One of Two Major Paralogs of *AVR-Pita1* Is Functional in Japanese Rice Blast Isolates

Mami Takahashi, Taketo Ashizawa, Kazuyuki Hirayae, Jouji Moriwaki, Teruo Sone, Ryoichi Sonoda, Masako Tsujimoto Noguchi, Susumu Nagashima, Kouji Ishikawa, and Michiyoshi Arai

First, second, third, and fourth authors: National Agriculture and Food Organization, National Agricultural Research Center, Hokuriku Research Center, 1-2-1 Inada, Joetsu, Niigata, 943-0193, Japan; fifth author: Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido, 060-8589, Japan; sixth author: National Agriculture and Food Organization, National Institute of Vegetable and Tea Science, Shimada, Shizuoka, 428-8501, Japan; seventh author: National Institute for Agro-Environmental Sciences, Kannondai, Tsukuba, Ibaraki, 305-8604, Japan; eighth author: Shimane Agricultural Technology Center, Ashiwata 2440, Izumo, Shimane, 693-0035, Japan; ninth author: Niigata Agricultural Research Institute, 857 Nagakura, Nagaoka, Niigata, 940-0826, Japan; and tenth author: National Agriculture and Food Organization, National Agricultural Research Center for Kyushu Okinawa Region, 2421 Suya, Koshi, Kumamoto 861-1192, Japan.

Current address of third author: National Agriculture and Food Organization, National Agricultural Research Center for Kyushu Okinawa Region, 2421 Suya, Koshi, Kumamoto 861-1192, Japan; current address of tenth author: National Agriculture and Food Organization, National Agricultural Research Center, Hokuriku Research Center, 1-2-1 Inada, Joetsu Niigata, 943-0193, Japan.

Accepted for publication 26 February 2010.

ABSTRACT

Takahashi, M., Ashizawa, T., Hirayae, K., Moriwaki, J., Sone, T., Sonoda, R., Noguchi, M. T., Nagashima, S., Ishikawa, K., and Arai, M. 2010. One of two major paralogs of *AVR-Pita1* is functional in Japanese rice blast isolates. *Phytopathology* 100:612-618.

We analyzed the avirulence gene *AVR-Pita1* in Japanese rice blast isolates to determine how they gain virulence toward rice cultivars containing the *Pita* resistance gene. An avirulent isolate, OS99-G-7a (G7a), from a Japanese commercial field contained two paralogs of *AVR-Pita1*, designated as *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*. Analysis of virulent, independent mutants derived from G7a, a single avirulent progenitor strain, indicated that *AVR-Pita1^{JA}* was functional but *AVR-Pita1^{JB}* was nonfunctional. The most frequent mutation was loss of *AVR-Pita1^{JA}*. Analyses of field isolates collected from diverse areas in Japan revealed

that most of the *AVR-Pita1* genes carried by Japanese isolates were identical to *AVR-Pita1^{JA}* or *AVR-Pita1^{JB}*. The relationship between these major paralogs in Japanese isolates and the virulence of the strains carrying them indicate that *AVR-Pita1^{JA}* is functional but *AVR-Pita1^{JB}* is not, as is the case in G7a. Isolates that show virulence toward rice cultivars containing the *Pita* gene are presumed to have evolved virulence from avirulent origins via loss of *AVR-Pita1^{JA}*, except for one case in which virulence resulted from a base substitution. In this study, we discuss the properties and specificities of Japanese rice blasts that relate to virulence against *Pita*-containing rice. Furthermore, we present a method to amplify *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* separately and, specifically, to monitor functional *AVR-Pita1* in Japan.

Additional keywords: *Magnaporthe oryzae*, *Pyricularia oryzae*.

Rice blast disease is caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) and is the most economically important rice disease worldwide. More than 40 resistance genes to rice blast disease have been identified and some have been bred into new cultivars (3). However, a number of resistant rice cultivars have become susceptible to the disease within 1 to 6 years of commercial production (2). These disease outbreaks were caused by spontaneous virulent blast mutants that arose as a result of the selection pressure imposed by resistant rice cultivars (17).

In Japan, almost all rice blast isolates from paddy fields are avirulent against rice cultivars containing the *Pita* gene (>99% of all blast isolates) (15). Thus, *Pita* is one of the most useful resistance genes in Japan, and several *Pita*-containing isogenic lines have been bred (1,23). However, production of new cultivars containing the *Pita* gene increases the possibility that new virulent mutants will arise, and the new cultivars will become susceptible.

Pita and its corresponding avirulence gene, *AVR-Pita*, have been identified and characterized. *Pita* is located on chromosome 12 of the rice genome and encodes a protein with a nucleotide-binding site and a leucine-rich repeat (LRR) domain (4). *AVR-Pita* is located in the telomeric region of chromosome 3 and encodes a putative zinc metalloprotease (20). The translated protein, *Pita*, binds directly to *AVR-Pita* and the interaction between them induces the defense response (10). Recently, it was found that *AVR-Pita* constitutes a gene family with functional and non-functional avirulence genes with similar sequences (*AVR-Pita2* and *avr-Pita3*) in *M. oryzae* isolated from diverse hosts. *AVR-Pita* was renamed *AVR-Pita1* (12).

The *AVR-Pita1* locus of five blast isolates from diverse hosts has been described in detail elsewhere (12) but can be summarized as follows. In the rice blast isolate O-137, the solo long-terminal repeat (LTR) of a retrotransposon, RETRO7-1 (5), is located in the 5' flanking region of the *AVR-Pita1* promoter, and a telomeric repeat is located 48 bp downstream from the stop codon of *AVR-Pita1* in its 3' flanking region. In the rice blast isolate 70-15 (5), the LTR of RETRO6-1 (5) is joined to a partial solo LTR of RETRO7-1 and is located in the 5' flanking region of the *AVR-Pita1* promoter. In its 3' flanking region, there are three linked solo LTRs, one from each of three retrotransposons: RETRO7-1, Pyret (19), and RETRO6-1. In the *Digitaria* isolate G-1, the 5'

Corresponding author: M. Takahashi; E-mail address: mamitaka@affrc.go.jp

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains a figure showing sequence comparisons among *AVR-Pita1* in O-137, *AVR-Pita1^{JA}*, and *AVR-Pita1^{JB}*.

