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Identification of virulent pathotypes causing rice blast disease (*Magnaporthe oryzae*) and study on single nuclear gene inheritance of blast resistance in F_2 population derived from Pongsu Seribu 2 × Mahshuri

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Abstract

Ten pathotypes of *Magnaporthe oryzae* found in Malaysia were tested against thirteen different Malaysian modern rice varieties/cultivars including resistant and susceptible to identify the most virulent pathotypes. The inheritance of blast disease resistance was studied using local cultivars by making normal and reciprocal crosses between resistance variety Pongsu Seribu 2 (*Oryza sativa* L. subsp. *indica*) and susceptible variety Mahsuri (*Oryza sativa* L. subsp. *indica*). Pathotype pathotype P7.2 followed by pathotype P5.0 was found as the most virulent against the cultivars. The disease scale of >2.5 was observed in six varieties against P7.2 and in five varieties against pathotype P5.0. Nineteen F_1 hybrids were confirmed using SSR marker RM168 and were selfed to produce F_2 populations. A total of 2560 F_2 plants from normal crosses and 3182 from reciprocal crosses were challenged with pathotype P7.2. A 3:1 (R:S) segregation ratio was observed in both types of crosses using the chi-square test, indicating the maternal effect which showed resistance to the blast disease caused by pathotype P7.2 is most likely controlled by a single nuclear gene. As a single resistance gene is able to retain the resistance for a short period, developing new breeding lines will be the alternative, rather than having no resistant material with multiple genes.

Keywords: Blast disease; Inheritance; Nuclear gene; Pathotypes; Virulence.
Abbreviations: AVR - Avirulence; PDA - Potato Dextrose Agarose; PS 2 - Pongsu Seribu 2.

Introduction

Rice blast disease occurs all over the world, causing damage to rice plants. With the increased usage of fertilizers, disease has become more abundant. Studies on factors that affect blast diseases revealed that a high nitrogen content resulted in increased susceptibility to blast disease in rice (Long et al., 2000; Snoeijers et al., 2000). Sufficient knowledge of the fungus and the host resistance is important to overcome this problem. A major resistance (R) gene confers specific resistance to a pathogen race that contains a specific avirulence (AVR) gene (Wang et al., 2008). Ellingboe et al. (1994) explained that the ability of a plant to express resistance depends on the pathogenicity. However, Valent and Chumley (1994) defined virulence as pathogenic ability on a particular cultivar, or as cultivar-specific. The tool for characterising this virulence diversity was pathogenicity assay of the fungus isolate. The isolates were sorted into pathotypes (races) by the symptoms they caused on each set of differential varieties/cultivars (Limpert et al., 1994). Earlier studies on the variability of *M. oryzae* relied mainly on the phenotypic characters and virulence test, using a set of host differential varieties. Most of these phenotypic traits are highly variable as this pathogen is genetically unstable (Ou, 1985). Such studies are labour-extensive and time-consuming, requiring a large greenhouse space and often leading to ambiguous results. Furthermore, they are influenced by environmental conditions, inoculation techniques and human errors in scoring (Srinivasachary et al., 2002). Blast classification studies have been conducted at the Malaysian Agricultural Research and Development Institute (MARDI) revealed that Mahsuri was the most susceptible cultivar, while Pongsu Seribu 2 was the most resistant variety against all 22 *M. oryzae* pathotypes tested. However, the virulence in *M. oryzae* can be changed due to its development into a new strain, as this pathogen keeps evolving according to time and environment. This phenomenon happened because the virulence of *M. oryzae* is influenced by environmental conditions (Srinivasachary et al., 2002). Thus, cultivars carrying a single resistance gene can only retain their resistance for a short period after deployment in the field because of the instability of *M. oryzae* avirulence genes (Bonman et al., 1989; Valent and Chumley, 1994; Zhou et al., 2004). A dynamic interaction exists between host plant (resistance gene) and pathogen (virulence gene). Therefore, screening for the virulence of *M. oryzae* pathotypes against differential varieties is important to ensure the stability of the pathotypes. Disease resistance is largely controlled by one or two pairs of genes (Chen et al., 2005; Notteghem et al., 1994; Padmavathi et al., 2005; Pan et al., 1999; Sharma et al., 2007; Yu et al., 1996). A single gene providing resistance against two races of blast was reported by Atkins and Jonston (1965) and Rath and Padrnanabhan (1973). Mackill et al. (1985) revealed from a genetic study that the traditional rice cultivars generally have one or two dominant resistance genes, which are effective against each

Table 1. Mean of blast disease score of 13 differential varieties against 2 local pathotypes .

