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SHORT COMMUNICATION

# Functional markers for bacterial blight resistance gene *Xa3* in rice

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**Abstract** Bacterial blight (BB) of rice (*Oryza sativa* L.) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a destructive disease in rice worldwide. *Xa3*, a gene conferring resistance to BB at the booting stage of the rice plant, has been characterized previously using map-based cloning. We cloned and sequenced the *Xa3/xa3* gene in the Korean cultivars Hwayeong, Ilmi, and Goun and conferred resistance or susceptibility to BB. We detected polymorphisms, and polymerase chain reaction-based functional markers were developed based on the single nucleotide polymorphism from the *Xa3* and *xa3* nucleotide sequence. Susceptible or resistant individuals from an F2 population developed from a cross between Milyang 244 and Ilmi, near-isogenic lines carrying BB resistance genes, were screened with functional markers. The BB3-RF and BB3-RR primers consistently amplified a resistance-specific fragment of 255 bp only in resistant plants, whereas the BB3-SF and BB3-SR primers were specific to susceptible plants. Genotyping results were co-segregated with phenotype by conducting the BB resistance test with the $K_3$ race. These markers could be effective for marker-assisted selection of the *Xa3* gene in rice breeding programs.

**Keywords** *Oryza sativa* · Bacterial blight · *Xa3* · PCR-based marker

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Rice (*Oryza sativa* L.) is an important cereal crop that supplies food for the world population, particularly in Asia. Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a destructive disease in rice. The disease may cause tiller wilting, resulting in yield loss. Disease resistance (*R*) genes and resistance quantitative trait loci that may regulate the *Xoo* resistance have been identified. Many attempts have been made to develop improved rice cultivars with BB resistance genes. Pyramiding different resistance genes in a one-crop cultivar has been attempted (Liu et al. 2000), and the pyramided lines showed durable resistance compared with that in a single gene in plants. Similarly, rice plants with various combination of BB resistance with various BB resistance genes showed increased resistance ability (Gnanamanickam et al. 1999; Sanchez et al. 2000). The *Xa3* and *xa3* nucleotide sequences have been identified (Sun et al. 2004; Xiang et al. 2006) and the genetic background that