doi:10.1094/PHYTO-100-6-0612

© 2010 The American Phytopathological Society

truncated RETRO6-1 adjoins a telomeric repeat and a partial solo LTR of RETRO7-1 in the 5' flanking region of the *AVR-Pita1* promoter, and the 3' flanking region contains RETRO7-1. The telomeric repeat is located 1,689 bp upstream from the start codon of *AVR-Pita1*. In isolate G-223 from *Pennisetum* spp., structures around *AVR-Pita1* are similar to those of G-1. However, the telomeric region and internal region of RETRO6-1 in G-1 are replaced by a sequence that was predicted to be nonfunctional. It was presumed that a telomeric repeat lies within a 30-kbp distance of the G223 *AVR-Pita1*. In *Pennisetum* spp. isolate G-78, the partial solo LTR of RETRO6-1 and the Pot2-like transposable element (612 bp upstream of solo LTR) are located in the 5' flanking region of the *AVR-Pita1* promoter, while the 3' flanking region contains RETRO7-1 with duplicated termini of the LTR. Thus, all studied *AVR-Pita1* genes are associated with sequences for transposable elements, which are dispersed throughout the genomes, and three of the five *AVR-Pita1* genes are located near telomeric repeats. In addition, the flanking regions of the five studied *AVR-Pita1* showed differences in the positions of telomeric repeats and in gene organization (i.e., the types, numbers, and lengths of LTRs and transposable elements).

Previously, rice blast mutants that gained virulence against *Pita*-containing rice cultivars have been isolated under laboratory conditions and their genetic alterations examined. There are various changes in the *AVR-Pita1* gene, including point mutations, transposon insertions, and deletions ranging in size from 100 bp to 12.5 kbp, which enable strains to gain virulence against *Pita*-containing rice (11,20,24). Likewise, analysis of field isolates from the United States showed that transposon insertion and deletions of *AVR-Pita1* resulted in a gain of virulence against *Pita*-containing cultivars. In the United States, isolates that were avirulent against *Pita*-containing rice always contained *AVR-Pita1*, which was confirmed by polymerase chain reaction (PCR) amplification (29).

In this study, we aimed to clarify how Japanese rice blast gains virulence against *Pita*-containing rice, and to develop a specific PCR method that can be used to monitor functional *AVR-Pita1*. Rice blast strains that are virulent against *Pita*-containing rice are very rarely isolated from the restricted area in which *Pita*-containing rice is cultivated in Japan. We investigated the presence and types of *AVR-Pita1* in Japanese field isolates, including virulent isolates. Our results differ from those of field isolates in the United States. Here, we report the specificity related to *Pita*-mediated resistance in Japanese rice blast strains, and we describe a PCR method to detect *AVR-Pita1* based on its specificity.

MATERIALS AND METHODS

Rice blast isolates. We used OS99-G7-a (G7a) and 13 independent virulent mutants derived from it to clarify the DNA alterations that affect virulence in *Pita*-containing cultivars. G7a is an isolate obtained from a commercial rice field in Japan. It has a unique DNA sequence that is used to identify it (8). All the mutants were independent and developed spontaneously. Of the 13 mutants, 5 (S06-1-1, S06-11-1, S06-13-1, S06-14-1, and S07-1) were isolated from the *Pita*-containing cultivar, Sasanishiki BL6 (1,22), inoculated with conidia of G7a. Fifty rice plants at approximately the seven- to eight-leaf stage were inoculated with 30 ml of conidial suspension (8.0×10^4 conidia/ml) and incubated in a moist chamber at 26°C for 20 h. After 10 days, susceptible lesions were collected (26). We isolated 8 of the 13 mutants from experimental paddy fields with severe infestations of G7a rice blast. The mutants P06-1, P07-2-1, P07-3-1, and P07-5-1 were isolated from Sasanishiki BL6 in paddy fields that were planted in alternate rows with the susceptible rice cultivar (Koshihikari or Sasanishiki) and Sasanishiki BL6 (22). The mutants 01-1-1-1, 01-26-2-1, 01-27-1-1, and 01-95-1-1 were isolated from a nursery in Yashimochi (21), from a seedling case that was placed in a

paddy field infested with G7a. This paddy field was planted with Niigata Koshihikari BL1 (*Pia*), Koshihikari BL2 (*Pii*), Koshihikari BL3 (*Pita*-2), and Koshihikari BL4 (*Piz*) (9). To cause epidemics, rice plants infected with G7a were transferred to paddy fields. Susceptible lesions were collected only once, when they were first found. This avoided harvest of lesions formed by replicated mutants. G7a-derived mutants were identified by PCR (8).

In addition to G7a, we used 32 Japanese field isolates collected in 19 prefectures in this study. Of the 32 isolates, 8 were virulent and 24 were avirulent. Of these 32 isolates, 14 isolates, including all the virulent isolates, were analyzed by sequencing and Southern hybridization. The 18 isolates that were all avirulent were analyzed by PCR amplification to detect the presence of *AVR-Pita1*^{JA} or *AVR-Pita1*^{JB}. IAI-1, P07-10, SYaP1, and SYaF1 were isolated from an experimental field planted with the rice cv. Yashimochi or Sasanishiki BL6. We isolated 031713 and 031719 from the rice cv. Koshihikari in a commercial field in Niigata prefecture. Sixteen rice blast strains (0115006 to 0132034) were isolated from commercial fields to investigate races in Japan in 2001 (15). GF0S8-1-1, 0528-2, Ken 54-20, Hoku 1, TH68-140, Ken 54-04, Kyu 92-22, 24-22-1-1, Kyu9439013, and Kyu89-246 were stored for genetic analyses at the National Agricultural Research Center (NARC) (7,14). Information on the 32 isolates besides G7a, their race codes in Japan (13,25), and their pathogenic or nonpathogenic interactions with rice cv. Yashimochi are shown in Table 1.

