Analysis of Genetic and Molecular Identity Among Field Isolates of the Rice Blast Fungus with an International Differential System, Rep-PCR, and DNA Sequencing

Junjie Xing, Longping Branch Institute, Postgraduate School, Central South University, Changsha, Hunan 410125, China; State Key Laboratory of Hybrid Rice, National Hybrid Rice Research and Development Center, Changsha, Hunan 410125, China; **Yulin Jia**, United States Department of Agriculture–Agricultural Research Service, Dale Bumpers National Rice Research Center, Stuttgart, AR 72160; **James C. Correll**, Department of Plant Pathology, University of Arkansas, Fayetteville 72701; **Fleet N. Lee**, Rice Research and Extension Center, University of Arkansas, Stuttgart; **Richard Cartwright**, Department of Plant Pathology, University of Arkansas, Fayetteville; and **Mengliang Cao** and **Longping Yuan**, State Key Laboratory of Hybrid Rice, National Hybrid Rice Research and Development Center, Changsha, Hunan 410125, China

Abstract

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 the rice blast fungus with an international differential system, Rep-PCR, and DNA sequencing. *Plant Dis.* 97:491-495.

The *Pi-ta* gene deployed in southern U.S. rice germplasm is effective in preventing the infection by strains of *Magnaporthe oryzae* isolates that carry the avirulence (AVR) gene *AVR-Pita1*. In the present study, 169 isolates from rice (*Oryza sativa*) cultivars, with and without *Pi-ta*, were analyzed for their genetic identity using an international differential system, repetitive element-based polymerase chain reaction (Rep-PCR), and sequence analysis of PCR products of *AVR-Pita1*. These isolates belong to the races IA1, IB1, IB17, IC1, and IC17 of *M. oryzae*. These isolates were further classified into 15 distinct groups by Rep-PCR. There was a predominant group within each race. Pathogenicity assays on ‘Katy’ (*Pi-ta*) and ‘M202’ (*pi-ta*) rice determined that IC1 was virulent to Katy and M202; IB17, IC17, and most of IA1 and IB1 were avirulent to Katy and virulent to M202, suggesting that

the *Pi-ta* gene in Katy is responsible for preventing infection by these isolates. Consistently, *AVR-Pita1* was not amplified from 28 virulent isolates. One *AVR-Pita1* allele was amplified by *AVR-Pita1*-specific primers in 78 avirulent isolates. Interestingly, different *AVR-Pita1* alleles were found in each of the 12 avirulent isolates, as determined by DNA sequencing. Sequence analysis of 90 PCR products revealed 10 *AVR-Pita1* haplotypes, 4 of which were new. In total, 12 amino acid changes were identified in the new variants when compared with the first described *AVR-Pita* sequence (AF207841). The finding of isolates with altered *AVR-Pita1* from rice cultivars with and without *Pi-ta* suggests that these virulent isolates were adapted to the field environments in the southern United States. Further research will be needed to verify this prediction.

Rice (*Oryza sativa*) feeds 50% of the human population and is under intensified cultivation in diverse geographic regions and climate conditions. Presently, rapid population growth, particularly in developing nations, has demanded increased production of rice. One of the most important bottlenecks for securing stable rice production is rice blast disease. Rice blast disease is caused by the ascomycete fungus *Magnaporthe oryzae* (formerly *M. grisea* (T.T. Hebert) M.E. Barr) (7). In commercial rice fields, *M. oryzae* is known to be transmitted via asexual spores by air or in close contact through dew or rain. Asexual spores germinate and infect rice tissue within a few hours of contact. The fungus absorbs nutrients from live rice cells for production of invasive mycelia and asexual spores. Disease symptoms can be observed as early as 3 days after infection in a greenhouse. Under high humidity and intensified rice production systems, blast can complete this infection cycle several times in a few months. As a result, rice

crop production is reduced and, sometimes, the entire crop can be lost.

