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Full Length Article

Rice Blast Resistance Analysis and Gene Identification of Restorer Line Mianhui357

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Abstract

Blast disease caused by the filamentous fungus *Magnaporthe oryzae* is one of the most serious rice diseases throughout the world. Mianhui357 (MH357) is an elite hybrid rice restorer line with high resistance to rice blast. To identify the major functional blast resistance genes in MH357, a disease assay was performed with 90 *M. oryzae* strains on MH357, Lijiangxin Tuan Heigu (LTH) and LTH-derived monogenic lines to determine their resistance levels. The disease phenotypes of MH357 were level 5 and level 3 symptoms for seedling blast and neck blast, respectively. MH357 had a blast resistance frequency of 83.3% to artificial inoculation and was classified as moderately resistant (MR) in the field, suggesting broad-spectrum resistance. The LTH monogenic lines with *Pi2*, *Pi9*, *Pi5* and *Pikm* exhibited high blast resistance in Sichuan Province. Additionally, we performed expression profiling by reverse transcription PCR (RT-PCR) on previously reported blast resistance genes in MH357. Genes at the Pita, Pid2, Pia, Pi5 and Pik loci showed high expression. Furthermore, molecular markers and blast resistance gene cloning were used to identify the *R* genes in MH357. Our data suggested that the broad-spectrum blast *R* genes *Pi5* and *Pid2-357* contributed to the rice blast resistance of MH357, and that *Pid2-357* was a novel functional allele conferring blast resistance. Thus, MH357 is a restorer line with broad-spectrum resistance containing multiple blast resistance genes, and is valuable for rice blast resistance breeding. © 2018 Friends Science Publishers

Keywords: Gene identification; Resistance analysis; Rice blast; Rice restorer line

Introduction

Rice blast is the most devastating fungal disease of rice worldwide, and is caused by the filamentous ascomycete *Magnaporthe oryzae* (Liu *et al.*, 2010; Lv *et al*., 2017). Rice blast seriously affects rice quality and rice yield, and is responsible for 20–50% of yield losses in susceptible rice varieties (Savary and Willocquet, 2000). As the use of chemical pesticides is harmful to the environment and human and animal health, developing resistant rice cultivars is the most effective and eco-friendly way to control rice blast (Wang *et al*., 1999). However, because of the rapid evolution of local *M. oryzae* races, rice varieties with race-specific resistance generally lose their resistance to rice blast within several years after their release (Line and Chen, 1995). Thus, it is important to identify rice germplasm genotypes with durable resistance for breeding.

In recent decades, research on rice blast resistance genes has become more popular due to the rapid development of molecular biological techniques. To date, more than 69 rice blast *R* loci have been identified, among which 16 loci with at least 30 *R* genes or alleles have been cloned and functionally analyzed in detail (Devanna *et al*., 2014; Xu *et al.*, 2014; Ma *et al.*, 2015; Zhang *et al*., 2015). In recent years, however, few *R* loci have been detected and a significant number of newly cloned rice blast *R* genes have been identified as being allelic to previously cloned rice blast *R* genes, with very few representing new rice blast *R* loci (Xu *et al.*, 2014; Vasudevan *et al.*, 2016). The majority of the cloned rice blast *R* genes form clusters (Qu *et al.*, 2006; Liu *et al.*, 2007; Ma *et al.*, 2015). For example, the Pi2 locus contains the cloned R genes *Pi2*, *Pi9*, *Pizt* and *Pi50*, and the Pik locus contains the cloned R genes *Pik*, *Pikp*, *Pikm* and *Pi1*. Thus, we believe that allele mining of cloned rice blast *R* genes in rice germplasms should reveal additional favorable *R* alleles for rice blast resistance breeding (Leung *et al.*, 2015).

