Medicon Agriculture & Environmental Sciences
Volume 3 Issue 3 September 2022
Research Article

Rice Blast Resistance Genes *Pi54* and *Pita* Show Single Nucleotide Polymorphisms in an Advanced 4-Way Crossed Segregating Population

Ankitta Mishra[1]*, Wickneswari Ratnam[1], Md Atiqur Rahman Bhuiyan[2] and Kshirod Jena[3]

[1]*Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*

[2]*Department of Agriculture, Faculty of Science, Noakhali Science and Technology University, Noakhali 3814, Bangladesh*

[3]*International Rice Research Institute, DAPO Box 7777, Metro Manila 1301, Philippines*

***Corresponding Author:** Ankitta Mishra, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.

Received: July 27, 2022; **Published:** August 06, 2022

DOI: 10.55162/MCAES.03.060

Abstract

Rice blast, caused by the fungal pathogen *Magnaporthe oryzae*, is a destructive disease in rice. The use of blast-resistant varieties is possibly one of the best economically and environmentally efficient ways to cultivate rice. Four-way crosses between two F_1s were performed from UKMRC2 × Teqing and UKMRC2 × Tetep. A segregating population of 30 4-way F_1 lines and 72 4-way F_4 lines were screened with a set of 51 single nucleotide polymorphic (SNP) markers representing *Pi9, Pi5, Pib, Piz, Pita*, and *Pi54* genes to evaluate the sequence variation. Field blast screening was carried out using six newly identified Malaysian isolates of *M. oryzae*. Blast disease score of 1 or 2 was observed in 28 4-way F_1 lines and 57 4-way F_4 lines. The 4-way F4 population showed the resistant alleles for *Pi54* and *Pita*. According to Sequenom data, both generated reproducible and aggressive calls. The SNP data revealed nine sequence variants, which showed the presence of two putative haplotype blocks for *Pi54* and *Pita* respectively. Qgene analysis of the 4-way F_4 population showed *Pi54* with LOD of 14 (R^2 = 56.6%) and *Pita* with LOD of 14 (R^2 = 72.4%). *Pi54* was mapped to a region of 312 kb on chromosome 11 flanked by SNPs *Pi54-3* and *Pi54-19* while, *Pita* was mapped to a region of 175 kb on Chromosome 12 flanked by SNPs *Pita-10* and *Pita-3*. The resistance alleles highlighted in this study can be used in development of rice blast disease resistant varieties having high yield through gene pyramiding.

Keywords: Rice blast; *Pi54*; *Pita*; Sequence variants; Qgene analysis

Key message

The 4-way F_4 segregating population, from Sequenom data shows the SNP resistant alleles for *Pi54* (R^2=56.6%) and Pita (R^2=72.4%) with LOD score of 14 each. *Pi54* was mapped to a region of 312 kb on chromosome 11 and *Pita* was mapped to a region of 175 kb on Chromosome 12.

Introduction

Rice is one of the most important food crops and a primary source of energy for more than half of the world's population. Approximately 154 million hectares or 11% of the world's cultivated area is used for rice production, of which above 90% of rice is produced and consumed within Asia (Sankar et al., 2011). Rice blast is one of the most destructive diseases and is caused by the fungal pathogen *Magnaporthe oryzae*, limiting crop production in various parts of the world. This disease causes considerable yield losses (60-80%) in almost all the major ecosystems where rice is grown (Talbot, 2003). The blast disease strikes all the aerial parts of the rice plant, with most infections occurring on the leaves. The disease causes diamond-shaped lesions with a grey or white centre and brown border that are approximately 1.0-1.5 cm long and 0.3-0.5 cm wide. Although many resistant rice varieties are already developed in several countries, sometimes they are unstable and tend to become susceptible after a few years of release (Kiyosawa, 1982). Sufficient knowledge of the fungus and the host resistance is important to overcome this problem.

Numerous genes contributing resistance to rice blast have already been found in both *indica* and *japonica* rice cultivars such as Tjina (*Pib*), Tetep (*Pib, Pi1,* and *Pita*), Moroberekan (*Pi5* and *Pi7*), 5173 (*Piz-5* = *Pi2*), TKM1 (*Piz-t*), Tadukan (*Pita*) and Zenith (*Piz*) (Campbell et al., 2004; Inukai et al., 1994; Wang et al., 1994). Wild relatives in the genus *Oryza* represent the majority of the existing genetic variations in rice. These wild relatives were used to broaden the genetic background of cultivated rice in breeding programmes (Xiao et al., 1998; Moncada et al., 2001; Septiningsih et al., 2003; Wickneswari and Bhuiyan, 2014). *Pi9* is a resistance gene that isoriginated from the wild species, *O. minuta* (Liu et al., 2002). *Pib* (Wang et al., 1999), *Pita* (Bryan et al., 2000), *Piz-5*, *Piz-t* and *Pi9* (Zhou et al., 2006) were all studied using map-based cloning techniques. Additionally, major genes *Pi1*, *Piz-5* (*Pi2*), *Pi5*, *Pi9*, and Pi40 were found showing broad spectrum resistance to *M. oryzae* (Jeon et al., 2003; Jeung et al., 2007). *Pi54* was associated with 'Tetep', an indica rice variety on chromosome 11, in vicinity to a set of other blast (*Pik, Pikm, Pikp, Piks* etc.) genes. *Pita* was cloned by map-based cloning method by Bryan et al. (2000) from a rice variety, Tadukan (Accession No. AF207842). *Pita* was found to be located near to the centromere of chromosome 12 and it is also one of the widely used blast resistance gene in many breeding programmes (Costanzo and Jia, 2010). This gene also confers broad spectrum resistance to different races of *M. oryzae* (Jia et al., 2004). However, the effective use of blast-resistant genes has been limited due to the development of new blast races. Hence, it is necessary that broad spectrumblast-resistant genes are identified so that effective protection against robust blast isolates of *M. oryzae* can be achieved.

The availability of a large number of sequence-based Simple Sequence Repeat (SSR) and SNP markers that saturate the rice genome would be of considerable value in fine mapping of target genes. Such markers are expected to eliminate the problems due to recombination and lack of polymorphism (Singh and Mohapatra, 2007). More recently, SNPs are preferred over other molecular markers because they have properties likeco-dominant inheritance, bi-allelic nature, chromosome specific location and genome wide distribution (Bhattramakki et al., 2002) and giving higher resolution (Nasu et al., 2002). Blast resistance genes and alleles have been studied in detail in rice from both wild and cultivated species (Yang et al., 2007; Geng et al., 2008; Huang et al., 2008; Das et al., 2012; Kumari et al., 2013; Thakur et al., 2013a, b). It is necessary to genotype and validate SNPs so that the available SNP information is best utilized.