Var/cultivars	P7.2					P5.2				
	Rep1	Rep2	Rep3	Rep4	Ave.	Rep1	Rep2	Rep3	Rep4	Ave.
Mahsuri	3.0	1.3	7.0	5.0	4.0	3.7	3.0	6.3	7.7	5.1
Bahagia	0.7	1.0	3.7	5.7	2.7	2.3	1.0	5.0	3.7	3.0
Setanjung	0.7	1.0	4.7	7.7	3.5	2.3	0.3	0.0	9.0	2.9
Pongsu Seribu 2	1.0	0.3	0.3	2.3	0.9	0.0	1.7	2.3	0.3	1.0
MR220	1.0	0.7	0.0	4.7	1.6	3.0	0.7	0.7	5.0	2.3
MR211	0.3	0.3	4.0	3.7	2.0	3.7	3.0	0.3	5.0	3.0
Tandukan	0.0	2.3	0.0	1.0	0.8	0.7	0.3	0.0	1.0	.05
Tetep	1.0	1.7	3.0	2.0	1.9	0.7	0.0	1.0	0.0	0.4
MR219	1.0	0.3	0.0	7.0	2.0	1.7	1.7	0.0	7.0	2.6
MRQ50	0.3	0.7	1.3	1.0	0.8	1.0	1.0	0.0	0.5	0.6
MRQ74	0.0	0.0	0.3	1.0	0.3	0.0	0.3	0.0	0.0	0.0
Pan Khari 203	2.3	0.0	1.7	3.0	1.7	1.7	1.7	0.0	2.3	1.4
MR232	0.0	1.0	1.7	2.3	1.2	1.0	1.7	0.7	3.7	1.7

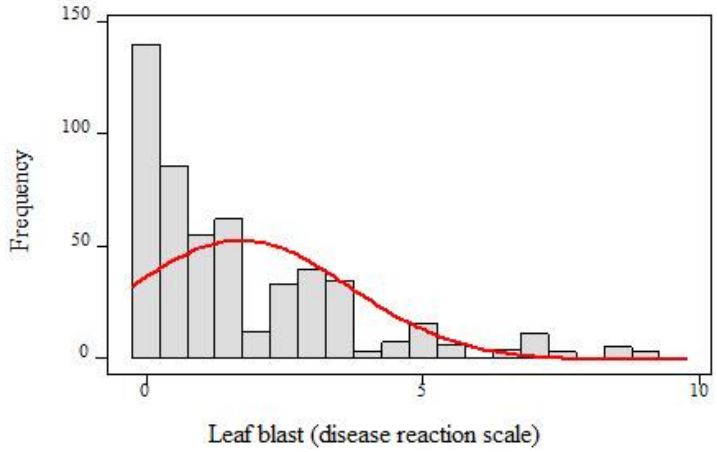

Fig 1. Frequency of leaf blast disease reaction scale, among the 13 differential varieties

fungal isolate. In most cases, *Pi*-ta genes, which originated from several traditional indica cultivars, including Tetep from Vietnam and Tadukan from the Philippines, have been used in rice breeding programmes worldwide (Jia et al., 2003). Currently, about 50 resistant genes that have been identified as dominant (Fukuoka and Okuno, 2001; Gowda et al., 2006; Hayashi et al., 2004; Zhou et al., 2004) have derived from indica land races. A few resistant genes have been used for cultivar development, but these are often not durable. Moreover, most of the resistant genes are race specific (Deng et al., 2006; Mackill and Bonman, 1992). Therefore, it is essential to identify broad-spectrum blast resistant genes for effective protection against dynamic blast isolates of *M. oryzae*. Pongsu Seribu 2, a Malaysian traditional variety that is resistant to blast diseases, has been used extensively in blast disease screening and as a blast resistant donor in cross breeding programmes (Supaad et al., 1980). Mahsuri, a susceptible variety, was derived by crossing Taichung 65 and Mayang Ebos 80 (Yamakawa et al., 1977). The present study was carried out to determine the most virulent *M. oryzae* pathotypes among the 13 Malaysian rice varieties for further genetic studies and the mode of inheritance of blast resistance to the most virulent *M. oryzae* pathotype, P7.2, in local varieties.

Results

Screening varities/cultivars against M. oryzae

The varieties were screened against *M.oryzae* and divided into 3 classes on the basis of the disease score scale (IRRI, 1996) as susceptible (S), moderately resistance (MR) and resistant (R). The cultivars Mahsuri and Setanjung were found as susceptible. Bahagia, MR211, MR220 and MR219 were found as moderately resistant, and MR50, MRQ74, Pan Khari 203, MR232, Pongsu Seribu 2, Tetep and Tadukan were found as resistant. Table 1 shows the actual mean of infection scale of each variety against 2 local pathotypes.

