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RESEARCH ARTICLE

Mapping Quantitative Trait Loci Conferring Blast Resistance in Upland Indica Rice (*Oryza sativa* L.)

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Abstract

A genetic analysis of blast resistance in upland rice variety is very crucial. In this study, we performed a linkage mapping of quantitative trait loci (QTLs) for blast resistance using an advanced backcross population from a cross between Way Rarem (susceptible *indica* variety) and Oryzica Llanos 5 (durable resistant *indica* variety). A transgressive segregation was observed in the advanced backcross population of Way Rarem//Oryzica Llanos 5. A total of 16 QTLs have been identified along chromosomes 1, 3, 5, 6, 7, 8, 9, and 11 against eight blast pathogen isolates. Each QTL accounted from 11.31 to 45.11% of the variation in blast resistance. Most QTLs showed race specificity, demonstrating the small effect of such QTLs. Unexpectedly, several superior blast resistance alleles were contributed by Way Rarem, the susceptible-recurrent parent. Among eight candidate defense response genes detected in several loci, a single gene (oxalate oxidase) present on chromosome 3 was found to be associated with blast resistance in upland *indica* rice. Ultimately, these advanced backcross lines with resistance to blast tagged by markers might be useful for pyramiding blast resistance alleles in upland rice.

Key words: blast resistance, *Oryza sativa*, QTL analysis, upland rice

Introduction

The rice blast caused by the fungus *Pyricularia oryzae* B. Couch (anomorph *Pyricularia oryzae* Cavara), which, in its sexual state, is known as *Magnaporthe oryzae* (Couch and Kohn 2002), is one of the most destructive diseases and major constraint of rice production worldwide. Blast was first reported in Asia more than three centuries ago and is now present in over 85 countries, including in the upland rice ecosystem in Indonesia (Rao 1994). A great deal of upland rice varieties show greater resistance to blast compared to lowland varieties. The infection of blast fungus is to be more conducive in upland conditions and sometimes causes many yield losses. Growing resistant varieties has been the most effective and economical way to control rice blast disease (Hirano 1994). Many resistant varieties have been deployed, however extensive application of resistant rice variety especially those with single dominant gene have led to the breakdown of their resistance as new pathogenic strains of the pathogen arise. Thus, to develop rice carrying durable resistance genes to control blast diseases both in upland and lowland is very important.

Durable resistance in other crops has been associated in some cases with major genes, in other cases with multiple genes accompanied with additive effects (Johnson 1983). Several major resistance genes which can provide complete host-plant resistance to a specific subset of races have been taken up by

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rice breeders. The breakdown of a major gene's effectiveness because of the emergence of new and highly virulent races of the pathogen, coupled with favorable environmental conditions conducive to blast development, can attribute to blast epidemics (Tabien et al. 2002). The major resistant genes and defense response genes act together in a coordinated manner to prevent the invasion of fungal pathogens (Wu et al. 2004).

Molecular markers closely linked with major genes or in gene position target can be used for selection in rice breeding programs. Selection for disease resistance using molecular markers is more favorable, in particular for avoiding the instability of resistant genes. Previous studies focusing on molecular markers especially simple sequence repeat (SSR) markers on rice blast investigation have been reported. SSR markers developed for rice chromosomes (Akagi et al. 1996) are widely used for marker-assisted breeding on blast disease (Li et al. 2008a). In the past few decades, more than 40 major blast resistance genes have been mapped through molecular marker technology (Zhou et al. 2004). Several QTL detection approaches were employed to map major and minor genes involved in blast resistance (Fukuoka and Ukuno 2001; Wu et al. 2005) mainly focusing on the resistance of upland rice (Miyamoto et al. 2001; Saka et al. 2005; Sato et al. 2006; Tabien et al. 2002). Therefore, identification and molecular mapping for blast resistance will help in effective use of its broad-spectrum resistance in rice breeding programs.

