Isolation and Characterization of Avirulence Genes in *Magnaporthe oryzae*

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ABSTRACT

Magnaporthe oryzae is a fungal pathogen contributing to rice blast diseases globally via their *Avr* (avirulence) gene. Although the occurrence of *M. oryzae* has been reported in Sarawak since several decades ago, however, none has focused specifically on *Avr* genes, which confer resistance against pathogen-associated molecular pattern-triggered immunity (PTI) in host. The objective of this study is to isolate *Avr* genes from *M. oryzae* 7' (a Sarawak isolate) that may contribute to susceptibility of rice towards diseases. In this study, *AvrPiz-t*, *AVR-Pik*, *Avr-Pi54*, and *AVR-Pita1* genes were isolated via PCR and cloning approaches. The genes were then compared with set of similar genes from related isolates derived from NCBI. Results revealed that all eight *Avr* genes (including four other global isolates) shared similar N-myristoylation site and a novel motif. 3D modeling revealed similar β-sandwich structure in *AvrPiz-t* and *AVR-Pik* despite sequence dissimilarities. In conclusion, it is confirmed of the presence of these genes in the Sarawak (*M. oryzae*) isolate. This study implies that Sarawak isolate may confer similar avirulence properties as their counterparts worldwide. Further R/Avr gene-for-gene relationship studies may aid in strategic control of rice blast diseases in future.

Keywords: Plant disease, rice blast, Sarawak, *Magnaporthe oryzae*

INTRODUCTION

Magnaporthe oryzae is a rice blast pathogen causing major harvest loss globally. It belongs to hemibiotrophic fungus which grows biotrophically on living plant tissue during initial life cycle and subsequently into necrotrophic mode causing the death of infected plant tissues (Horbach *et al*., 2011). It is known that *M. oryzae* encodes a variety of effector molecules to confer virulence (Li *et al*., 2009). Effectors deployed by pathogens interfere with PTI (pathogen-associated molecular pattern-triggered immunity), given that the PTI is overcome, the effectors will be recognized by specific R genes and followed by the action of ETI (effector-triggered immunity). The recognized effectors are described as AVR (avirulence) protein (Jones & Dangl, 2006). Hence, ETI brings about the development of disease resistance and often causes HR (hypersensitive response) at the site of infection. HR is a rapid and localized tissue necrosis at the penetration site and involves transcriptional activation of various defense genes of the plant which subsequently avoid further spread of pathogen through the plant tissue (Lamb, 1994; Joosten *et al*., 1997; Agrios, 2005). To date, several effector proteins encoded by *Avr* genes from *M. oryzae* have been cloned molecularly, namely *PWL2* (Sweigard *et al*., 1995), *AVR-Pita* (Orbach *et al*., 2000), *AVR-Pia* (Miki *et al*., 2009; Yoshida *et al*., 2009), *AVR-Pii* (Yoshida *et al*., 2009), *AVR-Pik* (Yoshida *et al*., 2009), *AvrPiz-t* (Li *et al*., 2009), *AVR1-CO39* (Ribot *et al*., 2013), *Avr-Pi9* (Wu *et al*., 2015), *AvrPib* (Zhang *et al*., 2015), and *Avr-Pi54* (Ray *et al*., 2016).

Malaysia is granted with temperature regime and rainfall distribution that favour year round rice cultivation, even if in the conditions of rainfed (Food and Agriculture Organization of the United Nations, 2002). According to Valera & Lee (2016), Malaysia achieved 63% self-sufficiency in rice production and relied the rest of it on rice import to compensate domestic production. With the ever increasing domestic demand in the country, ensuring a sustainable food supply is vital for the continuous development of the nation.

Several studies have been done on the blast resistance gene (*R* gene) in local rice varieties previously, however report on *Avr* gene in local *M. oryzae* which functions in complement with resistance genes is lacking (Fatah *et al.*, 2014; Tanweer *et al.*, 2015). This reflected an urge to bridge the knowledge gap in order to develop a more comprehensive and novel strategy in rice blast control. In this study, focus would be on preliminary finding of *Avr* genes from *M. oryzae* 7' isolate of Sarawak which has never been investigated.

