THE SIGNALING ROLES OF THE PREDICTED GPCR-ENCODING GENES REGULATED BY BIP1 IN THE RICE BLAST FUNGUS *Magnaporthe oryzae*

A Thesis

by

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ABSTRACT

Magnaporthe oryzae infects grass-family plants, such as barley and rice, by applying a special structure called the appressorium. A strain lacking the transcription factor BIP1 does not form infectious hyphae from mature appressoria and loses pathogenicity. Four of the BIP1-regulated genes are predicted to encode G-protein-coupled-receptor (GPCR)-like products, which may have potential functions in transducing environmental signals. One of the four genes, *plg1* (pth11-like gene 1) is crucial for appressorium differentiation and melanization in response to hydrophobic surface cues, while deletion mutants of *plg2* or *plg3*, and *plg4* do not affect pathogenicity or appressorium formation on hydrophobic surfaces. The predicted structure of PLG1 is a novel transmembrane protein with a predicted membrane topology similar to that predicted for PTH11. The different effects of exogenous cAMP and DAG on pathogenicity of Δ*plg1* spores on plants indicate that the cAMP- and DAG-dependent signaling pathways have different functions during appressorium development. As with wild-type spores on a hydrophilic surface, treatment of Δ*pth11* spores with cAMP induced appressorium development, although higher concentrations of cAMP were needed for induction of Δ*pth11* spores than for wild-type spores. In contrast, treatment of Δ*plg1* spores with cAMP did not induce appressorium development. DAG treatment of Δ*plg1* or Δ*pth11* spores inoculated on a hydrophilic surface did not induce appressorium development, while DAG treatment induced appressorium development by wild-type

spores. When both cAMP and DAG were added to Δ*plg1* or Δ*pth11* spores, they all showed increased appressorium formation. The reason why melanized appressoria of Δ*bip1* did not infect plants was investigated through cytorrhysis assay. Δ*bip1* and wild-type appressoria collapsed in response to treatment with similar concentrations of glycerol indicating that Δ*bip1* appressoria have normal turgor pressure. The failure of plant infection may be caused by disruption of the pore ring in appressoria. The addition of exogenous cAMP was not able to restore the formation of penetration pegs, indicating that the cAMP signaling pathway was not related to BIP1 regulation.

DEDICATION

I dedicate this work to Ruolin, my parents, and Kat who is always ready to help me in my research.

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The data analyzed in Figure 10C and D were provided by Dr. Daniel Ebbole. The experiment results of Δ*plg1* phenotypes in Chapter IV were provided by Dr. Kathrina Castillo. The data collected and analyzed in Figure 10A and B were done together with Dr. Kathrina Castillo.

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CHAPTER I

INTRODUCTION OF *Magnaporthe* AND RICE BLAST DISEASE, LITERATURE REVIEW

Life Cycle of *Magnaporthe oryzae*

Magnaporthe oryzae is a pathogenic fungus, that infects economically important grass family plants such as rice, barley, wheat and millet (Valent and Chumley 1991). The yield loss due to infection by *M. oryzae* is more than 10 million tons each year, which can feed about 60 million people (Saitoh et al. 2003). *M. oryzae* infects plants through a special structure, the appressorium (Bourett and Howard 1990). During its disease cycle, the

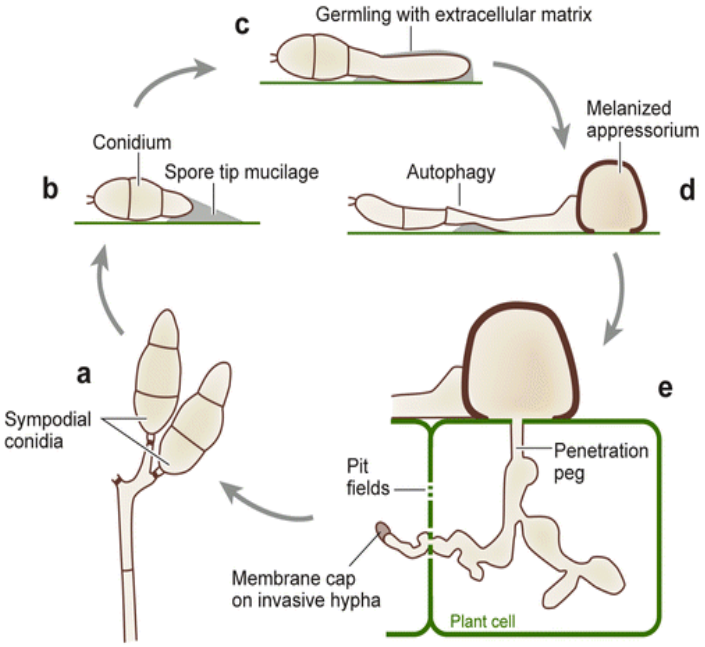

Figure 1 The infection cycle of *Magnaporthe oryzae* and its appressorium development.
(Reprinted with permission from "*Magnaporthe* as a model for understanding host-pathogen interactions" by Daniel J. Ebbole, 2007, Annual Review of Phytopathology 45, 437-56, Copyright 2007 by Annual Reviews.)

asexual spore attaches to the surface of plant leaves by secreting a spore tip mucilage (Hamer et al. 1988). Then the spore forms a germ tube and a hook structure from the tip, perceiving physical and chemical cues such as hardness, hydrophobicity and cutin monomers (Xiao et al. 1994; Lee and Dean 1994; Gilbert, Johnson, and Dean 1996). It has been reported that nutrient starvation also induces appressorium development by up-regulating the expression of a secreted hydrophobin, MPG1 (Talbot, Ebbole, and Hamer 1993). MPG1 is suggested to be involved in the recognition of hydrophobicity during the early stage of appressorium development (Beckerman and Ebbole 1996; Talbot et al. 1996). When the surface cues are appropriate, the hook structure develops into an appressorium with a melanized cell wall and a penetrating peg (Mendgen, Hahn, and Deising 1996; Talbot et al. 1996). Appressorium formation requires one round of mitosis after which a daughter nucleus migrates into the germ tube tip followed by a septum formation around the neck (Saunders, Aves, and Talbot 2010; Saunders, Dagdas, and Talbot 2010). The peg penetrates into leaf cells driven by turgor pressure generated from high intracellular glycerol concentration (de Jong et al. 1997). The fungus continues invasive growth in plants and forms leaf lesions where new conidia are produced, and spread to other plants (Figure 1) (Bourett and Howard 1990) (Ebbole 2007).

Signaling and Transduction Pathways in *Magnaporthe oryzae*

Several signaling pathways have been found to be important for pathogenicity of *M. oryzae* (Figure 2) (Li, Zhou, and Xu 2012). One of the most important pathways is mediated by cAMP, which is essential for surface recognition, appressorium development

and turgor generation (Wang et al. 2005; Lee and Dean 1993). Exogenous cAMP induces appressorium formation from conidia inoculated on hydrophilic surfaces (Lee and Dean 1993). The added cAMP analogs may be absorbed into fungal cells and work intracellularly as second messengers that signal to downstream pathways. MAC1, an adenylate cyclase, functions upstream of cAMP, regulating its intracellular biosynthesis (Choi and Dean 1997). The concentration of cAMP is down regulated through its hydrolysis by PdeH, a high-affinity phosphodiesterase (Ramanujam and Naqvi 2010).

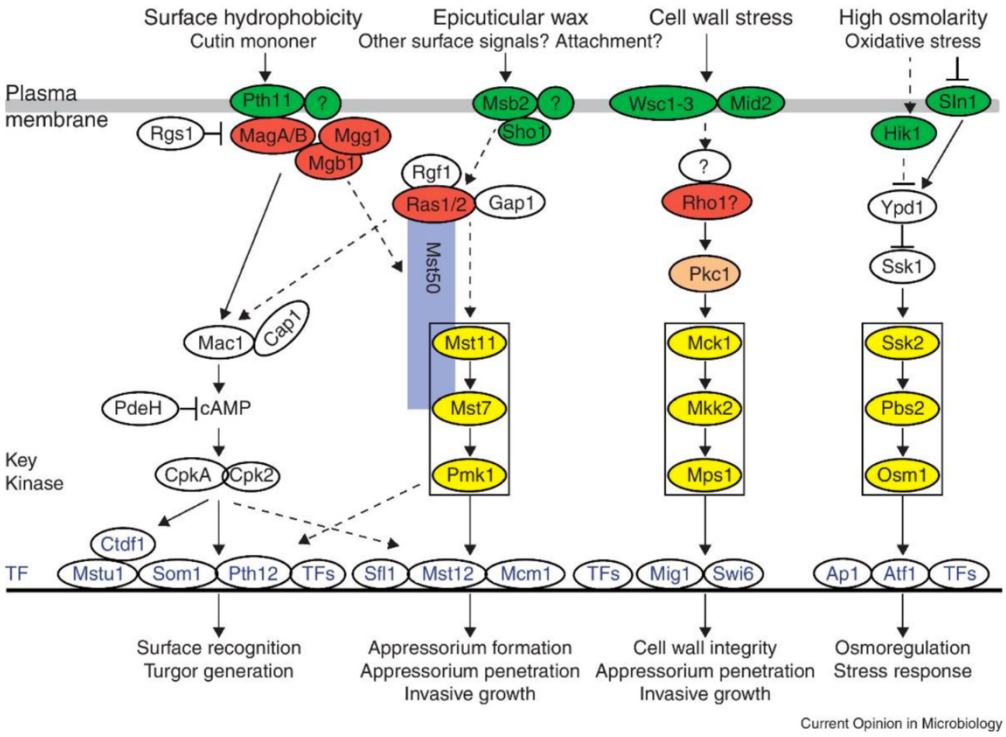

Figure 2 The important signaling pathways in *Magnaporthe oryzae* for pathogenic development.
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A regulatory subunit of PKA (Protein Kinase A), named SUM1, functions downstream of the cAMP pathway (Adachi and Hamer 1998). To date, CPKA is the only characterized PKA catalytic subunit, which is not relevant to appressorium formation but to plant penetration (Xu et al. 1997). Other PKA catalytic subunits may function downstream of cAMP and SUM1 to regulate appressorium formation (Mitchell and Dean 1995). MoRAS2, a small GTP-binding protein, functions upstream of cAMP pathways. Overactivation of MoRAS2 up-regulates intracellular cAMP level and bypasses the recognition stage of appressorium differentiation (Zhou et al. 2014).

The cAMP pathway responds to hydrophobic surfaces and the extracellular signal is transduced by a novel seven transmembrane domain protein, PTH11 (DeZwaan et al. 1999). The failure of Δ*pth11* mutants to form appressoria on hydrophobic surfaces is similar to the phenotype of wild-type on hydrophilic surfaces. The appressorial deficiency of the Δ*pth11* mutant can be rescued by adding exogenous cAMP. PTH11 is predicted to be a transmembrane protein similar to G-protein coupled receptors (GPCR) (Kulkarni et al. 2005; Talbot 2003). GPCRs are integral membrane proteins with seven alpha-helix transmembrane domains. The heterotrimeric G protein complex consists of Gα, Gβ and γ subunits. GPCRs bind with signaling molecules from the environment and activate downstream pathways through disassociation of the Gα subunit from Gβ and γ (Kulkarni et al. 2005).

Three new Gα homologs have been identified in *M. oryzae*, but only MagB is relevant to pathogenic development (Liu and Dean 1997). A Δ*magB* strain does not form appressoria under inducing conditions, but over-expressed MagB induces appressorium formation without leaf surface cues. The exogenous addition of cAMP is able to recover appressorium formation in Δ*magB* stains, indicating cAMP is downstream of MagB (Liu and Dean 1997).

The Gβ subunit MGB1, and Gγ subunit MGG1, have also been identified to be important for appressorium formation and host penetration. Exogenous cAMP induces appressorium formation in Δ*mgb1* or Δ*mgg1* mutants, indicating that cAMP is also regulated by MGB1 and MGG1 (Nishimura, Park, and Xu 2003; Li et al. 2015). However, the induced appressoria cannot penetrate plants, which means that other signals in addition to cAMP may work downstream of MGB1 or MGG1 (Nishimura, Park, and Xu 2003; Li et al. 2015). The G-protein regulator RGS1 directly interacts with all three Gα subunits in *M. oryzae*, and it was found to negatively regulate downstream pathways (Liu et al. 2007). GPCRs are common and vital in animal cells, but few classical GPCRs are found in *M. oryzae* (Kulkarni et al. 2005). Instead, a new protein family showing similar structures to PTH11 have a lot more members than GPCR homologs in *M. oryzae* (Kulkarni et al. 2005). The large group of PTH11-like proteins are specific to Ascomycota and are not found in other fungal groups. The role of PTH11 and PTH11-like proteins in pathogenesis of *M. oryzae* will be discussed later in this chapter.

Mitogen-activated protein kinases (MAPK) have been found to be crucial for appressorium formation and pathogenicity in *M. oryzae*. PMK1, the homolog of yeast MAP kinases FUS3/KSS1, is essential to arrest nuclear division during appressorium development and invasive growth in plants (Xu and Hamer 1996). PMK1 expression is increased when the appressorium is developing, and the protein is transported into appressorial nuclei (Bruno et al. 2004). MST12, the homolog of yeast STE12, is reported to function downstream of PMK1 (Park et al. 2002). MST12 is dispensable for appressorium formation and melanization, but is essential for host penetration and infectious growth (Park et al. 2002). PMK1 binds to MST7, the yeast STE7 homolog, specifically in appressoria (Zhao and Xu 2007). A complex of MST7 and MST11, the homolog of yeast STE11, regulate phosphorylation of PMK1 as a complex (Zhao et al. 2005). The homolog of yeast STE50, MST 50, is highly expressed in appressoria and works as an adaptor stabilizing the interaction between MST11 and MST7. The SAM domains in MST11 and MST50 are important for their interaction and for signal transduction (Park et al. 2006). MST11 and MST50 also have direct interactions with two upstream Ras homologs, RAS1 and RAS2, but they are not regulated by the two PAK (p21-activated kinase) kinases MST12 and CHM1 in *M. oryzae* (Li et al. 2004). MST50 also interacts with the Gβ subunit MGB1 and Cdc42 homolog MgCdc42 (Park et al. 2006). The PMK1 pathway may be regulated by MGB1 for host penetration and phytopathogenicity (Nishimura, Park, and Xu 2003). The PMK1 pathway is also regulated by RAS2, the overactivation of which up-regulates PMK1 phosphorylation and induces nonfunctional appressorium formation on hydrophilic surfaces (Zhou et al. 2014). Two

upstream receptors, MoMSB2 and MoSHO1, which are the homologs of yeast MSB2 and SHO1, have been found to recognize various surface signals and activate the PMK1 pathway (Liu et al. 2011). MoMSB2 may respond to hydrophobicity and cutin monomers, whereas MoSHO1 may react to leaf waxes (Liu et al. 2011). Another homolog of yeast MAP kinase SLT2, MPS1, is not relevant to appressorium development, but is important for appressorium function such as penetrating host cells because of its regulatory function on cell wall integrity (Xu, Staiger, and Hamer 1998). MPS1 interacts with MIG1, a MADS-box transcription factor, which is expressed in pathogenic structures and crucial for invasive growth inside plants (Mehrabi, Ding, and Xu 2008). The MPS1 pathway is downstream of MCK1, a MAPKKK homolog of yeast BCK1, important for cell wall integrity and appressorial penetration (Jeon et al. 2008).

Diacylglycerol (DAG) has been reported as another important second messenger in *M. oryzae.* Appressorium formation is induced on hydrophilic surfaces by adding exogenous DAG, and induction is not inhibited by glisoprenin A, a compound that inhibits induction by cAMP (Thines et al. 1997). The intracellular level of DAG is regulated by lipid phosphate phosphatases MoLPP3 and MoLPP5, which transform phosphatidic acid (PA) to DAG in *M. oryzae* (Sadat et al. 2014). Phospholipase C (PLC) may also regulate DAG levels by catalyzing phosphatidylinositol 4,5-bisphophate (PIP_2) into DAG and inositol 1,4,5-trisphosphate (IP_3) (Lee and Lee 1998). The pathways downstream of DAG are mediated through PKC (protein kinase C) which is also involved in regulating sporulation and cell wall integrity in *M. oryzae* (Penn et al. 2015).

The Ca^{2+} induced signaling pathway is also crucial for appressorium formation and pathogenicity in *M. oryzae* (Nguyen et al. 2008). The intracellular level of Ca^{2+} is up-regulated specifically in the germ tube tip when it senses the hydrophobic surface (Rho, Jeon, and Lee 2009). Addition of calcium chelators, calcium channel inhibitors or calmodulin antagonists inhibits appressorium formation (Lee and Lee 1998), showing that the Ca^{2+} signal is important for appressorium development. The Ca^{2+} signal is induced by IP_3, which is produced when phospholipase C1 (MoPLC1) transforms PIP_2 into DAG (Rho, Jeon, and Lee 2009). The inhibition of PLC synthesis by neomycin inhibits appressorium formation on hydrophobic surfaces. Neither exogenous Ca^{2+} nor IP_3 induces appressorium formation on hydrophilic surfaces, indicating Ca^{2+} is a second messenger necessary, but not sufficient, for appressorium development in *M. oryzae*. Calmodulin is a calcium-binding protein, which functions downstream of Ca^{2+} signals. Its expression is regulated by conidium attachment to plant surfaces during the early stage of appressorium development (Liu and Kolattukudy 1999). A putative calmodulin-dependent kinase, MoCMK1, is relevant to the efficient development of turgor pressure and pathogenic penetration (Liu et al. 2010). The calmodulin-dependent phosphoprotein phosphatase calcineurin also plays a role in appressorium formation, since its inhibition by the complex of CsA and cyclophilin A results in appressorial failure (Viaud, Balhadere, and Talbot 2002). MCNA, a catalytic subunit of calcineurin in *M. oryzae*, is crucial for appressorium maturation and pathogenicity (Choi, Kim, and Lee 2009). The calcineurin-responsive transcription factor MoCRZ1 interacts with MCNA, and its nuclear localization is

regulated by Ca^{2+}/calcineurin, which is essential for appressorial turgor pressure, cell wall integrity and Ca^{2+} homeostasis (Choi et al. 2009; Kim et al. 2010).

