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# Function of regulatory gene *PoLAE2* on appressorium formation in rice blast fungus *Pyricularia oryzae*

(イネいもち病菌の包括的制御遺伝子 *PoLAE2* の付着器形成における役割)

**Hokkaido University Graduate School of Agriculture**

**Division of Bio-system Sustainability Doctoral Course**

**Pradabrat Prajanket**

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# CHAPTER 1

## General overview

# CHAPTER 1

## General overview

### 1. 1 Rice demand

Rice is world's crucial crop that nourish more than half of world population. People around the world consume rice as a staple food because it's great source of carbohydrate including protein, vitamins minerals and fiber content that can produce energy for daily life. Moreover, rice is utilized extensively for health benefits such as ingredient for many kind of food, composition of cosmetics and dietary supplements. According to rice is monocot that able to grow under a variety or wide range of climatic conditions. Especially, submerged area and extreme of solar radiation. Therefore, rice is produced in almost world's region. In many countries that development base on agriculture produce rice for domestic consumption and supply for self-sufficiency. Furthermore, the other sector of rice product can trade by exports to make their income.

Base on department of Economic and Social Affairs, United Nations reported about world population prospect 2019 that the world's population is expected to increase by 2 billion persons in the next 30 years, from 7.7 billion currently to 9.7 billion in 2050 including the latest forecast of Food and Agriculture Organization (FAO) predicted world rice utilization expanding by an additional 5.2 million tonnes in 2018/19 to reach 509.1 million tonnes (FAO, 2018) including. Both predictions indicated that global rice demand is the parallel growth along with increasing of global population unquestionably. Therefore, rice supplying is a very important issue in the next future to provide sufficient amount of rice for consumers.

Although the rice market monitor by FAO showed the trend of rice production, utilization and stocks have been increasing continuously from 2008-2019. However, a tremendous problem that threaten rice cultivation for long last decade until present is the epidemic of rice disease that caused by several plant pathogens. One of the most important that impact to loss of the rice yield annually is rice blast disease. It is estimated that each year rice blast cause harvest losses of 10-30 % of the global rice yield which is enough rice to feed more than 60 million people (Talbot, 2003). For this reason, rice blast diseases become one of the most important disease that caused huge economic losses and need to control.

## 1.2 Rice blast disease

Among all diseases affecting rice production, rice blast disease is considered the most serious disease that threaten the cultivation of rice worldwide. This disease caused by plant pathogenic fungus, *Pyricularia oryzae*. The dissemination of disease caused by distribution of fungal conidia via airborne including rain and dew drop splash then attached to a part of rice plant and under favorable conditions, it will germination and form the special infection structure called appressorium to invade rice's tissue. The symptom will present obviously as necrotic lesion or spot at each part of host plant that depending upon the site of infection. For examples, lesions on leaf, node, stem, panicles and grain. Since the infection establishes, the lesions begin as small whitish or greyish spots then enlarge to be like a diamond shaped lesions with grey or white centers and narrow brown or reddish brown borders. The mature lesion develops greyish cottony centers due to sporulation of the fungus (Figure 1). Once fungus

sporulated, it can spread the disease to adjacent plant. The epidemic of the disease will expand in many area of paddy field caused the rice plant death rapidly.

The origin of rice blast emerged in China in 1637 as a rice fever disease. Subsequent discovery were reported in Japan, Italy and USA respectively. In 1912, the first report occurrence of the disease on rice was in Brazil (Maciel, 2011). From many last decades, rice blast disease is known to occur ongoing outbreak in several area of global rice cultivation. For example, There was reported that the demand for rice in Kenya has seen dramatic increase over while production has remain low because rice blast effect on rice production in 2009. Result of rice blast disease mapping in Mwea region showed that rice blast was the main contribute to yield loss in this area. The progression of rice blast disease in the farm units since the year 2006 to 2010 and its effect to the total production per acre indicated that during the year 2009 when rice blast occurrence was at 55.5% the average bags (90 kg.) that were produce per acre dropped to 10.5 from 21.9 produced in previous year (Kihoro *et al.*, 2013).

In recent years, use of chemical fertilizers has reduced to meet consumer demand for environmentally friendly agricultural products; however, this can lead to an increase in the occurrence of rice blast. Further, in 2016 and 2017, rice blast disease became a serious problem in Bangladesh and India. Moreover, blast disease affects not only rice but also wheat (Kim *et al*., 2017).

**Figure 1.** Rice blast lesions on the leaf are elliptical or spindle-shaped and whitish to gray centers with red to brownish or necrotic border. (www.knowledgebank.irri.org/traning/fact-sheets/pst-management/disease/item/blast-leaf-collar)

According to rice blast disease has the greatest impact as mentioned above. Thus, monitoring and precise prediction of the occurrence of this disease are important. Early prediction of the disease would be especially helpful for prevention this disease. Moreover, understanding to the disease mechanism is also necessary to sustainable control of rice blast fungus.

## 1.3 Rice blast fungus

*Pyricularia oryzae* (teleomorph: *Magnaporthe oryzae*) is a crucial phytopathogenic species in Magnaporthaceae, Ascomycete fungi that threat to a variety of crops worldwide. Its life style consists of a hemi-biotrophic infection cycle and predominantly asexual mode of reproduction. The sexual stage exist but is rarely found in nature rice-infecting population.

This fungus is a heterothallic ascomycete, and it has two mating types, *MAT*1-1 and *MAT*1-2. It produces nuclei that function as either male of female gametes. Thus, the sexual cycle involves fusion of male and female gamete, which must be opposite mating types. Under suitable environmental conditions, sexual reproduction initiates with the fusion of strains of opposite mating types and concludes with the production of mature perithecia with numerous eight-spore asci (Lengeler *et al*., 2000).

In 2002, The Fungal Genome Initiative (FGI) released a draft genome sequence of *P. oryzae* strain 70-15. The final genome sequence, obtained by whole genome shotgun sequencing, was published in 2005 (Dean et al., 2005). The total size of the *P. oryzae* genome is 41.7 Mb and organized in 7 chromosomes (Perez-Nadales *et al*., 2014).

The infectious life cycle of *P. oryzae* initiates when the fungal conidia settle on surface of rice plant. The conidium release an adhesive substance called spore tip mucilage from the conidial apex and attaches itself tightly to the hydrophobic plant surface (Fernandez and Orth, 2018). Conidium germination is triggered by favorable conditions and plant surface recognition. The germ tube extends for 10 - 15 µm. before flattening at its tip, hooking and beginning to differentiate into the unicellular appressorium (Ryder and Talbot, 2015). The appressorium is a unicellular, dome-shaped structure which generate cellular turgor that is translated into mechanical force to cause rupture of the rice cuticle and entry into plant tissue (Talbot, 2003; Ebbole, 2007). A penetration peg emerges at the base of appressorium and crosses the plant epidermal cell by combining physical force and secretion of cell wall degrading enzyme (Skamioti and Gurr, 2007) Subsequently, the infecting hyphae swell as they spread within and between plant cells. Within four days, a disease lesion is produced and the fungus sporulates to spread the disease to adjacent plant. (Harmer and Talbot, 1998).

*P. oryzae* can infect all the rice's growth phase. This fungus can also cause a similar disease in over 50 grasses, including economically important crops such as barley, wheat, and millet. Detailed studies of the pathogenesis of this fungus have established it as one of the model organisms among plant-pathogenic fungi (Lengeler *et al*., 2000). *P. oryzae*'s life cycle showed in figure 2.

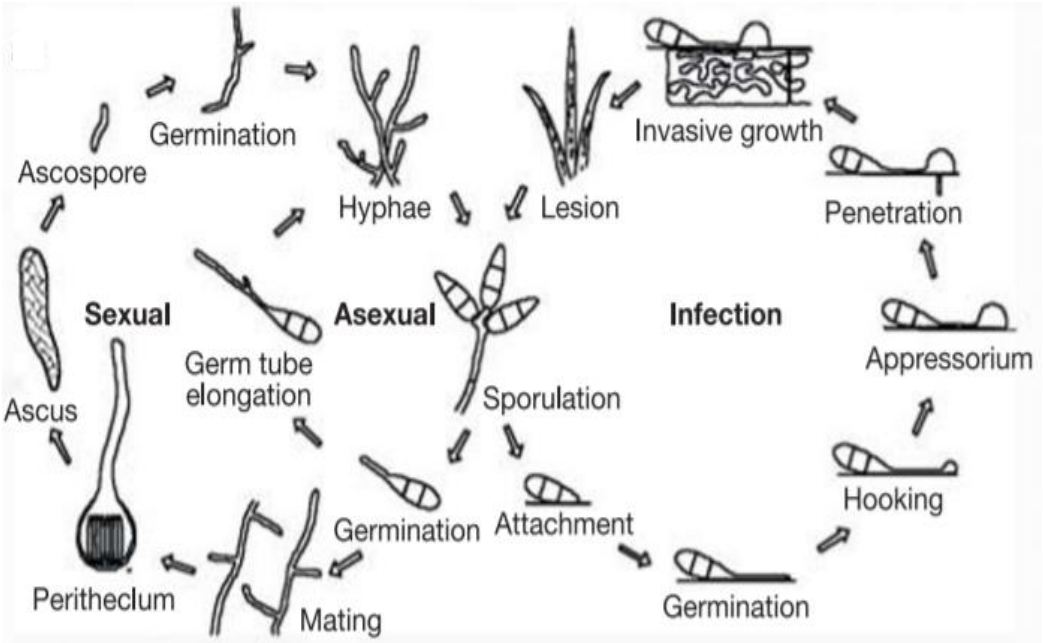



**Figure 2**. Overview of infectious life cycle of *P. oryzae*. (Dean *et al*., 2005)

## 1.4 Regulation of appressorium formation

In generally, pathogenic fungi have various strategies to invade and infect their hosts. The infection structure that rice blast fungus used to attack the host cell is a unicellular, dome shape structure called appressorium (Figure 3).

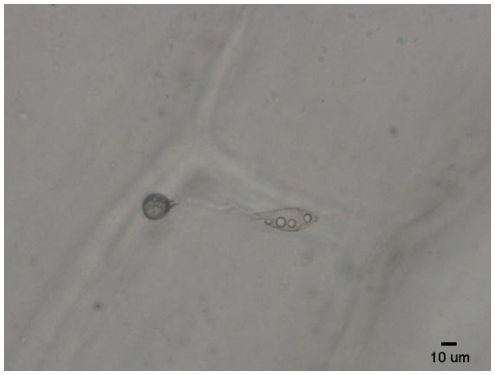



**Figure 3.** A three-celled conidium that formed a dome-shaped, Appressorium.

Formation of the appressorium implicate with sensing and responding to the physico-chemical cues from the host surface, Such as surface rigidity or hardness, hydrophobicity, plant surface waxes and cutin, etc (Kou and Naqvi, 2016). The recognition of physical cues leading the fungus to develop an appressorium (Fernadez and Orth, 2018). During germ tube differentiation, Pth11 is a G-protein-coupled receptor that encode plasma membrane protein to mediate appressorium in response to inductive substrate cues. Pth11 is required for completion of appressorium morphogenesis after the recognition phase. It is involved in sensing the host surface.

In rice blast fungus, there are several intracellular signaling pathways that regulate the differentiation during appressorium formation. For example, Heterotrimeric G-protein signaling is extremely important, and regulates growth and differentiation in eukaryotes. It consist of three subunits Gα MagA or MagB, Gβ Mgb1 and Gγ Mgg1. These heterotrimeric G-protein are involve in proper appressorium formation and function downstream of Pth11 receptor. Genetically, Regulator of G-protein signaling (Rgs1) modulates the Gα subunit MagA to enable *P. oryzae* to perceive and response to physical cues during appressorium formation (Liu *et al.*, 2007). After recognition the cues, Gα subunits binds to GTP and dissociates from the Gβγ

dimer that result in Gα-GTP and Gβγ complex are able to activate the downstream effector. Then the activated effector will regulate secondary messenger such as cyclic AMP and increasing of cAMP level leads to activation of signaling cascade to induce appressorium formation. (Figure 4).

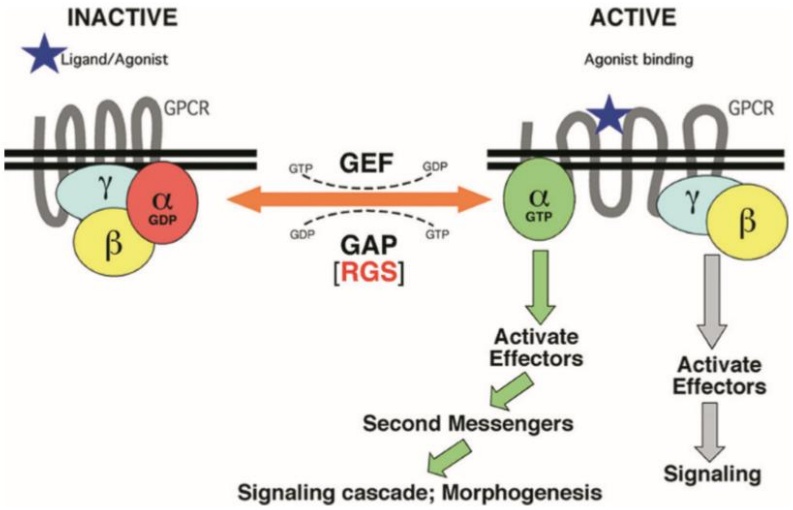



**Figure 4.** Schematic representation of canonical G protein signaling. (Liu *et al*, 2009)

In addition, there are two major signaling pathways have long been implicated in appressorium morphogenesis by *P. oryzae* )Ryder and Talbot, 2015; Yan and Talbot, 2016( that is the cyclic AMP-dependent protein kinase A signaling pathway is known to play an important role in regulating plant infection, controlling appressorium maturation an turgor driven infection (Xu *et al*., 1997; Yan and Talbot, 2016).

Mitogen active protein kinase (MAPK) signaling cascade have also been shown to play pivotal roles in infection-associated development in fungi. Such MAPKs function downstream of G-protein signaling and cAMP/PKA pathway during differentiation of infection structures. MAPK cascade comprise of three conserved kinases, MAP kinase (MAPKKK). MAP kinase kinase(MAPKK), and MAPK.

The three conserved kinases act sequentially in response to diverse environment signals. In Plant pathogenic fungi, all reported MAP kinases in *P. oryzae* play essential roles in fungal pathogenicity (Xu and Hamer, 1996; Kou and Naqvi, 2016).

However, not only the signaling pathway that mention above but appressorium formation in the rice blast fungus also concern with some other signal transduction pathways. For instance, Target of Rapamycin (TOR), Reaction of oxygen species (ROS), Calcium-dependent signaling pathway and etc. There are several reported revealed that during pre-penetration stages of *P. oryzae* to infect the plant cell are involved several genes of signaling pathway that show in Table 1 and Figure 5.

**Table 1** Key regulator of intracellular signaling pathways involved in infection-associated development during pre-penetration stages of *P. oryzae* (Table was adapted from Kou and Naqvi, 2016)

| Signaling pathway | Fungal pathogen | Genes |
|---|---|---|
| Heterotrimeric G-protein | *P. oryzae* | *MAGA, magB, MGB1, MGG1* |
| Small GTPases | *P. oryzae* | *MgRho3, MgRac1, MgCdc42, MoRAS2* |
| cAMP/Protein kinase A | *P. oryzae* | *Mac1, CPKA, PDEH* |
| MAPK cascade | *P. oryzae* | *MST11, MST7, MST50, PMK1* |

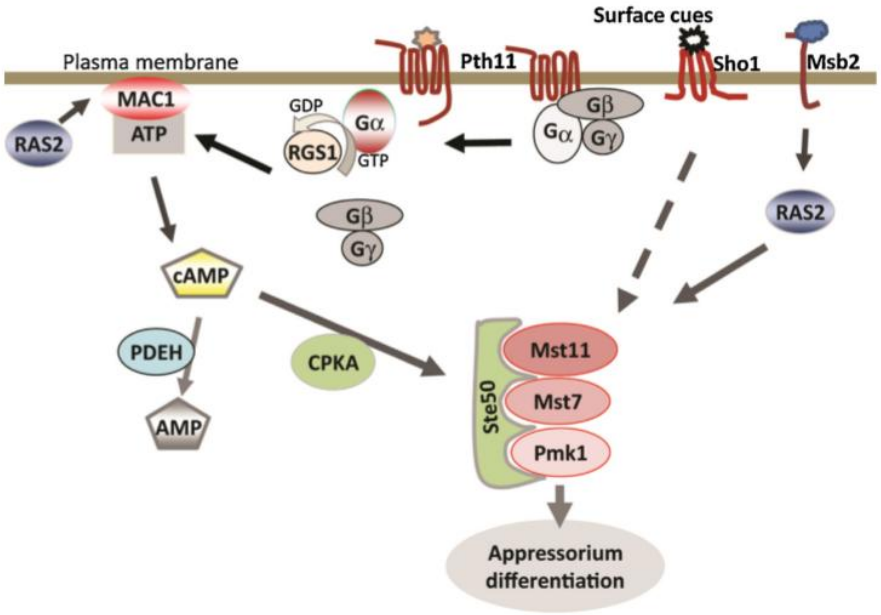



**Figure 5.** Schematic representation of cell signaling for appressorium differentiation in *P. oryzae*. ( Kou and Naqvi, 2016)

## 1.5 Research objectives

A global regulator of pathogenic ascomycete fungi including rice blast fungus that has been studied extensively in secondary metabolism and morphological development is *LaeA gene*. It is conserved in various species of ascomycete fungi including the rice blast fungus, *Pyricularia oryzae* that threaten rice production worldwide in the past until nowadays. However, the role of *LaeA* for appressorium formation remains unclear.

According to the former research in the laboratory of molecular applied microbiology, Graduate School of Agriculture, Hokkaido University was conducted to study on the function of *LaeA2* in *P. oryzae*. The result revealed that the *MolaeA2* deletion mutant reduce the rate of appressorium in onion epidermis. Therefore, this research purposed to further investigate function of regulator gene, *LaeA* by recover

this gene of the Ina 86-137∆*Lig4*∆*LaeA2* (*MoLaeA2* deletion mutant) that was disrupted the *LaeA2* including determine appressorium formation in non-host surface and fungal pathogenicity in the rice plant.

