

# MOLECULAR DETECTION OF RESISTANCE TO BACTERIAL LEAF BLIGHT ON CONDE INDONESIAN RICE VARIETY

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## Abstract

Rice bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) affected grain yield and decreasing rice production in rice growing countries. Conde, Indonesian rice variety, exhibits high resistance to most of the Indonesian races of (BLB) and has been used in Indonesia for cultivated rice. This study was aimed to conduct the molecular detection between proximal markers in chromosome 6 and relative expression of Conde rice variety compare to IRBB7 in *Xa7* region. The population screening, BLB evaluation and molecular detection around the *Xa7* region were conducted. The results showed from the collection of individual recombinants between resistant and susceptible parents narrow the region containing the BTBPOZ domain. The sequence alignment of *Xa7LD37* in two resistant and three susceptible cultivars demonstrated a perfect association. The sequence alignment in exon region of *Loc\_Os06g46240* in Nipponbare, IRBB7, and IR64 identified indel/SNPs in this region leading to nucleotide substitution and frameshift resulting in amino acid change between resistant and susceptible cultivars. It was predicted that Conde revealed the similar gene action with *Xa7* gene for BLB that encodes a *BTB POZ domain*.

**Keywords:** BTB/POZ domain, dominant gene, *Oryza sativa*, *Xanthomonas oryzae* pv. *oryzae*, *Xa7*

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## Introduction

BLB is extensively damaging disease particularly in tropical irrigated lowland rice environment under suitable condition. Yield losses was estimated at about 15% and up to 80% on severely infected field when the disease infected rice at maximum tillering stage (Sudir *et al.*, 2012). At present, most of the existing commercial rice varieties are susceptible to the existing *Xoo* pathotypes, causing damage to the rice production in the country. Triny (2009) reported that damage due to severe BLB attacks on IR64 and Ciherang varieties. Even though these varieties were released in 1986 and 2000 respectively as a BLB resistance crop however they have been broken down since 2006/2007.

Recently, the availability of molecular markers linked to genes for resistance to this disease has shown tremendous potential and would accelerate breeding programs,

especially when other traits are also being evaluated as in pyramiding gene approach. PCR-based markers closely linked to BLB resistance genes would be very useful for efficient marker-assisted breeding selection.

There are over 42 resistance genes to *X. oryzae* pv. *oryzae* (Busungu *et al.*, 2016). Seven of which have been cloned (*Xa1*, *Xa3*, *xa5*, *Xa10*, *xa13*, *Xa21*, *Xa26*, and *Xa27*) (Song *et al.*, 1995; Yoshimura *et al.*, 1998; Iyer and McCouch 2004; Tian *et al.*, 2014; Sun *et al.*, 2004; Gu *et al.*, 2005; Chu *et al.*, 2006; Xiang *et al.*, 2006; Gu *et al.*, 2004). *Xa1* encodes a member of the NBS-LRR class, *xa5* encodes the gamma subunit of transcription factor IIA (TFIIA $\gamma$ ). *Xa3*, *Xa21* and *Xa26* encode NBS-LRR proteins containing a protein kinase domain. *Xa10* encodes ER membrane protein. *xa13* confers resistance to the *Xoo* strains encoding PthXo1 effector (Antony *et al.*, 2010). *Xa27* encodes an apoplast protein of 113 amino acids (Wu *et al.*, 2008).

*Xa7* is a dominant resistance gene and was originally identified in rice cultivar DV85 (Sidhu *et al.*, 1978). The near isogenic line IRBB7 (containing *Xa7* gene) was developed by crossing DV85 to the susceptible cultivar IR24 (Ogawa *et al.*, 1991). *Xa7* is a potential source of durable and broad resistance to BLB (Vera Cruz *et al.*, 2000).

