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RESEARCH ARTICLE

A Relative Expression of *Xa7* Gene Controlling Bacterial Leaf Blight Resistance in Indonesian Local Rice Population (*Oryza sativa* L.)

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Abstract

Discovery of new alleles at important gene loci through allele mining could support the rice improvement program to sustain national food security. Evaluation of the existing Indonesian local rice landraces is an important point to detect the potential of functional alleles. One of the bacterial leaf blight (BLB) resistance alleles, *Xa7* was detected in Indonesian rice landrace germplasm, Parekaligolara. To validate this potential allele, field evaluation on the segregating population, expression analysis using real time RT-PCR, and sequencing were carried out. Two selected Parekaligolara progenies lines (F_4 and F_6) from double crosses with other selected landraces were clearly more resistant to a dominant Indonesian BLB, Race IV. Specific primers of *Xa7*-LD40 successfully amplified the alleles of F_4 and F_6 lines approximately 300 bp in length. The amplicon sequenced using vector-targeted primers, resulting 264 bp which were flanked between 602 and 866 bp sites. The translated sequence which produced 60 amino acids (open reading frame) ORF, showed homology with the encoding gene associated with the defense system to biotic stress, BTB/POZ. As integrated researches for many potential biotic and abiotic stresses alleles on Indonesian landraces germplasm, this outcome expectedly supports rice landraces utilization for developing of elite cultivars which survive on global changed conditions and benefiting to national food security.

Key words: bacterial leaf blight (BLB), gene expression, Indonesian rice landrace, *Xa7*

Introduction

Rice is the staple food for Indonesian and also for more than half the population in the world. In the fourth assessment report of the International Panel on Climate Change (IPCC), rice production in rice-producing countries could be greatly reduced due to global climate change (Metz et al. 2007). The steep reduction of rice productivity coupled with environmental problem is threatening global food security. Stresses caused by biotic factors become major issues endangering the global food security (Reynolds et al. 2010). In order to solve these issues, it is essential to enhance research and development on bio-resource production, utilization, and management, as well as to increase accessibility to the outcomes and to establish new technologies which may lead to improve food productivity and functionality. To support this idea, genetic resources should be well managed to maximize their use and to meet the needs of human being life.

A genetic diversity which more explicitly defined as variation within a species is clearly a precious heritage and very crucial asset for the survival of humankind. The genetic diversity of rice is mainly preserved in landraces or local varieties (Borba et al. 2009) and wild rice species (Barbier 1989) provides a valuable pool of genetic resources (Pervaiz

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et al. 2011). Identification and conservation of diverse alleles from local varieties are essential prerequisites for improving of rice traits, i.e productivity (Barry et al. 2007). It is also important to discover potential alleles for biotic stress resistance, including resistance to bacterial leaf blight (BLB).

BLB which caused by *Xanthomonas oryzae* pv. oryzae (*Xoo*) is considered one of the most serious bacterial diseases in rice-producing countries worldwide (Leung et al. 2004) including Asian countries such as China, Vietnam, India, the Philippines, South Korea, and Indonesia (Perez et al. 2008). *Xoo* is the main pathogen for BLB in rice could cause 15 - 25% of the yield loss each year (Yamamoto et al. 1997), or even more losses of 30 - 40% (Triny et al. 2007). Losses incurred by the bacterial leaf blight (BLB) is commonly higher in tropics than in subtropics. BLB attacks in Indonesia decrease rice yields both in the wet and dry season (Suparyono et al. 2004).

The prospects of molecular breeding including marker-assisted selection (MAS) and genetic engineering in tailoring allelic variation for genes of agronomic, particularly BLB resistance in rice look very promising. Several major resistance genes (*Xa*) have been detected and sufficiently fine-mapped or even cloned for MAS applications for the improvement of bacterial blight resistance via MAS (Chen et al. 2008). A high level, durable resistance against *Xoo* should be more efficiently achieved by pyramiding different types of *Xa* genes (Perez et al. 2008) that are being considered. One of these *Xa* genes, *Xa7* has been intensively investigated for improving rice variety resistance to BLB (Perez et al. 2008; Utami et al. 2010).