In addition, this research also focus on the role of *LaeA2* on cyclic AMP signal transduction in *Pyricularia oryzae* including the relation of regulator gene, *LaeA* and crucial genes that concern with maintenance of intracellular level of cAMP, *Mac1* and *Pde*.

# CHAPTER 2

## Function of *LaeA2* ortholog in *Pyricularia oryzae*

# CHAPTER 2

## Function of *LaeA2* ortholog in *Pyricularia oryzae*

## 2.1 Introduction

The regulatory gene, *LaeA* (loss of *aflR*-expression) is member of velvet complex that conserve in various species of phytopathogenic ascomycete fungi including the rice blast fungus. It was first described as a global regulator of secondary metabolism in *Aspergillus* spp.(Bok and Kelly, 2004)

*LaeA* gene was reported extensively about a key role in the regulation of secondary metabolites (SMs) in several genus of ascomycete fungi. For instance, *Aspergillus* sp. and *Penicillium* sp. Moreover, this gene implicate with the development and morphogenesis of these fungus.

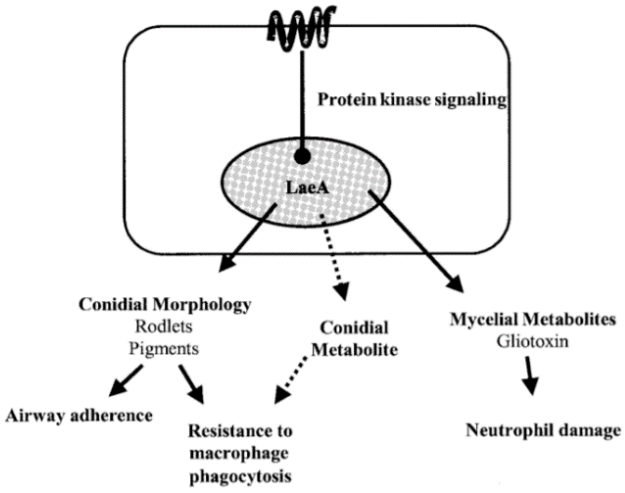



**Figure 6** Propose model of *LaeA* regulation of phenotypes that confer virulence.

(Bok *et al*., 2005)

*LaeA* well known in the role of fungal morphogenesis in ascomycete fungi. For example, most filamentous fungi exhibit both sexual and asexual development with a few exceptions. *LaeA* serves as a regulator of these processes and has been shown to

be involve in controlling the switch between sexual and asexual development in various ascomycete fungi (Jain and Keller, 2013). Bayram *et al*., 2008 explained that in *A. nidulans, LaeA* exhibit light dependent regulation of sporulation. In presence of light, *LaeA* promotes asexual sporulation while reducing the VelB and VeA levels in the nucleus (Bayram *et al*., 2008)

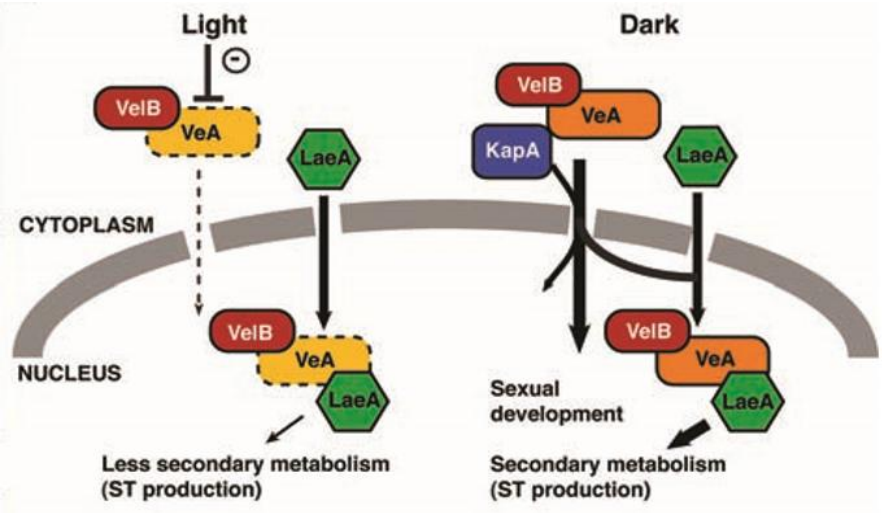



**Figure 7.** Scenario of velvet complex (*LaeA*, *VaeA* and *VelB*) that affects secondary metabolite clusters expression. (Bayram *et al*., 2008)

There are reports studied about function of *LaeA* in pathogenic ascomycete fungi and also indicated that loss of *LaeA* affects for fungal development and secondary metabolism of the fungus. For example, Chang *et al*, 2011 reported about characterization of *LaeA*'s function that *Aspergillus flavus* CA14 affects expression of genes involved in several aspects of conidiophore development. On potato dextrose agar culture medium, conidial chain elongation, production of conidiophores, and colony hydrophobicity were altered. These developmental and physical changes could affect the cellular environment conducive to secondary metabolite production and that *LaeA* may only indirect up regulate secondary metabolite gene expression.

In spot blotch disease that caused by the ascomycete fungus *Cochliobolus sativus.* It is one of the most common and economically important disease in barley. The deletion of *LaeA* produced more conidia under constant dark conditions than under constant light conditions whereas no differences were observed under the two conditions for wild type. Δ*CsLaeA* also showed significantly reduced conidiation under constant light conditions, but produced more small sized conidia under constant dark conditions compared to the wild type strain. Moreover, Δ*CsLaeA* showed some extent of reduction in virulence on susceptible barley plants compared to the wild type strain (Wang *et al*., 2016)

Wang et al., 2017 studied on secondary metabolite gene cluster regulated by LaeA in *Aspergillus niger*, FGSC A1297 by genome sequencing and comparative transcriptomics between the laeA deletion (ΔlaeA) and overexpressing (OE-laeA) mutants. RNA-seq data showed that 281 putative secondary metabolites were produced in OE-laeA mutants, including 22 secondary metabolite backbone genes. LC-MS chemical profiling illustrated that many secondary metabolites were produced in OE-laeA mutants compared to wild type and ΔlaeA mutants. Furthermore, 34 of 61 $Zn_2Cys_6$ transcription factors located in secondary metabolite clusters were differentially expressed between ΔlaeA and OE-laeA mutants.

In addition, there is report in ascomycete pathogenic fungi for *LaeA* along with velvet protein that play a significant role in the virulence. Wiemann et al., 2010 studied on characterization of two components of the *F. fujikuroi* velvet-like complex, *FfVel1* and *FfLae1*. The results showed that deletion of *Ffvel1* and *Fflae1* affect conidiation and virulence of *F. fujikuroi*. Virulence assays were performed with 7 day-old seedlings. The seedlings, grown in glass tubes filled with Vermiculite, were inoculated with agar plugs from plates containing the wild-type, Δ*Ffvel1*, *Ffvel1C* or Δ*Fflae1*

strains. Seedlings inoculated with the GA-deficient strain Δ*cps/ks* or with sterile water served as negative controls while seedlings inoculated with water containing GA3 served as a positive control. After two weeks, those plants infected with the wild-type and the *Ffvel1C* strains showed the typical bakanae symptoms like etiolation of the whole plant with chlorotic stems and leaves similar to the positive control. In contrast, the water control and the plants infected with Δ*cps/ks*, Δ*Ffvel1*, Δ*Ffvel2* and Δ*Fflae1* looked similar to each other with normal height and dark green stems and leaves. These findings clearly show that *FfVel1, FfVel2* and *FfLael1* are virulence factors of *F. fujikuroi* during infection, most likely due to their role as activators for GA biosynthesis.

In rice blast fungus also have report about velvet gene that has the role on fungal pathogenicity. Kim *et al*, 2014 identified and functionally characterized four gene, *MoVOSA*, *MoVELB, MoVEA* and *MoVELC* from the genome of *M. oryzae*. These genes were homologous to the velvet gene in *Aspergillus nidulans*. Deletion of *MoVEA*, *MoVELB*, and *MoVELC* resulted in a significant decrease in conidiation, indicating that their roles as positive regulators thereof. The *MoVELC* gene was involved in development of conidia morphology, while *MoVELB* and *MoVEA* appeared necessary for conidial germination, *MoVEA* further being indispensable for appressorial development and modulation of reactive oxygen species in disease development. Deletion of *MoVELC* affected the cell wall integrity of appressoria, resulting in failure to penetrate host cells. *MoVOSA* appeared dispensable for the development and pathogenicity of *M. oryzae*, even though its homologs play specific roles in other fungal species. This research demonstrate that the velvet genes are linked to *M. oryzae* infecton-related development and pathogenicity.

According to several reports of *LaeA* as mention. *LaeA* is considered as a regulatory gene that involve in various aspect of ascomycete fungi. Deletion or overexpression of this gene showed impact on fungal development, alteration of secondary metabolism and pathogenicity. Although function of *LaeA* orthologs was reported in several genus of filamentous ascomycete fungi that. However, the role of *LaeA* in rice blast fungus in the aspect of apressorium formation have not been clear. In this chapter, the *LaeA2* gene (MG0816) was restored to *P. oryzae* Ina86-137Δ*Lig4*Δ*LaeA2* (*LaeA2* deletion mutant). Function of *LaeA* on appressorium formation in the rice blast fungus was determined in onion epidermis (non-host surface) including fungal pathogenicity was tested in the rice plant (host surface).

## 2.2 Experimental procedures

### 2.2.1 Microorganism and culture conditions.

The mycelium stock of *P. oryzae*, Ina168-137Δ*Lig4*Δ*LaeA2* (*LaeA2* deletion mutant) was used in this study. *Escherichia coli* TOP10 (Invitrogen, Carlbad, CA, USA) was used for recombinant DNA experiment and plasmid construction.

### 2.2.2 Plasmid construction

The amplification of MG08161 was performed and the DNA fragment was inserted into pENTR/D-TOPO vector to produce pENTR-*LaeA* (See Appendix A for PCR composition and PCR condition) by added 2 µl of DNA sample to competent *E. coli* TOP 10 that contain in SOC medium (Appendix C) for electroporation. SOC medium was incubated at 37 $^{o}$C for 1 h. then 200 µl of SOC medium was spread on LB Agar with kanamycin. Thirty colonies of competent *E. coli* that growth on LB agar with kanamycin were picked up for Colony PCR that using GoTaq Green Master Mix (Pomega, Madison, USA), composition of reaction mixture and reaction condition was showed in Appendix A and after that five colonies of competent *E. coli* that contained pENTR-*LaeA* (entry vector) were extracted to check for the the result of insertion. After confirmation by sequencing analysis by using Big dye terminator. These clone were recombinated with PBLASTR (Destination vector) using Gateway®LR Clonase$^{TM}$ enzyme, following the manual's instruction (Appendix A) and the plasmid vector was extracted again to check for the result of transferring.

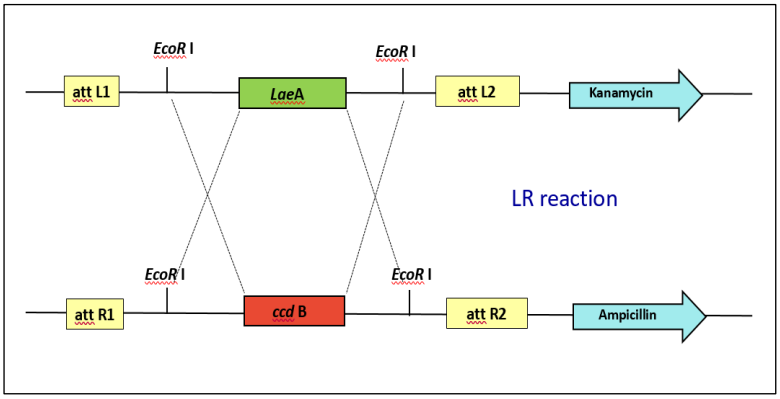



**Figure 8.** Schematic representation of *laeA2* restoration to *Pyricularia oryzea*, Ina86-137Δ*lig4*Δ*laeA2* (*LaeA2* deletion mutant)

**Table 1** Primers used to amplify DNA containing putative promoter and *MoLaeA2*.

| Primer | Sequence |
|---|---|
| *MoLaeA2*F | 5'- CACCCTCCCTGATGTCGTGGTTTT - 3' |
| *MoLaeA*2R | 5'- TGGGCTCTTCAGGATATTGG - 3' |

### 2.2.3 Fungal transformation

Fungal transformation was conducted by Protoplast-PEG method as previously described by Miki *et al*., 2009 and Abe *et al*., 2006. *P. oryzae* Ina36-137 Δ *Lig4*Δ*laeA2* culture was inoculate on oatmeal agar incubated at 25 $^{o}$C for 2 weeks under light condition. Then scrapped gently to eliminate the cloudy mycelia and re-incubated at 27 $^{o}$C for 3 days. Prepare the fungal conidia suspension by adding 5 ml

of 2YEG broth (Appendix C) to the culture plate for scrapped and filtrate through Miracloth (Calbiochem, San Diego, CA). This fungal conidia suspension was determined using haemacytometer and diluted the concentration to be $10^5$ cell/ml then inoculate to 200 ml of 2YEG for 3 days incubation at $27^o$ C. After this period, the mycelia was harvested by filtering through Miracloth and weighed for digestion of fungal mycelium.

Digestion buffer and enzyme mixed (Appendix B) was prepared to digest the fungal mycelium. When adding this buffer to fungal mycelium, incubated at 37 $^o$C for 1 h. by shaking gently. Protoplast suspension was filtered through Miracloth and the filtrate was centrifuged at 3,000 rpm for 15 min. at $4^o$C. 500 µl of STC buffer (Appendix B) 300 µl of STC buffer was required for washing step and centrifuged as mention. Finally, re-suspended by 300 µl of STC buffer and determined number of protoplast using haemacytometer.

The transformation step was initiated by adjust the fungal protoplast to $10^8$ protoplasts/ml. 10 µg of pBLASTR that contains *LaeA* was added to 100 µl of protoplast suspension and incubated on ice for 20 min. Then 2 ml. of polyethylene glycol (PEG) (Appendix B) was added, mixed gently and cooling on ice again for 20 min. 30 ml STC buffer was added, mixed gently and centrifuged at 3,500 rpm for 10 min at $4^o$C. The pellet was re-suspended in 200 µl STC buffer.25 ml of warm Bottom agar (Appendix C) was poured into suspension of the pellet, mixed thoroughly and aliquot to 2 petri dishes incubated at 27 $^o$C 24 h. After checked the regeneration of protoplast, overlay by Top agar (The bottom agar without glucose and mixed with 5 µg/ml blasticidin S). Incubated at 25 C with fluorescent for 5 days. Picked up colonies of putative transformant from top agar to inoculate on oatmeal agar plates.

In order to isolate single conidia of fungus as describe previously (Sone et al.,1997), a piece of oatmeal agar was cut and stamped on water agar (40 % agar) incubated at 27 $^{o}$C overnight. Then germinated conidium was isolated individually using sterile needle under stereomicroscope and 24 well plate of prune agar (Appendix C) incubate at 27 $^{o}$C until the fungus growth for the next step.

### 2.2.4 Screening of putative transformants

DNA of each strain of putative transformant that growth on 24-well plate of prune agar was extracted from mycelia. Fungal mycelia was cut from prune agar as a small piece and transferred to 1.5 ml. microcentrifuge tube. 500 µl. of lysis buffer (Appendix B) was added and the fungal mycelia were ground. DNA was precipitate by ethanol precipitation and DNA pellets were re-dissolved with 30 µl. of TE buffer. Each DNA of putative transformant was used as a template for PCR performed with KOD plus ver. 2 and designed primer (Table 1)(Appendix A for PCR condition)

### 2.2.5 Southern Hybridization

**- Fungal genomic DNA extraction**

Fungal genomic DNA extraction was done as previously described by Sornkom et al., 2015. The mycelia of selected putative transformants that cultured in 40 ml of 2YEG broth at 27$^{o}$C for 5 days with shaking were harvested and prepared for genomic DNA isolation. Mycelia were Freeze-dried for 12 h. then homogenized as a powdery by using multi bead shocker. 500 µl. of DNA extraction buffer (Appendix B) was added to homogenized mycelia and mixed by vortex mixer. 350 µl. of phenol and 150 µl. chloroform were added to mycelia suspension, mixed and centrifuged at 15,000 rpm for 1 h. The upper aqueous phase was pipette into a new

Eppendorf tube, added 25 µl. of RNase to prevent RNA contamination and incubate at 37$^{o}$c for 1 h. Then 500 µl. of phenol was added, mixed and centrifuged at 15,000 rpm for 5 min., and then pipette the upper part to a new tube, then repeated the previous step again. The phenol 250 µl. and chloroform 250 µl. were added into the tube. Mixed, and centrifuged at 15,000 rpm for 5 min., transferred the upper suspension to a new tube, follow by repeating this step again. Then 500 µl. of chloroform was added, mixed, and centrifuge to grey the upper aqueous phase again. Isopropanol 0.6 volume of solution in tube was added to precipitate the DNA for 20 min and centrifuged at 15,000 rpm for 15 min. The supernatant was discarded and the pellet was washed with 70 % ethanol and allow DNA pellets dry then re-dissolved in 50 µl. TE buffer.

**- DNA digestion and Gel electrophoresis**

Genome DNAs of transformants were digest with EcoRV-HF and XhoI restriction enzymes (TAKARA, Japan). The digested genomic DNAs were precipitated with ethanol and dissolved in 10 µl. Then Agarose gel (0.6% w/v; Seakem GTG) electrophoresis in TAE was performed. The restriction fragments were observed and photographed by using UV transilluminator.

