Evaluation of Bacterial Blight Resistance Using SNP and STS Marker-assisted Selection in Aromatic Rice Germplasm

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A molecular survey was conducted to identify the presence of the bacterial blight resistance genes (Xa1, Xa4, xa5, xa13 and Xa21) in 86 accesses of aromatic rice obtained from germplasm. The results revealed that the resistance gene Xa4 (32.5%), Xa21 (17%), and xa5 (16%) were widely observed in tested rice germplasm. Among tested rice germplasm, 49 accesses showed the presence of more than one of five R genes, and 37 accesses possessed none of the R gene. TALLi and 05-IRRI-M-46 showed the presence of Xa4, xa5, xa13 and Xa21. Rice race 415×1352 exhibited positive amplicon for the Xa1, Xa4, xa5 and Xa21. Hyangmibye01hos, 1R841-85-1-1-2 and Jasmine85 showed the positive amplicon for the Xa1, Xa4 and xa5 genes. Yekywil Yinkya Hmwe and Khao Dawk Mal105 showed the presence of Xa1, Xa4 and Xa21 gene. Masino Basmati showed the presence of xa5, xa13, Xa21 genes. Xa1 and Xa21 genes were noticed in Mihyangbyeo, Tarana Deshi, Mayataung and AZUCENA. Hyangmibyeo2ho, Basmati 6311 and Basmati405 possessed only two R genes such as Xa4 and xa5, and xa5 and xa13, respectively. The evaluation results of bacterial blight resistance genes in aromatic rice germplasm will help in breeding of multi disease resistant varieties.

Keywords: Aromatic rice, bacterial blight resistance, MAS

Bacterial blight (BB) disease is caused by Xanthomonas oryzae pv. oryzae (Xoo) and this has been one of the most serious biotic factors limiting rice production in worldwide (Ezuka and Kaku, 2000; Mew, 1987; Shin et al., 1992). The yield losses in severely infected fields generally range from 20 to 30% but may reach up to 80% (Ou, 1985; Singh et al., 1977). It is widespread throughout the world in many countries viz., Nepal (26 accesses), Myanmar (19 accesses), India (17 accesses), Sri Lanka (14 accesses), Philippines (10 accesses), China (10 accesses), Japan (6 accesses), Vietnam (6 accesses), Korea (5 accesses) (Adhikari et al., 1999; Fincks and Nelson, 1999; George et al., 1997; Lwin et al., 2007; Noda et al., 1996, 1999, and 2001; Noh et al., 2003; Ochiai et al., 2000; Sing et al., 2003). Recently, three races (K1, K2 and K3) are the most dominant races of Xoo in Korea. Pathogen populations of Xoo are highly variable, as revealed by virulence and DNA fingerprinting analysis (Adhikari et al., 1995, 1999; Leach et al., 1992; Nelson et al., 1994).

Over 30 resistance (R) genes exhibiting host resistance against various strains of Xoo have been identified and named from Xa1 to Xa28. Fourteen of the 30 resistance genes have been mapped to chromosomes 4, 5, 6, 7, 8, 11 and 12 (Chen et al., 2002; Ezuka and Kaku, 2000; Gu et al., 2004; Kinoshita, 1995; Lee et al., 2003; Lin et al., 1999; Sakaguchi, 1967; Singh et al., 2002; Wei et al., 2005; Yang et al., 2003; Zhang et al., 1998). The development of donor lines carrying major R genes have been incorporated into improved rice cultivars (Khush et al., 1990). Ten of the recessive R genes, xa5, xa8, xa13, xa15, xa16, xa19, xa20, xa24, xa26, and xa28, have been characterized as encoding different types of proteins and suggested that multiple mechanisms of R gene-mediated Xoo resistance (Chu et al., 2006; Gu et al., 2005; Lyr and Couch 2004; Song et al., 1995; Sun et al., 2004; Wei et al., 2005; Yoshimura et al., 1998).

