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Marker Assisted Selection for Bacterial Leaf Blight Rice Mutant Lines Resistant

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Abstract

Induction of mutation using gamma rays for improving of Mira-1 rice variety has been conducted. Rice mutant lines M2 generation have been obtained from mutation by the doses of 25, 50, 75, 100, 150 and 200 Gy of gamma rays. Selection of mutant lines tolerant to the disease was only observed in the field neither genetically. Marker assisted selection is a tool to obtain a new rice variety tolerant to the disease of bacterial leaf blight (BLB) genetically. *Xanthomonas oryzae* pv.*oryzae* (*Xa*) was the pathogen of BLB, and the identification of rice mutant lines which were containing of *Xa5*, Xa13 and *Xa21* genes have been done using Polymerase Chain Reaction (PCR) method. The result showed that one mutant line, and four mutant lines from mutation by the doses of 25 Gy and 150 Gy were containing *Xa5*, *Xa13* and *Xa21* genes the same as that of Code rice variety as positive control, and none in Kencana Bali rice variety as negative control. Mira-1 rice variety as the parent plant was only contains *Xa5* and *Xa21* genes. The doses of 50 Gy and 100 Gy were very affective on removing of all bands for identification of those genes. The purpose of this research was to obtain the mutant lines which were contain of those Xa genes as indicator for resistant to BLB disease genetically.

Keywords: bacterial leaf blight (BLB), marker assisted selection, PCR, *Xa*, rice mutant lines

Introduction

Indonesia is the 5th biggest country in the world, for populations of 250 million, the country needs 35.5 million ton rice per year. According to Central Bureau of Statistics, in the middle of 2013, Indonesia rice production was only 22.897 million ton, this figure is far below the Agriculture Ministry's target. The problem is not limited due to an imbalance of supply and demand, and the changing of agriculture area to non agriculture use, but also losses of grain yield by infection of diseases, pest and other causes.

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv.*oryzae (Xa)* is one of the major biotic destructive diseases throughout the world (Zhang and Wang 2013). The disease is known to occur in epidemic proportions in many parts of the world including Indonesia. Indonesia is a tropical region with high humidity which causes the disease develop easily. The peak of BLB disease attack to rice occurred in 2006 where 74,243 ha of rice damaged (Suryadi *et al.*, 2012). Crop loss assessment studies have revealed that this disease reduces grain yield to varying levels, depending on the stage of the crop. The degree of cultivar susceptibility to great extent, the conduciveness of the environment in which it occurs. Reported by Herlina and Silitonga (2011), showed that 30–40% decrease of rice production by this pathogen.

To develop a new rice variety tolerant against the disease, crossing between two parents which contain resistance to BLB have been conducted by some researchers, i.e, the presence of disease resistance genes in rice, has been developed by crossbreeding (Baehaki, 2012). Another tool to develop of rice tolerant to pathogen was by gamma

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rays. Gamma rays is physical mutagen which produce of free radicals from radiolysis of water, it will break of linkage genes and produce varies of plants genetically. The rice mutant traits were low amylose content, tillering ability, dwarf plants, and resistance to the pathogen.

Tanaka *et al.* (2010) mentioned that ionizing radiation could generate many kind of phenotypes, because it induces DNA damage relatively randomly, and therefore, induces a series of mutation. National Nuclear Energy Agency (NNEA/BATAN) has 20 rice varieties released since 1982, and the varieties should be resistant to diseases. All of new rice varieties were mutated by gamma rays. One of BATAN rice variety is Mira-1 which was released in 2006, this variety has less tasty when cooked, moderately resistant to Brown Plant Hopper (BPH) and Bacterial Leaf Blight (BLB) disease. For this reason, it is necessary to improve the Mira-1 plant traits. One of the steps to get a new rice variety is a selection. Selection of mutant lines based on genetic information is very valuable beside observation in the field. Marker assisted selection is a tool to find the mutant lines contains *Xhantomonas oryzae* (*Xa*) genes as an indicator of resistance to BLB.

The development of molecular markers diagnostic for the selection of resistant genes is the goal of many rice breeding programs. Several of the major resistance genes to the bacterial leaf blight (BLB) pathogen, *Xanthomonas oryzae* pv.*oryzae* have been tagged with restriction fragment length polymorphism (RFLP) or Simple Sequence Repeat (SSR) markers (Lukman *et al.*, 2013, Singh *et al.*, 2015). However, information of studies on molecular marker to differentiate between mutants and their parent plant are very limited.

