Analysis on pathogenesis-related morphogenesis in *Magnaporthe grisea*

（イネいもち病菌の感染に関する形態形成）

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INTRODUCTION

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Rice blast disease is one of the most devastating antagonists to rice production throughout the world, although it is especially prevalent in temperate and tropical areas. It has been estimated that rice blast disease may reduce global rice production by as much as 50 % every year, depending on prevailing weather conditions (http://agronomy.ucdavis.edu/uccerice/, UC cooperative extension rice project). This disease is a major threat to rice production in Japan (Fig. I-1), as numerous Japanese famines have been directly attributed to outbreaks of rice blast disease. The most recent and serious outbreak of rice blast disease occurred in 1993, a year that featured an unusually cool summer. Crop losses that year were estimated at 25% (http://www.toukei.maff.go.jp; Whitepaper 1994, Ministry of Agriculture, Forestry, and Fisheries of Japan). Control of the rice blast disease has largely relied on fungicides, but the use of such chemical agents has been declining in recent years due to many economical, ecological, and health issues that are associated with their application. It is desirable to develop new environmentally-friendly strategies that can supplement or replace the use of fungicides.

Magnaporthe grisea as a model system to study fungal-plant interactions

The rice blast disease is caused by the ascomycetal fungus *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Saccardo), a heterothallic hermaphroditic fungus whose mating type is controlled by two alleles at the *Mat1* locus (Kang *et al.*, 1994). Early attempts at understanding the genetics and molecular biology of *M. grisea* met with little success, since most of the *M. grisea* field isolates had heretofore been found to be female sterile (Itoi *et al.*, 1983; Kato and Yamaguchi, 1982; Yaegashi and Yamada, 1986). These difficulties have recently been overcome by the successful production of fertile laboratory strains (Kato and Yamaguchi, 1982;

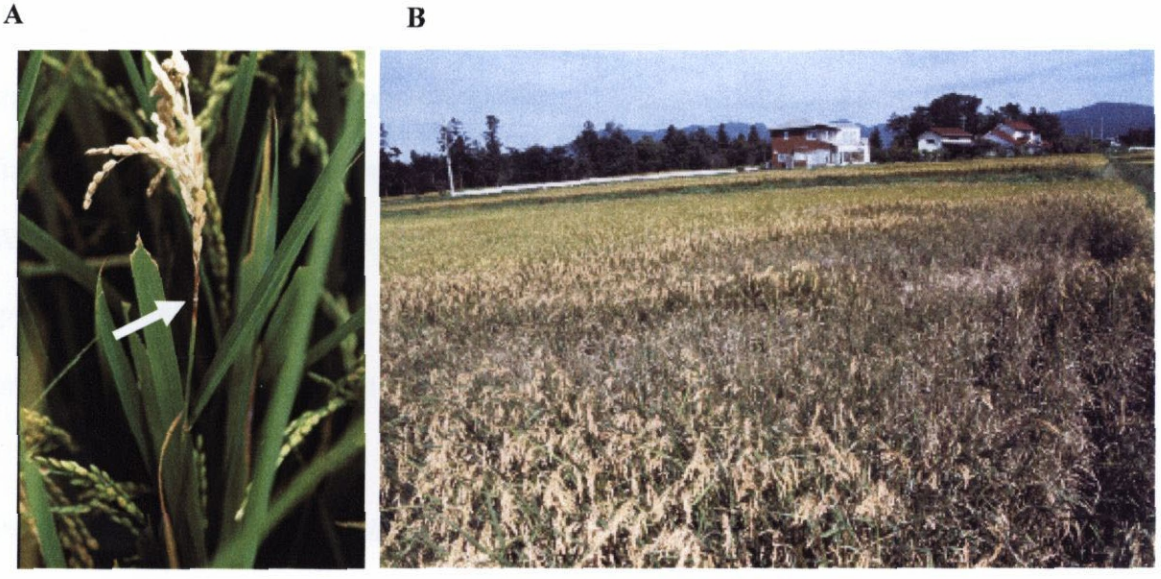


Fig. I-1. Rice blast damage on rice fields.

A. A typical neck blast on a rice plant. The arrow indicates the position of an infected node.

B. A rice field infected with the rice blast fungus showing the spread of the disease typified by the total decimation of plants in the foreground. Photos were taken in Kohriyama, Japan in 2003.

Yaegashi, 1977; Kolmer and Ellingboe, 1987; Chao and Ellinboe, 1991; Leung *et al.,* 1988; Valent and Chumley, 1991; Notteghem and Silue, 1992), and the development of reliable fungal transformation systems (Parsons *et al.,* 1987; Leung *et al.*, 1990). The study of the rice blast fungus has been further bolstered by the recent completion and annotation of the *M. grisea* genome. With the advanced tools and systems that are available for the extensive and detailed study of *M. grisea,* it is hardly surprising that this fungus has become one of the most popular experimental organisms among fungal plant pathogens.

Pathogenicity of *Magnaporthe grisea*

The genus *Pyricularia* has a wide host range among monocotyledonous plants that includes rice, wheat, barley, maize, millet, bamboo, ginger, and forage grasses. Individual strains within the genus *Pyricularia* have narrow host ranges, infecting only one or a small number of plant species. For example, rice pathogenic *Pyricularia grisea* (telemorph: *Magnaporthe. grisea*) can also infect barley and maize (Yamanaka and Yamaguchi, 1987). Rice pathogenic *M. grisea* strains are classified into races according to their compatibility with particular rice cultivars (Yamanaka and Yamaguchi, 1987). Avirulence (*avr*) genes exist in many fungi that have a gene-for-gene relationship with their host plant (Flor, 1971). They represent unique genetic determinants that prevent fungi from infecting plants that possess matching resistance (R) genes. Interactions between fungal elicitors and host receptors in resistant plants result in the induction of various defense responses, often involving a hypersensitive response (Lauge and de Wit, 1998).

The specificities between *M. grisea* and its host rice cultivars have recently been examined at the molecular level. Studies of the three cloned *M. grisea avr* genes *PWL2, AVR-Pita* and *ACE1* suggest that the corresponding gene products are specifically recognized by resistant plants as signs of pathogen invasion (Sweigard *et al.*, 1995; DeZwaan *et al.*, 1999; Boenert *et al.*, 2003). Pwl2, a glycine-rich, hydrophilic protein with a secretion signal peptide, induces a defense response in Weeping Lovegrass (Sweigard *et al.*, 1995). Avr-Pita is a metalloprotease with an

N-terminal secretion signal that binds specifically to Pi-ta, a resistance gene product of rice (DeZwaan *et al.*, 1999). Unlike Pwl2 and Avr-Pita, Ace1 does not possess any secretory signal peptide; Ace1 is associated with polyketides, products of secondary biosynthesis in many fungi (Yoder and Turgeon, 2001), in a polyketide synthase/nonribosomal peptide synthase cluster (Boehnert *et al.*, 1993). The varying mechanisms by which a rice plant detects and responds to invading pathogens are unknown, but it is likely that it detects primary or secondary products of *avr* genes.

Infectious life cycle of *Magnaporthe grisea*

The infectious life cycle of *M. grisea* begins when a conidium lands on the leaf surface of a rice plant and anchors itself by means of a complex carbohydrate known as spore tip mucilage (STM) (Hamer *et al.*, 1988). Germination is triggered by conidium moisture. First, the conidium extends a germ tube that terminates in an appressorium, a structure that is specific to the infectious stage. All of the cytoplasm in the conidial cell is transferred into the appressorium through the germ tube, and when completed, a specialized septum forms at the base of the appressorium (Hamer and Talbot, 1998). A mature appressorium is a dome-shaped structure with a melanin cell wall layer deposited between the plasma membrane and the chitin cell wall layer (Chumley and Valent, 1990). These cell wall layers are absent at the contact surface between the appressorium and leaf surface (Bourett and Howard, 1990). In a mature appressorium, glycerol synthesized from stored glycogen and lipids accumulates, generating a turgor pressure of 8.0 Mpa (Howard *et al.*, 1991; Thines *et al.*, 2000). The fungus penetrates the plant using this turgor pressure and a penetration peg that has formed at the appressorium-leaf contact site (Bourett and Howard). Colonization of plant cells by *M. grisea* is associated with a lesion on the leaf surface that is visible a few days after the infection event. A large number of conidia, produced by fungal conidiophores, emerge from the lesion to spread and infect new host plants (Yamanaka and Yamaguchi, 1987). A summary of the infectious life cycle of *M. grisea* is shown in Fig. I-2.

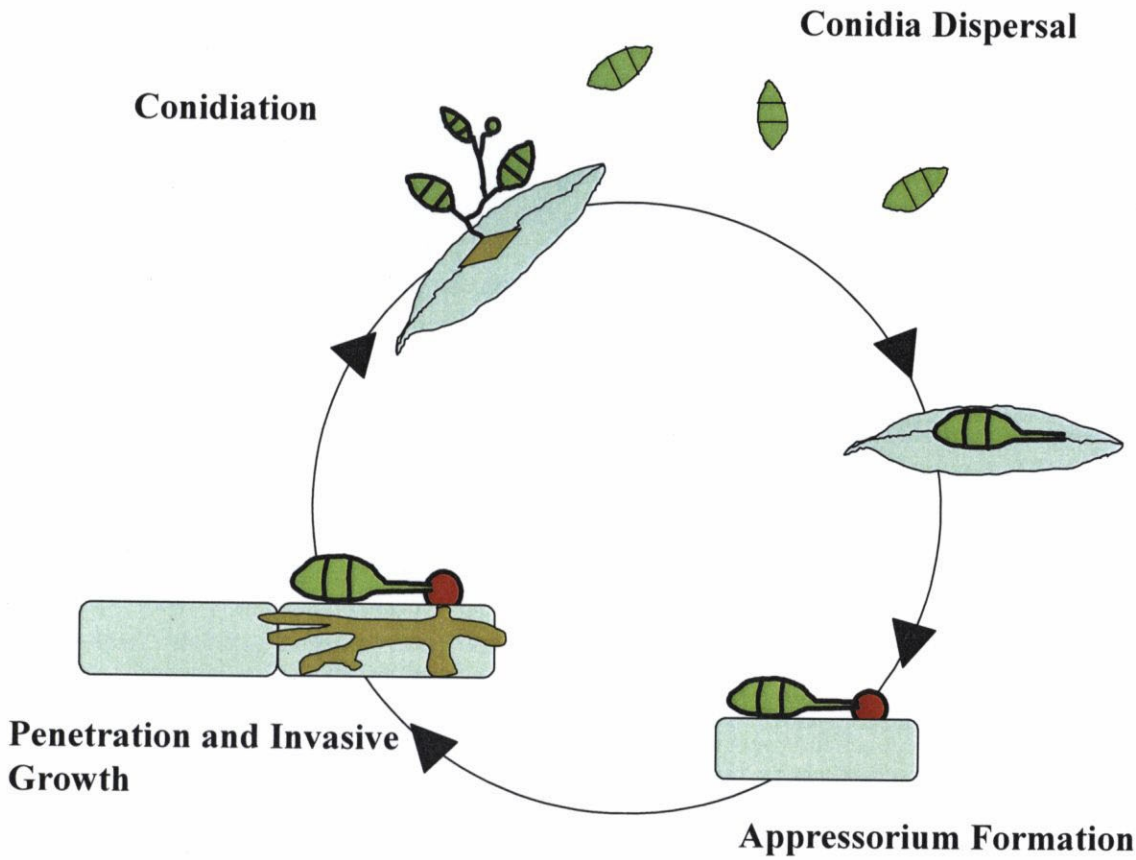


Fig. I-2. The infectious life cycle of *Magnaporthe grisea.* A description of the cycle is found in the accompanying text.

Signal Transduction Pathways in Appressorium Formation

Molecular techniques have been utilized to understand the mechanism of appressorium formation in *M. grisea*. It has been suggested that the fungus has surface sensors, since appressorium formation is regulated by surface hydrophobicity, substrate hardness, and plant wax components (Hamer *et al.*, 1988; Lee and Dean, 1993; Lee and Dean, 1994; Gilbert *et al.*, 1996). The cyclic AMP (cAMP) signaling pathway is also involved in appressorium formation, as this process is inducible on non-inductive surfaces in the presence of exogenous cAMP (Lee and Dean, 1993). Evidence accumulated from deletion mutant studies indicates the existence of at least three signal transduction pathways: cAMP; Pmk1 MAP kinase; and Mps1 Map kinase (Xu, 2000). The following is a short description of each of these pathways.

MAC1 encodes an adenylate cyclase that catalyzes the production of cAMP from ATP. The importance of a cAMP signal for appressorium formation in *M. grisea* is evident by the complete lack of appressoria in *mac1* mutants (Choi and Dean, 1997). Transmission of the cAMP signal proceeds via cAMP-dependent protein kinase A (PKA) activity and subsequent phosphorylation of target proteins. A deletion mutant *cpka*, which encodes a catalytic subunit of PKA, correlates with a delay in appressorium formation and a failure of the fungus to penetrate plant cells (Michel and Dean, 1995; Xu *et al.*, 1997). The production of appressoria by the *cpka* mutant, in contrast to the *mac1* mutant, indicates that there may be other PKAs that play a role in regulating appressorium formation (Adachi and Hamer, 1998; Xu *et al.*, 1997). It has been implied that *cpka* is defective in appressorium-mediated penetration primarily due to insufficient or retarded turgor generation (Thines *et al.*, 2000). The role of cAMP may be central to the development of functional appressorium as much as to the initiation of appressorium formation in *M. grisea*.

The Pmk1 signal transduction pathway plays a major role in appressorium maturation. *PMK1* encodes a functional homolog of yeast Fus3 and Kss1 MAP kinases (Xu and Hamer, 1996). In *Saccharomyces cerevisiae*, Fus3 and Kss1 are involved in a receptor-G-protein-coupled MAP

kinase cascade and in mediating pheromone response. Kss1 is also involved in invasive growth and cell wall integrity signal transduction pathways (Elion E A., 2000). A *pmk1* mutant is able to recognize hydrophobic surfaces and germinate, but is defective in appressorium differentiation. Instead of forming a functional, wild-type appressorium, the mutant develops a deformed structure at the tip of the germ tube. The mutant conidia respond to exogenous cAMP, as more structures form with increased amounts of exogenous cAMP, although these conidia are incapable of infecting rice. The Pmk1 MAP kinase pathway appears to regulate appressorium maturation and probably invasive growth of the fungus (Xu and Hamer, 1996).

The third known signal transduction pathway is the Mps1 MAP kinase pathway. Mps1 is functionally homologous to the yeast Mpk1 MAP kinase (Xu *et al.*, 1998). Mpk1 is activated by a PKC signal, which is important for the maintenance of cell wall integrity in *S. cerevisiae* (Elion, 2000). The Pkc1-Mpk1 signaling pathway of *S. cerevisiae* is required for bud emergence and polarization of the actin cytoskeleton (Zarzov *et al.*, 1996). The *M. grisea mps1* mutant has dramatically reduced aerial hyphal growth and conidiation. *Mps1* forms functional appressoria, and is able to colonize plants at wound sites, but the mutant is defective in appressorium-mediated penetration (Xu *et al.*, 1998). Mps1 is therefore essential for plant infection by *M. grisea* by functioning in the regulation of penetration peg formation (Xu *et al.*, 1998; Tucker and Talbot, 2001).

Genes Regulating *M. grisea* Conidiation

Conidiation in *M. grisea* is triggered by light and oxidative stresses. Conidiogenesis in *M. grisea* is a holoblastic process in which an initial conidium blows out from the conidiophore apex. A septum forms at the base of the conidium to delimit it from the conidiophore. A second conidium is produced on the tip of the next conidiophore, but extends from the side so that it lies below the first conidiophore. By repeating this conidiogenesis pattern, five to twenty conidia are arrayed on a zigzag arrangement of conidiophores (in sympodial). A mature conidium is tear-drop

shaped and consists of three compartments. Each compartment has an identical nucleus derived from a common mother cell (Cole, 1986).

The molecular mechanism of *M. grisea* conidiation is not well understood, as studies using random, insertional mutagenesis have identified only several conidiogenesis genes to date (Hamer *et al.*, 1989; Hamer and Givan, 1990; Shi and Leung, 1994; Shi and Leung, 1995; Shi and Leung, 1998). Mutations in *CON5* and *CON6* completely abolish conidiogenesis (Shi and Leung, 1995). *Con2,* and *chm1* mutants form conidia with only one or two compartments (Shi and Leung, 1995; Li and Xu, 2001). These results suggest Con2 and Chm1 are important in septum formation. Chm1, a homolog of yeast Cla4 PAK kinase (Li and Xu, 2001), also appears to play an important role in the morphogenesis of conidia, since *chm1* conidia are elongated (Li and Xu, 2001). Other mutants such as *con1*, *con4, con7* also form elongated conidia (Shi and Leung, 1994; Shi and Leung, 1995). Another conidial shape determinant is Smo1. A mutation in *SMO1* causes aberrant conidial shape with no visible axis of symmetry (Hamer *et al.*, 1989). All of the conidiation defective mutants are somewhat defective in the formation of functional appressoria (Hamer and Givan, 1990; Shi and Leung, 1995; Li and Xu, 2001). These mutant phenotypes indicate that several proteins probably contribute to a system that determines cell size (cytokinesis) and morphology (polarization of actin cytoskeleton) during conidiation.

Research Objectives

It is a tempting strategy to manage rice blast disease by blocking the fungal conidiation and appressorium formation stages, since they are critical steps in the infection process. Unfortunately, we have limited knowledge about regulation mechanisms of appressorium formation and conidiation.

In *M. grisea*, regulatory mechanisms of conidiation and appressorium formation appear to be linked, since most of the conidiation defective mutants described above fail to differentiate appressoria, and some of the appressorium formation defective mutants show a reduction in conidiation number.

To better understand the regulatory mechanisms of conidiation and appressorium formation in *M. grisea*, the functions of proteins Acr1 and Mgb1 have been studied in detail. Acr1 is predicted to play a critical role in the regulation of conidiation, due to the *ACR1* deletion mutant phenotype. Mgb1, the β subunit (Gβ) of the heterotrimeric G-protein, is similarly predicted to be a critical factor in the appressorium formation signal transduction pathway. Mgb1 may act upstream of Pmk1, since the corresponding yeast Gβ acts upstream of the MAP kinases in the Fus3/Kss1 signal transduction pathway.

In addition to probing the functions of the Acr1 and Mgb1 proteins in the isolated context of their respective predicted pathways, we have also attempted to reveal a possible cross-regulation mechanism of conidiation and appressorium formation in *M. grisea*.

SUMMARY

Rice blast disease, caused by the filamentous fungus *Magnaporthe grisea*, is one of the most devastating diseases to global rice production. The infectious life cycle of *M. grisea* starts when a conidium lands on the leaf surface of a rice plant. The conidium extends a germ tube that terminates in an infectious stage-specific structure known as the appressorium. A mature appressorium is a dome-shaped structure with a melanin cell wall layer. The mature appressorium accumulates glycerol, synthesized from glycogen and lipids stored within a conidium, to generate sufficient turgor pressure to penetrate the plant. Colonization of plant cells by the fungus is indicated by a lesion that develops on the leaf surface of the rice plant a few days after the infection event. A large number of conidia that are produced on the fungal conidiophores emerge from the lesion and spread out to infect new host plants. Previous studies have demonstrated that cAMP signalling and Pmk1 MAP kinase are essential for appressorium formation, and have indicated a few genes in *M. grisea* conidiogenesis, however most of the genes involved in regulatory mechanisms of appressorium and conidium formation and morphogenesis have yet to be revealed.

The fungus has seven chromosomes and approximately 11,000 genes spanning 38 Mb. The bacterial artificial chromosome (BAC) cloning system has been superior to other vector systems in the cloning of large genomic DNA fragments. In this regard, we have constructed an *M. grisea* BAC library. Our *M. grisea* BAC library consists of 5760 clones with the insert size ranging from 35 to 175 kb and averaging 120 kb. The library size is equivalent to approximately 18 genomes, thereby covering more than 99.999% of the genome. Total library clones were arranged onto two nylon membranes for efficient screening. Control hybridization with a single-copy RFLP marker result in ten positive clones, whose restriction patterns indicate no chimerality or deletions. This BAC library is exploited to facilitate the following studies of genes regulating conidiation and appressorium formation.

To understand the regulation mechanism of conidiation, we have isolated three *M. grisea* morphological mutants that form conidia in chains. These mutants have insertions of the LINE transposon MGL at the same position in the *ACR1* locus. Acr1 is a functional homolog of MedA, a conidiophore-developmental regulator in *Aspergillus nidulans*. The loss-of-function Acr1 mutants produce chains of elongated conidia in a head-to-tail array, while the wild type conidia are produced in a sympodial array. In *acr1*, appressoria production is greatly reduced and the appressoria are defective in plant penetration. The reductions in the appressorium formation and penetration rates in the mutant are not complemented by the addition of exogenous cAMP. To investigate the genes upregulated by Acr1, a nylon membrane arrayed with subtraction libraries constructed from conidial mRNA of *acr1* and its isogenic wild-type strain Guy11 were subjected to a reverse Northern dot blot hybridization analysis. The results from this and subsequent RT-PCR analyses indicate that the expression of the *MPCL1* and *MGPH1* genes is positively regulated by Acr1.

MPCL1 encodes the *Saccharomyces cerevisiae* Pcl2 subfamily homolog, and *MPGH1* encodes the glycogen phosphorylase gene. Since Pcl2 subfamily members play important roles in cell polarity and morphogenesis, it is likely that the defective morphology (mutant phenotype) conferred by *acr1* is partially due to the reduction of *MPCL1* expression. Glycogen phosphorylase catalyzes the degradation of glycogen and cytological analysis indicates that glycogen mobilization is significantly reduced in *acr1*conidia. These results imply that *acr1* is defective in penetration due to its inability to convert glycogen reserves in the conidia into sufficient amounts of glycerol required for the generation of appressorium turgor pressure.

In eukaryotes, trimeric G-proteins (Gα, Gβ, and Gγ subunits) transmit extracellular signals to various downstream effectors (e.g. MAP kinases). In *M. grisea*, the Pmk1 MAP kinase is essential for appressorium formation and infectious growth. The *PMK1* deletion mutant fails to form appressoria but still responds to exogenous cAMP for tip deformation. Since gene disruption mutants of three Gα subunits still form appressoria, it is likely that the

Pmk1 pathway is activated by Gβ subunit in *M. grisea*. In this study, we have isolated and characterized the *MGB1* gene that encodes the *M. grisea* Gβ subunit. Mutants disrupted in *MGB1* produce fewer conidia. Conidia from *mgb1* mutants are defective in appressorium formation and fail to penetrate or grow invasively on rice leaves. Exogenous cAMP induces appressorium formation in *mgb1* mutants, but these cAMP-induced appressoria are abnormal in shape and can not penetrate into plants. The intracellular cAMP level is reduced in *mgb1* mutants, and the defects in conidiation and hyphal growth are partially suppressed with 1 mM cAMP. Transformants expressing multiple copies of *MGB1* are able to form appressoria on hydrophilic surfaces, and have increased intracellular cAMP levels. Our results suggest that Mgb1 may be involved in cAMP signalling with regards to conidiation regulation, surface recognition, and appressorium formation. Mgb1 is essential for the fungus to proceed to the infectious stage.