The Study of *bip1* and *plg1* in *Magnaporthe oryzae*

The *bip1* gene, a novel basic leucine zipper (bZIP) transcription factor, identified by our lab is essential for the pathogenicity of *M. oryzae* (Tag unpublished). The mutant strain fails to cause brown lesions on barley and rice leaves, because its melanized appressoria do not form penetration pegs. The RNA expression of *bip1* is higher in spores and appressoria, but lower in mycelia. BIP1-GFP fusion proteins are located in the nuclei of appressoria. There are at least 44 genes whose transcription is down-regulated in a Δ*bip1* mutant. Four of these genes (*plg1*, *plg2*, *plg3* and *plg4*) encode PTH11-like proteins, and are being studied by our lab.

The protein structure encoded by *plg1* is predicted by TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM) (Krogh et al. 2001) to have seven transmembrane helices, one of which is a pore lining helix. Its C-terminal amino acid sequence shows weak interaction with MagB in a yeast two hybrid assay. But the interaction could not be confirmed by co-immunoprecipitation assays in vitro (Castillo 2015). The *plg1* mutant does not induce lesions on barley or rice, because it forms only 10% of wild-type appressoria on hydrophobic surfaces. But the mutant is able to infect plants when leaves are wounded. Appressorium formation by the Δ*plg1* mutant is restored by adding the cutin monomer 1,16-hexadecanediol, cAMP or DAG on Teflon membranes

or barley leaves. Adding of DAG restores full virulence to the Δ*plg1* strain, but adding cAMP does not, indicating that the DAG signal is downstream of PLG1. The conclusion is further supported by RNA expression result. The Δ*plg1* mutant has much lower levels of *pkc* RNA expression, which is in the DAG pathway, than wild-type, while transcripts of genes in other signaling pathways, such as *cpka* or *pmk1*, is not changed (Castillo 2015). However, the appressorium development of the Δ*plg1* strain is restored by treatment with both cAMP and DAG on hydrophilic surfaces, indicating that appressorium development may require multiple signaling pathways to be activated.

The fact that activation of more than one pathway is crucial for fungal pathogenicity has been discussed previously. For example, exogenous cAMP rescues appressorium development of Δ*pmk1* on hydrophobic surfaces, but only rescues germ tube differentiation on hydrophilic surfaces (Xu and Hamer 1996). Appressorium formation by a Δ*mst7* mutant is not rescued by cAMP on hydrophilic surfaces, either (Zhao et al. 2005). Constitutive expression of MST7 does not rescue appressorial penetration of Δ*mst11*, Δ*mst7* or Δ*mst50* mutants, whose products are involved in the PMK1 pathway, probably because of the down regulation of cAMP and MPS1 (Park et al. 2006). Exogenous cAMP only partially rescues the *mst50* mutant without RAD1 domain, but does not rescue the mutant without SAM1 domain (Park et al. 2006). MST12 was reported downstream of PMK1 and cAMP, indicating it is activated by both pathways (Park et al. 2002). Both the intracellular level of cAMP and PMK1 phosphorylation are up-regulated by RAS2 and MGB1, which are important for pathogenic development (Zhou et al. 2014). It is also

inferred that the PMK1 pathway regulates mobilization of glycogen and lipids to appressoria, while the cAMP pathway regulates degradation of glycogen and lipids for glycerol production (Thines, Weber, and Talbot 2000). An elevated Ca^{2+} signal is necessary but not sufficient to induce appressorium formation (Lee and Lee 1998).

The Project Research Aim

Four GPCR-like protein encoding genes regulated by BIP1 were identified, and are predicted to have roles in pathogenicity in *Magnaporthe oryzae*. One of them, *plg1* (*pth*11-like gene 1) has been demonstrated to be crucial for appressorium differentiation and melanization on hydrophobic surfaces (Castillo 2015). The functions of the other three genes need to be investigated.

There are multiple signaling pathways involved in pathogenicity in *M. oryzae*, and there is evidence of crosstalk between different pathways. When one pathway is activated in a mutant of another pathway, pathogenic development is only partially rescued. Constitutive activation of one pathway in most cases is only able to induce pathogenic morphogenesis but not full virulence. However, how these crucial pathways cooperate with each other is not clear. It can be inferred that the activation of one pathway may up-regulate or down-regulate another one at different time point to acquire full pathogenicity. More investigation should be done on the relationship between different signaling pathways to further understand pathogenic development in *M. oryzae*. Deletion of the *plg1* gene affects the formation and melanization of appressoria. While its predicted structure

shows similarities to PTH11 as a novel transmembrane protein, it may play an important role in transducing signals to develop appressoria during infection. The difference in the extent of pathogenicity between Δ*plg1* spores treated with cAMP or DAG on plants shows different roles of these two signaling pathways during appressorium development. The treatment with cAMP or DAG on Δ*plg1* spores inoculated on hydrophobic or hydrophilic surfaces results in different appressorium development. It is important to further investigate the relationship of the hydrophobicity signal and the pathway mediated by PLG1, in order to understand the role of PLG1 during appressorium development in *M. oryzae*.

CHAPTER II

THE FUNCTION OF BIP1 REGULATION DURING APPRESSORIUM DEVELOPMENT IN *Magnaporthe oryzae*

Introduction

Our lab and collaborator have found that *bip1* encodes a novel transcription factor with a leucine zipper (bZIP) domain binding with DNA strands (Tag unpublished). The insertional interruption of *bip1* (REMI) causes reduced plant infection on both barley and rice leaves. The complete deletion of *bip1* (Δ*bip1*) results in non-pathogenicity and the failure to form penetration pegs on leaf surfaces (Tag unpublished). However, why BIP1 is required for formation of penetration pegs is not clear.

When microarray analysis was used to compare the transcriptomes of the Δ*bip1* mutant and the wild-type, 44 down-regulated genes were identified in the Δ*bip1* mutant, including 4 genes encoding G-protein-coupled-receptor-like proteins (Tag unpublished). One of the four GPCR-encoding genes, *plg1* (*pth11*-like gene 1) is crucial for appressorium differentiation and melanization induced by hydrophobicity (Castillo 2015). The appressorium formation of the Δ*plg1* mutant can be restored by cAMP or DAG on hydrophobic surfaces, but the plant pathogenicity can only be restored by DAG, indicating that DAG works downstream of PLG1 (Castillo 2015). Since BIP1 regulates PLG1 expression, it is not clear yet that if BIP1 regulation is related to the cAMP or DAG

signaling pathways. This chapter further investigates the function of BIP1 and its functional relationship with PLG1 and its signaling pathway based on previous research.

Material and Methods

Fungal Strains and Media Culture

The wild-type strain used was *Magnaporthe oryzae* strain 70-15, which was obtained from the Fungal Genetics Stock Center (Kansas City, Missouri). It was grown on TNKYE plates with 1% glucose, 0.2% $NaNO_3$, 2% KH_2PO_4, 1% $MgSO_4$, 1% $CaCl_2$, 0.1% $FeSO_4$, 0.1% micronutrients, 0.2% yeast extract and 2% agar, and incubated under fluorescent light at 25°C (Castillo 2015). The strain was stored on desiccated and sterile filter discs at –20°C.

Bioinformatics

All nucleotide sequences were obtained from the *Magnaporthe oryzae* database at the Broad Institute (http:/www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html). The accession numbers for the sequences are listed in Table 1.

Gene	Identifier
bip1	MGG_08118
plg1	MGG_03584
plg2	MGG_06535
plg3	MGG_02160
plg4	MGG_11116
pth11	MGG_05871

Table 1 Gene identifiers from the Broad Institute website.

Split Marker Assay for the bip1 *Gene Deletion*

The *bip1* gene was deleted in *M. oryzae* using the split marker recombination protocol (Catlett et al. 2003). A sequence upstream of *bip1* was amplified by PCR and fused to one-half of the hygromycin B resistance gene (*hyg*). A downstream flanking sequence of *bip1* was amplified and fused to the other half of *hyg*. There was overlapping sequence between the two halves of *hyg* sequence (Figure 3A). The two fragments were then used for protoplast transformation.

Generation and Transformation of Fungal Protoplasts

A two-week-old wild-type 70-15 fungal culture was cut into cubes and homogenized. The fungus was then cultured in a 250 mL flask with liquid TNKYE medium with shaking overnight at 25°C. Mycelia were collected by filtering through a sterile Miracloth and resuspended in 50 mL 1M sorbitol solution containing 1-2 mg/mL NOVOZYM (lysing enzyme) and incubated at 30-32°C for 2h or less with shaking at 60 rpm. The condition of protoplasts was checked under a light microscope every 15 minutes. When clear protoplast cells were observed, the digestion was stopped. All protoplasts were collected by filtering through Miracloth and rinsed with 50 mL 1M sorbitol. Protoplasts were then pelleted by centrifugation at 4500 rpm at 10°C for 6 minutes. The supernatant was removed, and the rinse process was repeated one more time. Protoplasts were finally resuspended in 1X STC at a concentration of ~5 x 10^7 to 1 x10^8/mL for transformation.

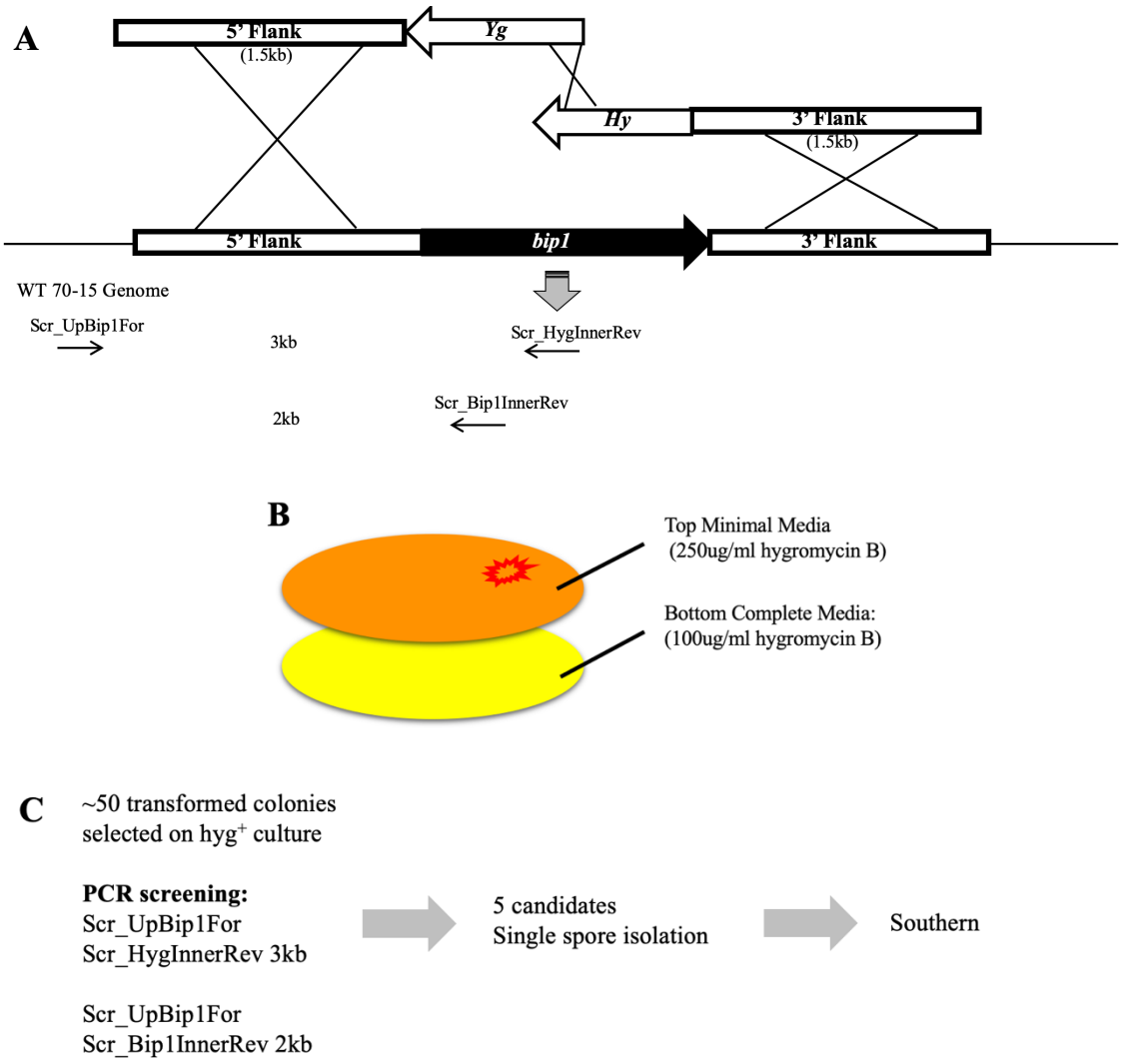


Figure 3 Generation of *bip1* KO in *Magnaporthe oryzae*.

A. Fragment design for deleting the *bip1* gene using split marker assay.
B. Screening *bip1* mutant strains on hygromycin selective plates.
C. Screening *bip1* mutant strains using PCR.

Two point five micrograms of the two PCR fragments generated for the split marker recombination protocol were transformed together into 300 µL of WT 70-15 protoplasts in a 15 mL Falcon tube and incubated at room temperature for 10 minutes. A solution of PEG 3550 buffer (dissolved in 25mM $CaCl_2$, 25mM Tris-HCl, pH 7.5) was added and incubated for another 20 min. Then complete medium (0.5% sucrose, 0.6%

yeast extract, 0.6% casein hydrolysate) was added. And the mixture was incubated overnight in a shaker at 25°C overnight.

Transformed protoplasts were plated in complete medium containing 100 ug/mL hygromycin B and 2% agar. After solidification, a layer of minimal medium (1% sucrose, 0.1% $Ca(NO_3)_2$, 0.02% KH_2PO_4, 0.025% $MgSO_4$, 0.015% NaCl) containing 250 ug/mL hygromycin B and 1.5% agar was poured on the top to select *hyg*$^+$ mutants (Figure 3B). During the first week of incubation, mutant candidates growing out of the top layer were picked individually onto TNKYE plates containing 250 ug/mL hygromycin B.

Genomic DNA was extracted from each mutant candidate for PCR screening using two primer pairs (Figure 3C). The forward primer for both PCR reactions was complementary to sequences upstream of *bip1*. The reverse primer was complementary to sequences either inside *bip1* or inside *hyg*. PCR results were examined by gel electrophoresis. The presence of a 3kb amplified band and the absence of a 2kb band indicates the *bip1* gene was deleted and replaced by the *hyg* gene. Single spore isolation for pure colonies was then performed for the putative deletion strains. Three isolates were picked from each mutant and screened by PCR again before Southern blot analysis.

Southern Blot Analysis

Isolates of mutant strains were examined by Southern blot analysis to see if they contained a single copy insertion of *hyg*. Genomic DNA from 70-15 wild-type and mutant strains was isolated from fungal hyphae using phenol-chloroform extraction as described by Sweigard et al. (1990). Twenty micrograms of genomic DNA from each strain were

digested by *Hind*III. All digested DNA samples were run on gel electrophoresis and blotted onto Hybond-N^+ membranes. The membranes were then hybridized to ^{32}P-radioactively labeled the probes for *bip1* or *hyg*. The membrane was exposed to a phosphor imaging screen in dark boxes overnight at room temperature. The films were then scanned using a Typhoon 9410 Variable Mode Imager.

Examination of Pathogenic Phenotypes

Three barley seeds were planted in each pot containing Redi-gro soil and grown for 3-4 weeks (15°C, 60% humidity). Barley leaves were cut into 2.5 inch pieces and placed in kinetin plates. Δ*bip1* and wild-type spores were collected after 15 days of incubation and concentrated to $1x10^4$/mL in 0.4% gelatin solution. Twenty microliters of spore suspensions were placed on barley leaf surfaces. All the plates were sealed with parafilm and incubated at 25°C. Lesions were evaluated by taking pictures after 7 days.

The infected leaves were processed to remove pigments to allow for microscopic examination of plant penetration. The infected leaves were transferred into a 2 mL microfuge tube and fixed with 1 mL lactophenol (1:1:1:1 volume ratio of lactic acid, glycerol, phenol and water) for 16h. After the liquid was removed, lactophenol and 95% ethanol were mixed at 1:1 v:v ratio and added. Tubes were incubated at 95°C for 1h to decolorize the leaves. The decolorization was repeated twice. Liquid was removed, and cotton blue (0.01% aniline blue in a 1:1:1 volume ratio of ethanol, lactic acid and phenol) was added to stain fungal hyphae. Tubes were incubated at 25°C for at least 24h (Oh and Lee 2000) before examining with light microscopy.

Δ*bip1* and WT 70-15 spores were collected in 0.4% gelatin solution after 15 days incubation and concentrated to $1x10^5$/mL. A pot of 3-4 weeks three barley seedings was placed in a bio-hazard plastic bag. Five milliliters of spore suspensions were sprayed by atomizer head onto all seedlings in each bag. Bags were sealed by rubber bands and incubated at 25°C for 7 days.

Δ*bip1* and WT 70-15 spores were collected in water solution after 15 days incubation and concentrated to $1x10^4$/mL. Twenty microliter of each suspension was placed on Teflon membranes and incubated in humidity chambers at 25°C for 12h and 24h. Appressoria were counted under a microscope.

Cytorrhysis Assay

Conidia were collected from 15-day old TNKYE cultures of *M. oryzae* WT 70-15 and the Δ*bip1* strains and concentrated to $1x10^4$/mL. Twenty microliters of conidia droplets were placed on Teflon membranes in humidity chambers and incubated at 25°C for 24 h. Then the liquid droplets were removed with a micropipettor, and replaced by 20 μL of 1 M glycerol, 3 M glycerol or 5 M glycerol to resuspend spores and appressoria (de Jong et al. 1997). At least 200 total appressoria including collapsed and uncollapsed ones were examined. The percentages of collapsed appressoria were determined.