Traditional rice varieties have been used extensively as genetic resources for biotic as well as abiotic stress studies in hybridisation programmes. Mahsuri is a traditional variety in Malaysia, which is highly susceptible to blast disease. Pongsu Seribu 2 also known as PS2 is a Malaysian traditional variety. PS2 is resistant to blast disease and has been widely used as a blast-resistant donor in breeding programmes (Rahim et al., 2013). IR64 is a blast resistant mega variety from the Philippines. IR64 has been involved in numerous backcross breeding programmes, leading to the introduction of many valuable genes (Mackill and Khush, 2018). Traditional varieties like Tetep and Tadukan, which originated from Vietnam and the Philippines, respectively, have been used as check varieties and donors in various Malaysian breeding programmes (Mohamad et al., 1988). Teqing is a Chinese *indica* cultivar, having high yield and is also been used in rice breeding programmes. The *Oryza rufipogon* accession from Malaysia is resistant to blast disease (Huang et al., 2008). An advanced backcrossed lines (BC_2F_2, BC_2F_5, BC_2F_6, and BC_2F_7 generations) were produced from a cross between the wild parent, *O. rufipogon* Griff. (IRGC105491) and *O. sativa* L. subsp. *indica* cv. MR219, a Malaysian high-yielding rice cultivar. This cross led to transgressive variants with high grain yield (Sabu et al., 2006; Wickneswari et al., 2012; Wickneswari and Bhuiyan, 2014). Six (UKMRC2,

UKMRC3, UKMRC4, UKMRC8, UKMRC9, and UKMRC10) out of seven genotypes tested in this study were registered as a *New Plant Variety* under the Malaysian Ministry of Agriculture (MOA) in 2009. The other genotype, UKMRC11, is similar to UKMRC9.UKMRC2 and UKMRC8 have been approved for the production of certified seeds for commercial cultivation in 2019 by MOA.

Hossain et al. (2014), reported that Tetep and Teqing had the least disease ratings for Sheath blight. Therefore, crosses were made between UKMRC2 and Teqing, and UKMRC2 and Tetep were made. These crosses were also confirmed and later 4-way crosses were made between the two F_1s in order to develop pyramidal lines. Tetep is already known to be used as a donor for blast genes in many studies and Tequing was shown to have moderate blast resistance while, UKMRC2, a high yielding variety is shown to carry some resistant alleles for blast (Mishra et al., 2021) and also shown to have some degree of resistance in field blast screening. Therefore, this 4-way population was considered interesting to understand the inheritance of blast resistant alleles for certain major *Pi* genes by genotyping and phenotyping. Hence, this study had the following objectives:

1. To map known blast disease resistance genes/, QTLs in advanced 4-way crossed populations using functional SNP markers.
2. To compare sequence variations among the SNPs in the segregating populations.

Materials and Methods

Development of 4-way Mapping Population

The mapping population used in this study was developed for sheath blight resistance study by Hossain et al. (2014) and Wickneswari et al. (2017) at Univesiti Kebangsan Malaysia. Sheath blight is caused by the soil-borne necrotrophic pathogenic fungus, *Rhizoctonia solani*. It is an emerging threat to rice cultivation. Only a few quantitative trait loci (QTLs) have been detected showing improved resistance against the disease. Teqing and Tetep both have showed improved resistance having QTLs, *qSB-9* and *qSBR11-1*, respectively. These QTLs demonstrate additive effects, therefore, pyramiding of these QTLs can be considered as an alternative to increase the sheath blight resistance in rice. Teqing and Tetep were detected as the most suitable sheath blight donors having major QTLs with improved resistance. On the other hand, UKMRC2 was found to have the lowest resistance ability against the disease. Hence, UKMRC2 was selected as recipient parent to pyramid resistance QTLs, *qSB-9* as well as *qSBR11-1* through advanced backcross breeding (Hossain et al., 2016). F_1 hybrids between UKMRC2 and Teqing, and UKMRC2 and Tetep were developed and confirmed and subsequently 4-way crosses were performed to understand inheritance of sheath blight and other disease resistance genes. Three sets of F_1s for both crosses (UKMRC2 x Teqing and UKMRC2 x Tetep) along with five sets of their parents were planted at 10 days intervals. At flowering stage F_1 plants were confirmed by comparing phenotypic traits such as days to 50% flowering, and plant height as well as by using two polymorphic SSR markers. After confirmation of F_1 progenies, 4-way F_1 crossing was conducted to combine the two QTLs (Fig 1). A segregating population of 30 4-way F_1 individuals and 72 4-way F_4 individuals were studied. UKMRC2, Tetep and Teqing were used as parental checks. Mahsuri acted as a negative control, while IR64 acted as the positive control.

SNP Genotyping

Isolation and Quantification of Total DNA

Leaf samples of all the plant materials were collected from 21-day old seedlings. Total DNA was extracted from the leaves (stored at -20°C in a refrigerator) according to the modified CTAB-based DNA extraction protocol previously developed by Murray and Thompson (1980). The extraction process was performed at the Plant Genetics Laboratory, School of Environment and Natural Resources Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). The DNA concentration was determined using 0.8% agarose gel electrophoresis in 1X TAE buffer at a fixed voltage of 60 V. The molecular ladder concentration for the agarose gel was 0.1 µg µL-1 λHindIII (Promega Corporation, USA) and 6X Ficoll dye was used as loading dye. The gel image was documented using the Alphaimager TM2200 (Alpha Innotech Corporation, USA). The DNA concentration was determined by comparing the gel image with λHindIII marker bands.

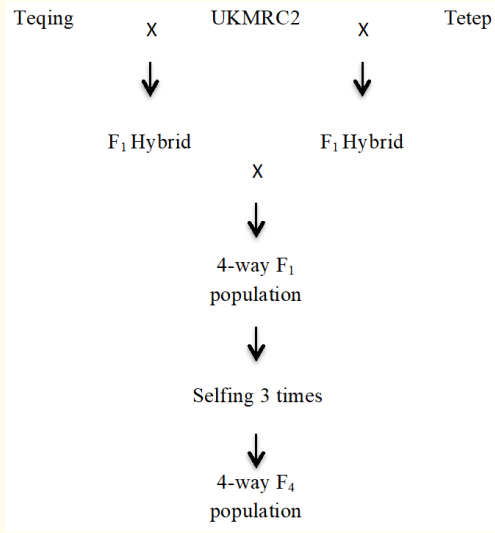

Figure 1: Development of 4-way F_1 and 4-way F_4 populations.

Genotyping

A total of 51 functional SNPs selected from a panel generated by Thakur et al. (2014) were used (Table 1). The SNP genotyping was outsourced to CMDV, Serdang, Malaysia. The SNP genotyping was carried out using a platform Agena massarray (manufactured by Agena Bioscience, Japan.) Fifty four SNPs spaning 6 major blast resistance genes were selected for genotyping. The sequence information was provided to the CMDV lab to check for compatibility with their Sequenom chip. These SNPs were then synthesized by the CMDV. All the segregating population's total leaf DNA was sent in plates. Three SNPs out of 54 were incompatible with the iplex designed, therefore only 51 SNPs were subsequently used. The DNA had to first qualify their quality check test before proceeding further. The iplex was run by the lab and the results were given back to us in form of cluster plots. The call was then assigned by us based on the cluster plots and the genotype data was derived for further analysis.

