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IDENTIFICATION OF A MAJOR QUANTITATIVE TRAIT LOCUS CONFERRING RICE BLAST RESISTANCE USING RECOMBINANT INBRED LINES

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ABSTRACT

Blast disease caused by *Pyricularia oryzae* is one of the limiting factors for rice production world wide. The use of resistant varieties for managing blast disease is considered as the most eco-friendly approaches. However, their resistances may be broken down within a few years due to the appearance of new virulent blast races in the field. The objective of the present study was to identify the quantitative trait locus (QTL) conferring resistance to blast disease using 126 recombinant inbred (RI) lines originated from a crossing of a durably resistant upland rice genotype (Laka) and a highly susceptible rice accession cultivar (Kencana Bali). The RI population was developed through a single seed descent method from 1997 to 2004. Resistance of the RI lines was evaluated for blast in an endemic area of Sukabumi, West Java, in 2005. Disease intensity of the blast was examined following the standard evaluation system developed by the International Rice Research Institute (IRRI). At the same year the RI lines were analyzed with 134 DNA markers. Results of the study showed that one major QTL was found to be associated with blast resistance, and this QTL was located near RM2136 marker on the long arm of chromosome 11. This QTL explained 87% of the phenotypic variation with 37% additive effect. The map position of this QTL differed from that of a partial resistant gene, *Pi34*, identified previously on chromosome 11 in the Japanese durably resistant variety, Chubu 32. The QTL, however, was almost at the same position as that of the multiple allele-resistant gene, *Pik*. Therefore, an allelic test should be conducted to clarify the allelic relationship between QTL identified in this study and the *Pik*. The RI lines are the permanent segregating population that could be very useful for analysing phenotypic variations of important agronomic traits possibly owned by the RI lines. The major QTL identified in this study could be used as a genetic resource in improvement of rice varieties for blast resistance in Indonesia.

[***Keywords***: *Oryza sativa*, *Pyricularia oryzae*, disease resistance, quantitative trait locus, recombinant inbred lines]

INTRODUCTION

Rice is the major staple food of nearly half of the world population, and more than 90% of this crop is grown in developing countries, where food supply is often shortage. With the forces of population growth and economic expansion, particularly in Asia, world rice productions are expected to increase by 1.7% annually between 1990 and 2025. With less land available to expand rice-growing areas and with competing demands from urbanization and industrialization on existing rice lands and irrigation water, rice production might be only reasonable to be increased through intensification of the existing lands. Consequently, more fertilizer, such as nitrogen will be required and less water be available per ton of rice produced. These can trigger development of serious disease, especially rice blast caused by *Pyricularia oryzae*.

Blast is one of the most explosive and potentially damaging diseases of the rice crop world wide. The disease often causes a significant yield loss, as high as 70-80% during an epidemic (Ou 1985). In Indonesia, the blast incidences reached 1.28 million ha or 12% of the total Indonesian rice growing area (Utami *et al*. 2005). This disease has been managed by combining applications such as host plant resistance, cultural practices, and fungicides. The use of resistant cultivars is considered as the most eco-friendly approach for managing blast disease. However, their resistances may be broken down within a few years due to the appearance of new virulent blast races in the field. Consequently, breeding for rice varieties with durable resistance to blast is a main concern in rice improvement programs.

Recent advances in genome mapping have resulted in high-density molecular marker linkage maps in many crop species including rice (Causse *et al*. 1994; Kurata *et al*. 1994; Harushima *et al*. 1998; McCouch *et al*. 2002). Recent studies demonstrated that genetic linkage maps constructed with various DNA markers are very useful for the analysis and detection not only of qualitative trait loci (Kubo and Yoshimura 2005) but also of quantitative trait loci (QTLs) (Sallaud

et al. 2003). Since most of the maps were independently constructed using different segregating populations, it is dificult to directly integrate the maps and accumulate new information. To minimize the difficulties, Tsunematsu *et al*. (1996) and Cho *et al*. (1998) attempted to integrate several maps using a permanent mapping population, recombinant inbred (RI) lines.