Appressorium Development and Plant Infection with cAMP Treatment

Δ*bip1* and WT 70-15 spores were collected in water after 15 days incubation and concentrated to $1x10^4$/mL in water or 0.4% gelatin solution. Twenty microliters of spore

suspensions treated with or without 10 mM cAMP were inoculated on 3-4 week old barley leaves in kinetin plates. All plates were sealed with parafilm and incubated at 25°C. Lesions were evaluated by taking pictures after 7 days. The infected leaves were processed to remove pigments as described previously to allow for microscopic examination of plant penetration.

Different aliquots of Δ*bip1* and WT 70-15 spore suspensions had cAMP added at 0, 10, 20 or 50mM. Twenty microliters of spore suspensions were placed on Gelbond membranes (Lonza Pharma & Biotech) and incubated in humidity chambers at 25°C. After 24h, the membranes were photographed and the numbers of melanized, abnormal, unmelanized appressoria and total spores were counted and classified. Twenty microliters of spore suspensions were placed on barley leaf surfaces.

Quantitative RT-PCR

Spores of WT 70-15 were incubated on Teflon membranes and collected at 0hpi, 4hpi, 6hpi, 12hpi, 15hpi, 24hpi and 36hpi. Total RNA was extracted from all time points as described (Castillo 2015). The cDNAs of *ef1α*, *bip1*, *pth11*, *plg1*, *plg2* and *plg3* were synthesized individually from 1ug extracted mRNA. The reagents of cDNA synthesis were from TaqMan reverse transcription kit (Life Technologies). Quantitative RT-PCR was done using SYBR Green real-time PCR Master Mix (Life Technologies). The Applied Biosystems 7600 Real-Time PCR System was used to analyze the process of RT-PCR. The results for *ef1α* were used as the control for normalization of other genes. Then results from other time points were normalized relative to the 0hpi sample.

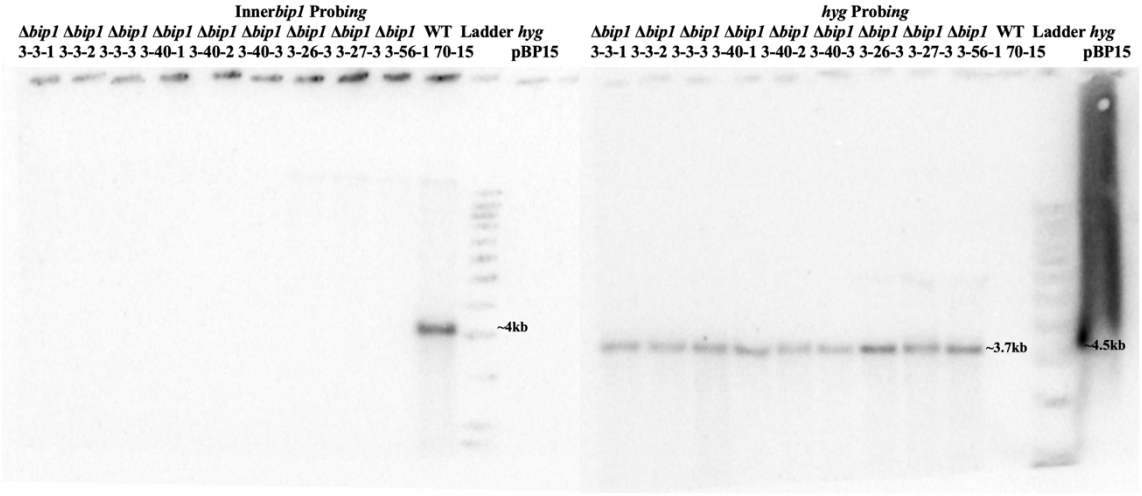


Figure 4 Southern blot result of *bip1* mutant strains.

Results

Generation of bip1 *Mutant Strains*

Five individual Δ*bip1* mutants were constructed as described in Material and Methods shown in Figure 3. Mutants were finally confirmed by Southern blot analysis. The expected size of the genomic fragment detected by probe Inner*bip1* was 4kb, and the fragment detected by probe *hyg* was 3.7kb (Figure 4). According to the Southern blot results (Figure 4), all tested isolates from five Δ*bip1* mutants had a deletion of *bip1* and insertion of *hyg*. Two independent mutants, 3-3-1 and 3-40-1, were selected for further study.

Pathogenic Phenotypes of bip1 *Mutant Strains*

The pathogenicity of Δ*bip1* mutant strains on barley was tested by placing droplets of suspended spores on detached leaves or spraying barley seedlings with the spore

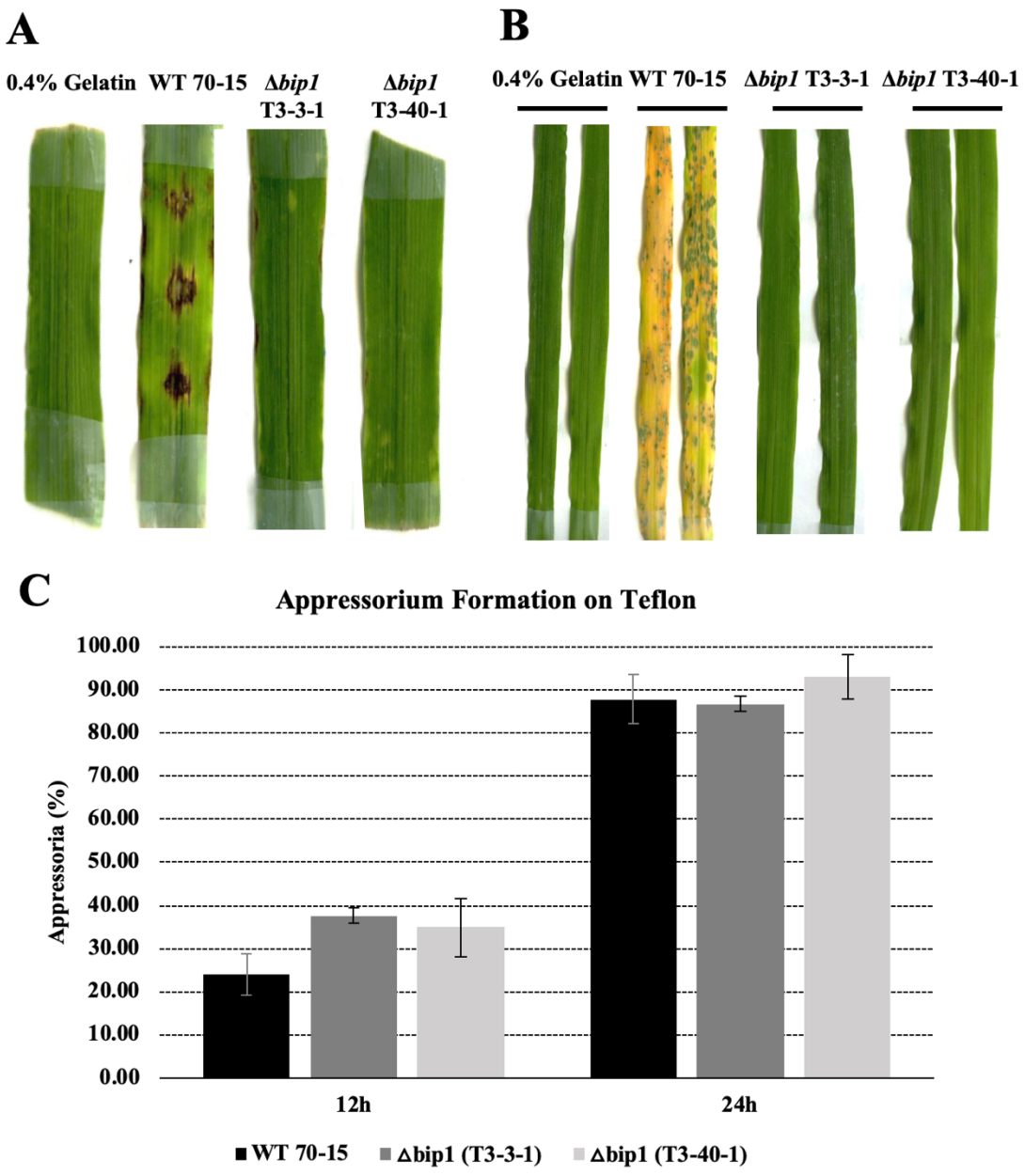


Figure 5 The phenotypes of *bip1* mutant strains.

A. Droplet inoculation of barley leaves with *Magnaporthe oryzae* spores.
B. Spray inoculation of barley seedlings with *Magnaporthe oryzae* spores.
C. Appressorium formation on Teflon membranes .

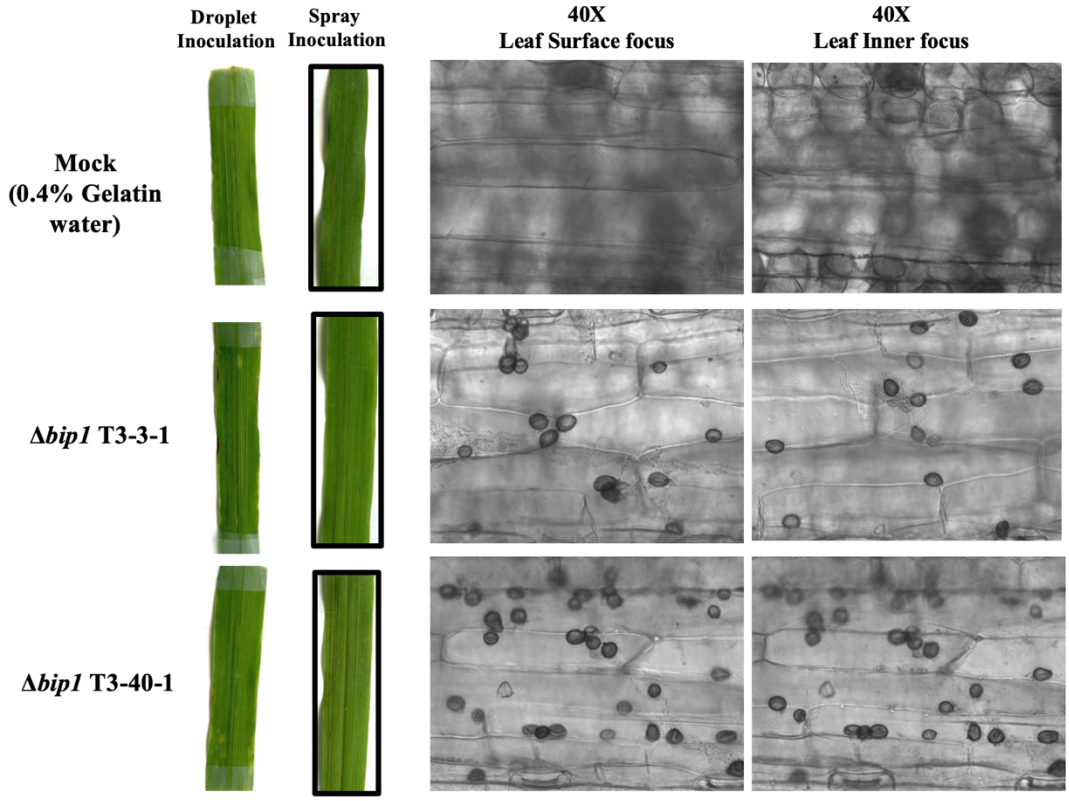


Figure 6 Appressorial penetration of bip1 mutant strains on barley leaves.

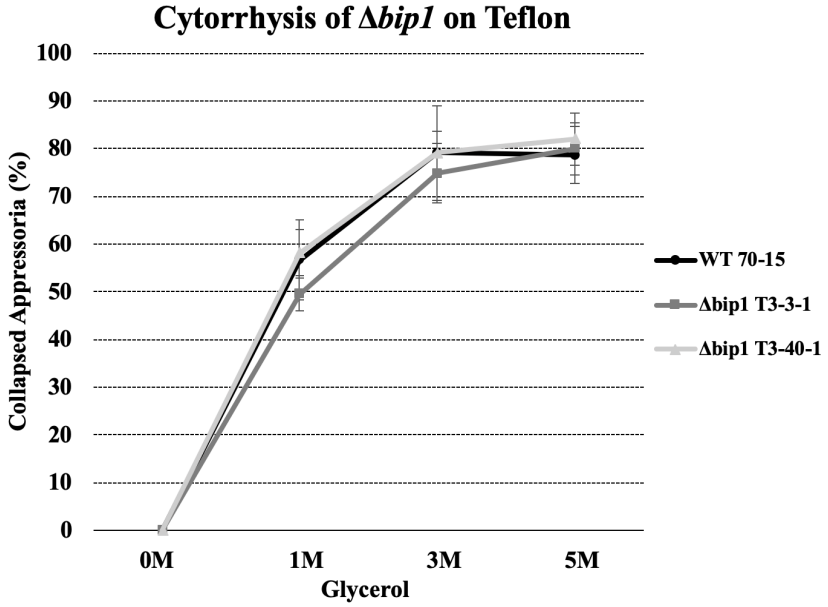


Figure 7 Cytorrhysis of *bip1* mutant strains on Teflon membranes.

suspension. The Δ*bip1* strains did not infect barley leaves or seedlings, while WT 70-15 formed brown lesions (Figure 5A and B). To determine if the Δ*bip1* mutants could form appressoria, WT 70-15 and Δ*bip1* spores were inoculated on Teflon membranes. The Δ*bip1* mutants formed similar amounts of appressoria as WT 70-15 (Figure 5C), indicating that the failure of Δ*bip1* infection may be due to the inability to penetrate the plant.

The ability of plant penetration was then examined under microscope, showing that Δ*bip1* mutants form melanized appressoria, but did not penetrate into the barley leaves (Figure 6). A possible cause of failed plant penetration is low turgor pressure inside the appressoria. However, the results of the cytorrhysis assay show that Δ*bip1* mutants had similar percentages of collapsed appressoria compared to WT 70-15 in 1M, 3M and 5M glycerol solution (Figure 7). This result indicated that Δ*bip1* mutants had the normal turgor pressure required for plant penetration. The nonpathogenic phenotype of Δ*bip1* mutants on barley may be due to other reasons, such as failure to form pore rings at the bottom of appressoria or the inability to overcome the immune system of plants.

Appressorium Development on Hydrophilic Surfaces with cAMP Treatment

To determine whether Δ*bip1* mutation affects cAMP-dependent signaling pathway in appressorium development, WT 70-15 and Δ*bip1* spores were treated with different amounts of cAMP and inoculated on Gelbond membranes. Appressorium development was evaluated and the results are shown in Figure 8. WT 70-15 and Δ*bip1* spores have similar levels of appressorium formation with the same cAMP treatment. This implies that BIP1 is involved in other signaling pathways important to appressorium development.

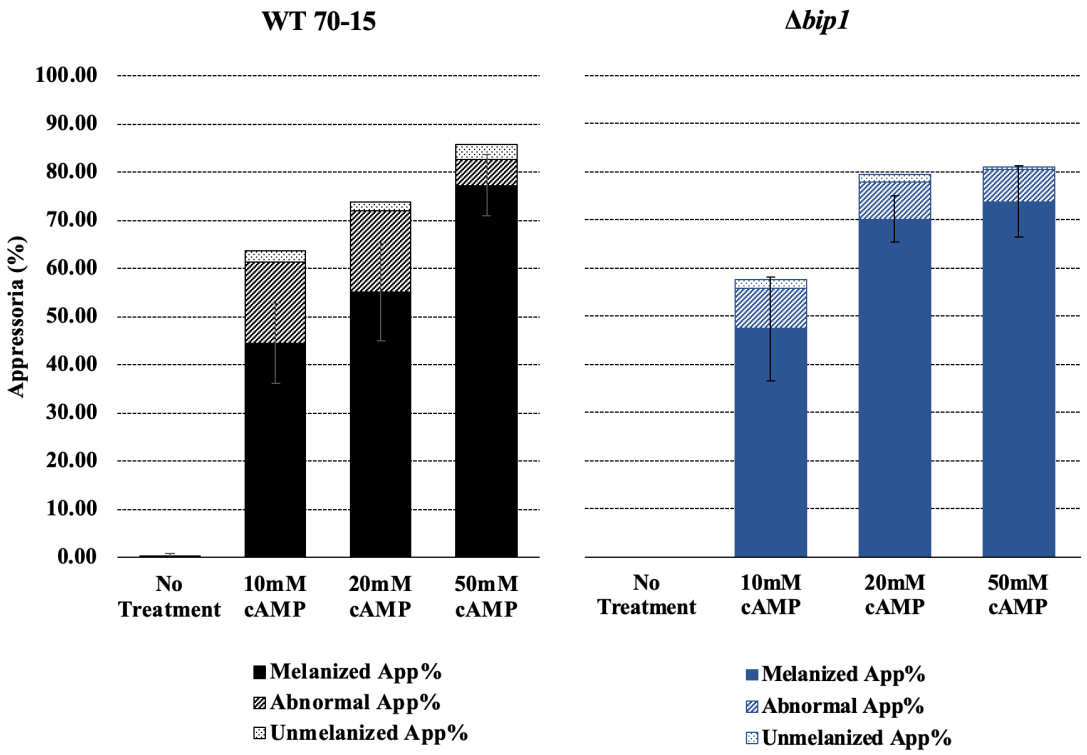


Figure 8 Development of appressoria on hydrophilic surfaces with cAMP treatments.

Pathogenicity on Barley with cAMP Treatment

To investigate whether cAMP treatment restores pathogenicity of the Δ*bip1* mutant, a suspension of Δ*bip1* spores was sprayed on barley leaves in either the presence or absence of 10mM cAMP. As a control, WT 70-15 spores with and without 10mM cAMP were also inoculated on barley leaves. The pathogenicity of Δ*bip1* on barley leaves was not restored by the addition of cAMP (Figure 9A). Microscopic examination of plant penetration showed that exogenous cAMP failed to restore penetration of Δ*bip1* mutants on barley leaves (Figure 9B).

