The *CPKA* Gene of *Magnaporthe grisea* Is Essential for Appressorial Penetration

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The rice blast fungus *Magnaporthe grisea* uses appressoria to penetrate into plant cells. Appressorium formation occurs following conidial germination on hydrophobic surfaces and may involve a cyclic AMP (cAMP)-dependent signaling mechanism. Recently, gene replacement mutants of *CPKA*, a gene encoding a proposed catalytic subunit of cAMP-dependent protein kinase A, were shown to be defective in appressorium formation, cAMP responsiveness, and lesion formation (T. K. Mitchell and R. A. Dean, *Plant Cell*, 7:1869-1878, 1995). Here we report a detailed phenotypic characterization of three *cpkA* mutants. *cpkA* mutants are dramatically reduced in pathogenicity toward healthy plants. However, the reduced pathogenicity does not appear to be due to a loss of appressorium formation. *cpkA* mutants are delayed in appressorium formation but form appressoria at the same level as wild-type strains over a 24-h period. Appressoria formed by *cpkA* mutants are fully melanized but are smaller than wild type and are defective in penetrating plant cells. *cpkA* mutants can produce infectious hyphae and cause lesion formation when inoculated through wounds. Finally, *cpkA* mutants are still responsive to exogenous cAMP for appressorium formation. These findings indicate an additional role for cAMP signaling involved in appressorial penetration and suggest the presence of additional cAMP-dependent protein kinase(s) involved in surface sensing in *M. grisea*.

The filamentous ascomycete *Magnaporthe grisea* is pathogenic to economically important crops such as barley, wheat, rice, and millet. Rice blast, caused by this heterothallic haploid fungus, is one of the most widespread and severe fungal diseases on rice throughout the world (Ou 1985). *M. grisea* initiates the disease cycle by attaching conidia to the rice leaf surface with spore tip mucilage (Hamer et al. 1988). With the appropriate temperature and humidity, the attached conidium germinates rapidly and forms a specialized infection structure called an appressorium. In mature appressoria, cell walls are melanized, presumably to provide a differential permeability barrier for turgor generation (Bourett and Howard 1990). The turgor pressure in appressoria can be as high as 8 MPa (Howard et al. 1991) and is essential for the mechanical penetration of the rice leaf cuticle. Melanin-deficient mutants that

cannot generate this high turgor pressure are defective in penetrating plant cell surfaces, but they can cause lesions if inoculated through wound sites (Chumley and Valent 1990).

The rice blast fungus can efficiently form appressoria on hydrophobic surfaces such as Teflon membranes (Hamer et al. 1988). On most hydrophilic surfaces, germinating conidia usually produce long, branched, vegetative hyphae (Lee and Dean 1994) but can be induced to form appressoria with high concentrations of exogenous cyclic AMP (cAMP) (Lee and Dean 1993). Additional studies with various pharmacological agents support a role for cAMP signaling during surface recognition and appressorium formation in *M. grisea* (Lee and Dean 1993). Recently, it was reported that disruption of a gene encoding the catalytic subunit of cAMP-dependent protein kinase A (*CPKA*) inhibited appressorium formation and the responsiveness of germinating conidia to exogenous cAMP (Mitchell and Dean 1995). *cpkA* mutants were found to be nonpathogenic even when inoculated through wounds.

Recently, we identified a mitogen-activated protein (MAP) kinase signal transduction pathway that regulates appressorium formation in *M. grisea* (Xu and Hamer 1996). A gene disruption mutation in the MAP-kinase gene (*PMK1*) inhibits appressorium formation and results in a loss of pathogenicity. This phenotype appeared somewhat similar to that of the *cpkA* mutants (Mitchell and Dean 1995). To clarify the role of *CPKA* in appressorium formation we have analyzed pathogenicity-related phenotypes in strains containing three different *cpkA* mutant alleles. Our results suggest that *CPKA* is dispensable for appressorium formation and invasive hyphal growth in plants, but may be essential for appressorial penetration.

RESULTS

Protein kinase mutants of *M. grisea*.

