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Identification of the blast resistance genes in three elite restorer lines of hybrid rice

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Abstract

Hybrid rice has the advantage to pyramid multiple resistance (*R*) genes because a hybrid rice cultivar is developed from the cross of a sterile line with a restorer line that can harbor different *R* genes. Thus, knowing the *R* genes in an elite line will help the combination of different *R* genes into a hybrid rice cultivar. Here, we identified the blast *R* genes in Shu Hui 548 (SH548), Shu Hui 882 (SH882), and Wu Shan Si Miao (WSSM), three elite restorer lines of hybrid rice that showed resistance to the rice blast fungus in the disease nurseries. At controlled laboratory conditions, the three elite restorer lines exhibited resistance to more than 20 China Rice Blast strains that harbor different avirulence genes, indicating their broad-spectrum resistance to blast disease. Expression analyses detected the transcripts of multiple known blast *R* genes. Sequencing of the expressed *R* genes indicated that, besides *Pid2*, SH548 also contains *Pi2* and *Ptr*, SH882 and WSSM also contain *Pikm* and *Pi9*-Type5, respectively. *Pi9*-Type5 is a novel functional allele of *Pi9*. Therefore, SH548, SH882, and WSSM can be exploited in combination with the sterile lines containing other *R* genes, and they can be used as blast resistance donors in disease-resistance breeding programs.

Keywords: *Pi2*, *Pikm*, *Pi9*, *Magnaporthe oryzae*, Broad-spectrum resistance

Background

Rice (*Oryza sativa*) is one of the most important staple food crops and feeds more than half of the world's population. Rice blast disease caused by *Magnaporthe oryzae* is the major constraint to rice production worldwide (Baldrich and San 2016). Under favorable environmental conditions, this disease can cause up to 50% yield loss (Khush and Jena 2009). Each year, rice blast destroys harvests that could feed more than 60 million people, at a cost of over $ 70 billion (Kaur 2019). To protect from pathogens, plants have developed a two-layered immune system. The first layer is pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) that occurs upon the recognition of PAMPs by pattern recognition receptors (PRRs) (Jones and Dangl 2006; Couto and Zipfel 2016). PTI is effective in protecting plants from being infected by potentially pathogenic microbes. However, adapted pathogens express effectors to aid infection of specific plants, resulting in the suppression of PTI (Toruño et al. 2016; Park et al. 2018). Detection of effectors by nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins leads to activation of the second layer of immunity, known as effector-triggered immunity (ETI) (Dou and Zhou 2012), resulting in the hypersensitive response and inhibition of pathogen infection (Alfano and Collmer 2004).

The utilization of functional rice blast *R* genes is the most eco-friendly approach to prevent huge losses (Wu et al. 2019). Up to now, more than 100 blast *R* genes have been identified followed by cloning of over 30 *R* genes, including *Pish*, *Pi35*, *Pi37*, *Pi64*, *Pit*, *Pi-b*, *pi21*, *Pi63*,

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PiPR1, *Pi9*, *Pi2*, *Piz-t*, *Pi50*, *Pizh*, *Pigm*, *Pi-d2*, *Pi-d3*, *Pi25*, *Pi36*, *Pi5*, *Pii*, *Pi56*, *Pb1*, *Pik*, *Pik-p*, *Pikm*, *Pike*, *Pi1*, *Pik-h*/*Pi54*, *Pi54rh*, *Pi54of*, *Pia*, *Pi-CO39*, *Pi-ta*, *Ptr*, and *Pi65* (Yin et al. 2021; Wang et al. 2022). Among them, *pi21* is a recessive mutant and the wild-type *Pi21* encodes a proline-rich protein with a heavy metal-binding domain (Fukuoka et al. 2009). *Ptr* encodes an atypical protein with four Armadillo repeats and is required for broad-spectrum blast resistance mediated by *Pi-ta* and *Pi-ta2* (Zhao et al. 2018). *Pi-d2* encodes a B-lectin receptor kinase protein conferring race-specific resistance (Chen et al. 2006). *Pi65*, a novel blast disease resistance gene that confers resistance to the rice blast isolates collected from Northeast China, encodes two transmembrane domains together with 15 LRR domains and one protein kinase catalytic domain (Wang et al. 2022). The other *R* genes encode NBS-LRR domain proteins. The rice blast *R* genes are present on 11 chromosomes in the rice genome except chromosome 3. Eighteen percent of these *R* genes are distributed on chromosome 6, 25% on chromosome 11, and 21% on chromosome 12 (Ashkani et al. 2016). Some *R* genes, such as *Pikm*, *Pi1*, *Pi5*, *Pi-CO39*, *Pia*, *Pikp*, *Pike*, and *Pik*, require a two-gene-paired partner to confer resistance to *M. oryzae*, one called sensor and the other called helper (Lee et al. 2009; Okuyama et al. 2011; Hua et al. 2012; Cesari et al. 2013). Pathogen infection induces the expression of *Pb1*, *Pi21*, *Pi63*, and *Pi5-1*, whereas, the remaining genes show constitutive expression (Xiao et al. 2020). *Pi2* and *Piz-t* share great sequence similarities with a difference of only eight amino acids in three leucine-rich repeats, and both of these genes confer broad-spectrum resistance against various *M. oryzae* isolates (Zhou et al. 2006). Because resistant rice varieties with a single *R* gene are relatively easy to lose their resistance upon the emergence of new virulent races, the key concern of rice blast resistance breeding is to integrate durable as well as broad-spectrum resistance genes (Wu et al. 2007). Hybrid rice relies on the combination of a male sterile line and a restorer line via cross-pollination, thus has the advantage to pyramid multiple *R* genes that can be introduced either in the male sterile line or in the restorer line (Zhao et al. 2017). For example, an elite hybrid rice cultivar, called Yi Xiang You 2115 (YXY2115) that is developed from the elite sterile line Yixiang1A (Y1A) and the elite restorer line Yahui2115 (YH2115), exhibited high blast resistance in the past ten years. YH2115 contains *Pi2* and Y1A contains a novel *Pid2* allele, *Pid2_Y1B* (Shi et al. 2015; Wang et al. 2017). Therefore, genotyping the *R* genes in different lines can help the breeding programs to better pyramid different *R* genes into a hybrid rice cultivar.

