Screening of Bacterial Leaf Blight Resistance Genes (*xa5, xa13, Xa21*) using Sequence Tagged Site (STS) Marker in Korean Varieties and Landraces

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ABSTRACT

Sequence-tagged site (STS) markers tightly linked to the bacterial leaf blight (BLB) resistance genes, *xa5, xa13* and *Xa21*, were used in this study. A survey was conducted to find polymorphisms between the resistant and susceptible germplasm in rice.

500 of Korean varieties and 100 of landraces were evaluated in this study. STS marker, RG207 was used to having *xa5* resistance gene of rice germplasm. 27 varieties of Korean germplasm showed resistant for *xa5* gene. The RG136 an *xa-13* marker resulted in a single band of approximately 1 kb in all the rice accessions studied. In order to detect polymorphism, digestion of the polymerase chain reaction (PCR) product was performed using a restriction enzyme Hinf I. The resistant lines resulted in two bands 0.5 kb on digestion with Hinf I, while the same enzyme did not digest the PCR product of susceptible lines. No polymorphism was detected in Korean varieties and landraces, indicating that they probably do not contain *xa13* gene. pTA248 an *Xa-21* marker detected a band of 1 kb in the resistant lines and bands of either 750 bp or 700 bp in the susceptible lines. Among germplasm tested, there are no varieties and landraces with *Xa21* resistant gene. The results of the germplasm survey will be useful for the selection of parents in breeding programs aimed at transferring these bacterial blight resistance genes from one varietal background to another.

Key words **:** Bacterial leaf blight, *Xanthomonas oryzae pv. oryzae,* STS marker, Rice.

INTRODUCTION

Bacterial leaf blight (BLB), caused by the rod-shaped bacterium, *Xanthomonas oryzae pv.oryzae* (*Xoo*), is the most important bacterial disease of rice. This disease, which occurs as vascular wilt at the early stages of crop growth (nursery to tillering) and as leaf blight at later stages (panicle initiation to flowering), severely affects production of rice cultivated in Asia, Australia, Latin America, Africa, and the United States (Khush and

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Virmani, 1985; Mew et al., 1993; Davierwala et al., 2001).

The most effective, fundamental and economical approach to control BLB is the use of resistant varieties. One way to delay such a breakdown of bacterial blight (BB) resistance is to pyramid multiple resistance genes into rice varieties. This approach can however, be very difficult or impossible using conventional breeding methods due to epistasis and/or the masking effect of genes, particularly when a breeding line already has a gene (Huang *et al.*, 1997).

The development of molecular markers diagnostic for the selection of resistance genes is a goal of many rice breeding programs. The identification of molecular markers linked to resistance genes made it possible to select plants harboring multiple resistance genes, even though the phenotypic effects of individual genes were masked in combination (Yoshimura *et al.*, 1995).

So far, 14 dominant resistance genes [(*Xa1, Xa2, Xa3, Xa4, Xa7, Xa10, Xa11, Xa12, Xa14, Xa16, Xa17, Xa18, Xa21,* and *Xa22* (t)) and six recessive (*xa5, xa8, xa13, xa15, xa19* and *xa20*) resistance genes for BB have been identified (Khush and Kinoshita, 1991; Kinoshita, 1995; Lin *et al.*, 1996).

The *xa5* gene is one of the recessively inherited resistance genes that provide race-specific resistance to bacterial blight. The gene is found at the end of the short arm of chromosome 5 and several researchers have located or developed using a set of NIL and RFLP markers, RZ390, RG556 and RG207, that are closely linked to it (McCouch, 1990; Yoshimura *et al.*, 1995; Blair and McCouch, 1997; Huang *et al.*, 1997; Sanchez *et al.*, 2000; Singh *et al.*, 2001) or constructed physical contigs around the gene (Saji *et al.*, 1996).

The *xa13* gene confers specific resistance to *Xoo* race 6 (PXO99). Originally identified in cultivar BJ1 (Ogawa *et al.*, 1987), *xa13* was found closely linked to a RAPD marker, AC5-900, and three RFLP markers, RG136, RZ28 and CDO116, in rice chromosome 8 (Zhang *et al.*, 1996). Resistance conferred by recessive genes such as *xa5* and/or *xa13* may represent very different and largely unknown biochemical pathways in the host defense system. If so, cloning and the molecular study of *xa13* may shed light on this important aspect of the plant defense response. Moreover, elucidation of the biochemical function of *xa13* may lead to more efficient strategies for combating *Xoo* (Sanchez *et al.*, 1999).

