Identification of *Magnaporthe oryzae* Avirulence Genes to Three Rice Blast Resistance Genes

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ABSTRACT

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The segregation of avirulence/virulence was studied in 115 F₁ progeny isolates of *Magnaporthe oryzae* from a cross of two field isolates on three Japanese race-differential rice cultivars Kanto 51, Fukunishiki, and Toride 1. The χ^2 tests of goodness-of-fit for a 1:1 ratio indicated that avirulence on cvs. Kanto 51, Fukunishiki, and Toride 1 was under monogenic control. The relationship between the avirulence (*Avr*) gene in the parental isolate and the *Avr* gene in the standard isolate was investigated by using 100 lines each of three F₃ families from the crosses of the rice cultivars Norin 3/Kanto 51, AK61/Fukunishiki, and Norin 3/Toride 1, respectively. Based on the resistant reactions of the F₃ rice lines to the parental isolates and the standard isolates harboring three known *Avr* genes, three genetically independent *Avr* genes, *AvrPik*, *AvrPiz*, and *AvrPiz-t*, were identified. The three identified *Avr* genes were mapped using random amplified polymorphic DNA (RAPD) analysis, and a partial linkage map was constructed with 17 RAPD markers closely linked to the *Avr* genes. Twelve markers and *AvrPik*, three markers and *AvrPiz*, and two markers and *AvrPiz-t*, as well as mating locus *MAT1*, constructed linkage groups A, B, and C, respectively.

Additional keywords: *Pyricularia oryzae*, rice blast fungus

Rice (*Oryza sativa* L.) is a major staple crop, particularly in Asia. The most disastrous and widespread disease of rice is blast caused by the ascomycetous fungus *Magnaporthe oryzae* B. Couch (anamorph = *Pyricularia oryzae* Cavara) [previously known as *Magnaporthe grisea* (Hebert) Barr] (1). One of the most effective means of controlling rice blast disease is the development and use of resistant rice cultivars; however, newly developed resistant cultivars often lose their resistance after a few years of commercial production. This suggests that a new approach is needed to maintain resistance to blast. A gene-for-gene relationship (4,5) has been proposed for cultivar specificity in the *O. sativa* and *M. oryzae* pathosystem, which is controlled by an interaction between a dominant major resistance (*R*) gene(s) in rice and its corresponding dominant major

avirulence (*Avr*) gene(s) in *M. oryzae* (11,15,22). Breakdown of resistance in cultivars is caused by failure of either the *Avr* gene or *R* gene to function properly. The breakdown of resistance in rice cultivars to blast is probably due to genetic instability of *Avr* genes. Identifying a genetic mechanism that governs the instability of *Avr* genes corresponding to the *R* gene would be indispensable for developing rice cultivars with lasting resistance.

Mendelian and molecular genetic analyses for the *Avr* genes have provided valuable information on the genetic nature of cultivar specificity of this pathogen (14,16,21,25). Kiyosawa (14,16) first reported a marked difference in mutation frequency of the rice blast fungus depending on not only the *Avr* genes used, but also the isolates possessing the same *Avr* gene. A difference was also confirmed in the mutation frequency of *Avr* gene *PWL2* (25). Orbach et al. (21) were successful in cloning *AVR-Pita* and its corresponding loci in spontaneous *avr-pita*⁻ mutants. Sequence analysis revealed that virulent loci (*avr-pita*⁻) suffered point mutations, insertions, or deletions, and these variations permitted the fungus to avoid triggering resistance responses mediated by *Pi-ta*

(21). They pointed out the possibility that the instability of *AVR-Pita* was an effect of its telomeric location. Except for *AVR-Pita*, almost all *Avr* genes analyzed were host species specific or cultivar specific, but corresponding *R* genes were not identified (2,3,12,20,25,26). Therefore, to determine the mechanism of genetic instability in cultivar specificity, it is necessary to describe more *Avr* genes, especially the genes corresponding to *R* genes that have already been identified and widely used in commercial rice cultivars. In Japan, the race-differential rice cultivars were genetically characterized, and nine rice blast *R* genes were identified in the differentials, based on host reactions to standard rice blast isolates established by Kiyosawa (15). Using combinations of the differential cultivars and standard isolates of *M. oryzae* would be a useful tool for genetic analyses of the *Avr* genes corresponding to the *R* genes in rice cultivars.

