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Screening of the Dominant Rice Blast Resistance Genes with PCR-based SNP and CAPS Marker in Aromatic Rice Germplasm

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ABSTRACT The objective of this study was to determine the genetic diversities of major rice blast resistance genes among 84 accessions of aromatic rice germplasm. Eighty four accessions were characterized by a dominant 11 set of PCR-based SNP and CAPS marker, which showed the broad spectrum resistance and closest linkage to seven major rice blast resistance (*R*) genes, *Pia*, *Pib*, *Pii*, *Pi5* (*Pi3*), *Pita* (*Pita-2*), and *Pi9* (t). The allele specific PCR markers assay genotype of SCAR and STS markers was applied to estimate the presence or absence of PCR amplicons detected with a pair of PCR markers. One *indica* accession, Basmati (IT211194), showed the positive amplicons of five major rice blast resistance genes, *Pia*, *Pi5* (*Pi3*), *Pib*, *Pi-ta* (*Pi-ta2*), and *Pik-5* (*Pish*). Among 48 accessions of the PCR amplicons detected with yca72 marker, only five accessions were identified to *Pia* gene on chromosome 11. The *Pib* gene was estimated with the NSb marker and was detected in 65 of 84 accessions. This study showed that nine of 84 accessions contained the *Pii* gene and owned *Pi5* (*Pi3*) in 42 of 84 accessions by JJ817 and JJ113-T markers, which is co-closest with *Pii* on chromosome 9. Only six accessions were detected two alleles of the Pita or *Pita-2* genes. Three of accessions were identified as the *Pi9* (*t*) gene locus.

Keywords : aromatic rice, rice blast resistance, *Pi* genes, SNP and CAPS marker

The rice blast fungus is highly variable and numerous; pathotypes (races) of blast fungus are present in most field conditions (Valent and Chumley, 1994; Xia *et al.*, 1993; Yang *et al.*, 2009). The identification and isolation of additional host resistance genes (R) and pathogen avirulence genes (S) is now required to deepen understanding of molecular mechanisms involved in the host-pathogen interaction (Inukai *et al.*, 1994; Kiyosawa *et al.*, 1986; Valent, 1990).

Most breeding programs of aromatic rice have a narrow genetic diversity of breeding resources. In Pakistan, out of seven basmati varieties which are currently under cultivation, five had 'Basmati 370' as one of the parent cultivar (Arif *et al.*, 2005). In Korea, only seven varieties of aromatic rice were bred and distributed as cultivars. Most of these varieties have a parent originated from Japan (Dohoku144 and Miyagaori) and IRRI (IR841-76-1) (Choi *et al.*, 1995; Ha *et al.*, 2003, 2006; Moon *et al.*, 2003; Shin *et al.*, 2001).

Recent advances in rice genomics research and completion of the rice genome sequence have made it possible to identify and map precisely a number of genes through linked to DNA markers (Basavaraj *et al.*, 2010; Koide *et al.*, 2009; Tacconi *et al.*, 2010). Out of 50 major blast resistance genes, 8 genes have been cloned: *Pib* (Wang *et al.*, 1999), Pita (Bryan *et al.*, 2000), *Pid2* (Chen *et al.*, 2006), *Pi9* (Qu *et al.*, 2006), *Pi2* and *Pizt* (Zhou *et al.*, 2006), *Pi36* (Liu *et al.*, 2005), and *Pi37* (Lin *et al.*, 2007). With the exception of *Pid2*, which was reported to encode a receptor-like kinase (Chen *et al.*, 2006), these cloned genes belong to the NBS-LRR class of resistance genes.

Monosi *et al.* (2004) determined the distribution of the NBS-encoding genes in the 'Nipponbare' rice genome and identified the approximate map position of blast resistance (designated *Pi*-) genes, which had been previously mapped

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to a chromosome region based on phenotype, using various mapping populations. At least 12 dominant genes conferring complete resistance to rice blast and 10 quantitative trait loci associated with partial resistance have been located via linkage to genetic markers (McCouch *et al.*, 1988).

Conventional genetic linkage maps of many crop species have been constructed by using morphological markers. But the morphological markers are usually difficult to apply in plant breeding program because they are few in number largely affected by environment (Causse *et al.*, 1994; Chen *et al.*, 1997; Gu *et al.*, 2005; Melchinger, 1990; Mohan *et al.*, 1997).

The present study was carried out to obtain information about genetic diversities of resistance genes against rice blast disease in aromatic rice germplasm for improving aromatic rice breeding efficiency using PCR-based markers including several candidate SNP (single nucleotide polymorphism) and STS (sequence-tagged site) markers.

MATERIAL AND METHODS

Plant material and DNA isolation

Eighty four of two hundred and sixty aromatic rice accessions were offered from National Agrobiodiversity Center of RDA, Korea (Table 2). Five rice seeds were imbibed for four hrs in 2 % NaOCl solution and washed overnight with tap water and placed on moist filter paper laid on the petri-dish at 30℃ for one-week in light condition.

