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SIGNALING PATHWAY IN APPRESSORIUM FORMATION IN

Magnaporthe grisea

A Dissertation

by

MARTA CRISTINA FILIPPI E SILVA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Plant Pathology

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Approved as to style and content by:

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August 2004

Major Subject: Plant Pathology

ABSTRACT

Signalling Pathway in Appressorium Formation in *Magnaporthe grisea*.

(August 2004)

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Chair of Advisory Committee: Dr. Daniel J. Ebbole

We identified a synthetic hexapeptide that blocks *Magnaporthe grisea* appressorium formation, in artificial hydrophobic surface. The results suggest that peptides interfere with surface recognition.

M. grisea non pathogenic *pth1* mutants were complemented by *N. crassa* orthologous gene suggesting that the biochemical function of *pth1* has not evolved specifically to play a role in appressorium development.

DEDICATION

To my father Prof. Dr. José de Filippi I devote every day of this journey.

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CHAPTER I

INTRODUCTION

Rice, O*ryza sativa*, is a principal food source for developing countries. It is the source of 20% of the calories and 13% of the protein consumed in the world (Soares et al. 1990). More than four-fifths of the world's rice is produced and consumed by small-scale farmers in low-income and developing countries. The amount of rice consumed by each of these people ranges from 100 to 240 kg per year, according to FAO (Food and Agricultural Organization of the United Nations). In 2030, the world population is expected to reach 8.2 billion compared to 6.2 billion today. A considerable quantity of rice will be required to meet future needs. In 2030, global demand is projected to be approximately 533 million tons of rice, compared to 472 million tons projected for 2015, and an average of 386 million tons consumed in 1997 to 1999 ((FAO 2002), Appendix A).

Rice blast, one of the most destructive diseases in rice fields, is caused by *Magnaporthe grisea* Barr (*Pyricularia grisea* anamorph Cav.). The fungus is distributed world-wide and causes losses of up to 100% of the yield depending on cultivar susceptibility, environmental conditions and management system. During 2003, in India, rice blast was responsible for losses of more than 266,000 tons of rice, which was about 0.8% of the total yield. In Japan, the disease affects approximately 865,000 hectares of rice fields each year. In the

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This dissertation follows Molecular Plant-Microbe Interactions format.

Philippines, rice fields may suffer more than 50% yield losses each year caused by rice blast (IRRI 2003).

M. grisea is a heterothallic ascomycete that parasitizes all rice plant growth stages; however, the critical stages are between 30 and 60 days after germination, when the disease occurs in the leaves, and during panicle emergence and grain formation. *M. grisea* also parasitizes other grasses and a large number of other monocot species (Table1).

Genetic resistance of rice cultivars against *M. grisea* combined with chemical control are the most commonly used measures to control rice blast. However, rice pathogen populations show a high degree of genetic variability resulting in the breakdown of previously resistant rice cultivars (Valent 1990). Genetic uniformity of modern rice varieties may render the crop more vulnerable to outbreaks of pests and diseases.

Table 1. Grasses parasitized by *M. grisea.*

Cientific name	Comum name	Cientific name	Comum name
Agropyron repens	Quackgrass	*Hierochloe odorata*	Vanilla grass
Agrostis palustris	Creeping bentgrass	*Holcus lanatus*	common elvet grass
A. tenuis	Colonial bentgrass	*Hordeum vulgare*	common barley
Alopecurus pratensis	Meadow foxtail	*Hystrix patula*	Eastern bottlebrush grass
Andropogon sp.	Bluestem	*Leersia hexandra*	Southern cutgrass
Anthoxanthum odoratum	Sweet vernalgrass	*Hierochloe odorata*	Vanilla grass
Arundo donax L.	Giant reed	*L. japonica*	
Avena byzantina	Oat	*L. oryzoides*	
A. sterilis	Oat	*Lolium italicum*	Italian ryegrass
A. sativa, f	Oat	*L. multiflorum*	
Brachiaria mutica	para grass	*L. perenne*	perennial ryegrass
Bromus catharticus	rescuegrass	*Muhlenbergia sp*	Muhly
B. inermis	smooth brome	*Musa sapientum*	French plantain
B. sitchensis	Alaska brome	*Oplismenus undulatifolius*	wavyleaf basketgrass
Canna indica	Indian shot	*Panicum miliaceum*	
Chikushichloa aquatica		*P. ramosum*	signalgrass
Costus speciosus	Canereed	*P. repens L.*	torpedo grass
Curcuma aromatica	Curcuma	*Pennisetum typhoides*	Pearl millet
Cynodon dactylon (L.) s.	Bermudagrass	*Phalaris arundinacea*	Reed canarygrass
Cyperus rotundus	nutgrass	*P. canariensis*	canarygrass
C. compressus L.	poorland flatsedge	*Phleum pretense*	timothy
Dactylis glomerata	Orchardgrass	*Poa annua L.*	annual bluegrass
Digitaria sanguinalis	hairy crabgrass	*P. trivialis*	rough bluegrass
Echinochloa crus-galli	Barnyardgrass	*Saccharum officinarum*	sugarcane
Eleusine indica	Indian goosegrass	*Secale cereale*	Cereal rye
Eragrostis sp.	Purple lovegrass	*Setaria italica*	foxtail bristlegrass
Eremochloa ophiuroides	Centipede grass	*S. viridis*	green bristlegrass
Eriochloa villosa	Cupgrass	*Sorghum vulgare*	grain sorghum.
Festuca altaica	Altai fescue	*Stenotaphrum secundatum*	St. Augustine grass
F. arundinacea	Tall fescue	*Triticum aestivum*	common wheat
F. elatior	meadow ryegrass	*Zea mays L.*	Corn
F. rubra	red fescue	*Zingiber mioga*	Mioga ginger
Fluminea sp.		*Z. officinale*	garden ginger
Glyceria leptolepis		*Zizania latifolia*	Manchurian wildrice

Source: Natural Resurce Conservation Service, USDA, 2004 (USDA 2004)

Systemics fungicides as Benomyl, Kasugamicina, Tebuconazole, Thiabendazole, Tricyclazole, and contact fungicides as Edifenfos, Fetin acetate, Fetin hydroxide, and Mancozeb offer an adequate level of disease control (Prabhu et al. 1995), however, the success of disease control depends on the genetic resistance of the rice cultivar, and on the number of applications of fungicide during the season (Prabhu 1989) However, chemical control measures tend to be expensive in countries where rice is the basic food crop because of the high production cost and low grain price, such as found in South-East Asia, India, Latin America, Africa, and the USA, creating the need for a less expensive chemical product. However, increased environmental concerns place restrictions on the use of such products (Froyd and Foeliger 1994).

The *M. grisea* life cycle is illustrated in Fig. 1 (Xu and Hamer 1996). The primary inoculum is dispersed by wind, water, or infected seeds. Conidia firmly attach to the wax-covered host surface using pre-formed mucilage packaged in the spore tip. The adhesive is thought to be composed of carbohydrate and glycoproteins rich in α-linked glucosyl and/or mannosyl residues (Howard and Ferrari 1989). A specialized cell, the appressorium is required to penetrate the host surface. Colonization of a susceptible host leads to the derangement of host metabolism and results in typical disease symptoms. Following lesion formation, asexual reproduction produces conidia and leads to further dispersal of the fungus. This infection cycle can be completed within 5 days and explains the rapid spread of an epidemic.

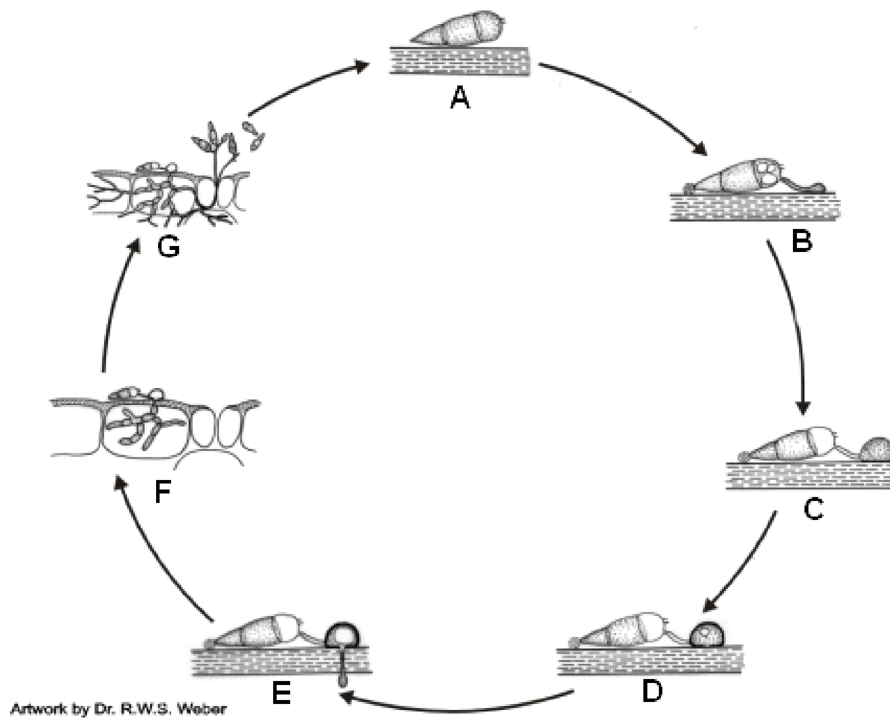


Figure 1. *Magnaporthe grisea* life cycle. A, Dispersal; B, attachment and germ tube enlongation; C, Appressorium formation; D, appressorium maturation; E, penetration peg formation and host tissue penetration; F, host colonization; G, asexual reproduction starting the second cycle**.**

BIOLOGY OF APPRESSORIUM FORMATION

In 1883, Frank (Deising et al. 2000) reported a new bean disease caused by *Colletotrichum lindemuthianum*, and described the germination process that is followed by a swelling of the tip of the germ tube and the development of an organ of adhesion. Frank coined the term appressorium to express the function of adhesion and pressure on the host surface. The appressorium is a structure formed after the germination of the conidium. It is employed by the fungus to generate hydrostatic turgor pressure used to penetrate the host surface (Howard and Ferrari 1989). Appressoria are unique fungal structures used by diverse groups of fungi to penetrate host surfaces (Lengeler et al. 2000).

During the first two hours after the *M. grisea* conidium attaches to an inductive host surface, the conidium germinates and the germ tube starts to elongate. During the next two hours, the elongating germ tube starts to differentiate to form a swollen hyphal tip referred to as the “hook” cell. In some species, such as *Venturia inaequalis,* the germ tube is short, and for other species, such as rusts and powdery mildews, the germ tube elongates until it senses a stomata on the leaf surface (Dean 1997). The nucleus originating from the conidium migrates to the germ tube and undergoes mitosis. One daughter nucleus migrates into the hook cell prior to septum formation and appressorium maturation. After septum formation a cell wall layer composed of melanin is produced covering the immature appressorium. In *Uromyces* spp., *Colletotrichum* spp., and *M. grisea,* the appressorium is separated from the germ

tube by a septum formed after movement of material from the conidium and germ tube into the nascent appressorium (Dean 1997). At the base of the maturing appressorium the cell wall is very thin and no melanin is present in this region that is in direct contact with the host cell (Howard and Ferrari 1989). An “O-ring” at the base of the appressorium appears to allow attachment to the host surface (Bourett and Howard 1990). During the next four to six hours, glycogen and lipid migrate into the forming appressorium. The accumulated glycogen and lipid in the nascent appressorium break down to generate high intracellular levels of glycerol (Talbot 1999), (Thines et al. 2000) The mature appressorium differentiates a penetration peg, a specialized hyphae that penetrates the plant tissue surface. The penetration peg gives rise to bulbous invasive hyphae that elongate during colonization of the tissue (Clergeot et al. 2001). Biotrophic fungi, such as rusts and powdery mildews, form appressoria over the stomata. Penetration hyphae form and enter the substomatal cavity producing the substomatal vesicle, a precursor of the haustoria mother cell (Dean 1997).

SIGNALING PATHWAYS

Saccharomyces cerevisiae is considered a model system for molecular biology studies including signal transduction. It undergoes a dimorphic transition to a pseudo-filamentous growth form in response to nitrogen limitation and abundant fermentable carbon source. These environmental cues lead to filamentous growth by inducing a switch from the normal pattern of cell growth to a unipolar budding pattern that allows invasion of the growth substrate. At least two conserved signal transduction pathways, the MAP Kinase cascade and the cAMP pathway, regulate filamentous growth. Homologous pathways regulating growth polarity also have been defined in the fission yeast, *Schizosaccharomyces pombe*. These pathways are conserved in the human pathogens *Candida albicans* and *Cryptococcus neoformans*, and in the plant pathogens *Ustilago maydis*, *M. grisea* and *Cryphonectria parasitica*, and in the model filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* (Lengeler et al. 2000). In every case, these pathways play important roles in growth and development.

Signaling between plants and foliar pathogenic fungi begins when they meet on the plant surface. Topographical features of the plant surface or chemical signals may trigger a programmed differentiation process leading to pathogenesis if the signals at the plant surface are sensed as favorable by the fungus (Kolattukudy and Roger 1995).

Formation of appressoria involves complex morphogenetic processes, which are influenced by multiple environmental signals. These signals may

include chemical cues from the host plant, such as wax monomers, or physical cues, such as surface hydrophobicity or topographical features (Lee et al. 2003).

Plants are normally covered by a wax layer, a complex mixture of long chain and very hydrophobic aliphatic compounds (Kolattukudy and Roger 1995), Gilbert et al. (1996), investigating the signal responsible for activating the appressorium formation pathway in *M. grisea,* tested the effects of specific cutin monomers, lipids compounds, and thigmotropic stimuli. It was found that: 1) *M. grisea* did not form appressoria on hydrophilic surfaces; 2) the most inductive cutin monomer was 1,16-hexadecanediol, when tested on the non inductive hydrophilic surface; 3) *M. grisea* formed appressoria on the artificial hydrophobic surface of GelBond film (Gilbert et al. 1996).

The mitogen activated protein kinase (MAP Kinase) cascade is conserved in eukaryotes and has been identified in many organisms, from fungi to humans (Xu 2000). MAP Kinases belong to a family of serine/threonine protein kinases involved in transmitting a variety of extracellular signals, allowing cells to adjust their activities, for example, regulating growth and differentiation processes. The kinase cascade starts by activation of a MAP Kinase Kinase Kinase which activates by phosphorylation a MAP Kinase Kinase which activates a MAP Kinase responsible for transmitting the signal to the nucleus by differential phosphorylation of transcription factors (Xu 2000), (Lengeler et al. 2000), (Lee et al. 2003).

The classical cAMP signaling pathway in filamentous fungi consists of a transmembrane cell surface receptor, which senses the extracellular environment and a heterotrimereic G protein, composed of an α subunit and a $\beta\gamma$ heterodimer. The exchange of GDP for GTP activates the Gα subunit to stimulate effector proteins, such as adenylyl cyclase. Adenylyl cyclase synthesizes cAMP, and cAMP-dependent protein kinases mediate the physiological effects of cAMP. When cellular levels of cAMP increase, the regulatory subunit of cAMP-dependent protein kinase dissociates from the catalytic subunits (Lee et al. 2003). Table 2 summarizes some of the processes regulated by cAMP signaling pathways in phytopathogenic fungi (Dean 1997), (Lee et al. 2003).

M. grisea senses hardness and hydrophobicity, adhesion, wax monomers, and other environmental factors to activate appressorium formation (Lengeler et al. 2000). *M. grisea*, *Uromyces* species, and *Colletotrichum* species respond to physical surface signals. In *C. gloeosporioides*, the stimulus provided by hard surfaces initiates a chain of molecular events that leads to the expression of hard surface induced genes, necessary for further activation of genes by plant compounds, such as ethylene or wax that are required for appressorium differentiation (Deising et al. 2000).

Table 2. Processes regulated by the cAMP signaling pathway during the life cycle of some phytopathogenic fungi.

Process	Microorganism
Mating	*Ustilago maydis*
Hyphal morphology	*Ustilago maydis*
Hyphal growth	*Fusarium* spp.
Virulence	*Ustilago maydis*
Infection structure formation	*M. grisea*
	Colletotrichum spp.
	Blumaria graminis f. sp. *Hordei*
Conidiation	*Fusarium* spp.
Conidial germination	*Colletotrichum* spp
	Fusarium spp
Sclerotia formation	*Sclerotinia sclerotium*
	Rhizoctonia solani
	Sclerotium rolfsii
Mycoparatitism	*Trichoderma* spp

The endogenous signaling molecule cAMP was shown to stimulate appressorium formation on normally non-inductive substrates (Lee and Dean 1993a). The same authors correlated hydrophobicity of the contact surface and induction of appressorium formation in *M. grisea* (Yong-Hwan and Dean 1994).

Conidia germination and appressorium development in *Blumeria graminis* f. sp. *Hordei*, the causal agent of powdery mildew of barley, was investigated. Conidia inoculated on an artificial inductive membrane displayed a transient increase in cAMP levels followed by a burst of PKA activity resulting in formation of the primary germ tube (PGT). PGT senses the surface causing a second release of cAMP and activation of the MAP kinase pathway. Both pathways interact to stimulate the emergence of the appressorium germ tube (AGT) (Kinane and Oliver 2004).

GENES INVOLVED IN REGULATING APPRESSORIUM FORMATION

Hydrophobins function to form self-assembling protein monolayers at hydrophobic/hydrophilic interfaces. *MPG1* encodes an amphipathic class I hydrophobin that assembles itself at air-water interfaces or at interfaces between hypha and hydrophobic surfaces, thereby promoting hyphal adhesion (Kinane and Oliver 2004). Fungi have to grow in close contact with the substrate in order to sense physical surface features. *M. grisea mpg1* mutants displayed reduced adhesion on hydrophobic surfaces (Talbot et al. 1993), (Beckerman and Ebbole 1996).

PTH11 is a gene in *M. grisea* predicted to encode a transmembrane protein. PTH11 was identified by REMI (Restriction Enzyme Mediated Integration) mutation. Pth11 mutants failed to form appressoria on inductive surfaces, and showed decreased pathogenicity. However these mutants were responsive to exogenous cAMP, forming functional appressoria and restoring pathogenicity. A Pth11-GFP fusion protein was localized to the cell membrane. Based on their results, the authors suggested the Pth11 protein plays a role in activating appressorium signaling as a receptor for inductive surface cues (DeZwaan et al. 1999).

To investigate new components in the cAMP signal transduction pathway the yeast two-hybrid system was applied to screen MAC1 and CPKA proteins against an appressorium cDNA library. A protein phosphatase-interacting domain in MAC1 was identified, and MAC1 was also able to interact with a MAP kinase kinase and a Ser/Thr kinase. ACI1 is a predicted membrane protein with an extracellular domain containing eight-cysteines, and is highly expressed during appressorium formation. ACI1 also interacted with MAC1. The N-terminal half of CPKA interacts with a putative transcriptional regulator and two different glycosyl hydrolases (Kulkarni and Dean 2004). The interaction of MAC1 with ACI1, and of CPKA with a transcription factor suggests that these proteins play a role in appresorium formation.

Protein kinase A (PKA) is a cAMP-dependent protein kinase composed of two catalytic and two regulatory subunits. The gene *cpkA* encoding the catalytic

subunits in *M. grisea* has been characterized. The mutant strains were defective in penetrating into plant cells. Although the mutants formed melanized appressoria, they are smaller than the wild type and non-functional. When inoculated into wounds, infectious hyphae and typical lesions were found. Induction of appressorium formation was responsive to exogenous cAMP. Thus, *cpkA* is required for production of functional appressoria, however, cells were still responsive to cAMP suggesting the existence of at least one more PKA involved in cAMP signaling in the the appressoria pathway. Probably, *cpkA* is involved in appressorium maturation, and the other PKA activity is involved in environmental sensing pathways (Mitchell and Dean 1995), (Xu et al. 1997). Mutation of a *Colletotrichum trifolii cpkA* ortholog generates strains able to form appressoria but unable to penetrate the host (Yang and Dickman 1999). *Bka1*, a gene in *B. graminis* similar to *cpka* in *M. grisea*, was described and introduced in the *M. grisea cpkA* mutant genome and expressed using the *MPG1* promoter. The gene restored pathogenicity and appressorium maturation kinetics in *M. grisea* mutants. This result further supported a role for cAMP signaling in *B. graminis* appressorium development (Bindeslev et al. 2004).

PMK1, a MAP Kinase gene in *M. grisea* was cloned, using PCR strategies, and mutants were generated to further understand the process of appressorium morphogenesis. *PMK1*, a homolog to FUS3/KSS1 in *S. cerevisiae*, was shown to be essential for pathogenesis and invasive growth. *pmk1*$^{-}$ conidia germinated and formed the hook cell on hydrophobic surfaces,

but did not swell to form a nascent appressorium. On artificial hydrophilic surfaces they did not form the hook cell. This suggests that *pmk1* mutants are able to sense the substrate surface, an event that requires the second messenger cAMP. The finding that cAMP activates hook cell formation of the *pmk1* mutant on non-inductive surfaces was interpreted to mean that the MAP kinase pathway is activated in response to cAMP signaling (Xu and Hamer 1996). In wound inoculations, *pmk1* mutants did not invade the host tissue, indicating a role for the MAP kinase signaling pathway in both appressorium maturation and host colonization.

The role for *PMK1* homologs in pathogenesis in other fungal species has been examined. *Colletotrichum lagenarium*, the causal agent of anthracnose of cucumber, was unable to differentiate an appressorium or to grow invasively inside host tissue when its homologous MAP kinase gene, *CMK1*, was disrupted (Takano et al. 2004) *Colletotrichum lindemuthianum*, cause of anthracnose on beans, was unable to infect bean leaves after the *CLK1* gene was disrupted by insertional mutagenesis (Dufresne et al. 1998). *Pyrenophora teres*, causal agent of barley net blotch, displayed reduced conidiation, a loss of appressorium differentiation and an inability to grow invasively in the host tissue when its *PMK1* homolog *PTK1* was disrupted (Deising et al. 2000). *Cochiobolus heterostrophus*, the causal agent of corn late blight, displayed reduced ability to infect leaves when *CHK1* was mutated by deletion (Lev et al. 1999).

MPS1, a second MAP Kinase in *M. grisea*, homologous to *S. cerevisiae SLT2*, was disrupted. The mutants formed appressoria, but did not penetrate and grow invasively. The *mps1* mutant was also shown to be more sensitive to cell wall-degrading enzyme than the wild type (Xu et al. 1998). The MAP kinase genes *OSM1* (Dixon et al. 1999), PMK1 (Xu and Hamer 1996) and *MPS1* (Xu et al. 1998) were identified and characterized. The *PMK1* MAP kinase was found to be required for appressorium morphogenesis and host colonization. The *MPS1* MAP kinase was found to be required for appressorium maturation and host colonization. The *OSM1* MAP kinase, homologous to MAP kinase (Hog1) is required to generate glycerol to counteract hyperosmotic stress in *S. cerevisiae*. However, mutation of *OSM1* did not alter the ability to produce a functional appressorium.

MST12, the homologue of *S. cerevisiae STE12*, was characterized and disrupted in *M. grisea*. *STE12* is a transcription factor regulated by *FUS3* and *KSS1* and *mst12* mutants were nonpathogenic on rice and barley leaves. However, the *mst12* mutant formed dome-shaped appressoria, in contrast to the *pmk1* mutant, but it was unable to penetrate onion epidermal cells and failed to grow invasively after wound inoculation. The authors concluded that *MST12* plays a role in appressorium maturation downstream of *PMK1* (Park et al. 2002).

Adenylate cyclase is a membrane bound enzyme that controls the levels of cAMP inside the cell. The adenylate cyclase encoding gene was cloned and disrupted generating the *mac1* mutant. The *mac1* mutant was unable to form

appressoria on an inductive surface, unable to penetrate susceptible rice leaves, was reduced in vegetative growth, conidiation and conidial germination, and failed to form perithecia when crosses with strains of the opposite mating type (Choi and Dean 1997).

Heterotrimeric G proteins are composed of an α subunit and a βγ heterodimer. Heterotrimeric G proteins are involved in the transduction of extracellular signals to an intracellular effector. Activation of a membrane receptor (usually a seven-transmembrane serpentine receptor) causes a conformational change leading to dissociation of the α subunit from the βγ heterodimer. When GDP is exchanged for GTP the α subunit activates the effector. In filamentous fungi, a known effector is adenylate cyclase. Hydrolysis of GTP to GDP converts the α subunit back to its inactivated form, allowing it to again form the heterotrimeric G protein. The genes *mag*A, *mag*B, and *mag*C, encode the three α subunits in *M. grisea.* The *magA* mutant displayed no defect in vegetative growth, conidiation, or appressorium formation. The *magC* mutant displayed normal vegetative growth and appressorium formation, but was reduced in conidiation. The *magB* mutant displayed reduced vegetative growth, conidiation, and appressorium formation. Appressorium formation of *magB* mutants was restored by addition of cAMP or 1,16-hexadecanediol (Liu and Dean 1997). These results showed that the heterotrimeric G protein encoded by *MAGB* acts upstream of adenylate cyclase to stimulate appressorium formation.

In *C. parasitica* the causal agent of chesnut blight, two genes encoding α subunits, *CPG1* and *CPG*2 were cloned and disrupted. A severe reduction in virulence was observed in the *cpg1* mutants. The disruption of the orthologous gene in the human pathogen *C. neoformans* resulted in mating defects and the inability to form melanin, a pathogenicity factor in this pathosystem. In *U. maydis*, only the disruption of *cpg3*, one of four α subunit genes, affected virulence (Deising et al. 2000).

MGB1, the *M. grisea* gene that encodes the heterotrimeric G protein β subunit, was cloned and disrupted. The mutants were reduced in conidiation, defective in appressorium formation, and failed to invasively colonize rice plants. The mutant's intracellular levels of cAMP were reduced and it was responsive to exogenous cAMP. However, it formed only defective appressoria. Thus, the *MGB1* gene affected conidiation, surface recognition and appressorium formation. The investigators concluded that the target of MGB1 is the PMK1 pathway. Exogenous cAMP rescued surface recognition (induced appressorium formation on a non-inductive surface) but not appressorium maturation (Nishimura et al. 2004). This result strongly suggests the participation of cAMP and MAP Kinase pathways in appressorium morphogenesis and maturation through the roles of different subunits of G proteins. Dissociation and activation of α subunits stimulates adenylyl ciclase, whereas the βγ subunits participate in the MAP kinase PMK1 pathway.

A proposed model for the appressorium formation signaling pathway in *M. grisea* is summarized in Fig. 2. *M. grisea* conidia attach to the hydrophobic surface and MPG1 hydrophobin protein is responsible for the close contact of the hyphal tip with the substrate in order to sense the physical surface features (Beckerman and Ebbole 1996). The hydrophobicity of the surface is sensed by a transmembrane receptor, encoded by the *PTH11* gene. The receptor is proposed to stimulate the heterotrimeric G protein containing the MagB α subunit. MagB is thought to activate adenylyl cyclase to control the cellular levels of the second messenger cAMP (Liu and Dean 1997), (Fang and Dean 2000). cAMP-dependent protein kinase, CpkA appears to be required for appressorium maturation, however, additional catalytic subunits must exist that are required for induction of appressorium development (Choi and Dean 1997), (Xu et al. 1997). Downstream of the induction step is activation of the Pmk1 MAP kinase cascade, which conducts the signal into the nucleus via MST12 and activates genes responsible for appressorium maturation and Pmk1 likely has additional transcription factor targets involved in appressorium morphogenesis. The CpkA-mediated maturation processes involve carbon source mobilization (Xu and Hamer 1996), (Xu 2000). *MGB1* is also potentially involved in activation of the *PMK1* Pathway.

