Journal of Genetics (2020) 99:45
https://doi.org/10.1007/s12041-020-01197-8

RESEARCH ARTICLE

High nucleotide sequence variation of avirulent gene, *AVR-Pita1*, in Thai rice blast fungus population

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Received 31 October 2019; revised 9 February 2020; accepted 24 February 2020; published online 27 May 2020

Abstract. Rice blast disease, caused by *Magnaporthe oryzae*, is one of the most importance diseases of rice production worldwide. The key role of defense mechanism to combat this fungus in rice follows the gene-for-gene concept, which a plant resistant (*R*) gene product recognizes a fungal avirulent (*AVR*) effector and triggers the hypersensitive response. However, the *AVR* genes have been shown to be rapidly evolving resulting in high level of genetic diversity. The aims of this study were to examine the nucleotide sequence variation of *AVR-Pita1* gene in Thai rice blast isolates and to identify the severity of blast disease using isogenic line of *Pita* gene. Seventy-six rice blast isolates collected from different parts of Thailand were used. Gene specific primers for *AVR-Pita1* gene coding sequence were designed and used for identifying the genetic diversity of *AVR-Pita1* gene by PCR amplification and sequencing. The obtained sequences were analysed for genetic variation and genetic relationship. Our results revealed the association between the sequence variations of *AVR-Pita1* and selective forces from *Pita* gene. This phenomenon demonstrated the coevolution between rice blast resistant gene in rice and avirulent gene in blast fungus. The information about variation and evolutionary mechanisms of *AVR* gene obtained from this study can be used in rice blast resistant breeding programme.

Keywords. avirulence gene; *AVR-Pita1* gene; genetic variation; resistant gene.

Introduction

Blast disease, caused by the filamentous ascomycetous fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*), is the most devastating diseases of rice (*Oryza sativa*) in all continents, where rice is cultivated (Couch and Kohn 2002). It has potential to attack all growth stages of rice under favourable conditions. Moreover, this disease can cause the loss of up to 100% rice production yield (Zeigler *et al*. 1994). *M. oryzae* is a haploid and heterothallic fungus. Mating type of rice blast fungus is controlled by a single locus with two alleles, *MAT1-1* and *MAT1-2* (Debuchy *et al*. 2010). The fusion of mycelium between opposite mating type leads to sexual reproduction, while asexual reproduction occurs in the field by conidiation (Zeigler 1998). The *M. oryzae* genome size is an ∼40 Mb packaged in seven chromosomes, with a frequency of one gene every 3.5 kb in the rice blast fungus genome (Dean *et al*. 2005).

Resistance to *M. oryzae* follows the concept of 'gene-for-gene interaction' (Flor 1971). The resistant gene (*R* gene) encodes for resistant protein usually contains nucleotide binding site (NBS) and leucine-rich repeat (LRR) domain. It

recognizes a unique corresponding effector encoded by an avirulent gene (*AVR* gene) in a pathogen. The consequence of the recognition will lead to the trigger of the defense response (Hulbert *et al*. 2001). However, the instability of *AVR* genes in *M. oryzae* plays an important role in generating new virulent forms of fungus and *R* gene products will gradually loss their recognition ability within a few years after the release (Huang *et al*. 2014). New virulent strain can emerge through sexual recombination and mutation which modify its *AVR* gene. The mutations including point mutation, deletion, translocation and transposable element insertion, lead to the avoidance of the inspection by the *R* gene products (Khang *et al*. 2008; De Wit *et al*. 2009; Fudal *et al*. 2009; Longya *et al*. 2019).

To date, 25 *AVR* genes of *M. oryzae* have been genetically mapped in rice blast genome (Dioh *et al*. 2000). Among them, 11 *AVR* genes (*PWL1* (Kang *et al*. 1995), *PWL2* (Sweigard *et al*. 1995), *AVR-Pita* (Orbach *et al*. 2000), *AVR1-CO39* (Farman *et al*. 2002), *ACE1* (Fudal *et al*. 2005), *AVR-Pizt* (Li *et al*. 2009), *AVR-Pia* (Miki *et al*. 2009), *AVR-Pii* (Yoshida *et al*. 2009), *AVR-Pik/km/k* (Yoshida *et al*. 2009), *AVR-Pi9* (Wu *et al*. 2015) and *AVR-Pib* (Zhang *et al*. 2015)) have been cloned and characterized. The first report on the direct interaction between a plant *R* gene product and a fungal AVR effector is between Pita protein and AVR-Pita1 effector (Jia *et al*. 2000). *Pita* is a single-copy gene located on rice chromosome 12. It encodes 928 amino acids, which predicted to be a cytoplasmic protein with a centrally located NBS domain. The LRR domain at the carboxyl terminus recognizes the corresponding avirulent gene product, AVR-Pita effector (Jia *et al*. 2000). *AVR-Pita* gene located on the telomeric region of chromosome 3 of *M. oryzae* genome and encodes a neutral zinc metalloprotease protein (Orbach *et al*. 2000). Previous studies showed that rice blast fungus has high level of genetic diversity, which promotes the ability to adapt to overcome the resistance of resistant rice cultivars (Bonman *et al*. 1989). *AVR-Pita1* was also revealed to have high level of the sequence and structural variation, which leads to the emergence of novel virulent isolates (Dai *et al*. 2010; Kasetsomboon *et al*. 2013). Single nucleotide substitutions and deletion of *AVR-Pita* coding sequence are common mechanisms for overcoming resistance in the field (Kang *et al*. 2001; Zhou *et al*. 2007; Dai *et al*. 2010; Takahashi *et al*. 2010). In addition, a frame-shift mutation in the first exon and insertion of Pot3 transposable element into the protease motif were also present in the variation of *AVR-Pita* (Kang *et al*. 2001; Takahashi *et al*. 2010).

