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The cAMP-Dependent Protein Kinase Catalytic Subunit Is Required for Appressorium Formation and Pathogenesis by the Rice Blast Pathogen *Magnaporthe grisea*

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Magnaporthe grisea*, the causal agent of rice blast disease, differentiates a specialized infection cell, an appressorium, that is required for infection of its host. Previously, cAMP was implicated in the endogenous signaling pathway leading to appressorium formation. To obtain direct evidence for the role of cAMP in appressorium formation, the gene encoding the catalytic subunit of the cAMP-dependent protein kinase (*cpkA*) was cloned, sequenced, and disrupted. Polymerase chain reaction primers designed after highly conserved regions in the same gene from other organisms were used to amplify genomic DNA fragments. The cloned amplification products were used to identify genomic clones. DNA blot analysis indicated that *cpkA* is present as a single copy in the genome. *cpkA* consists of 1894 bp, including three short introns sufficient to encode a protein of 539 amino acids with a predicted molecular mass of 60.7 kD. The deduced peptide shares >45% identity with other catalytic subunits and contains all functional motifs and residues with the addition of a glutamine-rich region at the N terminus. Two transformants, L5 and T-182, in which *cpkA* had been replaced with a hygromycin resistance gene cassette, were unable to produce appressoria, could not be induced to form appressoria by cAMP, and were nonpathogenic on susceptible rice, even when leaves were abraded. These results were confirmed by analysis of 57 progeny from a cross between transformant L5 and the wild-type laboratory strain 70-6. Other aspects of growth and development, including vegetative growth as well as asexual and sexual competence, were unaffected when measured in vitro. These results provide direct evidence that the cAMP-dependent protein kinase is necessary for infection-related morphogenesis and pathogenesis in a phytopathogenic fungus.

INTRODUCTION

Magnaporthe grisea infects a wide range of grasses, including barley and millet, but it is best known as the causal agent of rice blast disease, one of the most serious diseases of rice (Ou, 1985). Frequent, regional disease outbreaks have resulted in severe hardship for many millions of people in Asia, Africa, and parts of the Americas (Zeigler et al., 1994). The pathogen is highly polymorphic, and control through host resistance is not stable. Identification of cellular processes essential for infection may serve as a focal point for the development of durable intervention strategies. Infection is dependent on the elaboration of an appressorium, a specialized attachment cell that develops in response to specific environmental cues. Appressorium formation is initiated when the tip of the germ tube emerging from a conidium ceases apical extension and swells into a dome-shaped melanized structure, which becomes firmly attached to the plant surface (Hamer et al., 1988). Hydrostatic pressure increases inside this cell and provides the force necessary to drive a penetration hypha through the plant cuticle into the underlying tissues (Howard et al., 1991). Artificial surfaces with properties similar to the rice leaf surface are also conducive to appressorium formation (Lee and Dean, 1994). In contrast, surfaces with different properties are not conducive to appressorium formation, and the germ tube continues to extend and develop into the vegetative mycelium.

How the emerging germ tube recognizes an appropriate surface and relays the information to effect the developmental response is poorly understood. However, information on post-recognition cellular responses is beginning to emerge. The addition of cAMP to *M. grisea* conidia germinated in environments not usually conducive to appressorium formation, such as a hydrophilic surface, stimulates germ tube tips to differentiate into appressoria (Lee and Dean, 1993). The same effect is observed when 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cellular breakdown of cAMP, is added to conidia germinating on hydrophilic surfaces, suggesting that cAMP is necessary for appressorium development.

To establish a direct role of the cyclic nucleotide in infection-related morphogenesis in *M. grisea*, we isolated, sequenced, and disrupted the gene *cpkA*, encoding the protein kinase A catalytic subunit. Most cellular events associated with cAMP

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are the result of binding to and activation of protein kinase A (Gerisch, 1987; Taylor et al., 1993). In *M. grisea*, *cpkA* exists as a single copy in the genome, and targeted gene disruption results in loss of the ability to produce normal appressoria, even in the presence of cAMP and IBMX, and a complete loss of pathogenicity.

RESULTS

Isolation of *cpkA*

The *cpkA* gene was isolated using a polymerase chain reaction (PCR)-based strategy. Amplification products were obtained after using nested degenerate oligonucleotide primers designed on conserved sequences of the *cpkA* gene in other organisms (Hanks and Quinn, 1991). The 189-bp product of primers RDPK1 and RDPK4 was gel isolated and cloned into pBluescript KS+. One hundred putative *Escherichia coli* transformants were transferred to a master plate from which groups of 10 clones were pooled and subjected to PCR using nested primers RDPK2 and RDPK3. The predicted 60-bp fragment was observed in three pools (data not shown). Individuals of these pools were subjected to PCR; one clone from each pool exhibiting the 60-bp band was designated pRD1, pRD14, and pRD36, respectively. The DNA sequence of these clones was identical, and the deduced peptide sequence matched 52 of 63 amino acids from the corresponding region of the protein kinase A catalytic subunits from *Saccharomyces cerevisiae* (Toda et al., 1987).