A source of resistance (*Xa21*) was identified in the wild species *Oryza longistaminata* (Khush *et al.*, 1989). Unlike other *Xa* genes identified, the dominant resistant locus *Xa21* confers resistance to all Indian and Philippine races of *Xoo* tested (Ikeda *et al.*, 1990). *Xa21* is the first resistance gene to be cloned and transferred in any cereal crop plants. Due to its wide spectrum of resistance (Song *et al.*, 1995; Wang *et al.*, 1998), it is of great value in breeding rice for BLB resistance.

Abenes *et al.* (1993) used MAS for gene pyramiding for bacterial blight resistance for the first time. Many RFLP markers have been converted into PCR-amplifiable able sequence-tagged sites (STS) for specific locations in the rice genome (Inoue *et al.*, 1994; Williams *et al.*, 1991). STSs are short genomic sequences that can be amplified from DNA samples by means of a corresponding PCR assay (Wang *et al.*, 1998). Marker-aided selection (MAS) based on STS markers was used by Sanchez *et al.* (2000) to transfer three bacterial blight resistance genes *xa5, xa13* and *Xa-21* into three promising new plant types. Their studies indicated that MAS reached an accuracy of 95% and 96% in identifying homozygous resistant plants for *xa5* and *xa13* genes, respectively.

The use of markers linked to genes of interest will facilitate future efforts to transfer combinations of bacterial blight resistance genes into preferred rice varieties. Here we surveyed the polymorphism of the germplasms on RDA (Rural development Administration) in Korea using PCR-based markers for

the diagnosis of the *xa5, xa13* and *Xa21* resistance genes, and investigate to evaluate germplasm useful to BLB resistance breeding programs in rice.

MATERIALS AND METHODS

Plant material

600 rice accessions (500 Korean varieties and 100 landraces) were used for the PCR polymorphism survey. These accessions were obtained from the Gene Bank at RDA in Korea and represent a wide range of rice germplasm. The plants were grown in the greenhouse for DNA isolation.

The resistant isoline containing *xa5* (IRBB5), *xa13* (IRBB 13) and *Xa21* (IRBB21), and its susceptible line (IR24) were used as check lines to survey marker polymorphisms.

DNA isolation

A single piece of young rice shoot or leaf (5 cm long) was harvested 3 weeks after sowing and mechanically ground using liquid nitrogen. Genomic DNA was extracted from following the method by DNeasy Plant Mini Kit (Qiagen).

PCR amplification conditions

The PCR reaction mixture consisted of 50 ng template DNA, 4 pmol of each of the primers, 0.1 mM dNTP's, 1 × PCR buffer (10 mM Tris pH 8.0, 50 mM KCl, 1.8 mM $MgCl_2$, 0.01 mg/ml gelatin, *TaKaRa Taq*™) and 1 unit of *Taq* polymerase in a volume of 25 μl. Template DNA was initially denatured at 93℃ for 5 min, followed by 40 cycles of PCR amplification under the following parameters: 1 min denaturation at 93℃, 1 min primer annealing at 60℃ and 2 min extension at 72 ℃. A final 5 min incubation at 72℃ was allowed for completion of primer extension on a PTC-100 thermal cycler (MJ Research Inc.). The amplified products were electrophoretically resolved on a 1.2% agarose gel using 1 × TBE buffer (Hittalmani *et al.*, 1995)

Restriction digestion of PCR products

We initially used 5 μl of PCR product for gel electrophoresis to determine if PCR amplification was successful; the remaining 20 μl of PCR products was digested with restriction enzyme *Hinf* I. The total reaction volume was usually 25 μl. The reaction mixture consisted of 2 μl sterile distilled water, 2.5 μl restriction buffer (10u / μl), 0.5 μl reaction enzyme mixture (10 u/μl) and 20 μl of PCR products. The reaction mixture was incubated from 2 h to overnight at 37℃. The DNA fragments produced by restriction digestion were separated through gel electrophoresis (1.2% agarose) and visualized under UV light after

Table 1. Sequence tagged site and STS markers used for marker-assisted selection of resistance genes to *Xanthomonas oryzae pv. oryzae*