In this study, we investigated the nucleotide sequence variation of *AVR-Pita1* gene in Thai rice blast isolates and identified the severity of blast disease using isogenic line of *Pita* gene. Seventy-six rice blast isolates, collected from infected rice leaves in central, northern and northeastern rice production area of Thailand in 2006–2013 were used. Gene specific primers for *AVR-Pita1* gene coding sequence were designed. The obtained sequences were analysed for genetic variation and genetic relationship. Our results revealed the information linking sequence variation of *AVR-Pita* with selective forces from *Pita* gene. This phenomenon demonstrated the coevolution between resistant gene in rice, *O. sativa*, and avirulent gene in blast fungus, *M. oryzae*. Information about *AVR* gene variation and the evolutionary mechanisms obtained from this study is beneficial to the rice blast resistance breeding programme.

Materials and methods

Fungal materials, culture conditions and storage

Fungal isolates were collected from the infected leaves of rice with typical blast disease symptoms from central, northern and northeastern regions of Thailand in 2006–2013 (figure 1). The single spore isolates from each infected leaf sample were separated and cultured on filter paper. Seventy-six rice blast isolates used in this study were kindly provided by the Rice Blast Fungus Genetic stock at National center for Genetic Engineering and Biotechnology (Biotec, Thailand), Department of Agronomy, Kasetsart University and King Mongkut's Institute of Technology Ladkrabang. Two rice blast strains, 70-15 and Guy11 were used as reference strains.

Each fungal isolate was grown on rice flour agar (RFA) medium (RFA: 2.0% of rice flour, 2.0% of agar and 0.2% of yeast extract and 1 L distilled H_2O) at room temperature for seven days under fluorescence lighting to produce mycelia. Mycelium was transferred to filter paper of a new Rice Flour Agar Petri dish for 7–14 days. Filter papers were dried in a desiccator and were maintained at 4°C for working stock and at −20°C in a freezer as permanent stock.

DNA extraction and PCR amplification

Fungal mycelia of each blast isolate were transferred to 50 mL plastic tube containing potato dextrose broth (PDB) (potato dextrose flour 20 g, yeast extract 3 g and distilled water 1 L) and incubated at 28°C for 7 days with shaking 200 rpm. Total genomic DNA was extracted from the filtrating mycelia by using liquid nitrogen and cetyltrimethylammonium bromide (CTAB) method (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 2% SDS). Purified DNA sample was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and diluted with sterile-distilled water to a concentration of 50 ng/μL for PCR analysis.

The molecular characterisation of *M. oryzae* isolates were carried out using the polymerase chain reaction (PCR) system with gene specific primer of avirulent gene *AVR-Pita1*. These specific primers were designed by Launch Net primer program (http://www.premierbiosoft.com/netprimer/netprlaunch/Help/xnetprlaunch.html) to amplify the coding sequences, which covers from start to stop codon. Since *AVR-Pita1* gene is quite long, two primer pairs were designed to cover the whole gene designated as AVR-Pita1 up and AVR-Pita1 down primers (table 1; figure 2). One

microliter of 50 ng genomic DNA was used as the template in 20 μL reaction mixtures containing 1 U of *Taq* DNA polymerase, 1× Intron PCR buffer, 20 mM $MgCl_2$, 10 mM dNTPs, 1 μL of each 5 μM primer and distilled water in a final reaction volume of 20 μL under PCR following condition: including initial denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s of denaturation, 55°C for 30 s of annealing, 72°C for 50 s of extension and a final extension of 72°C for 5 min. The PCR products were resolved by electrophoresis on 1% agarose gel by stained with GelRed (Biotium, USA) and visualized under UV light. Amplified products were purified with a Qiaquick gel extraction kit (Qiagen, Valencia, USA) according to the manufacturer's protocol and verified by sequencing (Macrogen, Korea).