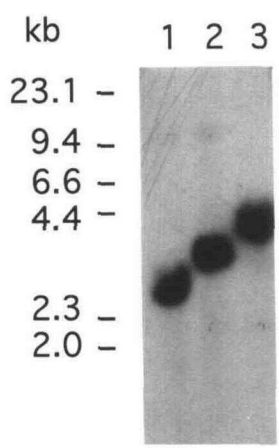

Figure 1. Genomic DNA Gel Blot Analysis of the *M. grisea cpkA* Gene.

Genomic DNA was digested with PstI (lane 1), KpnI (lane 2), and BamHI (lane 3). The blot was probed with pRD36 under low-stringency conditions. The presence of a single hybridizing band indicates that *cpkA* exists as a single copy in the genome.

pRD36 was used to probe a blot of *M. grisea* total DNA digested with PstI, KpnI, and BamHI. The probe hybridized to a single band under conditions of moderate stringency, indicating that the cloned DNA was derived from *M. grisea* and that the DNA exists as a single copy in the genome (Figure 1). This result was confirmed by extensive DNA gel blot analysis using either pRD36 or pTKM002 (a 3.1-kb HindIII fragment encompassing the 189-bp fragment) as hybridization probes.

Nucleotide Sequence and Features of *cpkA*

The nucleotide sequence was determined using a series of subclones from genomic clones pTKM001 and pTKM002 and several specific primers to bridge restriction sites. Figure 2 shows the nucleotide and deduced amino acid sequence of the encoded protein. An open reading frame of 1894 bp (including introns) was found. Excision of the three introns at nucleotide positions 963 to 1044, 1299 to 1410, and 1786 to 1866 allows a potential protein of 539 amino acids with a predicted molecular mass of 60.7 kD to be encoded. The three introns of *cpkA* all have the expected splicing elements found in fungal introns: a 5′ sequence of GTPrAGPy and a 3′ sequence of TAG (Py, pyrimidine; Pr, purine; Gurr et al., 1987). Introns 2 and 3 have the internal splicing sequence GCTAACPr necessary for lariat formation. Within this open reading frame, the catalytic core was identified between amino acids 228 and 483. This region shares 208 of 256 residues (81%) with the analogous proteins in *S. cerevisiae* (TPK1, TPK2, and TPK3) (Toda et al., 1987). However, a notable difference was the addition of a single amino acid between amino acid residues 427 and 430; the exact amino acid was not identifiable.

The deduced amino acid sequence of the kinase domain of CPKA was aligned with other protein kinases using the principle of maximum parsimony according to the PAUP software package developed by Swofford (1993). Figure 3 shows the single phylogenetic tree obtained. Branch lengths indicate the number of "weighted" characters that have altered since diverging from the preceding node; the smaller the number, the more closely related they are. CPKA clusters within the cAMP-dependent protein kinase family and is most closely related to TPK2 of *S. cerevisiae*.

The N-terminal region of CPKA contains a large number of glutamine residues. Similar but less pronounced regions of glutamines are found in TPK2. The glutamines in CPKA were not found in consecutive blocks but as strings alternating with proline or serine residues. No obvious alignments were apparent in protein data bases. This region is relatively hydrophilic but is devoid of other notable features.

CPKA contains motifs and residues commonly found in other catalytic subunits (Taylor et al., 1993). These include the ATP binding motif starting at position 236 Gly-Thr-Gly-Ser-Phe-Gly-Arg-Val-(16 residues)-Lys; a catalytic loop motif at position 350 Arg-Asp-Leu-Lys-Pro-Glu-Asn; a magnesium ion–cheating loop at position 368 Asp-Phe-Gly-Phe; and residues Glu-263, Asp-405, and Arg-466, which are important in proper protein folding.

Figure 2. Nucleic Acid and Derived Amino Acid Sequence of *cpkA*.

The conserved catalytic core is underlined, and introns are presented in lowercase letters. The GenBank accession number is U12335.

Two autophosphorylation sites, Thr-382 and Ser-474, are also conserved. No significant differences or missing conserved motifs have been identified in the catalytic core of CPKA other than the additional residue already noted.

Disruption of *cpkA*

To determine the function of *cpkA*, a gene replacement strategy was employed. A disruption plasmid, pTKM022, as shown in Figure 4A, containing the hygromycin resistance gene cassette flanked by 5′ and 3′ sequences from the *cpkA* gene, was constructed. Two (L5 and T-182) of the 62 hygromycin-resistant transformants examined were unable to form appressoria on artificial surfaces and onion scale segments. When analyzed on DNA gel blots, these were found to have undergone homologous recombination replacing the *cpkA* gene with the hygromycin resistance gene. Figure 4B shows the result of digesting genomic DNA from transformants L5 and T-182 with HindIII and probing with the pTKM002 HindIII insert (probe