The use of resistant cultivars is the most economical and effective method to control the bacterial blight. In modern crop plants breeding programmes, marker assisted selection (MAS) provides a opportunity to circumvent many problems associated with phenotypic selection of crops and increases the efficiency and flexibility of a breeding through selection of marker genotype linked to target genes or quantitative trait loci (QTL) (Melchinger, 1990; Mohan et al., 1997; Ogawa, 1993). The pyramiding of multiple resistance genes into rice varieties is one way to develop durable resistance to bacterial blight (Huang et al., 1997; Kinoshita, 1995; Sanchez et al., 2000; Yoshimura et al., 1995). Singh et al. (2001) reported that a three-gene combination (xa5, xa13 and Xa21) appeared to be the most effective of which Xa21 is contributing the largest component of resistance. In republic of Korea, the breeding program for resistant rice cultivars to bacterial blight was started in 1965 and japonica type of Seomjinbyeo carrying Xa1 gene was bred in 1982. Hwayeongbyeo was bred
from WAscAikou3 carrying Xa3 gene (Ezuka et al., 1974) in 1991 and had resistance to K1, K2 and K3 races. The aim of the present study was to get the information about the genetic diversities of bacterial blight resistance genes in aromatic rice germplasm to improve the aromatic rice breeding efficiency using several SNP and STS markers.

**Materials and Methods**

**Plant materials and DNA isolation.** Eighty six accessions of aromatic rice were obtained from National Agrobiodiversity Center, Rural Development Administration (RDA), and Republic of Korea and were listed in Table 2. Five rice seeds were imbibed for 4 h in 2% NaOCl further transferred to another container containing tap water for overnight and placed moist filter paper on Petri-dish at 30°C for one-week in light condition. Genomic DNA was extracted from young leaves of one-week-old seedlings by an improved CTAB (hexadecyl trimethyl ammonium bromide) method based on the procedure described by Murray and Thompson (1980). The extracted genomic DNA was examined on 1% agarose gel stained with ethidium bromide. The DNA was quantified by Nano Drop system (Nano Drop, USA), further concentration of DNA was adjusted to 10 ng/μl with sterilized distilled water and stored at 4°C.

**Bacterial Blight (BB) R genes specific DNA markers.** Previously reported five R gene specific DNA markers to develop SNP (Xa1 and xa13) and STS (Xa4, xa5 and Xa21) markers for the bacterial blight resistance genes from mapped various differential chromosomes were used for amplification of DNA fragments linked to BB resistant genes (Table 1). All the STS and SNP markers used in this study were obtained from the Oligo Synthesis Facility, Bioneer Co., Korea.

**Polymerase chain reaction (PCR) analysis.** The PCR analysis was conducted based on procedures described by Bioneer PCR Pre-mix kit manual (Bioneer Co. Ltd, Korea). The PCR reaction mixture contained 50 ng template DNA, 20 pmol of each primer set, 0.25 mM of each dNTPs, 1.5 mM MgCl2, 1X PCR buffer (10 mM Tris-HCl, pH 9.0, 30 mM KCl), and 1 unit of Taq DNA polymerase in a volume of 20 ul. Amplifications were carried out in a MyGenie96 Thermal cycler (Bioneer Co. Ltd, Korea) programmed that template DNA was initially denatured at 94°C for 5 min, followed by 30 cycles of PCR amplification with the following parameters; a 30 sec denaturation at 94°C, 30 sec primer annealing at at 42 to 62°C, and 1 min primer extension at 72°C allowed for completion of primer extension, with final extension at 72°C for 10 min. Initially 5 μl of the amplified products were electrophoretically resolved on a 1.5% agarose gel in 0.5X TAE (Tris-acetate-EDTA) buffer (pH 8.0) and visualized under UV light after staining with 0.1 μg/ml of ethidium bromide. The amplified fragments using STS markers (RM224, RG556 and pTA248) were scored as presence (R) or absence (S) of each gene linked DNA fragment.

**PCR-RFLP analysis.** The amplified products were digested with restriction enzymes such as EcoR V (16PFXa) and Hinf I (RG136 linked to the Xa1 gene). The total volume of the reaction for PCR-RFLP was 20 μl (2.0 μl of 10X restriction enzyme buffer, 0.5 μl of 10 u/μl restriction enzyme (Promega, USA), 7.5 μl of sterile distilled water, 10 μl of amplified products). The reaction mixture was incubated at 37°C for 5 h. The digested DNA fragments were separated through 2% agarose gel electrophoresis and visualized under UV light after staining with 0.1 μg/ml of ethidium bromide. The digested fragments were scored as described previously.

