Effectiveness and Durability of the Rice *Pi-ta* Gene in Yunnan Province of China

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ABSTRACT

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Rice blast is one of the most damaging diseases of rice worldwide. In the present study, we analyzed DNA sequence variation of avirulence (AVR) genes of *AVR-Pita1* in field isolates of *Magnaporthe oryzae* in order to understand the effectiveness of the resistance gene *Pi-ta* in China. Genomic DNA of 366 isolates of *M. oryzae* collected from Yunnan province of China were used for polymerase chain reaction (PCR) amplification to examine the existence of *AVR-Pita1* using gene-specific PCR markers. Results of PCR products revealed that 218 isolates of *M. oryzae* carry *AVR-Pita1*. Among of them, 62.5, 56.3, 58.5, 46.7, 72.4, and 57.4% of *M. oryzae* carry *AVR-Pita1* from northeastern, southeast,

western, northwest, southwestern, and central Yunnan province, respectively. The detection rate of *AVR-Pita1* was, in order: southwestern > northeastern > western > central > southeastern > northwestern Yunnan province. Moreover, in total, 18 *AVR-Pita1* haplotypes encoding 13 novel *AVR-Pita1* variants were identified among 60 isolates. Most DNA sequence variation was found to occur in the exon region, resulting in amino acid substitution. Six virulent haplotypes of *AVR-Pita1* to *Pita* were identified among 60 field isolates. The *AVR-Pita1* has evolved to virulence from avirulent origins via base substitution. These findings demonstrate that *AVR-Pita1* is under positive selection and mutations of *AVR-Pita1* are responsible for defeating race-specific resistance in nature.

Additional keyword: effector.

Rice blast, caused by the filamentous ascomycetous fungus (*Magnaporthe oryzae*), is one of the most damaging diseases of rice worldwide. Practically, blast disease is managed by the use of resistant cultivars carrying both major and minor resistance (*R*) genes and application of fungicides integrated with cultural practices. Among them, use of resistant cultivars is the most economical and environmentally benign method of reducing crop loss due to blast.

Major *R*-gene-mediated resistance is effective in recognizing the strains of *M. oryzae* that contain the corresponding avirulence (AVR) gene. Thus far, nine AVR genes in *M. oryzae* have been cloned: *AVR-Pita* (22), *AVR-Pizt* (20), *AVR1-CO39* (6), *ACE1* (8), *AVR-Pia* (32), *AVR-Pii* (32), *AVR-Pik/km/kp* (32), *PWL1* (15), and *PWL2* (28).

The effectiveness and durability of major *R*-gene-mediated resistance is determined by structural and functional integrity of AVR genes. The *AVR-Pita* gene of *M. oryzae* determines the efficacy for *Pi-ta*. *AVR-Pita* encodes a predicted metalloprotease with 223 amino acids (22). *AVR-Pita1*, renamed from *AVR-Pita*, is a member of the *AVR-Pita* alleles in isolates of *M. oryzae* (16). The variants of *AVR-Pita1*, including point mutations, insertions, and deletions, were found in the field isolates from the United States, China, Colombia, India, Egypt, and the Philippines (5). Specific alterations of the active site from glutamic acid to aspartic acid (E177D) and substitutions of methionine to tryptophan (M178W) in the putative protease motif of strain 4360-R-62 resulting in the loss of avirulence were observed (12).