An Indonesia upland rice variety, Way Rarem shows a high yielding and early maturing performance with high tolerance to blast when it was initially released but not durable. Oryzica Llanos 5, a well-known upland variety from Colombia with durable resistance to blast has been extensively used as a parent to improve blast resistance of Colombia rice. Despite the usefulness of field-upland resistance, its genetic analysis has not been performed. Thus, it is a crucial need to identify an alternative source for the improvement of blast resistance in upland rice. Both the upland rice varieties hopefully could be used as a genetic source material for this study. The objectives of this study were to evaluate blast resistance, and to identify genomic region controlling resistance to blast using QTL analysis and superior blast QTL allele. Additionally, this study was to identify candidate defense response genes associated with quantitative resistance to blast.

Materials and Methods

Plant materials and population development

An advanced backcross population derived from a cross between Way Rarem and Oryzica Llanos 5 (IRGC 117017) was used to conduct QTL analysis in this study. Way Rarem is an upland *indica* rice (*Oryza sativa* L.) variety which is popular in Indonesia (Suwarno et al. 2001) and Oryzica Llanos 5 is an upland *indica* rice and popular commercial variety that has excellent blast resistance properties from Colombia (Correa-Victoria et al. 2006). Way Rarem and Oryzica Llanos 5 were used as recurrent and donor parent, respectively.

Population development was carried out in a well-controlled greenhouse, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor, Indonesia. One hundred BC_1 seeds were obtained from backcross of F_1 to the recurrent parent, Way Rarem. Five seeds for each BC1 line which were used to produce the next generation (400 BC_2 seeds) were backcrossed again to the recurrent parent. Out of a total of 400 BC_2 seeds, 200 BC_2 seeds were randomly chosen, planted, and selfed to produce 200 BC_2F_2 families. For the QTL mapping study, a total of 123 BC_2F_2 lines were used, then these lines were selected randomly and also selfed to get BC_2F_3 and BC_2F_4 families (Suwarno et al. 2001).

Phenotypic evaluation

We used BC_2F_3 and BC_2F_4 progenies for phenotypic evaluation for blast resistance. Tested entries for phenotypic evaluation were conducted in greenhouse test, blast nursery test, and multilocation test. In the greenhouse test, the BC_2F_3 families were grown in tin trays (35 x 25 x 7cm^3) filled with a mixture of soil and farm yard manure (ratio of 1:1) inside the greenhouse, ICABIOGRAD, Indonesia. A total of eight Indonesia rice blast isolates (001, 033, 123, 133, 173, 04-223, 04-165, and 04-178) were used as inoculum treatments using a standard spore concentration (1 x 10^5 spore mL^{-1}) and applied to the 18-day-old seedlings by sprayer. Immediately after inoculation, seedlings in the trays were kept in a cage covered with water-soaked jute bags for 24 h in 90 - 100% of relative humidity and 25 - 26^{o}C to facilitate spore germination and penetration. After 72 h of inoculation, seedlings were transferred to growth chamber and maintained the well-grown conditions until disease evaluation. The population was inoculated in a randomized complete block design with three replications for 10 plants per line.

A blast nursery test was carried out in International Rice Research Institute (IRRI) using BC_2F_4 families in 2007. In this test, 30 to 40 seeds were sown in a row of 10 x 3 x 1 cm. Field experimental designs were performed during the wet season of 2003/2004 at disease hot-spots in Indonesia, i.e. Sukabumi, West Java and Tamanbogo, Lampung, Sumatra, using BC_2F_3 population. Both blast nursery and field experiments were laid out in randomized complete block designs with three replications. Field management activities mostly followed the normal agricultural practices. The percentage of disease leaf area (DLA) and lesion type (LT) scale ranging from 0 to 9 were estimated visually according to the standard evaluation system (SES) for rice (IRRI 1996). In the blast nursery test, blast disease was observed once a week beginning 14 days after sowing. However, only collected data at the 4th week were used for analysis. While in field testing, leaf blast incidence was observed at the plant stage showing 3 - 4 leaves (around 18 days after planting). Data were collected from 10 plants randomly chosen for each line.

