Introgression of bacterial blight resistance gene Xa7 into popular Indonesian rice varieties through backcross and molecular breeding

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Abstract. Bacterial blight (BB) caused by Xanthomonas oryzae pv. oryzae (Xoo) is one of major diseases in rice production. The BB resistance gene Xa7 is one of effective genes against Xoo strains in Indonesia. This study aimed to introgress BB resistance gene Xa7 into submergence tolerant rice variety Inpara 5 (IR64 Sub1) and brown plant hopper (BPH) resistant rice variety Inpari 13. Stable BB resistant rice lines have been successfully developed after three backcrossing and four generations of fixation (BC3F4). Screening for BB resistance were conducted in generative stage using Xoo strains IV and VIII. The reaction pattern of the backcross lines against these two Xoo strains were similar to the BB resistant parent Conde. The introgression of the Xa7 gene in Inpara 5 and Inpari 13 genome have been confirmed by using SSR markers RM20589 and RM20590; and Xa7 gene specific marker Xa7-LD34. Agronomic characters and grain quality properties of the backcross lines were comparable to the recurrent parents. Other desirable traits from recurrent parents including submergence tolerance and BPH resistance retained in the selected backcross lines. The BB resistance rice lines with popular rice varieties genetic background developed through this study have potential to be released for farmers adoption.

Keywords: Rice, bacterial blight, Xa7, IR64-Sub1, Inpari 13

1. Introduction

Bacterial blight (BB) is one of major diseases in rice production particularly in irrigated and rainfed lowland rice areas throughout Asia [1]. The disease is caused by Xanthomonas oryzae pv. orizae (Xoo) and infects rice plant both in vegetative and generative stages. Rice yield losses caused by the disease were commonly reported in the range of 20 to 30% [2], but under severe infection the yield losses could reach 50% [1, 3]. The degree of yield losses caused by the disease depends on growth stage, location, season, weather, and rice variety [1, 2].

Cultivation of BB resistant rice variety is considered as the most efficient and sustainable strategy to manage this disease and to prevent yield losses. More than 40 resistant genes for BB disease has been identified and used for the improvement of BB resistance of rice [4, 5]. Nine of the identified BB
resistance genes have been well characterized and cloned including Xa-1, Xa-3, xa-5, Xa-10, xa-13, Xa-4, Xa-23, xa-25(t), and Xa-27(t) [4].

Identification of numerous BB resistance genes allowed breeders to select effective and durable resistance genes which can be used in the breeding programs. The durability of BB resistance genes was reported to be vary among the genes and among the BB resistance genes Xa7 was considered to be durable [6]. The Xa7 is a dominant resistance gene and was firstly identified in rice cultivar from Bangladesh DV85 [7]. This resistance gene was reported to be effective against major Xoo isolates in Indonesia [8, 9] and Philippines [10]. The resistance gene Xa7 has been used for the development isogenic line IRBB7 [7], and in Indonesia the line has been used to develop BB resistant variety Conde through backcross with the genetic background of popular variety IR64 [11].

Backcross breeding is considered as the most efficient approach to improve BB resistance of popular rice cultivar by introducing one or more resistance genes into target cultivars. Availability of DNA markers for many BB resistance genes which can be used to assist backcrossing has made this approach become popular in the breeding to develop BB resistant cultivars. Marker assisted backcrossing approaches have been used to improve the resistance of popular aromatic rice cultivar Basmati from India [12, 13]; fragrance rice Khao Dawk Mali 105 (KDML 105) from Thailand [14], and popular cultivars Mahsuri and PR106 from India [15, 16]. A number of studies also reported improvement of BB resistance in parental lines of hybrid rice using backcross and molecular breeding [17-19].

