Identification of a New Blast Resistance Gene in the *indica* Rice Cultivar Kasalath Using Japanese Differential Cultivars and Isozyme Markers

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ABSTRACT

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Rice blast is one of the most destructive diseases of rice. This study was undertaken to identify the rice blast resistance gene(s) in the *indica* rice cultivar Kasalath. 'Kasalath' was crossed with 9 of 12 Japanese differential cultivars, each carrying a single resistance gene at one of seven known loci. Allelism tests were performed in the F_2 populations with rice blast races. The resistance of 'Kasalath' was controlled by two dominant genes at different loci. The two resistance genes were nonallelic to the nine known resistance genes at six loci, *Pi-a*, *Pi-i*, *Pi-k*, *Pi-ta*, *Pi-b*, and *Pi-t*, and one of the genes was an allele of the *Pi-z* locus. For the gene detected at the *Pi-z* locus, an allelism test was performed using race 433.5, which is virulent to *Pi-z*^{t} but avirulent to *Pi-z*. The results indicated that 'Kasalath' has the *Pi-z*^{t} gene. To determine the location of the other gene, a linkage test using isozyme markers was performed with an F_2 population segregating 3:1 (resistant/susceptible) when tested against race 477.1, which is virulent to *Pi-z*^{t}. This gene was linked to *Amp-3* (leucine aminopeptidase, EC 3.4.11.1) and *Pgi-2* (phosphoglucose isomerase, EC 5.3.1.9) genes on chromosome 6 with recombination values of 37.6% ± 3.0% and 27.3% ± 2.7%, respectively. This gene was, therefore, designated *Pi8*.

Additional keywords: avirulent race, *japonica*, *Oryza sativa*, *Pyricularia oryzae*, virulent race.

Rice blast, caused by *Pyricularia grisea* (Cooke) Sacc. (= *P. oryzae* Cavara) is one of the most devastating diseases of rice (*Oryza sativa* L.). Breeding blast resistant cultivars is considered the most desirable means to control blast. To effectively breed for blast resistance, genetic information about resistance genes is needed.

Systematic genetic studies on blast resistance in rice have been conducted in Japan. Kiyosawa and his colleagues identified a total of 14 resistance genes at eight loci: *Pi-a*, *Pi-i*, *Pi-k* (alleles: *Pi-k*, *Pi-k*^{s}, *Pi-k*^{m}, *Pi-k*^{h}, and *Pi-k*^{P}), *Pi-z* (*Pi-z* and *Pi-z*^{t}), *Pi-ta* (*Pi-ta* and *Pi-ta*^{2}), *Pi-b*, *Pi-t*, and *Pi-sh* (10). Among them, *Pi-a*, *Pi-i*, *Pi-k*^{s}, and *Pi-sh* were found in native Japanese cultivars; *Pi-k*, *Pi-k*^{m}, and *Pi-z* were found in Japanese cultivars that had blast resistance introduced from Chinese or American cultivars; and *Pi-k*^{h}, *Pi-z*^{t}, *Pi-ta*, *Pi-b*, and *Pi-t* were found in Japanese breeding lines that had blast resistance introduced from *indica* cultivars. Based on this work, Kiyosawa developed the term Japanese differential cultivars (JDCs); each JDC carrying a single blast resistance gene (10).

Recently, at the International Rice Research Institute (IRRI) in the Philippines, a set of near-isogenic lines (NILs) for blast resistance genes was developed (13). Through allelism tests using the IRRI NILs and JDCs, Inukai et al. (5) identified four resistance genes, *Pi-1*, *Pi-2*(t) (allelic or closely linked to *Pi-z*), *Pi-3*, and *Pi-4a*(t) (allelic or closely linked to *Pi-ta*). Yu et al. (19) identified *Pi-2*(t) and *Pi-4* genes on chromosomes 6 and 12, respectively, through restriction fragment length polymorphism (RFLP) analysis using two of six groups of the IRRI NILs; Wang et al. (16) identified *Pi-5*(t) on chromosome 4 and *Pi-7*(t) on chromosome 11 through RFLP analysis using recombinant inbred (RI) lines; and Zhu et al. (20) identified *Pi-z*^{h}(t) on chromosome 8 through random amplified polymorphic DNA (RAPD) analysis using doubled-haploid lines. Consequently, a total of 19 rice blast resistance genes have been identified.

