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Engineering of rice varieties with enhanced resistances to both blast and bacterial blight diseases via CRISPR/Cas9

Yanbiao Zhou[1,2,*,†], Shichong Xu[1,3,†], Nan Jiang[1], Xinhui Zhao[1,4], Zhenan Bai[1], Jinling Liu[4], Wei Yao[4], Qianying Tang[1], Gui Xiao[5], Chao Lv[1,3], Kai Wang[1], Xiaochun Hu[1], Junjie Tan[6,*] and Yuanzhu Yang[1,3,4,5,6,*]

[1]*Key Laboratory of Southern Rice Innovation & Improvement, Ministry of Agriculture and Rural Affairs/Hunan Engineering Laboratory of Disease and Pest Resistant Rice Breeding, Yuan Longping High-Tech Agriculture Co., Ltd, Changsha 410001, Hunan, China*

[2]*College of Life Sciences, South China Agricultural University, Guangzhou 510642, China*

[3]*College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China*

[4]*College of Agronomy, Hunan Agricultural University, Changsha 410128, Hunan, China*

[5]*State Key Laboratory of Hybrid Rice, Hunan Hybrid Rice Research Center, Changsha 410125, Hunan, China*

[6]*State Key Laboratory of Crop Genetics and Germplasm Enhancement, Innovation Center for Genome Editing and Engineering, Jiangsu Collaborative Innovation Center for Modern Crop Production, Nanjing Agricultural University, Nanjing 210095, China*

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**Correspondence*
(Tel +8613607442126; fax +86073187059179; e-mail: yzhuyah@163.com (YY); Tel +8615274918432; fax +86073187059179; zhouyanbiao2005@163.com (YZ); Tel +8616651618276; fax +86073187059179; tanjunjie@njau.edu.cn (JT))
[†]These authors contributed equally to this work.

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Summary

Rice blast and bacterial blight represent two of major diseases having devastating impact on the yield of rice in most rice-growing countries. Developments of resistant cultivars are the most economic and effective strategy to control these diseases. Here, we used CRISPR/Cas9-mediated gene editing to rapidly install mutations in three known broad-spectrum blast-resistant genes, *Bsr-d1*, *Pi21* and *ERF922*, in an *indica* thermosensitive genic male sterile (TGMS) rice line Longke638S (LK638S). We obtained transgene-free homozygous single or triple mutants in T_1 generations. While all single and triple mutants showed increased resistance to rice blast compared with wild type, the *erf922* mutants displayed the strongest blast resistance similar with triple mutants. Surprisingly, we found that *Pi21* or *ERF922* single mutants conferred enhanced resistance to most of tested bacterial blight. Both resistances in mutants were attribute to the up-regulation of SA- and JA-pathway associated genes. Moreover, phenotypic analysis of these single mutants in paddy fields revealed that there were no trade-offs between resistances and main agricultural traits. Together, our study provides a rapid and effective way to generate rice varieties with resistance to both rice blast and bacterial blight.

Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods feeding more than 50% population worldwide. However, its production and quality are severely threatened by a variety of pathogens. Among them, *Magnaporthe oryzae* (*M. oryzae*) and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) are two well-known destructive pathogens causing rice blast and bacterial blight, respectively (Yin *et al.*, 2018). The most economic and effective approach to control these diseases is to create the rice cultivars with broad-spectrum resistance against both of them (Deng *et al.*, 2017).

Hybrid rice with high yield plays a vital role in guarantee for staple food supply worldwide (Cheng *et al.*, 2007). Compared with conventional three-line hybrid rice, the two-line hybrid rice has attracted increasing attention as it is independent of restorer genes and more efficient in production of hybrid seeds (Yuan, 1994). Thermo-sensitive genic male sterility (TGMS) and photoperiod-sensitive genic male sterility (PGMS) lines are the central component for the two-line hybrid approach. LK638S is an *indica* (Xian) TGMS line developed in the 2000s with low critical sterility-inducing temperature (<23.5 °C), high outcrossing rate, good combining ability, but susceptible to rice blast and bacterial blight. Thus, establishing its variants with multiple diseases resistance is of great importance.

Plants have evolved a two-layered innate immune system to defend against pathogens (Zhao *et al.*, 2018). The first layer is activated upon perception of pathogen-associated molecular patterns (PAMPs) by cell surface pattern recognition receptors (PRRs), leading to PAMP-triggered immunity (PTI). The first layer could be overcome by pathogens through secreting PTI-inhibited effectors into plant cells, which activate effector-triggered susceptibility (ETS). To respond this virulence strategy, the second layer was activated by expressing plant resistance (*R*) genes, which specifically recognize pathogen effectors to activate effector-triggered immunity (ETI) (Dangl *et al.*, 2013). These innate immune responses trigger many molecular events, including induction of a large number of defense-responsive genes (Eulgem and Somssich 2007), production of defense signal molecules, reactive oxygen species (ROS) (Kaku *et al.*, 2006) and phytohormones (De Vleesschauwer *et al.*, 2013). Salicylic acid (SA) and jasmonic acid (JA) represent two most important phytohormones in regulation of plant immune responses (Liu *et al.*, 2016). Resistance to biotrophic pathogens is predominantly involved in SA-dependent pathway, whereas resistance to necrotrophic pathogens and insects is usually associated with JA- and ET-driven defenses (De Vleesschauwer *et al.*, 2013), e.g., overexpression of *OsWRKY13*, a WRKY-type transcription factor, enhanced rice resistance to rice blast and bacterial blight, which was associated with the activation of SA synthesis- and responsive genes (Qiu *et al.*, 2007); knock-outs of *MPK15* enhanced disease resistance in rice against blast and bacterial blight accompanied by up-regulation of SA- and JA-pathway-associated genes (Hong *et al.*, 2019).