To clarify the roles of MAP-kinase and cAMP-dependent protein kinase pathways in appressorium formation we obtained three mutant alleles of *CPKA* in different strain backgrounds. Strain I-27 is a progeny strain from a rice pathogen that contains the hygromycin phosphotransferase gene (*HPH*) in place of the *CPKA* gene (Mitchell and Dean 1995) and was generously provided by R. A. Dean at Clemson University. The mutation in I-27 was confirmed by Southern blot analysis (data not shown). Strain pTH4 was obtained through an insertional mutagenesis screen for nonpathogenic mutants, and it contains an *HPH* transforming plasmid inserted into the ter-

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minimal 3' region of the *CPKA* gene (J. A. Sweigard, unpublished results). pTH4 is derived by insertional mutagenesis of strain 4091-5-8, a weeping lovegrass and barley pathogen (Valent et al. 1986). Because the insertional mutation in pTH4 may not be null, we generated a third *cpkA* deletion/disruption allele in strain 4091-5-8 by replacing amino acid residue 333 to 517 (encoding protein kinase subdomains VI to XI) of the *CPKA* gene, with the *HPH* gene (see Materials and Methods). The pathogenicity-related phenotypes of this mutant, DF51, are almost identical to the phenotype of pTH4 (described below).

Appressorium formation in protein kinase mutants.

Figure 1 shows the kinetics of germination, germ tube hooking, and mature appressorium formation in wild-type (Guy11) and *cpkA* mutant strains. Spore germination was scored as the first visible sign of germ tube emergence. Germ tube hooking is the first visible indication that germ tubes have sensed an appropriate surface for appressorium formation (thigmotropism). We use the term "hooking" to describe the turning, slight swelling, and deformation of the germ tube tip (Bourett and Howard 1990). Appressorium formation was judged by the formation at the end of the germ tube of spherical, melanized structures delimited by a septum and containing a single nucleus (see Figure 2).

The *cpkA* mutants showed no defect in spore germination or germ tube hooking (Fig. 1). *cpkA* mutant strains I-27, pTH4, and DF51 were able to make appressoria after 24 h (Fig. 1). However, appressorium formation appeared to be delayed. In I-27, the initiation of appressorium formation was delayed at least 4 h compared with a wild-type strain; by 12 h, only about 40% of the I-27 germings had formed appressoria, compared with nearly 100% in the wild-type strain Guy11 (Fig. 1). In

pTH4, appressorium formation also initiated somewhat later, at least 2 h. In strain DF51, appressorium formation was slightly more delayed. After 24 h, over 90% of the germings of *cpkA* mutants I-27 and pTH4 were able to form appressoria (Fig. 1). A slightly lower percentage of appressoria was formed by the DF51 strain after 24 h. We conclude that appressorium formation is delayed but not blocked in *cpkA* mutants. This delay is not caused by any defect in germination or hooking, but may be caused in later stages of appressorium formation. Because germ tube hooking is a morphological landmark for thigmotropism, these data also indicate that *CPKA* is dispensable for surface recognition.

Appressoria produced by *cpkA* mutants.

Appressorium formation in *cpkA* mutants is delayed. Figure 2A shows that appressoria formed by I-27 and pTH4 are fully melanized, as judged by bright field microscopy. Under these conditions, unmelanized appressoria, such as those produced by *Buf*⁻ mutants, appear translucent and bright (Howard and Ferrari 1990). In addition, appressoria produced by *cpkA* mutants have a nearly normal, dome-shaped structure. Calcofluor and Hoechst 33258 staining demonstrate that nuclei migrate into the appressorium and that a septum is formed at the junction of the germ tube and the appressorium (Fig. 2B). Thus, examinations with light and fluorescent microscopy fail to reveal gross abnormalities in the appressoria formed by *cpkA* mutants.

When examining large numbers of samples we noticed that appressoria formed by *cpkA* mutants appeared to be somewhat smaller in size than those formed by relative wild-type strains. To avoid the influence of delayed appressorium formation in mutants, we measured the diameter of *cpkA* and wild-type appressoria that had formed on plastic coverslips after 48 h. We measured over 200 appressoria per strain and sorted the measurements into size groupings between 3 and 11 μ m. Figure 3 shows that all *cpkA* mutants form appressoria that are consistently smaller in size than the wild-type strains Guy11 and 4091-5-8.

cpkA mutants respond to cAMP.

