Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2023) **21**, pp. 1628–1641 doi: 10.1111/pbi.14064

Identification of two novel rice *S* genes through combination of association and transcription analyses with gene-editing technology

Yuchen Xu[1,2,†], Lu Bai[1,†], Minghao Liu[1,†], Yanchen Liu[1], Shasha Peng[1,2], Pei Hu[1], Dan Wang[2], Qi Liu[1], Shuangyong Yan[3], Lijun Gao[4], Xuli Wang[1], Yuese Ning[1], Shimin Zuo[5], Wenjing Zheng[6], Shiming Liu[1], Wensheng Xiang[1], Guo-Liang Wang[7] and Houxiang Kang[1,*]

[1]*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China*
[2]*Hunan Provincial Key Laboratory of Crop Germplasm Innovation and Utilization and College of Agronomy, Hunan Agricultural University, Changsha, Hunan, China*
[3]*Tianjin Key Laboratory of Crop Genetic Breeding, Tianjin Crop Research Institute, Tianjin Academy of Agriculture Sciences, Tianjin, China*
[4]*Guangxi Crop Genetic Improvement and Biotechnology Laboratory, Guangxi Academy of Agricultural Sciences, Nanning, China*
[5]*Key Laboratory of Plant Functional Genomics of the Ministry of Education/Jiangsu Key Laboratory of Crop Genomics and Molecular Breeding, Agricultural College of Yangzhou University, Yangzhou, China*
[6]*Rice Research Institute of Liaoning Province, Liaoning Academy of Agricultural Sciences, Shenyang, China*
[7]*Department of Plant Pathology, Ohio State University, Columbus, Ohio, USA*

Received 29 June 2022;
revised 4 April 2023;
accepted 16 April 2023.
**Correspondence* (Tel +8615801532342; fax 010-62817045; email kanghouxiang@caas.cn)
†These authors contributed equally to this work.

Keywords: 3′-UTR, CRISPR/Cas9 technology, domestication, genome-wide association study, rice blast resistance, susceptibility genes.

Summary

Traditional rice blast resistance breeding largely depends on utilizing typical resistance (*R*) genes. However, the lack of durable *R* genes has prompted rice breeders to find new resistance resources. Susceptibility (*S*) genes are potential new targets for resistance genetic engineering using genome-editing technologies, but identifying them is still challenging. Here, through the integration of genome-wide association study (GWAS) and transcriptional analysis, we identified two genes, *RNG1* and *RNG3*, whose polymorphisms in 3′-untranslated regions (3′-UTR) affected their expression variations. These polymorphisms could serve as molecular markers to identify rice blast-resistant accessions. Editing the 3′-UTRs using CRISPR/Cas9 technology affected the expression levels of two genes, which were positively associated with rice blast susceptibility. Knocking out either *RNG1* or *RNG3* in rice enhanced the rice blast and bacterial blight resistance, without impacting critical agronomic traits. *RNG1* and *RNG3* have two major genotypes in diverse rice germplasms. The frequency of the resistance genotype of these two genes significantly increased from landrace rice to modern cultivars. The obvious selective sweep flanking *RNG3* suggested it has been artificially selected in modern rice breeding. These results provide new targets for *S* gene identification and open avenues for developing novel rice blast-resistant materials.

Background

Rice blast, caused by *Magnaporthe oryzae* (*M. oryzae*), destroys rice crops worldwide (Pennisi, 2010). Recent evidence indicates that the *M. oryzae* Triticum pathotype also threatens the safety of global wheat production (Cruz and Valent, 2017). Planting resistant cultivars is the most effective way to prevent diseases and ensure stability and high yield of crops (Lu *et al.*, 2011). Typical resistance (*R*) genes play important roles in traditional rice blast resistance breeding. However, most of the known *R* genes are easy to lose their resistant functions due to the presence of large pathotypes of *M. oryzae* in the field as well as the high variability of the fungal population caused by the active transposable elements (i.e. MoTE-1) (Kang, Zhu *et al.*, 2016). The susceptibility (*S*) gene is a type of plant genes that could facilitate infection and support compatibility between pathogens and hosts (Van Schie and Takken, 2014). It was first explored in *Arabidopsis* in 2002 (Eckardt, 2002). The resistance conferred by the mutated *S* gene is usually genetically recessive. The mutation or down-regulation of *S* genes could confer a non-race-specific and potentially durable resistance (Van Schie and Takken, 2014). *Mildew resistance locus O* (*MLO*) is a widely studied *S* gene, whose knockout mutant can confer durable resistance to powdery mildew in several species (Li, Lin *et al.*, 2022; Schulze-Lefert and Vogel, 2000). In addition, *Bsr-d1* (Li *et al.*, 2017), *Pi21* (Yasuda *et al.*, 2015), *ERF922* (Wang *et al.*, 2016) and *SWEET14* (Li *et al.*, 2012) are also *S* genes that have been reported, and knocking out either of them can enhance resistance to rice blast or rice bacterial leaf blight disease. Nevertheless, only a few *S* genes have been applied in rice breeding due to the negative impact on other traits, such as reduced growth and tolerance to other stresses (Van Schie and Takken, 2014). Therefore, finding novel rice blast susceptibility-associated genes without impact on plant growth and yield in the diverse rice germplasms is important but challenging.

