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Molecular identification of blast resistance and pathogenesis-related genes in various traditional paddy varieties from different divisions of Sabah, East Malaysia

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Abstract

Paddy is the staple food of local people in Sabah, but its production is affected by a disturbing disease known as blast, which is caused by fungi (i.e., *Magnaporthe grisea* and *Magnaporthe oryzae*) infection. Knowledge regarding distribution of blast-resistant (BR) genes among various Sabah traditional paddy varieties in different divisions of Sabah is not clear; hence, molecular identification of BR genes in Sabah traditional paddies is urgently needed. In this study, genomic DNA was extracted from 21 different Sabah traditional paddy varieties collected from three divisions of Sabah including the West Coast Division (WCD), Sandakan Division (SD), and Interior Division (ID). The presence of 11 BR and 2 pathogenesis-related (PR) genes in their genome was molecularly screened using a PCR approach and validated using direct sequencing. Our results showed that high frequencies of *Pib* (95.2%), *pi-ta* (100.0%), and *RCC2* (100.0%) genes were obtained in all 21 Sabah traditional paddy varieties screened. Interestingly, one of the paddy varieties from ID of Sabah, PBT23, did not contain the *Pib* gene but carried the *Pita/Pita-2* allele in its genome. Besides, only three paddies (PBT08 and PBT07 from WCD, and PBT23) exhibited the *Pik-p* gene, and no varieties were found to carry the *Pik, Pik-m, Pit, Piz, Piz-t, Pi-ta, Pi9,* and *wwin2* genes. In conclusion, this study serves as the basis for improvement and effectual management of traditional paddies in different divisions of Sabah towards blast resistance with knowledge of BR or PR genes inherited in their genome.

Introduction

Paddy is one of the strategic crops in Malaysia and has been identified to be important for food security. Sabah, also known as North Borneo, is listed under the National Food Security Policy for paddy production initiatives (Tey, 2010) as more than 30,000 hectare of land in three main divisions including the West Coast Division (WCD), Sandakan Division (SD), and Interior Division (ID) of Sabah is allocated for paddy plantation (Department of Agriculture Sabah, 2016). Due to the geographical structure of Sabah, paddy plantation divides into two major groups comprising of lowland wet varieties and upland dry varieties (Low and Lee, 2012). The upland dry paddy varieties are usually cultivated for home consumption for people living in the rural areas of Sabah whereas lowland wet paddy varieties are commonly cultivated for consumption in urban areas of Sabah as well as for exportation.

The worldwide paddy production including in Sabah is affected by a disturbing disease known as blast which is caused by *Magnaporthe* species (i.e., *M. grisea* and *M. oryzae*) infection that results in a reduction of annual grain yield by around 10-30% (Couch and Kohn, 2002; Strange and Scott, 2005). In Malaysia, the production of both upland and lowland paddy has been dropping since 2010 until 2013 whereby blast disease is one of the major factors contribute to this unpromising situation (MOA, 2014). Therefore, molecular identifying of blast resistance (BR) and pathogenesis-related (PR) genes presented in the genome of Sabah traditional paddy is crucial for improvement and management of paddy resistance towards blast disease.

Resistance to *Magnaporthe* species is reported to follow a gene-for-gene system where a BR gene prevents infection by a specific strain of *Magnaporthe* species containing the corresponding avirulence *(Avr)* gene (Silue *et al*., 1992). To date, more than 100 BR genes (*Pi*-genes) have been identified (Tanweer *et al*., 2015) and are distributed throughout the 12 paddy chromosomes except for chromosome 3 (Yang *et al.*, 2009; Liu *et al.*, 2010). In addition to BR genes, previous study reported that PR genes such as

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chitinase *(RCC2)* and wheatwin2 *(wwin2)* enhance plant resistance to fungi infection by hydrolysis of chitin component in cell wall of fungi (Nishizawa *et al.*, 1999; Fiocchetti *et al.*, 2008), elucidating the great potential of these two genes for blast resistance in paddy.

The distribution of the BR and PR genes in Sabah traditional paddy varieties is not known. Hence, the present study molecularly identified the BR and PR genes in different varieties of Sabah traditional paddy that will be used to improve the breeding program and survival rate of our Sabah traditional paddies towards blast disease.

Materials and Methods

Plant materials and DNA isolation

Seeds from 21 different varieties of Sabah traditional paddy varieties were collected from different divisions of Sabah including the WCD (n = 9), SD (n = 4), and ID (n = 8). Genomic DNA from seed was isolated using method modified from Sharma *et al.* (2013) where cetyl trimethylammonium bromide (CTAB) was utilized instead of polyvinylpyrrolidone (PVP) and β-mercaptoethanol.

