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The Adenylate Cyclase Gene *MAC1* of *Magnaporthe grisea* Controls Appressorium Formation and Other Aspects of Growth and Development

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***Magnaporthe grisea*, the causal agent of rice blast disease, differentiates a specialized infection structure called an appressorium that is crucial for host plant penetration. Previously, it was found that cAMP regulates appressorium formation. To further understand the cellular mechanisms involved in appressorium formation, we have cloned a gene (*MAC1*) encoding adenylate cyclase, a membrane-bound enzyme that catalyzes the production of cAMP from ATP, by using a polymerase chain reaction–based strategy. The entire gene was isolated and subcloned from a large insert bacterial artificial chromosome library. Sequence characterization showed that *MAC1* has a high degree of identity with other adenylate cyclase genes from several filamentous fungi as well as yeasts. Gene deletion resulted in reduced vegetative growth, conidiation, and conidial germination. Transformants lacking *MAC1* were unable to form appressoria on an inductive surface and were unable to penetrate susceptible rice leaves. *mac1*$^-$ transformants were also sterile and produced no perithecia. Appressorium formation was restored in the presence of exogenous cAMP derivatives. These results confirm that cell signaling involving cAMP plays a central role in the development and pathogenicity of *M. grisea*.**

INTRODUCTION

The rice blast fungus *Magnaporthe grisea* is a major limiting factor in rice production throughout the world and has been developed as a model organism to study numerous aspects of the host–pathogen interaction (Ou, 1985; Valent, 1990; Dean, 1997). Infection occurs after a highly organized series of morphological events. The process is initiated by the attachment of a conidium. A germ tube then emerges and grows across the leaf surface. Subsequently, polar elongation ceases, and the tip of the germ tube becomes attached to the surface and begins to swell to form the dome-shaped, highly melanized infection cell, the appressorium (Hamer et al., 1988; Bourett et al., 1990). Hydrostatic pressure increases inside the appressorium that assists in driving a penetration hypha into the underlying tissues (Howard et al., 1991). The mechanisms triggering and regulating appressorium formation are being intensively investigated. Surface hydrophobicity (Lee and Dean, 1993; Jellito et al., 1994; Lee and Dean, 1994) and hardness of the contact surface (Xiao et al., 1994), as well as cutin monomers from the plant surface (Gilbert et al., 1996), have been implicated as primary environmental cues in triggering appressorium formation.

cAMP has been shown to have an essential role in regulating many of the biological activities of prokaryotic and eukaryotic cells (Pastan and Perlman, 1970; Robison et al., 1971). cAMP is an important regulator of development in fungi and slime molds, including *Dictyostelium discoideum* (Firtel et al., 1989; Firtel, 1991). In *M. grisea*, we have shown that cAMP plays a central role in appressorium formation (Lee and Dean, 1993). The addition of cAMP induces appressorium formation on a noninductive surface. cAMP functions primarily as a second messenger and activates a protein kinase. Activation occurs by the binding of cAMP and results in the release of regulatory subunits from the catalytic subunits (Taylor, 1989). Mitchell and Dean (1995) cloned the gene for the catalytic subunit of a cAMP-dependent protein kinase (*cpkA*) and have shown through gene disruption that the cAMP-dependent protein kinase is required for normal appressorium formation and pathogenicity. Xu et al. (1997) further demonstrated that upon prolonged incubation, *cpkA* mutants produce some appressoria, although they are defective as penetration fails to occur.

cAMP is produced from ATP by adenylate cyclase. This membrane-bound enzyme has been investigated in several fungal species. In *Neurospora crassa*, a morphological mutant, which lacks extensive mycelial growth, had reduced adenylate cyclase activity (Terenzi et al., 1976; Rosenberg and Pall, 1979). The mutation was complemented by the introduction of a wild-type adenylate cyclase gene (Kore-eda et al., 1991b). Similarly, the adenylate cyclase gene was cloned from *Podospora anserina* by complementation of mutants defective in vegetative compatibility (Loubradou et

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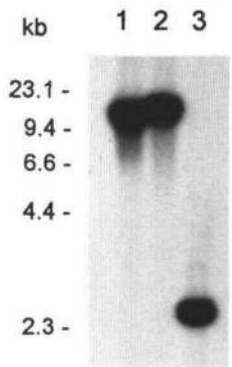

Figure 1. *M. grisea* Contains a Single Adenylate Cyclase Gene.

Genomic DNA from wild-type 70-15 was digested with BamHI (lane 1), EcoRI (lane 2), or HindIII (lane 3), size separated on a 1% agarose gel, transferred to a nylon membrane, and probed with pMAC383 under low-stringency conditions. Numbers at left indicate the size of DNA.

al., 1996). In *Ustilago maydis*, the corn smut fungus, disruption of the adenylate cyclase gene (*uac1*) resulted in a constitutive filamentous phenotype due to an inability to regulate the dimorphic growth cycle (Gold et al., 1994).

To further our understanding of the role that cAMP and the cAMP-dependent protein kinase pathway plays in *M. grisea*, an adenylate cyclase gene (*MAC1*) was cloned and characterized. We present the results of gene disruption on growth, development, and pathogenicity of this important phytopathogenic fungus.

RESULTS

Isolation of *MAC1*

The adenylate cyclase gene of *M. grisea* was isolated using a polymerase chain reaction (PCR)–based strategy. Nested degenerate oligonucleotide primers were designed from conserved sequences of the adenylate cyclase genes in other filamentous fungi (Kore-eda et al., 1991a; Gold et al., 1994; Loubradou et al., 1996). The DNA sequence of a 383-bp PCR product (pMAC383) showed a high degree of identity with other adenylate cyclase genes; the deduced peptide sequence matched 97 of 125 amino acids (positions 1777 to 1901 in MAC1) from the corresponding region of the adenylate cyclase of *N. crassa* (Kore-eda et al., 1991a). pMAC383 hybridized with single bands on genomic DNA gel blots under low-stringency conditions, indicating that the cloned DNA fragment was of *M. grisea* origin and exists as a single copy in the genome (Figure 1). A bacterial artificial chromosome (BAC) library (Zhu et al., 1997) was screened to isolate the entire *MAC1* gene by using the PCR product as a probe. Six BAC clones were identified; all contained three HindIII fragments (6.9, 2.6, and 2.5 kb) that hybridized with the entire *nac* gene from *N. crassa* (Kore-eda et al., 1991a; data not shown). These three HindIII fragments were cloned (pMAC4, pMAC17, and pMAC10) along with an EcoRI fragment (~18 kb; pMAC1) covering the entire *MAC1* gene. A restriction map of the *MAC1* gene is shown in Figure 2.