Several local rice accessions which originated in Indonesia and possess alleles contributing to tolerance to biotic and abiotic stresses have been successfully selected using allele mining approach. Those local rice germplasm accessions are IR54 (Indica, IRN:21165) possessing alleles for resistant to blast pathogens, *Pir/Pi9* and P deficiency, *PUP1*; Markuti (Indica, IRN:5754) containing tolerance alleles to Fe toxicity, *OsIRT1*; and Parekaligolara (Indica, IRN:1541) with resistance alleles to BLB pathogens, *Xa7* (Utami et al. 2010). These accessions have been used as parental lines for double crosses with another selected variety, Bio110 for developing new lines tolerant to biotic (blast and BLB) and abiotic stresses (Fe toxicity and P deficiency). This information leads to elucidate the expression pattern of *Xa7* allele from the parent, Parekaligolara in double crosses population. *Xa7* allele found in Indonesian local rice varieties seems to be resistant to the BLB races on generative stages (Utami et al. 2010). Since the high potency of *Xa7* for overcoming BLB in Indonesia, searching and utilization of the allele from Indonesian local rice germplasm need to be conducted. Therefore, the objective of this study was to validate the *Xa7* allele on assorted candidate germplasm by expression analysis the gene using real-time RT-PCR and field evaluation for BLB resistance on the progeny.

Materials and Methods

Plant materials

An Indonesian local rice variety resistant to BLB carrying *Xa7* allele (Parekacigolara), its susceptible variety as recurrent parent (IR54), a landrace and isogenic line with superior agronomical characters namely Markuti and Bio110, respectively, were double crossed with a scheme of IR54/Parekaligolara//Bio110/Markuti. A segregating F_2 generation derived from double crosses, the donor parent (Parekaligolara), the susceptible variety (IR64), and control varieties, i.e. Mahsuri-IRBB7 (*Xa7* isogenic lines) were used in the expression analysis. These varieties were chosen based on their genetic backgrounds with respect to BLB resistance.

Bacterial inoculation and evaluation of resistance to BLB

A selected Indonesian strain of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) belonging to race IV (*Xoo*-IV) which corresponds to *Xa7* protein virulence effector, *avrXa7sacB50* (Utami et al. 2010) was used in a single inoculation experiment to evaluate resistance and susceptible phenotypes. Bacterial *Xoo*-IV was cultured on media containing 20 g of sucrose, 5 g of peptone, 0.5 g $Ca(NO_3)_2$, 0.43 g Na_2HPO_4, and 0.05 g $FeSO_4$ in a total volume of 1L and was allowed to grow at 28 to 30ºC for 3 - 4 days. Bacteria were collected in sterile distilled water and adjusted to a concentration of an optical density at 600 nm = 1 (Iyer and McCouch 2004). All plants were grown in plastic pots in a well-controlled greenhouse and inoculated on plants at the booting stage (around 50-day-old rice seedlings) using the leaf clipping method. This experiment was conducted in two replication sets. Evaluation of BLB resistance was observed at 10 days after inoculation by scoring lesion length per leaf length for the total inoculated leaves to determine disease intensity. The isogenic line of the *Xa7* gene, IRBB7 was used as a positive control and IR64 as a susceptible control on this test. Criteria of resistance response were conducted according to System Evaluation Standard IRRI (1997).

DNA and RNA extraction

All varieties and the F_2 progenies were grown in a greenhouse under a well-controlled temperature, light, and humidity. Healthy leaf tissues were harvested at the tillering stage, ca. 4 - 5 week-old seedlings, and stored in -80ºC until DNA extraction. Genomic DNA was extracted using CTAB following the recommended protocol (Dellaporta et al. 1983; Sambrook and Russel 2001).