Exogenous cAMP can induce appressorium formation on non-inductive hydrophilic surfaces (Lee and Dean 1993). According to Mitchell and Dean (1995), appressorium formation cannot be induced by cAMP in *cpkA* mutants. After realizing that appressorium formation is delayed in these mutants and that the previous observations were made after 9 h of incubation (Mitchell and Dean 1995), we were interested in finding out whether *cpkA* mutants could still respond to exogenous cAMP following a longer period of incubation. Table 1 shows that all *cpkA* mutants were induced to form appressoria on hydrophilic surfaces after 24 h of incubation by the addition of cAMP. These results suggest that a cAMP-responsive signaling pathway for thigmotropic sensing and appressorium formation is still functional in *cpkA* mutants.

cpkA mutants are still pathogenic on wounded plants.

Similar to the previous report (Mitchell and Dean 1995), we did not observe any lesion on susceptible rice plants sprayed with the regular concentration (Valent et al. 1991) of I-27 conidia collected from 2-week-old oatmeal cultures (data not shown). This finding suggests that, like melanin-deficient

Fig. 1. Appressorium formation in protein kinase mutants of *Magnaporthe grisea*. Germination (■), hooking (◆), and appressorium formation (●) of *M. grisea* wild-type strain Guy11, and *cpkA* mutants I-27, DF51, and pTH4 on plastic coverslips. Each data point was determined as an average value from six samples evaluating 100 conidia per sample. Standard deviations are indicated. All *cpkA* mutants form appressoria.

mutants of *M. grisea*, *cpkA* mutants may form nonfunctional appressoria. Therefore, we undertook a careful examination of the ability of *cpkA* mutant I-27 to infect through wound sites. Wounding experiments were carried out by abrading rice plant leaves and inoculating the plants by either placing spore suspensions directly on the abraded sites or by spray inoculating plants with abraded leaves. We also inoculated by directly injecting spore suspensions into an area a few centimeters below the rice leaf whorl. Lesions were tested for their ability to produce mycelia and conidiophores when incubated on water agar plates. Control experiments with *Buf⁻* mutants were carried out to make sure rice leaves were appropriately wounded and inoculated. Consistent with previously published studies (Chumley and Valent 1990), *Buf⁻* mutants were able to form lesions on wounded plants and could produce conidiophores from the infection sites under high humidity (data not shown).

I-27 caused lesions after being inoculated onto abraded CO39 leaves to the same extent as *Buf⁻* strains. Lesions formed on abraded leaves in places where spore suspensions were placed (Fig. 4A) and on abraded leaves on plants that were spray inoculated (Fig. 4B). With injection assay, we also found that I-27 caused extended necrosis at the wound sites (Fig. 4C), but no lesions outside the wound sites. When wound sites inoculated with *cpkA* mutant I-27 were excised and incubated under high humidity on 2% water agar, mycelial growth and conidiation were observed (Fig. 4D). All the strains isolated from these wound-inoculation sites were hygromycin resistant and all 10 isolates analyzed by Southern blot analysis retained the *cpkA* gene disruption (data not shown). In infection assays with pTH4 and DF51 on weeping lovegrass and barley, rare lesions were observed. Figure 5 shows the typical rare lesions formed by pTH4 on barley

Fig. 2. Appressoria formed by wild-type strain Guy11 and *cpkA* mutants I-27 and pTH4. (A) Appressoria formed by Guy11, I-27, and pTH4 were dome shaped and melanized. Appressoria from *cpkA* mutants I-27 and pTH4 are smaller in size. Photos were taken under bright field microscopy after 48 h of incubation on plastic coverslips (bar represents 10 μm). (B) Epifluorescence microscopy after staining for cell walls with Calcofluor and nuclei with Hoechst 33258 (bar represents 10 μm). Appressoria of Guy11, I-27, and pTH4 contain a single nucleus.