Nucleotide Sequence and Characterization of *MAC1*

The nucleotide sequence of the three HindIII fragments was determined using a series of subclones from the BAC clones and several oligonucleotide primers to bridge restriction sites. The nucleotide sequence of *MAC1* contains a putative 6483-bp open reading frame with three introns (360, 236, and 75 bp). The introns were predicted by the existence of conserved splicing elements found in fungal introns (Gurr et al., 1987). The open reading frame of *MAC1* encodes a predicted 2160–amino acid protein named MAC1. This polypeptide sequence shows a high degree of similarity to adenylate cyclases of other fungal species, particularly *P. anserina* and *N. crassa* with 62 and 56% identity, respectively (Figure 3; Kore-eda et al., 1991a; Loubradou et al., 1996).

In other adenylate cyclases of fungi, at least four functional domains, consisting of the N terminus, the middle leucine-rich repeats, the catalytic region, and the C terminus, can be identified. The amino acid sequence of MAC1 shows a similar pattern of domain composition. The functional domains are highly conserved. The N terminus (amino acids 206 to 385), the leucine-rich repeat region (amino acids 844 to 1411), and the catalytic domain region (amino acids 1695 to 1993) are 81 and 76%, 70 and 66%, and 69 and 57% identical compared with the corresponding regions of *P. anserina* and *N. crassa*, respectively. A PEST domain, rich in proline (P), glutamine (E), serine (S), and threonine (T), is conserved in the N-terminal region (Rogers et al., 1986). Leucine-rich repeats are known to be required for interaction between adenylate cyclase and RAS protein in yeast (Colicelli et al., 1990; Suzuki et al., 1990). The consensus

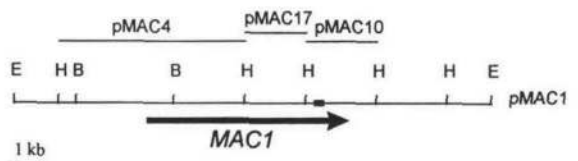

Figure 2. Structure of the *MAC1* Gene in *M. grisea*.

Restriction map of an 18-kb EcoRI fragment (pMAC1) covering the entire *MAC1* gene. pMAC4, pMAC17, and pMAC10 indicate the location of three HindIII fragments. The black box in pMAC1 indicates the location of the PCR product clone (pMAC383). H, HindIII; B, BamHI; E, EcoRI.

