A Rapid Survey of Avirulence Genes in Field Isolates of *Magnaporthe oryzae*

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Abstract

Magnaporthe oryzae is the causal agent for the devastating disease rice blast. The avirulence (AVR) genes in *M. oryzae* are required to initiate robust disease resistance mediated by the corresponding resistance (R) genes in rice. Therefore, monitoring pathogen AVR genes is important to predict the stability of R gene-mediated blast resistance. In the present study, we analyzed the DNA sequence dynamics of five AVR genes, namely, AVR-Pita1, AVR-Pik, AVR-Pizt, AVR-Pia, and AVR-Pii, in field isolates of *M. oryzae* in order to understand the effectiveness of the R genes, Pi-ta, Pi-k, Pi-zt, Pia, and Pii in the Southern U.S. rice growing region. Genomic DNA of 258 blast isolates collected from commercial fields of the Southern UNITED STATES during 1975–2009 were subjected to PCR amplification with AVR gene-specific PCR markers. PCR products were obtained from 232 isolates. The absence of PCR products in the remaining 26 isolates suggests that these isolates do not contain the tested AVR genes. Amplified PCR products were subsequently gel purified and sequenced. Based on the presence or absence of the five AVR genes, 232 field isolates were classified into 10 haplotype

groups. The results revealed that 174 isolates of *M. oryzae* carried AVR-Pita1, 225 isolates carried AVR-Pizt, 44 isolates carried AVR-Pik, 3 isolates carried AVR-Pia, and one isolate carried AVR-Pii. AVR-Pita1 was highly variable, and 40 AVR-Pita1 haplotypes were identified in avirulent isolates. AVR-Pik had four nucleotide sequence site changes resulting in amino acid substitutions, whereas three other AVR genes, AVR-Pizt, AVR-Pia, and AVR-Pii, were relatively stable. Two AVR genes, AVR-Pik and AVR-Pizt, were found to exist in relatively larger proportions of the tested field isolates, which suggested that their corresponding R genes Pi-k and Pi-zt can be deployed in preventing blast disease in the Southern UNITED STATES in addition to Pi-ta. This study demonstrates that continued AVR gene monitoring in the pathogen population is critical for ensuring the effectiveness of deployed blast R genes in commercial rice fields.

Keywords: rice blast, *Magnaporthe oryzae*, field isolates, avirulence gene, southern United States

Rice blast disease caused by *Magnaporthe oryzae* B. Couch (synonymous with *Pyricularia oryzae*) is one of the major constraints of rice production worldwide. The severity of blast disease varies each year depending on weather patterns, and is more pronounced under upland conditions and in areas where water is limited for irrigation. Contemporarily, blast disease has been managed using resistant cultivars and fungicides integrated into cultural practices. In the United States alone, approximately \$69 million are spent annually for fungicide applications to prevent blast disease (Nalley et al. 2016), and the cost to prevent blast disease is predicted to be greater in situations where there is a water shortage. Utilization of resistant rice cultivars with major blast resistance (R) genes is an economical and environmentally benign method to control blast disease. Thus far, over 100 major blast R genes in cultivated and wild rice relatives have been identified, and more than 30 of them have been cloned (Liu et al. 2010; Mekwatanakarn et al. 2000; Okuyama et al. 2011; Silue et al. 1992; Wang et al. 2017; Zhai et al. 2011; Zhao et al. 2018). Several major R genes have been used for improving blast resistance worldwide. These major R genes are effective in preventing infections by isolates/races of *M. oryzae* carrying the corresponding

avirulence (AVR) genes in a gene-for-gene fashion (Silue et al. 1992). Consequently, any AVR gene can be an indicator of the effectiveness of the cognate major R gene (Selisana et al. 2017).

To date, 40 AVR genes in *M. oryzae* have been genetically analyzed (Ma et al. 2006), and among them, 11 AVR genes (PWL2, AVR-Pita1, ACE1, AVR-CO39, AVR-Pizt, AVR-Pik, AVR-Pia and AVR-Pii, AVR-Pib, AVR-Pi9, and AVR-Pi54) have been cloned and characterized (Bohnert et al. 2004; Farman and Leong 1998; Li et al. 2009; Ray et al. 2016; Sweigard et al. 1995; Wu et al. 2015; Yoshida et al. 2009; Zhang et al. 2015). AVR-Pita1 controls avirulence in Pi-ta-containing rice cultivars. The product of AVR-Pita1 was determined to a putative mature protein AVR-Pita₁₇₆, which interacted with the Pi-ta protein in rice cells to trigger the resistance response (Jia et al. 2000). Different resistance Pi-k alleles directly recognize multiple AVR-Pik alleles encoding a protein with 113 amino acids. Five variants encoded by five alleles of AVR-Pik, AVR-Pika, AVR-PikB, AVR-PikC, AVR-PikD, and AVR-PikE were identified with single amino acid and single nucleotide differences (Yoshida et al. 2009). AVR-Pizt, recognized by Pizt, can suppress the BAX-mediated programmed cell death in *Nicotiana benthamiana*, suggesting that AVR-Pizt may be involved in pathogenicity of *M. oryzae* (Li et al. 2009). The other five AVR genes, including AVR-Pii and AVR-Pia, were identified by associations between putative secreted proteins, typical characteristics of AVR gene coding proteins, and AVR phenotypes, after aligning the genomes between avirulent and virulent *M. oryzae* strains (Ray et al. 2016; Yoshida et al. 2009).