Pi-ta has been effective in preventing blast disease in the United States since its release in 1990 (21). In China, the *Pi-ta* gene was found in 71 leading cultivars or hybrid rice parents in most rice-producing provinces (31) and in 72 landraces from Yunnan province (19,31). A single amino acid in the leucine-rich repeat (LRR) domain in one resistant *Pi-ta* haplotype is essential for *AVR-Pita* recognition (12,17). In contrast, 38 *AVR-Pita1* haplotypes encoding 27 *AVR-Pita1* variants were identified among 151 avirulent isolates collected worldwide. Most DNA sequence variations were found to occur in the exon region resulting in amino acid substitutions, suggesting that *AVR-Pita1* is under positive selection and mutations of *AVR-Pita1* are responsible for defeating race-specific resistance in nature (5). Thus far, the documented *AVR-Pita1* mutations in field isolates of *M. oryzae* from the United States also included the Pot3 transposon insertion (33); whereas, in field isolates from Japan, deletion of functional copies and base substitutions were observed (30). The *AVR-Pita* allele was detected in field isolates collected from Jilin province of China by *AVR-Pita*-specific markers. In total, 89 *AVR-Pita* alleles of 103 isolates have been amplified; however, structural and functional relations of the variants have not been analyzed (27).

The objective of the present study was to analyze DNA sequence variation of *AVR-Pita1* in field isolates of *M. oryzae* in order to understand the effectiveness and durability of *Pi-ta* in Yunnan province of China.

MATERIALS AND METHODS

Rice cultivars, fungal isolates, culture, and pathogenicity assays. The *Pi-ta*-containing rice monogenic line IRBLta-K1 and the susceptible backcrossing parent Lijiangxintuanheigu (LTH without *Pi-ta*) were used for pathogenicity assays. In total,

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366 isolates were collected, single-spore purified, and examined. All isolates were stored at -20°C on filter paper and grown at room temperature under blue and white fluorescent lighting on petri dishes containing oatmeal agar for spore production. Disease reactions were determined using a modified standard pathogenicity assay, as previously described by Jia et al. (13). Specifically, rice seedlings at the three- to four-leaf stage were placed in a plastic bag and spray inoculated with a spore suspension at 1 to 5×10^5 spores/ml. After inoculation, the plastic bags were sealed, to maintain high humidity, for 24 h before removing the plants from bags. Subsequently, plants were maintained in a greenhouse for an additional 6 days to allow the development of clear disease symptoms. The disease reactions were rated based on visual number and amount of lesions at the second youngest leaf using disease scale where 0 to 2 = resistant and 3 to 5 = susceptible.

DNA preparation, polymerase chain reaction amplification, and DNA sequencing. Fungal isolates were grown in complete liquid media at 25°C for 6 to 8 days to produce mycelium under dark conditions. DNA was then isolated from mycelia using the cetyltrimethylammonium bromide method (29). Primers YL169 (5'-CGACCCGTTTCCGCC-3') and YL149 (5'-TGACCGCG ATTCCCTCCATT-3') were used to amplify the *AVR-Pita1* allele and for sequencing using methods described by Dai et al. (5). Specifically, polymerase chain reaction (PCR) was performed using 2 $\times$ Taq PCR MasterMix (Tiangen Biotech Co. Ltd., Beijing). Each PCR consisted of the following components: 25 μl of Taq PCR Master Mix (containing 25 U of Taq DNA polymerase, 10 $\times$ Tiangen PCR buffer, 15 mM MgCl_2 , and 200 μM each dNTP), 1 μl of each 10 μM primer, 2 μl of fungal genomic DNA, and 21 μl of distilled water (provided by Tiangen Kit). Reactions were performed in a Bio-Rad Thermal Cycler (C1000; Bio-Rad Laboratories, Life Science Research, Hercules, CA) with the following PCR program: 1 cycle at 95°C for 3 min for initial denaturation; followed by 29 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 7 min. All PCRs were repeated three times (20 μl for detection, 50 μl for sequencing). The size of the amplified fragment was estimated by DL2000 DNA Ladder (Tiangen Biotech Co. Ltd.). PCR products were sequenced using the same primers as previously mentioned above for PCR amplification. DNA was sequenced by Shanghai Life Technologies Biotechnology Co., Ltd. (Shanghai, China). Each isolate was sequenced three times.