As a high-efficiency gene mapping method, genome-wide association study (GWAS) has been widely used for mapping plant resistance loci. In rice, hundreds of loci associated with blast resistance (LABR) have been identified through GWAS (Kang,

Wang *et al*., 2016; Liu *et al*., 2020; Wang *et al*., 2014; Zhu *et al*., 2016). However, presently, few studies focus on clarifying the expression patterns of genes in the LABRs, despite the fact that different expression patterns may be associated with disease resistance variations (Li *et al*., 2017). The untranslated region is important for post-transcriptional regulation, as it mediates the expression pattern by controlling the stability, transport and translation efficiency of mRNA (Srivastava *et al*., 2018). It has been reported that single nucleotide polymorphisms (SNPs) in UTR of genes could affect agronomical traits. For instance, one SNP located in the 5′-UTR of *GSN1* was associated with grain length and weight (Zhang *et al*., 2019). Similarly, a tandem repeat in the 5′-UTR of *OsSPL13* was revealed to enhance its expression level, resulting in larger grains (Si *et al*., 2016). Our previous study identified several allelic variations in the UTR, which were associated with tiller number variation (Jiang *et al*., 2019). However, limited studies have been carried out to investigate the impact of UTR on plant disease resistance. A transposon insertion in 3′-UTR of the wheat resistance gene *Pm41b* silenced this gene, causing the plant to become susceptible to wheat powdery mildew (Li, Dong *et al*., 2022). Thus, clarifying the UTR polymorphisms and their functions in plant disease susceptibility-associated genes is necessary for their extensive application in disease resistance breeding in the future.

In our previous study, we selected 586 rice cultivars from the Rice Diversity Panel 2 (RDP2) and conducted a GWAS of rice blast resistance (Liu *et al*., 2020), from which 27 loci were identified to be associated with rice blast resistance. However, about 81.5% (22 of 27) of those loci did not contain the typical NBS-type *R* genes, namely non-NBS loci. In this study, we re-analysed the GWAS data with the rice and rice blast interaction transcriptome data sets. Among those non-NBS loci, we identified eight genes whose expression levels were negatively associated with rice blast resistance. By sequence analysis, five 3′-UTR polymorphisms of two genes (*RNG1* and *RNG3*) were identified to be associated with both expression levels and rice blast resistance. Using these polymorphisms as the molecular markers, by spray inoculation on the randomly selected rice accessions from the rice 3K population (Wang *et al*., 2018), we confirmed that those genes were associated with rice blast resistance. Additionally, editing the 3′-UTRs of *RNG1* and *RNG3* altered their expression levels and rice blast resistance, while knocking out either of the two genes enhanced the rice blast and bacterial blight resistance. Furthermore, gene frequency and nucleotide diversity analyses indicated that these two genes have been artificially selected during rice domestication. Therefore, our results provide a new strategy for candidate *S* gene identification and pave the way for the development of novel rice blast-resistant materials.

Results

Gene expression patterns in the rice blast resistance-associated loci

We re-analysed the gene sequences of the 27 LABRs (Liu *et al*., 2020). Only 18.5% (5 of 27) of those loci contained typical *R* genes (a total of 20 NBS-LRR type genes) in the Nipponbare (NPB) reference genome (Figure S1 and Table S1). Because 96% (22 of 23) of the cloned rice blast *R* genes have their alleles or homologous in NPB reference genome in the corresponding genomic regions (Table S2), no typical *R* genes presented in those 22 LABRs indicated that other genes rather than *NBS-LRR* genes in those loci may confer rice blast resistance. Further investigation of the candidate genes in those 22 LABRs is necessary for both function and application analyses. Within the 22 LABRs, there are a total of 733 protein-coding genes. We analysed the expression patterns of all those 733 genes using the transcriptome data sets of rice and rice blast interaction 24 h post-inoculation (Kawahara *et al*., 2012). To describe their differential expression patterns, we hypothesized a 1-2-1 model (Figure 1a,b), where each number represents the different expression levels of genes in *S* (susceptible, the first number), *R* (resistant, the second number) and CK (control check, the third number) samples, respectively. '1' means low expression or no significant changes among different samples; '2' means at least two times higher expression than '1'. Using this model, the expression patterns of those 733 genes were classified into seven types (Figure 1a,b). Type 1 (1-1-1) included a total of 628 genes which showed either very low expression or no significant changes among the *S*, *R* and CK samples. Type 2 to 7 contained 105 genes that exhibited significant differential expression among the *S*, *R* and CK samples. Of these, nine genes were grouped to type 2 with the pattern of 2-1-1, indicating that the expression level of these genes in *S* was much higher than that in *R* and CK. Type 3 (2-1-2) contained 35 genes expressed higher in *S* and CK than in *R* samples. Expression patterns of genes in types 2 and 3 were both higher in the *S* samples and lower in the *R* samples, which suggests that expression of these genes displayed negative relationships with rice blast resistance. We speculate that *M. oryzae* might manipulate these genes to speed up its infection process, leading to more serious symptoms of rice blast disease.