The *indica* cultivar Kasalath is resistant to many Japanese blast fungus races and exhibits genetic polymorphism in many respects when contrasted to *japonica* cultivars. In Japan, 'Kasalath' has been extensively used as a model cultivar in the molecular genetics of rice; therefore, much genetic information about this cultivar has been acquired (12). To advance our knowledge of the molecular genetics of blast resistance, it is important to elucidate the blast resistance genes present in 'Kasalath'. In this study, two blast resistance genes in 'Kasalath' were analyzed through allelism tests with the JDCs, and a new resistance gene was identified through linkage tests with isozyme marker genes.

MATERIALS AND METHODS

Plant materials. Nine of the 12 JDCs were used in this study (Table 1). 'Kasalath' was crossed to these nine JDCs in a greenhouse in 1992 and 1993. The F_1 plants were grown in a paddy field to produce F_2 seeds in 1994. Seeds were pregerminated by soaking in water at 25°C for 48 h and were sown in a paper pot (36 files × 18 rows) filled with the granulated soil used in the commercial production of rice seedlings. Each paper pot was placed in a plastic tray (60 × 30 × 3 cm). To confirm the success of inoculation and the pathogenicity of tested races, 'Kasalath', the 12 JDCs, and a susceptible check cultivar Lijiangsintuanheigu were sown at both ends of each F_2 population in a tray. Seedlings

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were grown in a greenhouse at 20 to 35°C for about 3 weeks before inoculation.

Fungus races and inoculum production. Five *P. grisea* races, 007.0, 031.1, 031.5, 433.5, and 477.1, were used (Table 1). Races 007.0 and 031.1 are Japanese differential fungus strain races that were used with the other five differential races in Japan to identify the 14 known resistance genes. Races 031.5, 433.5, and 477.1 were used to distinguish *Pi-z*, *Pi-z*^{t}, and *Pi-t* genes, because these differential fungus races are avirulent to these three genes (10).

Inoculum was multiplied on a wheat seed medium (14). Wheat seeds were boiled until the seed coats broke open, and then they were rinsed with tap water. The seeds were put into a flask and autoclaved at 121°C for 40 min. An agar scrap from the maintenance culture medium (14) was seeded into the cooled wheat seed medium in a flask and incubated at 25 to 28°C for about 2 weeks until the surfaces of the seeds were entirely covered with aerial mycelia. The colonized seeds were put into a beaker and vigorously washed with sterile, distilled water. The seeds were then strained, wrapped with a sheet of sterile paper, and placed in a sterile plastic tray (23 × 32 × 5 cm). The seeds were incubated at 25 to 28°C in darkness for 18 to 24 h, and then transferred into an incubator at 10°C to maintain spore viability. Before inoculation, the seeds were put into a beaker with sterile, distilled water and stirred with a glass rod to release the conidia into the water. The concentration was adjusted to 10 to 50 × 10^4 conidia per milliliter, and five drops of Tween 20 or 80 per 100 ml were added to the suspension.

Inoculation and disease evaluation. At the four- to six-leaf stage, seedlings were transferred into an inoculation incubator with a temperature controller and humidifier and inoculated by spraying 50 to 70 ml of spore suspension per tray. After inoculation, the seedlings were kept in the inoculation incubator at 25°C and more than 95% relative humidity for 24 to 36 h, and then transferred to a moist vinyl tunnel at 25 to 35°C.

Disease reactions were scored about 7 days after inoculation, when typical lesions appeared on the leaves of susceptible plants. The scoring system of Kiyosawa and Ando (10) with slight modifications was used to rate each seedling: 0 = no lesions; 1 = small brown spot of pinhead size; 2 = brown spot with a large axis shorter than 2 mm; 3 = brown spot with a large axis longer than 2 mm; 4 = uncolored or purple spot with a large axis shorter than 2 mm; 5 = uncolored or purple spot with a large axis 2 mm or longer. For data analysis, the seedlings of classes 0 to 3 were regarded as resistant, and those of classes 4 and 5 were regarded as susceptible.

