Detection of Blast Resistant Gene in Rice by Host-pathogen Interaction and DNA-Marker

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Abstract Rice blast disease caused by *Pyricularia oryzae* is a major disease in Bangladesh. In the search of resistant source against *P. oryzae*, resistant gene (*R*-gene) was assessed by (i) host-pathogen interaction and (ii) PCR-based screening at the laboratory of Bangladesh Rice Research Institute. One hundred and forty four plant materials were classified into 10 cultivar groups (CG) based on their reaction patterns to four distinct blast isolates (SDBI; standard differential blast isolate) H-11-64, H-1-8, H-1-1 and H-11-67. The reaction patterns of plant materials to these four standard blast isolates indicated presence of *Pish*, *Pi9*, *Pita2* and *Pita* genes and their combination in the genetic background of tested plant materials. Gene specific primer 195R-1/195F-1, Pita440 (YL153)/Pita440 (YL154), OSM89 and AOL45/AOL48 were used to identify and confirm the presence of *Pi9*, *Pita*, *Pita2* and *Pish* genes in plant materials, respectively through PCR-based assay. Plant materials, H13, H23, H25, H35, H47, H49, H136, H138 harbored all three genes, *Pita*, *Pita2* and *Pish* in their genetic background. The *Pi9* gene together with *Pita2* was detected in local rice cultivar H100 and H129, which were confirmed by DNA analysis in PCR assay. These materials could be used in gene pyramiding in promising high yielding cultivar for durable blast resistance.

Keywords Rice, Standard Differential Blast Isolates, *R*-gene, DNA-Marker

1. Introduction

Rice (*Oryza sativa* L.) is the staple food for about half of the world's population. Bangladeshi people take Rice (*Oryza sativa* L.) as a major food which is central to the economy of the country accounting for about 20% of GDP (gross domestic product) [1, 2] and as more than 90% of all rice is produced in Asia it is called an Asian crop. Among

various factors responsible for low yield of rice, blast caused by *P. oryzae* plays an important role which causes yield loss of 11-15% worldwide annually [3]. Under climatic changes such as lacking in water, many rice diseases particularly, blast disease of rice may increase [4, 5].

Managing blast disease at farmers' field chemical control is mainly accomplished and other managing options, especially water is mostly difficult to practice [6]. Agricultural land in Bangladesh is declining but population is raising day by day, means that mounting of rice yield must be explored from different approaches. In such situation resistant cultivars is one of the potential ways to control blast pathogen. Innovation of blast *R*-gene in the genomic background of rice plant has created an opportunity to develop durable resistance in rice against blast pathogen [7]. Pyramiding of *R*-gene is needed for development of durable resistant rice cultivars against blast pathogen.

Blast isolates clarified on monogenic lines (ML; single gene resistance to blast pathogen) or differential varieties (DVs) through pathogenicity test indicated that *Pi9*, *Pish*, *Pita* and *Pita2* were the major genes responsible for blast resistance in Bangladesh which showed resistance frequencies of 80-90% against all tested blast isolates from the country [8, 9]. A set of SDBIs (Standard Differential Blast Isolates) has been developed [9] through pathogenicity test.

Huge number of rice entries has been screened in Bangladesh against blast pathogen to identify resistant entries but the gene(s) involved with those entries remains untested. Only one attempt by Khan et al. [8] has so far been made to identify the gene involved in fragrant rice, which is essential for maintaining and utilizing the known gene pool. Interaction between differential varieties and SDBIs (host-pathogen interaction) is used to detect blast *R*-gene in Japan and IRRI [9, 11]. Molecular technique is also useful to detect blast resistant gene(s) from a large number of rice germplasms [8, 9].

With this view in mind this project has been undertaken to find out source(s) of major blast resistant genes in rice land races through pathogenicity test with standard blast isolates for further breeding program. The study has been aimed to identify the resistant gene in different rice cultivars by gene specific primer through DNA marker analysis.