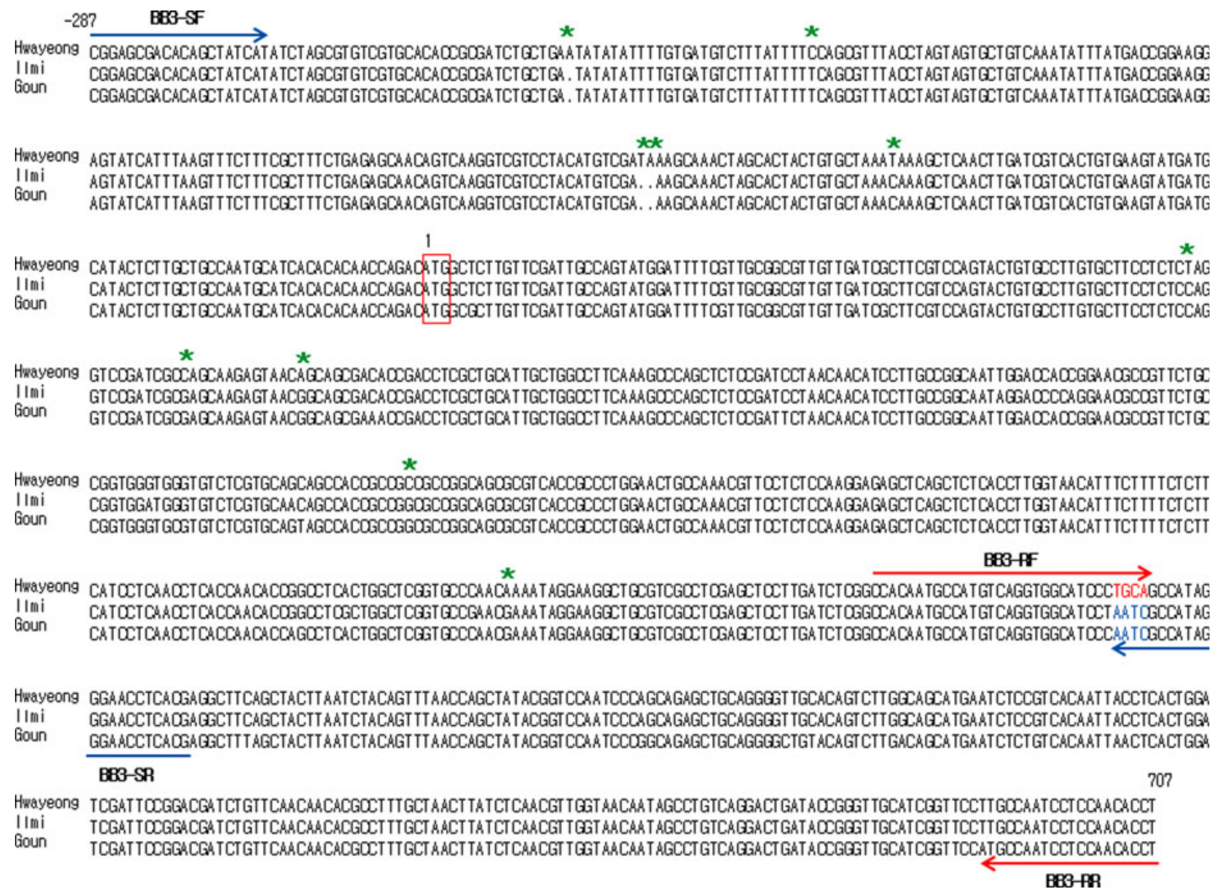


**Fig. 1** Sequence alignment of the *Xa3* gene amplified from Hwayeong, Ilmi, and Goun. Their positions are indicated above the sequences. The *red box* denotes the start codon location (ATG) and the *green asterisk* represents SNPs in Hwayeong corresponding to Ilmi and Goun. *Arrows* indicate the location of the susceptible allele (*blue*) and resistant allele (*red*) primers, respectively. (Color figure online)

affects the resistance spectrum and resistance level of *Xa3*/*Xa26* has been reported (Cao et al. 2007; Zhou et al. 2009). The genomic sequence of the *Xa3* gene on chromosome 11 (GenBank Accession No. DQ426646) of *japonica* rice (*Oryza sativa* ssp. *japonica* cultivar Nipponbare) was retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov). We designed four gene-specific polymerase chain reaction (PCR) primer pairs to amplify PCR fragments of the *Xa3* gene from Hwayeong, Ilmi, and Goun. Multiple sequence alignment of the *Xa3* alleles among Korean rice varieties and Nipponbare has been performed using the CLUSTALW program.

We compared the genomic sequences and showed that the resistant cultivar Hwayeong has the TGCA sequence at 456 bp from the start codon, whereas the susceptible cultivars Ilmi, Goun, and Nipponbare have the AATC sequence at the same site. This result was consistent with a previous study (Xiang et al. 2006) (Fig. 1). A sequence analysis showed that both the TGCA and AATC polymorphisms were independent of the *indica*–*japonica* classification. *Xa3* is involved in the receptor-like kinases, which contain an extracellular leucine-rich repeat (LRR) and an intracellular serine–threonine kinase domain. The LRR sequence of the LRR-containing R proteins is the major determinant of pathogen recognition (Dangle and Jones 2001) and produced differences between the resistant and susceptible proteins (Xiang et al. 2006). Therefore, the difference in this region could be the single nucleotide polymorphism (SNP) region for developing a functional marker. We designed functional markers (BB3-SR and BB3-RF) in which the 3′ terminal nucleotides corresponded to the SNP regions

**Table 1** Primer sequence information on functional markers developed in this study

| Primer | Product size (bp) | Annealing temperature used for PCR (°C) |
|---|---|---|
| BB3-SF: CGG AGC GAC ACA GCT ATC AT | 743 | 60 |
| BB3-SR: CGT GAG GTT CCC TAT GGC GAT T | | |
| BB3-RF: CCA CAA TGC CAT GTC AGG TGG CAT CCC TGC A | 255 | 55 |
| BB3-RR: AGG TGT TGG AGG ATT GGC AT | | |

(Table 1). BB3-SF was located in the upstream promoter region of the *Xa3* cDNA, and the BB3-RR primer was within exon 1 (Fig. 1). BB3-SF and BB3-SR primer-specific amplification showed a 743-bp fragment only in the susceptible genotypes, whereas BB3-RF and BB3-RR primers provided a 255-bp fragment in resistant genotypes. Rice genomic DNA was extracted from leaves using the modified CTAB method of Chen and Ronald (1999). Genomic DNA concentration and quality was checked by Nanodrop (Nanodrop Co., Wilmington, DE, USA). PCR amplification of the *Xa3* and *xa3* genes for testing PCR-based functional markers was performed under the following cycle conditions: 95 °C for 5 min, 40 cycles of (95 °C for 30 s, $T_a$ for 30 s, and 72 °C 1 min), and 72 °C for 7 min. The annealing temperatures $T_a$ for each primer pair are listed in Table 1. The PCR products were resolved on a 3 % agarose gel stained with ethidium bromide.

To confirm the functional markers, we screened the F2 population derived from a cross between Milyang 244 and Ilmi (Fig. 2). Milyang 244, which contains the resistant *Xa3* allele from Hwayeong, showed the resistance phenotype to BB, whereas Ilmi was highly susceptible to BB. The F2 population between Milyang 244 and Ilmi was developed using a marker-assisted strategy. The F2 population was examined for resistance by inoculating with *Xoo*. *Xoo* was grown on nutrient agar or WFP media as described previously (Iyer-Pascuzzi and McCouch 2007). Plants were inoculated by the leaf-clipping method (Kauffman et al. 1973), and the plant reactions to the pathogen were checked after 3 weeks. PCR results were co-segregated with phenotype by assaying the $K_3$ race with the BB resistance test (data not shown). The resistant phenotypic lines showed a 225-bp PCR-amplified fragment or a heterozygote (showing both the bands), whereas a single 743-bp fragment from the BB3-S primers was detected in the susceptible phenotypic lines.