Currently, rice blast disease is managed by the use of resistance (*R*) genes integrated with sophisticated cultural management systems, including the use of fungicides. However, host resistance is the most economical and powerful method of controlling rice blast disease. Genetic resistance to blast follows a gene-for-gene model where a single dominant *R* gene is effective in preventing *M. oryzae* strains that contain the corresponding avirulence (AVR) gene (24). To date, over 80 dominant *R* genes have been identified. Some *R* genes were deployed and dozens of *R* genes were molecularly characterized (2).

In the southern United States, the major blast *R* gene *Pi-ta* confers resistance to *M. oryzae* strains that contain *AVR-Pita*. *Pi-ta* was first deployed in the 1990s when ‘Katy’ rice was released (21). Subsequently, by using classic rice breeding methods, the *Pi-ta* gene has been transferred into a series of elite rice cultivars; namely, ‘Drew’, ‘Madison’, ‘Kaybonnet’, ‘Cybonnet’, ‘Ahrent’, ‘Banks’, ‘Springs’, and ‘Catahoula’ (10,19,20,28). These cultivars have been grown throughout the southern United States, where blast is known to be problematic. Thus far, resistance mediated by *Pi-ta* remains effective. However, virulent blast races, including IE1k and B2-B8, have been recovered from *Pi-ta*-containing rice cultivars in commercial rice fields in the southern United States (8,28). Historical surveys of blast field isolates revealed that at least 10 common *M. oryzae* races initially identified in the 1960s—namely, IA-45, IB-1, IB-45, IB-49, IB-54, IC-17, ID-1, IG-1, IE-1, and IH-1 (1)—are still present in contemporary rice fields (6). All of these races were shown to contain *AVR-Pita* alleles with minor modifications of their predicted products (8). Additionally, since it was discovered that *AVR-Pita* is a member of a small gene family, it was renamed as *AVR-Pita1* to distinguish it from other family members (17).

Corresponding author: Y. Jia, E-mail: yulin.jia@ars.usda.gov

This project is supported by the United States Department of Agriculture (USDA) Agricultural Research Service National Program 301 project “Responses of rice germplasm to biotic and abiotic stresses”. USDA is an equal opportunity provider and employer.

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary tables are included in the online edition.

Accepted for publication 18 October 2012.

<http://dx.doi.org/10.1094/PDIS-04-12-0344-RE>

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2013.

In order to monitor the genetic changes of *M. oryzae*, we have been studying genetic and molecular variations of *M. oryzae* strains. The fungi were isolated from rice cultivars with or without *Pi-ta*, random rice cultivars, and red rice at different growth stages of rice in rice fields, and crabgrass at the levee of rice fields. The objectives of this study were to determine (i) race identity using an international differential system, (ii) the genetic makeup of blast isolates using repetitive element-based polymerase chain reaction (Rep-PCR), (iii) disease reactions to rice cultivars with and without *Pi-ta*, (iv) the existence of *AVR-Pita* within a strain, and (v) the structural and avirulent function of *AVR-Pita1*.

Materials and Methods

Rice materials and growth. Rice plants were grown in a tray (20 9/16 by 10 3/16 in. [51 23/41 by 25 8/41 cm]) (Hummert) in a greenhouse at 25 to 30°C with 16 h of light for 3 weeks to reach the three- to four-leaf stage. Each rice cultivar—namely, ‘Raminad Strain 3’, ‘Zenith’, ‘NP 125’, ‘Usen’, ‘Dular’, ‘Kanto 51’, ‘Shatiao-tsao’, and ‘Caloro’—was used for race identification as described in an international race differential system (18). Katy, the *Pi-ta*-containing rice cultivar, was used to determine avirulence and ‘M202’, which does not carry *Pi-ta*, was used to determine virulence of all blast isolates, respectively.

Fungal isolates, spore suspensions, and pathogenicity assays. In the present study, 165 isolates of *M. oryzae* were collected from different rice cultivars in the rice fields of Arkansas in 1996 and 2009, and 4 isolates were collected from crabgrass on a levee of a rice field in 2008 (Supplementary Table S1). Among these isolates, 28 were collected from *Pi-ta*-containing cultivars; the remaining isolates were purified from random rice fields (cultivar unknown) in Arkansas. All isolates were purified, grown, and stored on desiccated filter papers at –20°C. Oatmeal agar and liquid complete medium were used for producing conidial inocula and mycelia, respectively. For all isolates, mycelia were produced in liquid complete medium broth for 7 days, shaken at 100 rpm at 23 to 25°C, and then separated using cheesecloth (Fiberweb) for DNA extraction.