The use of RI lines also provides several advantages for genetic analysis of quantitative traits. Since the RI lines are developed through inbreeding several times after a single cross, they are genetically homozygous and can be multiplied without further segregation. Therefore, they can be replicated and used over years with identical genotypes. The error of measurement of the traits can be minimized since a genotype was expressed by a line, rather than by an individual. Using the selected parental combination in development of RI lines and subsequent QTL analysis offers opportunities for direct breeding program. Marker-assisted breeding, guided by the QTL analysis could feasibly be used to efficiently produce new varieties from the experimental population.

The objective of the present study was to identify the QTL conferring resistance to blast disease in durably resistant rice variety. For this purpose, the RI lines were derived from an Indonesian landraces of an upland indica rice, i.e. Laka crossed with a tropical japonica rice, i.e. Kencana Bali. Laka is resistant to various Indonesian races of *P. oryzae*, while Kencana Bali is highly susceptible one (Sobrizal *et al*. 2002a). Using these lines, a DNA marker linkage map was constructed and the QTL conferring resistance to blast was identified. The map and the identified QTL can be expected to provide a reasonable starting point for high resolution QTL analysis, gene isolation, and molecular breeding.

MATERIALS AND METHODS

Preparation of Plant Materials

Parental lines, i.e. Laka and Kencana Bali were grown in the experimental farm of the Center for the Application of Isotopes and Radiation Technology, the National Nuclear Energy Agency (CAIRT-NNEA), Jakarta. The two rice genotypes were selected as parents because of their differences in blast resistances as well as their DNA polymorphisms (Sobrizal *et al*. 2002a). To analyze the blast resistance trait, 126 RI lines were developed by a single seed descent (SSD) method through inbreeding of the progeny of an F2 population derived from a cross of these two genotypes. In this study, the RI lines of F10 were used in the construction of DNA marker linkage map and for QTL analysis of blast disease. The RI lines were developed at the experimental farm of CAIRT-NNEA from 1997 to 2004.

Field Evaluation of Blast Disease

A total of 126 RI lines as well as their parents were evaluated for resistance to blast by natural infection at the experimental field of a blast endemic area in Sukabumi, West Java, during the wet season in January-April 2005. Disease infection was scored according to the standard evaluation system (IRRI 1996) in10 plants per line at 3, 4, 5, and 6 weeks after sowing. The obtained scores were converted to a percentage of disease severity calculated using the following formula: $S = \sum (n \times V) / (N \times V) \times 100\%$, where S = disease severity, n = number of plants with certain score, N = number of observed plants, and V = the highest scale of scoring.

Linkage Map Construction and QTL Analysis

Genomic DNA was extracted from a bulked sample of fresh leaves of 8 two-week-old seedlings per line by alkali method. A bulked sample was placed on a sterile 1.5 ml tube containing 100 µl of 0.25 N NaOH. After grinding, the sample was added by 400 µl of 100 mM Tris-HCl pH 7.5, and the mixture was mixed roughly. The tube was then centrifuged at 10,000 rpm for 10 minutes. Finally, 200 µl of supernatant containing crude DNA was removed to a new sterile tube and was used for PCR amplification.

A total of 453 DNA markers were used in this study consisted of 452 SSR markers and one insertion-deletion (in-del) marker on chromosome 9, AP6235. All SSR markers were developed by McCouch *et al*. (2002) and in-del marker, AP6235, developed in this study using the rice sequence database (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl). Primer pairs were designed using PRIMER 0.5 (http://www-genome.wi.mit.edu/ftp/pub/software/primer 05).

For PCR amplification, 5 µl of extracted DNA was used as a DNA template. The amplification reactions were performed in a total volume of 15 µl, containing 20 pmol of each primer, 0.03 U/µl of Taq polymerase (promega), 1 x buffer mix (gotaq), 40 mM of dNTPs, 1.5 µl of 0.15% bromophenol blue, and DNA tamplate.