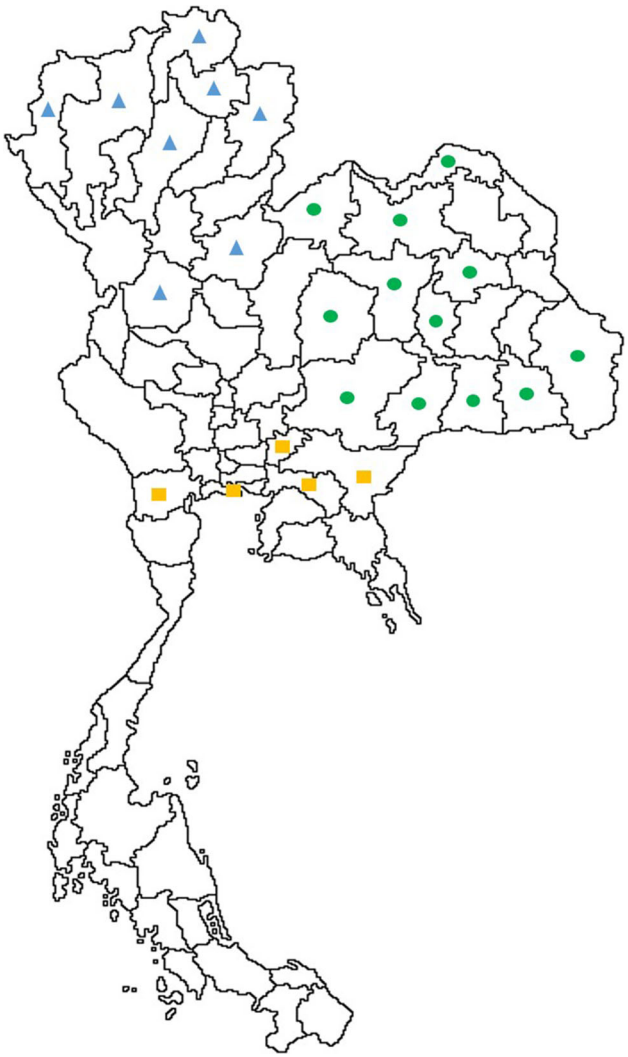

Figure 1. Geographic distribution of *M. oryzae* isolates from Thailand used in this study. (●) Blast isolates from central of Thailand; (■) blast isolates from northeast of Thailand; (▲) blast isolates from north of Thailand.

Data analysis

The nucleotide sequences of *AVR-Pita1* gene were assembled and aligned along with reference sequences of *AVR-Pita1* gene obtained from the GeneBank/EMBL/DDBJ databases (accession number: AB607340.1) with the CLUSTALW program (Thompson *et al*. 1994). The phylogenetic tree was constructed by the neighbour-joining, maximum-likelihood and maximum parsimony methods with the program MEGA v.6 (Kumar *et al*. 2008). The confidence values of individual branches in the phylogenetic tree were determined by using bootstrap analysis of Felsenstein (1985) based on 1000 samplings. The neutrality test was identified using DNA sequence polymorphism (DnaSP) v.5.0 program to indicate the polymorphic site. The number of nucleotide diversity per site was estimated and calculated by genetic parameters, Tajima's *D* test (Tajima 1989), Fu and Li's *D* test and Fu and Li's *F* test (Fu and Li 1993).

Pathogenicity assay

Pathogenicity assay was carried out using rice isogenic line with resistant gene, *Pita*, in genetic background of japonica rice accession Lijiangxin Tuan Heigu (LTH), from International Rice Research Institute (IRRI). Rice blast fungus isolates on desiccated filter paper were grown at room temperature under white fluorescence lights on Petri dish containing RFA medium (RFA, 2.0% of rice flour, 2.0% of agar and 0.2% of yeast extract) for producing mycelia. After seven days of incubation, the fungal mycelium surface was scraped using glass rod followed by incubation at room temperature for two days under black light to induce sporulation. The conidia were washed with 5 mL of sterile distilled water per Petri dish and the mycelial mats scraped

Table 1. Gene-specific PCR primers used in this study.

Primer	Primer sequences (5′–3′)	Annealing temp. (°C)	Expected size
AVR-Pita1 up	F: AGTGGACCCTTGTCCGATC R: CCGAAATCGCAACGGTGTG	56	614
AVR-Pita1 down	F: CGCCTTTTATTGGTTTAATTCG R: CCTCCATTCCAACACTAACG	60.5	1063

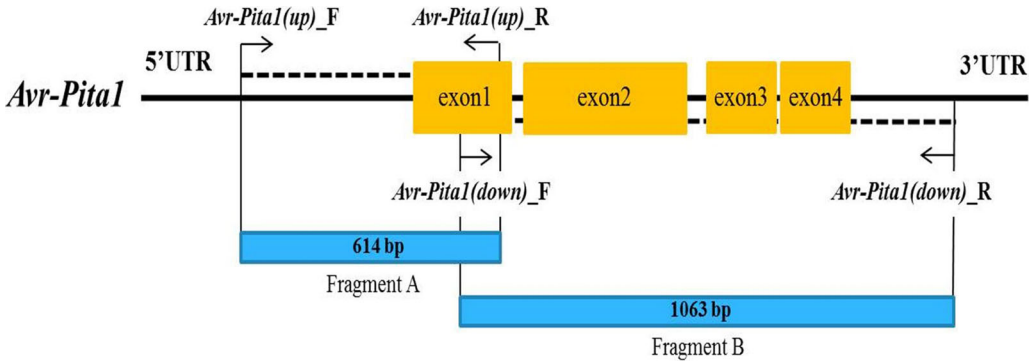

Figure 2. Gene structure of *AVR-Pita1* and location of primer binding site.