Hybrid rice breeding is an efficient method to develop new varieties with rice blast resistance. Mianhui357 (MH357) is a hybrid rice restorer line derived from a cross between Chenhui178 and Mianhui9938 (Mianhui138/Mianhui725) that exhibits dominant broad-spectrum resistance to rice blast. Several hybrid rice cultivars derived from MH357 have high yield with good grain quality. Thus, it is important to identify the major blast resistance genes in MH357 to make full use of this line. In this study, we performed a field resistance evaluation, expression profiling, molecular marker analysis and allele

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cloning, and found that the broad-spectrum resistance gene *Pi5* and a novel functional allele, *Pid2-357*, at the Pid2 locus were the main contributors to the blast resistance of MH357.

Materials and Methods

Rice Materials

We used the hybrid rice restorer line Mianhui357 (MH357) derived from a cross between Chenhui178 and Mianhui9938. MH357 exhibits dominant broad-spectrum field resistance to rice blast. The japonica rice variety Lijiangxin Tuan Heigu (LTH), which is considered to have no resistance genes, was used as a negative control for disease evaluation. Monogenic resistance gene lines in the LTH background were also used and are shown in Table 1 (Tsunematsu *et al.,* 2010).

M. oryzae Pathogen Strains

The *M. oryzae* strains used in this study (90 isolates in total) included strains collected from different rice growing areas in China and some strains previously stored in the lab. The single-spore isolation method was used to isolate strains (Gong *et al*., 2010).

Evaluation of Resistance against Rice Blast Disease

The rice blast resistance of MH357, LTH and the LTH monogenic lines was evaluated in Pujiang (30°20′ N, 103°29′ E; central Sichuan Province, China), which is seriously affected by rice blast, in 2016 and 2017. For each monogenic line, 30 plants were planted in three rows with 25 cm spacing between rows and 15 cm spacing within rows, using a completely random arrangement. No fungicide was used to prevent the disease during the whole growth period.

For rice blast resistance identification and RT-PCR under controlled conditions, the rice plants were grown in a controlled environment at 26°C and 70% relative humidity, with a 14 h light/10 h dark photoperiod. Rice seedlings at the three-leaf-stage were sprayed with the pathogen for inoculation as described (Li *et al*., 2014). Approximately 90 single-spore *M. oryzae* strains were used. Disease severity was evaluated on a scale of 1 (resistant, R) to 9 (susceptible, S) in accordance with the standard evaluation method of the IRRI (IRRI, 1996).

Gene Expression Analysis

Three-week-old rice seedlings were inoculated with mixed *M. oryzae* strains (Li *et al*., 2014) to analyze rice blast *R* gene expression. Leaves were collected at 0, 12, 24 and 48 h after inoculation and immediately frozen in liquid nitrogen. Disease symptoms were evaluated 1–2 weeks after inoculation.

Total RNA was isolated and cDNA was obtained from MH357 at 0, 12, 24 and 48 h after inoculation using ReverTra Ace qPCR RT Master Mix with gDNA Remover according to the manufacturer's instructions (Osaka, Japan). The cDNA was used as the template for RT-PCR. A pair of universal primers was designed for each *R* gene. The rice *OsActin* gene was used as the reference gene. The RT-PCR program was as follows: 94°C for 3 min, followed by 27 cycles of 94°C for 20 s, 58°C for 20 s and 72°C for 20 s, with a final extension at 72°C for 5 min. The PCR products were resolved on 1.5% agarose gels.

Pi5 Linkage Analysis and Sequencing of Pik and Pid2-357

DNA was extracted from leaves of each rice variety following the CTAB method (Doyle and Doyle, 1990). The full-length Pik and Pid2 loci genes and a *Pi5* DNA fragment were cloned, and the PCR products were sequenced by Sangon Biotech (Shanghai, China). The primers used in this study are shown in Table 2.

Results

Broad Resistance Spectrum of MH357 to Rice Blast

The rice blast resistance of MH357 and the LTH monogenic lines was investigated for two years. LTH is highly susceptible to rice blast, so it was used as a negative control for rice blast evaluation. The results of the resistance analysis are shown in Table 3.

The resistance frequency of LTH was 0 according to the artificial inoculation results. MH357 had a resistance frequency of 83.3%. In the natural disease investigation in the field, MH357 had level 5 leaf blast symptoms and level 3 neck blast symptoms, and was evaluated as moderately resistant (MR). Thus, MH357 displayed good resistance to the tested *M. oryzae* strains, showing broad-spectrum blast resistance.