For each inoculation, four to five filter discs containing mycelia and spores were removed from the freezer and then were grown on oatmeal agar plate for 7 to 10 days under blue and white fluorescent lighting (Atlanta light bulbs) at 23 to 25°C. The spores on the oatmeal plate were scraped into 0.25% gelatin (ICN Biomedical, Inc.) with sterilized H₂O under a flow hood. Spore suspensions were then filtrated using three to five layers of cheesecloth and concentration was determined using a hemacytometer (Hausser Scientific Company) as described by Sambrook, and Russell (23). A final concentration of spores of 1 to 8 × 10⁶ spores/ml was used for inoculating 200 to 300 rice seedlings in a tray (20 9/16 by 10 3/16 in. [51 23/41 by 25 8/41 cm]) (Hummert).

Standard pathogenicity assays were conducted as previously described (13,27). Specifically, rice seedlings at the three- to four-leaf stage were inoculated with spore suspensions inside a plastic bag. The bags were sealed to maintain 70 to 100% humidity for 24 h at 23 to 25°C in the laboratory. One day after inoculation, the inoculated bags were opened and rice seedlings were moved to a greenhouse for an additional 6 days to allow the full development of disease symptoms. The disease reaction was evaluated at 7 days after inoculation with a 0-to-5-scale rating system, where 0 to 2 indicates resistance and 3 to 5 indicates susceptibility (27). The race identity was determined based on the disease reaction of eight

previously named cultivars in an international differential system, as previously described by Ling and Ou (18). In total, 15 isolates purified from *Pi-ta*-containing Kaybonnet and Drew were retested to see whether the same disease reactions occurred using the above-mentioned procedure. This verified whether these isolates were virulent on *Pi-ta*-containing cultivars. All pathogenicity assays were repeated at least twice; in some cases, disease reaction was determined after a third test.

DNA extraction, Rep-PCR, *AVR-Pita1* amplification, cloning, and sequencing. The fungal DNA was prepared using a Qiagen DNeasy Plant Mini Kit following the manufacturer’s instructions (Qiagen Inc.). All PCR reactions were performed in a Peltier Thermal Cycler (PTC-200; MJ Research). Rep-PCR was performed as previously described (9). Briefly, primers Pot2-1 and Pot2-2 were used to detect genetic identity based on Rep-PCR (Table 1). Each Rep-PCR contained 25 µl of *Taq* PCR Master Mix (Qiagen Inc.), 1 µl of each primer (10 µM), about 30 ng of fungal genomic DNA, and distilled water to 50 µl. Rep-PCR was performed as follows: 1 cycle of 95°C for 2.5 min; 3 cycles of 94°C for 1 min, 62°C for 1 min, and 65°C for 10 min; 25 cycles of 94°C for 30 s, 62°C for 1 min, and 65°C for 10 min; and 1 cycle of 65°C for 15 min. Each Rep-PCR was repeated twice.

Primers YL169 and YL149 (Table 1) (30) were used to detect the presence of the *AVR-Pita1* gene in all isolates. PCR amplifications were performed with *Taq* DNA polymerase (Takara Bio Inc.). PCR components were 0.25 µl of Takara *Taq* (5 U/µl), 5 µl of 10× PCR buffer, 4 µl of dNTP mixture (2.5 mM), 1 µl of each primer (10 µM), about 30 ng of fungal genomic DNA, and distilled water to 50 µl. The PCR program for *AVR-Pita1* was as follows: 1 cycle of 94°C for 3 min; 29 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and, finally, 1 cycle of 72°C for 5 min. PCR products were purified from a gel with a QIA quick Gel Extraction Kit following the manufacturer’s recommendations (Qiagen Inc.) and sequenced using primers YL169 and YL149 (Table 1). The Primer JX-1 (Table 1) was designed based on GenBank sequence (AF207841) to verify DNA sequences of the 3’ region of the *AVR-Pita1* alleles. All other primers used in the present study are listed in Table 1. For DNA sequencing, 20 ng of PCR product and 0.5 µl of 1.6 µM primer were added into each well in an ABI 3730XL DNA Analyzer using 0.5 µl of BigDye v3.1 at the United States Department of Agriculture–Agricultural Research Service Mid South Area Genomics Laboratory at Stoneville, MS.