The purpose of this research is to obtain rice mutant lines containing the *Xanthomonas oryzae* (*Xa5*, *Xa13* and *Xa21*) genes as an indication of BLB resistance.

Material and Methods

Plant Materials. M2 plant mutant lines from Citayam-Indonesia rice field was used for this experiment. Mutant lines obtained by irradiation of 200 rice plants at stage of 10 days before anthesis. Gamma rays doses used were 25, 50, 75, 100, 150 and 200 Gy. The selected numbers of mutant lines were analyzed based on the agronomic trait in the field and seeds number per panicle. A number of individual plants of 30 mutant lines for each dose were used for the analysis.

Plant genomic DNA extraction. To obtain of DNA mutant lines, leaves were extracted by mini prepraparation using CTAB method, and DNA diluted in 50 µl of 1xTAE.

Polymerase Chain Reaction (PCR). PCR reactions were performed of 25 ul reaction volume consisting, 2.5 ul 10x buffer, 1.5 ul of 25 mM MgCl2, 5 ul 5Q, 1UL of 10 mM dNTP mix, 0.75 ul of primer R, 0.75 ul of primer F, 0.25 ul of Taq polymerase enzyme 5U/ul, 8.25 ul of DEPC and 5 ul of rice DNA. PCR reaction was carried out with the conditions of (i) denaturation of 5 min at 94°C, (ii) denaturation during 1 minute at 94°C, annealed 1 minute at the temperature of 55°C, extension 72°C for 2 minutes, extended extension 72 °C for 7 minutes. The number of cycles were 40 cycles, PCR products were

Table 1. List of bacterial leaf blight resistance genes and sequence of primers

No	*Gene*	*Chromosome locus*	**Primer Sequence**
1	*Xa5*	5	**F:**AGACGCGGAAGGGTGGTTCCCGGA **R:**AGACGCGGTAATCGAAGATGAAA
2	*Xa13*	8	**F:**GGCCATGGCTCAGTGTTTAT **R:**GAGCTCCAGCTCTCCAAATG
3	*Xa21*	11	**F:**TCCAACATGGCAAGAGAGAG **R:**GGTGGCATTCGATTCCAG

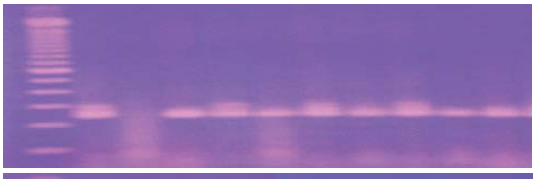
separated on 1.5% agarose by using of 100 bp DNA leader. The bacterial leaf blight resistance genes and sequence of primers is shown in Table 1.

Result and Discussion

It is expected that gamma – rays mutagenesis could cause many variations in the disease responses of rice plants. Mutations are changes in the genetic material, and various types of chromosomal DNA alteration in plants such as deletion, substitution, point mutation, and inversion could be occurred (Shu *et al.*, 2011). Genetic diversity is the basis for selection in order to obtain superior alleles in rice mutant plants with disease-resistant properties (Ishak, 2012). Various M2 plants of mutant lines have been observed in the field and genetically analyzed for the bacterial leaf blight resistance gene *Xa5* on the each dose of mutation induction is in Table 2.

The result displayed the only the numbers of mutant lines with positive and some of negative bands of *Xa* gene. *Xa5* is an important race-specific recessive gene in rice breeding due to its broad resistance spectrum to most Xoo strains.The results were shown that all mutant lines carry resistance alleles for *Xa5* by the doses of 25 Gy, 150 Gy and 200 Gy, equal to parent plant pattern and Code variety of the

Table 2. Pattern amplification of rice mutant lines by using *Xa5* gene

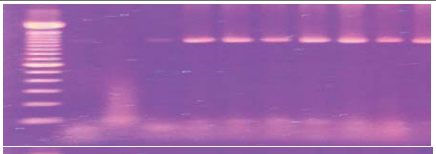
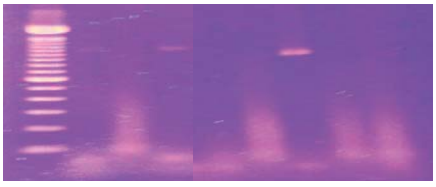
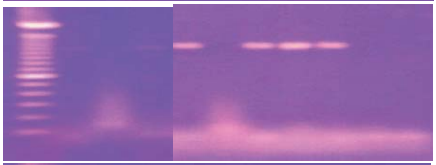
No.	Dose (Gy)	Mutant lines code	Agarose gel electrophoresis pattern
1	25	M, Cd, KB, Mira, 2, 3, 4, 6, 7, 8, 9	
2	50	M, Cd, KB, Mira, 15, 16, 18, 19, 26, 30, 32, 34	
3	75	M, Cd, KB, Mira, 35, 36, 37, 38, 39	
4	100	M, Cd, KB, Mira, 41, 43, 44, 46, 48, 50, 51	
5	150	M, Cd, KB, Mira, 52, 54, 57, 58, 60, 64, 67	
6	200	M, Cd, KB, Mira, 68, 70, 71, 72, 73, 74	