leaves. We also observed rare lesions on rice leaves sprayed with I-27 conidia at higher concentrations of 5×10^6 conidia/ml prepared from younger (6-day-old) oatmeal cultures (Fig. 5). Thus, we conclude that *cpkA* mutants are dramatically reduced in penetrating intact host leaves, but are able to form rare lesions. *cpkA* mutants are pathogenic on wounded plants and are still able to grow invasively in plant cells. When inoculated through wounds, *cpkA* mutants can complete the disease cycle by producing conidiophores and conidia from infected plant areas. Although it remains possible that *cpkA* mutants may be impaired in invasive growth to a certain degree, all three *cpkA* mutants can form lesions either on wounded or healthy host plants. Thus, *CPKA* is not essential for infectious growth or fungal pathogenicity after penetration.

Fig. 3. Appressoria size variation in *cpkA* mutants. Appressorium size was measured with a $\times 100$ objective after 48 h. More than 200 appressoria were measured for each strain. Appressoria were categorized by their diameter: 4 (3.6 to 4.5 μm); 5 (5.6 to 6.5 μm) etc. Heights of bars represent the percentage of appressoria in a certain size category. (A) Comparison of appressorium size between rice pathogens Guy11 and I-27. (B) Comparison between weeping lovegrass pathogens (4081-5-8, pTH4, and DF51).

Appressoria formed by *cpkA* mutants are impaired in penetration.

cpkA mutants may be defective in appressorium-dependent penetration or in early stages of infectious hyphae formation. We used an onion epidermal cell penetration assay (Chida and Sisler 1987) to distinguish between these defects. This assay has been used in *M. grisea* to demonstrate that melanin-deficient mutants are defective in penetration and to demonstrate the mode of action of tricyclazole fungicides that block penetration (Chida and Sisler 1987). As expected, most of the appressoria formed by wild-type strains could penetrate onion epidermal cells (Table 2) and form intracellular, bulbous, infectious hyphae (Fig. 6A). In contrast, appressoria formed by strain I-27 were smaller in size (Fig. 6B) and penetrated onion cells only in rare instances (Table 2). Out of 363 appressoria examined in strain I-27, we only observed five that formed infectious hyphae. However, we observed 11 instances in

Fig. 4. Invasive growth of *cpkA* mutant I-27 in rice plants. (A) Lesions formed in places where conidial drops were applied on leaves abraded with an emery board. (B) Lesions formed on abraded leaves spray-inoculated with I-27 conidia. (C) Necrosis around injection sites. (D) Mycelial growth and conidiation from these wound sites after incubation under high humidity.

which intracellular, invasive hyphae were formed without appressoria (Fig. 6C). In all of these cases we found that the fungus had penetrated through the cell junctions (Fig. 6C), most likely at wound sites generated during preparation of the onion epidermal strips. Figure 6C shows intracellular, infectious hyphae formed by strain I-27, and Figure 6D shows plant cell wall autofluorescence due to fungal penetration (Heath et al. 1990). Similar to those formed by strain I-27, appressoria formed by *cpkA* mutants pTH4 and DF51 rarely penetrated onion epidermal cells (Table 2). These findings suggest that *CPKA* is required for appressorium-dependent penetration and further demonstrate that *cpkA* mutants are capable of forming infectious hyphae.

DISCUSSION

Many plant-pathogenic fungi form appressoria to invade their hosts. Appressorium formation is a complicated process that involves surface attachment and recognition, spore germination, germ tube tip growth arrest, cytoskeletal reorganization (Kwon et al. 1991), melanin biosynthesis and localization (Howard and Ferrari 1990), turgor generation (Howard et al. 1991) and appressorium-specific gene expression (Talbot et al. 1993). Studies of melanin-deficient mutants of *M. grisea* and other appressorium-producing fungi show that appressorium morphogenesis and appressorium function are distinct processes and are separable by genetic analysis (Chumley and Valent 1990). Melanin-deficient mutants of *M. grisea* form appressoria that fail to penetrate, demonstrating that a melanized cell wall and turgor pressure generation are essential for penetration of plant cells but are dispensable for appressorium morphogenesis. Results presented here show that *CPKA* is also dispensable for appressorium morphogenesis but is essential for appressorium function.