Total RNA was extracted from rice leaves which were collected 10 days after inoculation of isolate *Xoo*-IV for expression analysis. Total RNA was isolated from 0.5 g of frozen tissue with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) using glassware and water treated by DEPC. The purity of DNA and RNA was determined before

further PCR amplification. Total RNA were analyzed by (Agilent) Bioanalyzer Pico6000 chip 18S/28S = 2, RIN (RNA integrity Number) (Agilent, Palo Alto, CA, USA) over 8 (Jahn et al. 2008). RNA integrity ranging from 1 to 10 is a useful feature whether the isolation process is successful. RIN values below 8 produces high variation and loss of statistical significance on gene expression analysis using real-time qRT-PCR.

Designed primer and PCR amplification

Primers were designed based on the region near and/or in the target gene. *Xa7* genes in the rice genome were mapped on chromosome (chr.) 6 with size of ~ 1,200 kb. To identify these genes, 60 primer pairs were initially designed which were marked by the primary name of *Xa7*-LD. The total of *Xa7*-LD primers were spread out from contig AP004744 (27.221.015-27.221.432 bp) to contig AP004797 (28.409.251 -28.409.822 bp). LD mapping association analysis showed that *Xa7*-LD34 and *Xa7*-LD40 significantly corresponded to *Xa7* allele (Utami et al. 2010). Both primers were mapped on the genomic position of 28.016.000-28.049.000 bp, on contig AP006454 for *Xa7*-LD34, and contig AP004989 for *Xa7*-LD40 in the rice genome.

Primers corresponding to *Xa7* gene, *Xa7*-LD40 were used for DNA amplification. The primers sequences for PCR amplification and real-time PCR analysis are listed in Table 1. PCR amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc.) in a total volume of 20 µL with the following genotyping PCR reagents: 2 µL of DNA at 20 ng μL^{-1}, 2 µL of 10 × buffer containing 25 mM $MgCl_2$, 1 µL of 2.5 mM dNTPs, 1 unit of *Taq* Polymerase (Invitrogen), and 1 µL each of forward and reverse primers (10 µM). The PCR reaction for further sequencing analysis consisted of 1 unit of Ex*Taq* polymerase (TaKaRa) in a total reaction of 50 µL. All amplifications were performed for a total of 35 cycles of 1 min at 95°C, 30 sec at 55°C, and 1 min at 72°C.

Table 1. The sequences of primers designated the target genes, *Xa7* and the housekeeping gene as a control for real time RT-PCR analysis

Primer Name	Sequences	Remarks
Xa7-LD40	F 5'-CTGGATACGGAACCTTCTAAC-3' R 5'-AGAGAACCTTCTCCTTCAGTG-3'	*Xa7* gene target
Elongation factor- 1α	F 5'-GTCATTGGCCACGTCGACTC-3' R 5'-TGTTCATCTCAGAGGATTCC-3'	Housekeeping gene

Cloning and sequencing

The amplicon produced by the primer corresponding to *Xa7* showing a single band was purified using PCR purification kit (Promega, Madison, WI, USA). The purified PCR product was cloned into pJET1.2-blunt cloning vector (Fermentas) and transformed into *E. coli* competent cells (Sambrook and Russel 2001). A single positive clone of transformants was selected: its plasmid was isolated and then verified by PCR. The PCR product containing a target fragment of *Xa7* gene was confirmed using the corresponding restriction enzyme site. At least three independent clones were chosen for each cloned PCR product and sequenced individually. A culture of the clone was used directly for amplification by a PCR program using universal primer M13. The purified cloned product were sequenced with Big Dye Terminator Cycle Sequencing Kit 2.0 (Applied Biosystem, Foster City, CA, USA) using either forward or reverse universal primer (M13) then used for sequencing on ABI 3700 DNA sequencer (Applied Biosystem, Foster City, CA, USA).

Preparation of poly(A) + RNA and cDNA synthesis

Poly(A) + mRNA (1 µg) was isolated from total RNA using an Oligotex mRNA Spin-Column (Qiagen, Valencia, CA, USA). Double-stranded cDNA was synthesized from poly(A) + mRNA using cDNA Synthesis System Kit (Roche Corp., Basel, Switzerland), fallawed by extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and resuspended in RNase-free water.