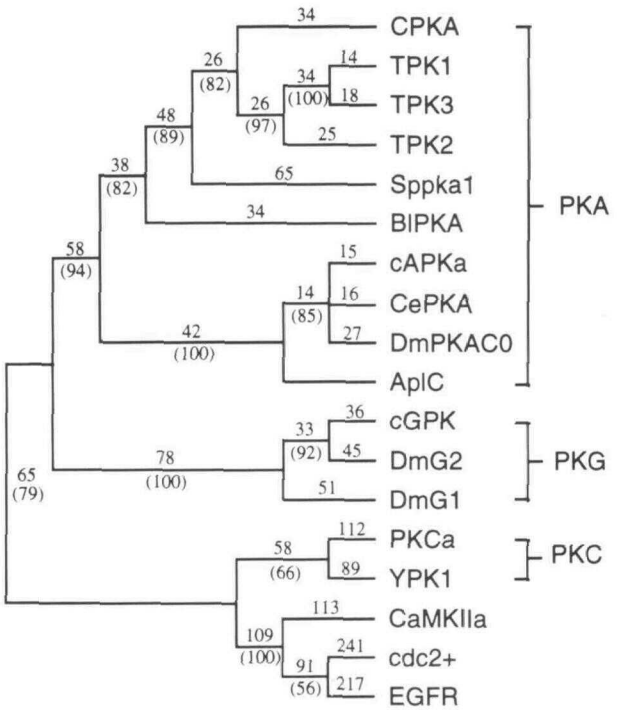

Figure 3. Phylogenetic Relationship of the *M. grisea cpkA* Gene Product to Other Eukaryotic Protein Kinases.

The amino acid sequence of the kinase domain from CPKA and representative members of each of the four main groups of protein kinases were analyzed by the Wagner parsimony method weighted according to Felsenstein's amino acid substitution rules. The analyzed sequences are TPK1 (GenBank (GB) accession number M17072), TPK2 (GB M17073), and TPK3 (GB M17074) from *Saccharomyces cerevisiae*; Sppka1 (GB D23667) from *Schizosaccharomyces pombe*; BIPKA (GB L17008) from *Blastocladiella emersonii*; cAPKa (GB X07767) from human; CePKA (GB U15983) from *Caenorhabditis elegans*; DmPKAC0 (GB X16969) from *Drosophila melanogaster*; AplC (GB X63420) from *Aplysia californica*; cGPK (GB Y07512) from human; DmG1 (GB M27114) and DmG2 (GB M30147) from *D. melanogaster*; PKCa (GB X52479) from human; YPK1 (GB M21307) from *S. cerevisiae*; CaMKIIa (GB L07043) from human; cdc2$^+$ (GB M129120) from *S. pombe*; and EGFR (GB Z27409) from human. The indicated cAMP (PKA)-, cGMP (PKG)-, and Ca^{2+} (PKC)-dependent kinase families are members of the AGC kinase group. CaMLIIa is a member of the CaMK group that includes the calcium/calmodulin family. cdc2$^+$ is a member of the CMGC group that includes the cyclin-dependent and MAP kinase families. EGFR is a member of the conventional protein–tyrosine kinase (PTK) group. The numbers above the horizontal branches indicate the numbers of weighted characters that are altered from the preceding node. The bootstrap confidence values that corroborate topographical elements are given below the branches.

1 shown in Figure 4A). These two transformants exhibited a hybridization band at 4.7 kb, consistent with the length expected from a gene replacement event. Other transformants exhibited bands at 4.7 and 3.1 kb (length of *cpkA*), suggesting a single crossover integration event or integration at an ectopic site (data

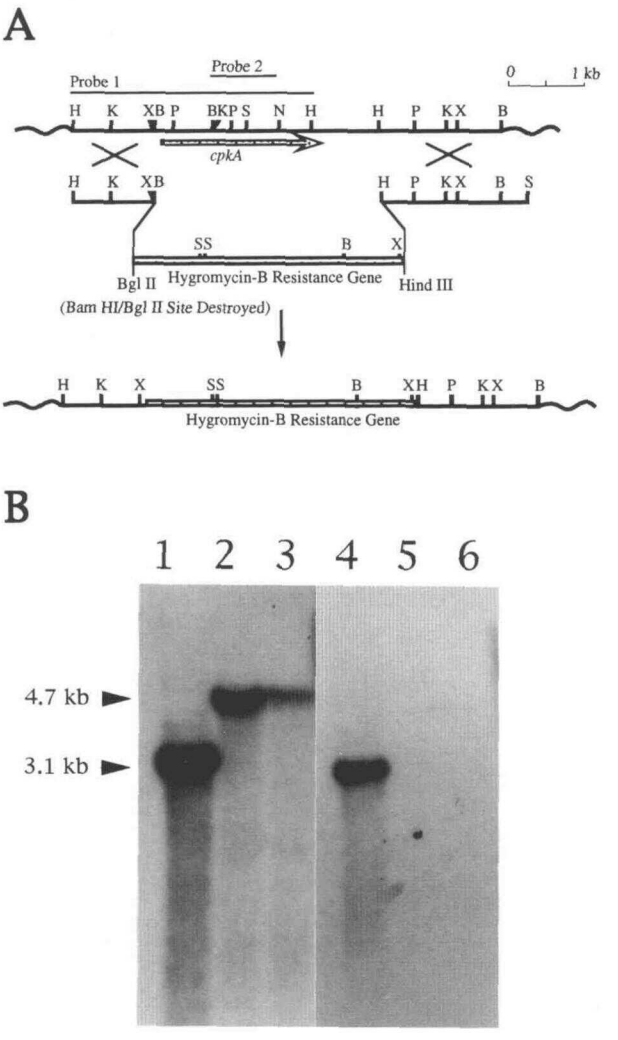

Figure 4. Targeted Disruption of *cpkA*.

