Regulation of cyclic AMP signaling during asexual development and pathogenesis in *Magnaporthe oryzae*

Ravikrishna Ramanujam

2013

Ravikrishna Ramanujam. (2013). Regulation of cyclic AMP signaling during asexual development and pathogenesis in *Magnaporthe oryzae*. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/55372>

<https://doi.org/10.32657/10356/55372>

NANYANG
TECHNOLOGICAL
UNIVERSITY

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*MAGNAPORTHE ORYZAE***

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ASEXUAL DEVELOPMENT AND PATHOGENESIS IN
*MAGNAPORTHE ORYZAE***

RAVIKRISHNA RAMANUJAM

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University

in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

2013

This thesis is dedicated to my parents

Krishnappa Ramanujam

and

Mythili Ramanujam

for their love, endless support

and encouragement.

Acknowledgements

Completion of this thesis would not have been possible without the help and support of many people. I am extremely grateful and would like to express my heartfelt thanks to my thesis supervisor Dr. Naweed Naqvi for his exemplary guidance, support and patience throughout the course of this work and for teaching me the skills necessary to become an independent researcher.

I would also like to thank Dr. Thirumaran Thanabalu, my co-supervisor at Nanyang Technological University, Prof. Mohan Balasubramanian, A/Prof Susana Geifman Shochat and A/P Richard Sugrue for their suggestions and comments. I also thank Dr. Snezhana Oliferenko, Prof David Siderovski and Dustin Bosch for their helpful suggestions. I am also extremely grateful to Dr. Fumio Motegi for his guidance, understanding and support.

I would like to thank all present and past members of the Naweed lab: Rajesh, Yizhen, Yang Fan, Poonguzhali, Yanjun, Yunlong, Ziwei, Liu Hao, Angayarkanni, Yangkui, Marilou and Chuanbao, for making a work environment that was fun and always stimulating and also for making my time during this PhD enjoyable. I thank Meredith for her help during image analysis and the scientific discussions. I thank Xu Yishi and Tanushree Bishnoi for their help.

I also thank Prof. P.N.Rangarajan for giving me an opportunity to work and learn in his laboratory at the Indian Institute of Science during the early days of my scientific career. I also thank Prof.G. Padmanaban for his support and guidance. I cherish the friendship I had at IISc, thanks Pushpa, Ravi, Anand and Abhishek. I thank Dr.S.Nagini for her encouragement and guidance. I thank all my friends during my master's course for their support and friendship, especially Ram Sudheer and Muthu Kumar.

I would like to thank the community (past and present) at Temasek Life Sciences Laboratory (TLL), administrative and support staff for all the help. I would also like to thank the imaging and sequencing facilities for their support. Financial support from TLL is duly acknowledged.

I thank my mom and my dad for their unconditional love, encouragement and blessings. Thank you for everything you have done for me, I couldn't have asked for better parents. I also thank my parents-in-law for their encouragement and blessings. Many thanks to Rajesh, Amit and Anup for their friendship and advice. I would like to especially thank my best friend, my wife Nayana for always being there for me and for being my pillar of strength. None of this would have been possible without her, her help and support, words of encouragement and companionship.

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List of Abbreviations

Aa	Amino acid
AMP	Adenosine monophosphate
AKAP	A-Kinase anchoring protein
cAMP	Cyclic adenosine monophosphate
CM	Complete medium
d	Day
DAPI	4',6-diamidino-2-phenylindole
dpi	Days post inoculation
Δ	Deletion
DEP	D isheveled, E gl-10 and P leckstrin
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
<i>E. coli</i>	<i>Escherichia coli</i>
FYVE	F ab1, Y OTB, V ac1 and E EA1
Gα	G-protein alpha subunit
Gβ	G-protein beta subunit
Gγ	G-protein gamma subunit
GAP	Guanosine triphosphatase activating proteins
GEF	Guanine-nucleotide exchange factors
GTPase	Guanosine triphosphatase
GIRK	G-protein coupled inwardly rectifying potassium
G-protein	Guanine nucleotide binding protein
GPCR	G-protein coupled receptor
GFP	Green fluorescent protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
h	Hour
hpi	Hours post inoculation

HOPS	Homotypic fusion and vacuole protein sorting
IBMX	1-methyl-3-isobutylxanthine
ILVs	Intra luminal vesicles
kDa	kilo dalton
kbp	kilo basepair
LTG	Lyso Tracker Green
min	Minute
MBC	methyl benzimidazole-2-ylcarbamate
MAPK	Mitogen-activated protein kinase
mC	mCherry
mg	milligram
mL	millilitre
mM	millimolar
MVBs	Multi vesicular bodies
ORF	Open reading frame
PA	Prune agar
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
RFP	Red Fluorescent Protein
RGS	Regulator of G-protein signaling
RPM	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
T-DNA	Transfer DNA
Tris	Tris(hydroxymethyl)aminomethane

μg	microgram
μl	microlitre
μm	micrometre
μM	micromolar
VPS	Vacuolar protein sorting
WT	wild type

Abstract

The conidial germ tube in *Magnaporthe oryzae*, the causal agent of the devastating rice-blast disease, responds to inductive surface cues and switches from polarized to isotropic growth to ultimately form the specialized infection structure known as the appressorium. The critical role played by signaling pathways, especially the G-protein/ cyclic AMP (cAMP) signaling in appressorium initiation was identified almost two decades ago, yet several fundamental questions remain unanswered. The purpose of this study was to 1) identify additional regulatory proteins that facilitate the maintenance of steady-state levels of cAMP; 2) decipher *in-vivo* mechanisms that function to control the activity of such regulatory proteins; 3) investigate the spatial and temporal dynamics of the cAMP signaling cascade and finally 4) understand how the signals are transmitted through the crowded intracellular network to specific sites of growth and/or signaling.

Utilizing a combination of reverse genetics, biochemical analyses and cell biology techniques, in Chapter I, I identified and functionally characterized two cAMP phosphodiesterases (a high affinity- PdeH and a low affinity PdeL) in *M. oryzae*. I also demonstrate that PdeH is the major enzyme hydrolyzing and regulating intracellular levels of cAMP during asexual, pathogenic and *in planta* development. More importantly, the study identifies that cAMP levels are modulated by PdeH in a biphasic manner, i.e. up regulation during early stages (appressorium initiation), and down regulation at the late stages (host invasion) of pathogenic development. The data further implicates the existence of two distinct pools of cAMP (cytosolic and nuclear) in *M. oryzae*, which are differentially modulated by PdeH and PdeL respectively.

Given that Regulator of G-protein signaling (RGS) proteins function as key negative regulators that control the intensity and duration of active G-protein signaling, in Chapter II, I sought to address and understand the mechanisms possibly involved in the regulation of a proto-typical member, namely Rgs1(a GAP for MagA). I found that Rgs1 was subjected to an endo-proteolytic cleavage yielding an N-terminal DEP-DEP fragment (that facilitated membrane targeting) and a C-terminal catalytic core RGS domain that was sequestered within vacuole in the absence of a functional DEP motif. Rgs1 cleavage likely represents an important means of ultimately

modulating the duration and intensity of G-protein signaling. Based on the physical interaction with Rgs1 specifically during early stages of appressorium initiation, I demonstrate Pth11 to be a *bona fide* GPCR (albeit non-canonical) for cAMP signaling in *M. oryzae*.

Finally, in Chapter III, I demonstrate that in the likely absence of AKAP-like anchor proteins in *M. oryzae*, the late endosomal compartments (comprising of a PI3P-rich dynamic tubulo-vesicular network) are capable of serving as scaffolds or platforms to anchor active MagA/G α_s , Rgs1, Adenylate cyclase and Pth11. Loss of HOPS component Vps39 and consequently the late endosomal function caused a disruption of adenylate cyclase localization, cAMP signaling and appressorium formation. Exogenous cAMP remarkably rescued the appressorium formation defects associated with *VPS39* deletion in *M. oryzae*, suggesting that the late endosomal HOPS complex potentially serves as a scaffold to anchor, compartmentalize and mobilize G-protein signaling components to sites of active growth and signaling, during early pathogenesis.

Taken together, the results presented in this thesis suggest that *M. oryzae* utilizes a variety of complex cellular mechanisms to tightly regulate the steady-state levels as well as modulate the spatio-temporal dynamics of cAMP signaling in response to inductive cues as well as in the context of changing cellular geometry

Chapter I

1.Introduction

1.1 Cyclic AMP signaling-a paradigm of signal transduction

At the cellular level organisms can sense and respond to changing environments through the action of conserved signaling pathways. Signaling cascades play an important role in regulating diverse processes from chemotaxis to differentiation including apoptosis [1-4]. Signal transduction or propagation more often than not involves second messengers such as cyclic nucleotides. Metazoans, fungi, protozoa and bacteria widely employ the second messenger cyclic AMP (cAMP) to relay signals to a host of downstream effectors [5-7].

cAMP was first described as a second messenger during the early 1970's [8]. cAMP is synthesized by adenylyl cyclases (ACs) from ATP, upon stimulation by heterotrimeric G-proteins. Phosphodiesterases (PDEs) catalyze its hydrolytic degradation and consequently facilitate signal termination [9] (Figure 1.1). The regulatory effects of cAMP are facilitated through interactions with downstream cyclic nucleotide binding proteins. An important outcome of cAMP signaling is the regulation of gene expression [10]. In general activation of transcription factors in eukaryotic cells necessitates the utilization of protein kinase A (PKA) as an intermediate, while in bacteria, transcription factors are directly activated upon binding cAMP. Currently in eukaryotes, cAMP signaling is known to regulate a wide array of biological processes ranging from metabolism to shaping memory and innate immunity [10, 11].

The near universal use of cAMP in diverse life forms and the identification of the importance of cAMP signaling in pathogen biology, i.e., during virulence and host-pathogen interactions has generated renewed interest in this second messenger [12]. Many such interactions are being discovered, thus providing a unique opportunity to study and understand such biological processes in detail.

Figure 1.1. The second messenger cyclic AMP transduces external environmental signals to downstream intracellular effectors.

Adenylate cyclases (ACs) catalyze the synthesis of cyclic AMP from ATP. Phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP to adenosine mono-phosphate (AMP). cAMP can relay signals to downstream intermediary effector proteins or directly to transcription factors. Adapted and modified from [12] .

1.1.1 Comparative overview of cAMP signaling in the yeast and phytopathogenic fungi

1.1.2 cAMP signaling in *S. cerevisiae*

The function of cAMP signaling has been best characterized in the budding yeast *S. cerevisiae* (Figure 1.2). It has a central role in sensing nutrients in addition to regulating a wide array of biological processes like stress resistance, energy metabolism, carbohydrate utilization, thermo- and osmotic shock tolerance, cell cycle progression, sporulation, bud site selection, pseudohyphal growth in response to limiting nitrogen conditions, autophagy and aging [13-15]. Transformation of diploid *S. cerevisiae* cells to a nutrient scavenging filamentous variety is referred to as pseudohyphal differentiation or filamentous growth. Pseudohyphal growth can also be stimulated by increased PKA activity.

Activation of the cAMP-PKA signaling pathway occurs upon binding of glucose or other fermentable carbon sources to the membrane anchored G-protein coupled receptor (GPCR) - *Gpr1*, which activates cAMP synthesis through the $G\alpha$ protein (*Gpa2*) mediated activation of adenylate cyclase (*Cyr1*). *Gpa2* was first identified based on its similarity to the mammalian $G\alpha$ subunits, and the $G\beta$ subunits *Gpb1* and *Gpb2* were found via their interaction with *Gpa2*. A $G\gamma$ subunit remains to be identified [15].

In *S. cerevisiae* and *C. albicans*, cAMP synthesis is also stimulated intracellularly, by the direct activation of adenylate cyclase by small G-proteins or Ras proteins [16-23]. Interestingly, it was discovered recently that bacterial peptidoglycan can directly stimulate adenylate cyclase in *C. albicans* [24]. It has also been further reported that, cAMP synthesis is dependent on the uptake as well as the intracellular phosphorylation status of glucose, mediated by the enzyme hexokinase. This puts forth an interesting possibility that extracellular nutrient levels/glucose is sensed by the GPCR- *Gpr1*, while the intracellular glucose /metabolic status by the enzyme hexokinase, although the exact role of hexokinase remains to be established [25, 26]

S. cerevisiae

Figure 1.2. The cAMP signaling cascade in *S.cerevisiae*.

The key proteins known to function in the signaling cascade are shown. Most of these proteins are highly conserved and found in various other fungi. Adapted and modified from [27].

Although Ras1 and Ras2 along with Gpa2 function to stimulate adenylate cyclase, single mutants defective in either Ras1/Ras2 or Gpa2 function are viable. While double mutants of *RAS* are inviable, a double mutant of either Ras1 or Ras2 in combination with Gpa2 exhibits a very slow growth phenotype. Remarkably, over expression of the adenylate cyclase gene *CYR1*, supplementation of exogenous cAMP or mutations in the *PDE2* gene, coding for the cAMP phosphodiesterase can suppress the Ras double mutant phenotype. In addition, expression of a dominant active *RAS2*^{val19} allele also causes accumulation of increased cAMP levels [28], and causes enhanced filamentation. Ras signaling mediated pseudohyphal growth in yeast is complex process involving the cAMP and MAP kinase signaling cascades [27, 29, 30].

In the absence of adenylate cyclase stimulation and cAMP synthesis, PKA holoenzyme is present as a non-active hetero-tetrameric complex consisting of two catalytic subunits (three isoforms, coded for by *TPK1*, *TPK2* and *TPK3*) and two PKA regulatory subunits Bcy1. Although the three PKA isoforms are by and large dispensable for viability, the presence of at least one of the isomers is required for normal growth [31-34]. Furthermore, there exist small variations in the conserved catalytic domains of the three isoforms, suggesting the possibility that these enzymes have different targets, and regulate different cellular processes in *S. cerevisiae* [15]. It has been reported that more than 87% of all PKA substrates is uniquely phosphorylated by just one of the Tpk's [35]. Tpk2 has been shown to positively regulate pseudohyphal or filamentous growth; Tpk1 and Tpk3 have opposing roles. Both PKA subunits are well conserved in fungi and eukaryotic cells [15, 31, 36]. In response to specific inducing conditions, the synthesized cAMP binds to the regulatory subunits Bcy1, causing them to dissociate from the complex as a homodimer, thereby releasing the two catalytic subunits as active monomers which function to phosphorylate downstream target substrates which include transcription factors as well as metabolic enzymes [31]. The most well characterized substrates/ targets of the catalytic subunit of PKA are as follows:

Transcription factors:

Rap1[37], Hsf1 [38], Adr1 [39], Msn2 and Msn4 - all of which are important regulators of stress response element controlled proteins [40-43].

Ssn2 [44], Sfl1 is an inhibitor of pseudohyphal growth, a transcriptional repressor of flocculation-related genes as well as an activator of stress response genes. Sfl1 has also been shown to be negatively regulated by cAMP-PKA signaling [45].

Flo8 and Flo11; In *S. cerevisiae*, Flo8 is a transcriptional regulator important for flocculation and invasive growth in haploid cells and pseudo-hyphal growth in diploids [46]. Flo8 acts downstream of the cAMP-PKA pathway, as *FLO8* mutants suppress the effects of a constitutively activated cAMP-PKA pathway [47]. Flo8 also regulates the expression of cell surface flocculin Flo11. *FLO11* codes for a cell surface flocculin implicated in cell-cell adhesion, whose expression is important for flocculation and invasive/filamentous growth [48]. Flo8 binds to the *FLO11* promoter in a Tpk2 dependent manner [49]. Furthermore, Tpk2 mediated phosphorylation of Flo8 is necessary for the interaction of Flo8 with *FLO11* promoter under *in vivo* as well as *in vitro* conditions [49].

Metabolic enzymes:

Cho1 [50], Pfk2 [51], Nth1 [52].

Other regulatory kinases:

Rim15 which plays a role in meiotic and stationary phase induced gene expression [53] and Atg1 [54].

For an extended period of time after PKA was discovered, the only substrates known for PKA included metabolic enzymes, especially those implicated in carbohydrate metabolism and transcription factors. However, in addition to protein targets, more recently PKA has been shown to have non-canonical functions. For example, Tpk2 has been shown to bind to and interact with promoters of genes encoding ribosomal proteins and also regulate genes involved in trehalose degradation, iron uptake and water homeostasis [55, 56]. Tpk2 has been shown to phosphorylate the APC activator Cdc20 (anaphase-promoting complex) thereby inhibiting the function of the APC [57, 58]. Finally, cAMP pathway, more specifically, adenylate cyclase or synthesis of cAMP is negatively controlled by PKA feedback loop, which inhibits cAMP synthesis. In addition, PKA acts to reduce intracellular cAMP levels by directly phosphorylating GTPase

Ras2 and the cAMP phosphodiesterase Pde1. Phosphorylation of Pde1 at the consensus phosphorylation PKA site supposedly enhances Pde1 activity [59-62].

1.1.3 cAMP signaling in *U. maydis*

U. maydis is a heterothallic basidiomycete that causes the smut disease in corn/maize, cereals and grasses. *U. maydis* is commonly known as the smut fungi or the corn smut fungus. The unicellular asexual haploid *U. maydis* cells are yeast-like, nonpathogenic and have the ability of growing saprophytically by budding. Infection is possible only after a mating event or fusion between compatible haploid yeast cells, that is followed by a transition to a filamentous dikaryotic hyphae that are obligatory biotrophic and pathogenic. Proliferation of filamentous hyphae in the plant stimulates gall formation that is pursued by the formation of diploid teliospores, which germinate to start a fresh infectious cycle. In *U. maydis*, mating-type compatibility and therefore virulence, is directed by allelic variations in the *a* and *b* mating-type loci [63].

The *a* locus encodes for *mfa1* and *mfa2* which are pheromone genes, and *pra1* and *pra2* which are pheromone receptor genes. The *b* locus codes for homeodomain proteins *bE* and *bW* that function as heterodimers and are necessary for growing filamentously and causing disease [64, 65]. Signaling events are initiated upon sensing of pheromone by cognate receptors present in opposite mating types that cause enhanced gene transcription of those that are specifically implicated in mating and conjugation tubes fusion from opposite mating-type cells to produce an dikaryon capable of causing disease [66-69]. In addition to the rice blast fungus *M. oryzae*, *U. maydis* is the only other phyto-pathogen that has been successfully and extensively used as a model for investigating signaling pathways regulating mating responses and pathogenicity [70]. A notable feature of the pathogenesis of *U. maydis* is that the processes of infection and mating are inter-connected and regulated partly by cAMP signaling. The role of cAMP pathway has been well studied and using gene deletion mutants key players involved in these processes have been identified and their roles established (Figure 1.3) [69, 71].

Figure 1.3. A diagram depicting components of cAMP signaling cascade in hytopathogenic fungus *U. maydis*.

The key proteins characterized to be involved in the pathway are depicted. Adapted and modified from [27].

Broadly speaking, mutants with enhanced or high PKA activity display a budding phenotype while those with lower PKA activities grow filamentously. Characterization of constitutively filamentous *U. maydis* mutants has led to the identification of the *uac1* adenylate cyclase gene. The *uac1*Δ mutant failed to cause disease and displayed a constitutively filamentous phenotype. Predictably, exogenous addition of cAMP to the *uac1*Δ suppresses the filamentation phenotype and restores budding growth. The gene encoding the regulatory sub unit (R-PKA) of PKA, *ubc1* (Ustilago bypass of cyclase), was identified in a genetic screen, as a suppressor of *uac1*Δ mutant (adenylate cyclase, [72]). *ubc1* strains display a yeast like morphology and show a budded phenotype, in a manner similar to cells exposed to exogenous cAMP. Interestingly, the *ubc1*Δ strains display a budding phenotype; it grows in a filamentous manner within host tissue when mixed with compatible *ubc1* strain although defective in tumor induction. Thus, even though a high PKA activity results in formation of filaments *in vitro*, the phenotype may be overcome in the host. The *ubc1*Δ is compromised in the severance of daughters from the mother cells and altered bud site selection. Recently it was demonstrated that the *ubc1*Δ accumulates elevated levels of transcript of genes concerned with phosphate acquisition and accumulation, implying a likely possibility of cross-talk between phosphate metabolism and cAMP signaling [73].

The *adr1*Δ (aromatic dicarboximide resistance), the catalytic subunit mutant (C-PKA) has defects identical to the *uac1* Δ mutant with respect to growth, mating and plant infection. The *adr1*Δ also displays a constitutive filamentous phenotype. The *adr1*Δ also rescues the yeast-like growth of *ubc1*Δ strains, consistent with catalytic subunit of *adr1* acting downstream of the *Ubc1* regulatory subunit [74]. Furthermore, strains deleted in *uka1*, (the second PKA catalytic subunit gene -Ustilago Kinase A), behave similar to and were phenotypically impossible to tell apart from *WT* cells in terms of morphology and pathogenicity. Consequently *Adr1* likely accounts for majority of PKA activity in *U. maydis* [75, 76]. Double mutants of *adr1 uka1* can survive, implying that the activity of PKA is not essential for growth in *U. maydis*. However, amongst the four *Gα* subunits, *Gpa3* is the sole *Gα* that is necessary for pheromone response, mating and pathogenesis in *U. maydis* [77].

Previously the role played by *gpa3* was unclear and based on experimental evidence. For example the *gpa3*Δ strain is sterile and upon pheromone induction, the *mfa1* and *b* genes failed

to express in this background, constitutive activation of the *gpa3*^{Q206L} mutant allele caused enhanced expression of pheromone inducible *mfa1* and *b* genes, it was suggested that *gpa3* was directly implicated in the pheromone sensing pathway. However, it is now clear that *gpa3* plays a fundamental role in the cAMP-PKA pathway. Evidence that support this conclusion is as follows; *gpa3*Δ strains are avirulent and display a filamentous phenotype similar to the *uac1*Δ mutant, which can be suppressed by the addition of cAMP. Cyclic AMP rescues sterility, mating defect and restores pheromone gene expression. The adenylate cyclase *uac1* mutant in the *gpa3*^{Q206L} background reduces *mfa1* gene expression, suggesting that *uac1* acts downstream of *gpa3*.

Interestingly, Pra1 and Pra2, the seven-transmembrane pheromone receptors are believed to interact with Gpa3. Unlike in yeast, where the Gβγ complex is primarily involved in the activation of MAP Kinase signaling, the Gβ subunit *bpp1* in *U. maydis* is also involved in cAMP-PKA signaling as well as mating. The *bpp1*Δ grows in a filamentous manner and shows decreased expression of genes coding for pheromones [78]. The phenotype of the *bpp1*Δ mutant is rescued by the *gpa3*^{Q206L} allele expression. However, unlike the *gpa3*Δ mutant that fails to cause disease, the *bpp1*Δ mutant successfully results in tumors on susceptible plants, implying that Gpa3 controls pathogenesis in a manner not independent on Bpp1. Although analysis of the *U. maydis* genome suggests the existence of gamma subunit of guanine nucleotide- binding protein, similar to yeast Gγ subunit Ste18p, the exact function of this protein remains unknown.

Unlike for the yeast PKA, where a wide range of downstream targets have been identified, only a few targets have been discovered in *U. maydis*. Furthermore, it appears that several of the yeast orthologous targets are not conserved in the smut fungus. The transcription factor Prf1 is among the few whose function is well characterized. Prf1 is an HMG-motif containing protein and is implicated in influencing the cAMP-PKA and MAP Kinase signaling pathways [64]. The *prf1*Δ mutant is sterile, non-virulent and fails to express genes encoding for *a* and *b* mating-type, upon pheromone stimulation. Prf1 has been shown *in vivo* to interact with PKA major catalytic subunit, Adr1. The PKA phosphorylation sites of Prf1 is necessary for inducing expression of genes coding for *a* and *b* mating type.

Sql1 and Hgl1 are putative transcription factors that are activated by cAMP signaling in *U. maydis*. Hgl1 is supposedly a downstream target of PKA and was recognized as a suppressor of the phenotype that caused filamentation of the *adr1*Δ mutant. Hgl1 is phosphorylated by PKA *in vitro*, which suggests that *hgl1* function is likely needs to be down regulated by PKA mediated phosphorylation, to support budding growth. *Hgl1* function is crucial teliospore formation during infection [75].

1.1.4 cAMP signaling in *Cryphonectria parasitica*

C. parasitica is a filamentous fungal plant pathogen that causes the devastating chestnut blight and belongs to the phylum ascomycota. Chestnut blight disease was first observed in North America and Europe during the early part of the twentieth century [79]. The disease spread to such a great extent that it almost caused the extinction of its natural host *Castanea dentate* (the American chestnut). Coincidentally, strains of *C. parasitica* with reduced virulence or hypovirulent strains were isolated and were found to contain an unknown cytoplasmically transmissible factor. These factors were subsequently identified as dsRNA mycoviruses, and placed under a new family, the hypoviridae [80]. A noteworthy feature of the infection of *C. parasitica* by the dsRNA virus is the attenuation of the disease-causing ability of the fungus [81]. Additionally viral infection also caused many phenotypic alterations in the fungi, which included modified colony morphology, decreased pigmentation and conidiation. Infection also caused variations in the expression levels of many genes, which included the *lac-1* gene (coding for a laccase), the *cbh-1* gene (coding a cellobiohydrolase), and importantly in context to cAMP signaling, *cpg-1* gene (a G protein) [82]. Further analysis and several independent lines of evidence suggested a connection between attenuated fungal virulence and a perturbed G-protein regulated cAMP-mediated signal transduction (Figure 1.4) [83].

Figure 1.4. cAMP signaling compared between *C. parasitica*, *Collectotrichum spp.* and *Fusarium spp.*

The components thus far identified and characterized are depicted. Although conserved, the components involved are not as extensively characterized, unlike *S. cerevisiae*.

The idea that signal transduction pathways were likely being disrupted upon viral infection in *C. parasitica*, originated from the initial observation by Choi et.al that the *lac-1*, a laccase encoded by the fungus was down-regulated upon viral infection. Additionally, *lac-1* is positively regulated by calmodulin and calcinurin, molecules actively involved in signal transduction pathways [84-86]. Following this hypothesis, signaling pathways that could potentially be disrupted upon viral infection were selected for further analysis. Initial focus was on the G protein pathway and in particular the G α subunits. Choi et. al isolated and cloned the *cpg-1* gene, an ortholog of *gna-1* in *N. crassa* and also a second G α -encoding gene, *cpg-2*. During their preliminary study of G-proteins, the authors observed that co-suppression of *cpg-1* function caused slow and poor growth on PDA (potato dextrose medium), non-uniform colony edges, reduced aerial hyphal growth and decreased orange pigment accumulation [82]. The *cpg-1* co-suppressed strains compared to the *WT* strains caused smaller lesions on chestnut stems, indicating reduced virulence. In addition, the authors also found that infection with a prototypic hypovirus, CHV1-EP713 led to a decreased accumulation of the gene product of *cpg-1*. Wang and Nuss, in another study, found that *cpg-1* Δ strains lacked in *cbh-1* expression, identical to hypovirus-infected fungal strains [87].

The function of the G α subunit *cpg-1* was studied in greater detail and utilized a differential display technique to assess the variations in transcript abundance between strains with suppression of *cpg-1*, a sample strain infected by virus, and control strain which was virus-free [88]. It was found that more than 400 transcripts to be differentially regulated after infection. In the same study they also observed that both viral infection of *C. parasitica* as well as the co-suppression of *cpg-1* resulted in a 3-5 fold increase in the levels of cAMP.

Furthermore, elevation of cAMP levels by pharmacological means yielded an identical pattern of transcriptional changes. Targeted disruption of *cpg-1* to cause accumulation of intracellular cAMP and phenotypic defects although similar, but more severe compared to the hypovirus-infected fungal strains [89]. In all, these results provided the first conclusive report that infection of *C. parasitica* by hypovirus led to phenotypes, at least in part due to the perturbation of the G-

protein signaling pathways that involved $G\alpha$ subunit *cpg-1* and that in *C. parasitica*, *cpg-1* negatively regulates adenylyl cyclase.

Deletion of the *cpg-2* gene did not result in any obvious defects but caused a minimal decrease in colony growth rate and mycelial mass. Asexual sporulation was also decreased. Interestingly, the *cpg-1* mutant failed to produce perithecia during the mating stage while the *cpg-2* deletion strain made 3-4 fold higher numbers of perithecia compared to the WT.

Kasahara et.al identified and disrupted *cpgb-1* gene, encoding the G-protein β subunit in *C. parasitica* [90]. The *cpgb-1* Δ similar to the $G\alpha$ subunit *cpg-1* Δ mutant was reduced in pigmentation, asexual sporulation, and virulence, although they differed in their growth rates. Targeted deletion of *cpg-1* resulted in poor growth, whereas deletion of *cpgb-1* enhanced vegetative growth.

The same authors also discovered a gene *bdm-1*, beta disruption mimic factor-1, which displayed sequence identity to mammalian Phosducin [91]. Phosducin, in mammals, supposedly through interactions with the $G\beta\gamma$ heterodimer functions to negatively regulate G-protein signaling. In *C. parasitica*, targeted deletion of *bdm-1* caused phenotypes that were similar to those in the *cpgb-1* ($G\beta$) deletion mutant, resulting in compromised infection, decreased conidiation, and enhanced growth rate indicating that *bdm-1* is necessary for proper $G\beta$ function. Simultaneous deletion of both genes caused poor or decreased buildup of *CPG-1* (to 10% of WT levels), implying that these genes probably function towards modulation of *CPG-1* post-translationally. In a more recent study, it has been reported that *bdm-1* is a phosphoprotein, which can potentially be phosphorylated by protein kinase 2. Absence of *bdm-1* phosphorylation, resulted in reduced fungal virulence and also decrease in the amount of $G\beta$ and *bdm-1* protein levels, thus implying a role for *bdm-1* in protein stability [92].

A regulator of G-protein signaling (RGS) was characterized, and its involvement in specific *cpg-1* regulation, conidiation, virulence and cryparin synthesis was demonstrated [93]. It is still not clear if the effect of hypovirus infection on fungal G-protein signal transduction is direct or

indirect, as yet, there is no proof for of a physical interaction between virus encoded factors and components of the fungal signaling cascade.

1.1.5 cAMP signaling in *Colletotrichum* spp.

A role for the cAMP signaling in pathogenicity and disease causing ability has been studied in several species of *Colletotrichum* (Figure 1.4). The genus *Colletotrichum* is amongst the most important genera of phyto-pathogenic fungi, and leads a ‘hemibiotrophic’ lifestyle that is a combination of an initial symptom free biotrophic phase followed by a necrotrophic phase with severe symptoms [94]. It is found worldwide, but mainly in the tropical and subtropical regions. *Colletotrichum* species cause economically important diseases (commonly referred to as anthracnoses) in grasses, cereals, vegetables, legumes and perennial crops, including fruit trees [95].

During the infection, *Colletotrichum* spores germinate giving rise to germ tubes that in turn develop into infection structures known as appressoria. In *C. lagenarium*, disruption of the function of the catalytic subunit of PKA, *CPK1* or the regulatory unit *RPK1* was found to have no effect on appressorium formation. Further characterization of the *rpk1* Δ mutant, revealed that the mutant possessed high PKA activity, but displayed significant reduction in vegetative growth, mycelial melanization, and conidiation [96]. Interestingly, the *rpk1* Δ mutant was non-pathogenic on whole plants cucumber assays, suggesting that hyper-activated PKA has negative effects on *C. lagenarium* disease causing ability. It was suggested that the inability of the *rpk1* Δ appressoria to penetrate the host most likely enhanced lipid metabolism during appressorium formation. However, on wounded cucumber plants the *rpk1* Δ strain successfully caused disease lesions. Takano et al. identified a growth-suppressor mutant from the *rpk1* Δ [96], which had lost its high PKA activity. However, the *rpk1*-suppressor was nonvirulent on cucumber, similar to the parent *rpk1* Δ . Genetic complementation assays indicated that the suppressor mutant was deficient in the gene encoding the PKA catalytic subunit.

In *C. lagenarium*, Yamauchi et.al studied the role of the adenylate cyclase gene (*CAC1*) and the catalytic subunit gene (*CPK1*) [97]. Interestingly, the *cpk1Δ* and *cac1Δ* mutants germinated poorly or hardly germinated at all, even after prolonged periods of incubation on suitable surfaces, indicating that cAMP or PKA signaling controls germination. Interestingly, the germination defect of the *cpk1Δ* and *cac1Δ* was partly suppressed when conidia were inoculated at lower concentrations or the conidia were less crowded. Although the conidia were capable of elaborating appressoria under less crowded conditions, the appressoria were non-functional. The authors also found that the *cpk1Δ* accumulated a greater number of lipid bodies compared to the WT and that deletion of *CPK1* slowed down the breakdown of lipid bodies within the appressoria, unlike the deletion of *RPK1* that enhanced it [97].

The presence of second PKA catalytic subunit has been suggested in addition to CpkA in *M. oryzae* [98]. In *C. lagenarium* the phenotype of the double mutant *cpk1Δ rpk1Δ* is similar to the single *cpk1Δ*, suggesting the absence of additional PKA catalytic subunits that are regulated by *rpk1*. Taken together, the phenotypes of the *cpk1Δ* and *cac1Δ* in *C. lagenarium*, suggest that while the cAMP-PKA signaling is important for appressorial functionality and *in-planta* infectious growth, it is dispensable for morphological development of appressoria.

In another study carried out in a different species namely *C. trifolii*, by Yang & Dickman, PKA activity was measured in various fungal tissues like the mycelium, conidia, germinating conidia and appressoria [99, 100]. PKA activity was found to be the highest in germinating conidia, but was lower in ungerminated conidia and basal in mycelia and appressoria. PKA inhibitor KT5720 inhibited conidial germination and appressorium formation. Furthermore elevation of endogenous cAMP levels through pharmacological agents caused appressorial development on water agar (1.5%, a non-inductive surface), thereby implicating a role for cAMP signaling in surface induced signaling responses. The authors further investigated and characterized the functions of the regulatory and catalytic PKA subunits in *C. trifolii* [100]. It was found that both genes existed as single copies and were developmentally regulated i.e., the *Ct-PKAC* transcript displayed greatest levels of transcript in conidia and lowest accumulation in mature appressoria [100].

Interestingly, although the *Ct-PKAC* mutant strain had negligible PKA activity, it did not show very strong defects. The *Ct-PKAC* mutant displayed a negligible decrease in growth and a minor delay in conidial germination and appressorium formation. Furthermore, addition of exogenous cAMP to the PKA catalytic subunit mutant did not restore wild-type phenotype, implying that in *C. trifolii*, *Ct-pakc* is accountable for the bulk of PKA activity. The *Ct-pakc* Δ strains also failed to infect intact host plants, although they formed appressoria. However, the *Ct-pakc* Δ could cause disease and colonize wounded plant tissues implying that the strain was compromised or defective at the stage of appressorial penetration.

Truesdell et al identified *ctg1*, in *C. trifolii*. The gene encoded a $G\alpha$ subunit that displayed high amino-acid similarity to *CPG-1* from *C. parasitica* and *MagB* from *M. oryzae*[101]. Deletion of *ctg1* caused slower growth and a reduced rate of spore germination compared to the WT. In addition, the mutants were also displayed poor efficiency of appressorium formation. It is not clear if Ctg1 influences adenylyl cyclase activity, although exogenous supplementation of cAMP failed to increase the frequency spore germination in the deletion mutants. Overall, the results suggest that in *Colletotrichum spp* appropriate control of PKA activity is important for growth, asexual development and appressorium function.

1.1.6 cAMP signaling in *Fusarium spp*.

Fusarium species are important maize infecting fungal pathogens and found extensively in the soil. It causes the economically important fusarium stalk rot, ear rot and seedling blights in maize. In addition, it produces potentially toxic mycotoxins such as fumonisin B₁, which are harmful to both humans and livestock. In order to decipher the role of cAMP signaling cascade in this species, Choi and Xu, identified orthologs of *M. oryzae* PKA catalytic sub unit, *CPKA* and adenylyl cyclase (*MAC1*) genes in *F. verticillioides* [102]. The orthologs from this *Fusarium* species were named as *CPK1* (for catalytic subunit of PKA 1) and *FAC1* (*F. verticillioides* adenylyl cyclase 1) respectively (Figure 1.4).

