Molecular identification of F1 population containing entC and pmsB genes to increase resistance for blast disease

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Abstract. Rice is the staple food of the Indonesian people. Many efforts have been made to improve rice productivity under biotic stress condition. Blast disease caused by Pyrcularia grisea is one of the main diseases in rice plants. This study aimed to assemble rice plants that have high productivity and resistant to blast disease by cross-breeding method between Rojolele transgenic containing entC and pmsB genes with Ciherang, Sintanur, Inpari 31, Inpari 33 and Tarabas varieties. The results showed that the salicylic acid coding gene was successfully derived in the F1 population, as indicated by the positive PCR analysis of the pmsB gene. Further analysis is needed on the next offspring to test stability of genes and bioassay for blast disease resistance.

1. Introduction

Rice (Oryza sativa L.) is one of the most important food crops in Indonesia. The demand of rice is very high but not followed with the high production. Efforts to increase rice production are constrained by biotic and abiotic stress. Blast disease caused by Pyrcularia grisea is one of the biotic stresses that can cause a decrease in production. Rice blast is the most important disease concerning the rice crop in the world. Since rice is an important food source for much of the world. However, the rice blast can occur in all the plant shoots, from the initial developing stages until the final seed production phase. The losses caused by the rice blast can be direct or indirect, whereas in the leaves it is indirect and affects the photosynthesis and respiration [4]. Pyrcularia grisea, also known as rice blast fungus, rice rotten neck, rice seedling blight, blast of rice, oval leaf spot of graminea and pitting disease that affecting torice production [1][2]. Pyrcularia grisea complex can also infect other agriculturally important cereals including wheat, and barley causing diseases called blast disease or blight disease. Rice blast causes economically significant crop losses annually [3].

Salicylic acid (2-hydroxybenzoic acid) is an important plant hormone. Although it has been most thoroughly studied with respect to its functions in pathogen resistance, SA sits within a complex regulatory network affecting signalling by other phytohormones and it directly or indirectly affects a wide range of plant responses [5]. After a hypersensitive response to invading pathogens, plants show elevated accumulation of salicylic acid (SA), induced expression of plant defense genes, and systemic acquired resistance (SAR) to further infection by a broad range of pathogens [6]. The research conducted on tobacco plants showed that the genes that increase the expression of salicylic acid
(entC and pmsB gene) that were successfully inserted, increase the resistance of these plants to pathogens, but did not affect the plant phenotype [6]. Salicylic acid content in rice plants was positively correlated with the level of rice resistance to *Pyricularia grisea* [7].

Development of technology for increasing rice productivity has been and continues to be carried out by national and international research institutions, such as IRRI. In the technological aspect, our researchers have succeeded in developing, among others, prospective rice varieties that have resistance to pests and diseases. Afterwards, it needs an effort in improving the character of the plant so the plants can grow in biotic stress conditions with high productivity. Plant breeding is an attempt to improve the character of the plant, so that plants are obtained that have better character than the parents. This study aimed to assemble rice plants that have high productivity and resistant to blast disease by cross-breeding method between Rojolele transgenic containing entC and pmsB genes with Ciherang, Sintanur, Inpari 31, Inpari 33 and Tarabas varieties.

2. Materials and Methods

The research was conducted in Genomics and Crop Improvement Laboratory, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI) and Indonesian Center for Rice Research, Ministry of Agriculture, Bogor, West Java, Indonesia. Planting materials used in this study were transgenic rice varieties Rojolele as female parents, rice varieties like Ciherang, Inpari 31, Inpari 33, Sintanur and Tarabas as male parents. Other materials include glacyne bags, label paper, nameplate, 70% alcohol, Urea fertilizer, SP36 fertilizer, and KCl fertilizer.

2.1. Cross Breeding

This activity was carried out at Biosafety clearing House, Cibinong in February-August 2017. The material used was Rojolele rice seeds which already contained entC and pmsB genes, Inpari 31, Inpari 33, Ciherang and Tarabas. The method of crossing (formation of F1) follows [8]. The F1 population begins with the planting of crosses elders. Other parent seeds are sown in tubs measuring 30 cm x 50 cm, then the seeds are transferred to the pot (bucket) or field after 21 days of age. Female elders are Rojolele transgenic containing entC and pmsB genes, planted in pots, two months earlier than other elders because of their long life. Other parent seeds are planted in the field with a spacing of 25 cm x 25 cm, one seed per planting hole. Planting parents is repeated three times with a two-week interval to synchronize flowering during crossing. The plants are fertilized with 200 kg ha⁻¹ Urea, 100 kg ha⁻¹ SP36 and 100 kg ha⁻¹ KCl. After the plants are flowering, the plants that are made into female elders are transferred to the pot and taken to the greenhouse to discard the male flowers (castration). A good flower to be encapsulated is when the stamens are in the middle of the flower. This Stadia shows that flowers will bloom in 1-2 days. The flower is cut with a slope of 600, then the stamens are removed by suction using a suction pump (the pistil cannot be damaged). The panicles that have been encapsulated are covered with oil paper and labeled.