(A) *pTKM022 contains the hygromycin resistance gene cassette flanked with border sequences from cpkA. The right and left flanking regions are 1.2 kb (HindIII-BamHI fragment) and 1.7 kb (HindIII-SacI fragment), respectively. The arrow indicates the position and direction of the cpkA gene. Restriction endonuclease cleavage sites are as follows: H, HindIII; K, KpnI; X, XbaI; B, BamHI; P, PstI; S, SacI; and N, NarI. Homologous recombination with a double crossover event as depicted results in the replacement of cpkA with the hygromycin resistance gene. The squiggly line represents the genome.*

(B) DNA gel blot analysis of *cpkA* disrupted transformants. Genomic DNA was digested with HindIII isolated from wild-type strain 70-15 (lanes 1 and 4), transformant L5 (lanes 2 and 5), and transformant T-182 (lanes 3 and 6). DNA in lanes 1 to 3 was probed with the 3.1-kb fragment from pTKM002, indicated as probe 1 in **(A)**. Strain 70-15 shows a 3.1-kb band that corresponds to the native version of *cpkA*, and L5 and T-182 exhibit the predicted band shift to 4.7 kb when *cpkA* was replaced with the hygromycin resistance gene. In lanes 4 to 6, the blot was stripped and probed with a 600-bp BamHI-NarI fragment from pTKM002, indicated as probe 2 in **(A)**. As expected, the native 3.1-kb band of strain 70-15 remains, and the 4.7-kb band from transformants L5 and T-182 is no longer present.

not shown). To confirm the deletion of *cpkA* in L5 and T-182, the blot was stripped and reprobed with an internal sequence of *cpkA*, a 600-bp NarI-BamHI fragment of pTKM002 (probe 2 shown in Figure 4A). As expected, the 4.7-kb band that previously hybridized did not appear in lanes containing DNA from L5 and T-182 (Figure 4B). In other transformants, the 3.1-kb band containing the native *cpkA* gene remained (data not shown).

Characterization of Transformants L5 and T-182 That Lack *cpkA*

Genetic Analysis of L5

CPKA strain L5 was subjected to genetic analysis to eliminate the possibility that the observed inability to form appressoria was due to a mutation introduced through the process of transformation. Two sister single conidial isolates of transformant L5 were crossed to wild-type isolate 70-6. From these two independent crosses, single progeny from 57 asci were obtained. Thirty-three were sensitive to hygromycin and 24 were resistant. The reduced ability to form appressoria segregated completely with resistance to hygromycin, and conversely, the ability to produce appressoria segregated with sensitivity to the antibiotic. The progeny were tested for their ability to form appressoria on the hydrophobic side of GelBond and on segments of onion. All progeny sensitive to hygromycin were able to produce appressoria at levels indistinguishable from wild type, whereas the progeny resistant to the antibiotic showed loss of this ability (Figure 5). These data show that the loss of the ability to form appressoria and hygromycin resistance is tightly linked, as would be expected if *cpkA* had been replaced by the hygromycin resistance gene cassette.

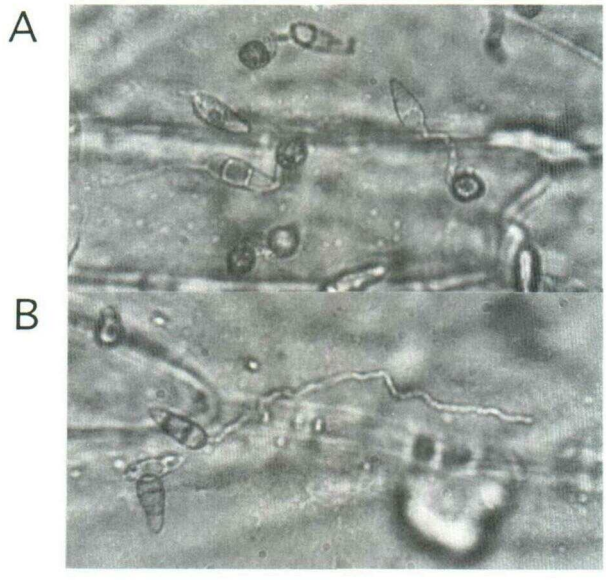

Figure 5. Germination of Conidia from Progeny of the Cross between Transformant L5 and Laboratory Wild-Type Strain 70-6 on Onion Epidermal Tissue.

(A) Hygromycin-sensitive progeny L5II-5 that germinated and differentiated into a melanized appressorium.
(B) Hygromycin-resistant progeny L5I-21 that germinated and continued hyphal elongation without the elaboration of an appressorium.
Conidia were observed and photographed at ×400 magnification after 7 hr.

Figure 6. Infected Rice Leaf Blades from Susceptible Cultivar S-201 Plants Infected with Progeny of the Cross between Transformant L5 and Laboratory Wild-Type Strain 70-6.

