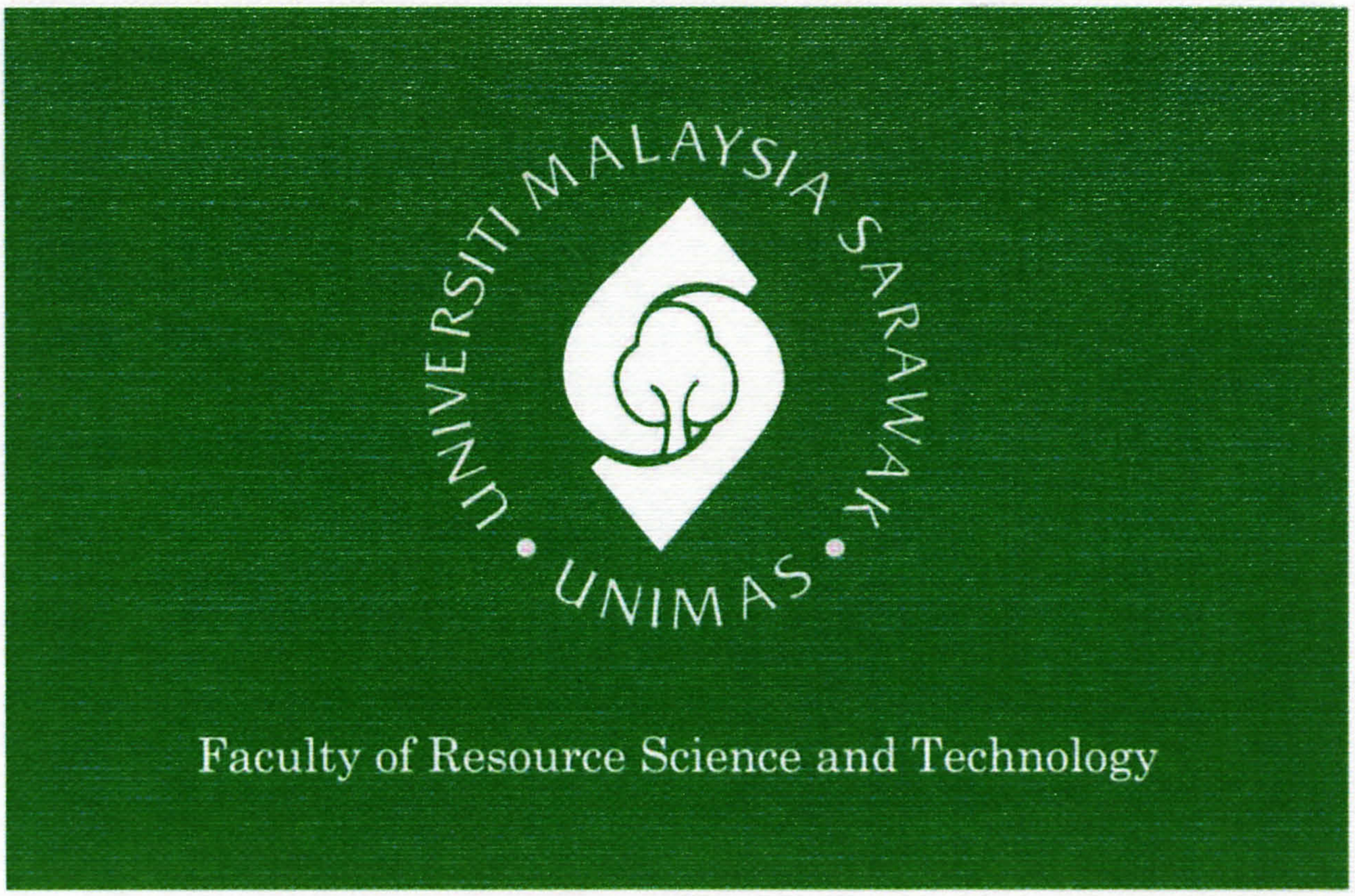

Cloning and Sequence Analysis of *Avr-Pik* gene from *Magnaporthe oryzae* Isolates from Sarawak

Martina Azelin Anak Dirum

Bachelor of Science with Honours
(Resource Biotechnology)
2016

Cloning and Sequence Analysis of *Avr-Pik* gene from *Magnaporthe Oryzae* isolate from

Sarawak

Martina Azelin Anak Dirum (42009)

A thesis is submitted in partial fulfilment of the Final Year Project 2 (STF 3015)

Resource Biotechnology

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Date (20/5/2016)

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Final Year Project Report ☑

Masters ☐

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DECLARATION

I hereby declare this thesis, entitled Cloning and Sequence Analysis of *Avr-Pik* gene from *Magnaporthe Oryzae* isolate from Sarawak is based on my original work except for quotations and citations, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted to any other degrees at UNIMAS or other institutions.