Near-isogenic lines (NILs) with a different BB resistance gene in an IR24 background were selected from the International Rice Research Institute to test for marker–phenotype association. Eight NILs, IRBB1 (*Xa1*), IRBB3 (*Xa3*), IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB10 (*Xa10*), IRBB13 (*xa13*), and IRBB21 (*Xa21*), and IR24 were used as a susceptibility confirmation, and those carrying *Xa3* or other *R* genes against *Xoo* were genotyped with functional markers (Fig. 3). All other NILs except IRBB3 showed the susceptible genotype. IRBB3 has

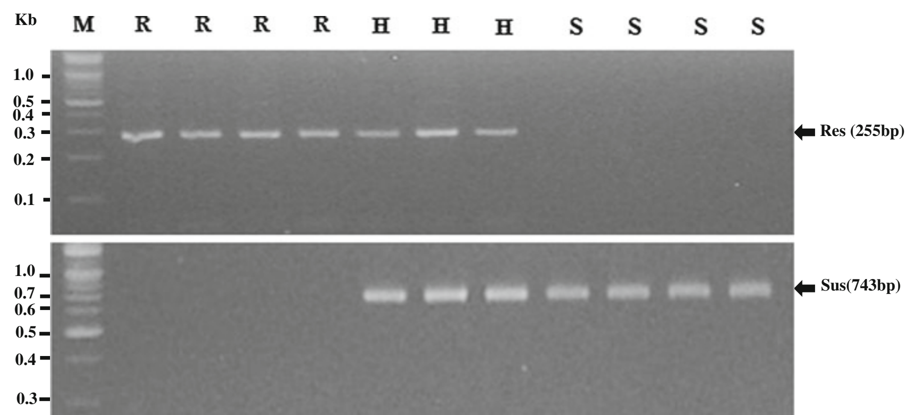


**Fig. 2** PCR amplification of F2 population derived from a cross between Milyang 244 and Ilmi with primers BB3-RF, BB3-RR (*top*) and with primers BB3-RF, BB3-RR (*bottom*). PCR amplicons were visualized on a 3 % agarose gel stained with ethidium bromide. *M* size marker, *S* susceptible allele, *R* resistant allele, *H* heterozygote, *Sus* susceptible allele, *Res* resistant allele

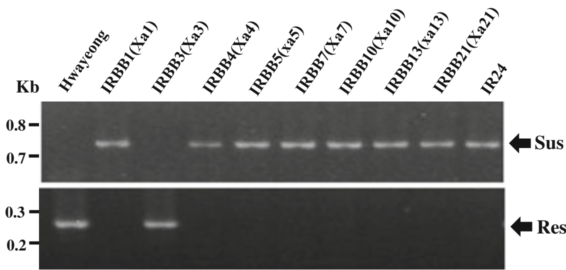


**Fig. 3** PCR amplification of near-isogenic lines with a different BB resistance gene using functional markers. *Sus* susceptible allele, *Res* resistant allele

the *Xa3* gene (Lee et al. 2003). This result suggests that *Xa3* functional markers could detect the *Xa3* or *xa3* gene, particularly among various genes for BB resistance. Gene pyramiding has been applied to increase the resistance level against *Xoo* (Yoshimura et al. 1995; Perumalsamy et al. 2010). However, pyramiding the *R* genes, including the *Xa3* gene, in one rice cultivar has not been attempted yet. Rice breeders could use functional markers to develop pyramided lines with the *Xa3* gene. This finding suggests that functional markers are a valuable tool in screening for *Xa3*-resistant rice cultivars. We also tested 80 Korean germplasms using the functional markers and classified 25 resistant-type alleles and 55 susceptible-type alleles (Supplementary Table 1). The use of functional markers is expected to contribute to directly identifying genetic diversity at the DNA level and to overcome the problem of recombination/linkage, and can be used for marker-assisted selection to improve crops (Andersen and Lübberstedt 2003). Non-synonymous SNPs result in amino acid sequence changes within the coding regions (Sunyaev et al. 1999) and these SNPs modify RNA splicing, resulting in phenotypic differences (Richard and Beckmann 1995). The functional markers designed at polymorphic sites in the gene sequences control phenotypic changes. The recent cloning of several agronomically important genes has facilitated the development of functional markers. Functional markers for identifying BB resistance genes have been developed. *xa5*, a recessive gene to BB, was identified and developed using a cleaved amplified polymorphic sequence marker based on a two-nucleotide SNP (Iyer-Pascuzzi and McCouch 2007). PCR-based sequence-tagged site markers, which were designed around the 48-base-pair deletion of the resistant allele *Xa38*, have been recently reported (Bhasin et al. 2011). These markers reside within the target genes themselves and can be used with great reliability to identify favorable alleles such as disease-resistant alleles in a breeding program. Additionally, these markers can be easily converted for use in a high-throughput system via the Illumina genotyping system.

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