Data analysis. DNA sequences of *AVR-Pita1* were assembled by Vector NTI software Suite V.10 (Invitrogen, Carlsbad, CA) and aligned using DNASTAR V7.10 software (<http://www.dnastar.com/>). The number of DNA haplotypes, polymorphic sites (π), and the sliding window were calculated using DnaSP v5.10.01 software (25). Haplotype network analysis was performed using TCS1.21 (<http://darwin.uvigo.es/>) (3). Diversity index was calculated as the frequency of haplotypes or protein types in the rice blast fungus population following Fontaine's method (7): diversity index = $(1 - \sum_{i=1}^n p_i^2)$ (where p_i is the frequency of the haplotype i in a population).

RESULTS

Effectiveness of the *Pi-ta* gene. Based on disease reactions, 239 of the 366 isolates tested were avirulent to the *Pi-ta*-containing rice monogenic line IRBLta-K1, while the remaining 127 isolates were virulent (Table 1). Of 366 isolates, 218 *AVR-Pita1* alleles were amplified by *AVR-Pita1*-specific primers (YL149/YL169) (Table 1); the average percentage of *AVR-Pita1* was 59.6%. The highest percentage of *AVR-Pita1* was 72.4% in the *M. oryzae* population collected from southwestern Yunnan whereas the lowest was 46.7% from northwestern Yunnan (Table 1). The percentages of *AVR-Pita1* were 56.3, 57.4, 58.5, and 62.5% in southeastern, central, western, and northeastern Yunnan province, respectively. These findings suggest that *Pi-ta* is still useful to most rice production areas in Yunnan province.

Haplotype diversity of *AVR-Pita1* of *M. oryzae*. In total, 18 *AVR-Pita1* haplotypes, including the original *AVR-Pita1*, were identified based on the DNA sequence assemblies of 60 isolates (Table 2). Nine haplotypes were detected in 25 *M. oryzae* from western Yunnan. Six, five, three, three, and two haplotypes were detected in 14, 7, 8, 4, and 2 *M. oryzae* from northeastern, southeastern, central, northwestern, and southwestern Yunnan, respectively (Table 3). Similarly, the diversity index was 0.78, 0.77, 0.76, 0.63, 0.50, and 0.41 for southeastern, western, northeastern, northwestern, southwestern, and central Yunnan, respectively (Table 3). In summary, the diversity index of *AVR-Pita1* was ordered in Yunnan province as southeastern > western > northeastern > northwestern > southwestern > central.

AVR-Pita1 is known to have three introns and four exons in the open reading frame (ORF) (22). High nucleotide variation in the exons of *AVR-Pita1* was observed (Fig. 1), and a haplotype network based on sequence variation was developed (Fig. 2). Four major evolutionary clusters (Fig. 2A to D) of *AVR-Pita1* were observed among 60 field isolates. The isolates of cluster C and other less frequent haplotypes of *AVR-Pita1* were avirulent to IRBLta-K1 (with *Pi-ta*) (Fig. 2; Table 4). The isolates in H11, H15, and H16 of cluster A; H17 of B; and H3 of D were virulent to rice cultivars with *Pi-ta* (Fig. 2; Table 4). These findings suggest that the A, B, and D clusters of *AVR-Pita1* were evolved to virulence from avirulent origins via nucleotide substitution, resulting in avoiding recognition of *Pi-ta* in nature. The virulent isolates of H11 and H16 were identified in southeastern, H15 in western, and H17 in northeastern and central Yunnan, China, suggesting that the virulent evolution of *AVR-Pita1* occurred in most rice-producing regions of Yunnan (Fig. 2; Tables 3 and 4). Among 18 haplotypes identified, 7 haplotypes were identified in multiple isolates whereas 11 haplotypes were identified only in a single isolate (Table 2). The original *AVR-Pita1* allele was found in 10 isolates (Table 2). Alignment of the *AVR-Pita1* DNA sequences from 60 isolates revealed that there were 22 polymorphic sites (excluding gaps), with 19 being in the exon region and 3 in the intron region. Of the 19 sites in the exon region, 17 resulted in amino acid substitutions (Table 4), indicating that most of the variation in the exon region resulted in alteration of the amino