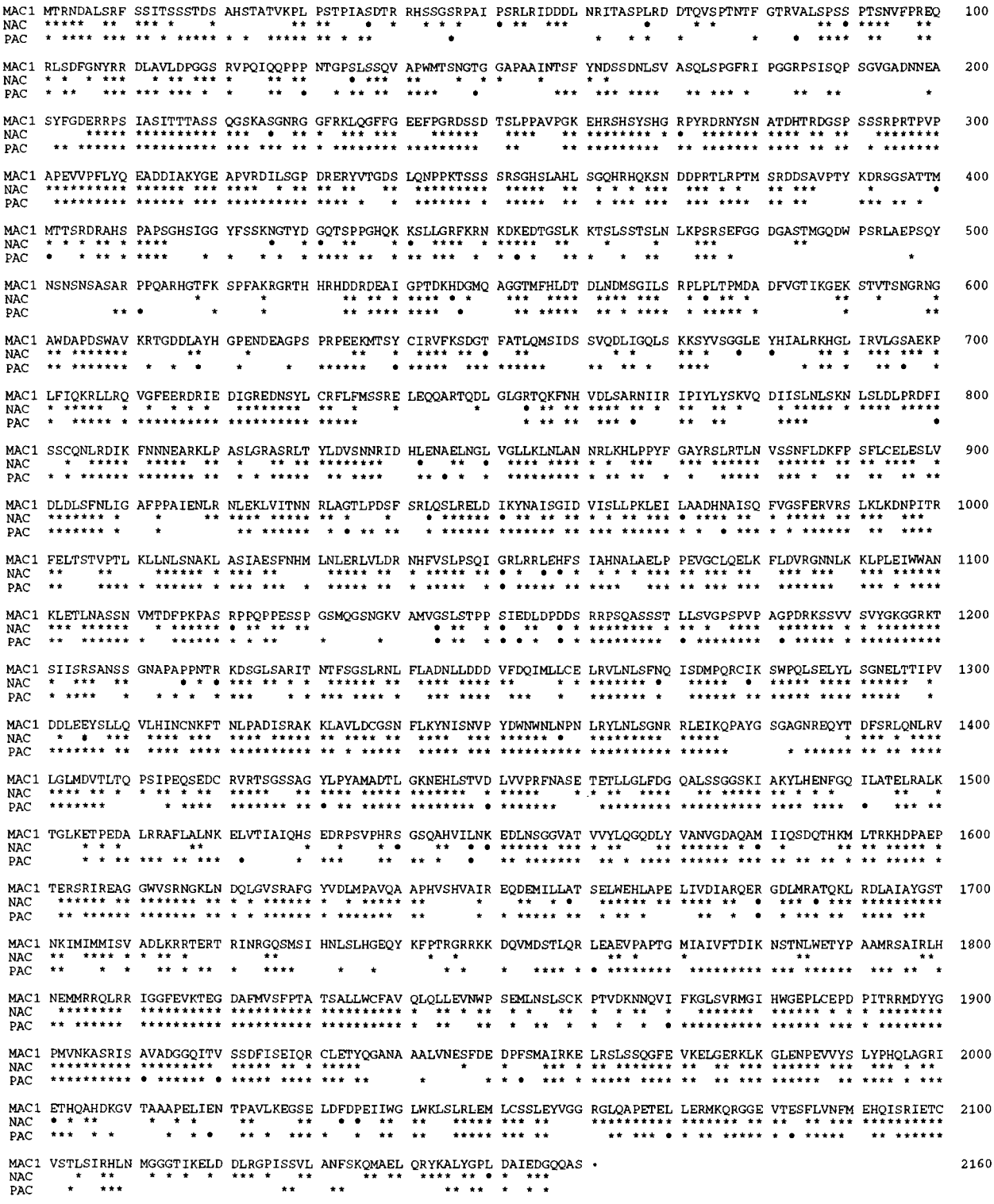

Figure 3. Alignment of Amino Acid Sequence of Adenylate Cyclases from *M. grisea* (MAC1), *N. crassa* (NAC), and *P. anserina* (PAC).

Gaps in the sequence alignment and nonidentical amino acids are not shown here. Asterisks indicate amino acid identity with MAC1. The MAC1 sequence has GenBank accession number AF012921.

sequence of the 23–amino acid repeat, as suggested by Kataoka et al. (1985), is as follows: PXXαXXLXXLXXLXLXXN-XαXXα (α indicates one of the three aliphatic amino acids, valine, leucine, or isoleucine; X indicates any amino acid). At least nine repeats are present in the central region (amino acids 844 to 1411) of *M. grisea* adenylate cyclase.

The catalytic activity region is located from amino acids 1695 to 1993 based on the alignment to the catalytic domain of the adenylate cyclase of *Saccharomyces cerevisiae* (Yamawaki-Kataoka et al., 1989). In the C-terminal region, two subdomains have been proposed: the cyclase-associated protein (CAP; Fedor-Chaiken et al., 1990; Field et al., 1990) binding site and a leucine zipper motif (Landschulz et al., 1988). The CAP binding site has been proposed to be essential for adenylate cyclase activity, and the short sequences around amino acids 1976 and 2011 appear to be critical for CAP binding in *S. cerevisiae* (Wang et al., 1993). We propose the amino acids SRIET and ANFSK (2095 to 2099 and 2131 to 2135) in MAC1 as the corresponding sequences. Also, the region from amino acids 2031 to 2070 is suggested as a leucine zipper domain based on the alignment to adenylate cyclase of *S. cerevisiae* (Yamawaki-Kataoka et al., 1989).

Disruption of *MAC1*

To determine the function of *MAC1*, a gene disruption strategy was employed. A disruption plasmid, pWBC1 (Figure 4A), containing the hygromycin resistance gene cassette flanked by 5′ and 3′ ends of the *MAC1* gene was constructed. Three (ACT2, ACT6, and ACT76; ACT stands for adenylate cyclase transformant) of 229 hygromycin-resistant transformants were determined by DNA gel blot analysis to have undergone gene replacement. Genomic DNA from 10 hygromycin-resistant transformants (three knockout and seven ectopic integration transformants) and wild-type 70-15 was digested with HindIII and probed with the pMAC17 (Figure 4B). The three *mac1*⁻ transformants (ACT2, ACT6, and ACT76) showed no hybridization with the middle part of the *MAC1* gene, whereas ectopic integration transformants and the wild-type 70-15 exhibited the expected hybridization band at 2.5 kb. To confirm the integration of the hygromycin resistance gene, the same blot was stripped and reprobed with pAN7-1 carrying the hygromycin resistance cassette (Figure 4B). The three *mac1*⁻ transformants exhibited a hybridization band at 10.7 kb, which is consistent with the expected length for a gene replacement event. Ectopic integration transformants showed a longer single band; ACT11 showed several bands, indicating that multiple integration (at least three sites) had occurred.

Characterization of Transformants Lacking *MAC1*

Disruption of *MAC1* significantly reduced the vegetative growth and conidiation of these mutants and rendered them

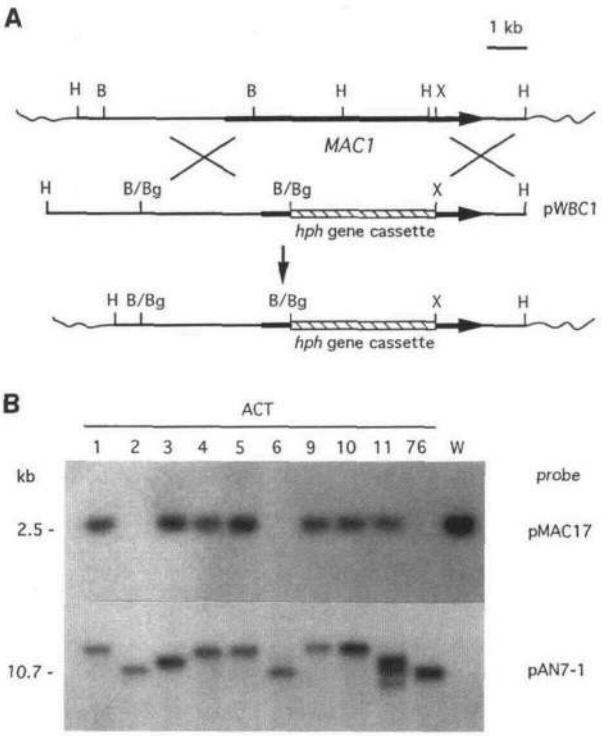

Figure 4. Targeted Disruption of *MAC1*.

(A) Disruption plasmid pWBC1 contains the hygromycin resistance gene (*hph*) cassette flanked by border sequences from *MAC1*. At left (a 3.7-kb BamHI fragment of pMAC4) and right (a 2.4-kb XbaI-HindIII fragment of pMAC10) are fragments from *MAC1* that were introduced into compatible restriction sites of pAN7-1 to flank the *hph* gene cassette and produce pWBC1. By double homologous recombination, the central region of *MAC1* was replaced by the *hph* cassette. The restriction enzymes are as follows: B, BamHI; Bg, BglII; H, HindIII; X, XbaI. B/Bg indicates the BamHI-BglII sites destroyed during construction. The wavy line represents the genome.