Wu Shan Si Miao (WSSM) is an indica inbred rice cultivar developed by the Rice Research Institute of Guangdong Academy of Agricultural Sciences and is released in 2009 (Huang et al. 2011). To date, WSSM has been widely exploited as an elite cultivar and an elite restorer line of hybrid rice (https://www.ricedata.cn/variety/varis/606814.htm). Shu Hui 548 (SH548) and Shu Hui 882 (SH882) are two elite restorer lines recently developed by the Rice Research Institute of Sichuan Agricultural University. All three lines exhibited high resistance to blast disease in our disease nurseries. Their resistance phenotypes were confirmed by inoculating with the China Rice Blast (CRB) strains (Fang et al. 2018). Moreover, the expression-based genotyping strategy (Wang et al. 2017) was exploited to identify the blast *R* genes in them. Our data indicate that WSSM, SH548, and SH882 contain the broad-spectrum *R* genes *Pi9*-Type5, *Pi2*, and *Pikm*, respectively. Therefore, they can be used to cross with the male sterile lines containing other blast *R* genes to pyramid multiple blast *R* genes in the hybrid rice.

Results

Three elite restorer lines of hybrid rice showed broad-spectrum resistance to *M. oryzae*

Hybrid rice restorer lines SH548, SH882, and WSSM showed high resistance to *M. oryzae* in the rice-planted areas of Sichuan, China. To confirm the high blast-resistant phenotype of these three lines, we performed spray-inoculation tests using three-week-old rice seedlings, and Lijiangxin Tuan Heigu (LTH) was used as a susceptible reference. The results showed that all three lines displayed complete resistance to the mixed *M. oryzae* strains including CRB1, CRB4, CRB9, CRB10, CRB13, CRB14, CRB15, CRB18, and CRB21 at 7 days post-inoculation (dpi). In contrast, LTH developed severe disease symptoms in the form of disease lesions (Fig. 1a). Consistently, the relative fungal biomass was significantly less in all three lines than that in LTH, and only minimal fungal biomass was detected in the three restorer lines (Fig. 1b), indicating that all these three restorer lines have a high resistance to rice blast.

To test whether the three restorer lines confer broad-spectrum blast resistance, we conducted a punch inoculation assay. The three restorer lines together with LTH were inoculated with seven *M. oryzae* isolates (including TT27, YS-A8, YS102, DZ11, TT3, DZ115, and YS4-1) collected from different locations across Sichuan Province, China and 24 CRB strains, which contain different avirulence (*Avr*) genes (Fang et al. 2018). All the lines showed high blast resistance to the seven isolates (Fig. 2) and CRB strains except that WSSM showed disease symptom to CRB11; SH882 to CRB11 and CRB19; SH548 to CRB4, CRB8, and CRB11 (Table 1). However, susceptible reference LTH was highly susceptible to all the tested rice blast isolates and exhibited typical disease symptoms (Table 1 and Fig. 2). These results indicate that the three restorer

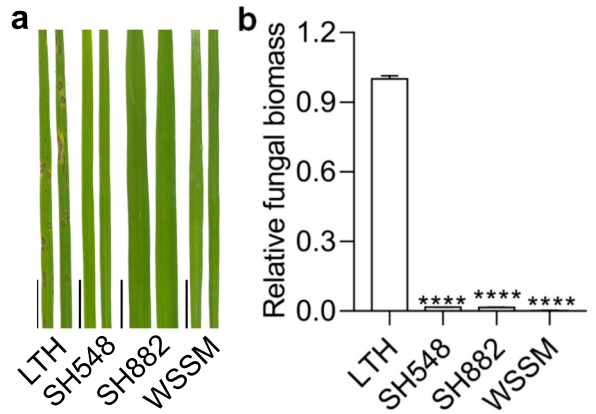

Fig. 1 Blast disease phenotypes in LTH, SH548, SH882, and WSSM. **a** The blast disease phenotype on the representative leaf sections of the indicated lines at 7 dpi. Three-week-old seedlings of SH548, SH882, and WSSM, together with susceptible reference LTH, were spray-inoculated with mixed spores (3×10^5 spores/mL) of CRB strains including CRB1, CRB4, CRB9, CRB10, CRB13, CRB14, CRB15, CRB18, and CRB21. Scale bars, 1 cm. **b** Relative fungal biomass was measured by RT-qPCR. Leaf samples were collected at 7 dpi for RNA extraction. Asterisks indicate significant differences (Student's *t*-test, ****$P < 0.0001$). Error bars indicate SD ($n = 3$)

lines have a broad-spectrum resistance against *M. oryzae* and are valuable germplasms for rice blast resistance breeding programs.