Our study demonstrates that Acr1 plays a critical role in conidiophore patterning and is important for the glycogen metabolism necessary to generate turgor pressure in appressoria. We also show that Mgb1, a major player in appressorium formation, is also a regulator of conidiogenesis. Our studies of *mgb1* and *acr1* raise the idea of cross-regulation mechanisms in conidiogenesis and appressorium formation in *M. grisea*. It seems likely, in light of these cross-regulation events, that Mgb1 and Acr1 are located at a point in the signal transduction pathway where the appressorium formation and conidiation regulatory signals intersect.

CHAPTER 1

Construction of a BAC Library of the *Magnaporthe grisea* Genome

INTRODUCTION

Magnaporthe grisea is a heterothallic fungus. Interestingly, throughout its evolution as a rice pathogen, most *M. grisea* strains have lost their female fertility (Yamanaka and Yamaguchi, 1987). The fungus has seven chromosomes, consisting of approximately 11,000 genes over 38 Mb (http://www-genome.wi.mit.edu/; *Magnaporthe grisea* database). Due to the large genome size, positional cloning is an ideal method to clone unknown genes of the fungus based on phenotype information.

The bacterial artificial chromosome (BAC) cloning system has become a dominant and critical tool for the physical mapping, positional cloning, and genome sequencing of large or complex genomes (Luo and Wing, 2003). The BAC system is superior to other systems due to the low rate of chimerism, high cloning efficiency, of large DNA fragments, easy handling, stability of the inserted DNA, and readiness for direct sequencing (Shizuya *et al.*, 1992). BAC libraries have been constructed using many organisms, from bacteria to human (http://www.ncbi.nlm.nih.gov/entrez, National Institute of Health; http://www.genome.gov/, National Human Genome Research Institute).

In this chapter, the construction of a BAC library of the *M. grisea* genome is described. This BAC library is constructed from the rice-pathogenic Chinese field isolate CHNOS60-2-3, which shows strong fertility over other rice pathogenic *M. grisea* strains (Hayashi *et al.*, 1997. This BAC library is used as a genetic tool in subsequent studies (see chapters 2 and 3).

MATERIALS AND METHODS

***Magnaporthe grisea* isolate, *Escherichia coli* strain, and plasmid:** Chinese *M. grisea* isolate CHNOS60-2-3 (*MAT 1-1*), which has strong fertility, was used to prepare High Molecular Weight (HMW) DNA for use in the construction of the BAC library. This isolate was obtained from Yunnan Province of China, where most isolates are highly fertile (Hayashi *et al.*, 1997). The *E. coli* strain DH10B (Invitrogen) was used for electroporation of the BACs. The modified BAC vector pBAC-*lac*, which carries a *lac Z* fragment of M13mp18, was used as a vector (Kim *et al.*, 1996). Purification, *Hin*dIII digestion, and dephosphorylation by calf intestine phosphatase (Takara) of pBAC-*lac* were performed as described by Nakamura *et al.* (1997).

Preparation of high molecular weight DNA: HMW DNA was prepared following the protocol of Shizuya *et al.* (1992) with some modifications. 100 ml of YG liquid medium in a 300 ml flask was inoculated with a small piece of mycelia (1 cm^2) from a three-day-old culture grown on YG agar (0.5% yeast extract, 2% glucose, 2% agar), and was incubated with gentle shaking (90 rpm) for four days at 25 °C. Mycelia were harvested by vacuum filtration on filter paper, resuspended in 50 ml of Novozyme treatment buffer (10 mg/ml $CaCl_2$, 0.7 M sorbitol, 2 mg/ml Novozyme 234, Novo Nordisk), and incubated at 28 °C with agitation at 100 rpm for 3 h to generate protoplasts. Protoplasts were filtered through Miracloth (Calbiochem) and pelleted at 1600 x *g* for 15 min. The protoplasts were rinsed three times with 0.7 M sorbitol, repelleted, and then mixed with an equal volume of 1% In Cert (FMC) in sorbitol buffer (0.7 M sorbitol, 0.5 M EDTA). The mixture was poured into plastic molds (Bio-Rad) and solidified at 4°C for 20 min. The final density of protoplasts in agarose was $2.4x10^9$ per ml. The DNA/agarose plugs were treated with proteinase K in 50 ml of digestion buffer (1% w/v sodium lauryl sarcosinate, 0.5 M EDTA, 1 mg/ml proteinase K, Clontech) at 50 °C for 48 h with gentle agitation and one change of digestion buffer after 24 h. The digested plugs were washed with 50 ml of 0.5M EDTA for 6 h and with 50 ml of TE, pH 8.0 for 6 h, with hourly exchanges for fresh wash buffer.

Partial digestion and size fractionation of DNA: For partial digestion, 25 mg of the DNA/agarose plug was equilibrated with 200 µl of *Hin*dIII digestion buffer (10 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM NaCl, 0.01% w/v BSA) containing 0.1 unit of *Hin*dIII at 4°C, overnight. One hour previous to the digestion, 10 mM $MgCl_2$ was added to the buffer on ice and diffused into the plug. The DNA in the plug was digested by incubating at 37°C for 1 h, and the reaction was stopped on ice by addition of EDTA to a final concentration of 50 mM. The digested DNA was fractionated in a single 1% Sea Plaque GTG agarose gel (FMC) by CHEF electrophoresis in 0.5 x TBE at 10 °C, with two-phase pulse time: a constant pulse time of 90 s for 5 h followed by 6 s for 12 h at 0.6 V/cm.

BAC library construction: A BAC library was constructed following the procedures of Nakamura *et al.* (1997) and Asakawa *et al.* (1997) with some modifications. The fractionated DNA fragments ranging from 200 kb to 500 kb were excised from the electrophoresed Sea Plaque GTG agarose gel and equilibrated with TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) for 6 h with an hourly buffer change. NaCl was added to a final concentration of 50 mM. The agarose slice was melted at 68 °C for 10 min, and digested with 1 U of GELase (Epicentre) per 25 mg of agarose gel at 42 °C for 1 h. The solution was directly used for ligation with dephosphorylated, *Hin*dIII-digested pBAC-*lac*. Insert DNA (added as 50 µl at 10 ng/µl with an average size of 350 kb) was ligated to the vector DNA at an insert to vector ratio of 1:1, using 0.042 Weiss units of T4 DNA ligase (NEB) in a final volume of 70 µl of 1x ligation buffer supplied by the manufacturer. After overnight incubation at 16 °C, the ligation mixture was drop-dialyzed at 4 °C against 0.2 x TE for 1 h on a VSWP02500 filter (Millipore). Electrocompetent *E. coli* DH10B cells were prepared as recommended by the supplier (Invitrogen). 40 µl of thawed DH10B cells was mixed with 5 µl DNA and placed in a 0.1 cm cuvette for electroporation using a Electro Cell Manipulator 600 M (BTX) at 129 Ohm, 1.25 kV and 25 µF. Following electroporation, cells were immediately incubated with agitation in 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl,

0.02M glucose, 0.01M $MgCl_2$, 0.01M $MgSO_4$) for 1 h at 37 °C and plated onto LB plates (1% NaCl, 0.5% yeast extract, 1% tryptone, 2% agar) containing 12.5 μg/ml chloramphenicol, 50 μg/ml X-gal and 12.5 μg/ml IPTG. After 24 h of incubation, white colonies were selected and placed in microtiter plates containing 150 μl LB freezing buffer (1% NaCl, 0.5% yeast extract, 1% tryptone, 36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 10% v/v glycerol, 12.5 μg/ml chloramphenicol). After 24 h of growth at 37 °C, the microtiter plates were stored at -80 °C.

Preparation and analysis of BAC DNA: The BAC DNA was prepared from 1.5 ml overnight LB cultures containing 12.5 μg/ml chloramphenicol using a PI 100 Sigma plasmid isolator (Kurabo). The DNA was dissolved in 100 μl TE. For insert size estimation, 10 μl BAC DNA was digested with *Not*I for 1 h and electrophoresed in a 1% CHEF gel in 0.5 x TBE at 6 V/cm with a ramped pulse time of 5 to 15 sec for 17 h.

Preparation of high-density replica membranes: For hybridization analysis, high-density replica membranes were prepared as described by Nakamura *et al.* (1997). A total of 6144 clones (5760 BAC + 384 pBR 328 clones) were replicated from 56 microtiter plates onto two sheets of Biodyne B nylon membrane (8 cm x 12 cm) using the BioMek2000 automated workstation (Beckman).

DNA probes: A single-copy RFLP marker NH165 was used to test the representation of the genomes in the rice blast BAC library.

Hybridization analysis: Labeling of probes and Southern hybridization were performed by ECL direct nucleic acid labeling and detection systems (Amersham) according to the manufacturer's instructions. Hybridized membranes were washed with primary wash buffer (6 M urea, 0.4% SDS, 0.5 x SSC) (1 x SSC: 0.015 M Na_3 citrate, 0.15 M NaCl pH 7.0) at 42 °Cand secondary wash buffer

(2 x SSC) at room temperature, and then exposed to X-ray film.

RESULTS AND DISCUSSION

Construction of a *Magnaporthe grisea* BAC library

A 5760-clone BAC library of the *M. grisea* genome was constructed from mycelial protoplasts of strain CHNOS60-2-3. The mycelia were harvested before melanization to allow a high efficiency of digestion with Novozyme 234. The partially digested HMW DNA was CHEF electrophoresed on a single gel with two phases of pulse time (90 sec and 6 sec) to fractionate the 200-500 kb range. The cloning efficiency using this size range for insertion was approximately $1x10^6$ clones/μg vector.

Insert size distribution of the BAC clones

The insert size of 100 randomly selected clones was estimated by *Not*I digestion (Fig. 1-1). Ninety-five percent of the clones contain inserts. The estimated insert size ranges from 35 to 175 kb, with an average of 120 kb (Fig. 1-2). The observation that the average insert size is lower than the size of fractionated DNA has been made by others (Nakamura *et al.*, 1997; Asakawa *et al.*, 1997; Zhu *et al.*, 1997).

Size fractionation of the digested HMW DNA greatly affected the insert size of the library. Before this study, we tested the relationship between average insert size and size fractionation conditions. Using HMW DNA compressed to more than 50 kb yielded an average insert size of 60 kb, while compressed DNA ranging from 200 to 500 kb yielded an average insert size of 120 kb.

Stability is one of the advantages of BAC libraries (Shizuya *et al.*, 1992). This *M. grisea* BAC library is also stable as shown by identical *Not*I digestion patterns after more than one hundred generations (data not shown).

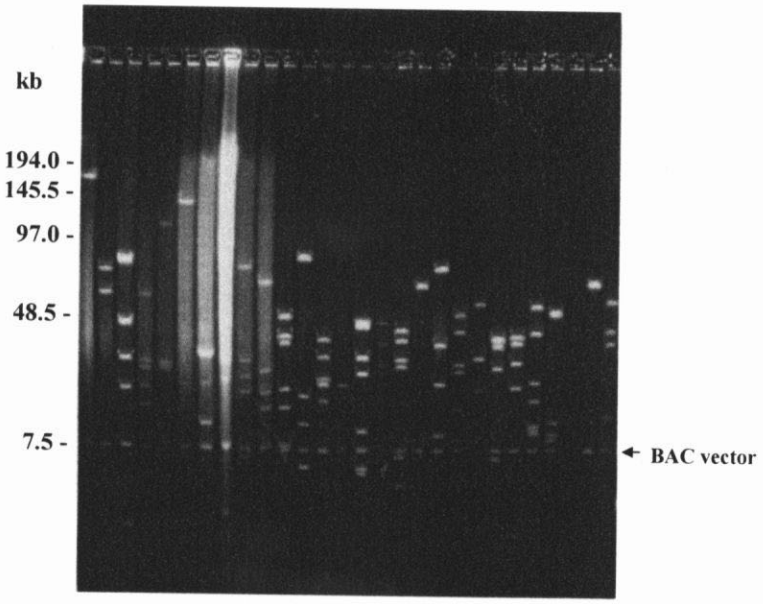


Fig. 1-1. Analysis of the insert size of the BAC clones by CHEF electrophoresis. BAC clones were digested with *Not*I. The BAC vector is indicated by an arrow.

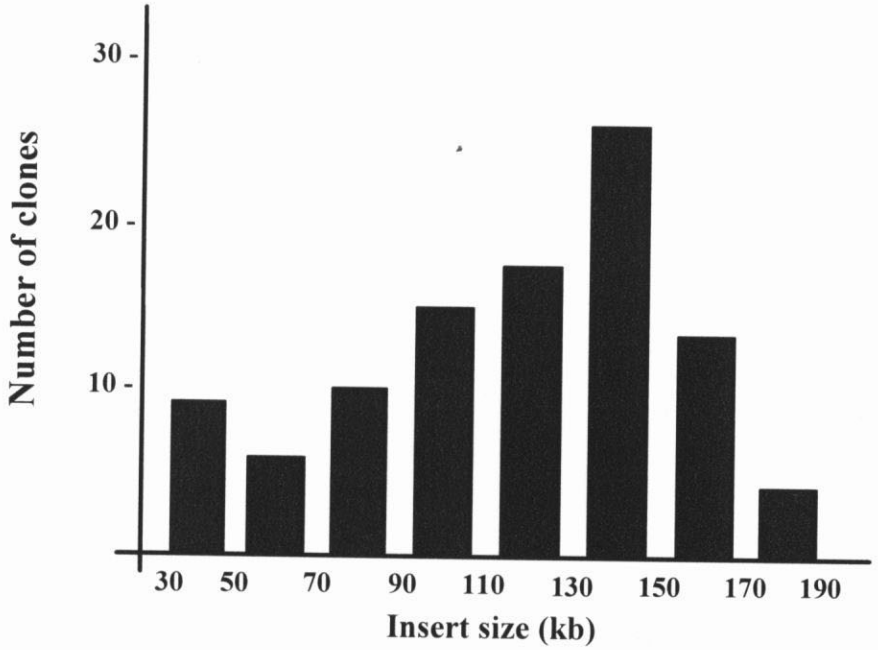


Fig. 1-2. Insert Size Distribution of the BAC Library. One hundred randomly selected BAC clones were digested by *Not*I. The insert size varies from 35 to 175 kb with an average of 120 kb.

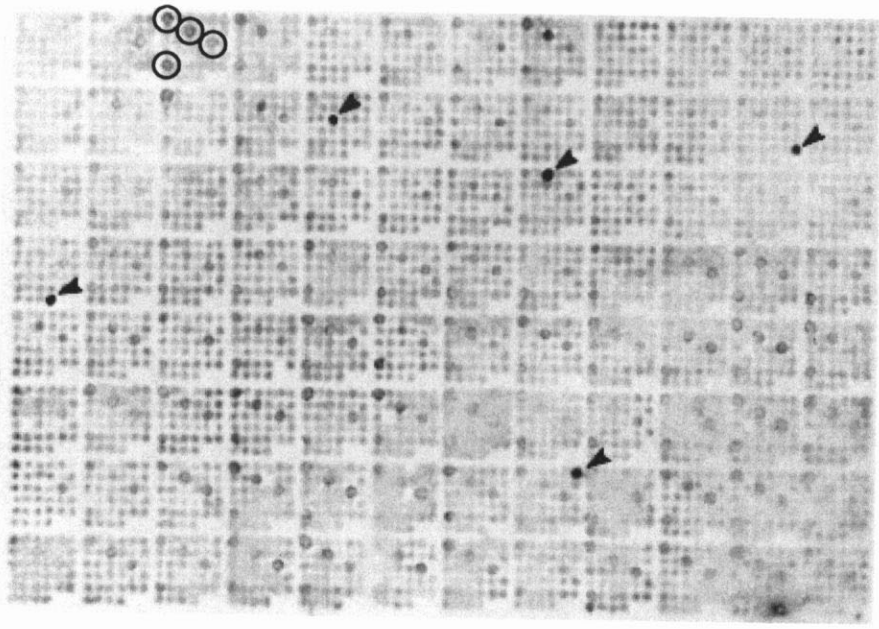


Fig. 1-3. Hybridization of the BAC Clones on the High Density Membrane with an RFLP Marker. On one membrane, 2880 BAC clones were arrayed in 6 x 6 point-matrices on the 96 well microplate. The size of the membrane is 8 x 12 cm. Clones hybridized with the single-copy RFLP marker NH165 are indicated by arrows. The content of DNA in one row of the matrices corresponds to the content of one genome of *M. grisea*. Circles in a minimatrix on the top row indicate marker positions.

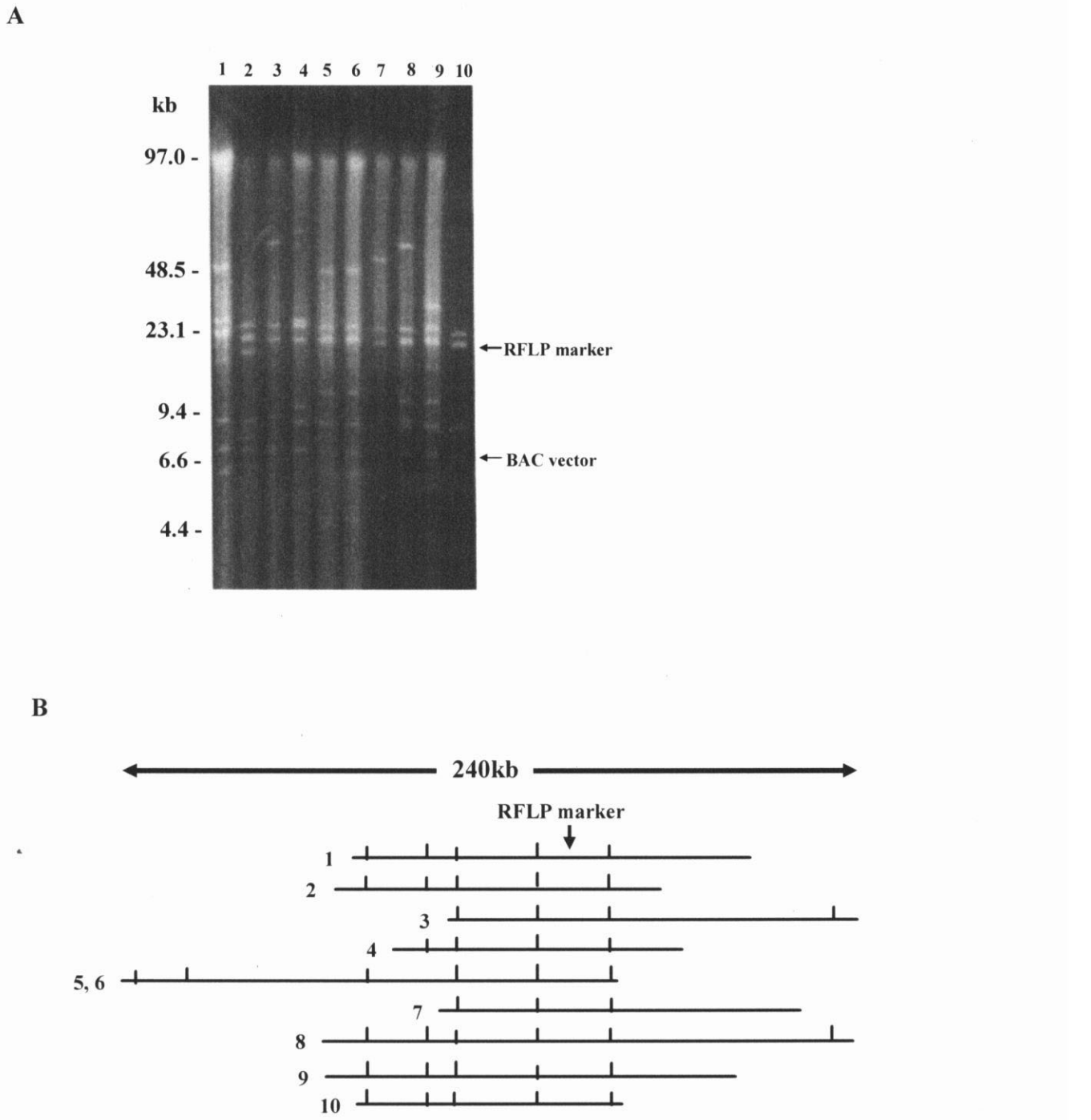


Fig. 1-4. **A.** *Not*I digestion patterns of the ten clones containing the RFLP marker NH165. The position of the 23-kb band hybridized with the RFLP marker is indicated by an arrow. The 7.5 kb BAC vector is also shown by an arrow. In lanes 1, 2, 5, 6, 8, 9 and 10, the 23-kb band and accompanying 21-kb band form a doublet. **B.** The *Not*I restriction map of the BAC clones containing NH165. The construction of the map is described in Materials and Methods. The numbers on the left of the bars correspond to the lane number in Fig. 1-4 A. The vertical bars represent *Not*I restriction sites. The 23-kb *Not*I fragment to which the RFLP probe hybridizes is indicated by an arrow. The ten clones cover 240 kb.

Library coverage

The size of the library is estimated to be equivalent to approximately 18 genomes, considering a genome size of 38 Mb (Hamer *et al.*, 1989). The probability of covering the entire genome is greater than 99.999%.

High-density replica membranes

For efficient colony hybridization, the BAC clones were replicated at high density on two nylon membranes of microplate size (8 x 12). Each membrane is composed of 96 matrices each of which contains a 6 x 6 point-matrix lacking two points. It is arranged with 2880 BAC clones at 30 clones per matrix and with four position markers of pBR328 in a matrix (Fig. 1-3). The two membranes represent a total of 5760 BAC clones. All BAC clones were easily screened by a one-step method using the membranes.

Representation of genome coverage in the BAC library

To test the representability of the library, the membranes were screened with a single-copy RFLP marker, NH165, linked to avr *ta*, which corresponds to the *Pi-ta* gene for blast resistance in rice (Fig. 1-3). Ten clones hybridized with the marker. This result is consistent with the estimated coverage of the library. The *Not*I digestion patterns of these clones show that they share 25-kb and 23-kb fragments (Fig. 1-4A). The RFLP marker hybridized to the 23-kb fragment. The *Not*I restriction maps of these clones were constructed with *Not*I digestion patterns and Southern hybridization patterns using these clones as probes. The maps show that the ten clones covered a 240-kb region around the RFLP marker (Fig. 1-4B). This result indicates the absence of chimerism and deletion.