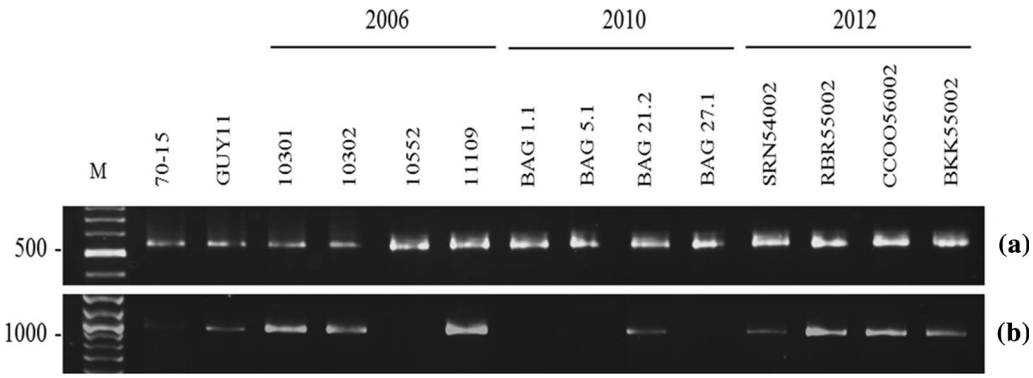

Figure 3. Specific amplification of (a) AVR-Pita1 (up) primer and (b) AVR-Pita1 (down) primer.

to collect the conidia for inoculation. Spore suspension was adjusted the concentration to 1×10^5 conidia per mL in 0.5% gelatin. The suspension was then sprayed onto leaves of 3-week-old plants. Inoculated plants were placed in tray at 25°C, 100% humidity for overnight and then transferred to the greenhouse. Disease evaluation was assessed at seven days post-inoculation using a 0–6 scale rating system (0–2: resistance; 3–6 susceptible) as described by Roumen *et al.* (1997).

Results and discussion

Presence of AVR-Pita1 in M. oryzae from Thailand

To identify the presence of *AVR-Pita1* gene in blast isolates from Thailand, two pairs of gene specific primer were designed and used. The 5′ end of the gene was successfully amplified in all 76 blast isolates with AVR-Pita1 up primer. On the other hand, the 3′ end of the gene was able to amplify in only 44 blast isolates (58%) with AVR-Pita1 down primer (figure 3). Two possible explanations for the PCR amplification failure were the nucleotide substitution at the primer binding site or the deletion/insertion within gene. Since, the *AVR-Pita1* primer down could not amplify the DNA fragment from 32 blast isolates, we could not obtain the whole gene sequence from these isolates for nucleotide variation analysis. Our results suggested that the 5′ region of the *AVR-Pita1* gene is more conserved than the 3′ region. The result was in agreement with the finding from Zhou *et al.* (2007) who reported that the portion of 5′ leader region of *AVR-Pita1* gene is conserved in 39 US blast isolates and there was an insertion of Pot3 transposon in the 3′ region in the virulent field blast isolates. The high level of genetic variability of *AVR-Pita1* gene may be due to the fact that it resides near the highly unstable telomeric region on the chromosome (Orbach *et al.* 2000; Khang *et al.* 2008). This finding suggested that the 5′ region of the gene may have significant function or very important for the function of the gene. Not only *AVR-Pita1* gene, other *AVR* genes includes *AVR-Pik*, *AVR-Pia* and *AVR-Pii* were also highly instable and were located closely to the unstable telomere regions on chromosomes (Yoshida *et al.* 2009; Dai *et al.* 2010; Chuma *et al.* 2011).

Variation of the AVR-Pita1 nucleotide sequence in M. oryzae from Thailand

To characterize the nucleotide variation of *AVR-Pita1*, 1.1 kb fragment nucleotide sequences of 44 rice blast isolates, which were successfully amplified, were examined using multiple alignments and the nucleotide sequences were

Table 2. Polymorphism and neutral test of the *AVR-Pita1* gene.