Molecular identification using PCR

A total of 11 BR genes (*Pib, Pik, Pik-m, Pik-p, Pit, Piz, Piz-t, Pita/Pita-2, Pi-ta, pi-ta,* and *Pi9*) and 2 PR genes (*RCC2* and *wwin2*) were studied (Table 2). β-actin *(ACTB)* was used as an internal control of PCR. A duplex PCR amplifying single BR/PR gene and *ACTB* internal control was performed in a final 20 µL reaction volume containing 1x PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of BR/PR genes forward and reverse primers, 0.1 µM of ACTB forward and reverse primers and 0.2 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif). The conditions of PCR were 1 cycle of initial denaturation for 3 min at 95°C, 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s according to respective primer's annealing temperature, extension of 1 min at 72°C except for the extension time of *Pi9* BR gene was 2 min; and a cycle of final extension for 5 min at 72°C. Amplified PCR products were assessed by 1% agarose gel electrophoresis and visualized with UV light.

Direct sequencing and blast

PCR products of BR (*Pib, Pik-p, Pita/Pita-2,* and *pi-ta*) and PR *(RCC2)* genes as well as *ACTB* internal control were purified using QIAquick PCR Purification Kit (QIAGEN, USA) according to manufacturer's instructions. The purified PCR products were sequenced using ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA), and blasted to the National Center for Biotechnology Information Database to validate the correct targeted genes have been amplified.

Results

Out of the 21 screened Sabah traditional paddies, 3 of them (PBT07, PBT08, and PBT23) contained the highest number of four BR/PR genes whereas 18 of the tested varieties have only three BR/PR genes (Figure 1 and Table 1). In BR gene analysis, there were high frequencies of *Pib* (95.2%) and *pi-ta* (100.0%) genes presented in all paddies. Interestingly, PBT23 is the only paddy variety from ID of Sabah that did not presented with *Pib* gene but carried the *Pita/Pita-2* allele in its genome. Only three paddies were found carrying the *Pik-p* gene (PBT08 and PBT07 from WCD, and PBT23) and no paddy was molecularly revealed to carry the *Pik, Pik-m, Pit, Piz, Piz-t, Pi-ta,* and *Pi9* genes in this study. In PR gene analysis, all screened Sabah traditional paddies were presented with *RCC2* gene (100.0%) but none of them carrying the *wwin2* gene (0.0%).

Discussion

The use of BR paddy varieties to overcome blast disease has been able to partially overcome the problem due to the high genetic variability of the pathogen towards virulence. Therefore, plant breeders have focused on combining multiple BR genes with the aid of molecular markers to produce highly BR paddy varieties (Wang *et al*., 2000). The use of DNA markers has allowed rapid identification of race-specific BR genes and selection of BR paddy varieties without the need to test the progeny or inexact phenotypic disease screening (Fjellstorm *et al.,* 2004), indirectly saving the time to produce new batch of paddy that greatly confer resistant to blast disease.

Pi-b is a member of a small gene family and the first cloned BR gene, encoding a 1251 amino acid sequence that consists of a nucleotide binding site and leucine-rich repeats (NBS-LRR) (Wang *et al*., 1999). *Pi-b* is one of the major BR genes that have been commonly used in paddy plantation programs in China, Indonesia, and Japan (Kiyosawa, 1972; Miyamoto *et al*., 1996; Monna *et al.*, 1997; Yokoo, 2005), but its expression will only be induced by the altered environmental conditions instead of *M. grisea* innoculation (Wang *et al*., 1999). Our study showed that 95.2% of screened Sabah traditional paddies

Table 1. List of genes presented in different varieties of Sabah traditional paddy.