Strains L5I-21 and L5II-8 are resistant to hygromycin, whereas strain L5II-5 is sensitive to hygromycin. Leaves were gently abraded with carborundum solution prior to inoculation. Typical leaves are shown 7 days after inoculation.

cpkA *Disruptants Are Unable To Infect and Colonize Susceptible Rice*

To assess the ability of isolates lacking the *cpkA* gene to cause disease symptoms on susceptible rice cultivar S-201, infection assays were performed. Five progeny from L5x70-6

Table 1. Appressorium Formation by Progeny from Transformant L5 (Lacking *cpkA*) Crossed with Laboratory Wild-Type Strain 70-6

	Hydrophobic	Hydrophilic				ANOVA
Progeny	H_2O	H_2O	IBMX[a]	cAMP[b]	10% Ethanol	by Progeny[c]
II-5	99.9(A)	0.4(A)	84.3(A)	41.8(A)	0.0(A)	A
I-10	98.6(A)	0.3(A)	87.5(A)	47.1(A)	0.0(A)	A
I-21	0.0(B)	0.0(A)	0.0(B)	0.0(B)	0.0(A)	B
I-27	0.0(B)	0.0(A)	0.0(B)	0.0(B)	0.0(A)	B
II-8	0.0(B)	0.0(A)	0.0(B)	0.0(B)	0.0(A)	B
ANOVA by Treatment[d]	A	B	C	D	B	

Progeny II-5 and I-10 are hygromycin sensitive, and I-21, I-27, and II-8 are hygromycin resistant. Numbers are averages of three experiments with three replicates per experiment and represent a percentage of germinated conidia that elaborated appressoria on the hydrophobic or hydrophilic surface of GelBond in the presence of various chemicals after 9 hr of incubation. Over 80 conidia were counted for each replicate.

[a] Five microliters of 25mM IBMX stock solution in ethanol was diluted in 45 μL of water containing conidia and used at a final concentration of 2.5 mM.

[b] cAMP was used at a final concentration of 10 mM (pH 7).

[c] Progeny with the same letter are not significantly different at P = 0.05 when analyzed across combined treatments.

[d] The same letter beneath each data column indicates no significant effect of treatment at P = 0.05 when progeny were combined.

(II-5 and I-10 sensitive to hygromycin and I-21, I-27, and II-8 resistant to hygromycin) were used in three independent experiments with three replicate plants per experiment. No visible symptoms were seen on plants inoculated with hygromycin-resistant progeny, even when blades had been abraded prior to inoculation. In contrast, the hygromycin-sensitive progeny caused extensive disease on all inoculated plants (Figure 6). Hygromycin-sensitive progeny and wild-type controls produced a greater size and number of lesions on the abraded leaves.

Disruption of cpkA *Affects Appressorium Formation*

The effect of disrupting *cpkA* on the rate of appressorium formation was carefully evaluated; each of the five progeny listed above was assayed at five time points over a period of 12 hr. These assays were performed on the noncoated, hydrophobic side of GelBond (trademark for an agarose-coated polyester film; FMC Bioproducts, Rockland, ME) and onion epidermal tissue. Analysis of variance (ANOVA) with P = 0.05 showed that the hygromycin-resistant progeny had a significantly different rate of appressorium formation from hygromycin-sensitive progeny. Sensitive progeny formed >90% appressoria by 6 hr, whereas the resistant progeny were unable to form any appressoria by that time. By 12 hr, the resistant progeny were able to form <1%. The few appressoria that did form from the hygromycin-resistant progeny appeared small and misshapen. No effects on rate of vegetative growth or conidiation in culture were observed (data not shown).

cAMP or IBMX Cannot Restore the Ability To Form Appressoria in Strains Lacking cpkA

If the mechanism of action of cAMP occurs through activating a protein kinase A, then treatments that result in the accumulation of endogenous cAMP would not be expected to induce appressorium formation in strains lacking the *cpkA* gene. Table 1 shows a significant difference between hygromycin-resistant and hygromycin-sensitive progeny with respect to the ability of cAMP and IBMX to induce appressoria formation. Hygromycin-resistant progeny were unable to form appressoria to a level >0.4% in the presence of cAMP or IBMX. Sensitive progeny formed appressoria in the presence of cAMP to a level of ~45%, and to even higher levels, 85%, in IBMX. In the presence of IBMX, <28% of the conidia of hygromycin-resistant progeny germinated, compared with >96% germination of the hygromycin-sensitive progeny. The percentage of germination was >95% for all other treatments.

DISCUSSION

Isolation of the Catalytic Subunit Gene for Protein Kinase A

Lee and Dean (1993) implicated cAMP in the series of cellular events leading to appressorium formation in *M. grisea*. To determine the function of cAMP, the primary cellular target of cAMP, the *cpkA* gene, was first cloned. Several lines of evidence indicate this cloning was accomplished. The two forward and two reverse PCR primers were designed with maximum homology with the cAMP-dependent kinase and little identity with the other serine–threonine kinases (Hanks and Quinn, 1991). PCR was first performed using the flanking primers, and the amplified products were cloned. PCR reactions of derived clones using the nested primer set amplified a DNA fragment of the appropriate size in several pools of clones. The DNA sequence of three clones was identical and very similar (>80% amino acid identity) to the corresponding region from analogous

genes in other microorganisms (Toda et al., 1987; Franco de Oliveira et al., 1994; Maeda et al., 1994). The predicted protein of the entire *cpkA* gene shared >80% amino acid identity in the catalytic core region, and conserved motifs such as ATP binding loops, magnesium binding domains, and autophosphorylation sites were present in the expected positions (Taylor et al., 1993). The presence of additional amino acids in the catalytic core is not without precedent. The DmC1 protein kinase A from Drosophila contains a two–amino acid addition at this location (Kalderon and Rubin, 1988).