CHAPTER 2

Appressorium Maturation in *Magnaporthe grisea* is Controlled by a Conidiophore Developmental Modifier Acr1

INTRODUCTION

In filamentous fungi, asexual reproduction (conidiation) is triggered by environmental stimuli such as nitrogen/carbon starvation, light signals, and exposure to air (Miller, 1990; Adams *et al.*, 1998). Under these stimuli, a fungus develops conidiophores that are capable of conidiation. Each fungus has a specific conidiophore-and-conidia developing pattern, termed 'conidiogenesis pattern', under a defined condition.

Like other fungi, conidiogenesis patterning is highly controlled in *M. grisea*. Conidiogenesis in *M. grisea* begins when the tip of the conidiophore blows out and grows to be a pyriform conidium with two septa. The next conidium swells outward from the apex of a new conidiophore, which originates from the side and below the first conidiophore. This conidiogenesis process is repeated several times to produce a cluster of conidia in a sympodial array on a mature conidiophore (Cole, 1986; Shi and Leung, 1995). A mature conidium is a three-celled structure, each containing a nucleus that is mitotically derived from a common mother nucleus (Shi and Leung, 1995; Adachi and Hamer, unpublished data). As such, the three nuclei are identical to one another.

To understand the regulation mechanisms of conidiogenesis patterning in *M. grisea*, several conidial morphogenetic mutants have been isolated and studied (Hamer *et al.*, 1989; Hamer and Givan, 1990; Shi and Leung, 1994; Shi and Leung, 1995; Lau and Hamer, 1998). The *Smo* mutants form irregularly shaped conidia (Hamer *et al.*, 1989; Hamer and Givan, 1990). A null mutation in *ACR1* causes an indeterminate growth of the conidial tip cell (Lau and Hamer, 1998). Mutations engineered in several of the epistatically related *CON* genes (e.g. *CON2, CON5, CON6, CON7*) (Shi and Leung, 1998) resulted in a number of altered conidial

phenotypes. These include the abolishment of conidiation (*con5* and *con6* mutants), the production of abnormally elongated conidia (*con1*, *con4* and *con7* mutants), and the requirement of direct illumination for conidiation (*con2* mutant) (Shi and Leung, 1995). Two features common among these conidial morphogenetic mutants are the inability to form appressorium and the loss of pathogenicity (Hamer and Givan, 1990; Shi and Leung, 1995; Lau and Hamer, 1998). Although it seems certain, given these observations, that these genes play significant roles in conidiogenesis, a core regulatory pathway in conidiogenesis remains poorly understood.

Aspergillus nidulans is a model fungus for the study of conidial development and the molecular mechanisms of conidiophore patterning and conidiation are well studied. In this fungus, the conidiophore vesicle produces two types of sterigmata known as metulae and phialides. Chains of conidia are budded off from the latter of the two by a process that is coupled to mitotic divisions (Miller, 1990; Ye *et al.*, 1999). Genetic and biochemical studies have led to the identification of the core regulatory pathway in conidiophore development, and it involves a process that is driven by BrlA, a Zinc-finger transcriptional factor (Adams *et al.*, 1998). BrlA is required for the transcriptional activation of developmentally regulated genes during conidiogenesis (Timberlake, 1993). The correct spatial and temporal expression of BrlA is required for normal conidiophore development (Adams *et al.*, 1988; Prade *et al.*, 1993; Busby *et al.*, 1996). The Null *BRLA* mutant fails to form conidiophores (Boylan *et al.*, 1987; Timberlake, 1993), while *brlA* alleles maintain partial BrlA functions characterized by the production of multi-tiered sterigmata and branching or secondary conidiophores (Miller, 1990). Recent studies have revealed that BrlA influences not only development related genes but also those related to cell cycle (Ye *et al.*, 1999; Schier *et al.*, 2001). In addition, BrlA is reported to upregulate the expression and activity level of $NimX^{cdc2}$, a yeast cyclin-dependent kinase (CDK) Cdc28 homolog (Ye *et al.*, 1999). PclA is a homolog of the yeast G1 cyclin Pcl1 in *A. nidulans*, and is induced by BrlA (Schier *et al.*, 2001). PclA interacts with $NimX^{cdc2}$ to likely modulate the kinase activity of $NimX^{cdc2}$during conidiogenesis (Schier and Fischer, 2002).

MedA is termed a developmental modifier because it modulates the correct temporal expression of BrlA (Busby *et al.*, 1996; Stephans *et al.*, 1999). Studies suggest that MedA interacts with BrlA to function in transcription complex stablization (Busby *et al.*, 1996). A mutation in the *MEDA* gene results in the formation of aberrant conidiophores that develop branching chains of metulae, a delay in conidial differentiation, and the frequent reinitiation of secondary conidiophores (Miller, 1990; Busby *et al.*, 1996). *MEDA* appears to be a fungal specific gene, as it has no obvious homology with other known genes (Adams *et al.*, 1998). This chapter focuses on the phenotypic analysis of loss-of-function mutants of the MedA homolog in *M. grisea*. In addition, the cross-regulation between conidiophore patterning and virulence is presented.

MATERIALS AND METHODS

Strains and growth media: The *M. grisea* strains used in this study are listed in Table 2-1. All cultures were maintained on oatmeal agar plates (3% oatmeal, 0.5% sucrose, 2% agar) at 25 °C under fluorescent light for the induction of conidiation, and stored on desiccated Whatman #1 filter paper at -20 °C as previously described (Xu and Hamer, 1996). Mycelia were collected from 5xYEG culture (0.5% yeast extract, 2% glucose) shaken and incubated at 150 rpm and 25 °C, respectively. Diameters of colonies formed from single conidia were measured following their incubation on oatmeal agar plates for 8 days at 25 °C.

Crossing and ascospore isolation: Crosses were carried out as described by Hayashi *et al.* (1997). Briefly, two parental strains were paired on oatmeal agar at 20 °C under a fluorescent light for three weeks to induce perithecia development. Matured perithecia were gently crushed on a glass slide with a drop of distilled water to release the asci. Each ascospore was isolated from the asci using a fine glass needle, and individually transferred to oatmeal agar plates.

Germination, appressorium formation, and penetration assays: Agar blocks containing mycelia were transferred onto 2% water agar plates and incubated at 25 °C to facilitate the observation of conidiation patterns. For germination and appressorium formation assays, 30μl of conidial suspension (10^3 conidia/ml) on hydrophobic surfaces (plastic coverslips) or hydrophilic surfaces (GelBond membrane; FMC Bioproducts) were incubated at 25°C up for 48 hours. To determine the effects of cAMP on germination and appressorium formation, exogenous cAMP was added to the conidial suspension at a final concentration of 10mM. For penetration assays, a conidial suspension was applied onto onion epidermal cells and incubated at 25 °C for 48 hours. Photomicrographs of fungal conidiation were taken using a BX-50 system (Olympus).

Infection assays: Infection assays were performed using four week-old rice seedlings (four leaves stage) of a susceptible rice cultivar LTH. Conidia were harvested from a conidial suspension of *M. grisea* (1×10^5 conidia/1ml of 0.1% Triton X 100) and sprayed on rice seedlings (10ml/plant) using a painter's brush. The infected seedlings were incubated at 24.5 °C in a dew chamber for 24 hours and moved to a greenhouse maintained at 26 to 28 °C. Five days post-inoculation, the rice seedlings were examined for lesion formation.

Nucleic acids manipulation: Fungal DNAs were extracted from 4-day-old mycelia obtained from a submerged culture using the CTAB protocol (Xu and Hamer, 1996). Southern and reverse Northern dot hybridizations were carried out using an ECL direct nucleic acid labeling and detection kit (Amersham). Plasmid DNAs were isolated by a Qiagen plasmid preparation kit (Qiagen) and sequenced using the BigDye^{TM} Terminator sequencing kit (PE Applied Biosystems). For RNA extraction, 4-day-old mycelia from a submerged culture or 5-day-old conidia collected from oatmeal agar plates were used. Total RNAs from mycelia and conidia were isolated with the TRIZOL reagent (Invitrogen). Poly $(A)^+$ RNA was isolated from the total RNA using the Oligotex mRNA purification kit (Qiagen) according to the manufacturer's instruction. First-strand cDNA was synthesized by Improne II Reverse Transcriptase (Promega). Standard molecular biology procedures were followed for Northern and Southern blot analyses, and the enzymatic manipulations of DNAs and RNAs (Sambrook *et al.*, 1989). Homolog searches of DNA/protein sequence databases were performed using the BLAST programs (Altschul *et al.*, 1997). DNA sequences were analyzed by the DNASIS program V3.0 (Hitachi Software).

Isolation of the *ACR1* gene: The clone pACR1-3, containing a partial ORF of *ACR1* (Lau and Hamer, 1998), was used as a probe to screen a *M. grisea* BAC library (Nishimura *et al.*, 1998). A 9.8 kb *Hin*dIII fragment, containing *ACR1*, *was* cloned into a pUC19 vector. The 5' RACE

system for Rapid amplification of cDNA Ends (Invitrogen) was used to amplify the 5' end of the message of *ACR1*.

Subtraction library construction and analysis: The PCR-select cDNA subtraction kit (Clontech) was used to construct subtraction libraries from the conidial mRNAs of *Guy11* and *acr1*, following the manufacturer's instruction. The subtracted gene fragments were amplified by PCR and cloned into a pGEM-T vector (Promega). The clones were arrayed onto Hybond N^+ nylon membrane (Amersham), as described by Nishimura *et al.* (1998), for further screening by reverse Northern dot hybridization.

Polymerase chain reactions: A foreign DNA fragment inserted in the *ACR1* gene was PCR amplified using the primers acr-INF(acgaggacaccactctcaag) and acr-INR (cagagatgggagttgttgac). A 970 bp fragment in the retrotransposon MGL was amplified by PCR using the primer mglF (cccacctttgttagcgattc) and mglR (cgtatgtgtgccaggttgtc). Expression levels of genes upregulated by Acr1 were detected by quantitative RT-PCR using the following PCR primers: mpcF1 (tcatgcacagtcccgaatta) and mpcR1 (tggaagccagtatcaaagca) (for the *MPC1* gene), mgphF1 (gtacttgcgacggtgcaaac) and mgphR1 (ccgaggtagctgctgaagtc) (for the *MGPH1*gene), as well as mpgF1 (ccaaatgctcaccatagcaa) and mpgR1 (acttctctagcgggcatcct) (for the *MPG1* gene). As a control, a β-tubulin gene was PCR amplified using the primers mbF1 (ctttgaccagccgtggtg) and mbR1 (ggcctcagtgaactccatct). Quantitative RT-PCR reactions were conducted using first strand cDNAs (10nM) synthesized from the conidial mRNAs of Guy11 and *acr1*. All PCR amplifications were performed using the Expand ^{TM} High Fidelity PCR System (Boehringer Mannheim) and the Gene Amp PCR System 2700 (Applied Biosystems).

Glycogen staining: The presence of glycogen in conidia was detected by staining a conidial suspension (10^4 conidia/ml) with 5 μl of Iode staining solution (60mg KI, 10mg I_2/ml), and

observing under bright-field optics using a Leica DM-IRB system (Thines *et al.*, 2000).

TABLE 2-1. Wild-type and the mutant *Magnaporthe grisea* strains used in this study.

Strain	Description	Reference
CHNOS60-2-3	Wild-type, *MAT 1-1*	Hayashi *et al.* (1997)
CHNOS59-9-1	Wild-type, *MAT 1-2*	Hayashi *et al.* (1997)
Guy11	Wild-type, *MAT1-2*	Notteghem and Silue (1992)
4000-R-67	*cac* mutant, *MAT 1-2*	This study
4000-R-69	*cac* mutant, *MAT 1-1*	This study
4000-R-102	*cac* mutant, *MAT 1- 2*	This study
4016-R-29	*cac* mutant, *MAT1- 2*	This study
acr1	*ACR1*defective mutant of Guy11	Lau and Hamer (1998)

RESULTS

Isolation of *cac* mutants displaying abnormal conidial morphology

In the process of mapping an avirulence gene of *M. grisea*, we isolated three morphological mutants among a collection of 113 random ascospores produced from the cross between rice-pathogenic field isolates CHNOS 60-2-3 and CHNOS 59-9-1 (Table 1). We could not obtain full tetrads because the viability of the ascospores was very low (<50%). The mutants, 4000-R-67, 4000-R-69, and 4000-R-102 produce conidia in head-to-tail arrays (Fig. 2-1A), whereas the wild type produce conidia in sympodial arrays (Fig. 2-1B).

Conidiophore-developmental pattern is abolished in the *cac* mutant

The *cac* mutants exhibit a sympodial branching pattern (Fig. 2-1B) similar to the wild type (Fig. 2-1A). Conidium formation in the wild type begins with a swelling at the apex of a conidiophore (Fig. 2-2A; 0 h), which grows to become a pyriform conidium (Fig. 2-2A; 4 h). The second and third conidia are formed at the tip of newly developed conidiophores, which grow from the side of the first and second conidiophore, respectively (Fig. 2-2A; 4 h, 8 h). This process of conidiogenesis happens repeatedly, resulting in the formation of a sympodial array of conidia (Fig. 2-2A; 12 h). Conidium formation in the *cac* mutant begins normally (Fig 2-2B; 0 h); however, the conidium becomes elongated as it matures (Fig. 2-2B; 4 h). Subsequently, the second conidium budded from the tip of the first conidium (Fig. 2-2B; 8 h). By repeating this concatenated conidiogenesis cycle, *cac* mutants form chains of more than twenty conidia. When mature, conidia formed by the mutant are separate (data not shown). It has been suggested that septa are formed between the conidia to delimit each conidium from the next.

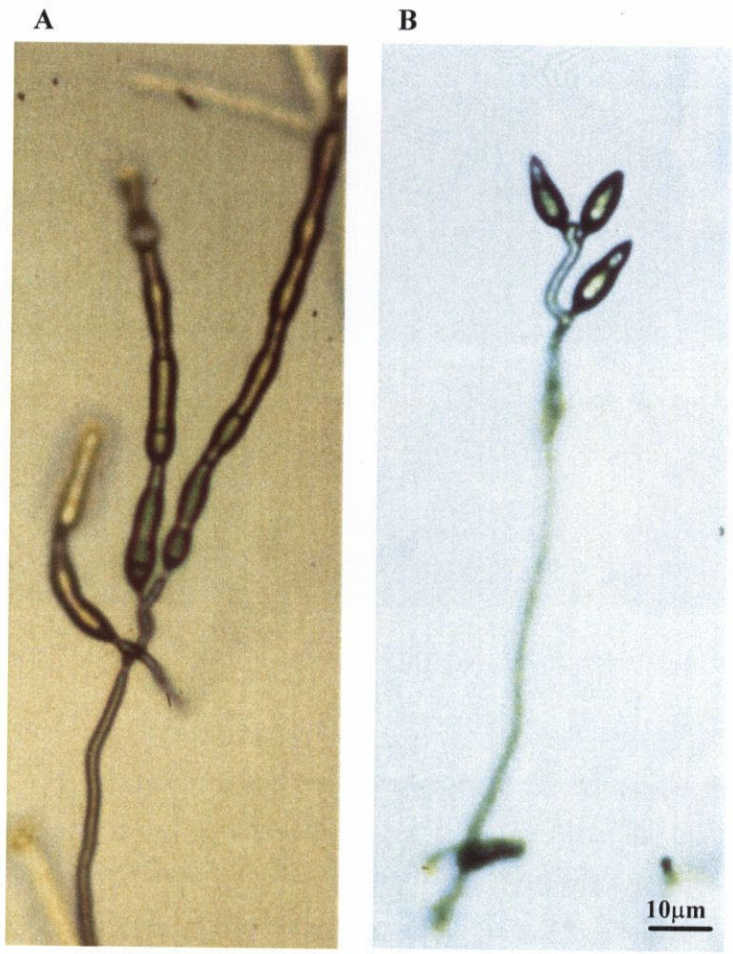


Fig. 2-1. Conidial morphology in the *cac* mutant and the wild-type strain.
A. Catenated conidia are produced in the *cac* mutant. **B.** Pyriform conidia are produced sympodially on a conidiophore in the wild-type strain.

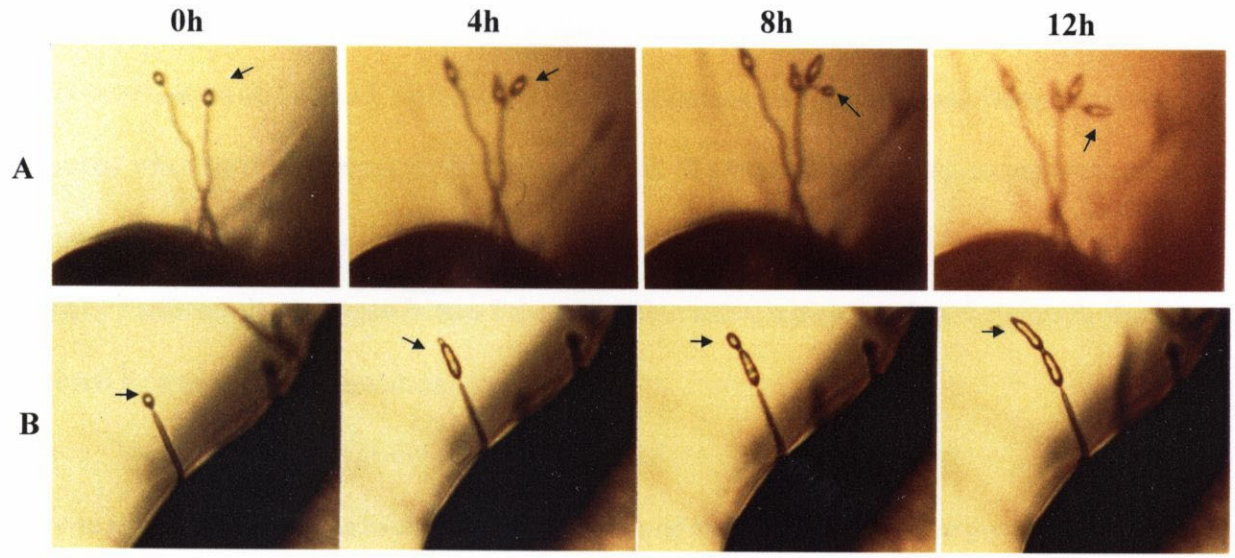


Fig. 2-2. The stages of conidia development in the wild-type strain and the *cac* mutant.
A. Conidiogenesis in the wild-type strain. 0 h: The tip of the conidiophore swells outward. 4 h: The swelling conidiophore grows to become a pyriform conidium. The second conidium is produced from the tip of the second conidiophore. 8 h: The third conidiophore develops from the side and below the second conidiophore. The conidiophore tip swells outward. 12 h: The third conidiophore tip completes swelling and becomes a mature conidium.
B. Conidiogenesis in the *cac* mutant. 0 h: The tip of conidiophore swells outward. 4 h: The swelling conidiophore grows to become an elongated conidium. 8 h: The second conidium buds from the tip of the first conidium. 12 h: The second conidium matures. Arrows indicate conidia.

The mutations occur in a single gene locus in *cac* mutants

To determine if the *cac* phenotype is due to a single gene mutation, a segregation analysis was performed on random ascospores obtained from the backcross between 4000-R-67 and CHNOS60-2-3. The first backcross between the acropetal mutant 4000-R-67 and its parental strain CHNOS 60-2-3 gave rise to the mutant strain 4016-R-29. The second backcross was between 4016-R-29 and CHNOS60-2-3. Random ascospores obtained from this second backcross were used in a segregation analysis. Out of 69 random progeny ascospores tested, 34 produced wild-type conidia, and 35 showed a mutant conidium formation pattern (data not shown). This result corresponds to a 1:1 segregation ratio, indicating that the mutant phenotype is caused by a single gene mutation.

The *cac* mutant phenotype is due to an insertion of the LINE retrotransposon MGL in the *Acr1* gene

Using an insertional mutagenesis screen, Lau and Hamer (1998) identified the *ACR1* (*acropetal*) gene that is essential for conidiogenesis and pathogenicity. The *acr1* mutant phenotype is characterized by the production of long spore chains in a head-to-tail array, a reduced number of appressoria, and the loss of pathogenicity. Since *cac* mutants exhibit morphologies similar to that of the *acr1* mutant, it is of interest to investigate if the corresponding *ACR1* gene locus in the *cac* mutants has been mutated. Southern hybridization was conducted with the *Sac*I-digested genomic DNA of the three *cac* mutants and their parental strains. When hybridized using pACR1-3 as a probe, a single 2.2-kb band could be seen in the parental strains. A similar band was absent in all *cac* mutants (Fig. 2-3); instead, a 5.1-kb and a 3.0-kb band were observed (Fig. 2-3). These observations strongly suggest that various phenotypic changes in these mutant strains might be caused by the insertion of an unknown, approximately 5.9-kb sequence into *ACR1*.

The unknown insertion in *ACR1* was amplified from the *cac* genomic DNA using PCR

primers, acr-INF and acr-INR, flanking the *ACR1* gene. For all three mutants, a 7.0 kb PCR product, containing the insertion and the flanking *ACR1* sequences, was obtained and sequenced. A search of the available databases for homologous DNA sequences indicates that 6.0 kb of the PCR product is identical to the *M. grisea* LINE retrotransposon (MGL; Hamer *et al.*, 1989; Kachroo *et al.*, 1997). In all these *acr1* morphological mutants, MGL is inserted at the same site in the *ACR1* gene. In addition, a 19-bp sequence is duplicated at the site of insertion (Fig. 2-6A). These data confirm that the conidial morphological changes in these mutants are the direct result of the MGL insertion into the *ACR1* gene. Interestingly, it appears that the same transposition event generated all three mutants. To test if these strains are clonally related, Southern analysis was performed on *Eco*R1 digested genomic DNA using a PCR amplified fragment MGL as the probe. The mutants show different restriction fragment length polymorphism (RFLP) patterns, demonstrating they are individual progenies (data not shown). Taken together, it seems most likely that these mutants are siblings, and that the MGL insertion event occurs pre-meiotically.