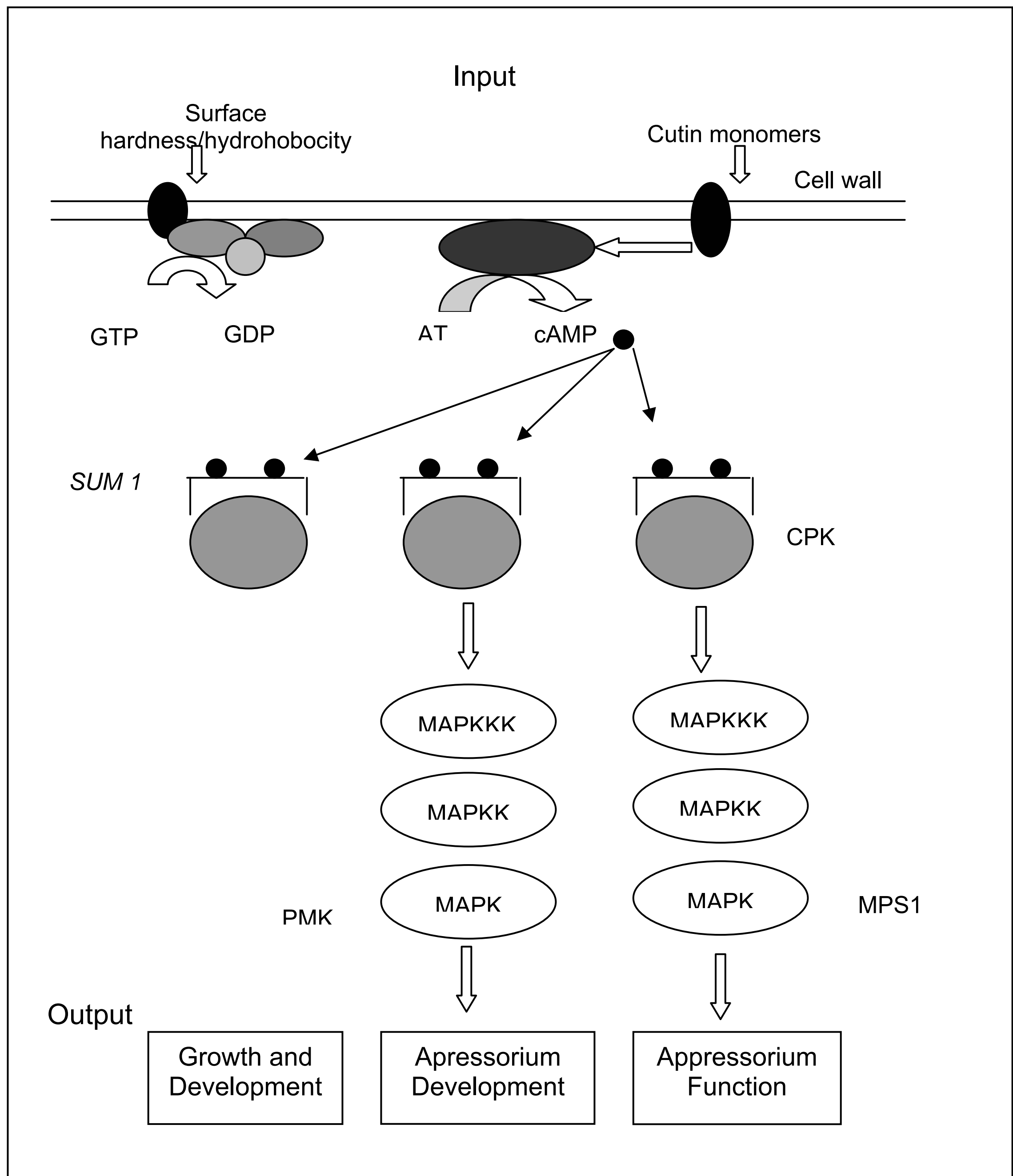


Figure 2. Proposed model for appressorium formation pathway. (Hamer and Talbot, 1998).

ANTIMICROBIOL PEPTIDES

Antimicrobiol peptides are considered one of the most important elements of the innate immune system, and are ubiquitous among eukaryotes and prokaryotes, including *S. cerevisiae* mammals, amphibians, insects, plants and protozoa (Kamysz et al. 2003). Antimicrobial peptides with low molecular weights (3000 to 5000) may be rapidly processed from larger precursors, and are highly diffusible for fast and efficient defense against microbes. Some are linear, and some contain highly conserved disulfide bonds (Gabay 1994). Over 800 antimicrobial peptides have been isolated and described. However, the species of origin is not the best means of classifying these peptides; rather, the amino acid sequence and structural information are more useful for classification. The primary amino acid sequences of some naturally occurring antimicrobial peptides are summarized in Table 3 (Gabay 1994). Almost all antimicrobial peptides are cationic or amphiphilic. The cationic part of the peptides interacts with the negative charged structure of lipopolysaccharides (LPS) in membranes leading to permeabilization of the membrane. Four main mechanisms of membrane permeabilization by proteins are known. Structural motifs that have been identified are classified as the barrel-stave motif, the worm-hole model, the carpet-like model, and structures that form selective ion channels.

Besides their antimicrobial activity, many peptides have functions that are not directly related to the defense system. The biological activity of antimicrobial peptides often depends on their concentration; at lower concentrations they may

influence signal transduction or proliferation, and at higher concentration they may cause cell lysis. Table 4 (Gabay 1994) summarizes the activities of some antimicrobial peptides.

Investigation of antimicrobial peptides has focused on medical applications, but some work has been done with respect to plant protection (Rao 1995) (Powell et al. 2000). Some of these investigations utilize a strategy of starting with known natural antimicrobial peptides and then designing synthetic peptides based on this parental sequence. This has been done with the magainins and it was possible to demonstrate growth inhibitory effects on both fungal and bacterial plant pathogens (Powell et al. 2000).

Chemical inhibitors, including synthetic peptides, provide potential tools to study appressorium formation. The inhibition of appressoria formation by the α-factor pheromone of yeast (Beckerman 1997) supported the concept of screening synthetic combinatorial libraries (Reed et al. 1997) to identify novel peptides that inhibit appressorium formation.

Table 3. The primary amino acid sequence of some natural antimicrobial peptides.

Peptides	Sequence of amino acid
Buforin	TRSSRAGOQFPVGRVHRLLRK
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR
Defensin HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
Histatin 5	DSHAKRHHGYKRKFHEKHSHRGY
Indolicidin	ILPWKWPWWPWRR-NH_2
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
Protegrin PG-1	RGGRLCYCRRRFCVCGGR- NH_2
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP

Table 4. Activities of antimicrobial peptides.

Activity	Peptide/reference
Inhibition of membrane protein synthesis	attacin (Carlsson et.al 1998)
Inhibition of DNA synthesis	PR-39 (Boman et al., 1993)
	magainins (Jacob & Zasloff, 1994)
Antitumor activity	defensins (Lichtenstein et al., 1986)
	cecropins (Moore et al., 1994)
Stimulation of cell proliferation	Defensins (Murphy et al., 1993)
Interference of signaling pathways	defensins (Charp et al., 1988)
	CAP 37 (Pereira et al., 1996)
	PR-39 (Tanaka, et al., 1996)
	ECAP (Hobta et al., 2001)
Chemoattractant for immune cells	defensins (Territo et al., 1989)
	PR-39 (Huang et al., 1997)
Stimulation of cytokine expression	defensins (Chaly et al., 2000)
	(van Wetering et al., 1989)
Stimulation of adheison molecule expression	defensins (Chay et al., 2000)
Angiogenesis	proline-rich peptide(Genmaro et al., 2002)
Chloride secretion	Cryptidins (Lencer et al., 1997)

An understanding of pathogen biology during its interaction with the host will lead to new strategies for disease control. Appressorium formation in *M. grisea* is crucial to establish a successful colonization. For these reasons, the goal of this study is to further understand the mechanism of appressorium formation including the signal transduction pathway that controls development.

In the present study I identified the hexapeptides D-CYRFTW as the best among the 400 samples, from the combinatorial peptide library, for blocking appressorium formation without direct growth inhibition of the strains. Differences in sensitivity to the peptides were noted between the isolates 4091 and CP 987. The peptides also blocked the appressorium pathway with high efficiency in isolates collected from commercial fields located in two representative areas of rice production in Brazil. In order to study the signaling pathway we also tested the hexapepetide activity against mutants with known genetic backgrounds. The isolate *magB*^{G42R} was more susceptible, while a strain carrying a suppressor of adenylate cyclase, SUM-99, was resistant to the inhibitory effect of both forms of the hexapeptide. This suggests that the peptide blocks at the activity of the G-protein or interferes with the sensing of surface characteristics needed to activate G-protein signaling.

Exogenous cAMP restored appressorium formation when combined with D-CYRFTW or L-WTFRYC. This result also suggested that the hexapeptides block the appressorium pathway upstream of cAMP synthesis. 1,6-hexadecandiol also

partially bypassed the effect of the peptides suggesting that the chemical sensing pathway was not a direct target of the peptides.

We quantified levels of PKA during the time course for appressorium formation. My data indicated that PKA activity is present at the time conidia are harvested. PKA activity then declined and subsequently increased at four hours after appressorium induction, when the hook cell forms. Levels of PKA activity were lower when cells were inoculated on non-inductive hydrophilic surfaces. Again this suggests that the hexapeptide blocks appressorium formation upstream the activation of adenylate cyclase.

The research effort described above focuses on early signaling events involved in the induction of appressorium morphogenesis. Work to date has demonstrated two important features of appressorium signaling. The first is signaling involved in induction of appressorium morphogenesis and the second is signaling involved in appressorium maturation. To gain a more complete view of the infection process, I also characterized a gene, PTH1 that is involved in appressorium maturation. This gene was identified as a pathogenicity mutant that failed to penetrate the leaf surface. Because both the cAMP pathway and the *PMK1/MST12* pathway are involved in appressorium maturation, it was of interest to determine what role *PTH1* plays during the maturation process. This work is detailed in Chapter IV.

CHAPTER II

CHEMICAL INHIBITION OF APPRESSORIUM FORMATION IN *Magnaporthe grisea*

INTRODUCTION

Commercial fungicides became routine as a method to control rice blast at the beginning of the 20th century. Rice growers started using inorganic copper-based fungicides around 1915, organic-mercurials in the 1950s, antibiotics and organophosphates in the 1960s, and finally systemic fungicides of diverse chemistries in the 1970s and 1980s. Today many blasticides provide a desirable level of disease control, however, the success of control depends on the number of applications during the growing season. Additional research is necessary to develop antiblast agents with new and specific modes of action and with environmental safety in mind (Froyd and Foeliger 1994).

Antimicrobial synthetic peptides

Antimicrobial peptides represent ancient host defense molecules present in organisms across the evolutionary spectrum (Yeaman and Yount 2003). Bioactive peptides can be either constitutive or inducible (Matsuzaki 1999). It is believed that the antimicrobial activity is due to the ability of the amphipathic peptides to interact with host cell surfaces, forming channels leading to membrane permeability (Rao 1995). The basis of the initial interaction with the membrane surface can be explained as an electrostatic interaction or receptor-

mediated membrane interaction, depending on the biochemical and biophysical properties of both the peptide and membrane. After the initial interaction several mechanisms have been proposed to target the peptide to the cytoplasmic membrane (Matsuzaki 1999), (Yeaman and Yount 2003).

Today, the idea that the activity of peptides is indiscriminant and that peptides act as membrane detergents is obsolete. New evidence points to targets that lie interior to the cytoplasmic membrane as being important in the antimicrobial mechanism of these peptides (Yeaman and Yount, 2003). Groups of amphipathic alpha-helical peptides, cercopins, magainins, and melittins have been widely investigated due to their inhibitory effect against a broad spectrum of microbes (Alan and Earle 2002). Synthetic peptides mimicking amphipathic alpha-helical motifs were shown to posses equal or increased activity, higher stability, and broader spectrum of antimicrobial activity (Alan and Earle 2002).

A natural example of the use of combinatorial peptide libraries is exemplified by cone snail toxins. Cone snails have evolved to use a combination of peptides to evolve defense molecules able to elicit specific physiological responses from their prey, predators, and competitors (Oliveira et al. 1995). In the past two decades, the hypermutation mechanism by which cone snails generate thousands of peptides with affinity to specific targets was used as a model for engineering bioactive peptide sequences using approaches such as positional scanning (Blondelle and Houghten 1996).

Synthetic combinatorial libraries have been screened to identify peptides with antifungal activity. Reed et al. (1997) identified a hexapeptide (D-FRLKFH) and a pentapeptide (D-FRLHF) with broad spectrum fungicidal activity that was effective against *Fusarium oxysporum f. sp. lycopersici, Rhizoctonia solani* (anastomosis group 1), *Ceratocystis fagacearum*, and *Pythium ultimum*. Use of a membrane impermeant dye indicated that fungal cytoplasmic and nuclear membranes were compromised by treatment with the peptide (Reed et al. 1997).

Gonzalez et al. (2002) identified and assessed two heptapeptides based on the hexapeptide described above and assessed the ability of these peptides to act synergistically with thiabendazole (TBZ) against TBZ-resistant *F. sambucinum* (Gonzalez et al. 2002). Oh et al. (2000) developed a rapid and target oriented screening system to identify candidate compounds for rice blast control. The compounds were derived from 1000 culture filtrates from members of the Actinomycetes. Fractions of the studied compounds inhibited appressorium formation *in vivo* and *in vitro*. The inhibitory activites were bypassed by addition of cAMP and 1,16-hexadecanediol suggesting that the mechanism of inhibition is related to surface sensing and the site in the pathway that was inhibited is located upstream of cAMP activation (Oh and Lee 2000).

Application in plant biotechnology

Plants respond to pathogens by producing defense related proteins, examples are the proteins that are directly involved with antimicrobial activity such as glucanases and chitinases, protease inhibitors, and enzymes associated with phytoalexin biosynthesis. Although plants produce antimicrobial peptides (Rao 1995), their precise functions are still under investigation. One approach to plant protection has been to engineer plants to express defense genes at higher levels and to introduce heterologous genes with antifungal properties. However, this approach faces the challenge of expressing proteins or antimicrobial peptides in the right tissue at the right physiological age, to increase the resistance of crop plants against diseases. Carmona et al. (1993) enhanced resistance to a bacterial pathogen of tobacco plants by transgenic expression of alpha-thionin. Jaynes et al. (1993) expressed a modified cecropin B peptide, Shiva-1, in tobacco, enhancing resistance of transgenic plants against *Pseudomonas solanacearum*. Montanelli (1991) transformed potato plants with the cecropin gene. The protein was expressed but did not enhance the resistance of the plant against phytopathogens (Montanelli and Nascari 1991), (Carmona et al. 1993), (Jaynes et al. 1993). Despite the challenge of expressing foreign genes in plants, antimicrobial peptides provide a source of new genes for engineering of plants for disease resistance (Rao 1995).

Disease life cycle and appressorium formation pathway

The proposed model for the signaling pathway leading to appressorium morphogenesis includes a central role for cAMP signaling (Lee and Dean 1993b) and the Pmk1 MAP kinase pathway (Xu and Hamer 1996). Detection of surface characteristics involves the MPG1 hydrophobin protein that may act as a bridge between the fungal cell wall and the substrate (Beckerman and Ebbole 1996), (Talbot et al. 1993), (Talbot 1999). The hydrophobicity (Yong-Hwan and Dean 1994) of the surface may be sensed by a transmembrane receptor (Lengeler et al. 2000). The receptor is proposed to stimulate the heterotrimeric G protein containing the MagB Gα subunit. MagB is thought to activate adenyl cyclase to control the cellular levels of the second messenger cAMP (Liu and Dean 1997), (Fang and Dean 2000). cAMP-dependent protein kinase, PKA appears to be required for appressorium maturation, however, additional catalytic subunits appear to exist that are required for induction of appressorium development (Mitchell and Dean 1995), (Xu et al. 1997). Chemical signaling via wax/cutin monomers leads to stimulation of the cAMP pathway but does not involve the Gα subunit protein (Gilbert et al. 1996). Downstream of this cAMP-dependent induction step is activation of the Pmk1 MAP kinase cascade required to activate genes responsible for appressorium morphogenesis and maturation (Xu and Hamer 1996).

To further explore the use of combinatorial peptide libraries as a sour ce of compounds to protect plants from fungal infection, we screened a

hexapeptide library to identify peptide inhibitors of appressorium formation in *M. grisea*. We identified that the peptide D-CYRFTW acts to block the recognition of inductive substrates, thereby preventing activation of cAMP signaling in response to surface cues. Sensing of wax/cutin monomers was less inhibited by the peptide suggesting that this Mag B-independent signaling pathway was not a direct target of the peptide.

MATERIALS AND METHODS

Strains and growth conditions

Wild type and mutant strains of *M. grisea* were stored by growing the fungus through sterile filter paper discs, followed by desiccation and storage at 20 °C. Strains utilized in this study are shown in Table 5. Fungal cultures were grown on PDA (potato dextrose agar, 100 μg/mL of ampicillin), and transferred to oatmeal agar (50 g of oatmeal per liter, 100 μg/mL of ampicillin and 25 μg/mL of chloramphenicol) at 25°C under continuous fluorescent light.

Biochemicals

8-Bromoadenosine 3’-5’ cyclic monophosphate and the cutin monomer 1, 16-hexadecanediol were obtained from Sigma Chemical Company.

Peptide synthesis

The first two positions of each hexapeptide pool was fixed for each of all 20 amino acid to provide an initial set of 400 pools of hexapeptides. The remaining positions in each pool were composed of an equimolar mixture of all 19 amino acids. Thus, each of the 400 pools has 19^4 (130,321) unique

hexapeptides. The amino acids present at the first and second positions of the hexapeptides in the combinatorial libraries are listed in Table 6.

Table 5. Description of *Magnaporthe grisea* strains utilized in this study.

Strain	**Description**	**Reference**
4091-5-8	Wild type laboratory strain. Goosegrass and weeping lovegrass pathogen. (Mat 1-2)	(Valent 1990)
Guy11	Wild type laboratory strain. Rice pathogen. (Mat 1-2)	(Silue et al. 1998)
CP987	Wild type laboratory strain. Rice pathogen. Spontaneous mutation form CP917.(Mat 1-1)	(Carroll et al. 1994)
70-15	Wild type -laboratory strain. Rice pathogen.(Mat 1-1)	(Choi and Dean 1997)
Sum 99	Isogenic to Guy-11. Bypass suppressor of adenylate cyclase (*mac1*) mutant. Carries a mutation in cAMP binding site of regulatory subunit of PKA (*sum1*). (Mat1-2)	(Adachi and Hamer 1998)
$MagB^{G42R}$	Isogenic to 70-15. Dominant mutation.Gprotein constitutively activate signaling. (Mat 1-1)	(Fang and Dean 2000)
408	Field isolate. Rice pathogenic. Isolate from Cica-8 cv., IRBN, Brazil, 1994	Embrapa[a]
409	Field isolate. Rice pathogenic. Isolate from Cica-8 cv., IRBN, Brazil, 1994	Embrapa
1600	Field isolate. Rice pathogenic. Isolate from Maravilha cv.,upland, Brazil, 1998	Embrapa
1605	Field isolate. Rice pathogenic. Isolate from Maravilha cv.,upland, Brazil, 1998	Embrapa
1444	Field isolate. Rice pathogenic. Isolate from Metica cv, Brazil, 1998	Embrapa

[a]Embrapa: Empresa Brasileira de Pesquisa Agropecuaria

Table 6. D-hexapeptide 2-6 libraries.

1. Q ^{1}Q^{2} XXXX	33. SDXXXX	65. ANXXXX	97. NKXXXX
2. QS XXXX	34. SCXXXX	66. AWXXXX	98. NLXXXX
3. QFXXXX	35. SGXXXX	67. AVXXXX	99. NEXXXX
4. QAXXXX	36. STXXXX	68. AMXXXX	100. NYXXXX
5. QNXXXX	37. SKXXXX	69. APXXXX	101. WQXXXX
6. QWXXXX	38. SLXXXX	70. AHXXXX	102. WSXXXX
7. QVXXXX	39. SEXXXX	71. ARXXXX	103. WFXXXX
8. QMXXXX	40. SYXXXX	72. AIXXXX	104. WAXXXX
9. QPXXXX	41. FQXXXX	73. ADXXXX	105. WNXXXX
10. QHXXXX	42. FS XXXX	74. ACXXXX	106.WWXXXX
11. QRXXXX	43. FFXXXX	75. AGXXXX	107. WVXXXX
12. QIXXXX	44. FAXXXX	76. ATXXXX	108. WMXXXX
13. QDXXXX	45. FNXXXX	77. AKXXXX	109. WPXXXX
14. QCXXXX	46. FWXXXX	78. ALXXXX	110. WHXXXX
15. QGXXXX	47. FVXXXX	79. AEXXXX	111. WRXXXX
16. QTXXXX	48. FMXXXX	80. AYXXXX	112. WIXXXX
17. QKXXXX	49. FPXXXX	81. NQXXXX	113. WDXXXX
18. QLXXXX	50. FHXXXX	82. NS XXXX	114. WCXXXX
19. QEXXXX	51. FRXXXX	83. NFXXXX	115. WGXXXX
20. QYXXXX	52. FIXXXX	84. NAXXXX	116. WTXXXX
21. SQXXXX	53. FDXXXX	85. NNXXXX	117. WKXXXX
22. SSXXXX	54. FCXXXX	86. NWXXXX	118. WLXXXX
23. SFXXXX	55. FGXXXX	87. NVXXXX	119. WEXXXX
24. SAXXXX	56. FTXXXX	88. NMXXXX	120. WYXXXX
25. SNXXXX	57. FKXXXX	89. NPXXXX	121. VQXXXX
26. SWXXXX	58. FLXXXX	90. NHXXXX	122. VS XXXX
27. SVXXXX	59. FEXXXX	91. NRXXXX	123. VFXXXX
28. SMXXXX	60. FYXXXX	92. NIXXXX	124.VAXXXX
29. SPXXXX	61. AQXXXX	93. NDXXXX	125. VNXXXX
30. SHXXXX	62. AS XXXX	94. NCXXXX	126. VWXXXX
31. SRXXXX	63. AFXXXX	95. NGXXXX	127. VVXXXX
32. SIXXXX	64. AAXXXX	96. NTXXXX	128. VMXXXX

Table 6. Continued.

129. VPXXXX	161. PQXXXX	193. HDXXXX	225. INXXXX
130. VHXXXX	162. PSXXXX	194. HCXXXX	226. IWXXXX
131. VRXXXX	163. PFXXXX	195. HGXXXX	227. IVXXXX
132. VIXXXX	164. PAXXXX	196. HTXXXX	228. IMXXXX
133. VDXXXX	165. PNXXXX	197. HKXXXX	229. IPXXXX
134. VCXXXX	166. PWXXXX	198. HLXXXX	230. IHXXXX
135. VGXXXX	167. PVXXXX	199. HEXXXX	231. IRXXXX
136. VTXXXX	168. PMXXXX	200. HYXXXX	232. IIXXXX
137.VKXXXX	169. PPXXXX	201. RQXXXX	233. IDXXXX
138. VLXXXX	170. PHXXXX	202. RSXXXX	234. ICXXXX
139. VEXXXX	171. PRXXXX	203. RFXXXX	235. IGXXXX
140. VYXXXX	172. PIXXXX	204. RAXXXX	236. ITXXXX
141. MQXXXX	173. PDXXXX	205. RNXXXX	237. IKXXXX
142. MSXXXX	174. PCXXXX	206. RWXXXX	238. ILXXXX
143. MFXXXX	175. PGXXXX	207. RVXXXX	239. IEXXXX
144. MAXXXX	176. PTXXXX	208. RMXXXX	240. IYXXXX
145. MNXXXX	177. PKXXXX	209. RPXXXX	241. DQXXXX
146. MWXXXX	178. PLXXXX	210. RHXXXX	242. DSXXXX
147. MVXXXX	179. PEXXXX	211. RRXXXX	243. DFXXXX
148. MMXXXX	180. PYXXXX	212. RIXXXX	244. DAXXXX
149. MPXXXX	181. HQXXXX	213. RDXXXX	245. DNXXXX
150. MHXXXX	182. HSXXXX	214. RCXXXX	246. DWXXXX
151. MRXXXX	183. HFXXXX	215. RGXXXX	247. DVXXXX
152. MIXXXX	184. HAXXXX	216. RTXXXX	248. DMXXXX
153. MDXXXX	185. HNXXXX	217. RKXXXX	249. DPXXXX
154. MCXXXX	186. HWXXXX	218.RRLXXXX	250. DHXXXX
155. MGXXXX	187. HVXXXX	219. REXXXX	251. DRXXXX
156. MTXXXX	188. HMXXXX	220. RYXXXX	252. DIXXXX
157. MKXXXX	189. HPXXXX	221. IQXXXX	253. DDXXXX
158. MLXXXX	190. HHXXXX	222. IS XXXX	254. DCXXXX
159. MEXXXX	191. HRXXXX	223. IFXXXX	255. DGXXXX
160. MYXXXX	192. HIXXXX	224.IAXXXX	256. DTXXXX

Table 6. Continued.

257. DKXXXX	289. GPXXXX	321. KQXXXX	353. LDXXXX
258. DLXXXX	290. GHXXXX	322. KSXXXX	354. LCXXXX
259. DEXXXX	291. GRXXXX	323. KFXXXX	355. LGXXXX
260. DYXXXX	292. GIXXXX	324. KAXXXX	356. LTXXXX
261. CQXXXX	293. GDXXXX	325. KNXXXX	357. LKXXXX
262. CS XXXX	294. GCXXXX	326. KWXXXX	358. LLXXXX
263. CFXXXX	295. GGXXXX	327. KVXXXX	359. LEXXXX
264. CAXXXX	296. GTXXXX	328. KMXXXX	360. LYXXXX
265. CNXXXX	297. GKXXXX	329. KPXXXX	361. EQXXXX
266. CWXXXX	298. GLXXXX	330. KHXXXX	362. ES XXXX
267. CVXXXX	299. GEXXXX	331. KRXXXX	363. EFXXXX
268. CMXXXX	300. GYXXXX	332. KIXXXX	364. EAXXXX
269. CPXXXX	301. TQXXXX	333. KDXXXX	365. ENXXXX
270.CHXXXX	302. TS XXXX	334. KCXXXX	366. EWXXXX
271. CRXXXX	303. TFXXXX	335. KGXXXX	367. EVXXXX
272. CIXXXX	304. TAXXXX	336. KTXXXX	368. EMXXXX
273. CDXXXX	305. TNXXXX	337. KKXXXX	369. EPXXXX
274. CCXXXX	306. TWXXXX	338. KLXXXX	370. EHXXXX
275. CGXXXX	307. TVXXXX	339. KEXXXX	371. ERXXXX
276. CTXXXX	308. TMXXXX	340. KYXXXX	372. EIXXXX
277. CKXXXX	309. TPXXXX	341. LQXXXX	373. EDXXXX
278. CLXXXX	310. THXXXX	342. LS XXXX	374. ECXXXX
279. CEXXXX	311. TRXXXX	343. LFXXXX	375. EGXXXX
280. CYXXXX	312. TIXXXX	344. LAXXXX	376. ETXXXX
281. GQXXXX	313. TDXXXX	345. LNXXXX	377. EKXXXX
282. GSXXXX	314. TCXXXX	346. LWXXXX	378. ELXXXX
283. GFXXXX	315. TGXXXX	347. LVXXXX	379. EEXXXX
284. GAXXXX	316. TTXXXX	348. LMXXXX	380. EYXXXX
285. GNXXXX	317. TKXXXX	349. LPXXXX	381. YQXXXX
286. GWXXXX	318. TLXXXX	350. LHXXXX	382. YS XXXX
287. GVXXXX	319. TEXXXX	351. LRXXXX	383. YFXXXX
288. GMXXXX	320. TYXXXX	352. LIXXXX	384. YAXXXX

Table 6. Continued.

385. YNXXXX	389. YPXXXX	393. YDXXXX	397. YKXXXX
386. YWXXXX	390. YHXXXX	394. YCXXXX	398. YLXXXX
387. YVXXXX	391. YRXXXX	395. YGXXXX	399. YEXXXX
388. YMXXXX	392. YIXXXX	396. YTXXXX	400. YYXXXX

Q[1] first position fixed; Q[2] second position variable; Amino acids are represented by the standard one-letter code.
Q glutamine; S serine; F phenylalanine; A alanine; N aspartic acid; W tryptophan; V valine; M methionine; P proline; H histidine; R arginine; I isoleucine; D asparegine; C cysteine; G glycine; T threonine; K lysine; L leucine E glutamine acid; Y tyrosine;

To define an optimal peptide sequence the most effective 2-6 D-hexapeptide was used as a starting point to synthesize a new set of 19 D-hexapeptide libraries with the first three amino acids fixed. The amino acids present at the third positions of the hexapeptides in the combinatorial libraries are listed in Table 7.

Table 7. The 3-6 D-hexapeptide pools.

1. CYQXXX	6. CYWXXX	11. CYRXXX	16. CYKXXX
2. CYSXXX	7. CYVXXX	12. CYIXXX	17. CYLXXX
3. CYFXXX	8. CYMXXX	13. CYDXXX	18. CYEXXX
4. CYAXXX	9. CYPXXX	14. CYGXXX	19. CYYXXX
5. CYNXXX	10. CYHXXX	15. CYTXXX	

The same protocol was used to define additional amino acid positions. The amino acids present at the fourth positions of the hexapeptides in the 4-6 pools are listed in Table 8.

Table 8. The 4-6 D-hexapeptide pools.

1. CYRQXX	6. CYRWXX	11. CYRRXX	16. CYRKXX
2. CYRSXX	7. CYRVXX	12. CYRIXX	17. CYRLXX
3. CYRFXX	8. CYRMXX	13. CYRDXX	18. CYREXX
4. CYRAXX	9. CYRPXX	14. CYRGXX	19. CYRYXX
5. CYRNXX	10. CYRHXX	15. CYRTXX	

The amino acids present at the fifth positions of the hexapeptides in the combinatorial libraries are listed in Table 9. The amino acids present at the sixth positions of the hexapeptides are listed in Table10.

