Characterization of Molecular Identity and Pathogenicity of Rice Blast Fungus in Hunan Province of China

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Abstract

The blast (*Magnaporthe oryzae*) resistance (*R*) gene is the most economical and environmental method to control rice blast disease. Characterization of molecular identity and pathogenicity of *M. oryzae* benefits the deployment of effective blast *R* genes. In order to identify blast *R* genes that would be effective in Hunan Province, 182 *M. oryzae* strains were analyzed with a Chinese differential system (CDS), repetitive element-based polymerase chain reaction (rep-PCR), and the presence and absence of avirulence (*AVR*) genes by PCR amplification with gene-specific primers. Identified blast *R* genes were validated with 24 monogenic lines (ML) carrying 24 major *R* genes. In total, 28 races

(isolates) of *M. oryzae* was identified with CDS, and classified into 20 distinct groups with rep-PCR. Interestingly, *AVR-Pia*, *AVR-Pik*, *AVR-Pizt*, *AVR-Pib*, and *AVR-Pi9* were detected in more than 86.8% of the isolates; *AVR-Pita1* was in 51.3% and *AVR-Pii* was in only 2.5%. In contrast, pathogenicity assays on 24 ML demonstrated that *Pi9*, *Piz5*, *Pikh*, and *Pikm* were more effective, with resistant frequencies of 91.6, 91, 87.9, and 87.3%, respectively; *Pia*, *Piks*, *Pit*, *Pi12*, and *Pib* were less than 15%. These findings revealed the complexity of a genetic basis of rice blast resistance, and shed light on useful blast *R* genes in Hunan Province.

Rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*), is one of the most destructive rice diseases in the world. Control strategies include the use of cultural practices, fungicides, and genetic resistance. Among them, genetic resistance is one of the best strategies because it is economical and environmentally friendly. Hunan Province, the origin of hybrid rice, ranks at the top in China for area of rice planted and produced (He and Qing 2012). However, over half of the rice-growing areas in Hunan are mountainous and the continental subtropical monsoon climate in Hunan often brings abundant rainfall during the rice-growing period. These high-humidity conditions often favor epidemics of rice blast disease. For a long time, because the main focus was on improving rice yield and less attention was paid to genetic resistance to blast, many rice cultivars released by the Hunan Provincial Seed Board have not contained sufficient genetic resistance to blast. Consequently, blast epidemics occurred in 9% of the total production areas in 2012 and 2014 in Hunan Province (<http://www.hnagri.gov.cn>). Therefore, genetic resistance to blast has now become an essential criterion for any new rice cultivar released by Hunan Province.

Major resistance (*R*) genes in rice effectively prevent blast disease only when *M. oryzae* strains contain corresponding avirulence (*AVR*) genes (Jia et al. 2000). Genetic resistance based on one or two major *R* genes is often broken down through changes of *AVR* genes in *M. oryzae* (Dai et al. 2010; Xing et al. 2013; Zhou et al. 2007), resulting in frequent shortages of major *R* genes. In Hunan, only a few of the rice varieties with considerable genetic resistance were deployed, such as Aihua 5 hao and Chuangfeng 1 hao (Xu et al. 2009). Because of the shortage of *R* genes, fungicides are still widely used in rice

fields. For example, the usage of fungicides in Hunan for rice disease prevention was over 60,000 t annually (He and Qing 2012). Continued use of fungicides has raised serious concerns for environmental safety for humans and wildlife.

Characterization of the molecular identity and pathogenicity of field blast strains has benefitted the selection of effective blast *R* genes. To date, field blast strains have been characterized with repetitive element-based polymerase chain reaction (rep-PCR) (Chen et al. 2006; George et al. 1998), amplified fragment length polymorphism (Thuan et al. 2006), *AVR* genes (Huang et al. 2014; Imam et al. 2015; Wang et al. 2014), differential lines (Le et al. 2010; Li et al. 2007; Xing et al. 2013), and monogenic lines (ML) with major blast *R* genes (Koide et al. 2011; Mekwatanakarn et al. 2000; Tsunematsu et al. 2000; Wang et al. 2013, 2015).

The objectives of the present study were to (i) identify genetic identity of 182 strains of field blast collected from different rice production areas in Hunan with a Chinese differential system and rep-PCR, (ii) screen the presence and absence of *AVR* genes by PCR with gene-specific primers, and (iii) analyze the resistance spectra of different *R* genes with International Rice Research Institute (IRRI) ML.