Isozyme assay. The inner tillers of plants were used for enzyme extraction with extraction buffer (1 M sucrose in 0.2 M Tris-HCl buffer, supplemented with 50 µl of 2-mercaptoethanol and five drops of Tween 20 or 80 per 10 ml before use). Electrophoresis was performed in a horizontal starch gel using a modified system (electrode buffer: 0.4 M Tris, adjusted to pH 7.8 with citric acid in deionized water; gel buffer: 0.005 M histidine-HCl, adjusted to pH 7.8 with Tris in distilled water and diluted 20 times) at 0 to 2°C with a constant current of 50 mA for 3 to 3.5 h according to the method of Glaszmann et al. (2). The following six enzymes were examined: leucine aminopeptidase (EC 3.4.11.1), phosphoglucose isomerase (EC 5.3.1.9), catalase (EC 1.11.1.6), esterase (EC 3.1.1.1), peroxidase (EC 1.11.1.7), and shikimate dehydrogenase (EC 1.1.1.25). Staining procedures were adopted from Ishikawa (7).

Data analysis. The number of resistance genes in 'Kasalath' was estimated from the F_2 populations inoculated with the races virulent to the JDCs and avirulent to 'Kasalath'. Allelism of the resistance gene(s) in 'Kasalath' to the known resistance genes in the JDCs was then tested in F_2 populations inoculated with races avirulent to both parents. Isozyme markers were used for linkage analysis of resistance genes in F_2 population(s) segregating 3:1 (resistance/susceptible). The recombination value was calculated by the maximum likelihood method (1,4).

RESULTS

Allelism test. When the races virulent to the JDCs and avirulent to 'Kasalath' were used, all the F_1 plants showed resistance, which indicated that the resistance of 'Kasalath' might be controlled by one or more dominant genes (data not shown). For F_2 segregation analysis, 26 experiments were performed with the nine F_2 populations using five races of *P. grisea* (Table 2). To estimate the number of resistance genes in 'Kasalath', the F_2 populations from the crosses of 'Kasalath' with 'Shin 2', 'Aichiasahi', and 'K 59' were inoculated with races 007.0 and 031.5, which are virulent to the JDCs and avirulent to 'Kasalath'. The segregation ratio of 15:1 was observed in each experiment with these F_2 populations (experiments 1, 5, 6, and 25), indicating that 'Kasalath' has two resistance genes at different loci. The two resistance genes were tentatively designated G1 and G2. Likewise, in the F_2 populations inoculated with races 433.5 and 477.1 (avirulent to *Pi-i*, *Pi-z*, *Pi-ta*, and *Pi-t*), the segregation ratio of 15:1 was observed in each experiment (experiments 10, 18, 21, and 26). These results indicated that one of the two genes in 'Kasalath' is susceptible to races virulent to *Pi-z*^{t} (Table 1), and we arbitrarily designated this gene as G1. To examine the allelic relationships of the two resistance genes in 'Kasalath' with known resistance genes at the six loci other than the *Pi-z* locus, races

TABLE 1. Reaction pattern of *indica* rice cultivar Kasalath and Japanese differential cultivars to two Japanese differential races and three additional races of *Pyricularia grisea*

Cultivar	Resistance gene	Cultivar code	*P. grisea* strain (race number)[a]				
			Ina72 (031.1)	Hoku1 (007.0)	TH67-22 (031.5)	Ai75-61 (433.5)	Ai74-134 (477.1)
Shin 2	*Pi-k*^{s}	1	S[b]	S	S	S	S
Aichiasahi	*Pi-a*	2	R	S	R	S	S
Fujisaka 5	*Pi-i*	4	R	S	R	R	S
Kusabue[c]	*Pi-k*	10	S	R	S	S	S
Tsuyuake	*Pi-k*^{m}	20	S	R	S	S	S
Fukunishiki	*Pi-z*	40	R	R	R	R	S
K 1	*Pi-ta*	100	R	R	R	R	R
PiNo.4[c]	*Pi-ta*^{2}	200	R	R	R	R	R
Toride 1[c]	*Pi-z*^{t}	400	R	R	R	S	S
K 60	*Pi-k*^{p}	.1	S	R	S	S	S
BL 1	*Pi-b*	.2	R	R	R	R	R
K 59	*Pi-t*	.4	R	R	S	S	R
Kasalath	?		R	R	R	R	R

[a] The race number of each fungus strain is the sum of the cultivar codes for cultivars susceptible to the race.
[b] S = susceptible; R = resistant.
[c] Not used in this study.

avirulent to both parents were used. The F_2 segregation ratio of 63:1 was obtained in each experiment (experiments 2–4, 9, 13, 14, 19, 20, and 22–24), indicating that both G1 and G2 are located at loci other than *Pi-a*, *Pi-i*, *Pi-k*, *Pi-ta*, *Pi-b*, and *Pi-t*.