Table 9. The 5-6 D-hexapeptide pools.

1. CYRFQX	6. CYRFWX	11. CYRFRX	16. CYRFKX
2. CYRFSX	7. CYRFVX	12. CYRFIX	17. CYRFLX
3. CYRFFX	8. CYRFMX	13. CYRFDX	18. CYRFEX
4. CYRFAX	9. CYRFPX	14. CYRFGX	19. CYRFYX
5. CYRFNX	10. CYRFHX	15. CYRFTX	

Table 10. The 6-6 D-hexapeptides.

1. CYRFTQ	6. CYRFTW	11. CYRFTR	16. CYRFTK
2. CYRFTS	7. CYRFTV	12. CYRFTI	17. CYRFTL
3. CYRFTF	8. CYRFTM	13. CYRFTD	18. CYRFTE
4. CYRFTA	9. CYRFTP	14. CYRFTG	19. CYRFTY
5. CYRFTN	10. CYRFTH	15. CYRFTT	

***In vitro* appressorium formation assay**

Conidia of *M. grisea* grown under continuous light were harvested from 10 to 12-day-old cultures in phosphate buffer (100 mM, pH 6.6), filtered once through Miracloth, and diluted to 1 x 10^5 conidia/mL. Drops of 5 μL of conidial suspension were placed on hydrophobic plastic microscope cover slips (Fisher Scientific) or on the hydrophilic surface of GelBond film (FMC Bioproducts). Five microliters of the peptides were added to a final concentration of 500 μg/ml and eight 2-fold dilutions were made to give final concentrations of 250 μg/ml, 125 μg/ml, 62.5 μg/ml, 31.3 μg/ml, 15.6 μg/ml, 7.8 μg/ml, 3.9 μg/ml, and 2.0 μg/ml. Addition of 8-Br-cAMP (Sigma), 1,16-hexadecanediol, and yeast extract (Difco, Inc.) were added to final concentrations of 10 mM, 1μM, or 2%, respectively. The percentage of appressoria formed was determined by microscopic examination after 16 h of incubation in a moist chamber at 25°C. All experiments consisted of two replicates per treatment and were run independently at least two times.

In vivo appressorium formation assay

Leaf appressorium assays were performed as described previous (DeZwaan et al. 1999). Briefly, 10 to 12-day old barley leaves, cv. Bonanza, were placed over filter paper in Petri plates, forming a humid chamber. Two 5 μl droplets of a freshly made conidial suspension ($1x10^5$ conidia/mL) were placed on the leaves 2 cm apart. Five microliters of the hexapeptide (D-CYRFTW) was added to the droplet from stocks solution to reach a final concentration of 250, 125, 62.5, and 31.3 μg/mL. The plates containing the leaves were incubated in a moist chamber at 25°C for 12 h. Inoculated plant tissue was fixed with lactophenol for 16 h, and decolorized with two treatments of 2 hours with lactophenol:ethanol (1:1, vol:vol) at 95°C. Fungal hyphae were stained with 0.01% aniline blue in a mixture of ethanol, lactic acid, and phenol (1:1:1). The leaves were stained for at least 24 h at 25°C (Oh and Lee 2000) and evaluated under light microscopy (Olympus BX60). Images were captured with a Q-fire digital camera (Olympus).

Statistical analyzes

The means were compared applying statistical t-test analyzes using SPSS software (SPSS 11.0).

RESULTS

Screening of a synthetic peptide combinatorial library for *in vitro* inhibition of appressorium formation in *M. grisea*

A synthetic combinatorial library (SCL) composed of 52 million unique hexapeptides was screened using an iterative approach. An initial set of 400 D-amino acid peptide mixtures, designated as the 2-6-series, was assayed for inhibitory activity against *in vitro* appressorium formation in *M. grisea* strains 4091-5-8 and CP987. Serial dilutions of the 2-6 series starting from 1000 μg/ml to 4 μg/ml were used to determine the IC_{50} and MIC values. Table 11 summarizes the pools that had the greatest activity for appressorium development and at the same time displayed low fungicidal activity. The D-CYXXXX sublibrary exhibited the lowest IC_{50} (16 μg/ml). The IC_{50} is defined as the concentration of peptide that was able to inhibit appressorium formation by 50%. A 3-6 series of 19 peptide pools was synthesized (cysteine was omitted) (Table 7). The IC_{50} was determined (Table 12) and the D-CYRXXX pool was found to have greatest activity against the strain 4091-5-8. This same strategy was used to screen 19 pools for the 4-6 series (Table 8), 19 pools for the 5-6 series (Table 9) and a final 19 peptides (Table 10) to define D-CYRFTW as the hexapeptide with greatest activity (8 μg/ml) for blocking appressorium formation on strain 4091-5-8 (Table 12,Fig. 3).

Table 11. Inhibitory activity of selected representative libraries on appressorium formation in *Magnaporthe grisea*.

Sublibrary	Peptide sequence	Activity (μg/mL)	
		[a]MIC (range)	[b]IC 50
42	[c]FSXXXX	1000	107.40
43	FFXXXX	1000-500	90.10
46	FWXXXX	1000-125	24.40
110	THXXXX	1000-250	97
117	TKXXXX	1000-250	59.30
146	MWXXXX	1000-250	59.50
186	HWXXXX	1000-500	80.20
280	CYXXXX	1000-250	15.40

[a]MIC= minimum inhibitory concentration
[b]IC_{50}= concentration necessary to inhibit 50% of appressoium formation as determined by *in vitro* assay;
[C]X is a close to equimolar mixture of 19 D-amino acids (cysteine omitted)

Table 12. Summary of the activity of CY-Based SCL.

Peptide Sequence	[a]IC 50 (μg/mL)	Total Number of Peptides
[b]CYXXXX	15.40	19x19x19x19 = 130,321
CYRXXX	52	19x19x19 = 6859
CYRFXX	42	19x19 = 361
CYRFTX	11.0	19
CYRFTW	7.7	1

[a] The activity is given in terms of the IC_{50} values (as defined in Table 11).
[b] X is a close to equimolar mixture of 19 D-amino acids (cysteine omitted).

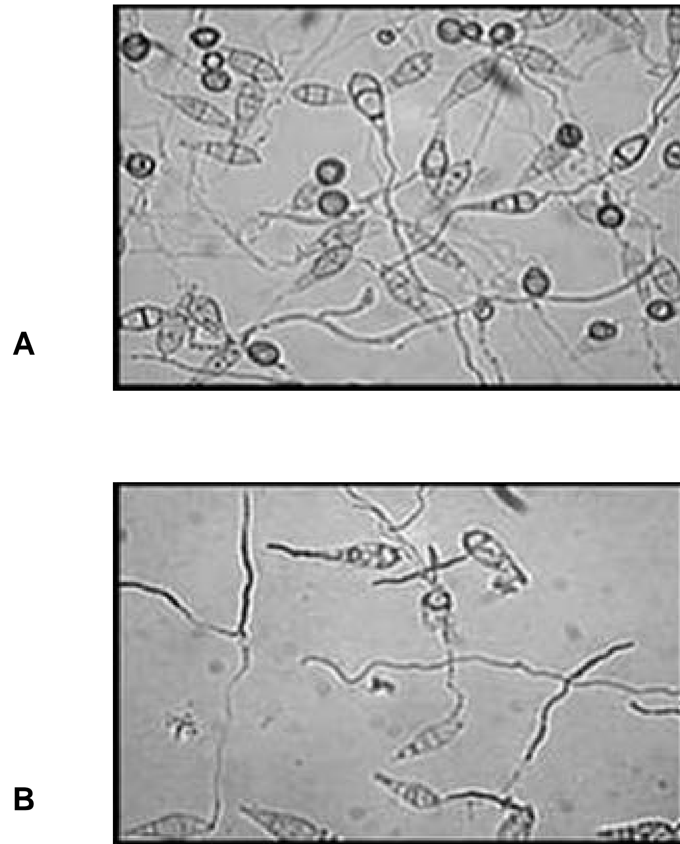


Figure 3. Inhibition of appressorium formation by hexapeptide D-CYRFTW. A Appressoria formed on artificial hydrophobic surface. B Inhibition of appressoria formation by hexapeptide D-CYRFTW(62 μg/mL).

Activity of D- CYRFTW and L-WTFRYC against field isolates of *M. grisea*

A collection of five *M. grisea* isolates from fields located in different regions (Table 5) in Brazil were assayed for inhibition by peptides. All isolates were inhibited by -CYRFTW (Table 14). An L-amino acid hexapeptide of reverse sequence L-WTFRYC was synthesized and tested for activity. This peptide is called the "retro-inverse" form of D-CYRFTW and the chemical secondary structure of the amino acid side chains are expected to adopt the same structure in both peptides. It has been observed that retro-inverse peptides have similar bioactive properties to the parental peptide (Barra and Simmaco 1995). We found this to be true for L-WTFRYC and it inhibited appressorium formation at a concentration similar to D-CYRFTW (Tables 13 and 14). The IC_{50} values are less than 10 for D-CYRFTW, and for L-WTFRYC, activity was observed with IC_{50} values ranging from 14 to 30 μg/mL. Overall, the field isolates displayed a similar level of sensitivity to the peptides as 4091-5-8. These isolates were collected from cultivars Metica-1, an irrigated cultivar, and Maravilha, an upland cultivar. Both cultivars are extensively planted every year and also utilized in rice breeding programs, but they lack stable resistance to rice blast. Thus, commercially important field isolates of *M. grisea* are sensitive to peptides. This prompted me to examine the ability of the peptide to inhibit appressorium formation when conidia were applied directly to the plant surface.

Strain 4091-5-8 was incubated with and without peptide on an artificial inductive surface and directly on the rice leaf surface in a detached leaf assay.

The hexapeptide D-CYRFTW showed no inhibitory effect when evaluated on rice leaves suggesting that the appressorium pathway was activated despite the presence of the peptide (Fig. 4). Potentially, induction of appressorium formation by leaf surface chemicals, such as cutin monomers could bypass the inhibition of surface sensing. Alternatively, proteases present on the leaf surface might be able to destroy the hexapeptide before it could act to inhibit appressorium formation. Experiments described below indicate that activation of appressorium formation by chemical cues is not readily inhibited by D-CYRFTW. This suggests that this particular inhibitory peptide would not be suitable as a direct commercial product.

Table 13. D-CYRFTW and L-WTFRYC against 4091 and CP987.

***M. grisea* strain**	**IC_{50}(μg/mL)**	
	D - CYRFTW	**L - WTFRYC**
4091	19.05	19.04
CP987	3.78	39.90

Table 14. Inhibitory activity of D-CYRFTW and L-WTFRYC of appressorium formation on field isolates of *M. grisea.*

***M. grisea* strain**	**IC_{50}(μg/mL)**	
	D - CYRFTW	**L - WTFRYC**
4091	15.08	9.65
409	2.27	13.34
1600	2.08	4.88
408	1.0	17.97
1444	1.0	28.18
1605	1.0	13.55

[a] The activity is given in terms of the IC_{50} values (as defined in Table II)

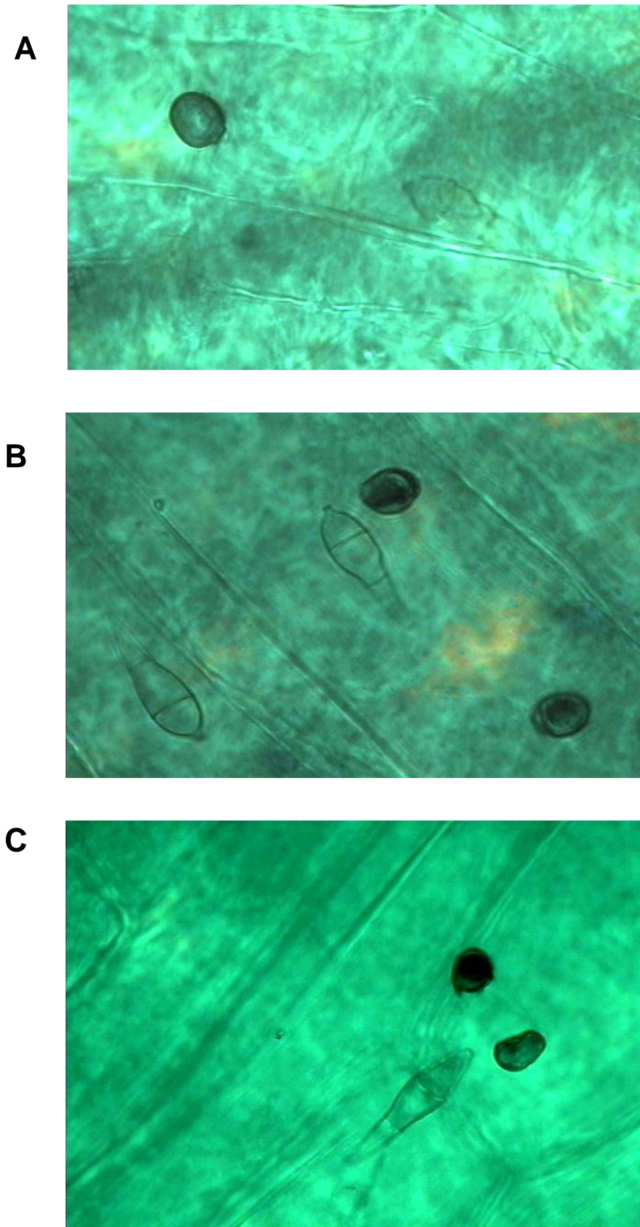


Figure 4. Appressorium formed on detached barley leaves assay. A-D-CYRFTW; B-Phosphate Buffer (50 125 ug/Ml; C-D-CYRFTW 250 ug/mL.

Effect of cAMP and 1,16-hexadecanediol on the activity of the defined hexapeptide

The inhibitory effect of the hexapeptide can be blocked by addition of cAMP (Fig.5). Incubation of strain 4091-5-8 with the hexapeptide D-CYRFTW at 31 (Fig.6A), 16 (Fig.6B) and 8 (Fig.6C) μg/mL and 8-bromo cAMP (10mM) formed significantly more appressoria than when it was combined only with the hexapeptide. However, strain CP987 was combined with the hexapeptide and cAMP the differences between the means with and without cAMP were not different.

The inhibitory effect of D-CYRFTW was also overcome by addition of 1 μM 1,16 hexadecanediol, a cutin monomer, with strain 4091-5-8. The statistical differences were significant only when the final concentrations of the peptide were 31 and 16 μg/mL respectively (Fig. 7). In the presence of 31 μg/mL hexapeptide, 1,16 hexadecanediol, was as effective as cAMP in restoring appressorium formation.

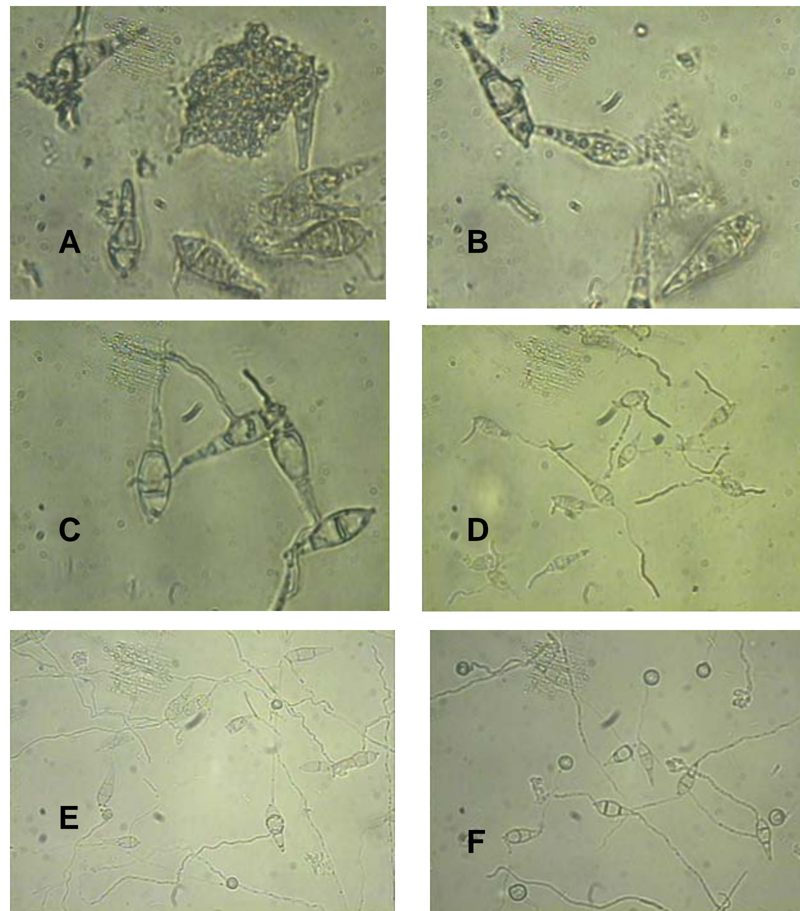


Figure 5. Inhibition of appressorium formation by hexapeptide D-CYRFTW and blocking of inhibition by cAMP when assayed on inductive surface. A D-CYRFTW 250 ug/mL; B- D-CYRFTW 250 ug/mL + cAMP(10Mm); C- D-CYRFTW 60 ug/Ml; D- D-CYRFTW 60 ug/mL + cAMP(10Mm); E- D-CYRFTW 15 ug/mL; F- D-CYRFTW 15 ug/mL

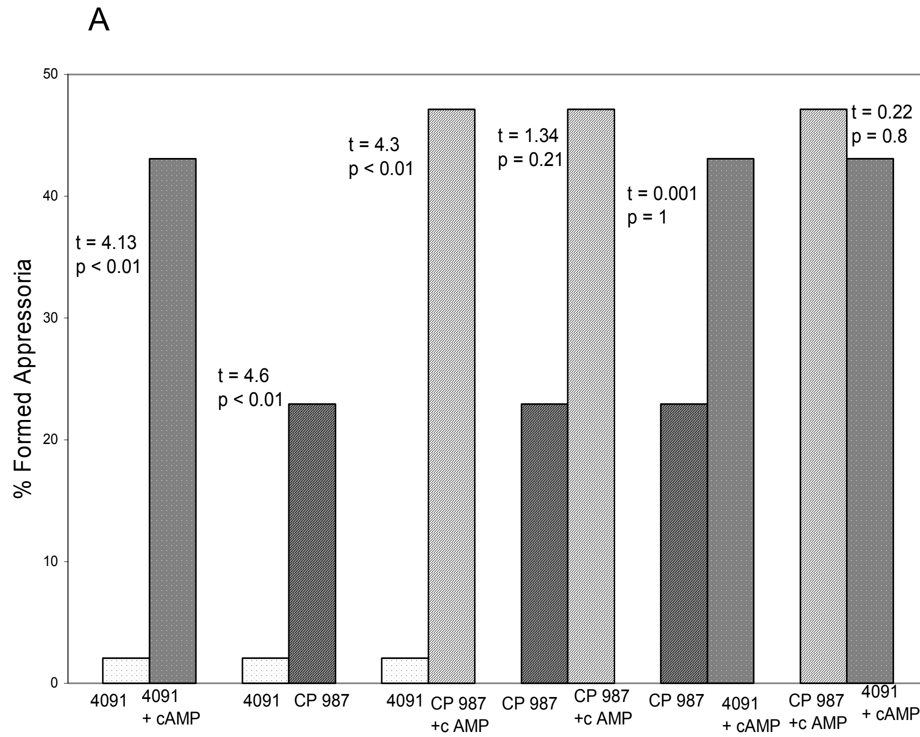


Figure 6. Means of appressoria formed when 8 Br-cAMP (10uM) was combined with *M. grisea* spore suspension and D-CYRFTW, 31 ug/mL (A), 16 ug/mL (B), and 8 ug/mL (C).

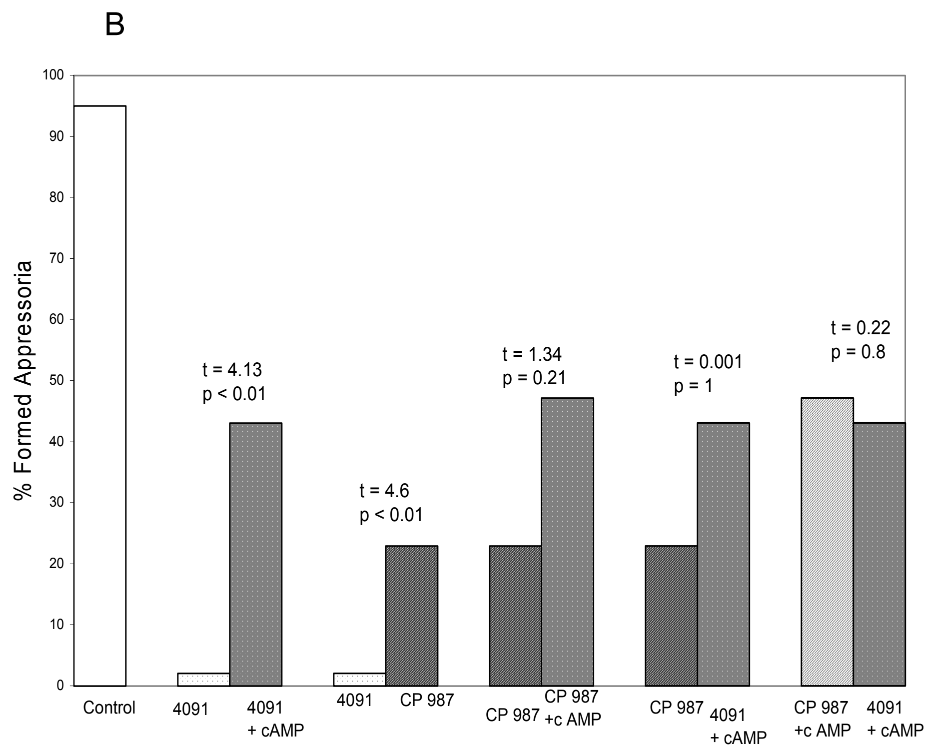


Figure 6. Continued

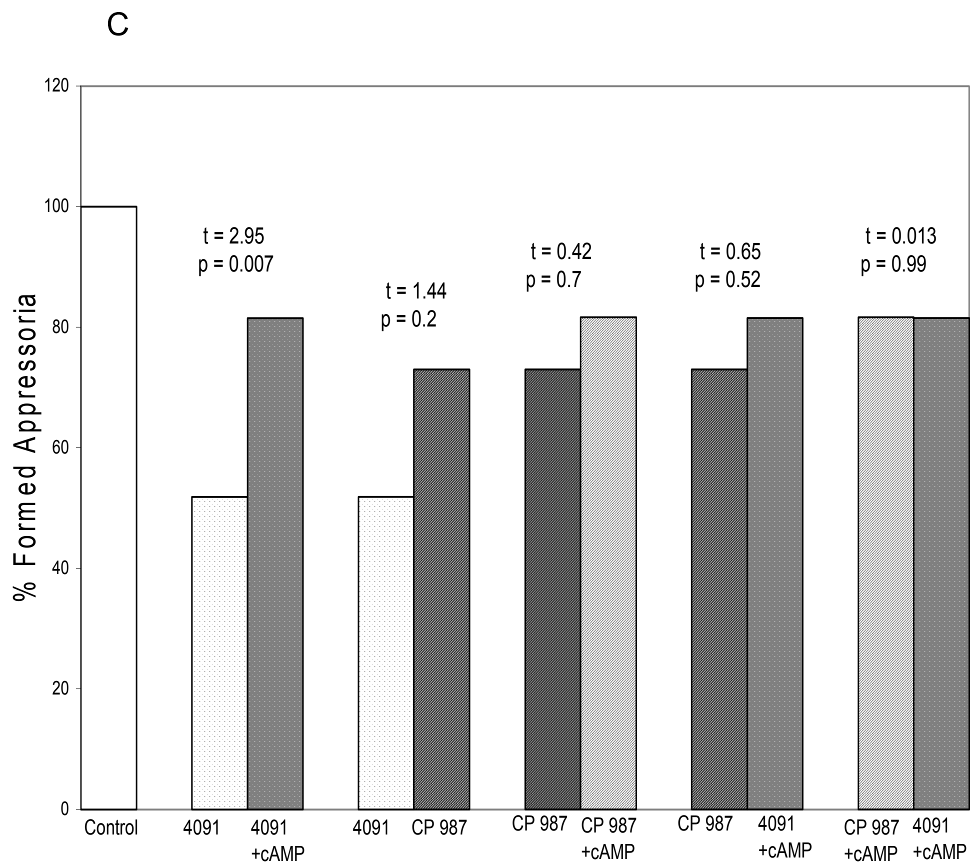


Figure 6. Continued.

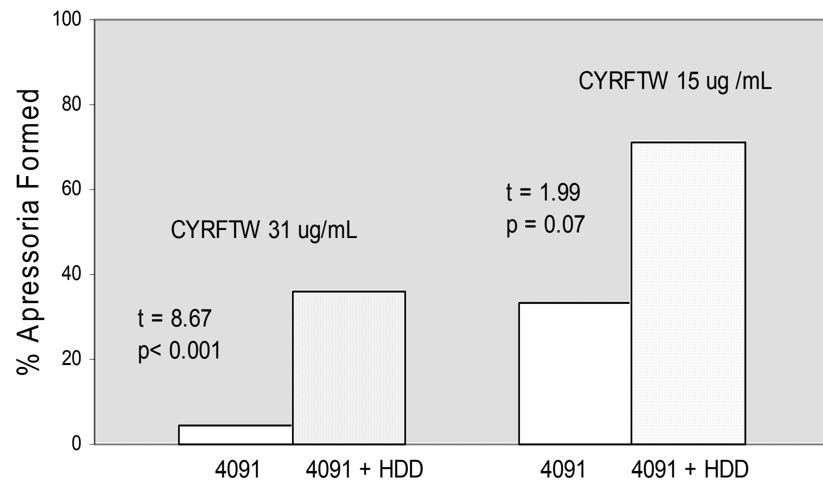


Figure 7. Means of appressoria formed after adding 1,16 hexadecandiol (100 uM) at *Mg. grisea* suspension and D-CYRFTW (31 and 16 ug/mL).

Activity of hexapeptide pools on inducing appressorium formation on hydrophilic surface

For those hexapeptide pools that displayed appressorium inhibiting activity, I also tested the effect of the peptides on *M. grisea* germinated on non-inductive surfaces (Table 15). Surprisingly, several of the hexapeptide pools that inhibited appressorium formation on inductive surfaces were found to induce appressorium formation on non-inductive surfaces. Appressoria formation on a hydrophilic surface with strain 4091 was observed in the presence of peptides 234, 278, 264, 239 and 121 at a final concentration of 1000 μg/mL and 500 μg/mL..

Thus, the peptide 2-6 libraries 234, 278, 264, 239 and 121 induced apressorium formation when assayed on a hydrophilic surface. In addition, the 264, 239 and 121 libraries showed response to the addition of hexadecanediol (1 μM) increasing the amount of appressoria formed on hydrophilic surface. The 234 and 278 libraries did not respond to the addition of hexadecanediol.

DISCUSSION

Any processes of the pathogen or in the plant-pathogen interaction that lead to disease provide potential targets for chemical control. Molecular analysis of plant and fungal biology can offer technology to address the issue of which processes and what genes are involved in pathogenesis. A major research emphasis in *M. grisea* has focused on appressorium morphogenesis.

Table 15. Effect of 2-6 series in appressorium formation when tested against strain 4091, on hydrophilic surfaces.

Hydrophilic surface					
2-6 library Peptide Sequence		**1000μg/mL**		**500μg/mL**	
		Peptide	Peptide + HDD	Peptide	Peptide + HDD
234	ICXXXX	7	9	6	12
278	CLXXXX	8	8	14	13
264	CAXXXX	46	74	62	97
239	IEXXXX	67	99	98	100
121	VQXXXX	86	97	99	100

Melanin is required to produce functional appressoria and a chemical that inhibits melanin synthesis, tricyclazole, is effective in protecting plants from *M. grisea*.

Therefore, I sought to identify a bioactive hexapeptide able to block appressorium formation in *M. grisea* to address two questions. First, can bioactive peptides be used directly to protect plants from infection by *M. grisea* or to identify targets as a basis for further development of anti-blast chemicals? Second, can peptide inhibitors be used to probe the mechanisms governing appressorium formation? Intracellular signaling systems for forming appressorium have been extensively studied, and the components of this pathway, composed by cAMP and MAP Kinase cascades, have been identified. These components represent potential targets for inhibition or activation. Specific inhibitors or activators of the system would represent important tools for understanding the biochemistry of pathogenesis in plant-pathogenic fungi.