A single conidium was isolated from lesions that we had collected, and was cultured on oatmeal medium. Conidia formation was induced by brushing of mycelia, and then incubating the culture for 3 days under white fluorescent lights. To retest the virulence of rice blast strains against *Pita*-containing rice, conidia

TABLE 1. Japanese rice blast isolates used in this study

Isolate	Reactions ^a	Origin		Race ^b
		Prefecture	Year	
IAI-1	+	Ibaraki	2006	117.1
P07-10	+	Niigata	2007	307.0
SYaP1	+	Shimane	2007	137.1
SYaF1	+	Shimane	2007	307.0
031713	+	Niigata	2007	103.0
0132034	+	Shimane	2001	101.0
GF0S8-1-1	+	Gifu	1993	303.0
0528-2	+	Akita	1976	333.1
OS99-G-7a	—	Saga	1999	007.0
0115006	—	Niigata	2001	001.0
0115018	—	Niigata	2001	003.0
0116020	—	Toyama	2001	047.0
0116030	—	Toyama	2001	001.0
0117011	—	Ishikawa	2001	001.0
0117012	—	Ishikawa	2001	041.0
0118009	—	Fukui	2001	037.1
0118025	—	Fukui	2001	003.0
0125029	—	Shiga	2001	003.0
0125030	—	Shiga	2001	007.0
0128112	—	Hyogo	2001	007.0
0128123	—	Hyogo	2001	001.0
0130114	—	Wakayama	2001	007.0
0131041	—	Tottori	2001	001.0
0132032	—	Shimane	2001	001.0
031719	—	Niigata	2007	003.0
Hoku 1	—	Hokkaido	1947	007.0
TH68-140	—	Yamagata	1968	035.1
Ken 54-04	—	Gifu	1954	003.0
24-22-1-1	—	Mie	1994	037.1
Ken54-20	—	Yamaguchi	1954	003.0
Kyu9439013	—	Kochi	1994	047.1
Kyu89-246	—	Miyazaki	1989	003.0
Kyu92-22	—	Kumamoto	1992	017.1

^a Reactions to Yashimochi (*Pita*): + = virulence and — = avirulence.

^b According to the method described by Yamada et al. (25) and Kiyosawa (13).

were inoculated onto Yashimochi rice plants at approximately the four- to five-leaf stage using a spray method (26).

PCR, cloning, and sequencing. Rice blast isolates were cultured in potato dextrose broth (PDB) for 2 or 3 days and DNA was extracted using an ISOPLANT II kit (Nippon Gene Co. Ltd., Tokyo). PCR was performed using a Gene Amp PCR System 9700 (Applied Biosystems Inc., Foster, CA).

For direct sequence analysis, *AVR-Pita1* was amplified with the primers AVR-PitaN89 (5'-CCGCTTTATTGGTTTAATTCGGA TA-3') and BamHIAVR-PitaR1 (5'-ATGGATCCTCCATTCC AACACTAACG-3'). For PCR, the 50- μ l reaction mixture contained $\approx$ 50 ng of genomic DNA, 25 pmol each primer, 0.2 mM dNTP, 5 μ l of 10 $\times$ buffer, and 1.25 units of *ExTaq* polymerase (Takara Bio. Inc., Otsu, Shiga, Japan). The PCR cycling program consisted of initial denaturation for 5 min at 94°C; 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 90 s of extension at 72°C; followed by final extension for 7 min at 72°C. Amplified fragments were purified using a QIA quick PCR purification kit (Qiagen Inc., Valencia, CA).

For cloning, *AVR-Pita1* of G7a was amplified with the primers BamHIAVR-PitaL1 (5'-AAGGATCCGAGCCGAGTCGTTCT GA-3') and BamHIAVR-PitaR1. For PCR, the 50- μ l reaction mixture contained $\approx$ 50 ng of genomic DNA, 25 pmol each primer, 0.2 mM dNTP, 1 mM MgSO₄, 5 μ l of 10 $\times$ buffer, and 1 unit of KOD Plus polymerase (Toyobo Co. Ltd., Osaka, Japan). The PCR cycling program consisted of initial denaturation for 2 min at 94°C; 35 cycles of 15 s of denaturation at 94°C, 30 s of annealing at 55°C, and 90 s of extension at 68°C; followed by final extension for 5 min at 68°C. Except in G7a, *AVR-Pita1* was amplified using the primers BamHIAVR-PitaN89 (5'-AAGGATCCGACCC GTTCCGCCTTTATT-3') and HindIIIAVR-PitaR1 (5'-ATAA GCTTTCCATTCCAACACTAACG-3'). PCR was carried out using KOD plus polymerase or Pyrobest polymerase (Takara Bio. Inc.). When KOD plus polymerase was used, the PCR procedure was exactly the same as that used to amplify *AVR-Pita1* from G7a. In the case of Pyrobest polymerase, the 50- μ l PCR reaction mixture contained $\approx$ 50 ng of genomic DNA, 25 pmol each primer, 0.2 mM dNTP, 2.5 units of Pyrobest polymerase, and 5 μ l of 10 $\times$ buffer. The PCR cycling program consisted of 30 cycles of 10 s of denaturation at 98°C, 30 s of annealing at 55°C, and 60 s of extension at 72°C. Amplified fragments were purified using a QIAquick PCR purification kit (Qiagen Inc.) and digested with restriction enzymes corresponding to additional restriction sites in the PCR primers. The fragments were purified from agarose gel slices and cloned into pUC19.

To examine the promoter region of *AVR-Pita1* in S06-14-1, we conducted PCR amplification using three sets of primers, as follows: BamHIAVR-PitaL1 and BamHIAVR-PitaR1; AVR-Pita-410-394 (5'-TTATGTAGTTATTAATAATATGGGCC-3') and BamHIAVR-PitaR1; and AVR-Pita-210-181 (5'-AGTCAAGCTG AAATAATAAATAAGTTGGCG-3') and BamHIAVR-PitaR1. To identify *AVR-Pita1^{JA}* in Ken54-20, we used the primer set AVR-PitaN89 and AVR-PitaApaLI (5'-CGTGACATTGTGCAAA AATAGCATAAC-3'). The PCR procedures for examination of promoter regions in S06-14-1 and for identification of *AVR-Pita1^{JA}* in Ken54-20 were the same as those used for amplification of *AVR-Pita1* for direct sequencing.

Sequencing reactions were carried out using the GenomeLab DTCS Quick Start Kit, and were analyzed using a CEQ8000 system according to the manufacturer's instructions (Beckman Coulter Inc., Fullerton, CA). Sequencing primers were as follows: AVR-PitaN89, BamHIAVR-PitaR1, BamHIAVR-PitaL1, AVR-Pita1161F (5'-TTCACACCGTTGCGATTTCG-3'); AVR-Pita454-478 (5'-GTTTATACGTGCGTCCGAGAAGAAG-3'); AVR-Pita758-733 (5'-CTTACTTGTGAATCCCATTCCATTTCG-3'); AVR-Pita1697R (5'-GGTTACCGGCAGTAATTTCC-3'); AVR-Pita-318-347R (5'-ATTATGCAGGTAATTAGGTCCACTT-3'); and the *BcaBEST* sequencing primer RV-M (Takara Bio. Inc.).