Coding sites	S	HD	π	θ	Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**
Coding	49	0.834	0.00537	0.0193	−2.52572**	−4.85028***	−4.78322***
Noncoding	16	0.208	0.00338	0.0176	−2.53945**	−5.25654***	−5.13452***
Entire gene	65	0.842	0.00487	0.0180	−2.63091**	−5.50655***	−5.32254***

S, number of segregating sites; HD, haplotype diversity; π, nucleotide diversity; θ, Watterson's estimator; asterisk indicates significant statistics $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

deposited in GenBank (GenBank ID: JQ409300–JQ409329 and LC110404–LC110390). Sequence alignment revealed 65 nucleotide variable sites (49 sites in the coding region and 16 sites in the noncoding region), which can be classified into 40 different haplotypes. Haplotype diversity index (0.834) showed high level of genotypic diversity. The nucleotide polymorphism (π) of the entire *AVR-Pita1* gene was 0.00487. High level of *AVR-Pita1* nucleotide diversity was observed more in coding region (π = 0.00537) than in noncoding region (π = 0.00338) (table 2). Our result was similar to the study by Dai *et al*. (2010), Kasetsomboon *et al*. (2013) and Huang *et al*. (2014) on variation of *AVR-Pita1*. They reported high levels of nucleotide substitutions and haplotype diversity in 62 China rice blast isolates and 151 isolates collected in US, China and Columbia.

Neutral selection tests of AVR-Pita1 gene in Thai rice blast isolates

To evaluate the genetic evolutionary rate of *AVR-Pita1* gene under the neutral theory, tests of neutrality were performed using three statistical parameters of Tajima's *D*, Fu and Li's *D** and *F** (table 2). The results revealed that all the statistical parameters were negative, which indicated significant deviation from the neutral model. The sliding window analysis clearly showed that most variations were observed in the coding region, at exons 3 and 4, and less variation was observed randomly across the entire coding region (figure 4). The ratio between nonsynonymous (K_a) and synonymous (K_s) polymorphic sites was 1.6321, which was more than 1 ($K_a/K_s > 1$) (table 3). Our results suggest that *AVR-Pita1* gene of Thai blast isolates were under positive selection pressure. Several reports provided evidences for a positive selection for nucleotide substitution in *AVR-Pita1* (Stahl and Bishop 2000; Zhou *et al*. 2007). All these findings suggested that selective pressure was a common mechanism for genetic variation of *AVR-Pita1* gene.

AVR-Pita1 amino acid diversification in blast isolates from Thailand

Forty-four nucleotide sequences of *AVR-Pita1* gene were translated into amino acid sequences. Amino acid sequences were aligned and compared with AVR-Pita1 protein of the

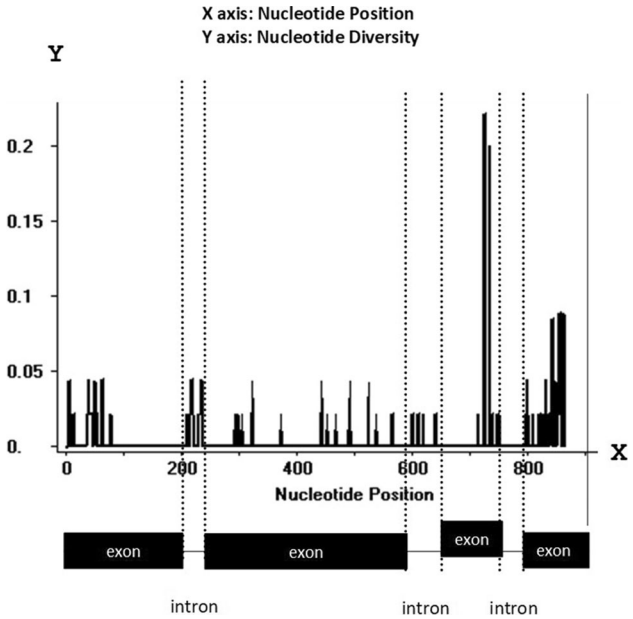

Figure 4. Distribution of the *AVR-Pita1* allele variation (π value) using sliding window showing three introns and four exons.

Table 3. Nonsynonymous (K_a) and synonymous (K_s) nucleotide substitutions at *AVR-Pita1*.

Population	K_a	K_s	K_a/K_s
AVR-Pita1	0.00488	0.00299	1.63210

Chinese isolate O-137 (Orbach *et al*. 2000). Amino acid alignment of 44 Thai blast isolates revealed 39 variable amino acid positions from 224 amino acids of AVR-Pita1 protein. The amino acid variation can be used to classify 27 haplotypes. There was deletion/insertion of leucine at positions 6 and 7, and amino acid substitutions of AVR-Pita1 protein scatter throughout (table 4). Among these 39 variable amino acid positions, 18 positions (46%) were found in exon 4 (last exon) of AVR-Pita1 protein. This result suggests that the encoded protein from last exon region is under influence of positive selection. Previously, Dai *et al*. (2010) reported that 23 of 28 polymorphic amino acid positions of AVR-Pita1 protein led to amino acid substitutions located in

Table 4. Protein variation of AVR-Pita1 sequences form 44 Thai rice blast isolates.