Recently, more than 100 major rice blast *R* genes have been identified and 37 of them have been cloned and characterized (Yin *et al*., 2021). Except for *Pid2*, *pi21* and *Ptr*, most known *R* genes encode typical nucleotide-binding domain leucine-rich repeat containing (NLR) domain, which directly or indirectly interact with fungal effectors to trigger ETI (Dangl *et al*., 2013; Yin *et al*., 2021). The comprehensive understanding of immunity mechanisms of *R* genes can enhance the rice breeding with durable resistance. So far, an atypical *R* gene *Pi21* and two defense-related genes *Bsr-d1* as well as *ERF922* have attracted much more attention due to their broad-spectrum resistance. *Bsr-d1* encodes a C_2H_2-type transcription factor that directly binds to the promoter of three peroxidase genes (*Os01g73170*, *Os05g04470* and *Os10g39170*), thereby affecting H_2O_2 accumulation and broad-spectrum resistance to *M. oryzae* (Li *et al*., 2017; Tao *et al*., 2021; Zhu *et al*., 2020). In addition, *Bsr-d1* was shown to be transcriptionally regulated by MYSB1 through directly binding to its promoter and a single base change (SNP33-G) in the *Bsr-d1* promoter leads to enhanced affinity with MYSB1, which suppresses *Bsr-d1* gene expression. *Pi21* encodes a proline-rich protein containing a putative heavy metal-binding domain and protein–protein interaction motifs (Fukuoka *et al*., 2009). Deletions of the proline-rich motifs cause a loss of function. Knockout of *Pi21* confers non-race specific and durable blast resistance (Tao *et al*., 2021). *ERF922* encodes an APETELA2/ethylene response factor (AP2/ERF) type transcription factor and is strongly induced by *M. oryzae* (Liu *et al*., 2012). Knockdown or knockout of *ERF922* resulted in enhanced resistance against rice blast, indicating the negative regulation role of this gene in disease resistance. To date, all the studies regarding *Bsr-d1*, *Pi21* and *ERF922* only separately explored their roles in resistance against rice blast and were mainly carried out in the *japonica* rice background. The rice blast resistance comparison of these three genes under the same rice variety as well as their roles in resistance to bacterial blight remains unknown.

CRISPR/Cas9 genome editing technology enables targeted mutagenesis of DNA (Baltes and Voytas,2015). Due to its simplicity, feasibility and versatility, it has been increasingly employed in a variety of organisms including crops that were edited for trait improvements. Moreover, because of its ability for multiplexable genome targeting, it could be used to improve multiple traits simultaneously (Oliva *et al*., 2019; Tang *et al*., 2017; Wang *et al*., 2016; Xu *et al*., 2019; Zhang *et al*., 2018; Zhou *et al*., 2016).

In this study, we have set an example of CRISPR/Cas9 applications in enhancing multiple disease resistance in a commercial *indica* TGMS line LK638S. We chose three broad-spectrum blast-resistant genes (*Bsr-d1*, *Pi21* and *ERF922*) and created their single as well as triple mutants. We found that *ERF922* mutants showed the strongest blast resistance level similar with triple mutants. Furthermore, we also explored their new roles in resistance to bacterial blight. Our study indicates CRISPR/Cas9 as a powerful tool in modern molecular breeding by targeted genes modifications that could significantly accelerate the breeding of rice varieties with multiple disease resistance.

Results

Targeted mutagenesis of *Bsr-d1*, *Pi21* and *ERF922* genes

To generate *Bsr-d1*, *Pi21* and *ERF922* single mutants, three sgRNAs were designed (Figure 1a-c). An expression vector that contained the Cas9 cassette driven by the ubiquitin promoter and single guide RNA (sgRNA) scaffold under the rice small nuclear RNA promoters was constructed based on previously described CRISPR/Cas9 vector (Yin *et al*., 2018). We took advantage of this vector to target one or multiple sites via a single construct, to create *bsr-d1*- or *pi21*- or *erf922*-targeted single gene mutants and triple *bsr-d1/pi21/erf922* mutants (Figure 1d, e). These recombinant expression vectors were used to transform the TGMS variety Longke638S (LK638S) via *Agrobacterium*-mediated transformation. Based on hygromycin-resistant selection, we obtained 34 resistant lines for *bsr-d1*, 22 for *pi21*, 45 for *erf922* as well as 35 for triple *bsr-d1/pi21/erf922*. The PCR-based sanger sequencing was performed to produce about 82% (28/34), 68% (15/22), 73% (33/45) and 71% (25/35) targeting efficiency for *Bsr-d1*, *Pi21*, *ERF922* and triple *Bsr-d1/Pi21/ERF922* genes, respectively (Table 1). Seven, five, eight homozygous single mutants for *Bsr-d1*, *Pi21*, *ERF922*, respectively, as well as three homozygous triple mutants were identified in the T_0 generations (Figure S1), but unfortunately no double-mutant plants were detected. In the T_1 generation, for each gene, we obtained two transgene-free mutants harbouring frameshift mutations (Figure 1f, g and Figure S2), and the reverse transcription quantitative PCR (RT-qPCR) showed that the expression of *Bsr-d1*, *Pi21*, *ERF922* were reduced in single or triple mutants (Figure S3), which could be explained by cellular nonsense-mediated mRNA decay (NMD) mechanism as described previously (Lykke-Andersen and Jensen 2015). These results indicated that both protein and transcriptional abundance were affected in these single and triple mutants.

Rice blast resistance was enhanced in mutants

To investigate their ability against rice blast, *bsr-d1, pi21 and erf922* mutants were evaluated for leaf blast resistance at seedling stages in the blast nursery of Dawei Mountain. The results showed that the LK638S leaves had much more lesions that were significantly decreased in all single and triple mutant lines (Figure 2a, b and Figure S4). To confirm these lesions were caused by *M. oryzae*, the amount of *M. oryzae* present in each inoculated leaf was measured by quantifying fungal-specific DNA. DNA-based quantitative PCR (q-PCR) showed that the mutant lines harboured less fungus than that in LK638S (Figure 2c). Importantly, we observed that the *erf922* mutants exhibited the strongest resistance than the other two single mutants but no significant difference with triple mutants. To determine if *ERF922* was transcriptionally regulated by *Bsr-d1* and *Pi21*, we carried out RT-qPCR to analyse the transcriptional abundance of *ERF922* in LK638S, *bsr-d1* as well as *pi21* (Figure S5). As the result showed, the expression levels of *ERF922* exhibited no significant difference between LK638S and each mutant, consistent with the previous report (Li *et al*., 2017). To test if enhanced blast resistance still existed at reproductive stage, the blast evaluation on rice panicles was conducted, and the results showed that percentage of diseased panicles in mutants were significantly lower than that in LK638S (Figure S6). These results demonstrated that the loss function of *Bsr-d1, Pi21* or *ERF922* improved the blast resistance at both seedling and reproductive stages. To further evaluate the rice blast resistance of the mutants in greenhouse, two blast isolates collected from different regions of China were used (110-2 and E2007046A2 from Hunan and Hubei, respectively). All mutant lines exhibited increased resistance to rice blast, as reflected by the significantly decreased lesion length and fungal biomass in the mutant lines compared with the LK638S at