(B) Gel blot analysis of *mac1*⁻ transformants (ACT2, ACT6, and ACT76), ectopic transformants (ACT1, ACT3, ACT4, ACT5, ACT9, ACT10, and ACT11), and wild-type 70-15 (W). All genomic DNA samples were digested with HindIII. The blot was probed with pMAC17 insert DNA first and then stripped and reprobed with pAN7-1 containing the *hph* gene. As expected, *mac1*⁻ transformants exhibited the predicted band at 10.7 kb when probed with pAN7-1 and showed no hybridization band at 2.5 kb when probed with pMAC17 insert DNA.

sterile (Table 1). All three knockout mutants showed reduced mycelial growth of ~60% relative to the colony diameter of the wild-type 70-15. *mac1*⁻ transformants produced dense aerial hyphae around the inoculation point with tight clusters of light gray–colored conidia, in contrast with the dark gray color of the ectopic integration transformants and wild type. No significant difference in the shape of the conidia and the number of cells per conidium among *mac1*⁻ disruption, ec-

topic integration, and wild-type strain was observed (Table 1). In mating experiments, *mac1*$^-$ transformants failed to form perithecia, whereas the ectopic integration transformants and the wild type formed normal perithecia with viable ascospores after 3 or 4 weeks of incubation with the opposite mating-type strain 70-6 (Table 1).

Germination and Appressorium Formation by *mac1*$^-$ Transformants

The three *mac1*$^-$ mutants were delayed in conidial germination compared with ectopic integration transformants and the wild type. *mac1*$^-$ transformants took between 12 and 24 hr to exceed 90% germination on either the hydrophilic or the hydrophobic surface of GelBond film, whereas ectopic integration transformants and the wild type typically took <6 hr to germinate to this level. The *mac1*$^-$ transformants failed to produce appressoria on the hydrophobic surface of GelBond film (Table 2), onion skin (Figure 5A), or rice leaves (data not shown) after 48 hr. No appressoria were observed at 72 hr.

Appressorium formation was restored by monobutyryl-cAMP (mb-cAMP; Table 2). However, neither 3-isobutyl-1-methylanthine (IBMX), an inhibitor of cellular degradation of cAMP, nor 1,16-hexadecanediol (diol), a cutin monomer triggering appressorium formation, was effective in restoring appressorium formation (Table 2). Chemically induced appressoria showed normal, short germ tubes and were well melanized on either GelBond film, onion skin (Figures 5B and 5C), or rice leaves (data not shown). The addition of mb-cAMP also increased conidial germination (90% at 6 hr of incubation on the hydrophobic surface of GelBond film). In the presence of IBMX, conidial germination of *mac1*$^-$ transformants on GelBond film was <10 to 20% at 24 hr, whereas ectopic transformants and the wild type appeared to be unaffected and germinated normally. To determine the effectiveness of mb-cAMP on restoration of appressorium formation in *mac1*$^-$ transformants, conidia of ACT2 were exposed to pulses of mb-cAMP treatment (Figure 6). For treatments ending at the same time point, 2-hr exposure to mb-cAMP was as effective at inducing appressorium formation on the hydrophobic surfaces of GelBond film as was a continuous exposure. Cells were most responsive to mb-cAMP between 8 and 10 hr.

Effect of Gene Disruption of *MAC1* on Pathogenicity

Infection assays were performed to assess the ability of *mac1*$^-$ transformants to cause disease on susceptible rice. In conidial spray assays, no symptoms were observed on plants inoculated with *mac1*$^-$ transformants (ACT2 and ACT6), compared with the typical diamond-shaped, gray-centered lesions caused by an ectopic transformant (ACT3) or the wild-type strain 70-15 (Figure 7A). In injection assays, disease symptoms by *mac1*$^-$ transformants were less severe compared with those of plants inoculated with an ectopic transformant or wild-type strain 70-15. Unlike the latter, *mac1*$^-$ transformants produced nonspreading lesions (Figure 7B).

DISCUSSION

We have cloned a gene, named *MAC1*, encoding adenylate cyclase in *M. grisea* by using a PCR-based strategy and

Table 1. Characteristics of *mac1*$^-$ Transformants and Wild-Type *M. grisea* Strain 70-15[a]

Strains	Disrupted *MAC1*	Growth Rate[b]			Conidiation[c]		Cells/Conidium[d]			Conidium Size[e]		
		4D	8D	12D	8D	12D	1	2	3	L	W	Mating[f]
ACT2	Yes	14A	33A	50A	69A	84A	6A	6A	88A	22A	8A	Sterile
ACT6	Yes	15B	33A	50A	174B	240B	6A	6A	88A	21A	8A	Sterile
ACT76	Yes	14A	33A	50A	51A	102A	6A	6A	88A	21A	8A	Sterile
ACT3	No	24C	52B	80B	456C	906C	5A	4A	91A	19A	8A	Fertile
ACT9	No	24CD	54C	81B	435C	975C	4A	5A	91A	20A	8A	Fertile
70-15	No	25D	54C	80B	480C	954C	5A	4A	91A	20A	8A	Fertile

[a] Means with the same letter in each column are not significantly different, as estimated by the Duncan's multiple range test ($P = 0.05$).
[b] A V8 juice agar plate was inoculated with a plug taken from the growing edge of a colony on the V8 agar plate. Colony diameter (millimeters) was measured after incubation of day (D) indicated. The data are the results of at least three experiments with three replicates in each.
[c] A V8 juice agar plate was inoculated as mentioned in footnote b. After incubation of 8 or 12 days (D), conidia were collected in water and counted with a hemocytometer under a microscope. The data ($\times 10^4$ conidia per plate) are the results of at least three experiments with three replicates in each.
[d] Percentage of conidia with one, two, or three cells. The data are the results of two experiments with >300 conidia examined in each.
[e] L and W represent length and width, respectively, of conidia in microns. The data are the results of two experiments with >50 conidia examined in each.
[f] Mating was performed on oatmeal agar plates, as described in Methods. After 3 or 4 weeks of incubation, the plate was observed for production of perithecia.