To identify the *Avr* genes that correspond to the specific *R* genes *Pik*, *Piz*, and *Piz-t*, we determined the mode of inheritance of avirulence on three race-differential rice cultivars through a genetic cross of rice blast isolates and then identified the *Avr* genes based on the reaction of F₃ families of rice to both the parental isolates and standard isolates harboring known *Avr* genes. We also constructed a partial linkage map containing the *Avr* genes using random amplified polymorphic DNA (RAPD) analysis.

MATERIALS AND METHODS

Rice plants. Rice cultivars used in this study are shown in Table 1. Three cultivars, Kanto 51, Fukunishiki, and Toride 1, were used as *R* gene donors in developing F₃ populations, and to evaluate the *Avr* gene in isolates of *M. oryzae* used. Three F₃ families of rice were generated for genetic studies of the *Avr* gene in the pathogen. In the Norin 3/Kanto 51 and Norin 3/Toride 1 families, the blast resistant cultivars Kanto 51 and Toride 1 were used as the pollen parents in a cross with the susceptible cultivar Norin 3 at the National Agricultural Research Center. Randomly selected F₁ seeds were grown in a field and self-pollinated to obtain F₂ seeds. The F₂

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seeds were randomly chosen and planted in a field. The F₃ seeds were harvested from each individual plant and established as F₃ lines, i.e., self-pollinated descendants from single F₂ seeds. Similarly, the AK61/Fukunishiki family was produced at the Aichi-ken Agriculture Research Center and Saga University. Each of the three families consisted of 100 F₃ lines, respectively. The rice cultivars Norin 3 and AK61, which have no *R* genes to *M. oryzae*, were used as the susceptible parents, and cv. Norin 3 was also used as a susceptible control in each inoculation test (Table 1).

Seeds were germinated prior to planting to ensure uniform emergence and facilitate disease ratings. In the genetic analysis of avirulence in the parental and progeny isolates of *M. oryzae*, seven plants of each rice cultivar were grown in a greenhouse in seedling boxes (5 × 15 × 10 cm). For *Avr* gene identification, 20 seedlings per rice line were grown in the seedling boxes.

Pathogen isolates. A total of 115 progeny isolates of *M. oryzae* were produced from a cross of two field isolates as previously described (19). Parental isolate 84R-62B (*MAT1-1*) of the rice blast fungus was isolated in 1984 in Aichi prefecture of Japan. The other isolate, Y93-245c-2 (*MAT1-2*), was isolated in 1993 in the Yunnan province of China. Three standard isolates, P-2b, Ken 54-20, and Ina 168, which were used by Kiyosawa (15) for determining the blast *R* genes in rice cultivars, were obtained from the National Agricultural Research Center for Kyushu Okinawa Region (Table 2).

Inoculation and evaluation. Isolates of *M. oryzae* were grown on oatmeal medium (1 liter of distilled water, 50 g of ground oats, 20 g of sugar, and 20 g of agar). After 14 days at 26 to 28°C, the surface of the oatmeal agar culture in petri dishes was rubbed with a sterile wet paintbrush to

remove aerial mycelia, then exposed to fluorescent light for 3 days at 25°C to induce sporulation. The cultures were flooded with distilled water containing 0.01% Tween 20, and conidia were dislodged with a wet paintbrush. The conidial suspension was poured through Kimwipe paper (Kimberly-Clark Corporation, Tokyo, Japan) and adjusted to approximately 2.0 to 5.0 × 10⁵ spores per ml. Seedlings at the four-leaf stage in 15 seedling boxes were sprayed with 100 ml of the spore suspension. Inoculated plants were incubated in a moist chamber for 18 to 20 h at 25 to 27°C and then returned to the greenhouse. The reactions of the rice cultivars were recorded 7 days after inoculation. Inoculation tests were repeated two or three times. The disease reaction scale used was as follows: 0 = no lesions; 1 = brown pinpoint lesions; 2 = less than 2 mm brown lesions, sometimes with gray centers; 3 = circular lesions with gray centers, lesion size variable; 4 = necrotic eyespot lesions with gray centers. Plants with reaction types 0, 1, and 2 were considered resistant, whereas reaction types 3 and 4 were considered susceptible.