Compared to the WT strain, the *cpk1Δ* and *fac1Δ* mutants displayed decreased radial growth, although the *fac1* delete strain was more severely affected. The *fac1Δ* mutant was completely abolished for macroconidiation production although it produced fewer micro conidia. The *cpk1Δ* was also defective in macroconidiation. Not all the phenotypic defects displayed by the *fac1Δ* mutant were displayed by the *cpk1Δ*, suggesting that additional catalytic subunits of PKA exist in *F. verticillioides*. In fact *CPK2* was identified as an ortholog of the second catalytic subunit *cpk2* present in *M. oryzae* [98]. Generally, *CPK1* homologs in various filamentous fungi seem to be causal for most of the PKA catalytic activity, while *CPK2* orthologs possess redundant or overlapping function. This was well exemplified in this study, where it was observed that macroconidiation was defective in both the *cpk1Δ* and *fac1Δ* mutants however, only the *fac1Δ* mutant was also defective in micro conidiation, suggesting that *CPK2* is more important for microconidiation than during macroconidiation [102].

Compared to the wild type, the *fac1Δ* mutant was more sensitive to carbon starvation, however it was more tolerant to heat and oxidative stress. In corn stalk rot assays, the *fac1Δ* caused limited necrosis only at the site of inoculation, failing to spread to nearby corn-stalk tissue, unlike the *cpk1Δ*, which was only slightly reduced in virulence. The phenotype of the *cpk1Δ* in *F. verticillioides* is in contrast to data from other plant pathogens, where the PKA catalytic sub unit homologs are required for full virulence [102]. In a related study, Zhao et.al independently characterized the gene *fpk1* that codes for the catalytic subunit of cPKA in *F. verticillioides*. The *fpk1Δ* strain showed reduced vegetative growth, fewer and shorter aerial mycelium, impaired conidiation and decreased spore germination rate. Supporting the findings by Choi and Xu, when the *fpk1Δ* was inoculated in susceptible maize plants, the infection was delayed and significantly reduced in pathogenicity compared to the wild- type [102, 103].

F. oxysporum, also known as the vascular wilt fungus, is also a soil borne fungus that causes economically important diseases such as vascular wilt, damping off and root rot disease in many crops. It is also an opportunistic human pathogen [104, 105]. The fungus typically invades the xylem, causing the disruption of the vascular system. Unlike foliar pathogens, which cause disease symptoms that are readily visible, the disease caused by *F. oxysporum* is concealed within the soil making it challenging to recognize disease symptoms and study its progression.

To analyze the function of G-protein related signal transduction in the regulation of pathogenicity and other physiological characteristics, Jain et al deleted *fga1*, the gene encoding a G α subunit in *F. oxysporum* [106]. *FGA-1* displayed high similarity to *CPG-1* in *C. parasitica*, Ctg1 from *C. trifolii*, and MagB in *M. oryzae*. When compared to WT, the *fga1* mutant displayed typical vegetative growth and germination frequency but was compromised for conidiation and pathogenicity. However, it was more resistant to heat stress. Moreover, the *fga1* Δ had decreased levels of intracellular cAMP, implying that the *FGA-1* plausibly facilitates adenylyl cyclase activation [106]. A second G α subunit *fga2*, belonging to the class III fungal G protein was also functionally characterized. The *fga2* mutant was more resistant to heat stress, but unlike the *fga1* mutant, was non-pathogenic. The *fga1/fga2* double mutants were similar to the *fga1* or *fga2* single disruptants, but were more heat resistance than each single disruptant [107].

The same authors also identified and characterized the G-protein β subunit *Fgb1* in *F. oxysporum*. The *fgb1* Δ mutant showed no obvious defect during vegetative growth, however showed a distinct alteration in colony morphology and had an elongated colony phenotype i.e., the wild-type strain showed a round center of hyphae at the colony center while the *fgb1* Δ disruptant showed a long bundle of hyphae at the center [108]. In addition, although the conidiation of the *fgb1* Δ mutant was compromised, the germination rate of the *fgb1* Δ conidia was surprisingly enhanced compared to the wild type. Finally, similar to the *fga1* mutant, the bud cells from the *fgb1* Δ were more heat resistant and accumulated lower levels of cAMP (50-60%) in comparison to the WT control strain. Interestingly, exogenous addition of cAMP failed to rescue any of the phenotypes displayed by the *fgb1* Δ strain. The pathogenicity was also markedly reduced in the *fgb1* disruptants [108]. It is worth noting that most of the physiological alterations displayed by the *fga1* Δ were also detected in the *fgb1* Δ strains, except for the enhanced germination rate. These results suggest that *FGA1* and *FGB1* have distinct but overlapping functions during the development and pathogenicity *F. oxysporum*.

Interestingly, it was found that Fgb1 acts together with its cognate G α subunit to activate the cAMP-PKA cascade, unlike in *S. cerevisiae* and *C. neoformans* in which the G β sub-unit functions to activate the MAP kinase pathway. Additionally, Fgb1 acts to maintain steady-state levels of Fga1 that subsequently activates adenylyl cyclase and cAMP signaling. Furthermore,

Fgb1 was found to regulate crucial processes like polarized tip growth, cell elongation and sub-apical hyphal branching [109]

The role of the catalytic sub-unit of PKA from *F. oxysporum* (*FoCPKA*) in growth, morphology, penetration, efficiency of root attachment and pathogenesis in *Arabidopsis thaliana* was studied by Kim et.al, *cpkA* mutant strains were less efficient in attaching to the root surface and were additionally unable to penetrate and enter the root vascular system and thus non-pathogenic. However, the mutant could successfully colonize wounded roots. The mutant was additionally compromised for hyphal growth and spore production [110].

Overall, the results suggest that cAMP-mediated G-protein signaling regulates characteristics of growth, asexual development and virulence in *Fusarium spp.*

1.2 Regulators of G-protein mediated cAMP signaling

1.2.1 G-protein Coupled Receptors (GPCRs)

Organisms have developed sophisticated means to sense and adapt to the surrounding environment in response to a range of external cues. Cell surface receptors that include GPCRs form the largest protein family of transmembrane receptors. In many fungi, GPCRs represent important conduits for pheromone, nutrient, amino acid, nitrogen and ion sensing.

Despite displaying remarkable diversity in terms of sequence and biological function, members of the family have a general structure consisting of a 7 α -helical transmembrane domain and mechanism for signal transduction. The extracellular N-terminal portion of the GPCR in general interacts with the ligand, while the C- terminus stimulates the exchange of GTP for GDP on the G-proteins upon ligand binding. Such a nucleotide exchange on the $G\alpha$, results in its dissociation from $G\beta\gamma$ and subsequent activation of various downstream signaling pathways including the cAMP pathway through the adenylate cyclase.

On the basis of sequence identity and similarity of function, GPCRs have been classified into 6 major classes (A to F), with each class having specific characteristics. For example the secretin

and adhesion GPCRs (class B), contain characteristic conserved cysteine residues or cysteine rich domains similar to the epidermal growth factor (EGF) domain at the N-terminus. The following is the classification of GPCR families:

Rhodopsin-like superfamily- Class A, Secretin receptor family- Class B, Metabotropic glutamate/pheromone receptors- Class C, Fungal mating pheromone receptors- Class D, cAMP receptors- Class E and Frizzled/Smoothed receptors- Class F. However, the atypical sugar sensor in yeast, Gpr1 and its homologs is not included in this classification but included in a new class.

The human genome encodes close to 600 potential GPCRs. However, only a few have been identified in fungal genomes. For example, in *S. cerevisiae* only three and in *S. pombe* only four GPCRs have been characterized. Sixteen and 10 potential GPCRs have been identified in *A. nidulans* and *N. crassa* respectively. Remarkably, the rice-blast fungus, *M. oryzae*, expresses seventy-six GPCR- like proteins, of which sixty-one belong to a new class associated to Pth11-like proteins containing the characteristic CFEM domain (Figure 1.5) and a further 15 GPCRs that are orthologous to GPCRs reported in other fungi. This group of Pth11-like receptors is conserved only in Pezizomycotina subphylum, but not in Basidiomycetes or other Ascomycota subphylum [111].

N- Terminal CFEM domain:

ivtktpsCamkCftdaitsrnCslesaqlvadCiCvsnptlsta
atCvqksCkfseqlvarnltlflslck

Figure 1.5. Schematic of *M. oryzae* PTH11 illustrating the unique N-terminal cysteine rich CFEM domain.

The region highlighted in blue marks position of the CFEM domain (31-110aa) in the protein. Adapted and modified from [111].

1.2.2 Heterotrimeric G-proteins

G-proteins function to integrate external signals sensed by a membrane spanning GPCR to downstream intracellular effectors that include but not limited to adenylate cyclase, phospholipases, phosphodiesterases and ion channels [112, 113]. Due to their characteristic 3-subunit configuration and the presence of a Ras-like motif in the G α subunit, which can bind either GDP or GTP, these proteins are known as heterotrimeric G-proteins [114]. G-proteins typically consist of non-identical α , β , and γ subunits. Generally this complex is made up of a single guanine nucleotide binding unit namely the G α subunit (33-55 kDa) which forms a complex with one G β (~35 kDa) and one G γ (~15 kDa) subunit. G-proteins function as molecular switches for signal transduction upon binding of G α subunit to GDP, downstream effector binding sites are blocked by the G $\beta\gamma$ complex. However, on binding GTP three switch regions in the G α undergo a conformational change so that the G β and G γ subunits dissociate thereby rendering the G α subunit free to initiate signaling by activating downstream effectors [115, 116]. Effectors activation typically leads to the production second messengers or other biochemical alterations that lead to the stimulation of a downstream protein kinase cascades [117]. The G α subunit has intrinsic GTP-hydrolyzing capabilities, which ultimately results in the G α to revert back to its GDP bound inactive state (Figure 1.6).

On the basis of similarity in sequence and function, mammalian G α subunits are classified into 4 main families namely, G α_s , G α_i , G α_q and G $\alpha_{12/13}$. G α_s subunits are involved in the stimulation and activation of adenylate cyclases; G α_i inhibits adenylate cyclases, but stimulates G-protein coupled inwardly rectifying potassium channels (GIRK). G α_q are involved in the stimulation of phospholipase C, and G α_{12} stimulates Rho guanine-nucleotide exchange factors [117].

Mammals have 20 heterotrimeric G-proteins, each constituting a unique α subunit, in combination with one of the 5 β subunits and any of the 12 γ subunits [112]. Over the years G-proteins have been identified to carry out important functions in almost all experimental models systems thus far used. So far, in fruit fly *Drosophila melanogaster*, 6 genes which code for G α subunits have been annotated, more than 10 in *Dictyostelium discoideum* (slime mould), *Xenopus laevis* (frog) and *Danio rerio* (zebra fish) and 20 in *Caenorhabditis elegans* (worm), mouse and humans. Among the best-characterized G-protein cascades is the rhodopsin-transducin visual

system in mammals and the pheromone response pathway in yeast. In filamentous fungi, G-proteins were identified in the early 1990s; however their functions remained unknown for a long time largely due to the lack of genome sequence information.

Fungal Gα subunits - Filamentous fungi possess three Gα proteins compared to yeasts, which have only two [118]. Each Gα subunit is a member of a distinct group. Group I Gα proteins are conserved in filamentous fungi and are believed to control a multitude of pathways. Most filamentous fungi have a single Group I Gα, but the *Rhizopus oryzae* genome codes for 4 such proteins [119]. The first member of this group to be identified in filamentous fungi was *GNA-1* from *N. crassa* with similarity to the mammalian Gα_i superfamily [120].

Group II Gα proteins are poorly conserved in comparison to either the Group I or Group III Gα proteins. Only *S. pombe* appears to contain a Gα protein (Gpa1) that is identical to Group II proteins. In most fungi the roles of the Group II Gα proteins is not fully understood [119].

Group III Gα proteins however are conserved and are identical to the mammalian Gα_s-analogous group, as members of this group mainly function to regulate cAMP levels [118]. For example, during primary stages of germination, *A. nidulans* *ganB* controls rapid and transient increase in cAMP levels while responding to glucose. The *ganB* mutants are defective in germination kinetics and trehalose metabolism; the latter event also linked to cAMP signaling [121]. In *C. neoformans* Gα- *gpa1* mediates cAMP synthesis in response to glucose. *Gpa1Δ* mutants are blocked for virulence factor (melanin) synthesis and capsule production, both of which are rescued by exogenous cAMP [122]. Group III Gα *GNA-3* in *N. crassa* involved in the modulation of cAMP levels and PKA signaling [123]. In *U. maydis* Gpa3 has been shown to be necessary for mating and to mediate cAMP signaling [124].

Figure 1.6. A schematic depicting canonical G-protein signaling highlighting the roles of a Regulator of G-protein signaling, RGS (as a GAP) and an upstream receptor or GPCR as a GEF.

Upon binding of ligand, G-protein-coupled receptors (GPCR) respond to extracellular ligands and function as a guanine nucleotide exchange factor (GEF) allowing $G\alpha$ to exchange GDP for GTP. GTP-bound $G\alpha$ undergoes a conformational change and dissociates from $G\beta\gamma$. The $G\alpha$ and $G\beta\gamma$ subunits are free to activate downstream effectors. Adapted and modified from [125].

Fungal G $\beta\gamma$ subunits - G β subunits are conserved in filamentous fungi, but share lower homology with counterparts in yeasts. The majority of fungi have one G β subunit unlike *R. oryzae* that codes for 4 proteins. In general disruption of G β function in filamentous fungi causes defects during asexual and sexual life cycles. Contrary to mammals, filamentous fungi contain one G γ subunit, however the genomes of fungi like *Cryptococcus neoformans*, *Rhizopus oryzae*, *Coprinus cinereus* encode for more than one, usually 2-5 G γ subunits [125].

1.2.3 Desensitization and Down-regulation of G-protein/ cAMP signaling

1.2.3.1 Regulator of G-protein Signaling

Studies focusing on understanding the mechanism of desensitization of G-protein signaling led to the identification of a family of guanosine triphosphatase activating proteins (GAPs) for G α subunits known as RGS proteins [126]. The proteins that belong to this family contain a conserved ~130 amino acids motif (RGS box) that specifically interacts with G α -GTP subunit to facilitate an increase in its GTPase activity. These proteins may contain other motifs like the G β binding, membrane targeting, Ras binding and GoLoco motifs. RGS proteins function to terminate G-protein signaling, by adhering to and stabilizing the transition state during GTP hydrolysis, thereby increasing the intrinsic GTPase activity of the G α subunit [127-129]. RGS proteins can also phosphorylate GPCRs to inactivate signaling. In addition to modulating G-protein cascades, RGS proteins can stimulate G-protein activation and function as effector antagonists. Furthermore, RGS proteins can act as scaffolds for receptors, G-proteins and other effector molecules [130].

So far greater than 50 members of the RGS family have been discovered in mammals, 19 in *C. elegans*, 11 in *D. discoideum*, 10 in *D. melanogaster*, 1 in *A. thaliana* and 4 in *S. cerevisiae* [112]. Evidence for regulators of G-protein signaling initially came from studies in lower eukaryotes. However, the first RGS was discovered in *D. melanogaster* and plays an important role during glial differentiation [131]. In yeast, when RGS protein Sst2 was mutated, the strain became supersensitive to G-protein directed pheromone responses [132]. FlbA, was identified in *A. nidulans* as a negative regulator of G-protein signaling [133]. In mammalian cells, two

independent studies identified *GOS8* and RGS-GIAP [134, 135]. *GOS8* shares a conserved motif with proteins of mammalian origin. It was not until the discovery of Egl-10 in *C. elegans*, that regulates movement and egg laying behavior through $G\alpha_o$ [136], that it was appreciated that each of these proteins, namely Egl-10, Sst2, FlbA and GOS8, belong to a larger superfamily of proteins involved in signaling that share a conserved 130 aa core domain. These proteins were christened regulators of G-protein signaling and the highly conserved domain was termed RGS domain.

1.2.3.2 Phosphodiesterases

cAMP levels inside a cell are regulated by a tight balance between its synthesis by adenylate cyclases and hydrolysis by phosphodiesterase (PDEs). In mammalian cells, the activities of adenylate cyclases and PDEs have been shown to be regulated positively as well as negatively by G-protein subunits, calcium signaling systems, inositol lipids and receptor tyrosine kinases. PDEs provide the only route for hydrolyzing cAMP, thus providing a pivotal means of regulating intracellular cAMP levels [137]. PDEs are modulated at the transcriptional and translational level (representing a long term and sustained mode of regulation) and through modifications like phosphorylation/ dephosphorylation, allosteric binding, binding of calcium, and different protein-protein interactions [9, 138].

PDEs are broadly classified into three classes I, II and III based on the presence of a conserved cyclic nucleotide hydrolyzing catalytic domain in the C-terminus of the protein. Class I cyclic nucleotide hydrolyzing activity is present in protozoans and metazoans; the class II cyclic nucleotide hydrolyzing function is found in fungi, slime mould and amoeba; and a class III β -lactamase-type motif found to exist only in PDEs from *D. discoideum*.

Mammalian PDEs belong to Class I, and so far 21 genes coding for PDEs have been discovered. These have been further categorized into 11 different families on the basis of their similarities in terms of sequence, kinetics, means of modulation and pharmacological characteristics. Three of the eleven PDE families selectively hydrolyze cAMP, three families are discerning for cGMP

and the remaining five families breakdown both nucleotides with differing efficiencies [9, 139]. Most sequenced fungal genomes contain two different phosphodiesterases. The first PDE belongs to the class I (signature sequence: -H-D-[LIVMFY]-x-H-x-[AG]-x(2)-[NQ]-x-[LIVMFY]), and the second one is a member of the class II (signature sequence: -H-x-H-L-D-H-[LIVM]-x-[GS]-[LIVMA]-[LIVM](2)-x-S-[AP]).

The fungal class I PDE is named as PDE2 whereas the class II PDE is named as PDE1. Pde2 family members are high-affinity/ low-K_m (for cAMP) phosphodiesterases that are conserved in organisms ranging from yeast to mammals [140, 141].

1.3 The endosomal system as a platform for signal anchoring

The output of any given signaling cascade depends not only on the activation of signaling but also on the location and duration of signaling, i.e., where and for how long the signal is emitted. In general, when and where signaling is activated inside a cell has a profound implication on downstream responses and cellular behavior. As a result, cells have evolved complex molecular mechanisms to control the spatial and temporal dynamics of signaling pathways.

The endosomal system represents a highly organized network of dynamic and biochemically specialized membranous compartments. The endosomal system can be broadly segregated into early, late MVBs (Multi Vesicular Bodies) and recycling compartments, with each compartment inherently programmed to carry out a specific function (Figure 1.7) [142]. Endocytosis *per se* has for a long time been considered as a means of down-regulating signaling events occurring at the plasma membrane. Previously, it was thought that one of the major functions of endocytosis was to internalize activated receptor-ligand complexes from the cell surface and target them for degradation via lysosomes and proteosomes. It is however clear now that signaling is not restricted only to the plasma membrane but takes place at various intracellular locations. It was observed in the early nineties that shortly upon ligand stimulation, a significant population of stimulated epidermal growth factor receptors (EGFRs) along with downstream effectors such as Grb2 (Growth factor receptor-bound protein 2), Shc (adaptor protein) and mSOS (a Ras guanine

nucleotide exchange factor), were associated with the early endosomes but not the plasma membrane [143]. Subsequently, it was also demonstrated that the nerve growth factor (NGF) interacted with phospholipase (PLC- γ) and its receptor TrkA and on endocytic organelles [144]. Together these observations led to the ‘signaling endosomes’ hypothesis.

Utilizing endocytosis mutants it was demonstrated that prolonged residence of activated EGFR receptor on the plasma membrane led to compromised activity of downstream components [145]. Daaka et.al additionally observed reduction in MAPK activation upon blocking β_2 -adrenergic receptor (β_2 -AR), strongly suggested the possibility of preferential activation of some downstream signaling cascades upon internalization of ligand-receptor complexes into endosomal compartments. Furthermore, supporting the possibility that signal transduction continues to take place post endocytosis was the observation that Grb2 (an initiator of Ras and MAPK signaling) interacted with EGFR on endosomes [146]. Additionally using biotinylated antibodies it was demonstrated that plasma membrane and endosomal populations of activated EGFR preferentially associate with different pools of downstream effectors [147].

Figure 1.7. The endo-lysosomal system is a dynamic continuum of phosphoinositide rich membranous compartments that facilitate signaling.

Endocytic vesicles deliver their contents and membranes to Rab5 positive early endosomal compartments, following which maturation from early to Rab7 positive late endosomes (or MVBs) takes place. The late endosomes undergo homotypic fusion to grow in size and acquire more PI3P rich ILVs. Fusion of the late endosome with the lysosome (vacuole) results in the formation of a transient hybrid organelle, the endolysosome, in which active degradation occurs. The endolysosome upon maturation is further converted to a classical lysosomal compartment in mammalian cells. The endosomes utilize microtubule cytoskeleton for movement. Adapted and modified from [142].

Recent findings also suggest that the late endosomal compartments also function as signaling anchors or platforms integrating membrane trafficking and intracellular signal transduction. For example, in the yeast *S. cerevisiae*, the constitutively active G-alpha ($G\alpha^{Q323L}$) interacts with PI3 kinase on endosomes containing Snf7 [148]. MVBs facilitate Wnt signaling, by sequestering the inhibitor (GSK3) within the Intra Luminal Vesicles (ILVs) [149]. In another example, the free RI α subunit of cPKA is sequestered onto the surface of MVBs by A-Kinase anchoring protein, AKAP11 in a cAMP dependent manner [150]. Late endosomal integrity has also been shown to be crucial for amino acid and insulin stimulated mTOR signaling [151]. Wunderlich et.al found that localization of MP1- MAP Kinase to the late endosomal via protein p14 was required for complete activation of ERK1/2 at the later stages of signaling post activation at the plasma membrane. Mislocalization of p14-MP1 to the plasma membrane failed to activate signaling suggesting that the late endosomes act as a platform for signal propagation.

Signaling on endosomes provides a cell with a dimension of temporal regulation, as the duration/period of active signaling is a crucial parameter influencing the biological output. The half-life of active receptor-ligand complexes depends on the kinetics of receptor internalization and fate subsequent to endosomal loading. The extent of the signaling also depends on the proportion of receptors being degraded to the number of receptors being recycled back to the plasma membrane [152].

Another role that the endosomal system can potentially play is to target active signaling complexes to their sites of action or to specific locations within the cell. Endosomes rely on microtubules, Rab5 and PI3Kinase for their movement [153]. By modulating these molecules, signaling complexes can potentially regulate intracellular distribution, channeling to specific target sites and motility. For example in neurons, signals from axon terminals must be transmitted over long distances to arrive at the cell body. A model of simple diffusion may not be sufficient to spread the signal. Instead, signaling is made possible by microtubule-associated retrograde transport of active signaling complexes, for example NGF bound to its receptor TrkA [154]. Such active transport can potentially generate different outputs from a given signaling response. Localized signals are generated at the site of stimulation while more widespread cellular responses are produced in the cell body [155]. Finally, in addition to acting as long distance transport vehicles, endosomes aid selective targeting of signaling molecules to specific

organelles, scaffolding proteins or segregate them into smaller sub-domains within a given organelle.

Thus, by helping sort and traffic active signaling complexes within defined time scales, endosomes provide a temporal control of signaling. By providing a signaling platform for specific interactions or by anchoring active signaling moieties, endosomes add a spatial dimension to the regulation of signaling processes. Thus the endosomal system *per se* can help the cell integrate and achieve modularity of signaling responses and ultimately regulate the amplitude and temporal duration of signaling in a precise manner [156, 157].

1.4 General Introduction to *Magnaporthe oryzae*

1.4.1 Economic Importance and Life Cycle of *M. oryzae*

M. oryzae is a filamentous ascomycete and causes the devastating and economically important blast disease of cultivated rice and many other crops such as barley, wheat and millet. Additionally, it can cause disease in about 50 species of grasses and sedges, including the commercially important turf varieties [158-160]. The disease is endemic to all rice growing regions worldwide, is found in more than 85 countries [161] and is extremely difficult to eradicate and control. The blast disease is a serious and persistent threat to global food security. Annual crop losses caused by *M. oryzae* vary from 10-30% of the harvest, and it is estimated that grain sufficient to feed 60 million people is destroyed by the blast disease annually. Between 1975 and 1990, the blast disease has accounted for the destruction of 157 million tons of rice annually internationally. The current day disease management strategies against rice blast include approaches such as burning of crop residues, planting disease free seeds, limiting the use of nitrogen fertilizer, water-seeding, growth under continuous flooding, use of blast-resistant rice cultivars and widespread employment of fungicides. However, these approaches, although valuable and useful, have proven inadequate as a long-term strategy to control the disease [158].

Breakdown of resistance in rice cultivars usually occurs after two or three seasons of planting due to the limited disease resistance incorporated and also due to the constant evolution of new fungal populations. Although the use of fungicides to control disease progression has demonstrated a certain degree of effectiveness in controlling blast disease, resistant isolates of *M. oryzae* have rapidly emerged, questioning the long-term feasibility of such a strategy [162, 163].

Figure 1.8. Vegetative and pathogenic development in *M. oryzae*.

M. oryzae can undergo either vegetative or pathogenic development depending on the surrounding environmental conditions. Germination of conidia on non-host or non-inductive surfaces and differentiation into mycelium comprises of the vegetative phase (depicted in the panel above) and the pathogenic phase (shown in the panel below), that constitutes the initiation and establishment of the infection process. C-conidia, A-appressorium and IH- infection hypha. The EM images were reproduced from [164].

M. oryzae is a haploid fungus and can either grow as vegetative mycelia or displays pathogenic phase where it infects and causes disease in susceptible host tissues (Figure 1.8). It reproduces mainly by asexual spores or conidia, which are dispersed primarily by rain and wind. The blast disease can occur at all stages of development (including seedling and adult stages) and infects plant tissues such as leaves, stems, nodes and panicles. It has also been shown to infect root tissues utilizing special haustoria like structures known as hyphopodia, for host entry [165-167]. Foliar infection or the pathogenic phase of the life cycle commences as soon as a three-celled conidium lands on and attaches to the surface of the leaf by means of an adhesive secreted or released by the spore tip. The secreted adhesive or mucilage like substance helps the spore anchor or attach itself to the hydrophobic surface of the rice leaf. In the presence of high atmospheric humidity, the spore germinates, usually from its tapering end, to form a slender polarized germ tube [168, 169].

The germ tube represents a specialized structure involved in sensing host surface cues. As a response to host surface cues such as hydrophobicity, hardness, waxes and cutin-monomers [170, 171], the germ tube tip undergoes a chronological/time-dependent sequence of morphogenic transitions, switching from polarized to isotropic growth, ultimately forming a spherical/dome shaped infectious cell known as the appressorium. Unlike the vegetative phase, where the fungus only forms hyphae, the pathogenic phase or the infection cycle of *M. oryzae* is comprised of a series of distinct developmental and timely morphogenic transition/switching events [168, 169]. The different morphogenic transitions that the germ tube tip undergoes in response to inductive surface cues are depicted in Figure 1.9.

The sequence of developmental events and an approximate time line are as follows: conidial germination and host surface sensing (for inductive signals) by the germ tube (1-3 hours post inoculation, hpi); switching from polarized to isotropic growth and hooking of germ tube (4-6 hpi); establishment of a specialized infection structure; the appressorium (6-8 hpi); streaming of cytoplasmic contents into the appressorium; turgor generation or build-up within the appressorium; and forceful mechanical entry into the host epidermis through elaboration of an

Figure 1.9. An illustration depicting the life cycle of *M. oryzae*, focusing on morphogenic transitions during the pathogenic phase.

The sequence of developmental events and approximate time line are also depicted: germination of the conidium (1-3 hpi); switching from polarized to isotropic growth and hooking of germ tube (4-6 hpi); elaboration of an appressorium (6-8 hpi); breaching of host epidermis by a penetration peg (12-24 hpi) and ensuing invasive growth within the host tissue (36-48 hpi). Adapted and modified [172].

infection peg (12-24 hpi) and subsequent invasive growth and spreading within the host tissue (36-48 hpi) [171, 173, 174].

The appressorium is a highly specialized dome-shaped cell capable of causing infection. The development of a mature and functional appressorium involves the synthesis and accumulation of high concentrations of glycerol within the appressorial cell [175] and also the simultaneous deposition of a thick melanin layer between the cell wall and the membrane [176]. The melanin layer is believed to help reduce the porosity of the appressorial plasma membrane. In response to the high internal glycerol concentration, influx of water from the surrounding environment generates an enormous hydrostatic pressure, estimated to be 8 MPa, within the appressorium [168]. The mature appressorium utilizes this massive internal turgor pressure and translates it into a mechanical force to push a thin penetration peg through the host cuticle [169]. Once inside the host, the penetration peg swells and develops into infectious hyphae, which ramifies and colonizes neighboring epidermal or parenchyma cells [177].

The first visible symptoms of infection may appear as early as 4 days post infection. As the disease progresses and fungal hyphae spread within the host tissue, typical blast lesions appear on the surface of the infected tissue. On leaves, lesions are typically large and ellipsoidal with grayish centers. Older lesions additionally have brown colored margins. The invading hyphae in the lesions, from aerial mycelia known as conidiophores, and under conditions of high relative humidity, produce spores that serve as inoculum for successive rounds of the disease cycle.

The *in-planta* growth stage or invasive growth phase of *M. oryzae*, has received a lot of attention in the recent past. On breaching the host epidermis, the penetration peg differentiates into a structure known as the infection hypha. Infectious growth or invasive phase involves an extended biotrophic stage, during which the fungus obtains its nutrients from the host tissue. Later as the disease lesions expand the fungus switches to a more aggressive necrotrophic mode of growth, ultimately killing the host. *M. oryzae* is therefore referred to as a hemibiotrophic fungus. During the initial stages of biotrophic growth, the invasive hypha within the host tissue is surrounded by or enclosed within an invaginated plasma membrane of the plant, known as the Extra Invasive Hyphal Membrane (EIHM). The EIHM becomes tightly sealed around the invasive hyphae, shielding it from the bulk apoplast [178]. Recently, the EIHM was observed to be tightly

opposed to the intracellular fungal hyphae as visualized by using transgenic rice plants in which the rice plasma membrane was tagged with GFP [179]. The primary infection hyphae transforms into secondary bulbous hyphae that continue to spread within the initial epidermal host cell until it is completely filled with fungal hyphae. *M. oryzae* makes use of the plasmodesmata at the pit field sites to spread to and colonize the neighboring host cells [178].

During the biotrophic phase *M. oryzae* secretes a number of effectors, either proteins or small molecules, to suppress host defense responses [180-182]. Based on software prediction tools and analysis it has been estimated that nearly 1500 effectors are secreted by *M. oryzae* and this represents almost 12% of the total number of proteins coded for by the *M. oryzae* genome [172]. Among the best studied effectors is Avr-Pita, a putative metalloprotease, which has been shown to directly interact with the corresponding resistance gene (R) product Pita in the host cytoplasm [183, 184]. Other secreted effectors, *PWL1* [185], *PWL2* [186], and *AVR-CO39* [187]. Recently, a group of effector candidates with no apparent avirulence activity have been described based on differential expression patterns during biotrophic growth. These genes have been referred to as Biotrophy Associated Proteins 1-4 (BAS 1-4) [181]. A commonality between all the effector proteins is their accumulation in an *in planta* structure called the biotrophic-interfacial complex (BIC), at designated sites within the EIHM compartment surrounding the infection hyphae [178]. BICs are believed to play a central role in the secretion of effectors into the host cell cytoplasm.

In addition to being an agriculturally and economically important pathogen, *M. oryzae* also serves as an excellent model system to study fungal phyto-pathogenicity and host-pathogen interactions. *M. oryzae* can easily be cultured, propagated and maintained under laboratory conditions on defined media facilitating biochemical, molecular genetics and cell biology related analyses. Although not commonly observed in nature, the sexual cycle can be induced in the laboratory permitting the use of classical genetic techniques to study fungal biology as well as analyze mutant phenotypes. The early stages of pathogenic development such as germination, appressorium formation and penetration can be readily assessed *in vitro* utilizing artificial substrates. Furthermore, the infection-deficient mutant phenotype can be analyzed under greenhouse conditions or under laboratory conditions using simple detached leaf assays or whole plant assays. It is also possible to cytologically and biochemically monitor and evaluate distinct

events of the pathogenesis cycle in the laboratory on artificial substrates or on leaf tissues. Extensive tools have been established for molecular genetic manipulation. DNA mediated transformation protocols are also well established. Whole genome annotation of *M. oryzae* along with the sequenced genome of rice is publicly available. In addition numerous genomic and cDNA resources are available along with collections such as ESTs, BACs and physical maps.

Furthermore, the attractive features of *M. oryzae* as being genetically tractable and amenable to molecular manipulations has been adequately demonstrated by the popularity of *M. oryzae* as a model phyto-pathogen and also corroborated through the extensive research carried out on pathogenicity genes identified using different approaches [188, 189].

1.4.2 Role of cell signaling during pathogenesis in *M. oryzae*

A detailed and comprehensive study of the development during *M. oryzae* pathogenesis has resulted in the identification of many proteins involved in key processes and has further helped delineate the important steps involved. Compared to the little that is known regarding the mode or mechanism of perception of external environmental stimuli *per se*, the intracellular signaling pathways involved in the development of appressorium initiation, morphogenesis and maturation are relatively better understood. Among the various steps involved in pathogenic development, one of the most critical steps takes place during the early phases of development and is known as the “hooking” stage where the germ tube tip switches from polarized to isotropic growth (4-6 hpi) and subsequently forms a mature functional appressorium. It has been suggested that germ tube hooking constitutes a “recognition phase” during which host or inductive surface cues/characteristics are sensed and assessed prior to commitment to appressorium formation [190].

Environmental cues in the form of either thigmotropic stimuli or chemical signals are believed to be transduced into the germ tube likely through upstream GPCRs via separate and distinct signaling pathways. Signaling cascades mediated by cAMP and mitogen activated protein kinases (MAPK) play a central role in appressorium initiation [191]. Experimental evidences

suggest that cooperatively the cAMP and MAPK pathways function to regulate appressorium formation in *M. oryzae*. The cAMP pathway is activated early on during the surface recognition or the appressorium initiation phase while the MAPK cascade regulates the later stages of appressorium formation, namely appressorium maturation and *in-planta* growth [192].

1.5 Intracellular signal transduction and appressorium formation

1.5.1 cAMP signaling and appressorium formation in *M. oryzae*

A landmark study identified the requirement of a cAMP-dependent signaling cascade during formation of appressoria [193]. It was subsequently reported that exogenous supplementation with cAMP induces appressorium formation in conidia inoculated on non-inductive surfaces [194]. Following these findings, the proteins implicated in cAMP signaling cascade were discovered by reverse genetic approaches and characterized in detail (Figure 1.10).

Firstly, the gene encoding adenylyl cyclase (*MAC1*) was cloned, and gene deletion strains were generated and characterized. *mac1Δ* resulted in non-pathogenic mutants that were not able to form appressoria on surfaces that are hydrophobic, such as artificial membranes as well as onion epidermis or rice leaves [195]. The inability of the *mac1Δ* mutant to form appressorium or cause disease was rescued via exogenous cAMP, confirming the role of the Mac1 enzyme in production of cAMP and the subsequent role of the cAMP in pathogenicity [98, 195].

MPG1 codes for a hydrophobin that is highly expressed and secreted during germ tube extension. It has been suggested that hydrophobins aggregate spontaneously at the periphery of the cell to form an amphipathic surface rodlet layers [196, 197]. Most hydrophobins including Mpg1 are implicated in conferring hydrophobicity to fungal surfaces [198, 199]. Furthermore, it has also been proposed that hydrophobins additionally aid in fungal spore attachment, by enhancing the wettability of the surface of the leaf and thereby enhancing the effectiveness of hydrophilic mucilage secreted at the germ tube-leaf interface [200]. *mpg1Δ* mutant shows reduced ability to form appressoria and were weakly pathogenic. Application of cAMP however

Figure 1.10. The cAMP signaling pathway is important for pathogenesis in *M. oryzae*.

The proteins and transcription factors characterized to act in the signaling cascade in *S. cerevisiae* and *M. oryzae* are compared and contrasted. Adapted and modified from [27].

restored the ability to form appressoria as well as pathogenicity related defects, strongly suggesting that germ tube surface attachment mediated by Mpg1 is a pre-requisite for the signaling cascades involved in the regulation of appressorial morphogenesis [201].