## - DNA transfer

Digested genomic DNA in the gel was transferred to a nylon transfer membrane (Amersham Hybond-N+, GE Healthcare UK, England) following the manufacturers's instruction with minor modifications. The gel was placed right side up on top of three pieces of filter paper (Whatman® 3MM,England) draped over and electrophoresis tray used as a plateform. The filter paper served as wick for the 20XSSC buffer (Appendix B). One piece of nylon member, three pieces of filter papers, 5 cm

stack of towel papers, electrophoresis cover and weight were laid over the gel. The set-up was left standing undisturbed overnight. After an overnight DNA transfer, the DNA bands blotted onto the membrane was mixed by UV irradiation using the spectrolinker (XL1000s UV Crosslinker; Spetronics Corp., Japan) This blotted membrane was kept in a hybridization bag at 4$^{o}$C

## - Probe preparation

The pBRAST-DEST-*LaeA* was digested by EcoRI (TAKARA, Japan) which cut at the 5' end and 3' end of *LaeA* fragment. Gel electrophoresis was performed. The gel was cut and purify the probe band in the gel using Nucleospin® Gel and PCR Clean-up (MACHERY-NAGEL GmbH&Co. KG, Germany). Examination for final concentration of probe was done and it should be 10 ng/ µl. for probe labeling in the next step.

## - DNA hybridization

Hybridization buffer (Appendix B) was warmed at 55oC was warmed to add into the hybridization bag in the volume of 0.25 ml/cm2 of membrane size and incubated at 55$^{o}$C with shaking. During the membrane had been incubating, the probe was labeled using AlkPhos Direct Labelling system (Amersham, GE Healthcare, England). The cross-linker solution. DNA probe was diluted to a concentration of 10 ng/ µl. using water supplied in the kit. 10 µl. of the diluted DNA was placed in 1.5 ml microcentrifuge tube and denatured by heating at 98$^{o}$C for 5 min. Immediately cooled in ice for 5 min. Reaction buffer (10 µl) was added to the cooled DNA, mixed gently, then added 2 µl. of labeling reagent and mixed gently. The diluted cross-linker solution (10 µl) was added and incubated at 37$^{o}$C for 30 min.

The labeled probe was added into a hybridization bag containing membrane with hybridization buffer and incubated at 55$^{o}$C with shaking overnight. After overnight hybridization, the membrane was washed by 250 ml. of primary wash buffer (Appendix B) which was pre-heated at 55$^{o}$C before use. The membrane was incubated at 55$^{o}$C for 10 min with shaking and washed again with primary wash buffer. The membrane was then washed with 250 ml secondary wash buffer (Appendix B) twice, gently shaking at room temperature for 5 min. Following pipetting (30-40 µl./cm2 of membrane size) CDP-Star$^{TM}$ detection reagent (Amersham, GE Healthcare UK Ltd.) over a membrane and leaved the membrane at the room temperature for 2 and 5 min, signal detection of chemiluminescence was conducted by luminescent image analyzer LAS-4000 (Fujifilm, Japan).

### 2.2.6 Onion epidermis assay

The host cell was represented by onion epidermis to analysis of appressorium formation. Onions were cut to be 2 cm$^{2}$. The epidermis was peeled out, soaked in distilled water and heat-killed for 45 second using microwave oven. Conidia suspension ($10^{5}$ spore/ml.) was dropped on the hydrophobic of epidermis layer. Incubated at 27 $^{o}$C for 24 hr. Percentage of appressorium formation was determined (Sornkom et al., 20017).

### 2.2.7 Spraying inoculation

Stock culture of wild type, *LaeA2* deletion mutants, Tranformant no.9 (T9) and no.11 (T11) were cultured on oatmeal agar. $10^{4}$ - $10^{5}$ cells/ml. of fungal spore suspension in 0.02 % Tween20 was used for spraying on to the fourth to fifth leaf stage of *Shin-2* rice (*Sativa oryza*). The inoculated plants were transferred to chamber at 100 % humidity at 25$^{o}$C in the dark for 24h and then using fluorescent light for 1 week

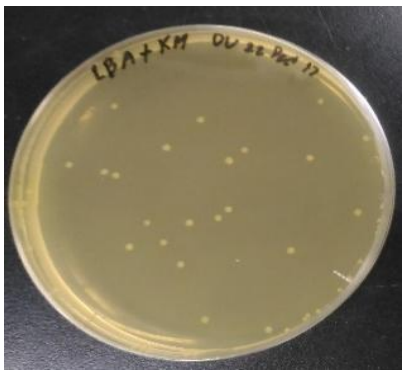
of incubation. Disease severity were scored after 7 days of inoculation. (Miki *et al.*, 2009)

### 2.2.8 Intact rice sheath assay

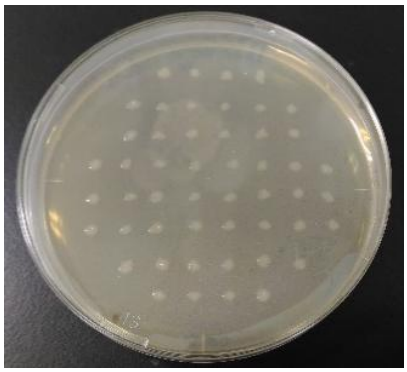
The inoculation of fungal spore suspension to leaf sheath was performed (Koga *et al*., 2004). Fungal spore suspension was diluted to be $10^4$ - $10^5$ cells/ml. and inoculated to *Shin-2* rice leaf sheath that grown in greenhouse for 2 months old. After 12 hr. of incubation, the inoculated inner layer of leaf sheath was prepared using wet slide technique and observed under microscope (Olympus, Tokyo, Japan).

## 2.3 Results

Result of plasmid construction for recover *LaeA2* gene (MG08161) to the *MoLaeA2* deletion mutant showed that the colonies of competent *E. coli* TOP 10 can growth on LB agar with kanamycin (Figure 9A) then 30 colonies were picked up for Colony PCR (Figure 9B) and result of colony PCR showed in Figure 10A and B. Five colonies of competent *E. coli* that contained pENTR-*LaeA2* (entry vector) were selected to extract for plasmid DNA and checked for the result of insertion (Figure 11) using EcoRI. The result of Gateway LR Clonase to transfer the entry vector to the destination vector showed that competent *E. coli* can growth on LB agar with ampicillin (Figure 12A). The plasmid vector was extracted to confirm the transferring (Figure 12B).



(A.)

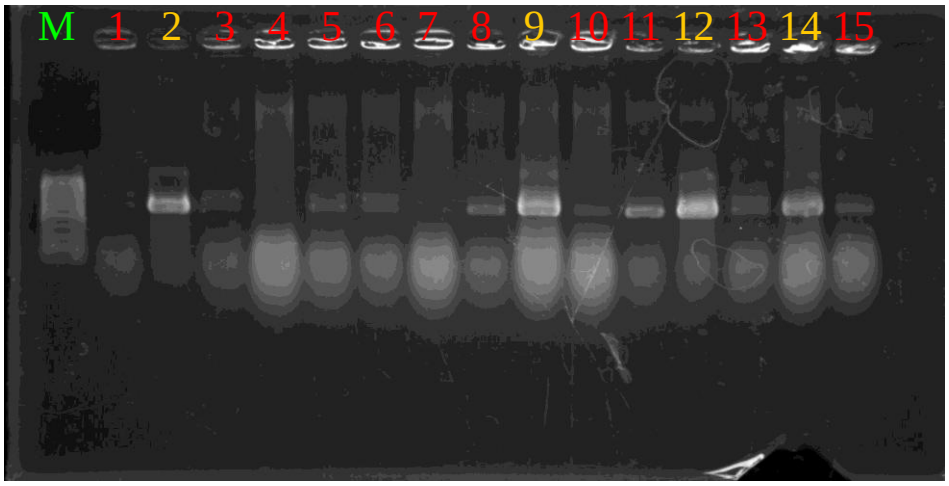


(B.)

**Figure 9 A.** Growth of competent *E. coli* TOP 10 on LB agar with kanamycin.

**B.** Selected competent *E. coli* TOP 10 for colony PCR.

(A.)




(B.)

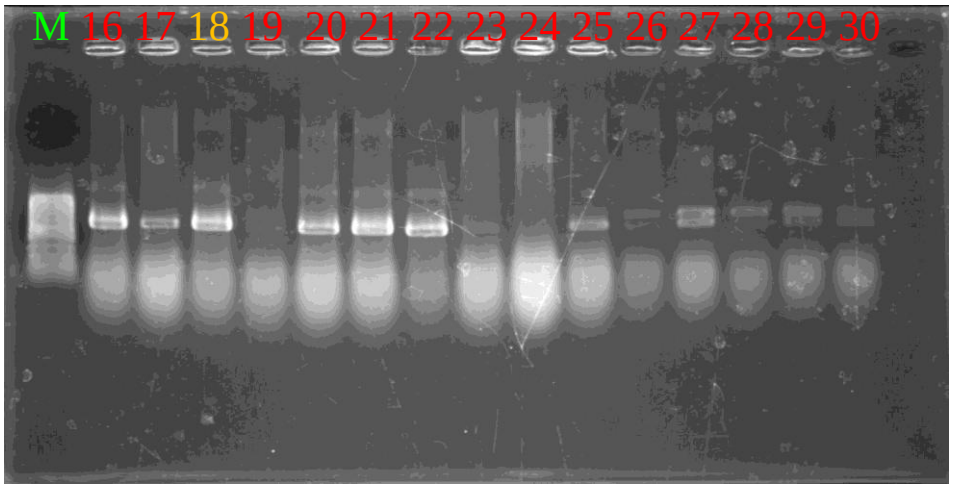



**Figure 10 A and B.** Colony PCR of competent *E. coli* TOP 10 that contained pENTR-*LaeA2*.

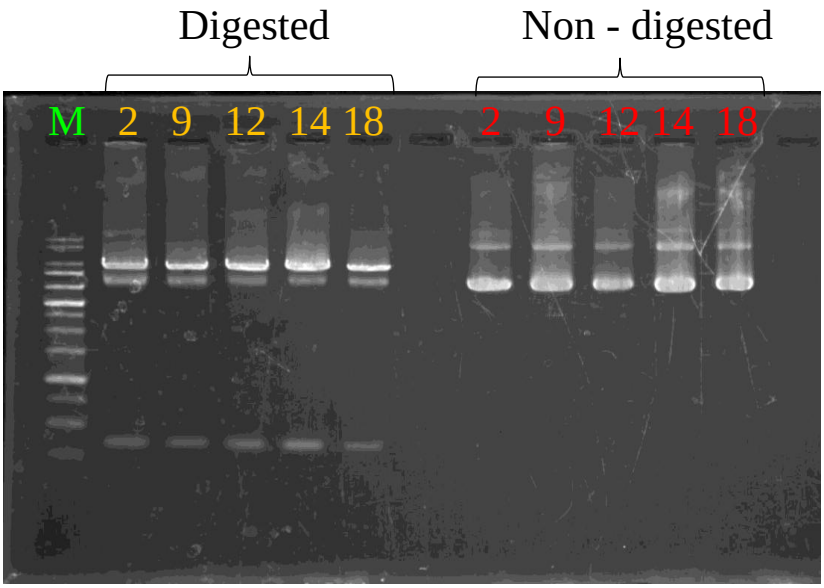
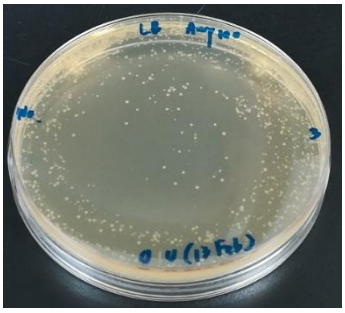
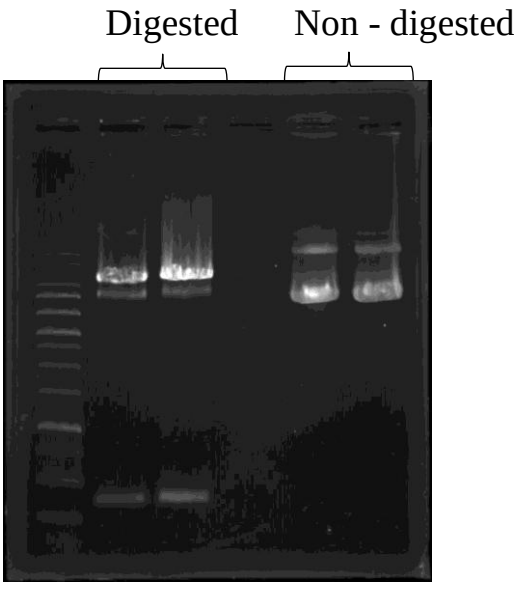



**Figure 11.** The entry vectors (pENTR-*LaeA2*) were digested by EcoRI.



(A.)



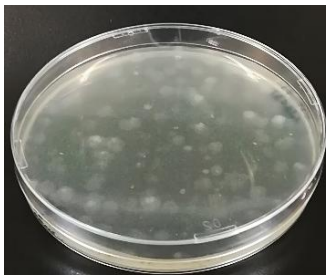

(B.)

**Figure 12 A.** Growth of competent *E. coli* TOP 10 on LB agar with ampicilin.

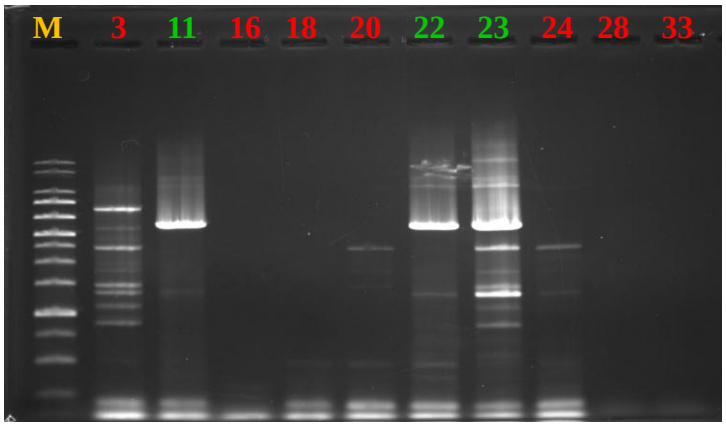
**B.** The destination vectors (pBLASR-*LaeA2*) were digested by EcoRI.

The result of fungal transformations by Protoplast-PEG method showed that colonies of putative transformant can growth on top agar with blasticidin S (Figure13 A). DNA extraction of each strain was extracted and determined by PCR, result showed in Figure 13 B and C.

(A.)



(B.)




(C.)

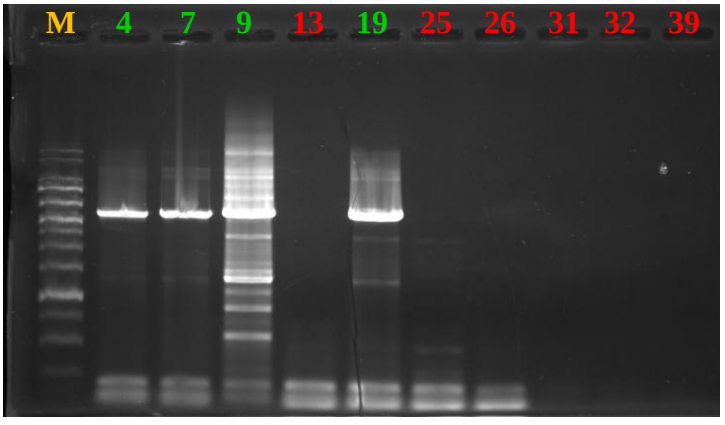



**Figure 13. A.** Growth of putative transformants on top agar with blasticidin S **B and C.** Screening of putative transformants by PCR.

Finally, southern hybridization was conducted to confirm the result *LaeA2* restoration to Ina 86- 137Δ*Lig4*Δ*LaeA2* (*LaeA2* deletion mutant, MT). The result of Southern hybridization showed in Figure 14.

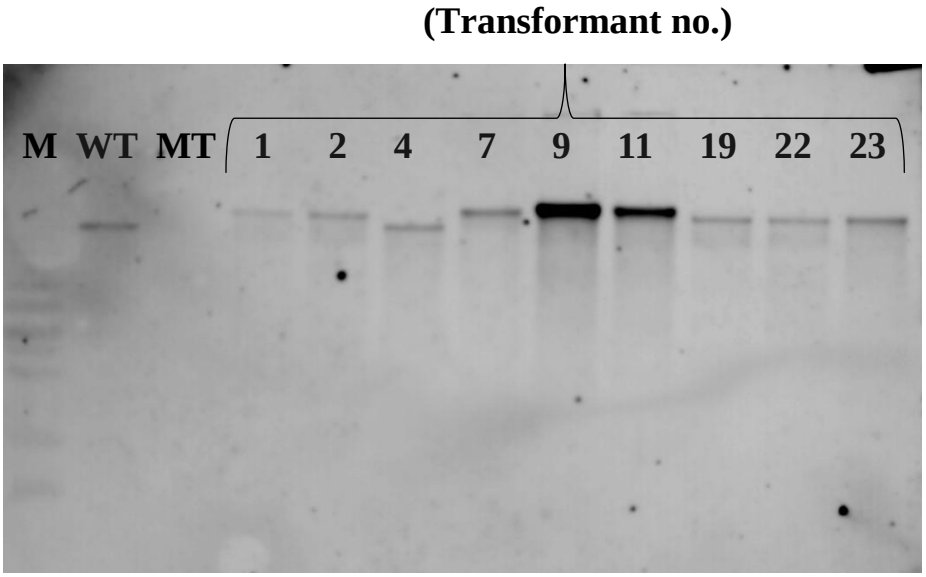



**Figure 14.** Southern analysis of Ina 86-17 (wild type, WT), Ina 86- 137Δ*Lig4*Δ*LaeA2* (*LaeA2* deletion mutant, MT), and the complementation strains (T1, T2, T4, T7, T9, T11 T19, T22 and T23).

## Onion epidermis assay

The result showed that more than 80% of appressorium formation rate was detected on killed onion epidermis after inoculated with wild type and each complementation strain. On the other hand, the appressorium formation rate of *MoLaeA2* was significantly decreased to less than 20% (Figure 15.)