Genomic DNA was extracted from frozen 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 estimated by 1% agarose gel staining with ethidium bromide for quality test. The quantity of extracted DNA was measured by Nano Drop system (Thermo, U.S.A.) and diluted to 10 ng/μL with sterilized distilled water and stored at 4℃.

Rice blast R genes specific DNA markers

Table 1 shows eight set of the major rice blast resistance (*R*) genes specific DNA markers, *Pia* (yca72*), *Pib* (NSb), *Pii* (JJ113-T), *Pi5* (JJ201-Pi5), *Pita* (*Pita-2*) (YL155/87, YL183/87), and *Pi9(t)* multiple-allele (NBS-O/U*, 195-1), previously reported by researchers to develop as SNP and CAPS markers for evaluation of the rice blast resistance genes (Cho *et al.*, 2007; Choi *et al.*, 1989; Jia *et al.*, 2002, 2003; Jin *et al.*, 2007; Koide *et al.*, 2009; Kwon *et al.*, 2008). All of the SNP and CAPS markers were synthesized at the oligo synthesis facility of Bioneer Co., in 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

Table 1. Details of SNP and CAPS markers used and linkage to the five major of rice blast resistant genes.

Gene	Chromosome Number	Marker	Primer sequence		Annaling Tm (℃)	Expected size (bp)	Reference
			Forward (5'-3')	Reverse (5'-3')			
Pia	11	yca72*	aggagaagaagccaccaagg	gagctgccacatcttcctt	54	905 (870,635)	Cho *et al.*, 2007
Pib	2	NSb	atcaactctgccacaaaatcc	cccatatcaccacttgttcccc	57	629	Kwon *et al.*, 2008
Pii	9	JJ113-T	ggatgatgtgatctgcagag	ctcttggtgatctttgttac	56	484	Jeon *et al.*, 2003
Pi5(Pi3)		JJ817-Pi5	gatatggttgaaaagctaatctca	atcattgtccttcatattcagagt	54	1450	Kwon *et al.*, 2008
Pita (Pita-2)	12	YL155/87	agcaggttataagctaggcc	ctaccaacaagttcatcaaa	60	1042	Jia *et al.*, 2002, 2004
pi-ta		YL183/87	agcaggttataagctagctat	ctaccaacaagttcatcaaa	60	1042	
Pi9(t)[a]	6	NBS-O/U*	tacaaccacctaccatcccat	ctcagaacctgcaagtctcg	60	928	Qu *et al.*, 2006
		195-1	atggtcctttatctttattg	ttgctccatctcctctgtt	42	2000	

*These PCR products were digested with *Hinf* I restriction enzyme
a: *Pi9(t)* multi allele gene have six family genes, *Pi9, Piz, Pik-5 (Pish)*, and *Piz-t (Piz-5)*

ng template DNA, 20 pmol of each primer set, 0.25 mM of each dNTPs, 1.5 mM $MgCl_2$, 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). The programmed DNA template was initially denatured at 94℃ for 5 min, followed by 30 cycles of PCR amplification with the following parameters; a 30 sec denaturation at 94℃, 30 sec primer annealing from 42 to 60℃, and a 1 min primer extension at 72℃ allowed for completion of primer extension, with final extension at 72℃ 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 (pH8.0) and visualized under UV light after staining with 0.1 ug/ml of ethidium bromide (Et-Br). The amplified fragment using SNP and CAPS markers, was scored as presence (1) or absence (0) of each gene linked DNA fragment.

PCR-RFLP analysis

For RFLP analysis of PCR-products, 10 μL of amplified products was digested with *Hinf* I restriction enzyme for two markers, yca72* (*Pia* gene) and NBS-O/U* (*Pi9(t)* multi genes, *Pi9*, *Piz*, *Pik-5* (*Pish*), and *Piz-t* (*Piz-5*)). The total volume of the reaction for PCR-RFLP was 20 μL with 2.0 μL of 10X RE buffer, 0.5 μL of *Hinf* I restriction enzyme (10 u/μl, Promega, U.S.A.), 7.5 μL of sterile distilled water, and 10 μl of amplified products. The reaction mixture was incubated at 37℃ for 5 hrs. The digested DNA fragments were separated through 2% agarose gel electrophoresis and visualized under UV light after staining with 0.1 ug/mL of ethidium bromide (Et-Br). The digested fragments were scored as presence (1) or absence (0) of each gene linked DNA fragment.

RESULTS

MAS of rice blast resistance accessions of aromatic rice germplasm

Eighty four accessions of aromatic rice germplasm were screened for the presence of six major rice blast resistance (*R*) genes, *Pia*, *Pii*, *Pi5*(*Pi3*), *Pib*, *Pita* (*Pita-2*), and *Pi9(t)* multiple allele, using eight set of SNP and CAPS markers. All of the eighty four accessions of aromatic rice germplasm possessed more than one blast resistance gene, except for one accession of *japonica* type 'Gerdeh' in Table 2. One accession of *indica* type, Basmati (IT211194), showed the positive amplicons of five major rice blast resistance genes, *Pia*, *Pi5* (*Pi3*), *Pib*, *Pi-ta* (*Pi-ta2*), and *Pik-5* (*Pish*) in Table 2. One accession of *japonica* type aromatic rice (TALLI) and six accessions of *indica* type aromatic rice (Basmati, Mayataung, Kala Namak, Masino Basmati, Basmati 198, and Basmati 5854) possessed four major rice blast resistance genes (Table 2). Eight accessions of *japonica* type and 31 accessions of *indica* type aromatic rice showed the positive bands of three major rice blast resistance genes in Table 2. Eleven accessions of *japonica* type and 13 accessions of *indica* type aromatic rice showed two major rice blast resistance genes in Table 2. Seven accessions of *japonica* type and five accessions of *indica* type aromatic rice possessed one major rice blast resistance gene, *Pi9*(*t*) multi-allele genes in Table 2.