Table 1. cAMP responses in *cpkA* mutants

Strain	No cAMP	10 mM cAMP
Guy11	3.1 ± 0.7% ^a	50.1 ± 11.7%
I-27	2.9 ± 1.8%	43.6 ± 8.0%
pTH4	8.0 ± 1.2%	48.6 ± 14.7%
DF51	5.0 ± 2.2%	50.9 ± 8.0%
4091-5-8	6.3 ± 4.0%	55.3 ± 12.8%

^a Percentage of germ tubes that formed appressoria on a hydrophilic glass surface in the presence or absence of cAMP after 24 h of incubation. The means and standard deviations were calculated from at least three repeats (n ≥ 100).

Table 2. Penetration assay on onion epidermis

Strain	Appressoria		Total examined
	With infectious hyphae	Without infectious hyphae	
Guy-11	254 (69.4 %) ^a	112 (30.6 %)	366
I-27	5 (1.4 %)	358 (98.6 %)	363
4091-5-8	490 (97.2%)	14 (2.8 %)	504
pTH4	15 (1.9 %)	769 (98.1 %)	784
DF51	12 (3.0 %)	387 (97.0 %)	399

^a Appressoria or infectious hyphae were counted with bright field microscopy after 48 h of incubation on onion epidermis. Percentage is given in parentheses.

cpkA mutants form appressoria.

In all *cpkA* mutants—I-27, DF51 and pTH4—similar to the result with wild-type strains, the vast majority of germ tubes make fully melanized appressoria after 24 h. Compared with the wild-type strain Guy11, appressorium formation is delayed in *cpkA* mutants. The delay in appressorium formation does not appear to be due to strain background differences and most likely accounts for the difference between our findings

Fig. 5. Rare lesions formed by *cpkA* mutants. Upper panels: Abundant lesions formed on barley leaves 5 days after infection with wild-type strain 4091-5-8 (left). Only rare lesions were formed by the *cpkA* insertion mutant pTH4 (right). Lower panels: Seedlings from rice cultivar CO39 were spray inoculated with conidia from either wild-type Guy11 (left) or *cpkA* mutant I-27 (right). Lesions are shown following 1 week of symptom development.

and those of Mitchell and Dean (1995). In their study, appressorium formation was measured after 8 or 9 h and rare appressoria were observed after incubations in vitro for 12 h. Similarly, cAMP responsiveness assays on hydrophilic surfaces were also conducted for short incubation periods. Other factors, such as different surfaces for assaying appressoria formation and different concentrations of conidia used in the assays, could explain more subtle variations in the data sets. We argue that longer incubation periods for appressorium formation in vitro are physiologically relevant for several reasons. First, measurements of turgor pressures in appressoria formed in vitro show that turgor continues to increase over a 46-h incubation period (Howard et al. 1991). Second, detailed studies of penetration on rice and weeping lovegrass leaves show that penetration by appressoria is highly asynchronous (Heath et al. 1990) and, thus, short incubation periods (<12 h) in vitro may not detect processes that occur over longer periods on plant surfaces.

***cpkA* mutants are defective in penetration but are capable of invasive growth.**

Several lines of evidence suggest that *cpkA* mutants are defective in penetration but may be capable of invasive growth. First, although *cpkA* mutants cannot infect intact host leaves

efficiently, we observed rare lesions in spray inoculation assays on relative host plants with all three *cpkA* mutants (Fig. 5). Second, I-27 forms appressoria and can infect through wounds. Third, all *cpkA* mutants—I-27, pTH4 and DF51—could form infectious hyphae in onion epidermal cell assays. Recent analysis of the mutant allele in pTH4 shows that the insertion in the *CPKA* gene occurred in the third intron, very close to the 3' end of the *CPKA* gene, potentially deleting only the terminal 8 amino acids of the protein. Thus, this mutation may be leaky and some appressoria formed by pTH4 may be able to penetrate intact leaves and form lesions. This explanation seems unlikely, because DF51, carrying a deletion/disruption allele of the *CPKA* gene, also produces rare lesions on weeping lovegrass and barley (data not shown).