The *Pi5* line had a resistance frequency of 72.2% according to the artificial inoculation results. In the natural disease investigation in the field, its leaf blast and neck blast levels were both 5, and it was evaluated as MR. Thus, *Pi5* displayed resistance to the tested *M. oryzae* strains, indicating broad-spectrum blast resistance.

The Pi2 locus includes the cloned genes *Pi2*, *Pi9* and *Pizt*. The resistance frequencies of the monogenic lines carrying these genes were 98.9%, 96.7% and 72.2%, respectively, according to the artificial inoculation results. In the field disease investigation, the leaf blast levels were 1, 3 and 4, and the neck blast levels were 1, 1 and 7, respectively. Thus, these lines were evaluated as R, R and moderately susceptible (MS), respectively. These results suggested that *Pi2* and *Pi9* have maintained their strong blast resistance, so should be used in resistance breeding, but that *Pizt* is gradually losing its resistance.

Table 1: Thirteen monogenic lines of blast resistance genes

No.	Monogenic lines	Carry genes	No.	Monogenic lines	Carry genes
IR2	IRBLa-C	*Pia*	IR16	IRBLsh-S	*Pish*
IR6	IRBLk-Ka	*Pik*	IR18	IRBL1-CL	*Pi1*
IR7	IRBLkp-K60	*Pikp*	IR20	IRBL5-M	*Pi5*
IR10	IRBLz5-CA	*Pi2*	IR22	IRBL9-W	*Pi9*
IR11	IRBLzt-T	*Pizt*	IR25	IRBLkm-Ts	*Pikm*
IR14	IRBLb-B	*Pib*	IR29	IRBLta-CP1	*Pita*
IR15	IRBLt-K59	*Pit*			

Table 2: Primers and sequences Sources

Primers		Sequence (5'-3')
RTPita	F: F: TACATCTTCACCAGCATCCC	R: AGACCCGAACCCCTCATT
RTPid2	F: GCCTGAGAATGTTCTACTTGACG	R: GCTCTTCCTCCCACCGA
RTPi2-loc	F: ATCACGACCTGGGGGCTGAA	R: TTCGTCGTCAACGTGATCA
RTPid3-loc	F: CCTGCTCTGTCCAAACCTG	R: CACCATTTCTGATGAACCCA
RTPia-RGA4	F: AGACGTTGATAGTGTAATGGAGG	R: CAGCAGGAGACATCTGAAAGC
RTPia-RGA5	F: TGAACTCTGCCTTGCTTTTATG	R: TGCTTGTTGACAGTTTCCTTG
RTPikl-loc	F: TCCTCATCAATGCTGGGTAT	R: CGATCTTGGGTTTCCTCTTC
RTPik2-loc	F: GGATCAGGACATAATAAAGGACA	R: CTCACGGAGATTTTCAAGGA
RTPi36	F: ATGTTCGGTTCCTAAAAGATGC	R: TGGACGGTTGGGATGGC
RTPi37	F: ATCTCACAGTTTCGCGTCC	R: CCTGGTGGTGACCTCATTTC
RTPit	F: AAGGAAGCAACATCGTTTACC	R: CAGCATTTACACCCACCGT
RTPi5-1	F: AGAAATGCGACAACACTCCC	R: AGGAACCAGGCTAACGGAC
RTPi5-2	F: AATAGACTACTCCCGTCCTCCC	R: TTCCTTGATAACCAATGTGCTGT
RTPish	F: AGGTTTCAAAGTTCCAGGGTT	R: AGATGTTATGTTGGGGCAGTC
OsActin	F: CCTCGTCTCGACCTTGCTGGG	R: GAGAACAAGCAGGAGGACGGC
Pi5-1-2	F: CGCTATCCAATCCAATGCTTCTG	R: ACATCAAGTGGCAAGGTTCCATG
Pik1	F: ATGGAGGCGGCTGCCATGGC	R: CTAGCTAGTAGTTTCTGTTTGAATTTCAATAT
Pik2	F: ATGGAGTTGGTGGTAGGTGCTTC	R: TCATGCAGTGACGATGCCATCAAC
Pid2	F: ATGCAAATGTGTGGATGGTTACTGAAG	R: TCATCTGGGACCAGAGAGCCTCA