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Table of Contents

LIST OF ABBREVIATIONS

Avr	Avirulence
BLAST	Basic Local Alignment Search Tool
DAMP	Damage-associated molecular pattern
ETI	Effector-triggered immunity
LAIX	Lb agar with ampicillin, IPTG and X-gal
LRR	Leucine rich repeat
MAMP	Microbe-associated molecular pattern
MATE	Multidrug and Toxic Extrusion
MIMP	Microbe induced molecular pattern
ml	Mili litre
NB	Nucleotide binding
ng	Nano gram
PAMP	Pathogen-associated molecular pattern
PRR	Pattern-recognition receptors
PTI	PAMP-triggered immunity
PCR	Polymerase Chain Reaction
R	Resistance gene
RLK	Receptor-like kinase
RLP	Receptor-like protein

LIST OF TABLES

LIST OF FIGURES

Cloning and Sequence Analysis of *Avr-Pik* gene from *Magnaporthe oryzae* isolate from Sarawak

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ABSTRACT

Avr-Pik gene found in *Magnaporthe oryzae* is one of the specific effector protein that interact with host resistance (R) gene that result in effective activation of innate immunity. *M.oryzae* is an ascomycete fungal pathogen that has been found infecting cultivated rice (*Oryzae sativa*) and become the major constraint in rice global production. The aim of this research is to isolate, clone and sequence *Avr-Pik* gene from *M. oryzae* originated from Sarawak. Prior to isolation and cloning, a set of primer consist of forward primer (5'-ATGCGTGTTACCACTTTTAACA-3') and reverse primer (5'-TTAAAAGCCGGGCCTTTT-3') was designed based on *Avr-Pik* sequences obtained from database. Gradient Polymerase Chain Reaction (PCR) was performed to optimize the annealing temperature. DNA fragments of ~342 bp were obtained from the amplification and were purified. Blue and white screening was conducted to grow colonies and distinguish between the recombinant and non-recombinant colonies. Subsequently, plasmid miniprep was conducted to obtain plasmid and it was sent for sequencing. The sequencing result was corroborated by using BLAST in which showed highest similarity with *M.oryzae* isolates from Japan. Based on this study, future identification and sequence analysis of this gene in understanding gene-for-gene resistance can be carried out, hence establishing *M.oryzae* as the fungal model to study fungal pathogenicity.

Keywords: *Avr-Pik*, *Magnaporthe oryzae*, resistance (R), rice blast disease, PCR

ABSTRAK

Gen Avr-Pik yang dijumpai dalam Magnaporthe oryzae adalah salah satu specifik protein efektor yang berinteraksi dengan gen resistant (R) yang menyebabkan pengaktifan immuniti semula jadi. M.oryzae merupakan patogen kulat yang menjangkiti padi (Oryzae sativa) merupakan kekangan utama dalam penghasilan global beras. Tujuan kajian ini dijalankan adalah untuk isolasi, pengklonan dan penjujukan gen Avr-Pik dari M.oryzae yang berasal dari Sarawak. Sebelum proses isolasi dan pengklonan, sepasang pencetus yang mengandungi pencetus ke hadapan (5'-ATGCGTGTTACCACTTTTAACA-3')dan pencetus ke belakang (5'-TTAAAAGCCGGGCCTTTT-3')telah direka berdasarkan 7 jenis jujukan gen Avr-Pik yang diperolehi dari pangkalan data. Tindak Balas Berantai Polimerase telah dijalankan untuk mengenalpasti suhu optimum penyepuhlindapan. Serpihan DNA yang bersais ~342 bp telah diperolehi dari proses amplifikasi dan dibersihkan. Permerhatian koloni biru dan putih dijalankan untuk mengenalpasti diantara koloni rekombinan dengan koloni bukan rekombinan. Proses plasmid miniprep dilakukan untuk memperoleh plasmid dan dihantar untuk penjujukan. Keputusan jujukan seterusnya dianalisa menggunakan BLAST menunjukan persamaan yang tinggi dengan M.oryzae isolasi dari Jepun. Berdasarkan kajian ini, identifikasi dan analisa jujukan gen dapat dijalankan untuk pemahaman hubungan gen-untuk-gen resistan, dan menjadikan M.oryzae sebagai model fungal untuk kajian patologi fungal.