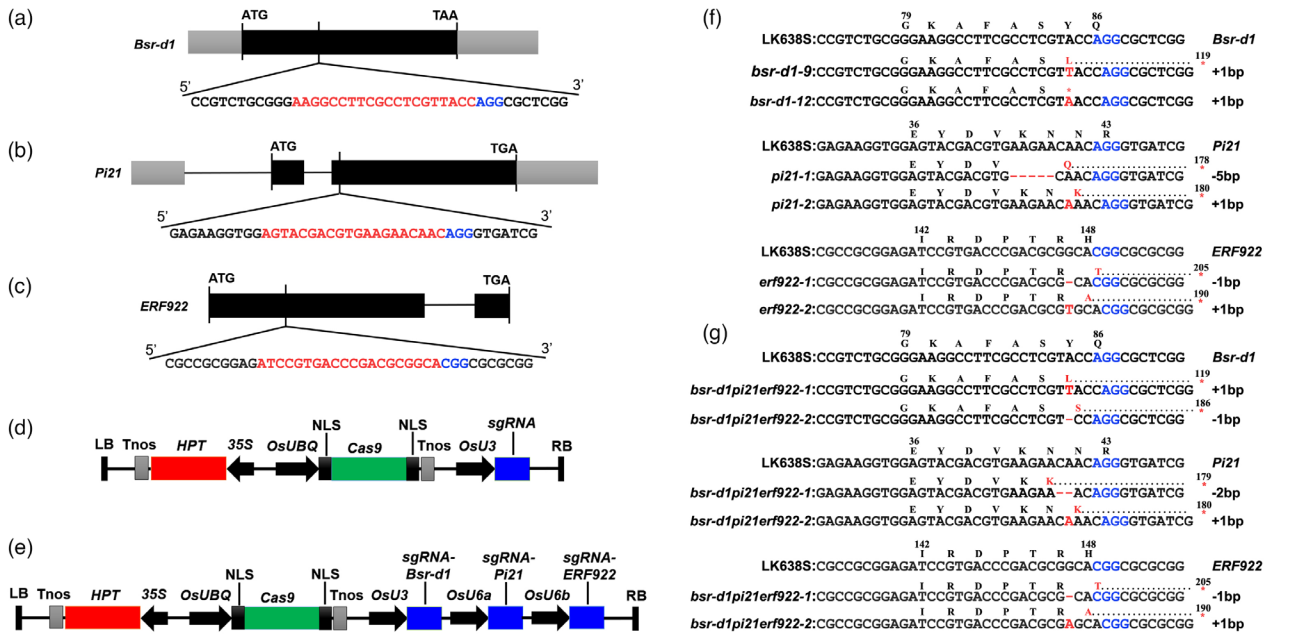

Figure 1 CRISPR/Cas9-induced mutations in the *Bsr-d1*, *Pi21* and *ERF922* genes. (a-c) Schematic of the *Bsr-d1* (a), *Pi21* (b) and *ERF922* (c) gene structures and target sites. Exons and introns are indicated with black rectangles and black lines, respectively. The spacer and PAM sequences were marked in red and blue. (d, e) Schematic diagram of the T-DNA structures including the Cas9 and single sgRNA (d) or three sgRNAs (e) in genome editing construct. The expression of *Cas9* is driven by the maize ubiquitin promoter (*OsUBQ*); the expression of the *sgRNA* scaffold is driven by the rice *OsU3* or *OsU6a* or *OsU6b* small nuclear RNA promoter; the expression of hygromycin (*HPT*) is driven by CaMV35S promoter. NLS, nuclear localization signal; Tnos, the terminator; LB and RB, left border and right border, respectively. (f, g) Homozygous mutations identified at the target sites of *bsr-d1*, *pi21*, *erf922* mutant lines (f) and triple *bsr-d1*/*pi21*/*erf922* mutant lines (g) in the T_1 generation. Amino acids were marked above the relative nucleotide triplets, and the first altered ones from the frameshift were indicated in red, with the number representing the order in the proteins. The stars represent premature stop codons

Table 1 Targeting mutagenesis efficiency in T_0 transgenic plants

Gene	T_0 plants	Mutant plants	Homozygous mutation plants	Targeting efficiency (%)
Bsr-d1	34	28	7	82
Pi21	22	15	5	68
ERF922	45	33	8	73
Bsr-d1/*Pi21*/ *ERF922*	35	25	3	71

10 days' post-inoculation (dpi) (Figure 3a, b). Notably, the lesion length and fungal biomass of *erf922-1* and *bsr-d1pi21erf922-1* were significantly lower than those of the *bsr-d1-9* and *pi21-1* (Figure 3a, b). All together, these results suggested that knockout of *Bsr-d1, Pi21* or *ERF922* enhanced rice blast resistance in the background of LK638S, and among them, *erf922* conferred the strongest resistance.

The expression analysis of defense-related genes in mutants

Rice *Bsr-d1* suppresses the expression of peroxidase genes through binding of the repressive MYB transcription factor (MYBS1) to the *bsr-d1* promoter, resulting in broad-spectrum resistance to rice blast (Li *et al.*, 2017; Zhu *et al.*, 2020). Thus, three *Bsr-d1* target peroxidase genes (*LOC_Os01g73170*, *LOC_Os05g04470* and *LOC_Os10g39170*) were used for determining the expression level by RT-qPCR. The results showed that these peroxidase genes in the *bsr-d1* mutant lines were reduced by 2- to 12-fold in comparison with wild-type LK638S, consistent with the previous study (Li *et al.*, 2017) (Figure S7a-c). The content of H_2O_2 in the *bsr-d1* mutant lines was significantly higher than that in LK638S (Figure S7d). These results indicated that loss function of *Bsr-d1* reduced the expression of the peroxidase genes and increased the amount of H_2O_2, thereby leading to the resistance to a broad spectrum of rice blast.

Activation of plant immune responses during pathogen attack is accompanied by defense hormone pathways, including the SA signalling pathway marker gene *OsPR1a* (Agrawal *et al.*, 2000), *OsPR1b* (Agrawal *et al.*, 2001), *OsWRKY45* (Tao *et al.*, 2009), and jasmonate acid (JA) signalling pathway marker gene *OsPR4* (Wang *et al.*, 2011). Therefore, the expressions of these defense-related genes were analysed in the mutant lines and LK638S at 0 h and 24 h after the leaves were inoculated with rice blast. The expression levels of these defense-related genes were markedly higher in mutant lines than those in LK638S at 24 h post-inoculation (hpi) (Figure 4). These results indicated that knockouts of *Bsr-d1* or *Pi21* or *ERF922* activated the SA and JA signalling pathway genes by rice blast fungi, which may have contributed to their improvement of rice blast resistance.