MATERIALS AND METHODS

Fungal Culture and Species Verification

Pure strain of *M. oryzae* 7' from Sarawak isolates was provided by Agriculture Research Centre Semenggok and cultured on oatmeal agar (instant oatmeal). The cultures were incubated at 28±0.5°C at dark for 5 days and were grown under continuous light for consecutive days. Fungal mycelia were harvested by using micropipette tip and subjected to direct PCR for species verification using ITS1/ITS4 universal primer pair with T100™ Thermal Cycler (Bio-Rad Laboratories, USA).

Direct PCR was carried out in a 20 µl reaction volume per PCR tube containing 10x EasyTaq® Buffer (with Mg^{2+}), 2.5 mM dNTPs, EasyTaq® DNA Polymerase, 10 µM ITS1 forward primer, 10 µM ITS4 reverse primer, and ddH_2O by referring to protocol as suggested by manufacturer of EasyTaq® DNA Polymerase (TransGen Biotech, China) in the following parameter: initial denaturation of 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 5 minutes. PCR product was run on a 1.5% agarose gel and visualized under UV transilluminator for band detection. Single, specific band containing gene fragment of interest was excised and subjected to purification using Wizard® SV Gel and PCR Clean-Up System (Promega, US).

Purified PCR product was then sent to First BASE Laboratories (Malaysia) for sequencing. The sequence received from First BASE Laboratories was checked by using MEGA 6 (Version 6.0) (Tamura *et al*., 2013). The identity of the gene sequence was analyzed by BLASTn (Version 2.3.1+) (Altschul *et al*., 1997).

Primer Design

Nucleotide sequences of *AvrPiz-t*, *AVR-Pik*, *Avr-Pi54*, and *AVR-Pita1* genes from other global isolates were retrieved from database of NCBI Genbank. From the identified conserved gene region using Clustal Omega (Version 1.2.1) (Sievers *et al*., 2011), forward and reverse primer pair was designed by using Primer3Plus (Untergasser *et al*., 2007) and Oligonucleotide Properties Calculator (Kibbe, 2007). Primer sequences were sent to First BASE Laboratories for subsequent synthesis. Details on the primer sequence were shown in Table 1.

Table 1. List of primers used and their details.

Target Gene	Primer	Primer Sequence (5'-3')	Optimized T_a (°C)	Amplicon Size (bp)
ITS1 region, 5.8S, and ITS2 region	ITS1	TCCGTAGGTGAACCTGCGG	57.0	~497
	ITS4	TCCTCCGCTTATTGATATGC		
AvrPiz-t	MOPIZTF1	ATGCAGTTCTCAACCATCATCA	60.7	327
	MOPIZTR1	CTATTGGCGCTGAGCCTGA		
AVR-Pik	MOPIKF1	ATGCGTGTTACCACTTTTAACA	58.6	342
	MOPIKR1	TTAAAAGCCGGGCCTTTT		
Avr-Pi54	MOPI54F1	ATGCAGTTCACCGCCACCAT	59.5	462
	MOPI54R1	CTAGCAGCCATAGGTGAGGA		
AVR-Pita1	MOPITAF1	ATGCTTTTTTATTCATTGTTATTTT	58.6	884
	MOPITAR1	TTAACAATWTTTATAACGTGCACAT		

PCR Optimization and Gene Amplification

EasyTaq® DNA Polymerase (TransGen Biotech, China) was used for gene amplification, protocol and components of reaction was slightly adjusted from Martin & Rygiewicz (2005). Gene-specific primer pairs were used and optimized separately in gradient PCR by using T100™ Thermal Cycler (Bio-Rad Laboratories, USA) (refer to Table 1). Positive control (ITS1/ITS4 primer pair) and negative control were carried out in parallel. Samples from PCR reaction were run on 2% agarose gel and visualized under UV transilluminator.