Validation of the types 2 and 3 genes expression patterns by qRT-PCR

To further confirm whether expression levels of those genes were negatively correlated with rice blast resistance, we analysed all the 44 type-2 and type-3 genes. Among these genes, 12 were annotated as 'expressed protein', 'hypothetical protein' and 'retrotransposon protein', and the other 32 genes had annotated function. In order to further validate whether these 32 genes were negatively related to rice blast resistance, we selected five susceptible accessions ('S', disease scale ≥5), five medium resistant accessions ('MR', 1 ≤ disease scale ≤3) and five resistant accessions ('R', disease scale = 0) to inoculate with rice blast strains of YN716 and RO1-1, respectively. Then, we sampled the rice leaves for the gene expression level validation using quantitative real-time polymerase chain reaction (qRT-PCR), with the sampling time points 0 and 24 hours post-inoculation (hpi). The qRT-PCR results indicated that except for three genes not detectable, at 24 hpi, compared with the incompatible interaction (i.e. MR and R), 52% (15/29) (Figure 1c and Figure S2) and 34% (10/29) (Figure 1d and Figure S3) genes were expressed significantly higher in susceptible interactions. Among them, eight genes had similar expression patterns after inoculation with different rice blast strains (Figure 1c,d). We named those eight genes as *RNG1-RNG8 (Resistance Negatively related Genes 1-8)*, respectively. These results validated that the expression levels of the eight *RNGs* were negatively correlated with rice blast resistance.

The association between polymorphisms in 3′-UTR of *RNG1* and *RNG3* and the gene expression levels

To further explore the mechanism of the negative correlation between the expression level and rice blast resistance, the eight genes, including 2000-bp upstream of the start codon and 500-