In spite of the large spread of BB disease incidence in Indonesia, the number of BB resistant rice cultivars remains limited particularly against dominant strains Xoo IV and VIII [20]. This study aimed to develop BB resistance rice cultivars by introgressing Xa7 gene into popular rice varieties from Indonesia through backcross breeding. Two popular rice varieties were selected for target improvement including submergence tolerant rice variety Inpara 5 which harbors Sub1 gene in genetic background of mega variety IR64 [21] and brown plant hopper (BPH) resistant rice variety Inpari 13 [22]. Molecular markers were used to confirm the introgression of Xa7 in the backcross progenies.

2. Materials and methods

2.1. Plant materials and backcross scheme

Two popular rice varieties were selected as recurrent parent for the BB resistance improvement including BPH resistant rice variety Inpari 13 and submergence tolerant rice variety Inpara 5 (IR64-Sub1). The BB resistant rice variety Conde harboring resistance gene Xa7 was used as donor parent. The BB resistance gene Xa7 was introgressed into Inpari 13 and Inpara 5 through backcross (BC) breeding (figure 1). Inpari 13 and Inpara 5 were crossed with Conde to obtain F1 seeds. F1s were backcrossed with respective recurrent parents to obtain BC1F1 seeds. Selection was performed in the BC1F1 generation to select individual plants that were resistant to BB and had comparable phenotype to the recurrent parents. The similar strategy was carried out in the second and third generation of BCs. Selected BC3F1 plants were then self-pollinated to obtained homozygous plants. Selected lines in BC3F4 generation were used for further analysis including two lines with genetic background of Inpara 5 (B14950-MR-17-1 and B14950-MR-24-1), and two lines with genetic background of Inpari 13 (B14954-MR-3-2 and B14954-MR-5-1).

2.2. Molecular marker analysis

DNA was extracted from healthy leaves of four weeks old rice seedlings using CTAB protocol [23]. Three primers which were reported to be associated to Xa7 gene were used including SSR markers RM20589 and RM20590; and Xa7 specific marker Xa7-LD34 [24, 25]. PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc.) in 20 µL reactions containing 2 µL of DNA at 20 ng µL⁻¹, 2 µL of 10 × buffer containing 25 mM MgCl₂, 1 µL of 2.5 mM dNTPs, 1 unit of Taq Polymerase (Invitrogen), and 1 µL of 10 µM forward and reverse primers. DNA amplifications were performed in 35 cycles of 1 min denaturation at 95°C, 30 sec annealing at 55°C, and 1 min extension.
at 72°C. DNA amplifications were completed with a final extension at 72°C for 5 min. The PCR product were analysed by electrophoresis on 2% agarose for genotyping.

2.3. Bacterial blight screening
Screening for BB resistance of segregating plants in BC1F1 to BC3F3 were performed in the field during primordia stage. The Xoo isolates were collected from infected rice leaves in Muara experimental station in Bogor. Infected leaves were cut into small pieces and dissolved in sterile water. The solution was then incubated for 24 h before used of BB screening. The rice plants were inoculated with Xoo isolate using clipping method [24].

Evaluation of the BB resistance of selected BC3F4 lines were performed in screenhouse using two Xoo races, Xoo IV and Xoo VIII. Rice plants were grown in plastic pots (16 cm diameter × 19 cm height) with three plants in each pot and three replicates. Xoo isolates were prepared in Wakimoto’s potato semisynthetic agar medium [25] and inoculated into rice plants using clipping method [24]. BB infection was scored on 15 days after inoculation following IRRI [26].

Figure 1. Scheme the development of bacterial blight resistant rice varieties using backcross breeding.

2.4. Evaluation of BPH resistance
Screening for BPH resistance was conducted in screenhouse using BPH biotype 1, 2, and 3 following the protocol described in Trisnaningsih and Nasution [27]. BPH resistance of rice was scored following IRRI [26].