Identification and nomenclature of *Avr* genes. One set of an F₃ family of rice (100 lines) was inoculated with the parental isolate of *M. oryzae*, which showed an avirulent reaction to a given cultivar, and another set of the same F₃ family (100 lines) was inoculated with corresponding standard isolate, which was selected by Kiyosawa (15) to classify Japanese rice cultivars on the basis of blast resistance. This strategy allowed the investigation of relationships between *Avr* genes in the parental isolate of *M. oryzae* and the *Avr* genes in the standard isolate. Based on the reactions of seedlings in each line, the plants of the individual F₃ lines were rated as either all resistant, showing segregation

for resistance, or all susceptible. The term avirulence was applied to an interaction that was demonstrated to be cultivar specific. An *Avr* gene, for which an allele determines avirulence on a specific rice cultivar, was named *Avr* with a symbol referring to the particular *R* gene of the cultivar on which the gene was effective (e.g., *AvrPik*).

Determination of mating type. Mating type was determined by pairing each ascospore isolate with the hermaphroditic testers. The standard tester isolates of *MAT1-1* and *MAT1-2* were Y93-164a-1 and SA98-4, respectively. Crosses were made by placing a mycelial plug of each isolate close together on oatmeal agar medium without sugar in petri dishes. The plates were incubated at 22°C with continuous illumination. After 30 days of incubation, the mating type of each isolate was determined (10,28).

DNA extraction. Each fungal isolate was grown in 50 ml of potato sucrose broth (200 ml of broth from 200 g of potato, 20 g of sucrose per liter) for 4 to 5 days at 25°C. Crude DNA of each isolate was extracted from resulting mycelia by the method of Hamer and Givan (6), and further purification was conducted by the following procedure. The crude DNA pellet was dissolved in 0.5 ml of TE buffer (10 mM Tris-acetate, 1 mM EDTA, pH 7.5) containing 20 ng of RNase A (Nippon Gene, Toyama, Japan) and then incubated at 37°C for 1 h. The solution was extracted once with phenol/chloroform (1:1, vol/vol) and twice with chloroform/isoamyl alcohol (24:1, vol/vol). The supernatant was mixed with 80 µl of 4 M NaCl and 70 µl of CTAB/NaCl (1.5%, 1.05 M), and then incubated at 65°C for 10 min. Further extraction was conducted twice with chloroform/isoamyl alcohol, and the DNA pellet was obtained by isopropanol precipitation. After washing with 70% ethanol, the pellet was dried and resuspended in TE buffer (pH 7.5).

RAPD analysis and mapping. A total of 780 primers (Kit A to Z and Kit AA to AM, Operon Technologies Inc., Alameda, CA) was used in RAPD analysis. All of the primers were 10-mer primers and were used as the primer combinations in each reaction. PCR was performed in a 20-µl reaction containing 0.5 units of Ex. *Taq* DNA polymerase (TaKaRa, Ohtsu, Japan), 1 × PCR buffer provided by the manufacturer, 125 µM of each dNTP, 0.5 µM of

Table 1. Reactions of rice cultivars to parental isolates of *Magnaporthe oryzae*

Cultivar	Resistance genotype ^a	Parental isolate of <i>M. oryzae</i> ^b	
		84R-62B	Y93-245c-2
Kanto 51	<i>Pik</i> , <i>Pi19(t)</i> *	Avr	Vir
Fukunishiki	<i>Piz</i> , <i>Pish</i> *, <i>Pi19(t)</i> *	Vir	Avr
Toride 1	<i>Piz-t</i> , <i>Pish</i> *, <i>Pi19(t)</i> *	Vir	Avr
Norin 3	+	Vir	Vir
AK61	+	Vir	Vir

^a Resistance genotypes according to Hayashi et al. (8), Imbe and Matsumoto (9), and Kiyosawa (15); * = isolates 84R-62B and Y93-245c-2 do not possess their corresponding *Avr* genes (19); + = does not possess any known *R* genes.