Cloning of *Avr* Genes

Single, specific band containing gene fragment of interest for each *Avr* gene was excised, and subjected to purification using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's protocol with slight modification. Purified PCR product was ligated into pGEM®-T Easy Vector (Promega, USA) and was transformed into *E. coli* XL1-Blue competent cell. Identification of recombinant bacterial colonies was carried out by blue/white screening followed by elimination of false positive colonies by secondary blue/white screening. Positive colonies were subjected to colony PCR for direct identification of vectors containing desired insert and purified by using PureYield™ Plasmid Miniprep System (Promega, USA). Verification of proper gene insertion into the multiple cloning site was performed by restriction digestion using FastDigest enzyme, *NotI* (Promega, USA). Purified plasmids each containing desired *Avr* genes were sent for sequencing.

DNA Sequencing and Data Analysis

The sequence file of *AvrPiz-t*, *AVR-Pik*, *Avr-Pi54*, and *AVR-Pita1* genes was accessed with MEGA 6 (Version 6.0) (Tamura *et al*., 2013). Open reading frame (ORF) of the gene was identified and the nucleotide sequence of vector was removed. The gene sequence was analyzed by BLASTn (Version 2.3.1+) (Altschul *et al*., 1997). Then, the nucleotide sequences were translated into protein sequence by using ExPASy translate tool (Artimo *et al*., 2012) for further sequence characterization. The nucleotide translated protein sequence was analyzed by BLASTp (Version 2.3.1+) (Altschul *et al*., 1997) for similarity search. On the other hand, the protein sequences of *AvrPi9* (AIS23643.1), *AVR-Pia* (BAH59484.1), *AVR-Pii* (BAH59485.1) and *AvrPib* (AKO62639.1) from *M. oryzae* of global isolates, each encoding for AVR type effector proteins were retrieved from GenBank database. ProtParam server (Gasteiger *et al*., 2005) was used for physico-chemical analyses such as length of amino acid residues, molecular weight, and theoretical pI. Subcellular localization was predicted by CELLO server (Version 2.5) (Yu *et al*., 2006). SignalP4.1 (Petersen *et al*., 2011) was used to identify signal peptide cleavage site. Apart from that, the presence of domains and motifs were detected through ScanProsite online software (de Castro *et al*., 2006), Interpro Scan 5 online software (Jones *et al*., 2014), Conserved Domain Database server (Marchler-Bauer *et al*., 2015), and MEME online software (Bailey & Elkan, 1994). Sequence variation in a novel motif detected was analyzed for conservation by using Clustal Omega (Version 1.2.1) (Sievers *et al*., 2011). Three-dimensional protein modeling was performed by using Phyre2 (Kelley *et al*., 2015), Jmol (http://www.jmol.org/) and ProFunc (Laskowski *et al*., 2005). Validation of modeled proteins was carried out by RAMPAGE for Ramachandran Plot analysis (Lovell *et al*., 2002). Then, Dali server (Holm & Laakso, 2016) was used to identify the structural neighbours.

RESULTS

Fungal culture and species verification

In order to verify the identity of fungus cultured, molecular characterization by direct sequencing appeared as a precise approach. Universal primer pair ITS1/ITS4 was used for the amplification of ITS1 region, 5.8S rRNA (ribosomal ribonucleic acid) gene, and ITS2 region from *M. oryzae* 7'. At an annealing temperature of 57°C, a single, specific band with an estimated size that range between 500 bp and 600 bp was obtained. After purification and sequencing, BLASTn sequence similarity

search verified the identity of fungal culture as *M. oryzae*. *M. oryzae* 7' shared a total similarity to that of *M. oryzae* MG1-1 (KJ766301.1) with a low E-value of 0.0 representing this result is highly significant.