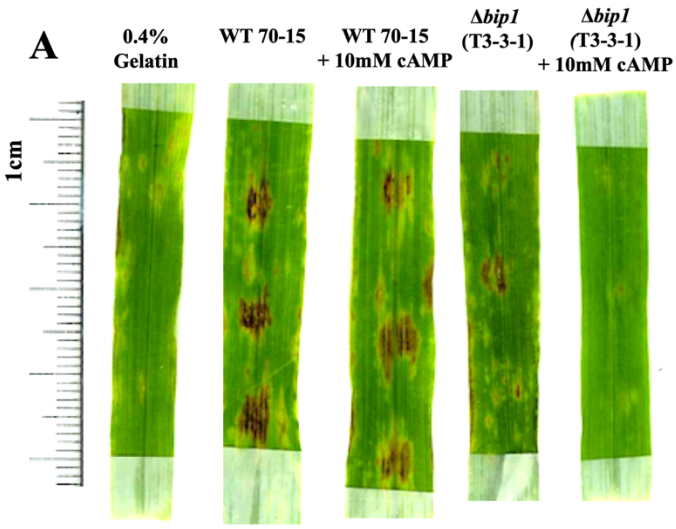
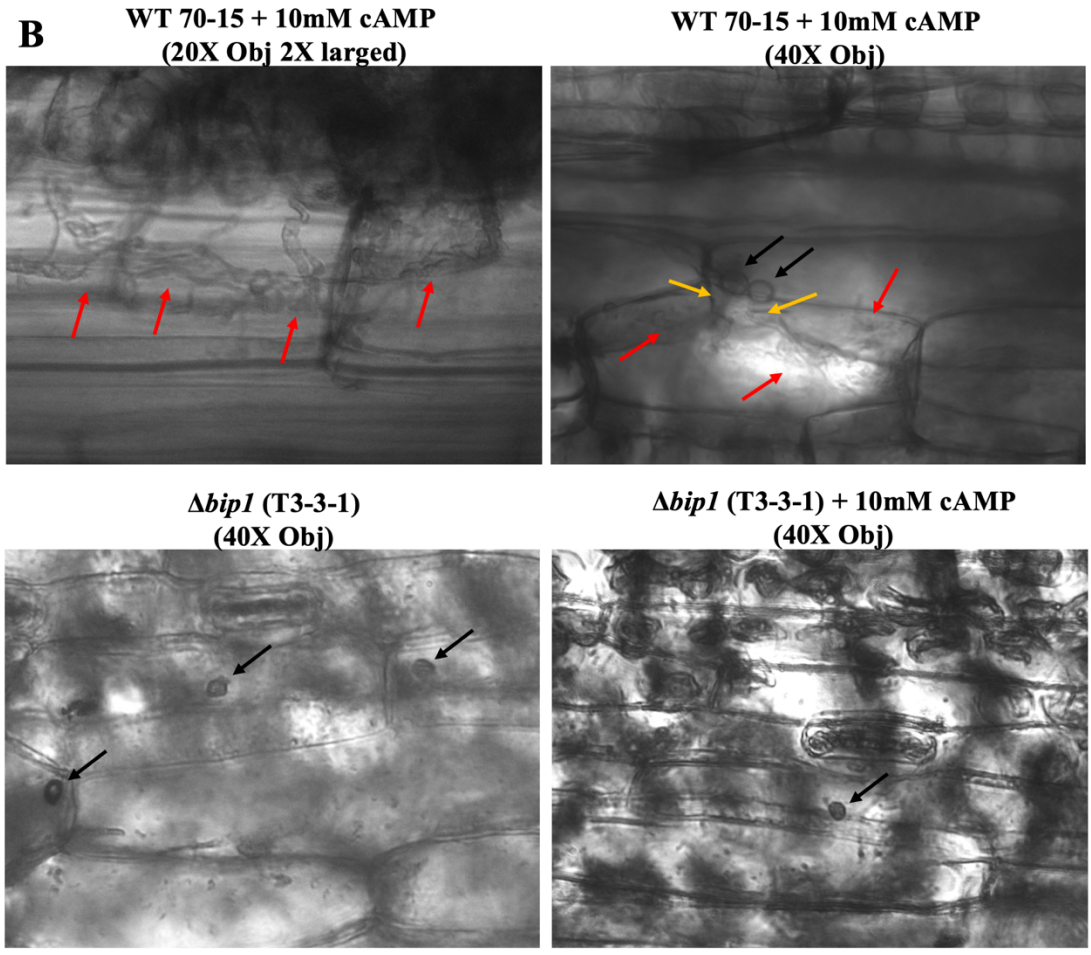


Figure 9 Infection on barley leaves with cAMP treatment.

A. Droplet inoculation of WT 70-15 and Δ*bip1* mutant strain on barley leaves with and without cAMP treatment.

B. Microscopic views showing appressorial penetration in the lesions shown in A. (Black arrows: melanized appressoria. Red arrows: invasive hyphae in plant cells. Yellow arrows: possible penetration pegs.)



Transcription of plg *Genes in Magnaporthe oryzae*

We looked at transcript levels of *plg1*, *plg2*, *plg3, bip1* and *pth11* during appressorium development on an artificial hydrophobic surface (Teflon membrane) of the WT 70-15 strain of *M. oryzae* by using RT-PCR (Figure 10A). Transcript levels of *bip1*, *pth11* and *plg3* increased (0hpi to 4hpi) during the early stage of appressorium development, then decreased (4hpi to15hpi) and increased again (15hpi to 36hpi) during later stages of appressorium development. The transcript level of *plg1* first increased (0hpi to 4hpi) and then decreased (4hpi to 6hpi) during the early stage of appressorium development, and started to increase again (6hpi to 36hpi) during later stages of appressorium development. The transcript level of *plg2* first decreased (0hpi-4hpi) and then increased (4hpi to 6hpi) during the early stage of appressorium development, and decreased (6hpi to 15hpi) and then increased again (15hpi to 36hpi) during later stages of appressorium development.

Transcript levels of all genes except *plg2* followed a similar pattern of changes where transcript levels at first increased, then decreased, and then increased again. The transcripts of *plg2* increased later than the other genes during the early stage of appressorium development, and decreased later than the other genes during later stages of appressorium development. The transcripts of *plg1* decreased earlier than all the other genes during the early stage of appressorium development, and increased earlier than all the other genes during later stages of appressorium development. These results indicate that all the genes tested may be important to appressorium development, and that PLG1

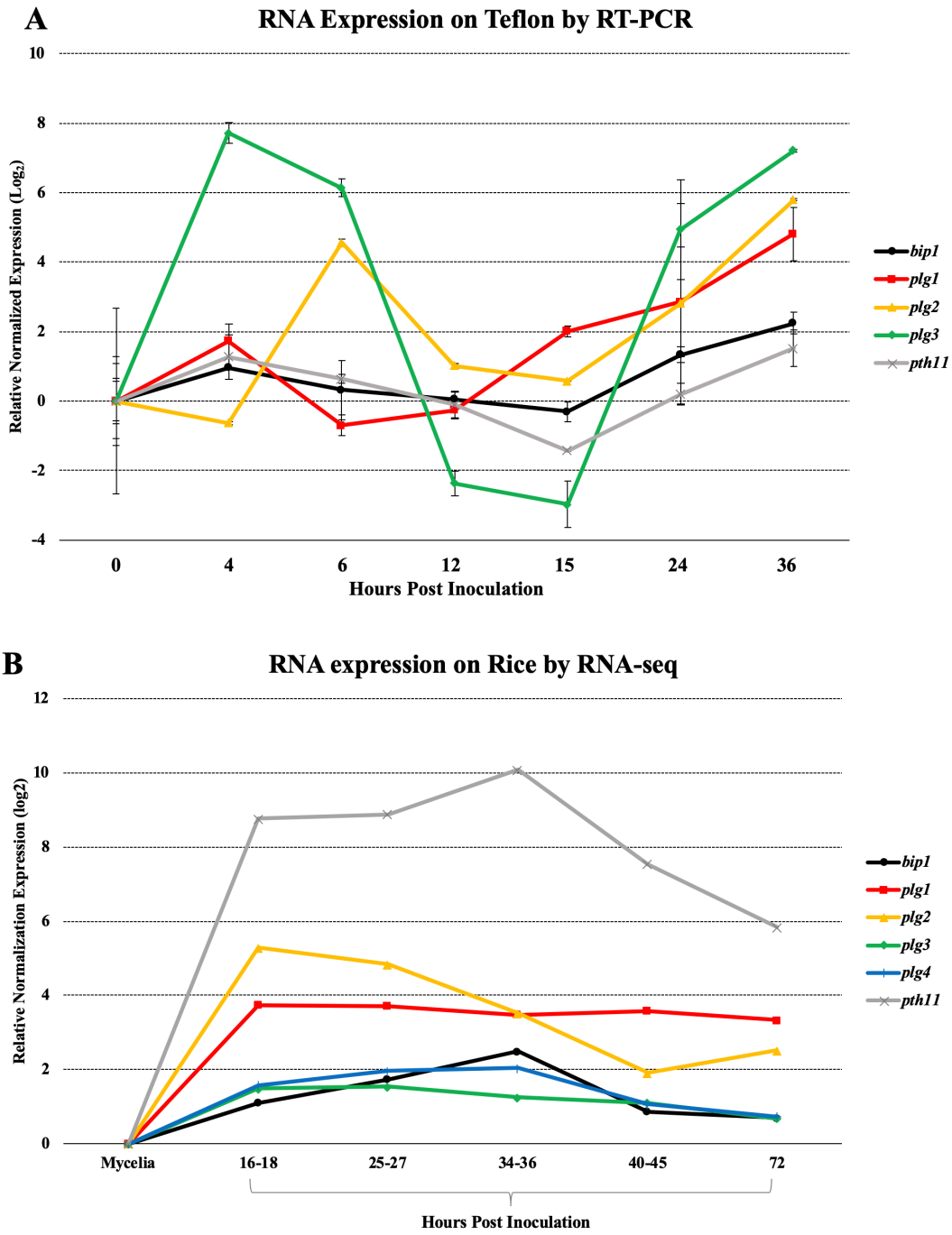


Figure 10 RNA expression levels in *Magnaporthe oryzae* during infection.

A. RNA expression during appressorium development on Teflon membranes analyzed by RT-PCR. The data were normalized first to *ef1α* and then to the 0h sample.

B. RNA expression during infection of rice plants analyzed by RNA-seq. The data were normalized first to *tubA* and then to the mycelial sample.

may regulate signaling pathways upstream of the other genes, while PLG2 may be regulated by signaling pathways downstream of the other genes.

To compare these results with transcriptome analysis during infection of plants, the sequences of the open reading frame for all four *plg* genes, as well as for *bip1* and *pth11* were used as queries in a BLAST-based search of the *M. oryzae* RNA-seq database at NCBI (SRX5076910-5076916, https://www.ncbi.nlm.nih.gov/sra). The query was performed by Dr. Daniel Ebbole using a script he had written. The data were normalized first to *tubA* and then to the mycelial sample. Relative RNA expression of each gene at different stages of development are shown in Figure 10B.

All genes examined had higher transcript levels during pathogenic stages (16-18hpi to 72hpi) than during vegetative growth (mycelia). Transcript levels of *plg1*, *plg2* and *plg3* were highest during appressorium development (16-18hpi), while transcript levels of *bip1* and *plg4* were highest during invasive growth (34-36hpi). The transcript level of *pth11* increased significantly during both appressorium development (16-18hpi) and invasive growth (34-36hpi). These results indicate that all the genes may be important for fungal pathogenicity: *plg1*, *plg2* and *plg3* may be more important for appressorium development than for other pathogenic stages; *bip1* and *plg4* may be more important for invasive growth in plants than for other pathogenic stages; *pth11* may be important for both appressorium development and invasive growth.

The RT-PCR results for all genes tested (Figure 10A) during appressorium development (0hpi to 15hpi) were consistent with their RNA-seq results in the NCBI SRA database (Figure 10B). All of their transcript levels increased during appressorium

development compared to vegetative stages, indicating that all of the genes are important to appressorium development. However, the RT-PCR results for *plg1*, *plg2* and *plg3* after most appressoria had developed (15hpi to 36hpi) were different from their RNA-seq results (16-18hpi to 34-36hpi), while the two results for *bip1* and *pth11* were consistent. This may be because the RNA used for RT-PCR were extracted from spores inoculated on artificial hydrophobic membranes where spores developed melanized appressoria, but no penetration pegs or invasive hyphae. The RNAs for RNA-seq were from spores inoculated on rice where spores developed melanized appressoria as well as penetration pegs, and continued to grow invasively inside rice. The transcript levels of *plg1*, *plg2* and *plg3* decreased after 24hpi on rice plants, while transcript levels of these genes increased after 24hpi on artificial hydrophobic surfaces, indicating that the three *plg* genes are more related to appressorium development than to plant penetration or invasive growth.

Conclusion and Discussion

Δ*bip1* mutants were generated using the split marker assay and confirmed by Southern blot analysis. As expected, the mutant strains were nonpathogenic on barley leaves or seedlings. They still developed melanized appressoria but no penetration pegs. The turgor pressure of Δ*bip1* appressoria was the same as the wild-type, so this was not the cause of abnormal plant penetration. The addition of exogenous cAMP did not make any difference between the appressorium formation of Δ*bip1* and wild-type on hydrophilic surfaces, and it did not restore the plant pathogenicity of Δ*bip1*. This indicated that BIP1 may not be involved in any cAMP-dependent signaling pathway in appressorium

development. The examination of transcript levels by RT-PCR and RNA-Seq in the database of *M. oryzae* showed that *bip1* is more related to invasive growth, *plg* genes are more related to appressorium development, and *pth11* is related to both appressorium development and invasive growth.

CHAPTER III

GENERATING MUTANT STRAINS OF THE OTHER GPCR-LIKE-ENCODING GENES REGULATED BY BIP1 (PLG2, PLG3, AND PLG4)

Introduction

G-protein-coupled receptors (GPCR) usually work as membrane proteins that transduce signals from outside to inside the cell and induce developmental or metabolic signaling pathways (Dohlman and Thorner 2001). It has been reported that *Ascomycota* fungi have a large number of G-protein-coupled-receptor-like (GPCR-like) proteins in addition to the classical GPCR proteins (Kulkarni et al. 2005) (Table 2). GPCR-like proteins may play important roles in the development of *Ascomycota* fungi (Kulkarni et al. 2005). One of the best known GPCR-like proteins in *M. oryzae* is PTH11, which is found to play an important role in fungal pathogenicity. PTH11 is crucial for developing appressoria on plant leaf surfaces and related to the cAMP signaling pathway (DeZwaan et al. 1999). Four genes regulated by BIP1 are predicted to encode PTH11-like proteins. These genes are *plg1*, *plg2*, *plg3* and *plg4*. The importance of *plg1* to pathogenicity of *M. oryzae* has been investigated by constructing and characterizing a Δ*plg1* deletion mutant

Classes of GPCR-like Proteins in *Magnaporthe oryzae*	**Numbers**
GPCR homologs of known classes (STE2-like, STE3-like, cAMP receptor-like)	8
Other GPCR-like homologs (GPR1-like, STM1-like, mPR-like, MG00532-like)	7
PTH11-related proteins (restricted to the subphylum Pezizomycotina of *Ascomycota*)	61

Table 2 Classes of GPCR-like proteins in *Magnaporthe oryzae*.

(Castillo 2015). The effects of deleting *plg2*, *plg3*, or *plg4* on *M. oryzae* pathogenicity need to be investigated. This chapter describes the construction of mutant strains of the three other *plg* genes (*plg2*, *plg3*, and *plg4*).

Material and Methods

Fungal Strains and Media Culture

Fungal strains were cultured and stored as described in Chapter II, pg. 13. All mutant strains with the resistant gene *hyg* were cultured on TNKYE agar plates supplemented with 250 μg/mL hygromycin B (PhytoTechnology Laboratories). All mutant strains were stored using the same method as the wild-type strains.

Bioinformatics

All DNA and amino acid sequences were obtained from the *Magnaporthe oryzae* database at the Broad Institute (http:/www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html) (Table 1, Chapter II). The prediction of membrane spanning domains in proteins encoded by *plg* genes was done by using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM) (Krogh et al., 2001).

Generation of plg *Mutant Strains*

All *plg* mutants were generated by deleting the target gene individually using split marker assay as described in Chapter II, pg. 14 (Figure 11). Generation of fungal protoplasts and their transformation was done as described in Chapter II, pg. 14-16. WT

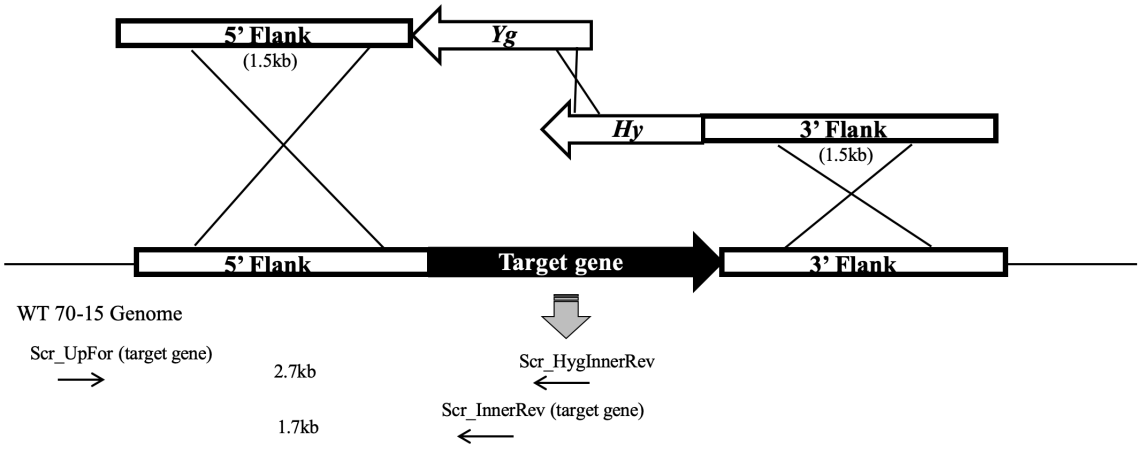


Figure 11 Generation of *plg* mutant strains using split marker assay.