Screening of Pia gene

Estimation of PCR based detection of the *Pia* rice blast resistance gene was determined by visualization of amplicons on 905 bp-positive fragment using yca72 primer. None of the five accessions of domestic aromatic rice germplasm were identified with *Pia* gene during PCR based detection of yca72 marker. The 905 bp- fragment of *Pia* gene on chromosome 11 was scored to 48 accessions of aromatic rice germplasm, seventeen accessions of *japonica* type and thirty one accessions of *indica* type (Fig. 1, Table 2). Among 48 accessions of the positive PCR amplicons, only five accessions (TALLI, Mayataung, Basmati, Basmati 198, and Basmati 5854) were cleaved with *Hinf* I restriction enzyme and separated on 870 bp and 635 bp of positive fragments linked to *Pia* gene (Table 2).

Genetic detection of Pib gene

The *Pib*-specific PCR primer set, NSb marker was developed to produce a 629 bp amplicon based on its sequence on chromosome 2. Screening of *Pib* blast resistance gene was determined by visualization of 629 bp-positive fragments from 64 accessions of aromatic rice germplasm using NSb marker set. Among the 64 accessions, two accessions

Table 2. Genotyping of 84 accessions of aromatic rice germplasm used SNP and CAPS marker linked to the five major rice blast resistant genes.

Lane	Varietie	Ecotype	*Pia*	*Pib*	*Pii*	*Pi5*	*Pi-ta (Pita-2)*	*pi-ta*	*Pi9(t)*	
			Yca72*	NSb-1	JJ113-T3	JJ817	YL155/87	YL183/87	NBS2-O/U*	195-1
1	Hyangmibyeo1ho	Tongil	0	*Pib*	*Pii*	0	0	*pi-ta*	*Piz-t, Piz-5*	0
2	Hyangmibyeo2ho	Tongil	0	*Pib*	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
3	Hyangnambyeo	Japonica	0	0	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
4	Aranghyangchalbyeo	Japonica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
5	Mihayangbyeo	Japonica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
6	A-2	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
7	A-3, Choh Chang	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
8	Muhyang99-8	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
9	Jahyangna861	Japonica	0	0	0	0	0	*pi-ta*	*Piz*	0
10	Jc149	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
11	Jc157	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
12	Iari 7447	Japonica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
13	Daebunhyangdo2	Japonica	0	0	*Pii*	0	0	*pi-ta*	*Pik-5, Pish*	0
14	Shiyayuuine	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
15	Rasomotrafotsy	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
16	TALLI	Japonica	*Pia*	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
17	Masino Basmati	Japonica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
18	Kalomasino Dhan	Japonica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
19	Gerdeh	Japonica	0	0	0	0	0	*pi-ta*	*0*	0
20	KINANDANG PAT	Japonica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
21	Milagrosa Mutant	Japonica	0	0	*Pii*	0	0	*pi-ta*	*Piz*	0
22	Inaguhu	Japonica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
23	Flores	Japonica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Piz*	0
24	Kung-ShanWu-Shen-Ken	Japonica	0	0	*Pii*	0	0	*pi-ta*	*Piz-t, Piz-5*	0
25	Daw Dam	Japonica	0	*Pib*	*Pii*	0	0	*pi-ta*	*Pik-5, Pish*	0
26	415 X Ir352	Japonica	0	*Pib*	*Pii*	0	0	*pi-ta*	*Piz-t, Piz-5*	0
27	Khau Nua Keo	Japonica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
28	Khau Tan Luong	Japonica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
29	Goolarath	Indica	0	0	*Pii*	0	0	*pi-ta*	*Piz*	0
30	Da13	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
31	Basmati 370	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
32	Hyanggaengdo	Indica	0	*Pib*	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
33	Seratus Malam	Indica	0	*Pib*	*Pii*	0	0	*pi-ta*	*Pik-5, Pish*	0
34	Basmati T3	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
35	Jc111	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
36	Arc 6011	Indica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
37	Kaminibhog	Indica	0	*Pib*	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
38	Tarana Deshi	Indica	0	*Pib*	0	0	0	*pi-ta*	*Pi9*	Pi9
39	Basmati	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	*Pi-ta (Pi-ta2)*	*0*	*Piz-t, Piz-5*	0
40	Iranbyeopssi	Indica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
41	Iranbyeopssi	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*0*	*Pik-5, Pish*	0
42	Domsiah	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*0*	*Pik-5, Pish*	0
43	Mulai	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
44	Tareme	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
45	Hyangdo	Indica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5,Pish*	0

Table 2. Continued.