Sources: (Zheng *et al*., 2014; Shi *et al*; 2015; Zhao *et al*., 2017)

Table 3: MH357, LTH and monogenic lines field resistance and inoculating resistance analysis

No.	Carry genes	Leaf blast	Neck blast	Resistance	Resistance spectrum (%)
MH357		5	3	MR	83.3
LTH	None	9	9	HS	1.1
IR2	*Pia*	9	9	HS	2.2
IR6	*Pik*	8	8	S	27.8
IR7	*Pikp*	8	8	S	35.5
IR10	*Pi2*	1	1	R	98.9
IR11	*Pizt*	4	7	MS	72.2
IR14	*Pib*	9	9	HS	5.56
IR15	*Pit*	9	9	HS	5.56
IR16	*Pish*	8	5	MS	44.4
IR18	*Pi1*	7	5	MS	55.6
IR20	*Pi5*	5	5	MR	72.2
IR22	*Pi9*	3	1	R	96.7
IR25	*Pikm*	5	3	MR	67.7
IR29	*Pita*	7	7	S	27.8

The Pik locus includes the cloned genes *Pik*, *Pikp*, *Pi1* and *Pikm*. The resistance frequencies of the monogenic lines carrying these genes were 27.8%, 35.5%, 55.6% and 67.7%, respectively, according to the artificial inoculation results. In the field disease investigation, the leaf blast levels were 8, 8, 7 and 5, and the neck blast levels were 8, 8, 5 and 3, respectively. Thus, they were evaluated as S, S, MS and MR, respectively. This suggested that *Pikm* has maintained its blast resistance, while the other genes have lost their resistance.

The other LTH monogenic lines, including those carrying *Pia*, *Pib*, *Pit*, *Pish*, and *Pita*, had all lost their blast resistance in Sichuan Province. The rest of the cloned *R* genes, including *Pid2, Pid3, Pid3-A4, Pi25*, *Pi21*, *Pb1*, *Pi36*, *Pi37*, *Pi50*, *Pi54* and *Pi56*, could not be evaluated, as LTH monogenic lines carrying these genes were unavailable.

Expression Profiling of Rice Blast *R* Genes in MH357 by RT-PCR

To identify the major functional blast *R* genes in MH357, RT-PCR was performed to examine the expression profiles of the cloned blast *R* genes in MH357 inoculated with *M. oryzae*. The results indicated that genes at the Pita, Pid2, Pi5, Pia, Pik (*Pikp*, *Pikm*, *Pi1*) loci had high expression levels at different time points after inoculation (Fig. 1). Conversely, transcripts of the Pib, Pi36, Pi37, Pi25 (*Pid3*, *Pid3-A4*), Pish, Pikh, Pi2 (*Pi9*, *Pizt*, *Pi50*), Pi1, Pi56, Pit and Pb loci genes were not detected in MH357.

***Pi5* Linkage Marker Analysis and Allele Cloning**

To identify whether the *R* gene *Pi5* was present in MH357, we used a *Pi5*-specific molecular marker. We obtained the same fragments (about 1066 bp) from both MH357 and the *Pi5* monogenic line *Pi5-NIL* (IR20) (Fig. 2). We also obtained a full-length fragment of *Pid2-357* from the *Pid2* locus in MH357 by allele cloning. To determine whether the amplified fragment from the Pik locus belonged to the resistant or susceptible allele, the full length of the Pik locus was amplified from genomic DNA of MH357.