2.5. Evaluation of submergence tolerance
Submergence tolerance evaluation was performed in screenhouse following the rapid screening protocol [28]. Seeds of BC3F4 lines, parental varieties, FR13A (submergence tolerant check) and IR42 (submergence susceptible check) were germinated in rows in plastic trays (35 cm × 25 cm × 10 cm). Complete submergence was imposed on four teen-day-old rice seedlings for 14 days. Scoring on the submergence tolerance was taken 14 days after de-submergence following IRRI [26].
2.6. Evaluation of agronomic performance
Selected BC3F4 lines, recurrent parents Inpara 5 and Inpari 13, and BB resistant donor Conde were evaluated for their agronomic performance in paddy field. Field trial was conducted in irrigated rice area at Muara experimental station in Bogor from May to August 2015. The experiment was arranged in randomized complete block design with four replications. Rice seeds were sown in seedbed and were transplanted to the paddy field after 21 days. Each genotype was grown in 4 m × 5 m plot with plant spacing of 25 cm × 25 cm. Fertilizers were applied to the plots consisting of urea (200 kg ha⁻¹), SP36 (100 kg ha⁻¹), and KCl (100 kg ha⁻¹). Rice plants were harvested when 80% of rice grains matured.

2.7. Grain quality evaluation
Grain quality properties of the selected BC3F4 lines, Inpara 5, Inpari 13, and Conde were evaluated following the standard protocol used in grain quality laboratory of Indonesian Center for Rice Research [29]. The grain quality properties analysis included milling degree, percentage of head rice, grain size, grain shape, amylose content and cooked rice texture.

3. Results
3.1. Bacterial blight resistance of backcross lines
The BB resistance of backcross rice lines were evaluated using two strains of BB disease, Xoo IV and Xoo VIII. The submergence tolerant rice variety Inpara 5 was moderately susceptible to Xoo IV and susceptible to Xoo VIII (table 1, figure 2). The BPH resistant variety Inpari 13 was susceptible to both Xoo IV and Xoo VIII. The backcross rice lines B14950-MR-17-1 and B14950-MR-24-1 were showed improved BB resistance compared to the recurrent parent Inpara 5 against both Xoo strain IV and VIII. Similarly, the BC lines derived from Inpari 13 i.e. B14954-MR-3-2 and B14954-MR-5-1 also showed resistant reaction against Xoo IV and Xoo VIII. Overall, bacterial blight resistance of developed BC lines was comparable to Conde, the donor parent for Xa7 gene.

| Lines                  | Xoo IV | Xoo VIII |
|------------------------|--------|----------|
|                        | Scale  | Reaction | Scale  | Reaction |
| B14950-MR-17-1 (Inpara 5+Xa7) | 1      | R        | 1      | R        |
| B14950-MR-24-1 (Inpara 5+Xa7) | 1      | R        | 1      | R        |
| Inpara 5               | 5      | MS       | 7      | S        |
| B14954-MR-3-2 (Inpari 13+Xa7) | 1      | R        | 1      | R        |
| B14954-MR-5-1 (Inpari 13+Xa7) | 1      | R        | 1      | R        |
| Inpari 13              | 7      | S        | 7      | S        |
| Conde                  | 1      | R        | 1      | R        |

R= resistant, S=susceptible, MS=moderately susceptible

3.2. Molecular analysis of the introgressed BB resistant gene Xa7
The integration of BB resistance gene Xa7 in the backcross lines genome was confirmed using three primers which were known to be associated to Xa7 gene. The alleles of SSR markers RM20589 and RM20590 in the BC lines derived from Inpara 5 and Inpari 13 were similar to the alleles of BB resistant variety Conde (figure 3). The susceptible parents Inpara 5 and Inpari 13 showed similar alleles of these markers. Using Xa7 spesific primer LD34, the backcross lines demonstrated the same alleles of Conde and can be differentiated to Inpara 5 and Inpari 13 alleles.
**Figure 2.** Typical leaf lesion of backcross lines, recurrent parents and donor parent for bacterial blight resistance gene *Xa7* inoculated by BB strains *Xoo IV* and *Xoo VIII*.