Lane	Varietie	Ecotype	*Pia* Yca72*	*Pib* NSb-1	*Pii* JJ113-T3	*Pi5* JJ201	*Pi-ta (Pita-2)* YL155/87	*pi-ta* YL183/87	*Pi9(t)* NBS2-O/U*	195-1
46	Seratus Malam	Indica	0	*Pib*	0	0	0	*pi-ta*	*Pik-5, Pish*	0
47	Mayataung	Indica	*Pia*	*Pib*	0	0	*Pi-ta (Pi-ta2)*	0	*Piz*	0
48	Yekywin Yinkya Hmwe	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pi9*	*Pi9*
49	Kala Namak	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	*Pi-ta (Pi-ta2)*	0	*Pik-5, Pish*	0
50	Masino Basmati	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	*Pi-ta (Pi-ta2)*	0	*Piz-t, Piz-5*	0
51	Basmati Dhan	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
52	Basmati	Indica	*Pia*	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	*Pi-ta (Pi-ta2)*	*pi-ta*	*Pik-5, Pish*	0
53	Basmati 370	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
54	Basmati 9-93	Indica	0	*Pib*	0	0	0	*pi-ta*	*Pi9*	Pi9
55	Basmati 198	Indica	*Pia*	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
56	Basmati 370	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
57	Basmati 5836	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Piz*	0
58	Basmati 5854	Indica	*Pia*	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
59	Basmati 5875	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
60	Basmati 6113	Indica	0	0	*Pi3, Pi5*	*Pi3, Pi5*	*Pi-ta (Pi-ta2)*	*pi-ta*	*Piz*	0
61	Basmati 1	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
62	Basmati 213 C	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
63	Basmati 372	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
64	Chahora 144	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
65	Pakistani Fine	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
66	Ir841-85-1-1-2	Indica	0	*Pib*	0	0	0	*pi-ta*	*Piz*	0
67	05-Irri-M-46	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
68	Basmati 107	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
69	Basmati 405	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
70	Basmati 5853	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Piz*	0
71	Basmati 5874	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
72	Basmati 6129	Indica	0	0	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
73	Basmati 6311	Indica	0	0	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
74	Basmati 6313	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Piz*	0
75	Basmati 6141	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Piz*	0
76	AZUCENA	Indica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
77	Binicol	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
78	Milfor 6	Indica	0	0	0	0	0	*pi-ta*	*Piz*	0
79	Dinorado	Indica	0	0	0	0	0	*pi-ta*	*Pik-5, Pish*	0
80	Khao Dawk Mali105	Indica	0	0	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
81	Jasmine 85	Indica	0	*Pib*	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0
82	Dellmont	Indica	0	*Pib*	*Pi3, Pi5*	*Pi3, Pi5*	0	*pi-ta*	*Pik-5, Pish*	0
83	Aroma	Indica	0	*Pib*	*Pii*	0	0	*pi-ta*	*Pik-5, Pish*	0
84	Ds20	Indica	0	*Pib*	0	0	0	*pi-ta*	*Piz-t, Piz-5*	0

*These PCR products were digested with *Hinf* I restriction enzyme
0 means the absence of amplicons linked to the rice blast resistant genes

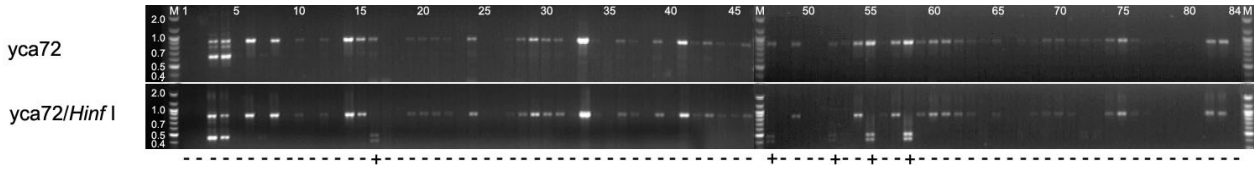

Fig. 1. Agarose gel photograph of 84 aromatic rice germplasms, to screen the presence (+) or absence (-) of 905 bp of the rice blast resistant gene amplified with CAPS marker yca72, linked to *Pia* gene (Upper). The PCR amplicons were cleavaged with *Hinf* I restriction enzyme and separated on 870 bp and 635 bp of positive fragments linked to *Pia* gene (Under). M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-84, eighty four accessions of aromatic rice.

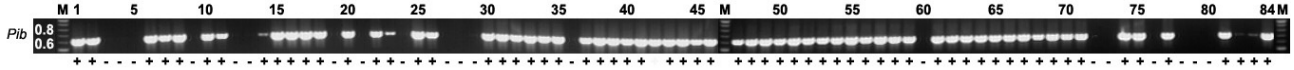

Fig. 2. Agarose gel photograph of 84 aromatic rice germplasms, to estimate the presence (+) or absence (-) of the 629 bp of *Pib* rice blast resistant gene amplified with SNP marker NSb. M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-84, eighty four accessions of aromatic rice.