For the *Pi-z* locus, the F_2 population from the cross between 'Fukunishiki' carrying *Pi-z* and 'Kasalath' was inoculated with races 007.0 and 031.5 (avirulent to both parents). No susceptible plants were observed in any of the experiments using this population (experiments 15–17). These results supported the hypothesis that G1 is an allele of the *Pi-z* locus. Since there are two alleles, *Pi-z* and *Pi-z*^{t} at the *Pi-z* locus (Table 1), the F_2 population of the cross 'Fukunishiki' × 'Kasalath' was inoculated with race 433.5, avirulent to *Pi-z* but virulent to *Pi-z*^{t}, to ascertain if G1 is *Pi-z* or *Pi-z*^{t}. The observed segregation ratio was 15:1 (experiment 18), indicating that G1 is *Pi-z*^{t} and not *Pi-z*. Results from the crosses 'Aichiasahi' × 'Kasalath' and 'Fujisaka' × 'Kasalath' also sup-

TABLE 2. Reactions to five races of *Pyricularia grisea* of F_2 populations from crosses between rice cultivar Kasalath and nine Japanese differential cultivars

Experiment no.	Differential cultivar[a]	Gene locus	Test race	Reaction of gene[b]			Reaction of F_2 plants			Expected ratio	*P* value[c]
				Known	G1	G2	R	S	Total		
1	Shin 2	*Pi-k*	007.0	S	R	R	200	11	211	15:1	0.90–0.80
2	Aichiasahi	*Pi-a*	031.5	R	R	R	314	1	315	63:1	0.20–0.10
3	Aichiasahi	*Pi-a*	031.5	R	R	R	332	2	334	63:1	0.30–0.20
4	Aichiasahi	*Pi-a*	031.1	R	R	R	364	7	371	63:1	0.80–0.70
5	Aichiasahi	*Pi-a*	007.0	S	R	R	312	25	337	15:1	0.50–0.40
6	Aichiasahi	*Pi-a*	007.0	S	R	R	170	11	181	15:1	>0.95
7	Aichiasahi	*Pi-a*	433.5	S	S	R	222	85	307	3:1	0.40–0.30
8	Aichiasahi	*Pi-a*	477.1	S	S	R	269	99	368	3:1	0.50–0.40
9	Fujisaka 5	*Pi-i*	031.5	R	R	R	355	9	364	63:1	0.30–0.20
10	Fujisaka 5	*Pi-i*	433.5	R	S	R	366	24	390	15:1	>0.95
11	Fujisaka 5	*Pi-i*	477.1	S	S	R	267	80	347	3:1	0.50–0.40
12	Fujisaka 5	*Pi-i*	477.1	S	S	R	270	103	373	3:1	0.80–0.70
13	Tsuyuake	*Pi-k*	007.0	R	R	R	260	4	264	63:1	0.90–0.80
14	K 60	*Pi-k*	007.0	R	R	R	212	4	216	63:1	0.90–0.80
15	Fukunishiki	*Pi-z*	007.0	R	R	R	275	0	275	1:0	
16	Fukunishiki	*Pi-z*	007.0	R	R	R	364	0	364	1:0	
17	Fukunishiki	*Pi-z*	031.5	R	R	R	265	0	265	1:0	
18	Fukunishiki	*Pi-z*	433.5	R	S	R	306	19	325	15:1	0.90–0.80
19	K 1	*Pi-ta*	007.0	R	R	R	332	8	340	63:1	0.40–0.30
20	K 1	*Pi-ta*	031.5	R	R	R	347	9	356	63:1	0.30–0.20
21	K 1	*Pi-ta*	477.1	R	S	R	367	28	396	15:1	0.60–0.50
22	BL 1	*Pi-b*	007.0	R	R	R	327	4	331	63:1	0.80–0.70
23	BL 1	*Pi-b*	007.1	R	R	R	198	5	203	63:1	0.50–0.40
24	K 59	*Pi-t*	031.1	R	R	R	399	5	404	63:1	0.80–0.70
25	K 59	*Pi-t*	031.5	S	R	R	373	21	394	15:1	0.60–0.50
26	K 59	*Pi-t*	477.1	R	S	R	358	20	378	15:1	0.60–0.50

[a] Japanese differential cultivars were used as the female parent in each cross.
[b] Known, G1, and G2 represent a known resistance gene in each Japanese differential cultivar and two tentative resistance genes in 'Kasalath', respectively; R = resistant; S = susceptible.
[c] Probabilities of chi-square test for the resistant/susceptible ratios.