PTH11 encodes a membrane-localized protein, initially predicted to contain nine transmembrane loops, and believed to function as a receptor for inductive surface cues. *PTH11* mutants are impaired in appressorium formation and disease-causing ability. Interestingly, exogenous cAMP suppresses such defects implying that Pth11 mediates appressorium differentiation by likely activating downstream intracellular signaling pathways [202]. Pth11 contains a N-terminal extracellular cysteine-rich EGF (Epidermal Growth Factor)-like domain known as the CFEM domain (Common in Fungal Extracellular Membranes), representing a novel category of G-protein coupled receptor-like proteins specific to filamentous ascomycetes [203]. *M. oryzae* possesses an expanded family of non-canonical GPCRs predicted to play a vital role in responding to a number of environmental cues [111, 172].

Lately, *CUT2*, a gene coding for a cutinase, was demonstrated to act upstream of the cAMP-PKA pathway [164]. The *cut2Δ* mutant was compromised during conidiation and virulence. Furthermore, on hydrophobic surfaces the *cut2Δ* mutant formed multiple germ tubes with aberrant appressoria. The defects of the *cut2Δ* were however restored by the addition of cutin monomers, cAMP or IBMX. It has been proposed that the products of hydrolysis produced by Cut2 may act as ligands recognized by signaling receptor(s) such as Pth11 in *M. oryzae* [164].

Heterotrimeric G-proteins function to transmit environmental signals relayed by upstream GPCRs to the downstream cAMP response pathway. As the name suggests trimeric G proteins are made up of α , β and γ subunits, which directly interact with a membrane spanning receptors [118]. On binding GTP, the $G\alpha$ subunit disassociates from a possibly inhibitory interaction with the $\beta\gamma$ subunits, and interacts with or activates downstream effectors [118]. Three $G\alpha$ subunits have been characterized in *M. oryzae* so far, namely MagA, MagB and MagC [204] belonging to group III ($G\alpha_s$ superfamily), I ($G\alpha_i$ superfamily) and II, respectively. Furthermore, one $G\beta$ subunit, Mgb1 [205] and one $G\gamma$ (Mgg1) subunit [206] have also been characterized. Surprisingly, characterization of gene deletion strain of *MAGA* revealed that its absence failed to affect

growth, formation of conidia and appressoria [204]. On the contrary, the mutant carrying the dominant active allele of *MAGA* elaborated appressorium on non-inductive surfaces, raising the possibility that MagA does contribute to regulating cAMP levels. Similarly, deletion of *MAGC* does not cause a strong defect. The *magCΔ* mutant is reduced in conidiation, but is not defective in vegetative growth or infection-related development. However, preliminary results suggest a role of MagC during vegetative growth in the maintenance of mycelial hydrophobicity [207]. Deletion of *MAGB*, belonging to the inhibitory $G\alpha_i$ subfamily, leads to significantly decreased vegetative growth, conidiation as well as appressorium formation [204]. However, the dominant active form of MagB forms appressoria on non-inductive surfaces. Interestingly, cAMP addition to *magBΔ* mutant restores appressorium formation to wild-type levels, suggesting that MagB may act upstream of the cAMP pathway, and may be important to repress the formation of appressorium on non-inductive surfaces. This observation also suggests that under certain circumstances MagB may possibly function as a regulator (likely negative) of adenylate cyclase, given its categorization to the inhibitory group I protein subfamily ($G\alpha_i$) [191, 204, 208]. Alternatively, it is possible that MagB stimulates cAMP synthesis rather than inhibits it.

MGB1 (*M. grisea* G-protein beta subunit) encodes a protein with 7 WD repeats characteristic of $G\beta$ subunits. *MGB1* mutants showed decreased conidiation and the conidia were compromised in appressorium formation, function and invasive growth. Interestingly, addition of cAMP rescued appressorium formation defects in the *mgb1Δ* strains. However, the appressoria formed were aberrantly shaped and did not penetrate. In addition, the *mgb1* mutants accumulated lower levels of intracellular cAMP. Based on the phenotype of the $G\beta$ mutant, it has been proposed that Mgb1 functions to activate the adenylate cyclase (Mac1) and furthermore regulates multiple aspects of infection-related morphogenesis by integrating cAMP and *PMK1*- MAPK signalling pathways [205].

MGG1 (*M. grisea* G-protein gamma subunit) codes for a protein with a prototypical G-protein gamma like domain (GGL) and a CAAX motif at the C-terminus. Gene deletion strains display phenotypes similar to those exhibited by the *mgb1Δ* mutant. The $G\gamma$ -disruption mutant was compromised in the ability to form proper appressorium as well as host penetration and *in-planta* growth. Furthermore, similar to the *magBΔ* and *mgb1Δ* strains, the *mgg1Δ* strain was rescued for

appressorium formation upon exogenous supplementation of 8-Br-cAMP. However, the appressoria were not functional. It has thus been speculated that *MGG1* may function in a manner identical to *MGB1* to regulate appressorial formation and *in-planta* growth [206].

In contrast, it has also been proposed that under certain conditions, the G $\beta\gamma$ complex in *M. oryzae* may function to inhibit adenylate cyclase activity, and furthermore in the absence of the G α subunit, the G $\beta\gamma$ subunit can potentially constitutively represses adenylate cyclase activity thereby preventing appressorium formation [171]. Bosch et.al has recently implicated the activation of MagB as a crucial step for surface hydrophobicity sensing, likely through the activity of a GPCR.

RGS proteins function to negatively regulate G-protein signaling [127, 134, 209, 210]. RGS proteins mainly function as GAPs which activate the conversion of GTP to GDP on the active G α subunit. In *M. oryzae*, the *RGS* gene (*RGS1*) deletion mutant was defective in conidiation, inductive surface signaling and pathogenicity [207, 211]. Rgs1 was also shown to directly interact with all the 3 G α subunits including the constitutively active form of MagA, implying that Rgs1 likely negatively regulates active MagA- GTP dependent adenylate cyclase activity [207]. Rgs1 interaction with MagB suggests that it negatively regulates MagB during asexual development or conidiation. A recent study has further identified seven additional RGS and RGS like proteins (MoRgs2 to Rgs8) in *M. oryzae*. In addition to previously characterized functions, Rgs1 was found to influence the integrity of the cell-wall as well as surface hydrophobicity. All other RGS proteins, especially Rgs4 influenced cAMP levels, conidiation, growth, appressorium development and pathogenicity to different extents [211].

Phosphodiesterases (PDEs) are a family of highly conserved enzymatic proteins which hydrolyze cAMP and whose primary role is to control the intensity, specificity and timing of cAMP signaling [139, 212]. In *M. oryzae*, two phosphodiesterases, namely a high- (PdeH) and a low- (PdeL) affinity PDE have been characterized [213]. Compared to the minor role played by *PDEL*, the *pdeH* Δ strain displayed enhanced conidiation, precocious appressorial formation, loss of surface dependency, defects in cell wall integrity, significantly decreased *in planta* growth and disease progression [213]. Additionally, the *pdeH* Δ strain accumulated increased levels of cAMP

in comparison to *pdeL* Δ or WT. While GFP-PdeH was predominantly cytosolic and peri-nuclear, GFP-PdeL was nuclear. Overall, PdeH plays a crucial and major role in regulating diverse facets of asexual and pathogenic development and the biphasic cycling of cAMP signaling that occurs during the early and late phases of appressorial formation [213]. Thus, together with Rgs1, PdeH serves as an additional negative regulator of cAMP signaling, thereby regulating important developmental events related to asexual and pathogenic development.

Recently, the function of Ric8 was characterized in *M. oryzae*. Ric8 acts as a non-GPCR GEF for some G α subunits. It is a highly conserved protein and its function has been previously studied in *C. elegans*, *Mus musculus* and *D. melenogaster* [214]. The *M. oryzae* mutant was non-pathogenic and disrupted for cellular differentiation related to sporulation, sexual development and plant infection. In *M. oryzae*, Ric8 acts along the cAMP pathway via interaction with MagB, thereby regulating appressorium formation on hydrophobic surfaces [215].

cAMP binds to the regulatory subunit of cAMP dependent protein kinase A (PKA), ensuing the separation and activation of the catalytic subunit, encoded by *CPKA* in *M. oryzae*. The PKA regulatory subunit in *M. oryzae* was identified as a by-pass suppressor mutant of the *mac1* Δ mutant, and termed *mac1* Δ *sum1-99* (*SUM1*). The mutation was traced to a single point in the cAMP-binding motif of the regulatory subunit of PKA, resulting in the continuous activation of the catalytic CpkA [98]. The constitutively active CpkA resulted in the complete restoration of appressorium formation in *mac1* Δ strain. The *mac1* Δ *sum1-99* showed faster conidial germination, germ tube growth as well as appressorium development. However, pathogenicity related defects were not restored in the suppressor mutant, suggesting that the existence of opposing cAMP signaling cascades regulating development and disease formation [98]. However, loss of CpkA leads to a significant delay in the formation and maturation of appressorium [194, 216].

Indeed, recent analysis of the *M. oryzae* genome sequence has revealed that a second putative PKA subunit exists, although the role of which is yet to be determined during pathogenic development [172]. Thus, activation of PKA is necessary for the production of functional

appressoria. Many transcription factors recently identified and proposed to act downstream of the cAMP-PKA pathway include Mst1, Som1, Cdt1 and Pth12. [217-219]

1.5.2 MAP kinase signaling and formation of appressorium in *M. oryzae*

In addition to G-protein mediated cAMP signaling, the MAPK signaling cascade also plays a fundamental role in *M. oryzae* appressorial development and maturation. The pathway has also been well studied and the components well characterized [192]. So far, three distinct MAPK-encoding genes *PMK1*, *MPS1* and *OSM1* have been identified in this fungus (Figure 1.11). Among them, *MPS1* and *PMK1* play important roles in infection-related development [192, 220].

The first *M. oryzae* MAPK-encoding gene to be identified was *PMK1* (pathogenicity MAP kinase-1), *PMK1* is highly similar to *S. cerevisiae* MAP kinases, Kss1 and Fus3. Moreover *PMK1* complements functionally the mating defects of *fus3Δ kss1Δ* [192]. Although unaffected in vegetative, sexual development and asexual reproduction, the *pmk1Δ* mutants failed to form appressoria and failed to cause disease in rice plants. However, *pmk1Δ* was responsive to hydrophobic surfaces or exogenously added cAMP, undergoing germ tube hooking and terminal swelling, but unable to form appressoria. These observations suggest that Pmk1 operates downstream of the cAMP cascade for appressorial development, though this remains to be verified [192]. Furthermore, when *pmk1Δ* spores were inoculated on wounded tissue, they failed to grow invasively and cause disease suggesting a role for Pmk1 in *in-planta* growth.

The activation of Pmk1 MAP kinase occurs through Mst7 and Mst 11 proteins, homologs of yeast Ste7 and Ste11 respectively. *M. oryzae* lacking orthologs of these genes do not form appressorium on surfaces that are hydrophobic. When a dominant active allele of Mst7 was expressed in the *mst11Δ* background, it restored appressorium formation defect of the *mst11Δ* strain, indicating that Mst7 acted downstream of Mst11 in the MAPK signaling pathway.

Figure 1.11. The MAP Kinase signaling pathway in *M. oryzae*.

MAPK pathways regulate appressorium development, penetration peg formation, invasive development and *in planta* spreading. In *M. oryzae*, the PMK1 and MPS1 modules play an important role during infection-related morphogenesis and development. TF- Transcription factors are depicted in blue. Adapted and modified from [221].

Ste12 is a transcription factor that functions downstream of the *fus3* and *kss1* kinases in *S. cerevisiae*. *MST12* a gene homologous to *S. cerevisiae STE12* was functionally characterized in *M. oryzae* by Park *et al.* *mst12Δ* mutants, unlike *pmk1Δ* mutants, produced melanized appressoria. However, the appressoria formed by *mst12Δ* mutant was unable to mediate penetration and was non-pathogenic. Even when inoculated onto wounded plants, *mst12Δ* mutants failed to cause disease, and appeared to be unable to spread within the host [222]. The Pmk1 MAPK is therefore probably involved in regulating many targets, playing crucial roles during early (appressorium initiation and formation) as well as late (appressorium function namely penetration) stages of pathogenesis [171].

The second MAP kinase-encoding gene identified in *M. oryzae* was *MPS1* (MonoPolar Spindle 1), which was homologous to MAP kinase *SLT2* from *S. cerevisiae* with almost 85% similarity. *SLT2* is necessary for integrity of the cell wall during conditions of low osmosis [223, 224]. Mps1 can rescue the thermo-sensitive growth of *slt2* null mutants as well as the autolysis defect of *slt2Δ* mutants [220]. Mutants lacking *MPS1* in *M. oryzae* display increased sensitivity to cell-wall-digesting enzymes and are affected in conidiation and fertility. In contrast to the *pmk1Δ* mutant, *mps1Δ* is capable of forming mature appressoria, however, the appressoria are non-functional and fail to penetrate host tissues, and consequently are non-pathogenic and defective in infectious growth [220]. This indicates that Mps1 function is dispensable for appressorium formation but is critical for host cell penetration. Furthermore, it is likely that re-modeling of the appressorium wall, during the penetration stage, takes place through an Mps1-dependent pathway [190].

The third MAPK encoding gene identified in *M. oryzae* is *OSM1*. An ortholog of the yeast high-osmolarity-glycerol 1 (*HOG1* gene), which functions to regulate cellular turgor [225]. When cultured under hyperosmotic conditions, mutants deleted for the *OSM1* gene were susceptible to osmotic stress and also displayed morphological deficiencies [226]. Surprisingly, *osm1Δ* strain showed no obvious defects in accumulation of glycerol, generation of appressorial turgor and mutants were fully capable of causing disease [226]. This demonstrates that *OSM1* fails to regulate appressorium turgor generation in *M. oryzae*, a function likely controlled in a manner that significantly differs from mechanisms of turgor generation in eukaryotic cells [171].

1.5.3 Cross-talk between cAMP and MAP kinase signaling

The components involved in the MAP kinase and cAMP signaling cascades have been isolated and functionally characterized in yeast and several filamentous pathogenic fungi. In fungi, levels of cAMP are controlled by a balance between synthesis of cAMP by adenylate cyclase and hydrolysis by cAMP-specific phosphodiesterases. It has been demonstrated in many fungal pathogens that the cAMP and the MAP kinase cascades function to influence differentiation, filamentous growth and virulence [63, 227, 228].

In *S. cerevisiae*, mutants with enhanced levels of basal cAMP, or cells supplemented with cAMP, show increased filamentation [229]. These filaments are comprised of more, rounded cells rather than the conventional elongated cells, suggesting that the cAMP pathway may negatively regulate or antagonize MAP kinase signaling in some way [230]. It has also been demonstrated that the small G-protein Ras2 activates adenylate cyclase and the MAP kinase pathway to regulate haploid invasive growth. The Ras2 regulated cAMP-PKA cascade acts alongside the MAP kinase cascade (Kss1) to stimulate invasive and filamentous growth through the activation of *FLO11* that codes for a cell surface protein necessary for pseudohyphal growth [47]. The MAP kinase pathway acted through Ste12 to induce *FLO11* transcription while the cAMP/PKA pathway acted on Flo8 to activate *FLO11* transcription. Furthermore, the Ste12 reportedly has been predicted to have multiple sites of PKA phosphorylation, and over expression of Ste12 can suppress pseudohyphal defects in a few cases, overall suggesting that Ste12 is a likely a target of the cAMP/PKA pathway.

The cAMP-PKA and the MAP kinase signaling play a role in dimorphic switching, mating, and pathogenicity in the plant pathogenic fungus *U. maydis* [72, 231-234]. However, in a manner opposite to that in *S. cerevisiae*, lower levels of intracellular cAMP leads to filamentation [72, 74] as exemplified by adenylate cyclase (*uac1*) mutant in *U. maydis* that grows filamentously instead of budding and is additionally compromised for mating response and virulence [72, 234, 235]. Mutations in the *ubc3* MAP kinase gene (Ustilago bypass of cyclase 3) rescue the filamentous growth that is constitutive but not the pathogenicity defect of the *uacΔ* exemplifying the cross-talk between cAMP/PKA and MAP Kinase cascades. cAMP-PKA and the *ubc3* (*kpp2*)

mediated MAP kinase cascades are possibly linked at the level of the transcription factor Prf1, as PKA mediated phosphorylation of Prf1 is necessary for the expression of mating type genes [232].

The cAMP signaling and MAPK signaling also integrate at Crk1, a novel MAP kinase [236] related to the *S. cerevisiae* Ime2p. crk1 mutant acts as a suppressor of the filamentation phenotype of either *gpa3* Δ or the CpkA -*adr1* Δ . Furthermore, the transcription of crk1 is negatively regulated by cAMP signaling [237]. Muller et.al have also demonstrated that an activated allele of the small GTPase Ras2 requires the MAP kinase ubc3/kpp2 to cause a filamentous phenotype in *U. maydis*.

The role of the Cek1 (Kss1 ortholog) MAP Kinase cascade in yeast-hyphae transition in *Candida albicans* has been well characterized. Interestingly however increasing intracellular levels of cAMP by exogenous addition or treatment with cAMP phosphodiesterase inhibitors can also induce yeast-hyphae transition [238]. Furthermore, mutants deleted for *TPK2*, a gene coding for the catalytic unit of PKA, are blocked for yeast-hyphal morphogenesis and displays reduced virulence. In contrast, overproduction of *TPK2* stimulates formation of hyphae and invasion of agar [239]. Additionally, the defects of *tpk2* Δ are rescued upon overexpression of Cek1 MAP kinase or the downstream transcription factor Efg1.

Figure 1.12. Crosstalk amid cAMP and MAP Kinase signaling cascades in *M. oryzae*.

The cross-talk involving the components of the cAMP-PKA and Pmk1-MAPK pathway may occur at the level of the upstream G-proteins or downstream Transcription Factors. Adapted and modified from [221].

In *M. oryzae*, the cAMP-PKA cascade is implicated in surface sensing and appressorium initiation. On the other hand the Pmk1 MAP Kinase cascade controls formation of appressoria, maturation and function [192] (Figure 1.12). Incidentally, both the pathways regulate invasive or *in-planta* growth. However it is yet unclear how surface characteristics determined by the cAMP-PKA module are transmitted to the Pmk1MAPK signaling for controlling the formation of appressoria. It has been proposed that Ras proteins (Ras1 and Ras2), the Rho GTPase Cdc42, the G β subunit Mgb1, and the G α subunit MagB may act upstream and function to activate Pmk1 pathway, thus forming the link between the two signaling modules. It is also possible that cross talk between the two pathways occurs at the level of the transcription factors, although it remains to be experimentally verified [221]. Additional studies need to be carried out to precisely establish how the cAMP-PKA and the PMK1-MAP kinase signaling cascades coordinate the course of appressorium development and the process of infection.

Chapter II

2. Materials and Methods

2.1 Strains, growth, culture conditions, infection assay and reagents

2.1.1 *M. oryzae* strains, growth conditions and culture preservation

M. oryzae wild-type strain B157 was obtained from the Directorate of Rice Research, Hyderabad, India. The B157 strain was used as the background strain to perform gene deletion, gene targeting and gene tagging experiments described herein. *M. oryzae* strains were cultured on Prune agar medium at 28°C (PA; per liter: 40 mL prune juice, 2.5 g lactose, 2.5 g sucrose, 1 g yeast extract, 20 g agar, pH 6.5) or liquid complete medium (CM; per liter: 6 g yeast extract, 6 g casein hydrolysate, 10 g sucrose). Gene deletion mutants and fluorescently tagged strains were generated using *Agrobacterium tumefaciens* Transfer DNA (T-DNA) mediated transformation and were selected on CM agar containing hygromycin (250 µg/mL) or basal medium (BM; per liter: 1.6 g yeast nitrogen base without amino acids, 2.0 g asparagine, 1.0 g NH₄NO₃, 10 g glucose, 20 g agar, pH 6.0) containing ammonium glufosinate (50 µg/mL), depending on the selection marker used. Conidiation was induced by exposure to continuous light in the PA medium grown cultures. For quantitative analysis of conidiation, mycelial plugs were subcultured onto PA medium and incubated in the dark for a day, and then exposed to constant illumination at room temperature for seven days. The conidia were harvested by scraping the surface of the colonies with inoculation loops in the presence of sterile water and the fungal biomass collected in Falcon tubes (BD Biosciences, USA). The suspension was vortexed thoroughly to ensure maximum detachment of conidia from mycelia, and then filtered through two layers of Miracloth (Calbiochem, USA). The conidia were by centrifugation at 3000 rpm for 10 minutes at 28°C. The pelleted conidia were collected and washed 2-3 times and finally re-suspended in sterile water.

The conidia produced by a given colony were enumerated or quantified using a hemocytometer and was reported as the total number of conidia per unit area of the colony [conidia (x100/cm²)]. For the isolation of genomic DNA, total RNA or proteins, fungal biomass was obtained from

cultures grown at 28°C for 2 to 3 days in liquid CM (gentle shaking at 160 rpm). The mycelial biomass was harvested from the spent medium by filtering the cultures through two layers of Miracloth. The fungal mass thus obtained was further washed and rinsed with sterile water and finally dried partially between paper towels. The partially dried mycelium was ground in a pestle and mortar with liquid nitrogen, to obtain a fine powder that was mixed with appropriate buffers determined by the downstream applications.

Magnaporthe strains were preserved by placing sterile 5 mm² pieces of Whatmann filter paper alongside mycelial plugs on freshly inoculated PA medium. The subcultured strains were incubated in the dark at 28°C for another 5-7 days to allow the fungal mycelium to colonize the filter paper pieces. The filter paper bits colonized by the fungus were aseptically removed and placed in sterile envelopes, which were dried using dehydrated silica gel. The dried filter paper pieces were eventually stored at -20°C.

2.1.2 *E. coli* strains and growth conditions

E. coli strain MC1061 and/or XL-1 blue was used for routine molecular cloning and plasmid propagation. The transformed bacterial strains were selected on solid or liquid standard Luria-Bertani (LB) medium (per liter; 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.0) at 37°C, with appropriate antibiotic selection [Kanamycin (100 µg/mL) or Ampicillin (100 µg/mL)].

2.1.3 *Agrobacterium tumefaciens* strain and growth conditions

Magnaporthe transformants were generated utilizing *A. tumefaciens* (AGL1) mediated transformation protocol. AGL1 transformed with requisite T-DNA constructs were grown on either solid or liquid LB medium at 28°C, with Kanamycin (100 µg/mL) selection.

The activation of *A. tumefaciens* with acetosyringone (ACS) was performed in liquid Induction Medium (IM, per liter: K salts 10 mL, M salts 20 mL, NH₄NO₃ (20%) 2.5 mL, CaCl₂ (1%) 1 mL Glucose 5 mM, MES 40 mM, Glycerol 0.5% w/v; K salts: K₂HPO₄, 20.5, KH₂PO₄ 14.5%; M salts: MgSO₄·7H₂O, 3%, NaCl 1.5%, (NH₄)₂SO₄ 2.5%), and the transformation of *M. oryzae* with *A. tumefaciens* was performed on solid IM (per liter: K salts 10 mL, M salts 20 mL, Z salts

5 mL, NH_4NO_3 (20%) 2.5 mL, CaCl_2 (1%) 1 mL, Glucose 5 mM, MES 40 mM, Glycerol 0.5% w/v, FeSO_4 (0.01%) 10 mL, Agar 20g; Z salts: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, H_3BO_3 , $\text{Na}_2\text{MgO}_4 \cdot 2\text{H}_2\text{O}$, each at 0.01%), according to previously described methodology [240].

Agrobacterium was selectively killed with 200 μM Cefotaxime after the co-cultivation with *M. oryzae*. The composition of solid induction medium (IM) was according to the protocols described for plant transformation [240].

2.2 Molecular Methods

2.2.1 DNA related methods

2.2.1.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) amplification using genomic or plasmid DNA as template was performed by using either PTC-100 (MJ Research Inc, USA) or Bio-Rad (MJ Research Inc, USA). High Fidelity DNA polymerase (Stratagene, La Jolla, CA, USA) was used for the PCR amplification of fragments required during cloning experiments, while *Taq* DNA polymerase (Promega, Madison, WI, USA) was used for routine PCR amplifications. Oligo nucleotide primers used in the course of this study are listed in Table 1. Required restriction enzyme (RE) sites were introduced in the primer sequence to facilitate cloning of PCR products into specific plasmid vectors. The annealing temperature and extension time during the PCR was adjusted according to the primer GC content and the length of target fragment to be amplified, respectively. All other components for PCR reaction were used according to the manufacturer's instruction. Typically for a 50 μL PCR reaction, primers were used at a final concentration of 10 μM . dNTPs at a final concentration of 10 μM . Template 50-100 ng, enzyme and PCR buffer was used as per manufacturer's instructions.

2.2.1.2 Agarose gel electrophoresis and gel extraction of nucleic acid fragments

Agarose gels (0.8-1%) containing 0.1 $\mu\text{g/mL}$ ethidium bromide (Sigma-Aldrich, USA) or Gel Red were prepared by dissolving 0.8 or 1 g agarose (Bio-Rad, USA) per 100 mL 1X TBE buffer (Tris base 89 mM, boric acid 89 mM, EDTA 2.6 mM, pH8.3). Typically 0.8% agarose gels were

used for samples that involved genomic DNA and 1% gels were used for all other routine purposes. The cast gels were submerged in a running tank containing 1X TBE buffer. The DNA samples from PCR amplification or restriction enzyme digestions were mixed with 6X DNA loading buffer (50% sucrose, 10% SDS and 0.2% Orange G), and loaded into preset wells. Typically the agarose gels were run at 100V, whereas they were run at 50-70V for genomic DNA samples. Nucleic acid bands separated on agarose gels were visualized by UV transillumination, and fragment sizes were compared to that of a 1 kb DNA marker (NEB, USA). The gels were photographed and documented using a Bio-Rad Gel Doc system. The bands of desired size were excised using sterile blades and DNA extraction and purification was performed using Nucleospin Extract II kit (Machery-Nagel, Easton, PA) according to the manufacturer's instruction.

2.2.1.3 Bacterial Transformation by heat shock

Plasmid DNA or ligation mixes were transformed into chemical competent *E. coli* strain XL1-Blue or MC1061 by heat shock treatment. XL1-Blue and MC1061 were made chemically competent by the calcium chloride method [241] and stored in 100-200µl aliquots at -70°C until use. For transformation,

1. *E. coli* competent cells (100-200 µl) were thawed on ice for 5-10 minutes.
2. Purified plasmid DNA (50-100 ng) was added to the competent *E. coli* cells and incubated on ice for 10 minutes. For the transformation of ligation reaction mixes, competent cells added to the pre-cooled ligation mix.
3. The mix containing competent cells and DNA/ ligation mix was heat shocked at 42°C for 60 seconds and immediately transferred to ice, and incubated on ice for 5 minutes
4. Cells were allowed to recover in 800 µl Luria-Bertani (LB; per liter: 10 g tryptone, 5 g yeast extract and 10 g NaCl) medium and incubated at 37°C incubator at 220 rpm shaking, for 45 minutes.

5. Recovered cells (100 µl) were plated directly onto LB plates containing appropriate antibiotics (100 µg/mL Kanamycin or 100 µg/mL Ampicillin) for selection and incubated at 37°C overnight. In the case of transformation of ligation mixes, the recovered cells are pelleted at 3000 rpm for 4 minutes, and the cell pellet is resuspended in 100 µL of LB, followed by plating on selection plates and overnight incubation at 37°C.

After overnight incubation at 37°C, isolated colonies were inoculated in 3 mL of LB medium plus kanamycin. The inoculated cultures were incubated at 37°C with 220 rpm shaking for 12-16 hours. The overnight grown cultures were used for plasmid DNA extractions according to manufacturer's instructions using the Gene Aid plasmid extraction kit (Taiwan).

2.2.1.4 Transformation of *Agrobacterium* by electroporation

Recombinant T-DNA plasmid clones were transformed into electro-competent *Agrobacterium* strain AGL1 by electroporation. AGL1 cells were made electro-competent by the calcium chloride method (Sambrook, 1989) and stored in 50µL aliquots at -70°C until use. For transformation,

1. Electro-competent *Agrobacterium* cells (50 µl) were thawed on ice for 5-10 minutes.
2. Plasmid DNA (1-2 µL) was added to the thawed *Agrobacterium* cells and mixed well.
3. The mixture was transferred to a pre-chilled electroporation cuvette (Gap 0.2 cm, Bio-Rad, USA).
4. An electric pulse at 1.8 kV for 4.5-5 msec was applied using the Micro Pulser (Bio-Rad, USA) to transform the *Agrobacterium* cells.
5. The cells were immediately supplemented with 1 mL of chilled LB medium to aid recovery, incubated at 28°C with agitation at 160 rpm for 1 hour.
6. The recovered cells were pelleted at 3000 rpm for 4 minutes, and the pelleted cells were plated on LB plates containing 100 µg/mL kanamycin. The plates were further incubated at 28°C until isolated colonies appeared (typically 1-2 days).

2.2.1.5 *Agrobacterium* mediated transformation of *M. oryzae*

Gene deletion mutants and gene tagging strains in *M. oryzae* were generated by utilizing an *Agrobacterium* mediated transformation/co-culture protocol. Fungal transformants were selected on CM agar containing hygromycin or basal medium containing ammonium glufosinate, depending on the selection marker used. The recombinant plasmid constructs were electroporated into AGL1 strain as described above. The co-culture protocol is as follows,

1. Transformed AGL1 (a single colony, with the appropriate recombinant plasmid) was grown overnight in LB medium containing 100 µg/mL kanamycin at 28°C with gentle shaking at 160 rpm.
2. The overnight culture was diluted to OD₆₆₀- 0.15 and activated using liquid induction medium [IM; per liter: K salts 10 mL, M salts 20 mL, NH₄NO₃ (20%) 2.5 mL, CaCl₂ (1%) 1 mL, glucose 5 mM, MES 40 mM, glycerol 0.5% w/v; K salts: K₂HPO₄ 20.5%, KH₂PO₄ 14.5%; M salts: MgSO₄-7H₂O 3%, NaCl 1.5%, (NH₄)₂SO₄ 2.5%] with 100 µg/mL kanamycin and 200 µM acetosyringone (ACS) and incubated with gentle agitation at 160 rpm at 28 °C for 6 hours.
3. Activated AGL1 (100 µl) culture was taken and mixed with an equal volume of the conidial suspension (from the appropriate background strain, at a concentration of 10⁶ /mL). This mixture of activated AGL1 cells and *Magnaporthe* conidia was then placed on a sterile 0.45 µm nitrocellulose membrane, which was in turn placed on a solid IM plate containing 200 µM acetosyringone, 60 µg/mL Streptomycin and 100 µg/mL Ampicillin or Carbenicillin.
4. This mixture of activated AGL1 cells and *Magnaporthe* conidia atop the nitrocellulose membrane that was allowed to dry within the laminar air-flow cabinet. Upon drying the plate was sealed with parafilm and incubated at 28°C for 48 hours.
5. Following the 48 hour incubation, all growth on the filter membrane was scrapped with an inoculation loop into 2 mL of 1X PBS buffer, containing 200 µM cefotaxime, 60 µg/mL streptomycin and 100 µg/mL ampicillin or carbenicillin. The mixture was vortexed for a minute to obtain a homogenous solution of cells.

6. The mixture (200 μ l) was then plated equally onto ten selection medium plates containing either CM hygromycin (250 μ g/mL) or BM plates containing 40 μ g/mL ammonium glufosinate in addition to 200 μ M cefotaxime (to selectively kill the *Agrobacterium*), 60 μ g/mL streptomycin, 100 μ g/mL ampicillin.

7. The plates were then incubated at 28 °C until transformed fungal colonies were evident (typically 3-5 days).

The putative transformants were picked up and subjected to a second round of selection to eliminate false positives. Correct gene replacement events were confirmed by locus-specific or gene-specific PCR and southern blot analyses.

2.2.1.6 Genomic DNA extraction from *M. oryzae*

1. Fungal mycelia was cultured in liquid CM at 28°C for 2-3 days with constant agitation at 160 rpm, in the presence of 60 μ g/mL streptomycin and 100 μ g/mL ampicillin.

2. Mycelia was harvested by filtering through two layers of Miracloth (Calbiochem, USA) and subsequently washed and rinsed thoroughly with sterile distilled water twice to remove any traces of the medium.

3. The mycelium was dried by blotting away excess water by placing the biomass between layers of paper towels, and subsequently ground into a fine powder in liquid nitrogen using a pestle and mortar.

4. Genomic DNA was extracted using the MasterPure Yeast DNA Purification Kit (Epicentre, USA) following the manufacturer's instructions.

2.2.1.7 Southern blot analysis

1. Genomic DNA (2-5 μ g) extracted from putative transformants was digested with appropriate restriction enzymes for 12 h (at a suitable temperature), followed by an RNase treatment for 30-45 min at RT.

2. The restriction enzymes were heat-inactivated, and the digested genomic DNA was mixed with DNA loading dye and subjected to agarose gel electrophoresis on a 0.85% gel, run at 50V for 2-3 h.
3. After completion of the electrophoresis, an image of the gel along with a ruler was taken in a Gel Doc (Bio-Rad, USA) and documented for further reference.
4. The gel was transferred to a clean plastic tray and rinsed thoroughly with distilled water to remove traces of the TBE running buffer.
5. The gel was now treated and covered with a depurination solution (250 mM HCl) and incubated with gentle agitation at RT for 8-12 min. Following the incubation, the depurination solution was discarded and the gel was rinsed thoroughly with distilled water 3 times, for 15s each.
4. The gel was next soaked in a denaturation solution (0.5 N NaOH, 1.5M NaCl) with gentle shaking for 45 min at RT. Following the incubation, the denaturation solution was discarded and the gel was again rinsed thoroughly with distilled water 3 times, for 15s each.
5. The gel was next treated and incubated in a neutralization solution (1.5 M NaCl, 0.5 M Tris HCl, pH 7.5) with gentle agitation at RT for 45 min.
6. The digested DNA was transferred from the agarose gel to a Hybond-N+ nylon membrane (Amersham Biosciences, USA) using an overnight capillary blot transfer procedure. The nylon membrane was pre-wet in distilled water and then soaked in 10X SSC for 10 min before use.
7. The following day, the membrane with the transferred DNA, was subjected to UV cross-linking using a UV Stratalinker 2400 (Stratagene, USA) at 1200J for 1 min.
8. The membrane was pre-hybridized using hybridization buffer at 45°C for 1 h in a hybridization oven.
9. DNA probes were labelled using the ECL kit (Amersham Biosciences, USA). Briefly, 100-200 ng DNA, to be used probe, in a volume of 10-12 µl, was denatured for 5-8 min in a boiling water bath, and immediately cooled down on ice for 10 min. Equivalent volume of the labeling

reagent was added to the DNA. The same volume of glutaraldehyde solution was then added to the above mixture, which was mixed thoroughly using a pipette and incubated at 37°C for 10 min.

10. The labeled DNA probe was mixed with 1 mL of pre-hybridization buffer, and then added to the membrane incubated with the pre-hybridization buffer. Hybridization was performed overnight at 42°C.

11. Following hybridization with the DNA probe, the hybridization buffer was discarded and the membrane was washed with primary wash buffer (0.4% SDS and 0.5X SSC, pH 7.0) at 55°C for 15 min, twice.

12. The membrane was transferred to a suitable container and then washed with a secondary wash buffer (2X SSC, pH 7.0) three times (5 minutes per wash) at RT.

13. The membrane was incubated with a solution containing equal volumes of detection reagent 1 and 2, and incubated for 1-2 min at RT.

14. With the excess detection solution drained, the membrane was placed in a autoradiography cassette and exposed to a x-ray film.

15. After the desired duration of exposure, the x-ray film was removed from the cassette and developed.

2.2.2 RNA-related methods

2.2.2.1 RNA extraction using RNeasy Plant Mini Kit

1. Fungal mycelia were grown in liquid CM with 60 µg/mL streptomycin and 100 µg/mL ampicillin for 2-3 days, with constant shaking at 160 rpm at 28°C.