Discussion

The rice three-line hybrid breeding system has significantly improved rice yields worldwide. Hybrid varieties developed using the WA (wild abortive) type of CMS (cytoplasmic male sterility) accounted for approximately 90% of the hybrid rice produced in China in the past (Yao *et al*., 1997). Restorer lines play an important role in improving the agronomic traits of hybrid rice. MH357 is an elite restorer line that has been used to generate numerous hybrid rice varieties. According to several years of field observations, MH357 displays dominant and strong rice blast resistance at different locations in Sichuan Province, China. In this study, we attempted to identify the major rice blast *R* genes in MH357.

Previously, most of the cloned rice blast resistance genes were introduced into the LTH background to form a series of monogenic lines (Tsunematsu *et al*., 2010). The monogenic lines carrying *Pi2*, *Pi5*, *Pi9* and *Pikm* were evaluated as "resistant" (R) or "highly resistant" (HR) to rice blast in our field disease investigation, and showed resistance frequencies of 98.9%, 96.7%, 72.2% and 67.7%, respectively, in our artificial inoculation experiment. MH357 was evaluated as MR to rice blast and had a resistance frequency of 83.3%. Therefore, the *R* gene in MH357 would not be *Pi2* or *Pi9*. The other LTH monogenic lines had all lost their field blast resistance in Sichuan Province. Thus, the Pi5 and Pik locus genes were identified as candidate blast *R* genes in MH357.

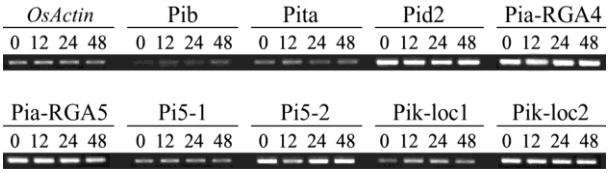

Fig. 1: RT-PCR analysis of cloned blast *R* genes expression profiling in Mianhui357. Rice *OsActin* gene was set as the control. Leaf samples were collected at 0, 12, 24, and 48 h post inoculation (hpi) for total RNA extraction

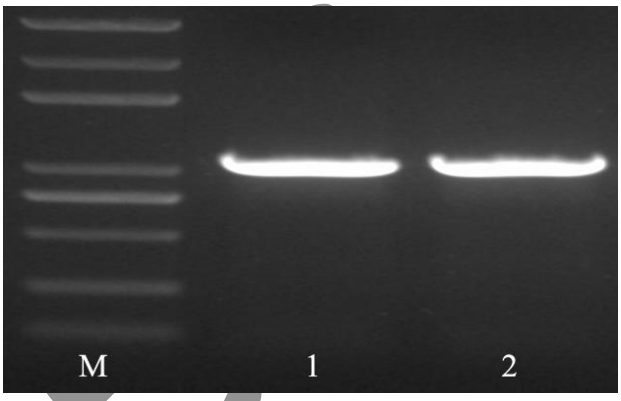

Fig. 2: Pi5 Molecular marker detection. M: marker 2K; 1: Pi5-NIL (IR20) 2: MH357

The Pi2 and Pik loci are the most important resistance loci, and many alleles have been cloned from them. However, *R* genes from the same locus may show different resistance characteristics. For example, *Pi2* and *Pizt* encode resistance proteins with eight amino-acid differences within three leucine-rich repeats (Zhou *et al.*, 2006). In our tests, *Pi2* and *Pi9* showed excellent blast resistance, with resistance frequencies of 98.9% and 96.7%, and were evaluated as R in the field disease assay. By contrast, the *Pizt*-NIL had a resistance frequency of 72.2% and was MS in the field. *Pikm* also showed good blast resistance, with a resistance frequency of 67.7%, and was MR in the field disease assay. However, *Pik* and *Pikp* only had resistance frequencies of 27.8% and 35.5%, and were both classified as S in the field disease assay, indicating they are susceptible to rice blast.

Previously, 23 rice restorer lines and 29 rice sterility lines from Sichuan Province were analyzed using molecular markers closely linked to rice blast *R* genes (Feng *et al.*, 2013). The results showed that none of them had the resistant genes *Pi9* or *Pi2*, while 11 of them probably contained the Pik locus resistance gene. Although MH357 has the *Pik* gene in the Pik locus, this gene has lost its field resistance. In the future, we need to collect and identify more germplasm resources containing *Pi2*, *Pi9*, *Pi5* and *Pikm* genes. Then, we can utilize molecular marker-assisted selection (MAS) and hybridization breeding to introduce these genes into rice restorer and sterility lines.