Determination of pathotypes virulence using different statistical tests

Statistical analysis using the Anderson Darling for normality test showed that the reaction scale departed from normal distribution (p = 0.000). Fig 1 shows a histogram of blast disease (disease reaction scale) versus frequency of rice plants. The normal curve was skewed to the left showing that most of the lesions were scored 5. Since the data distribution was not normal, a non-parametric test, the Kruskal–Wallis, was used to determine the significance of differences of the medians of the reaction scale from ten *M. oryzae* pathotypes. A ranking of the pathotypes is given in Table 2. The Kruskal-Wallis test statistic, H can be approximated by a chi-square distribution with (k-1) degree of freedom (Wardlaw, 2000). A significant result at $p<0.05$ level requires a chi-square of $\geq$ 16.9. Therefore the value of H= 25.8 with 9 degrees of freedom (df=9), is associated with a very low probability (p = 0.002). Thus, the difference in median of 10 virulent pathotypes was very highly significant. Pathotype P7.2 showed the highest virulence with a median value of 2.85 and an average ranking value of 315 followed by pathotype P1.0 with a median value of 2.0. The least virulent pathotypes were P1.2 and P1.4 (Median value = 0.3).

Table 2. Kruskal-Wallis test on disease reaction scale due to *Magnaporthe oryzae* pathotypes.

Pathotype	N	Median	Average Rank
P7.2	52	2.85	315
P1.0	52	2	304.4
P7.0	52	1	279.4
P5.4	52	1.15	275.7
P5.0	52	1	275
P7.6	52	1.3	256.8
P1.5	52	1	239.4
P1.1	52	1	226.4
P1.4	52	0.3	220.5
P1.2	52	0.3	212.3
Overall	520	1	260.5

Chi-square, H = 25.89 with df = 9 at p = 0.002

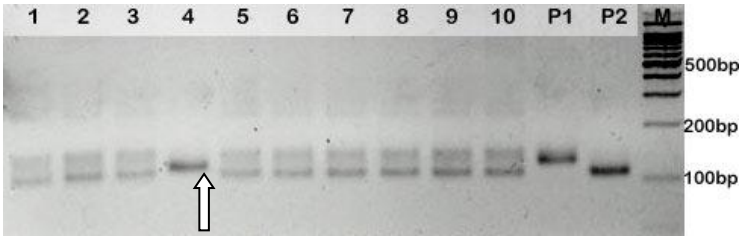

Fig 2. Evaluation 10 samples (Lanes 1-10) of F_1 plants derived from normal crossing between P1 (Pongsu Seribu 2) and P2 (Mahsuri) by using microsatellite marker RM168. Two bands were amplified in F_1 plants indicating that both sequence motifs originated from its parents. Lane 4 shows (marked with arrow) only single band indicating that this sample is not a hybrid.

The other pathotypes were considered as medium with median values ranging from 1 to 1.15.

Determination of pathotypes virulence through disease reaction pattern

The reaction pattern of the ten selected pathotypes to blast differential varieties/cultivars is given in Table 3. Resistant and susceptible reactions were categorised based on a disease reaction scale and a reaction scale greater than 3 was considered as susceptible. The resistance and susceptibility of each cultivar and line was determined by obtaining the disease reactions, using three plants in each tray with four repeats as recommended by Valent (1997). In this study, classification as described by Yu et al. (1987) was used, where *M. oryzae* isolates giving rise to lesion types 0, 1, and 2 were considered avirulent while those producing types 3 and 4 were considered as virulent. Therefore, the cultivar gave an average reaction scale of < 2.5 which was considered resistant to blast disease in this study, whilst cultivars with reaction scale ≥ 2.5 were considered as susceptible to blast disease. All varieties showed resistance to pathotypes P7.6 and P1.5, indicating that these pathotypes were the least virulent among all pathotypes tested. Some pathotypes showed similar patterns of infection, such as pathotypes P1.0, P7.0, P1.2, P1.1 and P1.0. These pathotypes showed their virulence/avirulence on similar differential varieties/cultivars. Similarly, pathotypes P1.0 and P7.0 showed similar virulence, where only Mahsuri, Bahagia and Setanjung showed to be susceptible against these pathotypes. Other pathotypes that showed similar virulence patterns against 13 differential varieties/cultivars were P1.2, P1.1 and P1.4. Pathotype P5.4 showed a different virulence pattern from the other pathotypes.

Evaluation of marker polymorphism using parents and screening F1s

Eight SSR markers were used to check polymorphism of two parents, Pongsu Seribu 2 and Mahsuri. Three (RM168 and RM166 and OSM89) out of the eight markers were found as polymorphic. Reconfirmation showed only RM168 was able to distinguish between the two parents with an allele size difference of 30 bp. Hence, F_1 plants from normal and reciprocal crosses were easily screened by RM168 (Fig. 2 and 3).