Table 2. Appressorium Formation of *mac1*⁻ Transformants and Wild-Type *M. grisea* Strain 70-15[a]

	Hydrophobic				Hydrophilic			
Strains	Water	Mb-cAMP (5 mM)	IBMX (2.5 mM)	Diol (1 μM)	Water	Mb-cAMP (5 mM)	IBMX (2.5 mM)	Diol (1 μM)
12 hr								
ACT2	0 ± 0	54 ± 7	0 ± 0	0 ± 0	0 ± 0	36 ± 4	0 ± 0	0 ± 0
ACT6	0 ± 0	45 ± 6	0 ± 0	0 ± 0	0 ± 0	41 ± 4	0 ± 0	0 ± 0
ACT76	0 ± 0	49 ± 4	0 ± 0	0 ± 0	0 ± 0	42 ± 4	0 ± 0	0 ± 0
ACT3	94 ± 2	95 ± 3	96 ± 1	96 ± 2	0 ± 0	57 ± 8	80 ± 7	88 ± 5
ACT9	95 ± 2	96 ± 2	95 ± 3	96 ± 2	0 ± 0	58 ± 3	84 ± 5	89 ± 4
70-15	96 ± 3	94 ± 3	95 ± 2	95 ± 2	0 ± 0	50 ± 1	87 ± 5	90 ± 4
24 hr								
ACT2	0 ± 0	85 ± 3	0 ± 0	0 ± 0	0 ± 0	67 ± 7	0 ± 0	0 ± 0
ACT6	0 ± 0	83 ± 5	0 ± 0	0 ± 0	0 ± 0	67 ± 4	0 ± 0	0 ± 0
ACT76	0 ± 0	80 ± 5	0 ± 0	0 ± 0	0 ± 0	67 ± 7	0 ± 0	0 ± 0
ACT3	96 ± 2	95 ± 2	97 ± 2	96 ± 1	2 ± 1	92 ± 4	92 ± 3	95 ± 2
ACT9	96 ± 2	97 ± 2	96 ± 2	95 ± 2	3 ± 1	89 ± 4	92 ± 1	93 ± 3
70-15	96 ± 2	95 ± 3	95 ± 3	97 ± 3	4 ± 1	90 ± 3	90 ± 3	94 ± 2
48 hr								
ACT2	0 ± 0	89 ± 3	0 ± 0	0 ± 0	0 ± 0	83 ± 3	0 ± 0	0 ± 0
ACT6	0 ± 0	91 ± 2	0 ± 0	0 ± 0	0 ± 0	83 ± 5	0 ± 0	0 ± 0
ACT76	0 ± 0	87 ± 3	0 ± 0	0 ± 0	0 ± 0	84 ± 5	0 ± 0	0 ± 0
ACT3	94 ± 3	96 ± 1	97 ± 2	96 ± 3	24 ± 4	94 ± 3	96 ± 2	97 ± 1
ACT9	94 ± 1	96 ± 2	96 ± 2	96 ± 1	24 ± 4	92 ± 2	97 ± 2	97 ± 1
70-15	96 ± 2	96 ± 2	96 ± 2	96 ± 2	20 ± 2	91 ± 3	97 ± 1	96 ± 3

[a] Conidia of each strain were harvested and adjusted to a concentration of 10^5 conidia per mL in distilled water. Data were recorded after 12, 24, and 48 hr of incubation on GelBond film. The experiments were repeated at least four times with three replicates each with similar results: percentages are of conidia with appressoria; mean ± SD.

have shown by targeted deletion that this gene is important for growth, development, and pathogenicity. The degenerate primers used were nested and designed from highly conserved regions of the catalytic region of adenylate cyclase genes of other filamentous fungi (*N. crassa* and *P. anserina*). An 18-kb EcoRI fragment containing the entire *MAC1* gene was cloned from a BAC library (Zhu et al., 1997). This large insert (>130 kb) library was particularly valuable for isolating genes of large size, such as *MAC1* (7.2 kb). Genomic gel blot analysis revealed *MAC1* to exist as a single-copy gene in the genome of *M. grisea* strain 70-15, thus facilitating functional analysis.

The phenotypic effects of disruption of *MAC1* were partially as predicted, given the previous evidence that cAMP plays a central role in appressorium formation (Lee and Dean, 1993; Mitchell and Dean, 1995; Xu et al., 1997). Adenylate cyclase–deficient transformants failed to form appressoria on inductive surfaces (the hydrophobic surface of GelBond, onion skin, or rice leaves). In addition, the genetic defect was restored by the addition of exogenous mb-cAMP but not by IBMX. The reason for reduced germination of conidia from *mac1*⁻ disruption strains in the presence of IBMX is unclear. A similar effect of IBMX on germination was observed for transformants lacking *cpkA*, the catalytic subunit gene of protein kinase A (Mitchell and Dean, 1995). Diol was also not effective at restoring appressorium formation, suggesting that this compound functions through adenylate cyclase. It is particularly interesting that diol restores appressorium formation to mutants lacking *magB*, a gene encoding a G protein α-subunit of *M. grisea* (Liu and Dean, 1997).