In the Southern UNITED STATES after the first resistant cultivar Katy with Pi-ta was released in 1990 (Zhou et al. 2007), at least 11 rice cultivars with Pi-ta have been developed to manage blast disease (Roychowdhury et al. 2012). Thus far, rice cultivars with different combinations of Pi-ta, Pi-b, Pi-ks, Pi-kh, and Pi-z have been effectively deployed (Khang et al. 2008; Roychowdhury et al. 2012) to provide overlapped resistance to 10 common blast races of *M. oryzae*, namely, IA45, IB-1, IB45, IB49, IB54, IC17, ID1, IE1, IG1, and IH1 (Dai et al. 2010; Jia et al. 2009). Blast disease was insignificant during the late 1970s. The severe drought in 1980 restricted blast

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epidemics until 1986, when approximately 60,000 acres of Newbonnet was damaged by “new” blast races IC-17 and IB-49. During 1987, blast was widespread in Arkansas, which produces 50% of the rice in the United States, and losses were estimated to be around 10%. In 1991, blast was exceedingly severe and widespread in all southern states, being described as the worst observed in many years (Hollier and Groth 2000; Lee 1992). Other outbreaks occurred in 2012–2015, and three blast races, IB-17, IB-49, and IC-17, were identified as the causal agents in Texas (Groth 2016; Zhou 2016).

The stability of blast resistance mediated by *Pi-ta* was determined by molecular structure and expression of *AVR-Pital* (Jia et al. 2000). Subsequent natural variation of *AVR-Pital* in selected field isolates was documented (Xing et al. 2013; Zhou et al. 2007). However, it is unknown if these field isolates also contain other cloned *AVR* genes.

The objectives of the present study were to: 1) develop *AVR*-gene-specific primers, 2) survey the presence of *AVR* genes in field isolates in the Southern United States, 3) determine sequence variation of protein coding region of *AVR* genes, 4) determine the relationship between races and *AVR* genes, and 5) identify effective blast *R* genes to enhance rice breeding in the Southern United States.

Materials and Methods

Fungal isolates and culture. A total of 258 isolates of *M. oryzae* recovered from diseased leaves of rice collected during 1975–2009 in the state of Arkansas, U.S.A., were used in the study (Supplementary Table S1). The isolates include wild-type field isolates with or without *AVR-Pital* from rice. A disc at 3–5 mm with fungal mycelia and spores of different isolates described in Dai et al. (2010) and Xing et al. (2013) stored at –20°C on desiccated filter papers were removed by forceps and grown in a 5-cm petri dish containing oatmeal agar (Difco Laboratories Inc., Sparks, MD, U.S.A.) in a 26°C dark incubator for 10 days to produce fungal mycelia for DNA extraction.

DNA extraction, PCR primers, amplification, and sequencing. A rapid DNA extraction method of Saitoh et al. (2006) was modified as follows: A patch of mycelia at 1 cm² removed from the oatmeal agar plate with a toothpick was transferred to a 1.5 ml micro tube with 500 µl lysis buffer (200 mM Tris-HCl, 50 mM EDTA, 200 mM NaCl, 1% SDS, pH 8.0). The mycelia blocks were dispersed in the buffer by vortexing, and then were incubated for 20 min at 70°C. The mixture was centrifuged at 18,000 × *g* for 5 min, and 300 µl supernatant were removed, and transferred to a new 1.5 ml micro tube and mixed with 750 µl ethanol (200 proof), the new mixture was centrifuged at 18,000 × *g* for 5 min to precipitate DNA. All liquid was removed gently with vacuum and tips. Resulting DNA pellets were washed with 1 ml 70% ethanol by centrifuging at 18,000 × *g* for 2 min, and were dried at room temperature for at least 4 h, and then dissolved in 20 µl TE for PCR amplification. Specific primers for four *AVR* genes, *AVR-Pizt*, *AVR-Pik*, *AVR-Pia*, and *AVR-Pii*, were designed and synthesized based on their genomic DNA sequences at 5′ leaders and 3′ trailers (GenBank accessions: JN639897, AB498875,

AB498873, and AB498874). Specific primers for *AVR-Pital* were from Zhou et al. (2007) (Table 1).

All PCR reactions were performed using *Taq* PCR Master Mix (Qiagen Inc., Valencia, CA, U.S.A.). Each PCR reaction consisted of the following components: 10 µl of *Taq* PCR Master Mix (0.5 U/µl of *Taq* DNA polymerase, 2× Qiagen PCR buffer, 3 mM MgCl₂, and 400 µM of each dNTP), 0.8 µl of each 10 µM primer, 0.8 µl fungal genomic DNA solution, and 8.4 µl distilled water (provided by Qiagen Kit) in a final reaction volume of 20 µl. Reactions were performed in a Thermal Cycler (PTC-200, MJ Research, Waltham, MA, U.S.A.) with the following PCR program: one cycle at 95°C for 3 min for initial denaturation, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and a final extension of 72°C for 8 min. All PCR reactions were repeated three times. The PCR products were separated by 1.0% (wt/vol) agarose gel electrophoresis and stained with SYBR safe (Invitrogen Inc., Grand Island, NY, U.S.A.), visualized using a Bio-Rad Gel Photographic System (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). The size of the amplified fragment was estimated by GeneRuler Express DNA Ladder (Fermentas Inc., Glen Burnie, MD, U.S.A.).

PCR products were gel purified and sequenced using the same primer for PCR amplification in an ABI 3730XL DNA Analyzer using 0.5 µl BigDye v3.1 at the United States Department of Agriculture–Agricultural Research Service Genomics and Bioinformatics Research Unit at Stoneville, MS. Each sample was sequenced three times with separate PCR products.

DNA and amino acid sequence analysis. The sequences of DNA and predicted amino acids were assembled by Vector NTI software (Invitrogen Inc., Carlsbad, CA, U.S.A.). DNA sequences consisting of the coding regions of four *AVR* genes were aligned using AlignX of Vector NTI 11.0 software (Zhou et al. 2007).