Segregation of resistance traits in F_2

Single Gene Model

The segregation ratio of the F_2 population with resistance to susceptible plants was expected to be 3:1 if only one gene is responsible for resistance against pathotype P7.2. Segregation patterns for blast resistance in the F_2 population are shown in Table 4 and 5, respectively. A Chi-square goodness of fit showed the F_2 population of normal crosses segregated in a 3:1 (R:S) ratio. For plants with resistance, eight families were susceptible. Out of 2560 F_2 plants from the normal crosses, 1879 plants were resistant and 681 were susceptible to pathotype P7.2 ($\chi^2_{3:1}$ = 3.5, P = 0.0613, Table 4). Similarly, the F_2 population from the reciprocal crosses showed similar results. From the 2156 F_2 plants of reciprocal crosses, 1600 were resistant while 556 were susceptible to pathotype P7.2 ($\chi^2_{3:1}$ = 0.715, P=0.398, Table 5). These results suggest that there may be a single gene controlling resistance to pathotype P7.2. The chi-square value for homogeneity for normal crosses was χ^2 = 12.88 (Table 4) and for reciprocal crosses it was χ^2 = 5.647 (Table 5).

Two Independent Genes

A two gene model was also analysed by classification of resistance to the *M. oryzae* pathotype P7.2 as Resistance (R), Moderate Resistance (MR), Moderately Susceptible (MS) and Susceptible (S). The plants with lesion scores 0-1 were considered resistant (R), 3 as medium resistant (MR), 5 as medium susceptible (MS) and 7-9 as susceptible (S)

Table 3. Disease reaction pattern of 10 selected pathotypes to blast differential varieties and cultivars.

Variety	Pathotypes									
	P1.2	P5.0	P1.1	P7.6	P7.2	P1.0	P1.5	P5.4	P1.4	P7.0
Mahsuri	S	S	S	R	S	S	R	S	S	S
MR211	R	S	R	R	R	R	R	S	R	R
Bahagia	S	S	S	R	S	S	R	R	S	S
Setanjung	R	S	R	R	S	S	R	S	R	S
MR220	R	R	R	R	S	R	R	R	R	R
MR219	R	S	R	R	R	R	R	S	R	R
MRQ50	R	R	R	R	R	R	R	R	R	R
MRQ74	R	R	R	R	R	R	R	R	R	R
Pan Khari 203	R	R	R	R	S	R	R	R	R	R
MR232	R	R	R	R	S	R	R	R	R	R
P. Seribu 2	R	R	R	R	R	R	R	R	R	R
Tetep	R	R	R	R	R	R	R	R	R	R
Tadukan	R	R	R	R	R	R	R	R	R	R

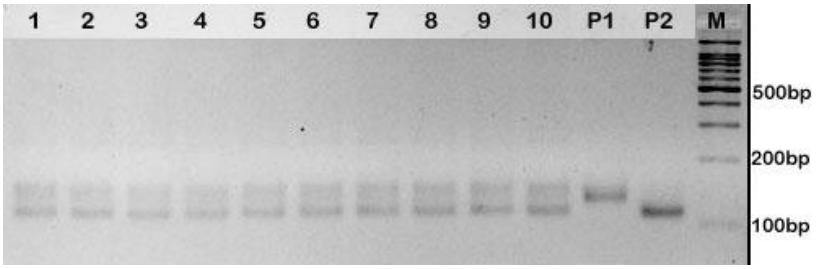

Fig 3. Evaluation 10 samples (Lanes 1-10) of F_1 plants derived from reciprocal crossing between P1 (Pongsu Seribu 2) and P2 (Mahsuri) by using microsatellite marker RM168. Two bands were amplified in F_1 plants indicating that both sequence motifs originated from its parents.

(Bonman et al. 1992). According to the Mendelian principle, the phenotypic segregation for a two gene model is 9:3:3:1 for R: MR: MS: S respectively. The segregation patterns of the F_2 populations treated with pathotype P7.2 of *M. oryzae* is shown in Table 6. The F_2 families did not show a good fit to 9:3:3:1 ratio ($\chi^2_{9:3:3:1}$ = 405.69, P=0.001, Table 6). This indicates that the resistance to blast disease caused by pathotype 7.2 in F_2 populations was most likely not controlled by two genes. The value for the homogeneity chi-square was χ^2 = 30.73. The critical value for the chi-square distribution was $\chi^2_{0.05,8}$ =15.51.

Two-locus Interaction/Epistasis

Testing for the epistasis was carried out by analysing F_2 populations from normal crosses, to determine whether they will segregate with 93.75% for resistant and 6.25% for susceptible, or a 15(R):1(S) ratio. The plants with a disease reaction score of 0, 1, 3 and 5 were considered resistant (R), while disease reaction scores of 7 and 9 were considered susceptible (S). The segregation patterns of the F_2 populations challenged with pathotype 7.2 of *M. oryzae* are shown in Table 7. The results indicated no locus interaction or epistasis occurred in the F_2 population.