2. Mycelia was harvested by filtering through two layers of Miracloth (Calbiochem, USA) and then washed thoroughly with sterile DEPC- treated water twice to remove traces of the medium.

3. The mycelium was dried by blotting away excess water and then ground into a fine powder in liquid nitrogen using a pestle and mortar.

3. Total RNA was extracted with using the RNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's instructions.

4. The quality of the RNA was verified by checking the absorbance of the sample $A_{260/280}$ using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA)

2.2.2.2 RNA extraction using Trizol reagent

1. Mycelia grown in liquid CM was essentially processes as previously described, the fungal biomass was ground into fine powder in liquid nitrogen, and then re-suspended in 1 mL Trizol (Sigma, USA) and incubated at room temperature for 5 minutes.

2. Chloroform (200 μ l) was added into the sample and incubated at room temperature for 5 minutes.

3. The sample was centrifuged at 12000 rpm at 4°C for 15 min, the supernatant containing RNA was transferred to a fresh 2 mL Eppendorf tube.

4. Total RNA was precipitated by adding 250 μ l isopropanol and 250 μ l buffer containing 0.8 M sodium citrate and 1.2 M NaCl to supernatant, and further incubated at RT for 10 min.

5. The RNA pellet was collected after centrifugation at 12000 rpm at 4°C for 15 min.

6. The pellet was washed with 1 mL 70% ethanol, and air dried and re-suspended in 50~100 μ l DEPC-treated water.

7. The quality of the RNA was verified by checking sample absorbance at $A_{260/280}$ using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA)

2.2.2.3 Reverse transcriptase PCR

1. Purified total RNA was treated with DNase (Roche Diagnostics, Germany) and were verified as DNA free by using it directly as template in a PCR assay.

2. First-strand cDNA was then synthesized using 2 µg total RNA and AMV Reverse Transcriptase (Roche Diagnostics, Germany) in the presence of oligo_{dT}18.
3. As a loading control, tubulin was amplified from the synthesized cDNA.

2.2.2.4 Quantitative real-time PCR (qRT-PCR)

First-strand cDNA was generated using 2 µg of DNase-treated total RNA and AMV Reverse Transcriptase (Roche Diagnostics, Germany) in the presence of oligo_{dT}18. Quantitative real-time PCR reactions were carried out in a final volume of 10 µl, containing 25 ng of cDNA, 0.5 µM of each primer and 5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems) using 7900HT Fast Real-Time PCR system. The following PCR/cycling parameters were used, 50°C 2 min, 1 cycle; 95°C 10 min, 1 cycle; 95°C 15 s, 60°C 1 min, 40 cycles. Relative abundance of transcripts was analyzed by the $2^{-\Delta\Delta C_t}$ method [242] and average threshold cycle (Ct) normalized to β -tubulin transcript for each condition as $2^{-\Delta C_t}$. Fold changes were calculated as $2^{-\Delta\Delta C_t}$.

2.2.3 Recombinant DNA techniques

2.2.3.1 Plasmid vectors for targeted deletion, genetic complementation, over expression and gene tagging

A single step gene replacement strategy using homologous recombination was used to construct the gene deletion mutants described throughout this study. Briefly, DNA fragments, nearly one kilobase pair (1kb) long representing the 5' and 3' flanking sequences of each target locus was amplified by PCR and cloned using appropriate restriction enzyme sites to flank the selection marker cassette of one of the three T-DNA vectors, namely *HPHI* (hygromycin phosphotransferase gene) cassette in pFGL44, or the *BAR* (bialaphos resistance gene) cassette in pFGL97, or *ILVI* (sulfonyleurea resistant gene) cassette in pFGL385. The plasmid containing the cloned fragments was introduced into *A. tumefaciens* by electroporation and further into the requisite background strain of *M. oryzae* using protocols described later. The genomic DNA extracted from the putative transformants was used to perform locus specific PCR analysis to confirm gene replacement events, and Southern blot was used to further confirm the gene

replacements. The primers used for the amplification of the 5' or 3' flanking sequences are given in Table 1 (the RE sites chosen for cloning are underlined).

For genetic complementation of the *pdeHΔ*, an in-frame translational fusion of RFP-PdeH under the native promoter was generated; the *PDEH* gene promoter fragment (about 1 kb) and a 3.9 kb fragment constituting the entire *PDEH* coding sequence as well as the downstream 1 kb region was amplified from genomic DNA of the wild type. The RFP coding sequence was amplified from pDsRED-Monomeric-N1 (Clontech, USA). All the three PCR products obtained were cloned into pFGL97 using appropriate restriction enzymes to get pFGL-NT-Comp construct with resistance to ammonium glufosinate (Cluzeau Info Labo, France) as a fungal selectable marker. This construct was introduced as a single-copy insertion into the *pdeHΔ* strain. The primers used for the amplification of the fragments are listed in Table 1.

For expression of *GFP-PDEH* or *GFP-PDEL* in the WT background, the entire coding region of eGFP was amplified from pEGFP-N1 (Clontech, CA, USA). A 3.0 kb fragment encoding the complete *PDEH* coding sequence and a 2.6 kb fragment coding the entire *PDEL* sequence was amplified from WT genomic DNA as template. In both the cases the amplified PCR products (eGFP and *PDEH*) or (eGFP and *PDEL*) were cloned in-frame, under the constitutive MPG1 promoter and TrpC terminator in pFGL275 to obtain pFGL-PRO_{MPG1}-GFP-*PDEH* and pFGL-PRO_{MPG1}-GFP-*PDEL*, representing the final constructs, which were subjected to sequence analysis and subsequently introduced into *pdeHΔ* and WT background strains respectively via *Agrobacterium*-mediated transformation. Fungal transformants were selected based on resistance towards ammonium glufosinate. Transformants were screened for GFP expression and confirmed by sequencing genomic DNA and Southern blot analysis. The primers used for the amplification of the fragments are listed in Table 1.

Plasmid constructs for the expression of *RGS1*-mCherry fusion

The *RGS1* gene was tagged with mCherry at the C-terminus since the N-terminal DEP-DEP domain is known to specify membrane targeting [243]. For the expression of *RGS1-mC* (mCherry) in the WT or requisite background strains, the mCherry fragment was PCR- amplified from p-mCherry (BD Biosciences Clontech, USA). The RP27 promoter and the *RGS1* gene (MGG_14517) were amplified using PCR from genomic DNA from the WT strain as template,

while the Ccg1 promoter was amplified from pMF272 (a kind gift from Prof. M. Freitag). The TrpC terminator was amplified using pFGL275. The PCR products obtained were digested with appropriate restriction enzymes (New England Biolabs, Beverly, MA) and purified using the gel elution Nucleospin Extract II kit (Machery-Nagel, Easton, PA). Using a three way ligation approach and suitable restriction enzyme sites the mCherry fragment (*SmaI/BamHI*) along with the TrpC terminator *BamHI/XbaI* were cloned into appropriate background vectors using appropriate enzyme sites [pFGL44 vector (encoding hygromycin phosphotransferase gene (*HPH1*) or pFGL97 (encoding ammonium glufosinate gene (*BAR*)] to obtain pFGL44/97-mCherry-TrpC terminator. The *RGS1* fragment (*EcoRI/SmaI* fragment) along with the RP27 (*EcoRI-EcoRI*) or Ccg1 (*EcoRI-EcoRI*) promoters was cloned next sequentially into to obtain pFGL44/97-*RGS1*-mCherry-TrpC, representing the final constructs. The orientation of the promoter fragment in the final constructs was confirmed using suitable restriction enzymes. The final clones were subjected to sequence analysis and were then introduced into the WT background strain via *Agrobacterium*-mediated transformation. Fungal transformants were selected based on resistance towards hygromycin (CM containing 250 µg/ml hygromycin, A.G.Scientific Inc, USA or BM containing 50 µg/ml ammonium glufosinate, Cluzeau Info Labo, France). Fungal transformants were screened for Rgs1-mCherry expression under a fluorescence microscope and confirmed by the sequencing of the genomic DNA. The primers used for the amplification of the fragments are listed in Table 1.

Construction of plasmid vector for *DEP-DEP* domain-mCherry

To specifically tag and express the N-terminal *DEP-DEP* domain of Rgs1 protein with mCherry, the *DEP-DEP* domain of Rgs1 fused to the *BAR* gene was produced by gene synthesis (GeneScript, USA), the mCherry fragment was PCR amplified from p-mCherry (Clontech, USA) The TrpC terminator was amplified using pFGL275 and construct was driven by the RP27 promoter. The TrpC terminator was first cloned into the pFGL44 vector, and the mCherry was cloned next into pFGL44-TrpC to yield pFGL44-mCherry-TrpC. Finally, the promoter and *DEP-DEP-BAR* fragment were inserted into pFGL44-mCherry-TrpC using appropriate restriction enzyme sites to give the final construct pFGL-*DEP-DEP* - *BAR*-mC-TrpC. The sequence of the final construct was confirmed by sequencing and subsequently introduced into the *rgs1Δ* strain

via *Agrobacterium* mediated transformation. Fungal transformants were selected based on resistance towards ammonium glufosinate. Transformants were visually screened for DEP-DEP - Bar- mCherry expression and confirmed by genomic DNA sequencing and western blot analysis.

Generation of plasmid vector for *RGS* domain-mCherry

Tagging and expression of only the C-terminal *RGS* catalytic domain of Rgs1 protein was carried out in the *rgs1Δ* background strain adopting an approach similar to the one described above. The gene encoding the *RGS* domain fused to the *BAR* gene was generated by gene synthesis (GeneScript, USA). The mCherry fragment was amplified from p-mCherry (Clontech, USA). The TrpC terminator was amplified using pFGL275 and construct was driven by the RP27 promoter. The promoter and *RGS*-*BAR* fusion fragment was cloned into pFGL44-mCherry-TrpC using a three-way ligation approach to yield the final construct pFGL-*RGS* -*BAR*-mCherry-TrpC. The sequence of the final clones was confirmed by sequence analysis. Transformants were selected based on resistance towards ammonium glufosinate. Fungal transformants were visually screened for *RGS*-Bar-mCherry expression and confirmed by sequencing of the genomic DNA.

Plasmid vector generation for DEP-A-mCherry and DEP-B-mCherry constructs

To further dissect the function of the DEP-A (aa 225–325) and DEP-B (aa 408–486) domains present in the DEP-DEP region of the Rgs1, plasmid constructs were generated by independently cloning and C-terminally tagging the individual domain fragments (DEP-A and DEP-B). The N-terminal portion of Rgs1 encoding amino acids 1–225 was retained in both the constructs. Briefly, the fragment encoding the RP27 promoter and the DEP-A domain and the *BAR* encoding fragment was PCR-amplified from the plasmid pFGL-*DEP-DEP* domain- *BAR*-mCherry-TrpC (described above) using appropriate primer pairs. Using a three-way ligation approach, the PCR fragments containing the promoter and the DEP-A domain (*EcoRI*-*SacI*) was cloned into pFGL44-mCherry-TrpC terminator, along with the *BAR* fragment (*SacI*-*SmaI*) to get the final construct pFGL-*DEP*-A domain-*BAR*-mCherry-TrpC. A similar approach was taken to tag the DEP-B domain with mCherry at the C-terminus. Essentially, the fragment encoding the promoter and the N-terminal (1–225 aa) region of Rgs1 was PCR amplified from pFGL-*DEP-DEP* domain-*BAR*-mCherry-TrpC and digested using appropriate primers and restriction

enzymes (*EcoRI-SacI*) respectively. The fragment encoding the DEP-B domain and the BAR fragments were PCR-amplified from the same template plasmid and digested using the restriction enzymes *SacI* and *SmaI*. Both the digested fragments were cloned into pFGL44-mCherry-TrpC terminator to yield pFGL-DEP-B domain-BAR-mCherry-TrpC. Both the final constructs were subjected to sequence analysis, and upon confirmation, introduced into the *rgs1Δ* strain via *Agrobacterium* T-DNA-mediated transformation. Transformants were selected based on resistance towards ammonium glufosinate (Cluzeau Info Labo, France). Transformants were visually screened for DEP-A-Bar-mCherry and DEP-B-Bar-mCherry expression and further confirmed by genomic DNA sequencing.

Construction of plasmid vector for GFP- α Tubulin

The α -tubulin gene was tagged at the N-terminus with eGFP driven by the RP27 promoter [180, 202]. The eGFP was PCR amplified from pEGFP-N1 (BD Biosciences Clontech, USA). The RP27 promoter and the α -tubulin gene (MGG_06650) were amplified using genomic DNA from extracted from WT *M. oryzae* as template. pFGL275 was used as a template for the amplification of the TrpC terminator. The PCR products obtained were digested with suitable restriction enzymes (New England Biolabs, Beverly, MA) and purified using the gel elution Nucleospin Extract II kit (Machery-Nagel, Easton, PA). The TrpC terminator as a *SmaI/BamHI* fragment was cloned into the *SmaI/BamHI* sites of pFGL97 vector [encoding ammonium glufosinate gene (*BAR*)] to obtain pFGL97-TrpC terminator. The digested and eluted eGFP (*NcoI/SmaI* fragment) and RP27 (*EcoRI/NcoI*) fragments were cloned using a three way ligation approach into pFGL97-TrpC to yield pFGL97-RP27-eGFP-TrpC. Finally the α -tubulin was cloned as a *SmaI/SmaI* fragment into pFGL97-RP27-eGFP-TrpC to subsequently yield pFGL97-RP27- α -tubulin-eGFP-TrpC, representing the final construct. The orientation of the α -tubulin fragment in the final construct was confirmed using *BamHI* restriction enzyme. The final clones were subjected to sequence analysis and were then introduced into the appropriate background (Rgs1-mCherry) strain via *Agrobacterium* T-DNA-mediated transformation. Fungal transformants were initially selected using ammonium glufosinate resistance (BM containing 50 μ g/ml ammonium glufosinate, Cluzeau Info Labo, France) and confirmed by nucleotide sequencing and eGFP epifluorescence.

Construction of plasmid vector for eGFP-Vps21/ Rab5 and eGFP-Ypt7/ Rab7

The Rab5 ortholog in *M. oryzae*, Vps21 gene (MGG_06241) was also tagged at the N-terminus with eGFP and driven by the RP27 promoter [180, 202]. C-terminal tagging of Vps21/Rab5 is known to disrupt protein function. The eGFP was PCR-amplified from pEGFP-N1 (BD Biosciences Clontech, USA). The RP27 promoter and the Vps21 gene (MGG_06241) were amplified using genomic DNA from WT *M. oryzae* as template. pFGL275 was used as a template for the amplification of the TrpC terminator. The PCR products obtained were digested with suitable restriction enzymes (New England Biolabs, Beverly, MA) and purified using the gel elution Nucleospin Extract II kit (Machery-Nagel, Easton, PA). First, the digested Vps21 gene, digested with *SmaI/BamHI* was cloned into pFGL97 vector [encoding ammonium glufosinate gene (*BAR*)] at the *SmaI/BamHI* sites, followed by the TrpC terminator as a *BamHI/BamHI* fragment to obtain pFGL97-Vps21-TrpC terminator. The orientation of the TrpC terminator was confirmed using *NcoI/PstI* restriction enzymes. Next, the digested and eluted RP27 (*EcoRI/NcoI*) and eGFP (*NcoI/SmaI* fragment) fragments were cloned using a three-way ligation approach into pFGL97-Vps21-TrpC, to yield pFGL97-RP27-eGFP-Vps21-TrpC, the final construct.

The Rab7 ortholog Ypt7 (MGG_08144) in *M. oryzae* was also tagged at the N-terminus, utilizing the pFGL97-RP27-eGFP-TrpC vector as the base for cloning. As mentioned above the Ypt7 gene was PCR amplified using wild type genomic DNA as template. The Ypt7 amplicon was digested with *SmaI* enzyme and cloned into the pFGL97-RP27-eGFP-TrpC vector at the *SmaI* site. This yielded the final construct pFGL97-RP27-eGFP-Ypt7-TrpC. The orientation of cloned Ypt7 fragment was checked and confirmed by digestion with *HindIII*.

In both cases, the final clones were subjected to sequence analysis and were then introduced into the appropriate background strains (*RGSI*-mCherry) via *Agrobacterium* T-DNA-mediated transformation. Requisite transformants were identified and confirmed as described above.

Construction of plasmid vector for eGFP-2X FYVE

The eGFP-2X FYVE fragment was released from pEGFP-2X FYVE (a kind gift from Prof Harald Stenmark) as an *NcoI-SmaI* fragment. This fragment (*NcoI-SmaI*) along with the RP27

promoter (*EcoRI-NcoI*) was cloned into pFGL97 vector using a three-way ligation strategy to yield pFGL97-RP27-eGFP-2X FYVE. The TrpC terminator was cloned next into the same vector as *SmaI/BamHI* fragments to yield the final construct pFGL97-RP27-eGFP-2X FYVE-TrpC. Transformants were identified and confirmed as previously described.

Construction of plasmid vector for active $G\alpha_s$ -GFP (MagA^{G187S}-GFP) and wild type $G\alpha_s$ -GFP (MagA-GFP)

Tagging at the C- or the N-terminus with fluorescent marker is known to compromise the function of the G- α subunit. In order to circumvent this problem, we introduced the eGFP coding sequence in frame into the α B- α C loop between amino acids 113-120 (a kind suggestion by D. Siderovski and D. Bosch, USA) of *M. oryzae* $G\alpha_s$ MagA. We used vector pFGL718 in which the fragment encoding ammonium glufosinate and eGFP is available as an *HpaI-MluI* fragment. Genomic DNA from the WT was used as template to amplify the regions flanking either side of the α B- α C loop region in the MagA protein. The upstream flank was amplified as an *EcoRI-MluI* fragment and the downstream flank as an *HpaI-HindIII* fragment. Both flanks were amplified such that they also incorporated a short six amino acid linker. In addition, another downstream flank incorporating the G187S point mutation was also amplified as an *HpaI-HindIII* fragment, using genomic DNA from the MagA^{G187S} strain [207]. The sequence of the *HpaI-HindIII* fragment incorporating the G187S point mutation was sequenced and verified. The single 5' upstream fragment and the two 3' flanks were digested with appropriate restriction enzymes and cloned into pFGL718 such that the upstream flank, the *BAR*-eGFP fragment and the downstream flank were all in frame. The constructs were confirmed and integrated into the Rgs1-mCherry strain such that the GFP-tagged MagA replaced the WT copy by homologous recombination. Site-specific replacement was confirmed using standard methods detailed above.

Construction of plasmid vectors for BiFC strain expressing (RP27_{PRO}-Pth11-YFP¹⁻¹⁵⁶) and (Ccg1_{PRO}-Rgs1-YFP¹⁵⁶⁻²³⁹)

A BiFC strain expressing Pth11-YFP¹⁻¹⁵⁶ and Rgs1-YFP¹⁵⁶⁻²³⁹ was generated to study at the interactions between the two proteins *in-vivo*. For this purpose two constructs were generated, one in which *PTH11* was cloned into pFGL44 [encoding hygromycin phosphotransferase gene

(*HPHI*)] and tagged C-terminally to YFP¹⁻¹⁵⁶ under the RP27 promoter, the other in which the *RGS1* was cloned in pFGL97 [encoding ammonium glufosinate gene (*BAR*)] and tagged at the C-terminus to YFP¹⁵⁶⁻²³⁹ under the Ccg1 promoter.

2.2.4 Protein extraction and related methods

2.2.4.1 Total cellular protein extraction from *M. oryzae* (Denaturation condition)

1. Fungal mycelium was grown in liquid CM at 28°C for 2-3 days with continuous agitation at 160 rpm.
2. Mycelia was harvested by filtering through two layers of Miracloth (Calbiochem, USA) and then washed thoroughly with sterile 1X PBS buffer (Phosphate Buffered Saline) to remove traces of the medium.
3. The mycelium was dried by blotting away excess water and then ground into a fine powder in liquid nitrogen using a pestle and mortar.
4. To obtain total cellular protein extracts, under denaturing conditions, 100 mg mycelial powder was re-suspended in 300 µl of SDS lysis buffer (10 mM Na₂HPO₄ pH 7.0, 0.5% SDS, 1 mM DTT, 1 mM EDTA, pH7.0) and incubated on ice for 10 min.
5. The lysates were clarified by centrifugation at 12000 rpm at 4°C for 20 minutes; the supernatant containing total protein was aspirated into a fresh Eppendorf tube and stored on ice for downstream applications.
6. Protein samples were mixed with 5X SDS loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) and denatured at 100°C on a heating block for 5-10 min prior to loading onto a SDS-PAGE.

2.2.4.2 Immuno-precipitation assay utilizing GFP-Trap

Total protein was extracted using non-denaturing NP40 buffer (10 mM Tris/Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40, 1mM PMSF and 1X Protease Inhibitor Cocktail). Total

protein was extracted from Pth11-GFP conidia inoculated on an inductive surface at the hooking stage (4 hpi) or from germ tubes during vegetative growth. The protein lysate was incubated with GFP-Trap agarose beads (ChromoTek, Germany) and immuno-precipitation carried out as per manufacturer's instructions. Immuno-precipitated samples was separated by SDS-PAGE, transferred to a PVDF membrane (Millipore Corporation, USA) and probed with α -DEP antiserum (1:1000 dilution). Secondary antibodies conjugated to horseradish peroxidase were used at 1:10000 dilutions. The Super Signal kit (Pierce, USA) was used to detect the chemiluminescent signal as instructed [207, 243].

2.2.4.3 Protein electrophoresis and immunoblot analysis

1. Protein sample from each extract, mixed with SDS loading dye, was fractionated on a SDS-PAGE (10 or 12%) under denaturing conditions at a voltage of 100V for about 1 h.
2. The separated proteins were transferred at 4°C onto a methanol pre-treated PVDF membrane (Millipore Corporation, USA), utilizing a wet-transfer protocol at a voltage of 80V for a duration of 2 h or at a voltage of 35V for 12-16 h.
3. The PVDF membrane with the transferred proteins was transferred to a suitable container and blocked in a solution of freshly prepared PBST buffer (0.05% Tween 20 in 1XPBS) containing 5% nonfat dry milk powder for 1 hour at RT. The blocking of the membrane can also be carried out at 4°C overnight.
4. Following incubation in blocking solution, the membrane was washed with fresh PBST (0.05% Tween 20 in 1XPBS) buffer five times 5 min each.
5. The primary antibody was diluted according to the manufacturer's instructions (usually 1:1000 dilution) in fresh PBST buffer containing 1% nonfat dry milk powder. Subsequently, the nitrocellulose membrane was placed in the primary antibody solution and incubated at RT for 2 h or at 4°C overnight.
5. The PVDF membrane was washed again with fresh PBST buffer five times for 5 min each.

6. The membrane was then incubated in PBST buffer containing HRP (Horse Radish Peroxidase) conjugated secondary antibody (1:10000 dilution) at RT for 1 h.
7. The membrane was washed with fresh PBST buffer three times for 15 min each.
8. Detection of the protein was performed using enhanced chemiluminescence system (Amersham Biosciences, USA) as instructed.

For re-probing of the immunoblots:

9. The PVDF membrane was stripped of all the proteins by incubating in a standard stripping buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β -mercaptoethanol) at 70°C for 45 min with constant gentle shaking.
10. The membrane was thoroughly washed with PBST buffer five times for 15min each.
11. The membrane was again blocked and re-probed essentially as described in steps 3-8 above.

2.2.5 cAMP extraction and analysis

2.2.5.1 Extraction of cAMP during early and late stages of pathogenic development in *M. oryzae*

1. Freshly harvested conidia (0 h) from the required strains, as well as conidia germinated for 6 h, and conidia that have formed mature appressoria on the inductive (hydrophobic) surface of GelBond membranes (Lonza Walkersville Inc., USA) following 21 h, 24 h, 28 h or 30 h incubation were harvested, frozen and lyophilized for 16 h.
2. The lyophilized fungal biomass was individually ground to a fine powder in liquid nitrogen in a pestle and mortar
3. Equivalent amounts (by wet weight) of the fine powder was re-suspended in 200 μ l of chilled 6% trichloro acetic acid (TCA) and incubated on ice for 10 min.

4. The supernatant was collected after centrifugation at 4000 rpm for 15 min at 4°C, and extracted four times with five volumes of water-saturated diethyl ether to remove the TCA. The organic ether layer was discarded after each extraction.
5. The remaining aqueous extract was again lyophilized for 16 h and dissolved in the assay buffer prior to quantification and analysis.
6. The cAMP levels in the samples of interest were quantified using the cAMP Biotrak Immunoassay System (Amersham Biosciences, USA).

2.2.5.2 Estimation of cAMP with cAMP Biotrak Immunoassay System

1. The assay buffer, working standard, antiserum and cAMP peroxidase conjugates, provided by the kit, were prepared and reconstituted according to the manufacturer's instructions (Amersham Biosciences, USA). All reagents were equilibrated to RT before use.
2. Required volumes of assay buffer, standard and samples were pipetted into the appropriate wells of the micro plate as directed.
3. The supplied antiserum (100 µl) was added into all wells except the blank and non-specific binding control wells.
4. The plate was covered with the lid and incubated in the dark with gentle agitation at 4°C for exactly 2 h.
5. Following incubation, the supplied cAMP-peroxidase conjugate (50 µl) was added into all wells except the blank and again incubated with gentle agitation at 4°C for exactly 1 h.
7. All wells were aspirated and washed with 400 µl wash buffer, 4 times and any residual volume from each well was removed.
8. The supplied enzyme substrate (150 µl) was immediately added into all wells. The plate was further incubated at RT for 25 min, with gentle agitation.

9. The reaction and the development of the color was terminated by adding 100 μ l of 1.0 M sulfuric acid and the optical density was determined immediately in a micro plate reader at 450 nm.

2.2.6 Evaluation of pathogenicity and related traits

2.2.6.1 Assessment of conidiation efficiency

Conidia were harvested by adding sterile distilled water to the light-exposed plates and scraping the surface of colonies with an inoculating loop. The suspension was filtered through two layers of Miracloth (Calbiochem, San Diego, USA), collected in Falcon tubes (BD Biosciences, USA). The spore suspension was vortexed for a minute to ensure complete detachment of conidia from the mycelia, and centrifuged for 10 min at 3,000 rpm at RT. The conidia pellet was resuspend in a fixed volume of distilled water. The radius of the colony was initially measured to calculate the surface area of the colony. Conidia produced by a given colony were quantified using a hemocytometer and reported as the total number of conidia present per unit area of the colony.

2.2.6.2 Assessment of appressorium formation and morphogenesis

The ability to form mature appressoria was assessed on the hydrophobic as well as hydrophilic surfaces of artificial polyester membrane, Gelbond (Lonza Walkersville Inc., USA). For appressorial assays and imaging using bright field optics, harvested conidia were resuspended in sterile water at a required concentration. Droplets (20-40 μ l) of the spore suspension containing the desired number of spores were inoculated on either the hydrophobic or the hydrophilic surface or glass bottom dishes (Mat Tek Corporation, USA). The samples were incubating in a moist chamber at room temperature. The total number of appressoria formed by the spores was quantified after an incubation period 16-24 h.

2.2.6.3 Surface hydrophobicity Assays

To test the surface hydrophobicity of the fungal colonies, the strains being tested were grown on PA plates for 7 days at 28°C in the dark. Three individual 20 μ l droplets of sterile distilled water

were placed on the colony surface, moving from the centre towards the periphery. The colonies with the water droplets were subsequently incubated at room temperature.

2.2.6.4 Evaluation of pathogenicity

To assess the pathogenicity of fungal transformants in the laboratory, two protocols were routinely used: the detached leaf assay in barley and the whole plant infection assay in rice. For the former, leaves from two-week old barley seedlings were cut into smaller pieces (2-3 cm long) and washed in sterile water, following which the leaf pieces were surface-sterilized for 45 s in 40% ethanol. The leaf pieces were then washed twice with sterile antibiotic containing distilled water (100µg/mL Streptomycin and 100µg/mL Ampicillin). The washed leaves were dried on paper towels and then mounted on kinetin agar plates (2 µg/mL kinetin, 1% agar). Conidia were quantified and a dilution series of the conidial suspension was inoculated on detached barley leaves at the required concentrations (usually 500-2000 conidia/ 20µl droplet). The samples were incubated in a humidified growth chamber with a 16 h light/8 h dark cycle at 22°C. Disease symptoms were assessed 5-7 days post inoculation.

For assessment of pathogenicity on rice, seedlings at the 3 leaf stage of cultivar CO39 (a susceptible variety) were spray-inoculated with 10^4 - 10^5 conidia/mL suspension containing 0.01% Tween-20, from the fungal strains to be tested. To maintain a high humidity (known to aid the infection process), the spray inoculated seedlings were kept covered with a moist plastic bag, and the inside surface of the bag was sprayed with water at regular intervals (usually 6-9 h intervals). The seedlings were incubated for 24 h in a humidity chamber prior to being transferred to a growth chamber. Disease symptoms were assessed 10-14 days post inoculation.

Quantitative assessment of appressorial function (as judged by the ability of the appressoria to penetrate the host surface) was performed on detached barley leaf explants. The experimental set-up was essentially as described for the infection assays, except that the inoculated barley leaves were sampled at earlier timepoints -24 hpi, 48 hpi, 72 hpi and 96 hpi (hours post-inoculation). The infected leaf tissue was dissected into smaller pieces and cleared of the chlorophyll content by incubation in 100% methanol with gentle agitation overnight. Cleared leaves bits were stained with aniline blue (0.1% aniline blue, 67 mM K_2HPO_4 , pH 9.0) to

visualize host leaf papillary callose formation, representing a defense response from the infected plant tissue. Callose formation underneath the appressoria was taken as a measure of a penetration peg formation. The penetration peg formation was quantified with WT as control. In addition, the number of infectious hyphae formed was also counted under a bright field microscope.

2.2.7 Chemical inhibitor, staining and rescue experiments

The cAMP analog, 8-Br-cAMP (BioLog, Germany) was first added at 0 h and again supplemented after 20 hpi (a final concentration of 10 mM or 50 mM). Stock solutions of 8-Br-cAMP (100 mM) were made in water, while IBMX (25 mM) (Sigma Aldrich, USA) was dissolved in 99% ethanol [164]. For tricyclazole (Cluzeau Info Labo, France) treatment, conidia from the requisite strain were inoculated on cover slips in the presence of 8 µg/mL tricyclazole [244]. Nuclear staining was carried out using DAPI (diamidino-2-phenylindole; Sigma Aldrich, USA) essentially as described [245], although with minor modifications to suit *M. oryzae* conidia or germlings. Freshly harvested conidia were appropriately diluted and inoculated on hydrophobic plastic cover slips in a moist chamber at RT for 1–2 h. The cover slips with the adherent germlings were then fixed with formaldehyde (3.7% final concentration), for 5–10 min at RT. The fixed samples were then washed gently with distilled water, prior to treatment with Triton X-100 (0.1% final concentration) for 1 min at RT. The samples were washed thrice with distilled water, and finally incubated in the dark for 10–15 min, with a solution of DAPI dissolved in water at a final concentration of 1 µg/ml, and visualized using a epifluorescence microscope. Nuclear staining during live imaging of GFP-PdeH was achieved using Hoechst 33342 (Sigma Aldrich, USA) at a concentration of 1 µg/ml (in water) for 20 min. Cell wall and septa were stained using calcofluor white in water at a final concentration of 10 µg/ml. Samples were washed twice with sterile distilled water prior to visualization by epifluorescence imaging using an epifluorescence microscope.

For chemical rescue experiments, samples of freshly harvested conidia from the fungal strains to be tested diluted to the appropriate final load, was supplemented with required chemical (i.e, 8-Br-cAMP and IBMX) at the required final concentration, prior to inoculation on leaf bits.

The Rgs1-mCherry strain was inoculated on hydrophobic plastic coverslips and treated for 30 min with MBC (final concentration 1 μ M) and LatA (final concentration 10 μ M) at the hooking stage. PI3 Kinase inhibitor LY294002 was added to Rgs1-mCherry conidia at a final concentration of 100 μ M at 4 hpi. Suppression of the *vps39* Δ mutant defects was tested by adding 8-Br-cAMP to a final concentration of 10 mM at 0 hpi. Wild type was treated with Bafilomycin-A1 2 hpi at a final concentration of 50 nM. A 0.1% DMSO solvent control was used in all assays.

2.2.8 Microscopy and related techniques

2.2.8.1 Live cell imaging and Colocalization analysis

Droplets (20-40 μ l) containing conidial suspension was placed on the desired surface and incubated in a moist chamber at RT. Imaging was performed at the desired time points. Bright field images as well as fluorescence images of required samples were captured at desired time points using either an Olympus BX51 or IX71 microscope equipped with a PlanAPO 100X/1.45 or UPlan FLN 60X/1.25 objective with appropriate filter sets. Images were captured using a CoolSNAP HQ camera (Photometrics, USA) and processed using MetaVue (Universal Imaging, USA).

Time-lapse or live cell fluorescence microscopy was performed using a Zeiss Axiovert 200M microscope (Plan Apochromat 100X, 1.4NA objective) equipped with an UltraView RS-3 spinning disk confocal system (PerkinElmer Inc., USA) including a CSU21 confocal optical scanner, 12-bit digital cooled Hamamatsu Orca-ER camera (OPELCO, Sterling, VA, USA) and a 491 nm 100 mW and a 561nm 50mW laser illumination under the control of MetaMorph Premier Software, Ver. 7.7.5 (Universal Imaging, USA). Typically, z-stacks consisted of 0.5 μ m-spaced planes for every time point. The maximum projection was obtained using the Metamorph built-in module. Alternatively, the Nikon TiE system (CFI Plan Apochromat VC 100XH 1.4 N.A. objective) with a Yokogawa CSU-X1-A1 spinning disk unit, a Photometrics CoolSNAP HQ2 camera and a DPSS 491nm 100mW and DPSS561nm 50 mW laser lines under the control

of MetaMorph Premier Software, Ver. 7.7.5 (Universal Imaging, USA). Typically, single z-plane or image stacks that consisted of 0.5 μ m-spaced sections every time point were captured. For quantitation of colocalization the raw .STK files were opened as stacks in Imaris, and the extent of colocalization between Rgs1-mCherry and the other GFP-tagged proteins or endosomal markers was measured using the Colocalization module of Imaris 7.5, 64-bit version (Bitplane AG, Saint Paul, MN, www.bitplane.com). Two-channel Z-stacks are assembled and the relative intensity of each wavelength is determined for each voxel. The minimum intensity threshold for the analysis was determined for the GFP-channel automatically using the iterative algorithm defined by Costes et al. [246]. A time-dependent analysis of colocalization was run for each sample. Both the percentage of material colocalized (as determined by the number of colocalized voxels relative to the total number of voxels with an intensity value of GFP signal above the minimum intensity threshold) and the Pearson's co-localization coefficient was calculated from each sample for a minimum of four time points, a minimum of four different cells and at least two independent experiments performed on different days. The data were pooled and the results represent the average with error bars corresponding to the standard error of the mean.

2.2.8.2 Image processing

Image processing and figure preparation was performed using Bitplane Imaris for 3D surface rendering and colocalization analysis (see above), Fiji (<http://fiji.sc/wiki/index.php/Fiji>), and Adobe Photoshop and Microsoft Excel (for figure preparation).

2.2.8.3 Statistical Analysis

Statistical analysis was evaluated by one-way ANOVA (analysis of variance) or the Student's T-test.

3. Results I

Chapter III

3.1 Role of cAMP phosphodiesterases in regulating asexual development and pathogenesis in *M. oryzae*

Introduction

The highly conserved cAMP signaling pathway controls many processes such as growth, morphogenesis and development. In response to ligand activated GPCRs, cAMP are produced through the stimulation of adenylate cyclases [137]. Numerous such sources of cAMP are made all over the cell, causing a steady buildup and increase in the intracellular cAMP levels. Upon reaching a critical threshold concentration, cAMP in turn activates multiple downstream effectors, the most important of which is a cAMP-dependent Protein Kinase A that in turn mediates a variety of physiological outcomes [117] (Figure 3.1).

The hydrolysis of active cAMP to inactive 5'-AMP is carried out by enzymes known as cyclic nucleotide phosphodiesterases (PDEs). The hydrolysis of cAMP is crucial not only for the regulation of the intensity and robustness of signaling but also for the termination and more importantly compartmentalization of signaling [212]. As a consequence, PDEs are specifically targeted to signaling complexes/platforms that localize to the cytosol and to various other subcellular locations [247-250]. Furthermore, within distinct regions in a cell, PDEs help generate and establish gradients of cAMP [9, 138, 251].