Phylogenetic analysis supports the conclusion that *cpkA* is a cAMP-dependent protein kinase (Figure 3). CPKA was found to cluster within the family of cAMP-dependent protein kinases using the principle of parsimony. The branch points were robustly supported by high bootstrap values. This analysis is commonly performed to provide statistical confidence in individual branch nodes. Furthermore, when the derived amino acid sequence was compared with sequences in GenBank using the BLAST alignment algorithm, 32 of the top 40 alignments were to other protein kinase As. The degree of similarity of CPKA with these proteins was high, ranging from 46% for bovine isoform C-β-2 to 65% in TPK2 of *S. cerevisiae* (Toda et al., 1987; Wiemann et al., 1991). A considerable level of similarity was observed with TPK1 (61%), human (47%), fruit fly (48%), *Blastocladiella emersonii* (52%), *Schizosaccharomyces pombe* (47%), and hamster (47%). The next groups of proteins with a high level of identity to CPKA were the cGMP-dependent protein kinases and other serine–threonine kinases; these had identities on the order of <23%.

A notable feature of the N terminus of the CPKA protein is the concentration of glutamines. The glutamines are not found in consecutive blocks but as strings alternating with proline or serine residues. Glutamine-rich regions are observed in proteins that have been identified as transcriptional activators (Gonzalez and Satre, 1991; Foulkes et al., 1992; Gerber et al., 1994; Gill et al., 1994; Tanaka et al., 1994). However, when comparing deduced sequences with CPKA, no obvious alignments were apparent. The function of these glutamines in CPKA remains to be determined.

Extensive genomic DNA blot analysis indicates that *cpkA* exists as a single copy in the *M. grisea* genome. This result is consistent with reports from several other organisms, including *S. pombe*, *B. emersonii*, *Caenorhabditis elegans*, and *D. discoideum* (Hanks and Quinn, 1991; Franco de Oliveira et al., 1994; Maeda et al., 1994). In *S. cerevisiae*, three *TPK* genes have been found. They apparently have a redundant function because only one is necessary for normal development (Toda et al., 1987). Disruption of *cpkA* was not expected to be lethal because disruption of analogous genes from *D. discoideum*, *S. pombe*, and *S. cerevisiae* affected development and sexual competency but was not lethal.

Disruption of *cpkA*

Using a gene replacement strategy with the hygromycin resistance gene cassette in plasmid pTKM022, two transformants, L5 and T-182, were obtained that had undergone the expected recombination event. DNA gel blot analysis confirmed the loss of the *cpkA* gene. To confirm that loss of the ability to produce appressoria was not an artifact of transformation, disruptant L5 was crossed with wild-type strain 70-6. We found that the inability to form an appressorium segregated completely with hygromycin resistance. No other gross differences were observed between progeny. The 24 resistant and 33 sensitive progeny all exhibited similar morphology, growth rate, and conidiation in culture. Conidia and colony morphology appeared to be no different from wild-type strains 70-15 and 70-6.

As expected, by disrupting the primary target for cAMP, we removed the ability of the fungus to respond to the cyclic nucleotide. In all cases, progeny resistant to hygromycin were unable to form appressoria in the presence of cAMP or IBMX. This provides further evidence that we have knocked out a functional cAMP-dependent protein kinase. The observation that hygromycin-resistant progeny germinated with reduced frequency in the presence of IBMX remains to be explained. The lack of responsiveness of transformants lacking *cpkA* to cAMP or IBMX is also consistent with *cpkA* encoding a downstream kinase, one that is regulated by the cAMP-dependent kinase. Although unlikely, because CPKA contains all of the conserved functional motifs and clusters within the cAMP-dependent kinase family, this possibility cannot be formally excluded.

The inability of strains lacking CPKA to cause disease on rice blades when the cuticle had been abraded was somewhat surprising. The requirement of CPKA for pathogenesis has recently been corroborated. A pathogenic deficient mutant obtained from a random insertional gene inactivation survey contained an insertion in the *cpkA* gene (J. Sweigard, personal communication). Our findings suggest that elaboration of an appressorium, or the cellular events that signal appressorium formation, may be necessary for inducing pathogenicity genes. A further possibility is that the disruption of *cpkA* has pleiotrophic effects, including affecting the ability of the fungus to colonize rice plants. However, other aspects of growth and development, including growth rate, sporulation, and sexual competence, appear to be unaffected when assayed in vitro.