Kata Kunci: Avr-Pik, Magnaporthe oryzae, resistan (R), penyakit blas padi, PCR

INTRODUCTION

1.1 Background

Rice is known as one of the essential food crops in the world. It is produced globally together with wheat and corn to meet the demand of nation food. According to International Rice Research Institute, human consume 85% of total rice production while wheat consumption is only 72% and corn is the least consume with only 19% of its production (Sasaki & Moore, 1997). In Asian, rice is a staple diet and a fundamental in their food pyramid. However, rice production is challenged due to diseases, insect, weed and environment factors.

Rice is prone to diseases that are caused by biotic factor or also known as plant pathogens. Usually, plant pathogens such as bacteria, fungi, virus and nematodes enter plants through plants pores (water and gas) such as stomata and hydathodes, and also through plants open wounds (Jones & DangI, 2006). Through all invasion trial, only several of potential pathogen will succeed to cause diseases in plants. In order to fight against diseases, plants present the basal defence mechanism.

Plants basal defence mechanism is layers of immunity defence that plant performed in order to fight against potential pathogens. The first line basal defence of plants comprises physical and chemical barriers that located on the surface of plants cells. Examples of surface structures are cuticle, trichome, and cell wall which serve as physical barrier to resist pathogens from penetrating into the cells (Lazniewska *et al.*, 2012). Detoxifying of potential pathogens also occur, conducted by multiple type of chemicals such as saponins, cyagonenic glucosides and glucosinolates (Iriti & Faoro, 2009). However, the first line defence cannot serve fully protection to plants as pathogens can generate strategies to breach these defences. As the first line defence cannot guarantee full

protection, the plants effector protein are able to detect pathogens and initiate induced defence.

After successfully breaching first line defence, potential pathogen will narrow the invasion and subsequently trigger plant induced defence which are PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). The induced defence mechanism use pattern-recognition receptors (PRRs) to recognize invaders. The PRRs recognize invaders directly through the perception of microbe-associated molecular pattern (MAMPs) which also known as pathogen-associated molecular pattern (PAMPs) (Ingle *et al.*, 2006). PAMPs/MAMPs are conserved molecules that are essential for pathogens for microbial fitness or survival of entire groups. Meanwhile, indirect recognition of invaders is also possible through the perception of damage-associated molecular patterns (DAMPs) (Dodds & Rathjen, 2010) which is also known as microbe-induced molecular patterns (MIMPs) (Mackey & McFall, 2006).Usually the recognition event brings to a rapid type of defence reaction described as the hypersensitive response which is characterized by rapid apoptotic cell death and local necrosis.

Rice blast disease is a destructive disease cause by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) that influence the global rice production. The avirulence genes in the *M.oryzae*, share a gene-for-gene relationship with the resistance genes in its host (Huang *et al*, 2014). According to Yuntao *et al.* (2010), evolution of race-specific plant resistance (R) genes enabled it to identify the product of the corresponding avirulence (Avr) genes and trigger rapid defence response to potential plant pathogens.

In general, this study focuses on *Avr-Pik* gene, on its functional value and characteristics. In fact, *Avr-Pik* gene acts as an avirulence gene and the resistance gene in the rice plant will interact and induced defence responses towards the potential pathogens.

Theoretically, emergent of new virulent races of the pathogen is through the evolution of *Avr* genes using various mechanisms (Yuntao *et al.*, 2010).

Research study of avirulence genes from fungi is vital for future information and better understanding in order to combat rice blast diseases. Currently, there is still insufficient understanding and information regarding *Avr-Pik* gene from *M.oryzae*. Often rice blast disease contributes to number of crop yield loss and due to its rapid evolution, the resistance (R) gene eventually be less effective to resist the new races of genes. On the other hand, research study in this project is promising to provide further understanding and approach to biological function of *Avr-Pik* gene.

1.2 Objectives

1. To isolate *Avr-Pik* gene from *Magnaporthe oryzae* isolate from Sarawak.
2. To clone *Avr-Pik* gene from *Magnaporthe oryzae* isolate from Sarawak.
3. To sequence *Avr-Pik* gene from *Magnaporthe oryzae*.