Linked gene	Chromo-some	Marker	Enzyme	Primer sequence	Distance cM	Reference
xa5	5	RG207	-	F: ATT GCC TAC GAC GAA GAT AGC	0.0	Blair and
				R: GCC ATG GCG ACT GTC AGT CG		McCouch, 1997
xa13	8	RG136	*Hinf*1	F: TCC CAG AAA GCT ACT ACA GC	3.7	Zhang *et al.*,
				R: GCA GAC TCC AGT TTG ACT TC		1996
Xa21	10	pTA248	-	F: AGA CGC GGA AGG GTG GTT CCC GGA	0.0	Ronald *et al.*,
				R: AGA CCG GTA ATC GAA AGA TGA AA		1992

F : Forward primer, R : Reverse primer.

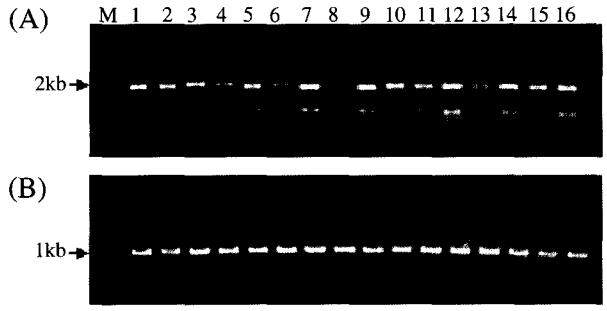

Fig. 1. PCR analysis of the used accessions. DNA amplified with primers RG207 and RG136 (digested with *Hinf* I) primers were linked with resistance genes *xa5* (A), *xa13* (B), respectively. For *xa5*, the resistant plants: 1, 2, 5, 7, and 9; for *xa13*, the susceptible plants are shown in lanes 1~16.
M : Kb ladder, lane 1~16 : Korean varieties.

staining with ethidium bromide (Huang, 1997).

Three STS markers, RG207, RG136 and pTA248, tightly linked to resistance genes *xa5, xa13* and *Xa21*, respectively, were used to confirm the presence of each gene.

The list of STS markers used and the restriction enzyme with which the PCR products were digested is given in Table 1.

RESULTS

A total of 600 plants were screened for resistance gene through molecular marker analysis. One allele was identified among the 500 accessions of rice germplasm surveyed with RG207, RG136 and pTA248 primers and the PCR products with RG136 digested with *Hinf* I (Fig. 1). The majority of the rice germplasms tested had an allele. The marker patterns for used lines are shown in Fig. 1 and 2.

Analysis of Recessive Resistance Genes, *xa5* and *xa13*

The PCR product of RG207 resulted in polymorphism between the lines which does not contain the *xa5* gene and which contains the *xa5* gene without restriction enzyme. Band patterns are similar to the obtained by Sanchez *et al.* (2000). Among 600 Korean varieties, 27 lines, such as Sujinbyeo, Pungsanbyeo, are shown resistance for *xa5*. None containing the *xa5* resistant gene was observed in 100 Korea landraces we tested. The STS marker, RG136, maps at a distance of 3.7 cM from xa13 (Zhang *et al.*, 1996). As shown in Fig. 1, all germlasms were found in a single band of approximately 1 kb. In order to detect polymorphism, the PCR product was performed using *Hinf* I for restriction digestion. The *Hinf* I could detect polymorphism between the near-isogenic line IRBB13 and other susceptible lines. All the resistant lines resulted in two bands of 0.5 kb on digestion with *Hinf* I, while the same enzyme did not digest the PCR product of susceptible lines (Davierwala *et al.*, 2001). No

Table 2. Evaluation of germplams in this studied

Germplasm	*xa5*		*xa13*		*Xa21*	
	R	S	R	S	R	S
Korean varieties	27*	473	0	500	0	500
Korean landraces	0	100	0	100	0	100

R : Resistant, S : Susceptible

* : Palkwangbyeo, Milyang 42, Namyeongbyeo, Jungweonbyeo, Milyang 95, Weonpungbyeo, Cheongcheongbyeo, Dasanbyeo, Chilseongbyeo, Gayabyeo, Gyehwabyeo, Yongjubyeo, Hyangnambyeo, Hyangmibyeo, Hwabongbyeo, Hwaseonchalbyeo, Heugnambyeo, Hwayeongbyeo, Baegyangbyeo, Singwangbyeo, Punsanbyeo, Nampungbyeo, Sujeongbyeo, Milyang 42, Arumbyeo, Suwon 345.