DNA extraction, PCR amplification, and marker analysis

Leaf tissue was harvested from each 123 BC_2F_2 individual

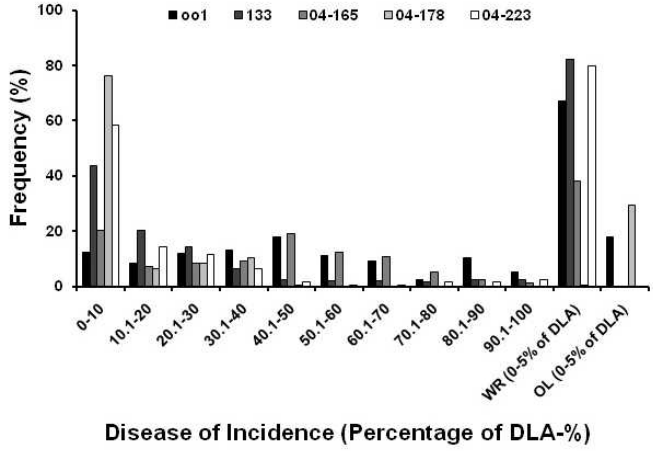

Fig. 1. The frequency distribution of leaf blast disease incidence to five rice blast isolates (001, 133, 04-165, 04-178, 04-223) on BC_2F_3 lines of Way Rarem//Oryzica Llanos 5 observed in greenhouse test, ICABIOGRAD, Indonesia in 2005. Disease incidence is represented by DLA (disease leaf area) in percentage. The DLA range of both parents, Way Rarem and Oryzica Llanos 5 are 0-5%.

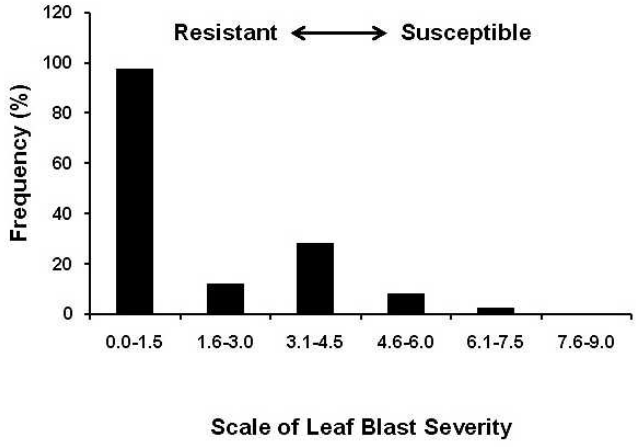

Fig. 2. The frequency distribution of lesion type scores of leaf blast severity on BC_2F_4 population of Way Rarem//Oryzica Llanos 5 observed at IRRI's blast nursery test in 2007. The score according to the standard evaluation system (SES) released by International Rice Research Institute (IRRI), ranging from 0 to 9. No lesion score of 7.6 to 9.0 detected on lines observed in this study (indicated by no bars on the scale score of 7.6 to 9.0.

grown in the greenhouse, ICABIOGRAD, and DNA was extracted using a chloroform-based DNA extraction protocol as described in McCouch et al. (1988). For molecular analysis, initially around 700 primers consisting of simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) primers searched in the available rice genomic database (www.gramene.org) were chosen and used to survey polymorphism between the two parental lines. On the basis of polymorphism analysis, a total of 112 primers comprising 110 SSR primers and two SNP primers showed polymorphism were selected and used in this study. Of the total SSR markers, 13 markers correspond to eight candidate defense response genes. The SSR primers were amplified according to the protocol from Chen et al. (1997).