Plant growth and disease evaluation. Rice seedlings were grown to V3–V4 stage for blast evaluation under greenhouse conditions. Specifically, 6–10 seeds/ per variety were sown in a tray containing inserts with 8 differential rice varieties, and plants were grown at 25°C with 8-h light and 16-h dark in a greenhouse like the conditions described by Chen et al. (2019). *M. oryzae* isolates were transferred from desiccated filter papers stored at –20°C to oatmeal agar (Difco Laboratories Inc., Sparks, MD, U.S.A.) plates for 7–10 days in the fungal chamber with 22°C, 49% humidity. Fungal spores were washed into a sterile 0.25% (wt/vol) gelatin (ICN Biomedical Inc., Costa Mesa, CA) solution by scraping fungal mycelia on the culture plates. The conidial suspension was filtered through 4 layered cheesecloth to remove mycelia and pieces of agar. The concentration of conidial suspension was determined using the method described by Sambrook and Russell (2001) under a microscope. Briefly, 5 µl of conidial suspension was delivered to a chamber of a hemocytometer by capillary action. The number of conidia in the large center square was determined and then multiplied by 10⁴ to give the number of conidia per ml. A spore concentration of 5 × 10⁴ to 1 × 10⁵ spores/ml was used to inoculate rice seedlings at V3–V4 stage as detailed in

Table 1. Primers for avirulence gene polymerase chain reaction (PCR)

Primer name	Sequence 5′-3′	AVR gene	Primer site ^a	Expected size of PCR product	NCBI accession
YL169 ^b	CGACCCGTTTCCGCC	<i>AVR-Pital</i>	966-980	1,086 bp	AF207841
YL149 ^b	TGACCGCGATTCCCTCCATT		2,032-2,051		
Z21	AATCCCGTCACTTTTCATTCTCCA	<i>AVR-Pizt</i>	1,434-1,456	637 bp	JN639897
Z22	GTCGCAAGCCTCGTACTACCTTT		2,048-2,070		
Z23	TCCAATTTATTCAACTGCCACTC	<i>AVR-Pik</i>	1,289-1,311	532 bp	AB498875
Z24	GTAACCTCGTCAAACCTCCCTA		1,798-1,820		
Z27	CCCATTATCTTACCAGTCGCTTGA	<i>AVR-Pia</i>	939-962	868 bp	AB498873
Z28	ATTCTCCCGTAAACAGTAAACC		1,784-1,806		
ZZ41	TGCAGGCCCAAATCCGTAGGAA	<i>AVR-Pii</i>	364-385	508 bp	AB498874
ZZ42	ACTGTCCGCCGCTCGTTTGG		852-871		

^a Locations of the five *AVR* genes were based on GenBank accession no. AF207841, JN639897, AB498875, AB498873, and AB498874.

^b These two primers were designed by Zhou et al. (2007).

Wang et al. (2015). The 0–5 rating system was used to evaluate disease reactions, where 0–2 was resistant and 3–5 susceptible. The race determination using eight international rice differentials was as described in Atkins et al. (1967) and Xing et al. (2013).

Results

AVR gene-specific markers. Gene-specific primers ZZ21/ZZ22 for *AVR-Pizt*, ZZ23/ZZ24 for *AVR-Pik*, ZZ27/ZZ28 for *AVR-Pia*, and ZZ41/ZZ42 for *AVR-Pii* (Table 1, Fig. 1) were successfully designed to amplify the corresponding AVR genes. As shown in Figure 1, DNA fragments were estimated to be 637, 532, 868, and 508 base pairs (bp) indicative of the presence of *AVR-Pizt*, *AVR-Pik*, *AVR-Pia*, and *AVR-Pii*, respectively. To verify the identity of four AVR genes, PCR products were sequenced. DNA sequences were blasted against the GenBank database (<https://www.ncbi.nlm.nih.gov>), the results showed 100% identity with DNA fragments from the coding region of four AVR genes (Table 1). These results suggest that all primer pairs developed were specific for the predicted AVR genes.

AVR genes in field isolates. A total of 258 field isolates collected from 1975 to 2009 were analyzed by PCR using the above-mentioned gene-specific markers. Based on the presence or absence of predicted PCR products for each of four AVR genes, the specific PCR amplifications for *AVR-Pizt*, *AVR-Pik*, *AVR-Pia*, and *AVR-Pii* were detected in 232 isolates. Among these isolates were 225 with *AVR-Pizt*, 44 with *AVR-Pik*, 3 with *AVR-Pia*, and one with *AVR-Pii* (Fig. 2).

By examining the presence and absence of 5 AVR genes, among which the information on *AVR-Pita1* was previously described (Dai et al. 2010; Xing et al. 2013), 258 field isolates were classified into 11 haplotype groups (Fig. 2). Among them, 7 haplotype groups consisting of 175 isolates have at least two AVR genes, 3 haplotype groups consisting of 57 isolates have single AVR genes *AVR-Pita1*, *AVR-Pizt*, and *AVR-Pik*. The last haplotype group consisting of 26 isolates has no detectable AVR genes.

Natural variation of AVR genes. A total of 40 different *AVR-Pita1* haplotypes were previously described (Dai et al. 2010; Xing et al. 2013), and DNA sequences of *AVR-Pizt*, *AVR-Pia*, and *AVR-Pii* in these field isolates were identical to their respective AVR gene sequences (Li et al. 2009; Yoshida et al. 2009).

A total of 6 variants of *AVR-Pik* were identified, among them, amino acid variants A to F differing in the amino acids in the protein at the 46th, 47th, 48th, 67th, and 78th positions (Yoshida et al. 2009). The haplotypes D, E, and A determine resistance specificities to *Pik/Pikm/Pikp*, *Pik/Pikm*, and *Pikm*, respectively (Kanzaki et al. 2012;

Yoshida et al. 2009). The corresponding *R* alleles as determined by other haplotypes are unknown. The change of amino acid H to N at the 46th site between haplotype D and E exchanged resistance specificity between *Pik/Pikm/Pikp* and *Pik/Pikm*. The amino acids at 67th and 78th were critical to maintain the function of *Pik/Pikm* and *Pik/Pikm/Pikp*, respectively. Among the 44 isolates amplified of *AVR-Pik*, 36 isolates obtained the consistent sequence of *AVR-Pik*. We found four polymorphic nucleotide sites resulting in four amino acid substitutions and attributing to three haplotypes in the other 36 isolates (Fig. 3). Eight of 36 isolates had the variant D, seven isolates had the variant A, and 21 isolates had a new variant F with amino acid K. For the other 8 isolates, double peaks showed in the chromatogram of DNA sequences (Fig. 3C), suggesting that these isolates resulted in 5 new *AVR-Pik* variants, suggesting on-going diversification within the gene (Fig. 3D).