2. Materials and Methods

The experiments were conducted at the experimental plots and Seed Pathology & molecular laboratory of Plant Pathology Division of Bangladesh Rice Research Institute (BRRI), Gazipur-1701 with the assistance of Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh.

2.1. Gene Assessment in Rice Land Races Based on Reaction Pattern to Standard Differential Blast Isolates (SDBIs)

2.1.1. Collection and Preservation of Standard Differential Blast Isolates

Four major blast resistant genes named *Pi9*, *Pish*, *Pita* and *Pita2* those were previously identified as blast resistant genes in Bangladesh [8, 9] was targeted for this research. Four standard differential blast isolates (SDBIs) viz. H-11-64 (incompatible to *Pita* gene; also *Pi9*), H-1-8 (incompatible to *Pish*; also *Pi9* gene), H-1-1 (incompatible to *Pita* -2 gene) and H-11-67 (incompatible to *Pi9* gene) were collected from Hossain [9] to identify those resistant genes in rice land races through pathogenicity test. Modified NIAS (National Institute of Advanced Studies) method [10] was used to preserve blast pathogen.

2.1.2. Preparation of Plant Materials and Inocula for Inoculation

Rice land races and susceptible checks (SC; susceptible to blast pathogen) were seeded in seeding cell trays (having 35 holes of seeding options in each tray) for each isolate. Modified NIAS method was followed to prepare plant materials and inocula for inoculation [10]. After growing 2-3 weeks, plants were ready for inoculation (5-6 leaf stage).

2.1.3. Inoculation of Plant Materials

The conidial concentration was determined using a hemacytometer and adjusted to 1×10^5 (100,000) conidia per ml. Inoculation was carried out by the NIAS method cited by Hayashi et al. [10]. The inoculated plants were incubated for approximately 24 hr at 25°C and then transferred to a screening house (humidity approximately 60%) at 25-30°C.

2.1.4. Disease Assessment Based on Infection Type on Plant

Phenotypic data from screening result: Susceptible (S) or resistant (R) reaction of the test plants to the SDBIs was assessed by examining the leaves for blast symptoms. Seven days after inoculation, infection type (0-5 scale) was rated where scale 0-3 were resistant for *Pish* and *Pita2*; 0-2 were resistant for *Pi9* and *Pita* genes [10, 11].

2.1.5. Sampling of Rice Plants and their Categorization

Rice plants were sampled based on symptoms on leaves produced by pathogen (susceptible or resistant reaction of plant to specific SDBI). Then plants were categorized or grouped (cultivar group, CG) based on presence or absence of resistant gene in the tested plant materials (Table 2, 3).

2.2. Gene Assessment in Rice Land Races Based on DNA Marker Analysis

Major blast resistant genes for Bangladesh [8, 9] were surveyed in the background of rice germplasm (including previous 100 rice land races). Gene-specific primers were used to identify and confirm (from section 2.1) the target gene in plant samples (Table 1).

2.2.1. Selection of Best Primers for Screening Plant Materials to Identify Blast R-Gene

Fifteen gene-specific primers were screened against the target gene of monogenic lines to select the best one. Among the 15 primers, 3 were for *Pita* gene, 3 for *Pita2*, 4 for *Pish* and 5 for *Pi9* (Table 1). The best primers were used in subsequent study to identify the target gene in studied plant materials.