To identify the major functional blast *R* genes in MH357, RT-PCR was carried out to detect the expression levels of cloned rice blast *R* genes in MH357 inoculated with *M. oryzae*. High gene expression levels were detected for the Pita, Pid2, Pia, Pi5 and Pik (*Pikp*, *Pikm*, *Pi1*) loci. Because the Pita and Pia loci have their lost field resistance and would contribute little resistance to MH357, we focused on the Pik, Pi5 and Pid2 loci. By allele cloning and sequencing, a full-length *Pik* DNA sequence was obtained. The Pik (*Pik-1* and *Pik-2*) sequence in MH357 was identical to a previously reported *R* allele (Zhai *et al*., 2011). However, the *Pik* gene has lost its field resistance, so *Pik* cannot be the major *R* gene in MH357.

The Pi5 locus gene was detected by RT-PCR, but a full-length fragment could not be obtained for *Pi5*. Thus, we used the *Pi5*-specific molecular marker Pi5-1-2 to amplify a *Pi5* fragment (Zheng *et al*., 2014). We obtained and sequenced fragments of the same size (1066 bp) from both MH357 and the *Pi5* monogenic line *Pi5-NIL* (IR20). The sequencing analysis indicated the 1066 bp DNA fragment was exactly the same as the *R* gene *Pi5* reported previously (Lee *et al*., 2009). These results suggested *Pi5* is functional in MH357. *Pi5* confers broad-spectrum blast resistance to *M. oryzae* strains from Korea, Philippines and most provinces in China (Wang *et al*., 1994; Chen *et al*., 2001; Han, 2010; Zheng *et al*., 2014). Our data indicated *Pi5* has a resistance frequency of 72.2% according to the artificial inoculation results. In the field resistance evaluation, it was classified as MR. Thus, *Pi5* should be the major *R* gene in MH357 and would be valuable for resistance breeding.

A full-length fragment of *Pid2-357* was amplified from the *Pid2* locus in MH357 by allele cloning. Compared with the previously reported allele *Pid2-Y1B* in Y1B, there was only one nucleotide substitution in *Pid2-357*, *i.e*., T2232C, which causes no amino acid change. *Pid2-Y1B* is resistant to a number of *M. oryzae* isolates, with a resistance frequency of 65%. Knocking-down *Pid2-Y1B* via RNAi in Y1B resulted in susceptibility. In contrast, overexpression of *Pid2-Y1B* in a blast-susceptible accession led to enhanced resistance to *M. oryzae* (Wang *et al*., 2017). In a previous report, a single amino acid difference at position 441 of the rice blast resistance gene *Pid2* in Digu was shown to distinguish resistant and susceptible alleles (Chen *et al*., 2006). *Pid2-357* had the same amino acid sequence as *Pid2* in Digu at position 441 (Table 4). Therefore, *Pid2-357* is a main contributor to the rice blast resistance in MH357. Additionally, we claim that *Pid2-357* is a novel functional allele.

Conclusion

Our findings suggest that the broad-spectrum blast resistance genes *Pi5* and *Pid2-357* are the major contributors to the blast resistance of MH357. Additionally, the *Pik* gene and the genes in the Pita locus provide some resistance in MH357.

Table 4: Sequence analysis of MH357 to Digu and Yixiang 1B in Pid2 locus

gene	locus				
	555bp	2057bp	2058bp	2232bp	686 AA
Pid2-digu	G	A	T	T	H
Pid2-Y1B	A	G	C	T	R
Pid2-357	A	G	C	C	R

Furthermore, *Pid2-357* is a novel functional allele conferring blast resistance. Thus, MH357 is a restorer line with broad-spectrum resistance containing multiple blast resistance genes and is valuable for rice blast resistance breeding.

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