My analysis shows that hexapeptide combinatorial libraries can yield inhibitors of appressorium development, in reasonable concentrations. However, as was found with inhibitory compounds discovered from Actinomycetes (Oh and Lee 2000), yeast extract and yeast alpha-factor pheromone (Beckerman 1997), the inhibition appears to occur at the level of substrate surface recognition and is overcome by chemical induces of appressorium development, such as cAMP and 1,16 hexadecanediol.

Thus, the hexapeptides identified here are not useful in identifying a target that would be directly effective in the field. However, my experience does point out that a new screen conducted directly on plants, or simultaneous *in vitro* and *in vivo*, together with an evaluation of the viability of the appressoria formed in the presence of the peptide, or in the presence of inducers such as 1,16 hexadecanediol or cAMP may be effective in identifying such peptides. The target for the hexapeptides is expected to lie upstream of adenylyl cyclase since cAMP is able to overcome the effect of D-CYRFTW (Fig. 8). Testing of this hypothesis could be addressed by analysis of mutants that are altered in the regulatory pathway governing appressorium development. This experiment is described in Chapter III.

Strain 4091-5-8 was the most sensitive to inhibition by hexapeptide, and responded to addition of cAMP and 1,16 hexadecandiol efficiently when compared to CP987. Strain CP987 besides exhibiting greater resistance to hexapeptide, did not show response with similar efficiency as that of 4091 to the treatment with cAMP. This leads me to conclude that the concentrations of the hexapeptide which inhibit the appressorium formation pathway in strain 4091, does not affect basic cellular levels of cAMP in strain CP 987. These results further suggest greater resistance of CP987 to the inhibitory effect of hexapeptide and higher cellular levels of cAMP than in 4091.

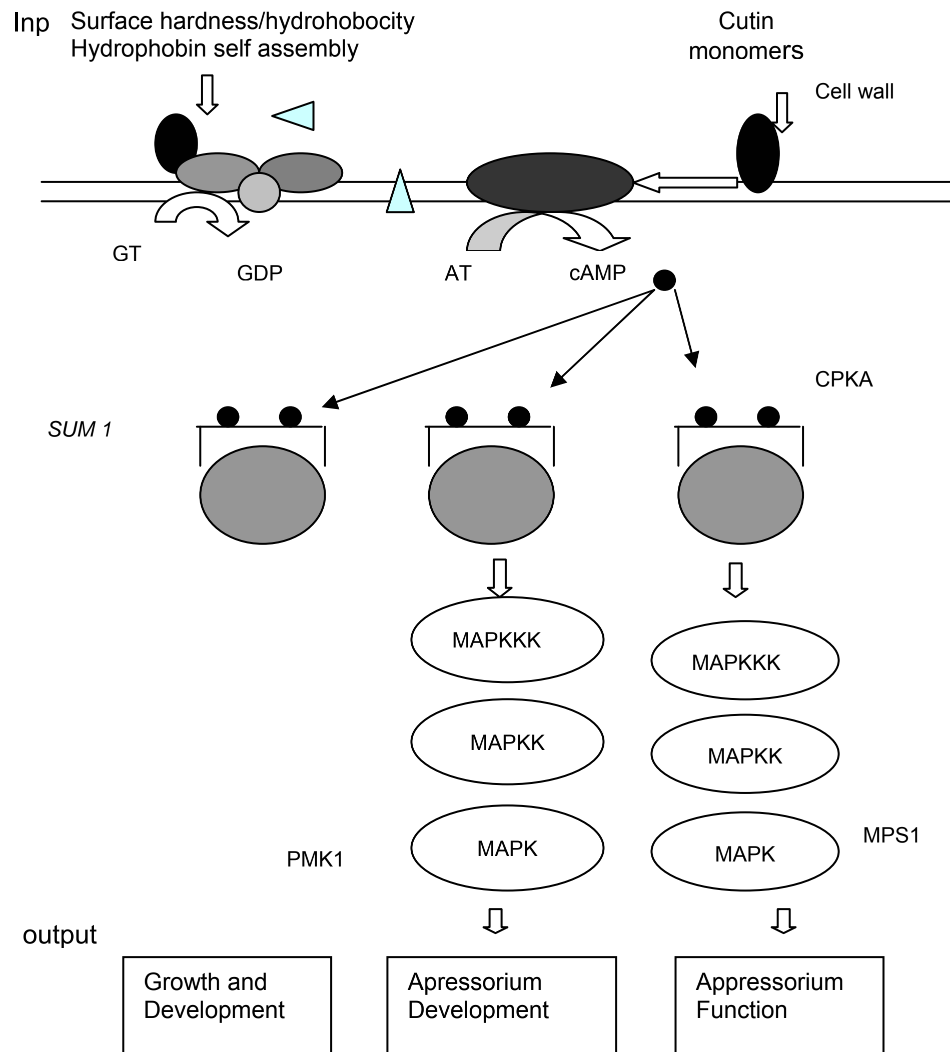


Figure 8. Hypothesis of mode site of action of the hexapeptide D-CYRFTW during appressorium formation on *M. grisea*, when tested on artificial hydrophobic surfaces.

CHAPTER III

SIGNAL TRANSDUCTION PATHWAY IN APPRESSORIUM FORMATION IN *Magnaporthe grisea*

INTRODUCTION

A key mechanism applied by eukaryotic cells to respond to external stimuli is reversible protein phosphorylation. The molecule 3', 5'-cyclic adenosine monophosphate (cAMP) was first identified as a heat-stable factor mediating the effect of glucagons on the phosphorylation status of glycogen phosphorylase. cAMP acts as a second messenger between the reception of an external signal (glucagon) and the activation of cellular events. The target for the cAMP signal was purified and identified as a cAMP-dependent protein kinase (PKA). PKA is a tetrameric holoenzyme consisting of two catalytic subunits (C) bound to a regulatory dimer subunit (R). The binding of four cAMP molecules to the regulatory dimer releases the two catalytic subunits that phosphorylate serine and threonine residues on specific substrate proteins. In the absence of cAMP, PKA is enzymatically inactive. PKA regulates many cellular processes such cell metabolism, cell growth, cell differentiation, and gene regulation (Skalhegg and Tasken 1997).

Saccharomyces cerevisiae serves as a model for signal transduction in fungal development. The pheromone response and invasive growth processes involve both MAP kinase and cAMP signaling pathways. The involvement of cAMP in developmental processes in filamentous fungi is also now being

explored at the molecular level (Wendland 2001). A number of different genes have been shown to influence the basic cAMP signaling pathway (Table 16).

The cAMP signaling pathway is activated during appressorium formation in *M.grisea*. (Choi and Dean 1997), (Lee and Dean 1993b), (Mitchell and Dean 1995), (Liu and Dean 1997). cAMP levels are thought to increase in germinated conidia in response to surface hydrophobicity and germ tube contact with hard surfaces (Talbot, 2003). Mutants summarized in Table 17 define genes that play a role in cAMP signaling in appressorium formation.

The findings described above (Table 16) show a large number of potential candidate genes that may play a role in appressorium morphogenesis and maturation. With the availabilty of the *M. grisea* genome sequence, it is anticipated that the roles of all of these genes in appressorium formation will be determined in the near future.

The MAP kinase genes OSM1 (Dixon et al. 1999), PMK1 (Xu and Hamer 1996) and *MPS1* (Xu et al. 1998) were identified and characterized. The *PMK1* MAP kinase was found to be required for appressorium morphogenesis and host colonization. The *MPS1* MAP kinase was found to be required for appressorium maturation and host colonization. The *OSM1* MAP kinase was hypothesized to be a likely candidate for involvement in appressorium maturation. In *S. cerevisiae*, the homologous MAP kinase (Hog1) is required to generate glycerol to counteract hyperosmotic stress. Glycerol production within appressoria is required to generate the turgor pressure required for appressorium function.

However, mutation of *OSM1* did not alter the ability to produce a functional appressorium. Clearly, there are many features of appressorium development left to be determined.

In this apressorium formation Chapter I further analyze the mode of action of the hexapeptides that inhibit appressorium (Chapter II). Mutants activated for G-protein and PKA signaling were tested for their sensitivity to D-CYRFTW. The results of these studies indicate that the peptide functions upstream of adenylyl cyclase and potentially upstream of, or at the level of the G-alpha subunit. Preliminary studies of total PKA activity during appressoria development reveled changes in PKA activity during time course of appressoria development.

MATERIALS AND METHODS

Strains, growth, and maintenance of *Magnaporthe grisea*

Strains of *M. grisea*, (Table 5) were stored by growing the fungus in sterile filter-paper discs, followed by desiccation and storage at –20 °C. Fungal cultures were grow first on PDA (potato dextrose agar + 100µg/mL of ampicilin), and transferred to oatmeal agar (50g of oatmeal per liter + 100µg/mL of ampicilin and 25 µg/mL of Chloramphenicol) at 25 °C under continuous fluorescent light to promote conidiation.

Table 16. Names and function of genes involved on cAMP pathway in filamentous fungi.

Species	Class	Gene name	Involvement
A. nidulans	Ras protein	*rasA*	Germination, development
	Gα protein	*ganB*	Unknown
	Gα protein	*fadA*	Secondary metabolite production, conidiation
	RGS domain protein	*flbA*	secondary metabolite production, conidiation
	Protein kinase A	*pkaA* (cat. subunit)	Growth, development, sporulation; secondary metabolite production
	Neutral trehalase	*treB*	Germination
M. grisea	Integral membrane protein	*pthll*	Host-surface recognition
	Ras protein	*rasl*	Unknown
	Gα protein	*magA*	Ascospore formation
		magB magC	Growth, conidiation, perithecia and appressorium formation. Conidiation
	Adenylate cyclase	*macl*	Growth, conidiation, sexual development, appressorium formation
	Protein kinase A	*cpkA* (cat.subunit)	Appresorium formation, pathogenicity
N. crassa	Ras protein	*smco7*	Hyphal growth, cell wall synthesis, conidiation
	Gα protein	*gna3*	Conidiation; cAMPlevel
	Adenylate cyclase Protein kinase A	*cr-1 Mcb* (reg. subunit)	Conidiation Growth polarity
	Neutral trehalase	*treB*	Trehalose mobilization
U. maydis	Gα protein	*gpa1 gpa2*	Unknown Unknown
		gpa3 gpa4	Sexual development, growth, pathogenicity Unknown
	Protein kinase A	*Ubcl* (reg. subunit) *ukal* (cat.subunit)	Growth, pathogenicity Unknown
		Adrl (cat. subunit)	Growth, pathogenicity
	MAP kinase	*ubc3*	Required for filamentous growth and full virulence; suppressor of *uacl*
	MAPKK kinase	ubc4	Suppressor of *uacl*
C.parasitica	Gα protein		Pigmentation, sexual and asexual reproduction,pathogenicity
		CPG1 CPG2	Vegetative growth, asexual reproduction
F.oxysporium	Gα protein	*fga1 bcg1*	Conidiation, pathogenicity
B.cinereus	Gα protein		Morphology, pathogenicity
		bcg2	Unknown
C.trifolli	Gα protein	*ctg1*	Germination, appressorium formation

Table 17. Effect of gene mutation on appressorium formation after surface induction in *M. grisea*.

Mutant	Gene	Appressoriun Phenotype		Reference
		HO	HY	
mac 1	Adenylate Cyclase	no	no	(Choi and Dean 1997)
Sum-1 DA 99	Supressor of mac1	yes	yes	(Adachi and Hamer 1998)
mag B	Gα Bsubunit	no	no	(Liu and Dean 1997)
Mag B G 42 R	Gα B activated	yes	yes	(Fang and Dean 2000)
Mag B G 203 R	Gα B inactivated	no	no	(Fang and Dean 2000)
Cpka	Catalytic subunit PKA	yes	no	(Xu et al. 1997)

Appressorium was induced in artificial hydrophobic (HO) and hydrophilic (HY) surfaces.

***In vitro* appressorium formation assay**

Conidia of *M. grisea* were harvested 10 to 12-day-old cultures (50 mM, and pH 6.6) filtered once through Miracloth, and diluted to 1 x 10^5 conidia/mL. Drops of 5 µL of conidial suspension were placed on hydrophobic plastic microscope cover slips (Fisher Scientific) or on the hydrophilic surface of GelBond film (FMC Bioproducts). The peptides were added to a final concentration of 500 µg/ml and eight 2-fold dilutions were made to give final concentrations of 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.3 µg/ml, 15.6 µg/ml, 7.8 µg/ml, 3.9 µg/ml, and 2.0 µg/ml.

Induction of appressorium formation

Conidia of *M. grisea* were harvested 10 to 12-day-old cultures in gelatin solution (0.25%) filtered once through Miracloth, and diluted to 3 x 10^6 conidia/mL. Appressorium formation was induced over hydrophobic (HO) surface, with and without hexapetide D-CYRFTW, and hydrophilic (HY) surfaces. Cuts of parafilm film (HO) washed with ETOH 70% followed by ddH2O, and GelBond (HY) (FMC Bioproducts) were placed over wet filter paper in a square (25 cm x 25 cm) plastic plate. Inoculum solution was sprayed (3x10^6 conidia/mL) over the surfaces and the plates were maintained in moist chamber at 25˚C, for 2 (Figure 17), 4 (Figure 18), 6 (Figure 19) hours after induction. For every time point forming appressoria was evaluated under an Olympus (BX6) light microscope and the image captured with an Olympus Q-Fire digital camera.

Protein extraction

Protein extraction buffer (10mM Tris-HCl, 150mM NaCl, 2mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10µg/mL Leupetinin, 10µg/mL Apotinin, pH = 7.4 at 4˚C) was sprayed over the plates containing the inoculum. Drops were brought together by crushing with a glass slide and centrifuged for 30 minutes at 13000 rpm. Supernatant was recovered and concentrated using a Centriplus YM-10 (Millipore) filter. Protein concentration was determined using a BioRad protein assay kit. PKA and MAP Kinase activity were detected during the appressorium formation time corse. MAP Kinase (0.5 µg/mL) was detected using a “Pep Tag Assay for non-Radioactive Detection” kit (Promega).

***Neurospora crassa* conidiation induction**

N. crassa wild type 74A, and the *ste12* mutant, was grown to induce conidiation under constant light at 32˚C during seven days, in Vogel’s minimal medium. Conidial suspension (1x 10^6 conidia/mL) was transferred to 50 mL of liquid Voguel’s (receita) medium under constant agitation, at 32˚C, during 16hours. The mycelium was harvested by filtration on Whatman filter paper and placed in Petri dishes containing Vogel's minimal medium with 1.5% agar.

The Petri plates were exposed to continuous light during 0, 2, 4, 6, 8, 10, 12, 16, 18, 20, 22, 24, and 28 hours, at 30˚C.

MAP kinase activity

MAP Kinase activity was detected during the *M. grisea* appressorium formation, during *N. crassa* conidiation, and after induction of vegetative growth in liquid medium of both microrganism, using a non radioactive detection kit (Cell Signalling), at 5ug/mL of the total protein extracted.

Microorganism *N. crassa*, wild type 74 A and *ste12* mutant, and *M. grisea*, wild type 4091, and *pmk1* mutant, were grown in liquid minimum medium during 24 hours, at 25˚C. The mycelium mass were vacuum filtrated and transferred to 50 mL liquid minimum medium with or without orthovanadate (100 u M), during 20 and 60 minutes.

PKA activity

PKA activity was detected for the appressorium formation time course using a “Pep Tag Assay for non-Radioactive Detection” kit (Promega), at 0.5 μg/mL of the total protein extracted.

RESULTS

Analyses of the activity of D-CYRFTW and L-WTFRYC in *M. grisea* strains altered in the appressorium signaling pathway

The strains 4091-5-8, CP987, Guy-11, DA 91 and MAGB^{G42R} were assayed with D-CYRFTW and L-WTFRYC. Both hexapeptides block the appressorium formation of strains 4091-5-8, CP987, Guy-11, and MAGB (G42R) (Fig.9 A and B). The means, and the t-values, of appressoria formed at the final concentrations (µg/mL) of 31.0, 16.0 and 8.00 µg/mL of D-CYRFTW are summarized at Fig. 10, 11, 12. The *M. grisea* strain DA99 was resistant to D-CYRFTW and L-WTFRYC. The mean values for appressorium formation by DA99 were significantly higher than for 4091-5-8, CP987, Guy 11 and MAGB (G42R) at all concentrations assayed.

The strain MAGB G42R was significantly more susceptible than 4091, and than the wild type Guy 11at 16.00 and 8.00 µg/mL. The data are summarized in Fig. 10 and Fig. 11. The means for appressoria formed for strains 4091 and CP 987 differ when the pathway is blocked by D and L forms of hexapeptide. CP 987 is less sensitive to the effect of the peptide based on the higher number of formed apressoria when compared to the others strains, under the same concentration.

A

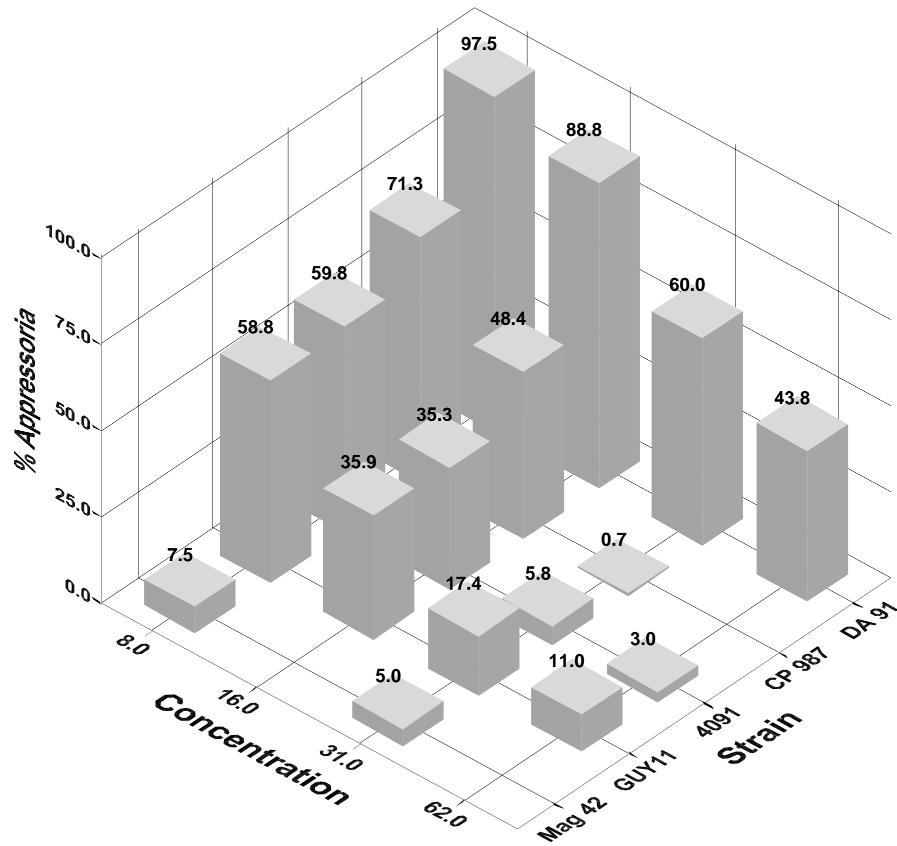


Figure 9. Means of appressoria formed for all strains at final concentrations of 8, 16 and 31 ug/mL of D- CYRFTW (A), and of 8, 16 and 31 ug/mL of L–WTFRYC (B)

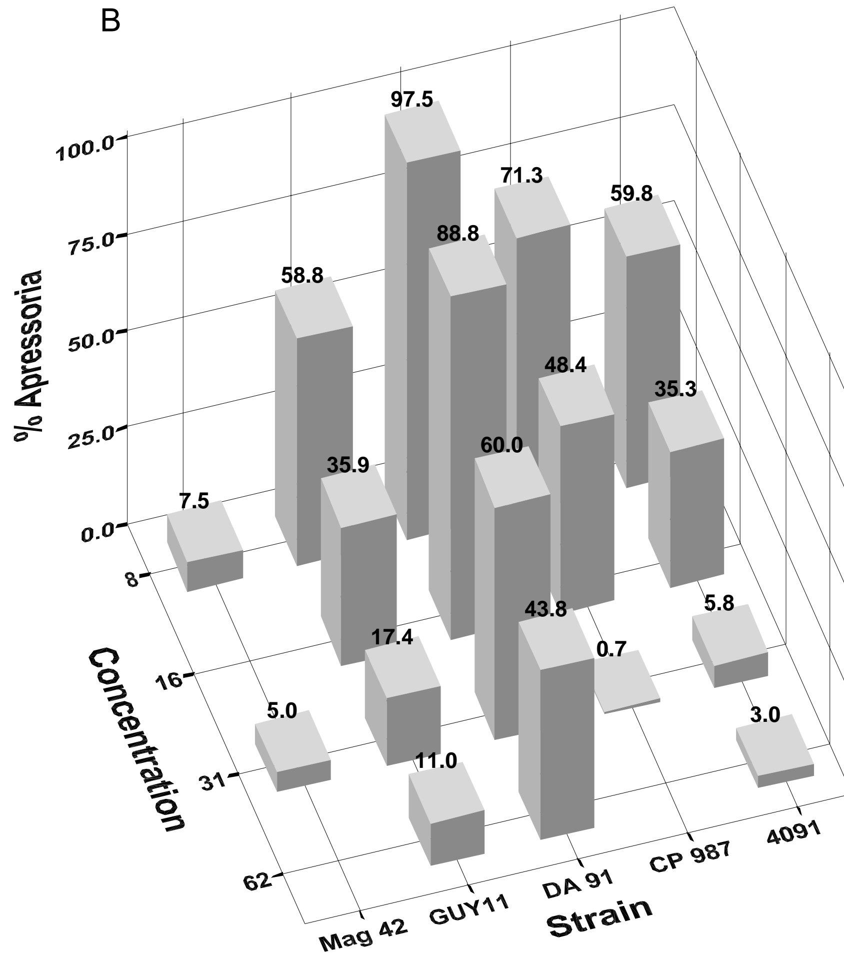


Figure 9. Continued

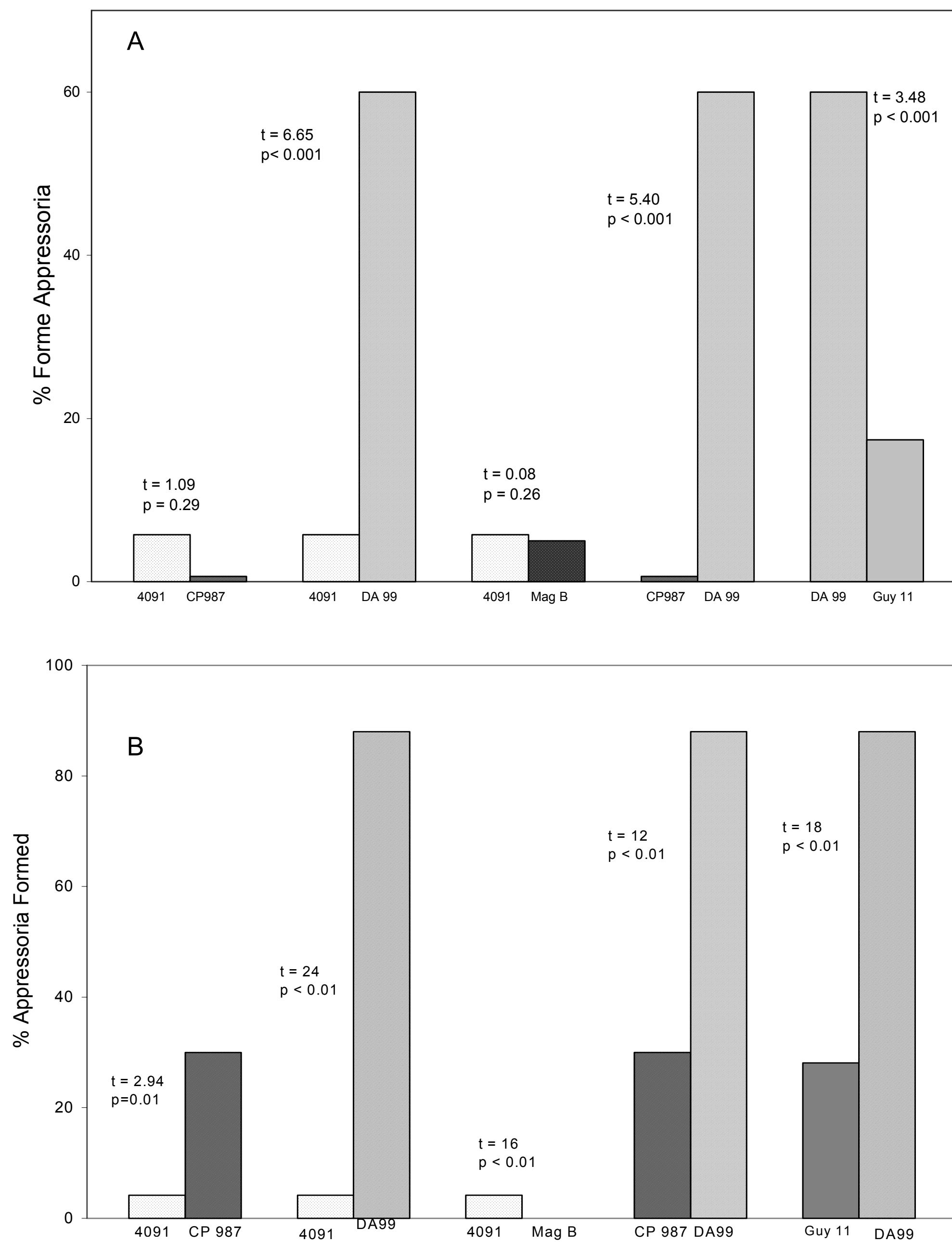


Figure 10. Means of appressoria formed at final concentration of 30 ug/mL of D- CYRFTW (A), and of L–WTFRYC (B).

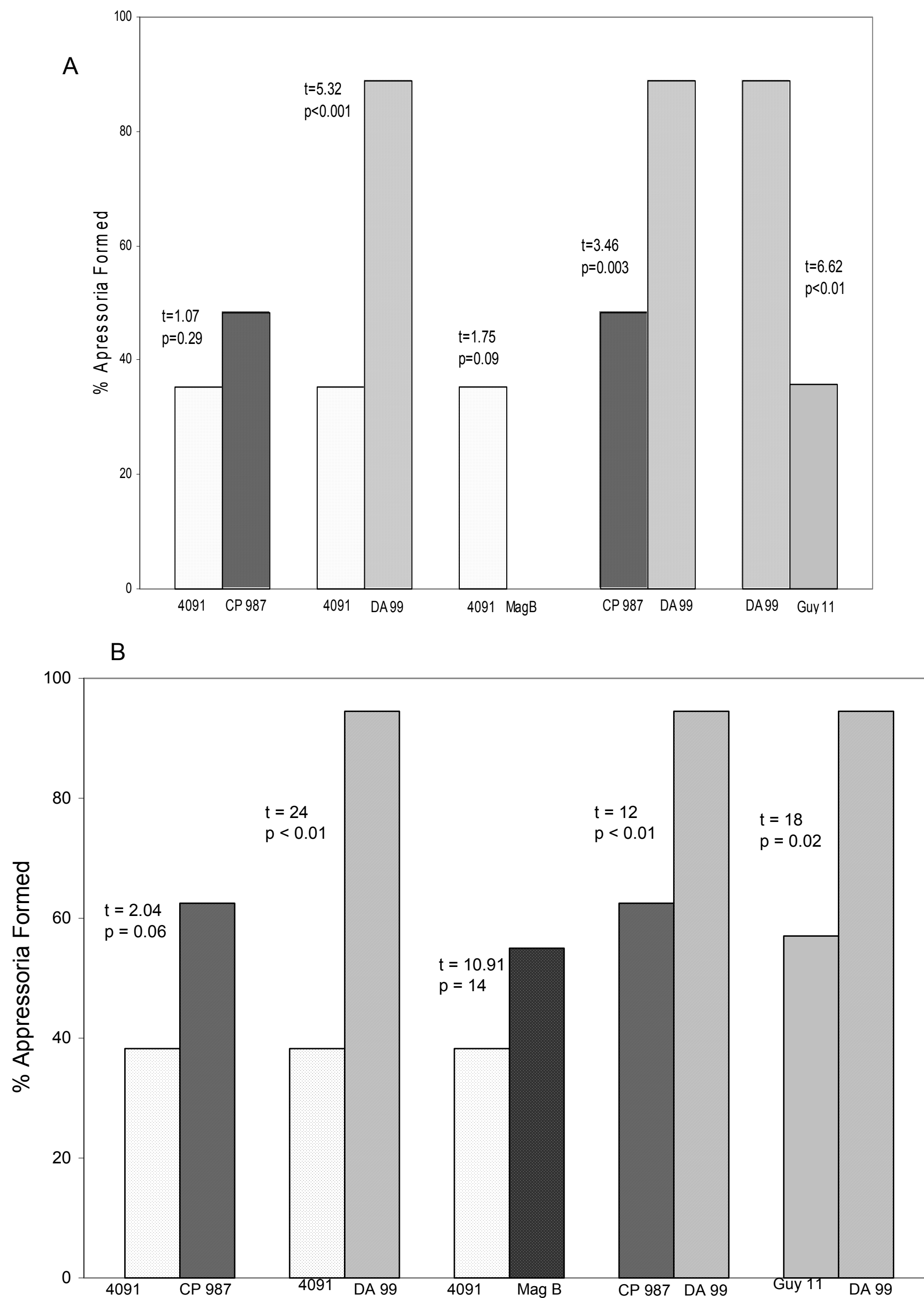


Figure 11. Means of appressoria formed at final concentration of 16 ug/m Lof D- CYRFTW (A), and of L–WTFRYC (B).