LITERATURE REVIEW

2.1 Types of Plant Pathogens

Plant disease is defined as any physiological abnormality or significant disruption in the normal health of a plant (Freeman & Beattie, 2008). In general, there are two major factors that lead towards plant diseases, which are biotic and abiotic factor. Biotic is defined as living organism such as fungi and bacteria; hence, biotic factor is referring to potential bacteria and fungi that give rise to plant diseases. The second factor is abiotic factor or abiotic components that refer to non-living chemical and physical parts of the environment that influence living things and normal ecosystem function. For instances, pollution, toxicities, nutrient deficiency, insufficient of oxygen, extreme temperature and exposure to ultraviolet radiation.

A large number of plant pathogen or phytopathogen exist in nature and use diverse life strategies to impaired plant health and growth (Park *et al.*, 2009). Example of plant pathogens are fungi, bacteria, oomycetes and viruses. However, not all phytopathogen can cause harm to plant because individual plant species is only susceptible to limited number of pathogen due to the presence of effective general mechanism (Park *et al.*, 2009). Phytopathogen infection may change the secondary metabolism regarding the induction of defence programmes and also affect the primary metabolism which leads to changes of growth and plant development (Berger *et al.*, 2007). Dissimilar to mammals, plants are lacking in mobile defender cells and adaptive somatic immune system (Jones & Dangl, 2006). These disadvantages allowed plant pathogens to proliferate into plant intracellular.

Different types of pathogens have different type strategies to attack plant immune system and different mode of feeding and reproduce in host (Berger *et al.*, 2007). As for biotrophic pathogens, it demand living tissue for growth and reproduction and due to many interaction the tissue will gradually die in late stages of infection (Berger *et al.*, 2007).

Unlike biotrophic, necotrophic pathogens cause the death of the host tissue at the early of infection and consume dead tissue as the nutrient source. Infection in plants, therefore, decreases the crop yield and even pathogens-plants interactions only also affect the yield. Agricultural research aimed to eradicate this disease but due to its variation and resistant pattern, this effort becomes more challenging.

2.2 General Plant Pathogenesis Mechanisms

Pathogens have to negate the plant defence to succeed the infection. Pathogens have developed specific infection structures or digestive enzymes to penetrate the physical barrier that is performed by defence on the plant surface (Łaźniewska *et al.*, 2012).Against the phytochemical defence, adapted pathogens acquired the ability to tolerate or detoxify the phytochemical compounds with antibiotic activity (Iriti & Faoro, 2009). The ability of pathogens to neutralize the preformed defences needs to be accompanied by the ability to escape or evade the recognition by the PRRs on the plant surface (Hoefle & Hückelhoven 2008; Łaźniewska *et al.*, 2012). Escaping or evading the recognition by PRRs is necessary especially for biotrophic pathogens which need to exploit plants without inducing PTI or with effective suppression of PTI (Laluk & Mengiste, 2010).

2.2.1 PAMP Triggered Immunity (PTI)

Plant immune systems are divided into two types. First type uses transmembrane pattern recognition receptors (PRRs) that respond to microbials or pathogens-associated molecular pattern (MAMPs or PAMPs) (Jones & DangI, 2006).Same signal may arise due to damage of the plant by pathogen, which is originally called endogenous elicitors and now it is named as damage-associated molecular patterns (DAMPs) (Boller & Felix, 2009). However, variations of recognition domain of PAMPs have been noticed, viz. bacterial flagellin and lipopolysaccharide which impaired the recognition PRRs to recognize without lowering the fitness of the pathogen (Pel & Pieterse, 2012). According to Jones and DangI,

PAMPs or MAMPs that is been recognized by PRRs, further generate in PAMP-triggered immunity (PTI). During pathogen trial to avoid the recognition by PRRs, the pathogen will secrete proteins that deter the PAMP recognition by PRR. The proteins that interfere with PTI are called effectors which interrupting the downstream of defense signaling pathways after PRRs recognition of pathogens (Pel & Pieterse, 2012). Effectors are "molecules secreted by plant-associated organisms that alter host-cell structure and function" (Win *et al.*, 2012), i.e. effectors are secreted by adapted and non-adapted pathogens, and mutualistic microorganisms.