Gene	*Markers*	*Chr*	*Location*	*Synonym*	*Donor*	*SNP*	*Call*
Pib	5	2	10.4cM	*Pib*	IR64	Seq-rs54174765	T/C
						seq-rs53169602	T/C
						seq-rs18701984	T/G
						seq-rs18703791	T/G
						seq-rs18701930	T/C
Piz-t	8	6	69cM	*Pi2*	Fukunishiki (japonica) *O. minuta*	piz_t_1	C/G
						seq-rs53791232	T/C
						piz_t_6	T/G
						piz_t_10	T/C
						piz_t_12	T/C
						piz_t_13	A/G
						piz_t_14	A/T
						piz_t_17	A/C

Pi9	11	6	58.7cM	*Pi9(t)*	*O. minuta*	TBG1284330	C/G
						TBGI284333	T/C
						TBGI284423	C/G
						TBGI284429	A/G
						TBGI284452	C/G
						TBGI284454	T/C
						TBGI284456	T/C
						TBGI284470	T/C
						TBGI284473	A/G
						TBGI284478	T/C
						TBGI284480	T/G
Pi5	9	9	8.5cM	*Pi3*	Pai-kan-tao (japonica)	seq-rs54200981	A/C
						seq-rs54309186	T/C
						seq-rs53750144	A/G
						seq-rs53933458	T/C
						seq-rs54393488	C/G
						seq-rs53074031	T/G
						seq-rs53938798	T/C
						seq-rs53454238	A/G
						seq-rs53413543	T/G
Pi54	10	11	101.9cM	*Pi-kh*	Taipei 309 (indica)	pi_54_4	A/G
						pi_54_2	A/C
						pi_54_3	T/C
						pi_54_10	T/C
						pi_54_11	T/C
						pi_54_13	T/C
						pi_54_14	A/T
						pi_54_18	A/G
						pi_54_19	T/C
						pi_54_16	C/G
Pita	8	12	50.4cM	*Pita-2*	Taducan (indica) *O. rufipogon*	pita 14	T/C
						pita 13	T/G
						pita 12	C/G
						pita 10	C/G
						pita 8	T/G
						pita 5	T/G
						pita 4	T/G
						pita 3	A/G

Table 1: Panel of functional Single nucleotide polymorphic markers.

Field Blast Screening

Blast isolates

Six newly identified Malaysian isolates of *M. oryzae* were isolated using the single spore isolation method (Mishra et al., 2015). Five isolates were obtained from infected leaf samples collected from Kompleks Latihan MADA, Kedah and the other isolate was obtained from Kelantan. The mixture of all six of blast isolates was used for field blast screening.

Field Screening

The field blast screening was performed at Terrace Q, UKM, from September to November 2016. The experiment was designed such that, each test line had five biological replicates, all test lines with five replicates each consisted of a test block. Two more complete test blocks were tested, at a staggering interval of two weeks, thus creating three technical replications. Therefore, for each test line, a total of 15 individual plants were screened.

Seeds were introduced with Bavistin DF (Carbendazim 0.5% w/w, BASF, Germany) for 24 hours to control seed borne pathogens. A ratio of 4:1 of top soil and cow-dung compost was used. Fifty mg of Furadan 3G (Carbofuran 3% w/w) insecticide was used to control soil borne nematodes. The field was watered with regular tap water and ploughed for 3 days to prepare for field blast screening. Urea (N), triple super phosphate (P_2O_5), and potassium chloride (K_2O) were applied. Fertilizers were applied thrice; at planting, 15 days after planting and one day before inoculation. The seeder material was prepared by infecting a susceptible check, Mahsuri at 2 leaf stage, by spraying concentrated inoculum of *Magnoporthe oryzae* spores. The disease was allowed to develop for a period of 3-4 weeks. The diseased leaves were cut and stacked in paper bags, left to dry. This dry infected straw was later used as the seeder material for infection in field blast screening. Planting was carried out in rows with at least a 3 cm distance from each other for better hand weeding and application of fertilizers. Each row consisted of five germinated seedlings of each test family. Five seedlings were sown per test line for each block. Test lines were sown in three blocks for infection. After every 2 rows, Mahsuri was planted in order to assist in establishment of disease. After every row of Mahsuri, an empty row was left to place the seeder material.

Seedlings were infected with the dry seeder material at 28 days of age (four leaf stage), in the evening. The plants were maintained in moist and dark conditions at 26–28^{0}C for 12 h by covering with polyvinyl sheets overnight (Fig 2). Disease was left to spread naturally via wind dispersal, seedlings were water sprayed 3–4 times per day and covered during the night to maintain a high humidity until disease development and progression was observed. Disease evaluation for the field blast screening was performed 10 days after inoculation. Five replicates from all three test blocks were evaluated for each test line using the SES of IRRI (1996) for screening leaf blast, and later Mean score was formulated. The following assessment scale was used: 0 = no evidence of infection; 1 = brown specks smaller than 0.5 mm diameter; 2 = brown specks about 0.5–1.0 mm in diameter; 3 = roundish to slightly of 1–2 mm diameter surrounded by brown margins; 4 = spindle-shaped lesions with reddish brown margins; 5 = spindle-shaped lesions with necrotic gray centers.

Figure 2: Field Blast Screening.

Data Analysis

QTL Analysis

QTL validation was performed using Qgene ver. 4.2 for detecting significant QTLs through permutation analysis and trait analysis for determining trait distribution in both subsets of the population. The mean value of the blast score and the genotype data of the population were prepared as text file. The text file was then converted into qdf file prior to loading into the Qgene software to detect any significance of the QTL through interval mapping and permutation analysis. The association between individual marker loci and QTLs for blast disease was evaluated by a single-marker analysis. The interval size was set at 2 cM (centimorgan), and the threshold value of the LOD score was considered as 3.0 for detecting the presence of a QTL (Shindo et al., 2003). The significant level of the LOD threshold was examined by permuting the data, as implemented by the permutation analysis module (Churchill and Doerge, 1994).

To identify an accurate and significant threshold for every trait, an empirical threshold was determined for interval mapping using 1000 permutations for each trait across all 12 chromosomes (Churchill and Doerge, 1994). To identify additional QTLs and to increase the resolution of QTL locations, composite interval mapping was employed (Zeng, 1994). Automatic cofactor selection using a forward/backward regression (forward $p < 0.01$, backward $p < 0.01$) was also performed with Qgene version 4.2. Significant thresholds for composite interval mapping were determined using 1000 permutations for each trait. If single marker analysis with either interval mapping or composite interval mapping showed a QTL at a LOD > 3.0, it was considered as a putative QTL. A putative QTL was reported if detected by Identified QTLs were named according to the method proposed by McCouch et al. (1997).