**Table 1.** Details of single nucleotide polymorphic (SNP) and sequence tagged site (STS) marker used for evaluation of BB resistance and linkage to bacterial blight resistant genes

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*Gene symbol with capital and small letter represent dominant and recessive gene, respectively.
Results

MAS of BB resistance accessions of aromatic rice germplasm. Two hundred and sixty accessions of aromatic rice germplasm introduced from 20 origins were preserved in Korean RDA genebank. One hundred and six of Two hundred and sixty accessions of aromatic rice were evaluated with agronomic traits, physico-chemical characteristics,

![Fig. 1. Agarose gel photograph of 86 aromatic rice germplasm, bacterial blight resistant (R), heterozygous (H) and susceptible (non-marked lanes) accessions amplified with SNP marker 16PFXa (upper), linked to Xa1 gene and the PCR products were digested by a restriction enzyme EcoRV (under). M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-86, 86 accessions of aromatic rice.]

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*a* Identity number in the Genebank of RDA, Korea; ID number with a prefix “K” is the temporary IT number. --; ID is not available in RDA Genebank.

*b* R, resistance; H, heterozygous; S, susceptible reactions linked to the bacterial blight resistant gene.
and analysis of aromatic compounds in 2007 (Kim et al., 2008a and Kim et al., 2008b).

Marker-assisted selection (MAS) was conducted to identify the presence of the bacterial blight resistance genes (Xa1, Xa4, xa5, xa13 and Xa21) in 86 accessions of aromatic rice obtained from germplasm. Bacterial blight resistance gene Xa4 (32.5%), Xa21 (17%) and xa5 (16%) is widely detected in tested rice accessions. Out of 86 accessions of aromatic rice germplasm, forty nine accessions showed the presence of more than one of bacterial blight resistance gene. Thirty seven accessions did not possess with anyone of the resistance gene (Table 2).

**Genetic diversity of Xa1 gene.** Detection of gene carrying of aromatic rice germplasm by SNP marker 16PFXA (Shin et al., 2006) showed the 350 bp fragment. Seven out of 86 accessions showed the BB resistant double fragments as 180 bp and 210 bp digested with restriction enzyme, EcoRV which corresponds to Xa1 gene (Fig. 1). Among 11 Xa1 positive rice races, 2 accessions such as Hydrobiophyto1ho (Choi et al., 1995) and MiHyangbye (Ha et al., 2003), were bred through domestic breeding program. Two japonica (MiHyangbye and 415×Ir352) and 3 accessions of indica type rice (Kebriri, Padi danat and Mayataung; originated from Indonesia and Myanmar) possessed Xa1 gene (Fig. 1, Table 2). The BB susceptible (S) fragments were not digested with restriction enzyme, EcoRV for overnight.

**Genetic diversity of Xa4 gene.** Amplification of Xa4 gene carrying of aromatic rice germplasm by STS marker RM224 (Chen et al., 1997) showed the BB resistant (R) fragment as 150 bp and the BB susceptible (S) fragments as 120 bp (Fig. 2). Thirty two of eighty six accessions of aromatic rice showed the appropriate amplification for BB resistant fragments of Xa4 gene (Fig. 2). Of which two tongil type rice accesses such as Hydrobiophyto1ho and Hydrobiophyto2ho were bred domestic breeding program (Moon et al., 1998) (Table 2). Out of 28 accessions of japonica type aromatic rice, seven accessions (Flores, Inaguhu, JC149, Kalo Masino Dhan, Milagrosa mutant, TALLII, and 415×Ir352) showed the BB resistant fragments linked to Xa4 gene. Positive amplion (150 bp) for Xa4 gene was observed in 19 of 56 races of indica type aromatic rice (Aci601, Aroma, AZUCENA, Basmati370, Basmati 9-33, Basmati198, Basmati 6129, Basmati6311, Da13, DS20, Jasmine85, Ir841-85-1-1-2, Khao Dawk Mali105, Tarana Deshi, Mayataung, Millotropic, Yekywin Yinkaya Hmwe, and 05-Irr1-M-46) (Fig. 2, Table 2).

**Genetic diversity of xa5 gene.** Presence of xa5 gene in aromatic rice germplasm was amplified by STS marker RG556 (Yoshimura et al., 1995) which showed the BB resistant (R) fragment as 500 bp and the BB susceptible (S) double fragments as 500 bp and 480 bp (Fig. 3). The results revealed that only 14 of 86 accesses possessed xa5 gene. Of which two accessions of tongil type rice such as Hydrobiophyto1ho and Hydrobiophyto2ho were bred domestic breeding program possessed xa5 gene (Fig. 3, Table 2). Three of twenty eight accessions of japonica type aromatic rice (Masino Basmat, TALLII, and 415×Ir352) showed the BB resistant fragments linked to xa5 gene. Nine of fifty six accessions of indica type aromatic rice (Basmati Dhan, Basmati405, Basmati5874, Basmati6311, Basmati5854, DS20, Ir841-85-1-1-2, Jasmine85, and 05-Irr1-M-46) showed the positive amplification for xa5 gene (Fig. 3, Table 2).