Real Time RT-PCR assay

Expression analysis of those selected germplasm and their progenies which were inoculated with *Xoo*-IV were performed using SuperScript™ One-Step RT-PCR (from Invitrogen). Before real-time RT-PCR was performed, the primer sets shown in Table 1 were confirmed with SuperScript™, One-Step RT-PCR using Platinum® Taq (Invitrogen) for RT-PCR, and amplification consisted of 40 cycles at 95°C for 1 min (denaturation), annealing at 55 - 60°C (10°C lower than melting temperature of each primer used) for 2 min, extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. Quantitative gene expression was measured by real-time RT-PCR using an iScript™, One-Step RT-PCR Kit with SYBR® Green (Bio-Rad Laboratories, Hercules, CA, USA) in three replications. PCR amplification was performed in a real-time PCR Detection System as follows: cDNA synthesis for 10 min at 5°C; iScript reverse transcriptase inactivation for 5 min at 95°C; PCR cycling and detection for 40 cycles [10 s at 95°C; 45 s at 55 - 60°C (depending on the primer used); extension for 1 min at 72°C]. Melt curve analysis parameters were 1 min at 95°C, 1 min at 55°C, and 10 s at a temperature ranging from 55 - 95°C (with an increase of 0.5 °C each cycle) for 80 cycles.

Data analysis

Sequences obtained from each forward and reverse primer were cleaned up from vector contaminants using available online tools (www.ncbi.nlm.nih.gov). The corrected sequences were assembled by overlapping them using global optimal alignment program (BioEdit). The sequences then were analyzed their homology by non-redundant protein using BLASTN. The nucleotides of those *Xa7* sequences were also translated to amino acids to check their functional sites facilitated by VNTI AdvanceII program. A housekeeping gene was considered for calibrating real-time PCR data that were used to calculate their relative expression of genes.

The cycle's threshold (Ct) is a relative quantitative method to differentiate the expression level by comparing the Ct value of the samples and the housekeeping gene, elongation factor-1α (Applied Biosystems). The threshold level for signal PCR baseline subtracted was established at 1000 CF RFU.

Results

Bacterial leaf blight (BLB) resistance

The selected local variety possessing the *Xa7* allele, its progenies and the controls confirmed their phenotypic performance following *Xoo* infection. Responses of Parekaligolara variety, and both positive (IRBB7) and negative controls (IR64) showed the expected phenotypes with disease intensity of 1.6, 1.2, and 3.8%, respectively. It indicated that Parekaligolara is considered as a resistant local variety to BLB, not so different from, a resistant control variety.

Responses of the local variety, parekaligolara the controls, and its lines to *Xoo* infection are presented in Fig. 2. Infection of *Xoo*-IV evidently differentiated the resistance and susceptible lines, which had lesions averaging 0.98 cm in length from the resistance lines (F_4 and F_6) with typical brown necrotic lesions of 0.45 cm in average. The average disease intensity of the susceptible and resistance lines was 4.93 and 1.5%, respectively. This phenotypic evaluation demonstrates that Parekaligolara is resistant to *Xoo* race IV which is in contrast to IR64, in addition to the progenies.

Sequence confirmation of *Xa7* gene

The developed markers, *Xa7*-LD40 were highly correlated with F_2 progenies approximately 25 - 35% co-segregated with BLB resistance. Consequently, to investigate variation of *Xa7* alleles between parents, Parekaligolara and IRBB7, the alignment of *Xa7*-LD40 amplicon product was analyzed. The results showed that out of a total length, nucleotide ranged frome 20 to 295 bp revealed the highest similarity, reaching 78% (Fig. 1). That position indicated the presence of some variants of 46 bp, consisting of deletion of 38 bp on Parekaligolara and 8 bp on IRBB7, while the indels (insertions/deletions) varied from 1 to 4 bases. This nucleotide variation of *Xa7* indicates the presence of novel *Xa7* alleles in Parekaligolara a local rice variety belongs to Indonesian germplasm.