METHODS

Strains, Growth, and Maintenance of *Magnaporthe grisea*

Strains 70-15 and 70-6, obtained from Dr. A. Ellingboe (University of Wisconsin, Madison), were used as wild-type controls. Strains 70-15 and 70-6 are domesticated, self-sterile hermaphroditic strains derived from field isolate GUY11, carrying the *MAT1-1* and *MAT1-2* mating-type alleles, respectively (Lau et al., 1993). All *M. grisea* cultures were maintained on a 4% V8 juice media containing 1.4% agar and incubated at 22°C ± 1°C with constant fluorescent lighting. These conditions allowed for conidial development within 5 days.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed on *M. grisea* genomic DNA using four primers based on conserved regions in the catalytic core of the protein kinase A catalytic subunit (*cpkA*) gene (Hanks and Quinn, 1991). The primers are as follows: RDPK1, 5′-CGGATCCTA(C/T, A/C)GIGA(C/T, C/T)TIAA(G/A)CCIGA(G/A)AA-3′; RDPK2, 5′-CGGATCCACIGA(T/C)TT(T/C)GGITT(T/C)GCIAA-3′; RDPK3, 5′-GAATTCGGIGTICC(A/G)CAIA(A/G)IGTCCA-3′; and RDPK4, 5′-GAATTCA(T/C)ICC(A/G)IG(C/A)CCA(A/G)TC-3′. The two outside primers were designated RDPK1 and RDPK4; the nested pair, RDPK2 and RDPK3. The two forward primers, RDPK1 and RDPK2, contained a terminal BamHI restriction site, whereas the reverse primers, RDPK3 and RDPK4, contained a terminal EcoRI site to facilitate cloning of PCR products. Each primer was used alone as well as in all forward and reverse pairwise combinations in a 50-μL PCR reaction volume that contained 5 μL 10 × PCR buffer (Perkin-Elmer Cetus), 1 μL each dATP, dCTP, dGTP, and dTTP (10 mM stock), 1 μL of *M. grisea* DNA (100 ng/μL stock), 1μL each primer (50 μg/μL stock), 37.8 μL distilled H_2O, and 0.2 μL AmpliTaq Polymerase (Perkin-Elmer Cetus). The PCR cycling parameters were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 2 min, with a final extension for 5 min at 72°C, which was then held at 4°C. PCR products were cloned into pBluescript KS+.

For PCR of bacterial colonies, bacteria were transferred by toothpick to 80 μL distilled H_2O and boiled for 5 min. Five microliters was added to a 50-μL PCR reaction mix with 0.1 μL of Taq polymerase and primers RDPK2 and RDPK3.

Isolation of Genomic Clones of *cpkA* and Construction of pTKM022

A 3.6-kb BamHI fragment, a 3.1-kb HindIII fragment, and a 3.8-kb XbaI fragment, which hybridized to PCR clone pRD36, were subcloned from genomic cosmid clone XXXC1 into pBluescript KS+ and designated pTKM001, pTKM002, and pTKM003, respectively. pTKM022 (Figure 4A) was constructed from a 4.1-kb BamHI-SacI fragment of pTKM002 containing 1.25 kb of left flank of *cpkA*, a 1.7-kb HindIII-SacI fragment from pTKM001 containing the right flank of *cpkA*, and a 3.5-kb HindIII-BglII fragment from pAN7-1 containing the hygromycin-B resistance gene under the control of the *Aspergillus nidulans trpC* promoter (Punt et al., 1987).

DNA Isolation and Manipulation

Plasmid DNA was isolated using the alkaline lysis method of Sambrook et al. (1989). Restriction site mapping and subcloning were performed according to standard methods (Sambrook et al., 1989). *M. grisea* DNA was isolated as described by Yelton et al. (1984), except that cells were freeze-dried and pulverized in microcentrifuge tubes. DNA digestion, agarose gel fractionation, radiolabeling, and hybridization were performed according to the manufacturers' instructions and standard methods (Sambrook et al., 1989). Hybridizations were carried out in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll 400, 0.02% PVP, 0.02% BSA), 0.1% SDS, and 50 mM phosphate buffer, pH 6.6, at 65°C. Low-stringency hybridization was carried out at 55°C. Membranes were washed down to between 2 and 0.1 × SSC, 0.1% SDS, before being exposed to autoradiographic film.

DNA Sequencing

Double-stranded DNA sequence was determined by Taq Deoxy Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA). A series of overlapping subclones and the following specific primers to bridge restriction sites were used: P79, 5′-GTGGCGGCCGCTCTAGAAC-3′; 768, 5′-GGATCAATAGTCCCCGG-3′; 958, 5′-CAGTCACAGCAGGACGC-3′; 1912, 5′-CCCCTTCTGGGATAGCG-3′; 1942, 5′-CTTCATAGGAGATCCGC-3′; P24, 5′-CCGTTCTGATCTGACGCG-3′; and P25, 5′-GCGACGCAAGCCAGTTT-3′. Sequencing reactions were fractionated on an Applied Biosystems auto-sequencer. Data were downloaded to GeneWorks 2.3 (IntelliGenetics, Mountain View, CA) for DNA and protein sequence analysis.

Analysis of Conserved Region in Protein Kinase Genes

Putative phylogenetic relationships between the deduced amino acid sequences of the kinase domain of representative protein kinase genes available from the GenBank data base (release 89.0) were performed using PAUP 3.1.1 (Phylogenetic Analysis Using Parsimony; Swofford, 1993) for Macintosh computers. This program finds the most parsimonious tree, that is, minimizing the number of character-state changes necessary to explain the available data. The sequence data were analyzed using the Wagner criterion, which permits reversibility of character states. However, state changes were weighted according to Felsenstein's amino acid replacement substitution rules. The most parsimonious tree was searched for by PAUP's "heuristic" search method with "tree-bisection reconnection" (TBR) branch point swapping. Bootstrap analyses consisted of 100 heuristic replications using the simple addition of the taxa branch swapping method. All minimal trees were saved from each bootstrap replication and used to calculate a consensus tree.