Materials and Methods

Rice materials. Seven Chinese differential cultivars—Tetep, Zhenlong 13, Sifeng 43, Dongnong 363, Guandong 51, Hejiang 18, and Lijiang-xintuan-heigu (LTH)—were used for race identification, as described by All China Cooperative Research on Physiological Races of *Pyricularia oryzae* (1980). Twenty-four rice ML with major blast *R* genes—IRBLA-A (*Pia*), IRBLi-F5 (*Pii*), IRBLks-F5 (*Piks*), IRBLK-Ka (*Pik*), IRBLkp-K60 (*Pikp*), IRBLkh-K3 (*Pikh*), IRBLz-Fu (*Piz*), IRBLz5-CA (*Piz5*), IRBLzt-T (*Pizt*), IRBLta-K1 (*Pita*), IRBLb-B (*Pib*), IRBLt-K59 (*Pit*), IRBLsh-B (*Pish*), IRBL1-CL (*Pi1*), IRBL3-CP4 (*Pi3*), IRBL5-M (*Pi5*), IRBL7-M (*Pi7*), IRBL9-W (*Pi9*), IRBL12-M (*Pi12*), IRBL19-M (*Pi19*), IRBLkm-Ts (*Pikm*), IRBL20-IR24 (*Pi20*), IRBLta2-Pi (*Pita2*), and IRBL11-Zh (*Pi11*)—along with LTH as a negative control, were used for pathogenicity assay and resistance spectrum analysis of 24 major blast *R* genes (Tsunematsu et al. 2000). Ten seeds were planted for each rice variety, resulting in 7 to 10 seedlings grown to the three- to four-leaf stage for pathogenicity assays in a greenhouse.

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Fungal isolates, growth, and pathogenicity assays. In this study, 182 isolates of *M. oryzae* were collected and purified from panicles from different rice varieties from 20 different regions of Hunan Province in 2012 following the method described by Chen et al. (2001) (Supplementary Table S1). Dried panicle blast samples were sterilized with 70% ethyl alcohol for 10 s and rinsed with distilled water three to four times to remove ethyl alcohol, then were soaked in distilled water for 2 h. The steeped panicle blast samples were laid on plastic plates with distilled water to keep humidity at 27°C for 24 h. Single spores were isolated with a sterilized needle under the microscope, then cultured on the oatmeal agar, and finally stored on sterilized filter paper at -20°C, as described by Xing et al. (2013).

In pathogenicity assays, for all isolates, seven Chinese differential cultivars and 24 rice ML were used for race classification and resistance spectrum analysis of *R* genes, respectively. Before inoculation, blast isolates were cultured on oatmeal agar plates for 7 to 10 days under dark and white florescent light at 25°C. Spores were scraped from oatmeal plates with a 0.25% gelatin solution and filtrated through six layers of cheesecloth to remove agar. The concentration of the spore suspension was determined with a hemocytometer (Sambrook and Russell 2001) before adjusting the concentration to 2×10^5 /ml with the 0.25% gelatin solution for inoculation.

For pathogenicity assays, 300 to 400 rice seedlings at the three- to four-leaf stage in a 56-by-23-cm plastic tray were sprayed with 20 ml of the spore suspension, then moved into a chamber with 95% humidity without light at 27°C for 24 h. Inoculated plants were maintained in the same room with light for the next 6 days. The disease reaction was evaluated at 7 days after inoculation with a 0-to-5-scale rating system, where 0 to 2 indicated resistance and 3 to 5 indicated susceptibility (Xing et al. 2013).

Race identity was determined according to the disease reactions on the Chinese differential cultivars (All China Cooperative Research on Physiological Races of *Pyricularia oryzae* 1980). Specifically, the same race designation of the international differential system was used based on seven Chinese differential rice cultivars; in total, 28 races of *M. oryzae* were separated into six groups: ZA, ZB, ZC, ZD, ZF, and ZG, in which Z stands for Zhongguo (China) and A to G were the abbreviation for each of Chinese differential rice cultivars. If A was susceptible, the isolate belonged to ZA group; if A was resistant and B was susceptible, then the isolate belonged to ZB group; the same rule was followed for the other groups. Pathogenicity assays were repeated once to confirm the disease reactions. The higher rating was used when ratings disagreed.