Protein[a]	Allele[b]	5	6	7	8	13	14	15	16	17	18	21	22	26	154	169	181	188	192
AVR-Pita1	**n/a**	**S**	**L**	**L**	**F**	**V**	**A**	**I**	**S**	**A**	**F**	**I**	**G**	**S**	**P**	**G**	**H**	**K**	**Y**
I	BAG2.3	S	-	-	F	V	A	I	S	G	F	I	G	R	P	G	H	K	C
II	BAG13.2, 20.2	S	-	-	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C
III	BAG16.1	S	-	-	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C
IV	BAG 19.2, 23.2, 10581	A	-	-	S	V	A	I	S	A	F	I	G	S	P	G	H	K	C
V	BAG17.2	S	-	-	F	V	A	I	P	A	F	I	G	S	P	G	H	K	C
VI	BAG21.2, 22.1, 25.1, 10302	S	-	-	F	V	A	N	S	A	F	I	G	S	P	G	H	K	C
VII	10551, 10732, 10837, 10985, RBR55002	S	-	-	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C
VIII	BAG24.1	S	-	-	F	V	A	V	S	A	F	I	G	S	P	G	H	K	C
IX	BAG32.1	S	-	-	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C
X	10100, 11108	S	-	-	F	V	A	I	S	A	F	I	G	S	P	G	H	K	Y

Protein[a]	Allele[b]	195	196	199	200	203	205	206	207	208	210	211	212	213	214	215	216	217	218	222	223	224
AVR-Pita1	**n/a**	**D**	**G**	**K**	**L**	**T**	**S**	**I**	**K**	**N**	**D**	**S**	**Y**	**A**	**I**	**F**	**A**	**Q**	**C**	**K**	**Y**	**C**
I	BAG2.3	H	G	K	L	T	S	I	Q	N	D	S	Y	V	I	F	A	Q	C	K	Y	C
II	BAG13.2, 20.2	H	G	K	L	T	S	I	K	N	D	S	Y	A	I	L	H	N	V	A	I	V
III	BAG16.1	H	G	K	L	T	S	I	K	N	D	S	Y	A	I	L	A	Q	C	K	Y	C
IV	BAG 19.2, 23.2, 10581	H	G	K	L	T	S	I	K	N	D	S	Y	A	I	S	A	Q	C	N	I	V
V	BAG17.2	H	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
VI	BAG21.2, 22.1, 25.1, 10302	H	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	N	I	V
VII	10551, 10732, 10837, 10985, RBR55002	H	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
VIII	BAG24.1	H	V	K	L	T	C	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
IX	BAG32.1	H	G	N	F	T	S	I	K	N	D	L	L	N	F	D	Q	L	N	N	I	V
X	10100, 11108	H	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C

Table 4 (*contd*)

Protein[a]	Allele[b]	5	6	7	8	13	14	15	16	17	18	21	22	26	154	169	181	188	192	195
XI	10301	S	-	-	F	V	A	I	S	A	F	I	G	S	P	G	H	K	Y	D
XII	10459	S	-	-	F	V	A	I	S	A	F	I	G	S	R	V	H	K	Y	D
XIII	10760	S	-	-	F	A	A	I	S	A	F	I	S	S	P	G	H	R	C	H
XIV	10812	S	-	-	F	V	P	I	S	A	S	N	G	S	P	G	H	K	C	H
XV	10941, 10971	S	-	-	F	V	A	V	S	A	F	I	G	S	P	G	H	K	C	H
XVI	10945	S	-	-	F	V	A	V	S	A	F	I	G	S	P	G	N	K	C	H
XVII	10993, 11109	S	-	-	F	V	A	I	S	A	F	I	G	S	P	G	H	K	Y	D
XVIII	SRN54001, CCOO56001	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C	H
XIX	SRN54002	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C	H
XX	SRN54005, SRN54009	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C	H

Protein[a]	Allele[b]	196	199	200	203	205	206	207	208	210	211	212	213	214	215	216	217	218	222	223	224
XI	10301	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
XII	10459	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
XIII	10760	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	H	C	K	Y	C
XIV	10812	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
XV	10941, 10971	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
XVI	10945	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
XVII	10993, 11109	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	C
XVIII	SRN54001, CCOO56001	G	K	L	T	S	I	N	P	D	S	Y	A	I	F	A	Q	C	K	N	S
XIX	SRN54002	G	K	L	A	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	Y	S
XX	SRN54005, SRN54009	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	N	I	V

Table 4 (*contd*)

Protein[a]	Allele[b]	5	6	7	8	13	14	15	16	17	18	21	22	26	154	169	181	188	192	195
XXI	CCOO56002	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	Y	D
XXII	CCOO56003	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C	H
XXIII	RBR55001	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	Y	D
XXIV	RBR55003	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C	H
XXV	NYK55001, CCOO56004	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	C	H
XXVI	BKK55001	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	Y	D
XXVII	BKK55002, BKK55003	S	L	L	F	V	A	I	S	A	F	I	G	S	P	G	H	K	Y	D