Relationship between races and AVR genes. Blast races determined by 8 international differential rice varieties have been used to guide breeders to develop blast-resistant rice varieties. To determine the relationship between races and AVR genes, most isolates of 7 races, IB-1, IC17, IA1, IC1, IE1, IB49, and IE1K (race K, Dai et al. 2010), among a total of 22, races were identified (Fig. 2, Xing et al. 2013). Among them, we found each race had more than one AVR gene. Namely, the isolates of the races IB-1 and IG1 had 4 AVR genes; the isolates of each of the races IB49, IC17, ID13, IE1, IH1, and IB45 had 3 AVR genes; and the isolates for each of 13 races had two AVR genes (Table 2). These findings demonstrated that more than one *R* gene can be deployed to prevent infections by each of these blast races.

Effective blast *R* genes. In the present study, we detected the existence of five AVR genes, *AVR-Pita1*, *AVR-Pizt*, *AVR-Pik*, *AVR-Pia*, and *AVR-Pii* blast strains, isolated from across different production areas in the Southern United States. Our findings suggest that *Pita*, *Pizt*, *Pik*, *Pia*, and *Pii* can be used to prevent blast disease in areas where these blast samples were collected. Among them, *Pizt*, *Pita*, and *Pik* are much more effective than *Pia* and *Pii* due to the presences of much higher proportions in the studied isolates.

Discussion

In this study, we developed a PCR-based method and predicted the effectiveness and durability of major blast *R* genes by analyzing natural variation of AVR genes in 232 isolates from commercial rice fields in the Southern UNITED STATES from 1975 to 2009. Our study validated the effectiveness of deployed blast *R* genes in the Southern UNITED STATES and provided insights into molecular changes for different AVR genes. In the Southern UNITED STATES, the race-specific *R* genes

Fig. 1. Polymerase chain reaction (PCR) detection of the four AVR genes. Genomic DNA was used as a template for each PCR amplification. The size of PCR amplicon was estimated by an identical kilobase marker - GeneRuler Express DNA Ladder with indicated sizes in base pair. **A**, PCR products of *AVR-Pizt*, *AVR-Pik*, and *AVR-Pia*. **B**, PCR product of *AVR-Pii*.

Fig. 2. Graphic presentation of AVR genes in 258 blast isolates. Name of AVR gene is indicated. Number on X axis indicates total number of isolates carrying each AVR gene; number on Y axis indicates total number of isolates carrying different AVR genes as indicated by different colors.

such as *Pi-ta/Ptr*, *Pi-kh*, and *Pi-ks* have been effectively used in blast resistance breeding (Fjellstrom et al. 2004). Among them, *Pi-ta/Ptr* provided the widest spectrum of resistance to *M. oryzae* races commonly found in this region, IA45, IB-1, IB49, IB54, IB45, IH1, IG1, IC17,

and IE1; whereas *Pi-kh* provided resistance to IB45, IB54, IH1, and IG1, and *Pi-ks* to one blast race, IB54.

Monitoring pathogen population dynamics in commercial rice fields is critical for guiding stable and effective *R* gene deployment.

Fig. 3. Natural variation at the AVR-Pik locus. Amino acids of AVR-Pik variants, A to F for the five variable sites. **A**, Amino acids of six AVR-Pik allelic products for the five variable sites. **B**, Nucleotide sequence of AVR-Pik in the present study for the five variable amino acid sites. For double peak pattern 1, there is a double peak in the 136th nucleotide site. For double peak pattern 2, there are 3 double peaks in the 136th, 139th, and 143rd nucleotide sites, respectively, **(C)** and new AVR-Pik variants deduced from double peaks **(D)**. Amplicons of double peaks using primer Z24 were shown. Single letter code was used for amino acid.

Table 2. Distribution of AVR genes in 22 international blast races

Name of AVR genes	Blast race ^a																			
	IA1	IB1	IB17	IB49	IC17	ID13	IE1	IG1	IH1	IA17	IA27	IB2	IB18	IB22	IB45	IC1	IC11	IC13	IC18	ID1
AVR-Pita1	14 ^b	64	4	13	28	3	15	9	4	1	1	2	3	2	1	2	1	1	1	4
AVR-Pizt	20	79	5	13	29	3	15	9	4	1	1	2	3	2	2	18	1	1	1	4
AVR-Pik		13		10	1	2	5	7	4						1					
AVR-Pia		3																		
AVR-Pii								1												

^a The race was determined using methods as described in Atkins et al. (1967) and Xing et al. (2013).

^b Indicates the total number of isolates in indicated race group.

The most durable blast *R* gene in the host often recognizes the most stable *AVR* gene found in the pathogen population. The *AVR-Pita1* gene is located near the highly unstable telomere region of the *M. oryzae* chromosome (Khang et al. 2008; Orbach et al. 2000). Genomic changes involved in insertion, deletion, and transposition were reported to occur at the *AVR-Pita1* locus in virulent field isolates, and 40 *AVR-Pita1* haplotypes encoding highly similar proteins with a few amino acid differences were identified in avirulent isolates (Dai et al. 2010; Takahashi et al. 2010; Xing et al. 2013; Zhou et al. 2007). In contrast, only one resistant *Pi-ta* haplotype with a single amino acid difference determining resistance specificity has been identified (Lee et al. 2009a; Wang et al. 2008, 2010). Discovery of 3 rice varieties, Amane, Patchaiperumal, and Hatadawee, having *Pi-ta* that were susceptible to two *M. oryzae* races that carry *AVR-Pita1* (Wang et al. 2010) raised the possibility that *Pi-ta* requires an additional gene to be fully functional. The durability of *Pi-ta* mediated resistance is now better explained by the linked helper gene, *Ptr* (Jia and Martin 2008). *Ptr* encodes a protein with 4 armadillo repeats

(Zhao et al. 2018). Interestingly, *Ptr* confers resistance to a wide range of blast races independent of *Pi-ta*. Knockout mutants of rice at the *Ptr* locus by fast neutrons and CRISPR-CAS9 rendered blast susceptibility in *Pi-ta/Ptr*-containing plants and validated the critical role that *Ptr* plays in *Pi-ta* mediated blast resistance (Zhao et al. 2018).