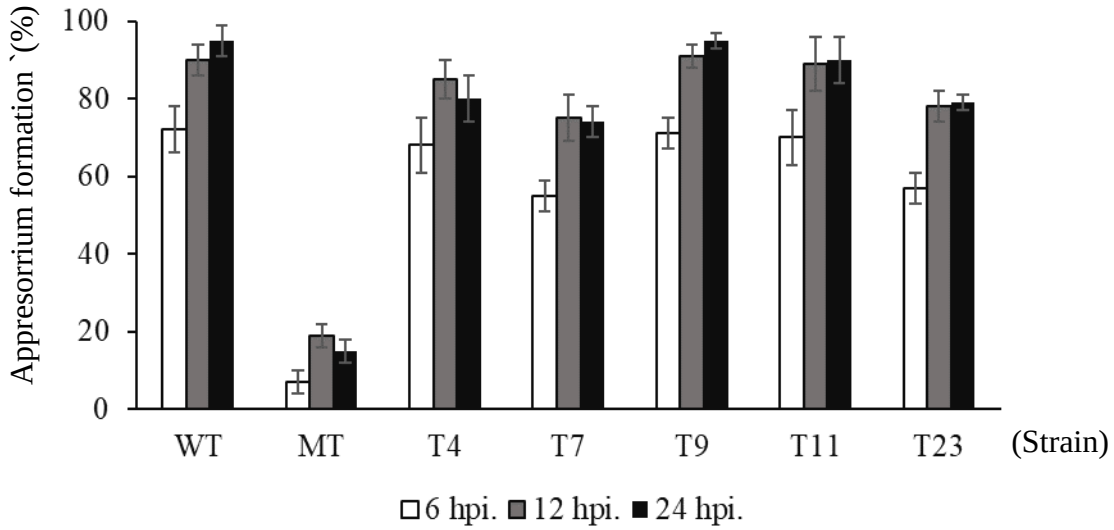



**Figure 15.** Percentage of appressorium formation in hydrophobic onion epidermis after inoculated at 6, 12 and 24 h. with Ina86-137 (wild type, WT), Ina86-137Δ*lig4*Δ*laeA2* (*LaeA2* deletion mutant, MT) and complementation strains (T4, T7, T9, T11 and T23).

## Spraying inoculation assay

The results of spraying inoculation assay indicated that all rice samples that were sprayed with *MoLaeA2* deletion mutant and complementation strains (T9 and T11) revealed the development of necrotic lesion on rice leaves that similar to wild type (Figure 16).

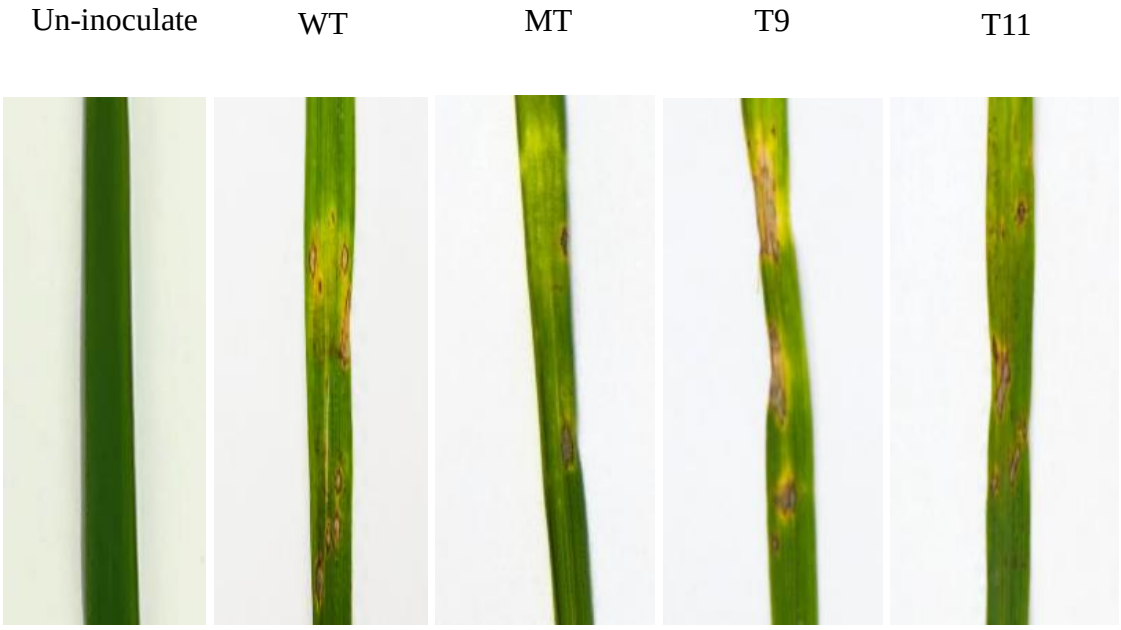


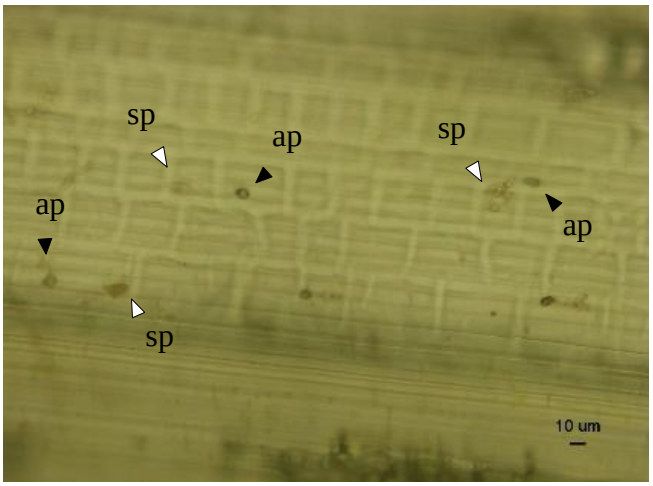

**Figure 16.** Susceptible of *Shin*-2-rice leave were sprayed with each fungal strain. Un-inoculated, *P. oryzae* Ina86-137 (WT), *P. oryzae* Ina86-137Δ*lig4*Δ*laeA2* (MT), Transformant no.9 (T9) and Transformant no.11 (T11) respectively.

## Intact rice sheath assay

The results of intact rice sheath assay confirmed the present of appressorium formation in all sample of rice tissue that was inoculated with each fungal strain. The results showed in Figure 17.

(A.)


(B.)

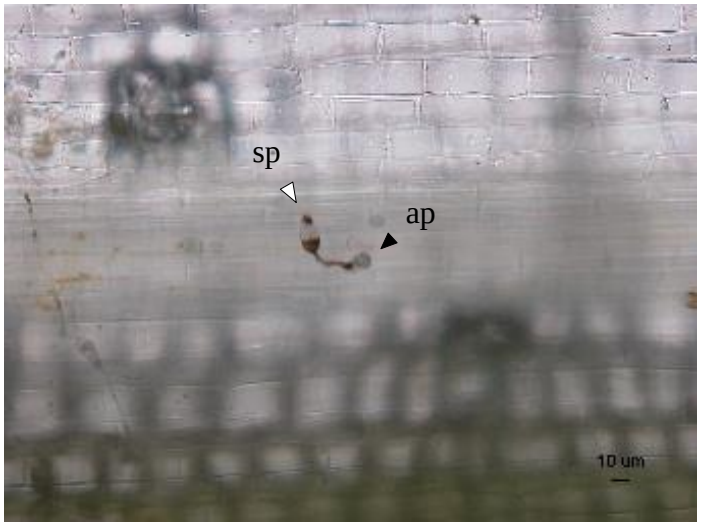



◄ appressorium (ap)
◅ spore (sp)

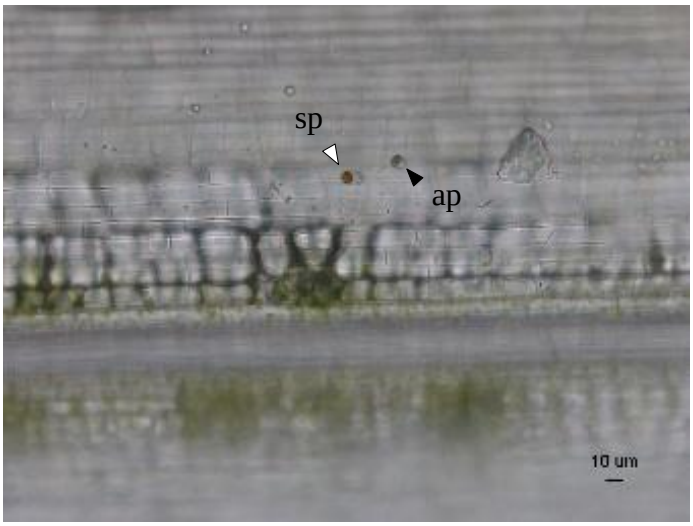
**Figure 17 A.** Appressorium formation in rice tissue after inoculated with wild type.

**B.** Appressorium formation in rice tissue after inoculated with *LaeA2* deletion mutant.

(C.)




(D.)




◄ appressorium (ap)
◁ spore (sp)

**Figure 17 (continued)** **C.** Appressorium formation in rice tissue after inoculated with T9.

**D.** Appressorium formation in rice tissue after inoculated with T11.

## 2.4 Discussion

In this chapter, purposed to study the function of the regulatory gene, *LaeA2* of *P. oryzae*. Deletion mutant, Ina86-137 Δ*lig4*Δ*laeA2* and its complementation strain were constructed and investigate the effect of *LaeA* on appressorium formation. Wild type and complementation strains exhibited high percentage of appressorium formation in killed onion epidermis but Ina86-137 Δ*lig4*Δ*laeA2* showed decrease in appressorium formation. However, result of spraying inoculation and intact rice sheath assay showed that all fungal strains can cause the lesions on rice leaves and formed appressoria in ricetissue. These assays indicated that lack of *LaeA2* leads to defective appressorium formation. Therefore, *LaeA2* is required for appressorium formation in non-host surface. However, deletion of *LaeA2* does not effect for fungal pathogenicity on rice.

In 2018, Yanyong et al. investigated proteomics analysis of *Aspergillus flavus* and found that deletion of *laeA* in *A. flavus* resulted in up-regulated of protein involved in chromation remodeling and modification, histone deacetylase including Izawa *et al*., 2009 revealed that using an antifungal antibiotic, Trichostatin A (TSA) to inhibit histone deacetylase causes the rate of appressorium formation in the rice blast fungus decreased significantly. Moreover, Lee *et al*., 2019 studied the role of gene in rice blast fungus, *MoHOS2* that concern with histone deacetylase. The results indicated that deletion of *MoHOS2* led to 25 % reduction in histone deacetylase activity. In addition, the deletion mutant exhibited defect in appressorium formation from both germ tip and hyphae. Therefore, it is possible that function of *LaeA* on appressorium formation in rice blast fungus might concern with regulation or alteration of histone deacetylase.

# CHAPTER 3

## The role of *LaeA2* on cyclic AMP signal transduction in *Pyricularia oryzae*

# CHAPTER 3

## The role of *LaeA2* on cyclic AMP signal transduction in *Pyricularia oryzae*

### 3.1 Introduction

As we know that a major pathway that concern with appressorium formation in rice blast fungus is cAMP signaling pathway. Cyclic AMP or cAMP (Cyclic adenosine 5'-monophosphate) is a well characterized secondary messenger in both prokaryotes and eukaryotes. It is also well known for its essential role in growth and morphogenesis in fungi as well as appressorium formation in rice blast fungus (Adachi & Hamer, 1998).

The intracellular levels of cAMP are regulated by the balance between the activities of two enzymes adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE). Adenylase cyclase are activated downstream from G-protein-coupled receptors (GPCRs). Then it can activate several effectors to trigger fungal morphogenesis and appressorium formation. cAMP signal transduction of rice blast fungus showed in Figure 18.

Lee and Dean, 1993 reported that cAMP are important components to induce and/or mediate appressorium formation in *M. grisea*. Infection structure formation is a cAMP-mediated process. The interaction between the emerging germ tube of *M. grisea* and the hydrophobic surface results in the accumulation of cAMP in the fungus. An elevated endogenous leve1 of cAMP in turn may either indirectly or directly trigger pathways leading to new gene expression and infection structure formation

Choi and Dean, 1997 showed that deletion of MAC1 gene results in mutant (Δmac1) which is non-pathogenic because the Δmac1 is unable to synthesize cAMP and impaired in differentiation into appressoria. Morover, addition of exogenous cAMP

allows appressorium development and restores pathogenicity of the mutant. This evidence confirms that the catalytic activity of the MAC1 gene is necessary for appressorium development.

In contrast, *Pde,* cAMP Phosphodiesterase is an important gene that maintenance of intracellular level of cAMP by hydrolysis cAMP. Naqvi and Ramanujam, 2010. Characterize gene-deltion mutants of a high-affinity (PdeH) and a low affinity (PdeL) in the rice blast fungus to gain insight the spatial and temporal regulation of the cAMP signaling. The result revealed that PdeH activity was found to be a key regulator of asexual and pathogenic development in *M. or*yzae. Loss of PdeH led to increased accumulation of intracellular cAMP during vegetative and infectious growth.

These previous reports showed that the level of intracellular cAMP is important for rice blast fungus in appressorium formation. Therefore, in this chapter, exogenous chemical treatment to each strain of rice blast fungus was performed. Then appressorium formation was examined to elucidate the role of *LaeA2* on cyclic AMP signal transduction. Moreover, the concentration of intracellular cAMP level also examined by High-performance liquid chromatography.

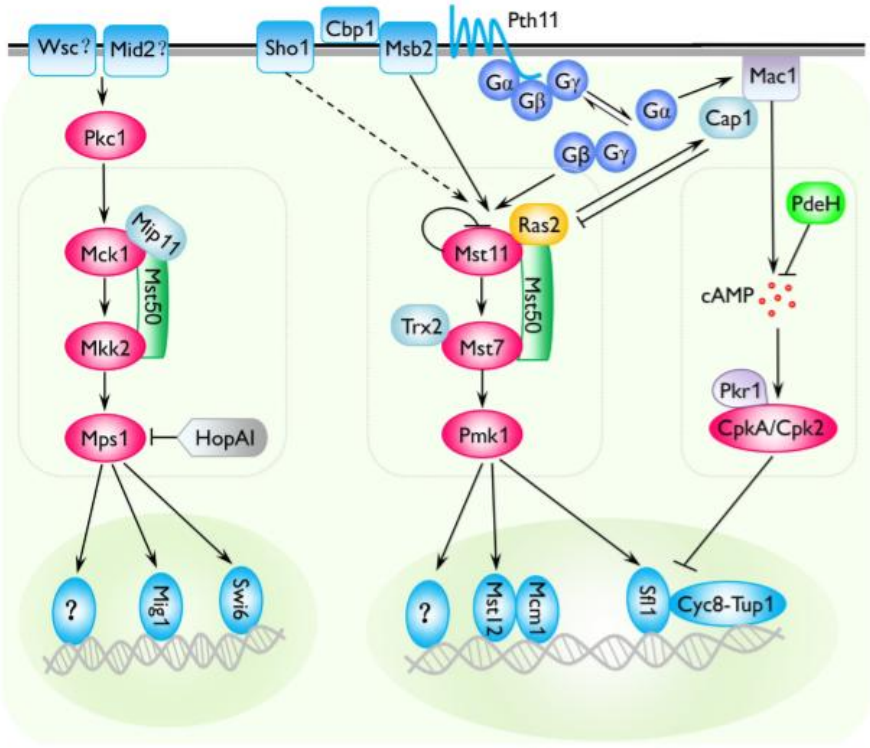



**Figure 18.** cAMP signaling pathway and its relationship with the Pmk1 and Mps1 pathways and in *P. oryzae* (Jiang *et al*, 2018).

## 3.2 Experiment procedures

### 3.2.1 Preparation of fungal conidia suspension

Four strain of rice blast fungus that consist of Ina 86-137 (wild type, WT) Ina 86-137Δ *Lig4ΔLaeA2* (Mo*LaeA2* deletion mutant, MT) and complementation strains (T9 and T11) were used to investigate appressorium formation on onion epidermis with treatment of exogenous chemical to fungal conidia suspension. Each strain was inoculated on oat meal agar and incubated at 25 $^{o}$C for 7 days in the light condition. Then cell scarper was used for scrape the mycelia gently to eliminate the cloudy mycelia and re-incubated for 3 days at the same condition. After this period, 5 ml of sterile distilled water was added to each culture plate then scrape gently to harvest fungal conidia. Fungal conidia suspension was collected and filter through Miracloth to get rid of debrish. Then adjust the fungal conidia suspension to be 10 $^{4}$ cell/ml. before use.

### 3.2.2 cAMP treatment

For the assays of appressorium formation that induced by chemicals, 10 mM exogenous cAMP (3′, 5′ cyclic Adenosine monophosphate) was added to conidial suspensions of each fungal strain as described by previous study (Choi and Dean, 1997). Each fungal conidia suspension as mention was inoculated in onion epidermis to assessed appressorium formation under microscope after incubating samples in a humid chamber at 27 °C for 6, 12 and 24 hpi. Microscopic and photograph were performed. Each test was repeated three times.

### 3.2.3 3-isobutyl-1-methylxantrine treatment

For the assays of appressorium formation that induced by chemicals, 10 mM exogenous 3-isobutyl-1-methylxantrine (IBMX, a phosphodiesterase inhibitor) was added to conidial suspensions of each fungal strain as described by previous study (Skamnioti and Gurr, 2007). Each fungal conidia suspension as mention was inoculated in onion epidermis to assessed appressorium formation under microscope after incubating samples in a humid chamber at 27 °C for 6, 12 and 24 hpi. Microscopic and photograph were performed. Each test was repeated three times.