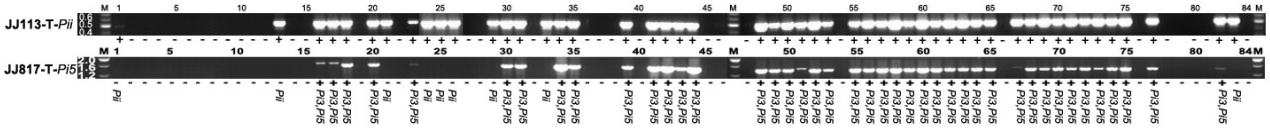

Fig. 3. Detection of *Pii* and *Pi5* (*Pi3*) rice blast resistance genes were shown as a positive DNA fragments amplified with three sets of SNP markers, JJ113-T (484 bp) and JJ817-*Pi5* (1450 bp), tightly linked to the *Pii* and *Pi5* (*Pi3*) genes. M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-84, eighty four accessions of aromatic rice.

(Hyangmibyeo1ho and Hyangmibyeo2ho) were included in *tongil* type and 15 accessions (A-2, A-3 (Choh Chang), Muhyan 99-8, Jahyangna 861, Jc 149, Jc 157, Shiyayuuine, Rasomotrafotsy, TALLI, Masino Basmati, Kalomasino Dhan, KINANDANG PAT, Inaguhu, Flores, Daw Dam, and 415 X Ir352) and 47 accessions included in *japonica* type and *indica* type aromatic rice germplasm, respectively (Fig. 2, Table 2).

Genetic diversity of *Pii* and *Pi5* (or *Pi3*) genes

The tightly linked *Pii* and *Pi5* (or *Pi3*) genes on chromosome 9 were screened with JJ113-T and JJ817-Pi5, respectively. The nine and forty two accessions of positive amplicons were identified as *Pii* gene and *Pi5* (or *Pi3*) genes, respectively (Fig. 3, Table 2).

Fortunately, one accession of domestic *tongil* type aromatic rice, Hyangmibyeo1ho, showed positive reactions of *Pii* gene. Also, five accessions of *japonica* type (Daebunhy-angdo2, Milagrosa Mutant, Kung-ShanWu-Shen-Ken, Daw Dam, and 415 X Ir352) and three accessions of *indica* type (Goolarath, Seratus Malam, and Aroma) aromatic rice possessed the *Pii* rice blast resistance gene.

Two *tongil* type and three *japonica* type of domestic aromatic rice did not identify *Pi5* (or *Pi3*) gene with PCR based detection of JJ817-Pi5 primer set. In 42 out of 84, *Pi5* (or *Pi3*) genes positive accessions were included five accessions of *japonica* type (TALLI, Masino Basmai, Kalomasino Basmati, KINANDANG PAT, and Flores) and 37 accessions of *indica* type aromatic rice germplasm (Fig. 3, Table 2).

PCR profiling of *Pi-ta* (or *Pi-ta2*) gene

For the PCR based screening of *Pi-ta* (or *Pi-ta2*) genes on chromosome 12, the experiment showed that six of the 84 accessions under study produced positive bands of 1042 bp with YL155/87 primer set which was tightly linked to the resistant *Pi-ta* (or *Pi-ta2*) allele. Six positive accessions of *Pi-ta* (*Pi-ta2*) genes possessed four homozygote resistant *Pi-ta* (or *Pi-ta2*) alleles (Basmati, Mayataung, Kala Namak, and Masino Basmati) and two heterozygote resistant *Pi-ta* (or *Pi-ta2*) alleles (Basmati (IT211194) and Basmati 6113). Five of domestic and 23 of foreign *japonica* type aromatic

rice germplasm did not have *Pi-ta* (or *Pi-ta2*) genes with allele specific marker YL155/87. But the 1042 bp-amplicon corresponding to the YL183/87 marker for the susceptible *pi-ta* allele amplified 78 accessions of aromatic rice including the two accessions of heterozygote *Pi-ta* (or *Pi-ta2*) allele (Fig. 4, Table 2).

Genetic diversity of *Pi9(t)* multi-allele genes

Screening of major rice blast resistance family genes *Pi9(t)* on chromosome 6 was determined by visualization of 928 bp and 2.0 kb of positive fragments using NBS-O/U and 195-1 primer, respectively. 928 bp-positive amplicon of scored to 83 out of 84 accessions of aromatic rice germplasm, expect for one accession of *japonica* type rice (Gerdeh) (Fig. 5). The gene-specific marker, 195-1, for *Pi9* of *Pi9(t)* multi genes produced 2.0 kb of positive bands in only three accessions of *indica* type aromatic rice germplasm (Tarana Deshi, Yekywin Yinkya Hmwe, and Basmati9-93) (Fig. 5, Table 2).

