Identifying a source of a bacterial blight resistance gene *xa5* in rice variety 'IR62266' and development of a functional marker 'PAxa5', the easy agarose based detection

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ABSTRACT

Bacterial blight (BB) caused by *Xanthomonas oryzae pv. oryzae (Xoo)* is one of the most important diseases in rice worldwide causing tremendous damage to rice yield in many countries. The use of resistant rice varieties is the most economical and effective method to manage and control this disease. A rice variety IR62266 developed by International Rice Research Institute (IRRI), was identified as a good genetic resource for broad spectrum BB resistance in Thailand. The genetic factor determining BB resistance was studied in the $F_{2:3}$ population developed from a cross between IR62266 and KDML105. A total of 190 $F_{2:3}$ individuals were challenged with three Thai *Xoo* isolates, TXO1, TXO2, and TXO5. The resistant reactions of the $F_{2:3}$ population showed the ratio of 3 susceptible: 1 resistant, thereby indicating a single recessive gene controlled BB resistance in IR62266. Genetic analysis indicated that RM122 was located on the short arm of chromosome 5 and was tightly linked to the resistance gene accounting for 66.03% of phenotypic variance. The RM122 was located very close to the cloned *xa5* gene, a broad spectrum BB resistance gene. Therefore, we developed PAxa5 as a new functional SNP marker for *xa5* and tested in the $F_{2:3}$ individuals. PAxa5 clearly differentiated the resistant and susceptible reactions in the population. PAxa5 was then validated in a wide range of germplasms and was found to clearly identify *xa5*. PAxa5 is currently implemented in the marker-assisted breeding program to facilitate the selection for the *xa5* gene.

Keywords: *xa5*; bacterial blight; rice; functional marker

INTRODUCTION

Bacterial blight (BB) is caused by the rod-shaped bacterium, *Xanthomonas oryzae pv. oryzae* (*Xoo*). The outbreak of this disease usually occurs in irrigated and rainfed lowland ecologies throughout Asia and worldwide. BB disease can cause yield loss typically ranging from 20-30% but in severe cases, it can cause as high as 80% yield reduction depending on rice growth stages, geographic locations or seasonal conditions (Singh *et al*., 1977; Ou, 1985). In Thailand, the prevalent of this disease was first reported in Pathum Thani Province in 1963 (Tabei and Eamchit, 1974). The most economical and effective approach to control BB is the use of resistant varieties. To date, more than 38 BB

resistance genes have been identified from cultivated rice and wild relatives (Niño-Lui *et al.*, 2006; Xia *et al*., 2012; Korinsak *et al*. 2009; Cheema *et al*., 2008). Fifteen of them were recessive resistance genes (*xa5,- xa5(t), xa8, xa9, xa13, xa15, xa19, xa20*, *xa24, xa26, xa28, xa31*, *xa32*, *xa33* and *xa34*) (Niño-Lui *et al*., 2006; Xia *et al*., 2012; Rao, 2003; Singh *et al*., 2007) and five of which (*xa15, xa19*, *xa20*, *xa26* and *xa28*) were induced by mutagenesis (Xia *et al*., 2012). Map-based cloning has been successfully employed for characterizing four dominant BB resistance genes (*Xa21, Xa1, Xa3/Xa26* and *Xa27*) and two recessive BB resistance genes (*xa5* and *xa13*) (Yoshimura *et al*., 1998; Iyer and McCouch, 2004; Song *et al*., 1995; Sun *et al*., 2004; Gu *et al*., 2005, Chu *et al*., 2006). *Xa1* resistance gene was tagged with RFLP marker XNpb125 and mapped to chromosome 4 (Yoshimura *et al*., 1996). The gene encodes a nucleotide-binding site leucine-rich repeat (NBS-LRR) protein (Yoshimura *et al*., 1998). *Xa21*, a broad spectrum BB resistance gene, was introgressed from *Oryza longistaminata* into *O. sativa* (Khush *et al*., 1990). The gene encodes for a receptor kinase domain carrying serine-threonine specificity (Song *et al*., 1995). STS marker pTA248 was developed from a tightly linked RFLP marker RG103 and has been used efficiently in marker-assisted selection (Ronald *et al*., 1992).