The *Xa7* allele using primer pair *Xa7*-LD40 was also successfully amplified in F_4 and F_6 lines with approximately 300 bp in length (Fig. 3). The insert was then sequenced with vector-targeted primers, resulting in 264 bp on the F_4 line. This consensus region of *Xa7* showed a single base mutation of T to C allele. The deduced amino acid sequence of this fragment showed position of *Xa7* in the genome. The translated protein revealing an open reading frame of 60 amino acids (using VNTIAdvanced11), showed homology with one of the genes of the defense system to biotic stress, BTB/POZ domain coding protein (LOC_os06g46240), in the contig AP003766 of chr. 6 positioned at 28 006 287-28 016 492 bp of the rice genome. Overall, the amino acid analysis demonstrated co-linear non-synonymous substitutions of *Xa7*-LD40 marker on the progenies with changes of lysine-cysteine-valine to serine-serine-threonine, respectively, between resistant and susceptible lines. This *Xa7* allele is obviously unique to Indonesian rice landraces and provides a valuable source of BLB resistance. Importantly, its ortholog in *Arabidopsis thaliana* located on chr. 1 (AT1G04390) shows a similar function of the *Xa7* gene investigated in this study, providing further evidence for the value of this prospective allele for BLB resistance on Indonesian rice landraces.

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                10        20        30        40        50
IRBB7    TTTTGTTTGT ATCCACTGTA AAAGGAATTT GGATGTGGGG ATTGT-GTGC
Parekali COGGGTTTGT ATCCACTGTA AA-GGAATTT G-ATGTGGTG ATTGTCGTGC
golara

                60        70        80        90       100
IRBB7     AAT--GATTG CAGAGATCAT TATAGTGAAG GTCTGATTAG AGTTCTTCAA
Parekali   AATCCGAT-G CAGAGATCAT T-TAG-GAAG GTC-GATTAG AGTTCTTCAA
golara

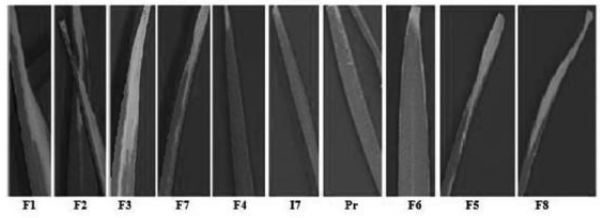
               110       120       130       140       150
IRBB7    AATGCGTCAT GTCAAAACTT GAGCCCAGGA CCGAAGTTGT ACATTTCACG
Parekali GCTCCGCCAC GTCA----TT GAGCCCA--A C----GT-GT ACACCT-ACA
golara

               160       170       180       190       200
IRBB7     T-ATACTGAG TTTGTTTGGC CTATGCGGTT TTCCAAGCAA GTTGGGAGGA
Parekali   TCATACTG-G TTTGTTTG-- CTATGCGC-- --CCAAGCAA GTTGGGAGGA
golara

               210       220       230       240       250
IRBB7    AAGATGAGAA GG-GCCTTAG ATGATAATGA GCTAGCTGAT CTGGAACTGT
Parekali--GATG-GAA GGCGCCTTAG GCGATA-TGA G-TGGC-G-- CGGG--CTGC
golara