Table 1. List of gene-specific primers used in the study

Gene name	Primer name	primer sequence (Forward)	Primer name	primer sequence (Reverse)
<i>Pi9</i>	195R-1	ATGGTCCTTTATCTTTATTG	195F-1	TTGCTCCATCTCCTCTGTT
	NBS3-F	AGATGTTAGTAGCAAGTTCC	NBS3-R	TGTCAGTTATGTCCAAAGTG
	NBS4-F	ACTTTGTTGTGCTTGATAAC	NBS4-R	ATGGTGAACGGTATCTGTAT
	NBS/ LRR-F2	CACTGTTGTAGCGGAGGAGA	NBS/ LRR-R2	CAGTACGCGATTTCATTGTTTC
	NBS2-O	TACAACCACCTACCATCCCAT	NBS2-U	TCTAGAACCTGCAAGTCTCG
<i>Pita</i>	Pita403 (YL100)	CAATGCCGAGTGTGCAAAGG	Pita403 (YL102)	TCAGGTTGAAGATGCATAGC
	Pita440 (YL153)	CAACAATTTAATCATAACG	Pita440 (YL154)	ATGACACCCTGC-GATGCAA
	Pita1042 (YL155)	AGCAGGTTATAAGCTAGGCC	Pita1042 (YL87)	CTACCAACAAGTTCATCAAA
<i>Pita2</i>	RM155	GAGATGGCCCCCTCCGTGATGG	RM155	TGCCCTCAATCGGCCACACCTC
	OSM89	TTGGTCAAAGTTAGCATGGGAGGG	OSM89	TTTGAACCGGTGGCCACATG
	RM7102	TTGAGAGCGTTTTTAGGATG	RM7102	TCGGTTTACTTGGTTACTCG
<i>Pish</i>	AOL45	TCAGAAATCCACAGTTGCATGCAC	AOL48	GGAGAGCTGCTCTATACAATTTATAC
	AOL54	GTGAAAGATTCCACCCAGGTTC	AOL71	TTGCGCTTCTCCGTAGTAGTG
	AOL51	AGCTGCAGTAGTGCTTCCATG	AOL52	CGTAAGATCATGAGCGAATG
	AOL351	ACTGCCCAACATAACATCTC	AOL353	CCTGCAGCTCTTCTTCAAAAG

2.2.2. Plant DNA Extraction and Preparation

Leaf samples were collected from young leaves of 21-day-old rice plants. DNA was extracted following modified Mini-scale method [12]. The quality and quantity of extracted DNA was assessed by using spectrophotometric method (NanoDrop spectrophotometer; Thermo Fisher Scientific, Wilmington, DE, U.S.A.). Dilution of concentrated DNA was done to make working concentration at 25 ng/μl.

2.2.3. Polymerase Chain Reaction (PCR) for Simple Sequence Repeats (SSRs)

The PCR was performed in 10μl reactions containing about 25ng of DNA template (3μl DNA with 10X dilution factor), 1μl 10X TB buffer (containing 200mM Tris-HCl pH 8.3, 500mM KCl), 1.35μl 25mM MgCl₂, 0.2μl of 10mM dNTP, 0.5μl each of 10μM forward and reverse primers and 0.1μl of *Taq* DNA polymerase (5 U/μl) using GStorm thermal cycler. The PCR machine was then be run with 103-112 °C lid temperature following SSR-PCR protocol [13]. Water was used as negative control for each primer instead of DNA sample. Reference monogenic lines were used as positive control.

2.2.4. Gel Electrophoresis, Staining and Visualization of the Gel

The PCR products were visualized by ethidium bromide (0.5 mg/ml) and agarose gel (1.5%; 1X TBE buffer) electrophoresis following the protocol used by Jean-Louis [14]. The stained gel was exposed to UV light in a gel

documentation system (BioDocAnalyze, Biometra, An Analytik Jena Company, Goettingen, Germany) and the gel image was saved as a JPEG file.

3. Results

3.1. Gene Assessment in Rice Land Races Based on Reaction Pattern to Standard Blast Isolates

Tested plant cultivars showed various patterns of reactions to the differential blast isolates. Twenty one cultivars and susceptible checks LTH and US2 were susceptible to all 4 selected isolates and classified as CG I (Table 2). None of the *Pita*, *Pish*, *Pita2* or *Pi9* was estimated in these cultivars. Seven cultivars and differential variety (DV), IRBLta-K1 (harboring *Pita* gene) showed resistance to a blast isolate H-11-64 (incompatible to *Pita* gene) and were classified as CG II (Table 2). From this reaction pattern 8 cultivars and differential variety (DV), IRBLsh-B (harboring *Pish* gene) were classified as CG III and considered to harbor *Pish* and not to harbor *Pita*, *Pita2* or *Pi9*. Fifteen cultivars and differential variety (DV), IRBLta2-Pi (harboring *Pita2* gene) were resistant to H-1-1 (incompatible to *Pita2* gene), but susceptible to other three blast isolates. This cultivar was classified as CG IV and estimated to harbor *Pita2* gene but not *Pita*, *Pish* or *Pi9* (Table 2). IRBL9-W (harboring *Pi9* gene) was susceptible to only 1 isolate H-1-1 (compatible to *Pi9* gene) and classified as CG V (Table 2).

Table 2. Cultivar classification based on reaction pattern to four selected blast isolates

Cultivar Group (CG)	Differential variety (DVs) and Cultivar	Reaction to SDBI				Estimated gene(s) harboring in the genetic background			
		H-11-64 ^A	H-1-8 ^B	H-1-1 ^C	H-11-67 ^D				
I	LTH,US2,H3,H9,H26,H27,H34,H41,H44,H46,H51,H53,H55,H64,H65,H66,H69,H82,H91,H93,H95,H133,H134	S	S	S	S	-	-	-	-
II	IRBLta-K1,H12,H29,H52,H63,H78,H77,H96	R	S	S	S	<i>Pita</i>	-	-	-
III	IRBLsh-B,H8,H38,H40,H61,H68,H80,H81,H84	S	R	S	S	-	<i>Pish</i>	-	-
IV	IRBLta2-Pi,H2,H7,H10,H24,H33,H43,H56,H60,H62,H73,H74,H85,H98,H99,H139	S	S	R	S	-	-	<i>Pita2</i>	-
V	IRBL9-W	R	R	S	R	-	-	-	<i>Pi9</i>
VI	H4,H28,H36,H45,H50,H67,H71,H141	R	R	S	S	<i>Pita</i>	<i>Pish</i>	-	-
VII	H1,H11,H14,H19,H31,H32,H39,H48,H86,H90,H144	R	S	R	S	<i>Pita</i>	-	<i>Pita2</i>	-
VIII	H15,H16,H18,H21,H22,H37,H42,H57,H58,H72,H79,H83,H135	S	R	R	S	-	<i>Pish</i>	<i>Pita2</i>	-
IX	H13,H23,H25,H35,H47,H49,H136,H138	R	R	R	S	<i>Pita</i>	<i>Pish</i>	<i>Pita2</i>	-
X	H100,H129	R	R	R	R	<i>Pita</i>	<i>Pish</i>	<i>Pita2</i>	<i>Pi9</i>

S' susceptible and R' resistant reaction of host plant to blast isolates; ^A Incompatible to *Pita* (also *Pi9*); ^B Incompatible to *Pish* (also *Pi9*); ^C Incompatible to *Pita2*; ^D Incompatible to *Pi9*

Eight cultivars were resistant to blast isolates, H-11-64 and H-1-8 and susceptible to H-1-1 and H-11-67. These cultivars were classified as CG VI and estimated to harbor *Pita* and *Pish* genes. Eleven cultivars were resistant to 2 isolates, H-11-64 and H-1-1, and estimated to have *Pita* and *Pita2* genes by primary estimation and classified as CG VII. Thirteen cultivars were resistant to H-1-8 in addition to H-1-1 isolates and estimated to have *Pish* and *Pita2* genes but not *Pita* or *Pi9* genes (CG VIII). Eight cultivars were classified as CG IX and estimated to have *Pita*, *Pish* and *Pita2* genes together in each of them. Resistance to the blast isolate, H-11-64, H-1-8, H-1-1 and H-11-67 in cultivars, H100 and H129 were recorded where all the 4 expected genes, *Pita*, *Pish*, *Pita2* and *Pi9* were expected to be present in those two cultivars. These two cultivars were classified as CG X (Table 2).

3.2. Gene Assessment in Rice Land Races Based on DNA Marker Analysis

3.2.1. Selection of Best Primers for Screening Plant Materials to Identify Blast R-Gene

The results of selecting primer(s) were presented in Figure 1-3. Four primers, Pita440 (YL153/YL154), OSM89, AOL45/AOL48 and 195R-1/195F-1 produced distinct single bands from the genomic DNA of monogenic lines, IRBLta-CP1, IRBLta2-Pi, IRBLsh-B and IRBL9-W, harboring *Pita*, *Pita2*, *Pish* and *Pi9* gene, respectively and were used in subsequent experiment to find out resistant gene in plant materials.

Figure 1. Selection of primer for *Pita* and *Pita2* genes. Lane 1 & 19 ladder M (1kb+); 2-4 primer Pita403, 5-7 primer Pita440; 8-10 primer Pita1042 for *Pita* gene; 11-13 primer RM155 and lane 14-16 primer OSM89 for *Pita2* gene; lane 17 and 18 negative control (LTH)

Figure 2. Selection of primer for *Pita2* and *Pish* gene. Lane 1 & 18 ladder M (1kb+); lane 2-4 primer RM7102 for *Pita-2* gene; 5-7 primer AOL45, 8-10 primer AOL54, 11-13 primer AOL51 and 14-16 primer AOL351 for *Pish* gene; 17 negative control (LTH)

Figure 3. Selection of primer for *Pita 2*. Lane 1 and 20 ladder (100 bp); lane 2-4 primer RM155, lane 5-7 primer OSM89 and lane 8-10 primer RM7102 where annealing temperature was 65°C; again lane 11-13 primer RM155, lane 14-16 primer OSM89 and lane 17-18 primer RM7102 where annealing temperature was 70°C; lane 19 negative control (LTH).

3.2.2. Genotyping of *Pita*, *Pish*, *Pita2* and *Pi9* Genes in the Tested Plant Materials Using DNA Markers

One hundred forty four rice plant materials of local land races were screened for the presence of 4 major rice blast resistant (*R*) genes, *Pita*, *Pish*, *Pita2* and *Pi9* using the gene specific primers.

Figure 4. Gel picture (partial) for screening rice land races for blast resistant gene, *Pish*. M 1kb⁺ and M¹ 100bp ladder, C(-) negative control (susceptible check, LTH for no blast resistant gene), C(+) positive control (resistant check for reference *Pish* gene), Serial number 1-16 indicates plant samples.

Figure 5. Gel picture (partial) for screening rice land races for blast resistant gene, *Pita2*. C(-) negative control and C(+) positive control (resistant check for reference *Pita2* gene), Serial 17-32 indicates plant samples.

Genetic diversity of *Pish* gene was assessed by DNA marker AOL45/AOL48 where forty accessions showed the positive reactions of *Pish* genes located on chromosome 1 (Figure 4 and Table 3). Out of 144 germplasms, the *Pita2* gene, present in chromosome 1 of rice, was detected in 75 accessions with OSM89 marker (Figure 5 and Table 3). The presence and absence of the *Pita* gene were estimated using the DNA marker Pita440 (YL153/YL154). The DNA fragments of positive control cultivar, IRBLta-K1 carrying *Pita* gene were amplified, while those of the

negative control, LTH or US2 (both having no blast resistant gene), were not amplified. The *Pita* gene on chromosome 12 was scored to 76 out of 144 tested plant materials (Figure 6 and Table 3). Genetic diversity of *Pi9* gene in tested plant materials was assessed by DNA marker 195R-1/195F-1. Only 2 plant materials showed DNA fragments with primer 195R-1/195F-1 indicating presence of *Pi9* in those materials (Table 3 and Figure 7).