The PCR (polymerase chain reaction) amplification reaction was conducted in a total volume of 20 μL consisting of 20 ng of genomic DNA, 2 μL of 10x buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 1 μL of 10 μM of each forward and reverse primer, 250 μM of each dNTP, and 1 U of *Taq* polymerase (Promega, Madison, Wis.). The reaction mixture was denatured at 94^{o}C for 4 min and subjected to 35 cycles of 94^{o}C for 45 sec, 55^{o}C for 45 sec and 72^{o}C for 45 sec, the final extension at 72^{o}C for 7 min on a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, Mass., USA). The PCR products were detected either on 2% agarose gels, on 6% non-denaturing polyacrylamide gels using ethidium bromide staining (model MGV, CBS Scientific Co.) (Wang et al. 2003) or on 4% denaturing polyacrylamide gels using silver staining following the manufacture's recommendation (Promega, Madison, WI, USA).

To detect SNPs, agarose-based EcoTILLING method was conducted according to Raghavan et al. (2007). Briefly, genomic DNA of individual BC_2F_2 line was combined with that of either parents and used as template for amplification of a candidate gene. Then, the PCR product was subjected to heteroduplex formation and digested with CEL 1 endonuclease. The digestion products were detected on 1.5% agarose. The absence of cleaved products indicates that the line carries the same allele as that of the parent was combined with, while the presence of cleaved products indicates the alternative allele. To identify heterozygous individuals, DNA of the BC_2F_2 was paired reciprocally with both parents to provide complementary data on the allelic state.

QTL analysis

QTLs was identified using single-point analysis (SPA), while the interval mapping (IM) was performed using QGene (Nelson 1997). For the IM analysis, 1-cM intervals, and the Kosambi function were chosen. The probability of a QTL for each interval was expressed as a LOD score using threshold of 3.0. The proportion of phenotypic variation explained by each QTL was calculated as a R^2 value.

Results

Phenotypic evaluation for blast resistance

Oryzica Llanos 5 showed more resistance to blast than Way Rarem as expected. In the greenhouse test, some lines demonstrated an opposite performance of leaf blast incidence in comparison with both parents. Only one isolate (04-178) did not infect Way Rarem, while two isolates (001 and 04-178) were virulent to Oryzica Llanos 5. The frequency of disease leaf area (DLA) to five blast pathogens on BC_2F_3 population of Way Rarem//Oryzica Llanos 5 in the greenhouse test is presented in Fig. 1. However, the frequency was not normal distribution detected in blast nursery test, showing around 70% of highly resistant lines. The frequency of leaf blast resistance on disease severity on BC_2F_4 population observed in blast nursery is presented in Fig. 2.

Table 1. QTLs for blast resistance detected on BC_2F_2 population of Way Rarem/Oryzica Llanos 5 based on all collected phenotypic data from field test, greenhouse test and blast nursery test

Trait	Marker	Chr#	LOD[a]	R^2 (%)	Favorable allele
IRRI	RM347	3	3.68	12.97	WR
IRRI	RM527	6	3.91	13.72	WR
IRRI	RM541	6	4.63	16.16	OL5
Lampung	RM149	8	3.17	11.46	WR
Sukabumi	RM8134	1	4.72	16.2	WR
Sukabumi	RM18082	5	3.97	13.93	WR
033	RM10825	1	3.53	12.96	WR
033	RM2	7	5.9	20.42	WR
033	RM149	8	4.9	17.15	WR
033	RM206	11	5.71	19.98	WR
123	RM14298	3	4.9	18.25	WR
123	RM148	3	3.27	11.69	OL5
123	RM2	7	4.66	16.51	WR
173	RM14298	3	4.17	16.15	WR
173	RM426	3	3.1	11.31	WR
173	RM342B	9	15.63	45.11	OL5

[a]Based on single point analysis implemented at QGene, Chr#:chromosome number
R^2: phenotypic variation

At the rice breeding screening site, Sukabumi, the reaction of breeding lines to rice blast incidence accumulated toward R (resistant) group, whereas the parents, Way Rarem and Oryzica Llanos 5 showed moderately resistant with 32.59 and 37.78% DLA, respectively. While in Lampung, the blast incidence on Way Rarem//Oryzica Llanos 5 population was to be more severe compared to Sukabumi, in contrast both parents showed more resistant than those in Sukabumi. Way Rarem was moderately resistant with DLA of 29.63% and Oryzica Llanos 5 was resistant with DLA of 8.15%. Both in Sukabumi dan Lampung, the DLA of lines did not show a normal distribution. Among 123 lines, 97.71 and 55.14% were arranged toward resistance in Sukabumi and Lampung, respectively. Some lines showed more resistant and more susceptible than both parents, indicating a transgressive segregation is arisen in advanced backcross population of Way Rarem//Oryzica Llanos 5. The rice blast incidence and the assortment of field blast resistance assessment on Way Rarem//Oryzica Llanos 5 progenies observed in Sukabumi dan Lampung are presented in Figs. 3 and 4, respectively.