Eighty three of *Pi9(t)* multi genes positive bands were cleavaged with *Hinf* I restriction enzyme and grouped as three patterns of *Pi9(t)* multi genes. Group I showed the *Piz* gene in three accessions of *japonica* type (Jahyangna 861, Milagrosa Mutant, and Flores) and nine accessions of *indica* type (Goolarath. Mayataung, Basmati 5836, Basmati 6113, Ir841-85-1-1-2, Basmati 5853, Basmati 6313, Basmati 6141, and Milfor 6). Group II had registered the *Pik-5* and *Pish* genes in two accessions of domestic *japonica* type (Aranghyangchalbyeo and Mihayangbyeo), 15 accessions of *japonica* type (A-2, A-3(Choh Chang), Jc 149, Jc 157, Iari 7447, Daebunhyangdo2, Rasomotrafotsy, TALLI, Masino Basmati, Kalomasino Dhan, KINANDANG PAT, Inaguhu, Daw Dam, Khau Nua Keo, and Khau Tan Luong), and 37 accessions *indica* type. Group III was identified as *Piz-t and Piz-5* genes in two accessions of *domestic tongil* type (Hyangmibyeo1ho and Hyangmibyeo2ho), one accession of *domestic japonica* type (Hyangnambyeo), four accessions of *japonica* type (Muhyang 99-8, Shiyayuuine, Kung-ShanWu-Shen-Ken, and 415 X Ir352), and seven accessions of *indica* type (Hyanggaendo, Kaminibhog, Basmati, Masino Basmati, Khao Dawk Mali 105, Jasmine 85, and DS20) (Fig. 5, Table 2).

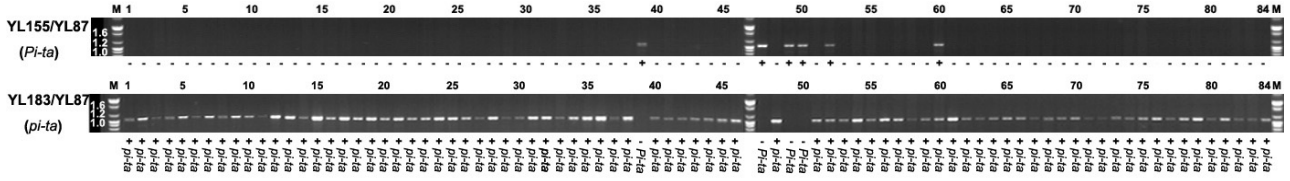

Fig. 4. Electrophoretical band patterns of 84 aromatic rice germplasms were scored as presence (+) or absence (-) of the rice blast resistance genes, *Pi-ta* (*Pi-ta2*), and the rice blast susceptible gene, *pi-ta*, were shown as a positive DNA fragments were amplified with three of SNP markers, YL155/YL87 (1042 bp) and YL183/YL87 (1042 bp), respectively. M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-84, eighty four accessions of aromatic rice.

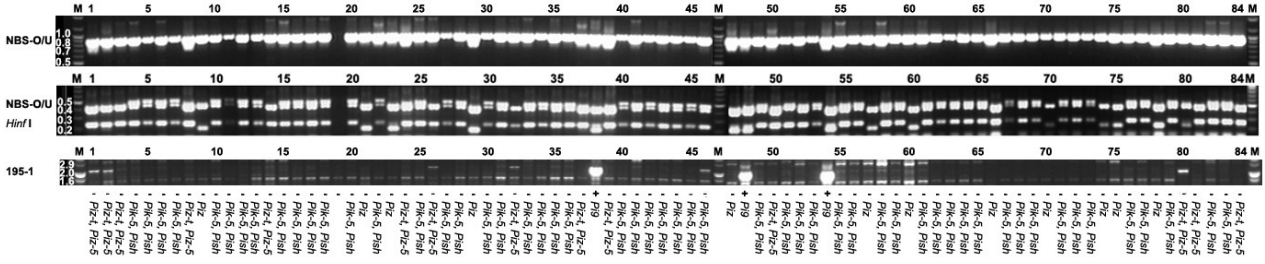

Fig. 5. Agarose gel photograph of 84 aromatic rice germplasms, showed the presence (+) or absence (-) of rice blast resistant genes amplified with CAPS marker NBS-O/U (928 bp) and SNP marker 195-1 (2.0 kb), linked to *Pi9(t)* multiple-allele (*Pi9, Piz, Piz-t, Piz-5, Pik-5*, and *Pish*). The PCR products amplified with NBS-O/U marker was digested with restriction enzyme *Hinf* I (Under). M, molecular marker (Bioneer 100 bp ladder plus); lanes 1-84, eighty four accessions of aromatic rice.

DISCUSSION

The rice blast fungus (*Magnaporthe grisea*) is an economically major cause of yield loss in rice (*Oryza sativa* L.) (Kiyosawa *et al.*, 1986; Xia *et al.*, 1993). The blast disease causes significantly crop losses and is annually estimated to destroy enough rice to feed more than 60 million people (Bonman *et al.*, 1986; Kiyosawa *et al.*, 1986; Kwon and Lee, 2002; Li *et al.*, 2007; Teng *et al.*, 1991; Yaegashi H., 1994).

Molecular marker-assisted selection (MAS) will enhance the efficiency of screening of disease resistance evaluation and the improvement of breeding program. Numerous studies have been carried out on the genetics of blast resistance in rice (Basavaraj *et al.*, 2010; Cho *et al.*, 2007; Dean *et al.*, 2005; Hayashi *et al.*, 2006; Koide *et al.*, 2009; Kwon *et al.*, 2008; Tacconi *et al.*, 2010).