Primer design for isolation of *Avr* genes

Four *Avr* genes were isolated from *M. oryzae* 7' isolate are, *AvrPiz-t* (KX459419), *AVR-Pik* (KX459420), *Avr-Pi54* (KY441415), and *AVR-Pita1* (KY441414), each encoding for effector protein that interacts with respective race-specific resistance protein in host rice plant. A number of replicates of each gene were sent for sequencing, and a consistent result was obtained. BLASTn revealed these four isolated *Avr* genes having a 99% similarity to previously deposited data from other global *M. oryzae* populations. This has proven the presence of these four *Avr* genes in *M. oryzae* 7' of Sarawak isolates. Similarly, BLASTp sequence similarity search using nucleotide translated protein sequence unveiled high degree of similarities to available database. Three of the *Avr* genes (*AvrPiz-t*, *AVR-Pik*, and *Avr-Pi54*) isolated were intronless whereas *AVR-Pita1* contains three segments of introns and was removed manually. Each of the isolated gene sequence having a complete open reading frame encoding from start codon until stop codon, with size of 108 aa, 112 aa, 153 aa, and 224 aa with respect to *AvrPiz-t*, *AVR-Pik*, *Avr-Pi54* , and *AVR-Pita1*.

Characterization of Avr genes

In order to further investigate the functional elements found in *Avr* genes, four other *Avr* gene sequences from *M. oryzae* of global isolates carrying out similar function has been retrieved from Genbank for further characterizations. From Table 2, it is known that each *Avr* genes encoded for a relatively small-sized protein molecules, where all of them except *Avr-Pita1*, were predicted to be secreted extracellularly, of which three of them *Avr-Pi54*, *AVR-Pia*, and *AvrPib* has other additional sites of subcellular localization in plasma membrane, mitochondrial, periplasmic, and cytoplasmic region; whereas *AVR-Pita1* was an exception predicted to be localized on plasma membrane. Information on the subcellular localization of these effector proteins would perhaps provide some clue on their functions within the highly compartmentalized eukaryotic cells. Result generated from SignalP4.1 reveals the presence of signal peptide and the absence of transmembrane segment in all of the analyzed sequences.

Table 2. Physico-chemical properties and subcellular localization of effector proteins encoded by avirulence genes.

Effector protein	Accession number	Number of amino acid residues		Molecular weight (kDa)	Theoretical pI	Subcellular localization
		Signal peptide	Mature chain			
AvrPiz-t	KX459419	1-18	19-108	11.70	9.17	Extracellular
AVR-Pik	KX459420	1-21	22-113	12.94	8.49	Extracellular
Avr-Pi54	KY441415	1-19	20-153	15.80	6.52	Extracellular Plasma Membrane
AVR-Pita1	KY441414	1-17	18-224	26.00	6.09	Plasma Membrane
AvrPi9	AIS23643.1	1-18	19-91	9.56	6.94	Extracellular
AVR-Pia	BAH59484.1	1-19	20-85	9.43	7.82	Extracellular Mitochondrial
AVR-Pii	BAH59485.1	1-19	20-70	7.50	6.00	Extracellular
AvrPib	AKO62639.1	1-22	23-74	8.12	9.22	Periplasmic Cytoplasmic Extracellular

Of all the eight genes, only *AVR-Pita1* contains a M35 deuterolysin like domain and is detected to be under M35 like superfamily. On the other hand, there is some high frequency motif such as N-myristoylation site, protein kinase C phosphorylation site, casein kinase II phosphorylation site, N-glycosylation site, and amidation site. As shown in Table 3, all effector proteins having the common presence N-myristoylation motif on their protein sequences.

Through multiple sequence alignment, these eight gene sequences was not found to share any significant sequence similarity to each other (data not shown). MEME server has however detected the presence of a novel motif across four genes (*AvrPiz-t*, *AvrPi9*, *AVR-Pia*, and *AVR-Pii*) out of the analyzed eight genes (*AvrPiz-t*, *AVR-Pik*, *Avr-Pi54* , *AVR-Pita1*, *AvrPi9*, *AVR-Pia*, *AVR-Pii*, and *AvrPib*) (refer to Figure 1). The motif consists of six amino acid residues at regions coding for signal peptide supported with an e-value of 3.9e-004.