PCR reactions were repeated three times to produce products for sequencing. For those isolates when *AVR-Pita1* DNA sequences could not be obtained from PCR products, their amplicons were cloned using a TOPO TA Cloning Vector (Invitrogen Corp.). After transformation, 10 individual colonies per isolate were selected (Supplementary Table S2) and sequenced with T7 (0.5 µl at 10 µM) (Table 1) using approximately 100 ng of plasmid DNA.

DNA sequence data analysis. The *AVR-Pita1* DNA sequences were analyzed by Vector NTI Suite V.11 (Invitrogen Corp.), and the haplotype network was constructed by MAGA 4.0 and TCS 1.21 (5,24). DNA sequences were aligned using contig expression and edited based on repeated sequencing results to generate the *AVR-Pita1* sequence assemblies. These sequence assemblies were then converted into FASTA format for MAGA 4, and sequence assemblies were aligned by ClustalW (25). The alignment was then exported as the FASTA format and converted to the Nexus format of the TCS on the website <http://sing.ei.uvigo.es/ALTER/>. To detect any correlation of virulent isolates with rice cultivars with or without *Pi-ta*, independent tests were performed using the IBM SPSS statistics (version 19; IBM Corporation).

Results

Race identification and disease reaction on Katy and M202. Based on disease reactions on the eight international differential rice cultivars, five races were identified among the 169 isolates of *M. oryzae*. In all, 26 isolates belonged to IA1, 98 to IB1, 5 to IB17, 24 to IC1, and 12 to IC17. The four isolates from crabgrass failed to infect eight international rice cultivars and M202; hence, race

Table 1. List of DNA primers used in this study

Primer	Sequence 5’ to 3’	Source
JX-1	ACA AATTGATAAAACATT TA	This study
YL149	TGACCGGATTCCCTCCATT	30
YL169	CGACCCGTTTCCGCC	30
Pot2-1	CGGAAGCCCTAAAGCTGTTT	9
Pot2-2	CCC TCATTCGTACACGTTT	9
T7	TAA TACGACTCACTATAGGGAA	Invitrogen

identity was undetermined. M202 was susceptible to all isolates collected from rice and red rice, whereas Katy was susceptible to 27 isolates, including 24 IC1, 2 IA1, and 1 IB1, but resistant to the remaining 142 isolates.

Initially, we were interested in examining the occurrences of virulent isolates on *Pi-ta*-containing rice cultivars. Of these isolates, 28 were purified from *Pi-ta*-containing cultivars Kaybonnet and Drew (Table 2); however, 20 were no longer infectious to Katy. A χ^2 test of independence of isolate and host genotype in Table 2 demonstrated that there was a significant difference (P value = 0.007) in the occurrence of virulence between isolates from *Pi-ta* and non-*Pi-ta* cultivars, and that the *Pi-ta* gene was related to the occurrence of the virulent isolates.

Genetic identity determined by Rep-PCR. In total, 15 different Rep-PCR patterns labeled as A to O were detected among all the isolates (Fig. 1; Supplementary Table S3; Table 3). There were 8 different patterns in IA1 group, 11 in IB1 group, 3 in IB17 group, 4 in IC1 group, and 3 in IC17 group. Among the isolates within each race group, there was one major pattern with a few other patterns (Table 3). Specifically, the D-type pattern was found in more isolates of the races IA1, IB1, and IB17 while the C pattern was found in more isolates of the races IC1 and IC17.

