Application and utilization of marker assisted selection for biotic stress resistance in hybrid rice (*Oryza sativa* L.)

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Abstract Development of disease resistant plant is one of the important objectives in rice breeding programs because biotic stresses can adversely affect rice growth and yield losses. This study was conducted to identify lines with multiple-resistance genes to biotic stress among 173 hybrid rice breeding lines and germplasms using DNA-based markers. Our results showed that one hybrid rice line [IR98161-2-1-1-k1-3 (IR86409-3-1-1-1-1/IRBB66)] possessed 5 bacterial blight resistance genes (*Xa4*, *xa5*, *Xa7*, *Xa13* and *Xa21*) while two hybrid rice lines [IR98161-2-1-1-k1-2 (IR86409-3-1-1-1-1/IRBB66) and 7292s (IR75589-31-27-8-33S(S1)/IR102758B)] possessed 3 bacterial blight resistance genes (*Xa4*, *Xa7* and *Xa21*, and *Xa3*, *Xa4* and *xa5*). Molecular survey on rice blast disease revealed that most of these lines had two different resistant genes. Only 11 lines possessed *Pib*, *Pi-5*, and *Pi-ta*. In addition, we further surveyed the distribution of insect resistant genes, such as *Bph1*, *Bph18(t)*, and *Wbph*. Three hybrid breeding lines [IR98161-2-1-1-k1-3 (IR86409-3-1-1-1-1/IRBB66), IR98161-2-1-1-k1-2 (IR86409-3-1-1-1-1/IRBB66), and 7292s (IR75589-31-27-8-33S(S1)/IR102758B)] contained all three resistance genes. Finally, we obtained four hybrid rice breeding lines and germplasms [IR98161-2-1-1-k1-2 (IR86409-3-1-1-1-1/IRBB66), Damm-

Noeub Khmau, 7290s, and 7292s (IR75589-31-27-8-33S(S1)/IR102758B)] possessing six-gene combination. They are expected to provide higher level of multiple resistance to biotic stress. This study is important for genotyping hybrid rice with resistance to diverse diseases and pests. Results obtained in this study suggest that identification of pyramided resistance genes is very important for screening hybrid rice breeding lines and germplasms accurately for disease and pest resistance. We will expand their cultivation safely through bioassays against diseases, pests, and disaster in its main export countries.

Keywords Hybrid, Rice, MAS, MAB, biotic stress, resistance

Introduction

Rice (*Oryza sativa* L.) is one of the most important and essential source of food crops for many people in the world. Maintaining stable rice production is extremely important to feed the constantly growing human population (Maclean et al. 2002; Sasaki and Burr 2000) in this ever changing climate. Improvement of disease resistance is an important objective in rice breeding programs because rice is exposed to various pests and several diseases including bacterial leaf blight (BB), rice blast (BL), sheath blight (ShB), tungro, and brown planthopper (BPH). Among diseases, bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the most destructive bacterial disease of rice that limits rice yield in Asia (Mew 1987) and most of the rice growing countries. Rice blast disease caused by the ascomycete fungus, *Magnaporthe oryzae* (Couch and Kohn 2002), is also one of the leading causes of great yield loss of rice worldwide and is the most devastating fungal disease on cultivated rice as well as other species of the Poaceae (Zeigler et al. 1994; Talbot and Foster 2001; Talbot 2003; Niño et al. 2015). Rice tungro disease (RTD) is caused by two viruses, *Rice tungro spherical virus*

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(RTSV) and *Rice tungro bacilliform virus* (RTBV), and is one of the most serious diseases of rice in South and Southeast Asia (Lee et al. 2010). Rice damaged by RTD show symptoms, such as small growth and yellow-orange discoloration of leaves (Hibino 1983). Among the rice-feeding insects, *Nilaparvata lugens*, the rice brown planthopper (BPH) is the most damaging pest (Jiang et al. 2012). In addition, a rice phloem sap-sucking insect, whitebacked planthopper (WBPH) (*Sogatella furcifera* Horvath), can cause serious reductions in plant height, number of productive tillers, filled grains, and yield (Yamasaki et al. 2003).

Breeding for resistant varieties is the most effective control approach to prevent rice production losses as well as reduce pesticides usage. However, development of rice resistant cultivars by conventional breeding without checking the incorporation of resistant genes may lead to the breakdown of the disease resistance after new resistant cultivar is released, due to complexity of genetic control with complementary or additive effects as well as their environment interactions (Yaegashi 1994; Han et al. 2001). Therefore, it is necessary to develop durable resistance cultivars by incorporating several resistant genes against highly variable pathogen or pests (Bonman et al. 1986; Hittalmani et al. 2000) using marker-assisted selection (MAS). Molecular marker techniques such as MAS may provide new ways for identifying and pyramiding valuable genes to enhance the disease resistance and overcome the breakdown of resistance that frequently occurs in rice breeding programs (Song et al. 2014; Ghulam et al. 2013). Conventional breeding approaches are difficult due to dominance and epistatic effects of genes governing disease resistance by gene pyramiding (Joseph et al. 2004; Rajpurohit et al. 2011). However, DNA-based genetic markers identified in accordance with the phenotypic traits or closed linked to each of the resistance genes are recently expected to play an important role in marker-associated breeding (MAB) for assessment of stress-tolerance and disease-resistance in hybrid breeding plants (Nogoy et al. 2016). According to Wang et al. (2005), developing super hybrid rice depends largely on the genetic resources of the parental lines and the conventional breeding technology. However, improving rice varieties based on this breeding technology is complicated, cumbersome and time-consuming. Uncovering the molecular genetic control of rice heterosis would further improve hybrid rice technology.

To develop rice varieties that can withstand the current insect pest and disease problem in the rice growing regions, we initiate to establish rice breeding programs based on conventional breeding and advanced molecular breeding techniques.

Materials and methods

Plant materials

We used a rice panel comprised of 173 hybrid rice breeding lines and germplasms (Table 1) and control varieties (Table 2), which can be compared by size and presence/absence of amplicon between resistant and susceptible lines.

DNA extraction

Total genomic DNA was extracted from fresh leaves of two-week-old rice seedlings using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method as previously described by Kump and Javornik (1996). DNA concentration was quantified using a spectrophotometer (NanoDrop One, Thermo Scientific). The DNA solution was then diluted to a working concentration with distilled water and stored at -20°C until use.