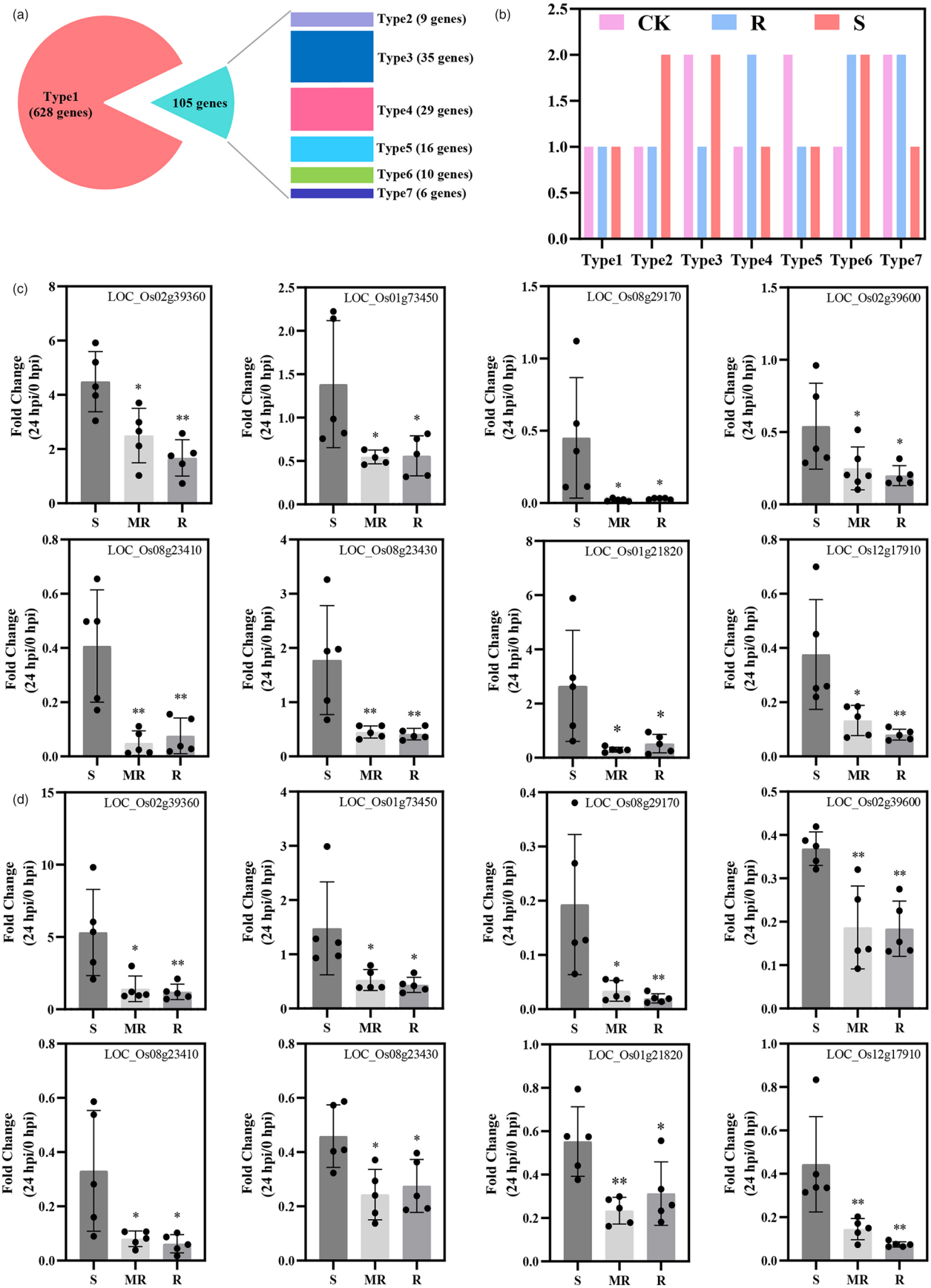


Figure 1 Gene expression patterns in the GWAS identified rice blast resistance-associated loci. (a) Seven types of gene expression patterns in the 27 LABRs, 628, 9, 35, 29, 16, 10, 6 genes in type 1 to type 7, respectively. (b) Different gene expression patterns of the seven types of genes. These expression patterns represented all genes from different types. Type 1 (1-1-1) indicated the genes have either very low expression or no significant change among the susceptible (*S*), resistance (*R*) and control check (CK) samples. And, type 2 to 7 represented the genes with significant expression differences among the *S*, *R* and CK samples 24 h post rice blast inoculation. Here we used 2-1-1 (type 2), 2-1-2 (type 3), 1-2-1 (type 4), 1-1-2 (type 5), 2-2-1 (type 6), 1-2-2 (type 7) to describe their differential expression patterns in *S*, *R* and CK samples, respectively. (c) qRT-PCR verification of the type 2 and type 3 genes' expression pattern after inoculation of 15 rice accessions with rice blast strain of YN716, we used 5 S rice accessions in addition to 5 MR (Medium Resistant) accessions and 5 R accessions. Each black dot represented a different accession. The *y*-axis represented the relative gene expression level at 24 hpi compared with 0 hpi, the *Osubiquitin* (LOC_Os03g13170) was used as the internal control. (d) qRT-PCR verification of the type 2 and type 3 genes' expression pattern after inoculation of 15 rice accessions with rice blast strain of RO1-1, similar to (c), 5 S accessions, 5 MR accessions and 5 R accessions were applied. Error bars represented the Standard Error of Mean (SEM). '*' and '**' represented significant differences ($P < 0.05$ and $P < 0.01$).

bp downstream of the stop codon, were cloned from *R* and *S* accessions. Sequence analysis results indicated that no association existed between the polymorphisms in coding/promoter regions and the expression levels of *RNG1-RNG8*. However, we identified that five polymorphisms in the 3′-UTR of *RNG1* (LOC_Os02g39360) and *RNG3* (LOC_Os08g29170) were tightly associated with *R/S* accessions (Figure 2a,b). In the 3′-UTR of *RNG1*, the three tightly associated polymorphic sites were located at 86-bp, 100-bp and 102-bp downstream of the stop codon (Figure 2a). In the 3′-UTR of *RNG3*, the two tightly associated polymorphic sites were located at 76-bp and 136-bp downstream of the stop codon (Figure 2b). Then, we did qRT-PCR to detect whether the polymorphisms were related to their expression level. The results showed that, although have one exception (accession of 121 571), the average expression levels of both *RNG1* and *RNG3* with *S*-type 3′-UTR were significantly higher than those of *RNG1* and *RNG3* with *R*-type 3′-UTR (Figure 2c,d). To further confirm the association between the 3′-UTR polymorphisms and gene expression levels, we did a transcription activity assay to test the contribution of different types of 3′-UTR to gene expression levels. The promoter of *RNG1* or *RNG3*, luciferase (Luc) and different types of 3′-UTR were fused and expressed transiently in rice protoplast for 24 h (Figure 2e,f). The results of luciferase activity assay showed that Luc fused with *S*-type 3′-UTR of both *RNG1* and *RNG3* had higher luciferase activity than that fused with *R*-type 3′-UTR (Figure 2g,h). *Mid*-type 3′-UTR was between *R* and *S*-type. The polymorphisms of *mid*-type 3′-UTR were consistent with the 3′-UTR in accessions 120 959 or 121 544 (Figure 2a,b). The luciferase activity of LUC with *mid*-type 3′-UTR was lower than that of *S*-type 3′-UTR and higher than that of *R*-type 3′-UTR (Figure 2g,h), demonstrating that *S*-type 3′-UTR increased the expression level of *RNG1* and *RNG3*. Thus, we conclude that polymorphisms in 3′-UTR are associated with the expression level of *RNG1* and *RNG3*.

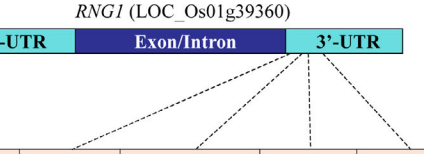
Polymorphisms at 3′-UTR of *RNG1* and *RNG3* could be used as the molecular markers for identifying rice-resistant accessions from diverse rice populations

To test the association between *RNG1/RNG3* genotypes and rice blast resistance phenotypes, eight cultivars carrying different types of *RNG1* and *RNG3* (four cultivars of each gene) were selected from the RDP2 population and inoculated with rice blast strain of RO1-1. For *RNG1*, the rice cultivars 120 983 and 120 959 carrying *R*-type polymorphisms in 3′-UTR were resistant to rice blast (Figure S4a). On the contrary, the rice cultivars 121 211 and 121 540, which carried *S*-type polymorphisms in 3′-UTR, were susceptible to rice blast (Figure S4a). Similar results were obtained for *RNG3*. The two rice cultivars, 121 368 and 121 538, which carried the *R*-type polymorphisms in 3′-UTR, were resistant to rice blast (Figure S4b), while the other two rice cultivars, 121 541 and 121 022, which carried the *S*-type polymorphisms in 3′-UTR, were susceptible to rice blast (Figure S4b). Furthermore, the disease area measurement results were consistent with the inoculation results (Figure S4c and S4d).