Evolution of AVR-Pita1. Using AVR-Pita1-specific primers YL169 and YL149 (Table 1), a predicted 1-kb fragment was amplified from 90 avirulent isolates. In contrast, multiple fragments of 0.5 to 3 kb were amplified from nine virulent isolates of the IC1 race and one avirulent isolate of the IB1 race; no PCR products were amplified from the remaining 69 isolates. Sequence analysis of 0.5- and 3-kb fragments indicated that both were not specific to AVR-Pita1, and they were not analyzed further. DNA sequence analysis of PCR amplicons in 90 isolates revealed 10 different haplotypes. Sequence alignment among these 10 haplotypes revealed 21 polymorphic sites (excluding gaps), 18 of which were in the exon region and three in the intron region. Of the 18 sites in the exon region, 17 had led to predicted amino acid substitutions, indicating that most sequence variations in the exon region had also resulted in amino acids alterations in the protein. In all, 6 of the 10 haplotypes were reported previously and 4 haplotypes were new findings (Table 4). Additionally, we found two or three AVR-Pita1 alleles in each of the 12 isolates using plasmids for sequencing (Supplementary Table S4). They were determined to be the haplotypes 1, 36, 40, 41, and 42.

Table 2. Disease reactions on 'Katy' of *Magnaporthe oryzae* isolates purified from *Pi-ta*- or non-*Pi-ta*-containing cultivars

Isolates	Disease reaction of Katy		Total
	Susceptible	Resistant	
<i>Pi-ta</i> -containing ^a	8	20	28
Non- <i>Pi-ta</i> -containing ^b	6	69	75
Total	14	89	103

^a *Pi-ta*-containing 'Kaybonnet' and 'Drew'.

^b Non-*Pi-ta*-containing 'Newbonnet', 'Bengal', 'CL151', 'CL142', 'CL1151', 'Francis', 'Jupiter', 'M206', 'Taggart', 'Wells', and 'RU0801145'.

Discussion

In the present study, we surveyed 165 field isolates of rice blast fungus in Arkansas, where most U.S. rice is grown. These isolates were determined to belong to the five races IA1, IB1, IB17, IC1, and IC17. Along with previous surveys, there are a total of 25 races among 291 isolates from the southern United States (8). Consistent with previous work, isolates of the races IB1, IB17, and IC17 were avirulent to Katy and virulent to M202, suggesting that the *Pi-ta* gene was responsible for preventing infections by these isolates. In addition, most IC1 isolates identified in this study were virulent to Katy. Rep-PCR were repeated for the IC1 and IC17 group using DNAs from newly grown fungus of the same isolate, and the results showed that the patterns were highly similar to one another (*data not shown*), suggesting that these Rep-PCR patterns can be used to rapidly estimate genetic identity of rice blast fungus.

Traditionally, the genetic identity of rice blast fungus was analyzed using restriction fragment length polymorphism based on the repetitive sequence MRG586 or Pot2 (11,29). Rep-PCR was based on the repetitive element Pot2, an inverted repeat transposon (16), which has been widely applied in analyzing the genetic identity of fungal genomes (3,6,12). In the present study, we found that Rep-PCR patterns of field blast isolates were reproducible even with different *Taq* polymerases or DNA preparations. Although there was not a perfect correlation between the race identified and the Rep-PCR pattern, one Rep-PCR pattern was found to predominate in each race group. The Rep-PCR pattern of IC1 was different from that of IELk, which also was known to infect Katy (30). The complexity of our Rep-PCR findings suggest that it may be a useful

Fig. 1. Different repetitive element-based polymerase chain reaction pattern in all rice blast isolates. Lanes A through O represent different pattern types. Patterns C and D predominate in all isolates. Molecular weights of each fragment are also estimated using Bio-Rad Gel Doc software and the data are summarized in Supplementary Table S3.