### 3.2.4 cAMP extraction

Each fungal strain as mention was culture into 2YEG broth and incubated at 27 $^{o}$C and shaking at 150 rpm for 5 days. Mycelia of each strain was harvested and lyophilized for 12 h. Then grind the mycelia to be powdery by using bead shocker. Intracellular cAMP extraction was performed according to the established procedures (Liu et al., 2007). 200 µl iced-cold 6% Trichloro Acetic acid (TCA) was added to the fungal mycelia and incubated on ice for 10 min. After centrifuge at 4,000 rpm for 15 min at 4 $^{o}$C, the supernatant was collected and washed four times with five volumes of water-saturated diethyl ether. The pellet was collected for quantification of the cAMP level by High-performance liquid chromatography (HPLC).

### 3.2.5 Analysis of intracellular cAMP level

The cAMP levels were quantified by HPLC analysis with programmable ChromNAV Chromatography Data System, Jasco. The solvent system used in this experiment as described previously (Spata *et al*., 1991), at teh flow rate of 1 ml per minute; 0.1 mg of cAMP solution per millilter was eluted through the column ($5C_{18}$, 4.6x150 mm.) and was detected at 254 nm. UV. Each sample was eluted through

the column in turn and peak values were detected. Standard concentration of cAMP in deionized water containing 0.005, 0.01, 0.03, 0.05, 0.07 and 0.10 mmol. were used in this study.

## 3.3 Results

### 3.3.1 cAMP Treatment

The results of exogenous cAMP treatment to fungal conidia suspension of Ina 86-137 (wild type, WT) Ina 86-137 Δ *Lig4*Δ*LaeA2* (Mo*LaeA2* deletion mutant, MT) and complementation strains (T9 and T11) showed up to 59 % of appressorium formation of *LaeA* deletion mutant was recovered in onion epidermis (Figure 19) and the phenotype of each strain when tested in onion epidermis showed in Figure 20 A to D.

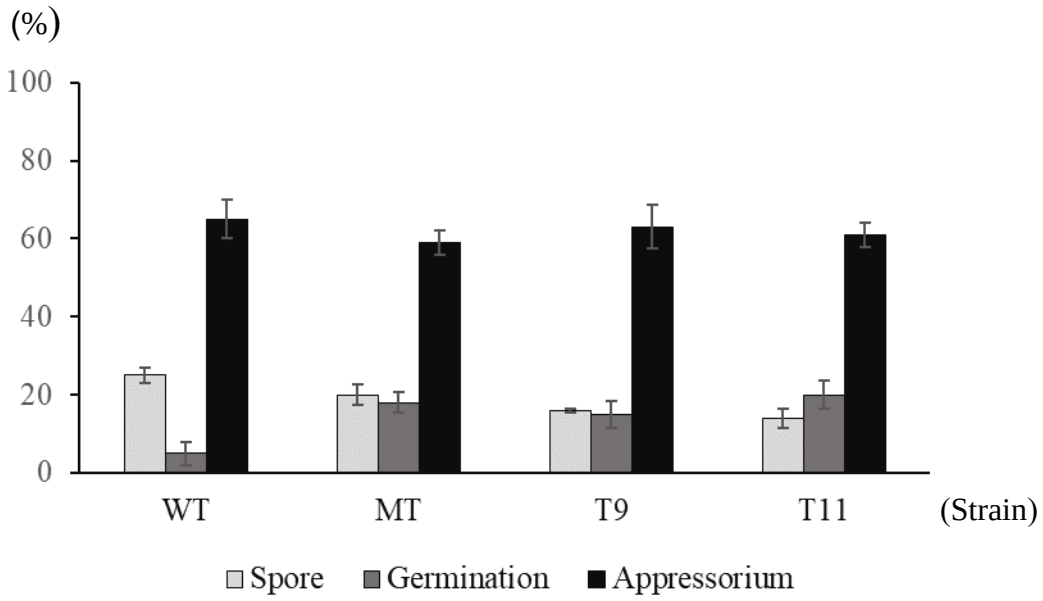
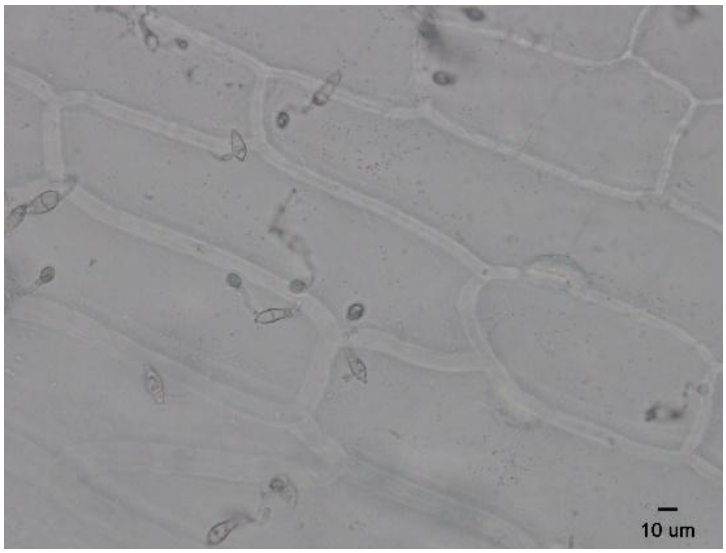



**Figure 19.** Appressorium formation of each fungal strain after applied with 10 mM exogenous cAMP to fungal conidia suspension and inoculated in onion epidermis.

(A.)




(B.)

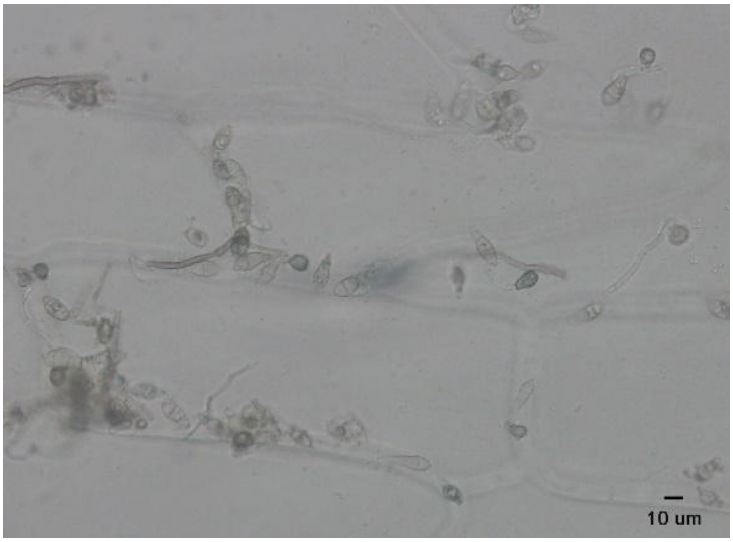
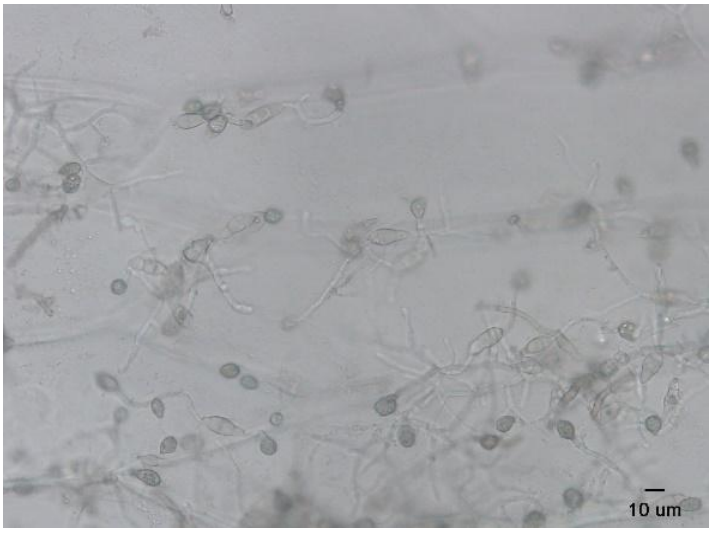



**Figure 20.** A. Phynotype of wild type after applied with 10 mM cAMP.

B. Phynotype of *LaeA2* deletion mutant after applied with 10 mM cAMP.

(C.)




(D.)

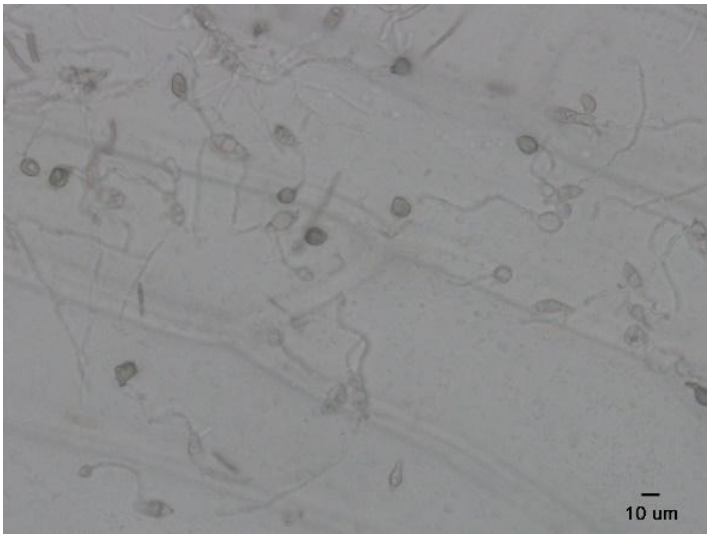



**Figure 20.** (continued) C. Phynotype of T9 after applied with 10 mM cAMP.

D. Phynotype of T11after applied with 10 mM cAMP.

### 3.3.2 3-isobutyl-1-methylxanthine Treatment

The results of exogenous 3-isobutyl-1-methylxanthine treatment to fungal conidia suspension of Ina 86-137 (wild type, WT) Ina 86-137 Δ*Lig4*Δ*LaeA2* (Mo*LaeA2* deletion mutant, MT) and complementation strains (T9 and T11) showed up to 70 % of appressorium formation of *LaeA* deletion mutant was recovered in onion epidermis (Fig 21) and the phenotype of each strain when tested in onion epidermis showed in Figure 22 A to D.

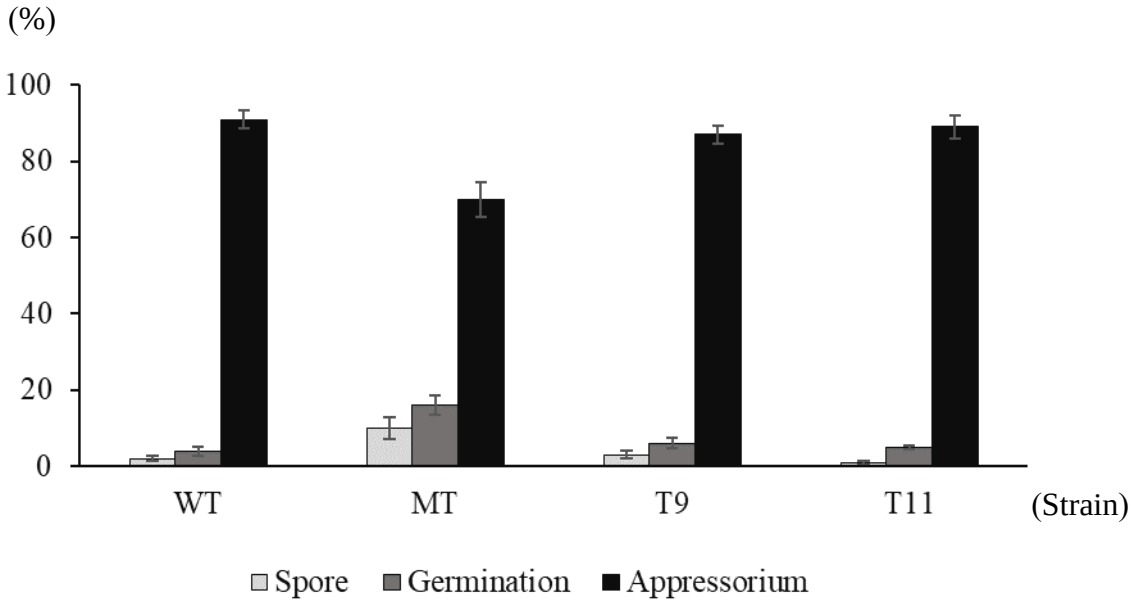
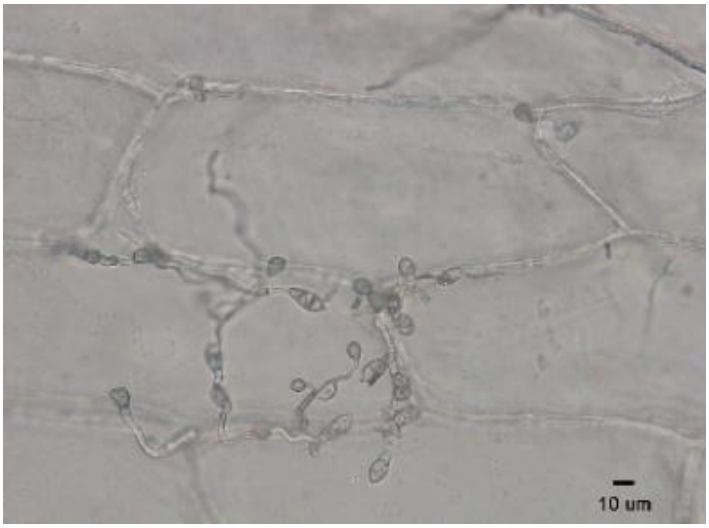



**Figure 21.** Appressorium formation of each fungal strain after applied with 2.5 mM exogenous 3-isobutyl-1-methylxanthine to fungal conidia suspension and inoculated in onion epidermis.

(A.)




(B.)

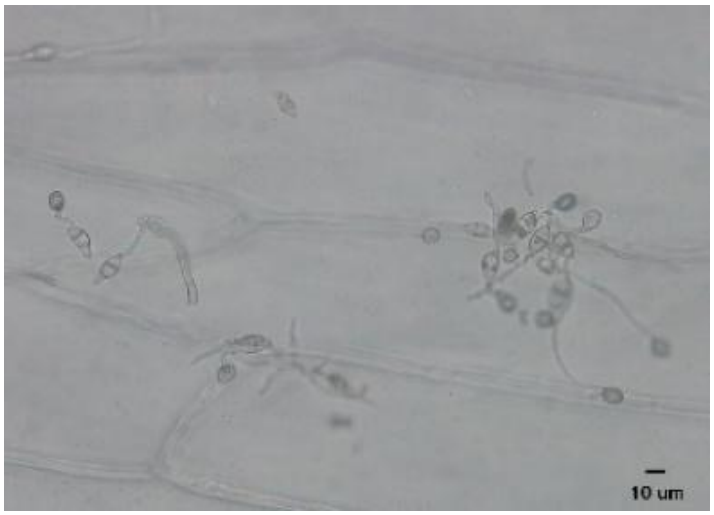
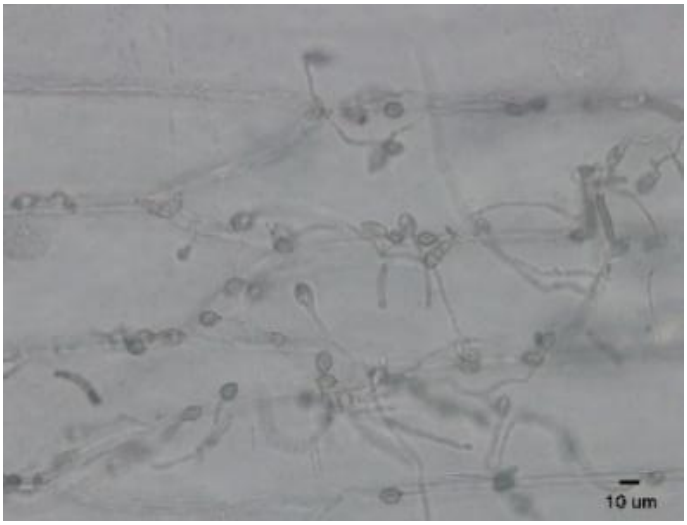



**Figure 22.** A. Phynotype of wild type after applied with 2.5 mM IBMX.

B. Phynotype of *LaeA2* deletion mutant after applied with 2.5 mM IBMX.

(C.)




(D.)

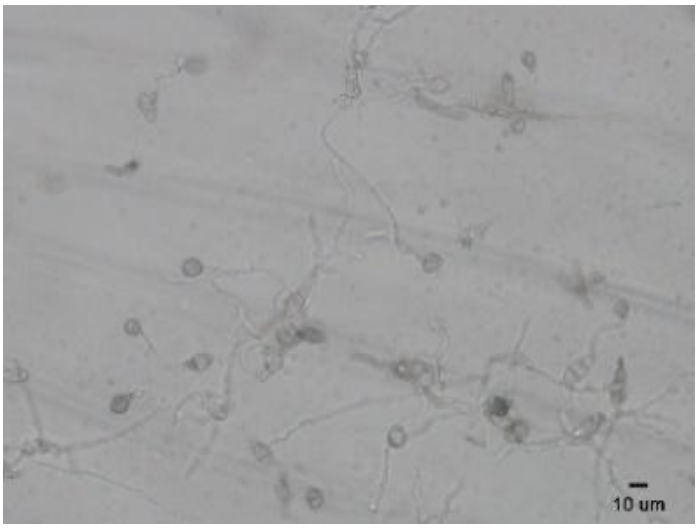



**Figure 22.** (continued) C. Phynotype of T9 after applied with 2.5 mM IBMX.

D. Phynotype of T11after applied with 2.5 mM IBMX.

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### 3.3.3 Analysis of intracellular cAMP level

The results of intracellular cAMP level analysis by high-performance liquid chromatography displayed intracellular cAMP in *MoLaeA2* deletion mutant is significantly lower than wild type and complementation strains. The results showed in Figure. 23

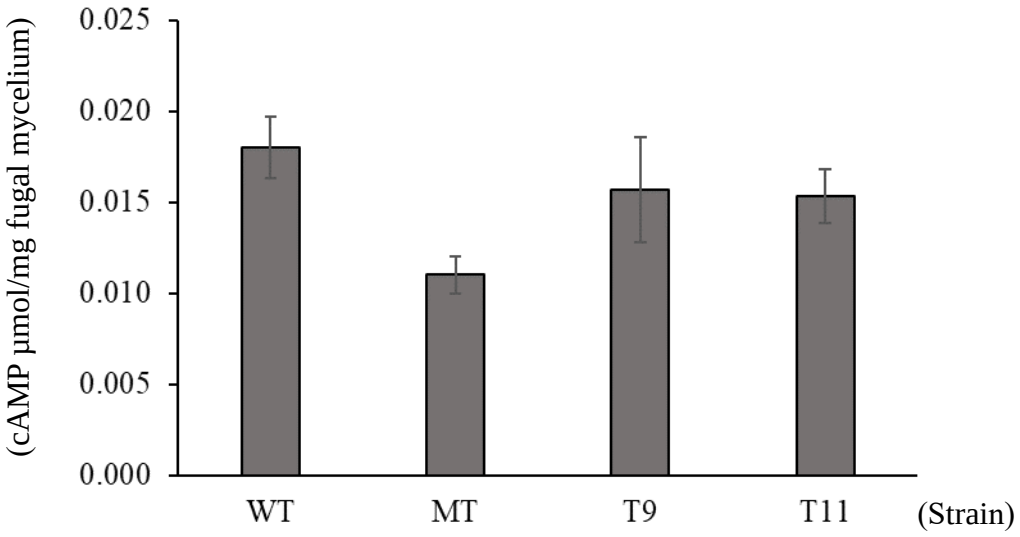


**Figure 23.** Quantification of cAMP in fungal mycelium cAMP was extracted from mycelia of Ina86-137 (wild type, WT), Ina86-137Δ*lig4*Δ*laeA2* (*MoLaeA2* deletion mutant, MT), and complementation strains (T9 and T11).