To test whether the 3′-UTR polymorphisms of the *RNGs* could be used as molecular markers for the identification of rice blast resistance accessions in other independent rice populations, we analysed those markers in the rice 3K population (Wang *et al.*, 2018). To ensure the high confidence of genotyping data sets, we used the 430 accessions, which had been re-sequenced with high coverage (depths > 20×), for further genotype analyses. Of these, seven accessions did not contain *RNG1* or/and *RNG3*, respectively (might be due to either incomplete genome sequencing data or gene absence). Of the remaining accessions, 27% carried the *R*-type 3′-UTR of *RNG1*, and 79% carried the *R*-type 3′-UTR of *RNG3* (Table S3). We further analysed how many accessions contained a single *R*-type *RNG1* or *RNG3* and how many contained both *R*-type or *S*-type genes. Results showed that, of the 423 accessions with both genes, 18% (76) had two *S*-type *RNGs* ('SS', $RNG1^S/RNG3^S$), 4% (15) carried *R*-type *RNG1* and *S*-type *RNG3* ('RS', $RNG1^R/RNG3^S$), 54% (229) carried *S*-type *RNG1* and *R*-type *RNG3* ('SR', $RNG1^S/RNG3^R$), the remaining 24% (103) had two *R*-type *RNGs* ('RR', $RNG1^R/RNG3^R$) (Table S4). To assay their rice blast resistance phenotypes, we randomly selected 16 rice accessions carrying the four types of combination of *RNG1* and *RNG3*, including SS (4 accessions), SR (4 accessions), RS (4 accessions) and RR (4 accessions), and performed the spray inoculation with two *M. oryzae* strains, YN716 and RO1-1. The results showed that the SS accessions were more susceptible than other types (Figure 3a,b). The accessions with one or both *R*-type *RNGs* (SR, RS and RR) were resistant to both rice blast isolates (Figure 3a,b). The lesion area on the leaves of different rice accessions was measured and the results were consistent with the inoculation results (Figure 3c,d). There was no statistically significant difference in the lesion area between SR or RS cultivars and RR cultivars, the mean lesion area of RR cultivars was 0.9% and 0.7% for YN716 and RO1-1 inoculation, respectively, which were slightly smaller than that of SR and RS type accessions (1.0% and 1.2% for YN716, 1.8% and 2.8% for RO1-1). All the above results indicated that *RNG1* and *RNG3* function quantitatively in rice blast resistance, and the 3′-UTR polymorphisms of *RNG1* and *RNG3* could be the molecular markers for identifying rice-resistant accessions.

(a)

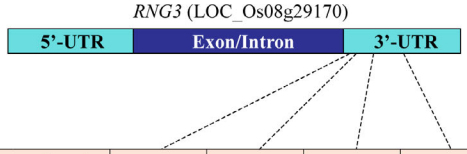
RNG1 (LOC_Os01g39360)



accessions \ location		+86	+100	+102	+126
R-Accessions	117456	+ACAAAA	+GCTGTGTAA	+T	G
	120892	+ACAAAA	+GCTGTGTAA	+T	G
	120983	+ACAAAA	+GCTGTGTAA	+T	G
	121390	+ACAAAA	+GCTGTGTAA	+T	G
	121392	+ACAAAA	+GCTGTGTAA	+T	G
	121429	+ACAAAA	+GCTGTGTAA	+T	G
	120959	+ACAAAA	+GCTGTGTAA	+T	A
	120976	+ACAAAA	+GCTGTGTAA	+T	A
	121434	+ACAAAA	+GCTGTGTAA	+T	A
S-Accessionss	121211	ref	ref	ref	A
	121447	ref	ref	ref	A
	121540	ref	ref	ref	A
	121544	ref	ref	ref	A
	121571	ref	ref	ref	A
	121403	ref	ref	ref	A

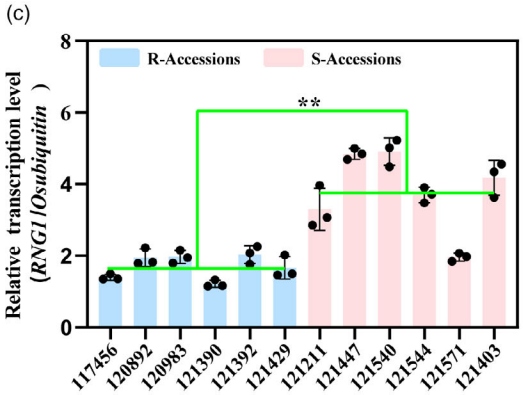
(b)

RNG3 (LOC_Os08g29170)

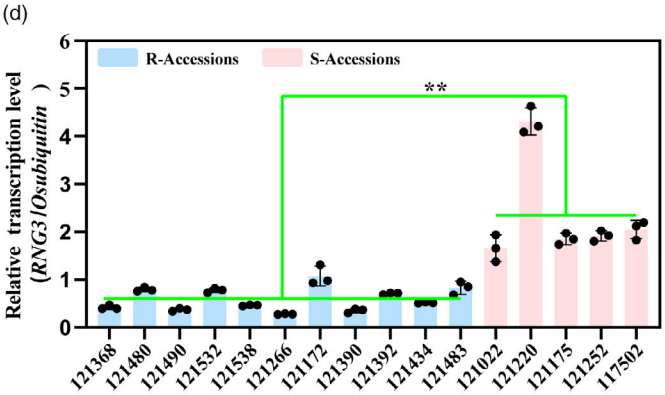


accessions \ location		+73	+76	+136	+246
R-Accessions	121368	T	G	C	T
	121480	T	G	C	T
	121490	T	G	C	T
	121532	T	G	C	T
	121538	T	G	C	T
	121266	T	G	C	T
	121172	T	G	C	T
	121390	T	G	C	T
	121392	T	G	C	T
	121434	T	G	C	T
	121483	T	G	C	T
S-Accessions	121544	T	A	T	T
	121211	T	A	T	T
	121541	T	A	T	T
	121571	T	A	T	T
	121022	C	A	T	C
	121220	C	A	T	C
	121175	C	A	T	C
	121252	C	A	T	C
	117502	C	A	T	C