**Genetic diversity of xa13 gene.** Existence of xa13 gene in aromatic rice germplasm was amplified through SNP marker (RG136) (Zhang et al., 1996) and showed the 1 kb positive fragment. None of the tongil type rice which was bred on domestic bred program showed the presence of xa13 gene (Fig. 4). Two accessions of japonica type aromatic rice (TALLII and Masino Basmati) and four accesses of indica type aromatic rice (Basmati405, Basmati5875, Chaohora 1144, and 05-Irr1-M-46) showed the presence of xa13 gene (Fig. 4, Table 2).

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**Fig. 2.** Agarose gel photograph of 86 aromatic rice germplasm, bacterial blight resistant (R) as 150 bp DNA fragment, susceptible (non-marked lanes) as 120 bp DNA fragment, and bacterial blight heterozygous (H) as double fragment with 150 bp and 120 bp amplified with STS marker RM224 (Chen et al., 1997), linked to Xa4 gene. M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-86, 86 accessions of aromatic rice.

**Fig. 3.** Agarose gel photograph of 86 aromatic rice germplasm, bacterial blight resistant (R) accessions as 500 bp of single fragment and susceptible (non-marked lanes) accesses as 500 bp and 480 bp of double fragments amplified with STS marker RG556 (Yoshimura et al., 1995), linked to xa5 gene. M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-86, 86 accessions of aromatic rice.
SNP and STS Markers of Bacterial Blight Resistance in Aromatic Rice Germplasm

Genetic diversity of *Xa21* gene. Examination of *Xa21* gene carrying aromatic rice by STS marker pTA248 (Ronald et al., 1992) showed the BB resistant (R) fragment as 800 bp and the BB susceptible (S) fragments as 700 bp (Fig. 5). *Xa21* was detected on five accessions of japonica type aromatic rice (Masino Basmati, Mihyangbyeo, TALLI, and 415SIR352) and 10 accessions of indica type aromatic rice (AZUCENA, Basmat6113, Basmati213C, DS20, Khao Dawk Mali105, Mayataung, Seratus Malam, Tarana Deshi, Yekywin Yinkya Himwe, and 05-Iri-M-46) (Fig. 5, Table 2).

Discussion

Numerous studies have been carried out on the rice-*Xanthomonas oryzae* pv. *oryzae* pathosystem, resulting in powerful advancement in the understanding of molecular basis of interaction (Nayak et al., 2008; Shen and Ronald, 2002). Due to continuous evolution of pathogenic races, breakdown of resistance has occurred in many improved varieties. Incorporation of resistance gene is very difficult using conventional methods of breeding due to epitasis or masking effects of other genes (Mew, 1987 and Mew et al., 1992). In Korea, seven varieties of aromatic rice, Hyangmibyeolho (IR841-76-1×Suwon334), Hyangnambyeo (Iri389×Dohoku144), Hyangmibyeo2ho (IR841-76-1×Suwon334), Aranghyangchalbyeo (Sinseonchalbyeo×Dohoku144), Mihyangbyeo (Seomjinbyeo×Dohoku144), Seolihyangchalbyeo (Miyagaori×Suwon357), and Heughyang (Sanghaehyanghyeolla, γ-300Gy) were bred from 1993 to 2000 (Choi et al., 1995; Ha et al., 2003; Ha et al., 2006; Moon et al., 2003; Shin et al., 2001).

In this study, eight of eighty six accessions of aromatic rice showed the presence of BB resistance gene linked to *Xa1*. and four accessions of aromatic rice (Kemiri, Khao Dawk Mali 105, Mayataung, and Padi danat) exhibited the presence the heterozygous BB resistance gene linked to *Xa1*. Bacterial blight resistance gene *Xa1* was first reported by Sakaguchi (1967) and mapped it on rice chromosome 4 and cloned recently by a map-based cloning strategy (Yoshimura, et al., 1999). The *Xa1* gene has widely been used in Japanese rice breeding program for BB resistance, since, race1 of *Xoo* is the most dominant race in Japan. In the current study, R gene linked to *Xa1* was observed in Hyangmibyeolho and Mihyangbyeo. Shin et al. (2007) reported the same result using SNP marker 16PFXa linked to *Xa1* gene. It is interesting to note that most of the rice varieties bred in South Korea showed resistant to BB Korean race K1.