Protein[a]	Allele[b]	196	199	200	203	205	206	207	208	210	211	212	213	214	215	216	217	218	222	223	224
XXI	CCOO56002	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	N	C
XXII	CCOO56003	G	K	L	T	S	I	K	N	D	S	Y	A	I	F	A	Q	C	K	N	C
XXIII	RBR55001	G	K	L	T	S	I	K	N	D	Q	L	C	D	F	A	H	N	K	N	C
XXIV	RBR55003	G	K	L	T	S	I	K	N	D	S	L	C	Y	F	A	H	V	K	N	C
XXV	NYK55001, CCOO56004	G	K	L	T	S	I	K	N	R	Q	L	C	Y	F	C	T	C	K	N	C
XXVI	BKK55001	G	K	L	T	K	Y	K	T	D	S	Y	A	I	F	A	Q	C	K	Y	C
XXVII	BKK55002, BKK55003	G	K	L	T	S	G	K	T	D	S	Y	A	I	F	A	Q	C	K	Y	C

The AVR-Pita1 reference sequence of the Chinese isolate 0-137 are in bold.
[a]Groups of AVR-Pita1 variants based on amino acid sequences.
[b]Groups of AVR-Pita1 variants based on nucleotide sequence.
N/a, not applicable.

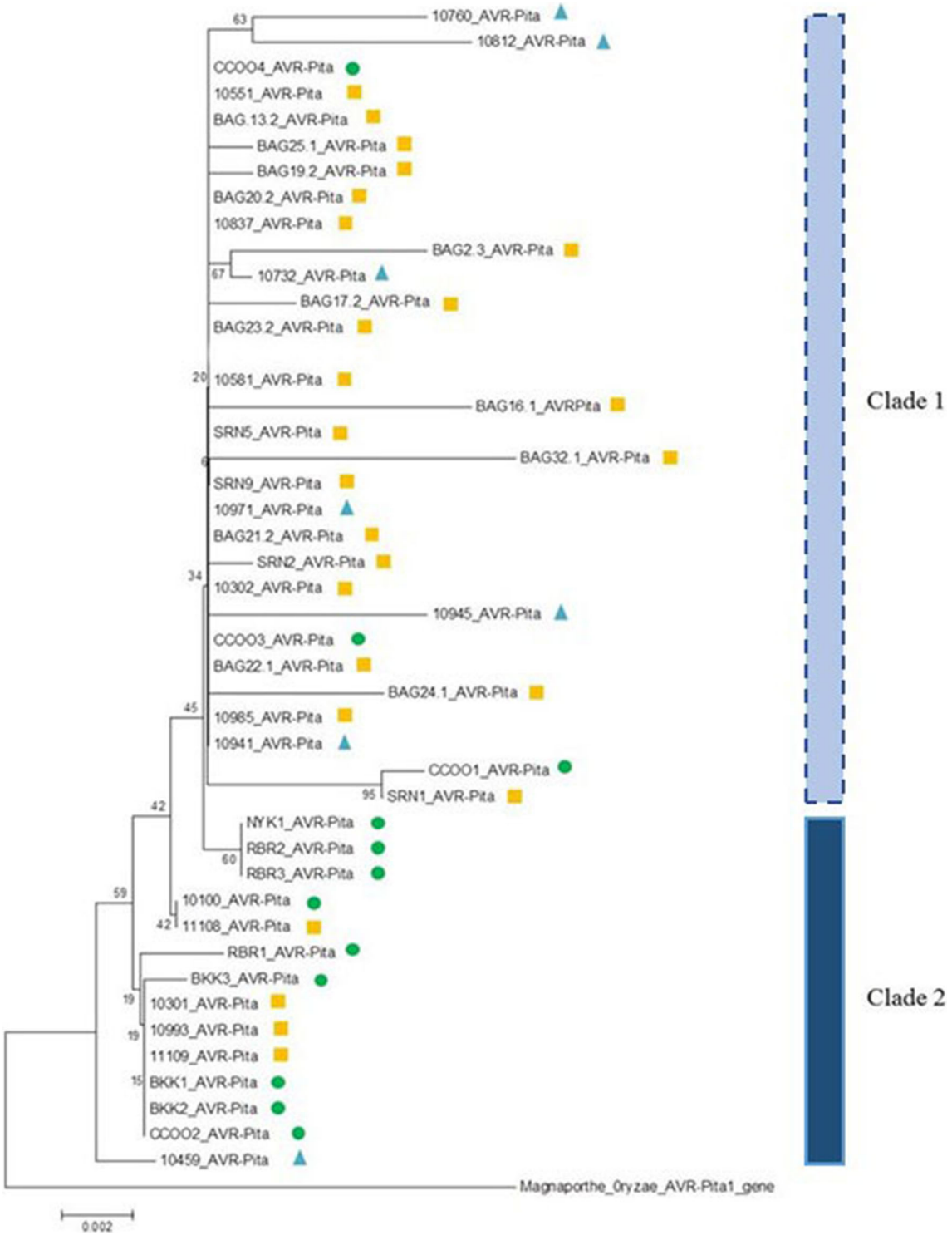

Figure 5. Neighbour-joining tree of *AVR-Pita1* gene of the 44 rice blast isolates constructed from nucleotide coding sequences with reference sequence using bootstrap 1000. (●) Blast isolates from central of Thailand; (■) blast isolates from northeast of Thailand; (▲) blast isolates from north of Thailand.

the exon region. Moreover, our result supported by recent studies also revealed that the most protein variation occurred in the exons 3–4 region of *AVR-Pita1* gene (Khang *et al.* 2010). These results suggested that *AVR-Pita1* gene especially in the last exon was under influence of positive selection.