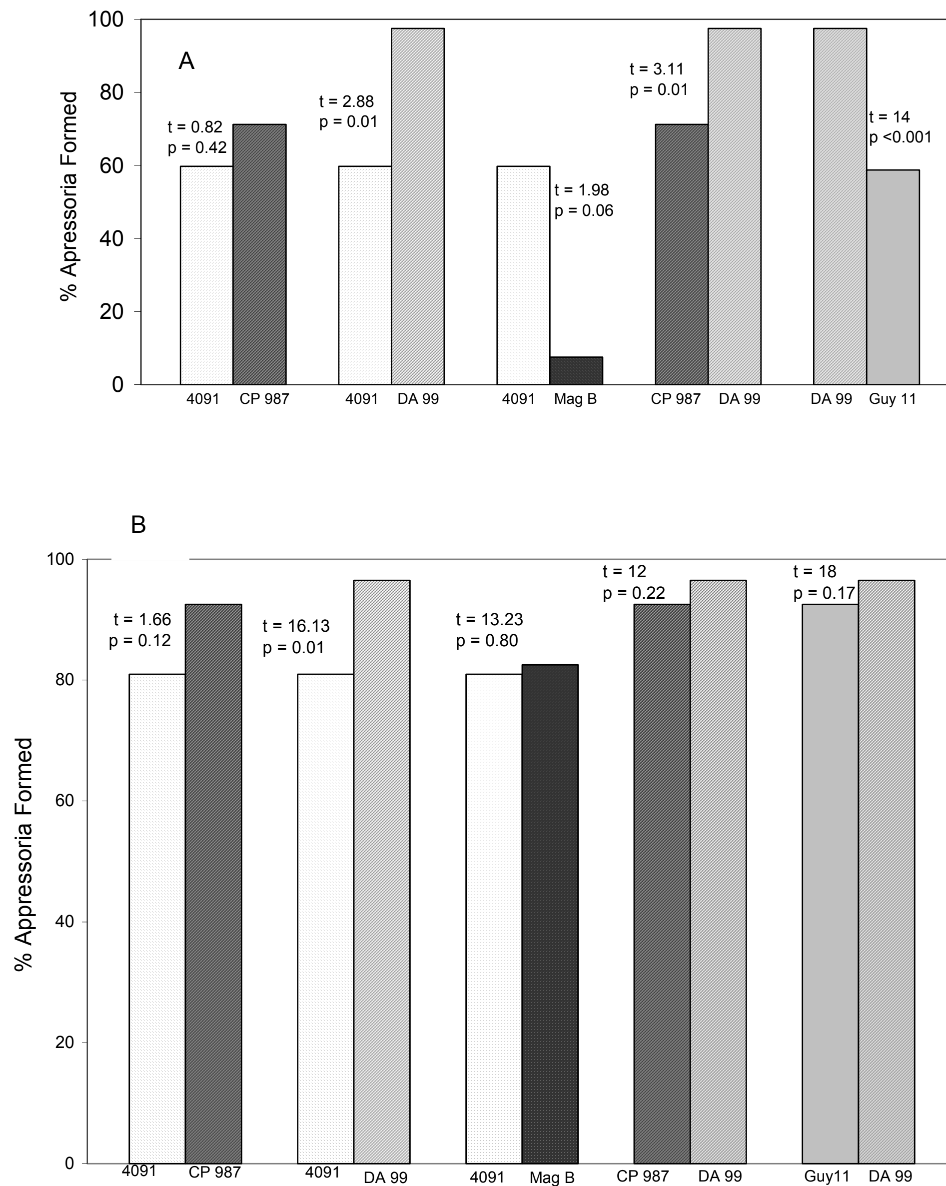


Figure 12. Means of appressoria formed at final concentration of 8 ug/m L of D- CYRFTW (A), and of L–WTFRYC (B).

MAP kinase activity detection

In order to compare MAP Kinase activity detection methods, the total proteins from mycilium of the microorganisms *N. crassa* (wild type and *mak*^{2} mutant), and *M. grisea* (wild type 4091, and *pmk1* mutant) were compared. From the same amount of mycilium, the total concentration of extracted protein from *N. crassa* was 15ug/ mL, and *M. grisea* was1ug/mL. Detection of phosphorolated MAP kinase was possible ony for *N. crassa* wild type, at 60 and 20 minutes after induction with orthovanadate (Figure 13). There was no expression of MAP kinase in the mutant mak2 (*Neurospora crassa*).

Total MAP kinase during apressorium formation, extracted from mycelium and from germlings of *M. grisea*, at a final concentration of 15ug/mL was compared. Wild type, 4091 and *pmk*^{1} had different pattern of expression of total MAP kinase expressed during vegetative growth (Fig.14). The wild type 4091 also had different pattern of expression during appressorium formation at time 0, 2 and 4 hours after induction (hook cell formation).

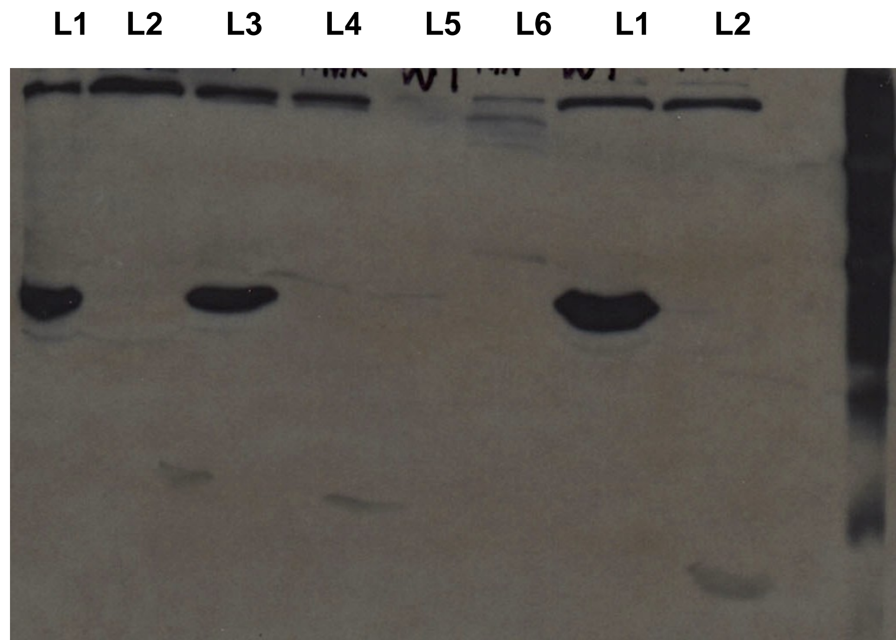


Figure 13. Detection of posphorylated MAP kinase in *N.crassa* (15ug/ mL) and *M. grisea* (1ug/mL). L1- *N. crassa* wild type 74A 60 minutes after induction in liquid medium; L2- *N. crassa* mak2 60 minutes after induction; L3- *N. crassa* wild type 74A 20 minutes after induction in liquid medium; L4- *N. crassa* mak2 20 minutes after induction; L5, *M. grisea* wild type 60 minutes after induction in liquid medium; L6 *M. grisea* pmk1 mutant nn95 60 minutes after induction in liquid medium.

L1 L2 L3 L4 L5 L6 L7 L8

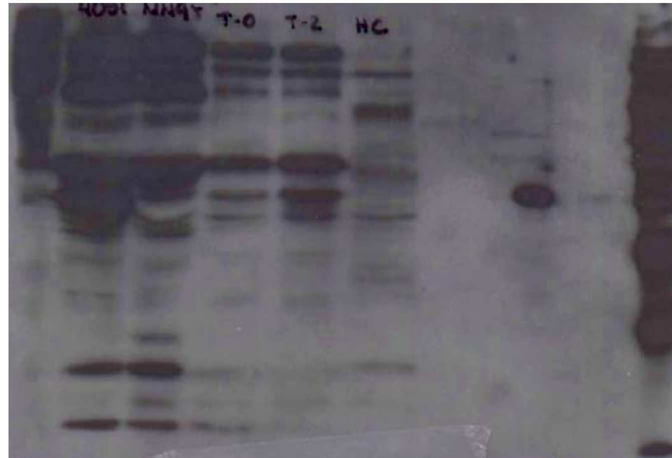


Figure 14. Detection of total MAP kinase (15ug/mL) extracted from *M. grisea* mycelium (line 2 and 3), and from germlings during appressorium formation at 0, 2 and 4 hours after induction (lines 4, 5 and 6). Lines 7 and 8 are positive and negative controls.

Detection of phosphorylated MAP Kinase during conidiation in *N. crassa* is in figure 15 and 16. Proteins samples (15ug/mL), wild type 74 A and *ste 12* were extracted from aerial hyphae and conidia formed at 0, 2, 4, 6, 8, 10, 12 , 16, 18, 20, 22, 24 and 28 hours after induction. *M.grisea* protein sample (15 ug/ mL) was extracted as described previously.

PKA activity during appressoria formation pathway

PKA activity was detected during apressorium formation (Fig. 17, 18, 19 and 20), and in conidia (lane 1). PKA showed to be active in conidia (line 1), and at 2 hours after induction in hydrophobic surface (line2), but not at 4h (line3). In the same experiment, samples in the presence of the D - CYRFTW peptide, 30 ug/mL, line 4, and in hydrophilic surface PKA showed to be active at 4 h after induction, line 5 (hook cells). Line 6 is the result of a second experiment, which showed that 2 hours after induction there is no activity of PKA. In line 7, 4 hours old cells germinated in hydrophobic surface presented PKA activity, different from the first experiment; In line 8, cells of 6 hours old, germinated in hydrophobic surface presented no activity of PKA; In the next line, cells of 2 hours old, germinated in hydrophilic surface showed no activity; at line 10 4h on hydrophilic surface there is PKA activity; line 11 6h on hydrophilic surface no activity of PKA; line 12 positive control

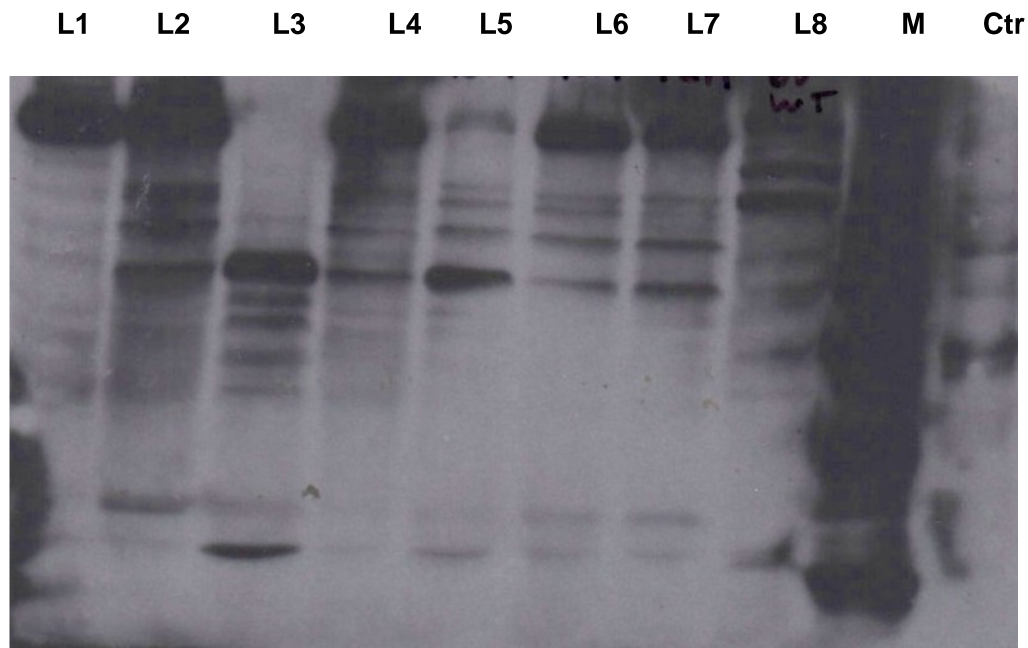


Figure 15. Phosphorylated MAP kinase in samples from *N. crassa* after conidiation induction, and from *M.grisea* mycelium mass protein sample. L1- *N. crassa* time 0; L2- *N. crassa* 2hours after induction; L3 - *N. crassa* 4hours after induction; L4 – *N. crassa* 6 hours after induction; L5 – *N. crassa* 8 hours after induction; L6 - *N. crassa* 10 hours after induction; L7- *N. crassa* 12 hours after induction; L8 – *M. grisea* 60 minutes after induction of vegetative growth; M-marker; Ctr - positive control.

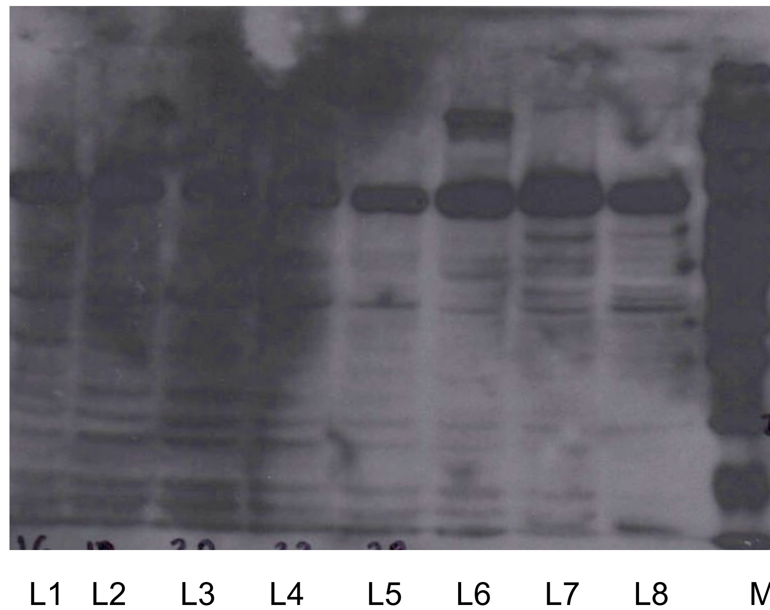


Figure 16. Phosphorilated MAP kinase in samples from *N. crassa*, wild type, after conidiation induction, and *ste12* (0, 2, and 4 hours after conidiation induction). L1 16 hours after induction; L2 18 hours after induction; L3 20 hours after induction; L4 22 hours after induction; L5 28 hours after induction; L 6 *ste12* 0 hours after induction; L7 *ste12* 2 hours after induction; L8 4 hours after induction. M marker.

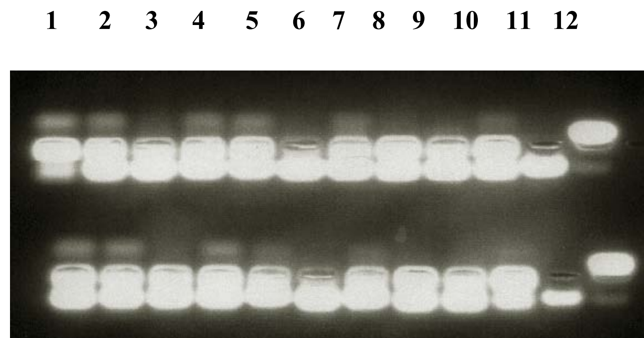


Figure 17. PKA activity of samples extracted from *M.grisea* after appressoria formation induction.1) Conidia; 2) 2h on hydrophobic surface; 3) 4h on hydrophobic surface; 4) 4h on hydrophobic surface + 30 µg/mL of CYRFTW; 5) 4h on hydrophilic surface; 6) 2h on hydrophobic surface; 7) 4h on hydrophobic surface; 8) 6h on hydrophobic surface; 9) 2h on hydrophilic surface; 10) 4h on hydrophilic surface; 11) 6h on hydrophilic surface; 12) Positive control

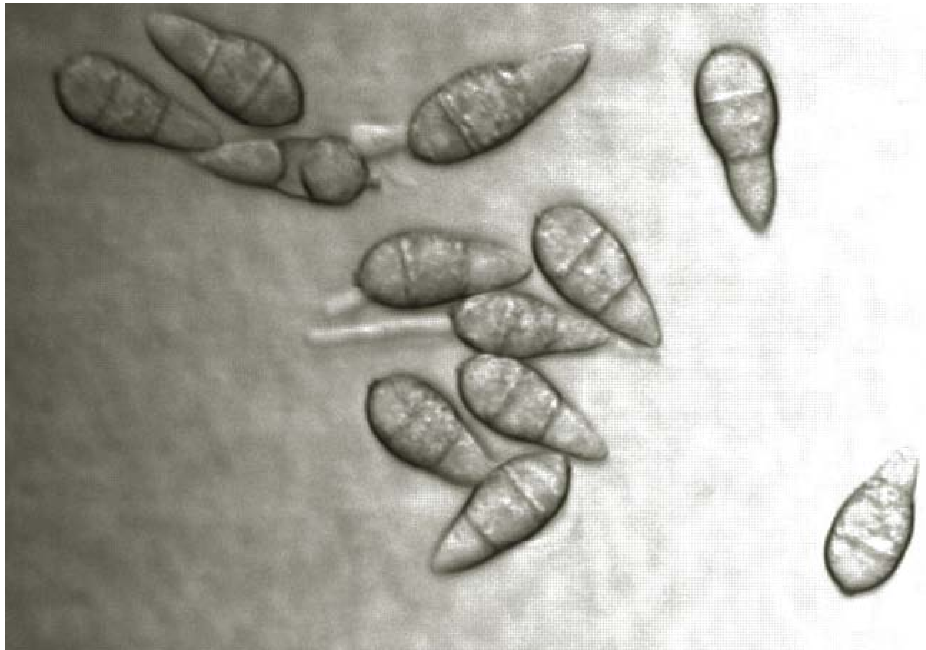


Figure 18. Germ tube elongation after 2 hours of induction on artificial hydrophobic surface.

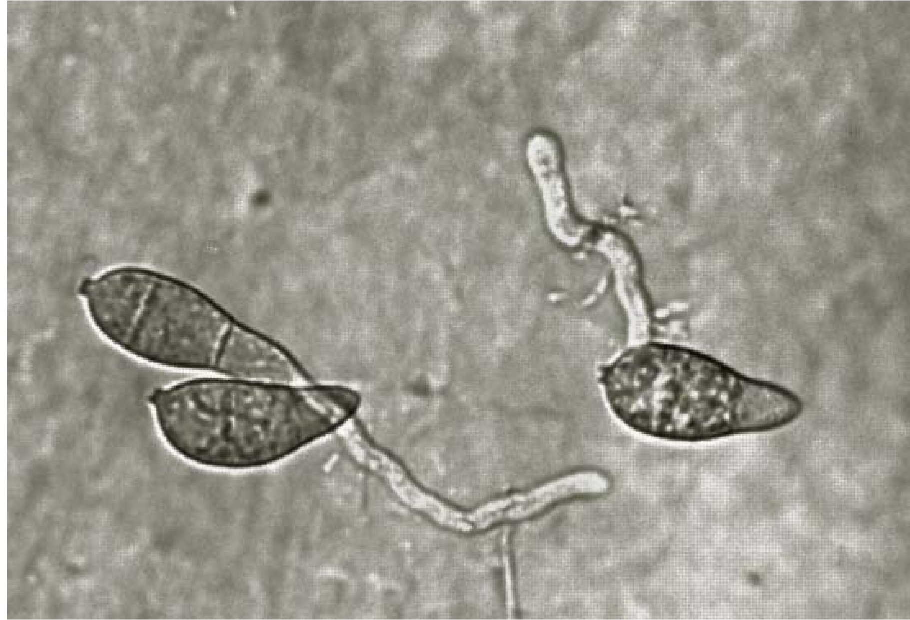


Figure 19. Germ tube elongated and tip swelling after 4 hours of induction on artificial hydrophobic surface.

Figure 20. Young appressoria formed after 6 hours of induction on artificial hydrophobic surface.

DISCUSSION

Strain DA99 found to have a regulatory suppressor of the adenylate cyclase mutant, *mac1*, that restored appressorium formation. The gene for the regulatory subunit of PKA is a candidate that should be involved in suppressing an adenylate cyclase mutant. Indeed, DA99 was found to contain a regulatory subunit gene that contains a missense mutation that alters the leucine at position 211 to an arginine. It was proposed that the interaction of the catalytic subunit of PKA interacted only weakly with this regulatory subunit. This leads to precocious activation of the PMK1 MAP kinase pathway, thereby restoring appressorium formation on both hydrophobic and hydrophilic surfaces (Adachi and Hamer 1998).

A strain containing the *MagB*^{G42R} allele was generated by site directed mutagenesis of the *MagB* gene, converting the glycine located at position 42 to arginine. This mutation was expected to disrupt the endogenous GTPase activity in the Gα subunit leading to constitutively active G protein signaling (Fang and Dean 2000). *MagB*^{G42R} strains were able to form appressoria on non-inductive surfaces, demonstrating the effect of the dominant activated allele. I hypothesized that if the hexapeptide acted to inhibit the pathway signaling appressorium formation at the G protein step or downstream of the G protein, that peptide would still be able to inhibit appressorium formation in the *MagB*^{G42R} strain. I found that the mutant was susceptible to the inhibitory effect of D-CYRFTW. Based on the expected effect of the point mutation in this strain,

constitutive activation of the G protein was disrupted or blocked by the hexapeptide D-CYRFTW.

According to the proposed model for appressorium formation (Fig. 21) CPKA is activated after internal levels of cAMP increased. DA99 does not rely on cAMP for activating the appressorium development pathway because it is constitutively activated. This strain displays resistance to the inhibitory effect of the hexapeptide, suggesting that the peptide interacts with a component of the pathway located upstream of CPKA.

MagB^{G42R} is constitutively activated because it has reduced GTPase activity and so it consitutively activates adenylate cyclase. In previous experiments (Fang and Dean 2000) this strain was shown to be able to form appressoria on non-inductive surfaces. *MagB*^{G42R} was susceptible to D-CYRFTW suggesting the target of this peptide is located at *MagB* or at any step of this proposed pathway upstream of adenylate cylase. Because the *MagB*^{G42R} likely interacts with a surface recognition receptor, its activity may also depend on surface sensing. Therefore, I can not exclude the possibility that the peptide acts at the membrane to alter surface sensor activity or acts as an antagonist of the surface sensor itself.

MAP kinase and PKA activity was very hard to detect. I had done many times these two experiments and the results were not consistent as we expected. Sometimes it was possible to detect phosphorylated MAP kinase, but

the quality, amount, period of conservation, together with the methods used to concentrate the sample were very important to get uniformity of the detection.

During appressorium formation the detection of MAP kinase would be clearer if the primary antybody used was specific for PMK1, and not general as a MAP kinase antibody. MAP kinase was difficult to extract as germlings, and was difficult to measure in *M.grisea* relative to *N.crassa*. From the same amount of mycelium, grown under the same conditions, the total protein extracted (ug) was very different from both microorganisms.

PKA activity is possible to be detected based on the physiological age of the cells. The physiological age that I refer are conidia germination (fig.18), elongation and size of germ tube (fig.19), and hook cell formation. According to my results PKA is active for conidia germination and germ tube elongation. During hook cell formation PKA is not active. Only after appressorium is formed and starts to maturate (fig.20) PKA gets active again. The activity of PKA detected on conidial cells germinated on hydrophilic surface, around 4 hours after induction, represents a delay in the beginning of a different physiological process, the hook cell formation. The same occurs in the presence of the peptide. The peptide did not inhibit the germination process, but it interferes in the recognition of the inductive surface delaying the hook cell formation.

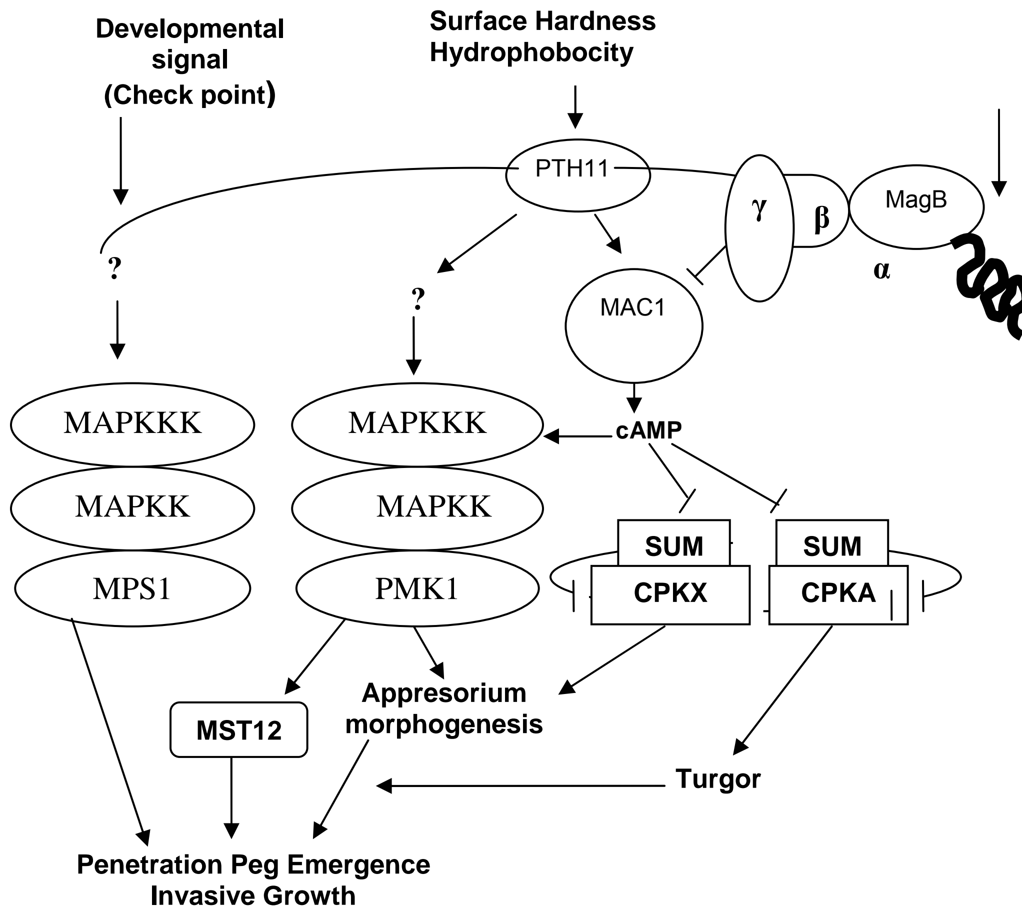


Figure 21. Hypothetical model for signaling pathway for appressoria formation in *M.grisea*. (Talbot 2003a) .

CHAPTER IV

ROLE OF THE *PTH1* GENE IN APPRESSORIUM MATURATION IN *Magnaporthe grisea*

INTRODUCTION

Appressoria of *M. grisea* generate very high internal turgor pressure to mechanically breach the rice cuticle. Melanin forms a darkly pigmented cell wall layer in the developing appressorium and reduces the porosity of the wall, affecting its permeability to small molecules. Jong and co workers, (Jong et al. 1997) extracted the contents of 24-48hs appressoria and biochemically characterized the compatible solutes in the cytoplasm of appressoria. Glycerol was found to be the most abundant solute. Glycerol is generated during germination and germ tube elongation, probably contributing to plasma membrane biosynthesis. Glycerol levels decrease just prior to appressorium development, during the the swelling of the hook cell, and later increase up to 3.5 M, generating an osmotic potential of 5.8 MPa in the newly formed appressorium. Glycerol might be derived from rapid degradation of lipids, glycogen, trehalose, mannitol, and other energy sources stored in the conidium. The reduced porosity of the melanized cell wall blocks efflux of metabolites and supports the increase in turgor pressure. Lipid droplets also move into the incipient appressorium and coalesce into a central vacuole before degradation

at the onset of turgor generation (Howard and Ferrari 1989), (Bourett and Howard 1990) .

The turgor pressure generated by glycerol biosynthetic enzymes during appressorium development, and the movement of storage reserves into the forming appressoria was investigated (Thines et al. 2000). Conidia contain an abundance of trehalose, a non-reducing dissacharide known to protect enzymes from thermal stress *in vivo* and thus providing protection for cells. The gene *TPS1*, encoding trehalose-6-phosphate synthase in *M. grisea* was characterized. Mutants of *tps1* display decreased pathogenicity due to their inability to fully generate turgor pressure and penetrate the leaf surface (Foster et al. 2003).