Different blast resistance genes were expressed in SH548, SH882, and WSSM

To genotype the blast resistance genes in the three restorer lines, we examined the expression of cloned blast *R* genes upon *M. oryzae* infection by reverse-transcription PCR (RT-PCR). We detected high expression of *Pi2*, *Pid2*, and *Ptr* in SH548, followed by relatively low expression of *Pii*, *Pb1*, *Pi64*, *Pi25*, and *Pish* (Fig. 3a), but not the other blast-resistant genes. In SH882, the expression of *Pikm2*, *Pid2*, *Pi63*, and *Pb1* was higher than that of *Pikm1*, *Pii*, *Pi64*, *Pish*, and *Pi25* (Fig. 3b). However, the expressions of *Pi63* were significantly lower at 12, 24, and 48 h post-inoculation (hpi) than that at 0 hpi (Fig. 3b). The expression of *Pb1* was induced at 24 hpi but relatively low at other time points (Fig. 3b). No expression was detected for the remaining *R* genes in SH882. In WSSM, *Pi9*, *Ptr*, and *Pid2* showed higher levels of transcript accumulation than *Pii*, *Pb1*, *Pish*, *Pi64*, and *Pi25* upon blast infection (Fig. 3c), whereas the expression of the other *R* genes was undetectable. Therefore, the broad-spectrum blast resistance may be attributable to *Pi2*, *Pid2*, and *Ptr* in SH548, *Pikm* and *Pid2* in SH882, *Pi9*, *Ptr*, and *Pid2* in WSSM, and these blast *R* genes were subjected to further analysis.

M. oryzae infection altered the expression of *Pi2*, *Pikm*, and *Pi9* in SH548, SH882, and WSSM

According to previous reports, *Pi2*, *Pikm*, and *Pi9* are functional and confer broad-spectrum resistance against *M. oryzae* (Shi et al. 2015; Wang et al. 2017; Zhao et al. 2017). Their high gene expression abundance in SH548, SH882, and WSSM prompted us to analyze the expression pattern of these genes by using reverse-transcription quantitative PCR (RT-qPCR) upon inoculation of the *M. oryzae* strain Guy11. Compared with that at 0 dpi, the expression of *Pi2* was highly induced at 12 and

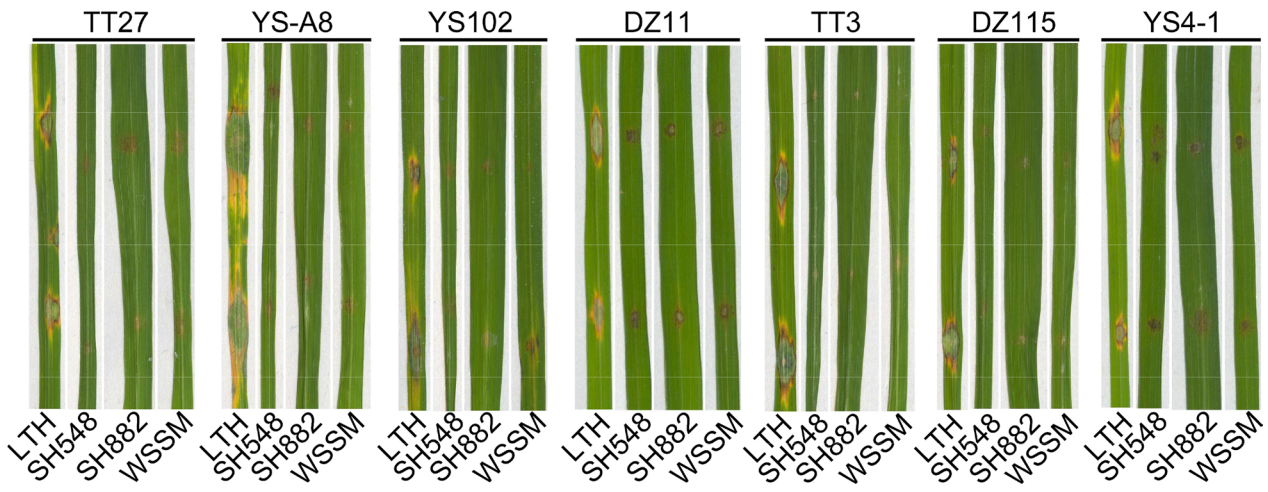

Fig. 2 Punch inoculation assay of LTH, SH548, SH882, and WSSM with individual *M. oryzae* isolates. Representative leaf sections show the responses of these rice lines/cultivars to the indicated *M. oryzae* isolates (4×10^5 spores/mL for each) at 6 dpi. Seven isolates were collected from different locations in China and used for punch inoculation

Table 1 Resistance spectrum analysis of SH548, SH882, and WSSM with CRB strains

M. oryzae strains	Rice lines/cultivars			
	SH548	SH882	WSSM	LTH
CRB1	R	R	R	S
CRB2	R	R	R	S
CRB3	R	R	R	S
CRB4	S	R	R	S
CRB5	R	R	R	S
CRB6	R	R	R	S
CRB7	R	R	R	S
CRB8	S	R	R	S
CRB9	R	R	R	S
CRB10	R	R	R	S
CRB11	S	S	S	S
CRB12	R	R	R	S
CRB13	R	R	R	S
CRB14	R	R	R	S
CRB15	R	R	R	S
CRB16	R	R	R	S
CRB17	R	R	R	S
CRB18	R	R	R	S
CRB19	R	S	R	S
CRB20	R	R	R	S
CRB21	R	R	R	S
CRB22	R	R	R	S
CRB24	R	R	R	S
CRB25	R	R	R	S

R, resistant; S, susceptible; CRB, China Rice Blast

48 hpi in SH548 (Fig. 3d). In SH882, *Pikm1* and *Pikm2* had much higher expression at 12 and 48 hpi than that at 0 hpi (Fig. 3e). In addition, *Pi9* showed higher expression abundances at 12, 24, and 48 hpi than that at 0 dpi in WSSM (Fig. 3f). These results indicate that rice blast fungus infection resulted in induced expression of broad-spectrum blast *R* genes, i.e. *Pi2*, *Pikm*, and *Pi9* in SH548, SH882, and WSSM, respectively.