Previous studies have described eighty six aromatic rice germplasm of agronomic traits, physicochemical characteristics, and analysis of aromatic compounds in 2008 (Kim *et al.*, 2008a; Kim *et al.*, 2008b). Also, we have previously reported the genetic diversities of blast resistance (*R*) genes form 84 accessions of aromatic rice with eight SNP markers, z4792, zt4792, z60510, zt6057, k6415, k6411, k39575 and t256, which showed a close-set linkage to 6 major genes, *Piz*, *Piz-t*, *Pik*, *Pik-m, Pik-p*, and *Pit* (Kim *et al.*, 2010).

To date, 96 genes and major QTLs have been reported and have been identified for the rice blast resistance (Koide *et al.*, 2009; Liu *et al.*, 2004; Pan *et al.* 1999; Sallaud *et al.*, 2003). However, a number of blast resistance genes below have recently been mapped, which should be regarded as an essential starting point to isolate these important genes through map-based cloning approach (Berruyer *et al.*, 2003; Bryan *et al.*, 2000; Chauhan *et al.*, 2002; Cho *et al.*, 3007; Goto *et al.*, 1981; Ise, 1993; Jia *et al.*, 2002; Lin *et al.*, 2007; Liu *et al.*, 2005; Zhu *et al.*, 2004).

In this study, evaluations were made on the molecular markers that can be used reliably in marker-assisted selection (MAS), and eight set of CAPS and SNP markers tightly linked to the six major resistance genes, *Pia*, *Pii*, *Pi5*(*Pi3*), *Pib*, *Pita* (*Pita-2*), and *Pi9(t)* multiple allele, from the 84 accessions of aromatic rice germplasm.

Blast resistance in rice is generally classified into two types, qualitative (complete) and quantitative (partial) (Bonman and Mackill, 1988; Jeon *et al.*, 2003; Wang *et al.*, 1994). Most of the partial resistance genes are non-race specific, quantitative and polygenic (Fukuoka and Okuno, 2001; Shinoda *et al.*, 1971; Zenbayashi, *et al.*, 2007). Partial or field resistance of rice blast has received much attention as a means of effective control of a parasite under natural field condition and conferring durable blast resistance when exposed to new races of that parasite (Hittalmani *et al.*, 2000; Wang *et al.* 1994).

Although many rice varieties with complete resistance to *M. grisea* have been developed, unfortunately in many cases, breakdown of rice blast resistance occurred within a few years of the initial cultivation because of the emergence of stronger virulent isolates of rice blast fungus (Bonman *et al.*, 1986; Mackill and Bonman, 1992; Yaegashi, 1994). Results of this study showed that all of the eighty four accessions of aromatic rice germplasm possessed more than one blast resistance genes except for one accessions of *japonica* type 'Gerdeh'. One accession of *indica* type, Basmati (IT211194), showed the broad spectrum of five major rice blast resistance genes, *Pia*, *Pi5* (*Pi3*), *Pib*, *Pi-ta* (*Pi-ta2*), and *Pik-5* (*Pish*). Cho *et al.* (2003) reported that the isogenic lines of Chucheongbyeo containing the *Pia* gene were more effective in field resistance, especially to neck blast, than those isogenic lines of Suweon 345 containing *Pib* gene.

This study had successfully detected 48 positive PCR amplicons of the *Pia* rice blast resistance gene with yca72 marker and identified 48 positive fragments cleaved with *Hinf* I restriction enzyme to only five accessions (TALLI, Mayataung, Basmati, Basmati 198, and Basmati 5854) as *Pia* gene. The *Pib* specific PCR primer set, NSb was developed to produce a 629 bp-positive amplicon in 64 accessions, two accessions of *tongil* type, 15 accessions of *japonica* type and 47 accessions of *indica* type aromatic rice germplasm.

It is well known that R genes share common sequence motifs, such as leucine-rich repeat domain (LRR), nucleotide-binding site (NBS) and kinase domains, reflecting related functions on their role in pathogen recognition and signal

transduction (Miyamoto *et al.* 1996). *Pib* (Wang *et al.*, 1999) and *Pita* (Bryan *et al.*, 2000) possessed sequences encoding NDS-LRR proteins.

Two dominant loci associated with qualitative resistance to five isolates of blast fungus *Pi-5(t)* and *Pi-7(t)* were mapped on chromosomes 4 and 11, respectively, in 287-F7 recombinant inbred lines (RILs) of Moroberekan/CO39. Jeon *et al.* (2003) efficiently constructed a genetic and physical map of the *Pi5*(t) locus, in which the locus is located in a 170-kb binary bacterial artificial chromosome contig on chromosome 9. In addition, they demonstrated that the *Pi5*(t) locus is identical to the *Pi3*(t) locus. The data indicated one accessions of domestic *tongil* type aromatic rice (Hyangmibyeo1ho), five accessions of *japonica* type (Daebunhyangdo2, Milagrosa Mutant, Kung-ShanWu-Shen-Ken, Daw Dam, and 415 X Ir352) and three accessions of *indica* type (Goolarath, Seratus Malam, and Aroma) aromatic rice possessed the *Pii* rice blast resistance gene. Also, five accessions of *japonica* type (TALLI, Masino Basmai, Kalomasino Basmati, KINANDANG PAT, and Flores) and 37 accessions of *indica* type aromatic rice germplasm possessed the *Pi5* (or *Pi3*) genes, but two *tongil* type and three *japonica* type of domestic aromatic rice did not have *Pi5* (or *Pi3*) gene with PCR based detection of JJ817-*Pi*5 primer set.