^b Avr = avirulent reaction, Vir = virulent reaction.

Table 2. Standard isolates of *Magnaporthe oryzae* used for identifying avirulence genes

Isolate	Avirulence genotype ^a										
	<i>Av-ks</i>	<i>Av-a</i>	<i>Av-i</i>	<i>Av-k</i>	<i>Av-km</i>	<i>Av-z</i>	<i>Av-ta</i>	<i>Av-ta2</i>	<i>Av-zt</i>	<i>Av-sh</i>	<i>Av-19(t)</i>
P-2b	+	+	<i>Av-i</i>	<i>Av-k</i>	<i>Av-km</i>	<i>Av-z</i>	+	+	<i>Av-zt</i>	+	+
Ken54-20	+	+	<i>Av-i</i>	<i>Av-k</i>	<i>Av-km</i>	<i>Av-z</i>	<i>Av-ta</i>	<i>Av-ta2</i>	<i>Av-zt</i>	+	+
Ina168	+	<i>Av-a</i>	<i>Av-i</i>	<i>Av-k</i>	<i>Av-km</i>	<i>Av-z</i>	+	<i>Av-ta2</i>	<i>Av-zt</i>	+	+

^a Avirulence gene designations according to the nomenclature of Kiyosawa (15); + = isolate contains the virulence gene that corresponds with the avirulence gene.

each primer, and 30 ng of template DNA. Amplification was carried out in a 9600 thermocycler (Perkin-Elmer, Norwalk, CT) programmed for 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, followed by 5 min at 72°C. Amplification products were separated by electrophoresis on 0.8% agarose gel containing ethidium bromide (2 µg/ml) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 50V for 1 h, and visualized under UV light. Molecular size markers, 50 to 2,500 bp DNA Maker (TaKaRa) and Smart ladder (Nippon Gene, Toyama, Japan) were included. Linkage analysis and construction of the genetic map were completed with the software Mapmaker version 2.0 for Macintosh (17). Parameters for map construction were a minimum LOD score of 3.0 and a maximum theta of 0.4. The Kosambi function was used to compute recombination distances in centimorgans (cM).

RESULTS

Segregation of mating types. Of the 115 ascospore progeny tested, 48 isolates were identified as *MATI-1* and 67 as *MATI-2*, indicating that the segregation of mating types fit a 1:1 ratio in the χ^2 test of a goodness-of-fit ($P_{1:1} > 0.05$).

Inheritance of avirulence in the parental isolates of *M. oryzae* on the three

rice cultivars. The segregation of avirulence/virulence of F_1 progeny isolates on three *R* gene donor cultivars is shown in Table 3. The parental isolate 84R-62B was avirulent to cv. Kanto 51, but virulent to cvs. Fukunishiki and Toride 1. The other parental isolate, Y93-245c-2, showed the opposite pathogenic reaction. All progeny isolates were virulent on the susceptible control cv. Norin 3 in each inoculation.

Among the 115 progeny isolates, the segregation ratio of avirulence/virulence on cv. Kanto 51 was 67:48, which fits a 1:1 ratio in the χ^2 test of a goodness-of-fit, suggesting that one *Avr* gene controls the avirulence on cv. Kanto 51. According to the pathogenic reactions of parental isolates, this *Avr* gene should have originated in the parental isolate 84R-62B (Table 3). The segregation ratio of avirulence/virulence on cv. Fukunishiki was 62:53, with a χ^2 test for goodness-of-fit with 1:1 ratio, indicating that avirulence on cv. Fukunishiki was determined by a single gene. The same segregation ratio for avirulence/virulence (62:53) was also observed on cv. Toride 1; however, it is apparent that the avirulence to cv. Toride 1 was controlled by different single gene, because the genes for avirulence to cvs. Fukunishiki and Toride 1 were inherited independently. These two *Avr* genes should have originated with the parental isolate