## 3.4 Discussion

As we know from the report of Choi and Dean, 1997 that showed the lacking *Mac1* in transformants of rice blast fugus were unable to form appressoria on an inductive surface, hydrophobic surface of gelbond film and appressorium formation was restored in the presence of exogenous cAMP derivatives. In addition, Ramanujam and Naqvi, 2010 found that in rice blast fungus, loss of *PdeH* led to increased accumulation of intracellular cAMP during infectious growth and significantly accelerated appressorium formation. These results indicated that when the cAMP level is not fine balance, it affects for appressorium formation and the result that found in *LaeA2* deletion mutant also correlated with the result of their reports. Exogenous cAMP that added to the fungal conidia of *MoLaeA2 deletion* mutant will increase the cAMP level to support the fungus to form appressorium. On the other hand, when applied IBMX, a phosphodiesterase inhibitor to the fungal conidia suspension. IBMX will inhibit phosphodiesterase that function to hydrolyze the cAMP level then cAMP level will increase until it is sufficient for the fungus to form appressorium. Therefore, *LaeA2* is essential for accumulation of cAMP in non-host surface to control level of cAMP for appressorium formation.

According to the Bok and Keller, 2004 presented the model of secondary metabolite regulation by *LaeA* in *Aspergillus* spp. (Figure 24) and showed that Adenylase cyclase activated Protein kinase A (PkaA) and leads to regulation of *LaeA*. The result of intracellular cAMP level analysis might related with this model. It might possible that *LaeA* play the role by feedback inhibition to control adenylase cyclase.

Therefore, a novel that found from this experiment is that homeostasis of intracellular cAMP level is required for proper appressorium formation, and *MoLaeA2* plays a role on the homeostasis. cAMP signaling and *MoLaeA2* can make a feed-back loop, and it might concern in the cAMP homeostasis.

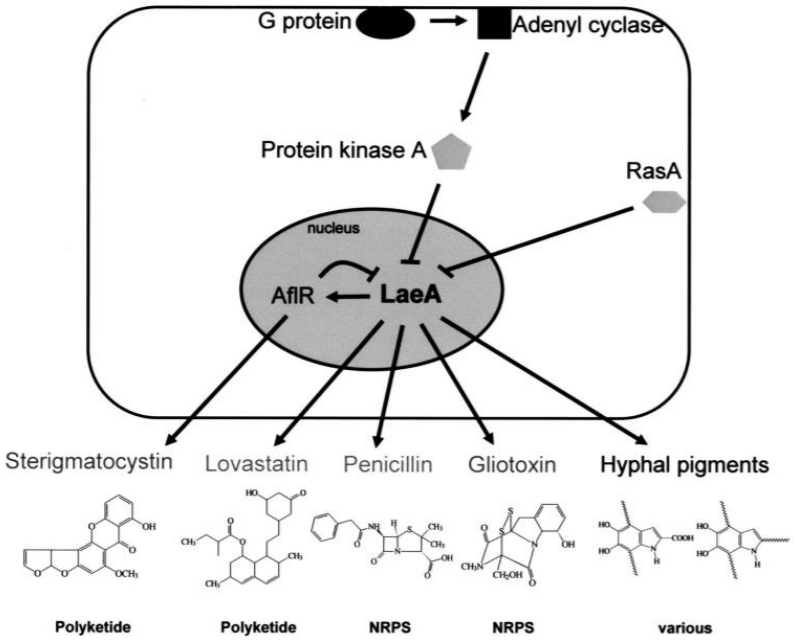


**Figure 24.** Propose model of secondary metabolite regulation by *LaeA.*

(Bok and Keller, 2004)

# CHAPTER 4

## The role of *LaeA2* on the expression of *Mac1* and *Pde*

# CHAPTER 4

## The role of *LaeA2* on the expression of *MAC1* and *Pde*

## 4.1 Introduction

As mention in chapter 3 that intracellular levels of cAMP are regulated by the balance between the activities of two enzymes, adenylyl cyclase that encode by *Mac1* and cyclic nucleotide phosphodiesterase, *PDE* including. In addition, there are reports that functions of these gene also interact with other genes elaborately. For instance, Zhou, *et al*., 2012 revealed that one of the the Mac1- interacting proteins is adenylase cyclase-assosiated protein named Cap1. CAP genes are well-conserved in phytopathogenic fungi also rice blast fungus. This research found that Deletion of CAP1 blocked the effects of a dominant *RAS2* allele and resulted in defects in invasive growth and a reduced intracellular cAMP level. The Δcap1 mutant was defective in germ tube growth, appressorium formation, and formation of typical blast lesions. Therefore. It is suggest that Cap 1 is important for Mac activation and plant infection in rice blast fungus. A hypothetical model of the function of Cap1 showed in Figure 25.

In case of *Pde*, Lui, *et al*., 2016 characterized putative protein phosphatase MoYvh1 in the rice blast fungus *Magnaporthe oryzae*. This research found that Deletion of MoYvh1 gene resulted in significant reduction in vegetative growth, conidial production, and virulence. Moreover, overexpression of the Phosphodiesterase *MoPDEH* suppressed the defects in conidia formation and pathogenicity of the Δ*MoYvh1* mutant, suggesting *MoYvh1* could regulate *MoPDEH* for its function. These result revealed that not only the importance of *MoYvh1* proteins ingrowth, differentiation, and virulence of the rice blast fungus but also a genetic link between *MoYvh1* and *MoPDEH*-cAMP signaling in this fungus. A propose model of MoYvh1

that functions upstream of the cAMP -PKA signaling pathway in rice blast fungus showed in figure 26.

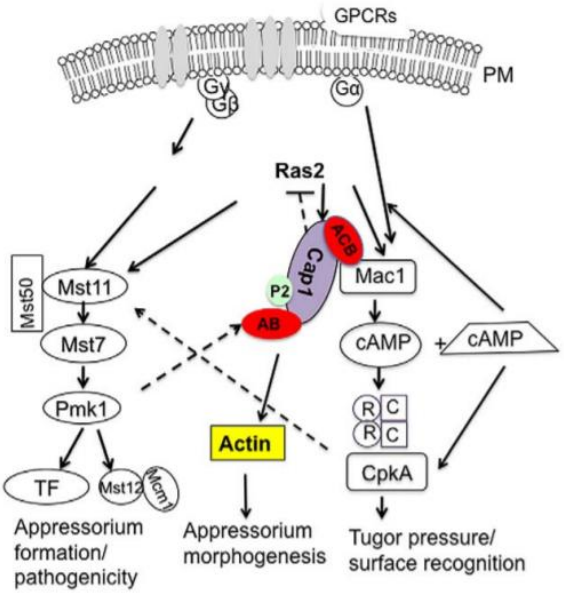


**Figure 25.** A hypothetical model of the function of Cap1. (Zhou *et al*., 2012)

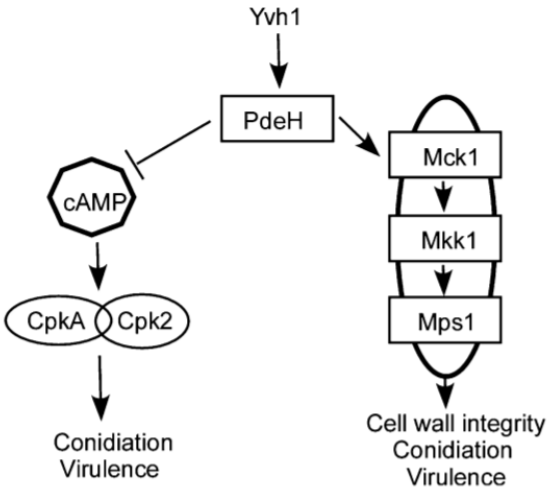


**Figure 26.** A propose model of MoYvh1 that functions upstream of the cAMP-PKA signaling pathway. (Liu *et al*., 2016)

According to report as mention showed that deletion or overexpression of some other gene affects for Mac1 and PdeH gene in rice blast fungus. Therefore, in this chapter, The role of *LaeA2* on the expression of Adenylase cyclase, *Mac1* and Phosphodiesterase, *Pde* was determine to elucidate the relationship of these genes in cAMP signaling pathway of rice blast fungus.

## 4.2. Experimental procedures

### 4.2.1 Fungal strains

- *P. oryzae* Ina 86-137 (wild type)

- *P. oryzae* Ina 86-137 Δ*lig4*Δ*laeA2* (*MolaeA2* deletion mutant)

- Complementation strain (T9)

- Complementation strain (T11)

### 4.2.2 Sample preparation and RNA extraction

Each strain of rice blast fungus as mention was inoculated on any surface to produce appressorium by using glass slide inoculation method, onion epidermis assay and intact rice sheath assay as describe in the previous chapter. Samples of appressorium at 6, 12 and 24 h. of each assay were collected for mRNA extraction. Samples of appressoium that collected from each method was lyophilized for 12 h. Then grind the samples to be powdery by using bead shocker. mRNA extraction using RNAiso (TAKARA, Japan) and purification by DNase. The extraction steps were followed by the procedure in appendix A

Platinum *Taq* DNA Polymerase (High Fidelity) (Invitrogen, USA.) was performed to check for DNA contamination before the step of Reverse transcription polymerase chain reaction. Database of *Mac1* and *Pde* gene for primer designed was obtained from Genbank database. Primers were designed for Reverse transcription of *Mac*1 and *Pde* as showed in Table 3

**Table 3** Synthetic oligonucleotide primers for amplification of each primer that consist of Actin, *Mac1 and Pde* in RT-PCR

| **Primer name** | **Sequence (5′-3′)** | **Target gene** |
|---|---|---|
| MG Actin-F | GACGACATGGAGAAGATCTGGC | *P. oryzae*'s actin gene |
| MG Actin-R | CGTCGTACTCCTGCTTCGAGAT | *P. oryzae*'s actin gene |
| *Mac1* RT-F1 | CGATGATTTGAACCGTATTA | *P. oryzae*'s *Mac1* gene |
| *Mac1* RT-R1 | GGGTTGCGAAATCGACGGTC | *P. oryzae*'s *Mac1* gene |
| *Mac1* RT-F2 | GACCGTCGATTTCGCAACCC | *P. oryzae*'s *Mac1* gene |
| *Mac1* RT-R2 | ATATTTTGCAATGTCATCAG | *P. oryzae*'s *Mac1* gene |
| *Pde* RT-F1 | TCATCGGTGATGTCCACCTC | *P. oryzae*'s *Pde* gene |
| *Pde* RT-R1 | TTCGATATCCGGCCGGATTT | *P. oryzae*'s *Pde* gene |
| *Pde* RT-F2 | AAATCCGGCCGGATATCGAA | *P. oryzae*'s *Pde* gene |
| *Pde* RT-R2 | TAAAGTCGTGCGCGCAGAAA | *P. oryzae*'s *Pde* gene |

### 4.2.3 Reverse transcription-PCR

The RT-PCR experiment were operated using the Superscript IV One-step RT-PCR system with Platinum Taq DNA Polymerase (Invitrogen, Carlbad, CA, USA). Composition of reaction mixture, and reaction condition for RT-PCR were showed in Table 4 and Table 5 respectively.

**Table 4** Composition of reaction mixture for RT-PCR

| **Components** | **Volume (µl.)** |
|---|---|
| 2X PlatinumTM SuperFiTM RT-PCR master mix | 10.0 |
| 10 µM forward primer | 1.0 |
| 10 µM reverse primer | 1.0 |
| SuperScriptTMRTMixTM | 0.2 |
| Template RNA | 1.0 |
| Nuclease free water | 6.8 |
| Total volume | 20.0 |

**Table 5** Reaction condition of RT-PCR

| **Step** | **Reaction** | **Temperature ($^{o}$ C)** | **Time** | **Number of cycle** |
|---|---|---|---|---|
| 1st | Reverse transcription | 50 | 10 min. | 1 |
| 2nd | Activation | 98 | 2 min | 1 |
| | Denaturation | 98 | 10 sec. | |
| 3rd | Annealing | 55 | 10 sec. | 20 - 40* |
| | Polymerization | 72 | 30 sec. | |
| 4th | Polymerization | 72 | 5 min | 1 |

* (20, 30 and 40 cycles were used for reaction condition)

## 4.3 Results

### 4.3.1 RT-PCR of *Mac1* gene

RT- PCR of fungal mRNA that extracted appressorium from glass slide inoculation and intact rice sheath assay showed that there were no significant difference in transcription of *Mac1* among strain tested. The results showed in Figure 27 and 28 respectively.

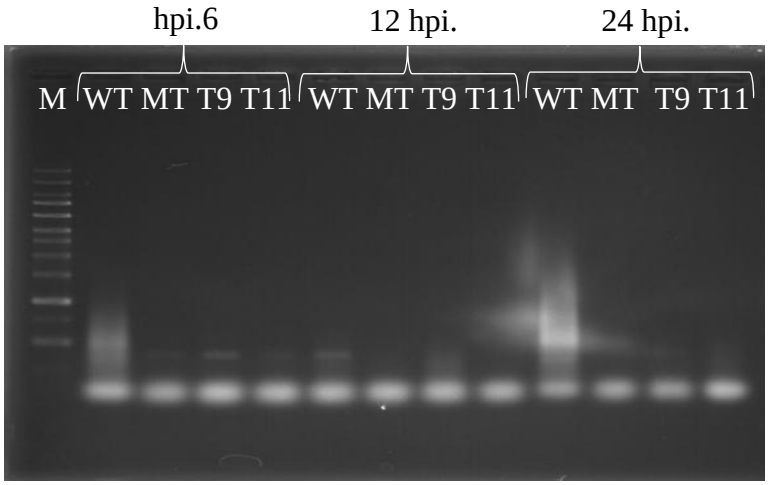


Figure 27. Reverse transcription PCR of fungal mRNA from glass slide inoculation by using *Mac1* RT-F1 and *Mac1* RT-R1 as primers for reaction. (40 cycles)

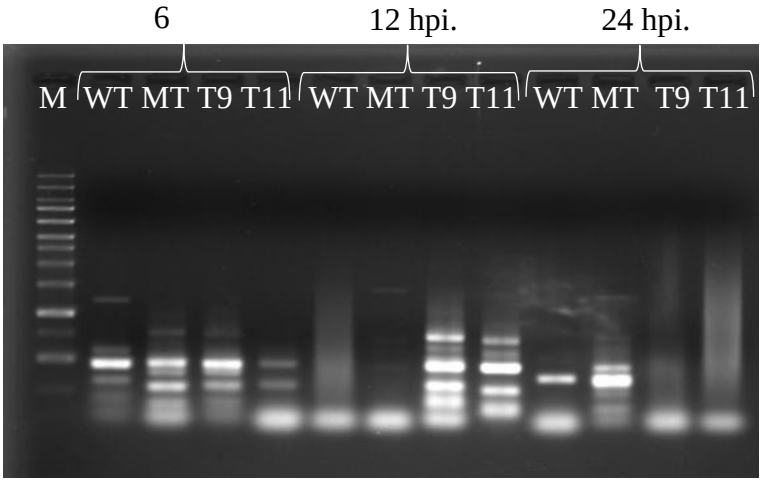


Figure 28. Reverse transcription PCR of fungal mRNA from intact rice sheath by using *Mac1* RT-F1 and *Mac1* RT-R1 as primers for reaction. (40 cycles)

RT- PCR of fungal mRNA that extracted appressorium from onion epidermis assay showed that there were no significant difference in transcription of *Mac1* among strain. The results showed in figure 29 to 31.