DNA extraction, rep-PCR, and AVR gene screening. Fungal mycelia were cultured in liquid complete medium (0.6% yeast extract, 0.6% casamino acid, and 1% sucrose) and shaken at 100 rpm at 25°C for 4 to 5 days (Xing et al. 2013). Before DNA extraction, fungal mycelia were freeze dried and pulverized into powder with liquid nitrogen. DNA was prepared following a protocol from the OMEGA fungal genomic extraction kit. For all isolates, rep-PCR was performed with primers Pot2-1 (CGGAAGCCC TAAAGCTGTTT) and Pot2-2 (CCCTCATTCGTCACACGTTTC), as described by George et al. (1998). Seven AVR genes (*AVR-Pia*, *AVR-Pik*, *AVR-Pii*, *AVR-Pizt*, *AVR-Pita1*, *AVR-Pib*, and *AVR-Pi9*) were screened with related specific primers (Wang et al. 2014; Wu et al. 2015; Xing et al. 2013; Zhang et al. 2015). Specifically, primers were designed to amplify a portion of the 5' protein coding region and

the 3' trailer region of each AVR gene (Table 1). PCR mixes included 12 µl of 2× EasyTaq SuperMix (TransGen Biotech), 0.5 µl of each primer (10 µM), about 10 ng of fungal genomic DNA, and distilled water for a final volume of 20 µl. Each rep-PCR contained 12 µl of 2× EasyTaq SuperMix (TransGen Biotech), 1 µl of each primer (10 µM), about 10 ng of fungal genomic DNA, and distilled water for a final volume of 20 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. For all samples, PCR assays were repeated once.

Results

Race classification. The ZA group has six races (ZA1, ZA5, ZA9, ZA13, ZA14, and ZA63), consisting of 25 isolates; the ZB group has 14 races (ZB1, ZB3, ZB5, ZB7, ZB9, ZB11, ZB13, ZB14, ZB15, ZB16, ZB19, ZB29, ZB30, and ZB31), with 117 isolates; the ZC group has five races (ZC5, ZC9, ZC13, ZC15, and ZC16), with 31 isolates; the ZD group has one race (ZD7), with 1 isolate; the ZF group has one race (ZF1), with 2 isolates; and the ZG group has one race (ZG1), with 6 isolates (Table 2). Among them, the ZB group has the most races and the ZD group has the least. For all isolates, ZB13 (29.1%) was the most predominant race, followed by ZB15 (11.5%), ZA13 (7.1%), and ZC15 (7.1%). The isolates from five

Table 2. Total number of isolates (*n*) for each race in Hunan Province

Group	Race	Isolates (<i>n</i>)
ZA	ZA1	1
	ZA5	8
	ZA9	1
	ZA13	13
	ZA14	1
	ZA63	1
ZB	ZB1	9
	ZB3	1
	ZB5	9
	ZB7	3
	ZB9	4
	ZB11	3
	ZB13	53
	ZB14	4
	ZB15	21
	ZB16	1
	ZB19	1
	ZB29	2
	ZB30	2
	ZB31	4
ZC	ZC5	3
	ZC9	1
	ZC13	8
	ZC15	13
	ZC16	6
ZD	ZD7	1
ZF	ZF1	2
ZG	ZG1	6

Table 1. List of primer sequences, predicted sizes, and references for avirulence (AVR) genes

AVR gene	Forward sequence	Reverse sequence	Size (bp)	Reference
<i>AVR-Pia</i>	TTATTGCTACCACCTTCCTC	GTAAGTAAGTGGCGTT	464	Wang et al. 2014
<i>AVR-Pik</i>	TCACCTTGGGAACGTGCG	GGAAGTCGCCCCGACAAAT	267	AB498875 ^a
<i>AVR-Pii</i>	CCTCGGCTTCTTGATATTACT	TAAATCGTGCGCTTTCAGAT	373	Wang et al. 2014
<i>AVR-Pizt</i>	GATCAAATGAACACCAGGAA	CGATGAAGAATGGAAGAATG	505	Wang et al. 2014
<i>AVR-Pita1</i>	TCACACCGTTGCGATTTC	ACTTGTGAATCCCATCCC	713	Xing et al. 2013
<i>AVR-Pib</i>	CGTTCTCAACCACTTT	ATTCCACGGTATATTTGC	220	Zhang et al. 2015
<i>AVR-Pi9</i>	CAGTTCTCTCAGATCCTCAC	TACCAGTGCGTCTTTTCGAC	338	Wu et al. 2015

^a GenBank accession number (<http://www.ncbi.nlm.nih.gov/genbank/>).

major regions (Chenzhou, Zhuzhou, Shaoyang, Yiyang, and Huaihua) were analyzed to determine regional race diversity. We found 14 races (52 isolates) in Chenzhou, 20 races (56 isolates) in Zhuzhou, 9 races (16 isolates) in Shaoyang, 6 races (13 isolates) in Yiyang, and 7 races (14 isolates) in Huaihua (Table 3). In the Chenzhou and Zhuzhou districts, where more isolates were collected, more races were identified and ZB13 was the dominant race among them. The lower diversity in other districts may be due to the limited number of blast isolates collected.