Knockout of *Pi21* or *ERF922* conferred robust resistance to bacterial blight

SA and JA are the most important plant hormones that play major roles in regulation of plant defense responses against *Xoo* (Bari

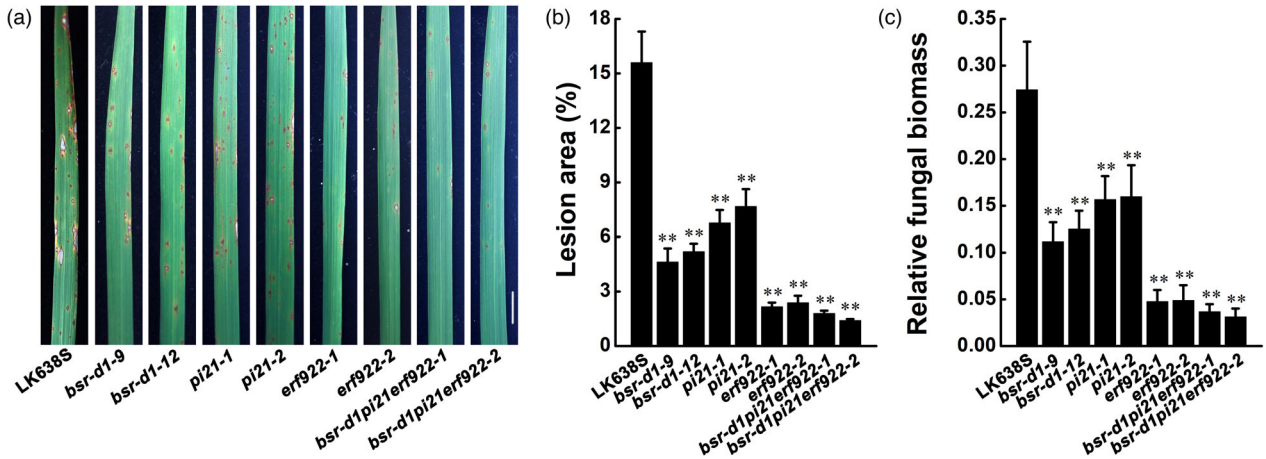

Figure 2 Enhanced blast resistance of the mutant lines in the blast nursery. (a) Rice mutant lines and wild-type LK638S were tested for resistance to *M. oryzae* at the seedling stage. Scale bar, 1 cm. (b) The percentage of lesion areas (disease index) were measured via image analysis using ImageJ software (n = 6 leaves). (c) Fungal growth was determined by the expression level of the *Magnaporthe oryzae MoPot2* gene in the inoculated leaves by RT-qPCR, and the levels were normalized to the expression level of the *OsUbi* gene (n = 3). Values and error bars represent the mean and standard deviation of three independent biological replicates. ** indicates a significant difference ($P < 0.01$ from Student's t-test)

and Jones 2009; De Vleesschauwer *et al*., 2013; Hou *et al*., 2019; Qiu *et al*., 2007). To investigate the bacterial blight resistance of the mutant lines, we inoculated 6 *Xoo* strains (Table S2) to mutant lines using the tip-cutting method (Ji *et al*., 2016). When the mutant lines were inoculated with FuJ, PXO61 and PXO71, respectively, the *pi21-1* and *erf922-1* showed significantly shorter lesions length than LK638S (Figure 5a, b). The lengths of the lesions on *erf922-1* and *pi21-1* caused by the PXO86 and PXO99 were significantly shorter than those observed in LK638S, respectively. Additionally, upon artificial inoculation of YN24, there were no significant difference of lesions length were detected between mutant lines and LK638S. These results demonstrated that knockout of *Pi21* or *ERF922* in LK638S background can improve resistance against bacterial blight disease. To further elucidate the regulation network, the transcriptional levels of SA and JA signalling pathway genes were examined by RT-qPCR. The results showed that all the tested genes were significantly up-regulated in *pi21-1* and *erf922-1* when the mutant plants inoculated with *Xoo*, whereas these genes remained unchanged in *bsr-d1-9*, except for *OsWRKY45*, which was induced in *bsr-d1-9* (Figure S8). These results suggest that the knockout of *Pi21* or *ERF922* in LK638S-enhanced resistance to bacterial blight disease, which may result from the activation of the SA and JA signalling pathway genes.

Characterizations of main agronomic traits in mutants

The above results showed that *bsr-d1-9*, *pi21-1*, *erf922-1* and *bsr-d1pi21erf922-1* mutants were more resistant to rice blast (Figure 2, 3), and the *pi21-1* and *erf922-1* mutants showed increased resistance to bacterial blight (Figure 5). We further investigated whether increased resistances against rice blast and bacterial blight could affect rice growth. At the mature stage, main agronomic traits including plant height, tillers per plant, panicle length and grain number per panicle were measured. Compared with that in wild-type LK638S, the plant height and tiller number in triple mutant *bsr-d1pi21erf922-1* were significantly lower, while there were not significantly different in the single mutant *bsr-d1-9*, *pi21-1* or *erf922-1* (Figure 6). In addition, there were no significant difference for the panicle length and grain number between all mutant lines and LK638S. These results suggested that knockouts of *Pi21* or *ERF922* exhibited increased resistance against both rice blast and bacterial blight without compromising major agricultural traits.

Discussion

Currently, the objective for rice breeding is diversified, and breeders consider not only high yields but also rice quality and disease resistance. Rice blast and bacterial blight are primary destructive diseases that badly affect not only rice production but also rice quality. In the current study, we took advantage of CRISPR/Cas9 technology to edit three known broad-spectrum blast resistant genes *Bsr-d1*, *Pi21* as well as *ERF922* in a very high efficiency to generate each single and triple mutant in T_0 generations (Table 1). We further carried out inoculation experiments and proved that all the single and triple mutants conferred enhanced resistance to rice blast (Figure 2 and 3). It is worth noting that the *erf922* mutants exhibited the strongest blast resistance among all single mutants similar with *bsd-d1pi21erf922* triple mutants indicating that *ERF922* is preferred to being employed to increase the blast resistance under the background of LK638S. In addition, similar blast resistances in *erf922* and *bsd-d1pi21erf922* triple mutants suggested possibility that ERF922 may act downstream of Bsd-d1 and Pi21. ERF922 may integrate Bsd-d1- and Pi21-mediated resistant signalling to respond pathogen attack. Expression levels of *ERF922* exhibited no significant difference between WT and each single mutant (Figure S5), suggesting the possibility that *ERF922* was post-transcriptionally regulated. The detailed post-transcriptional mechanism will be the subject of future work. Previously, all the studies regarding *Bsr-d1*, *Pi21* and *ERF922* only explored their roles in resistance against rice blast. It was unknown whether these three genes played roles in resistance to bacterial blight. In the present study, by testing six typical *Xoo* strains, we showed that *pi21-1* and *erf922-1* mutants exhibited resistances to four out of six *Xoo* strains with one Chinese isolates (FuJ) and three