Transformation of *M. grisea*

Transformation protocols were adapted from established procedures (Leung et al., 1990; Sweigard et al., 1992). Conidia (1.5×10^6) were germinated in liquid medium overnight at room temperature with agitation. Mycelium was harvested using Miracloth (Calbiochem), rinsed, and wrung dry. The pellet was suspended in 30 mL of 1.0 M sorbitol to which 1 mL of Novozyme 234 (20 mg/mL; BiosPacific Inc., Emeryville, CA) was added. After 90 min of agitation at room temperature, protoplasts were collected by filtering through cheesecloth and pelleted by centrifugation at 5000 rpm for 10 min. Protoplasts were suspended in STC (1.0 M sorbitol, 50 mM Tris-HCl, pH 8.0, and 50 mM $CaCl_2$), and centrifugation was repeated. Five micrograms of DNA was placed with 1.0×10^7 protoplasts in 200 μL STC for 10 min before 1 mL of PTC (40% polyethylene glycol 4000 in STC) was added. After 20 min, 3 mL of TB3 (3 g yeast extract, 3 g casein acid hydrolysate, 10 g glucose, and 200 g sucrose per liter) was added and incubated for 6 hr. Twenty milliliters of molten TB3 with agar containing hygromycin (200 μg/mL) was then added, and plates were poured. After ~7 days, hygromycin-resistant colonies were transferred to V8 plates. Conidia were streaked for single colonies.

Sexual Crosses

Crosses were performed using established procedures (Lau et al., 1993). Plugs of ~0.5 cm^2 of strains 70-6, 70-15, and a strain of interest were placed ~5 cm from each other on an oatmeal agar plate and incubated at 22°C ± 1°C under fluorescent and black lights. Asci were released from crushed perithecia on 2% distilled H_2O agar. Individual germinating asci were transferred to V8 plates and allowed to conidiate. Conidia were streaked for single colonies. A random single colony was chosen from each ascus and used for further investigation.

Pathogenicity Assays

Conidial suspensions were prepared from 7-day-old V8 plates and adjusted to 1.0 × 10^6/mL in a 0.25% Tween 20 solution. Approximately 8 mL were sprayed evenly onto 3-week-old rice plants with a fine mist aspirator or a pump-pressurized aerosol sprayer. Several leaves of each plant were gently rubbed with a carborundum solution prior to inoculation. Rice plants were placed inside a plastic bag, which was left unsealed, and incubated in an unlit dew chamber at 25°C ± 2°C with a relative humidity of >95%. After 24 hr, plants were incubated with a photoperiod of 15 hr using fluorescent lighting. Plants were rated for disease 5 to 7 days after inoculation.

Appressoria Assays

Appressorium development was assayed by germinating conidia on the hydrophobic surface of GelBond (a polyester film from FMC Bioproducts) and on onion epidermis. GelBond assays were performed by soaking sheets of the plastic polymer in sterile distilled H_2O for 30 min without agitation at room temperature. Three 50-μL drops of a conidial suspensions (~2 × 10^5 conidia/mL) from a 10-day-old culture were placed on each piece of GelBond. Following incubation at room temperature for designated time periods (as pertaining to the individual experiment), the germinated conidia were observed under a compound microscope. Appressoria from strain 70-15 developed in ~6 hr.

To monitor appressorium formation on onion epidermis, white or yellow onions were freshly cut into 3-cm^2 pieces and placed into a moisture chamber with the inner surface facing up. Fifty microliters of a conidia suspension (~2 × 10^5 conidia/mL) was placed on the onion surface and incubated as described for GelBond. To view the conidia, the epidermal layer was peeled away and laid face up on a microscope slide and overlaid with a coverslip.

cAMP and 3-Isobutyl-1-Methylxanthine Treatments

Forty-five microliters of conidial suspension was placed in each well of a clean 12-well glass plate. To the conidia, 5 μL of distilled H_2O, 100 mM cAMP, 25 mM IBMX, or 100% ethanol was added and allowed to incubate for 9 hr in a moisture chamber with constant light. Ethanol was used as a control because the 3-isobutyl-1-methylxanthine (IBMX) solution was prepared in 100% ethanol. Germination and appressorium formation were determined directly. Each compound—distilled H_2O, cAMP, IBMX, and ethanol—was tested in three experiments with three replicates per treatment.

Time Course Studies

The percentage of appressorium formation was determined at 2, 4, 6, 9, and 12 hr after 50 μL droplets of conidia (~2 × 10^5 conidia/mL) were placed on the hydrophobic surface of GelBond. The same conidia sample was observed at each time point. Additional experiments were performed on onion epidermal tissue; the destructive nature of preparing the sample for observation necessitated the use of different onion pieces for each time point. Time course experiments were conducted three times with three replicates per experiment.

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