A recessive resistance gene, *xa5*, was first reported by Murty and Khush (1972). The R gene was mapped to the telomeric region on the short arm of chromosome 5. RFLP markers RG556, RG207, RZ390 and SSR markers RM122 and RM390 were closely linked to *xa5* gene (Yoshimura *et al.,* 1995; Blair and McCouch, 1997). Subsequently, *xa5* gene was positionally cloned and found to encode the gamma subunit of transcription factor IIA (TFIIAγ) (Blair *et al*.,2003; Iyer and McCouch, 2004). Later, the functional marker, CAPS, was developed and has been employed for *xa5* resistance gene detection (Iyer and McCouch, 2007).

The rice improvement via marker-based selection requires the identification of molecular markers tightly linked to genes of interest. Several major resistance genes against bacterial blight pathogen, *Xoo,* have been tagged by RFLP and RAPD markers (McCouch *et al*., 1992; Yoshimura *et al*., 1992). However, both types of markers still have limitations. Maker-assisted selection based on RFLP markers is laborious, time-consuming, costly, involving the use of radiochemicals whereas that based on RAPD marker always faces reproducibility problems. Rice microsatellite markers have been developed and mapped (Wu and Tanksley, 1993; Panaud *et al*., 1996). They offer several advantages to plant breeding program since they are PCR-based markers, which represent single-loci, and provide high levels of polymorphism (McCouch and Doerge, 1995). Other effective PCR-based markers are CAPS (Cleaved amplified polymorphic sequence) and bi-PASA (bi-directional PCR amplification of specific alleles) (Konieczny and Ausubel, 1993; Liu *et al*., 1997). They are co-dominant genetic markers, but CAPS requires restriction endonuclease digestion to detect single nucleotide polymorphisms (SNPs) in the region of interest whereas bi-PASA can be amplified and directly ran on agarose gel. Generally, marker-assisted selection requires markers tightly linked to the traits of interest and the markers should be cost effective and easy to use. The functional marker, bi-PASA, was considered to be a suitable and powerful tool for breeding durable resistance in rice breeding program.

In this study, we aimed to identify a BB resistance gene in the promising line ‘IR62266’ and subsequently develop a new SNP functional marker

based on agarose gel for marker-assisted selection in breeding programs.

MATERIALS AND METHODS

Plant materials

The identification of BB resistance was studied in the $F_{2:3}$ population consisting of 190 plants derived from the cross between IR62266 and KDML105. IR62266 is an improved lowland rice variety from IRRI. It was used as a genetic source for BB resistance. It had shown a broad spectrum resistance against Thai *Xoo* isolates (unpublished data). KDML105, a popular Thai aromatic cultivar with excellent cooking and eating quality, was used as a susceptible parent. It is very susceptible to all *Xoo* isolates.

Pathogenic assay of BB resistance

The assessment of BB resistance was conducted at seedling stage (21 days-old plant) using the leaf-clipping method (Kauffman *et al*., 1973). Three *Xoo* isolates, TXO1, TXO2, and TXO5, were collected from farmer's fields at Kampang Saen district, Nakhon Pathom and Chainat provinces during 2001-2002, respectively (Pattawatang, 2005). They were grown in peptone sucrose agar (PSA: 5g peptone, 20g sucrose and 15g agar adjust to 1 litre with dH_2O) for 72 hours at 28^{o}C. For the preparation of inoculum, the bacterial cells were then suspended in sterile water adjusted to 10^{9} CFU/ml. The cell suspension was inoculated to two fully expanded leaves of each plant of the $F_{2:3}$ using clipped method. The inoculated plants were incubated in the moisture chamber for 24 hours and kept in the greenhouse for 14 days. Lesion length (LL) was measured for all inoculated leaves. The $F_{2:3}$ plants were classified as resistant (R) when the lesion length (LL) was longer than 3 cm and as susceptible (S) when it was shorter than 3 cm (Chen *et al*., 2001).