The following primers were designed to specifically amplify *AVR-Pita1^{JA}*: 5'AVR-Pita1^{JA}F (5'-GCTATCACGCCGTTAAAAAG TG-3') and 5'AVR-Pita1^{JA}R (5'-TAGCATAACTGTCCGGG TTTT-3'). To specifically amplify *AVR-Pita1^{JB}*, we used 5'AVR-Pita1^{JB}F (5'-GCTATCACGCCGTTAAAAAGTA-3') and 3'AVR-Pita1^{JB}R (5'-GCATAACTGTCCGGGTTTC-3'). The 3' terminal nucleotides of these primers corresponded to the nucleotides that differ between *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*. The PCR mixture (20 μ l) contained 50 ng of genomic DNA, 1 pmol each primer, 2 μ l of 10 $\times$ reaction buffer, 0.2 mM dNTP, and 1.25 units of *ExTaq* polymerase (Takara Bio. Inc.). The PCR cycling program consisted of an initial denaturation for 7 min at 94°C; 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 64°C, and 90 s of extension at 72°C; followed by a final extension for 7 min at 72°C.

Southern blot hybridization. Rice blast isolates were cultured in PDB for 14 days and genomic DNA was extracted using a DNeasy Plant Maxi kit (Qiagen Inc.). Approximately 3 μ g of DNA was digested with $\approx$ 4 to 12 units of restriction enzymes at 37°C overnight. DNA was separated by electrophoresis on a 0.67% agarose gel and blotted onto a Hybond N⁺ membrane (GE Healthcare, UK Ltd., Buckinghamshire, England) using a BacuGene XL Vacuum Blotting system (GE Healthcare, UK Ltd.). Hybridization was performed using an AlkPhos Direct Labeling and Detection System (GE Healthcare, UK Ltd.) according to the manufacturer's instructions. The fragment amplified from genomic DNA of G7a with BamHIAVR-PitaN89 and BamHIAVR-PitaR1 was digested with *Bam*HI and *Apa*LI and separated by electrophoresis on a 1% agarose gel to use as a DNA probe. The probe was hybridized to the blot at 55°C overnight.

RESULTS

***AVR-Pita1* in G7a.** *AVR-pita1* genes in G7a were amplified, cloned, and sequenced. Two paralogs were found in G7a. These paralogs shared >98% nucleotide sequence identity with *AVR-Pita1* cloned from O-137 (20). A phylogenetic tree was constructed by the neighbor-joining method using the nucleotide sequences of these two paralogs in G7a and those of other members of the *AVR-Pita* gene family described by Khang et al. (12). The tree revealed that the two paralogs in G7a were found in the *AVR-Pita1* clade, which was separate from the *AVR-Pita2* and *avr-Pita3* clades (data not shown). Therefore, the two paralogs in G7a were designated as *AVR-Pita1^{JA}* (accession no. AB520831) and *AVR-Pita1^{JB}* (accession no. AB520830). Sequences of *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* were identical except for three nucleotides at positions 303, 719, and 829 bp (numbering is relative to the translation start site of *AVR-Pita1^{JA}* or *AVR-Pita1^{JB}*). The three nucleotides that differed were G, A, and A in *AVR-Pita1^{JA}* and A, G, and G in *AVR-Pita1^{JB}*. These differences in DNA sequences were predicted to affect the amino acid sequences as follows: D (83), Y (192), and K (207) in *AVR-Pita1^{JA}*; and N (83), C (192), and R (207) in *AVR-Pita1^{JB}*. The amino acids D (83), Y (192), and K (207) in *AVR-Pita1^{JA}* were identical to the corresponding residues in *AVR-Pita1* in O-137. The sequences of promoter regions of the two *AVR-Pita1* genes in G7a were also determined. The upstream sequences (approximately -492 to -1 bp) of *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* were identical to each other and were >98% identical to those of *AVR-Pita1* in O-137.

Southern blot analyses were carried out to identify the copy number of *AVR-Pita1* in G7a (Fig. 1). We used *Apa*LI to digest genomic DNA and to produce the probe for the blot, because its cleavage site is located in the 3' terminal regions of *AVR-Pita1^{JA}* or *AVR-Pita1^{JB}*. This avoids detecting a band containing more than two *AVR-Pita1* sequences. Two bands were detected in all lanes, regardless of whether the DNA was digested with *Kpn*LI, *Apa*LI, or both enzymes. The two bands migrated faster in the lane of the double digestion compared with those in the lanes of

single digestions. This result indicates that both ends of each band in the lane of the double digestion had been digested by *Kpn*I and *Apa*LI, and that each band contained one *AVR-Pita1* paralog. This result supported the idea that G7a contains two paralogs. We tried to amplify the *AVR-Pita2* in G7a as described by Khang et al. (12); however, we did not obtain an *AVR-Pita2* fragment.

***AVR-Pita1* in G7a-derived mutants.** To clarify whether two paralogs were carried over into 13 G7a-derived mutants, we amplified and directly sequenced *AVR-Pita1* paralogs, taking particular note of the different nucleotides at 303, 719, and 829. In the mutant strains 01-95-1-1, S06-13-1, and S06-14-1, two signals of G and A were detected at positions corresponding to the three different nucleotides deduced from sequence data. In the other mutants, only one signal was detected at the position corresponding to the three different nucleotides. In addition, their amplicons shared 100% sequence identity with *AVR-Pita1^{JB}*. These results suggest that 01-95-1-1, S06-13-1, and S06-14-1 retain both *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*, but the other 10 mutants retain only *AVR-Pita1^{JB}* (Table 2).