70-15 was used to generate the Δ*plg2* and Δ*plg4* mutant strains. KU80 was used to generate the Δ*plg3* mutant strains. Southern blot analysis was done as described in Chapter II, pg. 16-17.

Results

The Predicted Transmembrane Structures of PLG Proteins

TMHMM 2.0 (Krogh et al., 2001) was used to predict transmembrane domains of the four GPCR-like proteins PLG1, PLG2, PLG3 and PLG4. As shown in Figure 11, they are all predicted to have transmembrane structures. PLG1, PLG2 and PLG4 are predicted to have seven transmembrane domains, similar to PTH11 (DeZwaan et al. 1999; Kulkarni et al. 2005). While PLG3 is predicted to have four transmembrane domains. The predicted membrane topology of PLG proteins is consistent with their predicted function as signal transduction proteins.

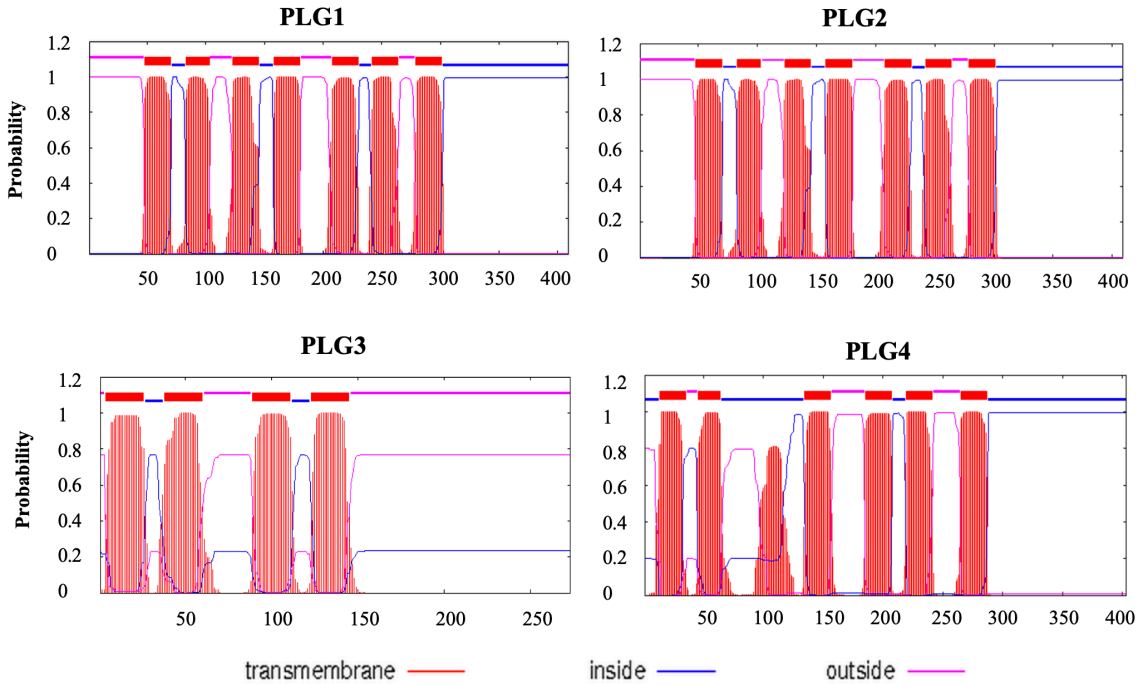


Figure 12 Predicted protein structures of *plg* genes by TMHMM 2.0.

Generation of plg *Mutant Strains*

The *plg2*, *plg3* and *plg4* genes were deleted and replaced with the hygromycin B resistant gene (*hyg*) as described in Material and Methods (Figure 11). The DNA fragments of *plg2, plg3* and *plg4* generated by split marker assay were transformed into WT 70-15 protoplasts individually. However, transformants of Δ*plg3* could not be isolated. The DNA fragments of *plg3* were then transformed into KU80 protoplasts. KU80 is a mutant displaying wild-type phenotypes, but has higher homologous recombination efficiency due to inactivation of the non-homologous-end-joining system (Villalba et al. 2008).

Confirmation of plg *Gene Deletion by Southern Blot*

Two single spore isolates from each individual Δ*plg2* mutants were performed Southern blot analysis. Genomic DNA from WT 70-15 and Δ*plg2* mutants was extracted and digested by *Hind*III. The genomic fragment detected by probe Inner*plg2* or probe *hyg* was 9kb (Figure 13). All tested isolates from Δ*plg2* mutants had a deletion of *plg2* and insertion of *hyg*. Two independent mutants, 1-1 and 2-1, were selected for further study.

Two single spore isolates from each individual *plg3* mutants were performed Southern blot analysis. Genomic DNA from WT 70-15, KU80 and Δ*plg3* mutants was extracted and digested by *Hind*III. The genomic fragment detected by probe Inner*plg3* was 4.2kb, and the fragment detected by probe *hyg* was 3.2kb (Figure 14). The 6-4 and 6-5 isolates had a deletion of *plg3* and insertion of *hyg*. However, the 5-1 and 5-2 isolates

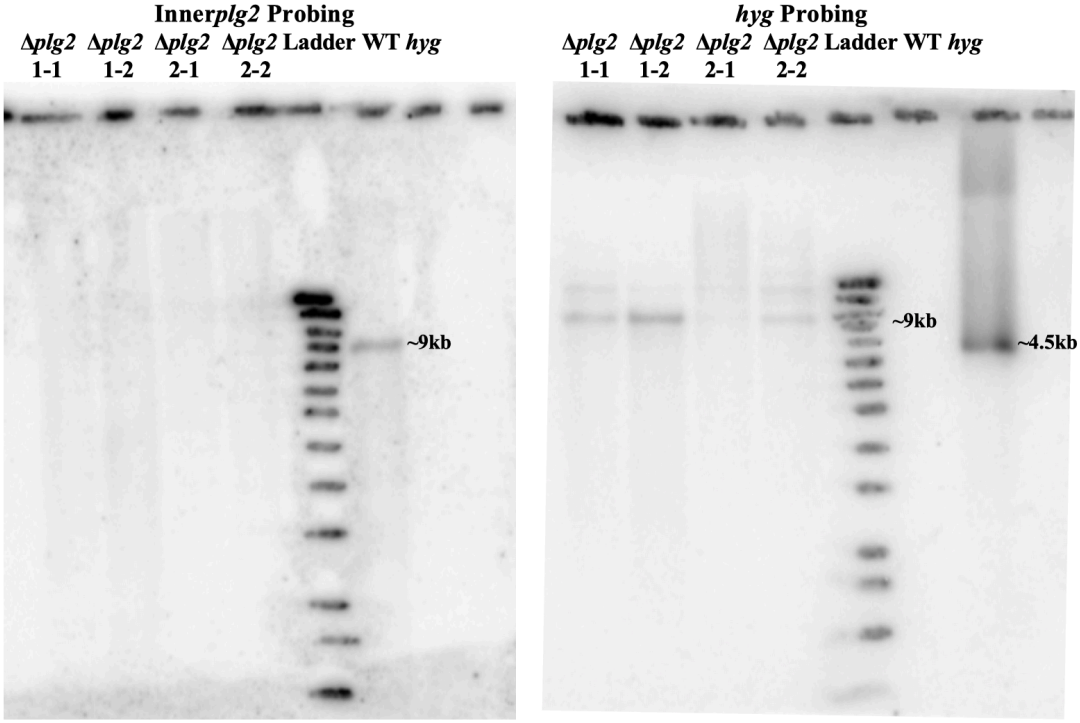


Figure 13 The Southern blot result of *plg2* mutant strains.

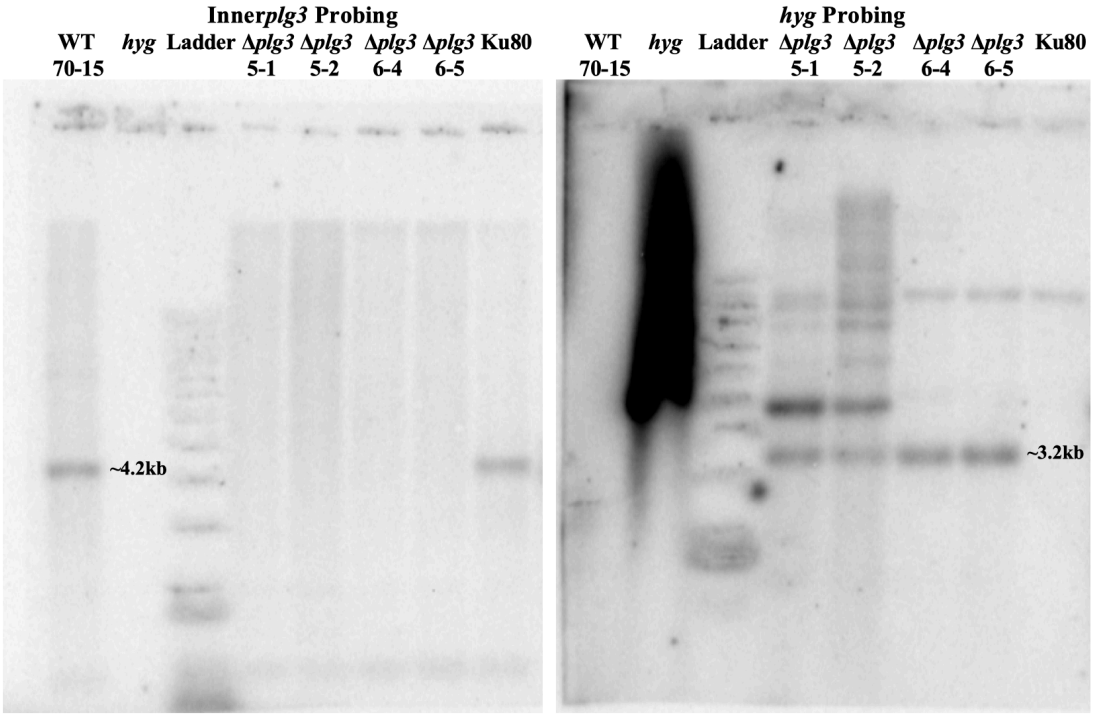


Figure 14 The Southern blot result of *plg3* mutant strains.

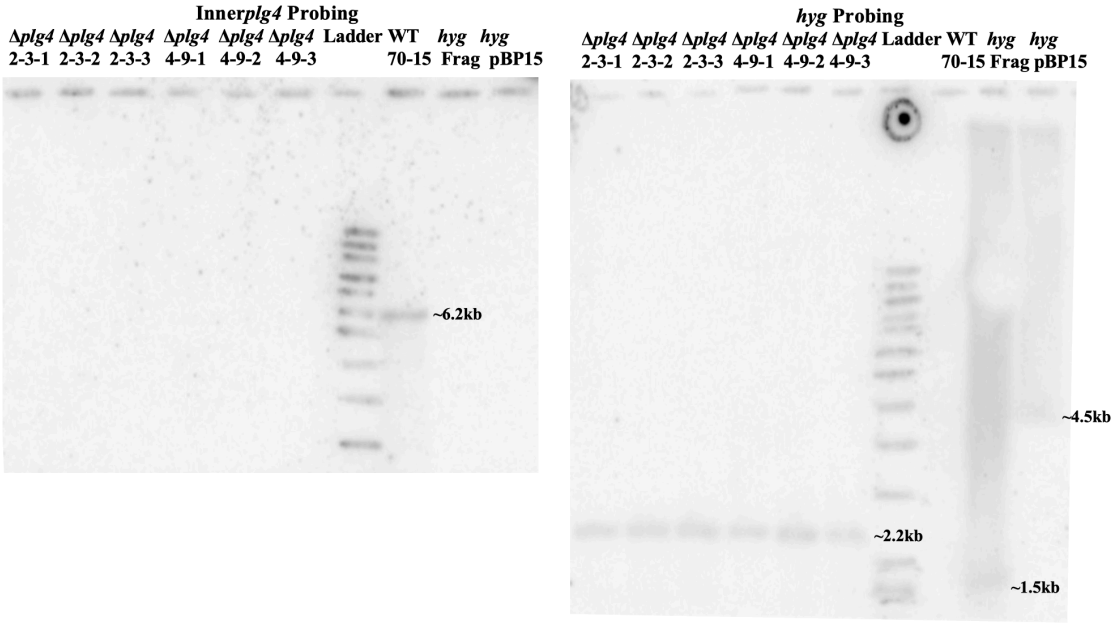


Figure 15 The Southern blot result of *plg4* mutant strains.

may have more than one copy of *hyg* beside a deletion of *plg3*. Two independent mutants, 5-1 and 6-4, were selected for further study.

Three single spore isolates from each individual *plg4* mutants were performed Southern blot analysis. Genomic DNA from WT 70-15 and Δ*plg4* was extracted and digested by *Sal*I. The genomic fragment detected by probe Inner*plg4* was 6.2kb, and the fragment detected by probe *hyg* was 2.2kb (Figure 15). All tested isolates from Δ*plg4* isolates had a deletion of *plg4* and insertion of *hyg*. Two independent mutants, 2-3-1 and 4-9-1, were selected for further study.

Conclusion and Discussion

In this chapter, *plg* genes were predicted to encode proteins with transmembrane structures. *plg* mutants were generated using split marker assay and confirmed by Southern blot. Each mutant has target gene replaced by the resistant gene *hyg*. One single spore isolate from each of two independent mutant strains was chosen for further study. The phenotypes of all *plg* mutants will be examined in the next chapter.

CHAPTER IV

THE FUNCTION OF THE FOUR GPCR-LIKE-ENCODING GENES REGULATED BY BIP1 (PLG1, PLG2, PLG3, AND PLG4)

Introduction

The four GPCR-like-encoding genes regulated by BIP are predicted to have transmembrane domains, consistent with their proposed roles of transducing signals from outside to inside in *M. oryzae*. A Δ*plg1* mutant of *M. oryzae* is defective in pathogenesis (Castillo 2015). It does not form any lesion on barley or rice and produces only 10% appressoria on hydrophobic surfaces as the wild-type strain. Appressorium formation by Δ*plg1* is restored by adding the cutin monomer 1,16-hexadecanediol, cAMP or DAG on Teflon membranes and barley leaves. However, cAMP does not restore virulence, indicating that DAG is downstream of PLG1 (Castillo 2015).

To investigate the roles of three other GPCR-like-encoding genes (*plg2*, *plg3*, and *plg4*), deletion mutant strains were generated (Chapter III). The effect of Δ*plg2*, Δ*plg3*, and Δ*plg4* mutations on fungal pathogenicity and appressorium development were investigated.

Material and Methods

Droplet Inoculation Assay

Spores of all mutant and wild-type strains were collected after 15 days incubation and resuspended in 0.4% gelatin at the concentration of $1x10^4$/mL. Three to four week old barley leaves were cut into 2.5 inches and placed in kinetin plates. Twenty microliter droplets of spore suspensions were placed on barley leaf surfaces. The experimental plates were sealed with parafilm and incubated at 25°C. Lesions were evaluated by taking pictures after 7 days. The infected leaves were then processed as described previously to remove pigments to allow examination of plant penetration by light microscopy.

Spray Inoculation Assay

Three barley seeds were planted in each pot containing Redi-gro soil and grown for 3-4 weeks (15°C, 60% humidity). Spores of all mutant and wild-type strains were collected after 15 days incubation, resuspended in 0.4% gelatin, and concentrated at $1x10^5$/mL. Each pot was placed in a bio-hazard plastic bag and sprayed by 5 mL spore suspension through atomizer heads. Bags were sealed by rubber bands and incubated at 15°C. Lesions were evaluated after 7 days.

Teflon Assay

Spores of all mutant and wild-type strains were collected in water after 15 days incubation and the concentration of each spore suspension was adjusted to $1x10^4$/mL. Twenty microliter droplets were placed on Teflon membranes and incubated in humidity

chambers at 25°C for 12h and 24h. The number of appressoria formed was counted under microscope.

Results

The Pathogenicity of plg *Mutant Strains*

Δ*plg2,* Δ*plg3* and Δ*plg4* mutant strains were generated to investigate their roles in fungal pathogenicity in *M. oryzae*. The previously generated Δ*plg1* strain (Castillo 2015) was also examined for comparison. Only Δ*plg1* and Δ*plg4-1* mutant strains failed to infect barley leaves, and Δ*plg4*-2 formed smaller lesions than WT 70-15 (Figure 16). Both independent mutant strains of Δ*plg2* (Δ*plg2*-1, Δ*plg2*-2) and Δ*plg3* (Δ*plg3*-1, Δ*plg3*-2) developed infective lesions on barley similar to those made by WT 70-15 or KU80 after 7 days of incubation (Figure 16).

Similar results were seen when barley seedlings were spray inoculated with suspensions of wild-type and mutant spores. The Δ*plg1* and Δ*plg4-1* mutant strains failed to infect barley seedlings, and Δ*plg4*-2 formed fewer lesions than WT 70-15 (Figure 17). Both independent mutant strains of Δ*plg2* (Δ*plg2*-1, Δ*plg2*-2) and Δ*plg3* (Δ*plg3*-1, Δ*plg3*-2) developed infective lesions on barley similar to those made by WT 70-15 or KU80 after 7 days of incubation (Figure 17).

Appressorium Development of plg *Mutant Strains*

The *plg* mutant spores were inoculated on a hydrophobic surface (Teflon membranes) to test for appressorium development. It was previously shown that spores of

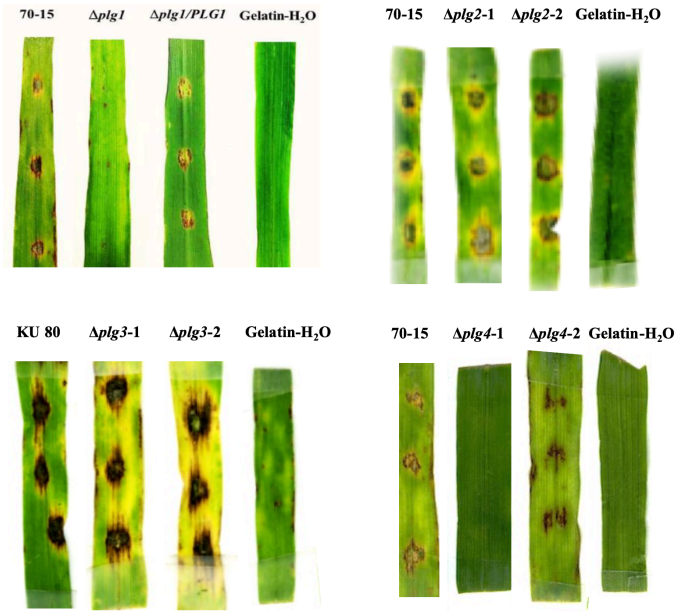


Figure 16 Droplet inoculation of barley leaves with *Magnaporthe oryzae* spores.