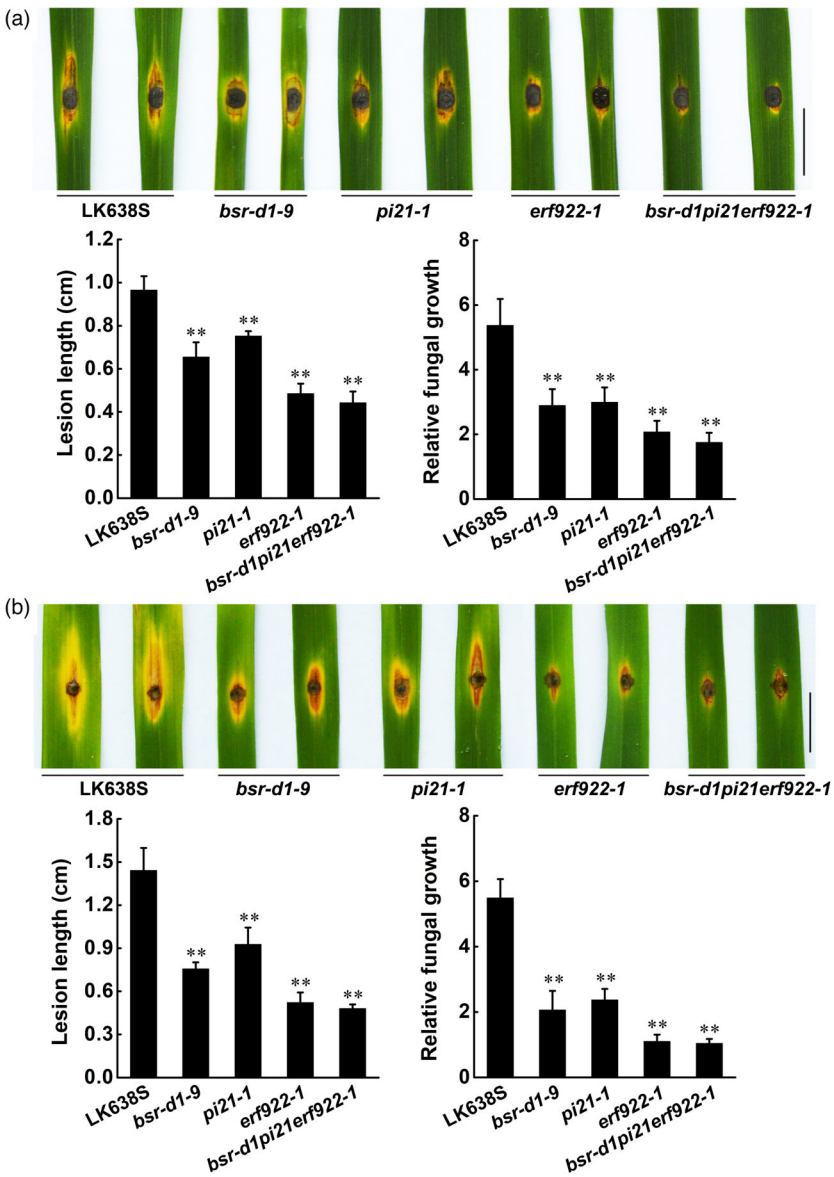

Figure 3 Blast resistance evaluation by punch inoculation. (a, b) The mutant lines were inoculated with the *M. oryzae* isolate 110-2 (a) and E2007046A2 (b), respectively. Two leaves for each of mutant lines and LK638S are shown. Lesion length and fungal growth were determined on inoculated leaves at 10 dpi. Scale bar, 1 cm. Values and error bars represent the mean and standard deviation of three independent biological replicates. ** indicates a significant difference ($P < 0.01$ from Student's *t*-test)

Philippine strains (PXO61, PXO71, PXO99 or PXO86) (Figure 5). More *Xoo* strains will be tested in these mutants to investigate their broad-spectrum resistance to the bacterial blight. A recent study showed that CRISPR/Cas9-mediated multiplex editing of the *Pi21*, *Bsr-d1* as well as a well-known *Xoo*-resistant gene *Xa5* generated *pi21-bsr-d1-xa5* triple mutant which conferred resistant to both blast and bacterial blight (Tao *et al*., 2021). In our study, on one hand, we further compared the blast resistance level among *Bsr-d1*, *Pi21* and *ERF922* single mutants and identified the *erf922* mutant with the strongest blast resistance. On the other hand, our study described the new roles of *Pi21* as well as *ERF922* in the resistance to *Xoo*, which could well explain why the *pi21-bsr-d1-xa5* triple mutant exhibited much stronger resistance to *Xoo* than *xa5* single mutant in the previous study (Tao *et al*., 2021).

It has been reported that many plant pathogenic *Xanthomonas* spp. secreted a class of proteins designated transcription activator-like (TAL) effectors that were injected into plant cells via the type III secretion system (TTSS) to activate the expression of host susceptibility genes that result in diseases (Hu *et al*., 2014; Xu *et al*., 2019). TALEs activate the expression of host gene through binding into specific promoters of host target genes (Wang *et al*., 2015). For example, the *Os8N3* was activated by the TAL effector PthXo1 from *Xoo* strain PXO99 by recognition of TAL effector binding elements (EBEs) located at the promoter region of *Os8N3* (Romer *et al*., 2010). Likewise, based on the enhanced resistances to *Xoo* strains in *pi21* and *erf922* mutants, we speculated that TALEs secreted by these *Xoo* strains might target the promoter region of *Pi21* and *ERF922* to activate *Pi21* and *ERF922* expressions and hence causing diseases, whereas the loss function of *Pi21* or *ERF922* was conducive to the bacterial blight resistances.

Plants response to pathogen attack by a combination of constitutive and inducible defense responses. Many of these responses are modulated through complex interconnecting signal transduction pathways, within which hormones fulfill central roles (De Vleesschauwer *et al*., 2013). Plant immunity is usually associated with SA and JA hormone signalling pathways (Bari and Jones 2009). For example, knockdown of *OsBON1* conferred enhanced resistance to rice fungal and bacterial pathogens, which was accompanied by the activation of SA- and JA-