Sequence comparison

The SNPs genotype data was studied in detail to examine the sequence variations. The Genotype calls were screened for sequence variations of genes contributing resistance to *M. oryzae*. The sequence variants were considered to be present, when a SNP allele was present, conferring resistance thus lowering the blast score, throughout the genotype data. This pattern of blast score modulation was observed throughout the SNP data.

Results

SNP Genotyping

The SNP data provided by CMDV was in the form of a detailed Microsoft Excel file depicting call details. This data was utilised by assigning the calls manually and generate call cluster plots. The example of call cluster plots representing two SNPs; *Pi_54_4* and Pita 10 are depicted in Fig 3. The total call rate achieved was 80.4%, accounting for aggressive calls (4.6%), moderate calls (12%) and conservative calls (73.8%). Resistant alleles for five genes (*Pi5, Pib, Piz, Pita*, and *Pi54*) except for Pi9was found based on the SNP genotype data. Dominant alleles were found representing *Pi54* and *Pita, SNPs* for *Pi54* and *Pita* were robust as they produced good scores and generated reproducible as well as aggressive calls as validated based on the Sequenom data. Hence, *Pi54* and *Pita* were characterised as the most reliable genes to be used in further screening studies.

Field Blast Screening

The parental materials Tetep, Teqing and UKMRC2 showed varied level of resistance to blast during the field screening. Tetep was found to be resistant showing a disease score 2, while UKMRC was moderately resistant showing a disease score 3 and Teqing was found to be moderately susceptible showing the disease score 4. IR64 was found to be resistant with a disease score 1, showing minimal blast symptoms. Mahsuri, was found to be susceptible, showing the most blast symptoms with a disease score of 5. The 4-way F_1 lines showed a high degree of resistance to the blast disease as compared to 4-way F_4 individuals. Twenty eight out of 30 4-way F_1 lines tested for blast, were found to have a disease score of 1 or 2. Fifty seven out of 72 4-way F_4 lines tested for blast, were found to have a disease score of 1 or 2.

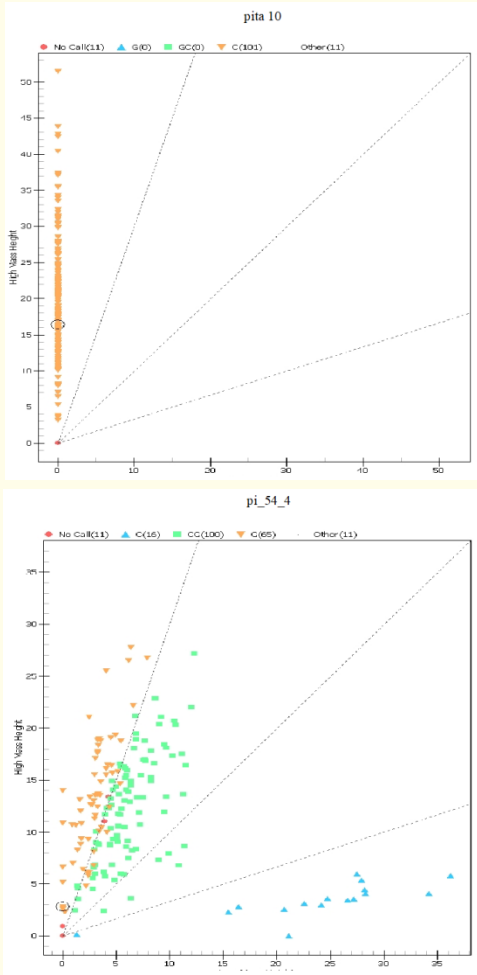

Figure 3: Call cluster plots for blast resistance genes based on SNP genotyping data.

QTL Analysis

QTLs for *Pi54* on chromosome 11 and *Pita* on chromosome 12 were found in the 4-way F_4 individuals. Both QTLs were identified with a LOD score of 14 (Fig 4; Fig 5) with phenotypic variations of 56.6% and 72.4% for *Pi54* and *Pita* respectively. However, the QTLs could not be detected with a good LOD score in 4-way F_1 individuals (Fig 6; Fig 7). The reason for this might be the low population size or the early stage of segregation. *Pi54* was mapped to a region of 312 kb on chromosome 11 flanked by SNPs *Pi54-3* and *Pi54-19* with a phenotypic variance of 56.6%, while *Pita* was mapped to a region of 175 kb on Chromosome 12 flanked by SNPs *Pita-10* and *Pita-3* with a phenotypic variance of 72.4%.

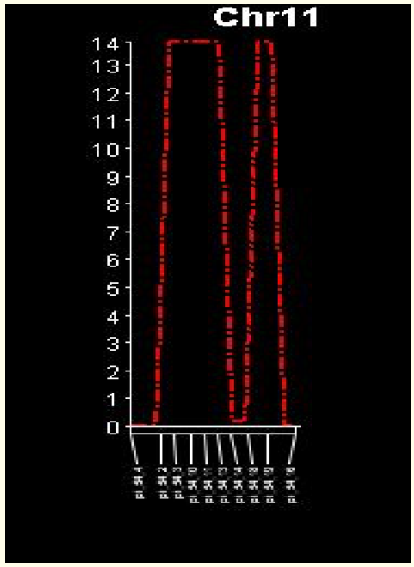

Figure 4: QTL Map of *Pi54* gene for 4-way F_4 linesv(X-axis: SNP loci, Y-axis: LOD).

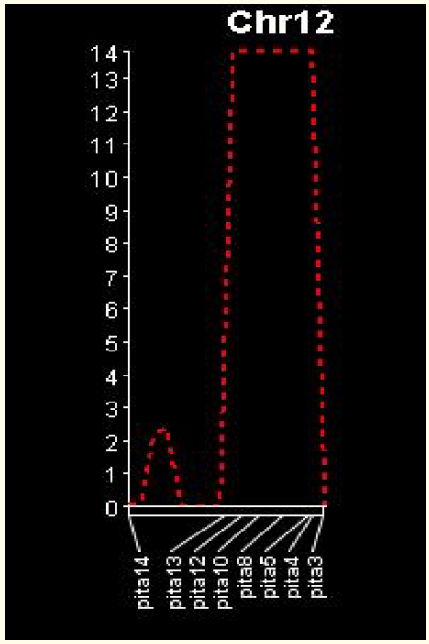

Figure 5: QTL Map of *Pita* gene for 4-way F_4 lines (X-axis: SNP loci, Y-axis: LOD).

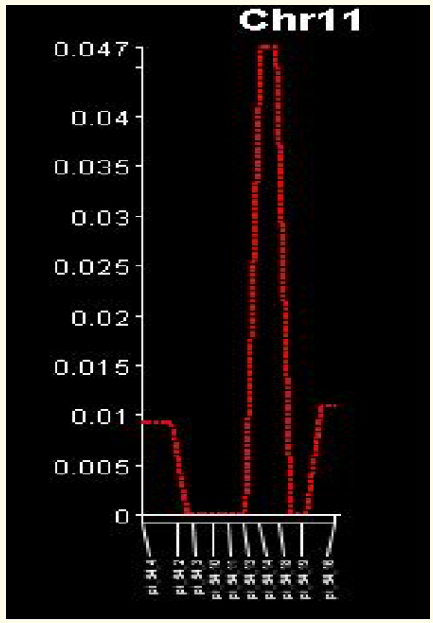

Figure 6: QTL Map of *Pi54* gene for 4-way F_1 lines (X-axis: SNP loci, Y-axis: LOD).