2.2.2 Effector Triggered Immunity (ETI)

The second type of plant immune system used the polymorphic NB-LRR products encoded by most R genes. They are named based on their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains (Jones & Dangl, 2006). Boller and Felix (2009) state that, plant gradually evolved perception system as the effector is recognized, either indirectly or directly and trigger (ETI). Under natural selection, pathogens will shed or will have new variant effectors to negate ETI. In turn, plants also will acquire new variance of R genes to reactivate the ETI.

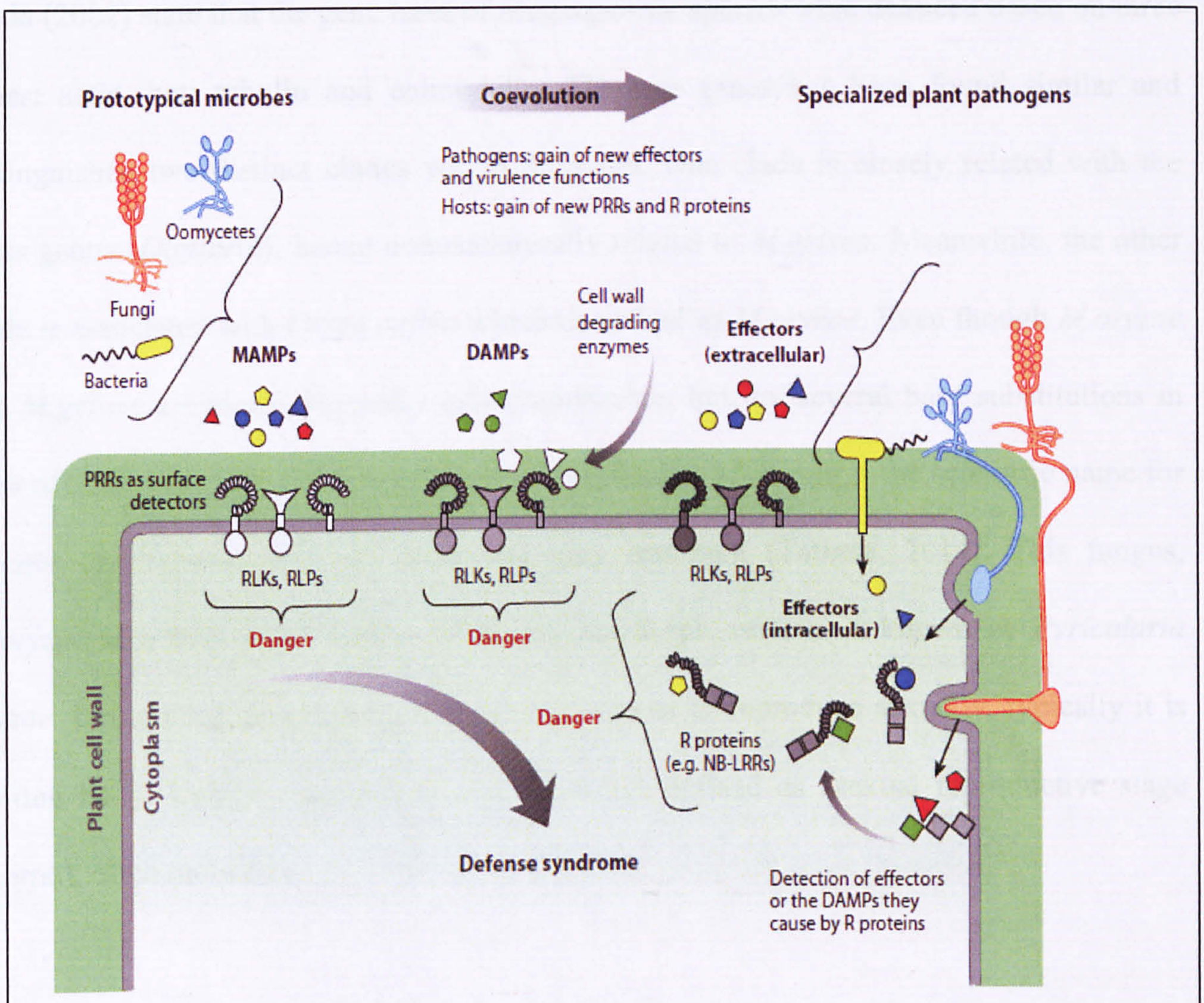

Figure 2.1: Microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and effectors are distinguished as signals of danger. Extracellular MAMPs of prototypical microbes and DAMPs released by their enzymes are recognized through pattern-recognition receptor (PRRs). RLK, receptor-like kinase; RLP, receptor-like protein; NB-LRR, nucleotide binding-site-leucine-rich repeat.