Taken together, in a wide range of organisms PDEs play a critical function in influencing the amplitude, precision and timing of signaling [139, 212].

In filamentous fungi and yeasts, cAMP signaling module has been selected to regulate many cellular and developmental processes. In *S. cerevisiae*, sensing of nutrient, differentiation of pseudohyphae, stress signaling and progression of the cell cycle is controlled by the cAMP pathway [230, 252-256]. In *S. pombe*, the cAMP pathway influences mating, sporulation and gluconeogenesis [257-260].

Figure 3.1. Proposed model indicating the putative roles of PDEs in cAMP signaling pathway in *M. oryzae*.

PDEs regulate intracellular or steady state cAMP levels by hydrolyzing cAMP to AMP. Adapted and modified from [261].

In human pathogens, like *C. neoformans* and *C. albicans*, cAMP signaling regulates virulence (a few aspects), stress tolerance as well as sexual differentiation [27, 141, 262-270]. In *U. maydis*, a phyto-pathogenic fungus, cAMP signaling influences dimorphic switching and virulence [73, 74, 77, 235, 271]. In *N. crassa* and *A. nidulans*, cAMP signaling regulates morphogenesis, asexual development and polarity of the cell [123, 272-281].

S. cerevisiae contains two cyclic nucleotide PDEs, namely a high-affinity PDE (Pde2; having a low K_m to cAMP) as well as a low-affinity PDE (Pde1; with a high K_m to cAMP) [282-285]. The low-affinity phosphodiesterase Pde1 is mainly involved in regulating increases of cAMP levels stimulated either stimulation by glucose or acidification of the cell. Pde1 activity is further controlled by a feedback mechanism involving phosphorylation by the downstream cPKA [59, 286, 287].

The high-affinity PDE, Pde2, besides shielding the yeast cells from excess cAMP, also functions to maintain basal levels of cAMP [59, 288]. Although not clear, Pde2 activity is also likely regulated by the cPKA pathway.

Interestingly, although *S. pombe* does not possess a Pde2 ortholog, it has a Pde1 that controls glucose stimulated cAMP levels in a cPKA dependent manner [258, 289]. A role for Pde1 has not yet been defined during development in *C. albicans* but the function of Pde2 has been well documented [264, 268, 290]. Deletion of Pde2 causes enhanced intracellular levels of cAMP and makes it sensitive to heat shock [263, 264, 268, 290]. Furthermore, in addition to being more sensitive to antifungal agents, *PDE2* mutant in *C. albicans*, show defects related to cell wall and membrane integrity [267, 268]. Additionally, although it displays enhanced filamentation, the *PDE2* mutant remains non pathogenic in a mouse model of systemic candidiasis [264].

In *C. neoformans*, also a human fungal pathogen, the functions of Pde1 and Pde2 have been well-studied. Deletion of *PDE2* resulted in mild phenotypic defects in contrast to *S. cerevisiae* and *C. albicans*. On the contrary, Pde1 regulated the basal levels of cAMP. Surprisingly, deletion of *PDE1* also caused subtle phenotypic defects. It has however been established that the activity of Pde1 is controlled by cPKA mediated feedback phosphorylation [141].

In this chapter, I identify and functionally characterize the deletion mutants of the two genes encoding PDE, namely a high-affinity PDE (PdeH) as well as a low-affinity PDE (PdeL) in *M. oryzae*, and their roles in modulating basal levels of cAMP during asexual development and pathogenesis. The results demonstrate that PdeH carries out a crucial function in controlling the intracellular levels of cAMP during pathogenic, asexual and *in-planta* development. Furthermore, analysis of the regulation of transcription as well as the localization of the PDEs demonstrate that in *M. oryzae*, the cAMP cascade is compartmentalized and can rapidly respond to maintain suitable levels of cAMP during development, morphogenesis and virulence.

Results

3.1.1 Classification of cAMP Phosphodiesterases in *M. oryzae*

Orthologs of the yeast PDEs were identified in rice-blast fungus utilizing a reverse genetics based approach. Using the yeast (*S. cerevisiae*) protein sequences as query, and performing a *TBLASTN* search (www.broadinstitute.org), two ORFs (open reading frames), namely MGG_07707 and MGG_05664 were identified. The ORF MGG_07707 encoded a 585 amino acid polypeptide with a 32% similarity to Pde1 in yeast, while the ORF MGG_05664 encoded an 893 amino acid long protein, showing 27% identity to Pde2. The entire cDNA sequence for each ORF was determined and the predicted amino acid sequence confirmed [Genbank accession GQ869476 for Pde1 (MGG_07707) and GQ869476 for Pde2 (MGG_05664)].

The Pde2 ortholog in *M. oryzae* was designated as a high-affinity PDE (Figure 3.2A) on the basis of the presence of a highly conserved consensus sequence for PDE class I [140]. Similarly, based on the presence of a conserved PDE class II consensus motif in Pde1 was similarly classified as a low affinity cAMP PDE (Figure 3.2B) [141]. Thus, based on the presence of highly conserved PDE domains, we propose that *M. oryzae* contains two PDEs, a low-affinity PdeL and a high-affinity PdeH.

Figure 3.2. Identification and comparison of PDE gene sequences in *M. oryzae*.

(A) Alignment of the catalytic sites of predicted high-affinity PDEs from *N. crassa*, *M. oryzae*, *A. niger*, *S. cerevisiae*, *C. albicans* and *C. neoformans*. The highly conserved signature sequence for Class I is underlined in red.

(B) Alignment of the region containing the predicted consensus sequence of the low-affinity PDE from *N. crassa*, *M. oryzae*, *S. pombe*, *A. niger*, *S. cerevisiae*, *C. albicans* and *C. neoformans*. The signature sequence for Class II is underlined. *S. pombe* does not encode the high affinity PDE variant. In both cases conserved amino acids are shown in black, while similar amino acids are marked in gray.

3.1.2 Generation of *PDEH*, *PDEL*, *PDEH PDEL* deletion and complemented *pdeHΔ* strains

PDEH or *PDEL* deletion mutant was generated in the WT background using the standard homologous recombination mediated one-step gene replacement strategy. The *pdeHΔ* strain was generated by replacing MGG_05665, with cassette encoding hygromycin (*HPHI*) resistance. In an identical manner, the *pdeLΔ* strain was generated by swapping MGG_07707, with the bialaphos (*BAR*) resistance encoding cassette. Specific gene replacement of *PDEH* or *PDEL* was verified by southern blot analysis and locus specific PCR (Figure 3.3A, B and Figure 3.4A, B).

To gain further insight into the relationship between *PDEL* and *PDEH*, a *pdeHΔ pdeLΔ* double mutant was generated. This mutant was created by substituting the *PDEH* ORF, with the cassette coding for hygromycin (*HPHI*) resistance in the *pdeLΔ* background. The *pdeHΔ* was also genetically complemented, by introducing a copy of the N-terminal RFP tagged full-length *PDEH* under the native promoter to obtain complemented *PDEH* strain (Comp-*pdeHΔ*; Figure 3.4C and D).

A **B**

Figure 3.3. Diagrammatic representation and Southern blot analysis of the *M. oryzae* High affinity phosphodiesterase gene (*PDEH*).

(A) Schematic representation of the *ScPDE2* ortholog *PDEH* (MGG_05664). Solid bars indicate coding regions while the short open boxes represent introns. The flanks of genomic DNA used for gene targeting are designated by the dashed lines. E: *EcoRI*, X: *XbaI*, P: *PstI* and H: *HindIII* are RE sites for cloning purposes. The gene cassette coding for Hygromycin resistance (*HPH1*) used for gene substitution is indicated. Scale bar equals 1 kb and demarcates the region used as probe used for southern blot.

(B) Verification of the *PDEH* deletion strains using southern blot analysis. Genomic DNA extracted from the WT or *pdeHΔ* strains was subjected to restriction enzyme digestion with *XhoI* and probed with a fragment from the 3' UTR, as depicted. Accurate *PDEH* replacement event was indicated by the presence of a 5.7 kb fragment in lane 1 representing the gene-deletion strain and a 2.7 kb in lane 2 representing the control WT. The same blot was stripped and re-probed with *HPH1* probe to identify a 5.7 kb confirmatory fragment in the *pdeHΔ*.

Figure 3.4. Diagrammatic representation and Southern blot analysis of the low affinity phosphodiesterase gene (*PDEL*) in *M. oryzae* and genetic complementation of the *pdeHΔ* strain.

(A) A schematic diagram of the *ScPDE1* ortholog *PDEL* in *M. oryzae*. Exons are indicated by solid bars, while introns are depicted by open boxes. The genomic flanks utilized for gene disruption are indicated by dashed lines. The sites used for cloning are shown as K: *KpnI*, B: *BamHI*, P: *PstI* and H: *HindIII*. *BAR* indicates the bialaphos-resistance gene cassette. Scale bar equals 1 kb and depicts the region used as probe for Southern blot.

(B) Confirmation the *PDEL* deletion by southern blot analysis. WT or *pdeLΔ* genomic DNA digested with *NcoI* was probed with 3'UTR fragment of *PDEL*. Accurate *PDEL* replacement was detected by the presence of a 4.3 kb fragment in lane 1 representing the mutant and a 1.8 kb fragment in lane 2, representing the WT. The membrane was probed again with *BAR* to confirm the presence of a diagnostic 4.3 kb fragment in the *pdeLΔ*.

(C) Verification of the double mutant *pdeHΔ pdeLΔ* strain. The *pdeHΔ pdeLΔ* strain was generated by deleting the *PDEH* gene in the *pdeLΔ* strain. Verification of the appropriate gene substitution was performed as previously described. The size (in kb) of the appropriate fragment is indicated.

(D) Southern blot analysis of the RFP-PdeH expressing strain. Genomic DNA from the aforementioned strain, lane 1 or the WT, lane 2 was digested with *NcoI* was subjected to Southern blot using RFP as a specific probe. The number of integrated copies /integrans was verified by Southern blot

3.1.3 Characterization of the *PDE* mutant strains - asexual development

3.1.3.1 Colony morphology, conidiation and aerial growth in *PDE* mutants

Two independent strains each representing the *pdeHΔ*, *pdeLΔ*, *pdeHΔpdeLΔ* and *Comp-pdeHΔ* were used for subsequent analysis and characterization. WT strain B157 was used as a control during all the experiments. Compared to the WT, the *pdeLΔ* displayed no apparent defects in colony morphology as well as under different conditions of radial or aerial growth (Figure 3.5). However, the *pdeHΔ* colony was flat, as a result of decreased aerial hyphae and showed increased pigmentation and radial growth defects at comparative points in time. The colony of the double deletion mutant also appeared flat with increased pigmentation and highly reduced aerial and radial growth, thus stressing the importance of PdeH function in *M. oryzae* (Figure 3.5). The complemented *pdeHΔ* strain was completely rescued for growth defects shown by the *pdeHΔ* and appeared similar to the WT, implying that the defects exhibited by the *pdeHΔ* were exclusively because of the absence of function of PdeH.

In conclusion, the results strongly suggest that in *M. oryzae*, PdeH plays a more important role during aerial hyphal growth and development compared to PdeL. Additionally, it is plausible that PdeL has a minor role during aerial and/or vegetative growth.

Figure 3.5. Colony characteristics of the *PDE* mutants and *pdeH*Δ complemented strain.

Colony morphology of the wild type, *pdeH*Δ, *pdeL*Δ, *pdeH*Δ *pdeL*Δ and the *pdeH*Δ complemented strains. The panel on the top represents images of the above mentioned strains grown on PA containing medium for a week in the dark. The panels below depict respective medial sections of the above colonies.

Asexual reproduction in *M. oryzae* commences with the development of special aerial conidiophores, which in turn generate spindle shaped, three celled, tri-nucleate spores known as conidia, which are usually arranged in a sympodial manner. The conidiation status of the *pdeHΔ* strain was determined. Remarkably, compared to *pdeLΔ* or the wild type, the *pdeHΔ* produced 2-3fold higher numbers of conidia (Figure 3.6A). Detailed microscopic observations revealed that unlike in the wild type strain, which typically produced three to four conidia per conidiophore, the *pdeHΔ* formed five to eight conidia for every conidiophore stalk assessed (Figure 3.6B). The *pdeHΔpdeLΔ* double deletion strain was severely defective in conidiation and failed to make proper conidia (98±1.0% reduction with respect to the wild type). Instead the double mutant made aberrant structures of varied morphologies that were highly pigmented and at a low frequency, two-celled conidia-like structures (2%, $P < 0.05$, Figure 3.6B and inset).

Next, the number of conidiophores produced by each strain was quantified. For every microscopic field observed, compared the wild types or *pdeLΔ* strains the *pdeHΔ* produced twice the number of conidiophores (Figure 3.6C). The *pdeHΔ pdeLΔ* strain was not only defective in producing conidia, but was also compromised during the stage of conidiophore development and differentiation. The double mutant instead produced structures that were highly pigmented and misshapen. Thus, in *M. oryzae* the concomitant loss of both the PDEs leads to serious defects during stages of asexual development and/or differentiation. The number of conidia and/or conidiophores produced by *pdeLΔ* and the complemented *pdeHΔ* strains was comparable to the wild type (Figure 3.6B).

In conclusion, the high-affinity phosphodiesterase, PdeH regulates facets of asexual development, mainly influencing conidiation including conidiophore differentiation. Furthermore we speculate that intracellular cAMP levels, regulated by PdeH, likely influences the number of conidia produced on each conidiophore stalk. The inability of the double mutant to differentiate proper conidia including conidiophores, suggests a regulatory although supplementary role for PdeL during asexual development.

Figure 3.6. Asexual development in *M. oryzae* is regulated by PdeH function.

Assessment of conidiation and conidiophores in the wild type, *pdeHΔ*, *pdeLΔ*, *pdeHΔ pdeLΔ* and the complemented *pdeHΔ* strain.

(A) To assess qualitatively and quantify the conidia produced, the aforesaid strains were subjected to growth in dark conditions for 24h after which they were exposed to constant light for a week. Data shows mean $\pm$ SE based on 5 independent replicates.

(B) Arrangement and number of conidia on conidiophores in the aforementioned strains. The strains being assessed were grown on a glass slide that supported a thin layer of PA medium. Prior to microscopic observations the strains were first incubated in the dark for 24h after which they were exposed to constant light for 3 days. The double deletion mutant formed 2-celled conidia-like-structure, depicted here in the inset. The arrowhead highlights a single septum in the conidia -like structure. Scale bar, 25 μ m

(C) The number of conidiophores produced per microscopic field was assessed and counted 1 day after photo induction. Three independent replicates were carried out and the results are shown as mean $\pm$ SE. The abnormally pigmented conidia or conidiophore-like structures generated by the *pdeH* Δ *pdeL* Δ are highlighted by the asterisk.

3.1.3.2 Conidial development is not accelerated in the *pdeH*Δ mutant

Given that conidiation in the *pdeH*Δ was two to three fold higher in contrast to *pdeL*Δ or the WT, we assessed if the rate at which the individual conidia are produced was accelerated and/or if multiple conidia arose simultaneously rather than sequentially in the *pdeH*Δ strain. To answer this question, time-lapse imaging was performed tracking the development and maturation of individual conidia over a period of 7-8 h. Compared to the wild type, there was no substantial variation in the rate or number of conidia produced in a given interval of time in the *pdeH*Δ (Figure 3.7A and B). The time taken for a complete conidium to be formed from respective conidial initials was also determined; in both the strains (*pdeH*Δ and the wild-type) this process lasted for approximately 2 hours. Thus, compared to the wild-type conidia formation or development is not altered in the *pdeH*Δ.

Figure 3.7. The *pdeHΔ* strain is similar to the wild-type with respect to the rate at which conidia are produced.

A sequence of time-lapse images depicting conidial development and maturation in the wild type (**A**) or *pdeHΔ* (**B**). The arrowheads indicate the point of conidial initiation and development into a mature conidium.

3.1.4 cAMP levels, regulation and homeostasis in *pdeHΔ*, *pdeLΔ* and *pdeHΔpdeLΔ* strains.

To determine if the phenotypic defects in *PDE* mutant strains, especially the *pdeHΔ* and the double mutant was because of increased levels of cAMP, the steady-state intracellular cAMP levels were measured and evaluated during conidiation in the all mutant strains. The WT was used as a control (Figure 3.8A). In the lack of stimulation by light, compared to the WT or the *pdeLΔ*, the *pdeHΔ* strain accumulated nearly 3 fold ($P < 0.005$) and nearly 2.6 fold ($P < 0.005$) higher cAMP levels respectively (Figure 3.8A). The *pdeHΔpdeLΔ* accumulated nearly 3 fold ($P < 0.001$) greater cAMP levels compared to *pdeHΔ* and nearly 10 fold increased levels compared to the WT or *pdeLΔ* ($P < 0.001$) under similar conditions.

In addition, it was observed that cAMP levels were significantly upregulated during conidiation (24 h light-induced) in all the strains except *pdeHΔpdeLΔ*: 2 fold ($P < 0.05$) in the WT and *pdeLΔ*, and by 1.4 fold ($P < 0.01$) in the *pdeHΔ* in contrast to the respective cultures grown in the dark. Nonetheless, in the *pdeHΔpdeLΔ*, the levels of cAMP were constantly high, irrespective of the growth conditions (Figure 3.8A).

Taken together, the *pdeHΔpdeLΔ* and *pdeHΔ* mutants maintain considerably increased levels of intracellular cAMP in contrast to the WT or *pdeLΔ*. In addition, cAMP levels are up-regulated in response to a light stimulus. Thus PdeH negatively regulates the cAMP pathway during asexual development in *M.oryzae*.

The basal levels of cAMP were next quantified in the *pdeHΔ*, *pdeLΔ* and the WT strains during pathogenesis (Figure 3.8B and 3.8 C). The following time points were chosen for the estimation of cAMP levels: ungerminated conidia at 0 h, appressorial initials at 6h, mature appressoria at 21h, penetration peg formation at 24h and formation stage of the infection hyphae *in planta* (28–30h) [173]. In freshly harvested conidia from the *pdeHΔ* strain, the cAMP levels were nearly 2.5 fold ($P < 0.005$) greater in contrast to the WT or *pdeLΔ* strains (Figure 3.8B). At 6h post inoculation (hpi), all the aforesaid strains showed enhanced cAMP levels, however the overall levels of cAMP were considerably higher in the *pdeHΔ* (1.5 fold; $P < 0.005$).

With the exception of the *pdeHΔ*, that maintained increased cAMP levels (nearly 2-fold) compared to WT or *pdeLΔ* (Figure 3.8B), levels of cAMP reduced across all the strains in mature appressoria (21 hpi). Furthermore, at 24 hpi, with the exception of the *pdeHΔ*, which maintained higher levels of steady-state cAMP, cAMP levels decreased further by almost two fold across all the other strains. At later time points, during penetration (24–30 hpi), the *pdeHΔ* continued to maintain significantly elevated levels of cAMP (nearly 2 to 5 fold; Figure 3.8C) contrasting the wild type or *pdeLΔ* strains. cAMP levels could not be reliably measured in the *pdeHΔpdeLΔ* double mutant during pathogenic development, as the strain failed to produce proper conidia.

Based on all the data obtained thus far from *in-vivo* cAMP measurements, it can be inferred or speculated that in *M. oryzae*, the steady-state or basal cAMP levels are controlled in two discrete stages or in a bi-phasic manner: during the early stages i.e. initiation and formation of appressorium, cAMP levels are upregulated, and during the late stages of pathogenesis i.e., during host invasion cAMP levels are down regulated. Nonetheless, based on levels of cAMP measured in *pdeHΔpdeLΔ* and the *pdeHΔ*, it can be hypothesized that function of PdeL is more important during the absence of a functional PdeH, like in the *pdeHΔ* strain.

A **B** **C**

Figure 3.8. PdeH regulates levels of intracellular cAMP during growth and development in *M. oryzae*.

Graphs illustrating cAMP levels present during stages of asexual development and pathogenesis in the *pdeH*Δ, *pdeL*Δ, *pdeH*Δ*pdeL*Δ and wild type.

(A) A bar graph depicting the cAMP levels during asexual development in the designated strains. Before the measurement of cAMP levels the indicated aforementioned strains were cultured for 3 days in the dark (represented by black bar), before light exposure for 24 h (represented by white bar). Values show mean $\pm$ S.E from 2 independent experiments with duplicates.

(B) *pdeH* Δ conidia (represented by black bar), *pdeL* Δ (represented by white bar) and WT (represented by gray bar) strains were inoculated on an inductive surface and cAMP estimations were carried out at the mentioned time points. The 6h and 21h represent the early pathogenesis. Values signify mean $\pm$ S.E from 3 independent experiments performed in triplicates.

(C) cAMP levels were measured at 24, 28, or 30h from conidia incubated on inductive surfaces signifying the late phases of pathogenesis. Values show mean $\pm$ S.E from three separate experiments performed in triplicates.

3.1.5 Characterization of the *PDE* mutant strains-pathogenic development

3.1.5.1 PdeH and surface dependency during appressorium development

To assess if the elevated levels of cAMP in the *pdeHΔ* could stimulate precocious formation of appressorium on non-inductive surfaces, the efficiency with which formation of appressorium occurred was quantified on both inductive and non-inductive surfaces in the *pdeHΔ*, *pdeLΔ* and in the WT strains. Interestingly, the *pdeHΔ* formed mature appressoria with a reasonably high frequency on inductive ($90\pm2.1\%$; Figure 3.9A and B) and non-inductive surfaces ($66\pm2.0\%$; Figure 3.9A and B). However, conidia from the WT or the *pdeLΔ* formed appressoria only on inductive surfaces (75–80%), but not on non-inductive surfaces (4–7%; Figure 3.9A and B). The *pdeLΔ* strain required proper inductive cues for appressorium formation similar to the WT. The inability *pdeLΔ* to form appressoria on non-inductive surfaces supports our estimations of *in-vivo* cAMP levels. Curiously, the aberrant 2-celled conidia elaborated by the *pdeHΔ pdeLΔ* formed appressoria on inductive as well as non-inductive surfaces. Because of the low numbers of such structures formed quantifications were not possible. Taken together, it can be speculated that the enhanced intracellular cAMP levels in the *pdeHΔ* and the *pdeHΔ pdeLΔ* mutants are adequate to uncouple surface dependency from pathogenesis.

Interestingly, prior to initiation of the appressorium on inductive surfaces we noticed that a considerable number of *pdeHΔ* conidia elaborated germ tubes that were extremely short or hardly visible. The lengths of these germ tubes was systematically quantified and grouped into three categories namely: short (short or barely visible), normal (equal to the length of the conidium) and long (longer than the conidium). Nearly $45\pm1.0\%$ of the conidia from *pdeHΔ* strain formed extremely short germ tubes (Figure 3.9 A; arrows), versus $25\pm1.8\%$ in *pdeLΔ* strain and $13\pm1.7\%$ in the WT. $49\pm1.7\%$ of the conidia from the *pdeHΔ* strain elaborated germ tubes with normal length, compared to $69\pm2.3\%$ in *pdeLΔ* and $74\pm2.2\%$ in the WT. Germ tubes which were long were seen in $6\pm1.7\%$ of *pdeHΔ* in comparison to $6\pm2.0\%$ and $13\pm1.8\%$ in conidia from *pdeLΔ* and WT respectively.

Figure 3.9. *PDEH* deletion uncouples surface dependency and pathogenesis.

Quantification of the efficiency of formation of appressorium on inductive as well as non-inductive surfaces.

(A) Conidia from the WT, *pdeH* Δ or *pdeL* Δ strains were inoculated on appropriate surfaces and the efficiency of appressorium formation were assessed post 12 h. The arrows indicate the germ tubes formed by the *pdeH* Δ conidia and were extremely short. Scale bar, 10 μ m.

(B) A graph showing the efficiency of formation of appressoria of the indicated strains on inductive (represented by a black bar) and non-inductive surfaces (represented by a white bar). Values show mean $\pm$ S.E from 3 independent replicates involving 1000 conidia per sample.

Taken together and based on the above observations we suppose that during the early phases of infection-related morphogenesis, PdeH (by regulating basal levels cAMP), guides germ tube growth and influences surface sensing.

3.1.5.2 Appressorial development and maturation is negatively regulated by PdeH

On an inductive surfaces, by 3-4 hpi, the germ tube tip from WT conidia hooked (Figure 3.10A; highlighted by white arrow), this was followed by swelling of the tip of the germ tube as well as isotropic growth giving rise to an immature appressorium by 5-6 hpi, ultimately forming a melanized dome shaped appressorium (Figure 3.10A; highlighted by black arrow) by 8 hpi. It was observed that the compromised PdeH function resulted in a significantly accelerated rate of appressorium formation on inductive surface. A systematic time-course analysis revealed that the *pdeHΔ* was considerably advanced during all phases of pathogenesis.

The tip of the germ tube arising from *pdeHΔ* conidia underwent hooking by 2 hpi (Figure 3.10B; white arrow). Incipient appressoria were formed as early as by 3-4 hpi and mature melanized appressoria were formed by 5 hpi (Figure 3.10B; black arrow). Therefore, conidia from the *pdeHΔ* initiated the process of formation of appressoria earlier and also formed mature appressoria rapidly, at least 3 h in advance in comparison to the WT strain, under identical conditions. The *pdeLΔ* behaved identical to the WT, taking about 8 h to form mature appressoria.

Taken together, these results imply that elevated levels of intracellular cAMP disrupt not only inductive surface based signaling (uncoupling of surface dependency and pathogenic differentiation), but also the temporal regulation of the processes that guide appropriate initiation, formation and maturation of appressoria.

Figure 3.10. Compromised PdeH function accelerates appressorial formation and maturation.

Sequential images of the process of germination and appressorial elaboration on an inductive surface in the wild-type (**A**) and *pdeHΔ* (**B**) strains. Conidia from the strains being tested were inoculated on inductive surfaces at RT. The samples photographed every hour over a period of 8 h. White arrows point to the stage of germ tube hooking and black arrows show a melanized mature appressorium. Scale bar, 10 μ m.

3.1.5.3 Appressorium formation precedes nuclear division in the *pdeHΔ* mutant

It is generally the terminal cell of the conidium that germinates to give rise to the germ tube. It is also the nucleus of the same geminating cell that undergoes a mitotic division within the germ tube, to contribute genetic material to the developing appressorium. In WT conidia inoculated on an inductive surface, mitosis usually occurs during 5 and 6hpi, and is continued by the movement of the daughter nucleus into the emergent appressorium (Figure 3.11A). Furthermore, it has been previously demonstrated that nuclear division or mitosis precedes appressorium development, and blocking replication of DNA or entry into mitotic does not allow formation of appressorium [291].

Since a majority of *pdeHΔ* conidia were accelerated for appressorium formation, the mitotic status of the nuclei in *pdeHΔ* during pathogenic development was determined. Curiously, in the *pdeHΔ*, up to 3 hpi time point, appressorial morphogenesis was rapid and occurred independent of nuclear division (Figure 3.11B; indicated by white arrows). However, by 4 hpi, nuclear division took place in between the terminal cell of the conidia and the newly forming appressorium (Figure 3.11B; asterisk). This was continued by the movement of one of the daughter nuclei inside the appressorium that is maturing. Thus, based on these observations it can be concluded that elevated levels of cAMP in *pdeHΔ* conidia can considerably advance appressorial morphogenesis/development in a manner independent of nuclear division.

A**Wild Type** **B*****pdeH*Δ**

Figure 3.11. Disruption of PdeH function advances appressorium morphogenesis in a manner independent of nuclear division.

Conidia from WT (**A**) or *pdeH*Δ (**B**) were incubated on an inductive surface, stained with DAPI and were evaluated at the mentioned times after incubation. Arrows indicate the hurried morphogenesis of the appressorium, while the asterisk (*) marks the nucleus undergoing mitosis in the *pdeH*Δ. Scale bar, 10 μm.

3.1.6 PdeH is essential for virulence in *M. oryzae*

It was evaluated if the *pdeH*Δ could cause disease earlier in contrast to the WT, since the *pdeH*Δ was accelerated for appressorium formation. This was tested by spray inoculating rice seedlings (susceptible variety CO39) at the three leaf stage with conidia from *pdeH*Δ, *pdeL*Δ and wild type control strains. Symptoms of the disease were assessed 9-10 days after inoculation. Leaves of seedlings inoculated with spores from the WT or *pdeL*Δ strains displayed several lesions that were typically spindle-shaped with gray centers. However, the *pdeH*Δ conidia failed to cause disease successfully, even after 14 days post inoculation. The *pdeH*Δ instead, formed only tiny brown speckles that did not coalesce and appeared more like a typical hypersensitive response (HR) similar to that displayed by a moderately resistant host plant (Figure 3.12A).

To verify our results, infection assays were also carried out on barley explants. Predictably, leaf pieces inoculated with conidia from either the WT or *pdeL*Δ at various dilutions displayed typical blast lesions unlike the *pdeH*Δ strain that failed to cause disease symptoms under identical conditions (Figure 3.12B). Similar outcomes from both the experiments strongly indicated that the *pdeH*Δ was indeed compromised for infection.

It was also tested if the aberrant structures (including, two-celled conidia) formed by *pdeH*Δ *pdeL*Δ were competent enough to cause disease. For controls, we used equal amounts of conidia from the WT. Curiously, unlike the control WT, *pdeH*Δ *pdeL*Δ did not lead to any symptoms of the disease on the leaves. In independent experiments on barley leaves, conidia from the complemented *pdeH*Δ (RFP-PdeH expressing strain) caused disease identical to the WT or the *pdeL*Δ.

Taken together, it can be concluded that PdeH function, by maintaining steady-state cAMP levels, is crucial for effective pathogenesis in *M. oryzae*.

Figure 3.12. Compromised PdeH function results in reduced pathogenicity on host plants.

(**A**) Conidia from the individual strains were sprayed on the rice seedlings and the symptoms were evaluated post 9 days. (**B**) Conidia harvested from the WT, *pdeHΔ* or *pdeLΔ* strains were spot-inoculated in triplicate onto explants of barley leaves and symptoms of disease were evaluated post 7 days.

3.1.7 *In planta* growth and development is regulated by cAMP signaling in *M. oryzae*

Plant cells react to microbial attack by by the quick synthesis and deposition of callose (a (1→3)-β-d-glucan) as plugs or plates near the site of invasion by the pathogen. These callose containing deposits are generally called as papillae and are believed to comprise of (1→3)-β-d-glucan, small quantities of additional polysaccharides, proteins, reactive oxygen intermediates and phenolic compounds. It has been hypothesized that these papillae function as a physical barriers to slow down penetration and spread by microbes. Callose deposits can be easily visualized under a microscope, by UV light–induced fluorescence upon staining with blue fluorochrome, aniline.

To determine whether the *pdeHΔ* mutant appressoria were compromised in its ability to either penetrate (form a penetration peg by 24 hpi) or elaborate primary infectious hyphae (between 36-48 hpi), host penetration was assessed and quantified utilizing aniline blue staining. By, 22 hpi, only 3±0.8% of the appressoria formed by WT conidia elaborated penetration pegs in contrast to 10±1.2% in *pdeHΔ*, suggesting a considerable progression ($P < 0.005$) in the capability of *pdeHΔ* appressoria to form penetration pegs. However, by 1 day post inoculation, there was no obvious difference between the two strains, with 75±2.8% of the WT and 73±3.8% of *pdeHΔ* appressoria forming penetration pegs (Figure 3.13A to D).

At 36 hpi, 90±5.0% of the WT penetration pegs developed into infectious hyphae, compared to only 14±1.7% in *pdeHΔ* strain (Figure 3.13C and D). Taken together, the quantifications as well as observations strongly imply that the *pdeHΔ* is fully capable in its ability to penetrate the host, but is compromised in differentiating infectious hyphae that aid subsequent *in-planta* spreading. Observations made at 48 hpi revealed that this defect persisted in the *pdeHΔ*, with 35±3.6% of *pdeHΔ* and 85±3.2% of WT penetration pegs forming *in planta* infectious hyphae respectively (Figure 3.13C and D). More importantly, the infection hyphae produced by the *pdeHΔ* was confined to the first invaded host cell. In sharp contrast, the *in planta* infection hyphae produced by the WT and the *pdeLΔ* strains had accomplished cross-wall penetration and ramified to neighboring cells (Figure 3.13E).

Even after 72 hpi the aberrant pigmented structures formed by *pdeHΔpdeLΔ* did not germinate or elaborate appressoria, and consequently did not elicit a host response (Figure 3.13F).

Surprisingly, the 2-celled aberrant conidia made by *pdeHΔpdeLΔ* effectively penetrated the surface of the host as soon as 22 hpi. However, tests at later time points i.e., 36, 48 and 72 hpi showed that *pdeHΔpdeLΔ* was also obstructed at the stage of penetration (Figure 3.13F).

Taken together, the results obtained thus far suggest that the principal cause for the inability of *pdeHΔ* to cause characteristic disease lesions is because of its poor capacity for formation of proper infection hyphae and additionally promote its development and spread within the host. In conclusion, regulation of cAMP levels and signaling in a manner influenced by PdeH is critical for colonization of the host by *M. oryzae*.

A **B** **C** **D**

E

F

Figure 3.13. The *pdeH*Δ mutant fails to colonize the host.

Comparison of penetration of the host and analysis of development in the plant by WT and *pdeH*Δ strains. Photographs showing host papillary callose deposits stained with aniline-blue in the WT (A) or *pdeH*Δ (B) at the mentioned points in time after inoculation. Scale bar, 10 μm.

(C and D) Graphs illustrating successful host penetration (marked by black bars) and its efficiency to subsequently develop into *in planta* infection hyphae (marked by gray bars) in the tested strains.

(E) Images following the advancement of the infection hyphae and its capability to ramify and colonize the host. Growth in the plant of the aforementioned strains 2 and 3 days post inoculation on barley leaf explants. Scale bar, 10 μm .

(F) Graph depicting successful penetration into the host (marked by black bars) and the development of infection hyphae (marked by gray bars) by the 2-celled conidia (n=25) in *pdeL* Δ *pdeH* Δ . Aniline-blue stained structures represent host papillary callose deposits at the mentioned points in time after inoculation. Scale bar, 10 μm .

3.1.8 Transcriptional regulation of *PDEH* and *PDEL* in *M. oryzae*

To measure the levels of *PDE* transcript during various phases of pathogenic development, quantitative Real-Time RTPCR analysis was carried out to elucidate if transcripts of *PDE* were differentially controlled due to altering levels of cAMP. The tissue types and time points used for the experiment were freshly harvested conidia at 0 h, germinated conidia at 3 h and incipient appressorial initials at 6 h. Additionally Real-Time RTPCR analysis was also carried out on infected leaves samples at varied stages of *in-planta* growth. It has been recently reported that the transcript levels of BAS1 (Biotrophy Associated Secreted protein-1, MGG_04795) is highly upregulated during infectious development *in-planta* [181]. We thus utilized BAS1 as a positive control during the RT-PCR analysis involving growth *in-planta*.

In the early phases of pathogenic differentiation, Real-Time analysis showed that the levels of the *PDEH* transcript was considerably down regulated (nearly 2.5 fold at 3hpi) and (nearly 2 fold at 6hpi) in comparison to WT conidia that were freshly harvested (0 h) (Figure 3.14A). An identical inclination of down regulation was followed by the *PDEL* transcript. The *PDEL* transcript was down regulated nearly 1.4 fold and 2.5-fold at 3 and 6 hpi respectively. Interestingly however, during the time points representing stages of infectious *in-planta* growth, the levels of the transcript of *PDEH* were constantly elevated at all the tested time points: 1.5-fold at 1 day post inoculation, 3-fold at 29hpi and 5-fold at 2 days post inoculation compared to the 21 hpi time point (Figure 3.14B). In contrast, *PDEL* transcript levels did not vary at the identical tested time points.

In conclusion, the experiments suggest that in *M. oryzae*, the *PDEH* transcript levels are regulated differentially during early and late stages of pathogenesis, in correlation to varying levels of cAMP at these stages.

Figure 3.14. Differential regulation of *PDEH* transcript during infectious development.