Enzymatic activities for glycerol generation from carbohydrate sources were present in appressoria but did not increase during development. In *S. cerevisae* the conversion from glycerol-3-phosphate to glycerol is carried out by three specific glycerol-3-phosphatase dehydrogenases. Two of these are encoded by the genes *GPD1* and *GPD2* present in the cytosol, and a third enzyme *GUT2* found in the inner part of the mitochondrial membrane. It is known that *GPD1* plays a role in osmoadaptation since its expression is induced by osmotic stress. Expression of *GPD2* is not affected by changes in external osmolarity, but is stimulated by anoxic conditions. The orthologs of the *Aspergillus nidulans* genes encoding glycerol-3-phosphate dehydrogenase and NADP-dependent glycerol dehydrogenase were identified in *M. grisea* and found

to be active in germinated conidia, but not in the forming appressorium (Talbot 2003a). The absence of these enzyme activities in appressoria is consistent with the view that glycerol present in the appressorium is not further metabolized and accumulates. Triacylglycerol lipase activity increased during appressorium maturation consistent with the view that lipids are central as a source of glycerol.

The involvement of signal transduction pathways in these processes has also been investigated (Thines et al. 2000). Glycogen and lipid mobilization does not occur in a *pmk1* mutant, and is retarded significantly in a *cpkA* mutant, which lacks the catalytic sub-unit of cAMP-dependent protein kinase A (PKA). The adenlyate cyclase mutant is defective in appressorium formation. However, suppressor mutants have been obtained that display constitutive appressorium formation. During appressorium formation in one of these suppressor mutants, *sum1-99*, glycogen and lipid degradation is very rapid and occurs before appressorium morphogenesis is complete, again providing evidence for a link between cAMP signaling and carbon mobilization (Thines et al. 2000).

Characterization of *PTH1*

Although a great deal is known about the physiological events that occur during appressorium development and the major signal transduction pathways involved, much less is known about the connection between signaling pathways and changes in enzyme activities. To identify additional genes involved in this process, Valent et al (Valent 1990) conducted an insertional mutagenesis (REMI) analysis in *M. grisea* and cloned seven *PTH* genes that play a role in pathogenicity

on barley, weeping love grass, and rice. *PTH1* was identified as a REMI mutant that was unable to produce functional appressoria. The gene was cloned (Beckerman 1997) and the sequence of *Pth1* revealed homology to *GRR1* of *S. cerevisiae*.

GRR1 encodes an F-box protein that plays a key role in cell cycle progression and nutrient sensing. F-box proteins are components of modular E3 (ubiquitin protein ligase enzyme) called SCFs, and are characterized by a 40 amino acid motif, the F-box. Three classes of F-box proteins have been characterized in humans based on additional protein-protein interaction motifs found in the proteins: Fbws contain WD-40 repeats, Fbls contains leucine-rich repeats (LRR), and Fbxs for which the protein-protein interaction motif has not yet been identified. The flexibility and specificity of ubiquitin-dependent proteolysis are mediated, in part, by the E3 ubiquitin ligases, which derives its specificity from F-box proteins, a heterogeneous family of adapters for target protein recognition. Grr1, the F-box component of SCF^{Grr1}, mediates the interaction with phosphorylated forms of the G_1 cyclins Cln1 and Cln2. Hsiung et al (Hsiung Y.G. et al. 2001) showed that a point mutation of *GRR1* converting the basic residues on the concave surface of the LRR protein binding motifs to neutral or acidic residues interfered with the capacity of Grr1 to bind to Cln2, and phenotypically caused hyperpolarization and enhanced pseudohyphal growth. This mutant allele did not interfere with the role of Grr1 in nutrient-regulated transcription of *HXT1* or *AGP1*. The authors concluded that the cationic nature of the concave surface of the Grr1

LRR is critical for the recognition of phosphorylated targets of SCF^{Grr1} but that other residues of Grr1 are involved in its other functions.

M. grisea pth1 mutants are unable to penetrate host tissue and to establish a successful host-parasite interaction. The mutants form defective appressoria, which are unable to generate sufficient cellular turgor to penetrate the host leaf surface (Beckerman 1997). It was proposed that *pth1* plays a specific role in appressorium maturation and likely affects carbohydrate metabolism and turgor pressure generation. To test this hypothesis I characterized the phenotype of the *pth1* mutant with respect to turgor pressure and carbon reserve mobilization. Because *pth1* appears to play a specific role in appressorium development, I also performed a comparative analysis by characterizing the *pth1* orthologue from *Neurospora crassa*.

MATERIALS AND METHODS

Strains

Wild type and mutant strains of *M. grisea* were obtained from Dr. Barbara Valent (DuPont) and stored by growing the fungus through sterile filter-paper discs, followed by desiccation and storage at –20 °C. Strains utilized in this study are showed on Table 18. Fungal cultures were grow first on PDA (potato dextrose agar + 100μg/mL of ampicilin), and transferred to oatmeal agar (50g of oatmeal per liter +100μg/mL of ampicilin and 25 μg/mL of Chloramphenicol) at 25 °C under continuous fluorescent light to promote conidiation.

Table 18. Name, origin and pathogenic phenotype of the strains used in this study.

Strain	Hyg^R	Pathogenic	Origin
4091-5-8**	S / S	+	laboratory strain; wt
CP2789	R / S	-	4091-5-8 *PTH1* ::Hyg insertion mutant
CP2791	R / S	-	4091-5-8 *PTH1* ::Hyg insertion mutant
CP2790*	R / S	+	4091-5-8 *PTH1* ::Hyg insertion, ectopically integrated
CP987***	S / S	+	laboratory strain; wt
CP2780	R / S	-	CP987 *PTH1* ::Hyg insertion mutant
CP2782	R / S	-	CP987 *PTH1* ::Hyg insertion mutant
CP 2783	R / S	+	CP987 *PTH1* ::Hyg insertion, ectopically integrated

Hygromycin resistant (R), hygromycin susceptible (S), ectopically integrated; ** Not pathogenic to rice, pathogenic to barley; *** Pathogenic to rice.

Pathogenicity assay

Three to four week old rice (cultivar M201) and barley (cultivar Bonanza) plants were spray and wound inoculated. Conidia were harvested in 0.25% gelatin solution from 10 to 12 days old *M. grisea* colonies, and the final concentration was adjusted to 3.0 x 10^5 spores/mL. Wound inoculation was performed by injecting 0.5 mL of inoculum into the leaf sheaths. Evaluation for symptoms was performed seven days after both inoculations. Leaves were evaluated under a Stereoscope (Olympus SZ 40) and the image captured with Panasonic digital camera (GP-KR 22)

Cytorrhysis experiments

Conidia of *M. grisea* were harvested from 10 to 12 day-old cultures in phosphate buffer (50 mM, and pH 6.6) and filtered once through Miracloth (Calbiochem) to remove hyphal fragments. The concentration of conidia was adjusted to 1 x 10^5 spores/mL in phosphate buffer. As a control for cells lacking the ability to generate high turgor pressure, tricyclazole (10 µg/mL) was added to inhibit melanin synthesis. Droplets (10 µL) of conidial suspension were placed on hydrophobic plastic microscope coverslips (Fisher Scientific), and incubated in a moist chamber for 16 h at 25˚C. Appressoria were submersed in a series of KCL solutions of different concentrations (0.5 M, 1.0 M, 1.5 M, 2.0 M, and 2.5 M) for three minutes. Evaluation for cell collapse and plasmolysis was performed by mounting on a glass microscope slide using a cover slips as spacers to prevent damaging cells. The number of appressorium formed and number of appressoria collapsed was quantified.

Detached leaves

Barley detached leaves were placed in a moist chamber and spray inoculated with conidial suspensions (3.0x10^5 spores/mL) of wild type, *pth1* mutant, and complemented mutant strains, and incubated at 25°C, for 96 h. Plant tissue was fixed with lactophenol for 16 h, and the leaves were cleared with two treatments with lactophenol:ethanol (1:1, vol:vol) of 2h at 95°C. Tissue was stained with 0.01% aniline blue in a mixture of ethanol, lactic acid, and phenol (1:1:1). The leaves were stained at least for 2 h at 25°C (Oh and Lee

2000). The number of appressoria that penetrated the leaf surface and initiated colonization of plant tissue was evaluated by light microscopy (Olympus BX60) after staining the leaves. Images were captured with a Q-fire digital camera (Olympus).

Cytological analysis

Conidia of *M. grisea* were harvested as described above for plasmolysis assays. Appressoria formation was induced in the presence or absence of tricyclazole. Droplets (10µL) of conidial suspension were placed on hydrophobic plastic microscope coverslips (Fisher Scientific), and incubated in a moist chamber for 4, 8, 12, or 24 h, at 25˚C. The occurrence of lipid in vacuoles in cells of freshly harvested conidia, germinating conidia, and appressoria was visualized by staining with Nile Red solution (Thines et al. 2000). Nile Red staining solution consisted of 50 mM Tris/maleate buffer, pH7.5, with 20 mg/mL polyvinylpyrrolidone and 2.5 µg/ml Nile Red Oxane (Sigma). Lipid droplets fluoresced when exposed to UV light and images were captured on the microscope (Olympus BX 60) with a Q-Fire digital camera as described above. Glycogen staining solution consisted of 60 mg/ml of KI and 10 mg/mL of I_2 in distilled water. Yellowish-brown glycogen deposits were visible in bright field optics of microscope as described above.

Statistical methods

The means of % of appressoria collapsed of three replications, containing 2 droplets of each treatment, for each concentration of KCl (3 M, 1.5 M, 1.0 M, 0.5

M) were analyzed (SPSS 11.0) by applying univariate analysis, and the means were compared with Tukey's method (α = 0.05).

RESULTS

Pathogenicity of *pth1* mutants

Rice plants were spray inoculated with the wild type, CP987, *pth1* mutants CP2780, CP2782, and ectopic transformant CP2783 (Table 19). Only the wild type and ectopic transformant were able to infect rice plants, producing typical disease symptoms 7 days after of inoculation. The *pth1* mutants CP2780 and CP2782 did not infected rice plants after spray inoculation. The same isolates were wound inoculated, and all of them were able to growth and colonize inside the rice plants tissue (Table 19), producing typical disease symptoms.

Barley plants, cv. Bonanza, were spray inoculated with the wild type 4091-5-8, *pth1* mutants CP2789, CP2790, and ectopic transformant CP2791 (Table 19). Only the wild type and ectopic transformant were able to infect barley plants, producing typical lesions 7 days after of inoculation. The *pth1* mutants CP2789 and CP2790 were not able to infect barley plants after spray inoculation. Seven days after wound inoculation with either wild type or ectopic transformants, barley plants presented typical disease symptoms (Table 19 and Fig. 22).

Table 19. Qualitative evaluation of rice and barley plants after 7 days of spray and wound inoculation with wild type and *pth1* mutants of *M. grisea.*

Isolate	Plant[1]	Rice[2]		Barley[2]	
		Spray	Wound	Spray	Wound
4091	Pl-1	R	HR (local)[3]	S	S
(Wild type)	Pl-2	R	HR (local)	S	S
	Pl-3	R	HR (local)	S	S
	Pl-4	R	HR (local)	S	S
	Pl-5	R	-		S
CP 2789	Pl-1	R	HR (local)	R	S
(*pth1* mutants)	Pl-2	R	HR (local)	R	S
	Pl-3	R	HR (local)	R	S
	Pl-4	R	HR (local)	R	S
	Pl-5	R	HR (local)	R	S
CP 2790	Pl-1	R	HR (local)	S	S
(ectopic)	Pl-2	R	HR (local)	S	S
	Pl-3	R	HR (local)	S	S
	Pl-4	R	HR (local)	S	S
	Pl-5	R	HR (local)	S	S
CP 2791	Pl-1	R	HR (local)	R	S
(*pth1* mutants)	Pl-2	R	HR (local)	R	S
	Pl-3	R	HR (local)	R	S
	Pl-4	R	HR (local)	R	R
	Pl-5	R	HR (local)	R	S
CP 987	Pl-1	S	S	S	S
(Wild type)	Pl-2	S	S	S	S
	Pl-3	S	S	S	-
	Pl-4	S	S	S	-
	Pl-5	S	S	S	-
CP 2780	Pl-1	R	S	R	S
(*pth1* mutants)	Pl-2	R	S	R	S
	Pl-3	R	S	R	S
	Pl-4	R	S	R	-
	Pl-5	R	-	R	-
CP 2782	Pl-1	R	S(local)	R	S
(*pth1* mutants)	Pl-2	R	S(local)	R	S
	Pl-3	R	S(local)	R	S
	Pl-4	R	S(local)	R	S
	Pl-5	R	S(local)	R	S
CP 2783	Pl-1	S	S	S	S
(ectopic)	Pl-2	S	S	S	S
	Pl-3	S	S	S	S
	Pl-4	S	S	S	-
	Pl-5	S	S	S	-
Control(H_2O)[4]	Clean	Clean	Clean	Clean	Clean

1-Plants were evaluated separately; 2-Reaction of each plant, R = resistant reaction, HR = hypersensitive reaction, S = susceptible reaction; 3-Local, the symptoms were observed only at the wound inoculation site; 4-Control: plants spray and wound inoculated with ddH_2O.

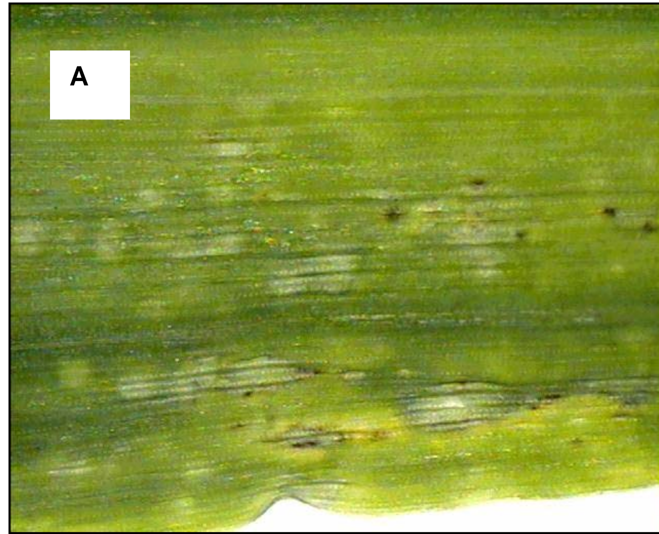


Figure 22. Disease symptoms four days after inoculation with pth1 mutant (A) and wild type (B).

Detached leaf assay

Detached barley leaves sprayed with a suspension of conidia of 4091-5-8 presented typical disease symptoms 4 days after inoculation (Fig. 23 A). After fixing the leaves in lactophenol and staining in aniline blue, growing infectious hyphae were visible underneath the cuticular layer, emerging from the appressorium on the leaf surface (Fig. 23 B). Leaves spray inoculated with the *pth1* mutant, CP2789 did not infect barley leaves (Fig. 23 C). The stained barley leaves contained no visible infectous hyphae emerging from the appressoria. Appressoria from the *pth1* mutant were apparently unable to penetrate the barley cuticilar layer (Fig. 23 D).

Cytorrhysis experiments

As was observed with the wild type strains 4091-5-8 and CP987, the ectopic transformants CP2790 and CP2783 formed typical melanized appressoria (Fig. 24A). *pth1* mutants CP2789, CP2791, CP2780, and CP2782 produced melanized appressoria, however, a significant fraction of these (10 - 30%) appeared to be deformed (Fig. 24B). This observation raised the question of whether the defect in appressorium function was a consequence of this morphological defect or if a defect in turgor pressure was also present. To test this idea, I measured appressorial resistance to osmotic pressure. High extracellular solute concentrations can cause water to be withdrawn from appressoria, causing the cells to collapse. This technique was used to show that appressoria can withstand a high concentration of extracellular solute without plasmolysis and that when

solute concentration is higher than intracellular solute concentrations the appressorium collapses (Howard et al. 1991). I observed that all strains exposed to 3 M KCl solution had a high percentage of collapsed appressoria (Fig. 25). The average percentage of appressoria collapsed at different KCl concentrations is shown in Fig. 26. At 3 M and 1.5 M KCl, appressoria of both wild type and pth1 mutants collapse at high frequency (Fig. 26 A, B). There is a statistically significant difference among the strains in their resistance to KCl observed in 1.0 M KCl solution (Fig. 26C). The fact that the difference between the strains was more apparent at this concentration indicates that *pth1* mutants have an intracellular solute concentration close to 1 M, whereas wild type cells have a higher intracellular solute level and so are more resistant to collapse. The *pth1* mutants CP2782 (97%) and CP2780 (81%), CP2789 (79%), CP2791 (75%) were the most sensitive to KCl, followed by ectopic transformants CP2783 (51%) and CP2791 (48%), and the wild types CP987 (48%), and 4091-5-8 (30%). The same tendency was observed when appressoria were exposed to 0.5 M KCl. *pth1* mutants had a significantly higher fraction of collapsed appressoria compared to the wild type and ectopic transformants (Fig. 26D). At 0.5 M KCl. The CP2782 and CP2780 strains were clearly more sensitive than the CP2791 and CP2789 strains. This suggests that in the 4091-5-8 strain background the pth1 mutation leads to a turgor pressure that can withstand 0.5 M KCl, whereas in the CP987 strain background the appressorial turgor pressure is lower.

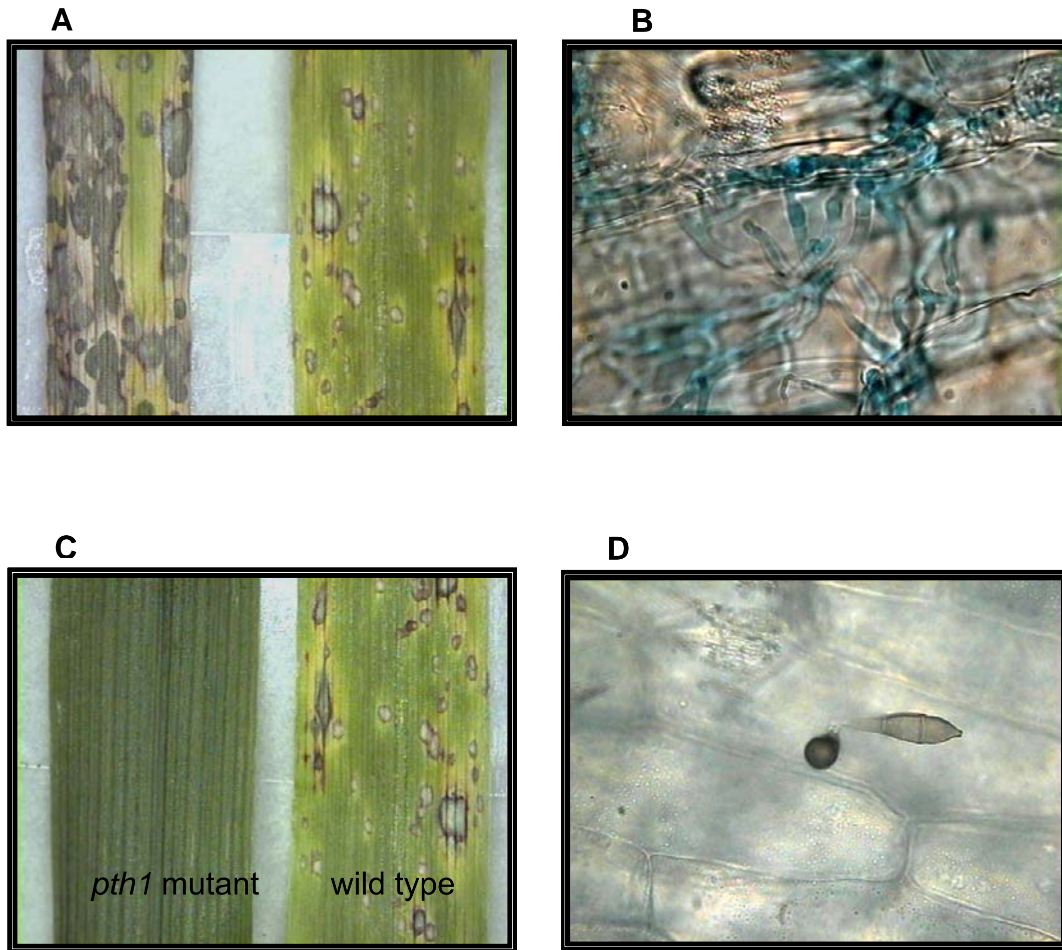
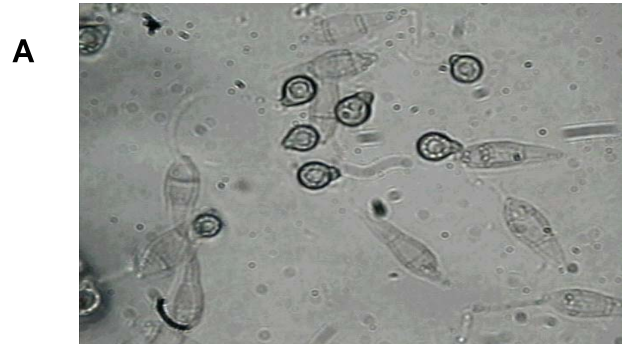


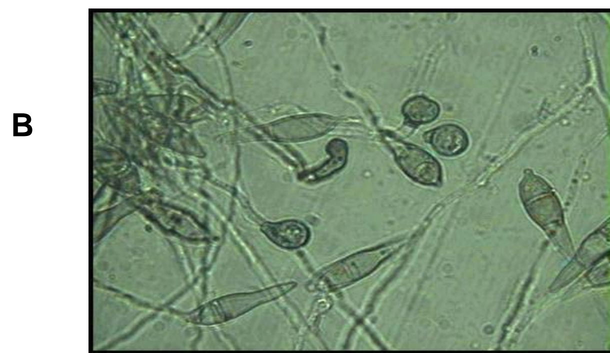
Figure 23. Wild type (4091) 96 h after inoculation on barley leaves (A,B), CP 2789 (*pth1* mutant) 96 h after inoculation on barley leaves (C,D).

A



Wild type normal appressoria

B



Deformed appresorria formed from *pth1* mutant

Figure 24. Wild type (4091) normal shaped appressoria (A), and pth1 mutant defective formed appressoria (B).

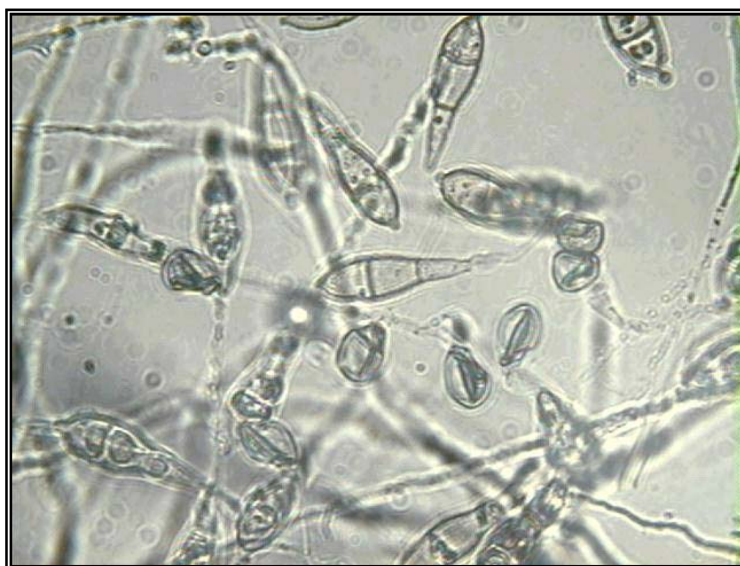


Figure 25. *pth1* mutant collapsed appressoria after treatment with KCL solution.

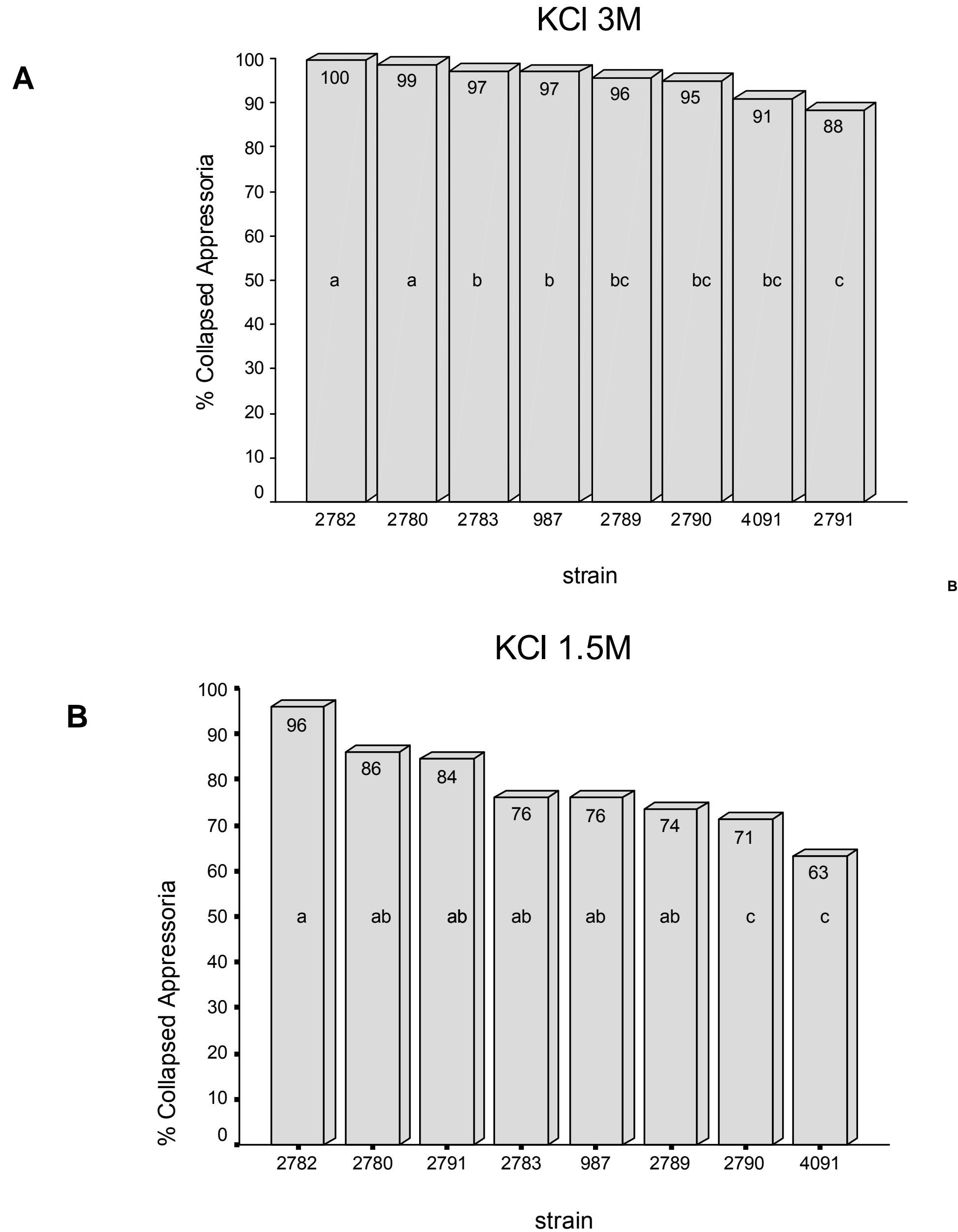
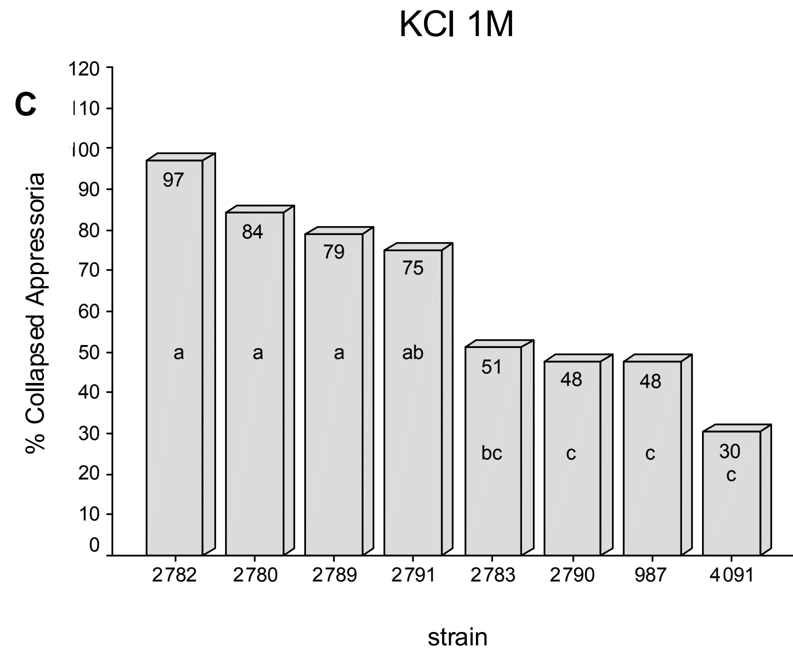


Figure 26. Means of collapsed appressoria in wild types, 4091 and CP 987, ectopic transformants 2790 and 2783, and pth1 mutants 2782, 2780, 2791, and 2789, at KCl solutions 3M (A), 1,5M (B), 1,0 M (C), and 0,5 M (D).