Recently, the *Xa7* gene has not been isolated, but the avirulence (Avr) gene *AvrXa7* corresponding to resistance gene *Xa7* has been cloned (Yang *et al.*, 2000). *Xa7* was previously located in a region between two markers M1 (2.2 cM) and M3 (0.5 cM) of chromosome 6 (Porter *et al.*, 2003). Chen *et al.*, (2008) further integrated to the region between two proximal markers GDSSR02 and RM20593, an interval of approximate 118.5 kb. Zhang *et al.*, (2009) had constructed the contig covering the genomic region between two markers RM20576 in contig OSJNBa0032M1 and MY4 in contig B1153E06, an interval of approximately 200 kb.

A previous report on the development of BLB resistance was conducted by Bustamam *et al.* (2002). They have already developed new lowland rice varieties using marker-assisted selection approach and nationally released as Angke variety containing *xa5+Xa4* gene derived from a cross between IRBB5 and IR64 cultivar and Conde rice variety containing *Xa7+Xa4* gene derived from a cross between IRBB7 and IR64 cultivar. They were named after the river's name in West Java and in Yogyakarta, respectively.

The aim of this research was to conduct molecular detection between proximal markers of *Xa7* region in chromosome 6 from the previously reported region and relative expression of Conde rice variety compare to near isogenic line IRBB7.

## Materials and Methods

**Population Screening.** A screen of approximately 869 BC<sub>1:2</sub>F<sub>2</sub> individuals from a cross between susceptible rice varieties of Ciherang, Inpari 13 and Nipponbare and resistant rice variety Conde. Total genomic DNA was extracted using young leaves collected from seedling stage as described by Dellaporta *et al.*, (1983). All 35 primers were amplified using Biorad Thermal Cycler PCR machine by following PCR conditions: (i) an

initial denaturation step of 2 min at 94 °C, (ii) 30 cycles of 45 second at 94 °C, 45 second at 55 °C (depend on the primer annealing), 1 min at 72 °C and (iii) a final extension step for 5 min at 72°C and run on 8% polyacrylamide electrophoresis gels and silver-stained. Seeds were planted and inoculated with BLB pathogen at 55 to 60 days after planting, and analyzed for phenotyping.

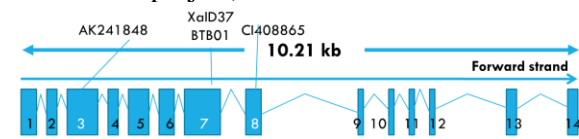
**Bacterial growth and inoculation.** *X. oryzae* pv. *oryzae* Race IV was cultured on media containing 20 g of sucrose, 5 g of peptone, 0.5 g Ca(NO<sub>3</sub>)<sub>2</sub>, 0.43 g Na<sub>2</sub>HPO<sub>4</sub>, and 0.05 g FeSO<sub>4</sub> per liter and allowed to grow at 28°C to 30°C for 3 to 4 days. Bacteria was collected into sterile distilled water and adjusted to a concentration of an optical density at 600 nm =1. For phenotyping, the plants were inoculated by the leaf-clipping method (Kauffman *et al.*, 1973) 55 to 65 days after planting and scored 14 days after inoculation. The symptoms were scored as resistant if the average attack intensity is 6% or less (score 1 & 2) and as susceptible if average attack intensity is greater than 6% (score 3 to 9) (IRRI, 1996).

**Molecular Detection.** Based on the previous report of *Xa7* gene, Porter *et al.*, (2003) identified three Bacterial Artificial Chromosome (BAC) clone sequences of *O. sativa* cv. Nipponbare, B1472F02, P0778G11 and B1153E06. Chen *et al.* (2008) employed twelve SSR markers on chromosome 6 that are linked to *Xa7*. Zhang *et al.* (2009) narrowed the *Xa7* gene to an approximately 200-kb segment in the subtelomeric region of chromosome 6. In addition, in this study, eight anchored primers and 15 primers pair based on DNA sequence around the *Xa7* region and were developed in the target region through bioinformatics analysis using the reference genome sequences of two subspecies, i.e. japonica (cv. Nipponbare; and indica (cv.93–11) (<http://www.gramene.org>) on chromosome 6 corresponding to the gene annotation released by the TIGR Rice Genome Annotation Version 4 (Figure 1).