Sequence analyses identified functional *R* genes in SH548, SH882, and WSSM

To check whether the transcript of each candidate blast *R* gene was from the resistant or susceptible allele, we amplified the coding sequence (CDS) of the candidates for sequence analysis, including *Pi2*, *Pid2*, *Ptr* in SH548; *Pikm* and *Pid2* in SH882; *Pi9*, *Ptr*, and *Pid2* in WSSM.

The resistant and susceptible alleles of *Pid2* are distinguished by a single amino acid substitution at position 441, i.e. I to M (Chen et al. 2006). As shown in Table 2, the CDS of *Pid2* has one or four nucleotide changes in SH548, SH882, and WSSM compared with that of *Pid2_Digu*, resulting in H666R amino acid substitution only, but not at position 441. This substitution is the same as that in the functional allele *Pid2_Y1B* (Wang et al. 2017). Therefore, *Pid2* in all three restorer lines is the functional *Pid2_Y1B*.

Compared with the susceptible allele, the resistant *Ptr* contains five single nucleotide polymorphisms (SNPs) causing non-synonymous mutations and a 12-bp deletion in the fourth exon (Zhao et al. 2018). To clarify whether *Ptr* in SH548 and WSSM is the resistant allele, we performed sequencing analysis of its CDS. Fortunately, we detected five SNPs and a 12-bp deletion in *Ptr* of SH548, but not in *Ptr* of WSSM (Fig. 4a). Consistently, the amino acid sequence of Ptr_SH548 is the same as that of Ptr in Katy (resistant variety), and Ptr_WSSM has the same amino acid sequence as Ptr in Amane (susceptible variety) (Fig. 4a). Therefore, *Ptr* contributes to the blast resistance of SH548, but not of WSSM.

Pikm1 and *Pikm2* are two closely linked genes required for broad-spectrum resistance of the *Pikm* locus on chromosome 11 (Ashikawa et al. 2008). To clarify whether the expressed *Pikm* in SH882 is functional, we performed sequencing analysis on the CDS of both *Pikm1* and *Pikm2*. Fortunately, again, the nucleotide sequences of *Pikm1* and *Pikm2* were the same as those of the reported resistant *Pikm1_TS* and *Pikm2_TS* alleles (Additional file 1: Figures S1, S2), indicating that *Pikm* contributes to the blast resistance of SH882.

At least eight blast *R* genes have been identified at the *Pi2*/*Pi9* locus on the short arm of chromosome 6 (Jiang et al. 2012). To date, five of them have been cloned, including *Piz-t*, *Pi2*, *Pi9*, *Pigm*/*Pi50*, and *Pizh* (Qu et al. 2006; Zhou et al. 2006; Jiang et al. 2012; Su et al. 2015; Deng et al. 2017; Xie et al. 2019). All these five cloned *R* genes encode NBS-LRR proteins and confer broad-spectrum resistance to rice blast. Resistant and susceptible alleles of *Pi2* are distinguished by an out-of-frame deletion of 3-bp at the position 1543–1545, resulting in cysteine-arginine loss and their replacement by glycine in susceptible alleles (Sheng et al. 2017). *Pi9* is an allele of *Pi2* with a difference of 46 amino acid residues (Qu et al. 2006). Moreover, thirteen novel alleles were identified at the *Pi9* locus, some of which showed better blast resistance than *Pi9* and *PigmR* (Zhou et al. 2020). To find out the *R* gene allele at the *Pi2*/*Pi9* locus in SH548 and WSSM, we performed sequencing analysis on the CDS at this locus of both lines. Fortunately, the *R* gene at the *Pi2* locus of SH548 had the same sequence as the resistant allele in the *Pi2* isogenic line C101A51 (Additional file 1: Figure S3) (Zhou et al. 2006), whereas the *R* gene at the *Pi9* locus of WSSM had the same sequence as *Pi9*-Type5 allele (Fig. 4b), a novel *Pi9* allele that has a difference of 36 amino acid residues as compared with *Pi9* but