Reaction conditions were initial denaturation for 5 minutes at 94°C, followed by 35 cycles at 95°C for10 seconds, 55°C for 20 seconds and 72°C for 20 seconds. Separation of PCR products was carried out on a 4% agarose gel and stained with ethidium bromide. Bands were visualized by typhoon 9410 scanner.

A total of 134 DNA markers (133 SSR + 1 in-del) showing polymorphism between Laka and Kencana Bali were used for genotyping of RI lines. Segregating markers were scored as either "A" (Kencana Bali homozygote) or "B" (Laka homozygote). Residual heterozygote and the absence of data were scored as "-" (missing). The X^2 test was performed to examine goodness of fit of the frequencies of the Kencana Bali or Laka alleles for each marker against expectation from Mendelian segregation. The null hypothesis of the test was that progenies segregated in a 1:1 ratio of which the alleles were derived from the Kencana Bali and Laka, respectively. Linkage map was constructed using the computer program of MAPMAKER/EXP 3.0 (Lander *et al*. 1987) at a minimum log of likelihood (LOD)-value of 3.0 as the threshold for grouping the genetic markers.

Percentages of disease severities at 6 weeks after sowing were used for QTL analysis. The presence of QTLs and their effects were calculated by interval mapping (IM) (Lander and Botstein 1989) and composite interval mapping (CIM) analyses (Zeng 1993). Both IM and CIM methods were applied using QTL Cartographer package v. 2.0 (Basten *et al*. 2001). A minimum LOD-value of 2.5 was selected to confirm the presence of a QTL in a given genomic region. When the peak was detected only by IM analysis, we considered this to be false QTL and it was not located on the linkage map. The LOD-value peak detected by both IM and CIM analyses or by CIM only was used to estimate the most likely QTL positions on the linkage map.

RESULTS AND DISCUSSION

Polymorphism Survey

Out of 453 DNA markers randomly selected across the 12 rice chromosomes, 165 of which showed polymorphism between Laka and Kencana Bali. The frequency of polymorphic markers was 36.0% distributed accross 12 chromosomes ranging from 22.4% to 50.0% (Table 1). This value is much lower compared to those of indica/japonica, Milyang 23/Akihikari (71.9%) (Fukuta *et al*. 2000) and Moroberekan/CO39 (68.3%) (Wang *et al*. 1994).

DNA Marker Linkage Map

A total of 134 clearly polymorphic markers were used in segregation analysis of the 126 RI lines. The genotypes at markers were scored for each line based on the banding patterns. Based on these data, the genetic linkage map was constructed as shown in Figure 1. The map covers all 12 rice chromosomes with a total map distance of 1313.8 cM. The order of markers in each linkage group mostly corresponded to that of previous map reported by McCouch *et al*. (2002). Due to lack of polymorphism, no marker was mapped on long arm of chromosome 6, 10, and 11 with total map distances of 39.2, 39.1, and 33.6 cM,

Table 1. Frequency of DNA marker polymorphism between Kencana Bali and Laka rice genotypes.

Chromosome	Number of markers showing		Total	Polymorphism (%)
	Polymorphism	Non-polymorphism		
1	24	24	48	50.0
2	20	29	49	40.8
3	21	26	47	50.0
4	16	20	36	44.0
5	11	34	45	24.4
6	11	38	49	22.4
7	11	32	43	25.6
8	14	31	45	31.1
9	10	10	20	50.0
10	9	17	26	34.6
11	8	16	24	33.3
12	10	11	21	47.6
Total	165	288	453	36.0