The most pronounced defect in *cpkA* mutants is in appressorial penetration. Currently, the role of *CPKA* in penetration is unknown. However, *cpkA* mutants were able to migrate a nucleus into the appressorium, form melanized appressoria, and form a septum at the base of the appressorium. In addition, we found that appressoria formed by *cpkA* mutants were tightly attached to the surface and could not be removed by shear forces (data not shown). Although detailed electron microscopy of the septum and the appressorial cell wall are still

Fig. 6. Penetration assays on onion epidermal strips. After 48 h, appressoria formed by wild-type strain Guy11 penetrated the onion epidermal cells and produced infectious hyphae (A). In contrast, the majority of appressoria formed by I-27 were smaller in size and did not penetrate (B). Infectious hyphae formed after rare opportunistic hyphal invasion of I-27 without appressoria (C) and subsequent plant autofluorescence (D). (Ap: Appressorium; G: germ tube; In: Infectious hyphae; S: Spore)

needed, these findings suggest that there are no major defects in appressorium morphogenesis in *cpkA* mutants.

The failure of *cpkA* mutant appressoria to penetrate plant cells may be related to an inability to generate sufficient turgor pressure. Preliminary findings suggest that high concentrations of a glycol may generate the hydrostatic pressure in *M. grisea* appressoria (Howard 1994). Since protein kinase A is known to play a role in carbohydrate mobilization in fungi and animal cells, *cpkA* mutants may be incapable of generating high levels of this glycol for turgor generation from stored glycogen (Howard 1994). The finding that *cpkA* mutant appressoria have variable sizes and can on rare occasions penetrate onion epidermal cells may be a manifestation of low to variable levels of cell turgor. It is worth noting that CpkA is highly homologous to the *Ustilago maydis* cAMP-dependent protein kinase Adr1. *ADR1* was first isolated as a gene conferring resistance to the dicarboximide fungicides (Orth et al. 1995). Interestingly, dicarboximide resistance has been related to abnormal osmotic sensitivity in several ascomycetes (Beever 1983). In *Neurospora crassa*, a dicarboximide resistant mutant produced 33% less glycerol and 40% less mannitol than a wild-type strain in response to 0.4 M NaCl (Ellis and Grindle 1991). We speculate that the CpkA protein kinase may be involved in accumulation of high levels of glycerol in appressoria.

We attempted to measure the turgor pressure in *cpkA* mutants and wild-type strains of *M. grisea* with incipient cytorrhysis methods (Howard et al. 1991). In one series of experiments, 50% of the wild-type appressoria collapsed at polyethylene glycol concentrations of 7 g per 10 ml, while the concentration for 50% appressorium collapse in I-27 was 5.5 g per 10 ml. However, we could not obtain a statistically reproducible result between experimental trials. In contrast, we could easily demonstrate that turgor pressure was reduced in Buf⁻ mutants (50% collapse at 3 g per 10 ml of polyethylene glycol). Thus, if there are reductions in turgor pressure in *cpkA* mutants, they may be small. Experiments by Howard et al. (1991) indicated that a slight reduction in turgor pressure was sufficient to alter the ability of *M. grisea* appressoria to penetrate artificial surfaces.

Additional protein kinase(s) may exist in *M. grisea*.

Earlier experiments implicated the existence of a cAMP-dependent signaling in surface recognition and appressorium formation in *M. grisea* (Lee and Dean 1993). Our finding that *cpkA* mutants are still responsive to exogenous cAMP strongly suggests that additional cAMP-dependent protein kinases may exist in *M. grisea*. Multiple cAMP-dependent protein kinases exist in other fungi such as *Saccharomyces cerevisiae* (Toda et al. 1987) and *U. maydis* (James Kronstad, personal communication). The level of nucleotide divergence between *CPKA* and additional protein kinase(s) in the *M. grisea* genome may be too high to allow detection by Southern blot analysis. The identification of additional protein kinase(s) by Mitchell and Dean (1995) may have been hampered by the use of genomic DNA as a polymerase chain reaction template. We notice that the reverse primers used by Mitchell and Dean (1995) fortuitously hybridized to a region in the second intron of *cpkA*. Additional protein kinases may be involved in thigmotropic sensing, and attempts to identify these genes are underway.