Next, we cloned and sequenced amplicons of 01-95-1-1, S06-13-1, and S06-14-1 (Table 2). In all three mutants, *AVR-Pita1^{JB}* was unchanged. *AVR-Pita1^{JA}* was unchanged only in the S06-14-1 mutant. In 01-95-1-1, *AVR-Pita1^{JA}* had a tandem duplication of 31 bp from 795 to 825 bp. In S06-13-1, *AVR-Pita1^{JA}* lacked a T at the 30-bp position. Both changes were predicted to cause a frame shift resulting in C-terminal-truncated *AVR-Pita1^{JA}*. Because *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* were unchanged in S06-14-1, their promoter regions were also analyzed. Fragments were amplified from genomic DNA of S06-14-1 using three primer sets: BamHIAVR-PitaL1 and BamHIAVR-PitaR1, AVR-Pita-410-394 and BamHIAVR-PitaR1, and AVR-Pita-210-181 and BamHIAVR-PitaR1. The resulting fragments were sequenced directly. Using the primer set AVR-Pita-210-181/BamHIAVR-PitaR1, both *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* were amplified and their sequences were unchanged from those in G7a. However, when the primer sets

BamHIAVR-PitaL1/BamHIAVR-PitaR1 and AVR-Pita-410-394/BamHIAVR-PitaR1 were used, *AVR-Pita1^{JB}* was amplified but *AVR-Pita1^{JA}* was not. Therefore, the primers BamHIAVR-PitaL1 and AVR-Pita-410-394 could not amplify *AVR-Pita1^{JA}*. The sequence analysis of PCR fragments suggested that the region containing the sequences of these primers is deleted from the promoter of *AVR-Pita1^{JA}* in S06-14-1.

We carried out Southern blot analyses to confirm the copy number of *AVR-Pita1* (Fig. 2). Two bands were detected in the lanes of 01-95-1-1, S06-13-1, and G7a. A band approximately 5 kbp in length, which corresponds to the lower band in G7a, was absent from the lanes of the other 11 mutants. These results supported the idea that two paralogs were carried over into 01-95-1-1 and S06-13-1, and that *AVR-Pita1^{JA}* was deleted from the genomes of 10 mutants. However, a hybridization analysis of

TABLE 2. Variations in *AVR-Pita1* in OS99-G-7a-derived mutants^a

Isolate	Mutation of	
	<i>AVR-Pita1^{JA}</i>	<i>AVR-Pita1^{JB}</i>
01-1-1-1	Deletion of gene	No change
01-26-2-1	Deletion of gene	No change
01-27-1-1	Deletion of gene	No change
01-95-1-1	31 bp of duplication ^b	No change
S06-1-1	Deletion of gene	No change
S06-11-1	Deletion of gene	No change
S06-13-1	Deletion of thymine base ^c	No change
S06-14-1	Partial deletion of promoter	No change
P06-1	Deletion of gene	No change
S07-1	Deletion of gene	No change
P07-2-1	Deletion of gene	No change
P07-3-1	Deletion of gene	No change
P07-5-1	Deletion of gene	No change

^a Confirmed by sequencing of amplified fragment with AVR-PitaN89 and BamHIAVR-PitaR1 or BamHIAVR-PitaN89 and HindIIIAVR-PitaR1, and Southern blot hybridization (except for mutation of S06-14-1). Deletion of *AVR-Pita1^{JA}* promoter in S06-14-1 was confirmed by amplification with specific primers for the promoter region and Southern blot hybridization.

^b Tandem duplication from 795 to 895 bp (numbering is relative to the translation start site of *AVR-Pita1^{JA}* or *AVR-Pita1^{JB}*).

^c Located at 30-bp position.

Fig. 1. Southern blot analysis of *AVR-Pita1* in rice blast isolate OS99-G-7a. Genomic DNA was digested with *Kpn*I, *Apa*LI, or both. Products were separated by electrophoresis on a 0.67% agarose gel and transferred to a nylon membrane. A labeled fragment that was amplified with BamHIAVR-PitaN89 and BamHIAVR-PitaR1 and digested with *Bam*HI and *Apa*LI was used as a probe. Hybridization was carried out using an AlkPhos Direct Labeling and Detection System according to the manufacturer's instructions (GE Healthcare, UK Ltd.). Sizes of markers are shown at left.

Fig. 2. Southern blot analysis of *AVR-Pita1* in virulent mutants derived from OS99-G-7a. Genomic DNA was digested with *Kpn*I and *Apa*LI, separated by electrophoresis on a 0.67% agarose gel, and then transferred to a nylon membrane. A labeled fragment that was amplified with BamHIAVR-PitaN89 and BamHIAVR-PitaR1 and digested with *Bam*HI and *Apa*LI was used as a probe. Hybridization was performed with the AlkPhos Direct Labeling and Detection System (GE Healthcare, UK Ltd.) according to the manufacturer's instructions. Names of mutants are shown above photographs. Sizes of markers are shown at left.

S06-14-1 suggested the presence of one paralog, which did not agree with the sequence analysis result. To consider the possibility that there were two overlapping bands, we digested genomic DNA of S06-14-1 with *Apa*LI and *Apa*I, because the *Apa*I site lies in the deleted region of the promoter in *AVR-Pita1^{JA}*. As expected, the Southern blot analysis revealed two bands in the lane of S06-14-1 (Fig 3). The 1.25-kbp band was identical in size to the band in G7a and was predicted to contain *AVR-Pita1^{JB}*. The other band was predicted to contain *AVR-Pita1^{JA}* with a partial deletion in its promoter.

Taken together, these results demonstrated that *AVR-Pita1^{JB}* was unchanged in G7a-derived mutants but *AVR-Pita1^{JA}* or its promoter was altered.

Fig. 3. Southern blot hybridization of S06-14-1. Genomic DNA was digested with *Apa*I and *Apa*LI, separated electrophoretically on a 0.67% agarose gel, and transferred to a nylon membrane. A labeled fragment that was amplified with BamHIAVR-PitaN89 and BamHIAVR-PitaR1 and digested with *Bam*HI and *Apa*LI was used as a probe. Hybridization was performed using the AlkPhos Direct Labeling and Detection System (GE Healthcare, UK Ltd.) according to the manufacturer's instructions. Sizes of markers are shown at left.

TABLE 3. *AVR-Pita1* paralogs in 14 Japanese isolates^a

Isolate	<i>AVR-Pita1^{JA}</i> ^b	<i>AVR-Pita1^{JB}</i> ^b	<i>AVR-Pita1^{JC}</i> ^d	<i>AVR-Pita1^{JD}</i> ^e
IAI-1	–	+	–	–
031713	–	+	–	–
P07-10	–	+	–	–
0132034	–	+	–	–
SYaP1	–	+	–	–
SYaF1	–	+	+	–
GFOS8-1-1	–	–	–	–
0528-2	–	+	–	–
031719	+	+	–	–
0132032	+	+	–	–
0131041	+	+	–	–
Ken54-20	+	–	–	–
Hoku 1	+	–	–	–
TH68-140	–	+	–	+

^a Symbols: – = not identified and + = identified by sequencing of amplified fragments with primer sets AVR-PitaN89 and BamHIAVR-PitaR1 or BamHIAVR-PitaN89 and HindIIIAVR-PitaR1.