(A) Total RNA extracted at the indicated time points from WT conidia that were incubated on inductive surface, was evaluated by quantification by Real-Time RTPCR. The black bar represents the *PDEH* transcript while the white bar represents the transcript of the *PDEL*. The levels of expression at 0 h were considered baseline and indicated as 1.0. (B) The transcript levels of the *PDEL* or *PDEH* were also analyzed during the later stage of pathogenesis (24h, 29h and 48h), with total RNA at the designated time points from barley leaf explants inoculated with WT spores. As a positive control, BAS1 was used for infectious growth. The level of expression at 21hpi was set as 1.0.

3.1.9 The transcripts of *PDEL* are regulated differentially in *pdeHΔ*

Our previous result indicates that *PDEL* transcript was more abundant during the early pathogenesis, 3-6 hpi, a time point during which cAMP signaling is enhanced, suggesting that PdeL, a known low-affinity phosphodiesterase, functioned optimally under such conditions. To support this observation and establish a function for PdeL under high cAMP conditions, the transcript levels of the *PDEL* in *pdeHΔ* (which is activated for cAMP signaling) was measured during pathogenic development. The level of the *PDEH* transcript was also ascertained in the *pdeLΔ* mutant. During the early stages, as previously demonstrated, in the wild type and *pdeLΔ* strains the *PDEH* transcript was down regulated (nearly 2-fold at 3h and nearly 2.5-fold at 6h). At the late stage, the *PDEH* transcript levels were upregulated: nearly 2-fold at 24h and nearly 5-fold at 48h (Figure 3.15A and previous experiment). Taken together, the levels or expression pattern of the *PDEH* transcript in the *pdeLΔ* was analogous to that in the wild type.

However, in a manner to corroborate its proposed role, in the *pdeHΔ* the *PDEL* transcript levels notably elevated during the early and later phases of pathogenesis, irrespective of the different conditions and time-points tested, unlike in the WT (Figure 3.15B).

In conclusion, it can be safely speculated that *PDEH* (and function of PdeHp) may respond to small variations in intracellular cAMP levels. On the other hand, *PDEL* and activity of PdeL plausibly reacts to significantly increased cAMP levels, similar to those common in *pdeHΔ*.

Figure 3.15. Expression analysis of transcripts of *PDEH* and *PDEL* in WT and mutant backgrounds.

(A) Real Time RTPCR analysis comparing the *PDEH* transcript levels in the WT (indicated by the black bar) and *pdeL*Δ (indicated by the white bar) strains at the specified time points. (B) *PDEL* transcript levels were also ascertained in the WT (black bar) and *pdeH*Δ (white bar) strains. Data was normalized to endogenous β tubulin transcript as controls (MGG_00604). Each experiment was repeated thrice with triplicates for every data set.

3.1.10 Wild-type conidia supplemented with cAMP or IBMX mimics *pdeHΔ*-like defects

Compromised PdeH function caused a derailment of cAMP signaling, leading to the accumulation of higher levels of intracellular cAMP. It was verified if the addition of exogenous 8-Br-cAMP, a variant of cAMP that is membrane permeable, or IBMX, an inhibitor of phosphodiesterase, to the wild type would cause it to mimic *pdeHΔ* like defects.

The wild-type strain displayed defects reminiscent of the *pdeHΔ* strain upon addition of either cAMP or IBMX (Figure 3.16A). Additionally, although the WT treated with 10 mM cAMP exhibited a minor rise in numbers of conidia, samples treated with 50 mM cAMP induced a greater i.e. nearly 1.6-fold ($P \leq 0.01$) increase in conidia production (Figure 3.16B) compared to the untreated control. Although significant, the conidiation was not as enhanced (2.3-fold; $P < 0.005$) as displayed by *pdeHΔ*. Compared to the control inoculations, WT conidia inoculated in when 10 mM cAMP was present on barley leaves displayed a dose-dependent decrease in size of the lesions and severity of the disease (Figure 3.16C). Surprisingly, conidia from WT treated with 2.5mM IBMX displayed no symptoms of disease (Figure 3.16C).

Quantification of penetration pegs by aniline blue staining revealed that nearly $75 \pm 4.0\%$ of wild type appressoria treated with cAMP, at 24 hpi, successfully formed penetration pegs, yet by 36 hpi, only $40 \pm 3.4\%$ of the penetration pegs developed into infection hyphae *in planta*. At 2 days post inoculation, there was an increase in the the number of infection hyphae formed to $47 \pm 3.0\%$ (Figure 3.16D and G). In contrast, at the corresponding time points, in the control untreated sample $90 \pm 3.6\%$ penetration pegs formed progressed into *in planta* infection hyphae (Figure 3.16E & H). Although WT conidia treated with IBMX efficiently formed appressoria on the leaves, these appressoria did not form penetration pegs by 36 hpi. Even at 3 days post inoculation, these treated samples did not develop infection hyphae (Figure 3.16F).

A **B** **C** **D**

Figure 3.16. Addition of 8-Br-cAMP or IBMX to WT causes *pdeHA*-like defects.

(A) Mycelial plugs from the WT colony were grown on PA medium having the designated amounts of 8-Br-cAMP or IBMX. The cultures were grown for 5 days in the dark before being analyzed.

(B) WT was grown in varying amounts of 8-Br-cAMP, and numbers of conidia were ascertained subsequent to a 6 day exposure to constant illumination

(C) WT conidia were incubated in the presence of indicate amounts of 8-Br-cAMP or IBMX. The symptoms of disease were scored 6 days after incubation

(D) WT conidia were inoculated on explants of barley leaf and incubated with 50 mM 8-Br-cAMP. The efficiency of formation of penetration pegs (indicated by black bars) and infection hyphae (indicated by gray bars) was evaluated at the specified time points.

(E) WT conidia were inoculated on barley leaves incubated with the solvent (ethanol). Graph illustrating the efficiency of penetration peg and infection hyphae formation

Barley leaf explants inoculated with WT conidia and incubated with 2.5 mM IBMX **(F)** or 50 mM 8-Br-cAMP **(G)** and photographed at specified time points. **(H)** WT incubated with the solvent (ethanol for IBMX) acted as control.

In conclusion, WT conidia treated with either cAMP- or IBMX- mimicked to a great extent the defects displayed by the *pdeHΔ* strain during growth of aerial hyphae, formation of conidia and progression of the disease. The above results are in support of our speculation that the deficiencies displayed by *pdeHΔ* is most likely due to increased endogenous cAMP levels, a consequence of compromised PdeH function.

3.1.11 Subcellular localization of RFP-PdeH

In order to follow the subcellular trafficking and to understand the temporal and spatial dynamics of PdeH, an N-terminal *RFP-PDEH* construct expressed under the native promoter in the *pdeHΔ* strain was generated. The strategy was aimed to additionally act as a tool for complementation that could in principle rescue the defects exhibited by *pdeHΔ* strain (Figure 3.17)

The localization pattern of RFP-PdeH was tracked at various time points and at different stages of asexual and pathogenic development. A weak cytosolic localization of RFP-PdeH was observed in the vegetative hyphae and developing conidiophores (Figure 3.17A and B). However in the conidial terminal cell (0h), PdeH localized to as punctate vesicular structures. At 2- 4 hpi on an inductive surface, PdeH localized to distinct punctate. Such structures were visible all over conidial terminal cell including the germ tube (Figure 3.17C; arrow). Additionally, we observed weak although definite signal, from the membrane of the germ tube (Figure 3.17C; arrow). Between 6 and 8 hpi, RFP-PdeH localized as arbitrarily distributed vesicles in the newly formed appressorium and weakly along the membrane of the developing appressorium. In mature melanized appressoria at 21 hpi, the RFP-PdeH localized predominantly to structures that resembled vacuoles (Figure 3.17C).

Figure 3.17. Subcellular distribution of RFP-PdeH during asexual and pathogenic development.

(A) Imaging of the hyphae from RFP-PdeH strain as carried out post 3 d growth on prune agar medium. Scale bar, 10 μm .

(B) Mycelial blocks from RFP-PdeH cultures grown in the dark were exposed for 24 h to light. Images of developing aerial (conidiophore) structures was captured the next day. Scale bar, 10 μm .

(C) Harvested conidia were inoculated on an inductive surface and photographed at the time points as indicated. The arrows point to the RFP-PdeH punctate at 2hpi and 4hpi. Scale bar, 10 μm .

Given that the *pdeH* Δ strain failed to cause disease in rice and the penetration hyphae formed were restricted to the first infected cell, the sub-cellular localization of RFP-PdeH in the infectious hyphae was determined. Microscopic examination at 36 hpi of rice leaf sheath revealed that RFP-PdeH was present all over the cytosol, with no specific localization pattern within the infection hyphae (Figure 3.18).

Thus, RFP-PdeH displayed a localization that was largely cytosolic at the stage of asexual development (in the conidiophores) and during the biotrophic phase in the infection hyphae. However, during appressorium morphogenesis on an inductive surface it localized to cytosolic vesicles and poorly to the germ tube plasma membrane.

Infection Hyphae

Figure 3.18. RFP-PdeH exhibits a uniform cytosolic localization during *in-planta* growth.

Conidia from the RFP-PdeH strain were incubated on the sheath of rice leaf and at 36 hpi the *in-planta* hypha was imaged utilizing epifluorescence microscopy. Scale bar, 10 μ m.

Next, to visualize better the subcellular distribution and dynamics, a strain expressing a fusion construct of GFP-PdeH expressed under the *MPG1* promoter [292] was generated in the *pdeHΔ* background. Another strain expressing a GFP-PdeL translational fusion also expressed under *MPG1* promoter was also generated. The subcellular localization of both the tagged proteins was examined during asexual and pathogenic development. The GFP-PdeH showed a moderate cytoplasmic signal in the mycelia as well as in the developing conidiophores in contrast to the poor RFP-PdeH signal (Figure 3.19A and B). At a identical stage of development, the pattern of GFP-PdeH localization was similar to that was exhibited by the RFP-PdeH expressing strain.

Next, live-cell imaging was carried out on the GFP-PdeH strain in the early and late phases of pathogenesis at the indicated time points: 2–3hpi (conidial germination; Figure 3.20A), 4–5hpi (hooking stage; Figure 3.20C), and 6–7hpi (mature appressorium; Figure 3.20D). Time-lapse imaging showed that the GFP-PdeH protein localized onto highly dynamic and mobile vesicular compartments (Figure 3.20A). Co-staining with Hoechst 33342, a nuclear dye, validated a peri- and/or extra- nuclear localization for PdeH vesicles (Figure 3.20B). At the hooking stage, 4–5hpi, PdeH marked the plasma membrane of the germ tube. Additionally PdeH was observed to localize to dynamic compartments trafficking between the conidia and the developing germ tube (Figure 3.20C). Furthermore, at 6 hpi, GFP-PdeH although vesicular, was predominantly enriched in the plasma membrane of the immature appressorium (Figure 3.20D).

Figure 3.19. Subcellular localization of the GFP-PdeH during conidiophore development in *M. oryzae*.

The GFP-PdeH was predominantly cytosolic in the vegetative mycelium (A) and conidiophores (B). Scale bar, 10 μm .

A**Germinating Conidium** **B** **Hooking Germtube****C**

D

Figure 3.20. Subcellular localization and dynamics of GFP-PdeH during appressorial formation in *M. oryzae*.

(A) Snapshots from a time-lapse movie, depicting dynamic GFP-PdeH structures at 2 hpi. Scale bar, 10 μ m.

(B) GFP-PdeH conidia were stained with Hoechst 33342 to mark the position of the nucleus and visualized utilizing epifluorescence microscopy. GFP-PdeH localized as foci at the perinuclear regions or existed as cytoplasmic punctae. Arrows point to the peri-nuclear localization of PdeH. Scale bar, 10 μ m.

(C and D) Snapshots from a movie illustrating the dynamic nature of PdeH during the hooking stage and appressorium formation. Scale bar, 10 μ m.

Figure 3.21 Nuclear targeting of GFP-PdeL during asexual development and pathogenesis in *M. oryzae*.

- (A)** Nuclear localization of GFP-PdeL (arrows) in the vegetative mycelium.
- (B)** GFP-PdeL in young and mature aerial conidiophores.
- (C)** Co-staining of GFP-PdeL conidia with nuclear stain DAPI at 0 hpi.
- (D)** A conidium incubated on an inductive surface for 2 h, followed by staining with DAPI.
- (E)** Localization of GFP-PdeL in appressorium at 12 hpi on an inductive surface. Scale bar, 10 μm in all the above panels.

Subsequently, the subcellular localization of the GFP-PdeL protein was determined in *M. oryzae*. Very curiously, the GFP-PdeL displayed a strong nuclear localization during all the phases of asexual development and pathogenesis (Figure 3.21A to E). The nuclear targeting of GFP-PdeL was validated by staining with a specific nuclear stain, DAPI (Figure 3.21C and D).

Thus, the predominantly compartmentalized localization of PdeH on vesicular structures (including the plasma membrane) in the cytosol and of PdeL to the nucleus implies that both the PDEs regulate differentially and probably control separate subcellular pools (nuclear vs cytosolic) of cAMP. Furthermore, the results strongly imply that time dependent modulation of cAMP signaling in *M. oryzae*, largely by PdeH, is important for controlling the disease causing capacity and progression.

3.2 Discussion

In the current chapter, the implication of PDE-mediated modulation of cellular cAMP levels during development and pathogenesis in the rice blast fungus were investigated. Overall, our results support a model in which PdeH, negatively influences cAMP signaling, albeit indirectly, by modulating the steady state or basal levels of cAMP. The consequences of gene-disruption of 2 cAMP PDEs-encoded by the *PDEH* (high-affinity) and *PDEL* (low-affinity) genes were assessed either independently or in combination. The results suggest an exclusive and non-redundant role for PdeH as well as PdeL; but emphasize the overall importance of the high affinity PDE, PdeH, as an important regulator of cAMP signaling in *M. oryzae*.

PdeH although cytosolic, was restricted to the perinuclear regions as punctate vesicular foci. Such an association of PDEs to perinuclear membranous compartments has been demonstrated in yeast previously through fractionation studies [293]. Furthermore, the results support a role for PdeH as a crucial modulator of intracellular cAMP levels during asexual development, unlike PdeL, whose function was found to be dispensable. This implies that PdeH is capable of recompensing for the absence of PdeL. Interestingly, concurrent deletion of both *PDEs* was detrimental to the growth and development, strongly indicating that surplus cAMP is deleterious above a certain threshold.

We found evidence that PdeH modulates cAMP signaling at 2 crucial phases during pathogenesis, viz. formation of appressorium and host invasion. This was further supported by the measurement of intracellular levels of cAMP in the WT and the *PDE* mutant strains, during the early and late stages of infection. An initial increase in cAMP levels in the WT during the early phase (i.e., appressorium formation) [193, 207], is possibly an outcome of a simultaneous reduction in the levels of the *PDEH* transcript, as judged by real-time PCR analysis (Figure 3.21). Studies on the regulation of PDEs in metazoans as well as in lower eukaryotes such as *Dictyostelium*, describes the existence of feedback loops between *PDE* gene expression and cycling cAMP levels [294, 295]. A decrease in PdeH function could also result from either modifications that occur post-translationally, as previously shown in yeast and mammalian cells

[59, 139, 141, 296] and the dynamic recycling of the PdeH vesicles away from the zones of active growth or signaling.

Another important aspect of this work is the finding that intracellular cAMP levels or cAMP signaling *per se* in *M. oryzae* necessitates being down-regulated during the stage of appressorium maturation (Figure 3.22). This is crucial as it greatly influences determination of the ability of *M. oryzae* to successfully colonize/infect the host. Supporting this is the result that demonstrates that exogenously supplemented cAMP retards *in planta* growth of the WT during late stages of infection.

Figure 3.22. Model illustrating a bi-phasic regulation of intracellular cAMP levels mediated by the PdeH, a high-affinity phosphodiesterase in *M. oryzae*.

The high-affinity phosphodiesterase facilitates the modulation of intracellular pools of cAMP at two distinct phases (upregulation during appressorial development and down-regulation during host invasion) is critical for proper pathogenic and infectious growth in *M. oryzae*.

Nevertheless, we suppose that there seems to be a critical threshold and dose-dependent response to elevated levels of intracellular cAMP, as loss of both PDEs concurrently, leads to the formation of abnormal fungal structures and a termination of spore production.

In the *pdeL* Δ strain the intracellular cAMP levels do not get affected considerably during conidiation or during formation of appressoria. However, the phenotype and the high cAMP levels of the *pdeH* $\Delta*pdeL* Δ double-deletion mutant suggest a function for PdeL in influencing cAMP cannot be ruled out. It is likely that PdeL function is more important under circumstances when there are higher levels of cAMP, as in the *pdeH* Δ strain. Given its subcellular localization, it is likely that PdeL responds and functions to regulate gradients of cAMP within the nucleus. Such a scenario was proposed in a previous study [297].$

A consequence of enhanced cAMP levels in the *pdeH* Δ was that significant populations of the mutant conidia were accelerated for appressorium formation. A high number of *pdeH* Δ conidia elaborated extremely short germ tubes on inductive surfaces. We hypothesize that although PdeH may not be directly implicated in the regulation of morphogenesis and cell shape, the short germ tube is likely a consequence of the premature activation of downstream signaling events. Our data also indicates that enhanced basal levels of cAMP can supersede the necessity for nuclear division before formation of appressorium. Nevertheless, further studies are needed to better understand the connection between cell cycle progression and enhanced cAMP signaling in *M. oryzae*.

The reduced or defective *in planta* growth of the *pdeH* Δ is probably a consequence of augmented sensitivity of the fungus due to conditions of stress experienced within the host. For example, experimental evidence from *C. albicans* indicates that cAMP cascade is an important modulator of stress signaling. *C. albicans pde2* Δ mutant is more susceptible to peroxide and cadmium induced stress [298]. Further studies would be focused on addressing the relationship between stress and cAMP signaling in *M. oryzae*.

4. Results II

Chapter IV

4.1 Structure-Function Analysis and Subcellular Localization of Rgs1 and its interaction with a non-canonical GPCR, Pth11, in *M. oryzae*

Introduction

The precise translation of signals into suitable and accurate responses in the cell is an outcome of appropriate regulation of the accuracy and strength of G-protein signaling. G-protein signaling is active until the G α subunit, by its intrinsic GTPase activity hydrolyses bound GTP to GDP. Thus, the duration of active G-protein signaling depends on the state of the guanine nucleotide state of G α subunit [128, 299].

Regulator of G-protein Signaling (RGS) proteins form an important element controlling the strength and duration of G-protein signaling as they function to accelerate the breakdown of GTP to GDP on the active G α subunits [214, 300]. RGS proteins act by stabilizing the “switch” regions on the G α subunits. These switch regions undergo conformational changes upon GTP hydrolysis (Figure 4.1).

Since their discovery in the 90's, RGS proteins have been found to be conserved in a wide range of species and have been identified as important regulators of G-protein signaling in multiple model organisms. In humans more than 50 RGS proteins have been identified. The first RGS protein to be identified was Sst2 that functions as a negative regulator of the pheromone response pathway.

The RGS proteins have been found to be conserved across species and have been functionally characterized in many fungi. RGS proteins have been found to be conserved in *S. cerevisiae*, *S. pombe* [301, 302] and filamentous fungi such as *A. niger*, *C. albicans*, *C. neoformans*, *C. parasitica*, *U. maydis*, *Fusarium spp*, *M. oryzae* and *Metarhizium* [207] [93, 133, 303-308].

Figure 4.1. Schematic representation highlighting the role of *RGS1* and a putative receptor (Pth11) in a canonical G-protein signaling cascade in *M. oryzae*. Adapted and modified from [309]

It has been previously demonstrated experimentally that deletion of RGS protein Sst2 in *S. cerevisiae*, increases the sensitivity to pheromone by 100 to 300-fold. Additionally, the *sst2*Δ cells arrest in the G1 phase of the cell cycle [127]. On the contrary, overexpression or mutations for gain-of-function in Sst2 severely reduces the effect of pheromone induced cell cycle arrest [127, 132]. The Sst2 ortholog, Rgs1, in *M. oryzae* was also identified to function as a negative regulator of G-protein signaling. Furthermore, it was found to facilitate responses to thigmotropic cues from the host [207]. The *rgs1*Δ strain was defective in conidiation, inductive surface based signaling, wettability, intracellular cAMP levels and disease causing ability [207, 310]. A recent study by Zhang et al in *M. oryzae* identified and characterized 7 more RGS family members. In addition to previously characterized functions of Rgs1, the authors further demonstrated that Rgs1 plays an important in maintaining integrity of the fungal cell wall, sexual development, hydrophobicity of colony surface, as well as *in planta* growth [211].

RGS proteins in fungi are generally composed of two N- terminal DEP domains (~ 80 aa): a degenerate DEP-A and a conserved DEP-B motif and further at the C-terminal region, a conserved catalytic RGS core motif (~120 aa) [307, 311]. The presence of repeated DEP domains is apparently exclusive to fungal RGS proteins, besides, the DEP motif is associated with targeting RGS proteins to intracellular organelles such as the Golgi as well as to the plasma membrane [312, 313].

Previous *in vitro* studies have shown that the catalytic core RGS domain to be essential and enough for the GAP function displayed by the RGS/Sst2 protein. However, *in vivo* studies have demonstrated that even moderate or small deletions of the protein at the N-terminus abolish RGS/Sst2 function [127, 314]. Therefore, an integral DEP-DEP domain is important for appropriate function of the RGS protein. It has been also experimentally demonstrated *in vivo* that Sst2 is subjected to endoproteolytic cleavage, resulting in the release of independent fragments of the N-terminal DEP-DEP and C-terminal RGS core motif [315].

PTH11 is an integral membrane protein and localizes to the plasma membrane. It is the only protein believed to function upstream of the cAMP signaling cascade in *M. oryzae* [202]. The N-terminal region of *PTH11* contains an EGF (Epidermal Growth Factor)-like cysteine rich CFEM (Common in Fungal Extracellular Membrane proteins) domain. Pth11 has been predicted to

contain two N-terminal transmembrane loops which include a non-membrane spanning signal sequence and a hydrophobic region within the extracellular CFEM domain, followed by 7-transmembrane domains. Interestingly the CFEM domain has been implicated to function during host–pathogen interactions as adhesion molecules, cell-surface receptors or signal transducers [203]. In *M. oryzae* 61 Pth11-like proteins having the characteristic CFEM domain have been identified based on the analysis of the genome [111, 172].

Furthermore, properties of the CFEM domain suggest that it is different from other known cysteine-rich domains, like those present in epidermal growth factor and hydrophobins. Hydrophobins are surface-active molecules known to be involved in cell adhesion and play a central role in the interaction with the environment. However, the structural features of hydrophobins with respect to domain size, spacing and pattern of cysteine residues are different when compared to those of CFEM domain [316, 317].

In this chapter, I demonstrate that Rgs1 in *M. oryzae* undergoes limited proteolysis or endoproteolytic cleavage to produce an N-terminal DEP-DEP motif and a $G\alpha$ -GTP interacting C-terminal RGS domain. Additionally, using mCherry-tagged DEP-DEP domains (DEP-A and DEP-B expressed individually or in tandem) or only the RGS catalytic core domain I also show that the DEP domain acts to mainly target the RGS motif to vesicles. In absence of the DEP motif, the RGS core is in turn mobilized to the vacuole. Furthermore, I show that the CFEM domain in *PTH11* is crucial for proper inductive surface-based cAMP signaling and that the physical interaction between Rgs1 and Pth11 validates Pth11 as a *bonafide* upstream activator and a likely receptor for cAMP signaling in *M. oryzae*.

Results

4.1.1 Organization of conserved domains and sequence alignment of *M. oryzae* Rgs1

To reiterate, the Rgs1 protein in *M. oryzae* has two tandem DEP domains (amino acids 225–325 and 408–486 respectively). A conserved G α -GTP interacting catalytic core RGS domain is located between the residues 532–687aa. A putative cleavage site for endoproteolysis is present in the stretch of 37 aa between the DEP motif and the RGS core (Figure 4.2A and B).

Previously, it has been shown that yeast Sst2 is subjected to endoproteolytic cleavage at the serine (S) and asparagine (N) residues at positions 415 and 417 respectively [315]. However, our sequence analysis and alignment of *RGS* proteins from filamentous fungi revealed that only the asparagine (N) was conserved. The polar uncharged residue serine (S), was substituted with aspartic acid (D), a polar amino acid (Figure 4B).

In conclusion, *M. oryzae* Rgs1, in addition to possessing conserved domains and functioning as a GAP for G-proteins, it likely retains an Sst2- like putative endoproteolytic cleavage site.

A

B

Figure 4.2. Schematic of conserved domains in *M. oryzae* Rgs1 and comparison of sequences from fungal RGS proteins and their organization.

(A) Representation of RGS proteins: containing N-terminal DEP-A (225-325aa) and DEP-B (408-486aa) domains and C-terminal RGS core (532-687aa) domain. The arrowhead indicates the putative endo proteolytic cleavage site.

(B) Alignment of RGS protein sequences from different fungal species: *M. oryzae*, *A. nidulans*, *S. pombe* and *S. cerevisiae*. The two DEP domains, DEP-A (marked in blue/black) and DEP-B (marked green) and the core RGS domains (marked red/gray) are conserved in yeast species and filamentous fungi. The asterisks show predicted cleavage sites (S-Serine and N-Asparagine) for *S. cerevisiae* Sst2. For purposes of alignment the RGS motif of Sst2 was shortened at positions # and ^. The conserved amino acids are colored in black and the similar amino acids are shown in gray.

4.1.2 Identification of post-translational endoproteolytic cleavage of Rgs1

To ascertain if Rgs1 undergoes post-translational endoproteolytic cleavage, we performed an analysis of the whole cell protein extracts by western blot utilizing the following strains: wild type, Rgs1-OE (this strain over expresses Rgs1 protein due to the presence of additional copies of the *RGS1* gene and has been previously characterized [310]) and Rgs1-GFP (in this strain, Rgs1 is tagged with GFP at the C-terminal and is driven by the native promoter).

To detect possible N-terminal fragments of Rgs1, Western blot analysis was performed with total protein extracts from the Rgs1-OE strain utilizing a polyclonal (α -DEP)antibody specifically to the DEP-DEP motif of Rgs1 [310] . We observed two bands, one at ~80 kDa (depicting the full-length protein, Rgs1) and one at ~ 55kDa, representing the cleaved fragment at the N-terminal (Figure 4.3A). An identical pattern of proteolysis was observed in the WT, thus confirming the occurrence of such a post-translational processing event (Figure 4.3A, lane 2). Further, to detect the cleaved RGS fragment, we used anti-GFP antibodies and performed a western blot with protein extracts of Rgs1-GFP (Figure 4.3B). We again observed two major bands, one at ~110 kDa, depicting the full-length Rgs1-GFP and another at ~50 kDa, representing the cleaved RGS motif fused to GFP, RGS-GFP (Figure 4.3B, lane 2). WT extracts acted as control (Figure 4.3B, lane 1).

In conclusion, we show that Rgs1 in *M. oryzae* undergoes limited proteolysis post-translationally, possibly at the conserved residues (Figure 4.2A and B), to give unique DEP-DEP and RGS motifs.

Figure 4.3. Rgs1 in *M. oryzae* is subjected to endoproteolytic processing.

(A and B) Total proteins extracted from the Rgs1 overexpression (Rgs1-OE) or Rgs1-GFP strains subjected to western blot utilizing antibodies raised against N-terminal DEP-DEP domain (α -DEP) and GFP (α -GFP). Definite bands corresponding to the N- and C- terminal cleavage products of Rgs1 were identified. The WT extract acted as control. A likely post-translationally modified form of Rgs1 is marked by an asterisk. Molecular mass standards are depicted in kDa. Images in this figure were contributed by Liu Hao.

Figure 4.4. Expression of fragments of the independent motif fails to rescue colony and aerial defects displayed by *rgs1Δ* strain.

(A) A diagram showing the DEP-A and DEP-B domains, marked in blue and green colors respectively. The RGS domain is depicted in red. The N-Rgs1 consists of 1-225 aa, including the tandem DEP domains (225-486aa). The C-Rgs1 fragment consists of only the RGS domain (532-714aa).

(B) Morphology of the C-Rgs1-mC, N-Rgs1-mC, *rgs1Δ*, Rgs1-mC and WT colonies. The indicated strains were grown on PA for a week in the dark and subsequently photographed.

(C) Sections of the above colonies (in B) at medial planes depicting the extent of aerial hyphal growth.

4.1.3 Analysis of individual domain expressing strains for suppression of Rgs1 deletion defects during asexual development

In order to identify the function of the independent motifs of Rgs1 in *M. oryzae*, strains in which the fluorescent protein mCherry (mC) was C-terminally tagged to the individual domain fragments, namely the DEP-DEP (hereafter referred to as N-Rgs1) and RGS motif (referred to as C-Rgs1) were generated. These fragments were expressed in the *rgs1*Δ background. Strains in which the expression of the fluorescently-tagged fragments was confirmed by Western blot were selected for further characterization. The domain-tagged strains appeared flat (due to reduced growth of aerial hyphae), in contrast to the WT and furthermore exhibited radial growth patterns identical to the parent *rgs1*Δ strain [207, 310] (Figure 4.4A, B and C).

In contrast to the WT, the *rgs1*Δ makes almost 3-fold higher number of conidia, thus acting as a negative regulator of asexual spore production [207]. We checked if the expression of the independent fragments could rescue the hyper-conidiation defect of *rgs1*Δ to wild type levels. Quantification of conidia formed by the individual domain expressing, *rgs1*Δ and wild-type strains revealed that the *rgs1*Δ strain produced nearly 2.4-fold ($P < 0.01$) greater number of spores as compared to the WT (Figure 4.5), the C-Rgs1 strain (without the DEP-DEP domain) and the N-Rgs1-mC strain (lacking the core RGS domain) also produced nearly 2–3-fold ($P < 0.01$ and $P < 0.05$ respectively) greater number of spores in contrast to the WT.

In conclusion, the results imply that the expression of the independent N- or C-terminal domain fragments is inadequate to rescue the growth (especially aerial) as well as conidiation defects of the *rgs1*Δ strain. Thus, we conclude that the functional expression of neither domain is able to provide the function associated with the full length Rgs1 protein, to overcome defects associated with conidiation or aerial growth.

Figure 4.5. Comparative quantification of conidia formation in the C-Rgs1-mC, N-Rgs1-mC, *rgs1*Δ and WT

The aforementioned strains were initially incubated in the dark prior to exposure to constant illumination. Conidia were harvested after a week. Data correspond to mean $\pm$ SE of triplicates for each strain.

Figure 4.6. Independent expression of domain motifs does not rescue surface wettability and hydrophobicity defects displayed by *rgs1*Δ strain.

Panels with the surface/water soaking ability of the colonies from C-Rgs1-mC, N-Rgs1-mC, *rgs1*Δ, Rgs1-mCherry and WT strains are shown. The aforementioned strains were first incubated in the dark, prior to inoculation of water droplets on the colony surface.

4.1.4 Surface hydrophobicity and wettability

The expression of independent domain motifs of Rgs1 protein failed to rescue aerial growth or conidiation in *rgs1* Δ . Next, we tested if the individual domain expressing strains retained the characteristic wettability defect of the *rgs1* Δ strain i.e., the ability to soak up water droplets which are incubated on the surface of the colony for extended periods of time (15–30 min) [211, 310]. We observed that the in strains lacking the DEP-DEP motif (C-Rgs1), RGS motif (N-Rgs1) and in the *rgs1* Δ , the inoculated water droplets were taken up within 30 min, unlike in the WT or the Rgs1-mC expressing strains, where the droplets continued to stay for long periods of time after inoculation (Figure 4.6).

Thus, surface hydrophobicity in *M. oryzae*, influenced by G-protein signaling, possibly needs the intact Rgs1 function.

4.1.5 Analysis of individual domain expressing strains for rescue of Rgs1 deletion defects during pathogenic development

Cues such as hydrophobicity and hardness associated with inductive surfaces are imperative in regulating the efficiency of appressorium formation. Previous studies have shown that the *rgs1* Δ accumulated higher levels of cAMP is activated for signaling [207, 211]. Appressorial assays were performed to verify whether the expression of the individual DEP-DEP or the RGS domains in the *rgs1* Δ background would change the way it responded to surface stimuli.

Quantification of the efficiency of appressorium formation with the aforesaid strains was carried out on inductive as well as non-inductive surfaces. On inductive surfaces both the Rgs1-mC and WT strains formed appressoria efficiently (~ 80% for both strains), while failing to elaborate appressoria on non-inductive surfaces (nearly 2% for both strains; Figure 4.7A and B). In contrast, the C-Rgs1-mC and the N-Rgs1-mC strains elaborated appressoria efficiently on the inductive (83% and 89% respectively) and on the non-inductive surfaces with an equally high efficiency (90% and 81% respectively). The *rgs1* Δ strain also elaborated appressoria on both surfaces with an efficiency of nearly 80% (Figure 4.7A and B).

Figure 4.7. Appressorium formation assays of the Rgs1 domain expressing strains on inductive and non- inductive surfaces.

(A) Quantification of the appressorium formation ability on inductive (shown as black bars) or non-inductive (shown as gray bars) surfaces of the C-Rgs1-mC, N-Rgs1-mC, *rgs1*Δ, Rgs1-mC or WT strains. Data corresponds to mean $\pm$ SE of three separate replicates.

(B) Conidia from the aforementioned strains were incubated on inductive as well as non-inductive surfaces and evaluated for appressorium formation after 16 h. Scale bar, 10 μ m.

Taken together, the results from our experiments demonstrate that the expression of the individual domain motifs (DEP or RGS) in the *rgsI*Δ is not sufficient to uncouple appressorium formation from surface dependency.

4.1.6 Infection assays on seedlings of barley and rice

Studies carried out previously in our lab and recently in other labs have unmistakably demonstrated that the *rgsI*Δ strain is unable to cause blast disease, and this is due to the inability of the infectious hyphae to spread into the neighboring cells [211, 310]. In order to find out if the expression of either the DEP or the RGS motif is adequate to rescue the *in planta* defects, barley leaf explants were incubated with conidia at various dilutions from all the strains being tested. The *rgsI*Δ and the wild type strains acted as controls for the assay. Seven days post inoculation, symptoms of the disease were assessed in all the samples (Figure 4.8A). The capacity of the strains to elaborate disease symptoms in rice cultivar CO39 was also evaluated. Ten days post inoculation, the symptoms of the disease were evaluated.

Unlike the Rgs1-mC and the WT strains that caused typical spindle-shaped lesions with gray centers, on both barley and rice hosts, the progression of the disease was comparatively slower in the domain deletion and *rgsI*Δ strains (Figure 4.8B). The poor capability of these strains to cause disease was not dependent on the conidial load.

Hence, we conclude that the expression of the tandem DEP or the RGS domains in the *rgsI*Δ background does not rescue defects related to pathogenicity in the *rgsI*Δ strain.

4.1.7 The DEP-DEP (Dishevelled, Egl-10, Pleckstrin homology) domain targets Rgs1 to vesicular structures

To get an insight into the physiological relevance of the endo-proteolytic cleavage of Rgs1 and additionally to further understand the roles of individual DEP-A and DEP-B motifs in the tandem DEP domain, we decided to follow the sub-cellular dynamics of the independent domains (tandem DEP, DEP-A, DEP-B and the RGS domains).

A

Barley

B

Rice

Figure 4.8. Expression of fragments of independent domains is not sufficient to rescue the compromised ability of *rgs1Δ* to cause disease.

(A) Barley leaves were inoculated with 2000, 1000 or 500 conidia in triplicate as indicated from the strains being tested. The symptoms of the disease on the leaf tissue were evaluated a week after inoculation.

(B) Conidia from the strains being tested were spray inoculated onto 14 day old rice seedlings and symptoms were assessed after two weeks.

To track the subcellular localization by fluorescence microscopy, constructs expressing the individual domain fragments fused to mCherry (mC) at the C-terminus were generated and transformed into the *rgs1*Δ (Figure 4.9A).

Observations were made on conidia at 0 hpi. Full-length Rgs1 (Rgs1-mC) trafficked to distinct punctate structures that were confined to the conidial terminal cell (Figure 4.9B). In a manner similar to the full-length Rgs1-mC protein, the N-Rgs1-mC fragment (which contains the tandem DEP motifs), including the separate DEP domain fragments (DEP-A or DEP-B) trafficked to punctate vesicles in the conidia (Figure 4.9C, Arrowhead).