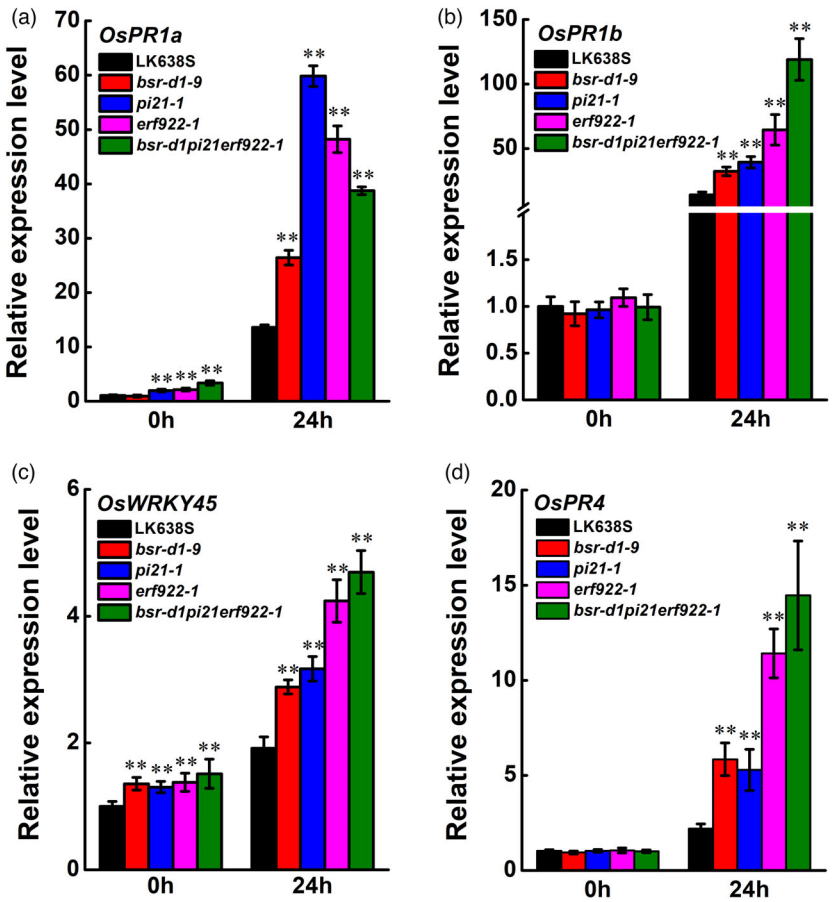

Figure 4 Transcriptional abundances of SA- and JA-regulated defense-related genes in mutant lines. (a-d) Expression levels of the genes involved in SA (a-c) and JA (d) signal transduction in *bsr-d1-9*, *pi21-1*, *erf922-1*, *bsr-d1pi21erf922-1* and wild-type LK638S were determined by RT-qPCR. Values and error bars represent the mean and standard deviation of three independent biological replicates. ** indicates a significant difference ($P < 0.01$ from Student's *t*-test)

responsive genes (Yin *et al*., 2018). Expressions of SA and JA pathway-associated genes were significantly upregulated in the *mpk15* mutant, resulting in enhanced disease resistance to rice blast and bacterial blight (Hong *et al*., 2019). The SA signalling marker gene *OsWRKY45* from *indica* rice varieties positively regulates rice resistance to rice blast and bacterial blight (Tao *et al*., 2009). The expression of *OsWRKY45* was induced not only by *M. grisea* but also *Xoo* (Ryu *et al*., 2006; Tao *et al*., 2009). *OsWRKY45*-overexpressing plants showed enhanced resistance to rice blast and bacterial blight. In this study, upon plants were infected with the rice blast, higher levels of SA signalling related genes *OsPR1a*, *OsPR1b*, *OsWRKY45* and JA signalling pathway gene *OsPR4* were detected in mutants than that in wild-type plants (Figure 4). In addition, when the mutant plants were inoculated with bacterial blight, the *OsPR1a*, *OsPR1b*, *OsWRKY45 and OsPR4* were significantly up-regulated in *pi21-1* and *erf922-1* (Figure S8). These results indicated that the enhancement of resistance to rice blast and bacterial blight may be associated with the activation of SA and JA signalling genes in mutants. However, detailed molecular mechanisms of *Pi21*- and *ERF922*-mediated immunity to bacterial blight still require further study. Of note, the inconsistence of gene expression in Figure 4 and Figure S8 at 0 h (e.g. expression of *OsWRKY45* is slightly higher in each mutant than LK638S in Figure 4 but similar in Figure S8) could be explained by the fact that the samples used for mRNA detection in the two experiments were different in terms of developmental stage, growth environment as well as the sampling area. Together, our work provides a convenient, rapid and effective way to obtain rice cultivars against the blast as well as bacterial blight by CRISPR/Cas9, which could significantly accelerate the breeding of rice varieties with multiple disease resistance.

Experimental procedures

Construct and rice transformation

The target sites of *Bsr-d1*, *Pi21* and *ERF922* were selected by the CRISPR-Plant Web server (Xie *et al*., 2014) and were constructed into the CRISPR/Cas9 vector as described previously (Ma *et al*., 2015). Briefly, the designed targeting sequence was synthesized and annealed to form the oligo adaptors. The oligo adaptors were inserted into the sgRNA expression cassette vectors at a *Eco31*I site. The integrated sgRNA expression cassette was then amplified by PCR using universal primers, and the amplicons were cloned into the CRISPR/Cas9 plant expression vector. The CRISPR/Cas9 plasmids were introduced into *Agrobacterium tumefaciens* EHA105. Rice transformation of LK638S was performed as described previously (Zhou *et al*., 2018). Specific primer pairs Cas9-F/Cas9-R and HPT-F/HPT-R were used to confirm T_0 transgenic-positive plants. Bsr-d1-CX-F/Bsr-d1-CX-R, Pi21-CX-F/Pi21-CX-R and ERF922-CX-F/ERF922-CX-R were used to amplify the genome regions containing each target site, and the resulting PCR products were followed by sequencing to detect mutations in T_0 and T_1 generations. Sequencing results were decoded by an online tool DSDecodeM (http://skl.scau.edu.cn/ dsdecode/) (Liu *et al*., 2015b). Transgene-free plants were identified using the primer pairs Cas9-F/Cas9-R and HPT-F/HPT-R and determined by both showing negative amplification. Sequences of the primers are listed in Table S1.