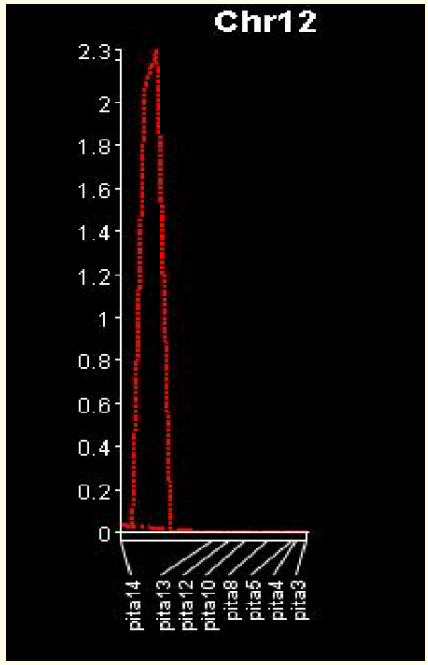

Figure 7: QTL Map of *Pita* gene for 4-way F_1 lines (X-axis: SNP loci, Y-axis: LOD).

Sequence comparison

Sequence variation of genes contributing resistance to *M. Oryzae* was identified using SNP genotyping. Nine sequence variants were reported as per the genotype and phenotype data for both 4-way F_1 and 4-way F_4 populations. These sequence variants, when present conferred resistance thus reducing the blast score. This pattern of blast score modulation, observed in the SNP data for 4-way F_1 population is highlighted in Table 2. The sequence variants for 4-way F_4 population are shown in Table 3. Each colour represents one putative haplotype block.

Locus	*Gene*	*Call*	*4wayF_1-1*	*4way F_1-2*	*4wayF_1-4*	*Mahsuri*	*IR64*	*Tetep*	*Teqing*	*UKMRC2*
seq-rs18701984	*Pib*	T/G	GT [1]	GT [1]	GT [1]	TT	GT[1]	GT[1]	GT[1]	GT[1]
seq-rs18701930	*Pib*	T/C	CT [2]	CT [2]	CT [2]	TT	CT[2]	CT [2]	CT [2]	CT [2]
piz_t_12	*Piz*	T/C	TT [3]	CC	CC	CC	TT[3]	TT[3]	CC	CC
piz_t_13	*Piz*	A/G	GG [4]	GG [4]	AA	AA	GG[4]	GG[4]	GG[4]	AA
TBGI284470	*Pi9*	T/C	TT [5]	TT [5]	TT [5]	CC	TT[5]	TT[5]	TT[5]	TT[5]
pi_54_4	*Pi54*	A/G	AA [6]	AA [6]	AA [6]	GG	**AA**[6]	**AA**[6]	GG	**AA**[6]
pi_54_11	*Pi54*	T/C	CC [7]	CC [7]	CC [7]	TT	**CC**[7]	**CC**[7]	TT	**CC**[7]
pita 4	*Pita*	T/C	CC [8]	CC [8]	CC [8]	CT	CC [8]	CC [8]	CC[8]	CT
pita 3	*Pita*	T/G	GG [9]	GG [9]	GG [9]	TT	GG [9]	GG [9]	GG [9]	TT
pita 10	*Pita*	C/G	CC	CC	CC	CC	GG	GG	CC	CC
Blast Score			1	1	1	5	1	2	4	3

[1-9]- Sequence variants identified, 2 putative haplotype blocks identified highlighted.

Blue: Block 1 and Yellow: Block 2.

Table 2: Sequence variations derived for 4-way F_1 population.

Locus	*Gene*	*Call*	*4wayF_4-2*	*4wayF_4-4*	*4way F_4-6*	*4wayF_4-14*	*Mahsuri*	*IR64*	*Tetep*	*Teqing*	*UKMRC2*
seq-rs18701984	*Pib*	T/G	GT [1]	GT [1]	GT [1]	GT [1]	TT	GT[1]	GT[1]	GT[1]	GT[1]
seq-rs18701930	*Pib*	T/C	CT [2]	TT	CT [2]	CT [2]	TT	CT[2]	CT [2]	CT [2]	CT [2]
piz_t_12	*Piz*	T/C	TT [3]	CC	CC	CC	CC	TT[3]	TT[3]	CC	CC
piz_t_13	*Piz*	A/G	AA	AA	AA	AA	AA	GG[4]	GG[4]	GG[4]	AA
TBGI284470	*Pi9*	T/C	TT [5]	TT [5]	CC	CC	CC	TT[5]	TT[5]	TT[5]	TT[5]
pi_54_4	*Pi54*	A/G	AA [6]	AA [6]	GG	AA [6]	GG	**AA**[6]	**AA**[6]	GG	**AA**[6]
pi_54_11	*Pi54*	T/C	CC [7]	CC [7]	CC [7]	CC [7]	TT	**CC**[7]	**CC**[7]	TT	**CC**[7]
pita 4	*Pita*	T/C	CC [8]	CC [8]	CC [8]	CC [8]	CT	CC [8]	CC [8]	CC[8]	CT
pita 3	*Pita*	T/G	GG [9]	GG [9]	GG [9]	GG [9]	TT	GG [9]	GG [9]	GG [9]	TT
pita 10	*Pita*	C/G	CC	CC	CC	CC	CC	GG	GG	CC	CC
Blast Score			1	1	2	1	5	1	2	4	3

[1-9]- Sequence variants identified, 2 putative haplotype blocks identified highlighted.

Blue: Block 1 and Yellow: Block 2.

Table 3: Sequence variations derived for 4-way F_4 population.

The SNP data analysis was done based on the 4-way F_4 population genotype and phenotype data as the 4-way F_1 population was smaller in size and in very early stage of segregation to give any conclusive results. The SNP data analysis showed a total of nine sequence variants. All nine of the sequence variants are considered individually in this study. Their segregation pattern revealed two putative haplotype blocks, one representing *Pi54* (*Pi54-4* to *Pi54-11*) and another representing *Pita* (*Pita-10* to *Pita-3*). The two SNPs for *Pi54* are 225 kb apart and co-segregating. The two SNPs for *Pita*, are 195 kb apart and also co-segregating.