Some plant materials consisted of more than one gene. Thirteen plant materials were observed with two genes viz. *Pita* and *Pish*. Thirty plant materials were recorded with *R*-gene *Pita* and *Pita2*. Resistant gene *Pish* and *Pita2* were present in twelve materials. Three *R*-genes, *Pita*, *Pita2* and *Pish* were found together in eleven plant materials. Two materials those were recorded only with *Pi9* gene also consisted of *Pita2* gene (Table 3).

Figure 6. Gel picture (partial) for screening rice land races for blast resistant gene, *Pita*. C(-) negative control, C(+) positive control (resistant check for reference *Pita* gene), Serial number 33-48 indicates plant samples.

Figure 7. Gel picture (partial) for screening rice land races for blast resistant gene, *Pi9*. C(-) negative control, C(+) positive control (resistant check for reference *Pish* gene), Serial number 129-144 indicates plant samples.

Table 3. Cultivar classification based on Presence (+) or absence (-) of resistant gene in rice land races assessed by DNA markers

CG	Fr	DVs and Cultivar	Gene(s) harboring in the genetic background			
			<i>Pita</i>	<i>Pish</i>	<i>Pita2</i>	<i>Pi9</i>
I	30	LTH, US2,H3,H9,H17,H26,H27,H31,H53,H54,H55,H65,H66, H69, H82, H91, H92, H93, H94, H109, H110, H111, H112, H114, H115, H118, H119, H121, H124, H126, H131, H132	-	-	-	-
II	22	IRBLta-K1,H12,H29,H30,H32,H41,H46,H51,H52,H63,H70,H75, H76,H77, H78, H90, H96, H107, H108, H113, H116, H117, H133	+	-	-	-
III	4	IRBLsh-B,H20, H40, H61, H80	-	+	-	-
IV	20	IRBLta2-Pi, H2, H5, H7, H10,H11, H24, H33, H34, H43, H44, H56, H60, H62, H64, H95, H98, H104, H128, H134	-	-	+	-
V	0	IRBL9-W	-	-	-	+
VI	13	H18,H28,H36,H38,H45,H50,H67,H68,H71,H81,H84,H127,H141	+	+	-	-
VII	30	H1, H6, H14, H19, H39, H48, H59, H73, H74, H85, H86, H87, H88, H89, H97, H99, H101, H102, H103, H105, H106, H120, H123, H125, H137, H139, H140, H142, H143, H144	+	-	+	-
VIII	12	H8, H15, H16, H21, H22, H37, H42, H57, H72, H79, H130, H135	-	+	+	-
IX	11	H13, H23, H25, H35, H47, H49, H58, H83, H122, H136, H138	+	+	+	-
X	2	H100, H129	-	-	+	+
10	144		76	40	75	2

4. Discussion

Tested rice plant materials of Bangladesh were classified into 10 cultivar groups (CG) based on reaction patterns to four distinct blast isolates, H-11-64, H-1-8, H-1-1 and H-11-67. Significant variations were found among tested plant materials in disease severity to blast, which indicated the existence of a genetic variability of blast resistance in the tested materials. It indicated that materials of these cultivar groups may contain blast-resistant useful genes *Pish*, *Pi9*, *Pita2* and *Pita* in their genetic background, because DVs having those similar genes were present in the same cultivar group (Table 2). Kobayashi et al. [15] presented the similar results. The cultivar group CG I having susceptible check indicated that materials of this CG are highly susceptible to all the 4 selected isolates. None of the *Pita*, *Pish*, *Pita2* or *Pi9* genes supposed to be present in these cultivars. Genotypes belonging to the different cultivar groups maintained genetic variation [16].