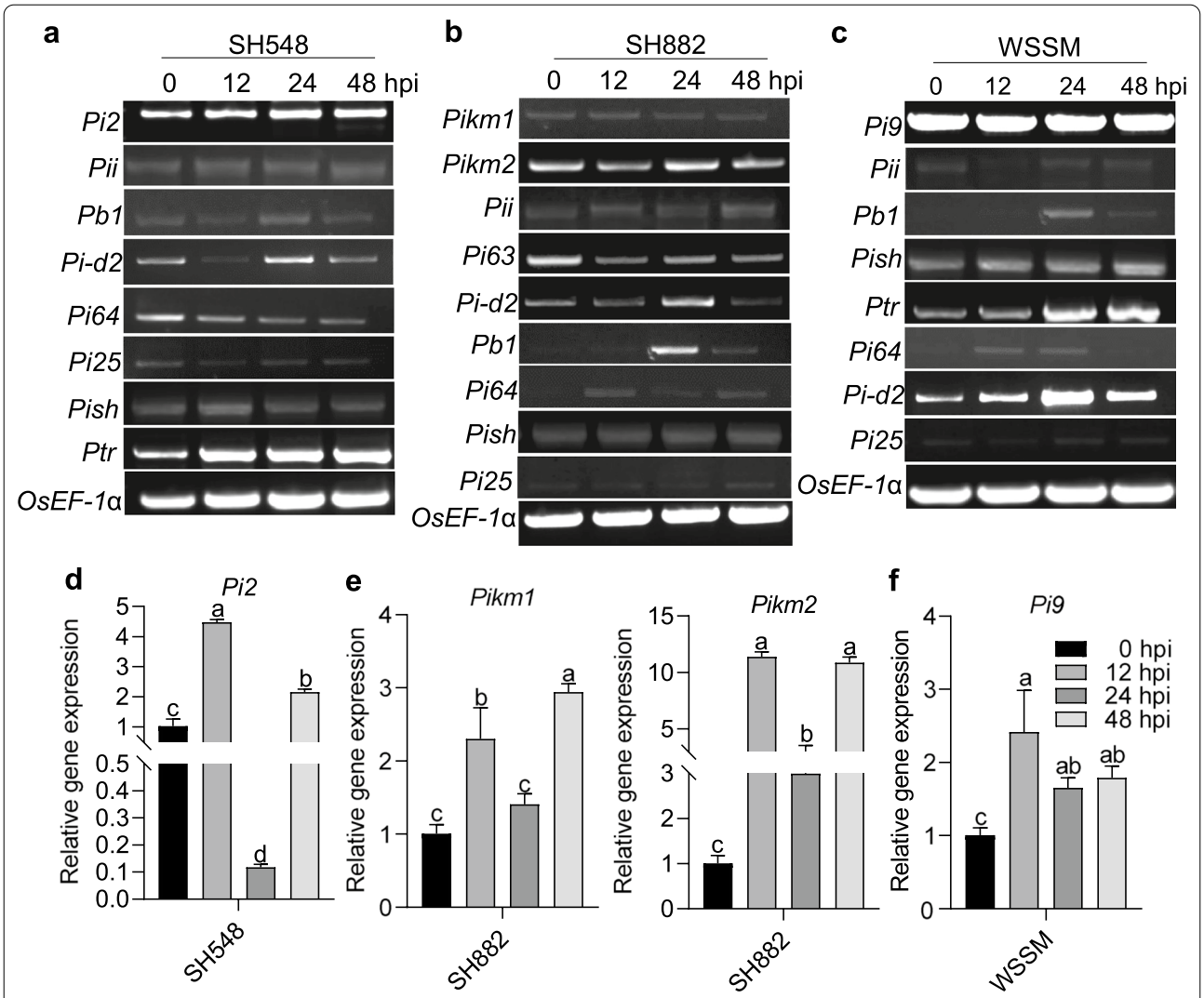

Fig. 3 Expression profiling of rice blast *R* genes in SH548, SH882, and WSSM. **a**–**c** RT-PCR analysis of the cloned rice blast *R* genes in SH548, SH882, and WSSM, respectively. Three-week-old seedlings of SH548, SH882, and WSSM were spray-inoculated with mixed spores (3×10^5 spores/mL) of CRB strains including CRB1, CRB4, CRB9, CRB10, CRB13, CRB14, CRB15, CRB18, and CRB21. The leaves of each line were collected at 0, 12, 24, and 48 hpi. *OsEF-1*α was used as an internal control. **d**–**f** RT-qPCR analysis of the rice blast *R* genes, including *Pi2* in SH548, *Pikm* (*Pikm1* and *Pikm2*) in SH882, and *Pi9* in WSSM. Leaf samples were collected at 0, 12, 24, and 48 hpi with Guy11 (4×10^5 spores/mL). Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA, post hoc Tukey HSD analysis). Error bars indicate SD ($n = 3$)**.** All the experiments were repeated two times with the same results

Table 2 Sequence analysis of *Pid2* in SH548, SH882, and WSSM

Rice lines/cultivars	Varied locus in CDS of *Pid2*				Amino acid substitution		
	495	1997	1998	2172	165	666	724
Digu	G	A	T	T	P	H	G
Y1B	A	G	C	T	P	R	G
SH548	G	G	T	T	P	R	G
SH882	A	G	C	C	P	R	G
WSSM	G	G	T	T	P	R	G

conferrs broader spectrum blast resistance than *Pi9* and *Pigm* (Zhou et al. 2020).

Taken together, we identified that all the three elite restorer lines of hybrid rice contain the functional *Pid2_Y1B*. Besides, SH548 contains *Pi2* and *Ptr*, SH882 contains *Pikm*, and WSSM contains the novel *Pi9* allele, *Pi9*-Type5.

Discussion

The hybrid rice breeding programs contribute successfully and greatly to rice blast disease control and yield increases during the past decades (Cui et al. 2020). The breeders obtain F_1 hybrid seeds by cross-pollination of a male sterile line with a restorer line. Therefore, functional blast *R* genes from either male sterile line or restorer line can be pyramided in a hybrid cultivar. The restorer line is a key component to improve disease resistance and quality of a hybrid rice cultivar in rice breeding. Knowing functional blast *R* genes in restorer lines will help their exploitation by crossing to sterile lines containing different *R* genes. In the paddy field observations of our blast disease nurseries over the past several years, we found that three elite restorer lines, namely SH548, SH882, and WSSM, displayed high blast disease resistance. Here, we reported the identification of the functional blast *R* genes in these lines so as to help their combination with the sterile lines containing the other *R* genes.