QTL analysis and identification of superior blast QTL allele

A total of 110 SSR and two SNP markers were used to construct the linkage map, detecting 16 QTLs. Of total marker used, 15 markers showing significant linkage with blast resistance were detected, revealing nine markers associated with partial blast resistance to single blast pathogen. The variance value of these QTLs ranged from 11.31 to 45.11%. The map position of these QTLs is shown in Fig. 5. The detected QTLs and the variance of each QTL are shown in Table 1.

Resistance alleles were mostly contributed from the susceptible parent, Way Rarem. Two loci (RM8134 and RM18082) showed association with field blast resistance in Sukabumi. One locus, RM149 on chromosome 8, was associated with resistance

Table 2. Defense response gene detected on BC_2F_2 of Way Rarem//Oryzica Llanos 5

Candidate Gene	Primer corresponding to candidate defense response gene	Chromosome
Oxalate oxidase	RM 426	3
	RM 126 , RM 544 , RM547, RM331, RM260	8
EAP	RM 3826	7
Aldose reductase	RM3870	5
Thaumatin	RM 340	6
HSP 90	RM176	6
DHAP	RM1330	7
PR1b	RM2887	10
Probenazole	PR10	12

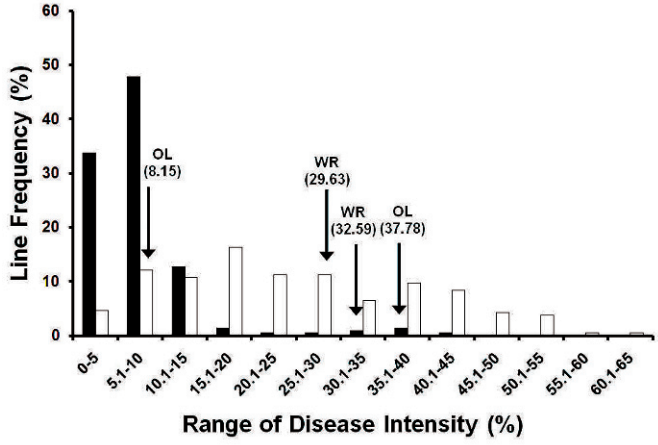

Fig. 3. The frequency distribution for field blast resistance on BC2F3 population of Way Rarem//Oryzica Llanos 5 based on leaf blast intensity during wet season of 2003/2004 in Sukabumi, West Java and Tamanbogo, Lampung. Disease intensity is represented in DLA (disease leaf area) in percentage. WR: Way Rarem, OL: Oryzica Llanos 5. In Lampung, the rate of disease intensity on Way Rarem and Oryzica Llanos-5 were 29.63 and 8.15% (indicated by arrows on blue bars), while in Sukabumi the DLA were about 32.59% for Way Rarem and 37.78% for Oryzica Llanos-5 (indicated by arrows on red bars).