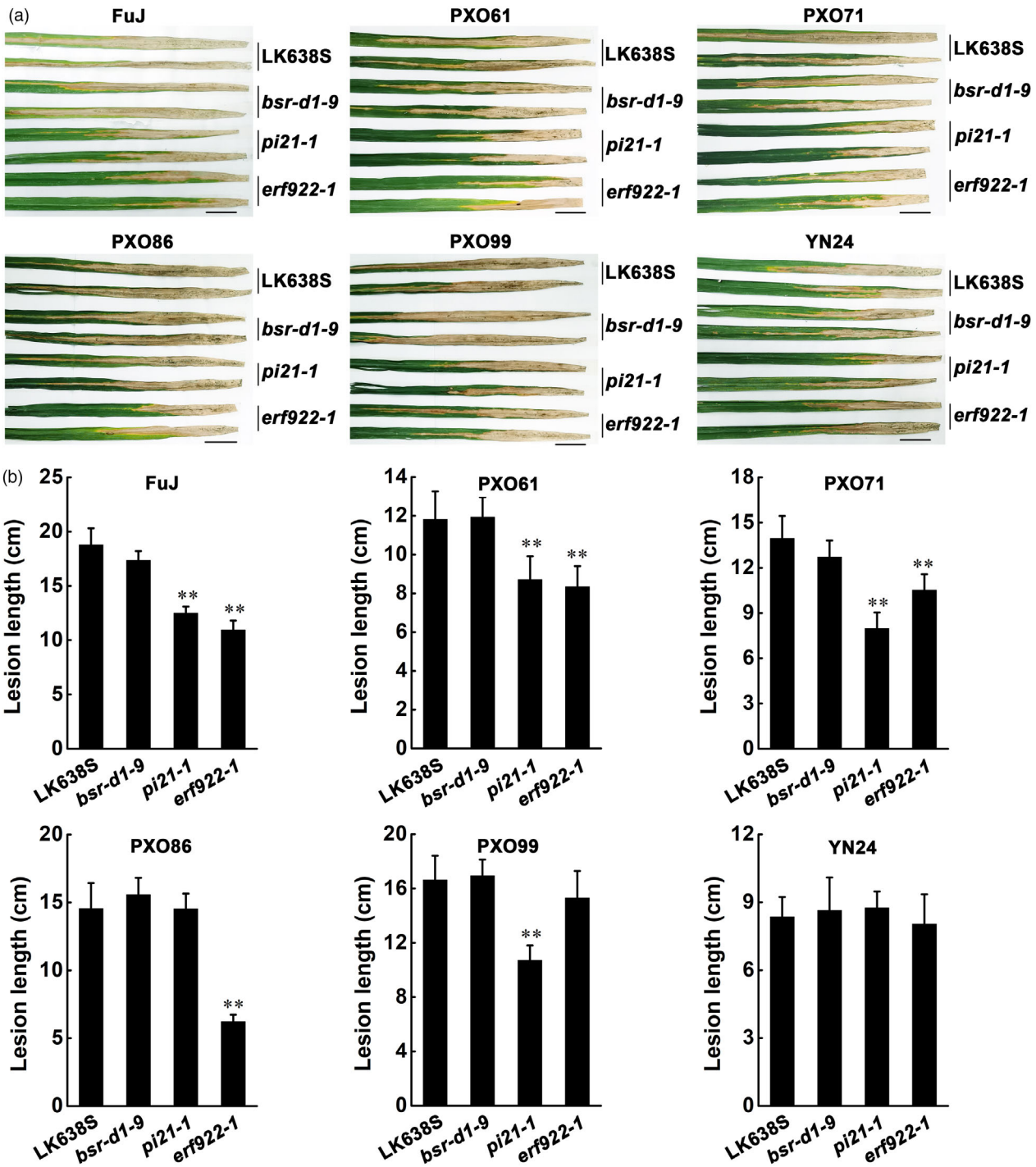

Figure 5 Phenotypical characterization of *bsr-d1-9*, *pi21-1*, *erf922-1* and wild-type LK638S against *Xoo*. (a) Phenotypes of disease reactions in the LK638S and the mutant lines after inoculation with *Xoo* strain FuJ, PXO61, PXO71, PXO86, PXO99 and YN24. Scale bar, 2 cm. (b) Measurements of disease lesion lengths (cm) in wild-type and mutant lines at 2 weeks after inoculation. Values and error bars represent the mean and standard deviation of six independent biological replicates. ** indicates a significant difference ($P < 0.01$ from Student's *t*-test)

Rice blast Inoculation

The rice blast nursery investigation was performed during April to May in two consecutive years (2019-2020) in Dawei Mountain (Hunan Province, 28°49′N, 113°99′E). Fifty seeds of LK638S and mutant rice plants were sown in single-row plots, and three extremely susceptible cultivars 9311, Xiangaizao7 and Xiangwanxian11 were sown on the plot borders used as an inducer to ensure uniform blast infection. The diseased straws were cut into small segments (2-3 cm) and sprinkled over the susceptible

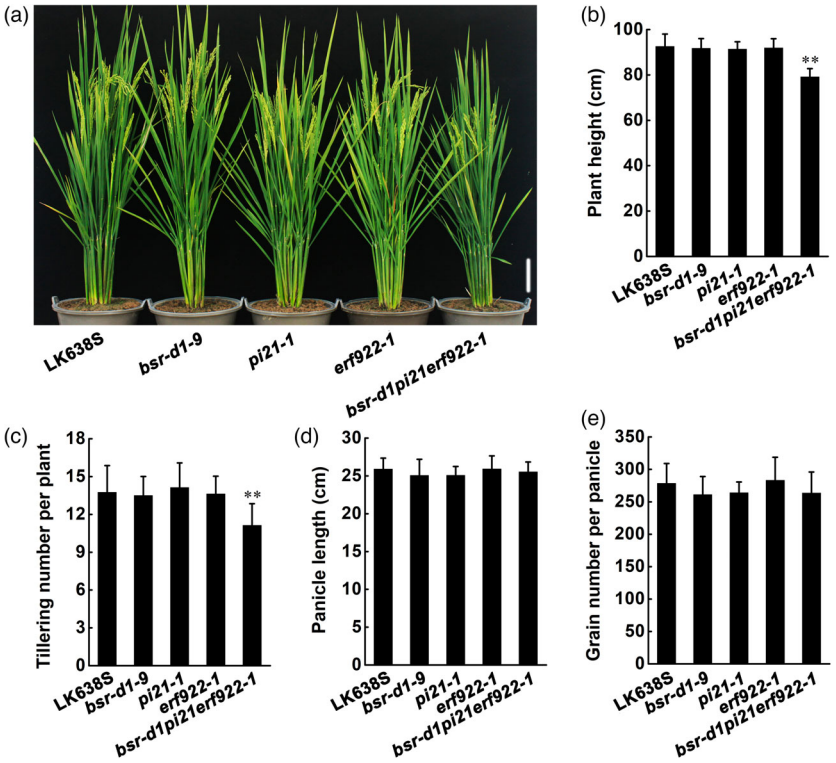

Figure 6 Comparison of major agronomic traits between wild-type LK638S and *bsr-d1-9*, *pi21-1*, *erf922-1* as well as *bsr-d1pi21erf922-1* rice plants. (a) Phenotypes of LK638S and each mutant plant at the heading stage. Scale bar, 10 cm. (b-e) The major traits including plant height (b), tillering number per plant (c), panicle length (d) and grain number per plant (e) are displayed in histograms. Agronomic traits were investigated in paddy filed located at Guanshan village (28°19′32′′N, 112°40′38′′E), Changsha, in 2020. Data are presented as mean ± SD (n = 20). ** indicates a significant difference ($P < 0.01$ from Student's t-test)

spreader cultivars at the two-leaf stage to induce natural infection. Twenty-five days later, flag leaves were harvested to analyse lesion area and relative fungal biomass. The relative fungal biomass was calculated using the threshold cycle value (C_t) of *M. oryzae Pot2* gene against the C_t of the rice genomic ubiquitin (*OsUbi*) gene (Li *et al*., 2017). Sequences of the primers are listed in Table S1.