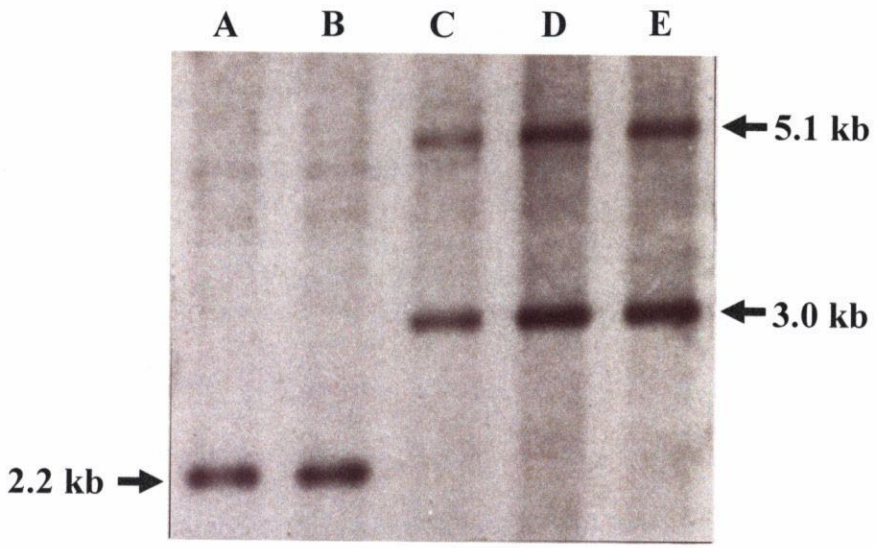


Fig. 2-3. Southern hybridization analysis of the wild-type strain and the *cac* mutants. A: the parental strain CHNOS59-9-1, B: the parental strain CHNOS60-2-3, C: the *cac* mutant 4000-R-67, D: the *cac* mutant 4000-R-69, E: the *cac* mutant 4000-R-102. Genomic DNA from these strains was digested by *Sac* I. pACR1-3, which covers the *ACR1* ORF, was used as a probe. A single 2.2 kb band is detected in the parental strains by Southern hybridization analysis. Band shifts (3.0 kb and 5.1 kb) are detected in these *cac* mutants, which indicate that the insertion events have occurred at the *ACR1* gene.

The *acr1* mutants show reduced levels of appressorium formation, penetration, and virulence

Since *cac* mutants have an active transposon insertion in *ACR1*, we took advantage of *acr1* and used it as a tool for gene function analysis. The average growth rate of the mutant *acr1* is comparable to that of the wild-type isogenic strain Guy11, although observations indicate that its germination rate is slightly reduced (Table 2-2). There is no significant difference in colony phenotype between *acr1* and Guy11 (data not shown). After 24 hours of incubation on plastic coverslips, while more than 95% of the wild type conidia form appressoria, only approximately 37% of the *acr1* conidia do the same. This reduction in appressorium formation rate in the mutant could not be complemented by the addition of exogenous cAMP (Table 2-2). Prolonged incubation of conidia on plastic coverslips (48 h) does not increase the appressorium formation rate in the mutant (data not shown). Both Guy11 and *acr1* fail to form appressoria on the hydrophilic surface of GelBond membrane (data not shown).

Penetration rate is markedly reduced in the mutant. In Guy11, approximately 58 % of the appressoria are successful in plant penetration. In contrast, only 8% of the mutant appressoria penetrate plant cells (Table 2-2). Exogenous cAMP does not affect appressorium formation or penetration in the *acr1* mutant (Table 2-2).

Spray inoculation assay was used to examine the pathogenicity of the mutant. The virulence of the *acr1* mutant is dramatically reduced. While infection by Guy11 results in the formation of many lesions on rice, infection by *acr1* results in the formation of only very few lesions, and this is most likely due to defective penetration (Fig. 2-4).

These results suggest that mutation in the *ACR1* locus affects appressorium formation and plant infection, and these processes are cAMP signalling independent.

Table 2-2. Phenotypes of *Magnaporthe grisea acr1* mutant

Strain	Colony diameter (cm)[a]	Appressorium formation (%) [b]		Germination (%) [c]		Penetration (%) [d]	Glycogen accumulation (%) [e]
		No cAMP	10mM cAMP	No cAMP	10mM cAMP		
Guy11	3.8 ± 0.1	92.7 ± 1.7	91.5 ± 2.3	96.5 ± 2.5	98.7 ± 0.9	57.6 ± 3.4	11.5 ± 2.5
acr1	3.8 ± 0.1	37.1 ± 2.6	32.3 ± 7.4	79.9 ± 6.2	82.3 ± 5.9	7.7 ± 0.7	69.3 ± 4.5

a. Colony diameter was measured after 8 day incubation at 25 °C on oatmeal agar.

b. Percentage of conidia that formed appressoria after 24 hour incubation.

c. Percentage of conidia that germinated after 24 hour incubation.

d. Percentage of appressoria that penetrated into onion epidermis cells by 48h.

e. Percentage of conidia that accumulated glycogen.

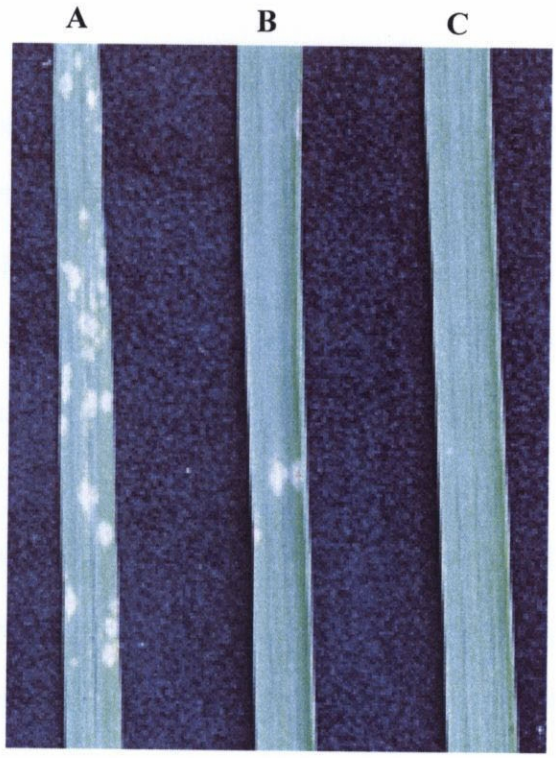


Fig. 2-4. Infection assays of *acr1* mutant.
Rice leaves are sprayed with conidia from the wild-type isolate Guy11 (A), the mutant *acr1*(B), or gelatin (Control; C). Many blast lesions were observed on the leaf inoculated with Guy11 (A), whereas few were observed on the one inoculated with *acr1*(B).

Acr1 is a MedA homolog in *Magnaporthe grisea*

The longest open reading frame (ORF) of *ACR1* is reported to have 593 amino acids, and has glutamine-rich domains that share a weak similarity with several transcriptional factors (Lau and Hamer, 1998). Using the internet-based PSORT program (http://psort.nibb.ac.jp), Acr1 was predicted to localize in the nucleus. Hydropathy analysis of Acr1 by the Kyte-Doolittle method indicated it has five hydrophobic regions (Fig. 2-5). However, some conidiophore-patterning regulators in *A. nidulans* have two ORFs (Wu and Miller, 1997; Adams *et al.*, 1998). Thus, to test the possibility of the presence of a second ORF, cDNA corresponding to a 1.5 kb region at the 5' end of *Acr1* was obtained by 5'-RACE and sequenced. The sequence was compared to the genomic DNA sequence of a 9.1kb-, *ACR1*- containing *HindIII* fragment subcloned from a *M. grisea* BAC library. The sequencing result reveals the presence of a second ORF in *ACR1*. This 2776 bp second ORF is interrupted by 3 introns (GenBank Accession No. BAC41196), and is predicted to encode an 852-amino-acid protein. The second intron is located at a position where the first ORF begins (Fig. 2-6B).

A comparison of the amino acid sequence of Acr1 and MedA of *A. nidulans* reveals the following: 1) They share a sequence identity and similarity of 55% and 67%, respectively, 2) The amino acids from position 448 to 658 in Acr1 are almost identical to those residing at the corresponding positions in MedA (Fig. 2-6B), and 3) MGL is inserted in the C-terminal of *ACR1* at amino acid position 425, a position located in the middle of the domain conserved between *M. grisea* and *A. nidulans* (Fig. 2-6B).

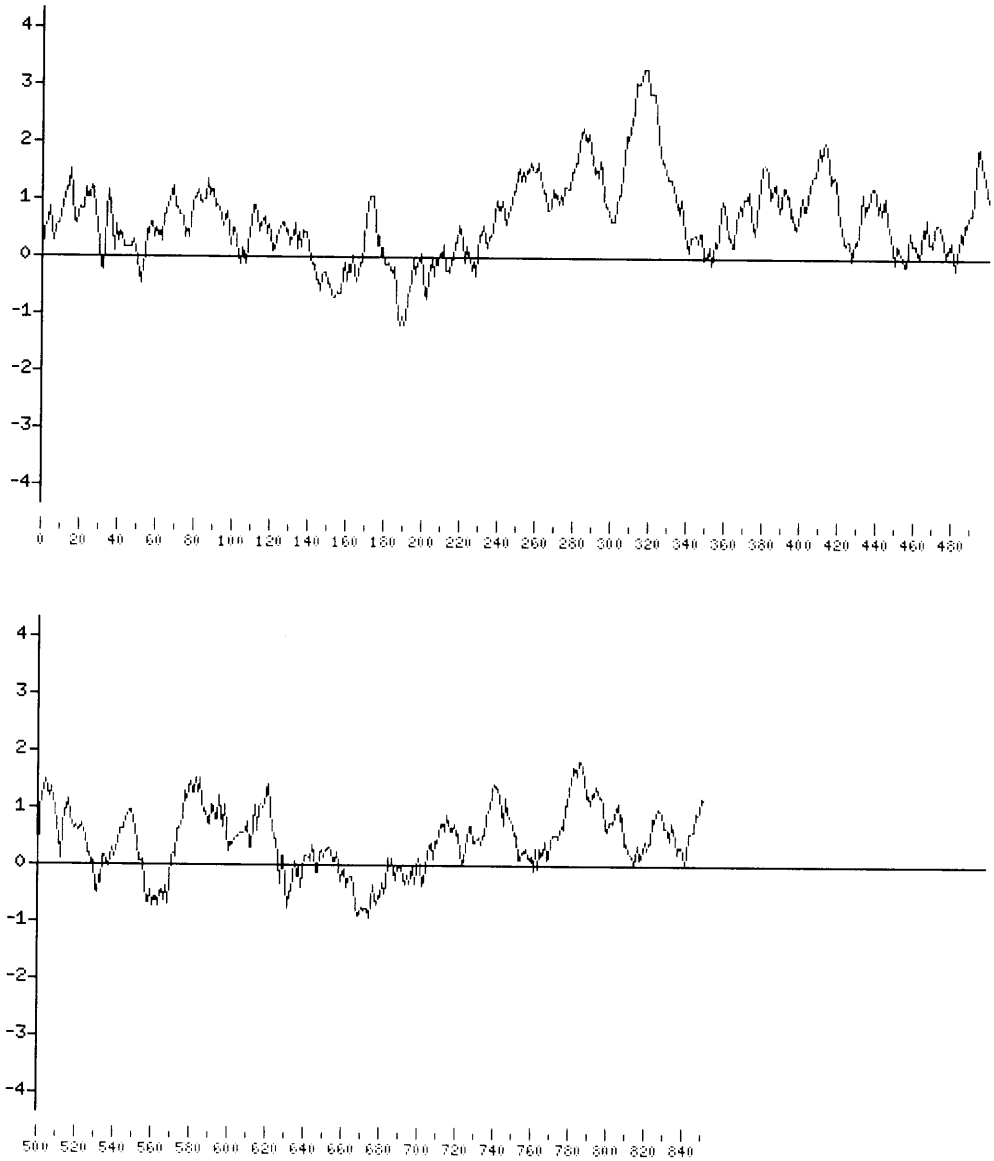
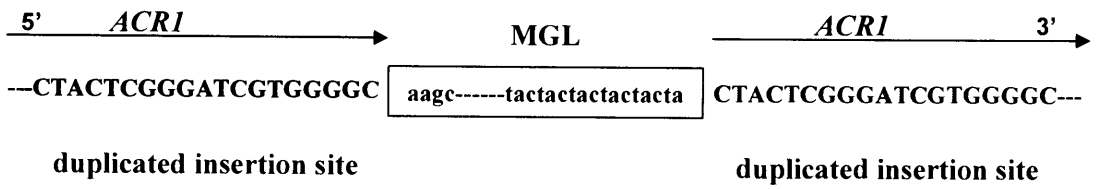


Fig. 2-7. Hydropathic profile of Acr1. The hydropathic profile is calculated by the Kyte-Doolittle method. Hydrophilic regions of the protein give positive values, while hydrophobic regions give negative values.

A.



B.

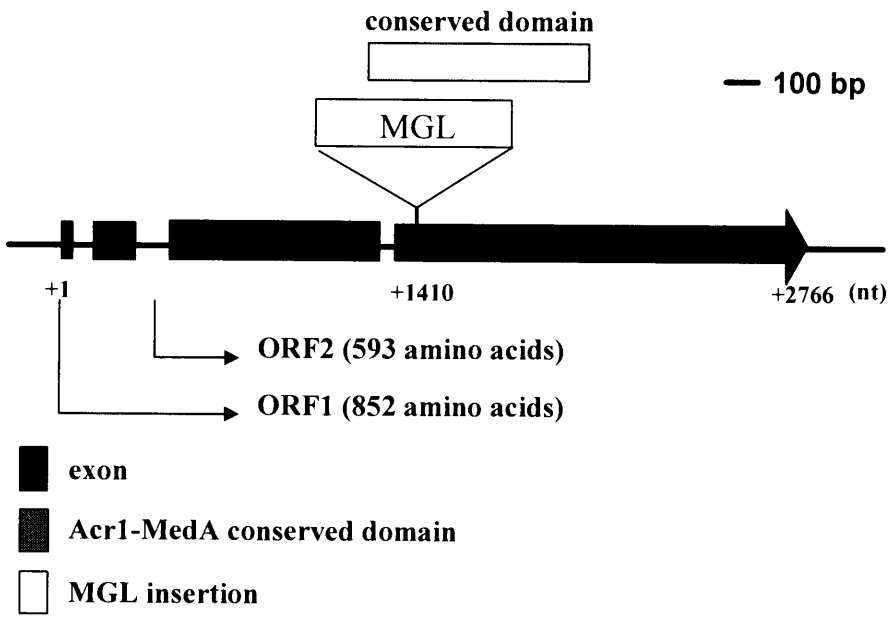


Fig. 2-6. Organization of *ACR1* locus. **A.** *M. grisea* LINE retrotransposon (MGL) insertion at the *ACR1* gene. MGL contains duplicated 19 nucleotide flanking regions and seven repeats of the trinucleotide TAC. **B.** Structure of *ACR1* gene. Three introns and two ORFs exist in *ACR1*. The large ORF encodes 852 amino acids. The second ORF (ORF2) begins in the second intron and encodes 593 amino acids. The domain (shaded box) that is highly conserved between Acr1 and MedA lies between nucleotides +1344 to +1924. The retrotransposon MGL is inserted behind the nucleotide +1410 in *ACR1*.

Potential genes that are upregulated by Acr1

To investigate the genes that are upregulated by Acr1, a forward and a reverse subtraction library was constructed from *acr1* and its isogenic wild-type strain Guy11. The forward library is enriched with the genes expressed in *acr1*, and the reverse library contains the genes suppressed in *acr1*. The sequence of 576 clones from the forward library and 192 clones from the reverse library were analyzed. The forward library is enriched with genes related to cell wall integrity, while the majority of the reverse library contains those related to glycogen phosphorylase. The clones from the libraries were arrayed onto a nylon membrane as described by Nishimura *et al.* (1998), and hybridized using a probe corresponding to the conidial mRNA of *acr1* or Guy11. Hybridization signals of varying magnitudes were observed for several genes (Fig. 2-7), indicating that Acr1 might have upregulated these genes. Expression levels of each conidial gene in *acr1* and Guy11 were further confirmed by quantitative RT-PCR (Fig. 2-7). *MPG1*, which encodes a class I fungal hydrophobin required for the synthesis of a conidial wall rodlet protein (Talbot *et al.*, 1996; Kershaw *et al.*, 1998), is most abundant in the forward library. A high level of *MPG1* expression can be detected in *acr1* during conidiation, as was previously reported by Lau and Hamer (1998). In this study, the expression level of *MPG1* in the mutant conidia is approximately double that of the wild type. In contrast to *MPG1*, expression of both the glycogen phosphorylase gene (*MGPH1*: *Magnaporthe* glycogen phosphorylase) and the *S. cerevisiae* G1-cyclin Pcl-1 homolog in *M. grisea* (*MPCL1*: *Magnaporthe PCL1* homolog) is reduced in the *acr1* conidia (Fig. 2-7). These data indicate that the expression of *MGPH* and *MPCL1* is most likely regulated in an Acr1-dependent manner.

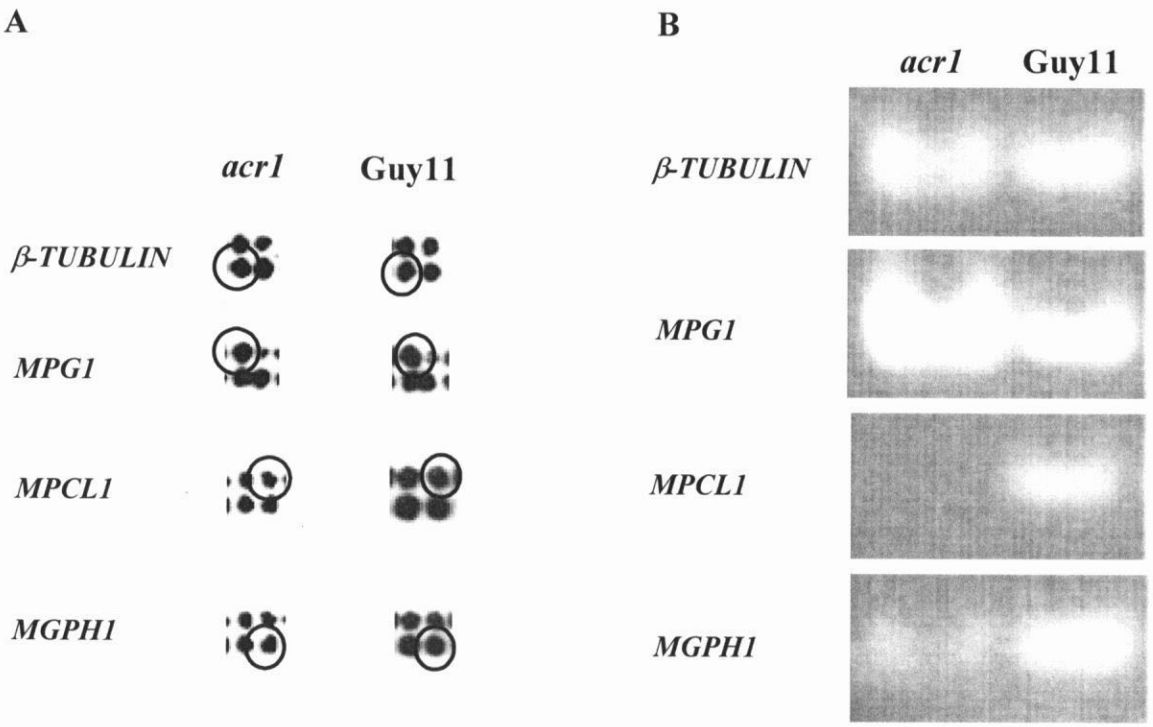


Fig. 2-7. Expression of genes upregulated by Acr1. The β-tubulin gene was used as a control. **A.** Reverse Northern dot hybridization. The clones from the *acr1* and Guy11 conidial mRNA subtraction libraries were arrayed onto nylon membrane, and hybridized with *acr1* conidial mRNA (left panel) or Guy11 conidial mRNA (right panel). The clones carrying the indicated genes (left) are circled. After hybridization with *acr1* mRNA, *MPG1* signal is enhanced, while the signal for *MPCL1* and *MGPH1* is reduced. **B.** RT-PCR expression levels of *MPG1*, *MPCL1* and *MGPH1* in conidia of *acr1* and Guy11. In *acr1* conidia, the expression of *MPG1* increases, while *MPCL1* and *MGPH1* expression levels decrease.

Glycogen degradation is reduced in the germinating conidia of *acr1*

M. grisea uses the cellular turgor generated in an appressorium to penetrate into plant cells (Howard *et al.* 1991). In order to generate sufficient turgor, an appressorium accumulates molar concentrations of glycerol synthesized from the lipid and glycogen reserves in the conidium (Thines *et al.*, 2000). Evidence for the rapid glycogen degradation, in the conidium, followed by the accumulation of glycerol and its dissolution before turgor generation, in the incipient appressorium, have been provided by Thines *et al.* (2000). Glycogen phosphorylase (*GPH*) plays a central role in the mobilization of glycogen in the glycerol synthesis pathway (Newgard *et al.*, 1989). Results from the current study indicate that a reduction in *MGPH* expression level is observed in addition to an impaired appressorium formation and plant penetration for the *acr1* mutant. We therefore hypothesized that *acr1* is deficient in glycogen degradation, and that in turn results in a reduced glycerol accumulation in the appressoria. Glycogen degradations have been examined in the conidia of Guy11 and *acr1* on the hydrophobic surface of plastic coverslips or onion epidermal cells. In Guy11, abundant glycogen is present in the conidia prior to germination (Fig. 2-8; Table 2-2). During appressorium formation, most of the glycogen, reserves in the conidia are degraded and 11.5% of the conidia retain glycogen following a 24h incubation period on a hydrophobic surface (Table 2-2). The degradation of glycogen in the conidia of Guy11 correlates with observations of fungal penetration into plant cells (Fig. 2-8). In contrast, glycogen is retained in 69.3% of the *acr1* following a 24h incubation period on a hydrophobic surface (Table 2-2). Furthermore, abundant glycogen is detected in the conidia that fail to penetrate into plant cells (Fig. 2-8).

These results imply that in *acr1, the* defect in appressorium penetration, caused by a failure to generate sufficient glycerol-induced turgor pressure, is due to a reduction in the enzyme activity of Mgph.