Sample	Division[a]	Genes[b]												Total number of genes
		Pib	*Pik*	*Pik-m*	*Pik-p*	*Pit*	*Piz*	*Piz-t*	*Pita/Pita-2*	*Pi-ta*	*pi-ta*	*Pi9*	*RCC2*	*wwin2*	
PBT08	WCD	1	0	0	1	0	0	0	0	0	1	0	1	0	4
PBT09	WCD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT12	WCD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT11	WCD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT19	WCD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT07	WCD	1	0	0	1	0	0	0	0	0	1	0	1	0	4
PBT10	WCD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT06	WCD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT02	WCD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT17	SD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT14	SD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT13	SD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT18	SD	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT20	ID	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT21	ID	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT22	ID	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT23	ID	0	0	0	1	0	0	0	1	0	1	0	1	0	4
PBT24	ID	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT25	ID	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT26	ID	1	0	0	0	0	0	0	0	0	1	0	1	0	3
PBT27	ID	1	0	0	0	0	0	0	0	0	1	0	1	0	3
Frequency (%)		95.2	0.0	0.0	14.3	0.0	0.0	0.0	4.8	0.0	100.0	0.0	100.0	0.0	

[a]WCD: West Coast Division; SD: Sandakan Division; ID: Interior Division.
[b]1 denoted as present and 0 denoted as absent of gene.

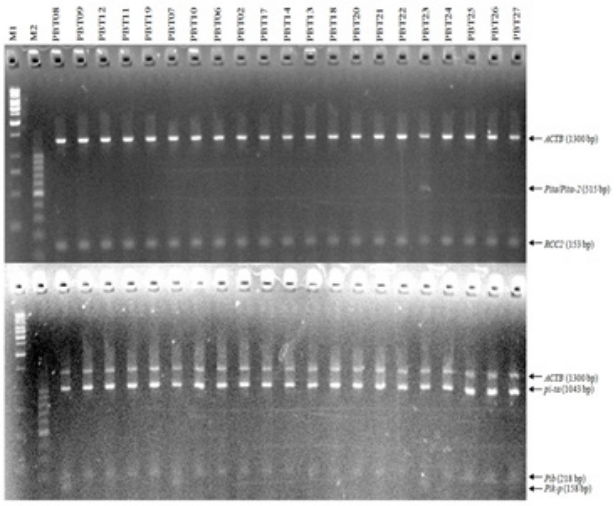

Figure 1. Agarose gel electrophoresis showing the amplified fragments for all 21 different Sabah traditional paddy varieties in this study. M1: Thermo Scientific GeneRuler™ 1 kb DNA Ladder. M2: Thermo Scientific GeneRuler™ 100 bp DNA Ladder

presented with this gene except for one paddy variety from ID of Sabah (PBT23). Interestingly, PBT23 is the only variety that carried the *Pita/Pita-2* BR gene. Since *Pita/Pita*-2 has a wide range of resistance continuum and presents resistance to all strains controlled by *Pi-ta* gene (Kiyosawa, 1967), cross-breeding of PBT23 with other paddy varieties in WCD and SD of Sabah that containing the *Pi-b* gene in this study may enhance the resistance level towards blast disease and further study will be needed for validation.

The *Pi-ta* (YL155/YL87) and *pi-ta* (YL183/YL87) primers used in this study was developed in order to distinguish the dominant *Pi-ta* allele from the recessive *pi-ta* allele (Jia *et al*., 2002). Our study revealed that all Sabah traditional paddies carrying the recessive *pi-ta* but none of them presented with dominant *Pi-ta* allele. The dominant *Pi-ta* consists of alanine amino acid at position 918 whereas serine amino acid was substituted at the same position for recessive *pi-ta* (Bryan *et al*., 2000). Previous study suggested that the alanine amino acid of the dominant *Pi-ta* but not the serine amino acid of the recessive *pi-ta* protein will correlate with the Avr-Pita protein in cytoplasm of paddy cells to stimulate a resistance response towards *M. grisea* infection (Bryan *et al*., 2000). Therefore, dominant *Pi-ta* gene should be introduced to the genome of Sabah traditional paddies to confer better resistance for blast disease.

Paddy chromosome 11 contains abundant of BR genes, including *Pik, Pik-m* and *Pik-p* genes in this study (Ashikawa *et al*., 2008; Wang *et al*., 2009). Our results revealed that three of the screened paddy varieties from WCD and ID of Sabah inherited with the *Pik-p* but none of them carrying the *Pik* and *Pik-m* BR genes. *Pik, Pik-m* and *Pik-p* are most likely allelic (Hayashi *et al*., 2006) and among them, *Pik-m* has the widest resistance spectrum when compared to *Pik* and *Pik-p* genes in Japanese and some Chinese

Table 2. List of primers used in this study.