Genotyping

Polymerase chain reaction (PCR) was performed using resistant and susceptible gene-specific primers reported in previous studies and developed in this study (Table 3). Approximately 40 ng of genomic DNA was used in a 20 µl PCR reaction containing 2 µl of primer pairs (10 pmol/µl), 2.0 µl of 10 x PCR buffer, 1.6 µl of dNTP (2.5 mM), and 0.2 µl of Taq polymerase (5 unit/µl; Promega, USA). The reaction mixture was subjected to the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55–60°C for 30 s and extension at 72°C for 45–60 s, and final extension at 72°C for 10 min. The PCR amplified products of bacterial blight (*Xa3*, *Xa7*, *xa13* and *Xa21*), blast (*Pi-b*, *Pi-ta* and *Pi-5*), and brown planthopper (*Bph1*) resistant genes were separated on 1.5–2.0 % agarose gel and stained with ethidium bromide. The amplified products of *Xa4*, *xa5*, and *Wbph* were run on a fragment capillary gel electrophoresis system (Fragment analyzer, USA) and fragments were sized and scored using PROSize 2.0 software (Fragment analyzer, USA). The amplification of *Bph18(t)* is performed on an EcoTM Real-Time PCR System (Illumina, San Diego, California, USA) according to the user guide manual (Illumina). Allele calling of amplified fragments of hybrid lines and control varieties were based on their respective resistance and susceptible controls.

Table 1 List of 173 hybrid rice breeding lines and germplasms used in disease resistance analysis in this study

No	Designation	Cross/Origin	Remark	No	Designation	Cross/Origin	Remark
1	Damm-Noeub Sark	Cambodia	Aromatic rice (Jasmine)	40	IR98178-8-2-1-k1-2	IR86603-15-2-1-1-1-1-1-1/ IR86515-4-7-2	Restorer
2	Srau Damm-Noeub Banh-Chras Kcharl	Cambodia	Aromatic rice (Jasmine)	41	IR98178-18-1-1-k1-2	IR86603-15-2-1-1-1-1-1-1/ IR86515-4-7-2	Restorer
3	Saigon	Vietnam	Restorer	42	IR98178-18-1-1-k1-3	IR86603-15-2-1-1-1-1-1-1/ IR86515-4-7-2	Restorer
4	Saigon Darat	Vietnam	Restorer	43	IR98187-30-1-1-k1-2	IR85508-6-1-1-1-1-1-1-1/ IR86409-3-1-1-1-1-1	Restorer
5	Rening	Seoul University germplasm	Restorer	44	IR98187-30-1-1-k1-3	IR85508-6-1-1-1-1-1-1-1/ IR86409-3-1-1-1-1-1	Restorer
6	Jasmin 85	Thailand	Aromatic rice (Jasmine)	45	IR98194-9-2-1-k1-2	IR86590-22-2-2-1-3-1-1-1/ IR86612-21-6-1-1-1-1-1	Restorer
7	OM 341	Vietnam	Local variety	46	IR98194-9-2-1-k1-3	IR86590-22-2-2-1-3-1-1-1/ IR86612-21-6-1-1-1-1-1	Restorer
8	OM 6312-1	Vietnam	Local variety	47	IR98200-25-1-1-k1-3	IR86590-22-2-2-1-3-1-1-1/ IR86612-21-6-1-1-1-1-1	Restorer
9	OM 6312-2	Vietnam	Local variety	48	IR98200-25-1-1-k1-4	IR86590-22-2-2-1-3-1-1-1/ IR86612-21-6-1-1-1-1-1	Restorer
10	OM 7347	Vietnam	Local variety	49	IR98212-18-2-1-k1-2	IR06A169/IR86612-26-9-5-1-1-1-1-1	Restorer
11	OM 9538	Vietnam	Local variety	50	IR98229-2-2-1-k1-2	IR08A138/IR72998-93-3-3-2R	Restorer
12	HHZ 12-DT10-SAL1-DT1	IRRI	Breeding line	51	IR98229-2-2-1-k1-3	IR08A138/IR72998-93-3-3-2R	Restorer
13	VD 20	Vietnam	Restorer	52	IR98229-9-2-1-k1-2	IR08A138/IR72998-93-3-3-2R	Restorer
14	Anhui collection 1	Anhui province, China	Restorer	53	IR98229-9-2-1-k1-3	IR08A138/IR72998-93-3-3-2R	Restorer
15	Anhui collection 2 PTGMS	Jilin Province, China	Restorer	54	IR98229-24-1-1-k1-2	IR08A138/IR72998-93-3-3-2R	Restorer
16	Anhui collection 3 R	Anhui province, China	Restorer	55	IR98229-24-1-1-k1-3	IR08A138/IR72998-93-3-3-2R	Restorer
17	Anhui collection 4 Bulk	Anhui province, China	P/TGMS	56	IR98241-24-2-1-k1-2	IR06N172/IR86612-38-2-2-1-1-1-1-1	Restorer
18	Aromatic rice 10-1	India	Aromatic rice (Basmati)	57	IR98241-24-2-1-k1-3	IR06N172/IR86612-38-2-2-1-1-1-1-1	Restorer
19	Aromatic rice 10-2	India	Aromatic rice (Basmati)	58	IR98272-5-1-1-k1-3	9311/FL478(from Dr. Ismail)	Restorer
20	IR101861-7-1-k1-2	MingHui63/IR03A550	Restorer	59	IR98272-5-1-1-k1-4	9311/FL478(from Dr. Ismail)	Restorer
21	IR101861-7-1-k1-3	MingHui63/IR03A550	Restorer	60	IR98305-10-1-1-k1-3	IR68897B/IRBB23	maintainer, BB
22	IR101861-28-1-k1-2	MingHui63/IR03A550	Restorer	61	Com collection 2-1	Cambodia	High yielding ability
23	IR101861-28-1-k1-3	MingHui63/IR03A550	Restorer	62	IR96632-1-1-2-1-k1-2	IR85592-7-1-1-1-5-1-1-1/ WeedTolerantRice1	Maintainer
24	IR101870-12-1-k1-2	MingHui63/IR08N103	Restorer	63	IR96632-1-1-2-1-k1-3	IR85592-7-1-1-1-5-1-1-1/ WeedTolerantRice1	Maintainer
25	IR101870-12-1-k1-3	MingHui63/IR08N103	Restorer	64	IR96701-28-5-2-1-k1-2	IR85552-37-1-1-1-2-1-1-1/IR80555B	maintainer, BB
26	IR101870-25-1-k1-2	MingHui63/IR08N103	Restorer	65	IR96701-28-5-2-1-k1-3	IR85552-37-1-1-1-2-1-1-1/IR80555B	maintainer, BB
27	IR101870-25-1-k1-3	MingHui63/IR08N103	Restorer	66	IR96701-28-5-2-1-k1-2	IR85552-37-1-1-1-2-1-1-1/IR80555B	maintainer, BB
28	IR101872-46-1-k1-2	MingHui63/ IR86590-22-2-2-1-3-1-1-1	Restorer	67	IR96701-28-5-2-1-k1-3	IR85552-37-1-1-1-2-1-1-1/IR80555B	maintainer, BB
29	IR101907-27-2-k1-2	IR85593-20-1-1-1-2-3-1-1-1-1/ IR86522-29-4-2-1-1-1-1-1	Restorer	68	IR 97727-11-1-2-2	HANAREUM/NSIC RC 158/HANAREUM	
30	IR101907-27-2-k1-3	IR85593-20-1-1-1-2-3-1-1-1-1/ IR86522-29-4-2-1-1-1-1-1	Restorer	69	IR 97727-82-1-2-2	HANAREUM/NSIC RC 158/HANAREUM	
31	IR98073-3-1-1-k1-3	IR72903-131-1-2-3R/ IR85485-106-B-B-1-1-1-1	Restorer	70	IR 97730-27-2-3-1	SAEGAEJINMI/IR 6 (PAKISTAN)/SAEGAEJINMI	
32	IR98116-7-2-1-k1-2	SACG7(GID: 2643634)/IRBB23	Restorer, BB	71	BK7 6089-74-40	Seoul University germplasm	Restorer
33	IR98116-7-2-1-k1-3	SACG7(GID: 2643634)/IRBB23	Restorer, BB	72	IR2768-11-133-e	IRRI	Restorer
34	IR98139-9-1-1-k1-2	IR86505-6-15-2-1-1-1-1/IRBB60	Restorer, BB	73	RPW6-13	Seoul University germplasm	Restorer
35	IR98139-9-1-1-k1-3	IR86505-6-15-2-1-1-1-1/IRBB60	Restorer, BB	74	GIIB-si 213	Seoul University germplasm	Restorer
36	IR98141-25-1-1-k1-2	IR86526-8-8-1-1-1-1-1/ IR86555-3-3-1-1-1-1-1-1-1	Restorer	75	IR20905-11-3-3	IRRI	Restorer
37	IR98141-25-1-1-k1-3	IR86526-8-8-1-1-1-1-1/ IR86555-3-3-1-1-1-1-1-1-1	Restorer	76	J. P. 5-IR 946-2-2-2	IRRI	Restorer
38	IR98161-2-1-1-k1-2	IR86409-3-1-1-1-1-1/IRBB66	Restorer, BB	77	Oksusu byeo	China	Restorer