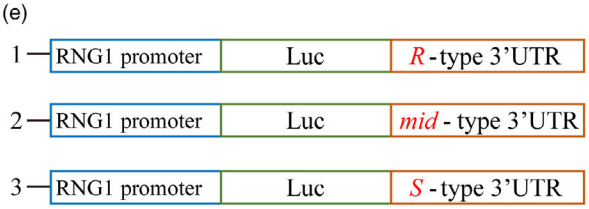
(c)



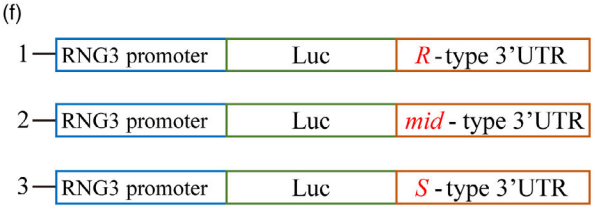
(d)



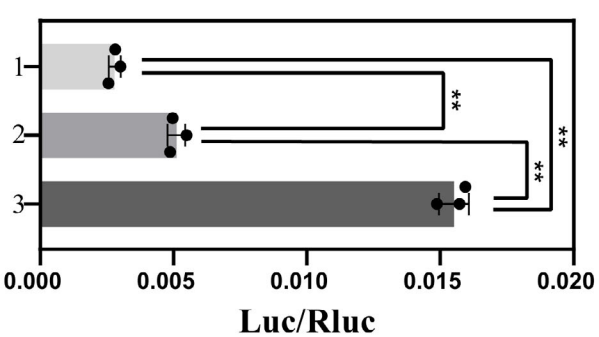
(e)



(f)



(g)



(h)

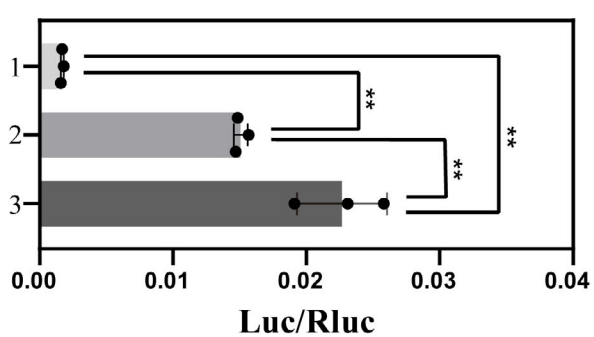


Figure 2 Expression level of *RNG1* and *RNG3* was related to the polymorphisms in their 3′UTR. (a) Four polymorphic sites in the *RNG1* (LOC_Os02g39360) gene's 3′-UTR. Numbers linked to 3′-UTR indicated the positions downstream of the termination codon. The green blocks represented the resistance genotypes, and the blue blocks represented the susceptible genotypes. (b) Four polymorphisms in the *RNG3* (LOC_Os08g29170) gene's 3′-UTR. (c) The expression level of *RNG1* in resistant and susceptible accessions. (d) The expression level of *RNG3* in resistant and susceptible accessions. (e) and (f) The structure of the vector for Dual-Luc assay. (g) and (h) Dual-Luc assay to detect the difference between *R*-type 3′-UTR, *S*-type 3′-UTR and *mid*-type 3′-UTR of *RNG1* and *RNG3*. Error bars represented the SEM of replications. '**' represented significant differences ($P < 0.01$).