positive control. Mira-1 has known contains *Xa5* and must be maintained in the rice mutant lines. However, by the doses of 50 Gy and 100 Gy, only 2 mutant lines carry resistance alleles of *Xa5* respectively, and by the dose of 75 Gy were smear band appeared even though analysis was done three times. In comparing Mira-1 as their parent plant, it is shown that mutation by the dose of 100 Gy affected on more disappearing of *Xa5* containing in mutant lines than other doses. From thirty four lines, twenty lines of them along with resistant checks amplified 219 bp size fragments indicating the presence of xa5 (Singh *et al.*, 2015).

The *Xa5* is a novel disease R gene, provides immunity to races of *Xhantomonas oryzae* pv.*oryzae*. The positional cloning identifies of this gene in an 8.1 kb region region of TFIIAγ in the subtelomeric region of chromosome 5, which probably confer resistance by modulating activation of other TALE targets (Leung *et al.*, 2015). FIIAγ is very essential for cell growth and has been shown to have several roles in transcription, including stimulation and stabilization of the interaction between the TATA-box binding protein, promoter selection, gene-specific regulation, and activator-dependent transcription.

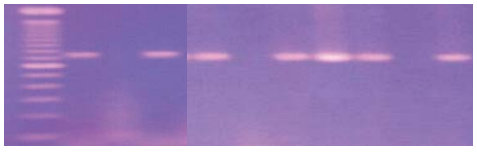
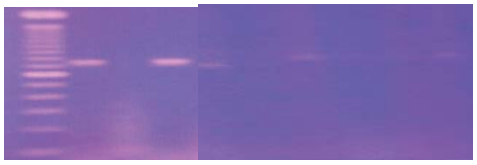
The rice resistance against diseases is highly important target in plant breeding, therefore, mutation induction produce

Table 3. Pattern amplification of rice mutant lines by using *Xa13* gene

No.	Dose (Gy)	Mutant lines code	Agarose gel electrophoresis pattern
1	25	Marker, Code, Kencana Bali, Mira-1, 2, 3, 4, 6, 7, 8, 9	
2	50	M, Cd, KB, Mira, 15, 16, 18, 19, 26, 30, 32, 34	
3	75	M, Cd, KB, Mira, 35, 36 37 38, 39	
4	100	M, Cd, KB, Mira, 41, 43, 44, 46, 48, 50, 51	
5	150	M, Cd, KB, Mira, 52, 54, 57, 58, 60, 64, 67	
6	200	M, Cd, KB, Mira, 68, 70, 71, 72, 73, 74	

mutant lines that have broad resistance trait is a challenge for plant breeders and pathologist. Rice mutant lines along with resistant check Code variety as positive control and Kencana Bali as negative control were analyzed for *Xa13* gene and the result is displayed in Table 3. Screening for disease responses revealed that various alteration have occurred in the gamma rays - mutant lines. It shows that by the dose of 25 Gy very clear band occured for all mutant lines, and found that four mutant lines by the dose of 150 Gy contain *Xa13*. Code shows smear band as also displayed by Mira-1 as the parent plant. Mutation induction by the doses of 50 and 100 Gy apparently have none band, and this only one was also found in mutant line number 37 and 70 by the doses of 75 and 200 Gy respectively. Han *et al.* (2004) found that 3 lines from 26 lines of gamma rice mutants were identified blast resistant. It was occured T base-pair deletion and early stopped coding at 60 and 67 bp after translation initiation region in hd(t) mutant compared to parent plant (Shang *et al.* 2012). Pradhan *et al.* (2015) mentioned that backrossing of Jalmagna rice was found 100 BC1F1 plants showed presence *Xa5*, and 91 of plants showed presence of *Xa13* and *Xa21* from 360 BC1F1 seeds. The blight attack is characterized by changes in the appearance of plants, starting immediately