**Figure 3.** DNA amplification of bacterial blight resistance backcross lines, recurrent parents and donor parent using SSR markers RM20589 and RM20590, and *Xa7* specific marker *Xa7-LD34*. 
3.3. Brown planthopper resistance of BB resistant backcross lines

BPH resistance of BC3F4 derived lines of Inpara 5 and Inpari 13 were determined using three biotypes of BPH, which were predominantly found in Indonesia (table 2). The BC lines B14950-Mr-17-1 and B14950-Mr-24-1 showed moderate resistant responses to BPH biotype 1, similar to the recurrent parent Inpara 5. However, these two lines showed moderate susceptible reaction to BPH biotype 2 while Inpara 5 was moderately resistant to this biotype. The reaction of Inpara 5 and its BC progenies against BPH biotype 3 were moderate susceptible.

Table 2. Reaction of bacterial blight resistance backcross lines, recurrent parents and donor parent against BPH biotype 1, 2, and 3.

| Lines                  | BPH Biotype 1 | BPH Biotype 2 | BPH Biotype 3 |
|------------------------|---------------|---------------|---------------|
|                        | Scale | Reaction | Scale | Reaction | Scale | Reaction |
| B14950-MR-17-1 (Inpara 5+Xa7) | 3    | MR      | 5     | MS       | 5     | MS       |
| B14950-MR-24-1 (Inpara 5+Xa7) | 3    | MR      | 5     | MS       | 5     | MS       |
| Inpara 5               | 3    | MR      | 3     | MR       | 5     | MS       |
| B14954-MR-3-2 (Inpari 13+Xa7) | 1    | R       | 3     | MR       | 3     | MR       |
| B14954-MR-5-1 (Inpari 13+Xa7) | 1    | R       | 3     | MR       | 3     | MR       |
| Inpari 13              | 1    | R       | 3     | MR       | 3     | MR       |
| Conde                  | 3    | MR      | 5     | MS       | 5     | MS       |

R= resistant, MR= Moderate resistant, MS= Moderate susceptible

The BPH resistance in Inpari 13 were retained in its BC progenies and was found to be consistent to all BPH biotype (table 2). BC3F4 of Inpari 13 were resistant to BPH biotype 1 similar to Inpari 13. These lines were moderately resistant to both BPH biotype 2 and biotype 3, while the donor of Xa7 Conde was moderately susceptible to those BPH biotypes.

3.4. Submergence tolerance of the BB resistant backcross lines

The submergence tolerance of the BC3F4 lines and their recurrent and donor parents were evaluated under 14 days complete submergence during vegetative stage. Submergence tolerant rice variety Inpara 5 and its backcross lines obviously showed higher survival rate after 14 days complete submergence compared to variety Inpari 13 and its backcross derived lines (figure 4). The survival rate of B14950-MR-17-1 and B14950-MR-24-1 were 63.3% and 72.6%, respectively. The submergence tolerance level of these backcross lines was slightly lower compared to their recurrent parent Inpara 5 which had survival rate of 81.5%.

3.5. Agronomic performance of the BB resistant backcross lines

The performance of the BB resistant backcross lines was evaluated in the paddy field in Muara experimental station Bogor. The BC3F4 lines showed comparable phenotype to the two recurrent parents used as genetic background, Inpara 5 and Inpari 13 (figure 5). Flowering times and maturity of BB resistant backcross lines were similar to their respective recurrent parents (table 3). The line B14950-MR-17-1 and B14950-MR-24-1 showed similar growing duration to Inpara 5, while B14954-MR-3-2 and B14954-MR-5-1 had comparable flowering times and maturity to Inpari 13. The donor parent for BB resistant Conde had longer growing duration compared to the backcross lines and the recurrent parents.

Number of filled grains of the BF3F4 line B14950-MR-17-1 was significantly higher than Inpara 5, while B14954-MR-3-2 had comparable number of filled grains to Inpara 5 (table 3). Number of filled grains of the backcross derived lines of Inpari 13 were comparable to the recurrent parent. Similarly, the number of empty grain character and grain weight of the backcross lines were not significantly different to their recurrent parents.
Figure 4. Survival rate of bacterial blight resistant BC3F4 lines, recurrent parents and donor parent under complete submergence for 14 days in vegetative stage.