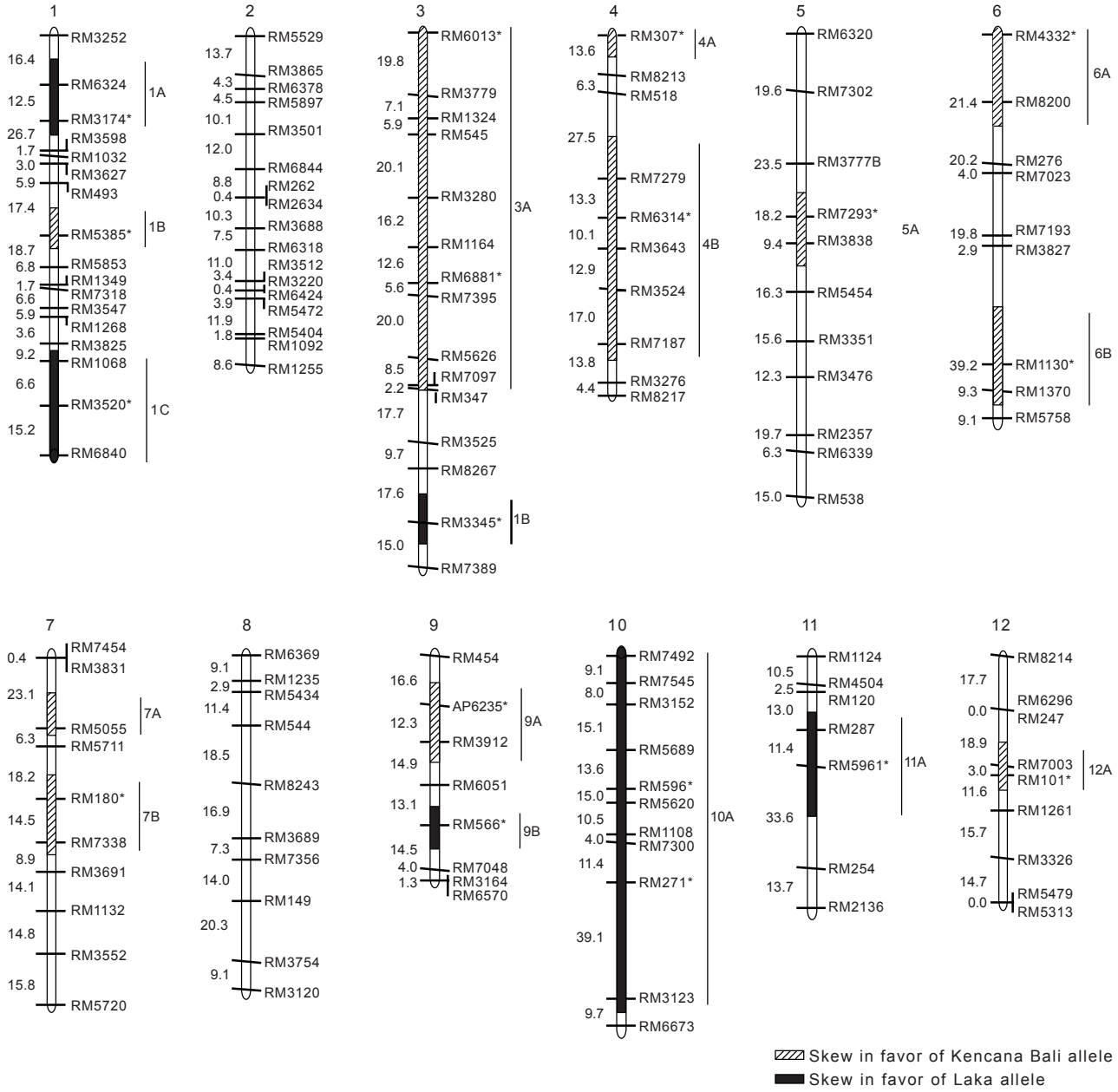

Fig. 1. DNA marker linkage maps of recombinant inbred lines originated from crossing of Kencana Bali and Laka rice genotypes and the chromosomal regions showing segregation distortions.

respectively. The segregation data for all of the markers could be very useful for analysis of the phenotypic variations observed in many traits owned by the RI lines. In this study, using the segregation data for all mapped markers and for rice blast incidence, we were able to identify a major QTL conferring blast resistance [Go to QTL analysis].

Segregation distortions were observed at 17 regions of chromosomes 1, 3, 4, 5, 6, 7, 9, 10, 11, and 12 (Fig. 1; Table 2). The segregation distortion is common in the progeny of wide crosses of rice. Zhao *et al*. (2006) reported that the segregation distortion in F2 population of indica/japonica, might be caused by gametophyte loci and sterility loci, since most gametophyte loci and sterility loci were mapped in segregation distortion regions. The reasons of distortions were speculated as follows.

