Identification and Characterization of a New Blast Resistance Gene Located on Rice Chromosome 1 Through Linkage and Differential Analyses

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ABSTRACT

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The Chinese native cv. Q14 expresses a high level of resistance to many isolates of *Pyricularia grisea* collected from Japan, Thailand, and China. Q14 was crossed to an *indica*-susceptible cultivar, Q61. To rapidly determine the chromosomal location of the major resistance gene present in the cultivar, a linkage analysis using microsatellite markers was performed in the F₂ population segregating 3R:1S (resistant/susceptible) through bulked-segregant analysis (BSA) in combination with recessive-class analysis (RCA). A total of 189 microsatellite markers selected from each chromosome equally (with ≈ 10 centimorgans) were tested with the BSA approach. Only two markers, RM151 and RM259, located on chro-

mosome 1 showed positive and negative polymorphisms, respectively, for a resistance gene segregating in the population. To confirm the polymorphic markers, a total of 155 viable susceptible individuals were tested with the RCA approach. The markers RM151 and RM259 were found to link to the resistance gene with recombination frequencies of $11.9 \pm 2.8\%$ and $9.7 \pm 8.0\%$, respectively. For further characterization of the resistance gene, 3 resistance genes mapped on chromosome 1, as well as 15 major resistance genes that might be employed in the breeding program, were selected for differential tests with 85 Chinese isolates. The resistance gene identified in this research conveys reactions distinct from those conditioned by the 18 resistance genes. This new resistance gene tentatively was designated *Pi27(t)*.

Additional keyword: Oryza sativa.

Blast, caused by the fungus *Pyricularia grisea* (Cooke) Sacc., is among the most destructive diseases of rice (*Oryza sativa* L.) in most rice-growing areas worldwide (27). Heavy losses occur frequently in both tropical and temperate countries under disease-conducive conditions (5,7,33). Utilization of genetic resistance is the most effective and environmentally friendly strategy for control of the disease.

Recently, certain progress has been made in mapping rice blast resistance genes with molecular markers, which forms the foundation for marker-aided selection (MAS) in resistance breeding program as well as for map-based cloning of the resistance genes. Imbe et al. (13) identified *Pi20* in the rice cv. IR24 on chromosome 12 through restriction fragment length polymorphism (RFLP) analysis using recombinant inbred lines (RILs). Leong et al. (18) identified a resistance gene, *Pi-CO39*, corresponding to avirulence gene *Avr1-CO39*, in cv. CO39 through linkage analysis in a F₂ population from a cross between cvs. CO39 and 51583 with microsatellite and RFLP markers. Zhuang et al. (41) identified two single resistance genes, *Pi24(t)* and *Pi25(t)*, in cvs. Zhong 156 and Gumei 2, respectively, through random amplified polymorphic DNA (RAPD) analysis using RILs. Ahn et al. (1) reported that Korean cv. Suweon 365 carries three major resistance genes, *Pi18*, *Pi21(t)*, and *Pi22(t)*, against Korean isolates KI-313, KJ-101, and KJ-201, respectively, through linkage analysis with RFLP and microsatellite markers in an F₂ population derived from a cross of cvs. Suweon and Chucheongbyeol. Fujii et al. (8) identified a panicle blast resistance gene, *Pb1*, in cv.

Asanohikari on chromosome 11 through RFLP analysis using an F₂ population derived from a cross between the cultivar and Koshikikari. Tabien et al. (35) identified *Pi-tq1*, *Pi-tq5*, *Pi-tq6*, and *Pi-lm2* on chromosomes 11, 6, 2, and 11, respectively, through RFLP analysis using RILs from a cross between cvs. Lemont (lm) and Teqing (tq). Fukuoka and Okuno (9) first identified a recessive field resistance gene, *pi21*, in Japanese upland rice cv. Owarihatamochi on chromosome 4 through quantitative trait loci (QTL) analysis with RFLP markers. Yang et al. (39) identified the resistance gene *Pi25(t)* in cv. Yunxi 2, which transferred resistance from a Yunnan upland cv. Sanbandqishiluo, on the long arm of chromosome 1, through RFLP and microsatellite analyses in an F₂ population from a cross of cvs. Yunxi 2 and 91F14-4. Li et al. (19) identified the first resistance gene, *PiD1(t)*, and Chen et al. (4) the second gene, *PiD2(t)*, in *indica* cv. Digu on chromosome 2 and 6, respectively, through RFLP, microsatellite, and resistance gene analog (RGA) analyses. To add to 27 resistance genes reviewed by Pan et al. (31), more than 43 major blast resistance genes have been identified so far (1,4,8,9,13,18,19,31,35,39,41).