Table 1 Continued

No	Designation	Cross/Origin	Remark	No	Designation	Cross/Origin	Remark
78	IR 68	IRRI	Restorer	127	Aromatic rice 11-1	India	Aromatic rice (Basmati)
79	IR5105-113-3	IRRI	Restorer	128	Aromatic rice 11-2	India	Aromatic rice (Basmati)
80	IR2006	IRRI	Restorer	129	Aromatic rice 11-3	India	Aromatic rice (Basmati)
81	IR2003-97-4-2	IRRI	Restorer	130	Jasponica H-B-B-8-B	Selected line	Restorer, Aromatic rice
82	IR2042-175-3-2-2	IRRI	Restorer	131	Jasponica H-B-B-7-B	Selected line	Restorer, Aromatic rice
83	IR1529-430-3	IRRI	Restorer	132	Jasponica H-B-B-16-B	Selected line	Restorer, Aromatic rice
84	IR1539-823-1-4	IRRI	Restorer	133	Jasponica H-B-B-21-B	Selected line	Restorer, Aromatic rice
85	Kianse-Wuan	China	Restorer	134	Jasponica H-B-B-26-B	Selected line	Restorer, Aromatic rice
86	Laxmi	India	Restorer	135	Jasponica H-B-B-7-1	Selected line	Restorer, Aromatic rice
87	Quella-Inia	Seoul University germplasm	Restorer	136	Jasponica H-B-B-9-1	Selected line	Restorer, Aromatic rice
88	IR9859-5-3-3	IRRI	Restorer	137	Jasponica H-B-B-12-1	Selected line	Restorer, Aromatic rice
89	CB435	Seoul University germplasm	Restorer	138	Jasponica H-B-B-17-1	Selected line	Restorer, Aromatic rice
90	IR23325-R-R-B-7-2-2	IRRI	Restorer	139	Jasponica H-B-B-18-1	Selected line	Restorer, Aromatic rice
91	IR4457-5-3-6	IRRI	Restorer	140	Jasponica H-B-B-19-1	Selected line	Restorer, Aromatic rice
92	VRH624	Seoul University germplasm	Restorer	141	Jasponica H-B-B-21-1	Selected line	Restorer, Aromatic rice
93	9019	China	Restorer	142	Jasponica H-B-B-27-1	Selected line	Restorer, Aromatic rice
94	Y4037	China	Restorer	143	IRRI-A29 B12	IRRI maintainer	Maintainer, High natural crossing
95	K100520	Seoul University germplasm	Restorer	144	IRRI-A29 B13	IRRI maintainer	Maintainer, High natural crossing
96	Conde	Seoul University germplasm	Restorer	145	IR1487-327-1-1-3	IRRI	Restorer
97	N22	India	Wide compatibility	146	DF-1	Seoul University germplasm	Restorer
98	Garu	Seoul University germplasm	Restorer	147	Utri Rajapan	IRRI_Indonesia	Restorer
99	Restorer 1	China	Restorer	148	IR4547-2-1-2	IRRI	Restorer
100	Restorer 2	China	Restorer	149	IR 01W105	IRRI	Restorer
101	Restorer 3	China	Restorer	150	IR 70	IRRI	Restorer
102	Pare panjang	Seoul University germplasm	Restorer	151	Tjempo Brondol	Indonesia	Restorer
103	ESMET126	Seoul University germplasm	Restorer	152	Burung Putar	Indonesia	Restorer
104	BD 43	Bangladesh	Restorer	153	Phkar Khgnei	Cambodia	Aromatic rice (Jasmine)
105	BR 26	Bangladesh	Restorer	154	Damm-Noeub Phka Roluoh	Cambodia	Aromatic rice (Jasmine)
106	IR OM CS 2102	IRRI- Vietnam	Restorer	155	Karn Dal Kbal	Cambodia	Aromatic rice (Jasmine)
107	IR7760-4-8-2	IRRI	Restorer	156	Thmar Ror Meal	Cambodia	Aromatic rice (Jasmine)
108	TGMS	China	TGMS	157	KaseKam	Cambodia	Aromatic rice (Jasmine)
109	Aromatic rice 1-1	India	Aromatic rice (Basmati)	158	Damm-Noeub Trar-sark	Cambodia	Aromatic rice (Jasmine)
110	Aromatic rice 1-2	India	Aromatic rice (Basmati)	159	Damm-Noeub Krar Muon	Cambodia	Aromatic rice (Jasmine)
111	Aromatic rice 1-3	India	Aromatic rice (Basmati)	160	Damm-Noeub Khmau	Cambodia	Aromatic rice (Jasmine)