**RT-PCR and qRT PCR.** Leaves tissue was collected from susceptible IR24 and its resistant IRBB7 and Conde variety at 2, 4, 8, 24, and 48 h after inoculation in two separate experiments. Uninoculated plants were used as



**Molecular Detection.** The graphical genotype analysis showed the significance of primer Xa7LD37 (2.6;  $pval=2.7 \times 10^{-3}$ ,  $R^2=0.86$ ). It was estimated the region located on BAC clone P0778G11 on *Loc\_Os06g46240* in region 28007285-28017490 bp (10.2 kb) on rice genome (Figure 3). It was known as BTB/POZ domain-containing protein, putative, expressed. The gene structure of *Loc\_Os06g46240* contained 14 exons, transcript length 3345 bps and translation length 1114 bp (MSU Rice Genome Annotation project).

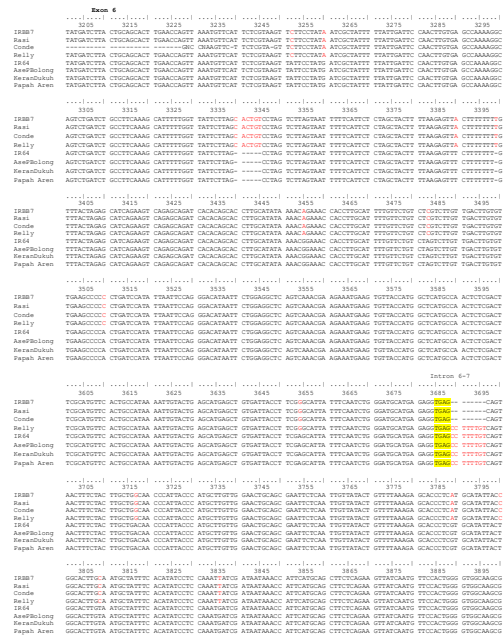


**Figure 3.** The gene structure of *Loc\_Os06g46240* (MSU Rice Genome Annotation project).

To further explore the association between this sequence polymorphism (genotype) and the BLB evaluation (phenotype) in local varieties, the sequenced varieties are listed in Table 1. The sequence of Xa7LD37 primer in the region of BTB/POZ domain revealed nucleotide substitutions in two resistant (Rasi and Relly) and 3 susceptible accessions (Ase Pulu Bolong, Keran Dukuh, and Ketan Papah Aren). The result showed two resistant haplotype conserved across all 2 resistant lines and three haplotypes among the 3 susceptible accessions. The Nipponbare and Ase Pulu Bolong contained the same susceptible haplotype as IR64 and other haplotype was Keran Dukuh and Ketan Papah Aren while Rasi the same resistant haplotype as IRBB7 and other haplotype was Relly (Figure 4).

**Table 1.** List of Indonesian local rice germplasm used in research.

No.	Accs. No.	Accs Name	Origin	Xoo (Race IV)	Haplotype
1	10221	Ase Pulu Bolong	South Sulawesi	S	S1
2	20514	Karang Dukuh	Central Kalimantan	S	S2
3	5205	Ketan Papah Aren	Gunungkidu 1, DIY	S	S3
4	4176	Rasi	Aceh	R	R1
5	3983	Relly	West Kalimantan	R	R2



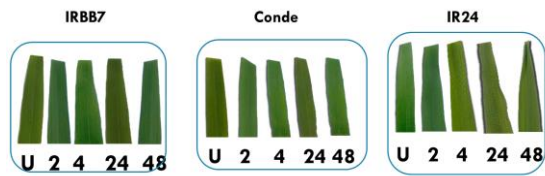
**Figure 4.** Sequence alignment of IRBB7, Conde, and two Indonesian rice germplasm (Rasi and Relly) as resistant accession and IR64 and three Indonesian rice germplasm Ase Pulu Bolong, Keran Dukuh, Papah Aren as susceptible accession using primer Xa7LD37 identified the SNPs difference between the resistant and susceptible cultivars. Xa7LD37 lied in region of Exon 6 and Intron 6-7 of *Loc\_Os06g46240*.