Y93-245c-2 (Table 3). The cv. Kanto 51 possesses *R* genes *Pik* and *Pi19(t)*, cv. Fukunishiki possesses *Piz*, *Pish*, and *Pi19(t)*, and cv. Toride 1 possesses *Piz-t*, *Pish*, and *Pi19(t)* (8,9,15) (Table 1). Previous research showed that both parental isolates have no *Avr* genes to each of the *R* genes *Pish* and *Pi19(t)*, because cv. Shin 2, which possesses *R* genes *Pish* and *Pi19(t)*, was susceptible to both parental isolates (19). Thus, the resistant reaction of cv. Kanto 51 to the parental isolate 84R-62B was most likely only attributable to the *R* gene *Pik*. Similarly, the resistant reactions of cvs. Fukunishiki or Toride 1 to the parental isolate Y93-245c-2 could only be attributable to either the *Piz* *R* gene or *Piz-t* *R* gene. In other words, the parental isolate 84R-62B probably has the *Avr* gene *Avr-Pik*, and the other parental isolate Y93-245c-2 has the *AvrPiz* and *AvrPiz-t* genes. To confirm this assumption, the *Avr* genes in both parental isolates were identified.

Identification of *Avr* genes. Three F_3 families of rice were analyzed for segregation of resistance to the parental isolates and the corresponding standard isolates, respectively (Tables 4 to 6). In the host reaction of the Norin 3/Kanto 51 family, the segregation ratio of 100 F_3 lines to the parental isolate 84R-62B was 20 all resistant: 47 segregating for resistance: 33 all susceptible. This segregation was consistent with a 1:2:1 ratio, indicating that the resistance of cv. Kanto 51 to isolate 84R-62B is due to monogenic control. Furthermore, the reactions of the parental isolate 84R-62B and the standard isolate P-2b were always identical on all 100 F_3 lines (Table 4). That is, each resistant line, segregating line, or susceptible line to the isolate 84R-62B was also resistant, segregating, or susceptible to the isolate P-2b. This indicates that the blast resistant gene

Table 3. Segregation of avirulence/virulence of the progenies from the cross of *Magnaporthe oryzae* isolates 84R-62B and Y93-245c-2 on three race-differential rice cultivars

Cultivar	Parental isolates		Progeny		χ^2	<i>P</i>
	84R-62B	Y93-245c-2	Avr:Vir ^a	1:1		
Kanto 51	Avr	Vir	67:48	3.14	0.08	
Fukunishiki	Vir	Avr	62:53	0.70	0.40	
Toride 1	Vir	Avr	62:53	0.70	0.40	

^a Avr = avirulent reaction, Vir = virulent reaction.

Table 4. Reaction of individual F_3 lines of rice to parental and the standard isolates of *Magnaporthe oryzae*

Standard isolate	Reaction to P-2b ^b	Number of F_3 lines ^a classified by reaction to 84R-62B				χ^2	<i>P</i>
		Resistant	Segregating	Susceptible	(Total)	1:2:1	
P-2b	Resistant	20	0	0	(20)		
	Segregating	0	47	0	(47)		
	Susceptible	0	0	33	(33)		
	(Total)	(20)	(47)	(33)	[100]	3.74	0.15

^a The F_3 lines of rice from the cross between Norin 3 (+) and Kanto 51 (*Pik*).

^b Resistant (or susceptible) = all individual plants in a given F_3 line had a resistant (or susceptible) reaction to the blast isolate used; segregating = host reaction to the blast isolate was segregating depending on individual plants of the same rice line.

Table 5. Reaction of individual F_3 lines of rice to parental and the standard isolates of *Magnaporthe oryzae*

Standard isolate	Reaction to Ken 54-20 ^b	Number of F_3 lines ^a classified by reaction to Y93-245c-2				χ^2	<i>P</i>
		Resistant	Segregating	Susceptible	(Total)	1:2:1	
Ken54-20	Resistant	25	0	0	(25)		
	Segregating	0	52	0	(52)		
	Susceptible	0	0	23	(23)		
	(Total)	(25)	(52)	(23)	[100]	0.24	0.89

^a The F_3 lines of rice from the cross between AK61 (+) and Fukunishiki (*Piz*).