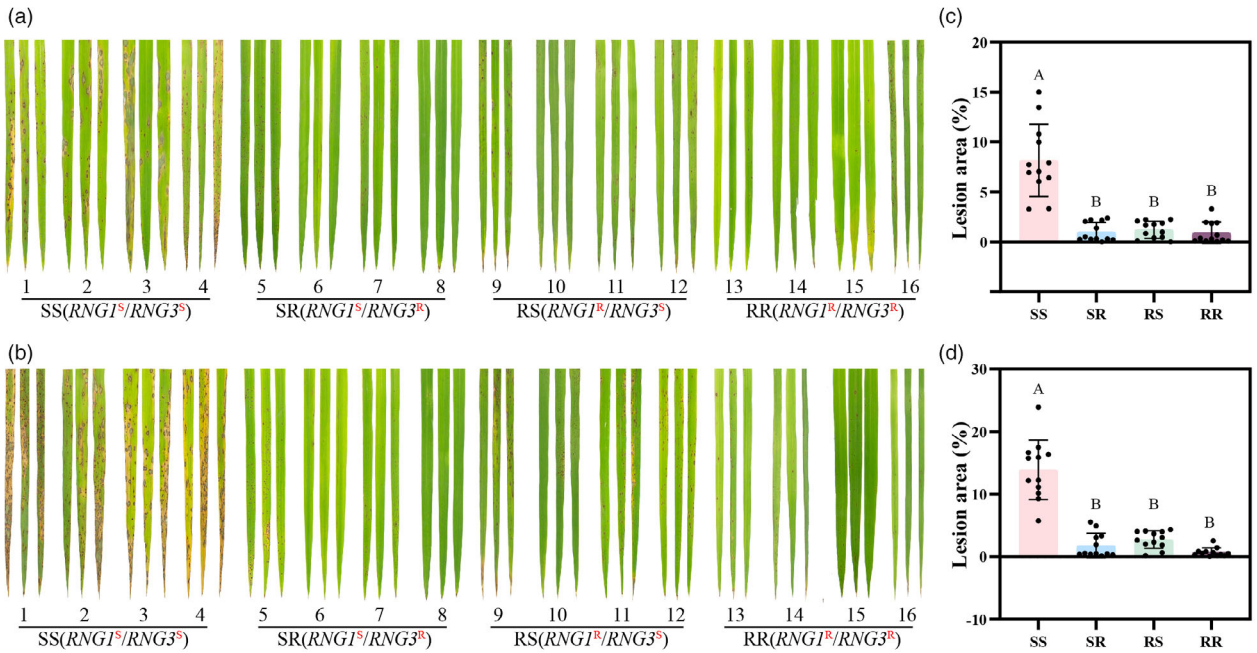


Figure 3 Rice blast disease evaluation of accessions carrying different genotypes of *RNG1* and *RNG3* in 3K rice population. 'SS', 'SR', 'RS' and 'RR' represented different haplotypes of '*S*-type *RNG1* and *S*-type *RNG3*', '*S*-type *RNG1* and *R*-type *RNG3*', '*R*-type *RNG1* and *S*-type *RNG3*' and '*R*-type *RNG1* and *R*-type *RNG3*', respectively. '1–16' represented different accessions with different types of *RNG1* and *RNG3*. (a) Spray inoculation of accessions with different types of *RNG1* and *RNG3* with rice blast strain of YN716. (b) Spray inoculation of accessions with different types of *RNG1* and *RNG3* with rice blast strain of RO1-1. (c) Lesion area of leaves showed in (a). (d) Lesion area of leaves showed in (b). Error bars represented the SEM of replications. 'A' and 'B' represent significant differences ($P < 0.01$) of lesion area among different haplotypes.

Editing the 3′-UTR of *RNG1/3* altered gene expression level and rice blast resistance

To further investigate the contribution of *RNG1* and *RNG3* to rice blast resistance, we first edited the 3′-UTR of two genes using CRISPR/Cas9 technology. Using NPB as the background material and the 3′-UTR polymorphism nearby region sequences as the targets for editing, we obtained four editing types of 3′-UTR for *RNG1*, they were insertion of 'A', 'T' or 'G' and 8-bp deletion (Figure 4a), and named as *rng1utr-11*, *17*, *8* and *10*, respectively. Subsequently, qRT-PCR results showed that insertion of 'A' (*rng1utr-11*) and 'T' (*rng1utr-17*) increased the expression level of *RNG1*, and insertion of 'G' (*rng1utr-8*) did not change the expression level; however, the 8-bp deletion mutant (*rng1utr-10*) significantly decreased *RNG1*'s expression level (Figure 4b). Then, we did punch inoculation of these mutants to evaluate their rice blast resistance. The results showed that *rng1utr-11* and *17* with increased expression levels were more susceptible, while *rng1utr-10* with decreased expression level was more resistant to rice blast, and the resistance of *rng1utr-8* was similar to that of the wild type (WT) (Figure 4c).

For *RNG3*, three editing types of 3′-UTR were obtained, including a deletion of 23 bp (mutations of *rng3utr-1* and *7*) and 'A' (*rng3utr-13*) or 'T' (*rng3utr-8*) insertion (Figure 4d). qRT-PCR detection results showed that the 23-bp 3′UTR deletion mutants increased *RNG3*'s expression, and the other two editing types (*rng3utr-13* and *-8*) did not affect *RNG3*'s expression (Figure 4e). Punch inoculation results showed that *rng3utr-1* and *7* were more susceptible to rice blast, while *rng3utr-13* and *8* were similar to that of the WT (Figure 4f).

Fungal biomass and lesion area of *rng1utr* and *rng3utr* mutants were consistent with inoculation phenotypes (Figure 4g–j). Further analysis showed that the expression levels of *RNG1* and *RNG3* were correlated with fungal biomass ($R^2 = 0.9894$ for *RNG1*, $R^2 = 0.9772$ for *RNG3*) and lesion area ($R^2 = 0.7829$ for *RNG1*, $R^2 = 0.0760$ for *RNG3*) (Figure 4k–n). These results demonstrated that editing 3′-UTR could alter the expression levels of both *RNG1* and *RNG3*, and these two genes' expression levels were negatively associated with rice blast resistance.