It was also known that *Pi-ta* can produce 12 transcripts, many of which could encode proteins with a typical NBS-LRR domain indicative of *R* genes (Costanzo and Jia 2009). Further studies of the function of these predicted proteins would help us understand the durability of *Pi-ta* mediated blast resistance and molecular evolution mechanisms of *Pi-ta*.

The resistance specificity was determined by direct interaction of products of both *Pi-k* and *AVR-Pik* (Kanzaki et al. 2012; Zhai et al. 2011, 2014). Changes of amino acids in the products of *AVR-Pik* affected the binding efficiency of the *Pi-k* protein (Kanzaki et al. 2012). The *AVR-Pik* gene encodes a protein with 113 amino acids. Five allelic *AVR-Pik* variants, A, B, C, D, and E, were previously identified

Table 3. Summary of race identity and *AVR-Pik* haplotype

<i>Magnaporthe oryzae</i> isolate ^a	Race	Disease reaction of Kanto 51 (<i>Pi-k</i>)	Origin	Year	The <i>AVR-Pik</i> variant
75A1	IB-1	S	U.S.	1975	D
75L26	IB-1	S	U.S.	1975	A
75A53	ID-13	R	U.S.	1975	D
75T51	ID-13	R	U.S.	1975	D
75L42	IG-1	R	U.S.	1975	A
75A41	IH-1	R	U.S.	1975	D
75A19	IH-1	R	U.S.	1975	D
75L2	IH-1	R	U.S.	1975	D
81L19	IB-1	S	U.S.	1981	A
81L14	IB-1	S	U.S.	1981	F
81F3	IB-49	S	U.S.	1981	A
85L2	IB-1	S	U.S.	1985	A
85F1	IB-49	S	U.S.	1985	F
85M5	IE-1	S	U.S.	1985	F
85A12	IG-1	R	U.S.	1985	D
91A38	IB-1	S	U.S.	1991	A
91A55	IE-1	S	U.S.	1991	F
A119	IB-49	S	U.S.	1992	F
ZN61	IB-49	S	U.S.	1992	F
92A8	IB-49	S	U.S.	1992	F
93M5	IB-49	S	U.S.	1993	F
93M2	IB-49	S	U.S.	1993	F
93M4	IB-49	S	U.S.	1993	F
93A17	IE-1	S	U.S.	1993	F
ZN25	IB-49	S	U.S.	1995	F
ZN04	IE-1	S	U.S.	1995	F
Newbonnet RN5-C 10-18-96	IB-1	S	U.S.	1996	F
Newbonnet RN2A 11-2-96?	IB-1	S	U.S.	1996	F
Newbonnet pine-tree RN5-C Segment 10-18-96	IB-1	S	U.S.	1996	F
Newbonnet PAN8C 10-30-96	IB-1	S	U.S.	1996	F
ZN14	IB-49	S	U.S.	1996	F
ZN27	IC-17	S	U.S.	1996	F
ZN26	IE-1	S	U.S.	1996	F
ZN39	IG-1	R	U.S.	1996	A
IH1	IH-1	R	U.S.	2001	D
Bengal-2	IB-1	S	U.S.	2009	F
75A49	IG-1	R	U.S.	1975	dpp2 ^b
75A13	IG-1	R	U.S.	1975	dpp2
75A49/3	IG-1	R	U.S.	1975	dpp2
24/1-2	IE-1K	S	U.S.	1992	dpp2
82T14	IB-45	R	U.S.	1982	dpp1 ^b
24/1	IG-1	R	U.S.	1992	dpp2
09S36-227R1-A	IB-1	S	U.S.	2009	dpp2
09S32-38R2-B	IB-1	S	U.S.	2009	dpp2

^a Name of original fungal isolates.

^b dpp represents double peak pattern. For dpp1, there is a double peak in the 136th nucleotide site. For dpp2, there are 3 double peaks in the 136th, 139th, and 143rd nucleotide sites, respectively.

(Yoshida et al. 2009). The resistance alleles, *Pi-k*, *Pi-km*, *Pi-kp*, *Pi-ks*, and *Pi-kh*, were mapped at the same location of chromosome 11, and these alleles may be a result of evolution of resistance specificity of the *Pi-k* gene (Ashikawa et al. 2008; Hayashi et al. 2006). It was demonstrated that nucleotide polymorphisms in *AVR-Pik* were all nonsynonymous substitutions providing evidence of positive selection at the *AVR-Pik* locus. Moreover, the amino acid differences among the variants A to E are functionally relevant in the *M. oryzae* and rice interaction (Costanzo and Jia 2010; Yoshida et al. 2009). The D allele is likely the ancestral allele, and the other alleles were derived from the D allele. The D allele was recognized by both *Pik/Pikm* and *Pikp*. The rice differential varieties including rice variety Kato 51 carrying *Pi-k* were used for race evaluation, and the data of blast reaction of Kato 51 supported that the D allele is recognized by *Pi-k* (Table 3). However, the isolate 75A51 carrying the D allele was susceptible to Kanto 51, suggesting that there exists an unknown mechanism of functional recognition of the D variant by *Pi-k*. One possibility is that expression of the D allele in 75A51 was hindered under conditions we used for race determination.

Further sequence analysis of the promoter region of the D allele and examination of expression of the D allele will provide clues to this discrepancy. The E allele was recognized only by *Pi-k/Pi-km*, the A allele was recognized by *Pikm*, and the B/C alleles were recognized neither by *Pik* nor *Pikp*. Difference of recognition specificities of *Pi-ks*, *Pi-kp*, *Pi-kh*, and *Pi-km* has yet not been found.