Gilbert et al. (1996) presented a model of appressorium formation in *M. grisea* that fits well with the data presented here. They envisioned a dual signaling pathway involving both thigmotropic and chemical stimuli from the environment being transduced into the germ tube via separate and distinct pathways. There are several lines of evidence supporting this model. Mutants at the *app2* locus, which do not form appressoria in response to a hydrophobic surface, formed appressoria in response to diol or cAMP treatment (Zhu et al., 1996). Furthermore, mutants lacking *cpkA* do not form normal appressoria in response to exogenous cAMP, diol, or a hydrophobic surface (Mitchell and Dean, 1995). Thus, the hydrophobic and diol signals were proposed to converge at or before protein kinase A activation. Recent work with *magB*⁻ mutants affected in an α-subunit of G protein supports these findings, because treatment with diol restored appressorium formation to wild-type levels, but

hydrophobicity alone was unable to trigger appressorium formation in these mutants (Liu and Dean, 1997). Our findings with *mac1*⁻ mutants allow us to further define this model of two parallel pathways in appressorium formation for *M. grisea* (Figure 8). The *mac1*⁻ transformants are unable to form appressoria on a hydrophobic surface or in response to diol. These data suggest a convergence of these two pathways at or before the activation of adenylate cyclase. This is in keeping with the previous model of Gilbert et al. (1996). Adenylate cyclase–deficient transformants also showed other phenotypic defects similar to loss of *magB*, including mycelial growth, conidiation, conidial germination, and sexual fertility (Liu and Dean, 1997).

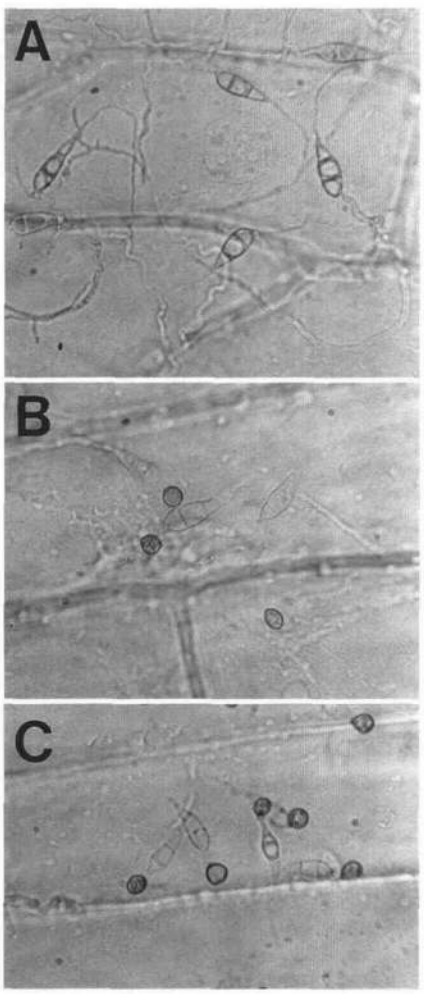

Figure 5. Defective Appressorium Formation in a *mac1*⁻ Transformant.

(A) ACT2 was germinated on an onion epidermal tissue, and hyphal growth continued without appressorium formation.
(B) Appressorium formation by ACT2 was restored in the presence of exogenous mb-cAMP.
(C) Wild-type strain 70-15 produced well-melanized appressoria on typical short germ tubes.
Cells were observed and photographed at ×400 magnification after 24 hr of incubation.

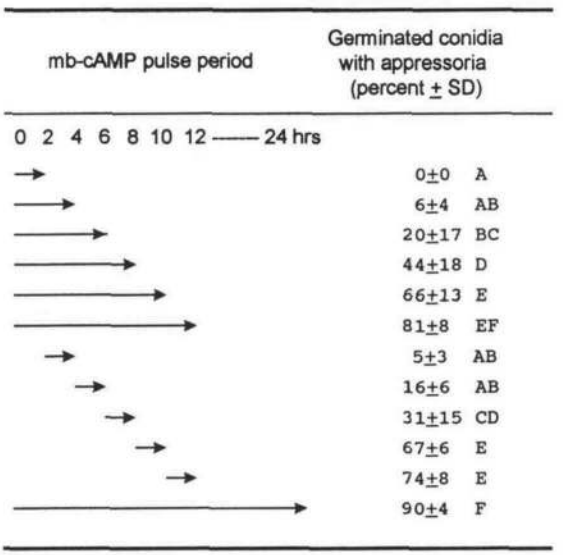

Figure 6. *Restoration of Appressorium Formation by mac1⁻ Transformant ACT2 after Pulse Exposure to mb-cAMP on the Hydrophobic Surface of GelBond Film.*

Arrows indicate the exposure period to 5 mM mb-cAMP. Appressorium formation was observed after a 24-hr incubation period. The average values plus or minus standard deviation of four independent experiments, each in three replicates, are presented. The letters to the right of the data indicate statistical significance according to Duncan's multiple range test (P = 0.05). Cells appear to become more responsive to cAMP with time.

A variety of phenotypic consequences have been noted for loss of adenylate cyclase activity in fungi, some of which correspond to the defects observed in *M. grisea mac1*⁻ strains. In *N. crassa*, mutants with low adenylate cyclase activity show small compact colonies with reduced hyphal elongation and tight clusters of dense conidia. These abnormalities were corrected in the presence of cAMP (Terenzi et al., 1976) and by the introduction of the *nac* gene encoding adenylate cyclase (Kore-eda et al., 1991b). Mutants showing vegetative incompatibility were complemented by introduction of an adenylate cyclase gene in *P. anserina* (Loubradou et al., 1996). In *U. maydis*, a mutation in the *uac1* gene, which encodes adenylate cyclase, resulted in constitutive filamentous growth rather than budding growth (Gold et al., 1994). The capability to form fruiting bodies was related to the adenylate cyclase activity in *Coprinus macrorhizus* (Uno and Ishikawa, 1973). Mutations in the adenylate cyclase gene in *S. cerevisiae* caused cells to arrest in the G_1 phase of the cell cycle, and normal growth was restored by addition of exogenous cAMP (Matsumoto et al., 1982, 1983). In *Schizosaccharomyces pombe*, disruption of the adenylate