^b Accession no. AB520831.

^c Accession no. AB520830.

^d Paralog is identical to *AVR-Pita1^{JA}* in G7a-derived mutant (S06-13-1) (i.e., *AVR-Pita1^{JA}* lacks T at 30 bp).

^e Sequence identical to that of *AVR-Pita1^{JA}*, except for C at the 411-bp position.

***AVR-Pita1* in Japanese field isolates.** We amplified and directly sequenced the *AVR-Pita1* gene from 14 isolates collected from diverse areas in Japan. However, the sequences of a virulent isolate and four avirulent isolates suggested that they contained more than two paralogs. Therefore, their amplicons were cloned and sequenced (Table 3). In 14 isolates, paralogs were identical to *AVR-Pita1^{JA}* or *AVR-Pita1^{JB}*, except for the paralogs in SYaF1 and TH68-140. Of the eight virulent isolates, one lacked *AVR-Pita1* (GFOS8-1-1), six contained only *AVR-Pita1^{JB}*, and one contained *AVR-Pita1^{JB}* and a paralog designated as *AVR-Pita1^{JC}*, which was identical to the altered *AVR-Pita1^{JA}* in S06-13-1 (SYaF1). Of the six avirulent isolates, two contained only *AVR-Pita1^{JA}*, three contained both *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*, and one isolate, TH68-140, contained *AVR-Pita1^{JB}* and a paralog designated as *AVR-Pita1^{JD}*, which had a sequence identical to that of *AVR-Pita1^{JA}* except for one base at the 411-bp position (Table 3). The cytidine base at the 411-bp position in *AVR-Pita1^{JD}* changes the amino acid sequence, resulting in Q (119).

Southern blot analyses were carried out to confirm the copy number of *AVR-Pita1* in each isolate (Fig. 4). No bands were detected in GFOS8-1-1, one band was detected in six virulent isolates and an avirulent isolate, and two bands were detected in a virulent isolate and five avirulent isolates. The copy numbers determined from Southern blot analyses agreed with the results of sequence analyses, except in Ken 54-20. Two bands were detected in the lane of Ken 54-20, although only *AVR-Pita1^{JA}* was amplified. DNA of Ken 54-20 was separated by agarose electrophoresis and the bands were extracted from agarose gel slices; then, *AVR-Pita1* genes were amplified and sequenced. The results showed that both bands corresponded to *AVR-Pita1^{JA}* in Ken 54-20.

Because almost all Japanese isolates contained *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*, a method for selective amplification of *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* was developed. Ken 54-20 and P06-1, which contained only *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*, respectively, were used to develop optimal conditions for specific amplification. We tested six primer sets that were designed using nucleotides that differed between *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*. Two primer sets, 5'AVR-Pita1^{JA}F/3'AVR-Pita1^{JA}R and 5'AVR-Pita1^{JB}F/3'AVR-Pita1^{JB}R, were the most useful for paralog-specific amplification.

Fig. 4. Southern blot analysis of *AVR-Pita1* in field isolates collected from diverse areas in Japan. Genomic DNA was digested with *Kpn*I and *Apa*LI, separated electrophoretically on a 0.67% agarose gel, and then transferred to a nylon membrane. A labeled fragment that was amplified with BamHIAVR-PitaN89 and BamHIAVR-PitaR1 and digested with *Bam*HI and *Apa*LI was used as a probe. Hybridization was performed using the AlkPhos Direct Labeling and Detection System (GE Healthcare, UK Ltd.) according to the manufacturer's instructions. Names of isolates are shown above photographs. Sizes of markers are shown at left. A, avirulent; V, virulent.

Annealing at 64°C enabled each primer set for *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* to amplify *AVR-Pita1^{JA}* in Ken54-20 and *AVR-Pita1^{JB}* in P06-1 exclusively (Fig. 5). We used this method to investigate 18 avirulent isolates, 13 of which were obtained from commercial fields in 2001 (15) and 5 of which were stored at NARC (7). The result revealed that 17 isolates contained both *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* and 1 contained only *AVR-Pita1^{JA}* (Fig. 5).

DISCUSSION

AVR-Pita1^{JB} was retained in all G7a-derived mutants whereas *AVR-Pita1^{JA}* was deleted or altered. This result indicates that *AVR-Pita1^{JA}* is functional in G7a but *AVR-Pita1^{JB}* is not. The presence of *AVR-Pita1^{JB}* in seven virulent field isolates confirms that *AVR-Pita1^{JB}* is not functional in those isolates, just as it is not functional in G7a. Furthermore, our results suggest that *AVR-Pita1^{JA}* and *AVR-Pita1^{JD}* would be functional in avirulent isolates. Gene expression confirmed that the upstream region (−489 to −9 bp) of *AVR-Pita1* functions fully as a promoter (12). Sequence analyses showed 100% identity of promoter sequences between *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*, which suggests that the transcriptional activity is the same in these two genes. The fact that *AVR-Pita1^{JB}* is nonfunctional may be due to differences in amino acid residues.

Analyses of G7a-derived mutants showed that the most frequent mutation was the loss of *AVR-Pita1^{JA}*. In addition, PCR and sequence analyses confirmed that 38 G7a-derived mutants lacked *AVR-Pita1^{JA}* (data not shown), except for those shown in Table 2. Likewise, Orbach et al. (20) reported that the most frequent mutation by which avirulent isolates became virulent in rice cultivars containing the *Pita* gene was the loss of *AVR-Pita1*. The high frequency of gene loss of this gene probably resulted from its telomeric location. Except for SaYF1, virulent isolates did not carry *AVR-Pita1* paralogs or carried only *AVR-Pita1^{JB}*. On the other hand, avirulent isolates, except for TH68-140, carried only *AVR-Pita1^{JA}* or carried both *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*. These results suggest that loss of *AVR-Pita1^{JA}* is the most prominent mechanism of acquiring virulence against rice cultivars containing the *Pita* gene in Japanese rice blast populations.

AVR-Pita1 was originally reported as a single-copy gene by analysis of O-137 (20). A number of isolates in the United States and Cameroon showed two bands in hybridization analyses of *AVR-Pita1* (12,29); however, the effects of two genes on pathogenesis in *Pita*-containing rice remained unclear. This study clarifies impacts of two *AVR-Pita1* genes by analyzing a Japanese isolate carrying two paralogs and its mutants.