The Yunnan upland rice cv. Q14 shows resistance to all the Japanese blast fungus races and many isolates collected from Thailand and China (Q. H. Pan, *unpublished data*). To effectively use its broad-spectrum resistance in a rice breeding program, it is important to tag the resistance gene or genes present in Q14 with molecular markers. In this research, we rapidly mapped a new resistance gene present in Q14 through bulked-segregant analysis (BSA) (23) coupled with recessive-class analysis (RCA) (40) approaches, and provided further evidence to distinguish the new resistance gene from other resistance genes mapped on the same chromosome, as well as 15 major resistance genes employed in the breeding programs through differential analysis with 85 Chinese isolates.

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MATERIALS AND METHODS

Plant materials. For tagging the resistance gene or genes, the *japonica*-resistant cv. Q14 was crossed to an *indica*-susceptible cv. Q61 (Table 1). F₂ seed were pregerminated by soaking in water at 25°C for 48 h, then sown in a plastic pot (34 files by 15 rows) filled with black organic soil treated with fungicide solution. Each pot was placed in a plastic tray (58 by 38 by 8 cm). To confirm the success of inoculation, the parental cultivars and a susceptible check, cv. Sariceltik, were sown at both ends of the F₂ population in a tray. After gene tagging, the parental cultivars, three cultivars each carrying a single resistance gene on the same chromosome, and 15 cultivars each carrying a single resistance gene were selected for subsequent differential tests of the resistance genes (Table 1). In this case, each cultivar was sown in a row in each tray. Seedlings were grown in a greenhouse at 20 to 35°C for ≈3 weeks before inoculation.

Conidial inoculation and disease scoring. *P. grisea* isolate CHL0335, which showed distinct reactions on the parents, was selected for constructing a mapping population. Eighty-five isolates collected from Guangdong Province, China, in 2002 were selected for subsequent characterization of the resistance gene or genes identified in Q14 with other, related resistance genes. Inoculum was prepared from cultures grown on rice polish agar

medium (20 g of rice polish powder, 2 g of yeast extract, and 11 g of agar per liter of tap water). Two agar scraps colonized by the isolates were placed symmetrically onto the new medium in a 10-cm glass petri plate and incubated at 25°C for ≈10 days, until the surface of the plate was almost covered with mycelial growth. The growth was gently scraped in sterilized distilled water with a metal spatula on a clean bench, and then the plate was placed on the sporulation incubator under dark blue light for 2 to 3 days to facilitate conidiation of the culture. The culture was flooded with distilled water and conidia were released by scraping. The suspension was filtered through gauze mesh, and 0.5% solution of gelatin (DGF STOESS, Germany) was added as a surfactant. The concentration of conidia ranged from 1×10^5 to 5×10^5 conidia ml⁻¹.

Seedlings were transferred into an inoculation incubator at the four- to six-leaf stage and inoculated by spraying 70 to 80 ml of spore suspension per tray. After inoculation, the seedlings were kept in the inoculation incubator at 25°C with saturated humidity for 20 h, then transferred to a moist vinyl tunnel at 25 to 30°C. Disease reactions were scored ≈7 days after inoculation by use of a 5-class scale described by Pan et al. (29).