Table 1 Continued

No	Designation	Cross/Origin	Remark	No	Designation	Cross/Origin	Remark
112	Aromatic rice 3-1	India	Aromatic rice (Basmati)	161	Damm-Noeub Trar-sark	Cambodia	Aromatic rice (Jasmine)
113	Aromatic rice 3-2	India	Aromatic rice (Basmati)	162	Damm-Noeub Chherng Meann	Cambodia	Aromatic rice (Jasmine)
114	Aromatic rice 3-3	India	Aromatic rice (Basmati)	163	Damm-Noeub Khmau	Cambodia	Aromatic rice (Jasmine)
115	Aromatic rice 5-1	India	Aromatic rice (Basmati)	164	Damm-Noeub youn	Cambodia	Aromatic rice (Jasmine)
116	Aromatic rice 5-2	India	Aromatic rice (Basmati)	165	Damm-Noeub youn	Cambodia	Aromatic rice (Jasmine)
117	Aromatic rice 5-3	India	Aromatic rice (Basmati)	166	Krar Harm	Cambodia	Aromatic rice (Jasmine)
118	Aromatic rice 6-1	India	Aromatic rice (Basmati)	167	Damm-Noeub Khmau	Cambodia	Aromatic rice (Jasmine)
119	Aromatic rice 6-2	India	Aromatic rice (Basmati)	168	7289s	IR75589-31-27-8-33S(S1)/IR102561B	TGMS
120	Aromatic rice 6-3	India	Aromatic rice (Basmati)	169	7290s	IR75589-31-27-8-33S(S1)/IR102568B	TGMS
121	Aromatic rice 9-1	India	Aromatic rice (Basmati)	170	7292s	IR75589-31-27-8-33S(S1)/IR102758B	TGMS
122	Aromatic rice 9-2	India	Aromatic rice (Basmati)	171	7293s	IR75589-31-27-8-33S(S1)/IR58025B	TGMS
123	Aromatic rice 9-3	India	Aromatic rice (Basmati)	172	7306s	IR75589-31-27-8-33S/IR105687B	TGMS
124	Aromatic rice 10-1	India	Aromatic rice (Basmati)	173	TGMS_bulks	IRRI	TGMS
125	Aromatic rice 10-2	India	Aromatic rice (Basmati)				

Table 2 Information of control varieties used in several disease resistance analyses in this study

Gene	Resistant Controls	Susceptible Controls
<i>Xa3</i>	IRBB3	IR24
<i>Xa4</i>	IRBB4	IR24
<i>xa5</i>	IRBB5	IR24
<i>Xa7</i>	IRBB7	IR24
<i>xa13</i>	IRBB13	IR24
<i>Xa21</i>	IRBB21	IR24
<i>Pi-b</i>	IRBL-b	LTH
<i>Pi-ta</i>	IRBL-ta(K1), IRBL-ta(CT2), IRBL-ta2(Pi)	LTH
<i>Pi-5</i>	IRBL_3(CP4), IRBL_5(M), IRBL_i(F5)	LTH
<i>Bph1</i>	Hangangchal1, IR26	Dongjin, IR24
<i>Bph18(t)</i>	Anmi, Anda	IR24, Ilpum
<i>WBPH</i>	Utri merah, N22	Nipponbare, TN1

Developing *Bph18(t)* primer for HRM

Bph18(t)(SNP23/SNP24) was designed based on a single nucleotide variation (G/C) found on the 18,379,251 region of the gene sequences in susceptible (HR 20654-39-3-5, IR 10 K153, IR 10K150, IR 10K152, Jinmi, Kanto 51 and Lemont) and resistant (IR65482, Anda, AG04208, SR14694-57-4-2-1-3-2-2, SR21733-48-1-12-3-2, Backunchal, Backyang and Chupung) cultivars. The forward and reverse primer sequences that flank the SNP region were generated using Primer 3 (v.0.4.0) (Untergasser et al. 2012; Koressaar and

Remm 2007) which were set to amplify 110 bp of product size. To test the primer, we used it to genotype a panel of 10 resistant and 31 susceptible varieties, as shown in Fig. 1.

Results

Estimation of Genotypes for BB Resistance Genes

A total of 173 hybrid breeding lines genotyped using PCR-based markers related to *Xa3*, *Xa4*, *xa5*, *Xa7*, *xa13*, and *Xa21*

Table 3 List of markers used for analysis of various diseases resistance

Disease/ Insect	Gene	Marker	Type	Primer sequence (5' → 3')	Expected Size (bp)	Reference
Bacterial blight (BB)	<i>Xa3</i>	BB3-Su	Fw	CGGAGCGACACAGCTATCAT	743	Hur et al. 2013
			Rv	CGTGAGGTTCCTATGGCGATT		
	<i>Xa3</i>	BB3-Re	Fw	CCACAATGCCATGTCAGGTGGCATCCCTGCA	255	
			Rv	AGGTGTTGGAGGATTGGCAT		
	<i>Xa4</i>	RM 224	Fw	ATCGATCGATCTTCACGAGG	150/120	Chen et al. 1997; McCouch et al. 2002
			Rv	TGCTATAAAAAGGCATTTCGGG		
	<i>xa5</i>	RM122	Fw	GCACTGCAACCATCAATGAATC	236/232	Chen et al. 1997
			Rv	CCTAGGAGAAACTAGCCGTCCA		
	<i>Xa7</i>	M5	Fw	CGATCTTACTGGCTCTGCAACTCTGT	294/1170	Porter et al. 2003
			Rv	GCATGTCTGTGTCGATTTCGTCCGTACGA		
	<i>xa13</i>	xa13 prom	Fw	GGCCATGGCTCAGTGTATTAT	1000/520	Zhang et al. 1996; Singh et al. 2011
			Rv	GAGCTCCAGCTCTCCAAATG		
	<i>Xa21</i>	pTA248	Fw	AGACGCGGAAGGGTGGTTCCCGGA	1000/750	Huang et al. 1997
			Rv	AGACGCGTAATCGAAAGATGAAA		
Blast	<i>Pib</i>	NSb	Fw	ATCAACTCTGCCACAAAATCC	629	Kwon et al. 2008
			Rv	CCCATATCACCACCTTGTTCCCC		
	<i>Pi5</i>	JJ817	Fw	GATATGGTTGAAAAGCTAATCTCA	1450	Kwon et al. 2008
			Rv	ATCATTGTCTTCATATTCAGAGT		
	<i>Pi-ta</i>	YL155/87	Fw	AGCAGGTTATAAGCTAGGCC	1042	Jia et al. 2002, 2004
			Rv	CTACCAACAAGTTCATCAAA		
BPH	<i>Bph1</i>	BeP18-3	Fw	CGCTGCGAGAGTGTGACACT	523	Kim and Sohn, 2005
			Rv	TTGGGTTACACGGGTTTGAC		
	<i>Bph18(t)</i>	SNP23	Fw	CGATGGATTACCCTATCACCTCAA	110	Developed in this study
			Rv	AACCCTCTGCACACCATCGG		
WBPH	<i>Wbph</i>	RM8213	Fw	AGCCCAGTGATACAAAGATG	177	Sun et al. 2005
			Rv	GCGAGGAGATACCAAGAAAG