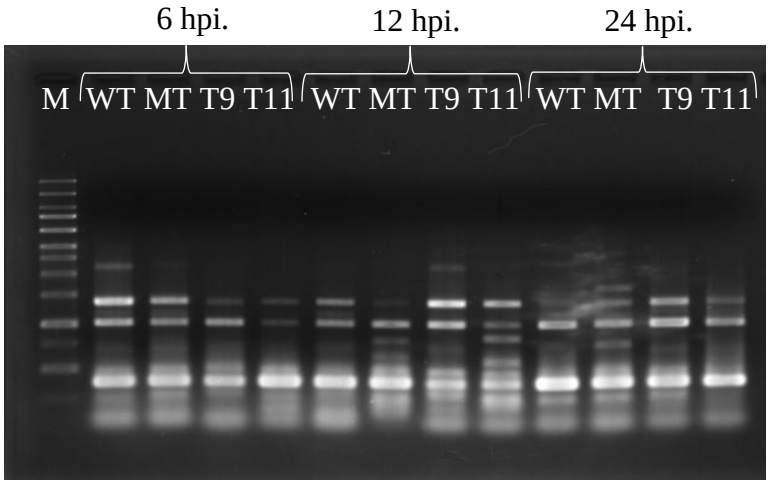


**Figure 29.** Reverse transcription PCR of fungal mRNA from onion epidermis by using *Mac1* RT-F1 and *Mac1* RT-R1 as primers for reaction. (40 cycles)

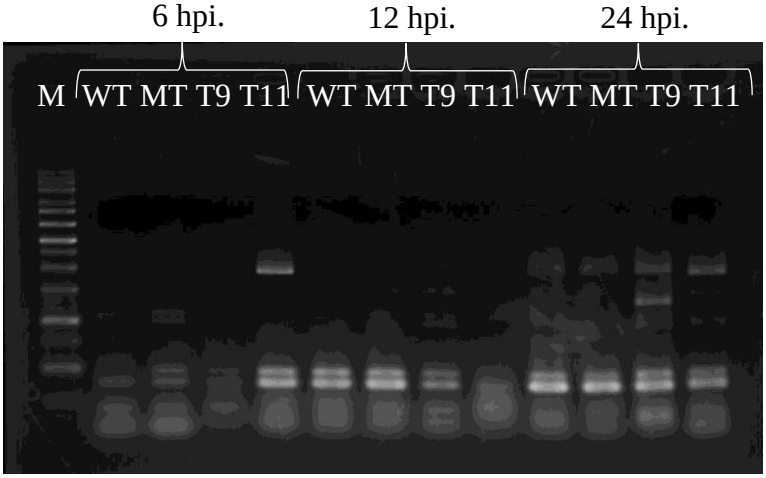


**Figure 30.** Reverse transcription PCR of fungal mRNA from onion epidermis by using *Mac1* RT-F2 and *Mac1* RT-R2 as primers for reaction. (30 cycles)

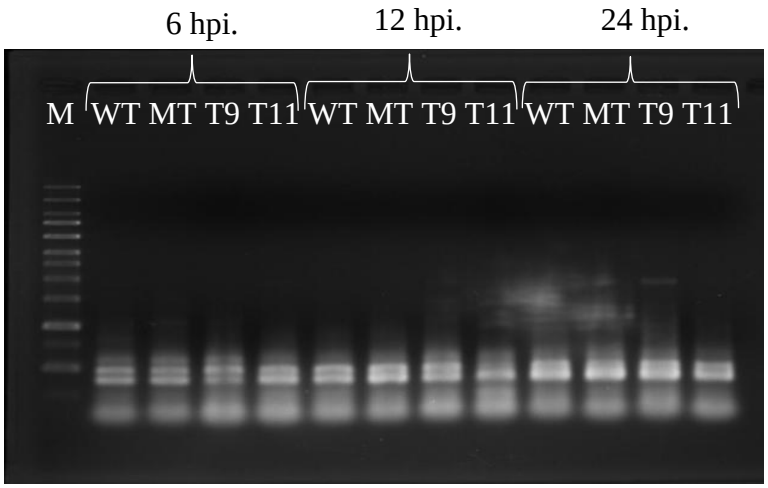


**Figure 31**. Reverse transcription PCR of fungal mRNA from onion epidermis by using *Mac1* RT-F2 and *Mac1* RT-R2 as primers for reaction. (40 cycles)

### 4.3.2 RT-PCR of *Pde* gene

RT- PCR of fungal mRNA that extracted appressorium from onion epidermis showed that there were no significant difference in transcription of *Pde* among strain tested. The results showed in Figure 32 to 37.

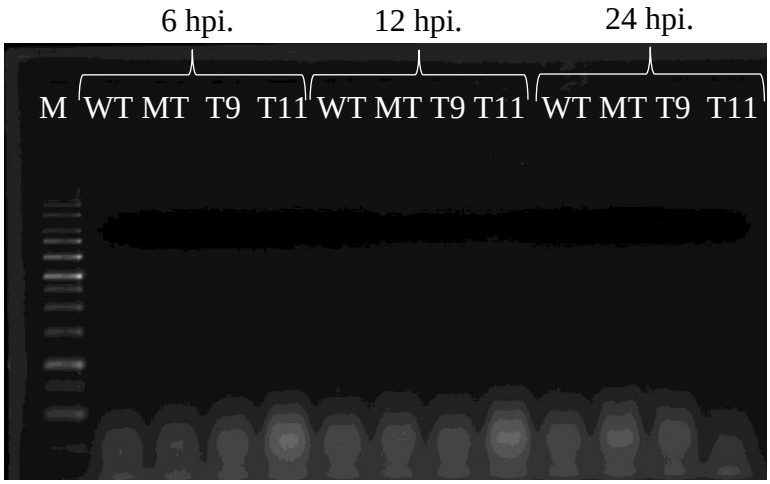


**Figure 32.** Reverse transcription PCR of fungal mRNA from onion epidermis by using *Pde* RT-F1 and *Pde* RT-R1 as primers for reaction. (20 cycles)

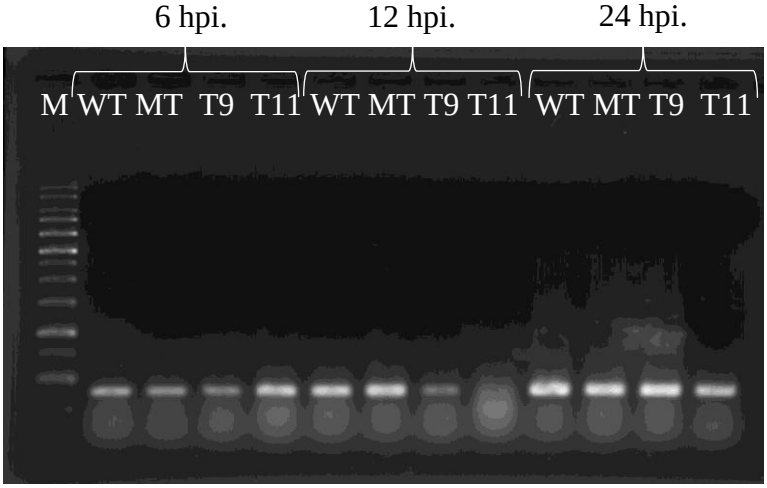


**Figure 33.** Reverse transcription PCR of fungal mRNA from onion epidermis by using *Pde* RT-F1 and *Pde* RT-R1 as primers for reaction. (30 cycles)

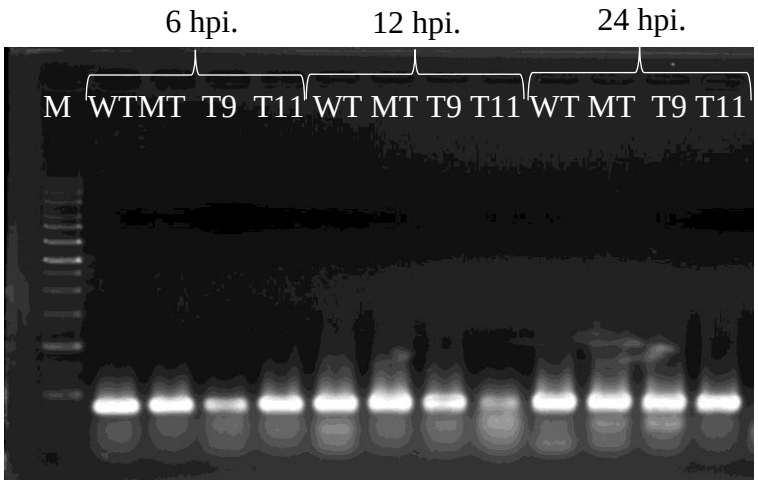


**Figure 34.** Reverse transcription PCR of fungal mRNA from onion epidermis by using *Pde* RT-F1 and *Pde* RT-R1 as primers for reaction. (40 cycles)

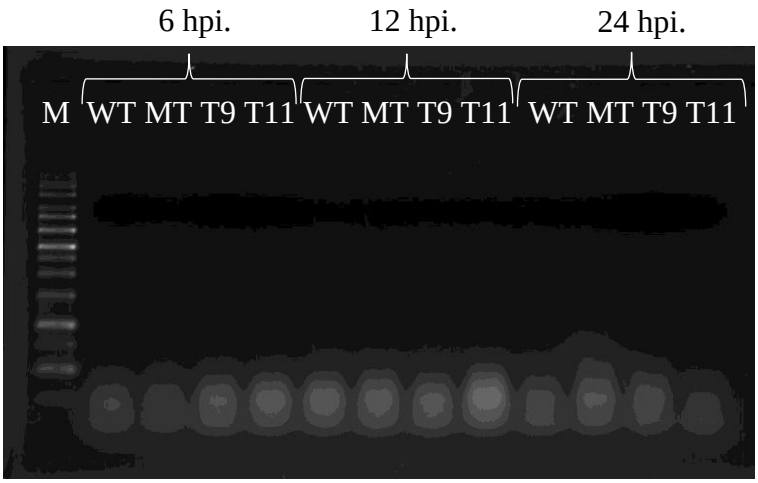


**Figure 35**. Reverse transcription PCR of fungal mRNA from onion epidermis by using *Pde* RT-F2 and *Pde* RT-R2 as primers for reaction. (20 cycles)

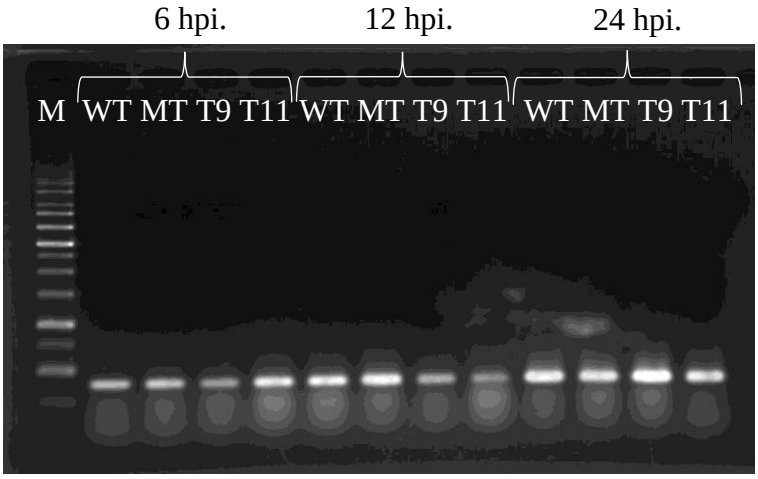


**Figure 36.** Reverse transcription PCR of fungal mRNA from onion epidermis by using *Pde* RT-F2 and *Pde* RT-R2 as primers for reaction. (30 cycles)

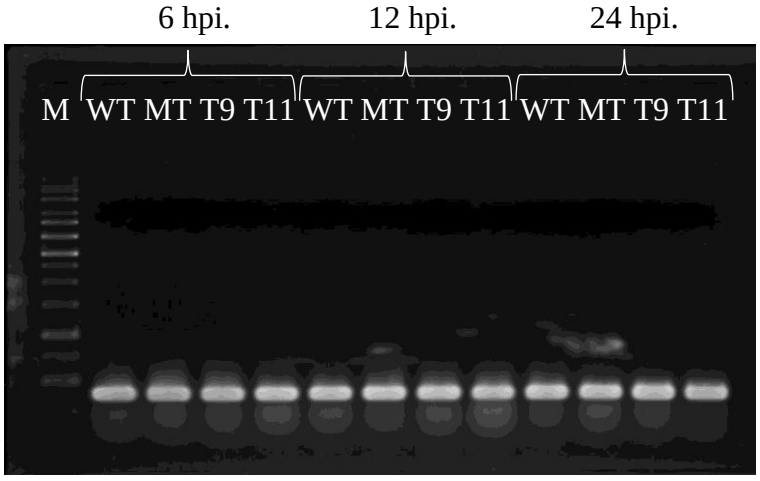


**Figure 37.** Reverse transcription PCR of fungal mRNA from onion epidermis by using *Pde* RT-F2 and *Pde* RT-R2 as primers for reaction. (40 cycles)

## 4.4 Discussion

According to the results of RT-PCR showed that there were no significant difference in transcription of adenylase cyclase, *Mac1* and cAMP phosphodiesterase, *Pde* among wild type, *LaeA2* deletion mutant and complementation strains. Therefore, *MoLaeA2* might not concerned directly with regulation of these genes at transcription level.

However, there was a report of *LaeA* in Ascomycete fungi, *Apsergillus* indicated that *LaeA* plays the role on transcription level of the fungus. Perrin et al., 2007 revealed that in *Aspergillus fumigatus, LaeA* plays a central role in regulation of chemical diversity. *LaeA* influences the expression of at least 9.5 % of the genome. Furthermore, genome regions that are transcriptionally controlled by *LaeA* are species and even strain specific, suggesting that they may serve as niche adaptation factors. The loss of *LaeA* results in a great decrease in repertoire of secondary metabolites which appears to impact the infection process. Therefore, *LaeA* constitutes a novel target for the production of an array of factors critical to success during pathogenesis. Furthermore, *LaeA* is a tool to identify metabolite gene clusters that may impact virulence.

For the results that found in chapter 3 indicated that *LaeA2* deletion affected cAMP signaling pathway, by decreasing the intracellular cAMP concentration including the report of Perrin et al, 2007 as mention. Therefore, it might possible that *LaeA2* in the rice blast fungus associates with crucial genes that function to balance intracellular cAMP level, *Mac1* or *Pde* gene indirectly. Although the result of RT-PCR did not show any clue. However, it is recommended for further study for more information to clarify this relation or to map *LaeA2* as a propose model.

# CHAPTER 5

# General discussion

# CHAPTER 5

## General discussion

Rice blast disease caused by *Pyricularia oryzae* is the pivotal plant-pathogenic fungi that threaten global rice production in many decades until present. This problem affects rice yields loss and world economics annually. The infection of rice blast disease initiates when fungal conidium germinates on the surface of the host and germ tube recognizes physical cues. Then surface signals by G-protein couple receptors activate adenylase cyclase through heterotrimeric G-proteins to generate secondary messenger cyclic AMP (cAMP) and result in activation of cAMP/PKA signaling leading the fungus develop a specialized infection structure called appressorium. *LaeA* is a regulatory gene that found and conserved in various species of pathogenic ascomycetes fungi including *P. oryzae*. It has been studied extensively in secondary metabolism, morphological development and virulence. However, the role of *LaeA* on appressorium formation has not been clear. In this research, *LaeA2* deletion mutant and its complemented strains were constructed and function of *LaeA* on appressorium formation especially on cAMP signal transduction pathway was investigated.

### 1. Function of *LaeA2* on appressorium formation and pathogenicity

*LaeA2* deletion mutant (Ina86-137Δ*lig4*Δ*laeA2*) and its complementation strains (T9 and T11) were constructed. Onion epidermis assay was performed to investigate appressorium formation and the result showed that wild type (Ina86-137) and complementation strains (T9 and T11) exhibited high percentage of appressorium formation in killed onion epidermis. On the other hand, *LaeA2* deletion mutant (Ina86-137Δ*lig4*Δ*laeA2*) showed decrease in appressorium formation. The same results were

observed on the plastic surface. Spraying inoculation assay on rice leaves showed that all fungal strains can cause the lesions on leaves. In addition, inoculation of fungal conidia onto intact rice sheath showed that all strains formed appressoria on rice tissue. These results indicate that *LaeA* is required for appressorium formation on non-host suface, whereas it does not affect fungal pathogenicity on the rice plant.

## 2. Role of *LaeA* on cAMP signal transduction for appressorium formation

In order to determine the role of *LaeA* on cAMP signal transduction, exogenous cAMP was added to fungal conidia suspension and inoculated on onion epidermis to examine appressorium formation. The appressorium formation of *LaeA* deletion mutant was recovered. Similar result was observed when 3-isobutyl-1-methylxanthine, which is potent inhibitor for phosphodiesterase, was applied. These results indicated that *LaeA* deletion affected cAMP signaling pathway, by decreasing the intracellular cAMP concentration. To examine function of *LaeA* on intracellular cAMP concentration, cAMP was extracted from liquid-cultured mycelia of each fungal strain. High-performance liquid chromatography (HPLC) was used to analyzed intracellular cAMP level. The result showed that intracellular cAMP concentration in *LaeA2* deletion mutant significantly lower than wild type, complementation strains no 9 and no.11 as 0.007, 0.003 and 0.004 mM/mg. fungal mycelium respectively. These results confirmed the relationship of *LaeA* and intracellular cAMP level in rice blast fungus.

## 3. Transcription analysis of genes related to cAMP level in *P. oryzae*

Relationship between *LaeA2* and genes that balance cAMP level in cAMP signal transduction, *Mac1* (adenylate cyclase) and *Pde* (phosphodiesterase) was investigated. RNA of each fungal strain were extracted and reverse transcription-polymerase chain

reaction (RT-PCR) was used for the transcription analysis. Result of RT-PCR revealed that there are no significant difference in transcription level of these genes in wild type, *LaeA* deletion mutant and complementation strains. These results suggest that *LaeA* might not concerned directly with regulation of *Mac1* and *Pde* at transcription level.

This research demonstrated that the regulatory gene, *LaeA2* is essential for appressorium formation on non-host surface of rice blast fungus. Moreover, *LaeA2* also concern with cAMP signaling pathway by regulates intracellular cAMP level. It is also suggested that at least low concentration of cAMP level is required appressorium formation on any surface, and *LaeA2* might compensate appropriate cAMP level to support the fungus to form appressorium on non-host surface. This is the first report to reveal the relationship between *LaeA2* and cAMP signaling in filamentous fungi.