TABLE 1. Distribution of avirulence (*AVR*)-*Pita1* genes and *AVR* isolates of *Magnaporthe oryzae* collected from Yunnan, China to IRBLta-K1

Locations	Number of isolates	PCR detection ^a		Pathogenicity assay ^b	
		Number of isolates with <i>AVR-Pita1</i>	Frequency (%)	Number of <i>AVR</i> isolates	Frequency (%)
Northeastern	72	45	62.5	58	80.6
Southeast	32	18	56.3	9	28.1
Western	164	96	58.5	110	67.1
Northwest	15	7	46.7	11	73.3
Southwestern	29	21	72.4	18	62.1
Central	54	31	57.4	33	61.1
Total	366	218	59.6	239	65.3

^a PCR = polymerase chain reaction.

^b Indicates pathogenicity assay on monogenic line IRBLta-K1 (containing *Pita*).

acids in the protein. Moreover, the *AVR-Pital* sequences among the 60 isolates were predicted to produce 14 functional proteins (Table 4). Among these 14 proteins, amino acid variations were predicted to occur at 17 positions, including an insertion/deletion. All variations occurred throughout the protein, including position 173 of the putative protease motif (Table 4). The protease motif in the 14 putative proteins was identical except for the 173rd amino acid, V173I, in the metalloprotease motif (22). Amino acid variations at D55N were found in six isolates, five of which were virulent on monogenic line IRBLta-K1 (with *Pi-ta*) (Table 4), suggesting that amino acid 55D is critical for avirulent function of *AVR-Pita*.

The *AVR-Pital* gene was not amplified from 61 of 127 virulent isolates and 87 of 239 avirulent isolates (Table 5). Nearly half of

virulent isolates were amplified with *AVR-Pital*, suggesting that there are additional factors that are critical for avirulence. Similarly, *AVR-Pital* was not amplified from 36.4% avirulent isolates, suggesting that *AVR-Pital* primers may be not specific for these isolates or *R* genes for these isolates are *Pi-ta* independent. There are 152 avirulent isolates on IRBLta-K1 with *Pi-ta* containing *AVR-Pital* and 61 virulent isolates without *AVR-Pital*. There are 58.2% of isolates that belong to both reaction types, which is consistent with the gene-to-gene theory (Table 5). In contrast, 87 avirulent isolates on IRBLta-K1 (*Pi-ta*) without *AVR-Pital*, 66 virulent isolates containing *AVR-Pital*, and 41.8% of isolates belonging to both reaction types, which is inconsistent with the gene-to-gene theory (Table 5). There were 29.2 to 66.7% of isolates from the six rice-producing regions of Yunnan, which