Discussion

Enhancing the host plant resistance is being considered as the best approach to handle the rice blast disease. Hence the combinations arising from various blast resistance genes, which are complementary to each other to impart resistance, are being incorporated in recipient plants in rice blast breeding programmes. In these pyramiding programmes, desired and robust alleles representing the resistance genes should be considered (Ramkumar et al., 2010). However, continuous cultivation of particular rice varieties and domestication of this crop by human selection for high yields has led to drastic reduction in genetic diversity. During the evolution and artificial selection processes, a significant portion of beneficial alleles are undiscovered in the landraces and wild species (McCouch et al., 2007), which can be used for the development of improved rice varieties.

Several rice genotypes were known to carry the Pi resistant genes for blast disease. MR219 and UKMRC lines were results of crossing with the variety IR64, which is a known to be blast resistant and also the most popular cultivated rice in Asia and as source of resistance to blast disease under irrigated lowland rice cultivation (Bonman et al., 1989). IR64 is widely grown in Asia; mainly in China, the Philippines, Thailand and Indonesia (Utami et al., 2008). In a study by Mishra et al. 2021, the same set of diverse rice germplasm was screened with various molecular markers such as SSR, InDel, SNP and gene specific markers that are reported for most of the mapped and cloned blast resistance genes. It was shown that many of the diverse germplasm carried resistant marker alleles. The check varieties of Tadukan and Tetep are widely known to be resistant to blast and possess Pita and *Pikh* (Pi54) genes, respectively (Padmavathi et al., 2005). PS2 was found to have similar resistance as Tadukan and Tetep. They were resistant to all pathotypes implying that they may have similar resistance genes as in Tadukan and Tetep. In a study involving the genetics of blast resistance in PS2, Tanweer et al. (2015) indicated that PS2 was strongly resistant to rice blast disease as opposed to MR219 that was found to be susceptible. It was also shown that PS2 contained at least two dominant genes, *Pi-b* and *Pi54*, which were involved in resistance against *M. oryzae* pathotype P7.2. The landraces preserve tremendous genetic variability, as they are not subjected to subtle selection over a long period of time, which is completely opposite to high yielding varieties. Therefore, landraces better adapt in wide agro-ecological conditions acting as depositories for important genetic resources.

Das et al. 2012 reported that *Pi54rha* rice blast resistant gene cloned from wild rice species provided broad spectrum resistance to *M. oryzae* and therefore can be innovatively used in rice improvement breeding programme. *Pi54* was cloned from *indica* rice Tetep and carries broad spectrum resistance against numerous *M. oryzae* strains (Sharma et al., 2002, 2005; Rai et al., 2011). Functional validation of cloned *Pi54rh* gene showed high degree of resistance to seven isolates of *M. oryzae* collected from different geographical locations of India (Rai et al., 2011). Two *M. oryzae* isolates from Taiwan, D41-2 and 12YL-DL3-2, were used to challenge a diverse set of rice accessions and the SNP genotyping was performed for 31 resistance genes. Three genes, *Pita, Pik,* and *Pib* were tested and the results indicated that the strongest resistance against two isolates tested was observed for *Pita* based on the QTLs (Lin et al., 2018). Landraces Tadukan and Tetep with the dominant *Pi-ta* gene have been used as donors for resistance breeding in Asia and USA (Jia et al., 2007; Rybka et al., 1997). Studies of *Pi-ta* gene in wild as well as cultivated rice indicated in conserved sequence before divergence (Wang et al., 2008). The *Pi-ta* alleles have been studied in detail among Indian landraces (Thakur et al., 2013b).

Pita and *Pi54* alleles have been found to be robust and reliable based on SNP genotyping results, which was further validated by field blast screening. The results showed that some of the resistant materials such as PS2 and IR65482-4-136-2-2 (NIL for *Pi40*) were able to with stand the disease during the greenhouse screening as reported by previous studies. Interestingly, this resistance was not exhibited during the field screening, although the marker alleles for the resistant genes were present. IR65482-4-136-2-2 (NIL for *Pi40*) has

been previously reported to have durable resistance (Jeung et al., 2007). However, *Pi40* NIL was unable to withstand the disease during the field screening. Nevertheless, IR64, Tetep, *and O. rufipogon* were shown to be resistant in field screening method and contained the resistant alleles when screened with SNP markers. Therefore, the rice genotypes, IR64, Tetep, and O. rufipogon, are thought to have broad spectrum resistance against the six local new isolates used in this study based on their resistance exhibited during the field screening. Tetep, Teqing, UKMRC2, IR64 and Mahsuri used in this study as parents and controls also showed varied level of resistance (score of 1 to 5). However, the 4-way F_1 and 4-way F_4 test lines showed a high degree of resistance to the blast disease as compared to the parents. This can be explained as the genes conferring resistance from all three parents Tetep, Teqing as well as UKMRC2 are introgressed in the 4-way segregating populations.

The resistance in rice varieties often diminishes within a few years of being released as *M. oryzae* can overcome plant resistance based on their race-specific resistant genes (Orbach et al., 2000). *M. oryzae* has continuously evolved due to environmental changes and natural mutation, in which the mutation rate is estimated to be high (Kiyosawa, 1996; Koizumi, 2007). Therefore, the study of blast virulence remains challenging. However, the high mutation rate in *M. oryzae*, largely due to transposon activity, repetitive genome and high selection pressure in its genome which often leads to an emergence of new strains resulting in an easy breakdown of single *R* gene mediated resistance response in rice (Khang and Valent, 2010; Srivastava et al., 2014). Resistance controlled by single genes may remain effective for many years, although this trait is typically not durable (Bonman and Mackill, 1988). Therefore, it is imperative to understand the regulation of plant defence mechanisms as well as determine the functional genes and controlling elements responsible for the effectiveness and breakdown of resistance.

The development of new *R* gene specific markers would benefit conventional breeding with MAS. This would assist in the possibility of pyramiding multiple genes in adapted germplasm toward realizing broader spectrum disease resistance. Stacking of multiple *R* genes acts as a buffer against breakdown of one or the other gene mediated resistance (Douglas and Halpin, 2010). Combining of multiple alleles of *R* genes might weaken the selection pressure on respective pathogen (Brunner et al., 2010; Fukuoka et al., 2015). This approach has been used successfully in rice to develop blast resistance lines with multiple *R* genes against *M. oryzae* using molecular breeding (Fukuoka et al., 2015; Tanweer et al., 2015; Xiao et al., 2017). Combining of multiple *R* genes could improve the durability of resistance against multiple strains of *M. oryzae* (Kumari et al., 2018). Wu et al. (2016) analysed the effective combination pattern of multiple blast R genes or their alleles and found that better combination of multiple genes or alleles could provide more dynamic and durable blast resistance in rice.

Molecular markers linked to major *R*-genes are important for MAS (Costanzo and Jia, 2010). Sequence variations in SNPs and InDels can be compared between genotypes to identify functional markers. *Pi-b* and *Pi-ta* as well as SNP markers for *Piz* locus and *Pik* locus (Hayashi et al., 2004; Jia et al., 2009; Zhai et al., 2011) are few of the examples. Sequence variations and allele mining for blast resistance in rice from wild and cultivated species have been widely studied (Yang et al., 2007; Geng et al., 2008; Huang et al., 2008; Das et al., 2012; Kumari et al., 2013; Thakur et al., 2013 a, b).