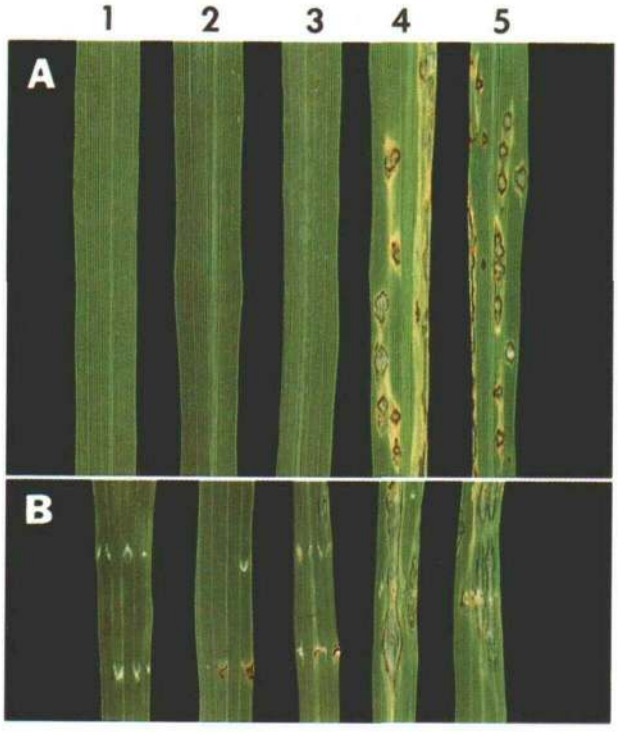

Figure 7. Pathogenicity Tests of *mac1*⁻ Transformants on Susceptible Rice Cultivar S-201.

(A) Plants were sprayed with conidial suspensions.
(B) Plants were injected into stems with conidial suspensions.
Treatments are as follows: 1, water control; 2 and 3, *mac1*⁻ transformants ACT2 and ACT6, respectively; 4, ectopic transformant ACT3; and 5, wild-type 70-15.

cyclase gene resulted in reduced growth and a tendency to enter the sexual reproduction pathway in rich medium (Maeda et al., 1990). Thus, it is perhaps not completely surprising that loss of *MAC1* in *M. grisea* affects numerous aspects of growth and development.

Adenylate cyclase–deficient *cr-1* mutants of *N. crassa* are known to accumulate modifier mutations periodically that change the morphology of the mutants to that of wild type (Garnjobst and Tatum, 1970), thus requiring periodic reisolation from stock cultures to maintain modifier-free *cr-1* mutants. In the case of *mac1*⁻ transformants, stability of the mutant phenotype has been demonstrated through at least 15 vegetative transfers over several months.

The major goal of this research is to further our understanding of the role of cAMP and the cAMP-dependent protein kinase pathway during development and pathogenesis in *M. grisea*. It will be interesting to explore how this pathway relates to the mitogen-activated protein kinase pathway that recently was shown by Xu and Hamer (1996) to be involved in regulating appressorium formation. With the adenylate cyclase and other genes in hand, we will focus further work on the interaction of gene products and additional dissection of the signaling pathways stimulated by physical and chemical cues.

METHODS

Fungal Strains and Cultural Conditions

Magnaporthe grisea 70-15 (MAT1-1) and 70-6 (MAT1-2), provided by A.H. Ellingboe (University of Wisconsin, Madison), were used as wild-type strains. All *M. grisea* cultures were maintained on V8 juice agar media (4% V8 juice and 1.5% agar, pH 7.0, with or without selection) with constant fluorescent lighting at 22 ± 1°C for conidiation. Mycelia for protoplast preparation and DNA isolation were prepared by culturing in CM liquid media (6 g of yeast extract, 6 g of casein acid hydrolysate, and 10 g of sucrose per liter) with shaking at 28°C in the dark for 2 or 3 days. After initial screening, all selected transformants were single spored, and dried cultures were prepared for long-term storage.

Polymerase Chain Reaction

Four degenerate primers were designed from highly conserved regions of adenylate cyclase genes from several filamentous fungi, and polymerase chain reaction (PCR) was performed with *M. grisea* 70-15

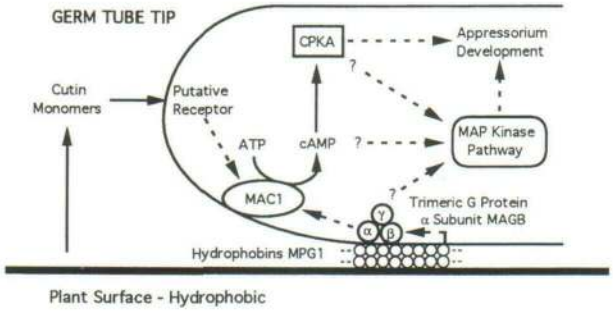

Figure 8. A Model of Signaling Systems Regulating Appressorium Development in *M. grisea*.

At least two different environmental cues trigger appressorium formation via separate and distinct signal transduction pathways: surface hydrophobicity and host cutin monomers. Surface hydrophobicity is detected by means of MPG1 (Beckerman and Ebbole, 1996), a hydrophobin protein, which conveys the hydrophobicity signal into the germ tube tip. This signal may then activate the trimeric G protein α subunit MAGB, leading to activation of the adenylate cyclase protein MAC1 and initiating the release of two active catalytic subunits, CPKA, which then presumably initiates a phosphorylation cascade. Alternatively, CPKA may directly phosphorylate transcription factors leading to new gene transcription. Elevated cAMP levels are also implicated in the activation of a MAP kinase cascade. Activation of both the PKA and MAP kinase cascades is required for the production of mature, fully functional appressoria.

genomic DNA. The primers are as follows: MAC1F, 5′-CGGATCCAC(A/C)GACATCAA(A/G)AACTC-3′; MAC2F, 5′-GA(A/G)GTCAAGAC(A/G)GAAGG-3′; MAC1R, 5′-GGAATTCATCTG(C/T)CC(G/T)CCATC(A/C)GC-3′; and MAC2R, 5′-T(C/G)GG(G/T)CCATA(A/G)TA(A/G)TCC-3′. The two outside primers, MAC1F and MAC1R, contain restriction sites (underlined) of BamHI and EcoRI, respectively. Each primer was used alone or in combinations of forward and reverse pairs. PCR was performed with Tfl polymerase (Epicentre Technologies Corp., Madison, WI) using the following cycling parameters: 5 min at 94°C, followed by 35 cycles (1 min at 94°C, 1.5 min at 50°C, and 2 min at 72°C), with a final extension of 72°C for 10 min. The PCR product was gel isolated using a GeneClean II kit (BIO-101, Inc., Vista, CA) and cloned using the pGEM-T Easy Vector system (Promega, Madison, WI).