DNA isolation and simple sequence repeat (SSR) analysis

Total genomic DNA from young leaves of the $F_{2:3}$ individuals and their parents were isolated using DNA trap kit® (DNA Technology Laboratory, Thailand). The SSR analysis was performed following the protocol described by Korinsak et al., (2009).

Identification of BB resistance

Bulked segregant analysis (Michelmore *et al*., 1991) was applied to identify the linkage relationship between markers and BB resistance gene. Eleven most resistant and nine most susceptible $F_{2:3}$ plants were selected based on their lesion lengths. They were genotyped with 34 SSR markers that were reported as closely linked markers to the known BB resistance genes (McCouch *et al*., 1992; Rao, 2003; Ronald *et al*., 1992; Yoshimura *et al*., 1992). All SSR markers were obtained from the Gramene database (http://www.gramene.org/). Two SSR markers, RM122 and RM153, that showed a clear difference between resistant and susceptible plants were used to genotype 190 $F_{2:3}$. The association between markers and LL was analyzed based on a simple linear regression model using STATGRAPHIC 2.1 program.

Functional marker development of *xa5:* PAxa5 marker

The sequence information of cloned *xa5* (GenBank accession 32975200,) was retrieved from www.ncbi.nlm.nih.gov. Bi-directional PCR amplification of specific alleles (bi-PASA) markers for *xa5* was designed and developed based on the guidelines proposed by Liu *et al*. (1997). Two sets of bi–PASA primers were developed to detect SNPs at position 39 of exon 2. Two outer primers were PAxa5-F1 (5´-GGC CAC CTT CGA GCT CTA CC-3´)

and PAxa5-R2 (5´- CAA CAT TGC AAC TCC GTG ATA AG-3´) and two inner primers were PAxa5-F2 (5´- GCT CGC CAT TCA AGT TCT TGT C-3´) and PAxa5-R1 (5´- CAG ATA CCT TAT CAA ACT GCT C-3´). These markers were determined on 2% agarose gel with ethidium bromide staining. The two inner primers PAxa5-F2 and PAxa5-R1 were designed to be specific to two nucleotides that are different between resistant (*xa5/xa5*) and susceptible (*Xa5/-*) alleles, respectively (Figure 1). The expected DNA fragment of resistance allele is 134 bp, whereas that of susceptible allele is 221 bp. Both primers generated a common allele (308 bp), which was used as a positive control of PCR amplification (Figure 1).

PCR amplification

The amplification reactions for PAxa5 markers were carried out in a volume of 10 μl containing 20-40 ng of DNA template, 1X PCR buffer, 0.2 mM of each dNTPs, 2.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Nanohelixes), 0.02 μM of PAxa5-F1, PAxa5-R1, and PAxa5-F2 primers, and 0.01 μM PAxa5-R2. PCR amplification was performed in a Perkin Elmer 9600 thermal cycler programmed as 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 57°C, and 1 min at 72°C) with a final extension of 5 min at 72°C. The amplification products were separated on 2 % agarose gel electrophoresis and stained with ethidium bromide.

Validation of PAxa5 functional marker

The marker PAxa5 functional was validated in 190 $F_{2:3}$ lines from the cross between KDML105 and IR62266, KDML105, IR62266, RD6 (susceptible check) and IRBB5 (resistance check).