The sequence comparison study was done based on the 4-way F_4 population genotype and phenotype data as the 4-way F_1 population was smaller in size and in very early stage of segregation to give any conclusive results. The SNPs genotyped in this study were used to study sequence variation of genes conferring resistance to *M. oryzae*, in which nine sequence variants were identified based on the SNP genotype results and blast scores obtained after field blast screening. When present, these sequence variants contributed resistance and thus reduced the blast score. All the nine sequence variants were evaluated individually in this study. Their segregation pattern showed the presence of two putative haplotype blocks. The SNP genotyping results identified the presence of robust and reliable *Pita* and *Pi54* alleles that were also validated by blast disease resistance in the field screening. Their segregation pattern shows the presence of two putative haplotype blocks, each for *Pi54* (*Pi54-4 to Pi54-11*) and *Pita* (*Pita-10 to Pita-3*). The two SNPs for *Pi54* are 225 kb apart and co-segregating. The two SNPs for *Pita*, are 195 kb apart and also co-segregating. Additionally, *Pi54* and *Pita* were mapped using SNP markers. Qgene analysis revealed QTLs for *Pi54* on chromosome 11 and *Pita* on chromosome 12 in the 4-way F_4 individuals. Both QTLs were identified with a LOD score of 14 with phenotypic variations of 56.6% and 72.4% for *Pi54* and *Pita*

respectively. However, the QTLs could not be detected with a good LOD score in 4-way F_1 individuals. The reason for this might be the low population size or the early stage of segregation. *Pi54* was mapped to a region of 312 kb on chromosome 11 flanked by SNPs *Pi54-3* and *Pi54-19* with a phenotypic variance of 56.6%, while Pita was mapped to a region of 175 kb on Chromosome 12 flanked by SNPs *Pita-10* and *Pita-3* with a phenotypic variance of 72.4%.

Conclusion

It can be concluded that this 4-way population showed good resistance to blast and can be deployed in marker assisted breeding and gene pyramiding studies. This population also showed the resistant alleles for SNPs representing *Pi54* and *Pita* on chromosome 11 and 12 respectively with LOD score of 14 each with phenotypic variations of 56.6% and 72.4% for *Pi54* and *Pita* respectively. *Pi54* was mapped to a region of 312 kb on chromosome 11 flanked by SNPs *Pi54-3* and *Pi54-19*. *Pita* was mapped to a region of 175 kb on Chromosome 12 flanked by SNPs *Pita-10* and *Pita-3*. Thus, these two genes are reliable for use in marker-assisted selection. These set of SNPs were also used for identifying a total of nine sequence variants within SNP alleles for genes contributing resistance to *M. oryzae*. These sequence variations showed the presence of two putative haplotype blocks. Additionally, the gene expression of both candidate genes, *Pi54* and *Pita*, should be investigated using RT-PCR techniques to verify if these sequence variants can be characterised as haplotype blocks and used in the selection process for marker-assisted breeding programmes.

The new resistance alleles identified would prove to be great resource for blast resistance breeding programmes. Resistance to blast is highly race-specific. Some cultivars may show resistance to certainraces of the pathogen but get infected by other races due to the breakdown of blast resistance. Therefore, periodic new races of the pathogen need to be re-examined to ensure that the known sources of blast resistance are still resistant to the new isolates.

Acknowledgements

Authors wish to thank Idris bin Haji Husin for field assistance.

Funding information

This research was supported by funding from the Ministry of Education (MOE), Malaysia for Long-term Research Grant Scheme on Food Security (Grant No. LRGS/TD/2011/UPM-UKM/KM/01 under the LRGS Food Security Grant (UPM/700-1/3/LRGS)) and research university grant, DIP-2012-019 from Universiti Kebangsaan Malaysia.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval

We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere. Informed consent was obtained from all individual participants included in the study.

Consent for publication

All authors have granted their consent for publication.

Availability of data and material

All supporting data has been made available as electronic supplementary files.

Author's contributions

AM designed and performed the experiments, analyzed data, and wrote the manuscript while WR reviewed and improved the manuscript.

References

1. Bonman JM and Mackill DJ. "Durable resistance to rice blast". Oryza 25 (1988): 103-110.
2. Bryan GT., et al. "A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistant gene Pi-ta". Plant Cell 12 (2000): 2033-2046.
3. Chen S., et al. "Genetic and physical mapping of Pi37(t), a new gene conferring resistance to rice blast in the famous cultivar St. No. 1". Theor Appl Genet 111 (2005): 1563-1570.
4. Costanzo S and Jia Y. "Sequence variation at the rice blast resistance gene Pi-km locus: implications for the development of allele specific markers". Plant Science 178.6 (2010): 523-530.
5. Das A., et al. "A novel blast resistance gene, Pi54rh cloned from wild species of rice, Oryza rhizomatis confers broad spectrum resistance to Magnaporthe oryzae". Funct Integr Genomics 12 (2012): 215-228.
6. Fjellstrom R., et al. "Development of DNA markers suitable for marker assisted selection of three Pi genes conferring resistance to multiple Pyricularia grisae pathotypes". Crop Sci 44 (2004): 1790-1798.
7. Geng XS., et al. "Cloning and analyzing of rice blast resistance gene Pi-ta allele from Jinghong erect type of common wild rice (Oryza rufipogon Griff) in Yunnan". Yi Chuan 30.1 (2008): 109-114.
8. Huang CL., et al. "Molecular evolution of the Pi-ta gene resistant to rice blast in wild rice (Oryza rufipogon)". Genetics 179 (2008): 1527-1538.
9. IRRI. Standard evaluation system for rice. 4th edn. The International Network for Genetic Evaluation for Rice, Genetic Resources Center. International Rice Research Institute, Manila 52 (1996).
10. Jena KK and Mackill DJ. "Molecular markers and their use in marker-assisted selection in rice". Crop Sci 48 (2008): 1266-1276.
11. Jeon JS., et al. "Genetic and physical mapping of Pi5(t), a locus associated with broad-spectrum resistance to rice blast". Theor Appl Genet 269 (2003): 280-289.
12. Jeung JU., et al. "A novel gene Pi40(t), linked to the DNA markers derived from NBS- LRR motifs confers broad spectrum of blast resistance in rice". Theor Appl Genet 115 (2007): 1163-1177.
13. Jia Y., et al. "Two major resistance genes confer resistance to race shift isolates overcoming blast resistance gene Pi-ta". Rice Research Studies 91 (2007).
14. Khang CH and Valent B. "Magnaporthe oryzae and rice blast disease. In Cellular and molecular biology of filamentous fungi". American Society of Microbiology (2010): 593-606.
15. Kiyosawa S. "Pathogenic variation of Pyriculaia oryzae and their use in genetic and breeding studies". SABRAO J 8 (1996): 53-67.
16. Koizumi S. "Durability of resistance to rice blast disease". JIRCAS Working Report 53 (2007): 1-10.
17. Kumari A., et al. "Mining of rice blast resistance gene Pi54 shows effect of single nucleotide polymorphism on phenotypic expression of alleles". Eur J Plant Pathol 137.1 (2013): 55-65.
18. Kumari M., et al. "Stacking of blast resistance orthologue genes in susceptible indica rice line improves resistance against Magnaporthe oryzae". 3 Biotech 8.1 (2018): 37.
19. Lin HA., et al. "Genome-wide association study of rice genes and loci conferring resistance to Magnaporthe oryzae isolates from Taiwan". Botanical studies 59.1 (2018): 32.
20. Liu G., et al. "Two broad-spectrum blast resistance genes Pi9(t) and Pi2(t) are physically linked on rice chromosome 6". Mol Genet Genomics 267 (2002): 472-480.
21. Mackill DJ and Bonman JM. "Inheritance of blast resistance in near-isogenic lines of rice". Phytopathology 82 (1992): 746-749.
22. McCouch SR., et al. "Development and mapping of 2240 new SSR markers for rice (Oryza sativa L.)". DNA Res 9 (2002): 199-207.