Figure 5. Performance of BC3F4 lines with genetic background of Inpara 5 (upper) and Inpari 13 (bottom), and the donor parent for bacterial blight resistance gene Xa 7 Conde.
Grain quality properties of BC3F4 lines derived from Inpari 13 and Inpara 5 were evaluated and compared to their recurrent parents (table 4). The milling degree of the line B14950-MR-17-1 similar to recurrent parent Inpara 5, while B14950-MR-24-1 had slightly higher degree of milling degree compared to Inpara 5. The milling degree of Inpari 13 backcross derived lines B14954-MR-5-1 was similar to Inpara 13, while B14954-MR-3-2 had slightly higher degree of milling degree than Inpara 13. The percentage of head rice of BC3F4 lines were in the range of 90-92% which were comparable to their recurrent parents.

The grain size and grain shape of the backcross lines were also similar to their parents. Among the BC4F3 lines, only the line B14954-MR-3-2 which had medium chalkiness which was similar to Xa7 donor parent Conde, while other lines had small chalkiness similar to Inpara 5 and Inpara 13. The amylose content of the BC3F4 lines were in the range of 21-23% which were classified as intermediate. Furthermore, the cooked rice textures of BC3F4 lines were also similar to their recurrent parents.

Table 4. Grain quality properties of bacterial blight resistance backcross derived lines, the recurrent and donor parents.

| Lines             | Milling degree (%) | Head rice (%) | Grain size | Grain shape | Chalkiness | Amylose content (%) | Rice texture |
|-------------------|--------------------|---------------|------------|-------------|------------|---------------------|--------------|
| B14950-MR-17-1    | 69                 | 92            | Long       | Medium      | Small      | 22.1                | Medium       |
| B14950-MR-24-1    | 71                 | 90            | Long       | Medium      | Small      | 22.8                | Medium       |
| Inpara 5          | 69                 | 93            | Long       | Medium      | Small      | 21.8                | Medium       |
| B14954-MR-3-2     | 70                 | 92            | Long       | Medium      | Medium     | 21.5                | Medium       |
| B14954-MR-5-1     | 69                 | 90            | Long       | Medium      | Small      | 22.8                | Medium       |
| Inpari 13         | 69                 | 89            | Long       | Medium      | Small      | 22.3                | Medium       |
| Conde             | 70                 | 90            | Long       | Medium      | Medium     | 21.7                | Medium       |

Interestingly, Inpara 5 derived backcross line B14950-MR-17-1 had significantly higher grain yield compared to Inpara 5, while the grain yield of B14950-MR-24-1 was not significantly different to Inpara 5. The grain yield of backcross derived line of Inpari 13, B14954-MR-3-2, was significantly lower than Inpari 13, while another backcross line B14954-MR-5-1 had comparable grain yield to the recurrent parent.

3.6. Grain quality of the BB resistant backcross lines

Grain quality properties of BC3F4 lines derived from Inpari 13 and Inpara 5 were evaluated and compared to their recurrent parents (table 4). The milling degree of the line B14950-MR-17-1 similar to recurrent parent Inpara 5, while B14950-MR-24-1 had slightly higher degree of milling degree compared to Inpara 5. The milling degree of Inpari 13 backcross derived lines B14954-MR-5-1 was similar to Inpara 13, while B14954-MR-3-2 had slightly higher degree of milling degree than Inpari 13. The percentage of head rice of BC3F4 lines were in the range of 90-92% which were comparable to their recurrent parents.

