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

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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 T1 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.

Keywords: rice, TGMS, CRISPR/Cas9, rice blast, 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 OsWRKY12, 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 (Qu 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 NLR gene Bsr-d1 and two defense-related genes Bsr-d1 and as well as ERF922 have attracted much more attention due to their broad-spectrum resistance. Bsr-d1 encodes a C2H2-type transcription factor that directly binds to the promoter of three peroxidase genes (Os05g04470, Os10g39170, and Os10g04470 and Os10g39170), thereby affecting H2O2 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 of 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 LK6385. 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 Longke6385 (LK6385) 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 T0 generation (Figure S1), but unfortunately no double-mutant plants were detected. In the T1 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 LK6385 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 LK6385 (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 LK6385, bsr-d1 as well as pi21 (Figure 5S). As the result showed, the expression levels of ERF922 exhibited no significant difference between LK6385 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 LK6385 (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 LK6385 at
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 H2O2 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 H2O2, 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

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Table 1 Targeting mutagenesis efficiency in T0 transgenic plants

| Gene       | T0 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.

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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 OsU6 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 T1 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.

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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 knockouts 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 bsd-d1-9, except for OsWRKY45, which was induced in bsd-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 bsd-d1-9, pi21-1, erf922-1 and bsd-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 bsd-d1pi21erf922-1 were significantly lower, while there were not significantly different in the single mutant bsd-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 T0 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-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 strains with one Chinese isolates (FuJ) and three...
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-

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**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 LK6385 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)
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 an Eco31I 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 T0 transgenic-positive plants. The oligo adaptors were inserted into the sgRNA expression cassette vectors at a Eco31I 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 T0 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 T0 and T1 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.
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, Xiangzaazao7 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 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)

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spreader cultivars at the two-leaf stage to induce natural infection. Twenty-five days later, flag leaves were harvested to analyze lesion area and relative fungal biomass. The 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). 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 $5 \times 10^5$ conidia/mL before punch inoculation. Dip 5 mL 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$_2$O$_2$ accumulation
The concentration of H$_2$O$_2$ 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 T0 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 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) H2O2 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 Fj 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