^b Resistant (or susceptible) = all individual plants in a given F_3 line had a resistant (or susceptible) reaction to the blast isolate used; segregating = host reaction to the blast isolate was segregating depending on individual plants of the same rice line.

of cv. Kanto 51 to the isolate 84R-62B is the same as the one controlling blast resistance to the isolate P-2b. The isolate P-2b has been shown to have an *Avr* gene *Av-k* (15) (Table 2), and this gene alone determined the incompatibility between the cv. Kanto 51 and the isolate P-2b by the interaction of *Pik* (*Pi-k*) and *AvrPik* (*Av-k*) (15). Therefore, the gene in the isolate P-2b controlling avirulence on cv. Kanto 51 is the gene *AvrPik* (18). As a result, the *Avr* gene of the isolate 84R-62B to cv. Kanto 51 was identified as the *AvrPik*.

In host reactions of the AK61/Fukunishiki family to isolates Y93-245c-2 and Ken 54-20, identified 25 lines had all resistant plants, 52 lines were segregating for resistance, and 23 lines had all susceptible plants (Table 5). Without exception, there was complete cosegregation for resistance to these two isolates on all 100 F₃ lines. Comparison of χ^2 distribution to the expected 1:2:1 ratio indicates that the *R* gene in cv. Fukunishiki was effective against isolates Y93-245c-2 and Ken 54-20. This suggests that the *Avr* genes controlling avirulence to cv. Fukunishiki in both isolates are identical. An *Avr* gene designated *Av-z* has been reported to exist in the isolate Ken 54-20 (15) (Table 2), and the incompatibility between cv. Fukunishiki and isolate Ken 54-20 was explained by the interaction of *Piz* (*Pi-z*) and *AvrPiz* (*Av-z*) (15). Because the *Avr* gene controlling avirulence on cv. Fukunishiki with isolate Ken 54-20 has been identified as *AvrPiz*, the *Avr* gene in isolate Y93-245c-2 that controls avirulence on cv. Fukunishiki was also identified as *AvrPiz* (7,18).

Similar results were obtained in the F₃ family generated by the cross of Norin 3/Toride 1. The segregation ratio of F₃ rice

lines to isolates Ina 168 and Y93-245c-2 was 25 all resistant plants: 53 segregating for resistance: 22 all susceptible (Table 6). This segregation ratio was a good fit to the 1:2:1 ratio expected for a character controlled by a single gene. Complete cosegregation of the reactions to the isolates Ina 168 and Y93-245c-2 indicated that both isolates possessed the identical *Avr* gene specific to cv. Toride 1. Isolate Ina 168 is known to have the *Avr* gene designated as *AvrPiz-t* (*Av-zt*) (15) that determines the incompatibility of cv. Toride 1 with *Piz-t* (*Pi-z'*) (15). Therefore, the *Avr* gene in the isolate Ina 168 conditioning avirulence in cv. Toride 1 is the gene *AvrPiz-t*, and the gene in the isolate Y93-245c-2 was identified as *AvrPiz-t* (7). The linkage analysis among these *Avr* genes showed that the three genes were not linked to one another in this cross (Table 7).

Construction of a partial genetic map of identified *Avr* genes. Of the 389 primer combinations tested, 273 combinations (70.2%) produced polymorphic fragments between two parental isolates. Using these 273 combinations, the polymorphic fragments were tested against parental and 10 progeny isolates in the preliminary analysis. As shown in Figure 1, amplified fragments linked with one of the three *Avr* genes were obtained from 25 of the 273 primer combinations. Further RAPD analysis was conducted for another 50 progeny isolates by using the 25 primer combinations identified. As a result, 17 amplified fragments were identified as linkage RAPD markers to these *Avr* genes. In addition to the RAPD markers, *MAT1* locus was also used for constructing a partial linkage map (Fig. 2). The three *Avr* genes were clearly separated into distinct linkage groups (A, B, and C) in the map.