TABLE 2. Haplotypes of *AVR-Pital* in rice blast fungus of Yunnan, China

Haplotype	N ^a	% ^b	Variant locus ^c																							
			17–18	18	63	163	234–235	249–250	260	276	298	300	316	319	365	456	460	517	559	573	716	724	758	759	778	827
AF207841	...	...	...	A	C	G	–	–	T	T	A	G	G	T	G	G	C	G	G	A	G	T	T	G	A	T
EF616489	...	...	GTT	·	·	·	–	–	C	·	G	·	A	·	T	·	A	·	A	·	·	A	–	A	·	·
H01	10	16.7	...	·	·	·	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
H02	1	1.7	...	·	A	·	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
H03	1	1.7	...	·	·	A	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
H04	1	1.7	...	G	·	·	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
H05	11	18.3	GTT	·	·	·	–	–	·	·	·	·	·	·	·	·	·	·	·	T	C	·	·	·	·	A
H06	1	1.7	GTT	·	·	·	–	–	·	·	·	·	A	C	·	·	G	T	·	·	·	·	·	·	·	·
H07	1	1.7	GTT	·	·	·	–	–	·	G	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	A
H08	9	15.0	GTT	·	·	·	–	–	C	·	G	A	A	·	T	·	·	·	A	G	·	A	–	A	·	·
H09	3	5.0	GTT	·	·	·	–	–	C	·	G	A	A	·	T	·	A	·	A	·	·	A	–	A	·	·
H10	1	1.7	GTT	·	·	·	–	–	C	·	G	A	A	·	T	·	A	·	A	G	·	A	–	A	·	·
H11	2	3.3	GTT	·	·	·	–	–	C	·	G	A	A	·	T	C	·	·	A	G	·	A	–	A	·	·
H12	12	20.0	GTT	·	·	·	–	–	C	·	G	A	A	·	T	·	A	·	A	G	·	A	–	A	G	·
H13	1	1.7	GTT	·	·	·	–	C	C	·	G	A	A	·	T	·	A	·	A	G	·	A	–	A	·	·
H14	1	1.7	GTT	·	·	·	A	–	C	·	G	A	A	·	T	·	A	·	A	G	·	A	–	A	·	·
H15	1	1.7	GTT	·	·	A	–	–	C	·	G	A	A	·	T	·	·	·	A	G	·	A	–	A	·	·
H16	1	1.7	GTT	·	·	A	–	–	C	·	G	A	A	·	T	C	·	·	A	G	·	A	–	A	·	·
H17	2	3.3	GTT	·	·	A	–	–	C	·	G	A	A	·	T	·	A	·	A	G	·	A	–	A	G	·
H18	1	1.7	GTT	·	·	A	–	–	C	G	G	A	A	·	T	·	A	·	A	G	·	A	–	A	G	·

^a Number of isolates.

^b Percentage of total.

^c Symbols: . indicates the same as AF207841 and – indicates deletion. AF207841 and EF616489 (GenBank accession numbers) of *AVR-Pital* were obtained from GenBank.

TABLE 3. Distribution of *AVR-Pital* haplotype in different rice-growing-regions

Haplotype	Isolates		Regions ^a					
	Number	%	Northeastern	Central	Southeastern	Western	Northwestern	Southwestern
H01	10	16.7	2 (14.3)	0	0	8 (32.0)	0	0
H02	1	1.7	0	0	0	1 (4.0)	0	0
H03	1	1.7	0	0	0	1 (4.0)	0	0
H04	1	1.7	1 (7.1)	0	0	0	0	0
H05	11	18.3	5 (35.7)	0	2 (28.6)	3 (12.0)	0	1 (50.0)
H06	1	1.7	0	0	0	1 (4.0)	0	0
H07	1	1.7	0	0	0	1 (4.0)	0	0
H08	9	15.0	0	0	0	8 (32.0)	1 (25.0)	0
H09	3	5.0	0	0	0	1 (4.0)	2 (50.0)	0
H10	1	1.7	0	0	1 (14.3)	0	0	0
H11	2	3.3	0	0	1 (14.3)	0	0	1 (50.0)
H12	12	20.0	4 (28.6)	6 (75.0)	2 (28.6)	0	0	0
H13	1	1.7	0	1 (12.5)	0	0	0	0
H14	1	1.7	0	0	0	0	1 (25.0)	0
H15	1	1.7	0	0	0	1 (4.0)	0	0
H16	1	1.7	0	0	1 (14.3)	0	0	0
H17	2	3.3	1 (7.1)	1 (12.5)	0	0	0	0
H18	1	1.7	1 (7.1)	0	0	0	0	0
Total	60	100	14	8	7	25	4	2
N ^b	...	...	6	3	5	9	3	2
DI ^c	...	...	0.76	0.41	0.78	0.77	0.63	0.50

^a Number and frequency (in parentheses) of isolates of each haplotype.

^b Number of haplotypes.

^c Diversity index (DI) was calculated as the frequency of haplotypes types in the *Magnaporthe oryzae* population following Fontaine's method (7). $DI = (1 - \sum_{i=1}^n p_i^2)$ (where p_i is the frequency of the haplotype i in a population).