In the present study, we identified *AVR-PikF* variant with amino acid M instead of K at 78th position in *AVR-PikA*. *AVR-PikF* was initially identified as a double peak in a PCR product (Longya et al. 2019). An impaired interaction between *Pi-k* and *AVR-PikF* resulting in blast susceptibility suggests that amino acid M/K at the 78th position of the *AVR-PikF* variant determines recognition specificity of *Pi-k* (Longya et al. 2019). These data suggest that integrity of the *AVR* protein is critical for triggering robust plant immunity. We identified 5 more *AVR-Pik* variants, G to K, in several field isolates collected from different fields and different collection periods resulting in a total of 11 *AVR-Pik* variants. Further studies will be required to determine if there is any interaction and resistant specificity between different alleles of *Pi-k* with these new *AVR-Pik* variants. Together we suggest that *AVR-Pik* is under duplication and mutation in U.S. commercial rice fields. Further research will be required to validate this prediction.

Extensive deployments of *R* genes often create pressure for the pathogen to change their corresponding *AVR* genes to avoid *R* gene-mediated disease resistance. Historically, rice varieties with different *Pi-k* alleles have been deployed in the United States since 1960 (Wang et al. 2015). As a result, structural changes of *AVR-Pik* in *M. oryzae* in the United States is not totally unexpected. In this study, the existence of 2 variants at the *AVR-Pik* locus were detected in 8 U.S. field isolates, suggesting that there existed an adaptive event (Fig. 3 and Table 3). To support this hypothesis, the double PCR amplicons resulting in a new variant, *AVR-PikF*, was validated by Longya et al. (2019). The presence of additional double peaks of PCR amplicons with *AVR-Pik* specific primer in blast isolates from Thailand were observed and validated by Tansian et al. (2019). Together these findings demonstrate that gene mutation and duplication occurred at *AVR-Pik* in commercial rice fields in Thailand and the United States. Further research will be required to validate these observations.

A total of 42 *AVR-Pital* alleles were found in avirulent blast isolates (Dai et al. 2010; Xing et al. 2013). Deletions of *AVR-Pital* were also found in some virulent isolates (Zhou et al. 2007). Both *AVR-Pital* and *AVR-Pizt* were found to lose the avirulence function due to the *Pot3* insertion at the promoter region (Chen et al. 2014; Kang et al. 2001; Li et al. 2009). Additionally, *Pot3* was found within the coding region of *AVR-Pital* in a virulent field isolate that caused disease on a *Pi-ta* containing rice variety (Zhou et al. 2007). *AVR-Pia* and *AVR-Pii* were cloned based on the presence/absence of *AVR* genes without nucleotide polymorphisms using blast isolates from Japan, China, and Brazil (Yoshida et al. 2009). In the present study, single haplotypes of *AVR-Pia*, *AVR-Pii*, and *AVR-Pizt* were identified in commercial rice fields from the United States. Lack of

molecular variation of these *AVR* genes may be due to the corresponding *R* genes not having been widely deployed in the United States. Deletions of both *AVR-Pia* and *AVR-Pii* may be more effective mechanisms to defeat their cognate *R* genes. Complete relationship between *AVR-Pizt* and *Pi-zt* has not been established; whereas the absence of PCR amplicons of *AVR-Pizt* in six isolates in the present study may not necessarily suggest that *Pi-zt* may not be effective (Selisana et al. 2017). Detailed analysis of reactions of rice varieties carrying *Pi-zt* and sequence analysis of the promoter regions of *AVR-Pizt* in these field isolates may provide more clues to these discrepancies.

The race of each blast isolate was determined with 8 international differential rice varieties, each with different genetic resistance (Atkins et al. 1967; Xing et al. 2013). The presence of several *AVR* genes in each race is not a surprise and is consistent with several studies showing that more than one blast *R* gene can be used to prevent the infection of a single race. For example, the race IB54 can be controlled with rice varieties carrying one of each *R* genes, *Pi-ta*, *Pir*, *Pi42(t)*, *Pi43(t)*, *Piks*, and other *Pik* alleles (Jia et al. 2009, 2019; Lee et al. 2009b; Zhao et al. 2018). This finding may benefit breeders in choosing which *R* gene to be deployed.

In summary, *M. oryzae* is one of the most destructive fungal pathogens for the rice crop, and resistance to this pathogen is a constantly moving target. In the present study, we developed four *AVR* gene specific markers from portions of the cloned genes. We then showed distinct molecular variation among the 5 *AVR* genes that contribute to different levels of instability in *M. oryzae*. Genomic methods and new knowledge gained in the present study will aid in the accurate prediction of effective *R* genes for breeding programs. For the long-term benefit, continued monitoring of natural variation of *AVR* genes in *M. oryzae* populations in commercial fields will accelerate the utilization of plant innate immunity for developing long-lasting resistant crop plants.