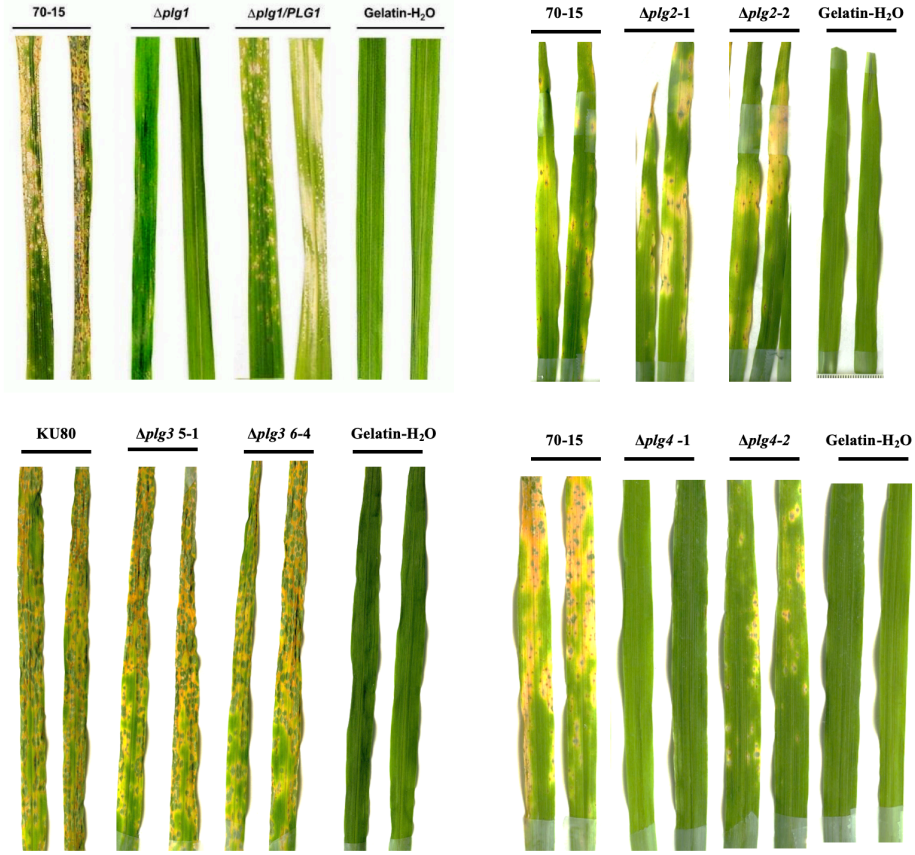


Figure 17 Spray inoculation of barley seedlings *Magnaporthe oryzae* spores.

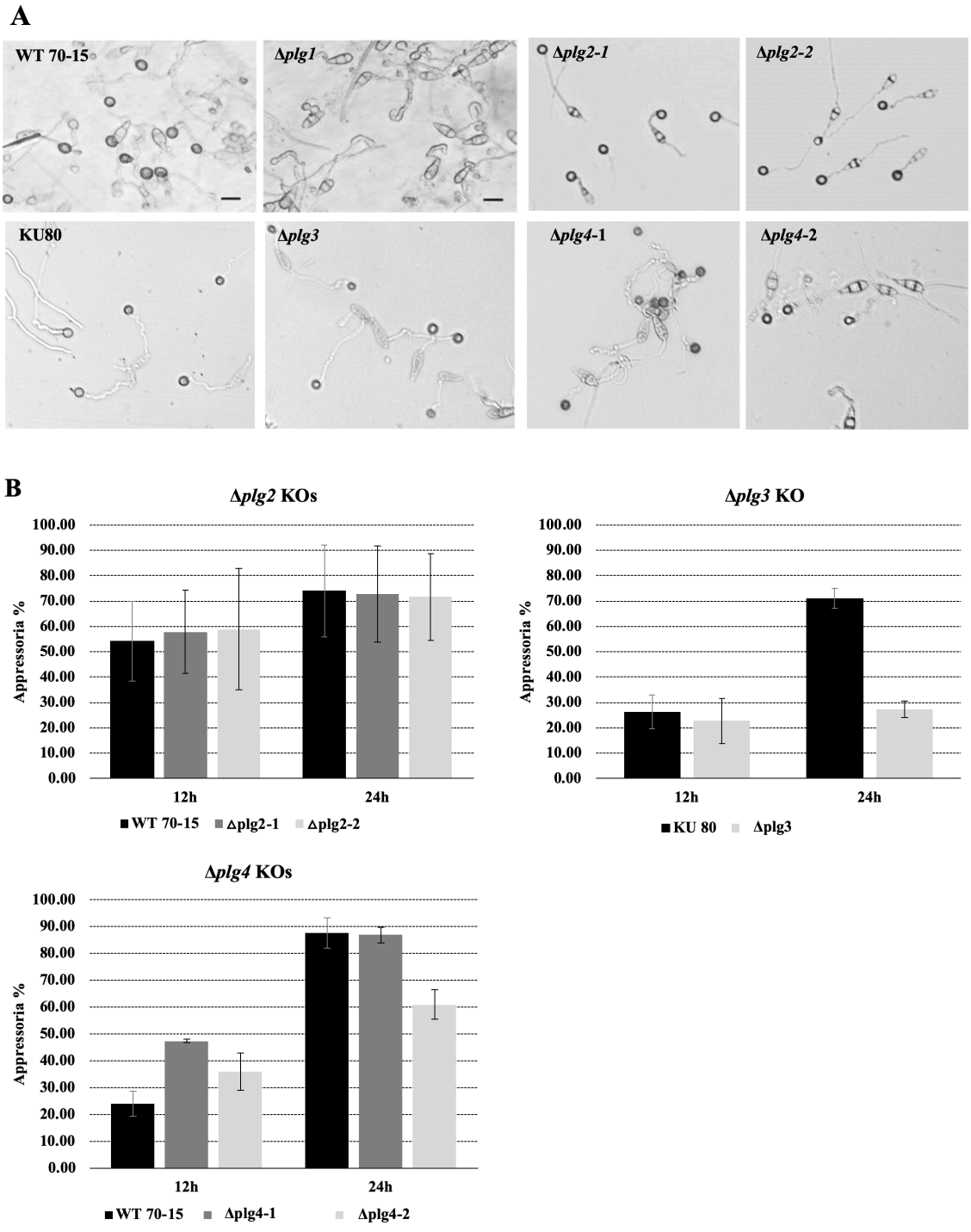


Figure 18 Appressorium development on Teflon membranes.

A. Micrographs of appressorium development on Teflon membranes.
B. Quantitation of appressorium formation on Teflon membranes.

the Δ*plg1* mutant strain formed many fewer appressoria after 24h compared to spores of the WT 70-15 (Castillo 2015). Spores of the Δ*plg2*, Δ*plg3*, and Δ*plg4* mutant strains all formed melanized appressoria (Figure 18A).

The Δ*plg1* mutant strain forms only 10% appressoria on Teflon membranes compared to WT 70-15 (Castillo 2015). The two independent mutants of Δ*plg2* (Δ*plg2*-1 and Δ*plg2*-2) developed appressoria on Teflon membranes at similar levels as WT 70-15 after 12h and 24h (Figure 18B). The Δ*plg3* mutant strain developed less appressoria than KU80 after 24h, but not as few as Δ*plg1* (Figure 18B). The two independent mutants of Δ*plg4* (Δ*plg4*-1 and Δ*pl4*-2) developed appressoria at similar levels as WT 70-15 after 12h (Figure 18B). However, Δ*plg4*-2 had slightly reduced appressorium formation at 24h compared to WT 70-15 and Δ*plg4*-1 (Figure 18B).

Conclusion and Discussion

The pathogenic phenotypes and appressorium development of *plg* mutant strains were examined. The Δ*plg1* mutant strain was nonpathogenic on barley and formed only 10% of spores formed appressoria on hydrophobic surfaces (Castillo 2015). The two independent Δ*plg2* mutant strains (Δ*plg2*-1 and Δ*plg2*-2) formed infective lesions on barley and appressoria at similar levels as WT 70-15. The two independent Δ*plg3* mutant strains (Δ*plg3*-1 and Δ*plg3*-2) formed infective lesions on barley at a similar level as KU80, but formed less appressoria on Teflon membranes than KU80 at 24h. The Δ*plg4*-1 mutant strain failed to infect barley, while Δ*plg4*-2 infected barley less seriously than WT 70-15. Δ*plg4*-1 formed appressoria on Teflon membranes at similar levels as WT 70-

15, while Δ*plg4*-2 formed fewer appressoria than WT 70-15 and Δ*plg4*-1 at 24h. The different phenotypes of the two independent Δ*plg4* mutant strains were probably caused by the process of homologous recombination during *plg4* gene deletion. The sequences upstream or downstream of *plg4* may have been replaced differently by the DNA fragments used in the split marker assay.

CHAPTER V

THE REQUIREMENT FOR BOTH PTH11 AND PLG1 DURING APPRESSORIUM FORMATION IN *Magnaporthe oryzae*

Introduction

Previous studies have shown that both of the two novel transmembrane proteins, PLG1 and PTH11, respond to surface hydrophobicity and are required for infection of barley leaves by *M. oryzae* (Castillo 2015; DeZwaan et al. 1999). The ability to form appressoria on hydrophobic surfaces is greatly reduced in Δ*plg1* and *pth11* mutants of *M. oryzae*. The deficiency of appressorium development on hydrophobic surfaces in these mutants is relieved by adding either exogenous cAMP or diacylglycerol (DAG). However, the Δ*plg1* mutant appressoria that form in the presence of DAG can infect barley leaves, while those that form in the presence of cAMP cannot (Castillo 2015). In contrast to Δ*plg1*, the *pth11* mutant appressoria that form in the presence of cAMP can infect barley leaves, while those that form in the presence of DAG cannot (DeZwaan et al. 1999). The full restoration of pathogenicity by DAG alone for the Δ*plg1* mutant and by cAMP alone for the *pth11* mutant indicates that the DAG-dependent signaling pathway is downstream of PLG1, and the cAMP-dependent signaling pathway is downstream of PTH11 (Castillo 2015; DeZwaan et al. 1999).

In wild-type *M. oryzae*, either exogenous cAMP or DAG induces appressorium formation on hydrophilic surfaces (Castillo 2015; DeZwaan et al. 1999). But the addition

of either cAMP (10mM) or DAG (20 ug/mL) to the Δ*plg1* mutant is not sufficient to induce appressoria formation under the same conditions (Castillo 2015). Only when both cAMP (10mM) and DAG (20 ug/mL) are added together, do appressoria develop (Castillo 2015). This implies that activation of only the DAG-dependent or the cAMP-dependent signaling pathway is not enough to induce appressorium formation in Δ*plg1*. However, the effect of exogenous cAMP or DAG on appressorium formation in *pth11* mutants on hydrophilic surfaces has not been investigated.

The results described above for Δ*plg1* and *pth11* mutants lead to the hypothesis that both DAG-dependent and cAMP-dependent signaling pathways are required for appressorium formation in *M. oryzae*. A model summarizing the hypothesis was proposed by Castillo (2015) and is shown in Figure 19. Both PLG1 and PTH11 respond to a hydrophobic signal. PLG1 is needed to induce a DAG-dependent pathway. PTH11 is needed for induction of a cAMP-dependent pathway. Both the DAG-dependent and cAMP-dependent pathways are needed for appressorium development in *M. oryzae*. The experiments described in this chapter were performed to test this model. A *pth11* deletion mutant was constructed in the WT 70-15 strain background, which is the parent strain for

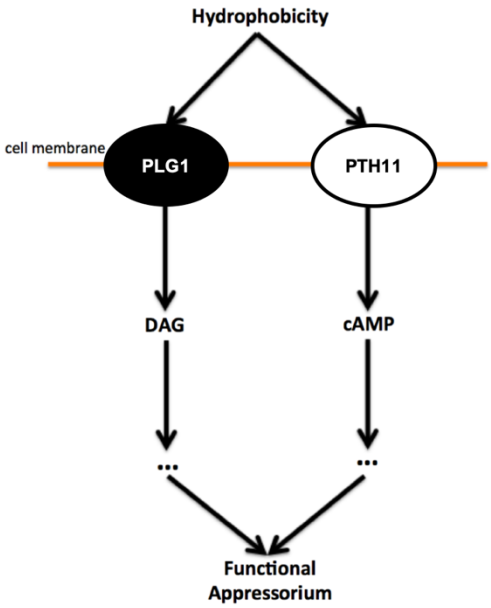


Figure 19 The model of two pathways involving PLG1 and PTH11 activated by hydrophobicity in appressorium formation.

Δ*plg1* mutants (Castillo 2015). Higher concentrations of cAMP and DAG than the ones tested by Castillo (2015) were applied to both Δ*plg1* and *pth11* mutants to further investigate their effect on appressorium formation on hydrophilic surfaces.

Material and Methods

Generation of pth11 *Mutant Strains*

Δ*pth11* mutant strains were generated by deleting the *pth11* gene in WT 70-15 using the split marker assay (see Figure 20A) as described in Chapter II, pg. 14. Generation of fungal protoplasts and their transformation was done as described in Chapter II, pg. 14-16. Southern blot analysis was done to confirm the *pth11* gene deletion as described in Chapter II, pg. 16-17.

Examination of Pathogenic Phenotypes

Infection of WT 70-15 and Δ*pth11* mutant strains on barley was tested by droplet and spray inoculation as described in Chapter II, pg.17-18. Appressorium development by WT 70-15 and Δ*pth11* mutant strains was examined on Teflon membranes as described in Chapter II, pg. 18. cAMP at a final concentration of 10 mM was added to the spore suspension of Δ*pth11* mutants and their appressorium development was also examined in the same way.

Appressorium Development with Treatment by cAMP or/and DAG

Δ*pth11,* Δ*plg1* and WT 70-15 spores were collected in water after 15 days incubation and concentrated to $1x10^4$/mL in water or 0.4% gelatin solution. Different aliquots had cAMP added at 0, 10, 20 or 50mM, DAG added at 20, 50 or 100 ug/mL, or both 10mM cAMP and 20 ug/mL DAG added. Twenty microliters of spore suspensions were placed on Gelbond membranes (Lonza Pharma & Biotech) and incubated in humidity

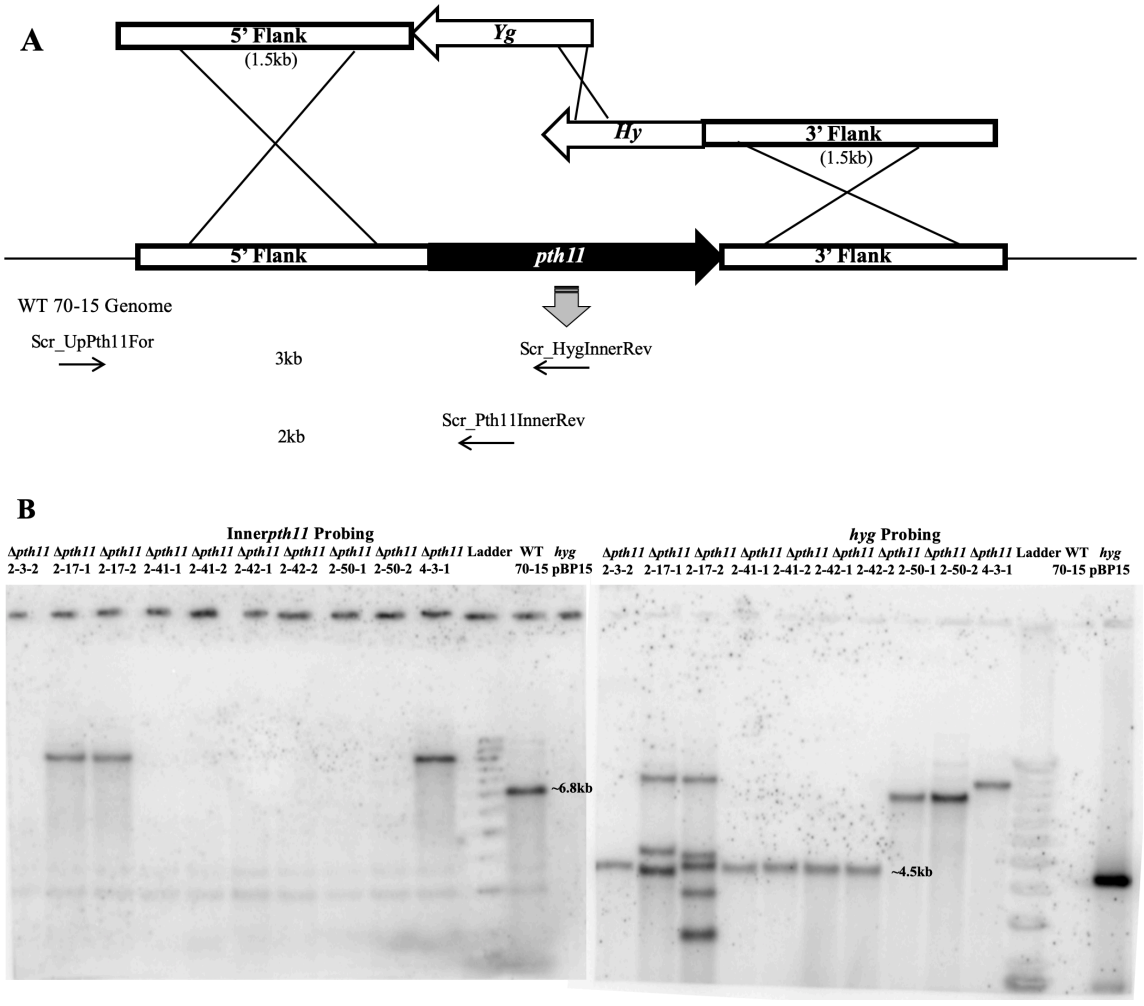


Figure 20 Generation of *pth11* mutant strains.