Fig. 1 HRM curve profiles of 10 resistant and 31 susceptible varieties using developed specific SNP marker for *Bph18(t)* in this study

genes were analyzed for fragment size differences and presence/absence of genes (Fig. 2). The percentage of rice panel identified to contain the gene was 7.5% with *Xa7*, 22.5% with *xa5*, 0.6% with *xa13*, and 1.2% with *Xa21* (Fig. 5A and Table 4). Among the hybrid breeding lines, IR98161-

2-1-1-k1-3 (IR86409-3-1-1-1-1-1/IRBB66) possessed five bacterial blight resistance genes (*Xa4*, *xa5*, *Xa7*, *Xa13* and *Xa21*) while two hybrid rice lines [IR98161-2-1-1-k1-2 (IR86409-3-1-1-1-1-1/IRBB66) and 7292s (IR75589-31-27-8-33S(S1)/IR102758B)] possessed three bacterial blight resistance genes

Fig. 2 PCR amplification patterns of markers that discriminate each of the six bacterial blight resistance genes in 173 hybrid rice breeding lines. A, *Xa3*; B, *Xa7*; C, *xa13*; D, *Xa21* shows the band patterns for BB resistance specific primer on gel electrophoresis; E, *Xa4* and F, *xa5* indicates the size differentiation by fragment analyzer gel electrophoresis. R and S in first and second lanes indicate resistant and susceptible control variety

(*Xa4*, *Xa7* and *Xa21*, and *Xa3*, *Xa4* and *xa5*). Eighty-one lines did not contain any of the BB resistance genes (Fig. 2 and Table 4). The three multiple gene containing lines were selected for developing resistant varieties using MAS.

Estimation of Genotypes for Rice Blast Resistance Genes

We examined the amplification patterns of three major rice blast resistant genes (*Pib*, *Pi-ta*, and *Pi-5*) in 173 hybrid lines (Fig. 3). The tightly linked *Pi-ta* on chromosome 12 and *Pi-5* genes on chromosome 9 were screened with respective primers, YL155/87 and JJ817 primers. *Pi-ta* gene was detected in 58 (33.5%) hybrid lines and *Pi-5* gene was amplified in 35 (20.2%) hybrid lines, while most of the lines (68.8%) were found to carry *Pib* (Table 4 and Fig. 5A). According to Ramkumar

et al. (2015), *Pib* gene is one of the major resistance genes located on chromosome 2 which confers resistance to wide range of isolates of rice blast pathogen. Of the total hybrid lines, only 11 were found to contain the three genes, *Pib*, *Pi-5* and *Pi-ta* genes (Table 4) while none was found to possess at least two genes combination. The three-gene containing lines may provide stable resistance to rice blast isolates.

Estimation of Genotypes for BPH and WBPH Genes

We further surveyed the distribution of resistant genes to brown planthopper (BPH) and whitebacked planthopper (WBPH) in 173 hybrid rice breeding lines and germplasms (Fig. 4). *Bph1* was present in 20.8%, *Bph18* in 27.2%, and *Wbph* in 13.3% of the total hybrid rice breeding lines and

Fig. 3 PCR amplification patterns of markers that discriminate each of the three blast resistance genes in 173 hybrid breeding lines. A, *Pib*; B, *Pi-ta*; C, *Pi-5* indicates the amplification patterns for each blast resistance genes in gel electrophoresis

Estimation of Genotypes for Gene Pyramiding

Distribution of different gene combination varied greatly among the lines. Number of lines with at least two genes was found highest (30.63%) followed by four gene containing lines (17.34%) and five gene containing lines (7.51%). Inter-

Table 4 Distribution patterns of disease and insect resistant genes in 173 hybrid rice breeding lines and germplasms

No	Entry	<i>Xa3</i> (<i>Su</i>)	<i>Xa3</i> (<i>Re</i>)	<i>Xa4</i>	<i>xa5</i>	<i>Xa7</i>	<i>xa13</i>	<i>Xa21</i>	No. of R genes	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	No. of R genes	BPH1	BPH18	WBPB	No. of R genes	Total no. of pyramiding R genes
1	611504	-	-	-	+	-	-	-	1				0	-	-		0	1
2	611506	-	-	-	+	-	-	-	1				0	-	-		0	1
3	611507	-	-	-	+	-	-	-	1				0	-	-		0	1
4	611509	+	-	-	+	-	-	-	1				0	-	-		0	1
5	611511	-	-	-	+	-	-	-	1				0	-	-		0	1
6	611513	+	-	-	+	-	-	-	1				0	-	-		0	1
7	611517	-	-	-	+	-	-	-	1				0	-	-		0	1
8	611531	+	-	-	+	-	-	-	1				0	-	-		0	1
9	611533	+	-	-	+	-	-	-	1				0	-	-		0	1
10	611535	+	-	-	+	-	-	-	1				0	-	-		0	1
11	611571	+	-	-	-	-	-	-	0				0	+	+		2	2
12	611589	+	-	-	-	-	-	-	0			+	1	-	-		0	1
13	611590	-	+	-	-	-	-	-	1				0	-	+	+	2	3
14	611611	+	-	-	-	-	-	-	0				0	-	-		0	0
15	611626	+	-	-	+	-	-	-	1				0	-	-		0	1
16	611631	+	-	+	-	-	-	-	1				0	-	-	+	1	2
17	611632	+	-	-	+	-	-	-	1				0	-	-		0	1
18	611639	+	-	-	-	-	-	-	0		+		1	-	-		0	1
19	611650	+	-	+	-	-	-	-	1				0	-	-		0	1
20	611674	+	-	-	-	-	-	-	0	+		+	2	-	-		0	2
21	611675	+	-	-	-	-	-	-	0			+	1	-	-		0	1
22	611677	-	-	-	-	-	-	-	0			+	1	-	-		0	1
23	611678	-	-	-	-	-	-	-	0	+		+	2	-	-		0	2
24	611680	+	-	-	-	+	-	-	1	+		+	2	-	-		0	3
25	611681	+	-	-	-	-	-	-	0			+	1	+	+		2	3
26	611683	+	-	-	-	+	-	-	1	+			1	-	-		0	2
27	611684	+	-	-	-	-	-	-	0				0	-	+		1	1
28	611686	-	+	-	-	-	-	-	1				0	-	-		0	1
29	611689	+	-	-	+	-	-	-	1				0	-	-		0	1
30	611690	+	-	-	+	-	-	-	1	+			1	-	-		0	2
31	611693	-	-	-	-	-	-	-	0		+	+	2	-	-		0	2
32	611697	+	-	-	-	-	-	-	0		+		1	-	-		0	1
33	611698	-	-	-	-	-	-	-	0		+		1	-	-		0	1
34	611700	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
35	611701	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
36	611703	+	-	-	-	-	-	-	0	+			1	-	-	+	1	2
37	611704	+	-	-	-	-	-	-	0	+			1	-	-		0	1
38	611707	+	-	+	-	+	-	+	3	+			1	-	-		0	4
39	611708	+	-	+	+	+	+	+	5	+			1	-	-		0	6
40	611710	+	-	-	-	-	-	-	0	+		+	2	-	-		0	2
41	611712	+	-	+	-	-	-	-	1	+			1	-	-		0	2
42	611713	+	-	+	-	-	-	-	1	+			1	-	-		0	2
43	611715	+	-	-	+	-	-	-	1				0	-	-		0	1
44	611716	+	-	-	+	-	-	-	1				0	-	-		0	1
45	611719	+	-	-	-	-	-	-	0				0	-	-		0	0
46	611720	+	-	-	-	-	-	-	0				0	-	-		0	0
47	611722	-	+	-	-	-	-	-	1				0	-	-		0	1
48	611723	-	+	-	-	-	-	-	1	+			1	-	-		0	2
49	611725	+	-	+	-	-	-	-	1			+	1	+	+		2	4
50	611728	+	-	-	-	-	-	-	0	+	+		2	-	+		1	3
51	611729	+	-	-	-	-	-	-	0	+	+		2	-	+		1	3
52	611731	+	+	-	-	-	-	-	1	+	+		2	-	-		0	3
53	611732	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
54	611734	+	-	-	-	-	-	-	0	+	+	+	3	-	-		0	3
55	611735	+	-	-	-	-	-	-	0	+	+	+	3	-	+		1	4
56	611737	+	-	-	-	-	-	-	0	+	+	+	3	-	-		0	3
57	611738	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
58	611740	+	-	-	-	-	-	-	0	+	+		2	+	+		2	4
59	611741	+	-	-	+	-	-	-	1		+		1	-	-		0	2
60	611743	-	+	-	-	-	-	-	1		+		1	-	-		0	2
61	611744	+	-	+	-	-	-	-	1	+		+	2	+	-	+	2	5
62	611747	+	-	+	-	-	-	-	1	+	+		2	+	+		2	5
63	611748	+	+	-	-	-	-	-	1	+	+		2	+	+		2	5
64	611750	+	-	-	-	-	-	-	0	+	+		2	+	+		2	4