Discussion

The results showed that the pathotype was only virulent on specific varieties or cultivars. For example, cultivars Mahsuri and MR211 were observed as susceptible and resistant against pathotype P7.2, respectively. In this case, the resistance gene that exists in cultivar MR211 was compatible with the avirulence gene that exists in pathotype P7.2. This result is in agreement with a statement explained by Wang et al. (2008) that the resistance (R) gene confers specific resistance to a pathogen race that contains a specific avirulence (AVR) gene. In addition, the ability of a plant to express resistance is dependent on the genotype of the pathogen (Ellingboe et al., 1994). Therefore, rice cannot be resistant to an isolate/pathotype of *M. oryzae* unless the pathogen has the genes that make it avirulent on the rice plant. An isolate/pathotype of *M. oryzae* cannot be avirulent on the rice plant unless the rice plant has genes that make it resistant to that isolate. Pathotype P5.0 was found as the second highest virulent among the ten pathotypes tested through the results of disease reaction patterns. Five out of 13 differential varieties were susceptible to this pathotype. The most virulent pathotype was P7.2, where six varieties were found to be susceptible to this pathotype, including two varieties ranked as resistant (Pan Khari 203 and MR232). The variability of pathotype virulence obtained in this study is in agreement with the statement that *M. oryzae* is found to have host-specificity as well as genetic instability (Ou 1985). The most virulent pathotype in this study that was found in the blast disease reaction patterns was also approved through statistical analysis. The result also revealed that the pathotype virulence could only be found on certain varieties/cultivars. Thus, variability in pathotype virulence is in agreement to the statement that most of the resistance genes are race specific (Deng et al., 2006; Mackill and Bonman, 1992). Three out of the 13 cultivars/varieties showed resistance to all pathotypes. They were not affected by any of the blast pathotypes tested, ultimately showing strong resistance to 10 selected Malaysian blast pathotypes. They are Pongsu Seribu 2, Tadukan and Tetep. Tadukan and Tetep are resistant varieties that possess the *Pi-kh* and *Pi-ta* genes, respectively (Jia, 2009; Padmavathi et al., 2005). This result gave a strong indication that Pongsu Seribu 2 may have a similar resistance gene to the Tetep and Tadukan varieties. The differential system is a useful method to study the resistance and virulence genes

interaction. It is possible to identify the genotype, which is resistant on certain pathotypes of blast isolate. This can be done with the selection of a suitable blast pathotype and differential varieties based on the reaction of its resistance gene. By using a standard set of differential varieties like near isogenic line (NIL) with a single resistance gene, it would be easier to differentiate the race of rice blast. A Chi-square of goodness of fit revealed the F_2 population of normal crosses segregated in a 3:1 (R:S) ratio for plants with resistance is susceptible to eight families. Similarly, the F_2 population from the reciprocal crosess of Mahsuri and Pongsu Seribu 2 were segregated in a similar manner, 3:1 ratio for plants with resistance that are susceptible. These results suggest that there might a single gene controlling resistance to pathotype P7.2. The value for the homogeneity chi-square was close to the total chi-square, indicating that the samples were homogenous. The critical value for the chi-square distribution, $\chi^2_{0.05,8}$, was 15.51. The F_2 populations from normal and reciprocal crosses showed similar segregation ratios, indicating that the segregation was not influenced by maternal effect; blast resistance in Pongsu Seribu 2 being solely due to a nuclear gene. This finding is in agreement with other inheritance studies conducted by many scientists where the F_2 populations were segregated in a similar manner (Mackill et al., 1992; Padmanabhan et al., 1973; Rath et al., 1972; Tanaka, 1986; Venkataswamy et al., 1963; Yu et al., 1987). A similar study has been carried out to determine the resistance of F_2 populations derived from traditional varieties/cultivars and the susceptible cultivar Mahsuri with a specific blast isolate. Sharma et al. (2007) crossed resistant (R) 'Laxmi' and susceptible (S) 'Mahsuri' cultivars. The F_2 population segregated into 3R:1S. Meanwhile, molecular mapping of rice blast resistance was carried out in the F_2 population derived from Mahsuri and a near isogenic line (NIL) C101LAC with resistant gene *Pi-1*(t). A Mendelian segregation ratio of 3:1 for resistance in susceptible plants was observed using SSR marker RM224 to the highly virulent blast isolate DRR 001 (Prasad et al., 2009).

An analysis of the chi-square goodness of fit showed that the F_2 families did not segregate in a 15R:1S ratio for plants with resistance, as given by the pooled chi-square, (χ^2= 21.02, with P = 0.001). Although five crosses i.e. N1, N2, N6, N7 and N8 showed chi-square values below 3.84 with P > 0.05, the total pooled chi-square was highly significant between the observed and expected number of resistant and susceptible plants. Therefore, the locus interaction or epistasis of blast resistance did not occur in the F_2 population derived from Pongsu Seribu 2 and Mahsuri.