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# APPENDICES

# Appendix A: Experimental Protocols

**Amplification of *LaeA* (MG08161)**

| Component | Volume | |
|---|---|---|
| KOD plus ver.2 | 1 | µL |
| DNTPs | 5 | µL |
| 10xbuffer | 5 | µL |
| Anti-sense primer (10 µM) | 1.5 | µL |
| $MgSO_4$ | 2.8 | µL |
| Template DNA | 1 | µL |

Sterile distilled water up to 50 µL

PCR conditions

94 $^{o}$C : 94 $^{o}$C : 55 $^{o}$C : 68 $^{o}$C : 68 $^{o}$C : 4 $^{o}$C

2.0 : 0.15 : 0.30 : 3.30 : 7.0 : ∞ (min.)

(35 cycles)

**pENTR™/D-TOPO® Cloning Procedure**

| Component | Volume | |
|---|---|---|
| Fresh PCR product | 0.5 - 4 | µL |
| TOPO® vector | 1 | µL |
| Water to a final volume of | 5 | µL |

2:1 molar ratio of PCR product: TOPO® vector.

**Plasmid extraction (pENTR-*LaeA2*) and gel electrophoresis**

| Component | Volume | |
|---|---|---|
| SDW | 6 | µL |
| Buffer (Smart cut) | 1 | µL |
| Plasmid DNA | 2 | µL |
| EcoRI | 1 | µL |

Incubate at 37 $^{o}$C for 1 h. then gel electrophoresis was performed.

**GoTaq Green Mater mix**

| Component | Volume | |
|---|---|---|
| GTG | 10 | µL |
| Forward primer (10 pmole/ µL) | 1 | µL |
| Reverse primer (10 pmole/ µL) | 1 | µL |
| Template DNA | 1 | µL |
| SDW up to 20 µL | | |

PCR conditions
95 $^{o}$C : 95 $^{o}$C : 55 $^{o}$C : 72 $^{o}$C : 72 $^{o}$C : 4 $^{o}$C
2.0 : 0.30 : 0.30 : 0.30 : 5.0 : ∞ (min.)
(35 cycles)

Primer used
F658 : MGG08161 r1F
R 1351 : MGG08161 r1R

**Big dye terminator sequencing**

| Component | Volume | |
|---|---|---|
| 5X seq buffer | 3 | µL |
| Big dye terminator | 2 | µL |
| 3.2pmol/primer | 1 | µL |
| Template | 1 | µL |
| SDW up to 20 µL | | |

PCR conditions
96 $^{o}$C : 96 $^{o}$C : 50 $^{o}$C : 60 $^{o}$C : 4 $^{o}$C
1.0 : 0.10 : 0.05 : 4.00 : ∞ (min.)
(35 cycles)

**LR reaction to obtain pBLASTR-DEST*LaeA2***

| Component | Volume | |
|---|---|---|
| *pENTR-LaeA2* plasmid (100-300 ng) | 1-10 | µL |
| Destination vector- pBLASTR-DEST (150 ng/µl) | 2 | µL |
| LR Clonase™ reaction buffer | 4 | µL |
| TE Buffer, pH 8.0 | 16 | µL |

**Plasmid extraction (pBLASTR-*LaeA2*) and gel electrophoresis**

| Component | Volume | |
|---|---|---|
| SDW | 6 | µL |
| Buffer (Smart cut) | 1 | µL |
| Plasmid DNA | 2 | µL |
| EcoRI | 1 | µL |

Incubate at 37 $^{o}$C for 1 h. then Gel electrophoresis was performed.

**PCR for genomic DNA of fungus**

| Component | Volume | |
|---|---|---|
| KOD plus version 2 | 1 | µL |
| DNTPs | 5 | µL |
| 10xbuffer | 5 | µL |
| Anti-sense primer (10 µM) | 1.5 | µL |
| $MgSO_4$ | 2.8 | µL |
| Template DNA | 1 | µL |

Sterile distilled water up to 50 µL

PCR conditions

94 $^{o}$C : 94 $^{o}$C : 55 $^{o}$C : 68 $^{o}$C : 68 $^{o}$C : 4 $^{o}$C

2.0 : 0.15 : 0.30 : 3.30 : 7.0 : ∞ (min.)

(35 cycles)

**Probe preparation**

| Component | Volume | |
|---|---|---|
| KOD plus version 2 | 1 | µL |
| DNTPs | 5 | µL |
| 10xbuffer | 5 | µL |
| Anti-sense primer (10 µM) | 1.5 | µL |
| $MgSO_4$ | 2.8 | µL |
| Template DNA | 1 | µL |

Sterile distilled water up to 50 µL

PCR conditions

94 $^{o}$C : 94 $^{o}$C : 55 $^{o}$C : 68 $^{o}$C : 68 $^{o}$C : 4 $^{o}$C

1.0 : 0.10 : 0.05 : 4.00 : 7.0 : ∞ (min.)

(35 cycles)

**Transformation of *E. coli* Top10 Transformation**

1. Melt SOC medium at 37$^{o}$C
2. Thaw *E. coli* Top10 in ice.
3. Add 5 µL of ligation product or 4 µL of TOPO® reaction product to thawed *E. coli* Top10, mix gently.
4. Set the condition at pulse controller
   - 200
   - Capacitance 25 µFD
   - 2.5 kV
5. Push Time constant.
6. Pipette the mixture into a cold electroporation cuvette (0.2 cm electrode, GenePulser®, Bio-RAD, CA), remove bubbles and place cuvette in the cold electroporation device.
7. Push two Pulse buttons for a while until beep sound. Check time constant (~4.6 is good condition).
8. Pipette a little amount of SOC media into cuvette by a disposable pipette to mix with transformation suspension, and pipette all to 5 ml of SOC tube.
9. Incubate at 37$^{o}$C for 1 h in water bath with shaking.
10. Plate electroporated cell onto LB with selective antibiotic plates and incubate at 37$^{o}$C, overnight.

**DNA purification by phenol-chloroform method**

1. Add 25 µL of phenol and 25 µL of chloroform : isoamyl alcohol (24:1) into DNA sample and mix well.
2. Centrifuge at 15,000 rpm for 5 min.
3. Pipette the upper layer of DNA solution to a new centrifuge tube.
4. Add 5 µL of 3 M Sodium acetate and125 µL off 99.5% ethanol into a DNA tube and mix it well.
5. Centrifuge at 15,000 rom for 10 min to precipitate DNA pellet.
6. Discard the liquid solution (in the step, DNA pellet should be seen in the bottom of a tube)
7. Add 500 µL of 70 % ethanol to clean DNA pellet.
8. Centrifuge at 15,000 rpm for 5 min and discard the ethanol carefully.
9. Dry the DNA pellet.
10. Add 10 µL of sterile distilled water or TE buffer to dissolve DNA pellet.

**RNA extraction: RNAiso Plus** (TAKARA Bio Inc., Shiga, Japan)

1. Frozen the tissue or sample for 24 h. and freeze dry for 12 h. Then grind the sample by bead shocker until powdery.
2. Add 1 ml. of RNAiso Plus to homogenized tissue (1 ml./50~100 mg of tissue sample). For a fresh tissue sample, add RNAiso Plus immediately after collecting the tissue, and homogenize completely.
3. Transfer homogenized tissue sample into a centrifuge tube, keep at the room temperature (15~30$^{o}$C) for 5 min.
4. Centrifuge at 12,000 g for 5 minutes.
5. Collect Supernatant and transfer it to a new centrifuge tube (do not collect pellet)

6. Add 0.2 times more chloroform than the amount of RNAiso Plus used above. Cap the centrifuge tube and mux until the solution become milky.
7. Keep the solution at room temperature for 5 min.
8. Centrifuge at 12,000 g for 15 min at 4$^{o}$C. Centrifuge the solution will separate it into three layers; liquid top layer (contains RNA), semisolid middle layer (mostly DNA), and the bottom organic solvent layer.
9. Transfer the top liquid layer to new centrifuge tube without touching middle layer.
10. Measure the amount of the top layer and add an equal amount or add up 0.5 times of the isopropanol of the top layer. Mix together well. Keep the mixture at room temperature for 10 minutes.
11. Centrifuge at 12,000 g for 10 min at 4$^{o}$C to precipitate RNA.
12. Carefully remove the supernatant, and do not touch the pellet. If some amount of isopropanol remains that is not a problem. Add an amount of 75% cold ethanol that was equivalent to the supernatant. Clean the precipitate by vortexing.
13. Centrifuge the solution at 7,500 g for 5 min at 4oC and discard supernatant. Be careful not to disturb the precipitate.
14. Dry the precipitate by leaving the tube open for several minutes. After precipitate is dry, dissolve it with appropriate amount of RNase-free water.

**NOTE**: Do not centrifuge to dry the precipitate or heat the precipitate to dry; it may cause difficulty with dissolving RNA.

# Appendix B: Buffers and solutions

## 1. Buffers

**DNA extraction buffer**

| | | |
|---|---|---|
| 1M Tris-HCl (pH 8.5) | 200 | ml |
| 0.5 EDTA (pH8.0) | 50 | ml |
| NaCl | 15 | g |
| SDS | 5 | g |
| SDW | 1 | Liter |

Dissolved all components and autoclave at 121$^{o}$C for 15 min.

**Lysis buffer, 100 mL**.

| | | |
|---|---|---|
| 1 M KH2PO4 | 0.3 | ml |
| 1 M K2HPO4 | 4.7 | ml |
| NaCl | 2.3 | g |
| KCl | 0.75 | g |
| Glyceral | 10 | ml |
| Triton X-100 | 0.5 | ml |
| Imidazole | 68 | g |

Dissolved all components with SDW and autoclave at 121$^{o}$C for 15 min.

**50X Tris Acetic acid EDTA (TAE) Buffer**

| | | |
|---|---|---|
| Tris | 242 | g |
| EDTA | 18.6 | g |
| Acetic acid | 57 | mL |

**Electrophoresis buffer**

1 X TAE (200 mL of 50X TAE in 9.8 L of distilled water)

**TE buffer**

10 mM Tris-Cl, pH 7.5

1 EDTA

Make from 1 M stock of Tris-Cl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).

10 mL 1 M Tris-Cl pH 7.5 per liter.

2mL 500 mM EDTA pH 8.0 per liter.

**DEPC-water**

Prepared by the addition of 0.l % diethylpyrocarbonate (DEPC, Sigma Chemical Co St. Louis MO, USA) to double-distilled water. The mixture is strongly agitated and left to stand overnight before autoclaving to inactivate the DEPC before use.

## 2. Buffers and solution for fungal protoplast fusion

**Digestion buffer**

| | |
|---|---|
| Sucrose | 205.5 g/L |
| Maleic acid monosodium salt ($C_4H_3O_4Na.3H_2O$) | 9.65 g/L |
| Distilled water | 100 mL. |

Dissolved all components, adjust to pH 5.5, add sucrose and autoclave at 121$^{o}$C for 15 min.

**Digestion buffer and enzyme mixed (5 mL)**

| | | |
|---|---|---|
| Cellulase | 0.025 | g |
| Yatalase | 0.1 | g |

Dissolve all component in 5 mL of digestion buffer and filter through membrane sized 0.45 µm for sterilization. 81

**STC Buffer**

| | | |
|---|---|---|
| Sucrose | 20 | g |
| 1 M Tris-HCl (pH 7.3) | 1 | ml |
| 2.5 M $CaCl_2$ | 2 | ml |

Dissolved all components in 100 ml of distilled water and autoclave at 121$^{o}$C for 15 min.

**Polyethylene glycol (PEG) solution**

| | | |
|---|---|---|
| PEG#4000 | 60 | g |
| 1 M Tris-HCl (pH 7.3) | 1 | ml |
| 2.5 M $CaCl_2$ | 2 | ml |
| Distilled water | 100 | ml |

Stir continuously to dissolve PEG while heating by hot plate stirrer.

## 3. Solutions and buffers for Southern hybridization

**Depurination solution**

| | | |
|---|---|---|
| 12N HCl | 20 | mL |
| Distilled water | 860 | mL |

**Denaturation solution**

| | | |
|---|---|---|
| NaCl | 43.83 | g |
| NaOH | 10.00 | g |

Adjust volume to 1 Liter with distilled water

**Neutralization Solution**

| | |
|---|---|
| NaCl (An. Grade) | 43.83 g |
| Tris (Mwt, 121.14) | 30.28 g |
| SDW | 1 Liter |
| Adjust to pH 7.5 with 12N HCl | |

**20x SSC**

| | |
|---|---|
| Trisodium citrate Dihydrate | 88.23 g |
| NaCl (An. Gnd) | 175.3 g |
| SDW 1 Liter | |
| Adjust to pH 7.0 with 12N HCl | |

**Primary wash buffer**

| | |
|---|---|
| 6.0 M Urea | 360.0 g |
| 0.4% SDS | 4.0 g |
| 0.5xSSC | 25 ml |
| Adjust volume to 1 L. with distilled water. | |

**Secondary wash buffer**

| | |
|---|---|
| 20xSSc | 100 ml |
| Adjust volume to 1 L. with distilled water. | |

# Appendix C: Culture media

## 1. Culture media for fungus

**Prune Agar**

| | | |
|---|---|---|
| Prune | 4.0 | g/L |
| Lactose | 5.0 | g/L |
| Yeast Extract | 1.0 | g/L |
| Agar | 17.0 | g/L |

Dissolved all components and autoclave at 121$^{o}$C for 15 minutes

**Oatmeal Agar (500 ml.)**

| | | |
|---|---|---|
| Oatmeal | 25.0 | g/L |
| Sucrose | 10.0 | g/L |
| Agar | 17.5 | g/L |

Add oatmeal to distilled water, boil at 90$^{o}$ C for 30 minutes. Filtrate for only oatmeal extracted. Then add 100 mL of sucrose solution and agar. Adjust the final volume to 500 mL. Autoclave at 121$^{o}$ C for 15 min. If required for mixing with antibiotic, wait until this medium cool down to 60$^{o}$ C.

**2YEG** (Yeast Extract and Glucose)

| | | |
|---|---|---|
| Yeast Extract | 2.0 | g/L |
| Glucose | 10.0 | g/L |

Dissolved all components and autoclave at 121$^{o}$C for 15 min.

**Bottom Agar**

| | | |
|---|---|---|
| Yeast Nitrogen base without amino acid | 6.7 | g/L |
| Glucose | 5.0 | g/L |
| Sucrose | 205.0 | g/L |
| Agar | 15.0 | g/L |

Dissolved all components and autoclave at 121$^{o}$ C for 15 min.

**Top Agar**

| | | |
|---|---|---|
| Yeast Nitrogen base without amino acid | 6.7 | g/L |
| Glucose | 5.0 | g/L |
| Agar | 10.0 | g/L |

Dissolved all components and autoclave at 121$^{o}$ C for 15 min and add Blasticidin S to a final concentration of 5 μg/mL.

**Water Agar**

| | | |
|---|---|---|
| Agar | 40.0 | g/L |

Dissolved into distilled water and autoclave at 121$^{o}$ C for 15 min

## 2. Culture media for bacteria

**LB-Ampicillin**

| | | |
|---|---|---|
| Polypeptone | 10.0 | g/L |
| Yeast Extract | 5.0 | g/L |
| Sodium Chloride | 10.0 | g/L |
| Distilled water | 1.0 | L |

Dissolved all components, adjust pH to 7.0 and autoclave at 121$^{o}$ C for 15 min. Finally, add ampicillin to a final concentration of 100 μg/mL when the medium cool down to 60$^{o}$ C.

**LB-Agar/Ampicillin**

| | | |
|---|---|---|
| Polypeptone | 10.0 | g/L |
| Yeast Extract | 5.0 | g/L |
| Sodium Chloride | 10.0 | g/L |
| Distilled water | 1.0 | L |

Dissolved all components, adjust pH to 7.0, add 15 g/L of Agar and autoclave at 121$^{o}$ C for 15 min. Allow to cool down to 60$^{o}$ C and add ampicillin to a final concentration of 100 μg/mL.

**LB-Agar/Kanamycin**

| | | |
|---|---|---|
| Polypeptone | 10.0 | g/L |
| Yeast Extract | 5.0 | g/L |
| Sodium Chloride | 10.0 | g/L |

Fill up with 1.0 L of sterile distilled water, adjust pH to 7.0, add 15 g/L of agar and Autoclave at 121$^{o}$ C for 15 min. Allow to cool down to 60$^{o}$ C and add kanamycin to a final concentration of 50 μg/mL.

**SOC Medium**

**Solution A:**

| | | |
|---|---|---|
| Tryptone | 20.0 | g/L |
| Yeast extract | 5.0 | g/L |
| Potassium chloride | 0.1864 | g/L |
| Sodium chloride | 0.5844 | g/L |
| Magnesium chloride hydride | 2.033 | g/L |

**Solution B:**

| | | |
|---|---|---|
| Glucose | 3.6 | g/L |

- Dissolve solution A in 900 mL, adjust to pH 7.0 and autoclave at 121$^{o}$C for 15 min. Then dissolve solution B in 100 mL and autoclave at 121$^{o}$ C for 15 min. After autoclaving, mix the solution A and solution B for using.

# List of Abbreviations

# Abbreviations

| | |
|---|---|
| Δ | Deletion mutant |
| % | Percentage |
| ATP | Adenosine triphosphate |
| cAMP | Cyclic 3’ ,5’ adenosine monophosphate |
| cDNA | Complementary DNA |
| cm | centimeter |
| $^{o}$C | Degree celsius |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleoside triphosphate |
| h | hour |
| hpi | hour post incubation |
| HPLC | High-Performance liquid chromatography |
| IBMX | 3-isobutyl-1-methylxanthine |
| µg | Microgram |
| µL | Microliter |
| µm | Micrometer |
| mg | Milligram |
| mL | Milliliter |
| mm | Millimeter |
| mmol | Millimole |
| mM | Millimolar |
| mRNA | Messenger Ribonucleic acid |
| PCR | Polymerase chain reaction |
| PEG | Poly ethylene glycol |

| | |
|---|---|
| RNA | Ribonucleic acid |
| rpm | Revolution per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SDW | Sterile distilled water |
| Sec | Second |
| TBA | Tetra butyl Ammonium Hydroxide |
| TCA | Trichloro Acetic Acid |

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