TABLE 4. Variation of the *AVR-Pita1* protein in rice blast fungus of Yunnan, China^a

Haplotype	Locus																		DR ^b
	Exon 1		Exon2											Exon3		Exon4			
	5–6	55	74	81	82	87	88	103	134	135	154	168	173	191	194	206	222		
AF207841	–	D	W	N	D	R	L	K	E	G	P	G	V	Y	D	K	Y	...	
EF616489	L	.	.	S	.	K	.	N	.	E	.	.	I	.	.	.	.	...	
H01	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	10R	
H02	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1R	
H03	–	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1S	
H04	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1R	
H05	L	.	.	.	.	.	.	.	.	.	.	.	.	F	H	.	N	11R	
H06	L	.	.	.	.	K	S	.	.	.	R	V	.	.	.	.	.	1R	
H07	L	.	G	.	.	.	.	.	.	.	.	.	.	F	H	.	N	1R	
H08	L	.	.	S	N	K	.	N	.	.	.	.	I	C	.	.	.	8M+1?	
H09	L	.	.	S	N	K	.	N	.	E	.	.	I	.	.	.	.	2R+1?	
H10	L	.	.	S	N	K	.	N	.	E	.	.	I	C	.	.	.	1R	
H13	L	.	.	S	N	K	.	N	.	E	.	.	I	C	.	.	.	1R	
H14	L	.	.	S	N	K	.	N	.	E	.	.	I	C	.	.	.	1R	
H11	L	.	.	S	N	K	.	N	Q	.	.	.	I	C	.	.	.	1S+1?	
H12	L	.	.	S	N	K	.	N	.	E	.	.	I	C	.	R	.	11M+1?	
H15	L	N	.	S	N	K	.	N	.	.	.	.	I	C	.	.	.	1S	
H16	L	N	.	S	N	K	.	N	Q	.	.	.	I	C	.	.	.	1S	
H17	L	N	.	S	N	K	.	N	.	E	.	.	I	C	.	R	.	2S	
H18	L	N	G	S	N	K	.	N	.	E	.	.	I	C	.	R	.	1?	

^a Symbols: . indicates the same as AF207841 and – indicates deletion.
^b Disease reaction (DR) indicates pathogenicity assay on monogenic line IRBLta-K1 (containing *Pita*). R, M, and S indicate that the DR was resistant, moderately resistant, or susceptible, respectively; Ex.10R indicates 10 isolates were avirulent to IRBLta-K1; and ? indicates unknown.

TABLE 5. Correlation of disease reaction of the isolate with or without *AVR-Pita1* of *Magnaporthe oryzae* by polymerase chain reaction (PCR) detection and pathogenicity assay

Isolates	Reaction types ^a			
	Consistent		Inconsistent	
	A+	V–	V+	A–
Number	152	61	66	87
Total	213		153	
Percentage (%)	58.2		41.8	

^a A+ indicates isolates with *AVR-Pita1* by PCR detection and avirulent to the corresponding resistance (*R*) gene; V– indicates isolates without *AVR-Pita1* by PCR detection and virulent to the corresponding *R* gene; V+ indicates isolates with *AVR-Pita1* by PCR detection and virulent to the corresponding *R* gene; and A– indicates without *AVR-Pita1* by PCR detection and avirulent to the corresponding *R* gene.

appeared as inconsistent with the gene-for-gene theory (Table 6), suggesting that virulent isolates to *Pi-ta* have been accumulated and genome organization at the *AVR-Pita1* locus is much more complex in Yunnan than predicted.