Table 3. Distribution of repetitive element-based polymerase chain reaction (Rep-PCR) pattern of isolates of *Magnaporthe oryzae* and *M. grisea*

Race	N ^b	Rep-PCR pattern ^a															Patterns ^c
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
IA1	26	...	...	...	9	1	7	3	1	3	1	...	...	...	1	...	8
IB1	98	...	1	3	49	5	16	4	...	4	...	2	9	4	1	...	11
IB17	5	...	...	...	3	...	...	1	...	1	...	...	...	...	...	...	3
IC1	24	...	3	16	...	...	...	...	...	...	1	3	...	...	...	...	4
IC17	12	...	...	8	...	...	...	...	...	...	...	3	1	...	...	...	3
Grass ^d	3	1	...	...	...	...	...	...	...	...	...	...	...	...	...	2	2
Total	168	1	4	27	61	6	23	8	1	8	2	8	10	4	2	2	...

^a Rep-PCR pattern shown in Figure 1.

^b Number of isolate in each race.

^c Total number of different Rep-PCR patterns.

^d Isolates of *M. grisea* purified from crabgrass.

way to look at genetic diversity, especially if *AVR-Pita1* and other virulent genes that affect race are under positive selection or horizontally transferred.

The *Pi-ta* gene in rice is known to recognize the *AVR-Pita1* allele in races of *M. oryzae* (14). The effectiveness of the *Pi-ta* gene in diverse genetic backgrounds was previously investigated (15). In total, 13 *Pi-ta*- and *Pi-ta2*-containing rice cultivars were resistant and 7 *pi-ta*-containing rice cultivars were susceptible to the races IC17 and IB49, respectively. With *Pi-ta*-specific markers, we showed that all *Pi-ta2* cultivars also contained *Pi-ta* (15). Whether or not *Pi-ta* and *Pi-ta2* are involved in recognizing the same pathogen signal molecule remains to be determined. The isolates that we characterized as avirulent and virulent toward Katy in this study would be useful for the investigation of pathogen recognition specificity using additional rice germplasm that carry *Pi-ta*. The monogenic lines with 24 major blast *R* genes developed by the International Rice Research Institute–Japan Collaborative Research Project (26) will be useful to verify *Pi-ta/AVR-Pita1* interaction, and identify additional *R* genes to blast isolates.

The specific marker YL149/YL169 was previously demonstrated to predict the existence of the *AVR-Pita1* gene using PCR. The

absence of PCR amplicons as indicative of the absence of *AVR-Pita1* was previously established using Southern blot analysis (8, 30). In this study, the *AVR-Pita1* allele was consistently amplified by YL149/YL169 from 90 avirulent isolates. Sequence analysis revealed 10 different *AVR-Pita1* haplotypes, of which 4 were new haplotypes designated as haplotypes 39 to 42 (Fig. 2). The other isolates that have *AVR-Pita1* belong to haplotypes 1, 11, 14, 16, 18, and 36. The haplotype sequences predict eight protein variants that are similar to the first reported *AVR-Pita1* sequence. Among these eight variant proteins, amino acid variations were predicted to occur at 15 positions, including the amino acid residue 173 that exists in the conserved region of *AVR-Pita1* (22). These findings support the idea that evolution of *AVR-Pita* alleles may involve protein modifications while maintaining their avirulence protein function (22). Interestingly, we found 12 isolates that contain two or three different *AVR-Pita* haplotypes. The chromosomal location and organization of these *AVR-Pita* haplotypes are undetermined; nevertheless, this finding is similar to the report that approximately two copies of *AVR-Pita* were found in some *M. oryzae* isolates collected from Asia (4). Both studies demonstrate that the *AVR-Pita* family has evolved through a highly complicated mechanism

Table 4. Amino acid variation among the avirulence (AVR)-Pita1 proteins

Protein	Amino acids ^a											
	*	59	81	87	103	118	135	173	191	194	206	222
H38 ^b	...	S	N	R	K	E	G	V	Y	D	K	Y
H39 ^c	L	S	S	K	N	Q	E	I	C	D	K	Y
H40 ^c	L	C	N	R	K	E	G	I	C	H	K	N
H41 ^c	L	C	N	R	K	E	G	V	C	D	R	Y
H42 ^c	L	S	S	K	N	E	E	I	C	H	K	N

^a Numbers above each column indicate amino acid position in the AVR-Pita1 protein (AF207841); * indicates the absence of amino acid. Single-letter code was used for each amino acid.
^b Indicates the original AVR-Pita1 protein (AF207841); the same number was used for each AVR-Pita1 haplotype in Figure 2.
^c GenBank accession numbers: H39, JX467664; H40, JX467665; H41, JX467666; and H42, JX467667.