Many studies have been conducted previously on the reaction of an F_2 population from the cross between resistant and susceptible parents. F_2 population tests of resistant parents such as IR56, Pan-kan-tao, Pan Khari 203 and Tetep showed a segregation ratio of 3R:1S. However, resistant parents Carreon and IR54 showed a segregation ratio of 15R:1S and 1R:3S, respectively (Yu et al., 1987). The F_2 population, derived from a cross between the Indian resistant traditional variety Laxmi (R) and the susceptible Mahsuri (S) cultivars (Sharma et al., 2007), segregated into a 3R:1S ratio, similar to those observed in F_2 populations derived from Pongsu Seribu 2 and Mahsuri. Previous studies showed resistance to blast is governed either by a single gene or a polygenic system, depending on the genotypes or cultivars, as well as their specificity to *M. oryzae* isolates, where resistance to blast disease is host specific and effective against only specific strains of *M. oryzae* (Zhou et al., 2006). However, studies conducted in IRRI revealed that most of the traditional varieties generally have one or two dominant genes (Mackill et al., 1985). Nevertheless, resistance to blast disease in Pongsu Seribu 2, particularly to pathotype *M. oryzae* P7.2, is controlled by a single gene.

Two genes can interact to determine one trait, in which two genes on separate chromosomes determine the resistance to blast. Hartl and Jones (2005) showed that F_2 families may segregate differently from the Mendelian principle for the single gene (3:1) and two gene models (9:3:3:1). This two-locus interaction, known as epistasis, describes any type of gene interaction where the classical phenotypic ratio of 9:3:3:1 is modified into subsets such as 9:7, 15:1, 9:3:4, 9:6:1, 12:3:1, and 13:3. It was reported that resistance genes for blast were controlled by three or more genes and differed from a single or two gene models (Fukuoka and Okuno 2001; Koizumi 2007; Mackill et al. 1985; Miyamoto et al. 2001; Tanaka 1986; Pan et. al, 1999; Persaud et al. 2007; Wang et al. 1994; Yu et al. 1987). Pan et al. (1999) observed segregations ratios of 15:1 and 63:1 while Persaud et al. (2007) also observed 57:7 for resistance in susceptible plants. Tanaka (1986) explained that segregation at a ratio of 63 resistant to one susceptible plants indicated that resistance in the cultivar is governed by three dominant gene pairs, while segregation at a ratio of 15:l indicated a duplicated dominant epistasis.

Materials and methods

Pathotypes of M. oryzae and different varieties/cultivars used in the experiment

Ten pathotypes were selected based on their virulence towards Mahsuri and Pongsu Seribu 2 (PS2), where Mahsuri was susceptible and PS2 was resistant to the selected pathotypes. Thirteen varieties including Mahsuri, MR211, Bahagia, Setanjung MR220, MR219, MR50, MRQ74, Pan Khari 203, MR232, Pongsu Seribu 2, Tetep and Tadukan were used in this experiment.

Media preparation for pathogen culture

Potato dextrose agar (PDA) was used as a media for growing the selected pathotype of *M. oryzae*. PDA was prepared by mixing 100 g of potato in 1.0 L of water and boiled at 70°C for 1 hour. The potatoes were then filtered and the solution was added up to 1.0 L with water. After that, agar (13 g per L) was added into the solution and was autoclaved. The solution was then poured in a 9 cm diameter Petri dish in the laminar flow cabinet and sealed with tape to avoid contamination.

Single spore isolation

Single spore isolation was done for 10 selected pathotypes. The spores were isolated from a single colony under the microscope using a sterile glass needle. Each colony was then eventually transferred to PDA slants and incubated at 28^{o}C - 30 ^{o}C for 7 days and used as a master culture.

Induction of Magnaporthe oryzae sporulation and inoculum preparation

The ten selected *M. oryzae* pathotypes were cultured at room temperature. Plate cultures were seeded from the master culture and conidia were induced by scraping the mycelia on the culture with a sterile spoon where the colonies were then exposed under the fluorescent light at room temperature and covered with wet cotton muslin cloth for 5 days to induce

Table 4. Chi-square test for 3:1 segregation of blast resistance in F_2 families of normal cross with pathotype P7.2 of *M. oryzae*

Family	R	S	Total	$\chi 2$	Probability
N1	254	71	325	1.72	0.1892
N2	111	49	160	2.70	0.1003
N3	212	88	300	3.00	0.0830
N4	220	75	295	0.03	0.8665
N5	228	70	298	0.36	0.5472
N6	225	72	297	0.09	0.7630
N7	218	80	298	0.54	0.4619
N8	203	84	287	2.79	0.0949
N9	208	92	300	5.14	0.0234
Observed	1879	681	2560	3.50	0.0613
expected	1920	640			
$\chi^2_{0.05,1} = 3.84$					
Total of chi-square				16.38	
Chi-square of totals (pooled chi square)				3.50	0.0613
Homogeneity chi -square				12.88	0.10<P<0.90
$\chi^2_{0.05,8} = 15.51$					

df=1 at p<0.05

Table 5. Chi-square test for 3:1 segregation ratio for blast resistance in F_2 families of reciprocal cross against pathotype P7.2 of *M. oryzae*.