Twelve RAPD markers and *AvrPik* constructed linkage group A. Three RAPD markers and *AvrPiz* constructed linkage group B. Group C containing *AvrPiz-t* was constructed by two RAPD markers and mating type locus *MAT1*. The most closely linked relationships were observed between eight RAPD markers (*AE6AF6/900* and seven RAPD markers in rectangular frame in Figure 2) and *AvrPik*, and between *AM13A13/1900* and *AvrPiz-t*. These markers were mapped at 1.7 cM (one recombination/60 progenies) from each *Avr* gene. The eight markers in group A were linked with each other at 0 cM. The most closely linked marker to *AvrPiz* was *P3Q2/900*, and the distance between them was 5.0 cM (three recombination/60 progenies).

Among the 17 RAPD markers, *P1O20/2400* and *X11Y10/4000* were amplified fragments from the isolate Y93-245c-2. The remaining markers were transmitted from isolate 84R-62B. All of the 17 markers segregated in a single locus Mendelian fashion (data not shown).

DISCUSSION

The pathosystem of *O. sativa*-*M. oryzae* has been demonstrated to be a classical gene-for-gene system (11,22). The identification of *Avr* genes has generally been used for describing the genetic analysis of avirulence in the pathogen (23,27). Recently, the molecular/genetic approach has been employed for studies, which include construction of a high resolution linkage map by using molecular markers and a map-based strategy for cloning of targeted *Avr* genes. So far, at least nine *Avr* genes have been identified in high-resolution linkage maps, and four *Avr* genes have been cloned and characterized at the se-

Table 6. Reaction of individual F₃ lines of rice to parental and the standard isolates of *Magnaporthe oryzae*

Standard isolate	Reaction to Ina 168 ^b	Number of F ₃ lines ^a classified by reaction to Y93-245c-2				χ^2	
		Resistant	Segregating	Susceptible	(Total)	1:2:1	P
Ina 168	Resistant	25	0	0	(25)	0.54	0.76
	Segregating	0	53	0	(53)		
	Susceptible	0	0	22	(22)		
	(Total)	(25)	(53)	(22)	[100]		

^a The F₃ lines of rice from the cross between Norin 3 (+) and Toride 1 (*Piz-t*).

^b Resistant (or susceptible) = all individual plants in a given F₃ line had a resistant (or susceptible) reaction to the blast isolate used; segregating = host reaction to the blast isolate was segregating depending on individual plants of the same rice line.

Table 7. Relationship among three avirulence genes identified in *Magnaporthe oryzae*

<i>AvrPik/AvrPiz</i>			<i>AvrPik/AvrPiz-t</i>			<i>AvrPiz/AvrPiz-t</i>		
<i>AvrPik</i>	<i>AvrPiz</i>	No. of progeny	<i>AvrPik</i>	<i>AvrPiz-t</i>	No. of progeny	<i>AvrPiz</i>	<i>AvrPiz-t</i>	No. of progeny
+ ^a	+	32	+	+	33	+	+	36
+	-	35	+	-	34	+	-	26
-	+	30	-	+	29	-	+	26
-	-	18	-	-	19	-	-	27
$\chi^2_{(1:1:1:1)}$		5.80			4.90			2.46
P value		0.12			0.18			0.48

^a + indicates the progeny possesses the corresponding avirulence gene, - indicates the progeny does not possess the corresponding avirulence gene.

quence level (2,3,12,20,21,24–26). Almost all *Avr* genes analyzed were named after the rice cultivar on which the avirulence inheritance was studied. For instance, *AVR1-MARA* controls the avirulence in the rice cultivar Maratelli (20). These *Avr* genes could not be identified in relation to corresponding *R* genes in rice cultivars, since the genetic background of those cultivars is not clear. The only exception is the