Fig. 2. Haplotype network for different avirulence (AVR)-*Pita1* alleles. H1 through H38 were reported by Dai et al. (8). Haplotypes in medium and dark gray were found in the present study. Those in dark gray (H39, 40, 41, and 42) were novel haplotypes and those in medium gray were previously found (8). The rectangle stands for the original haplotype, each circle stands for one unique haplotype, and there is 1-bp change between two connected circles. The size of circle represents the relative frequency of the haplotype in the population. Each black node on the line shows a missing haplotype.

(4,22). Additionally, *AVR-Pita1* can be amplified from all four isolates collected from crabgrass, and the *AVR-Pita1* allele from three of four isolates belonged to the haplotype 18. This was consistent with an earlier reported by Dai et al (8). All these crabgrass isolates were avirulent to the tested rice cultivars, suggesting that these isolates were not pathogenic toward rice. Because crabgrass in the present study was located near the levee of rice fields, an investigation of the role of *AVR-Pita1* in crabgrass would lead to a better understanding of the evolution of *AVR-Pita* in *Magnaporthe* spp. (17).

It is not difficult to predict high variability for any gene that is located at the telemetric region. This is the case for the *AVR-Pita1* gene. A sophisticated mechanism involving deletion, point mutation, and transposon insertion has been documented in virulent isolates. In avirulent isolates, more than 40 *AVR-Pita1* variants have been reported (4,8,30). The occurrence of virulent isolates in rice cultivars with or without *Pi-ta* suggests that blast fungus can survive in the southern United States. Moreover, the occurrence of virulent isolates was associated more with rice cultivars with *Pi-ta*. These findings suggest that virulent field isolates of *M. oryzae* in Arkansas are evolving by the selective pressure imposed by the *Pi-ta* gene in host plants.

Acknowledgments

We thank the Arkansas Rice Research and Promotion Board and China National Hybrid Rice Research and Development Center for supporting J. Xing's living and traveling expenses, E. McWhirter for proofreading the manuscript, and all other supporting staff of the Dale Bumpers National Rice Research Center for excellent technical support.