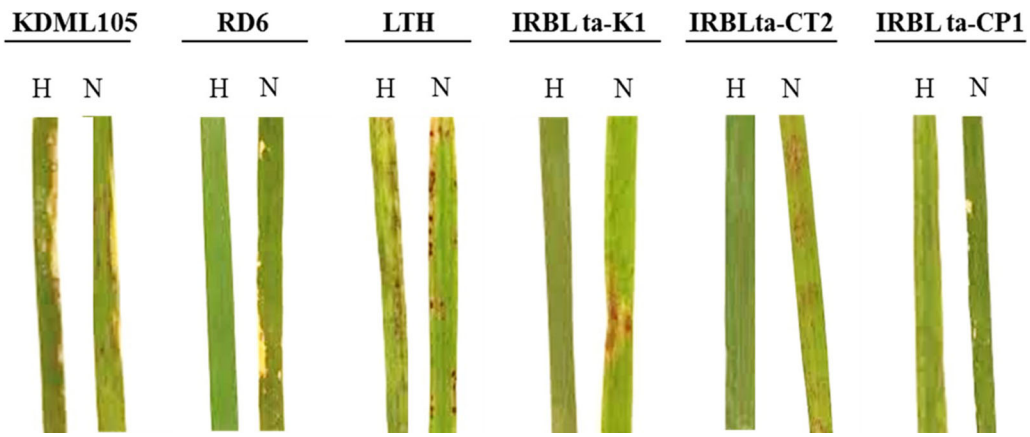

Figure 6. Pathogenicity assay of selection isolates on rice isogenic line of *Pita* resistance gene. H, the rice cultivar was infected with rice blast isolate carrying *AVR-Pita* gene; N, the rice cultivar was infected with rice blast isoalte without *AVR-Pita* gene.

Phylogenetic analysis

To assess the genetic relationships of 44 blast isolates from Thailand, a phylogenetic analysis was performed by neighbour-joining statistical analyses and neighbour-joining tree was constructed. The phylogenetic tree revealed two major clades. Rice blast isolates collected from different years were grouped together. Clade one composed of 27 isolates from north and north-east of Thailand while blast isolates from central of Thailand were mostly clustered together in clade two (figure 5). This suggests that the geographical location has influence the distribution of genetic variation. Our result was consistence with Kasetsomboon *et al*. (2013) that geographical location was the main factor in the fungal distribution. However, Huang *et al*. (2014) revealed that there was no significant clustering with the geographic structure of blast isolates from different parts of China and from different continents.

Pathogenicity analysis of Thai blast isolates

To evaluate the pathogenicity of blast isolates from Thailand based on the *AVR-Pita1* gene, disease screening assay was applied using rice cultivars LTH carrying *Pi-ta* resistant gene and a Thai susceptible rice cultivar, KDML105. Rice plants were inoculated with 33 rice blast isolates including 27 isolates (one isolate from each haplotype) and six isolates which could not obtained the gene sequence. As expected, six fungal isolates which do not contain functional *AVR-Pita1* gene were virulence to rice isogenic line LTH with *Pita* gene, LTH and KDML105. The 27 rice blast isolates from different haplotypes were not able to infect rice isogenic line LTH with *Pita* gene but showed virulence to LTH and KDML105. These results indicated that amino acid variations from 27 protein haplotypes exhibited avirulence function, which can still be recognized by *Pi-ta* gene (figure 6). These results indicated that the resistance to *M. oryzae* in rice follows a gene-for-gene concept where resistant R genes are effective in controlling infection by races of *M. oryzae* containing corresponding avirulence genes (Flor 1971).

In conclusion, the present study revealed that the genetic diversity of *AVR-Pita1* of *M. oryzae* in rice germplasm from many parts of Thailand linked with selective forces from *Pita* gene. The phylogenetic analysis of the *AVR-Pita1* sequences revealed that the geographical location has influence in the distribution of Thai rice blast population. The information obtained from this study can help us to understand the coevolution between rice and rice blast fungus and may lead to the development of strategies for improving the durability of resistance in rice breeding programmes.

Acknowledgements

This work was supported by Agricultural Research Development Agency (Public organization). Miss Katanyutita Damchuay received fellowship from the Capacity Building of KU Students on Internationalization Program: KUCSI, Kasetsart University and graduate scholarship from Faculty of Science, Kasetsart University. The authors would like to thank Mr Robert Harman II for critically and grammatically reviewing the manuscript.

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Corresponding editor: Manoj Prasad