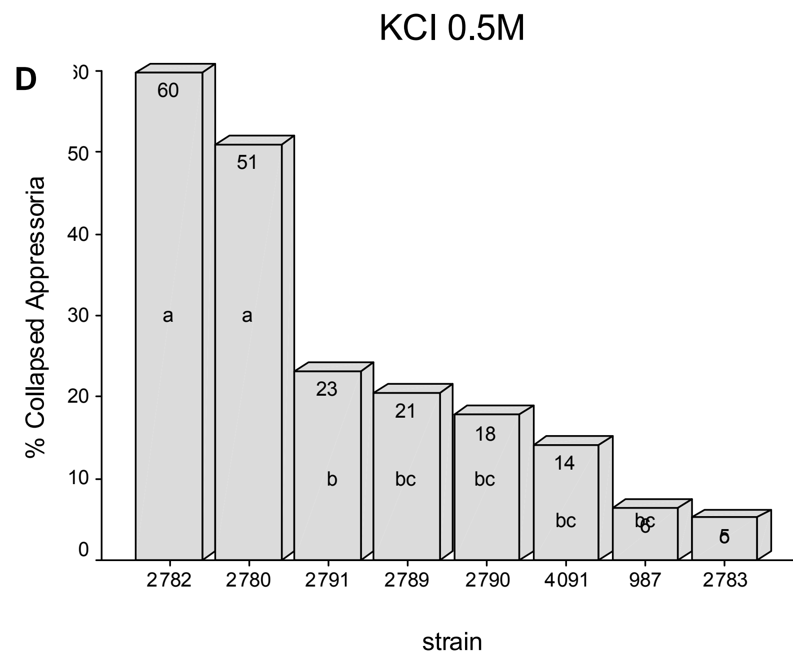


Figure 26. Contnued

Cytological analysis

The amount of glycogen and lipid present in conidia of wild type and *pth1* mutants can be qualitatively assessed by staining with glycogen and lipid specific dyes. Conidia of 4091-5-8 and the *pth1* mutant CP2789, show clear differences in the amounts of glycogen and lipid present in conidia (Fig. 27). The *pth1* mutant conidia have less intense glycogen staining and lipid abundance appears to be so low that fluorescence was not detected in some of the conidia.

Since I could detect a difference in the amount of these storage compounds in conidia I followed the movement of glycogen and lipid during appressorium development. Germination of *pth1* mutant was delayed relative to the wild type. At four hours after germination the wild type cells progressed to the stage of hook cell formation (Fig. 28A) and some immature appressoria were apparent. Observation of glycogen staining of germinated conidia with hook cells revealed that most of the glycogen was localized to the hook cell or nascent appressorium with lower amounts of glycogen in the germ tube (Fig. 28C). Little glycogen was observed remaining in conidia. Lipid was concentrated in the developing appressoria (Fig. 28E). The *pth1* mutant did not form hook cells four hours after germination and the glycogen was still most abundant in the conidia. However, some lipid staining was observed at hyphal tips (Fig. 28F). I conclude that some glycogen and lipids mobilization does occur in the *pth1* mutants however, the vast majority of glycogen has not been mobilized from the conidia of *pth1* mutants. One explanation for this

observation is that the reduced mobilization of glycogen simply reflects the delay in germination and appressorium development observed in the mutant.

Wild type appressoria are formed 8 hours after germination (Fig. 29A); the conidia stain lightly for glycogen whereas the appressoria are heavily stained (Fig. 29C). Intense lipid staining indicated the accumulation of lipids in the appressoria (Fig. 29E). In contrast, the *pth1* mutant showed a very small amount of glycogen and lipid staining in conidia and along the germ tubes.

At 12 hours after germination, both wild type and *pth1* mutant completed appressorium development (Fig. 30). Most of the glycogen and lipid staining in both wild type and *pth1* mutant was observed in the appressorium. At 24 hours after germination (Fig. 31), the amount of glycogen and lipid in appressoria appeared to be similar in both strains (Fig. 27C, 27D). However, *pth1* mutant appressoria appeared to have a more variable level of staining.

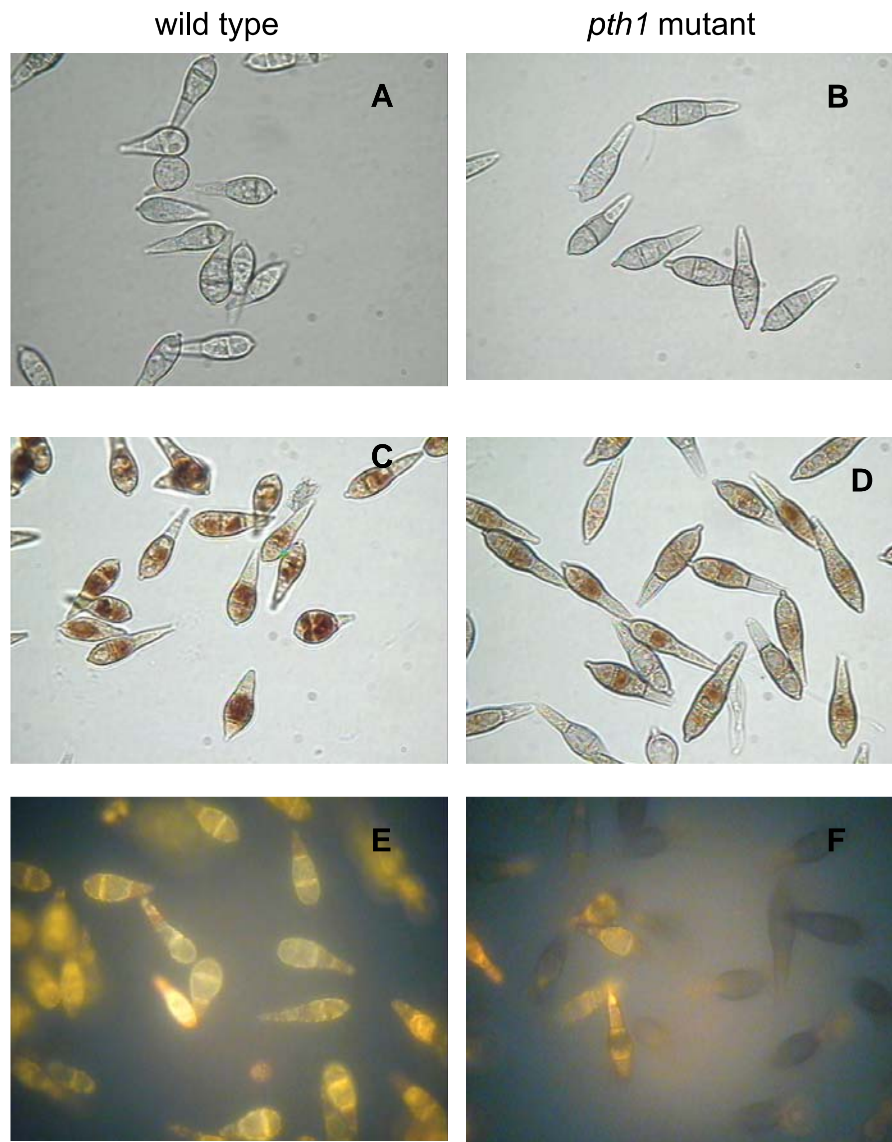


Figure 27. Control (A, B), glycogen (C, D), and lipid (E, F) stain during appressorium formation at 0 hours after induction in the strains 4091-5-8 (wild type) and CP2789 (*pth1* mutant).

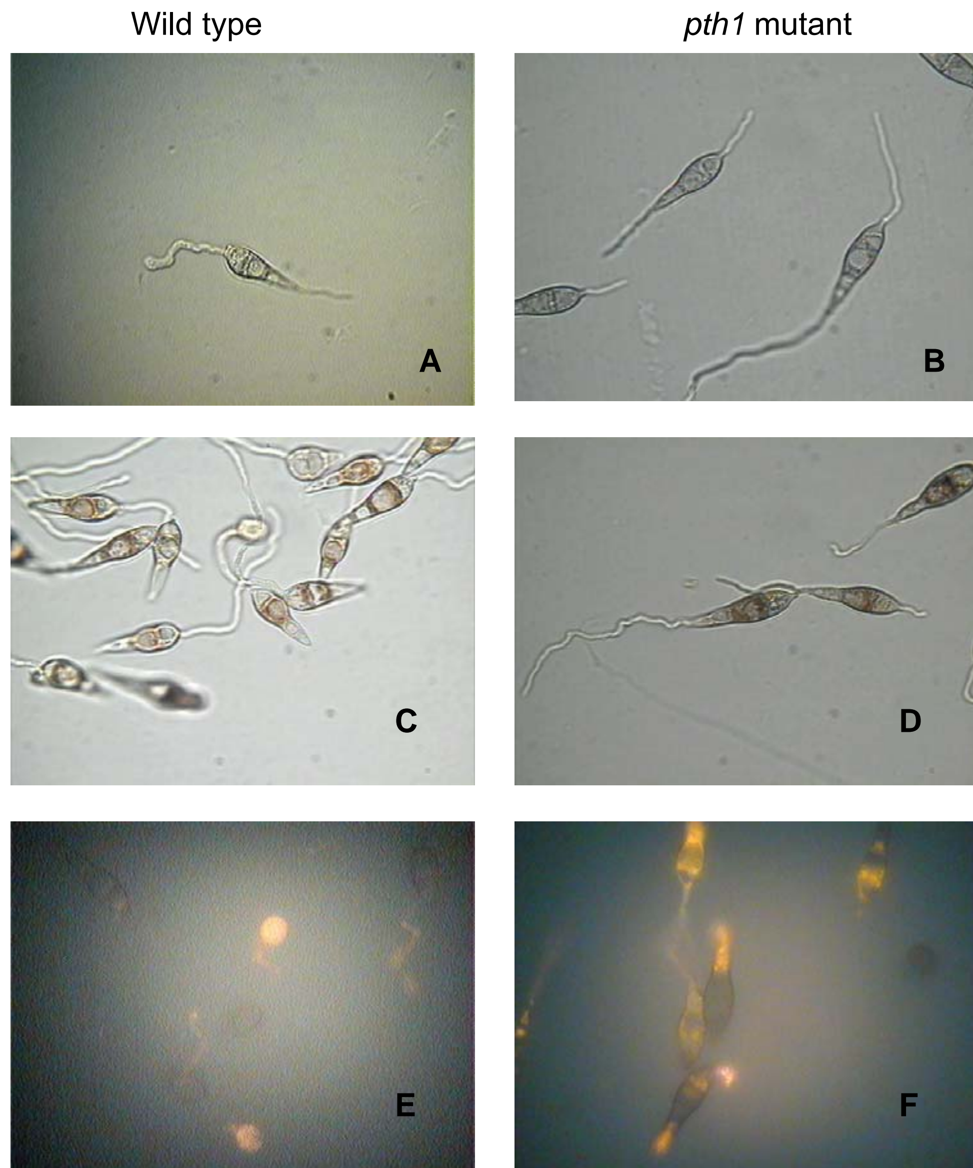
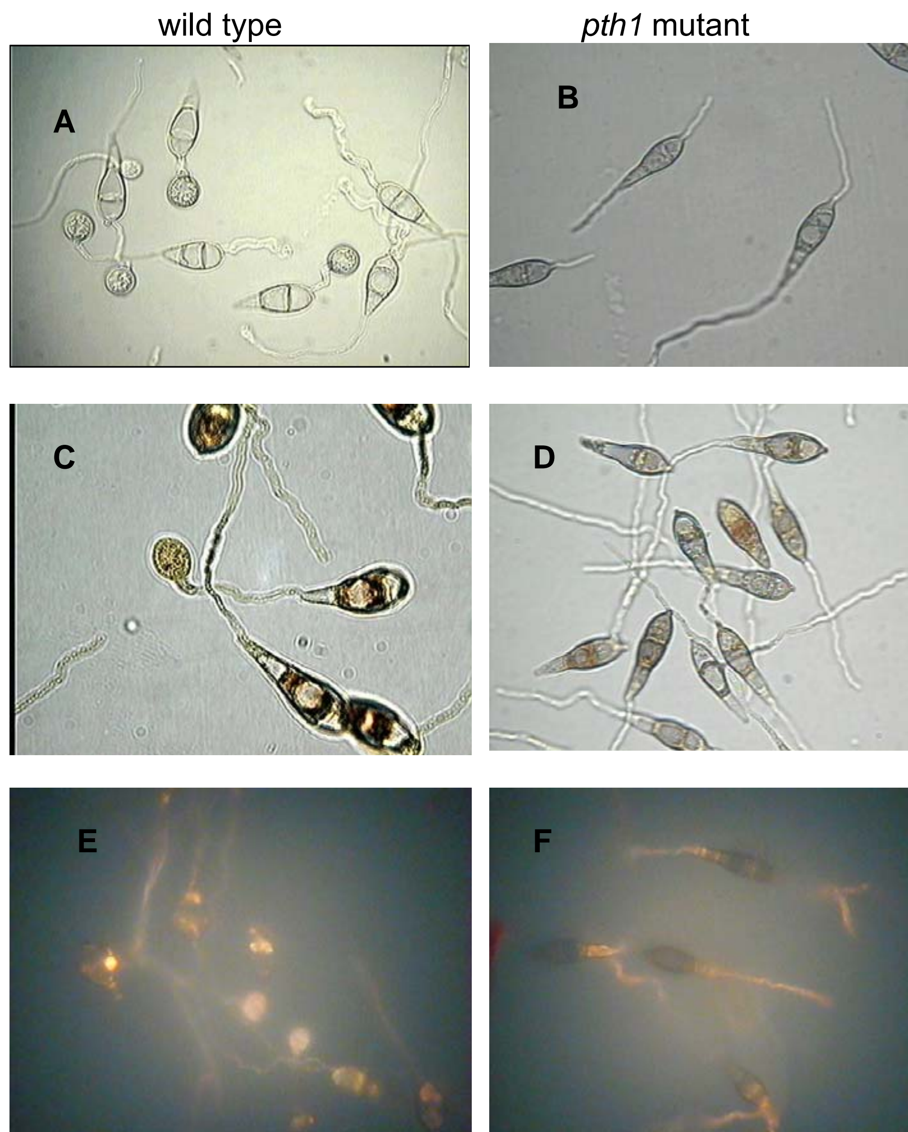


Figure 28. Control (A, B), glycogen (C, D), and lipid (E, F) stain during appressorium formation at 4 hours after induction in the strains 4091-5-8 (wild type) and CP2789 (*pth1* mutant).



A

Figure 29. Control (A, B), glycogen (C, D), and lipid (E, F) stain during appressorium formation at 8 hours after induction in the strains 4091-5-8 (wild type) and CP2789 (*pth1* mutant).

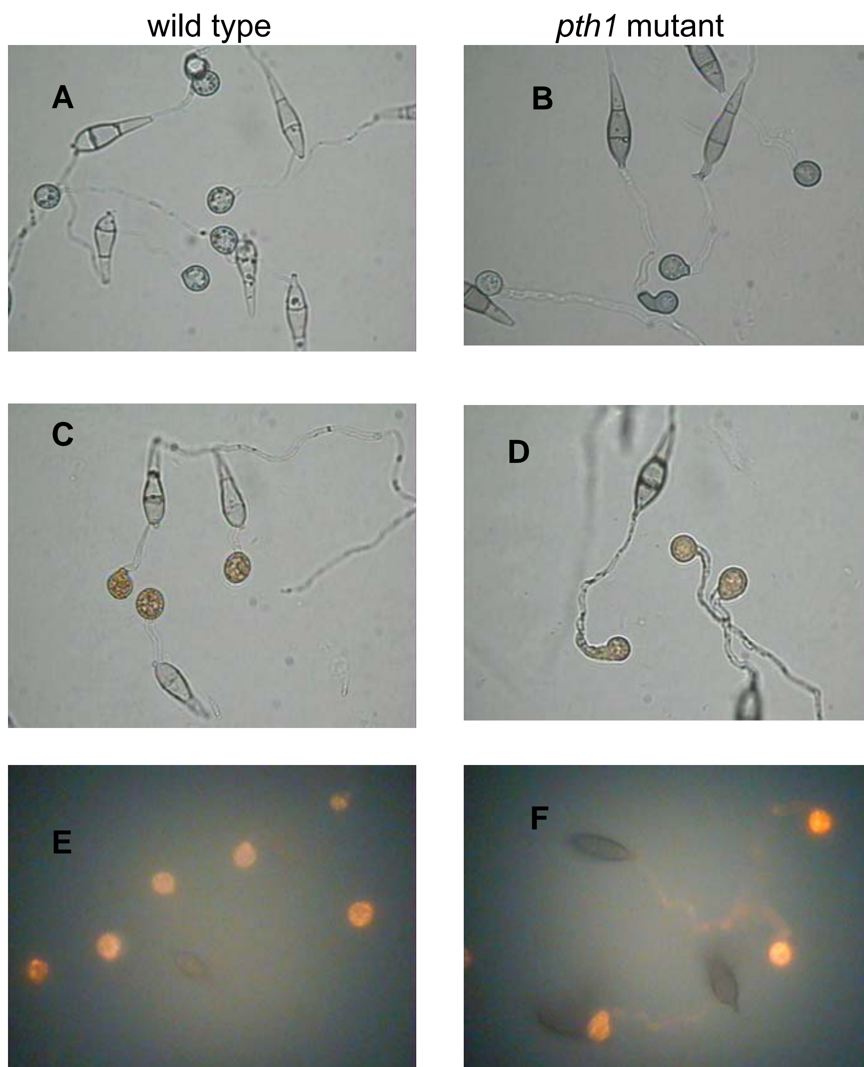


Figure 30. Control (A, B), glycogen (C, D), and lipid (E, F) stain during appressorium formation at 12 hours after induction in the strains 4091-5-8 (wild type) and CP2789 (*pth1* mutant).

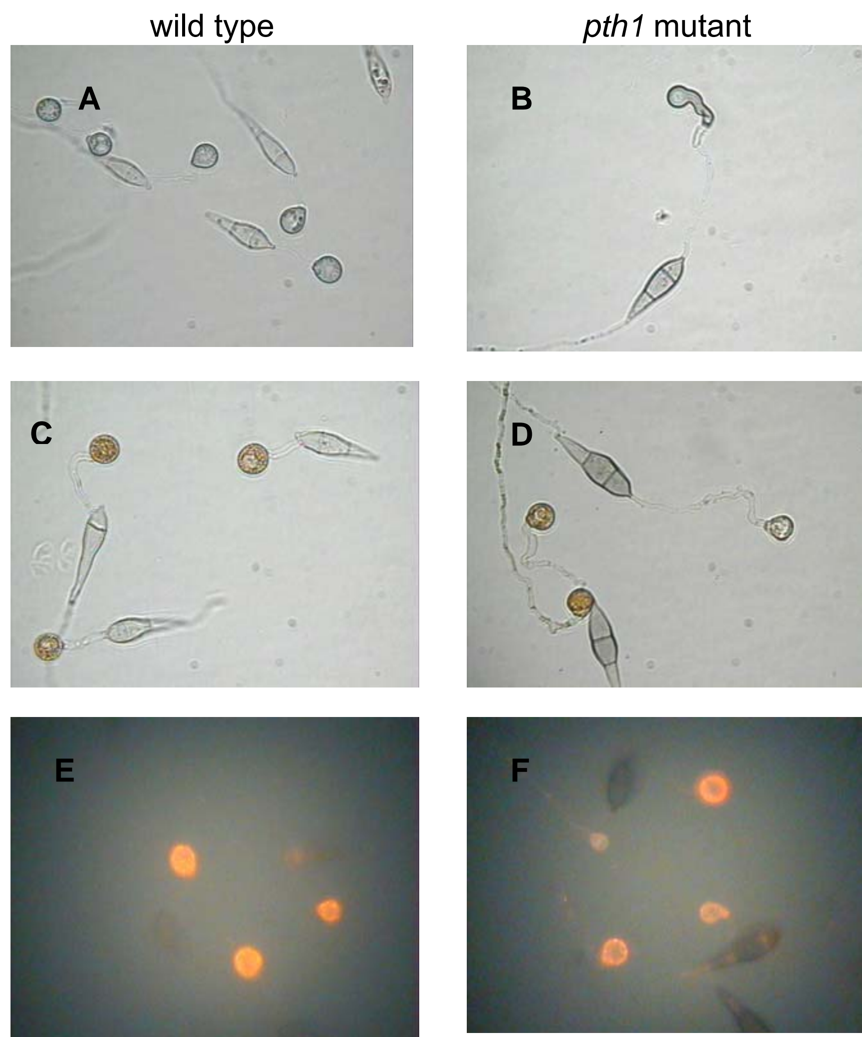


Figure 31. Control (A, B), glycogen (C, D), and lipid (E, F) stain during appressorium formation at 24 hours after induction in the strains 4091-5-8 (wild type) and CP2789 (*pth1* mutant).

The *Neurospora crassa pth1* ortholog complements the pathogenicity defect of *pth1* mutants

The genome sequence of *N. crassa* is available (Galagan et al. 2003) and a clear ortholog of *PTH1* is present (NCU 1216.1). There is no *M. grisea* ORF given until now. This gene was amplified and used to identify a cosmid clone containing the *N. crassa* gene (*nfb1*). The gene was cloned into pCB10004 (Carroll et al. 1994) and transformed into the *pth1* mutant strain CP2789 to produce strain CP1302. Construction of strain CP1302 was performed by Dr. Carlos Cortes. As expected, the *pth1* mutant transformed with the *N. crassa* orthologue, produced typical disease symptoms after wound inoculation (Fig. 32). CP1302 infected barley leaves after spray inoculation producing disease symptoms (Fig. 32). Stained leaves evaluated under light microscopy showed infectious hyphae emerging from appressoria over the cuticular layer (Fig. 32). Thus, the defect in appressorium development could be complemented by the homologous gene from a non-pathogenic fungus that does not produce appressoria.

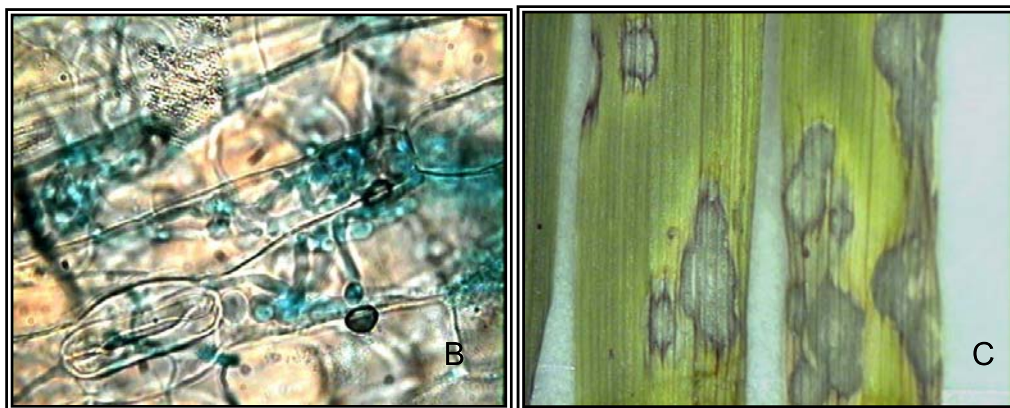


Figure 32. Symptoms of *M. grisea* infection after inoculation with complemented mutant CP1302. A-Barley leaves infected after wound inoculation. B and C-Barley leaves after spray inoculated with complemented mutant CP1302.

DISCUSSION

My results suggest that the *pth1* mutant forms defective appressoria, unable to generate enough turgor pressure to penetrate host tissue. The results obtained in pathogenicity assays indicated that *pth1* mutants are able to colonize host tissue and develop infectious hypha when inoculated into wound sites. Ubiquitin ligases target proteins for degradation. The ubiquitin ligase derives its target specificity from the LRR sequence motifs.

Conidia of the *pth1* mutants were found to accumulate less lipid and glycogen compared with conidia of the wild type. Conidial germination of *pth1* mutants is delayed about 4 hours, possibly because the process of carbon source mobilization requires the function of Pth1 acting as an E3 ubiquitin ligase. Responsibility for targeting substrate proteins to be ubiquitinated lies with E2 and E3 enzymes. *M. grisea* appears to contain three E3 ubiquitin ligases, each of which likely interacts with a different subset of substrate proteins. Also there are multiple genes encoding E2 components of the ubiquitin ligase (Stryer 1981). E2 components have been shown to be able to target proteins for degradation independently of E3. I propose a model in which *pth1* mutants lack the ability to target a subset of proteins for degradation and that proteins that interact with Pth1 define a subset of proteins that must be degraded to allow maturation of the appressorium.

This model proposes a new concept for understanding appressorium development. All previous studies have focused on signal transduction and genes that are induced in order to produce an appressorium. The work that I have done indicates that some proteins that are present in germinating conidia must be degraded in order to allow appressorium maturation to occur.

Turgor pressure generation is required for appressorium function carbon source mobilization is required for production of the glycerol needed to increase the intracellular osmotic potential. Consistent with the view that carbon mobilization from conidia is slower in the Pth1 mutant than in the wild type, I measured the amount of glycogen and lipids mobilized in developing appressoria and found that this is slower and possibly less than in the wild type. The morphological process of forming the hook cell is also delayed. However, once appressoria form, mobilization appeared to quickly "catch up" to the wild type. At 12 and 24 h, the amount of glycogen and lipids accumulated in *pth1* mutants and wild type appeared to be very similar, however, *pth1* mutants have a high number of deformed melanized appressoria, suggesting additional defects in *pth1* appressoria. It seems that by 24 h after induction, the amount of glycogen and lipids are almost the same in both strains, however, this does not lead to turgor pressure equal to the wild type.

Despite the finding that the amount of glycogen and lipid present after 12 to 24 h seems to be similar to the wild type, *pth1* mutants were found to be more sensitive to osmotic pressure. Sixteen hour-old *pth1* mutant appressoria collapsed

in KCl concentrations that had only mild effects on the the wild type. This demonstration is most evident when appressoria were exposed to 1.0 M KCl. If *pth1* mutants are 4 h delayed relative to wild type one might expect that even if turgor pressure is lower at 16 h, *pth1* appressoria might build up turgor pressure later than wild type, but achieve equivalent turgor pressure at the later time. However, the low efficiency of infection suggests that turgor pressure never reaches a level sufficient to allow host penetration.

The *N. crassa* orthologue, *nfb1,* complemented the *pth1* mutant. The complemented strain CP1302 recovered the ability to form normal appressoria, and penetrate the leaf surface for further colonization of the tissue. This suggests that the biochemical function of *pth1* has not evolved specifically to play a role in appressoria development.This points out the principle that pathogenic fungi may adapt functions used for non-pathogenic processes for roles in pathogenesis.

CHAPTER V

SUMMARY

Magnaporthe grisea, a heterothallic ascomycete, causes rice blast, a serious disease on rice plants, which causes losses all around the world. The degree of severity of rice blast, can cause yield losses up to 100% depending on cultivar susceptibility, environmental conditions and management system.

Rice blast is estimated to causes losses of an average of U$55 million/year in Asia. According to 1991 Rockefeller Foundation records, areas of South Asia infected with rice blast suffer losses of 8.8 kg/ha, corresponding to US $40.9 million/year. Fungicides for blast typically consume 6-50% of total crop protection costs. The Japanese market alone for blast fungicides is estimated to be US$400 million/year, only sustained by the high agriculture subsidies (70%) in this country, the highest in the world. A specific example is the cost of Azoxystrobin, a non specific blast fungicide. When used as recommended for blast control, it costs upwards of US$25-30.00/acres/application ((http://www.grain.org/docs/blast.pdf) Appendix B).

Fungicides and breeding have both been extensively applied to avoid rice blast, preparing the perfect scenario for epidemic to occur; combining chemical treatment, genetic uniformity, and high technology in irrigated land. Genetic engineering offers powerful tools to help with this problem. However, additional research is necessary to develop anti-blast agents with new and specific modes of action and with environmental safety in mind (Froyd and Foeliger 1994).

The signalling pathway controlling the mating process in *Saccharomyces cerevisiae* has many features in common with molecular mechanisms of other metabolic pathways in eukaryotic cells, including phytopathogenic fungi (Lengeler et al. 2000).