In U.S. isolates, virulence or avirulence against a *Pita*-containing rice cultivar could be determined using PCR amplifications using a primer set that was designed from the promoter sequence of *AVR-Pita1* and sequences from the 3' side of *AVR-Pita1* (29). In Japanese isolates, however, this PCR amplification is ineffective because both avirulent and virulent isolates contain *AVR-Pita1^{JB}*, and the primer sets used in that study could amplify both of the *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* paralogs. In this study, we developed a method to selectively amplify *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*. This method will be useful for predicting virulence of rice blast fungal strains in Japan. The amino acid sequence of *AVR-Pita1* in an avirulent isolate from the United States, A347, is identical to that of *AVR-Pita1^{JB}*. The reason why *AVR-Pita1* of A347 was functional in a previous study (29) remains unclear. One possibility is that A347 has a functional avirulence gene such as *AVR-Pita2* as well as the nonfunctional *AVR-Pita1*.

Isolates from the United States show higher diversity of *AVR-Pita1* than Japanese isolates (29). Don and coworkers (6) investigated the population structure of Japanese isolates by DNA fingerprinting using the probes MAGGY and MGR586, and showed that genetic diversity was lost in the period between 1960 and 1976. In Japan, there are two lineages, JL1 and JL2, with 97% of Japanese field isolates in the JL1 lineage. Our analysis suggested that the majority of the avirulent field isolates in Japan carried both *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*. Furthermore, Ken 54-04, which is in the JL1 lineage (6), contains *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*, like almost all the avirulent field isolates, but Ken 54-20, which is in the JL2 lineage (6), contains only *AVR-Pita1^{JA}*. These results suggest that the JL1 lineage may have carried two paralogs, *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*. Similar analyses of MGR586 revealed that rice blast isolates in the southern United States can be grouped into eight lineages and those in the Philippines can be grouped into six lineages (16,28). In Japan, the rice blast population is derived from only two lineages. This may explain the low diversity of *AVR-Pita1* in Japanese rice blast isolates.

On the basis of the results in this study, we suggest that the main reason for gain of virulence against *Pita*-containing rice in the field is deletion of the functional *AVR-Pita1*. Recently, the avirulence genes of rice blast, *AVR-Pii*, *AVR-Pia*, and *AVR-Pik*, were isolated and investigated (18,27). These results demonstrated that the presence or absence of these avirulence genes is consistent with the avirulence or virulence of blast isolates, as is the case in *AVR-Pita1^{JA}*. The instability of these newly isolated genes is probably because they are located near telomeric regions

Fig. 5. Specific amplification and identification of *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}* in Japanese isolates. **A**, Specific primers to amplify *AVR-Pita1^{JA}*, 5'*AVR-Pita1^{JA}*F, and 3'*AVR-Pita1^{JA}*R. **B**, Specific primers to amplify *AVR-Pita1^{JB}*, 5'*AVR-Pita1^{JB}*F, and 3'*AVR-Pita1^{JB}*R. Names of isolates are shown above photographs. Molecular weight markers are shown at left. For polymerase chain reaction (PCR), the 20- μ l reaction mixture contained 50 ng of genomic DNA, 1 pmol each primer, 2 μ l of 10 $\times$ reaction buffer, 25 mM dNTP, and 1.25 units of *ExTaq* polymerase (Takara Bio. Inc.). The PCR cycling program was as follows: initial denaturation for 7 min at 94°C; 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 64°C, and 90 s of extension at 72°C; and then final extension for 7 min at 72°C. Previous sequence analyses confirmed that OS99-G-7a contained both *AVR-Pita1^{JA}* and *AVR-Pita1^{JB}*, Ken54-20 contained only *AVR-Pita1^{JA}*, and P06-1 contained only *AVR-Pita1^{JB}*. These three isolates were used to validate that the method was paralog specific. All isolates except P06-1 were avirulent against *Pita*-containing rice.

or various transposons. The presence of two paralogs suggests that the instability of *AVR-Pita1* could result in either a decrease or an increase in copy number.

We identified a nonfunctional *AVR-Pita1* paralog that is carried in both avirulent and virulent Japanese rice blast isolates. Non-functional *AVR-Pik* paralogs were also found in a number of isolates (27). The presence of nonfunctional paralogs also raises the possibility that these avirulence genes have roles other than triggering defense reactions, and are being positively selected for in the field. Furthermore, if these nonfunctional paralogs also have roles other than triggering defense reactions, an isolate carrying one or more nonfunctional paralogs besides a functional paralog may evolve virulence more frequently than one carrying only a functional paralog, which would be completely inactivated by a deletion mutation.

ACKNOWLEDGMENTS

We thank S. Koizumi for invaluable suggestions and A. Goto for help in sampling of rice blast isolates.