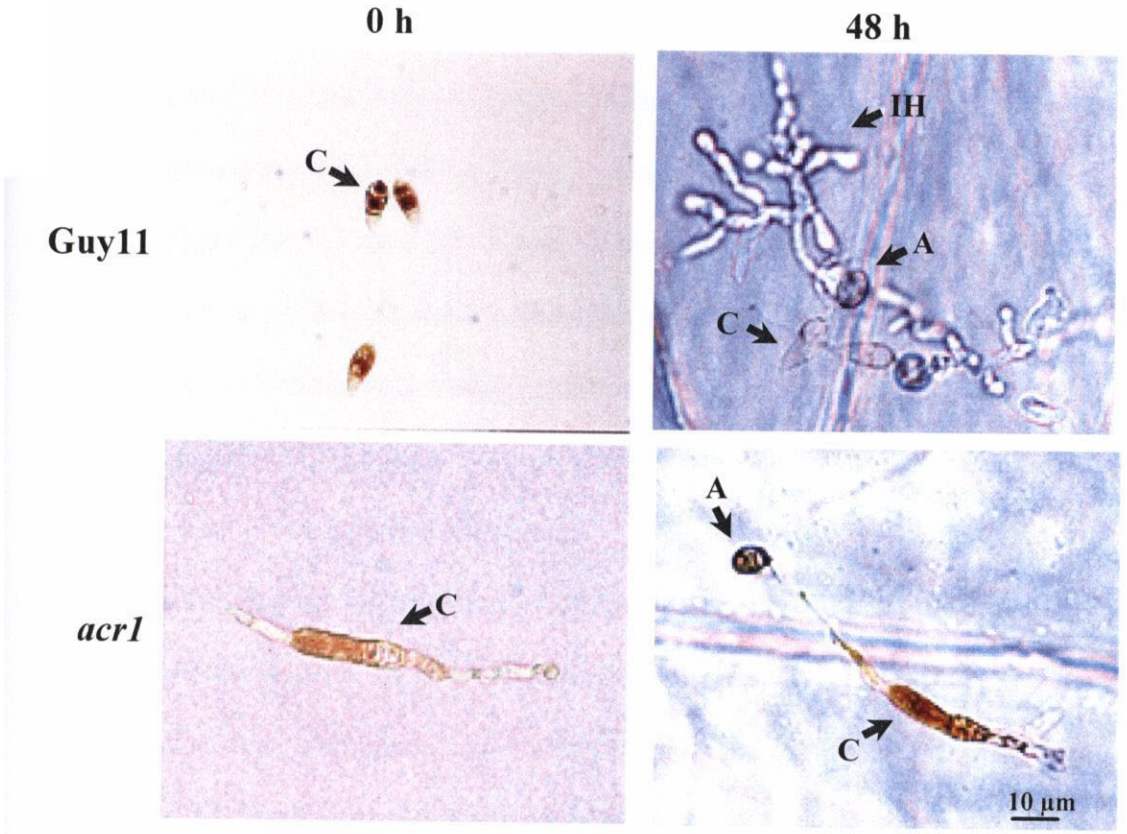


Fig. 2-8. Cellular distribution of glycogen in *acr1* mutants and the wild-type strain. The glycogen reserves in conidia were stained red by Iode staining solution. Conidia in both in Guy11 and *acr1* contain large amounts of glycogen before germination (0h). After 48 hours, most of the glycogen reserve has been depleted in the wild-type strain (Guy11) infectiously growing in onion epidermal cells (upper right). Large amounts of glycogen is still observed in *acr1* conidia after 48 hours, as the mutant forms appressoria, but fails to penetrate onion epidermal cells (lower right).

A: appressorium; C: conidium; IH: infectious hypha; GT: germ tube.

DISCUSSION

Many studies of *M. grisea* have demonstrated that mutations occurring in genes that are involved in conidiogenesis are quite often accompanied by defects in appressorium formation and infection (Hamer and Givan, 1990; Shi and Leung, 1995; Lau and Hamer, 1998). The *ACR1* gene is the first to be identified as one that affects normal conidial morphogenesis and infection (Lau and Hamer, 1998). In this study, we have further dissected its function by isolating and characterizing three morphological mutants, and demonstrating that the cause of mutation is due to an active transposon insertion in the gene.

ACR1 is the first fungal gene that shares a homology with the *MEDA* gene of *A. nidulans*. A total of 210 amino acids are encoded by the region conserved between the two genes, suggesting that this region might also be highly conserved in many other different groups of fungi. *MedA* and *acr1* mutants share a similar mutant phenotype in that the cells undergoing conidiogenesis are reiterated in chains. Furthermore, the defect in normal conidiophore development in *acr1* can be partially complemented by introducing a plasmid carrying *MEDA* (data not shown). Thus we conclude that Acr1 is a functional homolog of MedA. Additionally, because two transcription start sites are present in both *MEDA* and *ACR1*, initiating transcription at these two sites results in the generation of two mRNAs (Adams *et al.*, 1998). BrlA is a member of the core regulatory pathway of conidiophore development in *A. nidulans*. The *BRLA* gene is somewhat similar to *MEDA* and *ACR1*. The transcription of *BRLA* can, depending on the type of regulatory signals received, result in the generation of two overlapping transcripts that are essential for normal fungal development (Prade and Timberlake, 1993; Han and Adams, 2001). Similarly, the generation of the two kinds of messages from *ACR1* may also be in response to multiple regulatory signals. Although *ACR1* and *MEDA* are homologous to each other, they exhibit different expression patterns. *MEDA* is expressed throughout the fungal life cycle, and is important for fertility (Adams *et al.*, 1998; Vallim *et al.*, 2000), while the

expression of *ACR1* expression is limited to periods of conidiation, and is not required for fertility. These differences in expression patterns and functions might come from the non-conserved region at the N-terminus.

Cyclin–dependent kinases (Cdks) are known key regulators of cell cycle in eukaryotes (Huang *et al.*, 2002). Among them, Cdc28 is the only one essential Cdk; it associates with many cyclins to regulate cell cycle progression in *S. cerevisiae* (Meadenhall and Hodge, 1998). Cdc28, in association with G1 cyclins Cln1, Cln2 and Cln3, promote the G1 phase of a cell cycle. In the absence of Cln1 and Cln2, the Pho85-Pcl2 subfamily interactions are important for the progression of the G1 phase in a cell cycle (Measday *et al.*, 1997; Andrews and Measday, 1998). The Pcl2 subfamily is consists of Pcl1, Pcl2, Pcl5, Pcl9 and Clg1 (Measday *et al.*, 1997). Yeast strains lacking multiple Plc2 subfamily members display remarkable morphological defects such as elongated cells, connected chains of cells, and a pattern of growth consistent with unipolar budding. This indicates the important roles of the Pcl2 subfamily in cell polarity and morphogenesis (Measday *et al.*, 1997; Andrews and Measday, 1998; Moffat *et al.*, 2000). Recent functional genomics approaches have suggested that the Pho85-Pcl1, 2 interactions have a novel function in cell integrity (Huang *et al.*, 2002). PclA, a yeast Pcl1 homolog in *A. nidulans*, is induced by BrlA. Unlike yeast Pcl1, the function of PclA is independent of the *A. nidulans* Pho85 homolog PhoA during condiation (Schier *et al.*, 2001). Instead, PclA appears to regulate cytokinesis in phialides via $NimX^{cdc2}$ activity (Schier and Fischer, 2002). However, the PclA null mutant develops normal conidiophores, even though it displays a strong reduction in conidia production. PclA is responsible for the fast and repetitive cell divisions of phialides, leading to the formation of long chains of mitogenic conidia on the conidiophores (Schier and Fischer, 2001). The expression of *PCLA* and $NIMX^{cdc2}$ is controlled in a BrlA-dependent manner, evident by the presence of putative BrlA binding domains (BREs) in their 5'-upstream regulatory domains (Yun and Timberlake, 1992; Ye *et al.*, 1999; Schier *et al.*, 2001). In the *medA* mutant, the expression level of *BRLA* is reduced (Busby *et al.*, 1996). It is likely that the

PCLA expression level is also reduced in *medA* in parallel with the *BRLA* expression level. Our preliminary data indicate that the expression level of *MPCL1*, the *PCLA* homolog in *M. grisea*, is remarkably low for *acr1* (Fig. 2-7). Recently we have found four *MPCL1* homologs in the *M. grisea* genome, and four *PCLA* homologs in the *A. nidulans* genome (data not shown). The null phenotype of PclA suggests these PclA homologs have overlapping functions. It is plausible that Mpcl1 homologs also have redundant functions like the yeast Pcl2 subfamily members. Pcl2 and PclA subfamily members are similar in their relation to cell cycle events (Schier *et al.*, 2001; Moffat *et al.*, 2000). The mutant phenotype of *acr1* partially appears due to the reduction of *MPCL1* expression. Unlike *PCLA* and $NIMX^{cdc2}$, there is only one putative BRE within 1.2 kb of the 5'-upstream domain of *MPCL1* (data not shown). In addition, the amino acid sequence of a BrlA homolog in *M. grisea* has been found to exhibit low similarity to that of *A. nidulans* (Xu, personal communication). Thus it is possible that a consensus sequence of the BrlA-homolog binding domain in *M. grisea* is not the same as the BREs documented for *PCLA* and $NIMX^{cdc2}$. Experiments are underway to confirm the function and regulation mechanism of Mpcl1.

Glycogen phosphorylase catalyzes the degradation of glycogen, and its catalytic activity is controlled by transcriptional and allosteric regulators (Hudson *et al.*, 1993; Sunnarborg *et al.*, 2001). Our preliminary data indicate that the expression of glycogen phosphorylase gene (*MGPH1*) is reduced in the *acr1* mutant (Fig. 2-7). Although appressoria production can still be seen, they are greatly reduced in number and are defective in penetration. Cytological analysis indicates that glycogen mobilization is significantly reduced in the *acr1*conidia (Fig. 2-8). Therefore, we conclude that *acr1* is defective in penetration. This is due to insufficient appressorium turgor resulting from a failure to synthesize glycerol from the glycogen reserve in the conidia.

CHAPTER 3

The G-protein β Subunit Mgb1 is Important for Infection-Related Morphogenesis in *Magnaporthe grisea*

INTRODUCTION

In this chapter, the role of the M. grisea heterotrimeric G-protein β subunit (Gβ subunit) in regulating appressorium formation and conidiation is presented.

As with many fungal pathogens, M. grisea penetrates plants by means of specialized infection structures known as appressoria (Howard et al., 1991). The formation of appressoria occurs naturally in germinating spores that are attached to hydrophobic leaf surfaces of rice plants. Artificial hydrophobic surfaces which mimic that of rice leaves have been found to also induce appressoria formation. On hydrophilic surfaces, appressoria formation can be stimulated by cAMP (cyclic AMP) and IBMX (3-iso-butyl-1-methylxanthine), suggesting that cAMP signalling is involved in surface recognition (Lee and Dean, 1993).

MAC1 (adenylate cyclase gene) disruption mutants of *M. grisea* do not form appressoria, and are reduced in vegetative growth and conidiation (Adachi and Hamer, 1998; Choi and Dean, 1997). These defects can either be complemented by exogenous cAMP or suppressed by a mutation in the regulatory subunit of PKA (cAMP-dependent protein kinase A) (Adachi and Hamer, 1998). These data indicate that cAMP is a secondary messenger that triggers appressorium formation in *M. grisea*. The activation of adenylate cyclase is likely mediated by heterotrimeric G-proteins (Gα, Gβ and Gγ subunits) in filamentous fungi (Chen *et al.*, 1996; Kays *et al.*, 2000). Three Gα subunits, MagA, MagB and MagC have been characterized in *M. grisea*. While the *MAGA* or *MAGC* disruption mutants exhibit wild type level of appressorium formation and pathogenicity, *MAGB* disruption mutants show reduced levels of conidiation, appressorium formation and virulence (Liu and Dean, 1997). Gene replacement mutants of *CPKA*, a gene encoding the catalytic subunit of PKA, are reduced in virulence and delayed in

appressorium formation, but exhibit normal vegetative growth and conidiation (Mitchell and Dean, 1995; Xu *et al.*, 1997). Studies of several other fungal pathogens, including *Ustilago maydis*, *Cryphonectria parasitica*, and *Cryptococcus neoformans*, have also revealed a role for cAMP-mediated signal transduction in fungal infection and disease development (Kronstad *et al.*, 1998; D'Souza and Heitman, 2001).

In addition to the cAMP signalling pathway, a MAP kinase gene *PMK1* (Pathogenicity MAP Kinase) has been shown to be essential for appressorium formation and infectious hyphal growth (Xu and Hamer, 1996). The *pmk1* gene replacement mutants of *M. grisea* are nonpathogenic and fail to cause blast lesions on compatible rice plants. Closer observations indicate that germ tubes of *pmk1* mutants still recognize hydrophobic surfaces and form sub-apical swollen bodies (Xu and Hamer, 1996), but fail to arrest the germ tube tip growth and are unable to form appressoria on Teflon membranes or onion epidermal cells. These mutants remain incapable of appressoria formation under cAMP induction; response to cAMP occurs only in the form of germ tube tip deformation (Xu and Hamer, 1996). It is hypothesized that Pmk1 acts downstream of the cAMP signaling pathway for surface recognition and infection structure formation. Pmk1 homologues are essential for plant infection in many appressorium-forming and non-appressorium-forming phytopathogenic fungi. They are important for the regulation of not only appressorium formation but also other plant infection processes (Xu, 2000).

In *Saccharomyces cerevisiae*, Fus3 and Kss1 are two partially redundant MAP kinases that are involved in regulating mating responses (Dohlman and Thorner, 2001; Gustin *et al.*, 1998). Pheromone-receptor binding is coupled to the dissociation of an inhibitory Gα subunit (Gpa1) from stimulatory Gβγ subunits. The liberated Gβ subunit (Ste4) directly associates with a scaffold protein (Ste5) and a PAK kinase (Ste20), and is essential for activating the down stream MAP kinase cascade. Pmk1 is the only homolog of Fus3 and Kss1 in *M. grisea*, with the homology level being slightly higher with Kss1 than with Fus3. As such, it is capable of

functionally complementing the mating defect in a yeast *fus3 kss1* double mutant (Xu and Hamer, 1996). Since gene disruption mutants of three Gα subunits are phenotypically different from that of *pmk1* mutants (Liu and Dean, 1997; Xu and Hamer, 1996), it is possible that Gβ subunit regulates the Pmk1 pathway in *M. grisea* following a manner similar to that employed by Ste4 in regulating yeast mating responses via the Fus3 MAP kinase pathway.

The Functions of Gβ subunit homolog have been characterized in the filamentous fungi *Neurospora crassa* (Yang *et al.*, 2002), *Aspergillus nidulans* (Rosen *et al.*, 1999), *C. parasitica* (Kasahara *et al.*, 1997), and *C. neoformans* (Wang *et al.*, 2000). In these fungi, the Gβ subunit genes exist as single-copies in their genomes. Loss-of-function mutants of Gβ in *A. nidulans* and *C. parasitica* are reduced in conidiation (Rosen *et al.*, 1999; Kasahara *et al.*, 1997). In *N. crassa*, the mutation in Gβ gene induces profuse conidiation on solid medium and inappropriate conditions in submerged culture (Yang *et al.*, 2002). Therefore, these data suggest that Gβ subunit is a potential regulator in conidiation in *M. grisea*.

In this study, we report the isolation and characterization of the *M. grisea* G-protein β subunit gene, and discuss its role in infection-related morphogenesis.

MATERIALS AND METHODS

Stains and growth media: Wild type strains and all the transformants generated in this study (Table 3-1) were cultured on oatmeal agar plates (3% oatmeal, 0.5% glucose, 1.6% agar) at 25°C under fluorescent light for conidiation induction, and stored on desiccated Whatman #1 filter paper at -20°C as previously described (Xu and Hamer, 1996). Mycelia collected from three-day-old 5xYEG culture (0.5% yeast extract, 2% glucose) shaken at 150 rpm at room temperature were collected by filtration through Miracloth (Calbiochem) and used for extracting genomic DNA. Genetic crosses and progeny isolations were performed as described (Talbot *et al.*, 1993). To compare growth rate on complete (CM, Xu *et al.*, 1998), oatmeal, and 5xYEG agar cultures, the diameters of colonies formed by monoconidial cultures on 150 mm Petri plates were measured after incubation for 8 days at 25°C. The Number of conidia produced on oatmeal agar cultures was determined after incubating for 14 days at 25°C under fluorescence light.

Molecular manipulations with DNA and RNA: Fungal DNAs were extracted with the CTAB protocol (Xu and Hamer, 1996). Plasmid DNAs were isolated with Qiagen plasmid preparation kits (Qiagen) and sequenced with the BigDyeTM Terminator sequencing kit (PE Applied Biosystems). Total RNAs were isolated with TRIZOL reagent (Invitrogen). Standard molecular biology procedures were followed for Northern and Southern blot analyses and enzymatic manipulations with DNAs and RNAs (Sambrook *et al.*, 1989). Homolog searches of DNA/protein sequence databases were performed with the BLAST programs (Altschul *et al.*, 1997). DNA sequences were analyzed with the DNASIS program V3.0 (Hitachi Software). Amino acid sequence comparisons and alignments were made with the BESTFIT, PILEUP, and BOXSHADE programs in the GCG Wisconsin software package (Accelrys).

Isolating the *MGB1* gene: PCR products amplified with the degenerate primers described by Kasahara and Nuss (1997) were cloned in the pGEM-T vector (Promega) and sequenced. First

strand cDNA synthesized with RNA isolated from nitrogen-starved Guy11 mycelia was used as the template. One PCR-derived cDNA clone, pLR33, contained a 389 bp insert that was highly homologous to *C. parasitica CPGB-1*. The insert fragment of this clone was amplified and used to screen a BAC library (Nishimura *et al.*, 1998) and a Uni-ZAPII cDNA library (Xu and Hamer, 1996). The cDNA clone F2C10 and cosmid clone 1P20 were sequenced by primer walking.

***MGB1* gene replacement vector and mutants:** To construct the *MGB1* replacement vector, a 4.5 kb *Hin*dIII fragment containing the *MGB1* coding region and its flanking sequences were cloned into the pUC19 vector as pGbH2. The hygromycin phosphotransferase gene (*HPH*) was amplified with PCR primers Bg (gaagatcttcgctggagctagtggaggtcaacacatcaat) and Bm (cgggatcccggtcggcatctactctatt) from pCB1003 (Carroll *et al.*, 1993), and cloned into the *Bgl*II site of pGbH2 to generate pKI2 (Fig. 3-2A). A 2.5 kb fragment containing the hph gene and 3'-flanking sequence of *MGB1* was amplified from pKI2 with primers Bg and R20 (cccaagcttgggcccatgtgtcttgcctttgcc) and cloned into pGEM-T as pGpg3 (Fig. 3-2A). A 1.1 kb fragment containing the 5'-flanking sequence of *MGB1* was then amplified with primes 5F (gaagatcttctggtcaaaaaccagcaaggt) and 6R (cgggatcccgtgctcctgtcgttgatgaag) and cloned into the *Bgl*II site of pGpg3 as pKO1.1 (Fig. 3-2A). Vector pKO1.1 was transformed into Guy11 as previously described (Xu and Hamer, 1996). Putative *mgb1* gene replacement mutants were isolated by screening with PCR primers 7F (atcctctgcggaatcacatc) and 8R (gcagccgtcgagagtctagt) and further confirmed by Southern blot analyses. For complementation assays, a 8.0 kb XbaI-XhoI fragment containing the full-length *MGB1* gene was subcloned from cosmid 1P20 into pBSSK(+) as pXX30 (Fig. 3-2A). PXX30 was introduced into the *mgb1* mutant nw96 by co-transformation with pBF101 as described (Kimura *et al.*, 1995). Blasticidin S resistant transformants were screened by PCR with primers 7F and 8R to identify transformants carrying the wild type *MGB1* allele, and confirmed by Southern blot analysis with the *MGB1* probe amplified with primers 1F (gcattcgattaacgggcgccaacga) and 8R (Fig. 3-2A).

Appressorium formation and plant infection assays: Conidia were harvested from 14-day-old oatmeal agar cultures and resuspended to $5x10^4$ conidia/ml in sterile distilled water. Drops of conidial suspensions (30 ml) were placed on plastic microscope coverslips (Fisher Scientific) or GelBond membranes (Cambrex) and incubated in a moist chamber at room temperature. Conidial germination and appressorium formation were examined after incubating for 2, 4, 8, 12, and 24 h. Appressorial penetration and infectious hyphae formation were assayed with onion epidermal cells as described previously (Xu *et al.*, 1997). Exogenous cAMP (Sigma) was added to conidia suspension to a final concentration of 10 mM for assaying appressorium formation, penetration and plant infection as previously described (Xu *et al.*, 1997). For plant infection assays, conidia were resuspended to $1x10^5$ conidia/ml in a 0.25% gelatin solution. Two-week-old seedlings of the rice cultivar CO39 and 8-day-old barley seedling of Golden Promise were used for infection assays. Plant incubation and inoculation were performed as described (Valent and Chumley, 1991; Xu *et al.*, 1997). Lesion formation was examined 7 days after inoculation.

cAMP extraction and assay: Mycelia were collected from 3-day-old 5xYEG liquid cultures by filtering through Miracloth, or from 7-day-old oatmeal agar cultures by scraping off conidiophores together with conidia. All fungal samples were lyophilized and ground to powder in liquid nitrogen. Intracellular cAMP was extracted from the lyophilized samples and detected using the cAMP enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech) following the manufacturer's instructions.

Table 3-1. Wild type strains and mutants of *Magnaporthe grisea* used in this study.

Strain	Description	Reference
Guy11	wild type, *MAT1-2*	Leung *et al.*, 1988
3986-R-45	wild type, *MAT1-1*	Dr. N. Hayashi at NIAS
3986-R-10	wild type, *MAT1-2*	Dr. N. Hayashi at NIAS
nw49	ectopic transformant (transformed with pKO1.1)	This study
nw78	*mgb1* deletion mutant of Guy11	This study
nw96	*mgb1* deletion mutant of Guy11	This study
cmp9	Guy11 with multiple copies of ectopic *MGB1*	This study
cmp5	Guy11 with single copy of ectopic *MGB1*	This study

RESULTS

MGB1 encodes a G-protein beta subunit

A PCR-based strategy was used to isolate the Gβ subunit gene from the wild-type strain Guy11. A PCR-derived cDNA clone pLR33, containing a 389 bp insert homologous to the *C. parasitica CPGB-1*, was used to screen the cDNA and genomic libraries of Guy 11. Corresponding cDNA and genomic clones were identified and sequenced by primer walking. Sequence analysis revealed an open reading frame (ORF) interrupted with four introns. The corresponding gene, named *MGB1* (for *M. grisea* G-protein beta subunit), is predicted to encode a 359-amino-acid protein (GenBank Accession No. AB086901). Southern blot analysis indicated that *MGB1* is a single-copy gene in the Guy11 genome (data not shown). The amino acid sequence of *MGB1* is 95%, 91%, 76% and 57% identical to that of *C. parasitica CPGB-1*, *N. crassa GNB-1*, *A. nidulans SFAD*, and *C. neoformans GPB1*, respectively. In addition, a careful examination of the amino acid sequence of Mgb1 revealed the presence of seven WD repeats typical of Gβ subunits. Although the overall homology between Mgb1 and yeast Ste4 is low (26% identity), the putative Gα, Gγ and Ste20 association sites of Ste4, including D62, D224 and L65 (Whiteway *et al.*, 1994; Dowell *et al.*, 1998), are conserved in Mgb1 and its homologues from other filamentous fungi (Fig. 3-1). Our RT-PCR analysis did not detect any difference in the *MGB1* transcription level under different mycelia growth conditions (data not shown).