               260       270       280       290       300
IRBB7 TGCTTTCAAA TG-GTGAATC TTTAAAA-GC TCATA-CAGC CATCATTTCA
Parekali-GCGCCCAAA GCCGTGAA-C TACAAAACGC TCATAGCAGC CATC--TTCA
golara
```

Fig. 1. Sequences alignment of *Xa7*-LD40 PCR product on Parekaligolara and IRBB7. Nucleotide base marked green color indicates the differences between the two sequences analyzed.



Parameters	Parekali golara (Pr)	IRBB7 (I7)	IR64	F1	F2	F3	F4	F5	F6	F7	F8
Leaf length	38.2	32.8	17.1	23.5	20.3	16.0	23.7	33.3	26.6	26.9	20.2
Lesion length	0.64	0.4	0.7	1.1	0.6	0.9	0.4	0.5	1.7	1.2	0.4
Intensity (%)	**1.6**	**1.2**	3.8	4.7	3.0	5.7	**1.6**	6.1	**1.4**	4.1	2.2
Respond	R	R	S	S	S	S	R	S	R	S	S

R: resistance (R < 2); S: susceptible (S ≥ 2)

Fig. 2. Phenotype of BLB resistance on Parekaligolara and F_2 progenies (F_1-F_8). The percentage of disease intensity is indicated by lesions length per leaf length followed the criteria of resistance respond based on System Evaluation Standard IRRI (1997). The isogenic line of the *Xa7* genes, IRBB7 and the susceptible control variety, IR64 were used as positive and negative controls, respectively.

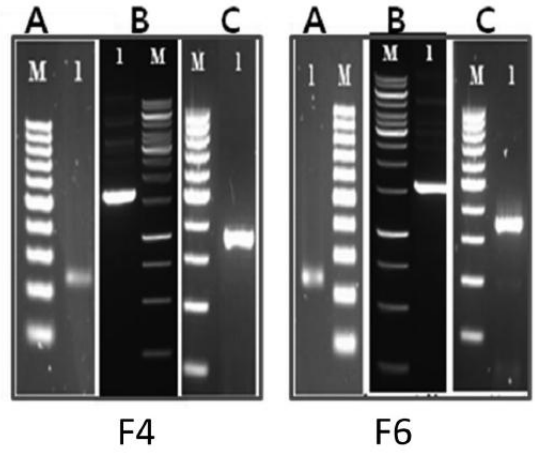


Fig. 3. Visualization of products of *Xa7* primer on agarose for cloning analysis. A. DNA amplicon produced by *Xa7* primer on lines of F_4 and F_6 for cloning and sequencing analysis. M, 100 bp DNA ladder (Fermentas); 1, DNA F_4 and F_6 lines. B. Recombinant plasmid containing the *Xa7* fragment on F_4 line. M, 1 kb DNA Ladder (Fermentas); 1, pJET1.2-F_4. C. PCR product of 300-400 bp amplified from the recombinant plasmid, indicating the presence of insert with expected size. M, 100 bp DNA ladder; 1, F_4 and F_6.

Relative quantitation of gene expression

Real-time (RT)-PCR analysis displays a comparative of the cycle's threshold (Ct) which is known as a relative quantitation method to differentiate the expression level by comparing the Ct value of the samples with a calibrator (housekeeping genes) (Applied Biosystems 2006). In real-time qRT-PCR, baseline is defined as PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument. Threshold, as an arbitrary level of fluorescence chosen on the basis of baseline variability can be adjusted (Heid et al. 1996). The threshold level for the signal baseline on RT-PCR) was established on 1000 CF RFU, and it showed a good quality and quantitative gene expression of the *Xa7* in this study (Fig. 4).

To confirm the expression patterns of *Xa7* gene on the local Indonesia varieties and the progenies, quantitative real-time RT-PCR was performed. Quantitative real-time RT-PCR analysis which is represented as the ratio between Parekaligolara together with the lines and IRBB7 (the control positive for *Xa7* gene) which was calculated after normalization with elongation factor-1-α, is shown in Table 2. Elongation factor 1-α was used as an internal control as it exists on rice genome. The expression pattern of the *Xa7* gene was preferentially higher expressed in the local Indonesian variety Parekaligolara ($2^{-\Delta\Delta Ct}$:1.23) than in the control variety, IRBB7 possessing *Xa7* allele ($2^{-\Delta\Delta Ct}$:1). Among lines, the two resistant lines to BLB, F_4 and F_6 showed a higher fold-change than the other susceptible ones as expected. Line F_6 ($2^{-\Delta\Delta Ct}$:2.143) displayed a higher fold-change than F_4 ($2^{-\Delta\Delta Ct}$:1.14869). Thus, it is worth noting that the *Xa7* is expressed at higher level in the resistant lines than that of the susceptible ones.

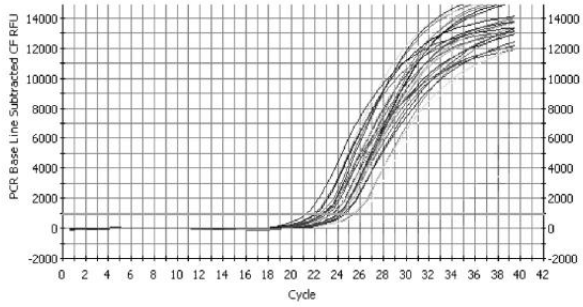


Fig. 4. Quantitative real time RT-PCR analysis of *Xa7* segment on total lines and control varieties (Parekaligolara and IRBB7) using SYBR Green detection.