DNA extraction and amplification. Total DNA was extracted from frozen leaves of rice plants according to Doyle and Doyle (6). Amplification reactions were performed with the GeneAmp

TABLE 1. Differential reactions of 4 rice cultivars carrying resistance genes on chromosome 1 and 15 cultivars carrying the main resistance genes on the other chromosomes, as well as an *indica*-susceptible cv. Q61, to some isolates of *Pyricularia grisea* selected from China

Cultivar ^a	Gene	<i>P. grisea</i> isolate ^b							
		CHL0335	CHL0888	CHL0918	CHL0955	CHL0957	CHL0977	CHL0979	CHL1008
A group									
Q14	<i>Pi27(t)</i>	R	R	R	R	R	R	R	S
Shin 2	<i>Pish</i>	S	S	S	S	S	R	S	S
K 59	<i>Pit</i>	S	S	S	S	S	R	S	R
Sanbangqishiluo	<i>Pi25(t)</i>	ND	S	S	S	R	S	R	R
B group									
Aichi Asahi	<i>Pia</i>	S	S	S	S	S	S	S	S
Fujisaka 5	<i>Pii</i>	S	S	S	S	S	R	S	R
Kusabue	<i>Pik</i>	S	S	S	S	S	R	S	R
Tsuyuake	<i>Pik^m</i>	R	R	R	R	R	R	S	R
K 60	<i>Pik^p</i>	S	R	S	R	S	R	S	S
Fukunishiki	<i>Piz</i>	R	R	S	S	S	R	R	R
Toride 1	<i>Piz^t</i>	R	R	S	S	S	R	R	S
K 1	<i>Pita</i>	S	R	S	S	S	S	R	S
PiNo. 4	<i>Pita²</i>	R	R	S	S	S	R	R	S
BL 1	<i>Pib</i>	S	R	S	S	S	R	R	S
C101LAC	<i>Pi1</i>	R	R	S	R	R	R	R	R
C101A51	<i>Pi2</i>	R	R	R	R	S	S	R	R
C104PKT	<i>Pi3</i>	S	S	S	S	S	S	R	R
C101PKT	<i>Pi4</i>	S	R	S	S	S	S	R	S
75-1-127	<i>Pi9</i>	R	S	S	R	S	R	R	R
Q61	?	S	S	S	S	S	S	S	S

^a A and B groups indicate resistance genes mapped on chromosome 1 and the other chromosomes, respectively.

^b R = resistant, S = susceptible, and ND = not determined.

TABLE 2. Linkage analysis of the resistance gene *Pi27(t)* of *Pyricularia grisea* in a homozygous recessive population consisted of 155 highly susceptible rice plants selected from an F₂ population derived from a cross between a Yunnan native resistant cv. Q14 and an *indica*-susceptible cv. Q61 using microsatellite markers

Gene pair	Linkage phase ^a	Segregation model ^b				Total	χ^2 segregation ^c	$r \pm \text{SE}^d$	Map (cM) ^e
		A-B-	A-bb	aaB-	aabb				
<i>Pi27(t)</i> –RM151	C	NT	NT	37	118	155	213.4***	11.9 ± 2.8	12.1 ± 3.0
<i>Pi27(t)</i> –RM259	R	NT	NT	125	30	155	253.0***	9.7 ± 8.0	9.8 ± 8.3
RM151–RM259	R	25	18	100	18	155	228.9***	27.7 ± 7.4	31.2 ± 12.1
<i>Pi27(t)</i> –OSR3	C	NT	NT	108	47	155	2.1	...	...

^a C = coupling phase and R = repulsion phase.

^b A- or B- = resistant or the marker present; aa or bb = susceptible or the marker absent; NT = not tested.

^c Marker segregation; *** indicates significance at $P = 0.001$ level for χ^2 .

^d Recombination frequency (percent) ± standard error.

^e Distance in centimorgans (cM).

polymerase chain reaction (PCR) System 9700 (PE Applied Biosystems, Foster City, CA). Primers of the microsatellite markers (36) were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The PCR procedures described by Temnykh et al. (36) were followed, with the exception that PCR products were separated on 6.0% polyacrylamide denaturing gels.