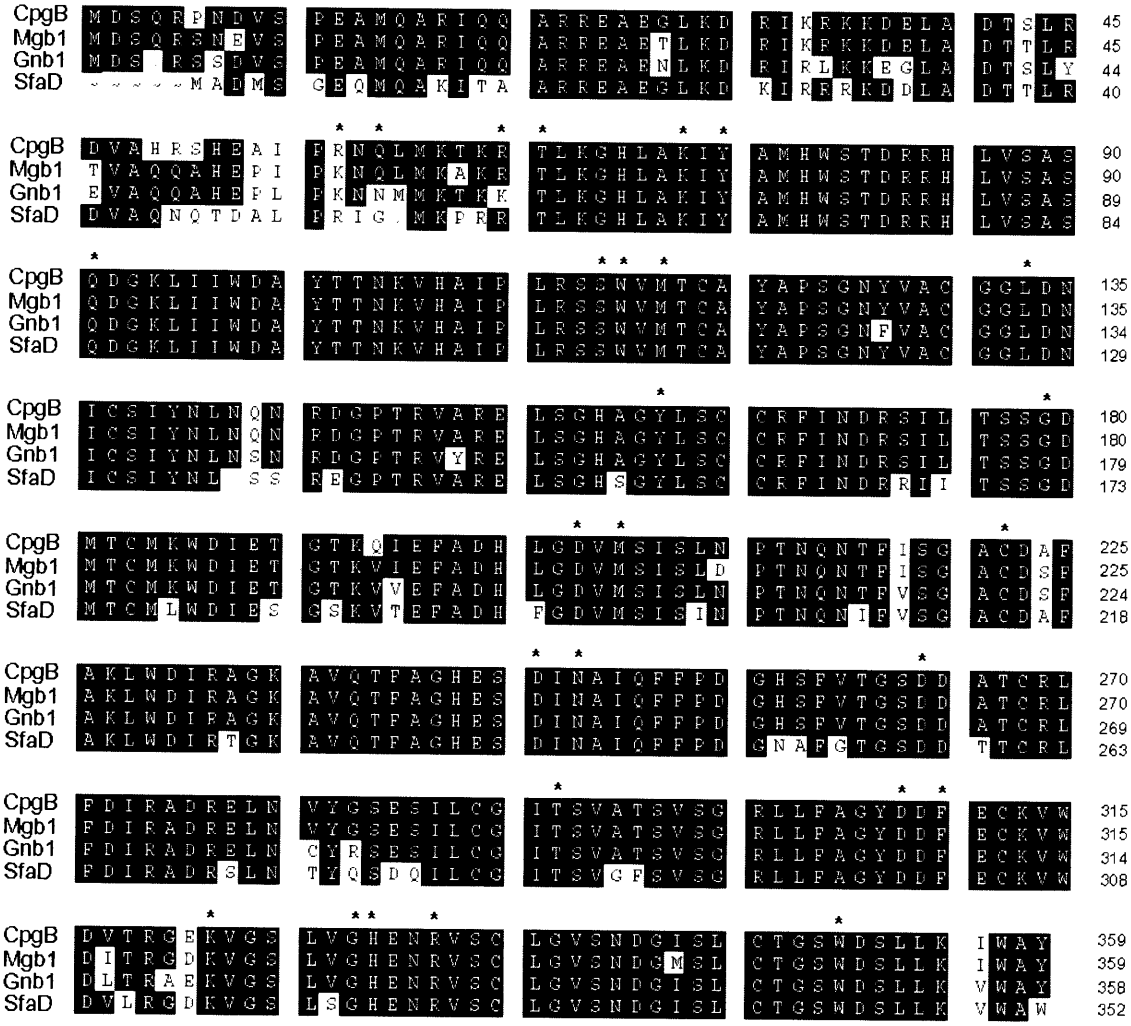


Fig. 3-1. Alignment of the predicted amino acid sequences of G protein beta subunits from fungi. With Mgb1 as the reference sequence, the locations of seven WD domains are mapped to amino acid positions 58-99 (WD1), 100-141 (WD2), 142-187 (WD3), 188-230 (WD4), 231-272 (WD5), 273-316 (WD6), and 317-359 (WD7). GenBank accession numbers: AAC49838 for C. *parasitica* CpgB, BAC01165 for *M. grisea* Mgb1, AAM53552 for *N. crassa* Gnb1, and AAC33436 for *A. nidulans* SfaD. Identical residues are shown in black boxes. RGS association sites are indicated as *.

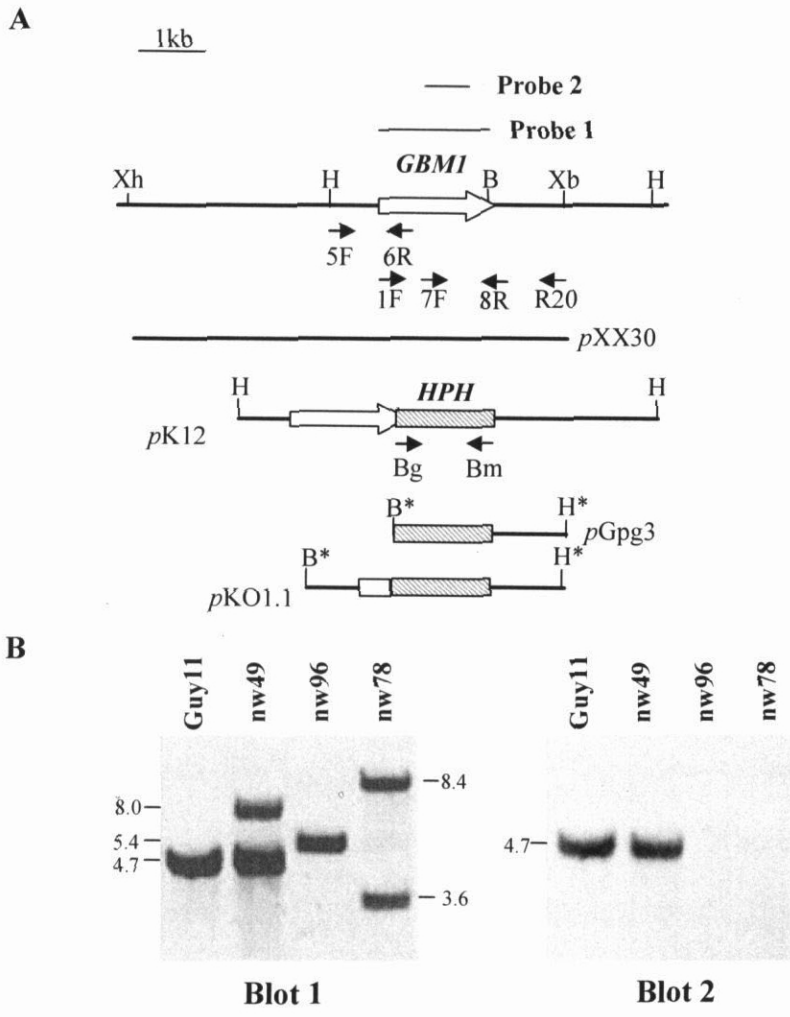


Fig. 3-2. The *MGB1* gene replacement vector and mutants.

A. Construction of the *MGB1* gene replacement vector. The *HPH* gene was inserted at the *Bgl*II site located 12 bp upstream from the stop codon of *MGB1* in pKI2. pGpg3 contained the fragment amplified with primers Bg and R20 from pKI2. The gene replacement vector pKO1.1 was constructed by cloning the fragment amplified with primers 5F and 6R from pKI2 into pGpg3. Locations and directions of PCR primers are indicated by arrows. Restriction enzymes are: B: *Bgl*II, H: *Hin*dIII, Xb: *Xba*I and Xh: *Xho*I. Symbol * indicated enzyme sites derived from primers or cloning vectors.

B. Southern blot of genomic DNAs from the wild-type strain Guy11, an ectopic transformant nw49, *mgb1* deletion mutants nw96 and nw78 were hybridized with probe 1 amplified with primers 1F and 8R (Blot 1 left) and stripped and re-hybridized with probe 2 amplified with primers 7F and 8R (Blot 2 right). All DNA samples were digested with *Hin*dIII.

Isolation of *mgb1* deletion mutants

The *MGB1* knockout vector pKO1.1 was constructed by replacing a 709 bp fragment of *MGB1* with the hph gene (Fig. 3-2A), followed by transformation of the construct into Guy11 protoplasts. Two putative *MGB1* deletion mutants, nw78 and nw96, were identified by screening with PCR primers 7F and 8R, and further confirmed by Southern blot analysis. When blots containing *Hin*dIII digested genomic DNAs were hybridized using a probe corresponding to a fragment containing the entire *MGB1* gene (probe 1, Fig. 3-2A), a single 4.7 kb band was observed for Guy11, but not for *mgb1* mutants nw78 and nw96 (Fig. 3-2B). Instead, a 5.4 kb band that functions as a marker for the gene replacement event was observed for nw96. Nw78 has the 8.4 kb band plus an extra 3.6 kb band. A 7.0 kb band in addition to the 4.7 kb band was observed for the ectopic transformant nw49 (Fig. 3-2B). When the same blot was stripped and tested using a probe corresponding to the deleted portion of the *MGB1* gene (probe 2, Fig. 3-2A), the 4.7 kb band appeared only for Guy11 and nw49. These data indicate that nw96 and nw78 are true *mgb1* mutants resulting from gene replacement. For nw78, in addition to the gene replacement event, the pKO1.1 DNA also is integrated into the flanking sequences of *MGB1* by a single homologous recombination because the 5.5 kb band is absent in nw78 (Fig. 3-2B). Mutants, nw78 and nw96 are phenotypically identical. They are slightly reduced in vegetative growth on oatmeal (Table 3-2), CM and 5xYEG agar cultures (data not shown). Interestingly, colonies formed by *mgb1* mutants on oatmeal agar cultures appear to produce more aerial hyphae than the wild-type strain Guy11 (Fig. 3-3A). Densely bundled hyphae are observed in hyphae of *mgb1* mutants grown in 5xYEG liquid cultures, similar to what has been reported for the *C. parasitica cpgb-1* mutant (Kasahara and Nuss, 1997)(data not shown). Conidia produced by *mgb1* mutants are morphologically indistinguishable from those of the wild-type strain Guy11. However, the number of conidia produced by *mgb1* mutants is reduced to approximately 10% of that of Guy11 (Table 3-2). Close examination indicates that *mgb1* mutants are reduced

in conidiophore development even though they produce abundant aerial hyphae. Each conidiophore still produces multiple conidia sympodially (data not shown).

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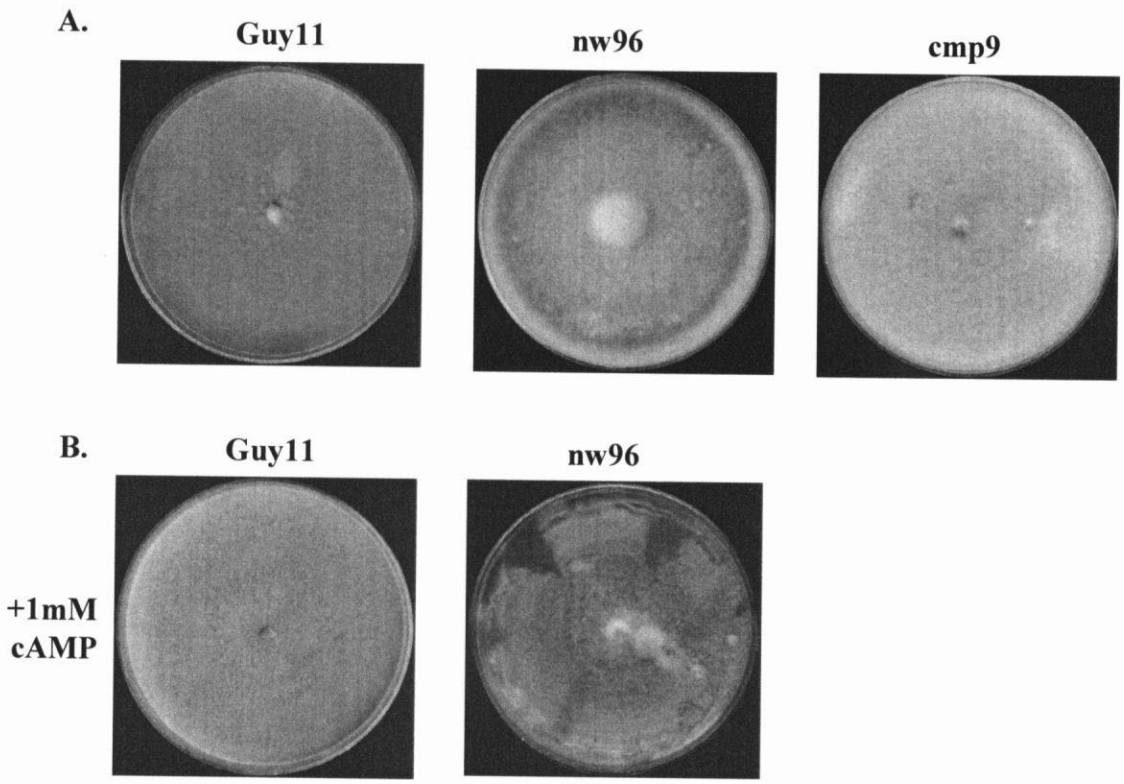

Fig. 3-3. Colony phenotypes of *mgb1* mutants.

A. Oatmeal agar cultures of the wild-type strain (Guy11), the *mgb1* mutant (nw96), the transformants expressing multiple copies of *MGB1* (cmp9).

B. Colonies of Guy11 and nw96 grown on oatmeal agar cultures supplemented with 1 mM of cAMP. Photos were taken after 14 days of incubation.

Table 3-2. Growth and conidiation in *mgb1* mutants.

Strain	Colony diameter (cm) [a]	Conidia/plate (x10^6) [b]
Guy11 (WT)	4.6 ± 0.1	50.1 ± 1.7
nw49 (ectopic)	4.5 ± 0.1	48.3 ± 2.4
nw96 (*mgb1*)	4.2 ± 0.1	4.6 ± 0.1
nw78 (*mgb1*)	4.0 ± 0.1	6.6 ± 0.2

[a] Colony diameter was measured after incubating for 8 days at 25°C.

[b] Number of conidia formed on each oatmeal plate examined after incubation for 14 days.

Means and standard errors were calculated from at least three independent experiments.

Table 3-3. Conidia germination and appressorium formation.

	Germination (%)[a]		Appressorium formation (%)[b]	
Time	Guy11	nw96	Guy11	nw96
2 h	65.3 ± 5.4	21.7 ± 2.2	0	0
4 h	90.6 ± 5.1	49.3 ± 3.4	33.8 ± 6.2	0
8 h	98.3 ± 2.3	95.0 ± 4.1	79.8 ± 6.8	0
12 h	98.7 ± 0.9	100.0 ± 0.0	93.2 ± 4.5	0
24 h	96.7 ± 0.9	99.7 ± 2.5	98.2 ± 3.6	0

[a] Percentage of conidia that produced germ tubes.

[b] Percentage of germ tubes that formed appressoria.

Means and standard errors were calculated from at least three independent experiments.

Table 3-4. Appressorium formation induced by exogenous cAMP.

Strain	No cAMP	10 mM cAMP
Guy11	98.2 ± 3.6*	95.3 ± 4.2
nw49	98.0 ± 1.5	98.3 ± 1.2
nw78	0	62.8 ± 2.0
nw96	0	62.7 ± 6.5

* Percentage of germ tubes that formed appressoria after 24 h of incubation at 25°C.

Mgb1 mutants show delayed conidial germination and are defective in appressorium formation

Conidia from *mgb1* mutants are able to adhere and germinate but fail to form appressoria on plastic coverslips, and on the hydrophobic side of GelBond membranes. In contrast to the melanized appressoria formed by Guy11, *mgb1* mutants produce long germ tubes that lack germ tube tip differentiation within 24 h of germination (Fig. 3-4). Even after a prolonged incubation of up to 60 h, tip deformation and appressorium formation are not observed (data not shown). Long germ tubes produced by *mgb1* mutants also fail to differentiate and are unable to form appressoria on onion epidermal cells (Fig. 3-4), indicating that *mgb1* mutants are defective in surface recognition and fail to produce appressoria on artificial hydrophobic surfaces or plant surfaces. Further examinations indicate that conidial germination is delayed in *mgb1* mutants (Table 3-3). While the majority of Guy11 conidia germinated within 2 h, less than 30% of nw96 conidia germinate under the same conditions. It is only after a 24 h incubation period that over 98% of nw96 conidia germinate. This phenotype of delay conidial germination is similar to that observed in the *cpkA* mutant (Xu *et al.*, 1997). In addition, appressorium formation is observed for over 90% of the wild-type Guy 11 germ tubes, whereas none is seen in those belonging to nw96 under the same conditions (Table 3-3).

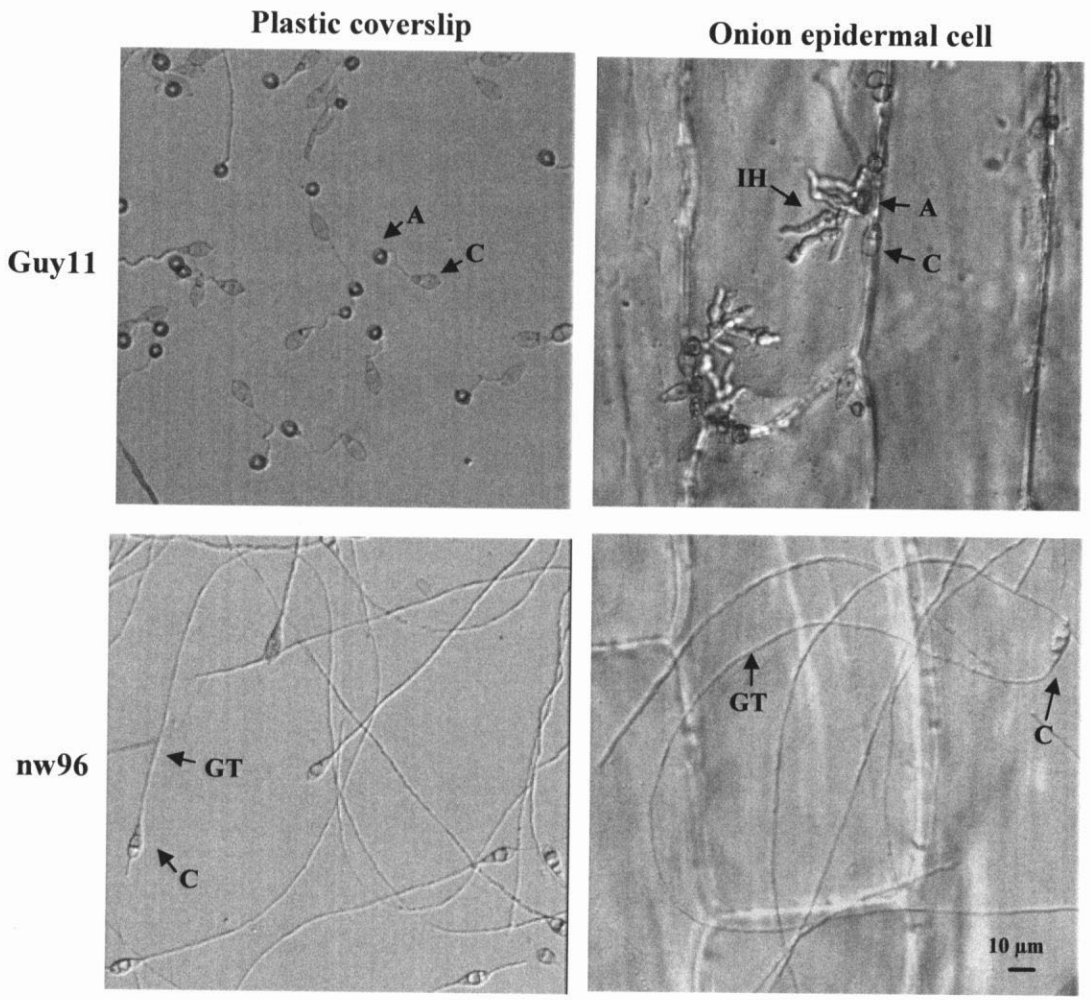

Fig. 3-4. Appressorium formation assay. While the wild-type strain (Guy11) produced melanized appressoria after incubation on plastic coverslips (left) or onion epidermal cells (right), the *mgb1* mutant nw96 only formed long germ tubes without tip differentiation under the same conditions. Guy11 developed infectious hyphae after penetrating onion epidermal cells. Photographs were taken after 48 h incubation. A: appressorium; C: conidium; IH: infectious hypha; GT: germ tube.

Mgb1 mutants remain responsive to exogenous cAMP

In *M. grisea*, cAMP signalling is an important process that controls surface recognition, and exogenous cAMP has been shown to induce appressorium formation (Lee and Dean, 1993). To test whether *mgb1* mutants are responsive to exogenous cAMP, a final concentration of 10 mM cAMP was added to a conidia suspension. Interestingly, up to 65% of the nw96 germ tubes form melanized appressoria by 24 h when incubated on plastic coverslips with supplemental cAMP (Table 3-4). However, these cAMP-induced appressoria are abnormal in shape and are often elongated (Fig. 3-5). On the hydrophilic side of GelBond membranes, cAMP induction stimulates the formation of these abnormal appressoria in nw96 as efficiently as in Guy11 (data not shown). Exogenous cAMP also induces appressorium formation in *mgb1* mutants on onion epidermal cells. However, while appressoria formed by Guy11 penetrate and develop infectious hyphae by 48 h, appressoria induced by cAMP in nw96 fail to penetrate onion epidermal cells (Fig. 3-5). No penetration peg and infectious hyphae is observed in the abnormal appressoria formed by *mgb1* mutants on onion epidermal cells in the presence of 10 mM cAMP, indicating that these irregularly shaped appressoria formed by nw96 are non-functional and defective in penetration.

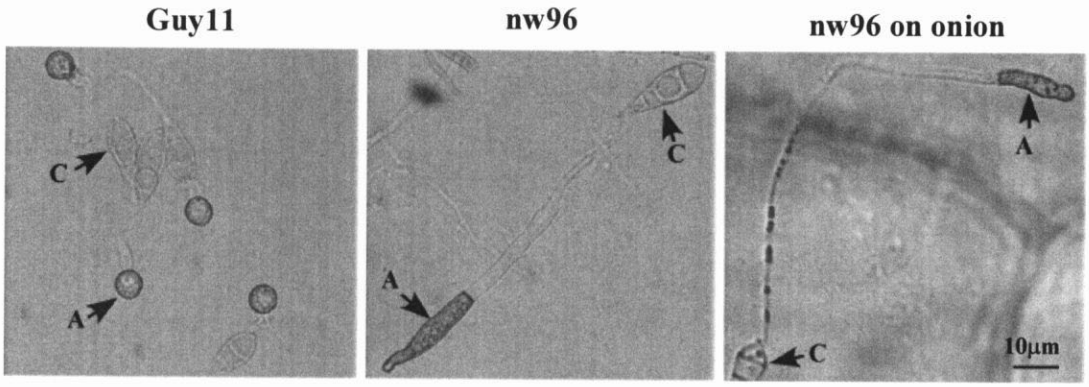

Fig. 3-5. Appressorium formation induced by cAMP. On hydrophobic surfaces supplemented with 10 mM cAMP, Guy11 forms normal melanized appressoria (left). The *mgb1* deletion mutant nw96 produces melanized but irregularly shaped appressoria on plastic coverslips (middle) and onion epidermal cells (right). A: appressorium, C: conidium.. Appressoria formed by nw96 on onion epidermal cells are non-functional and fail to penetrate and differentiate into infectious hyphae.