Avr gene *AvrPita*, that has been shown to correspond to the *R* gene *Pita* by molecular methods (11). To avoid confusion in the symbol of *Avr* genes, especially those where the symbol of corresponding *R* genes has been decided, it is important to identify *Avr* genes precisely by genetic analysis. On the other hand, the studies of *Avr* genes in *M. oryzae* and *R* genes in rice cultivars are complementary for clearly

identifying each *Avr* gene and *R* gene. It is unsafe to identify *Avr* genes based only on the genetics of the blast fungus, but it is acceptable to use the genetics of rice after inoculation with an experimental isolate and the standard isolate. In our study, the genetically analyzed *Avr* genes were confirmed as corresponding to known *R* genes using F_3 rice lines and standard fungal isolates. In Japan, it is possible to identify *Avr* genes corresponding to *R* genes even if the *Avr* genes and corresponding *R* genes have not yet been isolated. An attempt was made to identify the *Avr* genes *AvrPii* and *AvrPia* (30). A standard set of seven isolates, including P-2b, Ken 54-20, and Ina 168, has been established (15). Using these isolates, the genetic analysis of vertical resistance of rice cultivars to rice blast has been advanced, resulting in the establishment of a differentiating system of blast races with nine differential cultivars (29). The genetic backgrounds of these differential cultivars were fully studied (8,9,15). Out of nine differential cultivars, three, Kanto 51, Fukunishiki, and Toride 1, were identified as having the *Pik*, *Piz*, and *Piz-t* genes by using a standard set of isolates. It is clear that *AvrPik* in the standard isolate P-2b controls avirulence on cv. Kanto 51; similarly, the effective genes in Ken 54-20 and Ina 168 toward avirulence on cvs. Fukunishiki and Toride 1 are *AvrPiz* and *AvrPiz-t*, respectively. Therefore, based on investigation of the relationship between *Avr* genes in the parental isolate and *Avr* genes in the standard isolate, the effective *Avr* genes in parental isolates could be identified in relation to the corresponding *R* genes in rice cultivars if complete cosegregation for resistance to both the parental isolate and the standard isolate was observed in the F_3 lines from rice crosses.

In the present study, markers tightly linked to *Avr* genes were provided by using RAPD analysis. Especially, eight nearby markers were detected at 1.7 cM from *AvrPik*. For *AvrPiz-t*, the nearby marker, *AM13A13/1900*, was also detected at 1.7 cM. Biased distribution of one parental isolate was observed among RAPD markers. Out of 206 amplified fragments, which were easily resolved and scorable among the sample of the F_1 progeny isolates, 123 fragments were amplified from the parental isolate 84R-62B, and 83 were from parental isolate Y93-245c-2. Among 17 markers linked with *Avr* genes, almost all markers (15 out of 17) were amplified fragments from the isolate 84R-62B. This indicates that the RAPD markers were preferentially inherited from the parental isolate 84R-62B. It has been reported that RAPD markers amplified in fungi often contained repetitive sequences (2,13). The biased distribution of markers might be caused by the difference in distribution of repetitive sequences between the parental isolates. Single copy markers can be used for aids to locate *Avr* genes on chromo-

Fig. 1. Random amplified polymorphic DNA (RAPD) amplification with primer combinations OPE-AE6/OPE-AF6 (I), OPE-P1/OPE-O20 (II), OPE-AM13/OPE-A13 (III) in two parental and 10 progeny isolates of *Magnaporthe oryzae*. "A" and "V" indicated avirulence and virulence toward cvs. Kanto 51, Fukunishiki, and Toride 1 in I, II, and III, respectively. Arrows show amplified fragments linked to *AvrPik* (I), *AvrPiz* (II), and *AvrPiz-t* (III), respectively. These fragments correspond to RAPD markers *AE6AF6/900*, *P1O20/2400*, and *AM13A13/1900* in Figure 2.

Fig. 2. Genetic maps on regions of the *Magnaporthe oryzae* genome containing target *Avr* genes. Map was constructed using Mapmaker 2.0 with a minimum LOD score of 3.0 and a maximum theta of 0.4. Kosambi cM units were shown on the left, and *Avr* loci and markers were listed on the right. **A**, **B**, and **C** showed the *AvrPik*, *AvrPiz*, and *AvrPiz-t* linkage groups, respectively. The random amplified polymorphic DNA (RAPD) marker was designated as a primer combination/fragment size (bp).

somes and to clone these genes by positional cloning. It is necessary to characterize these RAPD markers and to find single copy markers for future study.

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