Punch inoculation was performed as previously described (Liu *et al*., 2015a) with slight modification. Detached leaf sheaths from the fourth leaf of five-leaf-stage seedlings were inoculated with two *M. oryzae* isolates collected from different regions of China, with 110-2 strain from Hunan and E2007046A2 from Hubei. *M. oryzae* isolates were grown on complete agar medium for 15 d before producing spores. Spores were collected via flooding of the fungal agar cultures with sterile water, and the spore concentration in the suspension was adjusted to 5×10^5 conidia/mL before punch inoculation. Dip 5 μL spore suspension for each drop using pipette tip at two spots on each leaf. Inoculated detached leaves were placed in 0.1% 6-benzylaminopurine (6-BA) in sterile water to keep moist. The lesion lengths of disease reactions were measured at 10 days' post-inoculation (dpi). Relative fungal biomass was calculated using the threshold cycle value (Ct) of *M. oryzae Pot2* gene against the Ct of the rice genomic ubiquitin (*OsUbi*) gene (Li *et al*., 2017).

Blast resistance at productive stage was also investigated in an experimental paddy field in Dawei Mountain. Twenty-four plants were transplanted for each line in early June. After flowering, the blast symptoms in rice neck were evaluated. The total number of panicles and the number of diseased panicles (with at least one black neck symptom) were counted for each plant. The panicle severity of a plot was defined as the mean of percentage of diseased panicle from 20 plants (Sester *et al*., 2014). Percentage of diseased panicle = % (the number of diseased panicles/ total number of panicles).

Bacteria strains and inoculation

To examine resistance of bacterial blight, *Xoo* strains were grown in TSA medium (10 g/L tryptone, 10 g/L sucrose, 1 g/L glutamic acid, 15 g/L agar and PH 6.8-7.0). Bacteria were suspended in sterile water to an OD_{600} = 1.0 for inoculation on rice leaves. Rice plants at the booting stage were inoculated with the leaf-clipping method as previously described (Xu *et al*., 2019). Disease phenotype was scored by measuring the lesion length at 2 weeks after inoculation. The sources of the *Xoo* strains tested in this study are listed in Table S2.

RNA Isolation and RT-qPCR

Total RNA isolation and reverse transcription quantitative PCR (RT-qPCR) were carried out as described previously (Zhou *et al*., 2015). To analyse the influence of *M. oryzae* infection on gene expression, three-week-old rice plants growing in the chamber were sprayed with 110-2 spores. The inoculated plants were kept in the growth chamber for 24 h in the dark at 25°C with 90% relative humidity. The inoculation leaves were harvested to analyse the gene expression. To examine the influence of bacterial blight infection on gene expression, flag leaves from 2-month-old rice plants in the paddy field were used. About 3 cm leaf fragments next to *Xoo* strain FuJ infection sites were used for RNA isolation. The primers used for RT-qPCR are listed in Table S1. The expression of rice *Actin* gene (LOC_Os03g50885) was used as an internal control. The relative expression levels were measured as previously described (Livak and Schmittgen 2001).

H_2O_2 accumulation

The concentration of H_2O_2 was measured according to the method described previously (Zhou *et al*., 2018).

Characterization of agronomic trait

The wild-type LK638S and each mutant were planted with three replications in paddy fields located at Guanshan village (28°19′32′′N, 112°40′38′′E), Changsha, in 2020. Each plot consisted of 7 rows with 8 plants per row at a planting density of 20 cm × 20 cm, and the field management was the same as that used in local paddy fields. Upon harvest, 20 plants in the middle of each plot were randomly selected for investigations of main agronomic traits, including plant height, tillers per plant, panicle length and grain number per panicle. Statistical analyses of these data were performed using Student's t test.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Y. Z. and Y. Y. designed the studies; Y. Z., S. X., N. J., X. Z., Z. B., J. L., W. Y., Q. T., G. X., C. L. and X. H. performed the experiments; Y. Z., S. X., N. J., K. W. and Y. Y. analysed the data. Y. Z. and J. T. wrote the manuscript. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Cas9-induced homozygous mutations in the T_0 generation rice plants. (a-d) homozygous mutations identified at the target sites of *bsr-d1*, *pi21*, *erf922* in corresponding single mutants (a-c) or triple mutants (d).

Figure S2 Amino acid alignment of LK638S and mutant protein sequences. (a-c) Alignment of the amino acid sequence of *Bsr-d1* (a), *Pi21* (b) and *ERF922* (c) between LK638S and mutant lines. The dark blue, red and light blue shading indicate 100%, ≥75% and ≥50% similarity, respectively.

Figure S3 Transcriptional abundance of *Bsr-d1*, *Pi21* and *ERF922* in LK638S and mutant lines. (a-c) Relative expression of the *Bsr-d1* (a), *Pi21* (b) and *ERF922* (c) in LK638S and mutant lines by RT-qPCR. Data are presented as mean ± SD (n=3, **P < 0.01, Student's *t-test*).

Figure S4 Natural nursery tests showed leaf blast resistance at seedling stage. (a-d) Phenotypes of single mutants *bsr-d1* (a), *pi21* (b), *erf922* (c) and triple mutants *bsr-d1pi21erf922* (d).

Figure S5 Transcriptional abundance of *ERF922* in *bsr-d1*, *pi21* and *LK638S*. Data are presented as mean ± SD (n=3).

Figure S6 Evaluation of panicle blast resistance at natural nurseries. Percentage of diseased panicle = % (the number of diseased panicles/total number of panicles). Data are presented as mean ± SD (n=20, **P < 0.01, Student's *t-test*).

Figure S7 Transcriptional abundance of the peroxidase genes by RT-qPCR in *Bsr-d1* knockout plants. (a-c) mRNA levels of the *Os05g04470* (a), *Os10g39170* (b) and *Os01g73170* (c) genes in LK638S and *bsr-d1* mutant lines under normal growth condition by RT-qPCR. (d) H_2O_2 content in LK638S and *bsr-d1* mutant lines under normal growth condition. Data are presented as mean ± SD (n=3, ***P* = 0.01, Student's *t-test*).

Figure S8 Transcriptional abundances of SA- and JA-regulated defense-related genes in mutant lines. (a-d) Expression levels of the genes involved in SA (a-c) and JA (d) signal transduction in LK638S, *bsr-d1-9*, *pi21-1* and *erf922-1* were determined at 0h as well as 72h after FuJ infection by RT-qPCR. Data are presented as mean ± SD (n=3, **P* = 0.05; ***P* = 0.01, Student's *t-test*).

Table S1 Primers used in this study

Table S2 *Xoo* strains used in this study