MGB1 deletion mutants are non-pathogenic

Spray and injection inoculation assays were used to determine if possible defects of *mgb1* mutants in plant infection exists. When 2-week-old rice CO39 seedlings are sprayed with conidia from the wild-type Guy11, numerous lesions are observed after 7 days. No lesions are observed on leaves sprayed with conidia from *mgb1* mutant nw78 or nw96 (Fig. 3-6A) even after prolonged incubation for up to 10 days. In injection infection assays with CO39 seedlings, typical spreading blast lesions are observed in and around the wound sites in leaves inoculated with Guy11 or the ectopic transformant nw49 (Fig. 3-6A). Leaves inoculated with *mgb1* mutants develop small areas of necrosis at the injection sites, but do not form any lesions outside the injection sites (Fig. 3-6B). The necrotic areas caused by injection inoculation with *mgb1* mutants are very limited (Fig. 3-6B). In addition, *mgb1* mutants fail to form lesions on rice leaves abraded with diatomaceous earth (data not shown). Because cAMP can stimulate appressorium formation, we also inoculated CO39 seedlings with conidia suspensions containing 10 mM cAMP. No lesions are observed on leaves sprayed (Fig. 3-6A) or injected (Fig. 3-6B) with nw96 conidia, indicating that these appressoria induced by cAMP are non-functional for infecting rice leaves. Exogenous cAMP cannot complement the defects of *mgb1* mutants in appressorial penetration and infectious growth. Thus, *mgb1* mutants are non-pathogenic and Mgb1 is involved in regulating appressorial penetration and infectious hyphae growth because *mgb1* mutants fail to infect through wounds.

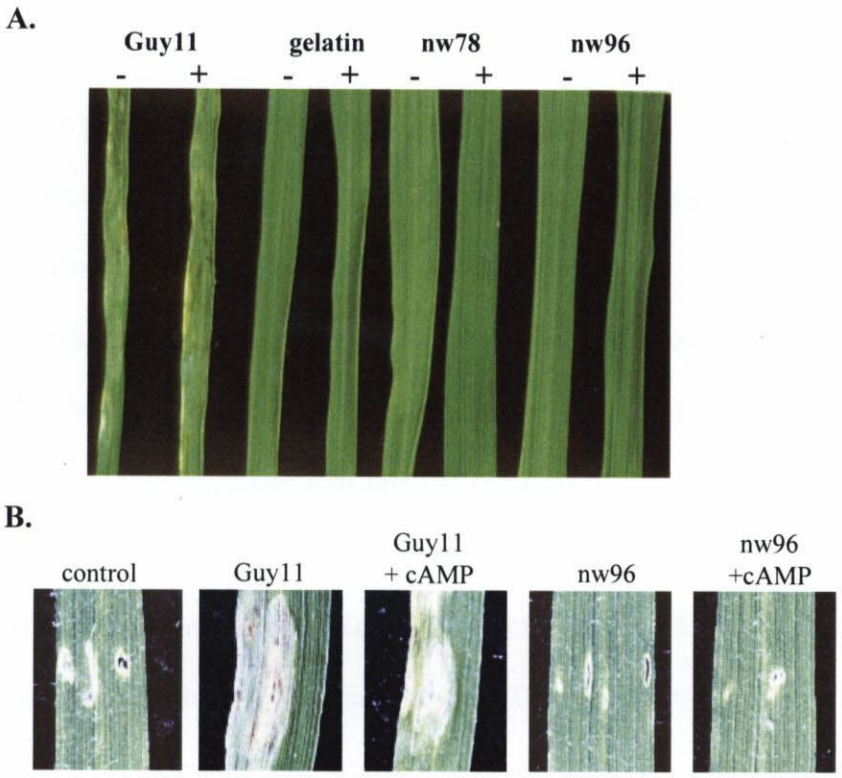

Fig. 3-6. Infection assays with *mgb1* mutants.

A. Rice leaves sprayed with conidia from the wild-type strain Guy11, or *mgb1* mutants nw78, or nw96 with (+) or without (-) 10 mM cAMP. Gelatin solution was used as the control. Typical blast lesions were only observed in leaves inoculated with Guy11. **B.** Leaves injected with 0.5% gelatin solution (control) or conidia from Guy11 and nw96 with or without 10 mM cAMP. Photographs were taken at 7 days after inoculation.

Co-segregation analysis and complementation assay

The *mgb1* deletion mutants are sterile when mated with compatible strains as the female. However, *mgb1* mutants have no defect in mating as the male, and produces abundant fertile perithecia (Fig. 3-7). Among 39 progenies isolated from a cross between nw96 x 3986-R-45, all 19 progenies that are resistant to hygromycin are non-pathogenic and formed fluffy colonies. In all four of these progenies examined, female fertility is restored. The remaining 20 hygromycin-sensitive progenies are virulent and form normal colonies, indicating that *mgb1* mutant phenotypes co-segregate with hygromycin resistance.

To further confirm that the phenotypes observed in *mgb1* mutants resulted from the deletion of *MGB1*, we also transformed pXX30, a clone containing the full-length *MGB1* gene, into the *mgb1* mutant nw96 together with pBF101 (Kimura *et al.*, 1995). Among 70 blasticidin S-resistant transformants isolated, four with single-copy integration of pXX30 (including cmp5; see below) have normal growth rate, conidial germination, female fertility, appressorium development and lesion formation (data not shown). Therefore, introduction of the wild-type *MGB1* gene rescued all the phenotypes observed in nw96.

Interestingly, one of the blasticidin S-resistant transformants, cmp9, has multiple copies of pXX30 integrated into the genome based on Southern blot analysis of genomic DNA (Fig. 3-8A). It forms appressoria on hydrophobic surfaces and causes lesions on rice leaves as efficiently as the wild-type strain Guy11 (data not shown). Colony morphology and conidiation in cmp9 are also similar to those of Guy11. However, more than 50% of cmp9 conidia collected from 7-day-old oatmeal cultures form appressoria on the hydrophilic surface of GelBond (Fig. 3-8B). These data indicate that expression of multiple copies of *MGB1* may over-activate the Gβ signalling and disturb surface sensing mechanisms in *M. grisea* for appressorium formation. In *S. cerevisiae*, overexpression of Ste4 results in the over-activation of the mating pheromone-response pathway and constitutive growth arrest in haploid cells. These phenotypes can be suppressed by overexpression of the Gpa1 Gα subunit (Whiteway *et al.*, 1990). Overexpression

of *MGB1* in the cmp9 mutant has no significant effect on plant infection as normal lesions are observed during the process. Intriguingly, only conidia collected from the 7 to 10 days old mycelia of cmp9 produce appressoria on hydrophobic surfaces (Fig. 3-9), whereas no age-dependent difference is detected in the Guy11 conidia grown on similar surfaces (data not shown).

Fig. 3-7. The *mgb1* mutant is female sterile. When nw96 (*MAT1-2*) was mated with 3986-R-10 (*MAT1-2*) and 3986-R-45 (*MAT1-1*), nw96 formed perithecia with its opposite mating type strain 3986-R-45 only as a male. Perithecia are indicated by arrows.

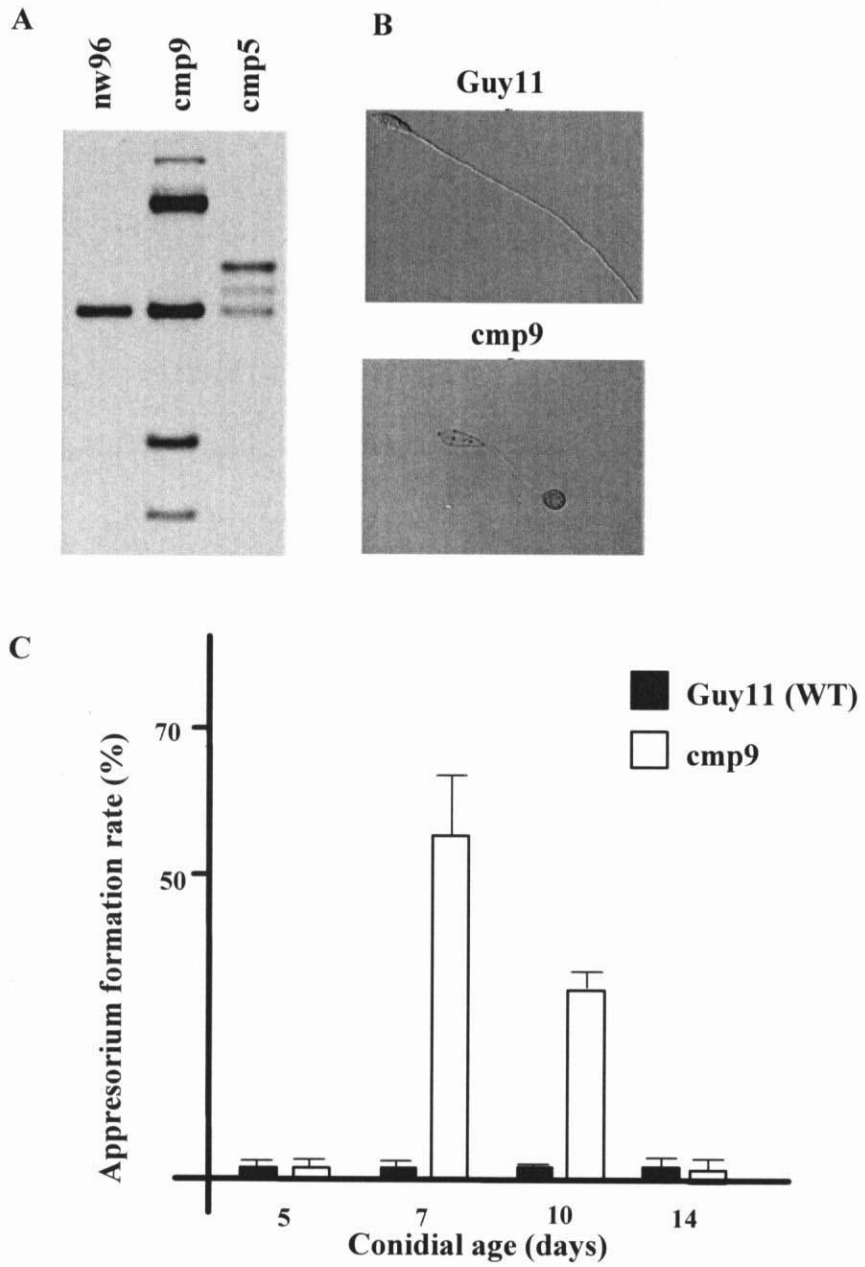

Fig. 3-8. Transformant expressing multiple copies of *MGB1*.

A. Southern blot of genomic DNAs of nw96, cmp9 and cmp5 hybridized with the probe amplified with primers 1F and 8R (Probe 1, Fig. 3-2). DNA samples were digested with *Hin*dIII. Both cmp9 and cmp5 originated from the *mgb1* mutant nw96 transformed with pXX30.

B. Appressorium formation on the hydrophilic side of GelBond membranes. While Guy11 conidia usually did not form any appressoria, over 50% of cmp9 conidia formed appressoria after 24 h of incubation.

C. Appressorium formation rate on hydrophilic surfaces was altered by conidial age. Only 7 to 10 days-old conidia of cmp9 showed efficient appressorium formation on hydrophobic surfaces. Results are averages of at least three experiments.

Intracellular cAMP level is altered in *mgb1* mutants

Because *mgb1* mutants still respond to exogenous cAMP and, deletion or overexpression of *MGB1* may affect the cAMP signalling, we measured the concentration of intracellular cAMP in the *MGB1* deletion mutant nw96 and transformant cmp9 carrying multiple copies of *MGB1*. In mycelia harvested from 7-day-old oatmeal agar cultures, the intracellular cAMP level is less than 61% of that observed in Guy11 grown under the same conditions (Table 3-5). Interestingly, the intracellular cAMP level of cmp9 is almost twice as high as that of Guy11 (Table 3-5). We also measured the intracellular cAMP concentrations in mycelia collected from liquid 5xYEG cultures. Similar to what is observed on oatmeal agar cultures, the cAMP level in nw96 is less than 28% that of Guy11. However, the amount of cellular cAMP in cmp9 is less than 69% that of Guy11 in liquid cultures (Table 3-5).

To determine whether the reduction in cellular cAMP is related to growth and conidiation defects of *mgb1* mutants, cAMP was added to oatmeal agar culture to a final concentration of 1 or 10 mM. While 1 mM cAMP has no obvious effect on the growth rate of nw96 (Fig. 3-10A), its conidia production is significantly increased (Fig. 3-10B). Colonies formed by nw96 on media with 1 mM cAMP are morphologically similar to those of Guy11, but they often form sectors that produce less aerial hyphae (Fig. 3-3B). In Guy11, conidiation is slightly increased with 1 mM cAMP, but is significantly reduced with 10 mM cAMP. In both Guy11 and nw96, 10 mM cAMP has an adverse effect on hyphal growth rate measured by colony diameter (Fig. 3-6A).

Table 3-5. Measurement of intracellular cAMP levels.

	Oatmeal agar culture		5xYEG liquid culture	
	fmol of cAMP/mg of mycelia and spores	% of Guy11 (wild type)	fmol of cAMP/mg of mycelia	% of Guy11 (wild type)
Guy11	4595.7 ± 703.31b	100	1350.0 ± 122.5	100
nw96	2820.7 ± 395.0	61	383.0 ± 82.6	28
cmp9	7691.7 ± 1046.9	167	933.3 ± 47.1	69

[a] Lyophilized weights of mycelia and spores.

[b] Means ± standard deviations were calculated from at least three independent experiments.

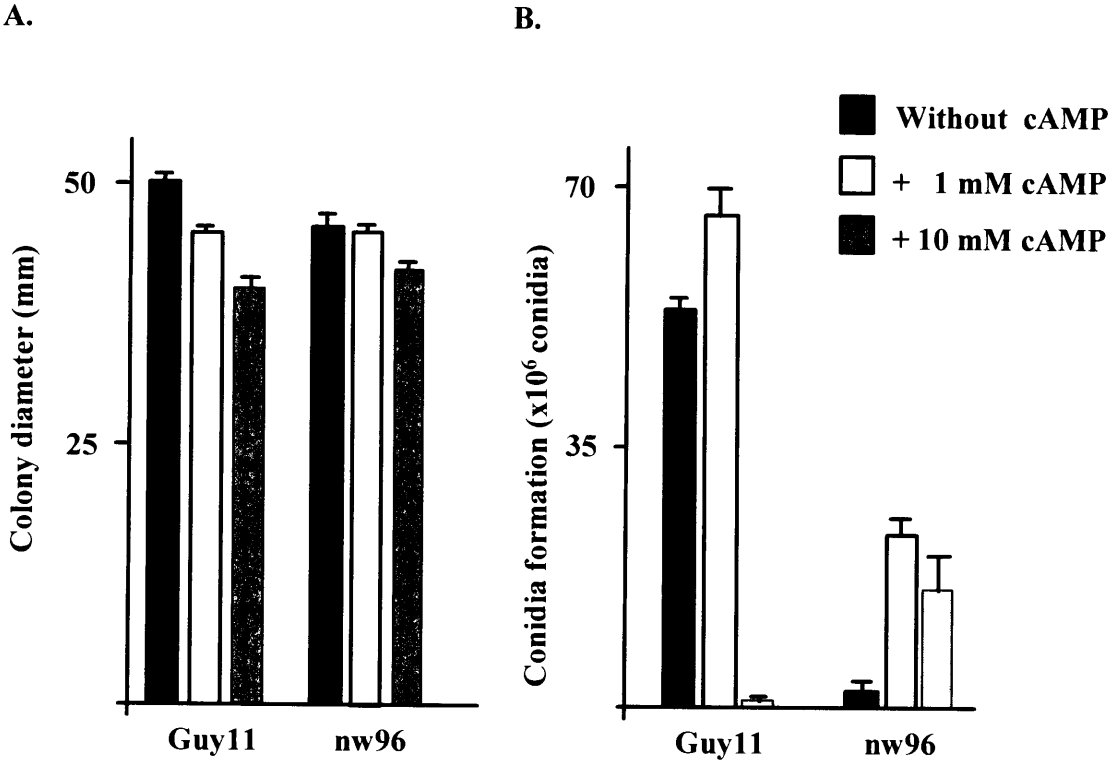

Fig. 3-10. Effect of exogenous cAMP on mycelial growth and conidiation. **A.** Radial growth, as measured by the colony diameter, after incubating on oatmeal agar culture for 8 days at room temperature. **B.** The number of conidia produced on each oatmeal agar culture (Φ 60 mm) after incubating for 14 days under fluorescent light. Each bar represents the mean value of three independent experiments, and error bars show standard deviations.

DISCUSSION

The *MGB1* gene is highly homologous to that encoding the Gβ subunits of heterotrimeric G-proteins characterized in other filamentous fungi, including *N. crassa GNB-1*, A. *nidulans SFAD*, *C. neoformans GPB1*, and *C. parasitica CPGB-1*. As in other fungi, *MGB1* is the only gene encoding a Gβ subunit in *M. grisea*. The *mgb1* mutants produce more aerial hyphae and form fluffy colonies, but they are reduced in conidiophore development and conidiation (Table 3-2). While deletion of *SFAD* causes hyperactive sporulation and severely reduces vegetative growth in *A. nidulans* (Rosen *et al.*, 1999), the *cpgb-1* mutant in *C. parasitica* has increased growth rate and reduced conidiation, and forms flat colonies with densely bundled hyphae (Kasahara *et al.*, 1997). In *N. crassa*, the *gnb-1* mutant has nearly wild-type growth rate, but it produces more conidia at the colony margins, and has abnormal submerged conidiation (Yang *et al.*, 2002). Based on these observations, it is likely that deletion of the Gβ gene has different effects on vegetative growth and conidiation in different fungi. Like the *gnb-1* mutant (Yang *et al.*, 2002), *mgb1* mutants in *M. grisea* are female sterile. In homothallic *A. nidulans*, the *sfaD* mutant is blocked in cleistothecium formation (Rosen *et al.*, 1999). The *C. neoformans gpb1* mutant is defective in mating and haploid fruiting (Wang *et al.*, 2000). Thus, Gβ signalling may be involved in regulating sexual reproduction in filamentous fungi.

The *mgb1* mutants fail to form appressoria on hydrophobic surfaces under normal conditions. In contrast to *pmk1* mutants that still recognize hydrophobic surfaces and form sub-apical swollen bodies (Xu and Hamer, 1996), *mgb1* mutants develop long, undifferentiated germ tubes (Fig. 3-4). Exogenous cAMP induces the formation of abnormal appressoria on hydrophobic or hydrophilic surfaces in *mgb1* mutants (Fig. 3-5) but not in *pmk1* mutants (Xu and Hamer, 1996). These data indicate that the deficiency of *mgb1* mutants in appressorium formation resulted from a defect in surface recognition and cAMP signalling (unrelated to activation of the Pmk1 pathway), and Mgb1 is dispensable for activating the Pmk1 pathway for appressorium formation (Fig. 3-11). In *S. cerevisiae*, Ste4 is essential for activating the Fus3

MAP kinase cascade for mating responses, but it is not involved in activating the Kss1 MAP kinase pathway for filamentous growth (Madhani and Fink, 1997; Dohlman and Thorner, 2001). Therefore, for appressorium formation in *M. grisea*, the Pmk1 MAP kinase pathway may be activated like the Kss1 pathway for filamentous growth in yeast (Fig. 3-11).

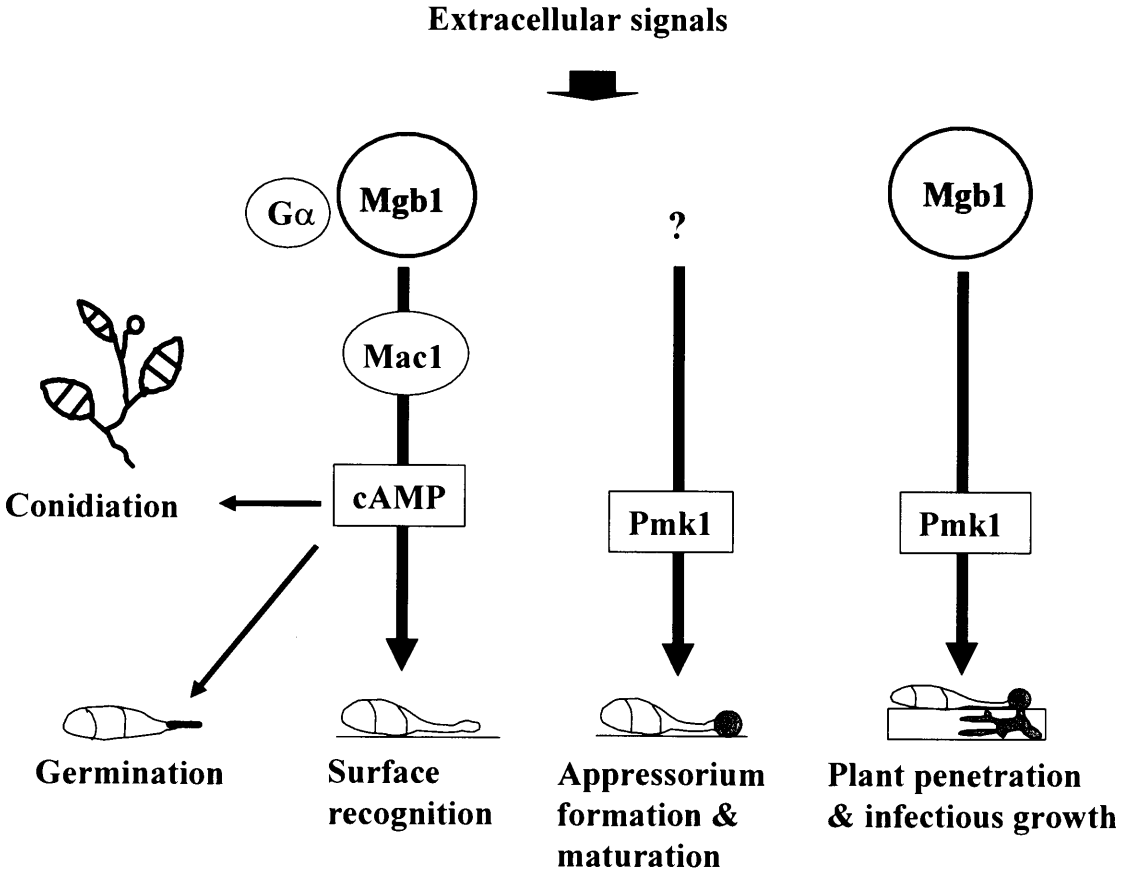

Fig. 3-11. A hypothetical model for Mgb1 function in *Magnaporthe grisea*. The rice blast fungus forms appressoria by attaching to hydrophobic surfaces or by cAMP induction on hydrophilic surfaces. Surface recognition signals are likely relayed by Mgb1 to activate adenylate cyclase (Mac1), which increases the intracellular levels of cAMP. Increased cAMP initiates appressorium formation by activating PKA. Conidiation and conidial germination may be also regulated by G-proteins via cAMP signalling. Downstream events in appressorium formation include arrest of germ tube tip growth, synthesis of specific cell wall layers and generation of turgor pressure. Mgb1 is dispensable to these downstream appressorium formation and maturation events that are regulated by the MAP kinase Pmk1. Pmk1 may be activated by other upstream elements. Mgb1 may function upstream of Pmk1 to regulate appressorial penetration and infectious hyphae growth since exogenous cAMP can only rescue appressorium formation, but not penetration and plant infection, in the *mgb1* mutant. However, direct proof of the activation of Pmk1 by Mgb1 has not been attained.