Identification of the resistance gene. BSA was performed to identify candidate markers linked to the resistance gene. Two contrasting bulks were prepared, each containing DNA from 25 resistant or susceptible F_2 individuals. Candidate markers were confirmed by the RCA with 155 viable susceptible individuals derived from the F_2 population. RCA was employed for verification because of the reliable reactions (diagnostic symptoms) and high efficiency to locate the gene on its chromosome (21,24,40). By assuming that all 155 susceptible plants were homozygous for the recessive allele at the target gene locus, the recombination frequency (r_{RM}) between a marker and the resistance gene was calculated by the maximum likelihood method (3). Based on the molecular data from the 155 susceptible plants: $r_{RM} = N_{ab}/2N_T$ in case of coupling phase and $r_{RM} = N_{ab}/2N_T$ in case of repulsion phase, in which N_T is the total number of the susceptible individuals tested and N_{ab} and N_{ab} are the numbers of the susceptible individuals with and without a marker, respectively (Table 2). Likewise, the recombination frequencies (r_{MM}) among the markers linked to the resistance gene were estimated by the balance $r_{MM} = N_{AB} + N_{ab}/2N_T$, in which N_{AB} is the number of the susceptible individuals representing markers A and B (Table 2). The variance is given by $V_r = 1/N_T I_r$, in which I_r is supplied according to Allard's Table 8 (3). The recombination frequency was transformed into centimorgans according to the Kosambi function (17).

RESULTS

Development of mapping population. In all, 1,147 F_2 progenies derived from the cross of Q14 with Q61 were inoculated with the isolate CHL0335, which is avirulent to Q14 and virulent to Q61. Segregation of resistant (R) and susceptible (S) progenies fitted a 3:1 ratio in the F_2 population (870R:277S, $\chi^2 = 0.40$, $P > 0.50$), confirming that Q14 carries a single resistance gene to the isolate CHL0335. A population consisting of 25 resistant and 155 viable, susceptible individuals was constructed for subsequent gene mapping using molecular markers, because many susceptible individuals died when they were transplanted into pots.

Molecular mapping of the resistance gene. To determine chromosomal position of the resistance gene, a total of 189 microsatellite markers selected from 12 chromosomes with an intervals of ≈ 10 centimorgans were tested through the BSA approach. The results showed that only two markers, RM151 and RM259, located on chromosome 1 showed positive (R) and negative (S) polymorphisms, respectively, for the resistance gene. To confirm the polymorphic markers, the plants consisting of the R and S gene pools were tested individually. The results showed that both markers cosegregated with the resistance gene (data not shown). For linkage analysis of the resistance gene with these two markers, a total of 155 S individuals, including those consisting of an S pool, were tested. The markers RM151 and RM259 were found to link to the resistance gene with recombination frequencies of $11.9 \pm 2.8\%$ and $9.7 \pm 8.0\%$, respectively (Table 2).

It is known that three other genes for blast resistance (e.g., *Pit*, *Pish*, and *Pi25(t)*) also were located in this region (12,16,39). A comprehensive map of the putative resistance gene with these three resistance genes was constructed based on the data published (Fig. 1). This study clearly suggested that the locus identified in the present study is different from the three loci above-mentioned. We, therefore, tentatively designated this new gene as *Pi27(t)*.

Characterization of the new resistance gene *Pi27(t)*. The resistance gene carrier Q14 showed high resistance to almost all Japanese and Thai isolates (Q. H. Pan, *unpublished data*), and has a broad resistance spectrum to Chinese isolates (a part of data shown in Table 1). To provide further evidence to distinguish the new resistance gene from the previously identified three resistance genes on chromosome 1, the first characterization of the new resistance gene was focused on the three resistance genes mentioned earlier. Four cultivars, including the parental cv. Q14, were subjected to differential tests with 85 isolates. The new resistance gene *Pi27(t)* showed distinct reactions from those conditioned by the three resistance genes (Table 1, A group).