Table 4 Continued

No	Entry	<i>Xa3</i> (<i>Su</i>)	<i>Xa3</i> (<i>Re</i>)	<i>Xa4</i>	<i>xa5</i>	<i>Xa7</i>	<i>xa13</i>	<i>Xa21</i>	No. of R genes	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	No. of R genes	BPH1	BPH18	WBPH	No. of R genes	Total no. of pyramiding R genes
65	611751	+	-	-	-	-	-	-	0	+	+		2	+	+		2	4
66	611525	+	-	-	-	-	-	-	0				0	+	+		2	2
67	611527	+	-	-	+	-	-	-	1				0	-	-		0	1
68	611809	+	-	-	+	-	-	-	1	+	+		2	-	+		1	4
69	611810	+	-	-	+	-	-	-	1	+	+	+	3	-	+		1	5
70	611811	+	-	-	-	-	-	-	0	+			1	-	-		0	1
71	611815	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
72	611529	+	-	-	+	-	-	-	1				0	-	-		0	1
73	611817	+	-	-	-	-	-	-	0	+			1	-	+		1	2
74	611818	+	-	-	+	-	-	-	1	+	+	+	3	-	+		1	5
75	611819	+	-	+	+	-	-	-	2				0	-	+		1	3
76	611820	+	-	-	+	-	-	-	1				0	-	-		0	1
77	611821	-	-	-	-	-	-	-	0	+	+	+	3	-	+		1	4
78	611822	+	-	-	-	-	-	-	0	+			1	+	+	+	3	4
79	611823	+	-	+	-	-	-	-	1	+			1	-	+		1	3
80	611824	+	-	+	+	-	-	-	2	+			1	-	+	+	2	5
81	611825	+	-	-	-	-	-	-	0	+			1	-	+		1	2
82	611826	+	-	-	-	-	-	-	0	+			1	-	-		0	1
83	611827	-	+	-	-	-	-	-	1	+			1	-	+		1	3
84	611828	+	-	-	-	-	-	-	0				0	-	-		0	0
85	611829	-	+	+	-	-	-	-	2	+			1	-	+	+	2	5
86	611830	+	-	-	-	-	-	-	0	+			1	+	+	+	3	4
87	611831	+	-	-	-	-	-	-	0				0	+	+		2	2
88	611832	+	-	-	-	-	-	-	0	+			1	-	-		0	1
89	611833	-	-	-	+	-	-	-	1		+	+	2	-	+		1	4
90	611834	+	-	+	-	-	-	-	1	+	+		2	-	+		1	4
91	611835	+	-	-	-	-	-	-	0				0	-	-		0	0
92	611836	+	-	+	-	-	-	-	1				0	-	-	+	1	2
93	611837	+	-	+	-	-	-	-	1	+			1	-	-		0	2
94	611838	-	-	-	-	-	-	-	0	+		+	2	-	-		0	2
95	611839	+	-	-	+	-	-	-	1	+	+		2	-	+		1	4
96	611840	+	-	-	-	-	-	-	0				0	-	-		0	0
97	611841	+	-	-	-	-	-	-	0	+			1	-	+		1	2
98	611842	+	-	-	+	-	-	-	1	+			1	-	-		0	2
99	611843	-	+	+	-	-	-	-	2	+			1	-	-		0	3
100	611844	-	+	-	-	-	-	-	1	+			1	-	-		0	2
101	611845	+	-	-	-	-	-	-	0	+			1	-	+		1	2
102	611846	+	-	+	-	-	-	-	1	+	+	+	3	+	-		1	5
103	611847	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
104	611848	+	-	-	-	-	-	-	0	+			1	+	+		2	3
105	611849	+	-	-	+	-	-	-	1	+	+		2	-	-	+	1	4
106	611850	+	-	+	-	-	-	-	1	+	+		2	-	+		1	4
107	611851	+	-	+	-	-	-	-	1	+		+	2	+	+		2	5
108	611852	+	-	-	-	-	-	-	0	+			1	-	-	+	1	2
109	611853	+	-	-	+	+	-	-	2	+		+	2	-	-		0	4
110	611854	+	-	-	+	+	-	-	2	+		+	2	-	-		0	4
111	611855	+	-	-	+	+	-	-	2	+			1	+	-		1	4
112	611856	+	-	-	+	-	-	-	1	+			1	+	-		1	3
113	611857	+	-	-	+	-	-	-	1	+			1	-	-		0	2
114	611858	+	-	-	+	-	-	-	1	+			1	-	-		0	2
115	611859	-	-	-	-	-	-	-	0	+			1	-	-	+	1	2
116	611860	+	-	-	-	-	-	-	0	+			1	-	-		0	1
117	611861	+	-	-	-	-	-	-	0	+			1	-	-		0	1
118	611862	-	-	-	-	-	-	-	0	+			1	-	-	+	1	2
119	611863	-	-	-	-	-	-	-	0	+			1	+	-	+	2	3
120	611864	+	-	-	-	-	-	-	0	+			1	+	+	+	3	4
121	611865	-	-	-	-	-	-	-	0	+			1	-	-	+	1	2
122	611866	-	-	-	-	-	-	-	0	+			1	-	-	+	1	2
123	611867	-	-	-	-	-	-	-	0	+			1	-	-	+	1	2
124	611868	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
125	611869	+	-	+	-	-	-	-	1	+	+		2	-	-		0	3
126	611870	+	-	+	-	-	-	-	1	+	+		2	+	-		1	4
127	611871	+	-	-	-	-	-	-	0	+	+		2	+	+		2	4
128	611872	+	-	+	-	-	-	-	1	+			1	-	-		0	2