Two blast resistance genes, *Pi-ta* and *Pi-ta2*, have been located at the *Pi-ta* locus on chromosome 12. These two genes were interacting in terms of their resistance specificity: *Pi-ta2* has a broader resistance spectrum than *Pi-ta* (Bryan *et al.*, 2000; Rybka *et al.*, 1997). That is, no *M. grisea* isolate has been found that is avirulent toward *Pi-ta* but virulent toward *Pi-ta2* (Bryan *et al.*, 2000; ISE, 1993).

In the present study, we found that six positive accessions of *Pi-ta* (or *Pi-ta2*) genes on chromosome 12 possessed four homozygote resistant *Pi-ta* (or *Pi-ta2*) alleles (Basmati, Mayataung, Kala Namak, and Masino Basmati) and two heterozygote resistant *Pi-ta* (or *Pi-ta2*) alleles (Basmati (IT211194) and Basmati 6113).

This resistance spectrum feature of *Pi-ta* and *Pi-ta2* suggested that *Pi-ta2* blast specificity was conferred by a combination of *Pi-ta* and at least one additional resistance gene (Jia *et al.*, 2002, 2003). *Pi-ta* has been cloned, while *Pi-ta2* has not been cloned, and it remains to be answered whether this hypothesis is valid (Bryan *et al.*, 2000).

Pik, Piz5, *Pi9* and *Pish* could be used as candidate resistance genes for rice breeding since their specific resistance differed from those of the backbone parents in Guangdong, China. Gene pyramiding of *Pikh* (or *Pi1*(t)), *Pi9* (or *Piz5*) and *Pish* (or *Pita2*) would be effective to obtain broad-spectrum blast resistance in rice breeding program in Guangdong, China (Goto *et al.*, 1981; Yang *et al.*, 2009). Liu *et al.* (2002) constructed a high-density map of the *Pi9*(t) locus, and demonstrated that the *Pi2*(t) and *Pi9*(t) was physically linked in about 100-kb interval on rice chromosome 6. *Pi9* (t) gene has been recognized to confer broad-spectrum resistance to many isolates collected from various countries (Chen *et al.*, 1996; Liu *et al.*, 2002). *Pi9* locus in a group of genetically distinct cultivars had revealed the complex and divergent genome organization of this locus (Zhou *et al.*, 2007).

Screening of major rice blast resistance *Pi9(t)* family genes on chromosome 6 was determined by NBS-O/U and 195-1 primers respectively. The gene-specific marker, 195-1, for *Pi9* detected only three accessions of *indica* type aromatic rice germplasm (Tarana Deshi, Yekywin Yinkya Hmwe, and Basmati9-93).

From the results, eighty three positive bands of *Pi9(t)* multi genes were cleaved with *Hinf* I restriction enzyme and possessed 12 accessions of *Piz* gene, 52 accessions of *Pik-5* and *Pish* genes, and 14 accessions of *Piz-t and Piz-5* genes. However, the Korean *japonica* and *Tongil* type varieties were grouped into four types: Koshihikari-type, *Piz-t* and *Piz-t*-type, *Piz* and *Pi9*-type, and null type, respectively, from two markers, pBA14 and NBS2-O/U (Cho *et al.*, 2007)

Characterization of additional rice germplasm was useful to U.S. rice breeders. Breeder conducted field evaluations on approximately 1000 rice germplasm accessions introduced into the USA. These accessions were evaluated for desirable agronomic characteristics and for resistance to the two major diseases, blast and sheath blight (Dilday *et al.*, 2001; Lee, 1994; Yan *et al.*, 2002, 2003).

Pyramiding different resistance genes using MAS provides opportunities to breeders to develop broad-spectrum resistance against diseases and insects. The use of

cost-effective DNA markers derived from the fine mapped position of the genes for important agronomic traits and MAS strategies will provide opportunities for breeders to develop high-yielding, stress-resistant, and better-quality rice cultivars (Jena and Mackill, 2008; McClung *et al.*, 1997; Suh *et al.*, 1987; Tabien *et al.*, 2000; Tabien *et al.*, 2002;).

In conclusion, the approach makes use of the successful identification of eight set of CAPS and SNP markers tightly linked to the six major resistance genes, *Pia*, *Pii*, *Pi5* (*Pi3*), *Pib*, *Pita* (*Pita-2*), and *Pi9(t)* multiple allele, from the 84 accessions of aromatic rice germplasm. It is possible that the marker-assisted selection of rice blast resistance genes will help in the breeding program for the production of multi disease resistant aromatic rice varieties from different genetic resources.

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