Apart from that, structures of the four locally isolated *Avr* genes (*AvrPiz-t*, *AVR-Pik*, *Avr-Pi54*, and *AVR-Pita1*) were *in silico* modeled and validated using Ramachandran plots (Figure 2 and Table 4). It is shown that >90% of residues in models of *AvrPiz-t*, *Avr-Pik*, and *AVR-Pita1* were in the favored and allowed regions and thus indicates for an overall good quality of modeled proteins. From the modeled 3D structures, *AvrPiz-t* and *Avr-Pik* contains antiparallel β-sheets that form a β-sandwich structure containing 6 strands. This is consistent with previous studies carried out by Maqbool *et al*. (2015) and Zhang *et al*. (2013). The significance in structural relationship between *AvrPiz-t* and *AVR-Pik* has been presented by the Dali Z-score of 3.7 in this study using locally isolated *Avr* genes. From the structural neighbours as indicated by Dali server, there are common structural neighbours shared between *AvrPiz-t* and *AVR-Pik* (Table 4). Structural neighbours of *AvrPiz-t* and *AVR-Pik* (PDB ID: 2MM0-A, 2MM2-A , 5A6W-C, 2MYV-A , 2LW6-A , 2MYW-A) having the common β-sandwich structure (de Guillen *et al*., 2015; Maqbool *et al*., 2015; Nyarko *et al*., 2014; Zhang *et al*., 2013).

In contrary, the modeled *AVR-Pita1* is relatively helix-rich based on the predicted secondary structure as compared to other *Avr* genes isolated. The structural neighbours of *AVR-Pita1* (2X3C-A, 1G12-A, 1EB6-A) are metalloendopeptidase. This could be due to the properties of *AVR-Pita1* as a member of M35 family containing motif encoded for zinc protease. Whereas the lack of support in Ramachandran plots and the absence of structural neighbour in *Avr-Pi54* could be due to the characteristics of *Avr-Pi54* as a twilight-zone proteins that complicates the modeling of the protein. On the other hand, the 3D structure of *AVR-Pita1* and *Avr-Pi54* do not display significant structural similarities to that of *AvrPiz-t* and *Avr-Pik*.

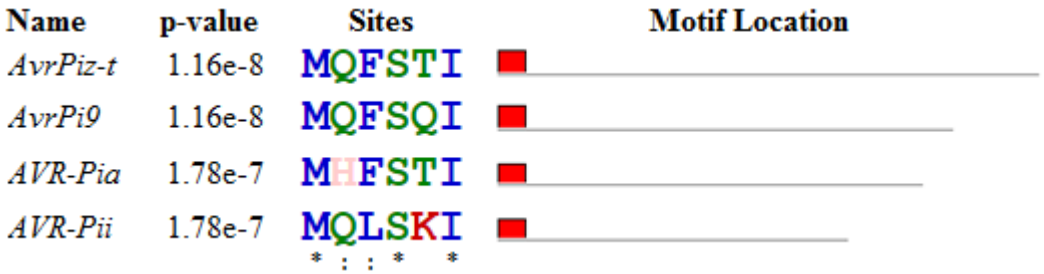

Figure 1. Location of novel motif detected on *AvrPiz-t*, *AvrPi9*, *AVR-Pia*, and *AVR-Pii.* (Asterisk (*) represents residue is fully conserved, whereas colon (:) represents residue is conserved in similar properties).