We first confirmed the broad-spectrum resistance of SH548, SH882, and WSSM via punch inoculation. The rice-blast fungus interactions follow the gene-for-gene paradigm. A cultivar exhibits resistance only when it carries one or more *R* genes that recognize their cognate *Avr* genes in the blast population (Fang et al. 2018). Conversely, if the blast population does not contain the cognate *Avr* genes, a cultivar will exhibit susceptibility even if it carries blast *R* genes. The CRB strains have distinct responses to the monogenic IRBL lines, because they contain the minimum number of *Avr* genes and could differentiate combinations of the major blast *R* genes (Fang et al. 2018). Thus, we performed blast disease assay using 24 CRB strains and seven isolates collected from different locations in the Sichuan Basin, China. Consistent with previous observations, all three restorer lines showed broad-spectrum resistance against the tested *M. oryzae* isolates (Fig. 2 and Table 1). We then performed RT-PCR analysis and found high expressions of *Pi2*, *Pid2*, and *Ptr* in SH548; *Pikm* and *Pid2* in SH882; *Pi9*, *Ptr*, and *Pid2* in WSSM upon *M. oryzae* infection (Fig. 3a–c). Sequencing analyses identified that all three lines contain *Pid2_Y1B* (Table 2), a novel functional *Pid2* allele detected in an elite maintainer line Yixiang 1B (Wang et al. 2017). Besides, SH548 contains *Pi2* and *Ptr* (Fig. 4a and Additional file 1: Figure S3), two broad-spectrum blast resistance genes at Chromosome 6 and 12, respectively, whereas SH882 and WSSM contain *Pikm* and *Pi9*-Type 5, respectively (Fig. 4b and Additional file 1: Figures S1, S2).

Following the genetic variability of avirulence effectors and pathogenicity of the rice blast fungus, the blast *R* genes in rice are co-evolved with high levels of allelic variations. Allele variations are critical events occurred in rice blast resistance gene loci. Sequence variations, such as the presence of SNPs, lead to significant differences in the function or/and resistance spectrum of the blast *R* genes. Such allelic variations increase the number of blast resistance genes and alter resistance spectrums, and thus are highly valuable for breeding programs aiming to improve blast disease resistance. For example, five orthologs of *Pid3* showed differential resistance spectra to all the tested rice blast isolates in a gradually descending order as follows: *Pid3-I1*, *Pid3-W5*, *Pid3-I3*, *Pid3-W4*, and *Pid3-W3* (Xu et al. 2014). Among them, the resistance spectrum of *Pid3-W5* is 17% wider than that of the original *Pid3-I3* from Digu. Sequence alignment of these *Pid3* alleles showed the sequence diversities (Xu et al. 2014), indicating that allelic variations of the *R* gene could expand the rice blast resistance and be valuable for breeding. To date, at least 10 haplotypes have been reported at the *Pid2* locus based on the nucleotide sequences of CDS in 42 cultivated and wild rice, namely H1, H2, H3, H4, H5, H6, H7, H8, H9, and *Pid2_Y1B* (Li et al. 2015; Wang et al. 2017). The amino acid residue Ile (I) distinguishes the resistant allele from the susceptible one that is substituted to Met (M) at postion 441 (Chen et al. 2006). The amino acid M was found at position 441 in the H1 and H6, suggesting they are susceptible alleles; whereas, amino acid I was found in the remaining 8 haplotypes, suggesting they are functional alleles. In this study, the *Pid2* haplotype in SH548 and WSSM is the same as H2, containing a point mutation (A to G) at the nucleotide position 1997 compared with that in Digu (Table 2). Moreover, the *Pid2* haplotype in SH882 is the same as H4, containing four base-pair mutations compared with that in Digu (Table 2). Interestingly, the amino acids encoded by H2 and H4 haplotypes are the same as that of Pid2_Y1B, which is encoded by *Pid2_Y1B*, a novel

(See figure on next page.)

Fig. 4 Sequence analysis of *Ptr* and *Pi9* in SH548 and WSSM, respectively. **a** An alignment of the *Ptr* sequence in SH548 and its alleles from Amane and Katy. The upper panel is the nucleotide alignment, the lower panel is the amino acid alignment. 'filled star' indicates SNP site, grey box shows the four amino acid deletion in Katy. The numbers at the top indicate the positions of amino acids. **b** Multiple sequence alignment of the predicted amino acid sequences of *Pi9_WSSM* and its alleles. Sequences were aligned by the CLUSTALW and displayed by using ESPript 3.0