Table 4 Continued

No	Entry	<i>Xa3</i> (<i>Su</i>)	<i>Xa3</i> (<i>Re</i>)	<i>Xa4</i>	<i>xa5</i>	<i>Xa7</i>	<i>xa13</i>	<i>Xa21</i>	No. of R genes	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	No. of R genes	BPH1	BPH18	WBPH	No. of R genes	Total no. of pyramiding R genes
129	611873	+	-	-	-	-	-	-	0	+	+		2	-	-		0	2
130	611874	-	+	-	-	-	-	-	1	+		+	2	-	-		0	3
131	611875	-	+	-	-	-	-	-	1	+			1	-	-		0	2
132	611876	+	+	-	-	-	-	-	1	+			1	-	-		0	2
133	611877	+	-	-	-	-	-	-	0	+			1	-	-		0	1
134	611878	+	-	-	-	-	-	-	0	+			1	+	+		2	3
135	611879	+	-	-	-	-	-	-	0	+			1	+	+		2	3
136	611880	+	-	-	-	-	-	-	0	+			1	-	-		0	1
137	611881	+	-	-	-	-	-	-	0	+			1	-	-		0	1
138	611882	+	-	-	-	-	-	-	0	+		+	2	-	-		0	2
139	611883	-	+	-	-	-	-	-	1	+			1	-	-		0	2
140	611884	+	-	-	-	-	-	-	0	+			1	-	-		0	1
141	611885	+	-	-	-	-	-	-	0	+		+	2	-	-		0	2
142	611886	+	-	-	-	-	-	-	0	+			1	-	-		0	1
143	611887	-	-	-	-	-	-	-	0	+			1	-	-		0	1
144	611888	+	-	-	-	+	-	-	1	+			1	-	-		0	2
145	611889	+	-	-	-	-	-	-	0	+			1	-	-		0	1
146	611890	+	-	-	+	-	-	-	1	+		+	2	-	-		0	3
147	611891	+	+	-	+	-	-	-	2		+		1	-	-		0	3
148	611892	+	-	+	-	-	-	-	1	+	+		2	-	+		1	4
149	611893	+	-	-	-	-	-	-	0	+			1	-	-		0	1
150	611894	+	-	-	+	-	-	-	1	+	+		2	-	+		1	4
151	611895	-	-	-	-	-	-	-	0	+	+	+	3	+	-	+	2	5
152	611896	+	-	-	-	-	-	-	0	+		+	2	-	-		0	2
153	611897	-	-	-	-	+	-	-	1	+	+	+	3	+	-		1	5
154	611898	-	+	-	-	+	-	-	2		+		1	-	-		0	3
155	611899	+	+	-	-	+	-	-	2		+		1	+	-		1	4
156	611900	-	+	-	-	-	-	-	1		+		1	-	-		0	2
157	611901	-	+	-	-	-	-	-	1			+	1	-	-		0	2
158	611902	-	-	-	-	-	-	-	0	+	+		2	+	-		1	3
159	611903	-	+	-	-	-	-	-	1	+	+		2	+	-		1	4
160	611904	-	+	-	-	-	-	-	1	+	+		2	+	-	+	2	5
161	611905	-	-	-	-	-	-	-	0	+	+		2	-	-	+	1	3
162	611906	-	+	-	-	+	-	-	2	+	+		2	+	-		1	5
163	611907	-	+	-	-	+	-	-	2	+	+		2	+	-	+	2	6
164	611908	+	-	-	-	-	-	-	0		+		1	+	-		1	2
165	611909	-	+	-	-	-	-	-	1		+		1	-	-		0	2
166	611910	-	-	-	-	-	-	-	0	+	+		2	+	-	+	2	4
167	611911	+	-	-	-	-	-	-	0	+	+	+	3	+	-		1	4
168	611912	+	-	+	-	-	-	-	1	+		+	2	-	+		1	4
169	611913	+	+	-	+	-	-	-	2	+			2	+	+		2	6
170	611914	+	+	+	+	-	-	-	3	+	+	+	3	-	-		0	6
171	611915	+	-	-	+	-	-	-	1	+	+		2	-	+		1	4
172	611916	+	-	+	+	-	-	-	2	+			1	-	+		1	4
173	611917	+	-	-	+	-	-	-	1	+			1	-	+		1	3

+: Positive response, -: negative response

estingly in this study, we were able to identify lines that contain up to six different resistance genes. These include IR98161-2-1-1-k1-3, Damm-Noeub Khmau, 7290s, and 7292s (Table 5). These results prove that it is possible to develop hybrid lines with gene pyramiding through marker-assisted breeding approach.