Family	R	S	Total	χ^2	Probability
R1	104	34	138	0.010	0.922
R2	224	76	300	0.018	0.894
R3	213	86	299	2.258	0.133
R4	100	40	140	0.952	0.329
R5	226	65	291	1.101	0.294
R6	188	65	253	0.065	0.799
R7	209	63	272	0.49	0.484
R8	1159	60	219	0.671	0.413
R9	177	67	244	0.787	0.375
Observed	1600	556	2156	0.715	0.398
expected	1617	539			
$\chi^2_{0.05,1} = 3.84$					
Total of chi-square				6.352	
Chi-square of totals (pooled chi square)				0.715	0.398
Homogeneity chi- square				5.647	0.10<P<0.90
$\chi^2_{0.05,8} = 15.51$					

df=1 at p<0.05

sporulation. Spores for inoculation were prepared as described by Chen et al. (2005) and the concentration was adjusted to 1 x 10^5 spores/conidia per ml. The aerial mycelia were slightly washed off by gentle rubbing with a water soaked paintbrush. Later, the brush was soaked in sterile distilled water. The spore suspensions were filtered through nylon gauze mesh and adjusted to a concentration of $1x10^5$ spores/ml using a haemocytometer. Before inoculation, 0.05% Tween 20 was added to the suspension to increase the adhesion of the spores to the plant.

Growing seedlings for virulence study of pathogen and screening of varities/cultivars

The seeds of 13 differential varieties/cultivars were soaked in water for one day and germinated on moist Whatman filter paper (size=1) in Petri dishes for 3 days in a 30°C dark incubator. The germinated seeds were then planted in plastic trays (36 cm × 23 cm x 10 cm) containing 3 kg of soil with NPK (5 g of 15:15:15) and 3 g of ammonium sulphate per 3 kg of soil as described by Prabhu et al. (2003). An additional 2 g of ammonium sulphate per tray was applied after 20 days of planting. Ten seeds of each variety/cultivar were sown in 10 cm long rows containing 21 rows per tray. The varieties/cultivars were completely randomised with four replicates. Plants were grown in a green house at 25-30°C for 2-3 weeks, until they were at the four-leaf stage (Filippi et al., 2001).

Inoculation and assessment of disease infection

Twenty-two day old plants, with three or four fully expanded leaves, were inoculated by spraying 20 ml aqueous spore suspension onto the leaves until run-off occurred. This was done by using an atomiser connected to an air compressor. Inoculated plants were incubated in a moisture/dew chamber and the relative humidity was maintain 100% for 24 h at 25 to 28°C and placed in a controlled environment at temperatures ranging from 25 to 30°C (Filippi et al., 2001). The relative humidity was maintained at above 90% by covering with a black net and watering four to five times during the daytime. Assessment of disease infection was scored at 7 days after inoculation. Scoring was carried out based on the Standard Evaluation System (SES) of the International Rice Research Institute (IRRI, 1996).

Table 6. Chi-square test for 9:3:3:1 segregation ratio for blast resistance in F_2 families of normal crosses to M. oryzae pathotype P7.2.

Family	R	MR	MS	S	Total	χ^2	Probability
N1	254	40	19	12	325	67.18	0.001
N2	111	23	17	9	160	12.27	0.001
N3	212	67	16	5	300	52.02	0.001
N4	220	50	15	10	295	51.37	0.001
N5	228	53	9	8	298	67.28	0.001
N6	225	45	14	13	297	55.02	0.001
N7	218	55	8	17	298	56.31	0.001
N8	203	50	14	20	287	40.66	0.001
N9	208	60	22	10	300	34.32	0.001
Observed	1879	443	134	104	2560	405.69	0.001
Expected	1440	480	480	160			
$\chi^2_{0.05,3} = 7.81$							
Total of chi-square						436.43	
Chi-square of total (pooled chi square)						405.69	0.001
Homogeneity chi-square						30.73	P<0.005
$\chi^2_{0.05,8} = 15.51$							

df=3 at p<0.05

Table 7. Chi-square test for 15:1 segregation for blast resistance to M. oryzae pathotype P7.2 in F_2 families of normal crosses.