Similar to *pmk1* mutants (Xu and Hamer, 1996), *mgb1* mutants are non-pathogenic and fail to penetrate and infect healthy or wounded plants (Fig. 3-6). Exogenous cAMP induces appressorium formation in *mgb1* mutants but has no effect on penetration and infectious growth (Fig. 3-6). In *M. grisea*, Mst12 is a putative transcription factor functioning downstream from Pmk1 that is dispensable for appressorium formation but essential for penetration and infectious growth (Park *et al.*, 2002). However, in contrast to *mst12* mutants, the appressoria formed by *mgb1* mutants induced by cAMP are morphologically abnormal. Although Mgb1 is not directly involved in activating Pmk1 for appressorium formation, it may be involved in regulating Pmk1 for appressorium penetration and infectious growth processes (Fig. 3-11). Pmk1 may be improperly regulated in *mgb1* mutants because appressoria formed by *mgb1* mutants are abnormal and non-functional. The specificity of the Pmk1 MAP kinase cascade for appressorium formation, penetration and infectious hyphae growth may be controlled by different upstream signals and different scaffold proteins (Park *et al.*, 2003; Fig. 3-11).

When cultured on agar plates, similar to the *gnb-1* mutant in *N. crassa* (Yang *et al.*, 2002), *mgb1* mutants have significantly reduced cAMP levels (Table 3-5). In submerged cultures, *mgb1* mutants continue to exhibit significantly reduced cAMP levels in contrast with the normal or slightly elevated cAMP levels observed for the *gnb-1* mutant (Table 3-5; Yang *et al.*, 2002). Conidiation is not observed in submerged cultures of *M. grisea* (data not shown). The difference between the cAMP observed for *mgb1* mutants grown on oatmeal agar and 5xYEG liquid cultures may result from conidiophores and conidia present in mycelia being scraped off from the oatmeal agar plates. In the presence of exogenous cAMP, Mgb1 becomes dispensable for appressorium formation. Thus, the defect in appressorium formation in *mgb1* mutants must have resulted from a defect in activating the cAMP signalling that is known to be important for surface recognition and initiation of appressorium formation (Lee and Dean, 1993). The *M. grisea mac1* mutant is reduced in conidiation and abolished in appressorium formation and plant infection (Choi and Dean, 1997; Adachi and Hamer, 1998). Its defects in

appressorium formation and conidiation can be complemented by exogenous cAMP or suppressed by a mutation in the regulatory subunit of PKA (Adachi and Hamer, 1998). These phenotypes are similar to what we observed in *mgb1* mutants. Germination of *mgb1* conidia also is delayed (Table 3-3) as observed in *cpkA* mutants (Xu *et al.*, 1997). These observations suggest that Mgb1 is involved in regulating conidiation, conidial germination and surface recognition via the cAMP signalling pathway (Fig. 3-11).

In *C. parasitica*, following disruptions of either one or both of the Gα subunits, *CPG-1* and *CPG-2*, the Gβ subunit *CPGB-1* increases in gene expression but reduces protein accumulation (Segers and Nuss, 2003; Parsley *et al.*, 2003). Similar results are observed in the *cpgb-1* mutant. In the absence of Cpgb-1, an increase in accumulation of *CPG-1* gene expression is accompanied by a reduction in protein accumulation (Kasahara *et al.*, 2000; Segers and Nuss, 2003; Parsley *et al.*, 2003). Interestingly, constitutively activated Cpg-1 also increases the expression level of *CPGB-1* gene, and reduces the accumulation of Cpgb-1 protein. These data indicate that the Gα and Gβ protein turnover rates are increased in the mutant strains in *C. parasitica* (Segers and Nuss, 2003; Parsley *et al.*, 2003). In Contrast to the results obtained for *C. parasitica*, a loss of either one of three Gα subunits, Gna-1, Gna-2 and Gna-3, in *N. crassa* does not affect Gβ protein Gnb-1 level (Ivey *et al.* 1999; Kays *et al.*, 2000). Furthermore, Gnb-1 level remains unaltered in constitutively active *gna-1* mutants (Yang and Borkovich, 1999). However, similar to *C. parasitica*, a loss of Gnb-1 leads to lower levels of the three Gα proteins (Yang *et al.*, 2002). These results may provide the explanation as to why common mutant phenotypes are shared among G-protein mutants in *N. crassa* and *C. parasitica* (Yang *et al.*, 2002; Parsley *et al.*, 2003). It is very tempting to speculate that several post-transcriptional regulation mechanisms of Gα or Gβ proteins exist in many filamentous fungi.

In *M. grisea*, some of the phenotypes of Gα mutants (*magB, magC*) and Gβ mutant, *mgb1* are observed to overlap. For example, *magB* mutant still forms conidia, appressoria and lesions even though they are significantly reduced (Liu and Dean, 1997). *Mgb1* completely loses

its ability to form appressorium and to infect, and is greatly decreased in conidiation (Fig. 3-4; Fig. 3-6). The appressorium formation defects in *magB* and *mgb1* are recovered by an addition of exogenous cAMP (Fig. 3-5; Fig. 3-10; Liu and Dean, 1997). These data indicate that deletion of either *MAGB* or *MGB1* leads to a reduced cAMP signalling activity required for surface recognition. Transformants expressing the putative constitutive-active *MAGB* ^{G42R} allele form appressoria on both hydrophobic and hydrophilic surfaces (Fang and Dean, 2000). This phenotype is similar to that observed in the cmp9 transformant carrying multiple copies of *MGB1*. When cultured on oatmeal agar plates, the intracellular cAMP level of cmp9 is higher than that of the wild type (Table 3-5). It seems that expressing *MAGB* ^{G42R} and multiple copies of *MGB1* both over-activate the cAMP signalling for surface recognition. The conidiation phenotype observed in the *MAGB* ^{G42R} mutation indicates a probable role of MagB as a negative regulator of conidiation. Interestingly, the *MAGB* ^{G42R} mutation results in mis-scheduled melanization at the tips of conidiophores (Fang and Dean, 2000). No such phenotype is observed in cmp9 or *mgb1* deletion mutants, suggesting that MagB may be involved in additional cellular processes in *M. grisea*. The lost of ability for the aged conidia (> 10-days old) of cmp9 to form appressoria on hydrophilic surface (Fig. 3-9), and the lost virulence in the *magB* ^{G42R} mutant (Liu and Dean, 2000) could both be related to a quick turnover of G-proteins.

Although it is likely that a similar post-transcriptional regulation mechanism of G-proteins exists in *M. grisea*, some of the *mgb1* phenotypes, such as the complete loss of pathogenicity and appressorium formation, cannot be explained by the decreased accumulation of Gα subunits. To date, 10 spontaneous mutants of *mgb1* with an increased conidiation phenotype have been isolated. All of these mutants are able to form appressoria but fail to infect healthy or wounded rice plants (M. Nishimura, unpublished data). The various *mgb1* deletion mutant phenotypes observed in this investigation indicate that *MGB1* plays an important role in cAMP signalling, appressorium formation and plant infection.

CONCLUSION

Infection-Related Morphogenesis of *Magnaporthe grisea*

Mgb1 functions in appressorium formation in *Magnaporthe grisea*

Environmental stimuli can alter the morphology of *M. grisea*. For example, light and oxidative stresses can drive *M. grisea* from mycelial growth to conidiophore and conidium development, while surface hydrophobicity can induce germinating conidia to produce appressoria (Shi and Leung, 1995; Lee and Dean, 1994). Our efforts have been directed at dissecting the mechanism of appressorium formation in *M. grisea*, as this represents one of the most important steps in the infection process of the fungus.

Our studies demonstrate that Mgb1 is essential for the triggering of appressorium formation. Mutants with deleted *MGB1* cannot form appressoria, and are defective in penetration and infectious growth. Mutants grown in the presence of exogenous cAMP develop appressoria, albeit abnormal ones that fail to penetrate plant cells. This suggests that Mgb1 is also required for penetration. This is made possible by integrating the cAMP signalling and Pmk1 MAPK pathways (Nishimura *et al.*, 2003).

Results from our studies on Mgb1 build on results from other laboratories to provide a better understanding of the signal transduction pathways involved in appressorium formation.

The cAMP signal transduction pathway regulates appressorium formation in *Magnaporthe grisea*

Appressorium formation (Lee and Dean, 1993) is triggered by cAMP, a secondary messenger and major component of the signal transduction pathway. A recent study indicates that Pth11 is likely a transmembrane receptor protein acting upstream of the cAMP signaling pathway. In that study a *PTH11* deletion mutant failed to form appressoria efficiently on hydrophobic surfaces, but responded to exogenous cAMP by forming functional appressoria

(Dezwaan *et al.*, 1999).

Within the cAMP signal transduction pathway, *MAC1* encodes an adenylate cyclase in *M. grisea* (Choi and Dean, 1997). Deletion mutations engineered in genes belonging to the cAMP signal transduction pathway are hypothesized to generate mutants with phenotypes similar to *mac1*. For example, the deletion mutant phenotypes of *PTH11*, *MAGB* (G-protein α subunit), *MGB1* (G-protein β subunit) and *MAC1* resemble each other in that they fail to form appressoria on hydrophobic surfaces, but are still capable of doing so in response to exogenous cAMP (DeZwaan *et al.*, 1999; Liu and Dean, 1997; Nishimura *et al.*, 2003). Changes in intracellular cAMP levels in the *MGB1* deletion mutant and in the *MGB1* over-expression variant suggest that the downstream target of Mgb1 is Mac1 (Nishimura *et al.*, 2003). It is likely that Pth11 transmits the signal to heterotrimeric G-proteins, and the resulting activated Mgb1 then transmits the signal to adenylate cyclase (Mac1). One of the intracellular targets of cAMP is CpkA (Mitchell and Dean, 1995; Xu *et al.*, 1997). A *cpkA* mutant infects wounded rice leaves but fails to penetrate via appressorium (Xu *et al.*, 1997). These data indicate the importance of cAMP signaling not only as a trigger in appressorium formation, but also in functional appressorium development.

Proteins up- and downstream of the Pmk1 signal transduction pathway

It is becoming increasingly evident with the discovery of more components that the Pmk1 signal transduction pathway plays a major role in appressorium maturation. The two genes *GAS1* and *GAS2*, which are up-regulated by Pmk1, have been isolated from a subtraction library of *pmk1*. The expression of these genes is appressorium-specific. Mutants with either *GAS1* or *GAS2* deleted exhibit a reduction in appressorial penetration and invasive growth (Xue *et al.* 2002). *MST12*, another member of this pathway, was cloned from *M. grisea* based on its homology with yeast *STE12*, the Fus3/Kss1 MAP kinases related transcriptional factors (Park *et al.*, 2002). An *mst12* mutant forms wild type appressoria on a hydrophobic surface, but is

unable to develop a penetration peg. *Mst12* also fails to undergo invasive growth through wounds. Experiments using a yeast two hybrid system indicate that Mst12 interacts with Pmk1 (Park *et al.*, 2002). The over-expression of *MST12* in a *cpkA* mutant complements the infection defective phenotype of the *mst12* mutant. These data indicate that Mst12 is essential for infection and likely acts downstream of both the Pmk1 MAP kinase and cAMP signal pathways (Park and Xu, 2003). Attempts were made to locate the upstream regulators of the Pmk1 pathway using information obtained from a closely related yeast system. *S. cerevisiae* proteins Ste7, Ste11 and Ste20 act upstream of the Fus3/Kss1 MAP kinases to regulate the pheromone and invasive growth signal transduction pathways (Elion, 2000). Mst7, Mst11, Mst20 are the *M. grisea* homologs of Ste7, Ste11 and Ste20, respectively. Knockout mutants of *MST7* and *MST11*, but not *MST20*, show a defective phenotype in appressorium formation (Xue *et al.*, 2003). This result suggests that Pmk1 is likely the downstream target of Mst7 and Mst11. Unlike in the yeast system, Mst20 does not appear to be involved in the Pmk1 pathway (Xue *et al.*, 2003).

The signal transduction pathway involved in the penetration of plants

MPS1 is essential for the maintenance of cell-wall integrity during specific phases of the life cycle and, most importantly, for turgor-driven penetration of plant cells (Xu *et al.*, 1998). Pls1 is a transmembrane protein encoding a tetraspanin-like protein and functions in penetration peg differentiation. Studies of a *punchless* mutant (*pls1)* indicate that although defective in pathogenicity, *pls1* produces normal melanized appressoria that exhibit wild type turgor levels. In mammals, proteins of the tetraspanin family are associated with other membrane proteins and are involved in cell differentiation, migration, and adhesion (Clergiot *et al.*, 2001). It is plausible that Pls1 is also involved in the formation of a receptor-effecter protein complex at the cell membrane.

The transmembrane protein Pls1, the Gβ subunit Mgb1, and the transcriptional factor

Mst12 are essential in facilitating the infectious growth of the fungus following its penetration into plants (Clergiot *et al.*, 2001; Park *et al.*, 2002; Nishimura *et al.*, 2003). The MAP kinase Mps1 is also essential for plant penetration but not for invasive growth in plants. It is plausible that both the Pls1-Mgb1-Mst12 and Mps1 pathways contribute to the signal transduction pathway involved in triggering the penetration process. A schematic model of the signal transduction pathway of appressorium formation in *M. grisea* is shown in Fig. C-1.

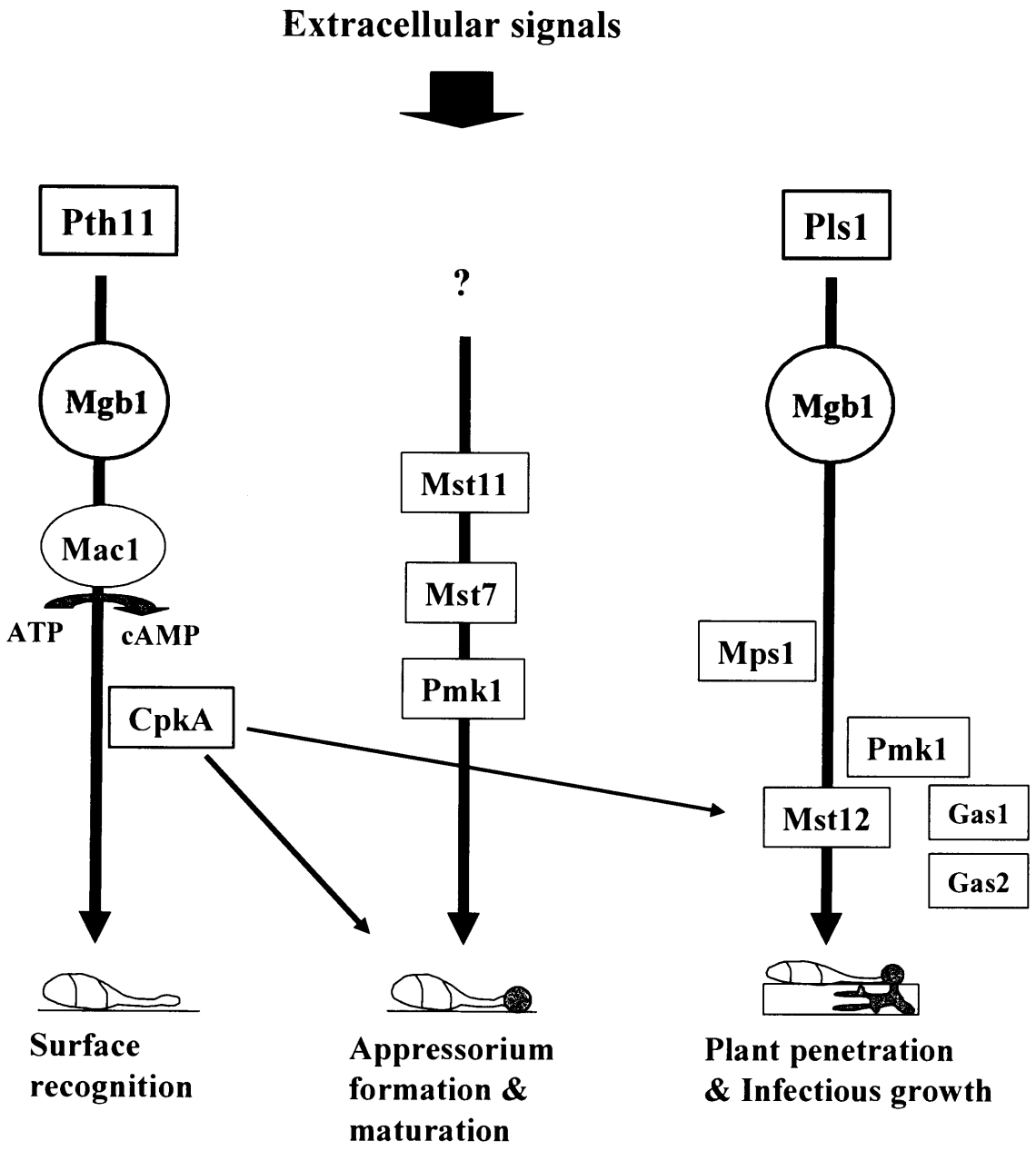

Fig. C-1. A schematic model for signal transduction pathways regulating infection related development of *M. grisea*. Descriptions of all the pathways are found in the accompanying text.

Acr1 functions in conidiogenesis of *Magnaporthe grisea*

Infection by the rice blast fungus begins with the attachment of the fungal conidia to the leaf surface of a rice plant. It is a tempting strategy to manage rice blast disease by targeting the development of *M. grisea* at the conidiation stage, since this is a critical step in the infection process.

Our studies demonstrate that Acr1 is essential for normal conidiophore development. The deletion of *ACR1* also results in defective appressorium formation, which correlates with abnormal conidiophore patterning and reduced glycogen degradation. Our preliminary data suggests that Acr1 is involved in regulating the expression of several genes including *MPCL1* and *MGPH1*. Acr1 might control conidiophore patterning and glycogen metabolism via Mplc1 and Mgph1 activities, respectively.

Acr1 is a homolog of *A. nidulans* MedA, a conidiophore development regulator (Nishimura *et al.*, 2000). Recently, new Acr1 homologs have been found in *Fusarium oxysporum* and *N. crassa* (Ohara and Tsuge, 2003; http://www-genome.wi.mit.edu, *Neurospora crassa* Database). It is evident that the counterparts of Acr1 (i.e. MedA in *A. nidulans* and Ren1 in *F. oxysporum*) have the same biological function of controlling conidiophore patterning, since the mutants exhibit a common phenotype characterized by the head-to-tail arraying of conidiogenesis cells. Thus Acr1 and MedA appear to represent founding members of a novel class of conserved regulators that control conidiogenesis pattern formation.

Table C-1. Summary of the mutant phenotypes related to conidiation and appressorium formation.

Gene	Mutant phenotypes			
	Appressorium formation		Conidiation	
	infection ability[1]	Number	Phenotype	Number
ACR1	Very reduced	reduced	Chain-like	Normal
CON1	NA[2]	0	Elongated	very Reduced
CON2	Very reduced	Reduced	Less septum	very reduced
CON4	very reduced	Reduced	Elongated	reduced
CON5	NA	0	NA	No
CON6	NA	0	NA	No
CON7	NA	0	Elongated	reduced
CHM1	Very reduced	Reduced	Elongated, less septum	very reduced
SMO1	Reduced	Increased	Round	Normal
PTH11	No	reduced	Normal	Normal
PLS1	No	Normal	Normal	Normal
MAGB	Very reduced	very reduced	Normal	Reduced
MGB1	No	0	Normal	Very reduced
MAC1	No	0	Normal	Very reduced
CPKA	Very reduced	Normal	Normal	Normal
PMK1	NA	0	Normal	Normal
MST11	No	0	?	Reduced
MST7	No	0	Normal	Normal
MST12	No	Normal	Normal	Normal
MPS1	No	Normal	Normal	Very reduced

[1]Infection abilities were determined based on the results of spray assays on rice plants.

[2]NA denotes Not Applicable.

Cross-regulation of conidiation and appressorium formation pathways in *Magnaporthe grisea*

Many of the *M. grisea* strains engineered with mutations in the appressorium formation signal transduction pathway also show a reduction in conidiation number. It is notable that most of the conidiation defective mutants described above are able to germinate, but have a reduced ability to differentiate appressoria under inductive conditions. These results suggest the probable existence of cross-regulation mechanisms in conidiogenesis and appressorium formation. A summary of mutant phenotypes related to conidiation and appressorium formation is shown in Table C-1.

Our studies of *mgb1* and *acr1* have led to a better understanding of the cross-regulation mechanisms in *M. grisea*. First, we have demonstrated that reduction of intracellular cAMP results in defective conidiophore development in *mgb1*. Second, as described above, cAMP signalling is essential for initiating the formation and proper functioning of appressorium. Taken together, our results clearly demonstrate that Mgb1 regulates both appressorium and conidiophore development via cAMP signalling. Our current study of *acr1* indicates that Acr1 plays a critical role in conidiophore patterning and is important for the glycogen metabolism, the product (glycerol) of which is necessary to generate turgor pressure in appressoria. It seems likely, in light of these cross-regulation events, that Mgb1 and Acr1 are located at a point in the signal transduction pathway where both the appressorium formation and conidiation regulatory signals intersect. A recent report of the co-expression of several genes triggered during conidiation, appressorium formation, and infection of *M. grisea* (Rauyaree *et al.*, 2001) lends support to our notion of the cross-regulation phenomenon seen with Mgb1 and Acr1.

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