The grain size and grain shape of the backcross lines were also similar to their parents. Among the BC4F3 lines, only the line B14954-MR-3-2 which had medium chalkiness which was similar to Xa7 donor parent Conde, while other lines had small chalkiness similar to Inpara 5 and Inpara 13. The amylose content of the BC3F4 lines were in the range of 21-23% which were classified as intermediate. Furthermore, the cooked rice textures of BC3F4 lines were also similar to their recurrent parents.
4. Discussion

Rice yield losses caused by bacterial blight disease threaten the sustainability of rice production in tropical countries including Indonesia. Improvement of rice cultivars, which currently exist in farmers' field, would help to minimize the losses and ensure the sustainability of the rice production. Using backcross breeding approach, we showed in this study that the BB resistance of two popular rice varieties from Indonesia, Inpari 13 and Inpara 5, were successfully improved. Stable BB resistant rice lines were obtained in BC3F4 generation. Theoretically 96.9% of the recurrent parent genetic background will be recovered after three generation of backcross. Huang et al. [19] also reported that stable and uniform progenies of backcross selection for BB resistance could be achieved in BC3F4 generation. Using molecular markers Ellur et al. [13] showed that in three generation of backcross recurrent parent genome recovered in the range of 90.79% to 93.75%.

The introgression of \(Xa7\) gene in BC3F4 lines have been confirmed by using three molecular markers including RM20589, RM20590 and LD34. In this study the molecular markers were only used for foreground selection to confirm the introgression of \(Xa7\). Background selection using molecular markers was not applied because the donor of \(Xa7\) is improved variety Conde. Background selection would be needed when the backcross involved donor parent with poor agronomic performance or use wild relatives [12]. Background selection to recover recurrent parents Inpari 13 and Inpara 5 genetic backgrounds were carried out by phenotype selection by growing the backcross progenies side by side to their respective recurrent parents. Bhatia et al. [12] demonstrated that combination of markers assisted foreground selection and phenotypic based background selection in rice recovered more than 90% of the recurrent parent background and were able to reduce the cost for molecular marker selection.

The resistance spectrum of \(Xa7\) gene has been investigated in previous studies and was found to be broad spectrum and durable resistance [6, 10]. The resistance reaction of the BC3F4 lines harboring \(Xa7\) developed in this study against \(Xoo\) IV and VIII indicated the effectiveness of this resistance gene to control BB pathotypes in Indonesia. Based on the report in 2016, \(Xoo\) IV was a dominant pathotype in Lampung, North Sumatra, and West Nusa Tenggara, while \(Xoo\) VIII were dominant in Banten, West Java, Central Java and East Java [30]. BC3F4 lines derived from Inpari 13 and Inpara 5 developed in this study were then potential to be deployed in such BB endemic areas.

Rice cultivar Inpari 13 which was selected as the recurrent parent in this study possess BPH resistance [22]. BPH caused by \textit{Nilaparvata lugens} [Stål] is considered as important pest in tropical Asia which could threat rice production in this region [30, 31]. Several attempts have been done to combine BPH and BB resistance in rice indicated the importance of combining these two traits [32, 33]. Selected BC3F4 BB resistant lines derived from BPH resistant cultivar Inpari 13 has been confirmed to have similar degree of BPH resistance to the recurrent parent and therefore has potential to be deployed in rice areas which have problems with BB and BPH.

Another popular variety used in this study Inpara 5 was developed through backcrossing of mega variety IR64 to improve its submergence tolerance using \textit{Sub1} gene [21]. The variety was targeted for flood prone rice areas which are currently increasing due to climate change [34]. Meanwhile, climate change would also exacerbate the disease threat on crop production [35]. Therefore, Inpara 5 backcross derived lines developed in this study are expected would become important materials in anticipating the impact of climate change.

Grain quality properties of the backcross derived lines of popular rice varieties were comparable to the recurrent parents. Grain quality is a major factor that influence the acceptance of rice by farmers and consumers. Popular varieties which are widely grown usually have excellent grain quality such as in case of mega variety IR64 [36]. More importantly, field observation on agronomic performance of BC3F4 lines derived from Inpari 13 and Inpara 5 indicated that there was no yield penalty resulted from the introgression of \(Xa7\) into these two varieties. Yield and its associated characters of the backcross lines were found to be comparable to the recurrent parents. It should be noted that the yield trial was done in one location. Further study to observe yield performance of the materials in multi
sites representing BB endemic areas of Indonesia would be important to determine the adaptability of the rice lines prior to farmer adoption.

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