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Literature Cited

- 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.
- Atkins, A., Robert, A., Adair, C., Goto, K., Kozaka, T., Yanagida, R., Yamada, M., and Matsumoto, S. 1967. An international set of rice varieties for differentiating race of *Pyricularia oryzae*. *Phytopathology* 57:297-301.
- Bohnert, H. U., Fudal, I., Dioh, W., Tharreau, D., Notteghem, J. L., and Lebrun, M. H. 2004. A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. *Plant Cell* 16:2499-2513.
- Chen, C., Chen, M., Hu, J., Zhang, W., Zhong, Z., Jia, Y., Allaux, L., Fournier, E., Tharreau, D., Wang, G.-L., Wang, Z., Shen, W.-C., Lu, G., Wang, B., and Mitchell, T. K. 2014. Sequence variation and recognition specificity of the avirulence gene *AvrPiz-T* in *Magnaporthe oryzae* field populations. *Fungal Genom. Biol.* 4:113.
- Chen, X., Jia, Y., and Wu, B. M. 2019. Evaluation of rice responses to blast fungus *Magnaporthe oryzae* at different growth stages. *Plant Dis.* 103:132-136.
- Costanzo, S., and Jia, Y. 2009. Alternatively spliced transcripts of *Pi-ta* blast resistance gene in *Oryza sativa*. *Plant Sci.* 177:468-478.
- Costanzo, S., and Jia, Y. 2010. Sequence variation at the rice blast resistance gene *Pi-km* locus: Implications for the development of allele specific markers. *Plant Sci.* 178:523-530.
- Dai, Y., Jia, Y., Correll, J., Wang, X., and Wang, Y. 2010. Diversification and evolution of the avirulence gene *AVR-Pital* in field isolates of *Magnaporthe oryzae*. *Fungal Genet. Biol.* 47:973-980.
- Farman, M. L., and Leong, S. A. 1998. Chromosome walking to the *AVR1-CO39* avirulence gene of *Magnaporthe grisea*: Discrepancy between the physical and genetic maps. *Genetics* 150:1049-1058.
- Fjellstrom, R., Conaway-Bormans, C. A., McClung, A. M., Marchetti, M. A., Shank, A. R., and Park, W. D. 2004. Development of DNA markers suitable