Three gametophyte genes, *ga7* (Maekawa 1982), *ga8* (Nakagahra 1981), *ga9* (Maekawa and Kita 1985), and three sterility genes, *msm18* (Suh *et al*. 1989), *S13* (Sano 1994), and *S16* (Ikehashi and Wan 1996) have been reported to be located on chromosome 1. Two gametophyte genes of *ga2* (Nakagahra 1972; Sobrizal *et al*. 1996) and *ga3* (Nakagahra 1972) and

Table 2. Segregation distortion of SSR markers in recombinant inbred (RI) lines derived from a cross of Laka and Kencana Bali rice genotypes.

Marker	Chromosomal region	KB/KB	LK/LK	Total	X^2 (1:1)[1)]	p
RM6324	1A	45	80	125	9.25	2.4×10^{-3}
RM3174[1]	1A	42	82	124	12.27	5.0×10^{-4}
RM5853[1]	1B	83	43	126	12.07	5.0×10^{-4}
RM1068	1C	48	77	125	6.27	1.2×10^{-2}
RM3520[1]	1C	39	84	122	15.74	7.3×10^{-5}
RM6840	1C	47	77	124	6.78	9.0×10^{-3}
RM6013[1]	3A	92	34	126	25.79	3.8×10^{-7}
RM3779	3A	82	40	122	13.78	2.0×10^{-4}
RM1324	3A	81	44	125	10.37	1.0×10^{-3}
RM545	3A	81	42	123	11.74	6.0×10^{-4}
RM3280	3A	86	40	126	16.07	6.1×10^{-5}
RM1164	3A	88	38	126	19.06	1.3×10^{-5}
RM6881[1]	3A	93	33	126	27.63	1.5×10^{-7}
RM7395	3A	83	39	122	15.16	9.9×10^{-5}
RM5626	3A	78	45	123	8.33	4.0×10^{-3}
RM3346[1]	3B	41	83	124	13.56	2.0×10^{-4}
RM307[1]	4A	50	74	124	4.27	3.9×10^{-2}
RM7279	4B	48	78	126	6.67	9.8×10^{-3}
RM6314[1]	4B	22	104	126	52.07	5.4×10^{-3}
RM6343	4B	35	91	126	24.01	9.6×10^{-7}
RM3524	4B	41	84	125	14.11	1.0×10^{-4}
RM7187	4B	46	79	125	8.19	4.0×10^{-3}
RM7293[1]	5A	80	44	124	9.88	2.0×10^{-3}
RM3838	5A	73	47	120	5.21	2.0×10^{-2}
RM4332[1]	6A	83	41	124	13.56	2.0×10^{-4}
RM8200	6A	77	47	124	6.78	9.0×10^{-3}
RM1130[1]	6B	87	36	123	20.33	6.5×10^{-6}
RM1370	6B	74	49	123	4.68	3.0×10^{-5}
RM5055[1]	7A	75	51	126	4.20	4.0×10^{-2}
RM180[1]	7B	86	39	125	16.93	4.0×10^{-5}
RM7388	7B	80	44	124	9.88	2.0×10^{-3}
AP6235[1]	9A	75	50	125	4.61	3.0×10^{-2}
RM3912	9A	75	51	126	4.20	4.0×10^{-2}
RM566[1]	9B	50	74	124	4.27	4.0×10^{-2}
RM7492	10A	49	76	125	5.41	2.0×10^{-2}
RM7545	10A	42	82	124	12.27	5.0×10^{-4}
RM3152	10A	32	93	125	28.80	8.0×10^{-8}
RM5689	10A	37	88	125	20.00	7.7×10^{-6}
RM596	10A	34	92	126	25.79	3.8×10^{-7}
RM5620[1]	10A	24	101	125	46.21	1.0×10^{-11}
RM1108	10A	39	87	126	17.53	2.8×10^{-5}
RM7300	10A	37	88	125	20.00	7.7×10^{-6}
RM271[1]	10A	26	98	124	40.65	1.8×10^{-10}
RM3123	10A	50	75	125	4.61	3.0×10^{-2}
RM278	11A	49	76	125	5.41	2.0×10^{-2}
RM5961[1]	11A	45	77	125	7.88	5.0×10^{-3}
RM7003	12A	74	50	124	4.27	4.0×10^{-2}
RM101[1]	12A	76	46	122	6.89	8.7×10^{-3}