The full-length Rgs1 protein localized exclusively to the conidial terminal cell, however in the strains expressing the N-Rgs1-mC, DEP-A-mC or DEP-B-mC the punctae localized all throughout the conidium (Figure 4.9B and C). In contrast to the DEP-A-mC containing vesicles, the DEP-B-mC vesicles were weaker, in terms of signal intensity and less in number (Figure 4.9C).

The RGS catalytic core (C-Rgs1-mC) located at the C-terminal, in contrast, localized largely to compartments that morphologically resembled vacuoles (based on DIC images) (Figure 4.9B and D). To verify if the catalytic RGS core localized to the vacuoles, co-staining with the vacuolar stain CMAC (7-amino-4-chloromethylcoumarin) was carried out [318].

The expression of the tagged domain fragments (C-Rgs1-mC and N-Rgs1-mC) and the full length protein (Rgs1-mC) was ascertained by immuno blot using α-DEP antibody (against the N-terminus) or α-RFP antisera (against the C-terminus). Specific bands of predicted molecular weights were observed in every sample; Rgs1-mC at ~110 kDa, N-Rgs1-mC at ~105 kDa and C-Rgs1-mC at ~70 kDa (Figure 4.9 E). Protein extracts from *rgs1*Δ was used as control.

Finally, we infer that the N terminal tandem DEP-DEP motifs of Rgs1, can facilitate independently (though with decreased efficiencies) or cooperatively (more efficiently) the precise targeting of the RGS motif to intracellular vesicles. In absence of the DEP domains, or upon proteolytic processing, the cleaved RGS domain is possibly rendered non-functional by targeting it for degradation to the vacuole.

A

B

C

D

E C-Rgs1-mCherry N-Rgs1-mCherry Rgs1-mCherry

Figure 4.9. The N-terminal DEP-DEP motif facilitates targeting of Rgs1-mC to intracellular vesicles.

(A) Schematic depicting the various constructs used for subcellular localization. DEP motif A (marked in blue), DEP motif B (marked in green), RGS domain (marked by a red pentagon) and mCherry (marked by a red rectangle).

(B) Microscopic observations were made on conidia harvested from the strains being tested at specified time points. The arrow indicates the punctate vesicles in the Rgs1-mC and N-Rgs1-mC strains. Scale bar, 10 μ m

(C) Conidia from the designated fluorescently tagged strains were observed with a epifluorescence microscope and photographed at the specified time point. The arrows point to the region magnified and shown as inset. Scale bar, 10 μ m.

(D) A co-staining experiment between the C-Rgs1-mC with CMAC, a specific dye for vacuoles. The merged panel depicts the trafficking RGS domain to the vacuole. Scale bar, 10 μ m

(E) Total protein extracts from the designated strains was subjected to SDS-PAGE and analyzed by western blot using specific antisera (α -DEP) and (α -mC). Immuno reactive bands corresponding to Rgs1-mC, DEP-DEP motif and RGS motif were observed. Protein extract from the *rgs1* Δ acted as control. A probable post-translationally modified form of Rgs1 is indicated by a white asterisk. Molecular weight markers (kDa) are indicated.

4.1.8 Pth11 facilitates inductive surface based cAMP signaling

Activation of cAMP is essential for the initiation of appressorium development and subsequent pathogenic development. However, very little is known about proteins that function upstream to regulate or modulate this pathway. So far, *PTH11* is the only protein believed to function upstream of the cAMP signaling cascade in *M. oryzae* [202]. To understand the role and functionally characterize Pth11 during pathogenic development in *M. oryzae*, gene-deletion mutants were generated in the WT background.

To assess the behavior of the *pth11Δ* strain and test if the mutant was defective in inductive surface based signaling, we performed appressorial assays on inductive surfaces with conidia from the *pth11Δ* strain. WT strain served as a control.

A characteristic of proper cAMP signal activation is the ability of the wild-type *M. oryzae* to elaborate mature appressoria on an inductive surface by 8-12 hpi. In comparison to the WT the *pth11Δ* did not elaborate appressoria at an equivalent time-point on inductive surfaces (Figure 4.10). The inability of the *pth11Δ* strain to form appressoria on inductive surfaces suggested that the mutant was disrupted for inductive surface signaling and likely for cAMP synthesis. To test this possibility, we exogenously supplemented to the inoculated *pth11Δ* conidia 8-Br-cAMP. Addition of 8-Br-cAMP suppressed the defects exhibited by *pth11Δ* on an inductive surface (Figure 4.10).

Taken together, the inability of the mutant to form appressoria on inductive surface as well as the ability of cAMP to rescue this phenotype, strongly suggests that *PTH11* acts upstream of the cAMP signaling cascade and is likely involved in the activation of inductive surface-based signaling.

Figure 4.10. Pth11 likely acts upstream of cAMP signaling pathway and facilitates inductive surface signaling.

The *pth11Δ* strain was inoculated on an inductive surface and observations were made 16 hpi. cAMP was exogenously added to the *pth11Δ* conidia prior to inoculation.

4.1.9 Pth11 localizes predominantly to the plasma membrane and intracellular vesicular structures

We next sought to investigate the subcellular localization of Pth11 during the early stages of pathogenic development. We performed a time-course analysis and followed Pth11-mCherry localization over a period of 6 hpi. Upon live cell imaging we observed during the early stages of germination (1-3 hpi), Pth11-mC localized to intracellular punctate and/or tubulo-vesicular structures (Figure 4.11A), however during the later time points (4- 6 hpi) Pth11-mC localized predominantly along the membranes of the germ tube and the developing appressorium (Figure 4.11B). Thus Pth11 localizes to the membrane of the germ tube as well as intracellular vesicular structures during early pathogenic development.

Figure 4.11. Subcellular localization of the G-Protein Coupled Receptor Pth11 during early stages of pathogenic development.

Pth11 localizes to tubulo-vesicular compartments (**A** and **B**) Confocal imaging at the indicated time-points of Pth11-GFP conidium inoculated on an inductive surface. The arrow head point to punctate as well as tubulo-vesicular compartments containing Pth11.

4.1.10 The CFEM domain is crucial for proper appressorium formation

To further dissect and characterize the function of Pth11, especially the role of the N-terminal 70- aa long CFEM domain, we generated a strain in which the only CFEM domain was deleted, *PTH11*^{ΔCFEM}, retaining the rest of the Pth11 protein intact, in the WT background. To track the subcellular localization of *PTH11*^{ΔCFEM}, RFP was introduced at the C-terminal end. Additionally, in the WT background we generated a strain in which the CFEM-GFP was constitutively expressed under the RP27 promoter.

Conidia from the aforementioned strains were inoculated on inductive surfaces. The WT was used as a positive control and the *pth11*Δ strain was used as a negative control. Surprisingly, both the domain deletion (*PTH11*^{ΔCFEM}-RFP) as well as Constitutive-Expression (*PTH11*^{CE}-GFP) strain did not form appressoria on inductive surfaces (Figure 4.12A and B), in a manner identical to the *pth11*Δ mutant (Figure 4.12C). Under identical conditions, the WT formed mature appressoria on inductive surfaces (Figure 4.12D). Furthermore, addition of exogenous cAMP rescued the appressorium formation defects of both the domain deletion as well as constitutive-CFEM domain expression strains.

To confirm that the tagged proteins were functional, the subcellular localization of proteins was determined. Both Pth11^{ΔCFEM}-RFP as well as Pth11^{CE}-GFP localized to the germ tube and the appressorial membranes as expected (in the cAMP rescue experiments) on inductive surfaces (16 hpi) (Figure 4.12A and B).

Taken together, the results indicate that the CFEM domain of *PTH11* plays a critical role in inductive surface based signal transduction. We infer that the CFEM domain is necessary for proper Pth11 function. Furthermore, the enhanced expression of the CFEM domain (only) leads to a dominant negative effect and compromised Pth11 activity in the wild type *M. oryzae*.

A **B**

Figure 4.12. The CFEM domain is crucial for *Pth11*-mediated surface signaling.

The requisite strains were inoculated on an inductive surface and observations were made 16 hpi using bright field and wide-field fluorescence microscopes. The wild type and the *pth11* Δ were used as controls.

4.1.11 Rgs1 physically interacts *in vitro* and *in vivo* with GPCR Pth11

Given that both Pth11 and Rgs1 localized to vesicular compartments, we were interested in deciphering if Rgs1 and Pth11 physically interacted. GFP trap based pull-down assay using whole cell lysates from the Pth11-GFP strain were carried out to answer this question. The pull down assay was carried out during the vegetative (mycelium grown in liquid complete medium) as well as the pathogenic stages (conidia inoculated on inductive surface for 4 hpi) of development.

Relevant immuno-precipitated as well as wild-type control samples were fractionated on an SDS PAGE and further evaluated by western blot analysis using α -Rgs1 antibody. Remarkably, pull down experiments revealed that Pth11 interacted with Rgs1 specifically during the pathogenic phase (Figure 4.13A and B). To further validate and visualize the interaction between Pth11 and Rgs1 *in vivo*, a BiFC (Bimolecular Fluorescence Complementation) experiment was carried out to observe the interaction between Pth11 and Rgs1. For this experiment, a strain in which Pth11 was tagged to nYFP (Pth11-nYFP) and Rgs1 to cYFP (Rgs1-cYFP) was generated. The confirmed strain showed a strong fluorescence signal (YFP) emanating from the membrane of the germ tube and developing appressorium and a weak signal from cytosolic tubulo-vesicular compartments (Figure 4.14).

Hence we conclude that, the putative upstream receptor Pth11 and Rgs1 localize to intracellular punctate tubulo-vesicular compartments. In addition, we provide evidence for the physical interaction between Pth11 and Rgs1, using *in vitro* as well as *in vivo* methods.

A

Vegetative

B

Pathogenic

Figure 4.13. Physical interaction between Pth11 and Rgs1.

(**A** and **B**) Immuno blots from a pull-down experiment depicting the stage specific interaction between Pth11 and Rgs1. Pth11 failed to associate with Rgs1 during vegetative growth (**A**), but interacted with Rgs1 during pathogenic differentiation (**B**). Total protein from an untagged wild-type strain served as a control.

Figure 4.14. BiFC assay between Pth11 and Rgs1 during early pathogenic development.

Fluorescence images from a BiFC experiment, illustrating the *in-vivo* interaction between Pth11-cYFP and Rgs1-nYFP at 2h and 4 h post inoculation on an inductive surface. Asterisk indicates colocalization at the plasma membrane. Scale bar 10 μ m.

4.2 Discussion

In addition to the MAP kinase cascade, the G-protein-mediated cAMP signaling pathway has been characterized in detail in *M. oryzae*. Genetic studies performed nearly over the last 20 years have identified a large number of proteins that function as key regulators of the cAMP pathway. These proteins are known to regulate multiple aspects of asexual and pathogenic development in *M. oryzae* [194, 195, 204, 205, 207, 213]. Among the important ones, particularly in the context of this study, include a putative non-canonical CFEM domain containing GPCR, Pth11, believed to function upstream of the cAMP cascade and Rgs1, a negative regulator of G-protein signaling.

Although Pth11 is hypothesized to play an important role upstream (likely during initiation and/or activation) of cAMP signaling cascade, there is no direct evidence supporting this hypothesis. Here, we sought to investigate among others, the role of the CFEM domain, and the subcellular localization during the early pathogenic development and finally validate its role as an upstream receptor, by probing for direct interaction with Rgs1.

RGS proteins are GTPase-activating proteins and control the strength of the signaling response. However, the mechanisms by which they are regulated in filamentous fungi are poorly understood and have not yet been addressed.

In addition to being regulated by transcription and phosphorylation [319], it has been demonstrated that the yeast Sst2, is subjected to *in vivo* endoproteolytic cleavage post translationally to regulate its function [315]. In this chapter I demonstrate that *M. oryzae* Rgs1 undergoes endoproteolytic cleavage to yield the N-terminal DEP-DEP domain and a C-terminal RGS core. Furthermore, I also show that the DEP-DEP domain mainly functions to target the catalytic RGS core to vesicle like structures. In the absence of the DEP domains the RGS core motif is localized to the vacuole.

Sequence alignment between RGS proteins in yeast and filamentous fungi was carried out to establish the extent of conservation of functional motifs and putative sites for proteolytic cleavage. Rgs1 from *M. oryzae*, FlbA from *A. nidulans*, Sst2 from *S. cerevisiae*, and the fission yeast Rgs1 were subjected to sequence alignment. All proteins were found to have a conserved

DEP-DEP domain at the N-terminal end and a catalytic RGS core motif at the C-terminal end (Figure 4.2B) as previously described [307, 311].

In Sst2 the serine (at position 415) and asparagine (at position 417) have been described as key residues where endoproteolytic cleavage occurs (Figure 4.2A and 4.2B, box and asterisks). We found that in the Sst2 orthologs in filamentous fungi, the Asn was conserved; however the Ser was replaced by Asp. This replacement possibly does not influence the efficiency of proteolytic cleavage of Rgs1 in *M. oryzae*.

Interestingly, mutations in the above mentioned Ser and Asn located at the cleavage site in Sst2p, suggests that the point mutants continues to undergo proteolysis in a manner similar to its WT Sst2 counterpart [315]. This is indicative of a recognition mechanism that is more complicated and also involves extra amino acids in close proximity of the site of proteolytic cleavage.

Our sequence alignments suggested that the site for proteolytic cleavage is most probably conserved in *M. oryzae* Rgs1. To verify the processing of Rgs1 and the also to prove the existence of cleaved N and C terminal fragments, we performed extensive western blot analysis with the different *M. oryzae* strains (viz. WT, Rgs1-OE and Rgs1-GFP). We were able to detect the cleaved fragments for the N-terminal DEP and C-terminal core motifs for Rgs1. Thus *M. oryzae* likely uses proteolytic cleavage in order to regulate Rgs1 function after translation. It still remains to be verified whether such a mechanism is conserved across other filamentous fungi.

A polypeptide of ~120 kDa, much higher than the size expected for full-length Rgs1-mCherry protein was observed. (Figure 4.3B and 4.9E, indicated by black and white asterisks respectively). Utilizing mass spectrometric analysis, it was confirmed that the protein was Rgs1 (data not shown) and likely represents a form of Rgs1, which is post-translationally modified. This aforementioned version of Rgs1 is probably hidden in the Rgs1-OE strain and undetectable in the WT (Figure 4.3A).

To address the importance endoproteolytic cleavage in Rgs1, we expressed in the *rgs1*Δ background, independent N- and C- terminal fragments and characterized the *rgs1*Δ and the independent motif expressing strains. The domain deletion strains exhibited similar defects as as the background *rgs1*Δ in all the experiments tested. A likely rationale for the inability to rescue

the *rgs1*Δ phenotype is the likelihood that the N and C terminal motifs perform specific and independent functions. Supporting our findings is an identical observation made for yeast Sst2. Here the expression of either N- or C-terminal motifs in the *sst2*Δ mutant, did not rescue its function *in vivo* [315]. Further studies have demonstrated that concurrent expression of fragments of the individual motif complements the *sst2*Δ phenotype poorly. It is suggested that this is probably because of the inability of the DEP domain to substitute for the catalytic function of the RGS core motif *in trans* [320]. Therefore, it is likely that cleavage and subsequent sequestration of the catalytic motif in the vacuole is a probable mechanism of down regulating Rgs1 protein function.

To validate that DEP-DEP and RGS motifs performed diverse functions, we tracked the subcellular localization of the fluorescently tagged fragments using fluorescence microscopy techniques. The tandem DEP-DEP fragment as well as individual DEP-A and DEP-B domain fragments trafficked to punctate subcellular structures in the conidia, thus supporting a role for the N-terminal motifs in membrane targeting. The relatively poor capacity of the DEP-B domain for vesicular targeting indicates a role of greater importance for the DEP-A motif in targeting of Rgs1 to specific vesicular compartments. Our findings are supported by other studies that have demonstrated the ability of DEP domains for membrane targeting. One study has shown that the N-terminal fragment of Sst2 protein localizes to microsomal membrane fractions [315]. It has also been shown that the DEP motif of Sst2 helps in its binding to the C-terminal tail region of its cognate GPCR (Ste2) at the plasma membrane [320]. In mice, the DEP domain of the RGS9 protein specifically targets the protein to the external sections of the rod photoreceptors [321]. In conclusion, membrane targeting of Rgs1 is crucial for the overall function of the protein and is a conserved process.

The activity of the RGS motif is important for the GAP activity of RGS proteins. Surprisingly, unlike the DEP-DEP motif, the RGS core motif (alone) was targeted to the vacuole. We suppose that the trafficking of the RGS motif to the vacuole alone is a probable outcome of the failure of the domain to be targeted independently to intracellular vesicular compartments and/or the plasma membrane. It is also likely that sequestration into the vacuoles assists in the down regulation of the GAP activity of Rgs1.

Pth11 is predicted to be a non-canonical GPCR and is believed to function upstream of the cAMP signaling cascade. However there is little experimental evidence supporting this hypothesis. The *pth11Δ* was completely rescued by the exogenous supplementation of 8-Br-cAMP, suggesting that Pth11 acted along the cAMP pathway. Live cell imaging experiments localized Pth11 to the membrane (physiologically relevant) of the germ tube as well as to intracellular punctate-vesicular structures, reminiscent of Rgs1 localization. To validate the interaction between Pth11 and Rgs1, pull down as well split-YFP or BiFC experiments were carried out. Both the experiments suggested that Pth11 physically interacted with Rgs1. Supporting our finding is an identical observation made in yeast where Sst2, physically interacts with its cognate receptor (Ste2), during the mating response [320]. We observed YFP signal from the plasma membrane as well as intracellular structures, suggesting that the two proteins likely interacted on subcellular vesicles in addition to the plasma membrane.

Finally, by generating mutants strains deleted only for the CFEM domain and constitutively expressing the CFEM domain, we found that the N-terminal CFEM domain in Pth11 is crucial for the proper functioning of the protein and subsequently for proper inductive surface based-signaling. Interestingly CFEM domain-containing proteins in *C. albicans* has been implicated in playing an imperative function in the development and maintenance of biofilms [322]. It is possible that Pth11 carries out a dual function in *M. oryzae*, firstly functioning as a transmembrane receptor for cAMP signaling and secondly as an adhesion molecule during host-pathogen interactions, a function likely mediated by the CFEM domain [203]. In the future, it would be interesting to check if the *pth11Δ* or the Pth11^{ΔCFEM} strains are compromised in their ability to adhere to artificial or inductive host surfaces.

In summary, in this chapter I show that in *M. oryzae* Rgs1 is subjected to endoproteolytic processing to give rise to an N-terminal DEP-DEP and a C-terminal core motif. The proteolytic processing and sequestration of the core RGS motif into the vacuoles possibly represents a conserved but incompletely understood mechanism engaged by yeast and filamentous fungi to regulate the function RGS protein post-translationally. This possibly represents an additional mechanism by which G-protein signaling is regulated in *M. oryzae*.

The inability of the *pth11* Δ , Pth11 ^{Δ CFEM} and the Pth11^{CE} to form appressoria on inductive surfaces strongly suggests a role for non-canonical GPCR, *PTH11* as a key upstream regulator and furthermore implicates the N-terminal CFEM domain as crucial for proper inductive surface based cAMP signaling. The ability of cAMP to rescue the phenotypes of the *pth11* Δ , Pth11 ^{Δ CFEM} and Pth11^{CE} as well the physical interaction between Rgs1 and Pth11 further authenticates *PTH11* as a *bonafide* upstream activator of cAMP signaling. Further research could focus on the identification and characterization of the vesicular compartments that traffic Rgs1 and Pth11.

5. Results III

Chapter V

5.1 The Late Endosomal compartment Anchors Active cAMP Signaling in *Magnaporthe oryzae*

Introduction

When and where proteins are activated can have a profound implication on the target cellular/biological processes. As a result, cells have evolved complex molecular mechanisms to compartmentalize and thereby control the spatial and temporal organization/dynamics of signaling pathways. In mammalian cells, signal compartmentalization is essentially brought about by either the sequestration and/or anchoring of key regulators and effectors on scaffold proteins such as A-kinase Anchoring Proteins (AKAPs) or on the dynamic endosomal network [323, 324]. Anchoring of signaling proteins on common platforms, endows the cell with a host of advantages, which include, the ability to specifically target and activate signaling pathways or components within a defined region of the cell, thereby preventing non-specific or off-target activation. Furthermore, signal anchoring *per se* can help the cell integrate and achieve modularity of signaling responses and ultimately regulate in a precise manner the amplitude and temporal duration of signaling.

The endo-lysosomal system represents a highly dynamic and biochemically specialized continuum of phosphatidyl-inositol-3-phosphate (PI3P) rich membranous compartments, that communicates with the plasma membrane, and is involved in the sorting of macromolecules, biological signals and maintaining cellular homeostasis [142]. The endosomal system can be broadly segregated into early, late and recycling compartments. Upon acquisition of ILVs (Intra Luminal Vesicles), late endosomal structures mature into MVBs (Multi Vesicular Bodies) and further fuse with lysosomes. Each compartment is inherently programmed to carry out a specific function. The role played by the endosomal system in signal attenuation through receptor down regulation, is well known [156]. However, recent studies in yeast and mammalian cells have implicated the late endosomes especially the MVB compartments in signal anchoring, extending beyond the conventional role of sorting and degradation. Such studies suggest that the late

endosomal compartments function as signaling anchors/platforms thereby facilitating the integration of membrane trafficking and intracellular signal transduction [149-151, 325].

One of the most critical steps during pathogenic differentiation in *M. oryzae*, occurs during the early phase and is known as the “hooking” stage wherein the germ tube tip switches from polarized to isotropic growth (3-4 hpi) and subsequently forms an appressorium (8-12 hpi). It is thought that hooking constitutes a “recognition phase” in which surface characteristics such as hydrophobicity, hardness, waxes and cutin-monomers are sensed and assessed prior to commitment to appressorium formation [190, 221].

In this chapter, I explore possible mechanisms of cAMP signal anchoring in the context of dynamically altering cellular geometry. Through extensive colocalization analysis with specific marker proteins, I have identified that Rgs1 localizes to the late endosomes. I further demonstrate that the late endosomes represent sites of active G-protein signaling and that the disruption of late endosomal integrity (by the deletion of homotypic fusion and vacuole protein sorting (HOPS) component Vps39, vacuolar protein sorting 39) results in mis-regulation of inductive surface- based cAMP signaling in *M. oryzae*.

Results

5.1.1 Inductive surface-induced dynamics of Rgs1-mCherry

Rgs1 in *M. oryzae* is an archetypal negative regulator of G-protein signaling, has two important and functionally well characterized protein motifs/domains: an N-terminal double DEP motif and a C-terminal catalytic RGS motif [243]. In the previous chapter, it was demonstrated that the vesicular targeting of Rgs1 was dependent on the presence of an intact DEP motif. Given that Rgs1 plays an important role in regulating the timing and duration of early G-protein mediated signaling events in *M. oryzae*, the spatial and temporal dynamics of Rgs1 was tracked to get a deeper insight into the regulation of Rgs1 during the early events in signaling and appressorium morphogenesis.

To answer this question, live cell imaging was performed on germinating conidia at 2 and 4 hpi on an inductive surface. Ungerminated conidia (0 hpi) were used as control. Remarkably, we found that, unlike in the conidia where Rgs1 localized predominantly to more or less static punctate vesicles [243] on an inductive surface, in germinating conidia, Rgs1 harboring vesicles were extremely dynamic and displayed a distinct tubulo-vesicular morphology within the polarized germ tube. In addition, these tubulo-vesicular compartments branched out from one another to form additional such compartments (Figure 5.1A).

Next we checked if the formation of the tubulo-vesicular structures *per se* or the observed dynamics of the Rgs1 compartments was influenced by inductive surface characteristics. On a non-inductive surface (1% agar) that fails to promote appressorium formation, Rgs1-mCherry did not localize to dynamic tubulo-vesicular structures, but was predominantly vacuolar or present as small puncta in developing germ tubes (Figure 5.1B).

Taken together, it can be concluded that in response to inductive cues, like hydrophobicity and hardness, Rgs1-mCherry localizes to dynamic tubulo-vesicular compartments. In the absence of such inductive cues or signals, Rgs1 is predominantly targeted to the vacuole.

A

B

Figure 5.1. Surface induced spatial and temporal dynamics Rgs1 during pathogenic development in *M. oryzae*.

(A) A sequence of confocal images at corresponding time points depicting the tubulo-vesicular compartments of Rgs1 in a developing germ tube (arrowheads at 2 h) as well as the incipient appressorium (arrowheads at 4 h). Rgs1-mCherry pseudo colored in yellow. The dotted line marks the boundary of the respective conidium. Scale bar, 10 μ m.

(B) Time course sequence of Rgs1-mC on a non-inductive 1% agar surface, 4 hpi. Rgs1-mCherry failed to localize to tubulo-vesicular compartments. Arrowheads indicate small puncta within the germ tube and a prominent vacuolar signal. In both the panels, images depict maximum intensity Z-projections of confocal stacks. The elapsed time is depicted in seconds. Scale bar, 10 μ m.

5.1.2 Rgs1- containing vesicles and tubules traverse bi-directionally

Time-lapse analysis of Rgs1-mCherry dynamics captured at 4 hpi revealed that Rgs1-harboring compartments traversed the length of the germ tube in both the directions. A subset population of Rgs1 structures moved towards the tip of the incipient appressorium or germ tube tip (Figure 5.2A), while a few punctae moved in the opposite direction towards the terminal cell of the conidium (Figure 5.2B). Thus, Rgs1 tubulovesicular structures were inherently bi-directional and traversed the length of the germ tube.

Figure 5.2. Bi-directional mobility of Rgs1-mCherry compartments.

(A and B) Confocal images exemplifying the highly dynamic nature of Rgs1 tubulo-vesicular compartments, 4 h post inoculation. The arrows show the direction of mobility of the compartments (towards the incipient appressorium and the terminal cell of the conidium respectively). GTT: Germ Tube Tip; and TC: Conidial Terminal Cell. The numbers on the top left corner indicates the time elapsed in seconds. Images are maximum intensity projections of confocal stacks. Scale bar, 10 μm.

5.1.3 Mobility of Rgs1 vesicles is facilitated by intact microtubule cytoskeleton

In polarized cells, either the microtubule and/or the actin cytoskeleton facilitate the transport of wide range intracellular vesicular cargo/compartments. We determined the nature of the cytoskeletal tracks engaged by Rgs1 for mobility by treating samples at the hooking stage, with either MBC (Methyl Benzimidazol-2-yl-Carbamate) to destabilize the microtubule tracks or latrunculin A (LatA) to disrupt actin filaments.

Compared to the DMSO control, the dynamics and morphology of Rgs1-mCherry tubulo-vesicular structures were completely disrupted upon treatment with MBC (Figure 5.3A). Additionally, MBC treatment caused an accumulation of the Rgs1-mCherry on the plasma membrane and as intracellular aggregates confined to the incipient appressorium and the terminal cell of the conidium. As expected, Rgs1-mCherry containing structures were coincident with microtubules (GFP- α Tub), supporting the microtubule-dependent trafficking of Rgs1 (Figure 5.3B) in *M. oryzae*. In contrast, LatA-treated samples showed typical mobile/dynamic tubulo-vesicular Rgs1 compartments although to a slightly reduced extent compared to the control sample (Figure 5.3C).

Collectively, our results show that the dynamic mobility of Rgs1 containing compartments depends on the integrity of the microtubule cytoskeleton.

A **B** **C**

Figure 5.3. Rgs1-harboring structures require an intact microtubule cytoskeleton for integrity and dynamics.

(A) Destabilization of the microtubule cytoskeleton with MBC resulted in the disruption of Rgs1 tubulation and dynamics along the germ tube. Treatment caused Rgs1 accumulation along the plasma membrane of the developing appressorium (marked by asterisk) and cytoplasmic aggregation or clustering (white arrowhead). The numbers indicate the time elapsed in seconds.

(B) Images depicting Rgs1-mCherry structures coincident with microtubule tracks (depicted in the inset), marked by GFP-tagged α tubulin. Scale bars, 10 μ m.

(C) Images from a time-lapse movie of Rgs1-mCherry strain treated with LatA at 4 hpi. The arrowheads highlight Rgs1-mCherry containing tubulo-vesicular compartments. The time elapsed in seconds is depicted. The dotted lines mark the boundary of the respective conidium. All images are maximum intensity Z-projections of confocal stacks. Scale bar, 10 μ m.

5.1.4 Rgs1 localizes to PI3P enriched tubulo-vesicular structures

In a vast majority of organisms, the endo-lysosomal system represents a dynamic continuum of tubulo-vesicular structures rich in the phosphoinositide PI3P. As a first step towards determining the identity of Rgs1-harboring compartments, we utilized LysoTracker Green (LTG) to stain the acidic endo-lysosomes. As evident from Figure 5.4, a large number of compartments with diverse morphologies were stained by LTG, however only a small population of these compartments colocalized with Rgs1-mC.

Thus, Rgs1-mC compartments essentially form a sub-population of the extensive network of the endo-lysosomal system.

Figure 5.4. Colocalization analysis of Rgs1-mCherry with lysotracker green marked endo-lysosomal compartments.

Panel portraying the partial colocalization between LysoTracker green (LTG) and Rgs1-mCherry on an inductive surface 4 hpi. Arrowheads indicate the compartments containing both LTG and Rgs1-mcherry and magnified in the inset. Scale bar, 10 μ m.

A hallmark of the endosomal compartments is the accumulation of the phosphoinositide PI3P therein. In addition to accumulating in membranes of early endosomes, PI3P was recently shown to be a component of the limiting membranes of vacuoles and the intraluminal vesicles (ILVs) of MVBs [326]. A GFP-FYVE probe has been extensively used to mark the PI3P-rich structures [327]. Co-localization analysis between GFP-FYVE and Rgs1-mCherry marked structures revealed a high degree of co-localization, suggesting a likely endosomal origin for Rgs1-containing vesicles and tubules (Figure 5.5A and B; Figure 5.13D).

Since several fungi including *M. oryzae* are known to have mitochondria and a sub-population of vacuoles that are filamentous, a colocalization analysis between Rgs1-mC and GFP-tagged mitochondria or the vacuole specific dye CMAC was carried out. Rgs1-mCherry structures were not related to mitochondria or filamentous vacuoles as judged by colocalization with mito-GFP and CMAC (7-amino-4-chloromethylcoumarin) respectively (Figure 5.6A and B). Taken together, our results strongly suggest that Rgs1 compartments P13P-rich endosomal compartments.

A **B**

Figure 5.5. Identity of tubulo-vesicular compartments containing Rgs1

(A) A montage from a time-lapse movie depicting Rgs1-mCherry colocalization with PI3P-rich endosomal compartments (marked by GFP-FVYE probe). Arrowheads mark the regions magnified in the inset. The elapsed time is indicated in seconds.

(B) A specific example (time point 1.2 s in Figure 5.5A) depicting Rgs1-mCherry colocalization with PI3P-rich endosomal compartments. Arrowheads mark the regions magnified in the inset and surface rendered in three dimensions. Images are maximum intensity projections of confocal stacks and the yellow asterisk marks the same compartment in the inset and the surface renderings.

Figure 5.6. Colocalization of Rgs1 with mitochondria and vacuoles.

(A) Rgs1-mC does not colocalize with filamentous mitochondria (mito-GFP). Arrowhead indicates the region magnified on the extreme right highlighting distinct Rgs1 structures and mitochondria. Scale bar, 10 μ m.

(B) Rgs1-mC tubulo-vesicular compartments do not colocalize prominently with CMAC-stained vacuoles (arrowhead). The conidial vacuole contains both CMAC and Rgs1. Rgs1-mC, top panel; CMAC, bottom panel. Images are single plane images captured on a confocal microscope. Scale bar 10 μ m.

5.1.5 Sustained Vps34 function is crucial for Rgs1 dynamics and tubulation

The PI3 kinase, Vps34, facilitates the synthesis of PI3P from phosphatidylinositol on endosomal membranes [148]. To determine if a continuous supply of PI3P was necessary to maintain the integrity/dynamic nature of Rgs1 compartments, Vps34 function was disrupted, using the PI3Kinase inhibitor LY294002. In comparison to solvent treated (DMSO) control, which displayed extensive dynamic tubulo-vesicular compartments, LY294002-treated Rgs1-mC conidia were devoid of tubulo-vesicular compartments at the hooking stage. Interestingly, in a manner identical to the MBC-treated conidia, there appeared to be an exclusive accumulation of mCherry signal in the plasma membrane of the incipient appressorium and vesicular aggregates in the terminal cell of the conidium (Figure 5.7).

Thus it can be concluded that Rgs1 localizes to the PI3P-rich endosomal network and in a manner dependent on sustained PI3 kinase activity.

Figure 5.7. Persistent supply of PI3P is necessary for Rgs1 dynamics and tubulation

Specific inhibition of PI3 Kinase function causes loss of Rgs1 association with tubulovesicular structures in the germ tube. PI3 Kinase inhibition also resulted in Rgs1 accumulation along the plasma membrane of the incipient appressorium (asterisk). DMSO treated sample was used as a control. Scale bar, 10 μm .

It has been previously demonstrated that deleting the C-terminal region (terminal ~50 amino acids) disrupts the ability of Vps34 to interact with Vps15, thus rendering the kinase non-functional and catalytically inactive [328]. A catalytically inactive mutant of Vps34 was generated, to confirm the role played by Vps34 (and by inference PI3P) in the stability and dynamics of Rgs1-mC compartments. Vesicular aggregates of Rgs1 were observed in the *vps34*^{ΔC48} strain, unlike in the control, where Rgs1-mC appeared as distinct punctate compartments (Figure 5.8).

In conclusion, proper Vps34 function, and by inference a persistent supply of PI3P, is crucial for the formation and/or sustenance of Rgs1 compartments.

Figure 5.8. Catalytic activity of Vps34 is required for Rgs1 vesicle formation.

Disruption of Vps34 function by C-terminal deletion causes aggregation of Rgs1 vesicles (arrow head) in the conidium, compared to distinct punctae present in the wild-type (arrowhead). Scale bar, 10 μ m.

5.1.6 Rgs1 co-localizes with Rab7 positive endosomes

The endosomal system is a complex network of inter-connected dynamic tubulo-vesicular structures. Although demarcating the limiting membrane for each endosomal compartment has been challenging, it is broadly classified into early and late compartments, based on the presence of characteristic markers in the form of small Rab GTPases. The Rab5 and Rab7 GTPases are widely used as markers for the early and late endosomes respectively [329].

In order to specify the endosomal compartment to which Rgs1 localized to, we first identified orthologs of specific Rab GTPases in *M. oryzae* and next generated GFP tagged strains marked for endosomal structures (Figure 5.9A and B). Systematic co-localization analysis between Rgs1-mC structures and GFP-tagged Rab5 or Rab7 was performed. Rgs1-mC structures associated significantly with Rab7 positive late endosomes (Figure 5.10A and B) and weakly with early endosomal marker Rab5 (Figure 5.11A).

Taken together these results demonstrate that Rgs1-mC specifically localizes to the late endosomes.