Table 4. Pattern amplification of rice mutant lines by using *Xa21* gene

No.	Dose (Gy)	Mutant lines code	Agarose gel electrophoresis pattern
1	25	M, Cd, KB, Mira, 2, 3, 4, 6, 7, 8, 9	
2	50	M, Cd, KB, Mira, 15, 16, 18, 19, 26, 30, 32, 34	
3	75	M, Cd, KB, Mira, 35, 36, 37, 38, 39	
4	100	M, Cd, KB, Mira, 41, 43, 44, 46, 48, 50, 51	
5	150	M, Cd, KB, Mira, 52, 54, 57, 58, 60, 64, 67	
6	200	M, Cd, KB, Mira, 68, 70, 71, 72, 73, 74	

with wilting in most tissues (especially the leaves), followed by chlorosis fast (in a few days), became brown and dead tissues on the surface could be seen.

BLB disease also causes the rice plants starting from seedling up to mature stage (Tasliah, 2012). Plants could defend themselves against pathogen because the plants have the

Table 5. Reaction of rice mutant lines toward *Xanthomonas oryzae* pv.*Oryzae*

No.	Dose (Gy)	Line codes	Xa5	Xa13	Xa21
1		Code (control positive)	+	+	+
2		Kencana Bali (Control negative)	-	-	-
		Mira-1 (parent plant)	+	-	+
3	25	2	+	+	-
4		3	+	+	-
5		4	+	+	-
6		6	+	+	-
7		7	+	+	-
8		8	+	+	+
9		9	+	+	-
10	50	15	+	-	-
11		16	-	-	-
12		18	+	-	-
13		19	-	-	-
14		26	-	-	-
15		30	+	-	-
16		32	-	-	-
17		34	-	-	-
18	75	35	+	-	-
19		36	-	-	-
20		37	+	+	-
21		38	-	-	-
22		39	-	-	-
23	100	41	+	-	-
24		43	-	-	-
25		44	-	-	-
26		46	-	-	-
27		48	-	-	-
28		50	+	-	-
29		51	+	-	+
30	150	52	+	+	+
31		54	+	-	-
32		57	+	+	+
33		58	+	+	+
34		60	+	+	+
35		64	+	-	-
36		67	+	-	+
37	200	68	+	-	
38		70	+	-	-
39		71	+	+	-
40		72	+	-	-
41		73	+	-	-
42		74	+	-	-

gene coding for disease-defense which is known as Xa genes. Detection of mutant lines containing of *Xa* genes is very helpful in the initial purification of rice mutant tolerant to BLB disease.

Rice mutant lines appearing with a band size of 750 bp of *Xa21*, displayed in Table 4. Mutation induction by the dose of 150 Gy was the best dose to obtain the mutant lines containing *Xa21* compared to other doses, only one mutant line with the same lines was found at the doses of 25 Gy and 100 Gy, and none at the doses of 75 and 200 Gy respectively. Gamma rays could affected on loss-of-function and gain-of-function of rice mutants (Wu *et al*., 2005). The rice gene, *Xa21*, encodes a receptor-like kinase with leucine-rich repeats in the extra cellular domain (Swamy *et al*., 2006). Wang *et al.* (2004) reported that from 23 mutants from irradiated of IRBB21 with fast-neutron 20Gy, 6 mutant lines found fully susceptible carried rearrangement at the *Xa21* as detected by PCR and southern blots, while 17 mutants with partial resistance had no visible deletions or rearrangements at the *Xa21* locus. Wang *et al.* (2004) also mentioned the possibility that point mutation or small deletions could have occurred at *Xa21* locus. According to Singh *et al.* (2015), during the polymorphic survey of thirty four rice cultivars, no amplicons specific to *Xa21* and *Xa13* allele were detected. Campbell and Ronald (2005) found that mutant line from irradiated rice by 15 Gy fast-neutron having enhanced resistance to only CA-1, may represent a mutation susceptible of factor for *M. grisea*. Furthermore, Yin *et al.* (2015) found that a deletion of 2446 bp from the 9th to the 14th exon of *Os08g19320* in the *vsl* mutant, and deletion occurred in the vegetative senescence of the lethal mutant.

Table 5 displayed the positive and negative response of rice mutant lines on three kinds of *Xanthomonas* resistance gene detection.

Conclusion

Marker assisted selection of rice mutant lines could help to identify the lines containing the *Xa5*, *Xa13* and *Xa21* which indicate them as tolerant to bacterial leaf blight (BLB) disease genetically. Mutation induction by the doses of 25 Gy and 150 Gy were better than other doses, it was found 5 rice mutant lines which were contains of *Xa5*, *Xa13* and *Xa21* genes with band size like Code rice variety as the control positive.

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