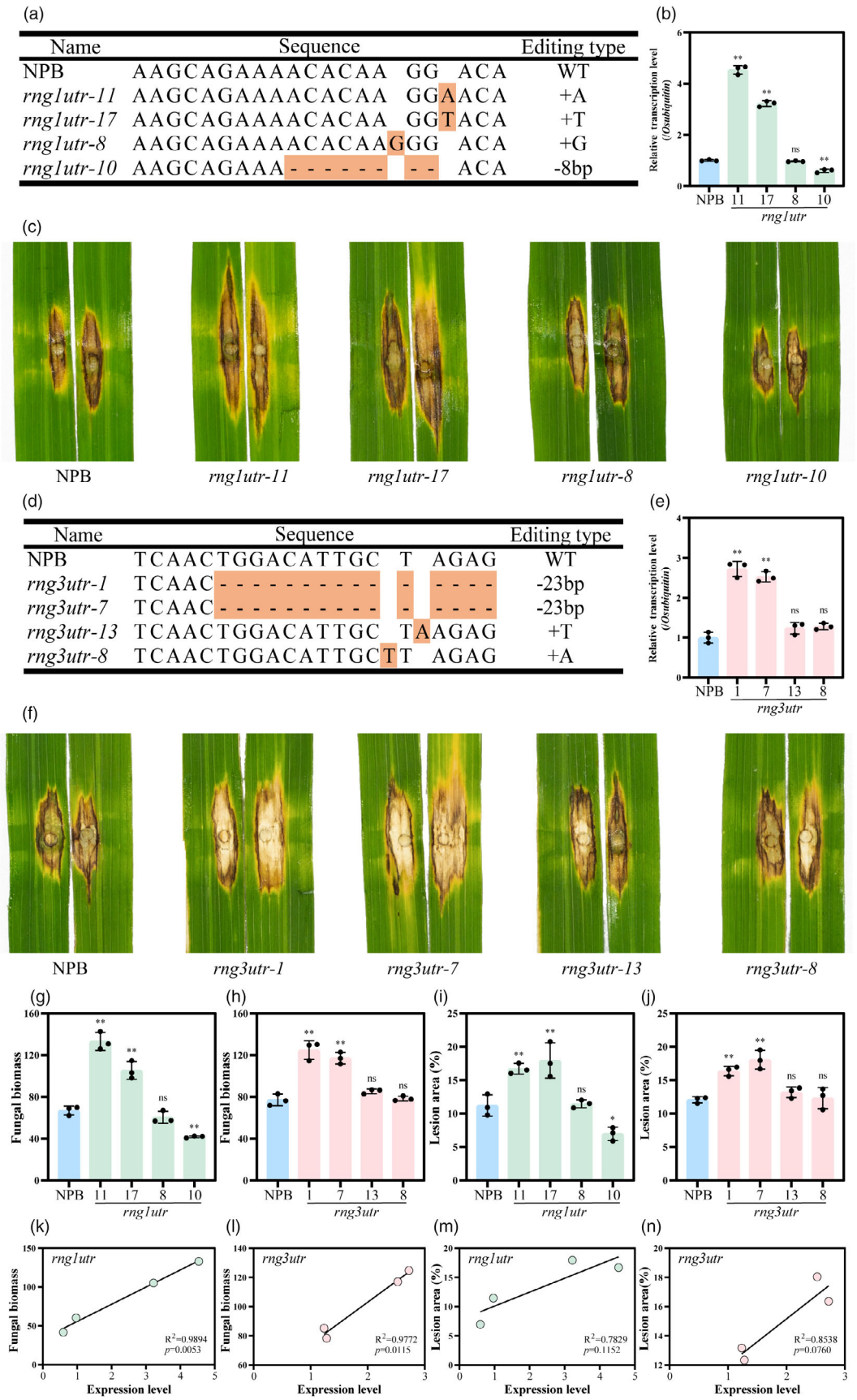


Figure 4 CRISPR-Cas9 edited 3′-UTR of *RNG1* and *RNG3* affected their expression levels, and *RNG1* and *RNG3* genes' expressions were associated with rice blast resistance. (a) Editing type of the 3′-UTR mutants of *RNG1*. (b) The expression level of *RNG1* in different mutant lines. (c) Punch inoculation of 3′-UTR mutants of *RNG1*. (d) Editing type of the 3′-UTR mutants of *RNG3*. (e) The expression level of *RNG3* in different mutant lines. (f) Punch inoculation of 3′-UTR mutants of *RNG3*. (g) and (h) Fungal biomass of 3′-UTR mutants of *RNG1* and *RNG3*. (i) and (j) Lesion area of 3′-UTR mutants of *RNG1* and *RNG3*.

Mutation of either *RNG1* or *RNG3* in rice increased the resistance to both rice blast and rice bacterial leaf blight diseases

We have demonstrated that 3′-UTR editing of *RNG1* and *RNG3* can alter gene expression and influence rice blast resistance; however, we still do not know the phenotype when *RNG1* or *RNG3* is mutated. Thus, we knocked out the CDS (coding sequence) of *RNG1* and *RNG3* in the NPB background through CRISPR/Cas9 gene-editing technology. Sequence analysis showed that two lines of *RNG1*, *rng1-4* and *rng1-7*, were homozygous mutants with 1-bp deletion or insertion (Figure 5a). *rng1-4* encodes a protein composed of 274 amino acids (aa), 2 aa longer than *RNG1*, the frameshift starts at 62nd position, and *rng1-7* led to a premature stop codon at 120 aa (Figure S5a). *rng3-2* and *rng3-6* were two homozygous mutant lines of *RNG3*. *rng3-2* had 1-bp insertion, and *rng3-6* had 16-bp deletion in the target region (Figure 5b). The frameshift starts at 91st aa in *rng3-2*, and *rng3-6* had a premature stop codon at 148 amino acids (Figure S5b). All of the above four editing types resulting in frameshift mutations (hereafter: we used knockout mutants to represent them). The transcription levels of *RNG1* and *RNG3* in their knockout mutants were examined. The results showed that, compared with the WT, the expressions of *RNG1* and *RNG3* were significantly reduced in their corresponding knockout mutants (Figure S6a,b). