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Article

Analysis of the Relationship between Blast Resistance Genes and Disease Resistance of Rice Germplasm via Functional Molecular Markers

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Abstract: Rice blast disease is one of the most devastating diseases of rice (*Oryza sativa L.*) caused by the fungus *Magnaporthe oryzae* (*M. oryzae*), and neck blast is the most destructive phase of this illness. The underlying molecular mechanisms of rice blast resistance are not well known. Thus, we collected 150 rice varieties from different ecotypes in China and assessed the rice blast resistances under the natural conditions that favoured disease development in Jining, Shandong Province, China in 2017. Results showed that 92 (61.3%) and 58 (38.7%) rice varieties were resistant and susceptible to *M. oryzae*, respectively. Among the 150 rice varieties screened for the presence of 13 major blast resistance (R) genes against *M. oryzae* by using functional markers, 147 contained one to eight R genes. The relationship between R genes and disease response was discussed by analysing the phenotype and genotype of functional markers. The results showed that the rice blast resistance gene *Pita* was significantly correlated with rice blast resistance. Our results provided a basis for the further understanding of the distribution of 13 major R genes of rice blast in the germplasm resources of the tested rice varieties, and were meaningful for rice disease resistance breeding.

Keywords: *Magnaporthe oryzae*; rice germplasm; rice blast resistance; functional marker

1 Introduction

Rice is one of the major staples in the world. The three main kinds of rice diseases are rice blast, leaf blight and sheath blight [1]. Rice blast disease is a fungal disease caused by *Magnaporthe oryzae*. Pathogens infect leaves at the vegetative stage, and infect ear branches, nodes and necks of rice plants at the reproductive stage, thereby posing a major threat to the sustainable development of global rice production, especially in Asia and Africa with the highest incidence rate [2]. Neck blast is the most destructive phase of rice blast, and its yield reduction is twice as severe as that of leaf blast [3]. Neck blast can also lead to rice grain sterility, reduce grain size and yield [4]. Therefore, breeding rice varieties with blast resistance (R) gene is the most effective and economical strategy for controlling rice blast.

Although breeders have developed many varieties with rice blast resistance, the resistance dose not last long due to the special reproductive mode of rice blast and the pathogenic diversity in the field. Resistant varieties with a single R gene may become susceptible after 3-5 years of widespread cultivation [5]. Hence, it is urgent to develop new rice varieties with broad spectrum and durable blast resistance. To address the above issue, we collected a variety of rice germplasms and analysed their resistance to rice blast.

Compared with marker-assisted breeding, traditional breeding programs are time consuming and inefficient. A new resistant variety can be bred via marker-assisted breeding by introducing the blast R gene into cultivated varieties. The rapid development of molecular biology, especially in molecular

marker technology, enables the large-scale screening of R genes for rice varieties. Thus far, approximately 85 main resistance genes were mainly distributed at 69 blast resistant sites [6]. The three main rice blast resistance gene clusters were identified on chromosomes 6, 11 and 12. At least 10 resistance genes (i.e., *Pi2*, *Pi9*, *Pi22*, *Pi25*, *Pi26*, *Pi40*, *Pi42*, *Pigm*, *Piz* and *Piz-t*) were distributed on chromosome 6, and *Pi2*, *Pi9* and *Piz-t* have been cloned. At least 21 resistance genes were identified on chromosome 11, and 13 of them were clustered near the Pik site. *Pik*, *Pik-m*, *Pik-h*, *Pik-p* and *Pik-s* have been fine mapped [7]. At least 16 resistance genes were located on chromosome 12, and 14 of them formed a large resistance gene cluster near the centromere. Most of the resistance genes were initially located, and at least 24 of them were successfully cloned which were *Pib*, *Pb1*, *Pita*, *Pi9*, *Pi2*, *Piz-t*, *Pid2*, *Pi36*, *Pi37*, *Pik-m*, *Pit*, *Pi5*, *Pid3*, *Pi54*, *Pish*, *Pik*, *Pik-p*, *Pia*, *PiCO39*, *Pi25*, *Pi1*, *Pi21*, *Pi63* and *Pi56* [8, 9]. *Pi-b* was the first blast resistance gene cloned in rice which was located on the long arm of chromosome 2 controling resistance to most of the small species of rice blast. This gene belongs to the resistance gene of NBS (Nucleotide binding site)-LRR (Leucine-rich repeat) type, and its expression is induced [10]. The subsequently cloned genes were *Pita*, *Pi9*, and *Pid2*, located on chromosomes 12, 6, and 6, respectively. In these genes, *Pi9* has a wide resistance spectrum, and *Pid2* is the only rice blast resistant gene with extracellular structure [11].

Numerous functional markers, including markers linked to *Pita*, *Pit*, *Pik-h*, *Pi1*, *Pib*, *Pid2*, *Pik*, *Pik-p*, *Pik-m*, *Pid3*, *Pi25*, *Pi9*, *Pi2* and *Pi5*, are tightly linked to the blast R genes and have been developed and widely applied [12]. Although, the aggregation of multiple resistance genes is thought to be one of the ways to develop a broad-spectrum, long-lasting resistant variety, however, the results were not consistent for the polymerization effect of different blast genes. Hittalmani et al. used C039 as background to establish monogenic lines with *Pi1*, *Piz-5* and *Pita*, and polygenic polymeric lines with 2-3 resistance genes [13]. The results showed that polygenic polymeric lines had strong resistance, but polygenic polymeric line with *Piz-5* and *Pita* had low blast resistance than monogenic lines. In recent years, scientists around the world have screened numerous blast genes in their local cultivars. Numbers of rice blast resistance genes identified have showed that the vast majority of rice germplasms had more or less a certain amount of rice blast resistance genes. However, why the resistance differences between different strains are so significant, whether there is a broad spectrum of resistance genes, and the combination of resistance genes may be the key to determine the relative strength of resistance. In 2012, the genetic diversity of rice blast resistance was assessed in a landrace set in India by using specific molecular markers. The 108 genes identified had 2-7 of the eight resistance genes, confirming the rich diversity of this important economic trait in these native species [14]. Similarly, Imam et al. detected the genetic diversity of nine major blast genes in 32 rice germplasms from northeastern and eastern India using 10 molecular markers [15]. In another study, approximately 289 genotypes with broad-spectrum blast resistance were identified using STS markers [16]. The presence of R genes in 195 varieties with high resistance to rice blast has been recently analysed using functional molecular markers [17]. Approximately 97 rice strains in Shanxi Province and 51 rice germplasms in the Hanzhong region of Shanxi Province were analysed with SNP and Indel markers, which were closely related to *Pib*, *Piz-t*, *Pi9*, *Pik-m* and *Pita* [18]. The R gene database was established using specific molecular markers to screen the rice blast genes *Pi9*, *Pita*, *Pib* and *Pik-m* in 70 varieties. Dai et al. evaluated the relationship between the existence of R genes and disease resistance, and the results suggested that the varieties were most resistant to blast when they contain the main R gene *Pi9* and the secondary genes *Pita* and *Pib* [19].

In 2014 and 2015, our lab detected blast resistance and major R genes in 358 rice varieties. Among the tested varieties, 124 varieties were resistant to rice blast under natural field conditions, and R gene *Pi2* was significantly correlated with rice blast resistance [20]. In the present study, we collected 150 main rice cultivated species in China to test the blast resistance and the distribution of major R genes by using functional markers. Additional reference lines were obtained for breeding blast resistant rice varieties, and the rule of change of rice blast resistance was obtained to provide a theoretical basis for breeding rice varieties with broad-spectrum, high blast resistance in the Huang-Huaihai area. We hope to improve the level and widen the spectrum of resistance by aggregating the positive and main effects of the microgene.

2 Materials and Methods

2.1 Cultivation of Rice Materials

The blast resistance of 150 rice varieties with different ecotypes in Jining, Shandong, was evaluated under natural field conditions. Rice blast has a long history in this area. Rice seeds were sown in early May 2017 and managed naturally throughout the growth period without administering any pesticides.

2.2 Evaluation of Rice Blast Resistance

Under natural conditions, all the rice varietieswere seriously infected with *M. oryzae*. The blast incidence score was from 0 (no lesions) to 9 (representing highly sensitive plants with panicle bases, complete or topmost internode lesions, or less than 30% of full grain near the base of the panicle axis) following the revised International Rice Research Institute (IRRI) standard evaluation method (IRRI 2002). In this study, 0-3 points of varieties were divided into resistance (R) group and 4-9 points of varieties were divided into susceptibility (S) group. A high score was used for analysis when the scores between the repetitions were different.

2.3 Identification of R Gene by PCR

Rice genomic DNA was extracted from fresh leaves by CTAB method. Expression of 13 blast resistance genes (e.g., *Pi9*, *Pita*, *Pib*, *Pik-m*, *Pik-h*, *Pit*, *Pid2*, *Pi2*, *Pi5*, *Pik*, *Pik-p*, *Pid3*, and *Pi25*) was analyzed by PCR using molecular markers (Tab. 1). A 20μl PCR mixture were used including 25 ng genomic DNA, 5 pmol primer for each and 10 μl 2x PCR reaction mix (Takara Bio). PCR amplification procedure including initial degeneration (95°C) for 5 min, 35 cycles of 95°C for 40 s; 55°C for 40 s; 72°C for 1 min and finally extend (72°C) for 10 minutes. Appropriate restriction enzymes were used for restriction digestion and PCR products were identified. For confirmation purposes, the PCR assay was repeated. The score is based on the presence (1) or absence (0) of the gene.

2.4 Data Association Analysis

Model fitting was conducted using the method of MLR, and the sum of squares of each marker, SS_reg, and the sum of squares of the model, SS_tol, was calculated using Anova [28]. The phenotype variance explained (PVE) of the marked marker was calculated as follows: PVE = SS_reg/SS_tol.

3 Results

3.1 Evaluation of Resistance to Rice Blast under Natural Field Conditions

In accordance with the improved IRRI evaluation method, varieties with 0-3 points were classified as R group, and those with 4-9 points were classified as S group. In 2017, the 150 rice varieties were screened for rice blast resistance under natural field conditions in Jining, Shandong Province, China. Ninety-two varieties (61.3%) were resistant to rice blast, and fifty-eight varieties (38.7%) were susceptible (Tab. 2).

3.2 Molecular Screening for the Rice Blast Resistance Genes

Thirteen blast R genes (*Pi9*, *Pita*, *Pib*, *Pik-m*, *Pik-h*, *Pit*, *Pid2*, *Pi2*, *Pi5*, *Pik*, *Pik-p*, *Pid3*, and *Pi25*) were analysed by PCR using marker primers as previously reported in 150 rice varieties (Tab. 1). For R gene *Pi9*, *Pita*, *Pib*, *Pik-h*, *Pid2*, *Pi2*, and *Pi5*, if 291 bp, 467 bp, 365 bp, 216 bp, 629 bp, 450 bp, and 206 bp PCR fragment could be obtained by PCR amplification using its gene-related primer seperately, the R gene could be regards exist (Figs. 1(A)-(C), (E), (G), (H), (I)). For R gene *Pik-m* and *Pit*, they need to use two gene-related primers for verification. If the 223-bp (Ckm1 primer) and 291-bp (Ckm2 primer) band could be amplified both in one germplasm, we prefered that *Pik-m* exist in rice genome (Fig. 1(D)). The R gene *Pit* could be regards exist if 733-bp (tK59-1 primer) or 530-bp (tK59-2 primer) band could be amplified respectively (Fig. 1(F)). For R gene *Pid3* and *Pi25*, enzyme digestion is required for validation after PCR amplification. *Bam*H I was used to perform enzyme digestion on the PCR products amplified

with by the dCAPS primer, and varieties producing 510-bp and 148-bp bands were considered to have the *Pid3* gene (Fig. 1(J)). The PCR product amplified by the CAPS primer CAP1 was identified by *Hinc* II digestion, and *Pi25* could be obtained as 406-bp band (Fig. 1(K)). The results showed that among the 150 varieties analysed, 147 (98%) had 1-8 major R genes. However, the *Pik* and *Pik-p* genes were not detected in all tested varieties. The R gene*Pi9*, *Pita*, *Pib*, *Pik-m*, *Pik-h*, *Pit*, *Pid2*, *Pi2*, *Pi5*, *Pik*, *Pik-p*, *Pid3*, and *Pi25* were detected in 83, 47, 52, 83, 74, 14, 26, 7, 21, 7, and 70 varieties seperately (Tab. 2), and the gene frequency is 55.3%, 31.3%, 34.7%, 55.3%, 49.3%, 9.3%, 17.3%, 4.7%, 14%, 4.7%, and 46.7%.

Table 1: Functional marker for the amplification of resistant genes

Gene	Marker	Forward Primer (5'-3')	Reverse Primer (5'-3')	Expected Size (bp)	Restriction enzyme	Reference
Pi9	Pi9	GCTGTGCTCCAAATGAGGAT	GCGATCTCACATCCTTTGCT	291/397		[21]
Pita	Pita	GCAGGTTATAAGCTAGGCC	CCACATTGATGAAGCCCATA	467		[21]
Pib	Pibdom	GAACAATGCCCAAACTGAGA	GGGTCCACATGTCAGTGAGC	365		[22]
Pik-m	Ckm1	CTGGAGAGCTTCCGTGTCGAC	TCTTCACGACGTCAATGGTGGC	223		[23]
	Ckm2	GTTGTTCACTCCGTATCTACTACGTC	TTCCTCCGTGATCTCAGCAACG	291		
Pik-h	Pikh	CAATCTCCAAAGTTTTCAGG	GCTTCAATCACTGCTAGACC	216/359		[24]
Pit	tK59-1	ATGATAACCTCATCCTCAATAAGT	GTTGGAGCTACGGTTGTTCAG	733		[8]
	tK59-2	ATGATAACCTCATCCTCAATAAGT	CCAAGGGATTAGGTCCTAGTG	530		
Pid2	d2In	GCGTCGAAGATGTCCTGAAGTCA	GGCAGTCGTATTGCTGTGAA	629		[24]
Pi2	Pi2	CAGCGATGGTATGAGCACAA	CGTTCCTATACTGCCACATCG	450/282		[22]
Pi5	Pi5	ATAGATCATGCGCCCTCTTG	TCATACCCCATTCGGTCATT	206/307		[22]
Pik	Pik	TTCGAGGCCCTACCAAGACA	CATGGAAGGCTATCCTTGGTA	2953	*Kpn* I	[25]
Pik-p	Pikp1	GCAGGTCAGCCAAGCAAT	CAACCGTTGTTTTGCCTCC	795	*Mse* I	[25]
Pid3	Pid3	TACTACTCATGGAAGCTAGTTCTC	ACGTCACAAATCATTCGCTC	148/510	*Bam*H I	[26]
Pi25	CAP1	TGAAATGGGTGAAAGATGAG	GCCACATCATAATTCCTTGA	406	*Hinc* II	[27]

Table 2: Genotyping of 150 rice germplasms estimated with special functional markers and resistance to rice blast

No.	Varieties	*Pi9*	*Pita*	*Pib*	*Pik-m*	*Pik-h*	*Pi2*	*Pid2*	*Pi5*	*Pit*	*Pik*	*Pik-p*	*Pid3*	*Pi25*	Resistance to neck blast
1	Yangjing 806	0	1	0	0	0	0	0	0	0	0	0	0	1	R
2	Yangjing 092-16	1	1	1	0	1	0	0	1	1	0	0	0	1	R
3	Fengjing 1	0	1	1	1	0	1	0	1	0	0	0	0	1	R
4	Fengdao 104	1	1	1	1	0	1	1	0	1	0	0	0	1	R
5	Jidao 2	1	1	0	1	0	0	0	1	0	0	0	0	1	R
6	Jidao 3	0	0	1	1	1	0	1	0	0	0	0	0	0	R
7	Longjing 12	0	1	1	1	0	0	1	1	0	0	0	1	1	S
8	Jidao 1	1	1	1	0	0	0	0	1	0	0	0	1	0	R
9	Runnong 1312	0	1	1	0	0	0	1	0	0	0	0	0	0	R
10	Runnong 5	1	1	0	1	0	0	0	1	0	0	0	0	1	R
11	Fengjing 2	0	0	0	1	0	0	0	1	0	0	0	0	1	R
12	T800	1	0	1	1	0	0	1	0	0	0	0	0	1	S
13	Shengdao 189	1	1	1	0	0	0	0	0	0	0	0	0	0	R

14	Shengdao 168	0	1	1	1	1	0	0	0	0	0	0	0	0	R
15	Shengdao 176	1	0	1	1	1	0	0	0	0	0	0	0	0	R
16	Jidao 6	1	0	1	1	1	0	0	0	0	0	0	0	0	R
17	Lindao 11	1	1	1	1	1	0	0	0	0	0	0	0	0	R
18	Lindao 10	0	1	1	1	0	0	0	0	0	0	0	0	1	R
19	Shengdao 17	1	1	0	1	1	0	1	0	0	0	0	0	0	S
20	Sheng 18	1	1	1	1	1	1	0	0	0	0	0	0	1	R
21	Suxiu 9	1	1	0	1	0	0	0	0	0	0	0	0	0	R
22	Linyi 5	1	0	1	0	0	0	0	0	0	0	0	0	1	R
23	Wuxiang 99075	0	0	1	0	1	0	0	1	0	0	0	0	0	R
24	Suxiu 10	0	1	0	0	0	0	0	0	0	0	0	0	1	S
25	Huodao 008	1	0	1	0	1	0	1	0	0	0	0	0	1	R
26	Zaojing 1	1	0	0	1	1	0	0	0	0	0	0	0	1	S
27	Rihuijing 18	1	0	0	1	0	0	0	0	0	0	0	0	0	S
28	Fudaowanjia 1	0	1	1	0	0	0	0	0	0	0	0	0	0	R
29	Lianjing 7	1	0	1	1	1	0	0	0	0	0	0	0	0	R
30	Suxiu 867	0	1	1	0	0	0	0	0	1	0	0	0	1	R
31	Yudao 518	1	1	0	1	1	1	0	0	0	0	0	0	1	R
32	Nanjing 9108	1	0	1	0	1	0	0	0	0	0	0	0	1	R
33	Lindao 16	1	0	0	0	1	0	0	1	1	0	0	0	0	R
34	Jindao 263	1	0	0	0	1	0	0	0	0	0	0	0	1	R
35	Yandao 10	1	1	1	0	1	0	0	0	0	0	0	0	0	S
36	Xinxiu 9	1	0	0	0	0	0	0	0	0	0	0	0	1	S
37	Zhongzhong 5	1	1	1	1	0	0	0	0	0	0	0	0	1	R
38	Longjing 968	0	0	0	0	1	0	0	1	0	0	0	0	0	R
39	Xudao 3	1	1	0	1	1	1	0	0	1	0	0	0	1	R
40	Xinxiu 326	0	1	1	1	0	0	0	0	0	0	0	0	1	R
41	Jindao 253	1	1	0	1	1	0	1	0	0	0	0	0	0	R
42	Jidao 5	1	0	0	0	0	0	1	0	0	0	0	0	0	R
43	Suxiu 10	0	0	0	0	0	0	0	0	0	0	0	0	0	R
44	Suxiang 10	0	1	0	1	0	0	0	0	0	0	0	0	1	R
45	Suxiang 9	0	0	0	0	0	0	0	0	0	0	0	0	0	R
46	Jindao 263	1	0	0	0	1	0	0	0	0	0	0	0	0	R
47	Wu 2743	0	0	0	1	0	0	1	0	0	0	0	0	0	R
48	Xiangnuo 701	0	1	0	1	0	0	0	0	0	0	0	0	1	R
49	Longjing 12	0	0	0	1	0	0	0	1	0	0	0	0	0	R
50	Fan 953	1	0	0	1	1	0	0	0	0	0	0	0	0	R
51	Jidao 7	1	0	0	1	0	0	0	0	0	0	0	0	0	S
52	Yanjing 11	0	0	0	0	1	0	0	0	0	0	0	0	0	S
53	Xindao 22	0	0	0	0	1	0	0	0	0	0	0	0	0	R
54	Wuyujing 23	1	0	0	1	0	0	0	0	0	0	0	0	0	R
55	Zhendao 19	0	0	0	1	1	0	0	0	0	0	0	0	0	R
56	Run 20	1	0	0	1	0	0	0	0	0	0	0	0	0	S
57	Suxiu 298	0	0	0	1	0	0	0	0	0	0	0	0	0	R
58	Lianjing 12	1	0	0	1	1	0	0	1	0	0	0	0	0	S

59	Ruihua 1704	0	0	0	0	1	0	0	0	0	0	0	0	0	S
60	Mingtian 019	1	0	0	1	0	0	1	0	0	0	0	0	0	S
61	Yangjing 516	0	0	0	0	1	0	0	0	1	0	0	0	0	S
62	Yanfeng 99	0	0	1	0	1	0	0	0	0	0	0	0	0	R
63	Kennuo 8	0	0	0	1	1	0	0	0	0	0	0	0	0	S
64	Yangjing 619	0	0	0	0	1	0	0	1	0	0	0	0	0	S
65	Wankenjinqing	0	0	0	1	0	0	0	1	0	0	0	0	0	S
66	Ruicheng 17058	1	1	1	1	1	0	1	0	1	0	0	0	0	R
67	Nongxuan 11	1	1	0	0	1	0	0	0	0	0	0	0	0	S
68	Wankennuo 124	1	0	0	0	0	0	0	0	0	0	0	0	0	S
69	Lvyixiangnuo	1	0	0	0	1	0	0	0	1	0	0	0	0	R
70	Xudao 6	0	0	0	1	1	0	1	0	0	0	0	0	0	S
71	Lingzhenyou 6	0	0	0	1	1	0	1	0	0	0	0	0	0	S
72	Fengdao 102	0	1	1	1	0	0	0	0	0	0	0	0	0	R
73	Yangnongjing 17-3168	1	1	1	0	1	0	0	0	0	0	0	0	0	S
74	Zhongyouhe 3	1	0	0	1	1	0	0	0	0	0	0	0	0	R
75	Fengjing 1	0	0	0	1	1	0	0	0	0	0	0	0	0	R
76	Ruicheng 6040	1	0	0	1	0	0	0	0	0	0	0	0	0	R
77	Hefengnuo 778	0	0	0	0	1	0	0	0	0	0	0	0	0	S
78	Daliang 317	1	0	0	1	1	0	0	0	0	0	0	0	0	S
79	Hefengnuo 316	0	0	1	1	0	0	0	0	0	0	0	0	0	S
80	Rihuijing 5	0	0	1	1	1	0	0	0	1	0	0	0	0	R
81	Fengrundao 169	0	0	0	1	1	0	0	0	0	0	0	0	0	S
82	Fengrundao 166	0	0	0	0	0	0	1	0	0	0	0	0	0	R
83	Guangwei 11	1	0	0	1	0	0	0	0	0	0	0	0	0	R
84	Rihuijing 6	0	0	1	0	0	0	0	1	0	0	0	0	0	S
85	Guoruijingnuo 2	1	0	1	1	0	0	0	1	0	0	0	0	0	R
86	Daliang 309	0	0	0	1	1	0	0	0	0	0	0	0	0	S
87	Lvxiu 19	1	1	0	0	0	0	0	0	0	0	0	0	0	S
88	Runnong 4	1	0	1	0	0	0	0	0	1	0	0	0	0	S
89	DH17500	0	0	0	0	1	0	0	1	0	0	0	0	0	R
90	Sidao 15-38	1	1	1	0	0	0	0	0	0	0	0	0	0	R
91	Kenxiang 48	1	1	1	0	0	0	0	0	0	0	0	0	0	R
92	Liannuo 1	1	0	0	0	1	0	1	0	0	0	0	0	0	S
93	Wankennuo 1	1	1	0	0	0	0	0	0	0	0	0	0	0	R
94	Xinjingnuo	1	0	0	1	1	0	0	1	0	0	0	0	0	S
95	Fengnuo 1246	0	1	1	1	0	0	1	0	0	0	0	1	1	S
96	Wuyunuo 16	1	0	0	1	1	0	0	0	0	0	0	0	0	S
97	Yangjing 3012	1	0	0	0	0	0	0	0	0	0	0	0	1	S
98	Jidao 8	0	1	0	1	0	0	0	0	0	0	0	0	1	R
99	Shengdao 1647	0	0	1	0	1	0	0	0	0	0	0	0	1	R
100	Shengxiangnuo 1	0	0	0	0	1	0	0	0	0	0	0	0	1	R
101	Yin 18	1	0	0	0	1	0	0	0	0	0	0	0	1	R
102	Shengdao 20	0	0	0	0	0	0	1	0	0	0	0	0	1	S
103	Yin 19	0	0	1	1	1	0	0	0	0	0	0	0	1	R

104	Shengdao 17	1	1	1	0	1	0	0	0	0	0	0	0	1	R
105	Yangguang 800	0	1	0	1	0	0	0	0	1	0	0	1	1	R
106	Shengdao 18	1	0	0	1	1	0	0	0	0	0	0	0	1	R
107	Daliang 203	1	0	0	0	0	0	0	0	0	0	0	0	1	S
108	Lin 11-D63	1	1	1	1	0	0	1	0	0	0	0	0	0	S
109	Runnong 11	0	0	1	0	0	0	0	0	0	0	0	0	1	S
110	Runnong 10	1	0	0	0	0	0	0	0	0	0	0	0	1	R
111	6J1605	0	0	0	0	0	0	0	0	0	0	0	0	0	R
112	Yin 20	1	0	0	1	0	0	0	0	0	0	0	0	1	R
113	Zhidao 21	1	0	0	0	0	0	0	0	0	0	0	0	1	R
114	SD505	0	0	0	1	1	0	0	0	0	0	0	0	1	R
115	Lin 10	1	0	0	0	0	0	0	0	0	0	0	0	1	S
116	Changlixiang	1	0	0	0	0	0	0	0	0	0	0	0	1	S
117	Lianheti 24	1	0	0	1	0	0	1	0	0	0	0	0	0	R
118	Yudao 518	1	0	0	1	0	0	0	0	0	0	0	0	0	S
119	Lianheti 28	1	0	1	0	1	0	0	0	0	0	0	0	0	S
120	Lindao 11-1	1	0	0	0	0	0	1	0	0	0	0	0	1	R
121	Lindao 19	0	0	0	0	1	0	0	1	0	0	0	0	0	R
122	Luzidao 8	0	0	0	0	0	0	0	0	0	0	0	0	1	R
123	Shengdao 19	0	0	0	1	1	0	0	0	0	0	0	0	0	R
124	Luzidao 10	1	0	0	1	1	0	1	0	0	0	0	0	0	S
125	Luzidao 11	1	0	0	1	1	0	0	1	1	0	0	0	1	S
126	Luzidao 12	1	0	0	1	0	0	0	0	0	0	0	0	1	R
127	Shengdao 22	1	0	0	1	1	0	0	0	0	0	0	0	1	R
128	Handao 277	0	0	0	0	1	0	0	0	0	0	0	1	1	S
129	Yin25	1	0	0	1	1	0	0	0	0	0	0	0	1	R
130	Xinxiu 326	0	0	0	1	0	0	0	0	0	0	0	0	1	R
131	Lianheti 23	0	0	0	1	1	0	1	0	0	0	0	0	1	S
132	Yin 23	0	0	0	1	0	1	0	0	0	0	0	0	1	S
133	Jinjing 818	0	0	0	1	1	0	0	0	0	0	0	1	0	S
134	Luzidao 6	0	1	1	1	0	0	0	0	1	0	0	0	1	R
135	Daliang 303	0	1	0	0	0	0	0	0	0	0	0	0	1	S
136	Jinjing 698	1	0	1	0	1	0	0	0	0	0	0	0	1	S
137	J1603	1	0	0	1	1	1	0	0	0	0	0	0	1	S
138	Jinjing 667-1	1	1	1	1	0	0	0	0	0	0	0	0	1	S
139	Lindao 21	1	0	0	0	0	0	1	0	0	0	0	0	1	S
140	Daliang 306	1	0	1	0	0	0	0	0	0	0	0	0	1	S
141	Zhenghan 9	1	0	0	0	1	0	0	0	0	0	0	0	1	R
142	Zhendao 458	1	0	1	0	0	0	0	0	0	0	0	0	1	S
143	Yangjing 516	0	1	1	1	1	0	1	0	0	0	0	0	1	R
144	Yanfeng 99	1	1	0	0	1	0	0	0	0	0	0	0	1	R
145	Fengnuo 158	0	0	1	1	1	0	0	0	0	0	0	0	1	R
146	Yangjing 619	0	0	1	1	1	0	0	0	1	0	0	1	0	R
147	Yangnongjing 16-2680	1	1	0	1	0	0	0	1	0	0	0	0	1	S
148	Daliang 307	1	1	0	0	0	0	1	0	0	0	0	0	1	R

149	Wankenjing G84	0	0	0	1	1	0	0	0	0	0	0	0	1	R
150	Yidao 206	0	0	1	1	1	0	0	0	0	0	0	0	1	R

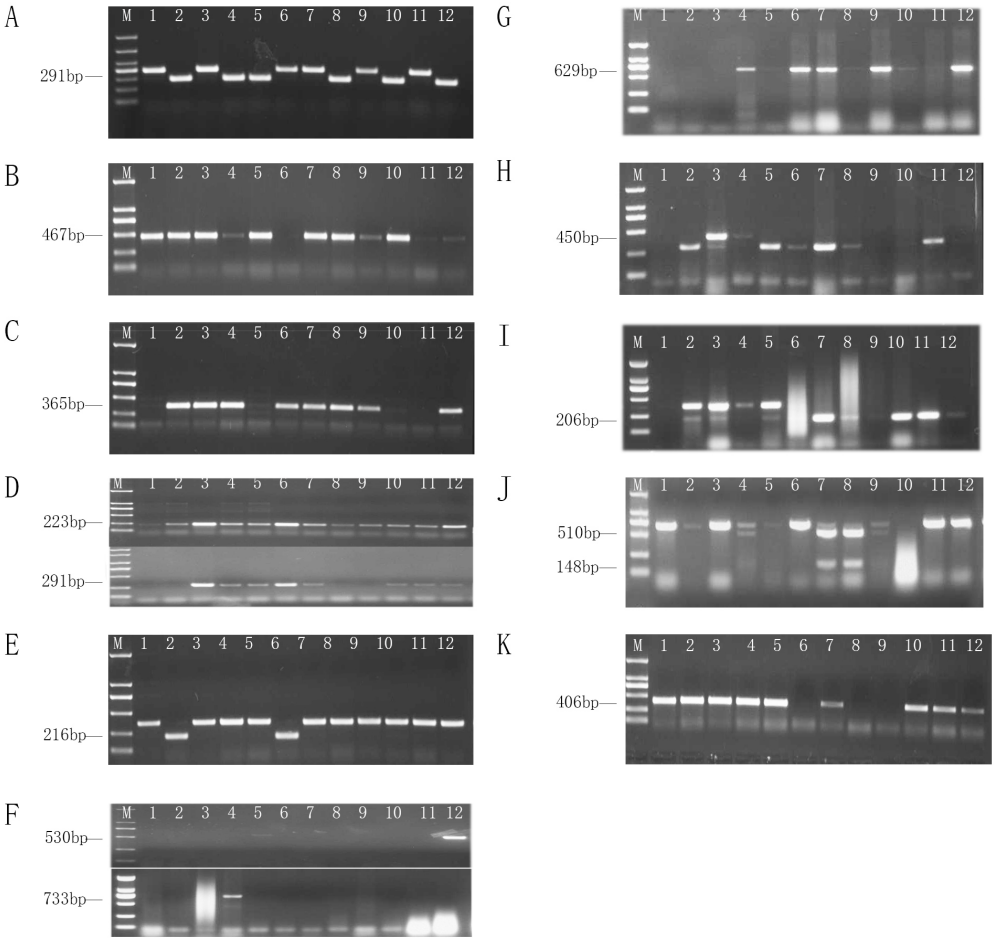

Figure 1: The existence of blast resistance (R) gene among rice varieties

PCR amplifiers were obtained from the genomic DNA of 12 rice varieties using gene-specific primers of 11 major R genes including *Pi9* (A), *Pita* (B), *Pib* (C), *Pik-m* (D; upper, Ckm1 primer; below, Ckm2 primer), *Pik-h* (E), *Pit* (F; upper, tK59-2 primer; below, tK59-1 primer), *Pid2* (G), *Pi2* (H), *Pi5* (I), *Pid3* (J), and *Pi25* (K). M, DL2000 DNA markers; 1, Yangjing 806 3; 2, Yangjing 092-16; 3, Fengjing 1; 4, Fengdao 104; 5, Jidao 2; 6, Jidao 3; 7, Longjing 12; 8, Jidao 1; 9, Runnong 1312; 10, Runnong 5; 11, Fengjing 2; 12, T800.

3.3 Correlation Analysis of R Gene and Disease Resistance

Among the 150 varieties, 92 and 58 were classified as resistant and susceptible, respectively, in accordance with the modified IRRI standard evaluation method.Furthermore, 147 varietiescontained 1-8 out of the 11 major rice blast R genes. In this regard, eight varieties contained only one R gene, 43 varieties contained two R genes, 40 varieties contained three R genes, 28 varieties contained four R genes, 17 varieties contained five R genes, 5 varieties contained six R genes, 5 varieties contained seven R genes, and only 1 variety contained eight R genes (Tab. 2). However, three germplasms, Suxiu 10, Suxiang 9

and 6J1605, showed good resistance in the field, but no resistance genes (13 R genes selected) were detected. Such germplasms may have other R genes that were not tested. On the basis of stringent phenotype coupled with genotyping using functional markers, the relationship between the presense of R genes and the blast resistance of varieties analysed via Anova. Correlation analysis showed that there was a significant correlation between R gene *Pita* and rice blast resistance in Jining, Shandong Province in 2017. The PVE of *Pita* was 3.5%, and the difference was significant.

4 Discussion

The outbreak of rice blast is a serious and recurring problem, and controlling this disease in all rice growing areas of the world is extremely difficult [29]. The rapid genetic evolution of fungus often overcomes the resistance conferred by major genes after a few years of intensive agricultural use. Therefore, disease-resistant breeding requires continuous effort to enrich the R gene pool. The resistance of rice varieties can be improved when breeders understand what and how many disease-resistance genes are carried by the varieties to ensure that they can develop a reasonable arrangement of the resistant varieties. He et al. analysed the genotypes of *Pita* and *Pib* resistance genes in some japonica rice varieties and strains in China, and determined the distribution of *Pita* and *Pib* in some japonica rice varieties [30]. Fan et al. conducted molecular marker detection of blast genes in Jiangsu rice varieties, and the results showed that *Pib*, *Pita*, *Pik-m* and *Pi54* are important disease-resistance genes in japonica rice bred in Jiangsu Province; among which, *Pita* is substantially correlated with resistance to ear and neck blast [31].

This study aims to evaluate 150 rice germplasms for rice blast resistance under natural field conditions and to identify the blast resistance genes in the tested rice germplasms. Although many disease-resistant varieties possessing a single R gene have been developed, however, disease-resistance can not be sustained for a long time due to the characteristics of rapid evolution of fungus in the field. In 3-5 years, disease resistance caused by a single R gene becomes ineffective [5]. Therefore, it is very important to develop cultivars containing broad spectrum resistance. Identification of blast resistant germplasm resources is the first step in this process. In this study, we obtained excellent potential disease-resistant donor varieties for the identification of blast resistance varieties by screening rice germplasms collected from different regions in China. In accordance with the improved IRRI standard evaluation method, we obtained 92 varieties with resistance to rice blast under natural field conditions. These varieties can be used as reference for breeding programs.

We screened 13 established blast R genes, i.e., *Pi9*, *Pita*, *Pib*, *Pik-m*, *Pik-h*, *Pit*, *Pid2*, *Pi2*, *Pi5*, *Pik*, *Pik-p*, *Pid3* and *Pi25*, in 150 rice varieties, and 147 of them contained 1-8 R genes. The distribution frequency of *Pik*, *Pik-p*, *Pid2*, *Pi2*, *Pi5*, *Pid3* and *Pit* was low, indicating that these blast R genes are not widely used in the main cultivated rice varieties in China. Molecular markers associated with the main blast R gene are used to identify specific genesand functional markers could be employed to identify R genes in diverse germplasms [8]. Functional markers closely related to the blast R genes of rice have been used to screen one or more R genes existing in breeding programs and to accelerate the identification of new R genes in different germplasm [21,28,31]. In this study, rice blast R gene, *Pita*, was significantly correlated with the rice blast resistance in rice varieties grown in Jining, Shandong Province. *Pita* is a single copy gene encoding a leucine-rich cytoplasmic membrane receptor protein consisting of 928 amino acid residues [32], which belongs to the NBS-LRR disease resistance gene. The disease-resistant response of *Pita* was triggered by the interaction between the Pita protein and the AVR-Pita protein which is the non-toxic gene form of rice blast fungus [33]. These results are not consistent with our previous results in 2014 and 2015 indicating that the dominant physiological species of blast fungus and the dominant genes for rice resistance in rice population have changed in the past 2-3 years [20]. These cultivars mainly used in China showed resistance to rice blast, indicating the feasibility of constructing polygenic polymeric broad-spectrum resistant varieties. *Pita* is not a major blast-resistant gene but plays the most important role in the rice population's blast resistance in the present study. Therefore, the function of any resistance gene should not be underestimated in the breeding of blast resistant varieties. Functional evaluation by incorporating the carrier lines of such genes into a rice breeding programme through gene pyramiding

must be first conducted. In general, the presence of multiple R genes usually leads to high resistance levels, but some susceptible varieties also contain multiple R genes. This discovery may explain the reasonable distribution of R genes with blast resistance of new rice varieties. As such, the genetic diversity in different regions and annual planting types for various blast-resistant varieties can be utilised to design a reasonable layout and rotation for resistance genes. This step can help avoid the resistance loss of these genes and prolong the use of fixed number of year of resistant varieties.

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References

1. Zhang, Q. F. (2007). Strategies for developing Green Super Rice. *PNAS, 104(42),* 16402-16409.
2. Sharma, T. R., Rai, A. K., Gupta, S. K., Vijayan, J., Devanna, B. N. et al. (2012). Rice blast managemet through host-plant resistance: retrospect and prospects. *Agricultural Research, 1(1),* 37-52.
3. Wilson, R. A., Talbot, N. J. (2009) Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. *Nature Reviews Microbiology, 7(3),* 185-195.
4. Puri, K. D., Shrestha, S. M., Chhetri, G. B. K., Joshi, K. D. (2009). Leaf and neck blast resistance reaction in tropical rice lines under green house condition. *Euphytica, 165(3),* 523-532.
5. Lang, N. T., Luy, T. T., Buu, B. C., Lang, N. T., Luy, T. T. (2009). Monogenic lines resistance to blast disease in rice (*Oryza sativa* L.) in Vietnam. *International Journal of Genetics and Molecular Biology, 1(7),* 127-136.
6. Deng, Y. W., Zhai, K. R., Xie, Z., Yang, D. Y., Zhu, X. D. et al. (2017). Epigenetic regulation of antagonistic receptors confer rice blast resistance with yield balance. *Science, 355(6238),* 962-965.
7. Hayashi, K., Yoshida, H., Ashikawa, I. (2006). Development of PCR based allele-specific and Indel marker sets for nine rice blast resistance genes. *Theor Appl Genet, 113(2),* 251-260.
8. Liu, W., Liu, J., Triplett, L., Leach, J. E., Wang, G. L. (2014). Novel insights into rice innate immunity against bacterial and fungal pathogens. *Annual Review of Phytopathology, 52(1),* 213-241.
9. Devanna, N. B., Vijayan, J., Sharma, T. R. (2014). The blast resistance gene Pi54 of cloned from Oryza officinalis inter-acts with Avr-Pi54 through its novel non-LRR domains. *PLoS One, 9(8),* e104840.
10. Rybka, K., Miyamoto, M., Ando, I., Saito, A., Kawasaki, S. (1997). High resolution mapping of the indica-derived rice blast resistance genes II. *Pi-ta*^{2} and *Pi-ta* and a consideration of their origin. *MPMI, 10,* 517-524.
11. Chen, X. W., Shang, J. J., Cehn, D. (2006). B-lectin receptor kinase gene confers rice blast resistance. *Plant Journal, 46,* 794-804.
12. Hayashi, K., Yasuda, N., Fujita, Y., Koizumi, S., Yoshida, H. (2010). Identification of the blast resistance gene *Pit* in rice cultivars using functional markers. *Theor Appl Genet, 121(7),* 1357-1367.
13. Hittalamani, S., Parce, A., Mew, T. V., Zeigler, R. S., Huang, N. (2000). Fine mapping and DNA marker-assistend pyramiding of the three major genes for blast resistance in rice. *Theoretical and Applied Genetics, 100,* 1121-1128.
14. Mahender, A., Swain, D. M., Subudhi, H. N., Rao, G. J. N. (2012). Molecular analysis of native Manipur rice accessions for resistance against blast. *African Journal of Biotechnology, 11(6),* 1321-1329.
15. Imam, J., Alam, S., Mandal, N. P., Variar, M., Shukla, P. (2014). Molecular screening for identification of blast resistance genes in north east and eastern Indian rice germplasm (*Oryza sativa* L.) with PCR based makers. *Euphytica*, *196(2),* 199-211.
16. Vasudevan, K., Vera Cruz, C. M., Gruissem, W., Bhullar, N. K. (2014). Large scale germplasm screening for

identification of novel rice blast resistance sources. *Frontiers in Plant Science, 5(5),* 505.

17. Liu, H., Meng, D. L., Zha, R. Y., Xu, D. Y. (2015). Identification and evaluation on blast resistance of rice varieties in Jiangsu. *Fujian Journal of Agricultural Science*s, *30(5),* 52-58 (in Chinese).
18. Zhang, Y., Feng, Z. F., Zhang, X. P., Li, X. G., Wang, B. J. et al. (2014). Study on genotyping and resistance evaluation for five rice blast resistance genes. *Southwest China Journal of Agricultural Sciences, 27(5),* 19-29 (in Chinese).
19. Dai, X. J., Yan, Y. Z., Zhou, L., Liang, M. Z., Fu, X. C. et al. (2012). Distribution research of blast resistance genes *Pita*, *Pib*, *Pi9* and *Pikm* in blast-resistant rice resources. *Life Science Research, 16(4),* 3400-3440.
20. Yang, Y. Y., Zhang, H., Xuan, N., Chen, G., Liu, X. et al. (2017). Idengtification of blast resistance in 358 rice germplasms (*Oryza sative* L.) using functional molecular markers. *European Journal of Plant Pathology, 148,* 567-576.
21. Xu, X. W., Wang, X., Huang, Y. X., Jiang, S. H., Li, W. et al. (2013) Advances in molecular markers of blast resistance genes and marker-assisted breeding in rice. *Jiangsu Journal of Agricultural Sciences, 29(4),* 403-410 (in Chinese).
22. Fjellstrom, R., Conawaybormans, C. A., Mcclung, A. M., Marchetti, M. A., Shank, A. R. et al. (2004). Development of DNA markers suitable for marker assisted selection of three Pi genes conferring resistance to multiple pyricularia grisea pathotypes. *Cropence, 44(5),* 1790-1798.
23. Costanzo, S., Jia, Y. (2010). Sequence variation at the rice blast resistance gene Pi-km locus: implications for the development of allele specific markers. *Plant Science, 178(6),* 523-530.
24. Ramkumar, G., Srinivasarao, K., Mohan, K. M., Sudarshan, I., Sivaranjani, A. K. P. et al. (2011). Development and validation of functional marker targeting an Indel in the major rice blast disease resistance gene *Pi54* (*Pikh*). *Molecular Breeding, 27(1),* 129-135.
25. Zhai, C., Lin, F., Dong, Z., He, X., Yuan, B. et al. (2011). The isolation and characterization of *Pik*, a rice blast resistance gene which emerged after rice domestication. *New Phytologist, 189(1),* 321-334.
26. Shang, J. J., Tao, Y., Chen, X. W., Zou, Y., Lei, C. L. et al. (2009). Identification of a new rice blast resistance gene, *Pid3*, by genomewide comparison of paired nucleotide-binding site leucine-rich repeat genes and their pseudogene alleles between the two sequenced rice genomes. *Genetics, 182(4),* 1303-1311.
27. Wang, H. M., Chen, J., Shi, Y. F., Pan, G., Shen, H. C. et al. (2012). Development and validation of CAPS markers for marker-assisted selection of rice blast resistance gene *Pi25*. *Acta Agronomica Sinica, 38(11),* 1960-1968.
28. Liu, G., Zhao, Y., Gowda, M., Longin, C. F. H., Reif, J. C. et al. (2016). Predicting hybrid performances for quality traits through genomic-assisted approaches in central European wheat. *PLoS One, 11(7),* e0158635.
29. Khan, M. A., Bhuiyan, M. R., Hossain, M. S., Sen, P. P., Ara, A. et al. (2014). Neck blast disease influences grain yield and quality traits of aromatic rice. *Comptes Rendus Biologies, 337(11),* 635-641.
30. He, Z., Chen, T., Zhang, Y. D. (2014). Genotype analysis of pita and *pib* resistance genes in some japonica rice varieties and strains in Jiangsu province. *Jiangsu Journal of Agricultural Sciences, 30(5),* 921-927 (in Chinese).
31. Zhang, Y., Feng, Z. F., Cui, M. Z. (2013) Distribution of *Pikm* resistance gene in rice seed resources in shanxi province. *Molecular Plant Breeding, 11(3),* 311-316.
32. Bryan, G. T., Wu, K. S., Farrall, L., Jia, Y., Hershey, H. P. et al. (2000). A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pi-ta*. *Plant Cell, 12(11),* 2033-2046.
33. Jia, Y., Mcadams, S. A., Bryan, G. T. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *Embo Journal, 19(15),* 4004.