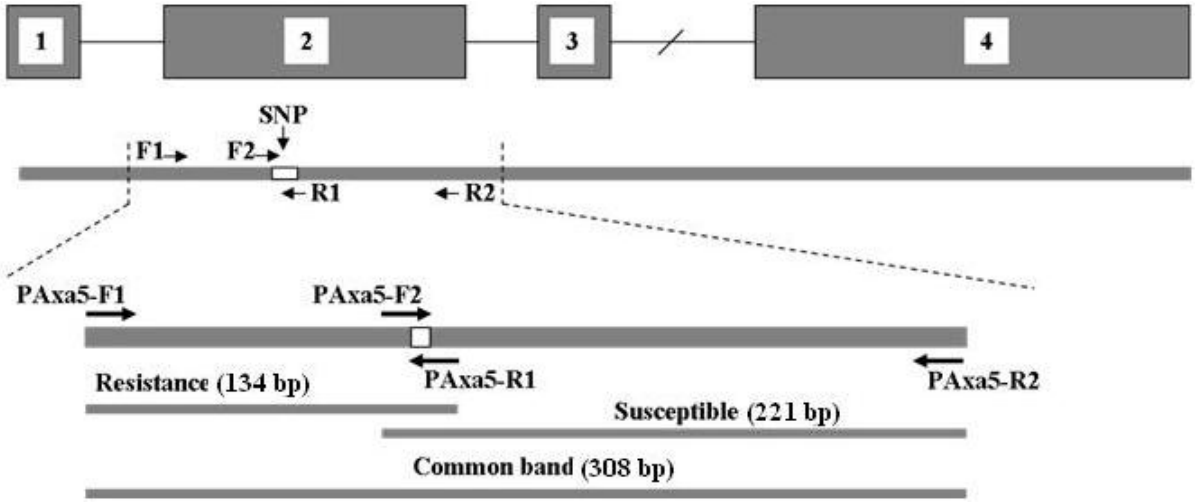

Figure 1 PAxa5 functional marker was designed to detect SNP at position 39 in exon 2 of *xa5.* Two nucleotide substitutions caused amino acid change from valine in susceptible variety to glutamic acid, resulting in resistance to BB. Resistance allele (134 bp) was amplified by primer PAxa5-F1 and PAxa5-R1 while susceptible allele (221 bp) was amplified by primer PAxa5-F2 and PAxa5-R2. The common allele was amplified by primer PAxa5-F1 and PAxa5-R2, which generated DNA fragment of 308 bp.

RESULTS

Segregation of BB resistance in $F_{2:3}$ population

IR62266 expressed strong resistant reaction (R) to three *Xoo* isolates TXO1, TXO2 and TXO5, whereas KDML105 showed a susceptible reaction (S). LL of IR62266 and KDML105 ranged from 2.0-2.3 cm and 6.0-14.3 cm, respectively (Figure 2). Continuous distributions of LL were observed in the $F_{2:3}$ progenies with skewing toward susceptible. However, distribution curves showed the possibility of two phenotypic groups (Figure 2). The segregation ratio was 3S:1R against three *Xoo* isolates, TXO1, TXO2 and TXO5 at $\chi 2$=1.27, p=0.26; $\chi 2$=0.83, p=0.36; $\chi 2$=1.39, p=0.24, respectively.

Identification of markers linked to the BB resistance

Tagging approach was used to identify markers linked to BB resistance. Two SSR markers RM122 and RM153 located on the short arm of chromosome 5 clearly differentiated the 11 R and 9 S $F_{2:3}$ plants (Figure 3). These two were used for genotype 190 $F_{2:3}$ individuals. Phenotype-genotype association indicated that LL was significantly associated with the markers. Multiple regression analysis convincingly showed that RM122 was probably the closest marker linked to the BB resistance gene in IR62266. This marker explained 66.03% of LL variation in $F_{2:3}$ population as shown in Table 1.