Genomic diversity based on rep-PCR. In total, 20 rep-PCR categories were detected, where categories A (19%), B (12%), D, (10%), and N (9%) were more abundant than the others (Fig. 1). There were 11 different categories in ZB13, 8 in ZB15, 5 in ZA13, and 8 in ZC15. The close correlation between blast races and rep-PCR categories was not found. However, category A was the most common in the ZA and ZC groups and category B was the most common in the ZB group. The remaining groups with few isolates were not analyzed for correlation. The genomic identity determined by rep-PCR was not diverse in comparison with that determined by pathogenicity on ML with major *R* genes, suggesting that *AVR* genes in *M. oryzae* were highly unstable.

The *AVR* gene profile. Presence and absence of *AVR* genes in all isolates are listed in Supplementary Table S2. The percentages of *AVR-Pia*, *AVR-Pik*, *AVR-Pii*, *AVR-Pizt*, *AVR-Pita1*, *AVR-Pib*, and *AVR-Pi9* in field isolates of *M. oryzae* were 98.8, 93.8, 2.5, 96.3, 51.3, 86.8, and 97.5%, respectively. The results revealed that *AVR-Pia*, *AVR-Pik*, *AVR-Pizt*, *AVR-Pib*, and *AVR-Pi9* were common in field isolates of Hunan Province, suggesting that the corresponding *R* genes may be useful for preventing blast disease. Based on race

information and presence of *AVR* genes in each isolate, there was no direct correlation found between race and *AVR* gene composition.

Disease reactions to ML. The pathogenicity results on ML are shown in Supplementary Table S3. The resistance frequencies of ML carrying *Pi9*, *Piz5*, *Pikh*, and *Pikm* were 91.6, 91, 87.9, and 87.3%, respectively. Resistance frequency of ML carrying *Pia*, *Piks*, *Pit*, *Pi12*, *Pib*, *Pi3*, *Pish*, *Pii*, *Pi19*, *Pi20*, and *Pi11* were less than 31%, and the remaining genes were between 40 and 73% (Fig. 2). These findings suggest that *Pi9*, *Piz5*, *Pikh*, and *Pikm* were more effective than other major *R* genes tested in controlling rice blast disease.

Discussion

Understanding race identity and pathogenicity of field blast isolates is useful for breeders to select a small number of representative isolates or races for breeding for improved blast resistance. In the present study, we characterized 182 field isolates belonging to 28 races with a Chinese differential system and 20 categories with rep-PCR, and the results suggest that a mixture of 20 to 28 blast isolates or races is adequate for breeders to use. However, 162 different disease reaction performances for 166 isolates on ML suggest that there is an extraordinary complexity of *AVR* genes in field isolates from Hunan.

To further investigate the avirulence profiles, we screened the presence and absence of *AVR* genes with gene-specific primers.

Table 3. Summary of isolate and race in each district in Hunan Province

District ^a	Number of	
	Isolates	Races
Chenzhou	52	14
Zhuzhou	56	20
Shaoyang	16	9
Yiyang	13	6
Huaihua	14	7

^a Main collecting sites in Hunan.

Fig. 2. Resistance ratio of 24 resistance (*R*) genes to isolates in Hunan. Resistance ratios of *Pi9*, *Piz5*, *Pikh*, and *Pikm* were around 90%.

Fig. 1. Graphic description of repetitive element-based polymerase chain reaction categories for all isolates. A to T represent each distinct category, in which A, B, D, and N were the major categories.

Seven AVR genes (*AVR-Pia*, *AVR-Pik*, *AVR-Pii*, *AVR-Pizt*, *AVR-Pita1*, *AVR-Pib*, and *AVR-Pi9*) were screened. *AVR-Pia*, *AVR-Pik*, *AVR-Pizt*, and *AVR-Pib* were detected in more than 86.8% isolates; however, pathogenicity assays of these isolates on the ML carrying the corresponding *R* genes showed that less than 86% were avirulent. This discrepancy may be due to the function loss of AVR genes. It was demonstrated that the single-nucleotide polymorphisms in AVR genes can lead to function variation of the AVR genes from avirulence into virulence (Orbach et al. 2000). For isolates from Liaoning Province, north China, *AVR-Pik*, *AVR-Pizt*, and *AVR-Pita1* were detected in most of the isolates but *AVR-Pia* and *AVR-Pii* were not detected. Sequence analysis of *AVR-Pik* and *AVR-Pita1* revealed significant variations that may be associated with loss of their avirulence functions (Wang et al. 2014). Therefore, sequence analysis of AVR genes in the isolates will help to resolve the discrepancy and aid in the development of gene-specific primers for these AVR genes. On the other hand, resistance frequencies of ML carrying *Pii* were higher than the frequencies of the presence of *AVR-Pii*, suggesting that there are additional minor *R* genes in the ML (Wang et al. 2015). This is due to the fact that the ML used in the study were pure lines consisting of two backcrosses to LTH from *R* gene donors of diverse rice germplasm (Tsunematsu et al. 2000). We found that five *R* genes (*Pi12*, *Piks*, *Pit*, *Pib*, and *Pia*) were less effective, which is similar to the situation in the southern United States, where *Pia* and *Pii* were less effective than other major blast *R* genes in ML (Wang et al. 2015).