Discussion

A number of rice varieties in tropical countries have been explored as a means to increase the yield to achieve rice self-sufficiency. However, the released varieties in Indonesia are still seriously damaged by BLB (Utami et al. 2010). Thus, improved BLB resistance carried by parents is crucial to realize the potential of high-yielding rice. Furthermore, Indonesia is well known to have abundant rice germplasm especially local varieties that already widely grown in the tropics. Exploration of local rice varieties with high resistance to BLB could help improve potential genetic materials and greater degree of genetic diversity in rice breeding pro-

Table 2. Quantitative real-time RT-PCR results for *Xa7* gene in Indonesia local rice varieties and the F_2 progenies from double crosse of IR54/Parekaligolara//Bio110/Markuti

Lines/varieties	*Ct of target	Housekeeping genes Ct as a normalizer	Δ Ct	$\Delta\Delta$ Ct	Ratio ($2^{-\Delta\Delta Ct}$)
Parekaligolara	35.9	23.8	12.1	0.3	1.231±0.013
IRBB7	35.7	23.9	11.8	0	1±0.011
F1	34.9	23.9	11	-0.8	0.574±0.023
F2	35.1	23.9	11.2	-0.6	0.659±0.031
F3	35	23.9	11.1	-0.7	0.615±0.009
F4	35.4	23.4	12	0.2	1.149±0.010
F5	34	22.6	11.4	-0.4	0.758±0.022
F6	36	23.1	12.9	1.1	2.143±0.008
F7	35.1	24	11.1	-0.7	0.615±0.017
F8	34.8	23.8	11	-0.8	0.574±0.014

*Ct = cycle threshold number, $\Delta\Delta$Ct = difference in cycle number between the sample and the standard (IRBB7), $2^{-\Delta\Delta Ct}$ Ratio was calculated after normalization with the housekeeping gene.

grams to reduce the vulnerability to the disease. In this study, we investigated the gene expression of the resistant gene *Xa7* found in Indonesian local varieties and screened F_2 lines harboring the gene. The tracked specific *Xa7* allele in Parekaligolara (Utami et al. 2010) enables early selection of desirable genes in a breeding population.

The broad BLB disease transmission is quite extensive which shows that races IV and VIII are known to dominate the attack of the BLB on rice in Indonesia (Suparyono et al. 2004). This effort is a part of Indonesian local rice germplasm conservation, as its potency has been deteriorated by using superior varieties (Crowder 1997). Phenotypic characterization of the F_2 segregating lines from the double crosses inoculated with of the *Xoo*-IV race showed transgressive lines, especially in comparison to Parekaligolara, one of the parental, an Indonesian local varietyies. Interestingly, the *Xa7* allele of the resistant lines (F_4 and F_6) was more similar to the allele of Parekaligolara. This was evidence of the frequency distribution of resistant and susceptible F_2 plants inoculated with *Xoo*-IV. The *Xa7* previously detected in IRBB7 variety has been resistant to PX086 (Porter et al. 2003) and a number of *Xoo* isolates from IRRI may contribute to the resistance phenotype in concert with the other *Xa* genes introgressed in the population, leading to a large number of plants expressing resistant response against specific races of *Xoo*.

Previous studies showed that the resistance gene to BLB, *Xa7* was durable and not easily broken down by several pathotypes of dominant BLB existed in several endemic areas in Indonesia (Suwarno et al. 2004). A variation of *Xa7* alleles found in Parekaligolara variety contributes to the resistance to BLB of races IV and VIII in the generative stage and in field conditions. Significant markers of *Xa7*-SNP8 and *Xa7*-SNP11 associated with the LD map of *Xa7* gene were located at 28.05-28.1 Mb of chr. 6 in the rice genome. These results were in accordance with the results of the *Xa7* genes mapping by Chen et al. (2008) which were mapped in contig AP006056, at 27.953.543- 28.132.662 bp.