Group CG II consisting of IRBLta-K1 (harboring *Pita* gene) was estimated to harbor *Pita* gene, because this gene was incompatible with the isolate H-11-64. Resistant gene *Pish*, *Pita2* and *Pi9* were thought to be absent in CG II because these materials were susceptible to other three isolates which are virulent to these genes. Materials in CG III group (having differential variety IRBLsh-B with *Pish* gene) were resistant to H-1-8 but susceptible to other three isolates and considered to harbor *Pish* gene and not to harbor *Pita*, *Pita2* and *Pi9* gene. Fifteen materials and differential variety, IRBLta2-Pi (harboring *Pita2* gene) were resistant to H-1-1 but susceptible to other three isolates and classified as CG IV. These materials were supposed to present *Pish* gene but not *Pita*, *Pita2* and *Pi9*

genes. IRBL9-W (harboring *Pi9* gene) was susceptible to isolate H-1-1 (compatible to *Pi9* gene) among the 4 isolate for primarily estimation of 4 genes. Since other three isolates were incompatible with *Pi9* gene, IRBL9-W also showed resistant reactions to them. This cultivar was classified as CG V and none of the tested materials matched with this cultivar group. Eight materials were resistant to blast isolates, H-11-64 and H-1-8 (Table 2) and classified as CG VI. The reaction pattern of CG VI was corresponded with that of additional effect pattern of IRBLta-CP1 with *Pita* and IRBLsh-B with *Pish*. Hence they are considered to harbor *Pita* and *Pish* genes. Eleven materials were resistant to 2 isolates, H-11-64 and H-1-1, and estimated to harbor *Pita* and *Pita2* genes by primary estimation of four genes and classified as CG VII. Thirteen materials were resistant to H-1-8 and H-1-1 isolates and classified as CG VIII. They showed susceptible reaction to other two isolates and thought to harbor *Pish* and *Pita2* genes. Eight cultivars were classified as CG IX as they showed resistance to 3 blast isolates H-11-64, H-1-8 and H-1-1 but showed compatible reaction to H-11-67. So they are considered to harbor three resistant genes, *Pita*, *Pish* and *Pita2*. Resistance to 4 blast isolate, H-11-64, H-1-8, H-1-1 and H-11-67 in cultivars, H100 and H129 (CG X) was recorded where all the 4 primarily estimated genes, *Pita*, *Pish*, *Pita2* and *Pi9* were expected to be present. Genotyping of *Pita*, *Pish*, *Pita2* and *Pi9* genes in tested plant materials was done using diagnostic DNA markers and confirmed the presence of expected genes in the plant materials (Figure 4-7 and Table 3). Similar experiments on the reaction patterns of cultivars and DVs to blast isolates were conducted by many other authors [17, 18]. Their findings were in agreement with the present findings. Kobayashi et al. [15]

observed various patterns of 42 IRRI-bred cultivars to the differential blast isolates and those cultivars were classified into 7 cultivar groups.

The results of Khan *et al.* [8] indicated that out of 140 test materials including 114 fragrant germplasms, 25 differential varieties (DVs) harbouring 23 blast-resistant genes, only 16 fragrant rice germplasms showed comparatively better performance against a virulent isolate of blast disease. The reaction pattern of single-spore isolate of *Magnaporthe oryzae* to differential varieties showed that *Pish*, *Pi9*, *Pita2* and *Pita* are the effective blast-resistant genes against the tested blast isolate in Bangladesh. However, the finding of effective blast resistant genes, *Pita*, *Pish*, *Pita2* and *Pi9* in this study agreed with those of previous reports. Pyramiding these four resistance genes into existing cultivars would lead to the development of highly resistant rice cultivars [19]. Liu *et al.* [20] proposed pyramiding (i.e. stacking) several *R*-genes with different resistance spectra into a single rice cultivar to prevent breakdown in blast disease resistance, so that the selection pressure on a single blast isolate would be reduced. However, its success depends largely on a detailed knowledge of both the regional and continental blast population diversity.