KB/KB = homozygous for Kencana Bali alleles.

LK/LK = homozygous for Laka alleles.

[1]The peak of distortion.

[1)]The null hypothesis of the tests was that progeny segregated in a 1:1 ratio.

three sterility genes, *ms7* (Ko and Yamagata 1987), *Sc* (Zang and Lu 1996), and *S19* (Taguchi *et al*. 1999) were located on chromosome 3. Three gametophyte genes, *ga6* (Mori *et al*. 1973), *ga10* (Kinoshita and Takemure 1984), and *ga12* (Maekawa and Inukai 1992), and three sterility genes, *s c2* (Oka 1957), *s e2* (Oka 1974), and *S28* (Sobrizal *et al*. 2002b) were located on chromosome 4. Two sterility genes, *ms14* (Ko and Yamagata 1987) and *S24* (Kubo *et al*. 2000) were located on chromosome 5. Three gametophyte genes, *ga1* (Iwata *et al*. 1964), *ga4* (Mori *et al*. 1973), and *ga5* (Mori *et al*. 1973), and thirteen sterility genes, *ms1* (Hara 1946), *ms9* (Ko and Yamagata 1987), *S A1* (Oka 1978), *S B2* (Oka 1978), *s a1* (Oka 1953), *s c1* (Oka 1957), *s d1* (Oka 1974), *S1* (Sano 1990), *S5* (Ikehashi and Araki 1986), *S6* (Sano 1989), *S8* (Ikehashi and Wan 1996), *S10* (Sano *et al*. 1994), and *S26* (Kubo and Yoshimura 2001) were located on chromosome 6. One gametophyte gene, *ga11* (Lin and Ikehashi 1993) and five sterility genes, *ms8* (Ko and Yamagata 1987), *S7* (Ikehashi and Wan 1996), *S20* (Doi *et al*. 1999), *S21* (Doi *et al*. 1999), and *S23* (Sobrizal *et al*. 2000), were located on chromosome 7. One sterility gene, *ms10* (Ko and Yamagata 1987) was located on chromosome 9. One sterility gene, *S18* (Doi *et al*. 1998) was located on chromosome 10. One gametic lethal gene, *gal(lt)* (Tomita *et al*. 1989) and one sterility gene, *S11* (Sano 1993) were located on chromosome 11. Finally, gametophyte gene *ga13* (Rha *et al*. 1994) and two sterility genes, *S15* (Ikehashi and Wan 1996) and *S25* (Kubo *et al*. 2001) were found to be located on chromosome 12.

Segregation of Blast Resistance in RI Lines

For assessment of blast resistance, the RI lines as well as their parents (Laka and Kencana Bali) were grown in field of endemic blast area in Sukabumi, West Java, during the wet season of 2005. Natural infection of the leaf blast was appeared 3 weeks after sowing and the disease developed rapidly within 4-6 weeks after that. At 5 weeks after sowing, disease severity on the susceptible parent, Kencana Bali, reached 100%, but on the resistant parent, Laka, was less than 10% (Fig. 2 and 3). A wide phenotypic distribution of resistance with two peaks was observed in the RI lines. The distribution at 4, 5, and 6 weeks after sowing showed the same tendency (Fig. 2). This result strongly suggests that major gene(s) govern(s) the segregation.