Discussion

Cultivated crops are exposed to different stress factors. Climate change by increasing temperature affects the habitat range of

pathogens and pests, which can facilitate spread of pathogens (Luck et al. 2011; Madgwick et al. 2011). Biotic stress adversely affect plant growth, yield losses, and nutritional values (Porter and Semenov 2005), as well as seed quality of crops. Although conventional breeding is an effective method to develop resistance rice variety, it takes a lot of time to develop a new rice variety. Cross-breeding via marker-assisted selection (MAS) is expected to play a role for the development and improvement of broad-spectrum stress-tolerant crops (Nogoy et al. 2016). Therefore, we investigated the amplification patterns of resistance genes against various biotic stresses such as bacterial blight, blast and brown planthopper pests to

Table 5 List of hybrid breeding lines with six different disease resistance genes

Gene combination	No. of breeding lines	Resistance genes					
6 genes	39	<i>Xa4</i>	<i>xa5</i>	<i>Xa7</i>	<i>xa13</i>	<i>Xa21</i>	<i>Pi-b</i>
	163	<i>Xa3</i>	<i>Xa7</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Bph1</i>	<i>Wbph</i>
	169	<i>Xa3</i>	<i>xa5</i>	<i>Pi-b</i>	<i>Pi-5</i>	<i>Bph1</i>	<i>Bph18(t)</i>
	170	<i>Xa3</i>	<i>Xa4</i>	<i>xa5</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>
5 genes	61	<i>Xa4</i>	<i>Pi-b</i>	<i>Pi-5</i>	<i>Bph1</i>	<i>Wbph</i>	
	62	<i>Xa4</i>	<i>Pi-b</i>	<i>Pi-5</i>	<i>Bph1</i>	<i>Bph18(t)</i>	
	63	<i>Xa3</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Bph1</i>	<i>Bph18(t)</i>	
	69	<i>xa5</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	<i>Bph18(t)</i>	
	74	<i>xa5</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	<i>Bph18(t)</i>	
	80	<i>Xa4</i>	<i>xa5</i>	<i>Pi-b</i>	<i>Bph18(t)</i>	<i>Wbph</i>	
	85	<i>Xa3</i>	<i>Xa4</i>	<i>Pi-b</i>	<i>Bph18(t)</i>	<i>Wbph</i>	
	102	<i>Xa4</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	<i>Bph18(t)</i>	
	107	<i>Xa4</i>	<i>Pi-b</i>	<i>Pi-5</i>	<i>Bph1</i>	<i>Bph18(t)</i>	
	151	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	<i>Bph1</i>	<i>Wbph</i>	
	153	<i>Xa7</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Pi-5</i>	<i>Bph1</i>	
	160	<i>Xa3</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Bph1</i>	<i>Wbph</i>	
	162	<i>Xa3</i>	<i>Xa7</i>	<i>Pi-b</i>	<i>Pi-ta</i>	<i>Bph1</i>	

genotype 173 hybrid rice lines. It is important to screen candidate genetic resources in rice cross breeding lines through genotyping because it will help breeders to maintain several small base lines having resistance to biotic stress. Furthermore, the use of markers enables the usage and maintenance of diverse resistance genes, analysis of variation, and selection for pyramided genes (Lammerts van Bueren et al. 2010).

In this study, we report the distribution of biotic stress resistance genes such as dominant (*Xa3*, *Xa4*, *Xa7*, and *Xa21*) and recessive (*xa5* and *xa13*) BB-resistance genes, rice blast (*Pib*, *Pi-5*, and *Pi-ta*), and BPH (*Bph1*, *Bph18* and *Wbph*) resistance genes in hybrid breeding lines. A total of 173 hybrid rice breeding lines and germplasms were used for the screening of those lines with different resistance genes through MAS-based pyramiding approaches. We analyzed the distribution and genetic differences of BB resistant genes using six major resistant markers related to *Xa3*, *Xa4*, *xa5*, *Xa7*, *xa13* and *Xa21* genes. The results revealed that only 0.6% and 1.2% of the hybrid lines have *xa13* and *Xa21*, respectively. However, we were also able to identify lines such as IR98161-2-1-1-k1-3 which contained five genes (*Xa4*, *xa5*, *Xa7*, *xa13* and *Xa21*), and IR98161-2-1-1-k1-2 and 7292s which possessed three respective gene combinations (*Xa4*, *Xa7* and *Xa21*, and *Xa3*, *Xa4* and *xa5*) (Table 4). Kinoshita (1995) reported that the combination of resistance genes into rice varieties is good way to develop durable resistance to BB disease. Recently, Pradhan et al. (2015) cited that a three-gene combination appeared to be the most effective with *Xa21* contributing the largest component of resistance to BB. Therefore, in our future studies, we will use the identified hybrid lines with at

least three gene combinations for phenotypic selection.

According to the report of Singh et al. (2015), several genes for blast resistance were found and effectively used to control rice blast disease in rice breeding and genetic studies (Chen et al. 2005; Ballini et al. 2008; Koide et al. 2009), and many blast resistant varieties have been developed. We used three major rice blast resistant genes, *Pib*, *Pi-ta*, and *Pi-5* to confirm the distribution of resistance genes for improving rice blast resistance and multiple-resistance efficiency. Our results showed that *Pi-ta* on chromosome 12 was present in 33.5% of the hybrid rice lines while *Pi-5* gene on chromosome 9 was amplified in 20.2% of 173 hybrid rice lines. Moreover, *Pib* gene in chromosome 2 was present in 68.8% of the total hybrid lines. Ramkumar et al. (2015) reported that *Pib* gene is a major resistance gene and offers resistance to wide range of isolates in India. Out of these hybrid lines, only 11 lines possessed three genes, *Pib*, *Pi-5* and *Pi-ta*, which may be expected to provide high level of resistance against rice blast isolates, because *Pib* is one of significant rice blast resistant genes (Ramkumar et al. 2015; Wang et al. 1999).

Previous results have been reported that brown planthopper is a destructive phloem sap sucking pest of rice which has negative effect both qualitative and quantitative (Jena et al. 2006). In this study, we searched for hybrid lines having resistant genes for brown plant hopper, namely, *Bph1*, *Bph18(t)*, and *Wbph* in 173 hybrid rice lines. *Bph1* was present in 20.8%, *Bph18* in 27.2%, and *Wbph* in 13.3% of the total hybrid lines. Among these lines, IR68, Laxmi, and Aromatic rice 6-3 have all three resistance genes (Table 4), which will be used in the future for phenotypic analysis.

In summary, we investigated hybrid rice lines with multiple-

resistance to biotic disease and insect pests using DNA-based markers. Our results showed that most of the hybrid breeding lines contained one to four different resistant genes. We identified four lines harboring the maximum number of six resistance genes out of 12 resistance genes used and 13 lines with five resistant genes. The four lines were IR98161-2-1-1-k1-3, Damm-Noeub Khmau, 7290s, and 7292s lines (Table 5). These lines carrying a stack of six-genes are expected to provide higher level of multiple- resistance to biotic stresses. The information obtained and the selected lines with various resistance genes could be used to further facilitate rice disease resistance breeding especially in hybrid rice breeding programs. Available molecular analysis and transcriptome analysis of the various different genes present in each hybrid line may be able to propose a generalized stress response or points of cross-talk between signaling pathways (Atkinson and Urwin, 2012). Lastly, field performance and phenotypic reactions of the identified lines carrying multiple stack of genes in this study need to be verified to provide strong recommendations for use in hybrid rice breeding programs.

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