DNA Isolation and Manipulation

Bacterial artificial chromosome (BAC) DNA was isolated by a BAC DNA minipreparation method (Woo et al., 1994). Genomic DNA of *M. grisea* was isolated as described previously (Yelton et al., 1984). Restriction digestion, agarose gel fractionation, cloning, and DNA gel blotting were performed according to the standard methods (Sambrook et al., 1989). Hybridization was carried 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 performed at 55°C. Membranes were washed down to between 2 and 0.1 × SSC, 0.1% SDS, before being exposed to autoradiographic film.

Nucleotide Sequencing of *MAC1*

BAC clones hybridizing with the PCR clone were digested with HindIII, and the three fragments hybridizing with the *nac* gene were subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA). Nucleotide sequence of these three HindIII fragments was determined using a DNA sequencing kit (Perkin-Elmer Corp., Norwalk, CT) on an AB373 DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) by using a strategy of subcloning and primer extension.

Construction of *MAC1* Gene Disruption

A gene disruption plasmid, pWBC1 (Figure 4), was constructed by inserting a 2.4-kb XbaI-HindIII fragment of pMAC10 containing the 3′ flank end of *MAC1* into the XbaI-HindIII sites of pAN7-1, which contains the hygromycin B resistance gene (Punt et al., 1987), to produce plasmid pANXH. A 3.7-kb BamHI fragment of pMAC4 containing the 5′ flank end of *MAC1* was then inserted into the BglII site of pANXH, resulting in pWBC1. The orientation of the BamHI fragment was determined by restriction analysis.

Transformation

Protocols for protoplast formation and transformation were adapted from established procedures (Leung et al., 1990; Sweigard et al., 1992). Mycelia were prepared as described above and harvested using sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA). After washing with sterile water and then 1 M sorbitol, the mycelium was suspended in 30 mL of 1 M sorbitol with Novozyme 234 (100 mg/g mycelium; InterSpex Products Inc., Foster City, CA). After 2 hr of agitation at room temperature, protoplasts were collected using sterile cheesecloth and pelleted by centrifugation (4500*g* for 5 min). Protoplasts were resuspended in STC (1 M sorbitol, 50 mM Tris-HCl, pH 8.0, and 50 mM $CaCl_2$) and adjusted to a concentration of 5×10^7 protoplasts per mL. Five micrograms of plasmid DNA (either linearized with HindIII or nonlinearized DNA) was mixed with 1.0×10^7 protoplasts in 200 μL of STC for 10 min before 1 mL of PTC (40% polyethylene glycol 4000 in STC) was gently added. After 20 min at room temperature, 3 mL of TB3 (3 g of yeast extract, 3 g of casein acid hydrolysate, 10 g of glucose, and 200 g of sucrose per L) was added and incubated for 6 hr at room temperature to allow the cells to recover. Twenty milliliters of molten TB3 agar with 200 μg/mL hygromycin B was mixed with the treated protoplast mixture and plated onto a Petri dish. After incubation for 1 week at 28 ± 2°C in the dark, transformants were transferred to V8 juice agar plates with selection and purified by single spore isolation.

Mating

Sexual crosses of transformants were performed by placing a mycelial plug of the test strain 4 cm from a mycelial plug of 70-6 on an oatmeal agar plate. A plug of 70-15 was also placed equidistant from the other plugs as a control. Plates were incubated at 22 ± 1°C with constant fluorescent lighting for 3 to 4 weeks until mature perithecia were evident.

Appressorium Formation Assay

Conidia were harvested from 8- to 10-day-old cultures on V8 juice agar plates in sterile distilled water and adjusted to 10^5 conidia per mL. Drops (50 μL) were placed on hydrophobic and hydrophilic sides of GelBond film (FMC Corp. BioProducts, Rockland, ME), onion skins, or rice leaves supported by slide glass, then placed in a moistened glass box and incubated at 22 ± 2°C (Mitchell and Dean, 1995). The percentage of germinated conidia forming appressoria was determined by microscopic observation after 12, 24, and 48 hr, unless otherwise indicated. For the monobutyryl-cAMP (mb-cAMP) assay, 5 μL of a 50 mM mb-cAMP stock dissolved in water was mixed with 45 μL of conidial suspension and then placed on an assay surface. For 3-isobutyl-1-methylanthine (IBMX) and diol assays, 0.5 μL of concentrated stocks (250 mM and 100 μM in ethanol, respectively) was mixed with 49.5 μL of conidial suspension and then placed on the assay surface. One percent ethanol was used as a control in IBMX and diol assays.

Pathogenicity Assays

For the spray inoculation test, 10 mL of conidial suspension (2×10^5 conidia per mL) containing Tween 20 (0.05% final concentration) was sprayed onto 2-week-old susceptible rice plants (variety S-201). Inoculated plants were placed in a dew chamber (>95% of relative humidity) at 25 ± 5°C for 24 hr in the dark, then kept for 5 to 10 days with a photoperiod of 15 hr using fluorescent lights. For injection inoculations, 100 μL of conidial suspension was injected into stems of 2- or 3-week-old rice, using a 26G1/2 needle (Becton Dickinson Labware, Franklin Lakes, NJ), and the development of lesions was examined after 5 to 10 days of incubation as described above for the spray

inoculations. In the case of *mac1*$^-$ transformants, the lesions were excised, surface-sterilized with 70% ethanol, and placed on V8 juice agar with selection (hygromycin) to confirm that the lesion was caused by the hygromycin-resistant adenylate cyclase–deficient mutants.

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