BB resistance gene *Xa4* is one of the most widely exploited resistance gene in many Asian rice breeding programs and conferred durable resistance in many commercial rice cultivars (Mew et al., 1992). *Xa4* gene offers resistance to bacterial blight at all stages of plant growth (Khush, 1981; Mew et al., 1992). In the present study also among 86 accessions of aromatic rice, thirty two accessions were positive for *Xa4* gene.

*Xa21* was reported to confer resistance to pathotype from the Philippines and India at post-seedling growth stages (Ikeda et al., 1990). Besides, this has been reported as the single most effective gene against 17 isolates of *Xoo* from Punjab (Singh et al., 2001). The *Xoo*-X*a21* interaction provides a model system in which to study the molecular basis for disease resistance (Song et al., 1995). *Xa21* gene is the only known resistance gene that encode three structural features (leucine-rich repeats (LRR) in the putative extracellular domain, a single-pass transmembrane domain whereas other resistance gene products possess cytoplasmic serine/threonine kinase (STK) intracellular domain) found in various combinations in other resistance gene products (Song et al., 1995).

The presence of two major types of disease resistance to plant pathogens, vertical resistance (complete whole-life resistance) and horizontal resistance (quantitative resistance), have been recognized in interactions between rice and *Xoo*. 

Fig. 4. Agarose gel photograph of 86 aromatic rice germplasm, bacterial blight resistant (R) and susceptible (non-marked lanes) accessions amplified with SNP marker RG136 (upper), linked to *xa13* gene and the PCR products were digested by restriction enzyme *Hin1* (under). M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-86, 86 accessions of aromatic rice.

Fig. 5. Agarose gel photograph of 86 aromatic rice germplasm, bacterial blight resistant (R) accessions as 800 bp DNA fragment and susceptible (non-marked lanes) accessions as 700 bp DNA fragment amplified with STS marker pTA248 (Ronald et al., 1992), linked to *Xa21* gene. M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-86, 86 accessions of aromatic rice.
(Zhang and Mew, 1985). In this study, two accessions of aromatic rice (TALLI and 05-IRRI-M-46) showed the presence of four multi bacterial blight resistance gene such as Xa4, xa5, xa13 and Xa21. The multiple resistance genes Xa1, Xa4, xa5 were detected on single rice accessions such as Hyangmibyeo1ho, lr841-85-1-1-2, Jasmine85, Khao Dawk Mali105, and Yekywin Yinkya Hmwe.

The pyramiding of Xa4 and other bacterial blight resistance genes offered a broad range of resistance to Xoo strains besides exhibited a higher level of resistance than the single resistance gene (Huang et al., 1997; Yoshima et al., 1995; Zheng et al., 1998). It could be concluded that the presence of multiple resistance genes in a single accession of aromatic rice probably useful for gene pyramiding. The advent of molecular markers tagged to different resistant genes enabled convergence breeding and pyramiding of more than two different genes into an agronomic variety. Sanchez et al. (2000) reported that recessive genes such as xa5 and xa13 are difficult to select through conventional breeding in the presence of a dominant gene such as Xa21. Xa21 gene was governing resistance to all known races of X. oryzae pv. oryzae. It is impossible to distinguish between plants having Xa21 alone and those having Xa21 and other genes due to masking effects of former (Huang et al., 1997; Singh et al., 2001).

Similarly, a molecular survey was conducted by Ramalingam et al. (2001) to examine the presence of BB resistance genes such as xa5, xa13 and Xa21 in Chinese rice germplasm. Although conventional approach for the identification of different resistance genes in rice germplasm is also being used (Kihupi et al., 2001; Lee et al., 2003), however, it is time consuming and need artificial inoculation of all the lines with different pathotypes of the Xoo.

When single gene resistant varieties were continuously cultivated in wide area, the resistance to the specific BB race would be broken down through pathogen’s variation (Bai et al., 2000; Mew et al., 1992; Noh et al., 2003). A MAS has been advocated as a highly efficient breeding method, because it can offer rapid and precise selection of target gene. The present study demonstrated the presence of multiple bacterial blight resistance genes in aromatic rice germplasm stored at National Agro-biodiversity center of Republic of Korea. In future, the results of the present study will be exploited for breeding programs to develop a multi disease resistant aromatic rice varieties against Xanthomonas oryzae pv. oryzae.

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References