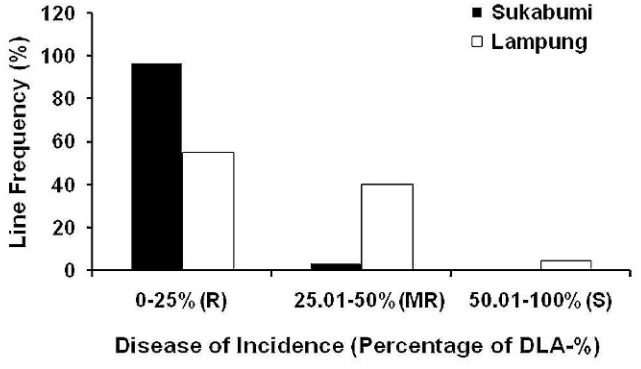

Fig. 4. Assortment of field blast resistance assessment based on leaf blast intensity on BC_2F_3 population of Way Rarem//Oryzica Llanos 5 in the screening sites, Sukabumi, West Java and Tamanbogo, Lampung during wet season of 2003/2004. Leaf blast intensity is represented by disease leaf area in percentage ranging from 0 to 100. R: resistant, MR: moderately resistant, S: susceptible.

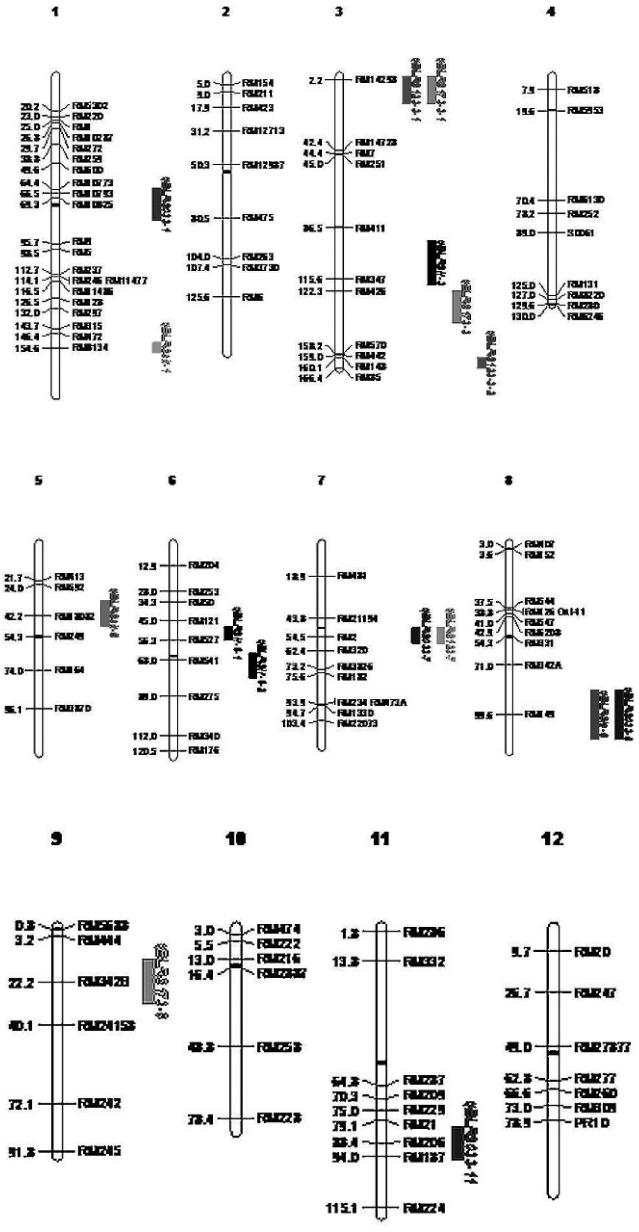

Fig. 5. Linkage map and QTL positions for blast resistance. All collected phenotypic data for blast evaluation in the greenhouse, field experiment (Sukabumi and Lampung) and blast nursery tests were included in the QTL analysis. The number on the left-hand side of each linkage group indicates map distance using Kosambi function. Bars highlighted with colors represent putative regions of QTLs for the disease score with a LOD value of more than 3.0 in an advanced backcross population of Way Rarem//Oryzica Llanos 5.

to isolate 033 and also for field blast resistance in Lampung, with Way Rarem parental line contributing the favorable allele. To check the favorable allele contributed by the parent, one QTL for blast resistance at blast nursery on chromosome 6 (*qBLRSir-6-1*) using eight individual lines belonging to a heterogeneous inbred family (#93) was verified. All four moderate lines (blast severity scale = 5) had Oryzica Llanos 5 allele, whereas the resistant lines (blast severity scale = 1) either have Way Rarem allele or heterozygous. This verifies that Way