Gene (marker)	Primer sequences (5'- 3')	Annealing (°C)	Expected size (bp)	Reference
ACTB internal control	F: CGT CTG CGA TAA TGG AAC TGG	60	1300	Reece *et al.*, 1990
	R: CTG CTG GAA TGT GCT GAG AGA T			
Pib(b213)	F: GCA TTA GAT AGT GAT GAA AGC CGG	60	218	Hayashi *et al.*, 2006
	R: TGT TCA TCC AGG CAA TTG GC			
Pik(k6415)	F: CTA ATG GAA TTA AAC GGT TGA GCT A	60	140	Hayashi *et al.*, 2006
	R: ATC CCG ATG TCA TCG ATC AC			
Pik-m (k6441)	F: TGT AAA ATA CTT TCT ATG CGC AGG C	60	404	Hayashi *et al.*, 2006
	R: GTT TAT GGA GAG AGT AGT CGC TG			
Pik-p (k39575)	F: GGT GTT TGG GAA CCT GAA CCC TA	60	158	Hayashi *et al.*, 2006
	R: TTT CTG TTC GTC GGA TGC TC			
Pit (t256)	F: GGA TAG CAG AAG AAC TTG AGA CTA	60	322	Hayashi *et al.*, 2006
	R: CAT GTC TTT CAA CAT AAG AAG TTC TC			
Piz (z565962)	F: AAG AAA TAA TAT TTT TGA AAC ATG GCA AAG	60	267	Hayashi *et al.*, 2006
	R: CCA TGG TGG TAA CTG GTA TGT G			
Piz-t (zt56591)	F: TTG CTG AGC CAT TGT TAA ACA	60	257	Hayashi *et al.*, 2006
	R: ATC TCT TCA TAT ATA TGA AGG CCA C			
Pita/Pita-2 (ta5)	F: CAG CGA ACT CCT TCG CAT ACG CA	60	515	Hayashi *et al.*, 2006
	R: CGA AAG GTG TAT GCA CTA TAG TAT CC			
Pi-ta (YL155/YL87)	F: AGC AGG TTA TAA GCT AGG CC	55	1042	Jiaet *al.*, 2002
	R: CTA CCA ACA AGT TCA TCA AA			
pi-ta (YL183/YL87)	F: AGC AGG TTA TAA GCT AGC TAT	55	1043	Jiaet *al.*, 2004
	R: CTA CCA ACA AGT TCA TCA AA			
Pi9 (195F-1/195R-1)	F: ATG GTC CTT TAT CTT TAT TG	56	2000	Qu *et al.*, 2006
	R: TTG CTC CAT CTC CTC TGT T			
RCC2	F: GTA CGG CGT GAT CAC CAA C	56	153	Present study
	R: GGC CTC TGG TTG TAG CAG TC			
wwin2	F: CTA CTG CGC GAC CTG GGA	65	230	Present study
	R: CGT TGG TGT CGA TCT TGG TG			

isolated blast pathogen populations (Kiyosawa, 1987). In addition, previous study reported that these three genes are independently trigger differential responses against a variety of blast pathogen isolates (Wang *et al.*, 2009), and a great concern is needed to investigate the resistance of these three genes towards blast pathogen isolates in Sabah for advance BR management in Sabah traditional paddies.

RCC2 and *wwin2* are two PR genes that recently attract attention of plant pathologists worldwide due to their ability in enhancing resistance towards fungal infections in plants (Nishizawa *et al*., 1999; Fiocchetti *et al*., 2008). In this study, we had designed primers that specifically targeting the *RCC2* and *wwin2* genes in plant's genome, and our results showed that all Sabah traditional paddies screened in this study presented with *RCC2* but none of them inherited with the *wwin2* gene. Nishizawa *et al.* (1999) reported that transgenic paddy which expressed *RCC2* at 12-fold higher than wild paddy had a dramatically resistant to *M. grisea* infection. Besides, over-expression of *wwin2* has been reported with specific antifungal activity in tobacco plants (Fiocchetti *et al.*, 2008), but remain unclear in paddy. Taken together, *RCC2* and *wwin2* are two PR genes that have immense potential for blast disease resistance and our next step is to study their expression towards blast resistance in Sabah traditional paddies.

Conclusions

In summary, this is the first study to molecularly identify BR and PR genes distribution of traditional paddy varieties in different divisions of Sabah. Our study revealed that high frequencies of *Pib, pi-ta* and *RCC2* genes were presented among the 21 screened Sabah traditional paddies. With the knowledge of BR and PR genes inherited in their genome from the present study, we recommend one of the traditional paddy varieties from ID of Sabah (PBT23) should be cross-breed with other varieties in WCD and SD; and introduction of *Pi-ta* allele into the genome of all screened paddy varieties for highly resistant and confer better survival rate towards blast disease in Sabah.

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