In conclusion, our findings suggest that at least two cAMP-dependent signaling pathways are required for plant infection

in the rice blast fungus. One pathway is involved in appressorial penetration and the second in surface sensing. These pathways most likely involve different protein kinase(s), one of which may be involved in relaying the signal generated by surface recognition to the downstream *PMK1* MAP kinase signal transduction pathway that regulates appressorium formation (Xu and Hamer 1996).

MATERIALS AND METHODS

Strains and media.

Wild-type Guy11 (Nottingham and Silué 1992) and mutant strains were cultured at 25°C on oatmeal plates under fluorescent light (Romao and Hamer 1992). Mutant pTH4, which has the *HPH* gene inserted in the *HindIII* site of intron 3 of *cpkA* gene, was isolated as a reduced pathogenicity mutant in an insertional mutagenesis screen (J. A. Sweigard, manuscript in preparation). Mutant DF51 was generated by replacing the *EagI-EcoRV* fragment of the *CPKA* gene with the *HPH* gene in 4091-5-8 (Valent et al. 1986).

Assays for appressorium formation.

Conidia were prepared and assayed for appressoria formation as previously described (Hamer et al. 1988) with the following modifications. Conidia were harvested from 7-day-old cultures, resuspended in water, filtered once through Miracloth, and diluted to 2×10^4 conidia/ml. Droplets (90 μ l) of conidial suspension were placed on plastic microscope cover slips (Fisher Scientific Co., Pittsburgh, PA) and incubated in a moist chamber. Germinating conidia and appressoria were stained with 10 μ g of Calcofluor and 100 ng of Hoechst 33258 per ml as described (Harris et al. 1994) and examined by epifluorescence microscopy. Photographs were taken with Kodak Technical-Pan film. To assay for cAMP responsiveness, conidia were resuspended in 10 mM cAMP and tested for cAMP responsiveness as described (Lee and Dean 1993) but with longer incubation periods.

Plant infection assays.

Conidia were collected from 10- to 14-day-old oatmeal agar cultures and resuspended to 5×10^5 /ml in 0.25% gelatin solution. Eight-day-old barley seedlings (cv. Bonanza), and 2-week-old seedlings of the rice cultivar CO39 and weeping lovegrass (*Eragrostis curvular*) were used in the infection assays. Plant incubation, spray infection, and lesion examination were as described (Valent et al. 1991). For abrasion infection assays, rice leaves were abraded with an emery board (Chumley and Valent 1990), then inoculated by either spraying or applying small drops of spore suspensions onto abraded leaves. For injection inoculation assays, conidial suspensions were injected into leaf sheaths of rice seedlings with a 26G_{1/2} needle on a 1-ml syringe. Lesion formation and necrosis around the inoculation sites were examined when the injection-wounded leaves unfolded 4 to 7 days after injection. Wound inoculation sites were excised, surface sterilized with 70% ethanol for 1 min, and incubated on 2% water agar with fluorescent light and high humidity to assess sporulation.

Penetration assays on onion epidermal strips.

Appressorial penetration was studied as previously described (Chida and Sisler 1987) with the following modifica-

tions. The second outer layers of yellow onions were prepared and rinsed with water. The onion bulb scale was not washed with chloroform. The inner epidermis was peeled off, cut into 1-cm² strips and floated on distilled water. About 400 conidia in 20 µl were placed on the strips. Appressorium formation and infectious hyphae growth were examined after 48 h of incubation at room temperature.

NOTE ADDED IN PROOF

During the review of our manuscript it occurred to us to repeat our appressorial assays on the *cpkA* mutants, using conditions more closely resembling the conditions reported by Mitchell and Dean (1995). We repeated the appressorium formation assay and used, as a hydrophilic surface, the hydrophilic side of Gelbond membranes (FMC, Rockland, ME). All three *cpkA* mutants were stimulated to the same extent as wild type, with either 10 mM cAMP or 2.5 mM IBMX (3-iso-butyl-1-methyl-xanthine).

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