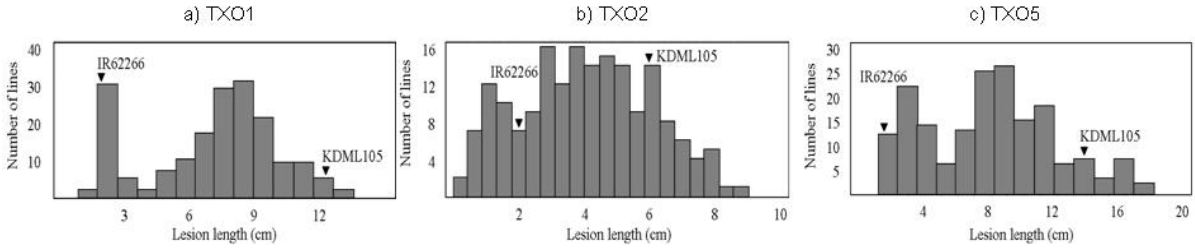

Figure 2 Distribution of LL after inoculation with *Xoo* isolates in a set of F_3 population derived from a cross between KDMl105 and IR62266. a) TXO1, the average LL of KDML105 and IR62266 were 12.5 ± 3.4 cm and 2.0 ± 1.0 cm, respectively. b) TXO2, the average LL of KDML105 and IR62266 were 6.0 ± 2.1 cm and 2.1 ± 2.0 cm, respectively. c) TXO5, the average LL of KDML105 and IR62266 were 14.3 ± 3.5 cm and 2.3 ± 0.5 cm, respectively.

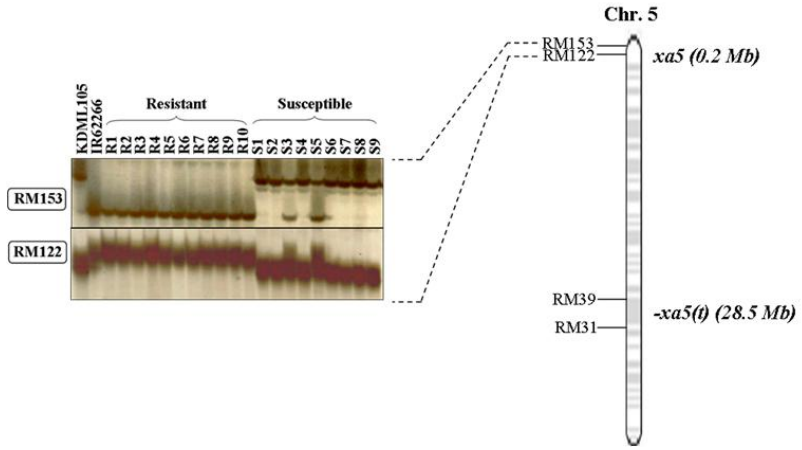

Figure 3 Banding pattern of two SSR markers RM122 and RM153 in R and S plants derived from a cross between IR62266 and KDML105. These markers were located in the vicinity of reported *xa5*.

Table 1 Phenotype-genotype association analysis using ANOVA and regression analysis in the F_3 population from the cross between KDML105 and IR62266. Mean of LL was significantly associated with three SSR markers. IR = homozygous IR62266, H = heterozygous and KDML105 = homozygous KDML105.

Isolate	Marker	R^2	Mean of LL			
			IR	H	KDML105	
TXO1	RM507	47.39**	2.51a	7.71b	9.5c	**
	RM153	65.09**	2.53a	7.64b	9.34c	**
	RM122	66.03**	2.39a	7.67b	9.38c	**
TXO2	RM507	30.62**	1.52a	4.14b	5.54c	**
	RM153	53.56**	1.53a	4.12b	5.54c	**
	RM122	53.85**	1.44a	4.20b	5.50c	**
TXO5	RM507	27.90**	3.15a	8.96b	9.89b	**
	RM153	32.95**	3.18a	8.88b	9.65b	**
	RM122	60.75**	3.15a	8.96b	9.89b	**

** Significance at P = 0.01

a, b and c stand for the clustering value on traits which are significant or not significant

A bi-PASA marker testing

PAxa5 marker clearly identified resistant, susceptible and heterozygous genotypes of 190 $F_{2:3}$ rice lines from the cross between KDML105 and IR62266 using electrophoresis on a 2% of agarose gel. The size of resistance allele generated from IR62266 was 134 bp, which was the same as that from IRBB5, a rice variety known to carry the *xa5*, whereas susceptible allele in KDML105 and RD6 generated a 221 bp fragment and all samples have a common fragment of 308 bp. The heterozygous lines showed three bands as expected **(Figure 4).**