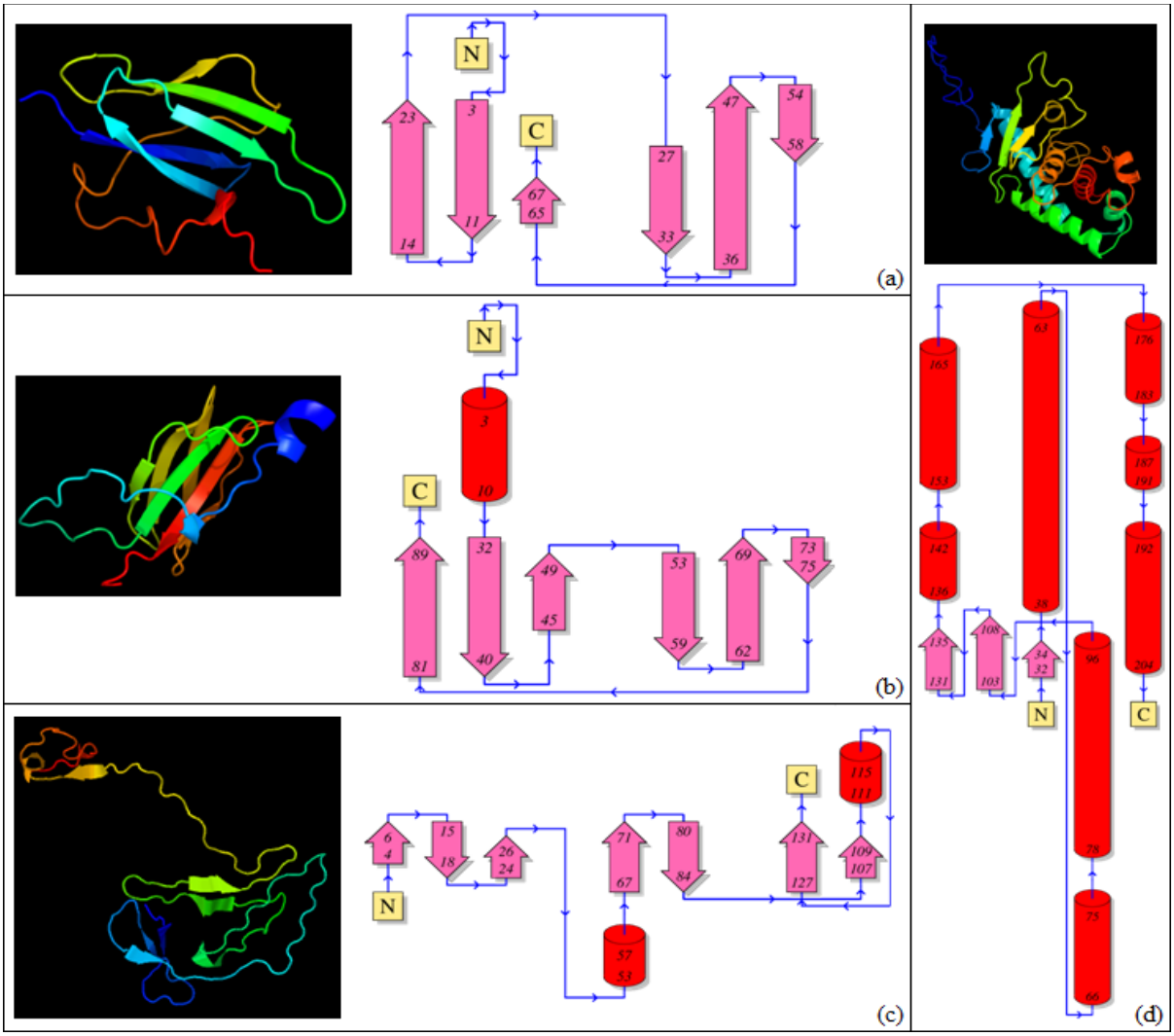

Figure 2. Three dimensional protein modeling and topology diagram of *Avr* genes isolated from *M. oryzae* 7'. (a) *AvrPiz-t*, (b) *AVR-Pik*, (c) *Avr-Pi54*, and (d) *AVR-Pita1*.

Table 3. High frequency motif detected in *Avr* sequences.

Gene	N-myristoylation site	Protein kinase C phosphorylation site	Casein kinase II phosphorylation site	N-glycosylation site	Amidation site
AvrPiz-t	✓	✓	✓	×	×
AVR-Pik	✓	✓	×	✓	✓
Avr-Pi54	✓	×	✓	×	×
AVR-Pita1	✓	✓	✓	×	×
AvrPi9	✓	✓	×	×	×
AVR-Pia	✓	✓	×	×	×
AVR-Pii	✓	×	✓	×	×
AvrPib	✓	×	✓	×	×

Table 4. Ramachandran plot statistics and stuctural neighbours of modeled AVR proteins.