23. Mishra A., et al. "Single spore isolation and morphological characterization of local Malaysian isolates of rice blast fungus Magnoporthe grisea". In AIP Conference Proceedings. AIP Publishing 1678.1 (2015): 020019.
24. Mishra A., et al. "Broad spectrum blast resistance alleles in newly developed Malaysian rice (Oryza sativa L.) genotypes". Euphytica 217 (2021): 8.
25. Mohamad O., et al. Mutant PS1297. Teknologi Padi 4 (1988): 15-22.
26. Nasu S., et al. "Search for and analysis of single nucleotide polymorphisms (SNPs) in rice (Oryza sativa, Oryza rufipogon) and establishment of SNP markers". DNA research 9.5 (2002): 163-171.
27. Orbach MJ., et al. "Atelomeric avirulence gene determines efficacy for the rice blast resistance gene Pi-ta". Plant Cell (12) (2000): 2019-2032.
28. Rahim H., et al. "Identification of quantitative trait loci for blast resistance in BC". Genetics and Molecular Research 11.3 (2012): 3277-3289.
29. Rahim HA., et al. "Identification of virulent pathotypes causing rice blast disease ('Magnaporthe oryzae') and study on single nuclear gene inheritance of blast resistance in F2 population derived from Pongsu Seribu 2 x Mahshuri". Australian Journal of Crop Science 7.11 (2013): 1597.
30. Rai AK., et al. "Functional complementation of rice blast resistance gene Pi-k h (Pi54) conferring resistance to diverse strains of Magnaporthe oryzae". Journal of Plant Biochemistry and Biotechnology 20.1 (2011): 55-65.
31. Ramkumar G., et al. "Development and validation of SNP-based functional codominant markers for two major disease resistance genes in rice (O. sativa L.)". Molecular Breeding 35.6 (2015): 129.
32. Rybka K., et al. "High resolution mapping of the indica-derived rice blast resistance genes II. Pi-ta2 and Pi-ta and a consideration of their origin". Molecular plant-microbe interactions 10.4 (1997): 517-524.
33. Sabu KK., et al. "Development and evaluation of advanced backcross families of rice for agronomically important traits". Communications in Biometry and Crop Science 1.2 (2006): 111-123.
34. Sankar DP., et al. "Rice breeding for salt resistance". Research in Biotechnology 2 (2011): 1-10.
35. Sharma TR., et al. "High-resolution mapping, cloning and molecular characterization of the Pi-kh gene of rice, which confers resistance to Magnaporthe grisea". Molecular Genetics & Genomics 274 (2005): 569-578.
36. Srivastava D., et al. "Morphological and molecular characterization of Pyricularia oryzae causing blast disease in rice (Oryza sativa) from North India". International Journal of Scientific and Research Publications 4.7 (2014): 2250-3153.
37. Tanweer FA., et al. "Current advance methods for the identification of blast resistance genes in rice". Comptes rendus biologies 338.5 (2015): 321-334.
38. Thakur S., et al. "Molecular diversity in rice blast resistance gene Pi-ta makes it highly effective against dynamic population of Magnaporthe oryzae". Funct Integr Genomics 13.3 (2013a): 309-322.
39. Thakur S., et al. "Positive selection pressure on rice blast resistance allele Piz-tmakes it divergent in Indian land races". J Plant Interact 8.1 (2013b): 34-44.
40. Thakur S., et al. "Genotyping and development of single-nucleotide polymorphism (SNP) markers associated with blast resistance genes in rice using Golden Gate assay". Mol Breeding 34.3 (2014): 1449-1463.
41. Utami DW., et al. "Blast resistance genes in wild rice Oryza rufipogon and rice cultivar IR64". Indonesian Journal of Agriculture 2 (2008): 71-76.
42. Wang X., et al. "Haplotype diversity at the Pi-ta locus in cultivated rice and its wild relataives". Phytopathology 98 (2008): 1305-1311.
43. Wickneswari R., et al. "Identification and validation of quantitative trait loci for agronomic traits in advanced backcross breeding lines derived from Oryza rufipogon x Oryza sativa cultivar MR219". Plant Molecular Biology Reporter 30 (2012): 929-939.
44. Wickneswari R and Bhuiyan MAR. "Exploiting wild accessions for development of high yielding new rice genotypes". Malaysian Applied Biology 43 (2014): 89-95.
45. Wickneswari R, Ankitta Mishra and Md Kamal Hossain. "Breeding for durable resistance to blast and sheath blight diseases in

rice". In: Wickneswari R. Nadarajah K. and Choong C.Y. (eds.) 2017. Applications of High Technology in Varietal Improvement of Rice. Penerbit UKM, Bangi, Malaysia (2017): 36-57.

46. Yang MZ., et al. "A rice blast resistance genetic resource from wild rice in Yunnan, China". Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao 33 (2007): 589-595.
47. Zhao B., et al. "The eight aminoacid differences within three leucine-rich repeats between Pi2 and Piz-t resistance proteins determine the resistance specificity to Magnaporthe oryzae". Mol Plant Mcirobe Interact 19 (2006): 1216-1228.
48. Zheng TQ., et al. "Genetic effects of background-independent loci for grain weight and shape identified using advanced reciprocal introgression lines from Lemont × Teqing in rice". Crop science 51.6 (2011): 2525-2534.
49. Zhou B., et al. "The eight amino acid differences within three leucine-rich repeats between Pi2 and Piz-t resistance proteins determine the resistance specificity to Magnaporthe grisea". Mol Plant Microbe Interactions 19 (2006): 1216-1228.
50. Zhu W., et al. "Development of chromosomes segment substitution lines derived from backcross between two sequenced rice cultivars, indica recipient 93-11 and japonica donor nipponbare". Plant Mol Biol Rep 27 (2009): 126-131.

Volume 3 Issue 3 September 2022