ubc1-4* and *ubc1-5*, respectively.

The PCR assay employed the following primer sets ONK002 (5′-GATCGAACAAGGCGCTCAGG-3′) and O-SG011 (5′-ACGCATCGCAGATAGGAGAAG-3′) amplifying the region of the *ubc1* gene from base 855 to 1800. We found no large insertions indicative of transposable elements.

Plant Inoculations

Golden Bantam (Hill Seed and Garden & Turfgrass supplies, Florence, KY) seedlings were grown in UGA soil mix (consisting of two parts top soil, one part sand, one part vermiculite, one part perlite, one part peat moss, one–one hundredth parts lime, one–two hundredth parts 10-10-10-fertilizer mix, one–two thousandth parts ammonium nitrate, and one–two thousandth parts triple phosphate) and injected into the culm just above the soil line with cell suspensions of mixtures of 10^6 cells per mL of each of the mating strains. Plants were inoculated 7 days after planting, except in the competitive inoculation experiments described below in which they were inoculated at the specified times. All of the *ubc1* mutants used in this study possess a multicellular phenotype; when quantifying inoculum with a hemocytometer, each cell in a cluster was counted. When *ubc1-1* mutants were inoculated at 10^7 cells per mL, identical results were obtained (data not shown).

Plants were maintained in a growth chamber (model E15; Conviron, Inman, SC) with 12-hr day/night cycles at a constant 30°C. Disease symptom data were collected at 7, 10, and 14 days postinoculation (dpi). Disease ratings were as described previously (Gold and Kronstad, 1994), except that a rating of 1 included discrete chlorosis as well as anthocyanin production. Briefly, these ratings are as follows: 0, no symptoms; 1, anthocyanin production and/or chlorosis; 2, small leaf galls; 3, small stem galls; 4, large stem galls; and 5, plant death. Experiments to analyze pathogenicity were performed a minimum of three times, with the exception of the competitive inoculation experiment, which was performed twice.

For competitive inoculation experiments, compatible *ubc1-1* mutants were inoculated into plants on day zero, and compatible wild-type strains were subsequently inoculated into these preinoculated plants on day 0, 3, 5, or 7 after the initial inoculation. At each of these times, uninoculated control plants of the same age as those previously inoculated with the *ubc1-1* mutants were inoculated with compatible wild-type strains.

Microscopy

Micrographs of symptomatic leaf discs were taken with a Wild M-5D stereomicroscope (Heerbrugg, Switzerland) at ×6 magnification. Fungal hyphae in the leaf tissue were stained by removing 5-mm leaf discs in symptomatic areas with a hole punch. Leaf discs were cleared by boiling in 70% ethanol for 20 min. After clearing, 1/10 volume of 10% aniline blue in lactophenol was added and mixed with the tissue. Samples were left at room temperature for 2 to 3 days and then destained for 5 to 15 min in boiling 70% ethanol and observed. Photographs of stained tissue were taken using Ektachrome 64T film (Kodak) and an Olympus BH-2 microscope (Tokyo, Japan) with differential interference optics. Photographic figures were compiled with the software application Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA) and printed with on a dye sublimation printer (model UP-D7000; Sony, Cypress, CA) with Power Macintosh (Apple Computer Inc., Cupertino, CA) text editing.

Plate Mating Assay

A plate mating assay was used to determine the effect of cAMP on the filamentous phenotype associated with the mating reaction. Strains were grown in PDB with or without 25 mM cAMP and then plated onto PDA with or without 25 mM cAMP. The only exception to this was that the *uac1* disruption strain 1/9 was always grown in liquid with added cAMP to promote the budding form. Compatible wild-type haploid strains 1/2 and 2/9 form yeastlike colonies; upon mixing, they produce the dikaryotic filaments typical of the successful mating reaction. The diploid strain 2/21 *a1a2 b1b2* was also grown in PDB with or without cAMP and plated on PDA-ink plates with or without cAMP.

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