	AvrPiz-t	*AVR-Pik*	*Avr-Pi54*	*AVR-Pita1*
Ramachandran plot statistics:				
Number of residues in favoured region	67 (76.1%)	86 (95.6%)	83 (62.9%)	181 (88.3%)
Number of residues in allowed region	13 (14.8%)	3 (3.3%)	19 (14.4%)	16 (7.8%)
Number of residues in outlier region	8 (9.1%)	1 (1.1%)	30 (22.7%)	8 (3.9%)
Structural Neighbours	2MM0-A 2MM2-A 5A6W-C 2MYV-A	2MYV-A 2MM0-A 2MM2-A 2LW6-A 2MYW-A		2X3C-A 1G12-A 1EB6-A

(PDB IDs and protein names of structural neighbours are as follow: 2MM0-A: Host-selective toxin protein; 2MM2-A: *Toxb*; 5A6W-C: *AVR-Pik* Protein; 2MYV-A: *AVR1-CO39*; 2LW6-A: *AvrPiz-t* protein; 2MYW-A: *AVR-Pia* protein; 2X3C-A: Extracellular Toxic Zinc Metalloendopeptidase; 1G12-A: Peptidyl-Lys Metalloendopeptidase; 1EB6-A: Neutral Protease II).

DISCUSSION

Occurrence of *M. oryzae* has been reported in Sarawak since several decades ago. However, this is the first study focusing specifically on *Avr* genes of this rice blast fungus from local isolates. The successful molecular cloning of *Avr* genes has detected and confirmed their presence in the *M. oryzae* 7' of Sarawak isolates. The cloning involved full-length coding sequence and would provide useful information for subsequent functional and expression studies. However, the lacks of significant sequence similarity among *Avr* genes under study complicate the efforts to identify components accounted for their functional similarity in triggering host defense. Understanding on the domains and motifs present in AVR effector proteins is important for their functional prediction.

AVR-Pita1 predicted under the M35 family has two signature zinc-binding histidines as well as a catalytic glutamate (Markaryan *et al*., 1994). Additionally, a motif encoding for zinc protease is detected, which may imply the function in metalloendopeptidase activity. Neutral metallopeptidase NMP1 has recently found to play a major role in mycotropic interactions and self defence of avirulent plant symbiont, *Trichoderma* species which is capable of producing compounds that induce resistance response in plants (Harman *et al*., 2004; Zhang *et al*., 2016).

The motif of N-myristoylation presents in all the eight analyzed sequences. Such appearance might give a clue on common biochemical activities performed by the *Avr* genes. N-myristoylation, is one form of protein fatty acylation that serves as a targeting strategy between plants and pathogens (Boyle *et al*., 2016). Protein modification involving N-myristoylation occurs mainly on cytoplasmic proteins while rarely found on integral membrane proteins. They are usually involved in the regulation of cellular structure and function which encompassed proteins associated with cellular signal transduction pathways (Moriya *et al*., 2013). The importance of consensus myristoylation sites has been highlighted in ensuring efficient localization of AVR proteins and HR elicitation in different plants (Nimchuk *et al*., 2000; Tampakaki *et al*., 2002).

According to Devanna *et al*. (2014), the presence of phosphorylation site in a protein implied the role of the protein in signal transduction and transmittance to other genes for downstream activities. For example, Protein kinase C (PKC) phosphorylation sites having significant role in the regulation of catalytic activity, stability and intracellular localization of enzyme (Freeley *et al*., 2011). It has been noted that proteins, which possess this particular phosphorylation site, were highly expressed during appressorium formation in *Magnaporthe* species (Thines *et al*., 1998; Xue *et al*., 2002). The role of appressorium has long been noted as an infection cell employed by *M. oryzae* to invade rice plant upon maturation in the form of hyphae (Talbot & Wilson, 2009).