A

Conserved Switch I and II regions in *M. oryzae* Rab5 ortholog

Switch I

```

Mo  MADG-AAKPSSSVKLVLLGEAAVGKSSLVLRVVNNDIQENKEPTIGAAFLTQKCNLPART 59
Nc  MADTNAPKPSSSVKLVLLGEAAVGKSSLVLRVVNNDIQENKEPTIGAAFLTQKCNLPTRT 60
Sc  ----MNTSVTSIKLVLLGEAAVGKSSIVLRVVSNDEAENKEPTIGAAFLTQRVITINEHT 55
    .. :*:*****:*****:*** *****: .. :*:

    Switch II
Mo  IKFEIWDTAGQERFASLAPMYRNAQAALVVDLTQKPTSLIKAKHWAE LQRQASPGIVI 119
Nc  IKFEIWDTAGQERFASLAPMYRNAQAALVVDLTQKPTSLIKAKHWAE LQRQASPGIVI 120
Sc  VKFEIWDTAGQERFASLAPMYRNAQAALVVDVTKQSF IKARHWKELHEQASKDIII 115
    :*****:*** *:***:*** **:..*** .*:

```

Conserved Switch I and II regions in *M. oryzae* Rab7 ortholog

Switch I

```

Mo  MSSRKKVLLKVII LGDSGVGKTS LHMNYVNKKFSASYKATIGADFLTREVLVD-DRQVTM 59
Nc  MSSRKKVLLKVII LGDSGVGKTS LHMNYVNKKFSASYKATIGADFLTREVLVD-DRQVTM 59
Sp  MAGKKKHLLKVII LGESGVGKTS IMHNYVNRKFSKDYKATIGADFLTKEVLVD-DKVVTL 59
Sc  MSSRKKNI LKVII LGDSGVGKTS LHMRYVNDKYSQQYKATIGADFLTKEVTVDGDKVATM 60
    *:..** :*****:*****:*:*** *:* ..*****:*** ** *:..*:

    Switch II
Mo  QLWDTAGQERFQSLGVAFYRGADCCVLVVDVNNKSF DALDSWRDEFLIQASPRDPDNFP 119
Nc  QLWDTAGQERFQSLGVAFYRGADCCVLVVDVNNKSF DALDSWRDEFLIQASPRDPDNFP 119
Sp  QLWDTAGQERFQSLGVAFYRGADCCVLVVDVNNKSF ET LDSWRDEFLIQASPSNPETFP 119
Sc  QVWDTAGQERFQSLGVAFYRGADCCVLVVDVTNASSF ENIKSWRDEFLVHANVNSPETFP 120
    *:*****:*****:****:..***: ..*****:..* .*:..**

```

B

Figure 5.9. Comparative sequence alignment of Rab5 and Rab7 orthologs in *M. oryzae*.

(A) Sequence alignment Rab5 (Vps21) and Rab7 (Ypt7) orthologs from *M. oryzae*, *N. crassa*, *S. cerevisiae* and *S. pombe*. All the orthologs contain the highly conserved switch regions. (B) Confocal images depicting early endosomal (GFP-Rab5) compartments marked by FM4-64. The arrowheads highlight a few such structures amongst a vast majority of endosomes.

Figure 5.10. Colocalization between Rgs1 structures and late endosomal compartments. (A and B) GFP-Rab7 colocalizes with Rgs1-mCherry during early pathogenesis. The arrowhead marks the regions magnified in the inset and surface rendered in 3D (Asterisk). Images are maximum intensity Z-projections of confocal stacks. Scale bar, 10 μ m.

Figure 5.11. Colocalization analysis between Rgs1 structures and early endosomes.

GFP-Rab5 marked structures do not significantly associate with Rgs1-mCherry compartments during germ tube hooking. The arrowhead marks the regions magnified in the inset and surface rendered in 3D (Asterisk). Images are maximum intensity Z-projections of confocal stacks. Scale bar, 10 μm .

5.1.7 Rgs1 co-localizes with constitutively active $G\alpha_S$ on the late endosomes that likely represent sites of active signaling

Given that Rgs1 negatively regulates $G\alpha_S$, and is closely associated with the GTP bound form of $G\alpha_S$, we were interested in understanding the physiological relevance of Rgs1 localization to the late endosomal compartments in the context of active G-protein signaling. A *M. oryzae* strain expressing Rgs1-mCherry and a GFP-tagged constitutively active $G\alpha_S$ subunit ($G\alpha_S^{G187S}$) was generated. The wild-type $G\alpha_S$ -GFP was used as control. The GFP was introduced within the alpha B-alpha C loop region of the protein, to avoid any possibility of disruption of MagA function.

Remarkably, we observed a differential pattern of localization between the GFP-tagged $G\alpha$ variants. The active $G\alpha_S$ (MagA^{G187S}-GFP) predominantly localized to distinct punctate vesicles, while the wild-type $G\alpha_S$ (MagA^{WT}-GFP) primarily localized to the plasma membrane in the vegetative mycelium (Figure 5.12).

Figure 5.12. Subcellular localization of MagA^{G187S}-GFP ($G\alpha_S^{G187S}$) and wild-type MagA-GFP ($G\alpha_S$) during vegetative growth.

Mycelia from the indicated strains were subjected to confocal microscopy. Scale bar, 10 μ m.

Next, we tested if the active $G\alpha_s^{G187S}$ associated with Rgs1 during early pathogenesis, and specifically during the hooking stage (4 hpi). Indeed, active $G\alpha_s^{G187S}$ -GFP vesicles significantly co-localized with a high degree of correlation with Rgs1-mCherry (Figure 5.13A and C). In freshly harvested conidia, we found only a partial/minimum overlap between the two proteins (Figure 5.13B).

In conclusion, our results suggest that Rgs1-harboured compartments and by inference the late endosomes, likely represent sites of active G-protein signaling. Furthermore, we infer that a preferential association exists between the $G\alpha_s$.GTP and Rgs1 on such dynamic structures during early stages of surface recognition and appressorium initiation.

A **B**

C

Figure 5.13. Rgs1 co-localizes with active $G\alpha_s$ on tubulo-vesicular structures.

(A) Association between active MagA^{G187S} ($G\alpha_s^{G187S}$) and Rgs1 during appressorium initiation. Colocalization of active MagA^{G187S} -GFP (green) and Rgs1-mCherry (red) compartments during the hooking stage. Active $G\alpha_s^{G187S}$ localizes to cytoplasmic vesicular structures and the plasma membrane (asterisk). The arrow highlights compartments co-populated by MagA^{G187S} -GFP and Rgs1-mCherry and the yellow asterisk highlights the same compartment in the inset and the corresponding 3D surface rendering (panel on the right). Scale bar, 10 μm .

(B) Partial colocalization between MagA^{G187S} ($G\alpha_s^{G187S}$) and Rgs1 in conidia. The dotted line demarcates the respective conidium. All images are maximum intensity Z-projections of confocal stacks. Scale bar, 10 μm .

(C) Pearson's coefficient analysis for overlap or colocalization between Rgs1 and Rab5 or Rab7 or $G\alpha_s^{G187S}$ or FYVE. A graph illustrating the quantification of the Pearson's colocalization coefficient for early endosomes (Rab5), late endosomes (Rab7), active $G\alpha_s^{G187S}$ or the GFP-FYVE with Rgs1.

5.1.8 Disruption of Late Endosome formation or function leads to signaling defects in *M. oryzae*

Vps39 (vacuolar protein sorting 39), a key member of the HOPS (homotypic fusion and vacuole protein sorting) complex, plays an important role in the conversion of early endosomes into late endosomal compartments. Disruption of Vps39 function blocks endosomal conversion producing merged organelles with characteristics of both early and late endosomes [330]. Our results so far support a model in which the late endosomes function as a scaffolding platform for active G-protein signaling. To verify our hypothesis and to understand the physiological relevance of late endosomal function in cAMP signaling, we disrupted the *VPS39* function in *M. oryzae*. The *vps39* Δ mutant showed significant defects in appressorium initiation and appressorium formation and maturation, when compared to the WT at identical time points (Figure 5.14A).

One of the hallmarks of proper cAMP signal activation or signaling is the ability of the WT conidia to form mature appressorium on an inductive surface within a specific time-period. To test if the *vps39* Δ was disrupted in cAMP signaling, we carried out an appressorium formation assay with the *vps39* Δ strains and followed appressorium formation over a period of 24 h. Remarkably, the *vps39* Δ strain was completely dis-regulated for cAMP signaling and failed to form proper appressoria but formed only extensively long germ tubes. In addition, the germ tubes formed by the mutant showed a multiple hooking phenotype on an inductive surface, suggestive of disruption of proper cAMP signal activation and regulation (Figure 5.14A, arrowhead). Only 5% and 40% of *vps39* Δ conidia formed abnormal appressoria by 10 hpi and 24 hpi respectively (Figure 5.14B).

A**Inductive Surface** **B**

Figure 5.14. The late endosomal/HOPS component Vps39 is necessary for appropriate cAMP synthesis and signaling in *M. oryzae*.

(A) Time course analysis depicting delayed appressorium formation and multiple hooking phenotype (white arrowheads) as demonstrated by the *vps39Δ* strain upon inoculation on an inductive surface. The WT strain was used as control. Scale bar, 10μm.

(B) Graph depicting the efficiency of appressorium formation in the *vps39Δ* strain at 10 and 24 hpi respectively on an inductive surface. The bars represent the efficiency of appressorium formation. Values correspond to mean ± S.E taken from three independent experimental replicates each involving 300 conidia per sample.

Additionally, to validate the importance of late endosomal organization/function in the context of cAMP signaling and appressorium formation, endo-lysosomal acidification and hence late endosomal function was disrupted by employing the V-ATPase inhibitor - Bafilomycin A1 [331]. The germ tubes of Bafilomycin-treated WT conidia displayed defects associated with disruption of cAMP signaling, such as multiple hooking and failure to form proper appressoria (Figure 5.15).

Surprisingly, we found that addition of 8-Br-cAMP was sufficient to completely suppress the multiple hooking/ surface sensing and appressorium formation defects in the *vps39Δ* (Figure 5.14B and Figure 5.15).

The rescue of *vps39Δ* mutant phenotype by cAMP strongly suggested that the mutant was likely disrupted for adenylate cyclase function. The subcellular localization of adenylate cyclase (the enzyme responsible for the synthesis of cAMP) in *vps39Δ* background was therefore assessed. Remarkably, we found that compared to the wild-type control, majority of Adc-GFP (Mac1-GFP) was cytoplasmic and sequestered within the vacuoles (Figure 5.16A). Likewise, Rgs1-mCherry localized as intracellular aggregates and to the vacuoles in the *vps39Δ* strain (Figure 5.16B).

Taken together, our results strongly support the hypothesis that the late endosomal compartments act as signaling platforms/anchors and facilitate cAMP signaling in *M. oryzae*. Disruption of LE formation/integrity or stability leads to breakdown of cAMP signal synthesis and/or regulation, leading to defects in cell signaling necessary for appressorium formation.

Figure 5.15. The late endosomal function is essential for proper cAMP signaling.

Time course analysis depicting multiple hooking phenotype (white arrow heads) as demonstrated by the wild type upon treatment with bafilomycin A1 (row one). *vps39*Δ defects are completely rescued by exogenous addition of 8-Br-cAMP. Scale bar, 10μm

Figure 5.16. Subcellular localization of adenylate cyclase (Adc-GFP) and Rgs1-mC in the wild type and *vps39* Δ background strains respectively.

The bar (in panel B) highlights the absence of tubular compartments/dynamics in the germ tube. The arrowhead in panel A illustrates the localization of Adc-GFP to tubular cytosolic compartments. The white arrowhead in panel B indicates the failed hooking attempt of the *vps39* Δ mutant. Scale bars, 10 μ m.

5.1.9 The *vps39* mutant fails to cause blast disease in rice and barley

To test if Vps39 function or the integrity/formation of LE for is essential for causing blast disease, we carried out infection assays on barley explants as well as susceptible rice cultivar (CO39). Symptoms of the disease were evaluated after one and two weeks post inoculation in barley and rice, respectively. Surprisingly, the *vps39* Δ failed to cause disease or caused mild hypersensitive like response, both on barley and rice seedlings. On the other hand, the wild type caused disease with characteristic spindle shaped lesions with gray centers (Figure 5.17A and B).

In conclusion, anchoring of cAMP signaling via Vps39 activity, and by inference the late endosomal/HOPS function, is essential for pathogenesis and disease progression during *M. oryzae* infection.

Figure 5.17. Infection assay on rice and barley with the Vps39 gene-deletion mutant.

The *vps39*Δ strain did not cause typical disease lesions on susceptible rice cultivar and Barley leaf explants, unlike the wild type.

5.2 Discussion

Cellular geometry and the spatial segregation of signaling molecules within the cytoplasm influence how molecules interact to produce a cellular response. Spatial isolation of interacting molecules, by localization to different subcellular compartments/organelles, is a widespread mechanism of regulating pathway activity [323, 332]. In this chapter, I demonstrate that absence of authentic AKAP-like homologs in fungi [333], *M. oryzae* utilizes the dynamic late endosomal compartment as a common platform to anchor, transmit and likely regulate G-protein signaling during stages of surface sensing and pathogenic development.

Time-lapse imaging of Rgs1-mCherry conidia inoculated on an inductive surface revealed that the more or less static Rgs1 compartments change into dynamic tubulo-vesicular structures. We infer that this transformation is likely in response to inductive host cues and also a consequence of successful activation of G-protein signaling. The ability of the late endosomes to navigate and bi-directionally traverse the length of the germ likely facilitates the transport and recycling of signaling components to or away from the sites of endocytosis or signaling on the plasma membrane. However, our data demonstrate the preferential localization of active $G\alpha_s$ ($G\alpha_s^{G187S}$) on Rgs1-containing compartments; suggest that the late endosomes function as additional sites of active G-protein signaling in the cytosol. Such a scenario, in addition to facilitating signal transmission through the complex cytoplasmic milieu provides the cell with the flexibility to generate G-protein signaling hot spots as well as shape cAMP gradients at desired locations within the cell, and away from the plasma membrane.

The physical interaction between Pth11 and Rgs1 specifically during early stages of signal activation strongly indicates and validates the status of Pth11 as a *bona fide* upstream receptor of cAMP signaling in *M. oryzae*. Furthermore, based on the BiFC experiment, we propose that the interaction between Pth11 and Rgs1 occurs additionally on late endosomes, likely in the context and close proximity to the active $G\alpha_s$. It remains to be seen whether such an association requires post-translational modifications as has been demonstrated for the orthologous Sst2 and Ste2 in budding yeast during the mating response [320].

Incidentally, we also observed that Rgs1 accumulated on the membrane of the incipient appressorium upon treatment with either MBC or PI3Kinase inhibitor, suggesting that the trafficking of Rgs1 and by inference $G\alpha_s$ likely initiates at the outer membrane upon receptor (Pth11) activation. We found that the phosphoinositide PI3P is a pre-requisite for the maintenance of the integrity and dynamics of the Rgs1 containing compartments and by inference the late endosomes and $G\alpha_s$. Colocalization between Rgs1 and the PI3P binding FYVE probe indicates that Rgs1 is targeted to PI3P enriched endosomal structures. We have recently demonstrated that the N-terminal DEP domain of Rgs1 facilitates specific targeting to vesicular compartments [243]. Although we have not tested if Rgs1 can directly interact with phospholipids or PI3P, it has been demonstrated previously that the DEP domains of Sst2 has the propensity to bind phospholipids *in vitro* [320].

Figure 5.18. A simplified model depicting the proposed role of late endosomal compartments as signaling scaffolds that anchor key activators and regulators of the G-protein signaling in the rice-blast fungus *M. oryzae*.

Deletion of Vps39 function, a key member of HOPS complex, resulted in the complete mis-regulation of surface signaling and appressorium formation in *M. oryzae*, suggesting that late endosomal function and integrity is crucial for proper cAMP signaling. In the *vps39Δ*, a significant population of Adc-GFP as well as Rgs1-mCherry localized to the vacuole implying that in context of compromised late endosomal function, key signaling proteins are targeted to the vacuole either for sequestration, recycling and/or degradation. At the same time, the extensively delayed appressorium formed by the *vps39Δ*, suggests the possibility of active G-protein signaling initiating from other cellular locations, such as the plasma membrane. Such residual activity is likely insufficient to allow for proper cAMP synthesis and appressorium formation, implying that the anchoring of G-proteins to the late endosomes is important for sustained and requisite activation of cAMP signaling in *M. oryzae*.

Our results are in accordance with a number of recent studies implicating a role for the endosomes, and especially the late/MVB compartments in signal anchoring. In *S. cerevisiae*, the constitutively active G-protein (Gpa1^{Q323L}) has been shown to colocalize and interact with PI3kinase on endosomal compartments marked by Snf7 [148]. In mammalian cells, the MVBs play a positive role in activating Wnt signaling, by sequestering the inhibitor (GSK3) within the ILVs [149]. In another example, the free RIα subunit of cPKA is sequestered onto the surface of MVB's by AKAP11 in cAMP dependent manner [150]. Finally, it has also been shown recently that the integrity of the late endosomes is crucial for amino-acid and insulin- stimulated mTOR signaling [151].

Collectively, these findings suggest clearly that in addition to the canonical roles played by the late endosomal compartments, they also function as legitimate signaling anchors/platforms integrating trafficking and intracellular signal transduction during pathogenesis, as illustrated in our simplified model (Figure 5.18).

6. Chapter VI

Conclusions and future directions

Signaling research carried out over the last two decades has shown that cAMP plays a fundamental role in regulating a wide range of cellular events as well as changes in gene transcription in various model organisms. The cAMP signaling cascade is highly conserved across distant taxa including fungi and represents one of the best-characterized signal transduction cascades with most of the components functionally characterized. For example in fungi, cAMP signaling regulates aspects of growth, polarity, morphogenesis, differentiation, cell movement, response to stress, virulence, mating, sporulation, cell cycle progression and gene expression [12, 27]

Heterotrimeric G-proteins transduce extracellular stimuli into intracellular signals leading to changes in intracellular concentrations of cyclic nucleotides such as cAMP. The intracellular levels of cyclic nucleotides can vary or change dramatically depending on the type of tissue and the nature of stimulation, as reported for mammalian cells. These changes can either last a few milliseconds [334] or a few hours [2]. Variations in durations of the cAMP signals, as well as the subcellular targeting of the components of the cascade implicate that cAMP signaling is a complex process that is temporally and spatially regulated. Temporal regulation of cAMP signaling is achieved by a delicate balance between cAMP synthesis by adenylate cyclases, and its hydrolysis by PDEs [9]. Likewise, spatial control is accomplished by the association of key regulators (enzymes) with anchoring or scaffolding platforms (in the form of proteins or organelles). This facilitates signaling specificity by placing regulators in proximity to cognate effectors or substrates [137, 139].

The tip of the conidial germ tube in *M. oryzae* senses physico-chemical cues or signals from the plant surface and responds by switching the course of growth from polarized to isotropic, ultimately form a dome shaped infection structure-the appressorium. Changes in cellular geometry are ultimately in response to the activation of MAP kinases but is initiated and mediated by the activation and action of the upstream G-proteins and cAMP [192].

Brief Summary of findings

Given the key role of cAMP signaling in regulating a variety of processes in *M. oryzae* [190, 221], I first attempted to investigate and understand the effects and outcome of enhanced levels of intracellular cAMP on asexual growth and pathogenesis in *M. oryzae*. Towards this end, I identified two orthologs of phosphodiesterases in *M. oryzae*, and on the basis of the presence of highly conserved signature motifs therein, the proteins were categorized as high-affinity (PdeH) and low-affinity (PdeL) phosphodiesterases. On the basis of data obtained from experiments involving the characterization of the *pdeHΔ* and the double *pdeHΔpdeLΔ* strains, cAMP measurements, as well as *pdeHΔ*-like defects of the wild-type strain (upon treatment with exogenous cAMP), I further demonstrate that in *M. oryzae* PdeH is the major enzyme hydrolyzing and regulating intracellular levels of cAMP during asexual, pathogenic and *in planta* development. The low-affinity PDE possibly functions exclusively to regulate cAMP signaling within the nucleus, or in the absence of a functional PdeH or under conditions of excess intracellular cAMP.

Another important aspect of this work is the finding that cAMP levels are regulated by PdeH in a biphasic manner, i.e. up regulation during early stages, and down regulation at the late stages of pathogenesis. This model is supported by results from the measurements of intracellular cAMP and real-time PCR analysis of PDE mRNA, during the early and late phases of infection in the WT.

In *M. oryzae*, the RGS family of proteins consists of eight members [211], with one prototypical member, Rgs1. Rgs1 was found to regulate intracellular cAMP levels, asexual, pathogenic and *in planta* development. Given that RGS proteins function as key negative regulators that control the intensity and duration of active G-protein signaling, I next sought to understand the mechanisms of Rgs1 regulation. For this, the function of, as well as the subcellular localization the full length protein as well as the individual protein domains was determined. Rgs1 was found to undergo an endo-proteolytic cleavage to yield a DEP-DEP fragment and a core RGS domain. The DEP domain functioned to target Rgs1 to punctate vesicular structures and the catalytic core was trafficked to and likely sequestered within vacuole in the absence of a functional DEP motif.

The proteolytic processing and the vacuolar targeting of RGS domain in all probability represents a means of inactivating and down-regulating Rgs1 function, thus directly impacting the timing or duration of G-protein signaling in *M. oryzae*. Based on the physical interaction with Rgs1 specifically during early stages of appressorium initiation, we validate Pth11 to be a upstream modulator of cAMP signaling in *M. oryzae* and further more suggest Pth11 to be a *bona fide* GPCR (albeit non-canonical) for cAMP signaling in *M. oryzae*.

Long after the discovery of the requirement of cAMP signaling for appressorium initiation and formation in *M. oryzae*, a question that still persists is – What are the mechanisms by which signals that are likely initiated at the plasma-membrane are transmitted through the crowded intracellular network to specific sites of active growth and/or signaling? An initial clue towards answering this question was the subcellular localization of Rgs1. Rgs1 localized to dynamic tubulo-vesicular structures during early pathogenic differentiation on an inductive surface. These structures were capable of engaging the microtubule cytoskeleton to traverse bi-directionally along the length of the germ tube. Through colocalization with specific endosomal markers I determined that the identity of the structures as late endosomal compartments. Colocalization of late endosomes with constitutively active G-protein mutants implied that the late endosomes represented sites of active G-protein signaling. Disruption of the integrity and function of the late endosomes through mutants and treatment with specific chemical inhibitors resulted in the disruption of cAMP signaling, supporting the role of late endosomal compartment in influencing cAMP signaling.

Thus, sequestration or anchoring of key G-protein signaling components on dynamic late endosomes plausibly provides an effective means to compartmentalize and actively control the spatio-temporal organization of cAMP biogenesis amidst changing cellular geometry during pathogenic development.

In conclusion, the work carried out in this thesis identifies a PdeH dependent biphasic regulation of cAMP signaling in *M. oryzae*. Additionally the work also unravels an elegant mechanisms adapted by *M. oryzae* for regulating (through proteolytic processing), anchoring and trafficking of G-protein signaling components/regulators of cAMP synthesis during initiation of infection-related growth and development.

Future Work

cAMP signaling in the nucleus and during biotrophic development

In contrast to the PdeH, the function of the low-affinity PDE (PdeL) appeared to be not essential for asexual development or pathogenesis. However, the distinct nuclear localization of PdeL during asexual as well as pathogenic stages of development raised the possibility that PdeL functioned within the nucleus. Furthermore the observation strongly suggested the existence of two distinct (although not exclusive) pools of cAMP (cytosolic and nuclear) in *M. oryzae*. The possibility that PdeL responds and functions to regulate enriched pools of cAMP within the nucleus, while the PdeH functions to maintain cytosolic cAMP gradients, remains to be tested. In the future additional experiments may be carried out to confirm the specific roles and contributions of nuclear pools of cAMP in *M. oryzae*. Also, of interest is to understand the mechanism/s by which PdeL is trafficked to the nucleus in the absence of obvious nuclear localization signals (NLS).

The finding that intracellular cAMP levels or cAMP signaling *per se* necessitates being down-regulated during the stage of appressorium maturation is imperative since it strongly influences the ability of *M. oryzae* to successfully colonize/infect the host tissue. Future experiments could focus on understanding the relationship between cAMP signaling and *in planta* development in the context of sensitivity towards stress conditions (reactive oxygen species), such as those encountered within the host during biotrophic growth.

The expression level of PdeH tagged at the C-terminus with RFP (driven by the native promoter) was very weak and difficult to image. In order to circumvent this problem and visualize better the subcellular localization of PdeH, we decided to use the promoter of a highly expressed hydrophobin gene, namely *MPG1* to drive the expression of PdeH-GFP. The GFP tagged strain showed no obvious defects during asexual growth or pathogenic development; however it does raise an important question about the effects of overexpression or constitutive expression of PdeH on steady-state cAMP levels and *M. oryzae* development.

In the *pdeHΔ*, appressorial development was advanced and partially independent of the nuclear division, compared to the wild-type (where nuclear division or mitosis precedes appressorial initiation), suggesting that enhanced cAMP levels could support appressorial development independent of nuclear division. This scenario provides an opportunity to study further the relationship between cAMP levels, appressorial morphogenesis and nuclear division.

Given that Rgs1 primarily functions as a negative regulator of GTP bound G-protein, any post translational modifications (in this case proteolytic processing of the protein as well as the vacuolar targeting of the core RGS motif) probably represents a means of inactivating the function of Rgs1, thus directly influencing the duration and intensity of active G-protein signaling in *M. oryzae*. In the future, experiments may be performed to decipher the role of the DEP-DEP domain, in targeting Rgs1 in close proximity to either G-proteins and/or upstream GPCRs. An interesting possibility is to validate if the N-terminal DEP-DEP of Rgs1 facilitates interaction with Pth11 through the C-terminal tail or cytoplasmic loops. The individual domain fragments did not restore any aspect of functionality of the full-length protein, strongly indicating that the N- and C-terminal motifs perform specific and independent functions. Expression of both the domains may be necessary for the complete rescue of asexual differentiation and development in the *rgs1Δ*; however it remains to be tested.

Structure Function analysis of the non-canonical GPCR, Pth11

Based on data from phenotypic characterization of *PTH11* deletion strains, chemical genetics experiments, biochemical interactions and sub cellular localization studies, we propose Pth11 to be an upstream GPCR for cAMP signaling in *M. oryzae*. However, a number of questions regarding the mode of action of Pth11 and the exact function of the N-terminal cysteine rich CFEM domain within Pth11 remain to be answered. For example, what is the ligand that binds to and activates Pth11? Does Pth11 respond to thigmotropic cues or lipids present on the host surface? Does Pth11 or the CFEM domain contribute towards adhering to the host surface? Does the CFEM domain play a role in sensing extracellular ligands? What is the function of the cysteine residues within the CFEM domain? In a manner identical to the DEP-DEP domain in the Rgs1 protein, does the CFEM domain function to target Pth11 to the membrane? What are

the effects of overexpression of the CFEM domain on inductive surface signaling and signal activation? In an effort towards answering a few of the above questions, preliminary results, obtained from a strain deleted only for the CFEM domain, suggest a crucial role for this domain in inductive surface based signaling and surface recognition.

Roles of late endosomal anchored cAMP signaling during *in-planta* development

We have through a series of experiments established that *M. oryzae* makes use of the dynamic late endosomal system as a platform or scaffold to anchor, transmit and directly regulate G-protein signaling during early stages of infection-related development. Although this finding by itself answers an important and fundamental question that has persisted in the field of *M. oryzae* biology, many interesting questions that arose during the course of the study remain to be answered. For example: Do components of MAP Kinase pathway also utilize the late endosomal system as a scaffold for signal anchoring and transmission? Does the late endosomal system facilitate G-protein (if any) or MAP Kinase signaling *in -planta*?

Contribution of phosphoinositide signaling during pathogenic development and effector secretion

Our experiment, especially the one utilizing the PI3 kinase inhibitor, LY294002, suggests a crucial role played by PI3 kinase/ phosphoinositide signaling in *M. oryzae* biology. In the future, experiments may be directed towards understanding in greater detail the contribution of phosphoinositide signaling during the late stages pathogenic development especially during or in the context of *in-planta* growth and/or effector secretion.

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7. Appendix

Table 1. Oligonucleotide primers used in this study (5'- 3'). Restriction enzyme sites introduced for cloning purposes are underlined.

Chapter III

PDEH (MGG_05665) gene deletion constructs

1kb upstream

F- CAGAGAGAAATTCAGCACCAGCATGGCACCACACTATC

R-CAGAGATCTAGACAAAGAGCGTCCAGTCATAAGACT

1kb downstream

F- CAGAGACTGCAGGTTTCAGTACTACTGTTCACTCAGAT

R- CAGAGAAAGCTTACGCATTACCCAATGTTGGCATC

PDEL (MGG_07707) gene deletion construct

1kb upstream

F-CAGAGAGGGTACCCCGTTTGCTACCTGTGGCCAACG

R-CAGAGAGGGATCCCCGCCCGTCCCGTCTAGCCCAGCTGGGCT

1kb downstream

F-CAGAGACTGCAGTGCGACCCTATGACAGTCCCCT

R-CAGAGAAAGCTTGAGGCCGCCAATGCCACGAGCGC

RFP-*PDEH* complementation construct

PDEH Promoter

F-CAGAGAGAAATTCGTTTCGGCTCAATTCAATTCGA

R-AGAGAGAGCTCCGTGGGCCCAAAGAGCGTCCA

RFP coding sequence

F- CAGAGAGCGCTCATGGACAACACCGAGGAC

R- CAGAGACCCATGGCCTGGGAGCCGGAGTG

PDEH coding sequence + downstream 1kb

F- CAGAGACCCATGGAGAATGCTGCCTGCAAT

R-AGAGAGGATCCTGCCAAGTTGTCACTTTCCAAGT

GFP-*PDEH* and GFP-*PDEL* constructs

PDEH coding sequence

F-CAGACATATGGGAGAATGCTGCCTGCAAT

R- CAGATCTAGATCAACCAGCAGTGTC

PDEL coding sequence

F-CAGACATATGGGCGAGGGCAGCGCCGAA

R-CAGATCTAGATCACAAGTACAATGCCTCGCCA

eGFP

F-CAGACCCATGGTGAGCAAGGGCGAGGA

R- CAGACATATGCTTGTACAGCTCGTCCAT

Real-time RT PCR

BAS1 (MGG_04795)

F- TTTGATCAGCGTTACCAAGG

R- CGGTGACCAACATTCTCTTG

α -Tubulin (MGG_00604)

F- CATGATGGCTGCTTCTGACT

R-CGACGAGTTCTTGTCTGGA

PDEH (MGG_05664)

F- GCTTGAGCGCTGGAGAATGT
R-TAACGAGCCGATCTGTACCA

PDEL (MGG_07707)

F- CTTCTACAGCAGAGACACC
R- GCTCCTGCATAATAATGTCC

Chapter IV

Rgs1-mCherry construct

RP27 promoter

F-CAGAGAGAATTCATAAATGTAGGTATTACCTG
R-CAGAGAGAATTCCTTTGAAGATTGGGTTCCTAC
F-CAGAGAGAATTCATAAATGTAGGTATTACCTG
R-CAGAGACATATGTTTGAAGATTGGGTTCCTAC

RGS1

F-CAGAGAGAATTCATGGACGACACCTCCCGCC
R-CAGAGACCCGGGTAACCGTTGCGAGCGGCTT
R-CAGAGAGAGCTC CGTAGCGAACAAATCC

mCherry

F-CAGAGACCCGGGATGGTGAGCAAGGGCGAGG
R-CAGAGAGGATCCTTACTTGTACAGCTCGTCCATGCCG

DEP-A mCherry and DEP-B mCherry constructs

DEP-A

R-CAGAGAGAGCTCGTTACGTGAAGCAAAAG

DEP-B

F-CAGAGAGAGCTCGACTCTTTTGCTTCACG

BAR

F-CAGAGAGGAGCTCAGCCCAGAACGACGCCCCG

R-CAGAGACCCCGGGGATCTCGGTGACGGGCAG

TrpC terminator

F-CAGAGAGGGATCCACTTAACGTTACTGAAATCATCAA

R-CAGAGATTCTAGACGAGCCCTCTAAACAAGTGT

Chapter V

Rgs1-mCherry construct

RGS1

F-CAGAGAGAAATTCATGGACGACACCTCCCGCC

R-CAGAGACCCCGGGTAACCGTTGCGAGCGGCTT

mCherry

F-CAGAGACCCCGGGATGGTGAGCAAGGGCGAGG

R-CAGAGAGGGATCCTTACTTGTACAGCTCGTCCATGCCG

TrpC terminator

F-CAGAGAGGGATCC/CCCGGGACTTAACGTTACTGAAATCATCA

R-CAGAGATTCTAGA/GGATCCCGAGCCCTCTAAACAAGTGT

GFP-VPS21 construct

VPS21/RAB5

F-CAGAGACCCCGGGTCCGGAGGAGGAGGATCCGCCGACGGAGCCGCCAAAC

R-CAGAGAGGGATTCTCAACACGCGCATCCATCC

eGFP

CAGAGACCCATGGTGAGCAAGGGCGAGGAGCTGT (F)

CAGAGACCCCGGGCTTGTACAGCTCGTCCAT (R)

GFP-YPT7 construct

YPT7/RAB7

F-CAGAGACCCCGGGTCCGGAGGCGGTGGCTCGTCCAGAAAGAAGGTTCT

R-CAGAGACCCCGGGTTAGCAGGCGCATCCATC

MAGA-GFP construct

MagA upstream

F-GAGCAGGAAATTCTCCCCACTCCGACGACGT

R-TCACTCACGCGTAGACCCTCCGCCACCACTCGACTCGACCATGTAGTC

MagA downstream

F-GAGCAGGTTAACAGCGGCGGAGGTGGCTCGGGACCACAGGCAGAGATAG

R-GAGCTGAAAGCTTAACATCGTTGCCTCCAGAG

α -Tubulin -GFP construct

α TUB

F-CAGAGACCCCGGGTCCGGAGGAGGAGGATCCCGCGAGATTATCAGCATC

R-CAGAGACCCCGGGCTAGTACTCCGCCTCAATAC

VPS39 (MGG_01054.6) gene deletion constructs

1kb upstream

F-CAGAGAGAAATTCGAGGATAAGCCTTGTGAAG R-

CAGAGAGGATCCTGCGACGTCGGGCTCGCG

1kb downstream

F-CAGAGACTCGAGGCTCGGTGTTGAGAGTTGAC

R-CAGAGAAAAGCTTCTGTTACCCGTTCAGCCGTC

***PTH11*-mCherry construct**

PTH11

F-CAGAGAGAATTCTTGCATTCACCCGGTTGCT

R-CAGAGAGATATCGATGAGACCACCGGGCAGT

F-CAGAGAGGGTACCAGTTGTGATCTTAAG

R-CAGAGAGGGTACCGTGACAGCGAATC

BiFC construct

YFP (1-156)

F-CAGAGGGTACCCGCTCCATCGCCACGATGGTGAGCAAGGGCGAGGAGCTGTTCAACC

R-CAGAGAGGGATCCTTAGGCCATGATATAGACGTTGTG

YFP(156-239)

F-CAGAGACCCCGGGTCTAGAAAACAGAAGGTCATGAACCACGACAAGCAGAAGAAC

R-CAGAGACCCATGGACTGTACAGCTCGTCCATG

8. List of Publications

- **Ramanujam R** and Naqvi NI. (2010) PdeH, a High-Affinity cAMP Phosphodiesterase, Is a Key Regulator of Asexual and Pathogenic Differentiation in *Magnaporthe oryzae*, **PLoS Pathogens** 6:e1000897
- **Ramanujam R**, Xu Y, Liu H and Naqvi NI. (2012) Structure-function analysis of Rgs1 in *Magnaporthe oryzae*; Role of DEP domains in sub-cellular targeting. **PLoS One** 7(7): e41084
- Bosch DE, Willard FS, **Ramanujam R***, Kimple AJ, Willard MD, et al (2012) A P-loop mutation in G alpha subunits prevents transition to the active state: implications for G-protein signaling in fungal pathogenesis. **PLoS Pathogens** 8: e1002553 (* Second Author)
- **Ramanujam R**, Calvert M, Selvaraj P, Naqvi NI. (2013) The Late Endosomal HOPS complex Anchors Active G-protein Signaling Essential for Pathogenesis in *Magnaporthe oryzae*. **PLoS Pathogens**, 9(8), e1003527

Book Chapter

- Liu, H, **RK Ramanujam** and NI Naqvi. (2009) Surface sensing and signaling during initiation of rice blast disease. In Advances in Genetics, Genomics and Control of Rice Blast Disease (eds. GL Wang and B Valent) Springer Chapter 2: 23-32.

9. Posters, awards

- A Non-canonical GPCR Required for Surface Signaling and Pathogenicity in *M. oryzae*, 2007 (Poster), The 4th International Rice Blast Conference, Changsha, Hunan, China
- The High-Affinity cAMP Phosphodiesterase, Is a Key Regulator of Asexual and Pathogenic Development in *Magnaporthe oryzae*, 2010 (Poster), The Gordon Research Conference on Cyclic Nucleotide Phosphodiesterases, New Hampshire, USA
- The Late Endosomal compartment Anchors active Cyclic AMP Signaling in *Magnaporthe oryzae*, 2012 (**Best poster award among graduate students**), The Scientific Advisory Board Meeting, Temasek Lifesciences Laboratory, Singapore