2.3 *Magnaporthe oryzae*

2.3.1 Taxonomy and nomenclature

Magnaporthe oryzae is a well-known ascomycete fungal pathogen with the ability to devastate plant such as rice, grass, barley and wheat (Dai *et al.,* 2010). *Magnaporthe oryzae* is described as a new species distinct from *M.grisea* (Brett & Linda, 2002). Taxonomic research proved that *M.grisea* is morphologically identical with *M.oryzae*, a species that infects crabgrass (*Digitaria*) (Tatiana, 2014). Previous study by Brett and

Linda (2002) state that the gene trees of *Magnaporthe* species were deduced based on three genes: actin, beta-tubulin and calmodulin. The tree genes has been found similar and distinguished two distinct clades within *M.grisea.* One clade is closely related with the grass genus (*Digitaria*), hence nomenclaturally related to *M.grisea*. Meanwhile, the other clade is associated with *Oryza sativa* which described as *M.oryzae*. Even though *M.oryzae* and *M.grisea* are morphologically indistinguishable, but, by several base substitutions in each of three loci, they exhibit difference. Specifically, *M.oryzae* is the scientific name for isolates that related with rice blast and grey leaf spot (Tatiana, 2014). This fungus, *M.oryzae* is a telemorph fungus while the anamorph version is known as *Pyricularia oryzae.* Being a telemorph fungus enabled *M.oryzae* to reproduce sexually, typically it is fruiting body. Unlike anamorph fungus, they are defined as asexual reproductive stage (morph), often mold-like.

2.4 Avirulence Genes

2.4.1 General Concept

Avirulence gene (Avr) is detected from a rust or powdery mildew fungus and oomycete (Cataranzi *et al.*, 2007) that have the ability to trigger immune system of plants as the race-specific plant resistance (R) gene able to recognize the products of the correspond avirulence (Avr) genes (Dai *et al.*, 2010). In the host plant cytoplasm, the avirulence gene is recognizable by the resistance proteins. Matching of the Avr-R pair generates rapid plants defense responses (Chang *et al.*, 2011). The resistance gene can be very effective during early treatment but due to high variable of Avr gene involves mutation of the avirulence effector, the resistance gene product lose the ability to detect it (Izuma *et al.*, 2007).

There are nine Avr genes inside rice blast pathogens which are *Avr Pita, Avr-C039, PWL1, PWL2, ACE1, Avr-Pizt, Avr-Pia, Avr-Pii, and Avr-Pik/km/kp* (Huang *et al.*, 2014). All of these avirulence genes encode protein of unknown function. In term of host specificity, three avirulence genes have been cloned which are *PWL1, PWL2 and Avr1-C039* (Izumi *et al.*, 2011). Meanwhile, in rice cultivator specificity the cloned avirulence effectors are *Avr-Pita, ACE1, Avr-Pizt, Avr-Pia, Avr-Pii, and Avr-Pik/km/kp*.

2.4.2 *Avr-Pik*

One of the avirulence genes that have been discovered in *Magnaporthe oryzae* was *Avr-Pik*. Research has found that *Avr-Pik* physically binds the N-terminal coiled-coil domain of Pik alleles by the various Pik in yeast two-hybrid assay as well as in planta co-immunoprecipitation assay (Wang *et al.*, 2014). *Avr-Pik* gene is a 113 amino acid protein with 21 amino acid signal peptide (Yoshida *et al.*, 2009). 21 isolates of *Magnaporthe oryzae* from Japan were identified five alleles of *Avr-Pik (Avr-Pik-* A, B, C, D and E) (Kanzaki *et al.*, 2012). The *Avr-Pik* DNA sequence is highly variable with a nucleotide diversity (Nei, 1987) of 7.1×10^{-3}, which is two orders higher than the mean value for the entire genome (8.2×10^{-5}) as revealed by EcoTILLING (Dai *et al.*, 2009). Tight recognition specificity of *Avr-Pik* alleles was observed in the various Pik alleles (Wang *et al.*, 2014).