The pheromone response in yeast *S. cerevisiae* (Fig. 33), and the environmental and chemical response on *M. grisea* leading to appressorium formation (Fig 34), share the same pathways based on cAMP and MAP Kinase. These pathways have been compared in the two organisms and others, *Candida albicans* and *Cryptococcus neoformans*, and in the plant pathogens *Ustilago maydis*, *M. grisea, Cryphonectria parasitica*, and in the model filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* (Lengeler et al. 2000). In every case, these pathways play important roles in growth and development.

Many plant pathogenic fungi differentiate a highly specialized infection structure, called an appressorium, allowing fungi to penetrate the host and derive nutrients and energy from the host cells. The process of forming the appressorium is considered a well adapted strategy based on the fact that fungi from different taxa are able to form appressoria (Deising et al. 2000).

In *S. cerevisiae,* the whole mating process is well understood in terms of genetics and biochemistry. *S. cerevisiae* has two mating types, **a** and α. Each mating type produces a specific pheromone (**a** and α factor, respectively) and, on its plasma membrane, bears a receptor for the pheromone of the opposite

mating type. The binding of a pheromone to the corresponding receptor on a partner cell triggers a sequence of cellular events by means of the G-protein coupled receptor, which in turn leads to activation of the transcription of genes involved in the production of the pheromone itself, pheromone receptor and the proteins of the signaling pathway, ultimately resulting in morphological changes. The inhibition of appressorium formation by the α-factor pheromone of yeast (Beckerman et al. 1997), supported the concept of screening synthetic combinatorial libraries (Reed et al. 1997) to identify novel peptides that inhibit appressorium formation, thus providing a powerful tool to study the signaling pathway of appressorium formation.

The main objective of this work was to identify a bioactive hexapeptide, screened from a combinatorial library that is capable of blocking appressorium formation on an artificial inductive surface. The specific objectives were to identify chemicals able to overcome the inhibitory effect of the hexapeptide; test the effect of the hexapeptide on mutants for appressoria formation with known genetic lesions; study the effect of the hexapeptide on the signalling pathway for appressorium formation; quantify the activity of protein kinase A; and analyse the role of *Pth1* in appressorium formation and maturation.

We identified D-CYRFTW, a hexapeptide that is able to block appressorium formation in artificial hydrophobic surface at low reasonable concentrations. The peptide L-WTFRYC also blocked appressorium formation.

D-CYRFTW projected the same blocking effect when tested against field isolates.

The effect of both forms, D-CYRFTW and L-WTFRYC can be blocked by the chemicals cAMP and 1, 16-hexadecanediol suggesting that the interaction of the hexapeptides and fungal cells is very specific, and they are able to prevent activation of cAMP signaling in response to surface cues.

Among the mutants for appressorium formation, DA-99 (suppressor of Adenylate cyclase mutant) was shown to be resistant to the blocking effect of D-CYRFTW and L-WTFRYC. $MagB^{G42}$ (G protein dominant activated) proved to be susceptible to the blocking effect of D-CYRFTW. The results of these studies indicate that the peptide functions upstream of adenylate cyclase and potentially upstream of, or at the level of the G-alpha subunit preventing activation of cAMP signaling in response to surface cues.

Among the combinatorial libraries tested during these studies, some displayed an inductive effect for appressorium formation on hydrophilic surface. This suggests that peptides are interfering with the recognition of the surface, or interacting with the receptor, which can not recognize the non inductive surface signal. Preliminary studies of total PKA activity revealed changes in PKA activity during the time course of appressorium development.

M. grisea pth1 mutants are unable to penetrate host tissue and to establish a successful host-parasite interaction; However, *pth1* mutants are capable of colonizing plant tissue following wound inoculation. *pth1* conidia have a germination lag that results in a delay in appressorium formation of about 4 hours. This may be related to the finding that *pth1* mutant conidia have reduced amounts of glycogen and lipids. However, accumulation of glycogen and lipid in appressoria appears similar to wild type after about 12-16 hours after germinaton. Appressoria turgor pressure is reduced in the *pth1* mutants and a fraction of *pth1* appressoria are deformed. Since the orthologous gene of *N. crassa* is capable of complementing the pathogenesis defect of *pth1* mutants the biochemical function of *pth1* has not evolved specifically to play a role in appressorium development; this is consistent with the view that *Pth1* is part of E3 ubiquitin ligase, required to control the levels of a variety of cellular proteins.

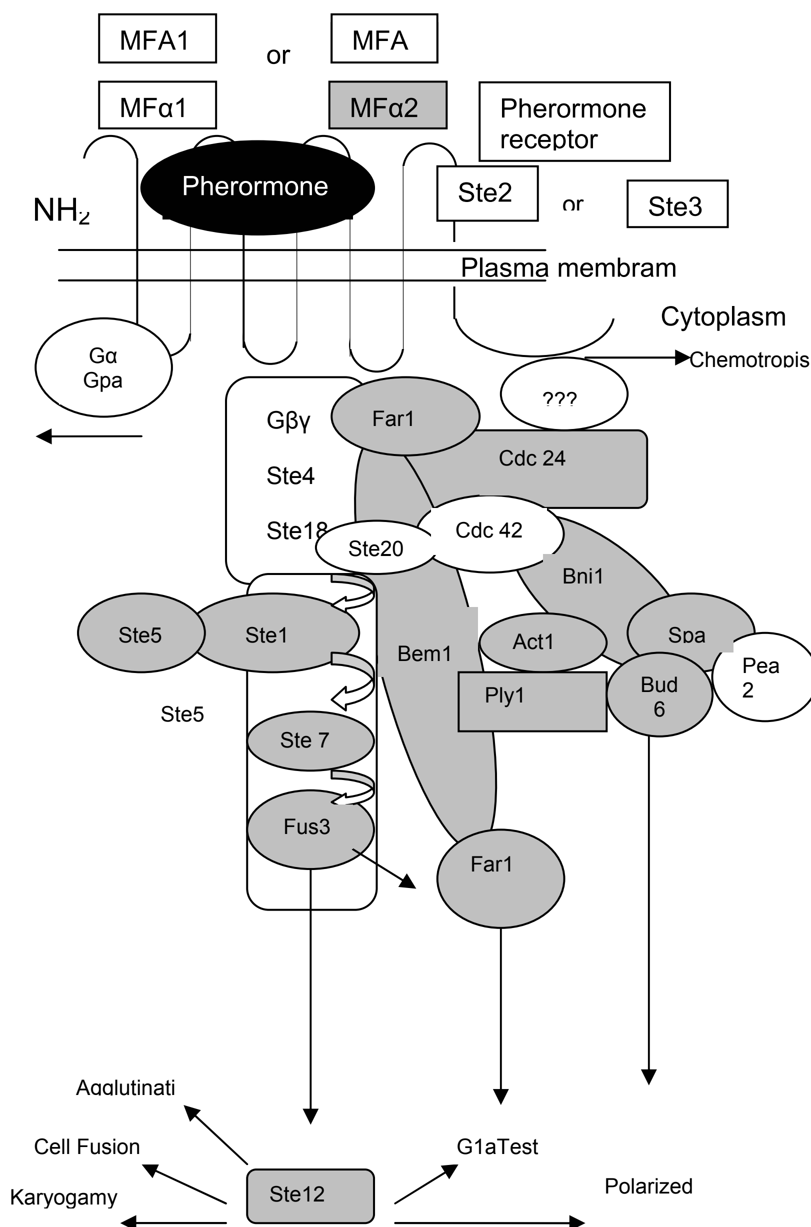


Figure 33. Signaling pathway for pheromone response in *S. cerevisiae*. The G protein alpha-subunit is Gpa1p. The G protein beta subunit is Ste4p. Together with Ste18p, the gamma subunit, it makes up the positive signalling element of the G protein; mutants in either beta or gamma lead to sterility; The Ste20p kinase serves as a link between the G protein and the kinases of the pheromone response MAP kinase cascade. In addition, Ste20p functions in the pseudohyphal growth pathway; Ste5p apparently serves as a scaffold for the kinases of the MAP kinase cascade (Ste11p, Ste7p, and Fus3p/Kss1p). IThe amino terminus of Ste5p associates with the G protein beta-gamma subunits. Ste12 encodes a transcription factor that governs the expression of many genes in the pherormone response pathway (Lengeler et al. 2000).

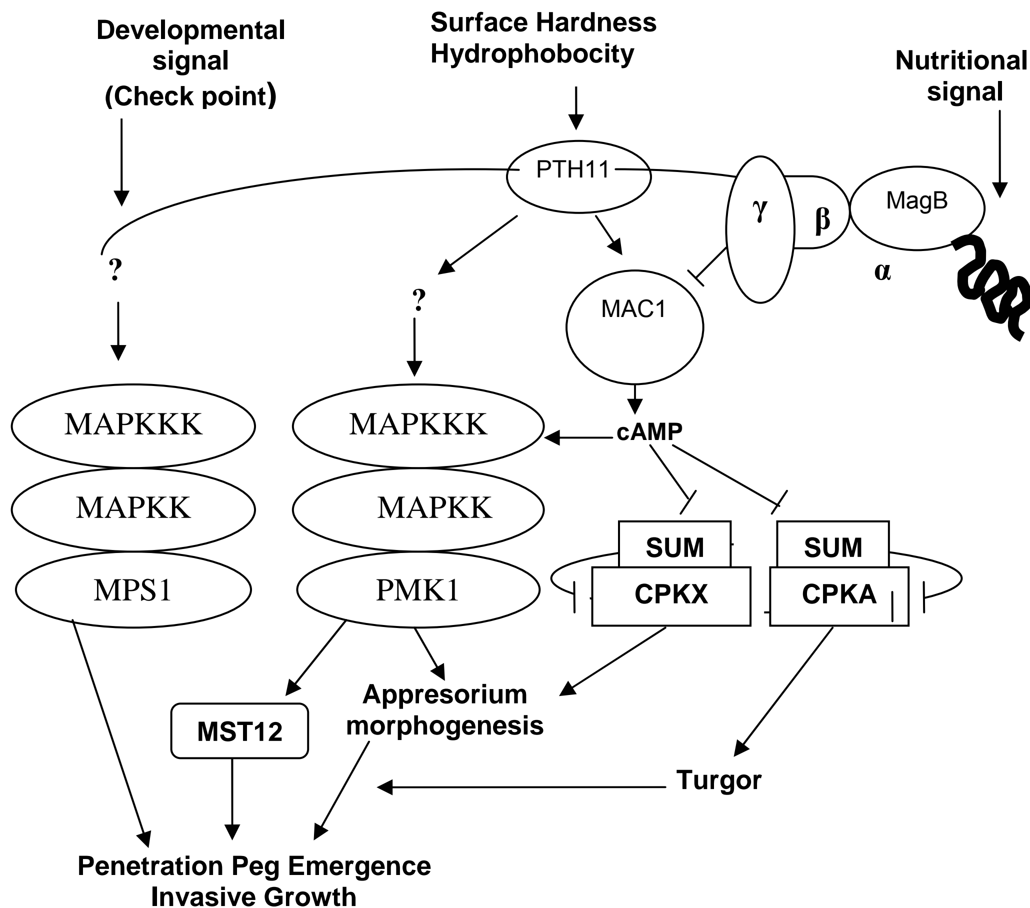


Figure 34. Model for signaling pathway for appressorium formation in *M. grisea*. The hydrophobicity and chemical signals of the surface may be sensed by a transmembrane receptor. The receptor is proposed to stimulate the heterotrimeric G protein containing the MagB α subunit and MGB1, ? subunit. MagB is thought to activate adenylyl cyclase to control the cellular levels of the second messenger cAMP. cAMP-dependent protein kinase, CpkA appears to be required for appressorium maturation, however, additional catalytic subunits exist that are required for induction of appressorium development. Downstream of the induction step is activation of the Pmk1, MAP kinase cascade, which phosphorylates MST12, a transcription factor homologous to STE12 (Talbot 2003b) (Nishimura et al. 2004).

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APPENDIX A

ROME, 17 July 2002 -- "There is an increasing concern about the current rice production practices meeting demands, contributing effectively to rural poverty alleviation and minimizing environmental degradation," the UN Food and Agriculture Organization (FAO) warns in advance of the 20th session of the International Rice Commission (IRC) to be held in Bangkok, Thailand, 23-26 July 2002.

Productivity of rice is now increasing at a slower rate than during the height of the Green Revolution," FAO expert Dat Tran says. "Yield stagnation in many Asian countries, limited possibilities for arable land expansion, and fewer water resources for expanding rice planted areas are the main constraints to expanding production. Other concerns are related to environmental degradation, genetic erosion and nutritional quality of rice," Mr. Tran adds

Rice yield growth rate decelerated from 2.3 percent per year during the 1980s to 1.1 per cent per year during the 1990s (or approximately equal to population growth) due to the difficulty of sustaining the growth of rice productivity as yield has advanced, according to FAO.

In 2001, world production of milled rice reached 397.2 million tonnes as compared to 381.1 million tonnes in 1996. Milled rice represents 67 per cent of paddy rice (i.e. 592.8 million tonnes in 2001 and 568.5 million tonnes in 1996). However, a considerable quantity of rice will be required to meet future needs. In 2030, global demand is projected to be approximately 533 million tonnes of milled rice, as compared to 472 million tonnes projected for 2015 and 386 million tonnes in 1997/99. In 2030, the world population is expected to reach 8.2 billion as against 6.2 billion today.

Rice is the world's most important staple food crop. More than four-fifths of the world's rice is produced and consumed by small-scale farmers in low-income and developing countries. More than half of the world's population relies on rice as the major daily source of calories and protein. The amount of rice consumed by each of these people ranges from 100 to 240 kg per year, according to FAO.

In recent years, the world's rice production has especially suffered from the lack of investment in irrigation development and research work. This has slowed down the adoption of existing high yielding varieties (hybrid rice, for instance) and improved crop management techniques.

Genetic uniformity of modern rice varieties may render the crop more vulnerable to outbreaks of pests and diseases. "The erosion of genetic diversity due to the adoption of few improved varieties may limit the success of rice varietal improvement for higher yield, quality and resistance," FAO experts say

he current approaches to intensification of rice production have caused considerable damage to the environment and related natural resources, including the building up of salinity/alkalinity, water pollution and health hazards caused by excessive use of agro-chemicals and emission of important

greenhouse gases. Proper management practices will certainly minimize these negative effects and will increase productivity, according to FAO.
Rice is the main source of food energy and is an important source of protein providing substantial amounts of the recommended nutrient uptake of zinc and niacin. It is very low in calcium, iron, thiamine and riboflavin and nearly with no beta-carotene. However there appears to be some genetic variation for iron and zinc content in rice, which may offer an opportunity for improving its nutritional value, FAO experts say. During the Green Revolution (1966-1990), the increase in the world rice production has resulted in more rice being available for consumption despite the continued increase in population. However there are still some 815 million people in the world suffering from hunger and malnutrition, and most of them live in areas that are dependent upon rice production for food, income, and employment, FAO underlines.The FAO's International Rice Commission is a forum where senior policy makers and rice specialists from 61 countries review their national rice research and development programmes. Its objective is the promotion of national and international action in matters relating to the production, conservation, distribution and consumption of rice. Its member countries have grown from 15 in 1949 to 61 at present. The last 19th Session of the IRC was convened in Cairo, Egypt in 1998.

APPENDIX B

Blast, biotech and big business
IMPLICATIONS OF CORP CORPORATE STRATEGIES ON ORATE RICE RESEARCH IN ASI ASIA
AUGUST UGUST 2000
http://www.grain.org/publications/reports/blast.htm
Rice blast is a problem almost everywhere that rice is grown. This fungal disease *(see box)*is estimated to cause production losses of US$55 million each year in South and Southeast Asia. The losses are even higher in East Asia and other more temperate ricegrowing regions around the world.1 Blast is gaining interest among agricultural biotechnology companies because of the potential genetic engineering offers to generate dual profits for their chemical and seed departments. The rice blast problem and industry's approaches to dealing with it provide a clear example of how corporate research and development (R&D) strategies are diverging further and further from the needs and means of farmers, particularly in the poorer countries of South and Southeast Asia.

What is blast?

The rice blast disease is caused by the fungus *Pyricularia grisea*, which, in its sexual state, is known as *Magnaporthe grisea*. The disease can strike all aerial parts of the plant. Most infections occur on the leaves, causing diamond-shaped lesions with a gray or white center to appear, or on the panicles, which turn white and die before being filled with grain.2 *P. grisea* is highly specific to rice, although certain strains that don't attack rice can harm weeds in the rice field. Once on a rice plant, the fungus rapidly produces thousands of spores, which are carried readily through the air, by wind or rain, onto neighboring plants.3 Blast was first reported in Asia more than three centuries ago and is now present in over 85 countries. It is highly adaptable to environmental conditions and can be found in irrigated lowland, rain-fed upland, or deepwater rice fields.4 Jim Correll, a scientist from the University of Arkansas, who has worked on blast for years, speculates that the disease originated in Asia, where rice itself originated, and was spread throughout the rest of the world by the exchange of seeds. Blast can survive on seeds and can easily move over borders if proper safety checks are not in place. In 1996 in California, USA, despite the enforcement of strict safety measures to prevent the entry of blast, the disease managed to find its way into the state's paddy fields for the first time.5 While it is present nearly everywhere rice is grown, blast is more of a problem in the temperate flooded and tropical upland cropping systems, marked by cooler climates.6 Rainy periods or periods of high humidity also favor the disease. Certain cultural practices encourage blast: excessive use of nitrogen (through chemical fertilizers) increases susceptibility of rice to the fungus, as does inadequate spacing (often practiced under rice intensification programs).

Blasting a moving target

Chemicals are somewhat effective against blast, and a number of the major pesticide manufacturers market commercial pesticides targeted at the disease. Breeders have also spent years looking for resistant varieties of rice that farmers have selected over generations. They have not only collected many traditional rice varieties that are resistant to blast, but have also identified a number of rice genes that they believe are responsible for the resistance. Neither chemicals nor breeding provide a totally effective approach, however. Due to the pathogen's ability to rapidly adapt, crops remain vulnerable. These conditions make blast a very attractive candidate for genetic engineering in corporate labs. Genetic engineering offers the perfect means for pesticide companies to protect and expand their earnings, as the limitations of – and the risks involved with – chemically-intensive agriculture to control the disease become increasingly obvious. Not surprisingly, a number of giants in the industry, with vested interests in pesticides and seeds, are eagerly pouring millions of dollars into producing rice seed-chemical packages to “manage” the disease. In the long-term, the technology for blast will only form one small component of a much larger corporate program for disease management that will have deep implications for nearly all sectors of agricultural production. Yet genetic engineering will not provide poor rice farmers in Asia a solution to the blast problem. Looking at it from their situation, the GE approach is impractical, expensive and unwarranted, as there are much more affordable and effective ways to control the disease. In this sense, there is a fundamental conflict within agricultural research and development – between an agenda that caters to the needs of industry and one that addresses the needs of resource-poor farmers, the bulk of Asia's population.

In most rice growing areas of South and Southeast Asia, blast remains less of a problem than some other diseases, such as tungro and bacterial blight. Few studies have been done to examine the intensity of the problem.7 The Rockefeller Foundation found in 1991 that 3.8% of the rice area in Southeast Asia was affected by blast, causing yield losses of 3.1 kg/ha resulting in production losses valued at US$14.3 million. In South Asia, yield losses were almost three times as high: rates climbed to 8.8 kg/ha at a cost of US$40.9 million.8 More recent figures from Pesticides Action Network (PAN) Indonesia show that during the October 1999 - March 2000 planting season, rice blast infested some 15,000 hectares across 60% of the provinces in the Indonesian archipelago.9 Over in the Philippines, a nationwide survey conducted in 1996 among an extensive sampling of farmers by the National Crop Protection Center showed that blast is not much of a problem. The 6 S.C. Scardaci et al., “Rice Blast: A New Disease in California,” Agronomy Fact Sheet Series 1997-2, Department of Agronomy and Range Science, University of California, Davis, retrieved from http://agronomy.ucdavis.edu/uccerice/AFS/agfs0297.htm, on 18 May 2000. 7 P.S. Teng, C.Q. Torres, F.L. Nuque, and S.B. Calvero, “Current knowledge on crop losses in tropical rice”, *Crop Assessment in Rice*, IRRI, 1990, p. 39. 8 Robert W. Herdt, “Research Priorities for Rice Biotechnology”, in *Rice*

Biotechnology, G.S. Khush and G.H. Toenissen (eds.), Alden Press Ltd., London, 1991. (Personal communications, July 2000). More important problems for the farmers were stem borer, brown plant hopper, green leafhopper, rice bug, leaf folder, golden apple snail and tungro.10 Another recent study *(see box)* demonstrated that, in a country like the Philippines, diseases such as blast are among the least significant factors affecting the country's rice supply, even if they are seen as hot targets for genetic engineering.

Nice landing, wrong airport

A study conducted by the Swiss Federal Institute of Technology in Zurich (ETH), in cooperation with the University of the Philippines Los Baños, investigated the perception of problems affecting the Philippine rice economy and the potential of genetic engineering to solve them.11 The questionnaire was answered by 65 respondents from 46 organizations, all active in the field of genetic engineering: NGOs, including consumer organizations (28%); government institutions (23%); business sector (12%); international research institution, the International Rice Research Institute (IRRI, 9%); academia (8%); legislators (6%); media (6%); international foundations (5%); international NGOs (3%). Respondents were asked to assess the importance of the problems of the Philippine rice economy according to a ranking of 1 (least important) to 5 (most important). The same scale was used to assess the potential of genetic engineering to solve the problems. The most serious problems affecting rice production were assessed to be market conditions, lack of irrigation facilities, inadequacy of post harvest facilities, and indebtedness due to high input costs, weak support services, typhoon, inefficient transport network and unequal land distribution. Meanwhile the potential of genetic engineering for solving production problems was rated highest in controlling plant diseases and pest infestation, improving food quality, reduced use of pesticides, stabilizing yields and developing drought tolerance.

The study showed that there is a serious mismatch between the perceived problems affecting rice production and the potential of genetic engineering to solve them. The potential of genetic engineering is highest for problems that are perceived to be least important, such as pest infestation. The result of the study stresses the fact that the amount of money and time being invested in biotechnology is disproportionate to its importance, at least in the case of rice in the Philippines.

Pesticide pitfalls and breeders' block

For industry, meanwhile, blast is a big money spinner. It is one of the few crop diseases that justifies the development of single-target fungicides: the Japanese market alone for blast fungicides is estimated at US$400 million per year.12 However, chemical fungicides present hazards to human health and the environment, and farmers in Asia are already rejecting them in favor of more sustainable approaches. Some researchers are looking into alternative, nonchemical fungicides. Studies suggest that there are many substances naturally occurring in plants that are toxic to the blast fungus, although, to date, there has been little research and development in this area. Biological agents,

such as micro-organisms or botanical pesticides, are also available to control the disease.13 But the Asian market is still studded with a range of chemical weapons, some of them so hazardous that their sales are restricted by several governments. Despite the sales figures, the blast fungicide market is still compromised. Blast fungicides are expensive products for a generally insolvent set of customers – Asian rice farmers. Fungicides for blast typically consume 6%-50% of total crop protection costs.14 The big Japanese market is only sustained by the highest agricultural subsidies (70%) in the world.15 Syngenta's Quadris (azoxystrobin).
A second limitation stems from the fungicides themselves. When the fungicides are used intensively, they place enormous selection pressure on blast, and the pathogen rapidly develops resistance. Given that it costs up to US$100 million to develop a new fungicide and bring it to market, companies are rarely willing to develop new products when resistance to the older brands develops. The only fungicide recently introduced specifically for blast is carpropomid, which was introduced as Win in 1998 by Bayer's Japanese subsidiary Nihon Bayer Agrochem. Farmers are therefore left with the choice of using fungicides in moderation, which leaves the crop vulnerable to blast, or beginning a cycle of heavier and heavier dosages of chemicals. Neither option is appropriate. Not only is chemical protection too expensive but, even in moderation, there are indirect costs from the use of fungicides to the health of the farming family and the surrounding ecosystem, which put great strain on the family's limited resources. As these problems have become more widely recognized, the international agriculture research institutions have responded by shifting their focus to breeding. Breeding efforts, however, have also been quite limited in their success. Blast is highly variable, especially under intensive, large scale monoculture conditions, and breeders simply can't keep up with it. As reported by CIAT, *"New blast strains mutate rapidly, rendering resistant varieties susceptible within 2 or 3 years of release – sometimes, even before the breeding lines reach the farm."*17 The result is a neverending race for breeders to keep ahead of the disease with new varieties.
Fungicides and breeding have both been deployed against blast within a specific model of intensive rice production that was promoted by the 'Green Revolution' – chemical-greedy varieties, uniform crops and irrigated lands. Fungicides and breeding can be used to patch up problems, such as blast, that intensified under the Green Revolution, without requiring any fundamental change in direction. However, neither fungicides nor breeding are capable of sustaining the fight against blast. Some believe that genetic engineering can resolve this dilemma.

The hunt for durable resistance

Scientists are now trying to use genetic engineering to create what is called *"durable resistance"* – plant resistance to disease that lasts for long periods of time. The initial idea was to isolate the genes responsible for blast among resistant plant varieties, clone these genes, and then incorporate them into

susceptible high-yielding varieties. In theory, the transgenic varieties could benefit the pesticides industry, seed breeders, and farmers.

Looking back to the future?

As geneticists struggle in biotech laboratories, others are enjoying successes in the field using traditional methods to control the disease. Dr Christopher Mundt of Oregon State University in the United States is working with IRRI and the Yunnan Agricultural University on a rice blast project in Yunnan, a southwestern province of China. The project utilizes a *"multi-line system",* where different varieties of rice are planted in the same field to control blast.23 In Yunnan, blast is a severe problem and farmers often resort to eight applications of fungicides per season to try to control the disease. According to Mundt, the multi-line system has had an immediate impact: the severity of blast decreased by 95% and farmers did not have to apply any fungicides. IRRI claims that farmers participating in the project earned an additional US$150 per hectare from their harvests. By the end of 2000, up to 60,000 hectares in Yunnan will be planted to the multi-line scheme.24. Despite the project's success, there is resistance to the idea. According to Mundt, *"It's the people with the PhDs that have the biggest problem with it."* Farmers have been quite willing to try it out. This is logical, since farmers have used similar principles to manage blast for generations. Mundt says that the varieties that they chose to use for the project were actually suggested by a local farmer who was mixing the varieties successfully in his own field. In Vietnam, where Mundt is working on another multi-line project, the participating farmers told him that they remembered using similar strategies years ago, before the Green Revolution production model came in. The multi-line system draws criticism because it goes against the basic tenets of industrial agriculture. It utilizes diversity, whereas industrial agriculture needs and breeds uniformity. By planting a variety of crops, the multi-line system prevents the intensification of the disease, keeping it at manageable levels for the rice plants to exert their own natural defences. It is only where uniformity is widespread that blast becomes dominant and is capable of causing severe damage. This was recently acknowledged by IRRI, one of the leading promoters of the Green Revolution: *"Simplification, or lack of diversity, has created a fragile biotic environment, which made crops vulnerable to pest and disease outbreak."*

"Traitor Technology"

The days of tighter seed-chemical packaging are now approaching.27 Syngenta, a recent mergerbetween the agriculture divisions of Novartis and Zeneca, has already developed ways tocombine the application of agrochemicals with the incorporation of disease resistance genes toenhance the overall resistance of plants. The obvious advantage of the combination for Syngenta is that any farmer growing its genetically engineered blast-resistant rice seed may also have to purchase and apply Syngenta's agrochemicals. Critics call this "Traitor Technology" becausefarmers enjoy the trait – blast resistance, in this case – only if they use the company's chemical triggers. Syngenta has already received at least two "Traitor Tech" patents. US patents 5,614,395 and 6,031,153 are for

chemically-induced or -enhanced disease resistance in plants engineered with a specific gene sequence linked to disease resistance. The latter patent covers the application of a number of widely used fungicides that can act as inducers, including mancozeb, metalaxyl, ridomil, fosetyl, and azoxystrobin. Syngenta is not the only company pursuing this strategy. Mitsui, in Japan, has a patent on a method for chemically inducing resistance to bacterial diseases, such as bacterial blight in rice. These patented technologies have been developed at a time when TNCs are moving into the rice market through the acquisition of hybrid rice companies.29. Clearly, they intend to take over a portion of the breeding process from the public sector, which currently stands behind much of the rice seed market in Asia. One important difference between transgenic blast-resistant rice and conventional blast-resistant rice is that in the transgenic scenario, the genes will be patented (Personal communications with MASIPAG staff, 27 July 2000).

VITA

Marta Cristina Filippi e Silva was born in 1963, at São Paulo city, located at São Paulo State, Brazil. She is married with Reginaldo and has a 13 year old daughter, Vitoria and a 7 years old son named João Antonio.

On December 1984 she finished her Bacharel degree in Agronomy from “Agronomia e Zootecnia Manoel Carlos Gonçalvez” University, São Paulo State.

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