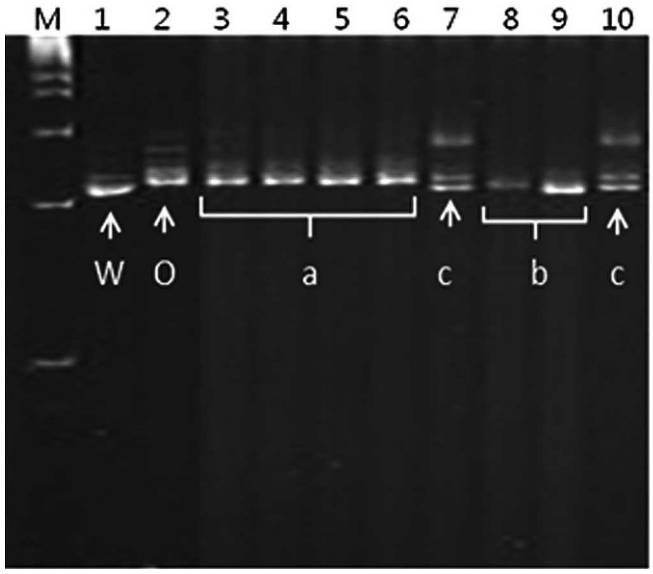

Fig. 6. Verification of one QTL for blast resistance blast nursery on chromosome 6 (*qBLRSir-6-1*) based on RM527 marker for blast resistance using eight individual lines belonging to a heterogeneous inbred family (#93). M. DNA ladder, 1. Way Rarem, 2.Oryzica Llanos 5, 3-10. Lines belong to HIF #9, 3-6. Lines with leaf blast severity scale of 5 (moderate) 7-10. Lines with leaf blast severity scale of 1 (resistant; W: allele of Way Rarem, O: allele of Oryzica Llanos 5, a: alleles belong to Oryzica Llanos 5, b: alleles belong to Way Rarem, c: alternative/heterozygous alleles.

Rarem contributes the favorable allele for blast resistance in this locus (RM527). Verification of QTL based on RM527 marker for blast resistance in blast nursery is presented in Fig. 6.

Identification of candidate defense response genes

Thirteen markers associated with candidate defense response genes showed polymorphism in both parents. Importantly, we detected five markers on chromosome 8 and one marker, (RM426) corresponding to oxalate oxidase on chromosome 3. Only single marker (RM426) contributed to partial resistance under greenhouse, especially resistance to single pathogen, isolate 173. Defense response gene detected in BC_2F_2 of Way Rarem//Oryzica Llanos 5 is listed in Table 2. Analysis of variance suggested that the marker of RM426 encoding oxalate oxidase individually contributed 11.31% of the variation in DLA in breeding lines of Way Rarem//Oryzica Llanos 5.

At the present time, we have been developing near isogenic lines (NILs) and simultaneously molecular analysis. Two lines with high blast resistance in the field trial of eight hot spots out of 10 rice central productions in Indonesia (recent progress, data not shown) were successfully selected. These two lines would be the improved new variety candidates that could be released. More multi-locations trial would be needed to get the fixed performance of the variety candidates.

Discussion

To improve upland varieties resistant to blast, here, we present a novel population originating from a cross between upland

indica rice varieties. Oryzica Llanos 5, a durable blast resistant variety, was developed by combining highly resistances from several cultivars with different sources to different genetic lineages of the pathogen (Lopez-Gerena 2006). Thus, Oryzica Llanos 5 might be an alternative genetic source for blast resistance by introgressing to Way Rarem.