Blast resistance in rice is generally classified into two types, complete and partial resistances (Bonman and Mackill 1988). Complete resistance is qualitative and characterized by prevention of blast fungus reproduction in compatible combinations of the host

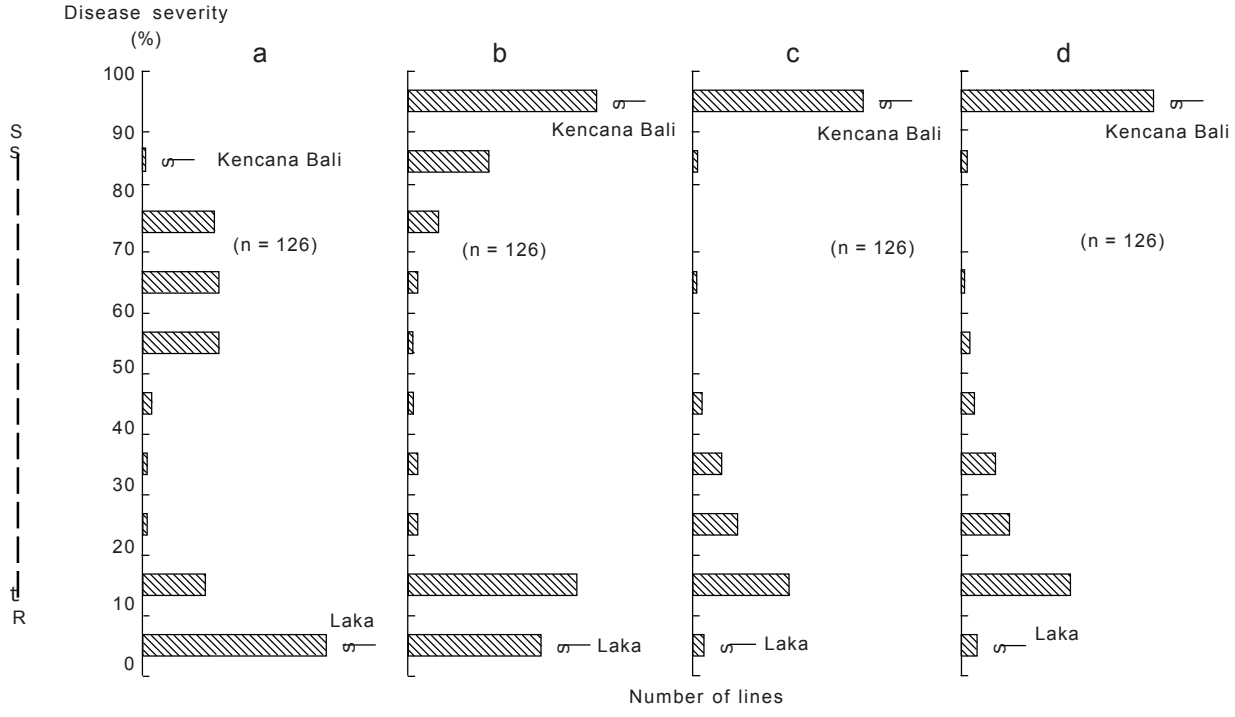

Fig. 2. Distribution frequency of disease severity of recombinant inbred lines derived from crossing of Laka and Kencana Bali rice genotypes at 3 weeks (a), 4 weeks (b), 5 weeks (c), and 6 (d) weeks after sowing.

Fig. 3. Performance of blast disease resistance in the parental genotypes, Laka and Kencana Bali (A), and segregation for blast resistance in the recombinant inbred (RI) lines (B). Arrows point small disease lesions found in the resistant RI lines.

and pathogenic races. It is usually controlled by a single gene. Partial resistance, on the other hand, reduces the extent of pathogen reproduction in the compatible interaction (Jonson 1983). Most of partial resistance is non-race specific, quantitative and polygenic (Maruyama *et al*. 1983; Higashi 1995). However, there are some exceptions such as *Pif* (Toriyama *et al*. 1968), *Pb1*(t) (Fujii *et al*. 1999) and a partial gene in Chubu 32 (Zenbayashi *et al*. 2002), which are single genes conferring partial resistance to blast. In this experiment, compatible interaction between pathogen and resistant lines was occurred as marked by small lesions in resistant lines (Fig. 3) suggested that the resistance in these lines might be a partial resistance.