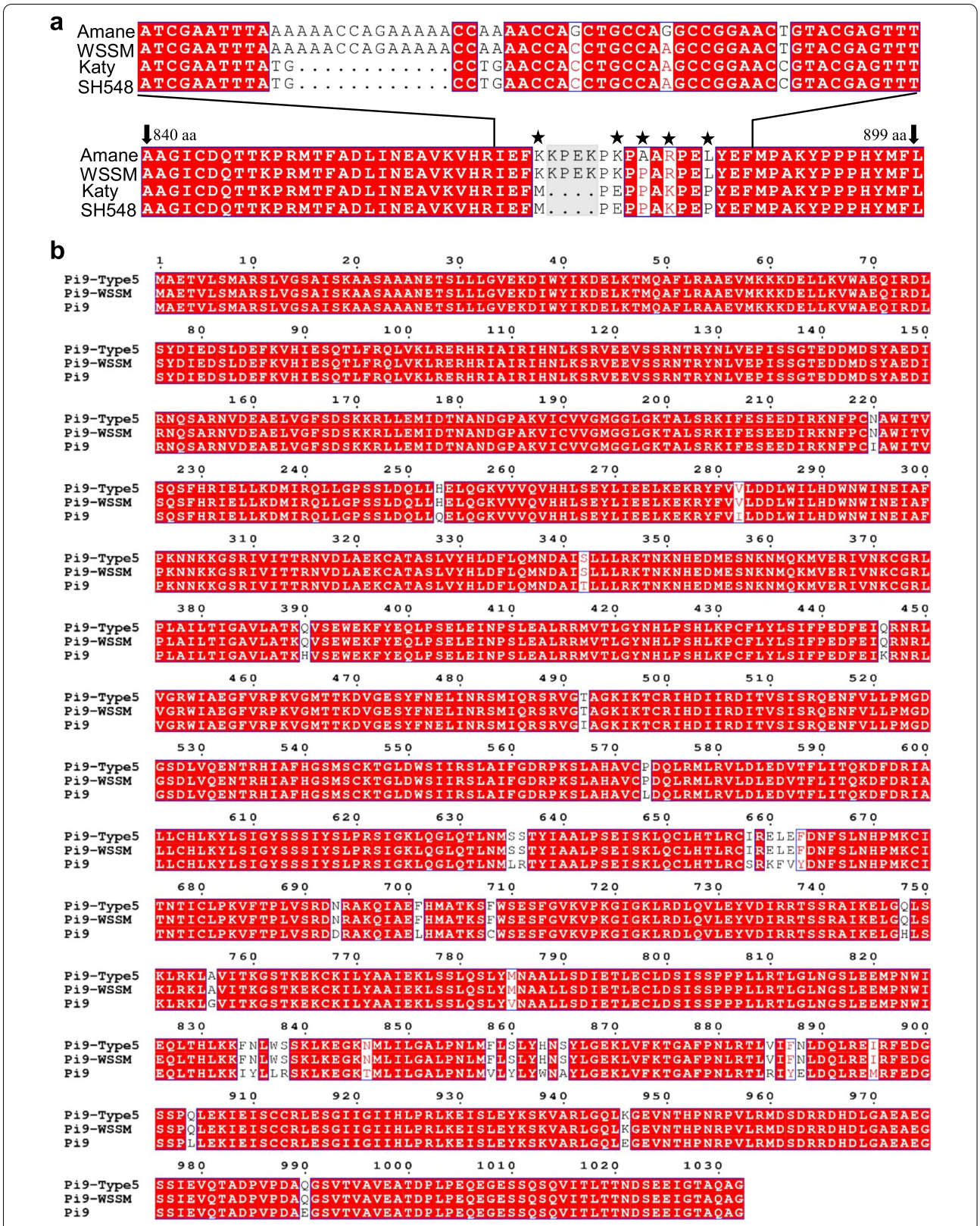

Fig. 4 (See legend on previous page.)

functional allele of *Pid2*, cloned from an elite maintainer line Y1B using allelic mining (Wang et al. 2017). *Ptr* is an atypical resistance gene located on the short arm of chromosome 12 and is required for the broad-spectrum blast resistance mediated by *Pita* and *Pita2*, but its function is independent of *Pita* (Zhao et al. 2018). Katy carrying *Ptr* was resistant to 348 out of 389 genetically diverse isolates, while the Katy mutant with nonfunctional *Ptr* was only resistant to 17 isolates (Zhao et al. 2018), indicating its broad-spectrum resistance. Here, SH548 contains *Ptr* and thus is valuable for blast disease-resistance breeding.

The *Pik* locus contains one of the most abundant variations in blast resistance gene loci that require two-paired R genes to mediate resistance. At least nine resistance allelic loci have been isolated and functionally characterized with differential resistance to *M. oryzae* isolates, including *Pikm*, *Pik*, *Pikp*, *Pi1*, *Pikh*, *Piks*, *Pi7*, *Pike*, and *Pikg* (Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011, 2014; Hua et al. 2012; Chen et al. 2015; Meng et al. 2021). Among them, Pikg is different from Pike by one amino acid substitution at position 229 (D to E) in Pike-1, resulting in a unique reaction pattern that was different from that of other *Pik* alleles against rice blast (Meng et al. 2021). However, among the allelic loci, the *Pikm* monogenic line showed the highest resistance frequency to blast isolates (Shi et al. 2015), indicating that *Pikm* is the most critical allelic locus for the resistant phenotype. Here, SH882 contains *Pikm* and thus is valuable for blast disease resistance breeding.

At the *Pi2*/*Pi9* locus, five blast resistance genes have been cloned, i.e. *Piz-t*, *Pi2*, *Pi9*, *PigmR*/*Pi50*, and *Pizh* (Qu et al. 2006; Zhou et al. 2006; Jiang et al. 2012; Su et al. 2015; Deng et al. 2017; Xie et al. 2019). Recently, thirteen novel *Pi9* alleles were identified from 107 rice varieties by using sequence-based allele mining, named *Pi9*-Type1 to *Pi9*-Type13 (Zhou et al. 2020). Resistance evaluations showed that *Pi9*-Type3/4/5/6/9/10/11 alleles exhibited a broad spectrum of blast resistance. Among them, the resistance ratio of *Pi9*-Type5/9 was higher than those of *Pigm*, *Pi2*, and *Pi9* as they exhibited 100% resistance frequency (Zhou et al. 2020). Thus, these alleles are highly valuable in blast disease resistance breeding. In the current study, the restorer line WSSM contains *Pi9*-Type5 allele at the *Pi2*/*Pi9* locus (Fig. 4b), suggesting the valuability of this line as a resistance donor germplasm.