Literature Cited

- 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.
- Ballini, E., Morel, J. B., Droc, G., Price, A., Courtois, B., Notteghem, J. L., and Tharreau, D. 2008. A genome-wide meta-analysis of rice blast resistance genes and quantitative trait loci provides new insights into partial and complete resistance. *Mol. Plant-Microbe Interact.* 21:859-868.
- Chen, Q. H., Wang, Y. C., and Zheng, X. B. 2006. Genetic diversity of *Magnaporthe grisea* in China as revealed by DNA fingerprint haplotypes and pathotypes. *J. Phytopathol.* 154:361-369.
- Chuma, I., Isobe, C., Hotta, Y., Ibaragi, K., Futamata, N., Kusaba, M., Yoshida, K., Terauchi, R., Fujita, Y., Nakayashiki, H., Valent, B., and Tosa, Y. 2011. Multiple translocation of the *AVR-Pita* effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species. *PLoS Pathog.* 7:1-20.
- Clement, M., Posada, D., and Crandall, K. A. 2000. TCS: A computer program to estimate gene genealogies. *Mol. Ecol.* 9:1657-1659.
- Correll, J. C., Harp, T. L., Guerber, J. C., Zeigler, R. S., Liu, B., Cartwright, R. D., and Lee, F. N. 2000. Characterization of *Pyricularia grisea* in the United States using independent genetic and molecular markers. *Phytopathology* 90:1396-1404.
- Couch, B. C., and Kohn, L. M. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94:683-693.
- Dai, Y., Jia, Y., Correll, J., Wang, X., and Wang, Y. 2010. Diversification and evolution of the avirulence gene *AVR-Pita1* in field isolates of *Magnaporthe oryzae*. *Fungal Genet. Biol.* 47:973-980.
- George, M. L. C., Nelson, R. J., Zeigler, R. S., and Leung, H. 1998. Rapid population analysis of *Magnaporthe grisea* by using Rep-PCR and endogenous repetitive DNA sequences. *Phytopathology* 88:223-229.
- Gibbons, J. W., Moldenhauer, K. A. K., Gravois, K., Lee, F. N., Bernhardt, J. L., Meullenet, J. F., Bryant, R., Anders, M., Norman, R. J., Cartwright, R., Taylor, K., Bulloch, J., and Blocker, M. M. 2006. Registration of 'Cybonnet' rice. *Crop Sci.* 46:2317-2318.
- Hamer, J. E., Farrall, L., Orbach, M. J., Valent, B., and Chumley, F. G. 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. *Proc. Natl. Acad. Sci. USA* 86:9981-9985.
- Javan-Nikkhah, M., McDonald, B. A., Banke, S., and Hedjaroude, G. A. 2004. Genetic structure of Iranian *Pyricularia grisea* populations based on Rep-PCR fingerprinting. *Eur. J. Plant Pathol.* 110:909-919.
- Jia, Y., and Martin, R. 2008. Identification of a new locus, *Ptt(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.
- Jia, Y., Wang, Z., Fjellstrom, R. G., Moldenhauer, K. A. K., Azam, M. A., Correll, J., Lee, F. N., Xia, Y., and Rutger, J. N. 2004. Rice *Pi-ta* gene confers resistance to the major pathotypes of the rice blast fungus in the United States. *Phytopathology* 94:296-301.
- Kachroo, P., Leong, S. A., and Chattoo, B. B. 1994. *Pot2*, an inverted repeat transposon from the rice blast fungus *Magnaporthe grisea*. *Mol. Genet.* 245:339-348.
- 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.
- Ling, K. C., and Ou, S. H. 1969. Standardization of the international race numbers of *Pyricularia oryzae*. *Phytopathology* 59:339-342.
- Moldenhauer, K. A. K., Gibbons, J. W., Lee, F. N., Bernhardt, J. L., Wilson, C. E., Cartwright, R. D., Anders, M. M., Norman, R. J., Blocker, M. M., Boyett, V. A., Tolbert, A. C., Taylor, K., and Bulloch, J. M. 2007. Registration of 'Banks' rice. *Crop Sci.* 47:445-446.
- Moldenhauer, K. A. K., Gravois, K. A., Lee, F. N., Norman, R. J., Bernhardt, J. L., Wells, B. R., Dilday, R. H., Blocker, M. M., Rohman, P. C., and McMinn, T. A. 1998. Registration of 'Drew' rice. *Crop Sci.* 38:896-897.
- Moldenhauer, K. A. K., Lee, F. N., Norman, R. J., Helms, R. S., Wells, B. R., Dilday, R. H., Rohman, P. C., and Marchetti, M. A. 1990. Registration of 'Katy' rice. *Crop Sci.* 30:747-748.
- 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.
- Sambrook, J., and Russell, D. W. 2001. Pages 3:8.46-48.53 in: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Silué, D., Nottenghem, J. L., and Tharreau, D. 1992. Evidence for a gene-for-gene relationship in the *Oryza sativa*-*Magnaporthe grisea* pathosystem. *Phytopathology* 82:577-580.
- Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.
- Tsunematsu, H., Yanoria, M. J. T., Ebron, L. A., Hayashi, N., Ando, I., Kato, H., Imbe, T., and Khush, G. S. 2000. Development of monogenic lines of rice for rice blast resistance. *Breed. Sci.* 50:229-234.
- Valent, B., Farrall, L., and Chumley, F. G. 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127:87-101.
- Wells, B. R., Dilday, R. H., Rohman, P. C., and Blocker, M. M. 1995. Registration of 'Kaybonnet' rice. *Crop Sci.* 35:587-588.
- Xia, J. Q., Correll, J. C., Lee, F. N., Ross, W. J., and Rhoads, D. D. 2000. Regional population diversity of *Pyricularia grisea* in Arkansas and the influence of host selection. *Plant Dis.* 84:877-884.
- 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.