2.2 Isolation of DNA Genome

Plant samples can be prepared by cryogenically grinding tissue in a mortar and pestle after chilling in liquid nitrogen. Freeze dried plants can be ground at room temperature. In either case, a fine powder is best for extracting DNA. For each 100 mg homogenized tissue use 500 µl of CTAB Extraction Buffer. Mix and thoroughly vortex. Transfer the homogenate to a 60°C bath for 30 minutes. Following the incubation period, centrifuge the homogenate for 5 minutes. at 14,000 x g. Transfer supernatant to a new tube. Add 5 µl of RNase solution A and incubate at 32°C for 20 minutes. Add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex for 5 seconds then centrifuge the sample for 1 min. at 14,000 x g to separate the phases. Transfer the aqueous upper phase to a new tube. Repeat this extraction until the upper phase is clear. Transfer the upper aqueous phase to a new tube. Precipitate the DNA by adding 0.7 volume cold isopropanol and incubate at -20°C for 15 minutes. Centrifuge the sample at 14,000 x g for 10 minutes. Decant the supernatant without disturbing the pellet and subsequently wash with 500 µl ice cold 70% ethanol. Decant the ethanol. Remove residual ethanol by
drying in a SpeedVac. Dry the pellet long enough to remove alcohol, but without completely drying the DNA. Dissolve DNA in 20 µl TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The pellet may need warming 37°C 1 hour in order to dissolve. PCR analysis was used to detect the integration of pmsB and entC gene in rice genome.

2.3 PCR Analysis
DNA isolation was carried out by modified CTAB method [9]. The primers to detect pmsBin the population of F1 has the following base sequence: forward 5’ ATGCTGCCGCTAAAACCGCCACAA 3’ and reverse 5’ TGACTTGGCCTGCGCGAGTACGT-3’. The PCR amplification conditions were as follow: one cycle of 5 minutes denaturation at 95°C, followed by 40 cycles of amplification steps of 5 seconds denaturation at 95°C; 30 seconds primer annealing at 60°C, and 30 seconds primer elongation at 70°C; and one cycle of final extension at 70°C for 5 minutes [10]. The electrophoresis was conducted with 100 volt for 60 minutes and the results were observed and photographed using Bio Rad Gel Doc UV 1000.

3. Result and Discussion
The assembly of high yielding and tolerant superior varieties of major pests is carried out starting from the selection of potential elders, creating diversity among others through crossing, selection and fixation of lines according to those expected to use the pedigree and bulk methods, and with anther culture tools, observation of yield power, as well as yield strength tests carried out in accordance with plant breeding procedures [11][12].

Seeds produced from the crossing of transgenic with several rice varieties were planted in soil media. After 10 days the leaves were taken for DNA isolation. The PCR was applied to F1 population. A total of one hundred and sixty one sample from 5 crosses variety parental were selected for PCR detection (Table. 1). The PCR result from the 5 crosses variation, between ciherang and rojolele showed percentage pmsB gene is 37%, Tarabas and Rojolele is 35,7%, Sintanur and Rojolele is 60,9 %, Inpari 31 and Rojolele is 58,3%, Inpari 33 and Rojolele is 37,5%. The percentage indicates that the pmsB gene is successfully inserted to F1 population. The PCR results showed the specific band of 335 bp in size generated from specific primer amplification for pmsB gene (Figure 3).
Table 1. Integration of \textit{pmsB} insertion in first generation (F1) using primer specific \textit{pmsB} gene

| No | Cross                        | Number of sample | Positive \textit{pmsB} |
|----|------------------------------|------------------|------------------------|
| 1  | Ciherang x Rojolele          | 62               | 23                     |
| 2  | Tarabas x Rojolele           | 28               | 10                     |
| 3  | Sintanur x Rojolele          | 23               | 14                     |
| 4  | Inpari 31 x Rojolele         | 24               | 14                     |
| 5  | Inpari 33 x Rojolele         | 24               | 9                      |
|    | Total Number                 | 161              | 70                     |

The result of plant preview in the green house had shown that different harvest ages were obtained and from cross breeding result showed varieties resemble with their parent plants Rojolele except Tarabas (Figure 2). Therefore it is necessary to do selection on phenotypic resemblance to the recurrent parent with the backcross breeding scheme. The main target of back crossing method was include the donor parent that has a gene of interest \textit{entc} and \textit{pmsb} genes encoding the salisilic acid resistance to blast disease.

Figure 2. Growth of vegetative and generative of cross-bred rice plants in Green House located in Research Center for Biotechnology LIPI Cibinong.

Figure 3. PCR with specific primer \textit{pmsB} M) Marker 200bp, 1) positive control, 2) negative control, 3) water, 4-38) samples
4. Conclusion
In this study, we obtained 60 progeny of lowland rice plants (Ciherang, Inpari 31, Inpari 33 and Sintanur varieties) and 10 progeny of upland rice (Tarabas varieties) containing \textit{pmsB} gene based on PCR analysis. Further studies will be conducted to determine transgene copy number, and to evaluate the resistance to blast disease.

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