- for marker assisted selection of three genes conferring resistance to multiple pathotypes. *Crop Sci.* 44:1790-1798.
- Groth, D. E. 2016. Louisiana rice blast update and management. Page 58 in: Proceedings of 36th Rice Technical Working Group meeting. M. E. Salaasi, L. Tarpley, and E. P. Webster, eds. Galveston, Texas, March 1 to 4, 2016.
- Hayashi, K., Yoshida, H., and Ashikawa, I. 2006. Development of PCR-based allele-specific and InDel marker sets for nine rice blast resistance genes. *Theor. Appl. Genet.* 113:251-260.
- Hollier, C. A., and Groth, D. E. 2000. Characteristics of rice blast epidemics in Louisiana. Page 79 in: Proceedings of 28th Rice Technical Working Group meeting. Biloxi, Mississippi, February 27 to March 1, 2000.
- Jia, Y., Jia, M. H., Wang, X., and Zhao, H. 2019. A toolbox for managing blast and sheath blight diseases of rice in the United States of America. *Protecting Rice Grains in the Post Genomic Era*. Y. Jia, ed.
- Jia, Y., Lee, F., and McClung, A. 2009. Determination of resistance spectra of the *Pi-ta* and *Pi-k* genes to U.S. races of *Magnaporthe oryzae* causing rice blast in a recombinant inbred line population. *Plant Dis.* 93:639-644.
- Jia, Y., and Martin, R. 2008. Identification of a new locus, *Pir(t)* required for rice blast resistance gene *Pi-ta*-mediated resistance. *Mol. Plant-Microbe Interact.* 21: 396-403.
- 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.
- Kanzaki, H., Yoshida, K., Saitoh, H., Fujisaki, K., Hirabuchi, A., Allaux, L., Fournier, E., Tharreau, D., and Terauchi, R. 2012. Arms race co-evolution of *Magnaporthe oryzae* AVR-Pik and rice *Pik* genes driven by their physical interactions. *Plant J.* 72:894-907.
- 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.
- Lee, F. N. 1992. Rice blast: Current status. Page 35 in: Proceedings of 24th Rice Technical Working Group meeting, Little Rock, Arkansas, February 23-26, 1992.
- Lee, S., Costanzo, S., Jia, Y., Olsen, K., and Caicedo, A. 2009a. Evolutionary dynamics of the genomic region around the blast resistance gene *Pi-ta* in AA genome *Oryza* species. *Genetics* 183:1315-1325.
- Lee, S., Wamshie, Y., Jia, Y., and Liu, G. 2009b. Identification of two major resistance genes against race IE-1k of *Magnaporthe oryzae* in the indica rice cultivar Zhe733. *Mol. Breed.* 24:127-134.
- Li, W., Wang, B., Wu, J., Lu, G., Hu, Y., Zhang, X., Zhang, Z., Zhao, Q., Feng, Q., Zhang, H., Wang, Z., Wang, G., Han, B., and Zhou, B. 2009. The *Magnaporthe oryzae* avirulence gene *AvrPiz-t* encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene *Piz-t*. *Mol. Plant-Microbe Interact.* 22:411-420.
- Liu, J., Wang, X., Mitchell, T., Hu, Y., Liu, X., Dai, L., and Wang, G. L. 2010. Recent progress and understanding of the molecular mechanisms of the rice-*Magnaporthe oryzae* interaction. *Mol. Plant Pathol.* 11:419-427.
- Longya, A., Chaipanya, C., Franceschetti, M., Maiment, J. H. R., Banfield, M. J., and Jantauriyarat, C. 2019. Gene duplication and mutation in the emergence of a novel aggressive allele of the AVR-Pik effector in the rice blast fungus. *Mol. Plant-Microbe Interact.* 32:740-749.
- Ma, J. H., Wang, L., Feng, S. J., Lin, F., Xiao, Y., and Pan, Q. H. 2006. Identification and fine mapping of *AvrPi15*, a novel avirulence gene of *Magnaporthe grisea*. *Theor. Appl. Genet.* 113:875-883.
- Mekwatanakarn, P., Kosiratana, W., Levy, M., and Zeigler, R. S. 2000. Pathotype and avirulence gene diversity of *Piricularia grisea* in Thailand as determined by rice lines near-isogenic for major resistance genes. *Plant Dis.* 84:60-70.
- Nalley, L., Tsiboe, F., Durand-Morat, A., Shew, A., and Thoma, G. 2016. Economic and environmental impact of rice blast pathogen (*Magnaporthe oryzae*) alleviation in the United States. *PLoS One* 11:e0167295.
- Okuyama, Y., Kanzaki, H., Abe, A., Yoshida, K., Tamiru, M., Saitoh, H., Fujibe, T., Matsumura, H., Shenton, M., Galam, D. C., Undan, J., Ito, A., Sone, T., and Terauchi, R. 2011. A multifaceted genomics approach allows the isolation of the rice *Pia*-blast resistance gene consisting of two adjacent NBS-LRR protein genes. *Plant J.* 66:467-479.
- Orbach, M. J., Farrall, 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.
- Ray, S., Singh, P. K., Gupta, D. K., Mahato, A. K., Sarkar, C., Rathour, R., Singh, N. K., and Sharma, T. R. 2016. Analysis of *Magnaporthe oryzae* genome reveals a fungal effector, which is able to induce resistance response in transgenic rice line containing resistance gene, *Pi54*. *Front. Plant Sci.* 7:1140.
- Roychowdhury, M., Jia, Y., and Cartwright, R. D. 2012. Structure, function, and co-evolution of rice blast resistance genes. *Acta Agron. Sin.* 38:381-393.
- Saitoh, K. I., Togashi, K., Arie, T., and Teraoka, T. 2006. A simple method for a mini-preparation of fungal DNA. *J. Gen. Plant Pathol.* 72:348-350.
- Sambrook, J., and Russell, D. W., eds. 2001. Estimation of cell number. A8.6 in: *Molecular Cloning: A Laboratory Manual*, Vol. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Selisana, S. M., Yanoria, M. J., Quime, B., Chaipanya, C., Lu, G., Opulencia, R., Wang, G. L., Mitchell, T., Correll, J., Talbot, N. J., Leung, H., and Zhou, B. 2017. Avirulence (AVR) gene-based diagnosis complements existing pathogen surveillance tools for effective deployment of resistance (*R*) genes against rice blast disease. *Phytopathology* 107:711-720.
- Silue, D., Notteghem, J. L., and Tharreau, D. 1992. Evidence of a gene-for-gene relationship in the *Oryza-sativa-Magnaporthe-grisea* pathosystem. *Phytopathology* 82:577-580.
- Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F. G., and Valent, B. 1995. Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. *Plant Cell* 7:1221-1233.
- 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.
- Tansian, P., Jaihom, N., and Parinthewong, N. 2019. Diversity analysis of Avr-Pik effectors of rice blast population collecting in Thailand between year 207 to 2013. Page 76 in: Proceedings of the 8th International Rice Blast Conference, Chengdu, China, May 27 to 31, 2019.
- Wang, B., Daniel, J. E., and Wang, Z. 2017. The arms race between *Magnaporthe oryzae* and rice: diversity and interaction of *Avr* and *R* genes. *J. Integr. Agric.* 16:2746-2760.
- Wang, X., Fjellstrom, R., Jia, Y., Yan, W. G., Jia, M. H., Scheffler, B. E., Wu, D., Shu, Q., and McClung, A. 2010. Characterization of *Pi-ta* blast resistance gene in an international rice core collection. *Plant Breed.* 129: 491-501.
- Wang, X., Jia, M. H., Ghai, P., Lee, F. N., and Jia, Y. 2015. Genome-wide association of rice blast resistance and yield related components of rice. *Mol. Plant-Microbe Interact.* 28:1383-1392.
- Wang, X., Jia, Y., Shu, Q., and Wu, D. 2008. Haplotype diversity at the *Pi-ta* locus in cultivated rice and its wild relatives. *Phytopathology* 98:1305-1311.
- Wu, J., Kou, Y., Bao, J., Li, Y., Tang, M., Zhu, X., Ponaya, A., Xiao, G., Li, J., Li, C., Song, M. Y., Cumagun, C. J., Deng, Q., Lu, G., Jeon, J. S., Naqvi, N. I., and Zhou, B. 2015. Comparative genomics identifies the *Magnaporthe oryzae* avirulence effector *AvrPi9* that triggers *Pi9*-mediated blast resistance in rice. *New Phytol.* 206:1463-1475.
- Xing, J., Jia, Y., Correll, J. C., Lee, F. N., Cartwright, R., Cao, M., and Yuan, L. 2013. Analysis of genetic and molecular identity among field isolates of rice blast fungus using an international differential system, Rep-PCR and DNA sequencing. *Plant Dis.* 97:491-495.
- Yoshida, K., Saitoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., 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.
- Zhai, C., Lin, F., Dong, Z., He, X., Yuan, B., Zeng, X., Wang, L., and Pan, Q. 2011. The isolation and characterization of *Pik*, a rice blast resistance gene which emerged after rice domestication. *New Phytol.* 189:321-334.
- Zhai, C., Zhang, Y., Yao, N., Lin, F., Liu, Z., Dong, Z., Wang, L., and Pan, Q. 2014. Function and interaction of the coupled genes responsible for *Pik-h* encoded rice blast resistance. *PLoS One* 9:e98067.
- Zhang, S., Wang, L., Wu, W., He, L., Yang, X., and Pan, Q. 2015. Function and evolution of *Magnaporthe oryzae* avirulence gene *AvrPib* responding to the rice blast resistance gene *Pib*. *Sci. Rep.* 5:11642.
- Zhao, H., Wang, X., Jia, Y., Minkenberg, B., Wheatley, M., Fan, J., Jia, M. H., Famoso, A., Edwards, J. D., Wamshie, Y., Valent, B., Wang, G. L., and Yang, Y. 2018. The rice blast resistance gene *Pir* encodes an atypical protein required for broad-spectrum disease resistance. *Nat. Commun.* 9:2039.
- Zhou, E., Jia, Y., Singh, P., Correll, J. C., and Lee, F. N. 2007. Instability of the *Magnaporthe oryzae* avirulence gene *AVR-Pita* alters virulence. *Fungal Genet. Biol.* 44:1024-1034.
- Zhou, X. G. 2016. Texas rice blast update. Page 57 in: Proceedings of 36th Rice Technical Working Group meeting. M. E. Salaasi, L. Tarpley, and E. P. Webster, eds. Galveston, Texas, March 1 to 4, 2016.