Family	R	S	Total	χ^2	Probability
N1	313	12	325	3.63	0.057
N2	151	9	160	0.11	0.744
N3	295	5	300	10.76	0.001
N4	285	10	295	4.12	0.042
N5	290	8	298	6.47	0.011
N6	284	13	297	1.78	0.182
N7	281	17	298	0.15	0.697
N8	267	20	287	0.25	0.615
N9	290	10	300	4.356	0.037
Observed	2459	104	2850	21.02	0.001
expected	2305	7			
$\chi^2_{0.05,1} = 3.84$					
Total of chi-square				31.62	
Chi-square of totals (pooled chi square)				21.02	0.001
Homogeneity chi-square				14.95	0.05<P<0.10
$\chi^2_{0.05,8} = 15.51$					

df=1, at P<0.05

Development and evaluation of F_1 plants

Normal and reciprocal crosses were done between Mahsuri and Pongsu Seribu 2 to obtain F_1 seeds following the procedure described by Virmani & Sharma (1992). The parent and the F_1 seedlings from both normal and reciprocal crosses were raised in the Malaysian Agricultural Research and Development Institute (MARDI). Leaves from each F_1 plant were collected for DNA isolation. The DNAs from the parents was used to identify the best polymorphic markers with microsatellites (SSR) associated to blast resistance. The best polymorphic SSR marker was used to verify 20 F_1 plants from normal and reciprocal crosses of Pongsu Seribu 2 and Mahsuri.

Genomic DNA extraction and purification

Total genomic DNA was extracted from young and fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Gawel et al., 1991). RNA was removed by adding RNAse (10 µg/ml) into the dissolved DNA, and incubated for 30 minutes at 37°C. The DNA was re-precipitated by adding 1/10 volume of sodium acetate 3M (pH 6.8) followed by two volumes of 70% ethanol into the mixture. The mixture was then incubated on ice for 30 min prior to centrifugation at 13000 rpm in a room temperature for 1 minute. The DNA pellet was then dried at 37°C for 10 min and re-suspended in a 250 µl 1X TE buffer and incubated at 4°C overnight to dissolve the DNA. It was finally quantified by using Nano-Drop spectrophotometry (ND1000 Spectrophotometer).

Genotyping of F_1 Plants

Eight simple sequence repeat (SSR) markers (RM138, RM140, RM155, RM166, RM168, RM212, OSM89 and OSM91) associated with resistance to blast were evaluated to determine the most suitable polymorphic marker to identify 20 F_1 plants derived from normal and reciprocal crosses of Pongsu Seribu 2 and Mahsuri. These primers were amplified based on the method described by Chen et al. (1997) with the following modifications: initial denaturation at 94°C for 4 min; 40 cycles consisting of 1 min at 94°C, 1 min at 42°C and 1.5 min at 72°C; and a final extension of 8 min at 72°C. The PCR products were separated in 1.5 % Methapor gel at 70V for 3 h. All PCR amplifications were carried out in a PTC-100 thermal controller (MJ Research, Waltham, Mass.).

Screening for F_2 Populations

About 300 plants representing the F_2 generation of each cross and a total of 19 crosses were analysed to study segregation patterns of resistance to *M. oryzae* pathotype P7.2. The seeds of each cross were planted in plastic trays. Plants were grown in a green house at 25-30^{o}C for 2-3 weeks, until they reached the four-leaf stage (Filippi et al. 2001). Twenty-day F_2 seedlings were challenged with pathotype P7.2 of *M. oryzae* to determine the segregation pattern of blast resistance. Pathotype P7.2 was identified as the most virulent pathotype based on its reaction against 13 Malaysian differential varieties. The blast inoculation was carried out as described by Chen et al. (2005) and described in the previous section of the present study. The lesion degree (LD) was determined based on the standard evaluation system (IRRI, 1996) and standardised protocol of Mackill and Bonman (1992).

Statistical Analysis

Data for lesion scale scores was analyzed using MINITAB Release 13.2. The non-parametric Kruskal–Wallis test was used to determine the significance of differences of the median among the *M. oryzae* pathotypes. The Kruskal–Wallis test does not assume a normal population, unlike other one-way analysis of variances. The test does assume an identically shaped and scaled distribution for each pathotype, except for any difference in the medians. Therefore, the data distribution was determined using the Anderson Darling method before using the Kruskal-Wallis test (Wardlaw, 2000). The observed segregation ratio of R (resistant) and S (susceptible) was tested for goodness of fit to the expected Mendelian ratio using a chi-square test (Samuels and Witmer, 1999). Homogeneity among the replicates was tested using a homogeneity chi-square analysis (Zar, 1999).

Conclusion

Pathotype P7.2 was found to be the most virulent among the ten selected pathotypes after observing their reaction patterns on a set of selected differential varieties, although the category of pathotype virulence based on disease reaction patterns was not in agreement with the median ranking through statistical analysis. Pathotype P7.2 was found as the most virulent pathotype in both analyses. Hence, pathotype P7.2 was used in experiments to understand the inheritance of blast resistance. The inheritance of blast resistance gene in F_2 populations derived from Pongsu Seribu 2 and Mahsuri showed a segregation ratio of 3R:1S, in both the normal and reciprocal crosses. Chi-square tests did not fit with the independent two-genes and epistasis segregation ratios. Therefore, resistance to blast pathotype P7.2 in Pongsu Seribu 2 is controlled by a single nuclear gene.

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