A. Fragment design for deleting the *pth11* gene using split marker assay.
B. Southern blot result of *pth11* mutant strains.

melanized, abnormal, unmelanized appressoria and total spores were counted and classified.

Results

Generation of pth11 *Mutant Strains*

The previous studies were done on *pth11* mutant strains generated by insertion mutation in the background of wild-type 4091-5-8 (DeZwaan et al. 1999). The *pth11* gene was deleted and replaced with the hygromycin B resistant gene (*hyg*) in WT 70-15 as described in Material and Methods (Figure 20A). Six independent Δ*pth11* mutants were selected and single spore isolation was performed before Southern blot analysis. Genomic DNA from WT 70-15 and Δ*pth11* strains was extracted and digested by *Hind*III. The expected size of the genomic fragment detected by probe Inner*pth11* was 6.8kb, and the fragment detected by probe *hyg* was 4.5kb (Figure 20B). According to the Southern blot results (Figure 20B), the isolates from three independent Δ*pth11* mutants (2-3-2, 2-41-1, 2-41-2, 2-42-1, and 2-42-2) had a deletion of *pth11* and insertion of *hyg*. Two independent mutant strains, 2-3-2 and 2-41-1, were selected for further study.

Pathogenic Phenotypes of pth11 *Mutant Strains*

The pathogenicity of two Δ*pth11* mutant strains on barley leaves was tested by both droplet and spray inoculation. The results were the same as previously reported (DeZwaan et al. 1999) that Δ*pth11* mutants do not infect barley leaves or seedlings (Figure 21A and B). Appressorium formation by Δ*pth11* mutant spores on hydrophobic surfaces was highly

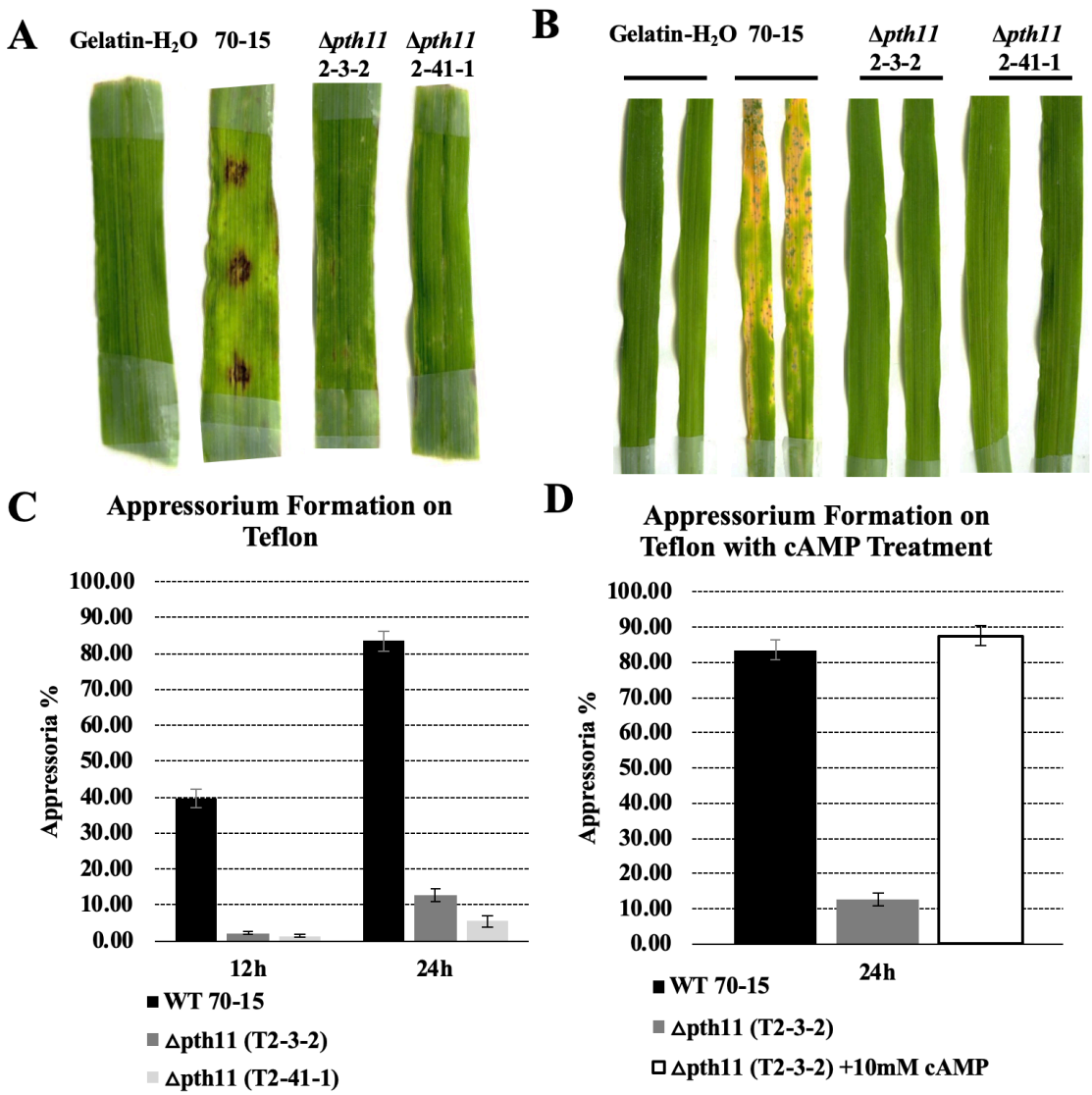


Figure 21 Pathogenic phenotypes of *pth11* mutant strains.

A. Droplet inoculation of barley leaves with *Magnaporthe oryzae* spores.
B. Spray inoculation of barley seedlings with *Magnaporthe oryzae* spores.
C. Appressorium development on Teflon membranes.
D. Appressorium development on Teflon membranes with cAMP treatment.

reduced compared to WT 70-15 (Figure 21C), and could be restored to the same level as wild-type by adding 10mM cAMP exogenously (Figure 21D), which was consistent with the results previously reported (DeZwaan et al. 1999).

Appressorium Development on Hydrophilic Surfaces with cAMP or/and DAG Treatments

To test the model shown in Figure 19, Gelbond membranes were inoculated with spores of Δ*plg1*, Δ*pth11* and WT 70-15 strains that had been treated with cAMP at concentrations of 0, 10, 20 or 50 mM. The percentage of spores that formed appressoria after 24h inoculation was determined and the results are shown in Figure 22A.

WT 70-15 spores developed 65% appressoria (45% melanized) on Gelbond membranes with 10 mM cAMP treatment, while Δ*plg1* spores developed 0.3% appressoria (0% melanized) and Δ*pth11* spores developed 12% appressoria (7% melanized) under the same conditions. WT 70-15 developed 74% appressoria (55% melanized) on Gelbond membranes with 20 mM cAMP treatment, while Δ*plg1* developed 7% appressoria (1% melanized) and Δ*pth11* developed 54% appressoria (43% melanized). WT 70-15 developed 86% appressoria (77% melanized) on Gelbond membranes with 50 mM cAMP treatment, while Δ*plg1* spores developed 8% appressoria (1% melanized) and Δ*pth11* developed 89% appressoria (79% melanized).

Appressorium development by Δ*plg1* spores on hydrophilic surfaces with cAMP treatment (10 mM) was significantly reduced compared to WT 70-15 spores. Even when the cAMP concentration was increased to 50 mM, appressorium development by Δ*plg1*

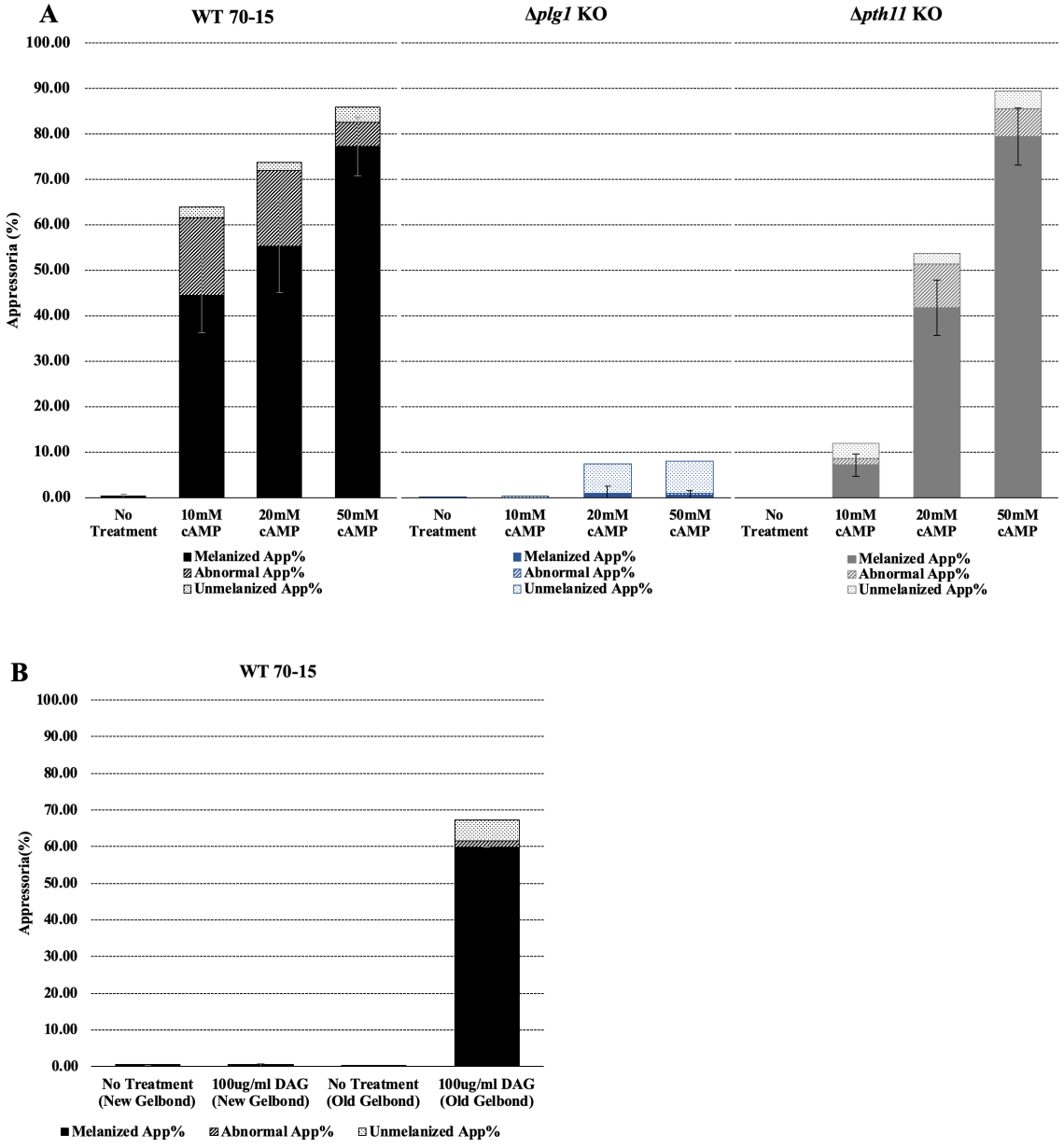


Figure 22 Appressorium development on hydrophilic surfaces with cAMP or/and DAG treatments.

A. Appressorium development on Gelbond membranes (new) with cAMP treatments.
B. Appressorium development on Gelbond membranes (new and old) with DAG treatments.

spores was not restored to wild-type levels. Appressorium development by Δ*pth11* spores on hydrophilic surfaces with cAMP treatment (10 mM) was also highly reduced compared to WT 70-15 spores, but not as reduced as Δ*plg1* spores. When cAMP treatment was increased to 50 mM, appressorium development by Δ*pth11* spores was restored to wild-type levels.

Spores of WT 70-15 strains were inoculated on Gelbond membranes and treated with exogenous DAG (Figure 22B). However, WT 70-15 spores did not develop any appressoria, which was inconsistent with the previously reported results (Castillo 2015). The method of DAG dilution was optimized, and multiple biological trials were done. The results of DAG treatment by Castillo (2015) were still not able to be repeated. The company (Lonza Pharma & Biotech) producing the Gelbond films used in these experiments was contacted and confirmed that the composition of Gelbond films had been changed in June 2014. Even though this change did not affect the use of Gelbond films as a backing for agarose gels, the change of formula appeared to affect appressorium development by *M. oryzae*. Gelbond films produced before the formula change were found and used for testing appressorium development by WT 70-15 spores with DAG treatment. Spores of WT 70-15 developed 67% appressoria (60% melanized) with 100 ug/mL DAG treatment (Figure 22B), indicating that the formula change of Gelbond membranes was the cause of inconsistency with the results of Castillo (2015). The experiments looking at appressorium development with cAMP treatment were repeated using old-formula Gelbond membranes, and the results (data not shown) were consistent with the results seen using the new-formula Gelbond membranes (Figure 22A).

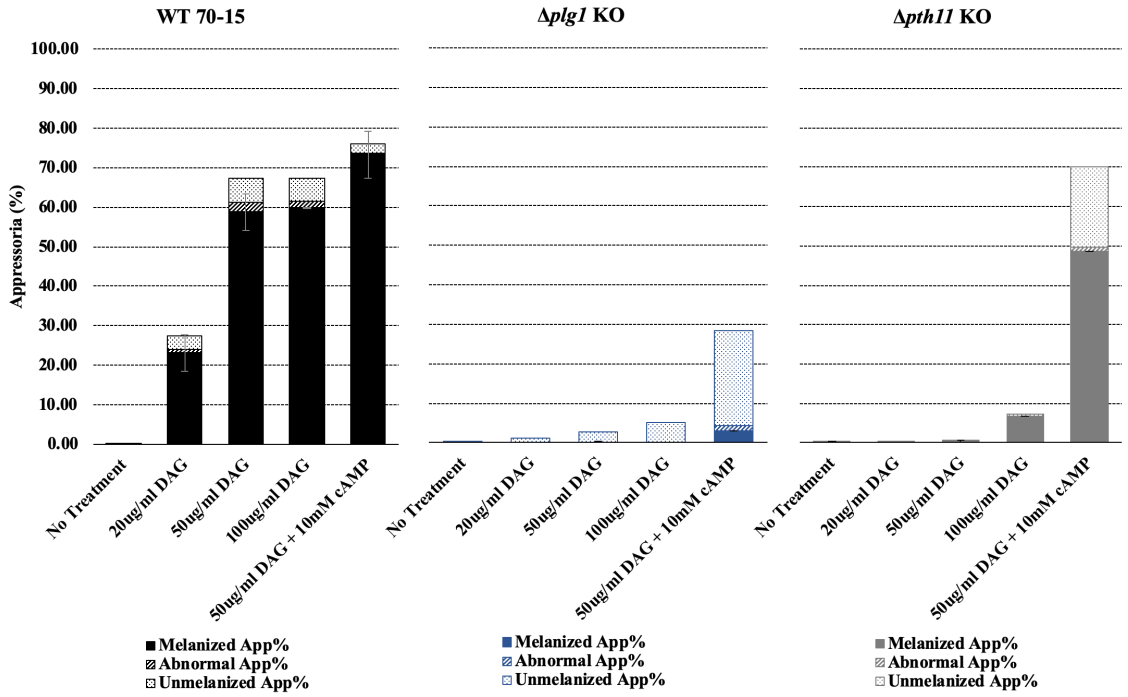


Figure 23 Appressorium development on Gelbond membranes (old formula) with DAG treatment.

Spores of Δ*plg1*, Δ*pth11* and WT 70-15 strains were inoculated on old-formula Gelbond membranes and treated with exogenous DAG at concentrations of 0, 20, 50, or 100 ug/mL DAG. The number of appressoria that formed after 24h inoculation was determined and the results are shown in Figure 23.

WT 70-15 spores developed 27% appressoria (23% melanized) on old-formula Gelbond membranes with 20 ug/mL DAG treatment, while Δ*plg1* spores developed 1% appressoria (0% melanized) and Δ*pth11* spores developed 1% appressoria (0.3% melanized) under the same conditions. WT 70-15 spores developed 67% appressoria (59% melanized) on old-formula Gelbond membranes with 50 ug/mL DAG treatment, while Δ*plg1* spores developed 1% appressoria (0.1% melanized) and Δ*pth11* spores developed

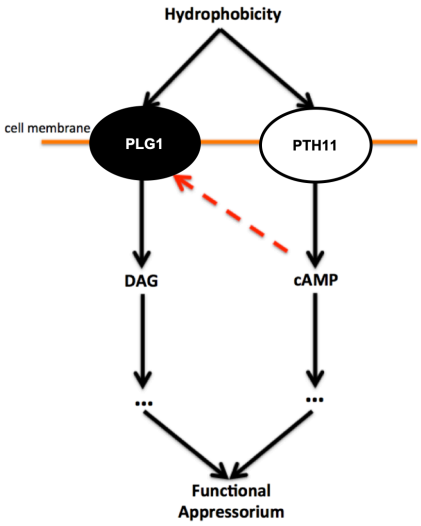
0.3% appressoria (0% melanized). WT 70-15 spores developed 67% appressoria (60% melanized) on old-formula Gelbond membranes with 100 ug/mL DAG treatment, while Δ*plg1* spores developed 5% appressoria (0% melanized) and Δ*pth11* spores developed 7% appressoria (7% melanized).

The treatments with 20 ug/mL DAG were unable to induce appressorium development by either Δ*plg1* or Δ*pth11* mutant strain spores compared to WT 70-15. When DAG treatment was increased to 100 ug/mL, appressorium development by Δ*plg1* and Δ*pth11* spores was still not restored to wild-type levels.