METHODOLOGY

3.1 Fungal Culture

Pure strain of *Magnaporthe oryzae* was obtained from Agricultural Research Centre Semenggok, Sarawak. Firstly, oat meal agar was prepared as the medium to culture the fungus. 500ml of water was warmed in the microwave, while waiting, 15g of quick oat meal was grinded in the blender. Water was added to the blender and mixed for 1 minute. Next, the mixture was transfer to 500ml bottle and 12g of agar was added. The media was undergo autoclaving for 2 hours and left to cool down, then 200µL of Carbenicilline (100mg/ml) was added in the media and was pour into the petri dishes.

After preparing the culture medium, the fungal was inoculated and isolates using stocks on filter paper onto oatmeal agar media and was incubated for 5 days in the dark at 28°C. Further, the fungus culture was moved under fluorescent light. It was incubated for another 7-9 days under continuous light. Condensate water was removed from the petri dish to maintain culture dry.

3.2 Primer Design and Sequence Alignments

Multiple alignments was conducted between four different isolates of *Avr-Pik* gene from *Magnaporthe Oryzae* with the accession numbers of AB498875.1, AB498876.1, AB498877.1, and AB498878.1 using Clustal Omega software (Siever et al., 2011). The region of the sequence from *Magnaporthae oryzae* that is highly conserved was picked for designing primer pairs using Primer 3 (version 0.4.0) online program.

3.3 Agarose Gel Electrophoresis (AGE)

To prepare 1% of agarose gel, 0.25 g of agarose powder was added into 25 ml of 1X TAE (Tris-acetate EDTA) buffer. The mixture then heat up in a microwave oven for a minute to dissolve the agarose. Cooling process of the gel to approximately 60°C take about 5 minutes. After that, the gel solution was slowly poured to a gel tray.

Bubbles was being pushed using pipette tip and the comb was inserted. Then, the gel was left for about 30-40 minutes to allow it solidifies. 1 µl of loading dye (6X) and 4µl of pure water was added to 2 µl of DNA sample. The sample was loaded in the gel and run at 90V for approximately 30 minutes in 1X TAE buffer. After electrophoresis, the gel was submerged in staining solution with ethidium bromide (EtBr). The gel staining solution was allowed to be incubated at room temperature (22°C to 25°C) for 45 minutes. Staining time depends on size, thickness and the percentage of agarose in the gel. Then, the gel was viewed using an UV light transilluminator.

3.4 Gradient Polymerase Chain Reaction

Prior to determine the optimum annealing temperature of forward and reverse primer and to amplify *Avr-Pik* gene, Gradient Polymerase Chain Reaction was carried out. A final volume of 20µl of PCR mixture per tube was prepared by master mix (Table 3.2). A little of scrapped mycelium was added in every PCR tubes accept for negative control. The components were combined in order listed and were shock spin to mix well. The PCR was carried out by using Thermal Cycler PCR machine while cycles of PCR amplification was performed for 35 cycles as shown in Table 3.2. To indicate the functionality of DNA sample, positive control was prepared for each sample. ITS primer was used as the positive control.

Table 3.1: Component of Gradient PCR reaction for *Avr-Pik* gene

Components	Final Concentration	1X reaction (Volume per 20 µl reaction)	Master Mix (4X)
Nuclease-free water	n/a	14.6µl	58.4µl
10X Easy Taq Buffer (with Mg2+)	1X	2.0µl	8.0µl
2.5 mM dNTPs	0.2mM	1.6µl	6.4µl
Template (DNA)	As required	Scrap from culture	Scrap from culture
10µM Forward Primer	0.2 µM	0.4µl	1.6µl
10µM Reverse Primer	0.2 µM	0.4 µl	1.6µl
Easy Tag® DNA Polymerase	2.5 units	0.2 µl	0.8 µl
Final Volume		**20.0 µl**	**80.0 µl**

Table 3.2: PCR cycle

Initial denaturation	94°C for 2 minutes
Denaturation	94°c for 30 seconds
Annealing	50-60°C for 1 minute
Extension	72°C for 1 minute
Final Extension	72°C for 5 minutes

3.5 Gel Extraction and Purification

Gel extraction was done using Wizard® SV and PCR Clean-Up System Protocol (Promega, USA). DNA sample was extracted from agarose gel run with 1X TAE buffer. A 1.5ml microcentrifuge tube was weighed and recorded for each DNA fragments to be isolated. The DNA fragment of interest was excised in a minimal volume of agarose gel using a clean, sharp scalpel. The gel slice was transferred to the weighed microcentrifuge