DISCUSSION

In the present study, we found new changes in the *AVR-Pita1* sequences among field isolates of *M. oryzae* from various rice-producing regions in Yunnan, China, and a mass of virulence isolates to *Pi-ta* gene were identified in field isolates collected in Yunnan, suggesting that *Pi-ta* was defeated in some rice production areas due to extensive development of *Pi-ta* in China. *Pi-ta* was effectively deployed in the United States over two decades ago (14,21) and is still effective in the United States (Y. Jia, unpublished data). Partial deletions, complete deletions, frame-shift mutations, and sequence variations have occurred in *AVR-Pita1* sequences among field isolates of *M. oryzae* from various rice-producing countries (5). However, a majority of isolates surveyed from commercial rice fields, thus far, still contain a functional *AVR-Pita1*. Rice cultivars with *Pi-ta* were resistant to 75.1% of isolates (282 isolates) in Yunnan (18) and 18.5% of isolates (146 isolates) from Guangdong province, China (34), suggesting that *Pi-ta* has a limited effect in these rice production

areas. Continued analysis of *AVR-Pita1* in these isolates will help us to predict the stability and effectiveness of *Pi-ta* mediated resistance.

Gene-for-gene theory predicts that *Pi-ta* specifically prevents the infections by isolates of *M. oryzae* that contain *AVR-Pita1*. *M. oryzae* isolates without *AVR-Pita1* are avirulent to IRBLta-K1 (with *Pi-ta*), suggesting that IRBLta-K1 also contains *Pi-ta*-independent blast *R* genes (Table 1). However, nine isolates with *AVR-Pita1* in the southeastern region of Yunnan infected IRBLta-K1. One possibility is that expression of these *AVR-Pita1* alleles may be hindered. Further examination of the promoter regions and expressions of these *AVR-Pita1* alleles should help to verify this prediction.

Most DNA sequence variation was found in exon regions of *AVR-Pita1*, and 18 haplotypes were identified based on the 25 variant nucleotides (included insertion/deletion) among 60 isolates collected from Yunnan province (Table 2). Five novel variant amino acids (D55N, W74G, L88S, E134Q, and Y222N) of the *AVR-Pita1* variants in the 60 isolates were identified. Among them, 55D may be another critical amino acid for avirulent function because the isolates were virulent to IRBLta-K1 (*Pi-ta*) when 55D was replaced with 55N (Table 4). The amino acid V or I at position 173 was a critical amino acid residue for metalloprotease (9). We also identified V in 26 isolates and I in 34 isolates at position 173 of the metalloprotease motif of *AVR-Pita1* in an isolate population (Tables 3 and 4). Similarly, the structures of the *AVR-Pita* alleles in 39 U.S. isolates of *M. oryzae* were analyzed. Five alleles were predicted to encode metalloprotease proteins highly similar to the *AVR-Pita* protein (5). An avirulent isolate, OS99-G-7a (G7a), from a Japanese commercial field containing two paralogs of *AVR-Pita1* (*AVR-Pita1*^{JA} and *AVR-Pita1*^{JB}) was identified. Isolates that showed virulence toward rice cultivars containing the *Pi-ta* gene were presumed to have evolved virulence from avirulent origins via loss of *AVR-Pita*^{JA}, except for one case in which virulence resulted from a base substitution (30).

Various mechanisms, including the mobility of active transposable elements, have been theorized to explain the instability of this fungus. A Pot3 transposon was identified in the DNA region encoding the putative protease motif of the *AVR-Pita* protein from a field isolate B2 collected from a *Pi-ta*-containing ‘Banks’ rice.

TABLE 6. Distribution and correlation of disease reaction of the isolate with or without *AVR-Pita1* of *M. oryzae* by polymerase chain reaction (PCR) detection and pathogenicity assay

Locations	Number of isolates	Reaction types of isolates and frequency(%) ^a				Total
		A+	V–	V+	A–	
Northeastern	72	41	10	4 (5.6)	17 (23.6)	21 (29.2)
Southeast	32	7	12	11 (34.4)	2 (6.3)	13 (40.7)
Western	164	70	28	26 (15.9)	40 (24.4)	66 (40.3)
Northwest	15	4	1	3 (20.0)	7 (46.7)	10 (66.7)
Southwestern	29	13	3	8 (27.6)	5 (17.2)	13 (44.8)
Central	54	17	7	14 (25.9)	16 (29.6)	30 (55.5)
Total	366	152	61	66 (18.0)	87 (23.8)	153 (41.8)

^a A+ indicates isolates with *AVR-Pita1* by PCR detection and avirulent to the corresponding resistance (*R*) gene; V– indicates isolates without *AVR-Pita1* by PCR detection and virulent to the corresponding *R* gene; V+ indicates isolates with *AVR-Pita1* by PCR detection and virulent to the corresponding *R* gene; A– indicates without *AVR-Pita1* by PCR detection and avirulent to the corresponding *R* gene.