Conclusions

In the present study, we demonstrated that three restorer lines of hybrid rice have a broad-spectrum resistance against rice blast caused by *M. oryzae*. Sequence analysis demonstrated that all the restorer lines have *Pid2_Y1B*. Besides, SH548 contains *Pi2* and *Ptr*, SH882 and WSSM contain *Pikm* and *Pi9*-Type5, respectively. However, we can't exclude that other unknown *R* genes may contribute to the broad-spectrum blast resistance of SH548, SH882, and WSSM based on the genotyping strategy. As functional characterization of novel *R* genes is time-consuming and labor-intensive, future research can be performed to clarify whether these lines harbor the other *R* genes. Because each restorer line contains at least two functional *R* genes, they are valuable as resistance donors for rice breeding programs aiming to improve blast disease resistance via crossing to elite sterile lines containing other *R* genes.

Methods

Plant materials and growth conditions

Three elite restorer lines of hybrid rice were genotyped for blast resistance genes, including SH548, SH882, and WSSM. Lijiang-xin Tuan Heigu (LTH) was used as a susceptible reference. All the rice lines were grown in a growth chamber under a photoperiod cycle of 12 h light /12 h dark, 26 °C, and 70% relative humidity.

Pathogen inoculation and blast disease assay

All the rice blast isolates used in this study were prepared as previously described (Zhao et al. 2020). Briefly, blast strains were cultured for 7 days on oatmeal tomato agar (OTA) (40 g oatmeal, 150 mL tomato, and 15 g agar in 1 L medium) at 26 °C under a photoperiod cycle of 12 h light /12 h dark, then aerial mycelia were removed and maintained another four days at the same condition for spore generation. For spray-inoculation, 3-week-old rice seedlings were inoculated with mixed spores (3×10^5 spores/mL) from different *M. oryzae* strains according to the method described previously (Li et al. 2013). Inoculated seedlings were kept in dark for 24 h and then transferred to the growth chamber. The inoculated leaves of each accession were collected at 0, 12, 24, and 48 hpi and were used for *R* gene expression analysis by RT-PCR and RT-qPCR. Disease symptoms were analyzed at 7 dpi, meanwhile, the fungal biomass assay was calculated as a ratio of the *M. oryzae Pot2* level against the rice *ubiquitin* level by RT-qPCR. For punch inoculation, detached leaves from 6-week-old plants were inoculated with spore suspension (5×10^5 spores /mL) of each CRB isolate. After floating inoculated leaves on 0.1 mmol/L 6-benzyladenine buffer for 6 days, the size of lesions was measured with LTH as a susceptible reference (Park et al. 2012).

RNA extraction and reverse-transcription PCR

Total RNA was prepared as previously described (Zhao et al. 2021). Brief, total RNA was extracted from the leaves collected at 0, 12, 24, and 48 hpi by TRIzol reagent

(Invitrogen). The concentration of RNA was determined by using Nanodrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized by using NovoScript Plus All-in-one 1st Strand cDNA Synthesis SuperMix (Novoprotein, China). RT-PCR was done to examine the expression levels of reported blast *R* genes upon *M. oryzae* infection by using Easy-*Taq* DNA polymerase (TransGen Biotech, China). RT-qPCR was performed to check the expression abundance of the candidate key *R* genes. *OsEF-1α* was used as an internal control (Wu et al. 2015). All the RT primers are listed in Additional file 2: Table S1.

Sequence analysis

Amplification of *R* genes was done by using cDNA and Phanta Max Super-Fidelity DNA polymerase (Vazyme Biotech). For sequence analysis, gel-purified PCR products were cloned into the *p-EASY* blunt vector (TransGen Biotech, China). Individual positive colonies for each gene were picked up to prepare plasmid DNA for sequencing using the gene-specific primers (Additional file 2: Table S1). Sequence results were analyzed by using SnapGene 4.3.6.

Abbreviations

Avr: Avirulence; CDS: Coding sequence; CRB: China Rice Blast; dpi: Days post-inoculation; ETI: Effector-triggered immunity; hpi: Hours post-inoculation; LTH: Lijiangxin Tuan Heigu; *M. oryzae*: *Magnaporthe oryzae*; NBS-LRR: Nucleotide-binding site leucine-rich repeat; OTA: Oatmeal tomato agar; PAMPs: Pathogen-associated molecular patterns; PRRs: Pattern recognition receptors; PTI: PAMPs-triggered immunity; R: Resistance; RT-PCR: Reverse-transcription PCR; RT-qPCR: Reverse-transcription quantitative PCR; SH548: Shu Hui 548; SH882: Shu Hui 882; SNPs: Single nucleotide polymorphisms; WSSM: Wu Shan Si Miao; Y1A: Yixiang1A; YH2115: Yahui2115; YXY2115: Yi Xiang You 2115.

Supplementary Information

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Additional file 1: Figure S1. An alignment of the *Pikm1* sequence. Sequences were aligned by the CLUSTALW and displayed by using ESPript 3.0. **Figure S2.** An alignment of the *Pikm2* sequence. Sequences were aligned by the CLUSTALW and displayed by using ESPript 3.0. **Figure S3.** An alignment of the *Pi2* sequence. Sequences were aligned by the CLUSTALW and displayed by using ESPript 3.0.

Additional file 2: Table S1. Primers used in this research.

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Author contributions

BH performed the experiments with support from YTP, SL, XXY, CC, FG, SXZ, MP, YPJ, YPW, WZ, YLP, and FH. WMW and ZXZ conceived the project and designed the experiments. BH, ZXZ, and WMW analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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