To evaluate the effect of the new gene for blast resistance breeding, a further characterization of the new resistance gene was focused on comparison of the resistance spectrums between the new resistance gene and the main resistance genes that might be employed in the breeding program in China. Fifteen cultivars, each carrying a single resistance gene, were selected for differential tests using the 85 isolates. The results revealed that the new resistance gene conditions a broader and complementary resistance to the 15 important resistance genes (Table 1).

DISCUSSION

Yunnan Province in southwestern China is affluent in rice genetic resources (25,37). Rice cultivars native to this region recently were investigated as a new source of blast resistance (20,22,38; Q. H. Pan *unpublished data*). Preliminary screening showed that many cultivars confer high resistance to many isolates that are virulent to cvs. carrying the *Pik^m*, *Piz*, *Piz^l*, *Pita²*,

Fig. 1. Genetic map of four genes, *Pit*, *Pi27(t)*, *Pish*, and *Pi25(t)*, which confer resistance to blast on rice chromosome 1. The locations of three genes other than *Pi27(t)* identified in the present research were estimated based on the data from a, Kaji et al. (16); b, Akagi et al. (2); c, Imbe and Matsumoto (12); and d, Yang et al. (39). Map distances are in centimorgans (17).

Pib, *Pi2* (*Piz⁵*), and *Pi9* genes (22; Q. H. Pan, *unpublished data*). Based on these studies, at least six new resistance genes, *Pi13* and *Pi14* (30), *Pik⁸* and *Pi15* (32), *Pi25(t)* (39), and the current resistance gene, *Pi27(t)*, have been identified so far.

In order to rapidly identify markers linked to resistance genes, Micheltore et al. (23) developed BSA to identify markers in the target genomic regions. With this method, DNA samples from resistant or susceptible plants from a segregating population are bulked separately. The comparison of two parental cultivars and bulks using molecular markers allows the identification of candidate markers linked to the target genes. However, the candidate markers should be confirmed by the phenotypic analysis of the segregating population. Following this, Zhang et al. (40) proposed a more cost-effective approach, RCA, which is particularly useful for mapping genes controlling expression of a complex trait. In this approach, chromosomal regions containing the target genes are identified through a bulked-extremes survey, and the chromosomal loci of the target genes are determined through linkage analysis using recessive-class individuals. Lin et al. (21) also applied this approach in mapping the chromosomal location of the gene *Xa22*, conditioning resistance for rice bacterial blight disease, which cannot be distinctly separated into two contrasting groups. The recent work from our laboratory, including this study, provides further suggestions that the combination of BSA with RCA approaches may be especially useful for mapping resistance genes (28). First, there is a risk of mis-scoring of inoculation-escaped plants as resistant individuals, even though the reactions controlled by target genes are identified clearly. Second, some susceptible plants which were heavily infected might not survive for subsequent gene mapping. In this study, out of 277 susceptible plants, only 155 plants were viable to take leaves for DNA extractions for linkage analysis using molecular markers.

Resistance genes tend to be clustered together on particular chromosomes (14,15,34). Therefore, it is necessary to illustrate the relative map locations of resistance genes when a new resistance gene is mapped into the cluster. We carefully reviewed other loci of rice blast resistance genes mapped on chromosome 1 and constructed a comprehensive map of rice blast resistance genes located on chromosome 1 (Fig. 1).

The new resistance gene *Pi27(t)* conditions distinct reactions from those conditioned by the known resistance gene located on chromosome 1. Moreover, *Pi27(t)* also conditions clear resistance against isolates that are virulent to the main resistance genes employed in the current breeding program in Guangdong Province, indicating that *Pi27(t)* might be a new resistance source for the resistance breeding program. As an application to the breeding program through an MAS approach (10,11,26), we are undertaking to finely map the resistance gene with other molecular markers, such as sequence-tagged site (STS) and cleaved amplified polymorphic sequence (CAPS) markers.

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