Based on homology analysis, this *Xa7* gene in this study belongs to one of defense genes involved in immunity to leaf blight (Kump et al. 2011). Importantly, this *Xa7* is dominant gene providing resistance to *Xoo* race with broad spectrum (Zhang et al. 2006), particularly Indonesian *Xoo* races. The insertion-deletion allele variation was found in the 300 bp amplicon of co-segregation marker of RM20589 in the Indonesian local rice variety, Parekaligolara (Indica, 15141) (Utami et al. 2010). The variation was different from *Xa7* allele variation in the isogenic lines of *Xa7* gene, IRBB7, demonstrating that a novel *Xa7* allele existed in Indonesian local rice. This current data conclusively determines that this *Xa7* gene is involved in BLB resistance to specific *Xoo* races in Indonesia. Genetic studies on resistance to BLB have been conducted mainly in several countries, in particular *Xa* genes which have been identified using *Xoo* isolates from different origins. Therefore, resistant genes to BLB identified at different sites can not be compared to each other. From this point, the tester varieties and *Xoo* isolates are important for identification of genes, including *Xa7*.

Quantitative RT-PCR proved that *Xa7* genes were up-regulated in resistant rice plants, in contrast to the susceptible ones which were down-regulated. The higher expression of *Xa7* gene in resistant plants may indicate that this allele has high association with BLB resistance. In additional to Parekaligolara as a potent Indonesian local variety resistant to BLB, the two lines (F_4 and F_6) could be a prospective genetic material assisting rice breeding programs for BLB. This *Xa7* gene expression study here provided an important clue for guiding further functional studies of BLB resistance mechanism in rice. A single mutation of *Xa7* gene found between Parekaligolara and other varieties is able to detect lines with alleles belonging to Parekaligolara and shows a greater BLB resistance. It demonstrates that real-time RT-PCR could provide a rapid, sensitive, and easier method for analyzing quantitative gene expression of *Xa7* among Parekaligolara, IRBB7, and the lines. In addition, the technique is able to analyze a differential gene expression that could be useful for this study.

The functional molecular marker specific for *Xa7* gene with high expression contributing to rapid evaluation of resistance phenotype could be a marker-aided analysis and has enabled detection of potential new resistance breeding lines to BLB. Some rice plants that might carry genes other than *Xa7* could be detected with other molecular markers for different *Xa* genes, as some *Xa* genes are closely linked (Perez et al. 2008). Previous studies proved that pyramiding these dominant *Xa* genes was a useful approach for improving BLB resistance in hybrid rice (Zhang et al. 2006). Combining *Xa* genes such as *Xa7* with *Xa5* (Wang et al. 2005), *Xa7*+*Xa4*+*Xa21* (Du and Loan 2007), as well as *Xa7* and *Xa21* (Zhang et al. 2006) showed a higher resistance to BLB. This study illustrates that markers developed from certain double crosses may not necessarily be linked to *Xa7* as target gene in different genotypes. However, the use of markers linked to loci controlling phenotypic (traits) must be implemented for the benefit of using diagnostic alleles of resistance genes that would essentially guarantee the selection of plants with appropriate phenotypes. By association test, the functional marker for the *Xa7* gene was validated for broader application to BLB resistance evaluation on rice germplasm.

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