Based on this result, tightly linked gene based marker AOL45/AOL48 for *Pish*, 195R-1/195F-1 for *Pi9*, OSM89 for *Pita2* and *Pita440* (YL153/YL154) for *Pita* were selected for molecular analyses. Tested rice plant materials exhibited DNA band with specific primers on agarose gel electrophoresis. The DNA banding patterns of these cultivars were compared with those of positive and negative control cultivars. These cultivars were also classified into 10 cultivar groups (CG) based on DNA banding patterns with four gene specific primers which was similar to the classification of cultivar group based on reaction patterns to standard blast isolates. From these DNA banding patterns, presence or absence of these 4 genes was estimated in the cultivars for each CG. Based on both the methods of gene estimation, i) reaction to blast isolate and ii) DNA analysis, CG I comprised of fourteen plant materials LTH, US2, H3, H9, H26, H27, H53, H55, H65, H66, H69, H82, H91 and H93 those didn't harbor any resistant gene of expectation. Seven materials IRBLta-K1, H12, H29, H52, H63, H78 and H96 in CG II harbor only *Pita* gene. In CG III, IRBLsh-B, H40, H61 and H80 harbor *Pish* gene. Resistant gene *Pita2* was present in IRBLta2-Pi, H2, H7, H10, H24, H33, H43, H56, H60, H62 and H98 in CG IV. Similarly no single *Pi9* gene was obtained in CG V. Both *Pish* and *Pita* genes were grouped in CG VI comprising of 7 materials H28, H36, H45, H50, H67, H71 and H141. Both of the gene detection methods gathered H1, H14, H19, H39, H48, H86 and H144 materials in CG VII comprising of *Pita* and *Pita2* genes. Ten materials H15, H16, H21, H22, H37, H42, H57, H72, H79 and H135 with both *Pish* and *Pita2* genes grouped in CG VIII. Similarly three resistant genes, *Pita*, *Pish* and

Pita2 were confirmed for H13, H23, H25, H35, H47, H49, H136 and H138 materials. Finally, two materials H100 and H129 exhibited *Pita2* and *Pi9* genes those showed resistant reaction to all of the tested 4 virulent blast isolates. Resistant frequency of these two materials attributed to 100% (Table 3) might be due to the presence of broad spectrum *Pi9* gene together with *Pita2* gene.

The detection of DNA fragments in agarose gel electrophoresis for *Pi9*, *Pita*, *Pish* and *Pita2* genes and their combinations using specific DNA markers were consistent for above mentioned plant materials with the results of gene estimation using virulent blast analysis. These findings are in accordance with the findings of Fujita *et al.* [21], Yanoria *et al.* [22], Mokate *et al.* [24] and Khan *et al.* [8]. Koide *et al.* [23] carried out a detailed study on the resistant genes and selected DNA markers for blast disease in rice. They found effective markers that were tightly linked to the blast resistant genes. Kobayashi *et al.* [15] confirmed the presence of seven genes through genetic analysis of ten cultivars. Their studies supported the present study.

As expected, single *Pi9* gene was only detected in two accessions; therefore, more rice entries were needed to screen to detect this gene separately. These germplasms could be extremely useful as the experimental material to develop blast-resistant rice cultivars.

5. Conclusions

Source of rice blast resistant gene (*R*-gene) was identified by host-pathogen interaction and confirmed the presence of those *R*-genes in tested plant materials by PCR-based screening. Four distinct blast isolates (SDBI) having incompatibility reactions to four blast *R*-gene viz. *Pish*, *Pi9*, *Pita2* and *Pita* genes classified tested plant materials into 10 cultivar groups (CG). The reaction patterns of those SDBIs indicated presence of those resistant genes and their combination in the genetic background of tested plant materials. Gene specific primers were used to identify and confirm the presence of those *R*-genes in plant materials through PCR-based assay. Three genes, *Pita*, *Pita2* and *Pish* were observed together in each of the plant materials of H13, H23, H25, H35, H47, H49, H136, H138 in their genetic background. Only two materials, H100 and H129 were identified having *Pi9* gene together with *Pita2* through both of the methods. This variability would greatly help researchers in selecting suitable donors in the breeding-for-resistance program meant for transferring particular sets of genes into a desirable background for durable resistance against blast pathogen.

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