To investigate the possible function of the polymorphisms in the *Loc\_Os06g46240*. Two possible exons were chosen. The first exon of *AK241848* was in exon 3 while the second exon was *C140886* in exon 8. The first and second exon were analyzed by RT-PCR and qRT-PCR.

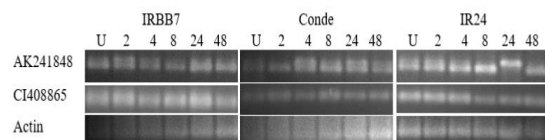
**Relative expression analysis.** The expression patterns of *AK241848* and *C140886* were compared to susceptible and resistant plants, with and without inoculation with race IV of *Xoo*. RT-PCR analysis showed that transcripts from *AK241848* and *C140886* were detectable in resistant and susceptible cultivars at all time points (Figure 5 and 6). The resistant line IRBB7 and Conde, *C140886* showed high intensity in IRBB7 rather than in Conde at all time points while in IR24 the expression was higher in 2h and 4h PI rather than 8h, 24h, and 48h PI. In *AK241848* the expression in IRBB7



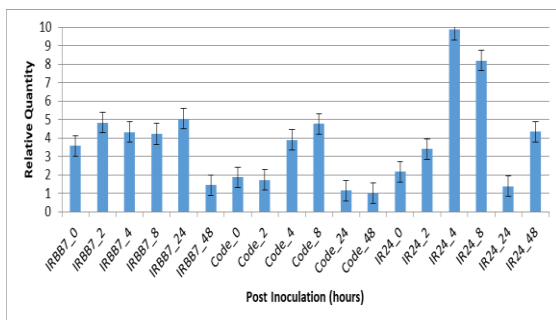
and Conde was higher in 4h, 8h, 24h and decrease in 48h while in susceptible line IR24 the expression was high in all time points. Then, the analysis of *AK241848* was pursued for relative expression analysis using qRT-PCR (Figure 7).



**Figure 5.** Leaf phenotype of IRBB7, Conde and IR24 as an example showing reaction to *Xoo* Race IV. Leaf 1, uninoculated plants; leaf 2, 2 h postinoculation (PI) with *Xoo* race IV; leaf 3, 4 h PI; leaf 4, 8 h PI; leaf 5, 24 h PI; and leaf 6, 48 h PI. Leaves were photographed hours after inoculation.



**Figure 6.** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *AK241848* and *CI40886*. The experiment was repeated two times, and typical results are shown. Lane 1, uninoculated plants; lane 2, 2 h postinoculation (PI) with *Xoo* race IV; lane 3, 4 h PI; lane 4, 8 h PI; lane 5, 24 h PI; and lane 6, 48 h PI.



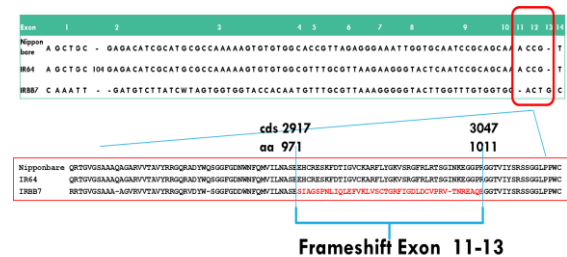
**Figure 7.** The histogram of qRT-PCR analysis of *AK241848*. The experiment was repeated three times, 0: uninoculated plants; 2: 2 h postinoculation (PI) with *Xoo* race IV, 4: 4 h PI; 8: 8 h PI; 24: 24 h PI; and 48: 48 h PI.