Similarly, the insertion of retrotransposon in *ACE1* was hypothesized to result from avirulence to virulence (8). Virulent isolates were found to exhibit either a Pot3 insertion in the promoter region or single-nucleotide substitution resulting in an amino acid change in *AVR-Pizt* (20). Transposon and telomeric sequences in the genomic regions neighboring *AVR-Pia* and *AVR-Pii* led to the enhanced likelihood of gene loss and horizontal transfer (24,26). These findings demonstrated that transposons can contribute to the instability of *AVR* genes in *M. oryzae* and is one important molecular mechanism for defeating *R* genes in rice cultivars.

Plants and pathogens have coexisted and coevolved. There are two predominant hypotheses for the co-evolution of *R* genes in plants and *AVR* genes in pathogens. The foremost hypothesis is the “arms race” that predicts that both *R* and *AVR* genes are under diversified selection, whereas a “trench warfare” hypothesis predicts that either *R* or *AVR* is under balanced selection and the other is under diversified selection. To date, direct interactions of *AVR-Pita* with *Pi-ta* and *AVR-Pik* and *Pi-k*, and indirect interaction of *AVR-Pizt* and *Pi-zt*, have been reported (1,12,23). Directional selection was found in the *Pi-ta* region in accessions of *Oryza rufipogon*, whereas significant deviation from neutral evolution was not found in accessions of *O. sativa* (10,17). *AVR-Pita* is located near the telomere, an unstable region (16,22); the presence of multiple *AVR-Pita1* variants in avirulent field isolates appears to suggest the ‘arms race occurs between *Pi-ta* and *AVR-Pita1* if similar numbers of resistant and susceptible alleles can be found at the *Pi-ta* locus. Sequence variation observed in flanking regions around *Pi-ta* in *O. sativa* suggest that the size of the resistance *Pi-ta* introgressed block was at least 5.4 Mb in all elite resistant cultivars but not in the cultivars without *Pi-ta*. These findings suggest that the *Pi-ta* region has evolved under extensive selection pressure during crop breeding for resistance (11,17). Despite this, it is still difficult to determine whether a balanced or an unbalanced selection has occurred at the *Pi-ta* region. The absence of multiple resistant *Pi-ta* haplotypes suggests that *Pi-ta* engages trench warfare with *AVR-Pita1*. Similarly, *RGA4* and *RGA5* encode two predicted nucleotide-binding LRR proteins that were demonstrated to recognize the two unrelated effector molecules, *AVR1-CO39* and *AVR-Pia* of *M. oryzae*, indicating that the *R* proteins may possess dual recognition specificity (2). *Pi-ta* was predicted to encode 12 transcripts, most of which could encode nucleotide-binding site LRR proteins (4), suggesting that they may recognize different *AVR-Pita1* haplotypes or different *AVR* genes. Continued analysis of the *Pi-ta* allele in rice germplasm and examination of functionality of these *Pi-ta* alternative transcripts will lead to a hypothesis that *Pi-ta* engages in an arms race with *AVR-Pita1* at a transcriptional level.

In summary, we demonstrated that the *Pi-ta* gene is effective in significant portions of rice production areas in Yunnan province. Our findings support the idea that a functional *AVR-Pita1* possesses diversified sequence structures and is under positive selection pressure in nature. Our results are consistent with previous

findings in field isolates, and declaim the molecular mechanisms of *AVR-Pita1* diversification in nature.

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