On the other hand, casein kinase II (CK2) activity mediates signal responsible for virulence in a fungal plant pathogen, *Cryphonectria parasitica* in which the elimination of CK2 phosphorylation site lead to a lack of virulence (Salamon *et al*., 2010). Apart from that, glycosylation regulates how proteins fold, their biological activity as well as their half-life (Kotz *et al*., 2010). Study shown that *Slp1* effector protein from *M. oryzae* undergo N-glycosylation is capable of evading plant immune response (Chen *et al*, 2014).

A novel motif is detected in *AvrPiz-t* gene isolated from local *M. oryzae* isolate. The motif is located at gene region coding for signal peptide. Signal peptide carries the information that direct targeting and translocation of secretory and membrane proteins. Translocation of AVR proteins into host cells could be performed on the basis of intracellular recognition by the host resistance proteins signaled by their N-terminal regions in the absence of the pathogen itself (Rafiqi *et al*., 2010).

Li *et al*. (2009) has revealed the importance of the secretion signal at the N terminus of *AvrPiz-t* in triggering host immune response mediated by *Piz-t*, whereby the removal of secretion signal caused the loss of function in the avirulent phenotype. This indicated information carried in signal peptide is vital for recognition by cognate resistance gene in the host plant and therefore the lack of pathogenicity. Finding of the motif in these four *Avr* genes might be useful in further study on plant-pathogen recognition mechanism and in the sequence pattern of effector proteins. Thus, functional significance of this motif in the regulation and expression of *AvrPiz-t, AvrPi9, AVR-Pia*, and *AVR-Pii* has to be validated and characterized experimentally.

AvrPiz-t to as structural neighbour of *AVR-Pik*, at a z-score of >2.0 implying the significance in structural similarity. Previous study supported by experimental data showing that *AvrPiz-t*, *AVR-Pia* from *M. oryzae*, *AVR1-CO39* from *M. grisea*, and *ToxB* from *Pyrenophora tritici-repentis* are sequence-unrelated structural homologs having a common β-sandwich structure and similar topology was suggested to be categorized under family MAX-effectors (*Magnaporthe* Avrs and ToxB like) (Zhang *et al*., 2013; Nyarko *et al*., 2014; de Guillen *et al*., 2015). Notably, separate study by Maqbool *et al*. (2015) also reported the presence of characteristic β-sandwich structure of MAX-effectors family in *AVR-PikD* from *M. oryzae*. Thus, this is consistent with findings of study whereby the predicted 3D structure of *AvrPiz-t* and *AVR-Pik* have the β-sandwich structure and structural similarity validated by the Dali z-score of 3.7.

Each of this MAX-effectors function in recognition to NLR (nucleotide-binding leucine-rich repeat) immune receptors in host rice plant: *AvrPiz-t* to *Piz-t* by indirect interaction, *AVR-Pik* to *Pik* by direct binding, as well as *AVR1-CO39* and *AVR-Pia* to the same RGA4/RGA5 by direct binding (Cesari *et al*., 2013; Maqbool *et al*., 2015; Park *et al*., 2016). NLR proteins are the largest class of R genes that play the key role as receptors that function in recognition to the pathogenic secreted effector proteins. Upon recognition between the plant effector proteins and pathogen effector proteins, HR associated with local cell death is triggered to avoid further spread of disease caused by the pathogen (Jones & Dangl, 2006).

CONCLUSION

In conclusion, several *Avr* genes were isolated in this study which were *AvrPiz-t*, *AVR-Pik*, *Avr-Pi54*, and *AVR-Pita1*. Further structural studies have been conducted based on the ORF of these genes, which a novel signal peptide motif was found in *AvrPiz-t*. This study serves as an approach to fill up the knowledge gap on the distribution and variation of *Avr* gene. The discovery of these genes in *M. oryzae* 7' implied their possible presence in other closely related *M. oryzae* isolates. Further study in this gene to include more isolates would enhance understanding in *Avr* gene sequence diversity. In a wider prospect, there is a need to investigate the presence and dynamics of their cognate resistance gene in rice plant which would provide useful information on selection pressure characterizing the *Avr* gene profile of *M. oryzae*.

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