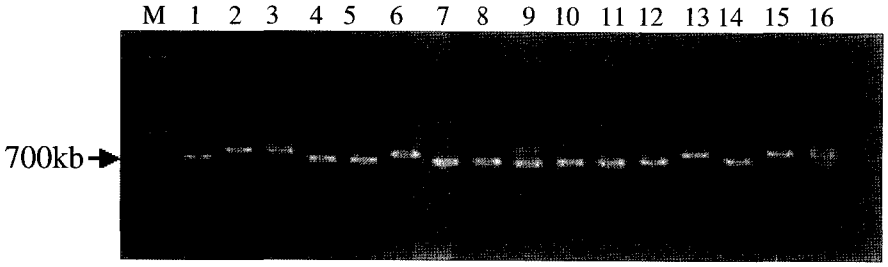

Fig. 2. Banding patterns of PCR products amplified with STS primer, pTA248. Lane M is a 1 kb ladder. For *Xa21*, the susceptible plants: 1~16.
1~16 : Korean varieties and landraces.

polymorphism was detected all of the used accessions (Fig. 1), indicating that they probably did not contain the *xa13* gene, or if present, it could not be detected by the RG136 marker, due to some recombination in the 3.7 cM region between the *xa13* gene and RG136 marker. Most of the lines except the near-isogenic line IRBB13 showed the absence of the 0.5 kb bands, indicating the possibility of absence of this gene in all these lines.

Analysis of Dominant Resistance Gene, *Xa21*

The presence of the *Xa21* gene was detected by the STS marker pTA248 located within 0.0 *cM Xa21* and originally obtained by sequencing the genomic clone of the RAPD248 fragment using the same primer (Ronald *et al.*, 1992). pTA248 detected a band of 1 kb in the resistant line such as IRBB21, and most of the accessions studied showed 700 bp or some were observed with band of 750 bp in band patterns (Fig. 2). These accessions are susceptible to the *Xa21*.

In the case of dominant genes, individuals homozygous and heterozygous for the gene show the same phenotypic reaction and identification of homozygous plants is possible only by use of codominant DNA markers, closely linked to the gene of interest (Davierwala, 2001). To develop new *Xa21*-specific markers based on published sequences of *Xa21* (Song *et al.*, 1995), it will be used SNP (Single nucleotide polymorphism) to marker assisted selection in future.

DISCUSSION

DNA markers have been used for mapping and tagging of major genes of agronomic and economic importance in rice. Since DNA markers are not affected by environmental variations or growth stage of the plant, they can be used to accurately select plants carrying the target gene (Abenes *et al.*, 1993; Hittalmani *et al.*, 1994; Tanksley *et al.*, 1989; Yoshimura *et al.*, 1995; G. Zhang *et al.*, 1996), which will provide a potentially powerful tool to improve conventional breeding efficiency.

STS markers are easily developed once sequence data are available for cloned genomic DNA (Inoue *et al.*, 1994). However, they are usually less polymorphic than the RFLPs from which they were derived (Ghareyazie *et al.*, 1995). The use of STS markers for the selection and incorporation of resistance genes for rice blast has been recommended (Hittalmani *et al.*, 1995). We found that polymorphisms among accessions for two STS markers RG 207 and pTA248, used in this study were detected directly, however, an STS marker, RG136, could be detected after digestion with restriction enzyme. Therefore mapping of the STS markers required the additional costly and time-

consuming step of digesting PCR product.

The analysis of genetic variation at resistance gene loci using PCR-based markers has been recommended for the screening of wild or cultivated germplasm for alternate resistance sources (Michelmore, 1995). Marker-aided selection is useful to increase breeding efficiency. MAS is especially useful in selecting for recessive genes, such as *xa13*, where the presence of the gene in the heterozygous condition cannot be detected though traditional approaches without progeny testing, and/or for cases where different genes have the same effect on a character and their genotypes cannot be identified through conventional approaches (Zhang, 1996).

We report the results of the screen of the BLB three genes, *xa5, xa13, Xa21*. The results using the STS markers (RG136, pTA248) do not show resistance gene to *Xoo* in Korean varieties and landraces. The *xa5* gene identified in this study, however, is useful for developing resistant varieties. The materials having *xa5* gene will be used to incorporate into improved germplasm with resistance to bacterial blight.

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