TABLE 3. Linkage analysis of a rice blast resistance gene with isozyme markers in the F_2 population of a cross between rice cultivars Aichiasahi and Kasalath using race 477.1 of *Pyricularia grisea*

	Loci		Locus	Locus B[b]					χ^{2c}			Recombination value (%)
	A[a]	B[a]	A[b]	BB	B–	Bb	b–	bb	Locus A	Locus B	Linkage	
Chromosome 2	G2	*Amp-1*	A–	78		121		64	0.8	2	1.6	
			aa	23		48		27				
Chromosome 6	G2	*Amp-3*	A–	106		123		35	0.8	20.8***[d]	21.2***	37.6 ± 3.0
			aa	17		54		27				
	G2	*Pgi-2*	A–	102		134		27	0.8	9.5**	58.0***	27.3 ± 2.7
			aa	7		50		41				
	Amp-3	*Pgi-2*	A–	76		44		3	20.8***	9.5**	187.8***	19.6 ± 1.7
			A–	30		122		24				
			aa	3		18		41				
	G2	*Cat-1*	A–	72		139		52	0.8	6.5*	0.7	
			aa	30		50		18				
Chromosome 7	G2	*Est-9*	A–	84		119		60	0.8	4.2	2.8	
			aa	23		48		27				
Chromosome 12	G2	*Pox-2*	A–		217		46		0.8	13.5***	0.8	
			aa		84		14					
	G2	*Sdh-1*	A–	73		138		52	0.8	8.6*	8.2*	
			aa	16		66		16				

[a] A and B represent a tentative resistance gene G2 of 'Kasalath', or isozyme genes.
[b] A–, B–, and b– are used when the heterogeneous genotypes cannot be identified because of segregation of a dominant allele.
[c] χ^2_A, χ^2_B, and χ^2_L represent the calculated chi-square values for loci A, B, and linkage, respectively.
[d] *, **, and *** represent significance at $P = 0.05$, $P = 0.01$, and $P = 0.001$ levels, respectively, for a χ^2.

ported this conclusion. When these F_2 populations were inoculated with a race avirulent to 'Kasalath' and virulent to the other parents and to the *Pi-z*^{t} gene, the segregation ratio of 3:1 was observed in each experiment (experiments 7, 8, 11, and 12).

Linkage tests. The allelism tests revealed that 'Kasalath' carries two resistance genes, *Pi-z*^{t} and an unknown resistance gene. To determine the location of the newly detected resistance gene, the F_2 population exhibiting a segregation ratio of 3:1 using a race virulent to the *Pi-z*^{t} gene (experiment 8) was selected. The F_2 seedlings were transplanted into a field after evaluating the disease response, and then fresh tillers from each plant were sampled for isozyme analysis at the maximum tillering stage of plant development. A total of 361 viable F_2 plants were tested. The newly detected resistance gene G2 was linked to *Amp-3* and *Pgi-2* on chromosome 6 (Table 3). The recombination values between G2 and *Amp-3* loci, G2 and *Pgi-2* loci, and *Amp-3* and *Pgi-2* loci were 37.6% ± 3.0%, 27.3% ± 2.7%, and 19.6% ± 1.7%, respectively. In conformity with the new rules of gene designation for rice blast resistance (8), the newly detected blast resistance gene G2 was designated as *Pi8*. The linkage map of the two resistance genes *Pi-z*^{t} and *Pi8* of 'Kasalath' is shown in Figure 1, in which the location of the *Pi-z*^{t} gene was inferred according to the latest rice linkage map (17).