In the resistant line of IRBB7, similar expression of 2,4,8, and 24h PI and decreased in 48h PI while in variety of Conde the relative expression increased in 4 h and 8 h PI and

decreased in 24 h and 48h PI. In the susceptible line of IR24, the relative expression increased in 4h and 8h PI, however, it decreased in 24h PI and continued increasing in 48h PI. The expression of IRBB7 was stable rather than that of Conde. Although Conde giving similar pattern (increasing in 4h and 8h PI) with IR24, however, the relative expression was lower in Conde (2 times) rather than IR24 (5 times) (Figure 7).

### Sequence alignment of *Loc\_Os06g46240*.

The start codon, splice junctions, and 3' UTR of the *Loc\_Os06g46240* of Nipponbare to IR64 (susceptible) BAC and aligned to IRBB7 (resistant). This identified ~78 nucleotide substitutions resulting in an amino acid substitution, in *AK241848* from Arginine to glutamic acid to glutamine in the resistant cultivar while in *CI40886* from valine to glycine and glycine to serin, a significant change from a hydrophobic to semi-hydrophilic amino acid. The deletion of A in resistant cultivar leading to frameshift at the end of exon 11, the whole part of exon 12 and at the beginning exon 13, from 971 to 1011 amino acid. There is 104 bp deletion in resistant cultivar in exon 1, however, the deletion was identical in Nipponbare (susceptible) and IRBB7 (resistant), indicating that they do not play a role in *Xa7* resistance (Figure 8).



**Figure 8.** Sequence alignment of indel/SNPs in exon region of *Loc\_Os06g46240* in Nipponbare, IRBB7, and IR64 leading to amino acid substitution and frameshift.

### Discussion

The resistance gene that identified in Conde variety was located on the long arm of chromosome 6. In this region, two dominant genes (*Xa7* and *Xa27*) (Sidhu *et al.*, 1978; Gu *et al.*, 2004) and recessive gene *xa33(t)* (Korinsak *et al.*, 2009) have been reported.

Even though *Xa7* and *xa33(t)* shared commonly linked markers, however, *Xa7* and *xa33(t)* had shown different gene actions and specificity in responding to BLB isolates. *Xa27* was reportedly located between RFLP markers RG424-RG162 (70.4-104.6 cM, Cornell map) on the long arm of chromosome 6, which was about 22.1 cM away from *xa33(t)* and *Xa7*. In the *xa33(t)*, the segregation ratio was 1R:3S in which gene governing the resistance acted as recessive.

Bric-a-brac, Tramtrack and Broad Complex/Pox virus and Zinc finger (BTB/POZ) domain that is known to mediate both homo- and heterodimerization (Bardwell *et al.*, 1994; Zollman *et al.*, 1994). The *Arabidopsis* genome encodes at least seventy-six BTB domain proteins that can be classified into eleven major families according to their domain architecture (Dieterie *et al.*, 2004). BTB proteins seem to be involved in a broad range of processes, such as systemic acquired resistance (Cao *et al.*, 1997) and targeted proteolysis (Pintard *et al.*, 2003; Figueroa *et al.*, 2005). Jiang *et al.* (2006) reported the molecular characterization and functional investigation on OsBTB gene induced by *Magnaporthe grisea* in Rice blast disease.

Based on some experimental observations, in this report, it was predicted that Conde rice variety revealed the similar gene action with *Xa7* gene for BLB that encodes a *BTB POZ domain*. First, a collection of individual recombinants between resistant (Conde) and susceptible parents (Ciherang, Inpari 13 and Nipponbare) allowed to narrow the *Xa7* region in resistant rice variety (IRBB7 and Conde) to an approximately 10.2-kb interval containing the BTBPOZ domain. Second, sequencing alignment of *Xa7* gene using Xa7LD37 primer in two resistant (Rasi and Relly) and three susceptible accessions (Ase Pulu Bolong, Keran Dukuh, and Ketan Papah Aren). demonstrated a perfect association between the haplotype and resistance to race IV of *Xoo*. Third, sequence alignment in exon region of *Loc\_Os06g46240* in Nipponbare, IRBB7, and IR64 cultivars identified indel/SNPs in this region leading to nucleotide substitution and frameshift that resulting in amino acid change between resistant and susceptible cultivars.

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