In this study, using several condition tests to evaluate blast resistance with artificial inoculation and natural population are able to elucidate an insight into the genetic control and agronomic performance in this advance backcross population. The frequency distributions of DLA and LT scores were examined to determine their approximated normality. All isolates were virulent to the breeding lines, but showing isolate specificity, in which a differentiation of the race of rice blast corresponding to the varieties of rice has occured (Fig. 1). As expected, Oryzica Llanos 5 showed its blast resistance with less blast disease incidence (8.15%). It could be understood because Oryzica Llanos 5 contains major blast resistance genes, including 4-5 *Pi*-genes with major effect. Several QTL linked to blast resistance and 29 candidate resistance genes have also been detected on the progenies (Lopez-Gerena 2006). The different performance of blast incidence on both parents and the lines between Lampung and Sukabumi indicates that there is dependence of rice variety and segregated lines to blast race. Thus, the environment for assaying this disease builds a noticeable distinction in resistance to blast race.

These results also specify that diseases severity on susceptible lines has higher blast incidence then both of their parents. This condition indicates that the load of spore in the atmosphere during the investigation is high enough to cause serious blast incidence in susceptible plants. On the other hand, some lines performed good resistance to rice blast, revealing more prospective than both of their parents. These transgressive segregants might be produced by an interaction of favorable alleles from both of their parents. Specifically, it might be a response of epistatic effects between alleles and over dominance caused by heterozygosity at specific loci (Rieseberg 1999). Some isolates might carry factors that modify the interaction of their avirulence genes with major resistance genes (Hittalmani et al. 2000). Thus, the performance of the lines in this study challenged with pathogens is very intriguing.

QTL for blast resistance traits have been mapped to several loci (Sallaud et al. 2003; Tabien et al. 2002) including in upland rice populations (Lopez-Gerena 2006; Sato et al. 2006). Of a total of 16 QTLs identified in this study, two QTLs were already mapped in the same loci on the cross of Oryzica Llanos 5//Fanny (Lopez-Gerena 2006) and a cross of Shenshan 97//Minghui 63 (Li et al. 2008b). While other QTLs were recently detected in this study, they have not mapped in upland rice populations yet. The genes controlling these resistances might be allelic to those controlling the QTL in these other crosses.

A previous study reported that the genetic factors controlling blast field resistance of upland *japonica* varieties were located on chromosome 4 (Miyamoto et al. 2001), but it is not in a good agreement with this study. This study revealed that QTLs responsible for field blast resistance was found on chromosomes 1, 5, and 8. It indicates that QTLs responsible for blast resistance for *japonica* and *indica* upland rice is not located in the same chromosome. In this study, most of the QTLs appeared to be race-specific in their effects but it is possible some of the QTL with smaller effects are non-specific, which is consistent with the results of the population of Oryzica Llanos 5//Fanny (*indica//japonica*) (Lopez-Gerena 2006).

The accumulation of favorable alleles from both parents could be a reason for this positive skewed distribution. Oryzica Llanos 5 is considered to be the blast-resistance donor, however, the favorable alleles resistant to blast are largely Way Rarem alleles, the susceptible recurrent parent in the backcross scheme. It might be caused by the highly blast resistance ability of Way Rarem in those initially released in Indonesia. The beneficial alleles for blast resistance might still be present in Way Rarem. Accordingly, the large numbers of QTLs for blast resistance indicate that the durability of Oryzica Llanos 5 is due to many genes with variable effects (Lopez-Gerena 2006).

Only single candidate defense response genes, oxalate oxidase associated with resistance to single blast pathogen (isolate 173) were identified in this study. Oxalate oxidase might play an important roles in disease response, especially single blast pathogen isolate, PO6-6 (Wu et al. 2004), leaf rust, and tan spot in wheat (Faris et al. 1999), and powdery mildew fungus in barley (Zhang et al. 1995). Oxalate oxidase is likely to play a role in different disease resistance in several plant species (Wu et al. 2004). Thus, this study demonstrates the association between a QTL and a member of a multigene family, showing consistence with a previous report from Zhuang et al. (2002). Since we progressively selected lines with high blast resistance, further studies to elucidate the genetic aspect, such as identification of major genes, molecular mechanism, and effectiveness of blast resistance genes would be needed.

Acknowledgments

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