Spores of Δ*plg1*, Δ*pth11* and WT 70-15 were also inoculated on old-formula Gelbond membranes and treated with both 10 mM cAMP and 20 ug/mL DAG. WT 70-15 spores developed 78% appressoria (73% melanized), while Δ*plg1* spores developed 28% appressoria (3% melanized) and Δ*pth11* spores developed 70% appressoria (49% melanized). The addition of both cAMP and DAG induced much higher appressorium development in the Δ*plg1* and Δ*pth11* mutants than adding cAMP or DAG alone.

Appressorium Development Pathways Involving PLG1 and PTH11

Both a summary of the results above and a revised model are shown in Figure 24. According to this new model, both the "PLG1→DAG" and "PTH11→cAMP" signaling pathways are required for appressorium development in *M. oryzae*. The activation of either signaling pathway is not enough for appressorium development. In the absence of a hydrophobicity signal, the "PTH11→cAMP" signaling pathway may be able activate the "PLG1→DAG" signaling pathway through PLG1.



Hydrophobicity	PLG1	PTH11	DAG	cAMP	Appressoria
+	+	+	-	-	+ (pathogenic)
-	+	+	-	-	-
-	+	+	+	-	+
-	+	+	-	+	+
-	+	+	+	+	+
+	-	+	-	-	-
+	-	+	+	-	+ (pathogenic)
+	-	+	-	+	+ (non-patho.)
-	-	+	+/++	-	-
-	-	+	-	+/++	-
-	-	+	+	+	+ (unmelanized)
+	+	-	-	-	-
+	+	-	+	-	+ (non-patho.)
+	+	-	-	+	+ (pathogenic)
-	+	-	+/++	-	-
-	+	-	-	+/++	-/+
-	+	-	+	+	+

Figure 24 The modified model showing predicted interaction of the PLG1 and PTH11 pathways in appressorium formation.
(The experimental results that led to the new model are summarized in the figure.)

Δ*plg1* mutant spores form functional appressoria with induction by hydrophobicity and exogenous DAG, but form nonpathogenic appressoria with induction by hydrophobicity and exogenous cAMP. Hydrophobicity and exogenous cAMP only activate the "PTH11→cAMP" signaling pathway in Δ*plg1*, which is enough to induce appressoria formation but not enough to induce pathogenicity. Similarly, Δ*pth11* mutant spores form functional appressoria with induction by hydrophobicity and exogenous cAMP, but form nonpathogenic appressoria with induction by hydrophobicity and exogenous DAG. Hydrophobicity and exogenous DAG only activate the "PLG1→DAG"

signaling pathway in Δ*pth11* spores, which is enough to induce appressoria formation but not enough to induce pathogenicity.

Δ*plg1* mutant spores do not form appressoria with induction by exogenous cAMP or DAG alone (no hydrophobic signal), because only one of the "PTH11→cAMP" and "PLG1→DAG" signaling pathways is activated, which is not enough to induce appressoria development. Δ*pth11* mutant spores do not form appressoria with induction by exogenous DAG alone (no hydrophobic signal), because only the "PLG1→DAG" signaling pathway is activated, which is not enough to induce appressoria development. Δ*pth11* mutant spores develop a few appressoria with induction by exogenous cAMP alone (no hydrophobic signal), but the deficiency can be restored by increasing cAMP induction. The restoration may be because not only the "PTH11→cAMP" signaling pathway is activated by exogenous cAMP, but the "PLG1→DAG" signaling pathway is also activated by exogenous cAMP. If this is the case, it implies that the "PTH11→cAMP" signaling pathway may regulate the "PLG1→DAG" signaling pathway through PLG1. The activation of the "PLG1→DAG" signaling pathway induced by 10 mM cAMP may be too weak to reach the wild-type level of activation, resulting in reduced appressoria formation compared to the wild-type. When the cAMP induction signal is increased (50 mM), the activation of the "PLG1→DAG" signaling pathway can reach the wild-type level of activation, resulting in the same level of appressorium development by Δ*plg1* and wild-type spores.

Δ*plg1* and Δ*pth11* mutant spores develop more appressoria with induction by both exogenous cAMP and DAG (no hydrophobic signal) than with induction by either

exogenous cAMP or DAG alone. This may be because both the "PLG1→DAG" and the "PTH11→cAMP" signaling pathways can be activated by adding exogenous cAMP and DAG together. However, in the case of the Δ*plg1* mutants, the increase in appressorium development is mostly unmelanized appressoria, probably because the "PTH11→cAMP" signaling pathway cannot increase the activation of the "PLG1→DAG" signaling pathway in the absence of PLG1.

Conclusion and Discussion

The signaling pathways of appressorium development involving PLG1 and PTH11 were investigated in *M. oryzae*. The Δ*pth11* mutant strains were generated in the same genomic background as the Δ*plg1* mutant. Pathogenic phenotypes of Δ*pth11* mutants were examined and found to be the same as previously reported for *pth11* mutants in a different strain background (DeZwaan et al. 1999). Appressorium development by Δ*plg1*, Δ*pth11* and WT 70-15 spores was tested on hydrophilic surfaces with various cAMP or/and DAG treatments. The results helped modify the model proposed by Castillo (2015) that both the "PLG1→DAG" and "PTH11→cAMP" signaling pathways are required independently for appressorium development in *M. oryzae*. The modified model hypothesizes that the "PTH11→cAMP" signaling pathway activate the "PLG1→DAG" signaling pathway through PLG1. This activation is required to induce appressorium development at a similar level as the wild-type.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The previous chapters described my investigation of signaling pathways involved in pathogenesis of the fungus *Magnaporthe oryzae*. I investigated the functions of *bip1*, which encodes a novel transcription factor (Tag unpublished), and of four of the genes it regulates: *plg1*, *plg2*, *plg3* and *plg4*. I also investigated the roles of the PLG1-dependent and PTH11-dependent signaling pathways and identified possible interactions between the two pathways. This chapter summarizes the conclusions and proposes future experiments that could be done.

To investigate the function of *bip1* in pathogenesis, derivatives of wild-type strain 70-15 with Δ*bip1*::*hyg* mutations were constructed and shown to be unable to infect barley leaves, consistent with the results of previous studies performed in a different background (Tag unpublished). Although unable to infect plants, Δ*bip1* spores can form appressoria. The turgor pressure in Δ*bip1* appressoria was shown to be the same as in wild-type appressoria, indicating that the inability to penetrate plants is not due to abnormal turgor pressure. Both wild-type and Δ*bip1* spores formed appressoria on a hydrophilic surface in the presence but not the absence of exogenous cAMP, indicating that BIP1 is not involved in cAMP-dependent signaling pathways of appressorium development.

Transcript levels during the course of plant infection of *bip1*, *plg1*, *plg2*, *plg3*, and *pth11*, another GPCR-like protein important for pathogenesis, were investigated by RT-PCR experiments and also by examining the results of an RNA-seq study of *M. oryzae* during infection (SRX5076910-5076916, https://www.ncbi.nlm.nih.gov/sra). The RNA expression patterns seen in both studies indicate that: BIP1 is important during invasive growth; PLG1, PLG2 and PLG3 may be needed only during appressorium development; and PTH11 is important during both appressorium development and invasive growth.

The proteins encoded by the *plg* genes are predicted to be GPCR-like signaling proteins with 4 to 7 membrane-spanning domains (Tag unpublished; Castillo 2015; Kulkarni et al. 2005). Previous work has shown that *plg1* is essential for appressoria development and plant invasion (Castillo 2015). To determine the roles of *plg2*, *plg3*, and *plg4* in pathogenesis, strains with deletion::substitution mutations were constructed and characterized. On barley leaves, the Δ*plg2* and Δ*plg3* strains developed infective lesions similar to wild-type. One of two independent Δ*plg4* strains formed smaller lesions than wild-type, while the other Δ*plg4* strain, like Δ*plg1*, was not able to form any lesions. The phenotypes of the two independent Δ*plg4* mutants have not been complemented with a copy of the wild-type *plg4* gene. The ability of the plg mutants to form appressoria on a hydrophobic surface was also examined. It is previously reported that appressorium formation of Δ*plg1* mutants is highly reduced (Castillo 2015). By comparison, the appressorium formation of Δ*plg2* and Δ*plg4*-1 was the same as wild-type. The appressorium formation of Δ*plg3* and Δ*plg4*-2 was reduced, but not as significantly as

Δ*plg1*. Taken together, these results indicate that, unlike *plg1*, *plg2*, *plg3*, and *plg4* are not essential for plant infection.

The GPCR-like proteins encoded by *plg1* and *pth11* are both required for formation of appressoria induced by hydrophobicity (Castillo 2015; DeZwaan et al. 1999). PLG1 is upstream of a DAG-dependent signaling pathway needed for appressorium development (Castillo 2015), while PTH11 is upstream of a cAMP-dependent signaling pathway needed for appressorium development (DeZwaan et al. 1999). The model shown in Figure 19 was proposed by Castillo (2015) based on her studies of Δ*plg1* and the published studies of the *pht11*-insertion mutants (DeZwaan et al. 1999).

Before I could further study the roles of PLG1 and PTH11 in *M. oryzae* pathogenesis, it was necessary to construct a *pth11* deletion-substitution mutation in the same genetic background as the Δ*plg1* mutation. The Δ*pth11* mutation in the 70-15 strain background caused the same pathogenic phenotypes as reported previously for the *pth11* insertion mutation in 4091-5-8 (DeZwaan et al. 1999). Spores of the Δ*pth11* strain did not infect barley plants and, on an artificial hydrophobic surface, formed <15% as many appressoria as the wild-type strain. In the presence of exogenous cAMP, appressorium development by the Δ*pth11* mutant was restored to wild-type level.

Once isogenic wild-type and Δ*plg1* and Δ*pth11* strains were constructed, it was possible to further characterize the function of the signaling pathways involving PLG1

or/and PTH11. How appressorium development of the mutant strains on a hydrophilic surface was affected by the addition of various concentrations of cAMP or/and DAG was determined. Δ*plg1* spores did not develop appressoria with induction by cAMP or DAG alone (no hydrophobic signal), but appressorium formation was increased with induction by both cAMP and DAG. Δ*pth11* spores formed fewer appressoria with induction by cAMP or DAG alone (no hydrophobic signal) than with induction by both together. However, increasing the concentration of cAMP added to the Δ*pth11* mutant restored appressorium development to wild-type levels.

The results of these experiments led to the modified model shown in Figure 24. Both the "PLG1→DAG" and "PTH11→cAMP" signaling pathways are required to induce appressorium development in *M. oryzae*. The "PTH11→cAMP" signaling pathway may regulate the "PLG1→DAG" signaling pathway through PLG1 during appressorium development. The regulation may be necessary to induce appressorium formation at a similar level as wild-type.

In summary, *plg1* has important function(s) during appressorium formation and fungal pathogenicity on plants. Its expression is regulated by *bip1* and PLG1 functions upstream of DAG-dependent signaling pathways needed for appressorium development. Appressorium formation by *M. oryzae* requires not just the activation of the "PLG1→DAG" signaling pathway, but also the activation of "PTH11→cAMP" signaling pathway, which may activate the "PLG1→DAG" pathway through PLG1.

Future Work

The conclusion that the "PTH11→cAMP" pathway may activate the "PLG1→DAG" pathway should be tested further. First, to confirm that the phenotypes of the Δ*plg1* and Δ*pth11* mutant strains are due to the absence of the respective wild-type genes, complemented strains should be tested for restoration of the ability to form functional appressoria on hydrophilic surfaces with either cAMP or DAG treatment. The appressorium formation is expected to be restored to the wild-type level with the same treatment applied to wild-type. A complemented strain of the Δ*plg1* mutant has been made (Castillo 2015), but a complemented version of the Δ*pth11* mutant needs to be constructed.

If the complemented strains give the expected results, a mutant strain lacking both the *plg1* and *pth11* genes should be constructed and tested for appressorium formation under the same conditions. The model predicts that spores of the double mutant will not form appressoria when treated with either cAMP or DAG alone, but treatment with cAMP and DAG together will restore appressorium formation.

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APPENDIX A

PRIMERS USED IN THIS STUDY

All primers are written in the direction of 5' to 3'

***bip1* Knock-out**

Bip_UpFor: CCG AAT TTC ATC ATA CCT GCC ACA GT

Bip_UpRev_SMA: ACT GGC CGT CGT TTT ACA ACG TCG TGA CGG TTG GAG ATG GTT ATG ATG

Bip_UpRev: GGT TGG AGA TGG TTA TGA TGA GTG AAG

Bip1_DownFor_SMA: GGT CAT AGC TGT TTC CTG TGT GAA ATT GGC GGC TAA GGG TCA TTT GGC

Bip1_DownFor: GCG GCT AAG GGT CAT TTG GC

Bip1_DownRev: GTC CGA GTT TCA CTT TCA CTT GCC G

Scr_Bip1UpFor: CCG TTC TCG CAT TTC AGT GAC GAT G

Scr_HygInnerRev: GGC GAA GAA TCT CGT GCT TTC AGC

Scr_Bip1InnerRev: GGG AAG ATA GCT CCC CAA GTC ATG A

***plg2* Knock-out**

Plg2_UpFor: GGG TGG CCT GAC AGC TTG AAT C

Plg2_UpRev (NotI): ATA GCG GCC GCC GAC GTC GGA ATC G

Plg2_DownFor (XbaI): GCT TCT AGA GCC ATC TTA TGT TGT TAT

Plg2_DownRev: GGT CGC GAT AGA GTG AAG CTG C

Plg2_For: ATG GCT TCT CTA TAC TCG TTC CTC G

Plg2_Rev: TTA CAC AAC CAT CGC ACG

Plg2+Up_For: TTT CTG CAC ATC ATC ACC AC

Plg2+Up_Rev: CCA GCA ATA GCC CTC TAT CA

Plg2+Down_For: AAA AAT CAG ACG ACG GAG TA

Plg2+Down_Rev: AGT CTA CCA TGC TCA TCA CC

Plg2_JunctionFor: CGT CGA AAA GAA AAT GGC TTC

Plg2_JunctionRev: TTT TTT TTT GTT ACA CAA CCA TCG C

Hyg_For (NotI): TAG GCG GCC GCT GAT ATT GAA GGA G

Hyg_Rev (XbaI): TGG TCT AGA CTA TTC CTT TGC CCT CG

***plg3* Knock-out**

Plg3_For: ATG TTC GTC ATC CAG CTG ACG TAC G

Plg3_Rev: TCA TCG CTG CTG TGC CTG CT

Plg3_InnerFor: CGC ATC CCC ATC TGG ATC AT

Plg3_InnerRev: ATC GGC TTT AGG GTT GGG AG

Plg3_UpFor: CGC CGC CCA CCA GGT GGT AGC CCG G

Plg3_UpRev (SPA): ACT GGC CGT CGT TTT ACA ACG TCG TGA CTC CAA TCG CCC AGG CCC TCG

Plg3_DownFor (SPA): GGT CAT AGC TGT TTC CTG TGT GAA ATT GCG GCG CGC GGT CCG ACG TGT

Plg3_DownRev: GGG GTC CTC GGG AGA AAG GGT GCC AGG C

Plg3_Down2_For: GGT CAT AGC TGT TTC CTG TGT GAA ATT GCT TAG TCT AAA ATC TTT GTT

Plg3_Down2_Rev: GCT GCG CGG CGC TCT CGA AAC G

Scr_UpPlg3For: CCC TTT CCG TTA GCT TTT GGT TGC C

Scr_UpPlg3For2: GGA CAA CCC AAT GCA GAG TAA GC

Scr_Down2Plg3Rev: CGG CTT CGA CGT CTG GTT CG

pPlg3Up+YG: TAC TTC GAG CGG AGG CAT CC

pHY+Plg3Down: GGT ATG ACC GGG TCG TCC AC

HY_For: GTT GGT CAA GAC CAA TGC GGA GCA

HY_Rev: CGA CAG CGT CTC CGA CCT GAT G

YG_For: GTT GGT CAA GAC CAA TGC GGA GCA

YG_Rev: CGA CAG CGT CTC CGA CCT GAT G

***plg4* Knock-out**

Plg4_For: ATG TAC TAC GAT GGC TTG AGC TAT CGA G

Plg4_Rev: TCA CAC CCA ATC CGG ACG CAT TT

plg4_UpFor: GCT GTA CCG CTC ATT CGG GAC CAT CG

plg4_UpRev: ACT GGC CGT CGT TTT ACA ACG TCG TGA CAT TTG TGG AAA CAA GGA AAC

plg4_DownFor: GGT CAT AGC TGT TTC CTG TGT GAA ATT GAG CTG AAT CTC TTG ATG TAT

plg4_DownRev: CCT CTT GCT GGC ACG AGA CGT TGT CC

Scr_UpPlg4For: GTC ATG TCG CCC GAG CTA CTG

Scr_HygInnerRev: GGC GAA GAA TCT CGT GCT TTC AGC

Scr_Plg4InnerRev: GAA CGG ACG AGG ATT GTG GGA C

***pth11* Knock-out**

Pth11_For: CCA CGA TGC CTT TCT CGC TG

Pth11_UpRev: TTT CGA GAT GCC GTT CAA ATG TGA TAT TAG

Pth11_UpRev_SMA: ACT GGC CGT CGT TTT ACA ACG TCG TGA CTT TCG AGA TGC TCA AAT

Pth11_DownFor_SMA: GGT CAT AGC TGT TTC CTG TGT GAA ATT GAA GCC GGA CCA TGG TTG TAT

Pth11_DownRev: GTC CGC CCG AGC CTC TAC CT

Scr_UpPth11For: CTC AGT CCA GCC ACG TCA ACT C

Scr_HygInnerRev: GGC GAA GAA TCT CGT GCT TTC AGC

Scr_Pth11InnerRev: GAC TGA AGC CGC TCC GCA TAT C

pPth11_For: CAT CTG CGT CTC CAA TCC GA

pPth11_Rev: GAT TGG CAA ATC TGG CAC GG