Overall, race ZB13 was the most commonly found race and the presence of this race has increased gradually in surveyed years. The most commonly found races in Hunan in different years were previously reported (Huang et al. 2010; Li et al. 2007). In the present study, we found that there was a variation in the most commonly found races in different years. The research in 2004 and 2010 showed that ZG1 occurred with a frequency of 26.5 and 17.49%, respectively, and ZB13 occurred with a frequency of 11.55% in 2010 and 29.1% in 2012. Similar findings of changes in most commonly found races have been described (Huang et al. 2010; Li et al. 2007). Interestingly, more isolates belonging to the ZA group were found, with increased percentages from 6.6 to 12.6% in 2 years, suggesting that they are more virulent isolates toward Tetep, the most resistant variety in this Chinese differential system used. Tetep was reported to contain at least four major *R* genes: *Pi3*, *Pi5*, *Pi-kh*, and *Pi-ta* (Jeon et al. 2003; Jia et al. 2003; Sharma et al. 2005; Yi et al. 2004). *Pi-ta*, inherited from Tetep, has been effective in the United States for over 20 years (Jia et al. 2003, 2004). Consistently, more than half of the isolates in the ZA group did not have *AVR-Pita1*, suggesting that the *Pi-ta* gene was not effective in rice production areas where isolates in the ZA group were collected.

rep-PCR has been widely used to analyze the genetic identity of rice blast fungus (Javan-nikkhah et al. 2004; Xing et al. 2013). In the present study, 20 rep-PCR categories have been found, and most of the rep-PCR types belong to the dominant race ZB13. Poor correlations between race and rep-PCR category may be due to the complex fungal population in Hunan. Based on rep-PCR, we suggest that the genetic background of the field blast isolates in Hunan is not diverse. Further genotyping analysis using simple sequence repeats (Wang et al. 1998) or genome-wide sequencing (Chen et al. 2013; Xue et al. 2012) will be required to confirm our prediction. Despite discrepancies between AVR gene profiles and disease reactions to ML carrying the corresponding *R* genes, our data demonstrated that the AVR genes in the field isolates in Hunan were much more complicated than previously thought. Further investigation of the historical record of deployed *R* genes in Hunan will increase understanding of the molecular basis of coevolution of *R* and AVR genes.

The *Pi9* gene was previously shown to be an effective *R* gene in different countries and districts in China (Qu et al. 2006; Wang et al. 2015; Wu et al. 2015). Our results suggest that, in addition to *Pi9*, *Piz5*, *Pikh*, and *Pikm* are also effective blast *R* genes that can be immediately deployed to prevent rice blast disease in Hunan Province. Most test isolates can be controlled by *Pi9* with *Pi5* or *Pikm* with *Piz5*. However, we have identified two strains that were virulent

to all four *R* genes. The first is HNB61 (race ZB3, category H), which was virulent on 22 of the 24 *R* genes; HNB61 was only avirulent toward *Pii* and *Pita*. The second is HNB120 (race ZC15, category G), which was virulent on all *R* genes except *Pi5* and *Pi7*. Additionally, HNB62 was virulent on seven *R* genes (*Piz5*, *Pii*, *Piz*, *Pita*, *Pi3*, *Pi5*, and *Pita2*) and has already overcome three of the four most broadly effective *R* genes. Although 12 isolates have overcome two of the four recommended genes, 6 isolates would still be controlled by *Piz5* with *Pi9*, 2 would be controlled by *Piz5* and *Pikh*, 2 would be controlled by *Piz5* and *Pikm*, and 2 isolates would be controlled by *Pi9* and *Pikm*.

In conclusion, we demonstrated that the blast population in Hunan Province was extremely diverse in pathogenicity, suggesting that challenges lie ahead in preventing rice blast. For a short-term benefit, two gene pyramids, as mentioned above, should be considered for different rice production areas and, eventually, stacking four *R* genes (*Pi9*, *Piz5*, *Pikh*, and *Pikm*) will lead to more effective blast resistance in Hunan Province.

Acknowledgments

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