LITERATURE CITED

- Abe, S. 2004. Breeding of a blast resistant multiline variety of rice, Sasanishiki BL. JARQ 38:149-154.
- Asaga, K. 1995. Resistance in rice cultivars. Pages 216-249 in: Rice Blast Disease, 2nd ed. T. Yamanaka and T. Yamaguchi, eds. Yokendo Co. Ltd., Tokyo. (In Japanese)
- Ashikawa, I., Hayashi, N., Yamane, H., Kanamori, H., Wu, J., Matsumoto, T., Ono, K., and Yano, M. 2008. Two adjacent nucleotide-binding site-leucine-rich repeat class genes are required to confer *Pikm*-specific rice blast resistance. Genetics 180:2267-2276.
- Bryan, G. T., Wu, K.-S., Farrall, L., Jia, Y., Hershey, H. P., McAdams, S. A., Faulk, K. N., Donaldson, G. K., Tarchini, R., and Valent, B. 2000. A single amino acid difference distinguishes resistant and susceptible paralogs of the rice blast resistance gene *Pi-ta*. Plant Cell 12:2033-2046.
- Dean, R. A., Talbot, N. J., Ebbole, D. J., Farman, M. L., Mitchell, T. K., Orbach, M. J., Thon, M., Kulkarni, R., Xu, J. R., Pan, H., Read, N. D., Lee, Y. H., Carbone, I., Brown, D., Oh, Y. Y., Donofrio, N., Jeong, J. S., Soanes, M. D., Djonovic, S., Kolomiets, E., Rehmeier, C., Li, W., Harding, M., Kim, S., Lebrun, M. H., Bohnert, H., Coughlan, S., Butler, J., Calvo, S., Ma, L. J., Nicol, R., Purcell, S., Nusbaum, C., Galagan, J. E., and Birren, B. W. 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature 434:980-986.
- Don, L. D., Kusaba, M., Urashima, A. S., Tosa, Y., Nakayashiki, H., and Mayama, S. 1999. Population structure of the rice blast fungus in Japan examined by DNA fingerprinting. Ann. Phytopathol. Soc. Jpn. 65:15-24.
- Hayashi, N. 2005. Rice Blast Fungus. MAFF Microorganism Genetic Resources Manual (18). National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan. (In Japanese)
- Hirayae, K., Fujimoto, T., Yamaguchi, J., Nakajima, T., and Fujita, Y. 2008. Specific DNA markers of *Pyricularia grisea* isolates, Kyu9439013 and OS99-G-7a. Kyushu Plant Prot. Res. 54:1-6. (In Japanese)
- Ishizaki, K., Matsui, T., and Harasawa, R. 2005. Practical application of multiline variety with blast resistance gene of Koshihikari in Niigata prefecture. Jpn. J. Crop. Sci. 74:438-443. (In Japanese)
- Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. 19:4004-4014.
- Kang, S., Lebrun, M. H., Farrall, L., and Valent, B. 2001. Gain of virulence caused by insertion of a *Pot3* transposon in a *Magnaporthe grisea* avirulence gene. Mol. Plant-Microbe Interact. 14:671-674.
- Khang, C. H., Park, S.-Y., Lee, Y.-H., Valent, B., and Kang, S. 2008. Genome organization and evolution of the *AVR-Pita* avirulence gene family in the *Magnaporthe grisea* species complex. Mol. Plant-Microbe Interact. 21:658-670.
- Kiyosawa, S. 1984. Establishment of differential varieties for pathogenicity tests of rice blast fungus. Rice Genet. Newsl. 1:95-97.
- Kiyosawa, S., and Cho, C. I. 1973. Relation of the sporulating ability on the rice plant to some other characters in blast fungus strains. Jpn. J. Breed. 23:239-244.
- Koizumi, S., Iwano, M., Zenbayashi, K., dela Pena, F. A., Sonoda, R., Nakajima, T., Arai, M., Nakajima, T., Miyasaka, A., Ashizawa, T., Yasuda, N., and Noguchi, M. T. 2007. Distribution of pathogenic races of rice blast fungus in Japan in 2001. Misc. Publ. Natl. Agric. Res. Cent. 7:1-63. (In Japanese)
- Levy, M., Romao, J., Marchetti, M. A., and Hamer, J. E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95-102.
- Marchetti, M. A. 1994. Race-specific and rate-reducing resistance to rice blast in US rice cultivars. Pages 231-244 in: Rice Blast Disease. R. S. Zeigler, S. A. Leong, and P. S. Teng, eds. CAB International, Wallingford, UK.
- Miki, S., Matsui, K., Kito, H., Otsuka, K., Ashizawa, T., Yasuda, N., Fukiya, S., Sato, J., Hirayae, K., Fujita, Y., Nakajima, T., Tomita, F., and Sone, T. 2009. Molecular cloning and characterization of the *AVR-Pia* locus from a Japanese field isolate of *Magnaporthe oryzae*. Mol. Plant Pathol. 10:361-374.
- Nakayashiki, H., Matsuo, H., Chuma, I., Ikeda, K., Betsuyaku, S., Kusaba, M., Tosa, Y., and Mayama, S. 2001. *Pyret*, a *Ty3/Gypsy* retrotransposon in *Magnaporthe grisea* contains an extra domain between the nucleocapsid and protease domains. Nucleic Acids Res. 29:4106-4113.
- Orbach, M. J., Farall, L., Sweigard, J. A., Chumley, F. G., and Valent, B. 2000. A telomeric avirulence gene determines efficacy for the rice blast resistance gene *Pi-ta*. Plant Cell 12:2019-2032.
- Rybka, K., Miyamoto, M., Ando, I., Saito, A., and Kawasaki, S. 1997. High resolution mapping of the *indica*-derived rice blast resistance gene II. *Pi-ta*² and *Pi-ta* and a consideration of their origin. Mol. Plant-Microbe Interact. 10:517-524.
- Takahashi, M., Ashizawa, T., Hirayae, K., and Moriwaki, J. 2008. Estimating the mutation rate of the rice blast fungus from avirulence to virulence in the paddy field. Proc. Assoc. Plant Prot. Hokuriku 57:11-17. (In Japanese)
- Tomita, K., Horiuchi, H., Terada, K., Kobayashi, A., Tanaka, I., Tanoi, M., Minobe, T., Furuta, H., Yamamoto, A., Masaki, N., Minami, T., Sugimoto, A., Kagoshima, C., and Horiuchi, K. 2006. "Hanaechizen BL1", "Hanaechizen BL2", "Hanaechizen BL3", "Hanaechizen BL4", new rice isogenic lines with true resistant genes to blast. Bull. Fukui Agric. Exp. Stn. 43:1-16. (In Japanese)
- Valent, B., and Chumley, F. G. 1991. Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. Annu. Rev. Phytopathol. 29:443-476.
- Yamada, M., Kiyosawa, S., Yamaguchi, T., Hirano, T., Kobayashi, T., Kushibuchi, K., and Watanabe, S. 1976. Proposal of new method for differentiating races of *Pyricularia oryzae* Cavara in Japan. Ann. Phytopathol. Soc. Jpn. 42:216-219.
- Yamaguchi, T. 1980. Experimental methods for study of rice blast fungus. Pages 140-174 in: Rice Blast Disease and Breeding for Resistance. Y. Yamasaki and T. Kosaka, eds. Hakuyusha Publishers, Tokyo. (In Japanese)
- Yoshida, K., Saitoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., Yoshida, K., Tosa, Y., Chuma, I., Takano, Y., Win, J., Kamoun, S., and Terauchi, R. 2009. Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen *Magnaporthe oryzae*. Plant Cell 21:1573-1591.
- Zeigler, R. S., Couc, L. X., Scott, R. P., Bernardo, M. A., Chen, D. H., Valent, B., and Nelson, R. J. 1995. The relationship between lineage and virulence in *Pyricularia grisea* in the Philippines. Phytopathology 85:443-451.
- Zhou, E., Jia, Y., Singh, P., Correll, J. C., and Lee, N. F. 2007. Instability of the *Magnaporthe oryzae* avirulence gene *AVR-Pita* alters virulence. Fungal Genet. Biol. 44:1024-1034.