DISCUSSION

In this study, we identified a new genetic resource of *xa5* gene in rice variety IR62266. This variety showed a broad spectrum resistance against all *Xoo* isolates in Thailand (Sripakhon, 2009). IR62266 is also a high yielding variety with good agronomic characteristics. A recessive gene in IR62266

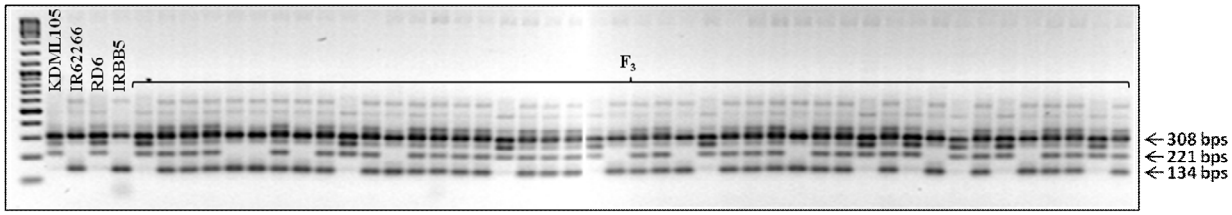

Figure 4 Polymerase chain reaction-based screening for BB resistant in $F_{2:3}$ lines (only 44 out of 190 lines were shown) from the cross between KDML105 and IR62266 using bi-directional marker, PAxa5 on a 2% agarose gel. Products amplified by PAxa5 reveal polymorphism among resistant carrying 134 bp allele (IR62266 and IRBB5), heterozygote carrying both of 221 and 134 bp alleles, and susceptible cultivars (KDML105 and RD6) carrying 221 bps allele. All samples were carrying positive control allele or common allele of 308 bp.

identified in this study was tightly linked to RM122 marker and corresponded well with information reported by Pattawatang in 2005. To confirm that a recessive gene in IR62266 was *xa5*, the functional marker PAxa5, which was designed to cover the same position as CAPS markers reported by Iyer and McCouch (2007) was used in this study. Our results confirmed that the recessive gene in IR62266 was the same as *xa5*. Furthermore, IRBB5 and IR62266 were developed from the same ancestor rice line, IR1545-339. IR1545-339 is a parental line of a well known rice cultivar DZ192, an *Aus*-boro variety originated in Bangladesh. DZ192 is the *indica* subspecies, carrying *xa5* gene (Glaszmann, 1987; Ogawa *et al.*, 2004). This suggested that, a resistance gene *xa5* identified from IR62266 was introgressed from IR1545-339.

PAxa5 is a co-dominant functional marker (FM) that is completely predictive of the functional nucleotide polymorphism that differentiates resistant and susceptible alleles of the *xa5* (Iyer and McCouch 2004). FM is superior to random DNA markers such as RFLP, SSR and AFLP because it is completely linked with the trait of interest. The primers of FM are normally designed from the genomic region within the gene where it causes the phenotypic trait variation. FM can be applied across germplasm and genetic background. This would be a major advance in marker applications, particularly in plant breeding. Additionally, FM can be used to avoid genetic drift at characterized loci (Andersen and Lübberstedt, 2003).

PAxa5 is ideally suited as a tool for MAS in rice breeding for BB resistance. This primer can identify clear-cut resistant, susceptible and herterozygous genotypes. Restriction enzyme is not needed and PCR product can be ran on a 1 to 2 % agarose gel. This can help breeders to save time and cost for the selection process. In this study, PAxa5 was as reliable as CAPS marker reported by Iyer and McCouch (2007) but it is cheaper than CAPS marker because the enzymatic digestion is not needed. This marker can be used for MAS in rice breeding program.

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