DISCUSSION

The present study clarifies that 'Kasalath' possesses two blast resistance genes: one is a known resistance gene, *Pi-z*^{t}, and the other is a new resistance gene, *Pi8*. Compared with *japonica* cultivars, *indica* cultivars are genetically complex for blast resistance (9–11,13,15,18). As suggested in this study, the use of JDCs and marker genes will make it possible to identify the resistance genes in *indica* cultivars without using breeding lines, NILs, and RI lines. The present study also suggests that JDCs are quite helpful in identifying the resistance gene(s) not only in *japonica* but also in *indica* cultivars, if a more diverse set of pathogen races can be used (5). An ideal set of differential cultivars should carry not only single resistance genes, but also many marker genes that breeders can easily use, such as morphological and isozyme marker genes. Thus, we plan to introduce marker genes from the *indica* cultivars Kasalath and IR36 into JDCs and the Chinese cultivar Lijiangsintuanheigu, which is susceptible to many races of the blast pathogen.

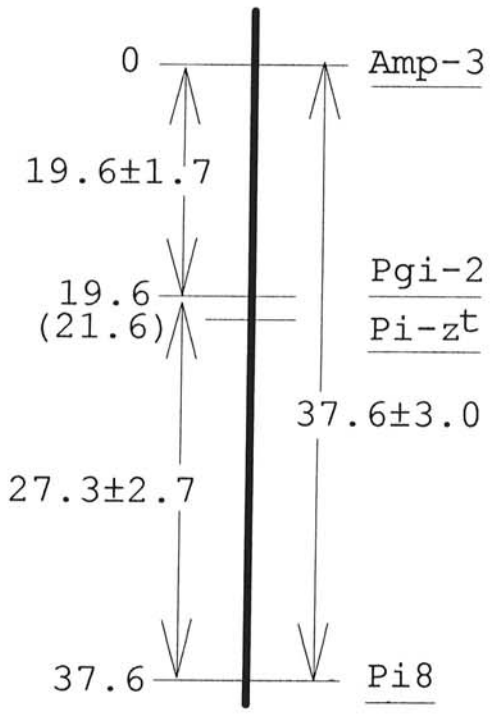

Fig. 1. Linkage map of two rice blast resistance genes, *Pi-z*^{t} and *Pi8* of 'Kasalath' on chromosome 6. *Pi-z*^{t} was identified by allelism tests using Japanese differential cultivars (Table 2), while *Pi8* was identified by an F_2 ('Aichiasahi' × 'Kasalath') linkage test using isozyme markers and a race virulent to *Pi-z*^{t} (Table 3). The map distance in parenthesis was determined by the latest rice linkage map (17). Genetic distances are in recombination values.

From the results in this study, we infer that the new resistance gene *Pi8* confers intermediate resistance to the highly virulent Japanese races 433.5 and 477.1. The disease reaction of a resistance gene is dependent on not only the genetic background of cultivars, but also test races, inoculation and evaluation methods, and environment (5,10,13,18). The difficulty of identifying resistance genes in *indica* cultivars might be because many *indica* cultivars carry several major, as well as minor, genes (10,15,18). Although the existence of minor genes makes it difficult to distinctly divide the segregating population into resistance or susceptible groups, this difficulty can be overcome by using appropriate test races and inoculation and evaluation methods.

Based on the latest rice linkage map (17), *Pi8* should be linked to *Pi-i* at about 10 centimorgans on chromosome 6. However, the data obtained in the F_2 population from the cross between 'Fujisaka 5' (*Pi-i*) and 'Kasalath' (*Pi8* and *Pi-z*^{t}) showed that these two genes are inherited independently of each other (experiments 9 and 10). As for the *Pi-i* locus, Goto et al. (3) pointed out that *Pi-i* is not located on chromosome 6, and Iseu (6) found that *Pi-i* is not linked to either *Pi-z* or the photoperiod sensitivity gene *Se-1*^{u} (*Lm*^{u}) on chromosome 6, but linked to a pentachlorobenzyl alcohol sensitive gene, *pcs* (possibly on chromosome 7). The results in the present study support this conclusion.

Compared with developing breeding lines, NILs, and RI lines to identify resistance genes in the cultivars with complex resistance (5,10,16,19), the method used in this study could save a great deal of time and labor. However, for the final goal of cloning a new resistance gene, it is necessary to isolate the new resistance gene from 'Kasalath' and to detect some molecular marker genes tightly linked to it. For this purpose, we selected resistant F_2 plants for further study.

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