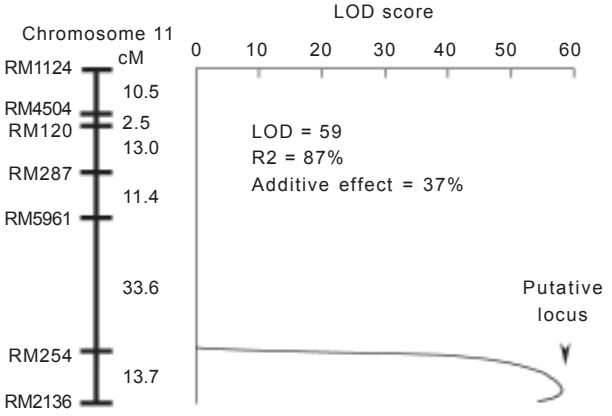

Fig. 4. The likelihood location of a major rice blast resistance QTL detected on the long arm of chromosome 11.

QTL Analysis

To locate the QTL(s) associated with partial resistance on a linkage map, interval mapping was performed using the phenotypic data of 5 weeks after sowing and analyzed with a computer software of MAP MAKER/QTL. The result showed that only a major QTL detected near the SSR marker *RM2136* on chromosome 11 (Fig. 4). This QTL explained 87% of the phenotypic variation having additive effect of 37%. Since this QTL was estimated as a partial resistance based on performance of the RI lines against rice blast incidence in the field, it is obligatory to confirm their reaction by artificial inoculation with various blast fungal races.

In the previous study, a partial resistance QTL was identified on the long arm of chromosome 11 (Zenbayashi *et al*. 2002). However, our newly identified QTL reported here is located at different chromosomal region, of chromosome 11. Moreover, Fjellstrom *et al*. (2004) reported that the multiple alleles of resistance gene, *Pik,* were located on chromosome 11, and such locus located near the QTL identified in this study. An allelic test, therefore, should be conducted to clarify the allelic relationship between the gene and QTL.

The nearest marker to the QTL is RM 2136 (Fig. 5). Most of the resistant lines carried Laka alleles at this locus, while the susceptible lines carried Kencana Bali alleles (Fig. 5). This result confirmed that the source of resistance in this population is originated from Laka genotype.

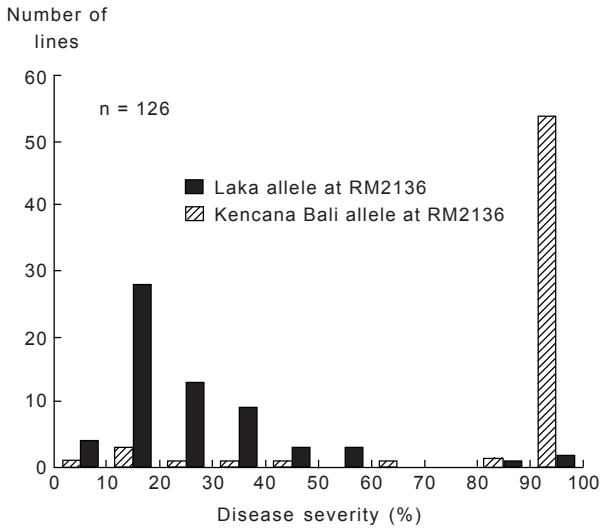

Fig. 5. The relationship between disease severity and recombinant inbred line (RIL) genotypes at the SSR marker locus RM2136.

CONCLUSION

A major QTL conferring rice blast resistance was identified in RI lines of crossing between the resistant rice genotype Laka and susceptible genotype Kencana Bali. The QTL was located near SSR marker of *RM 2136* at the end of long arm of chromosome 11. The QTL explains 87% of phenotypic variation with 37% of additive effects. This finding provides a new genetic resource for blast resistance improvement of rice varieties. Information on the closely linkage markers should be very useful for transferring resistance genes in a breeding program.

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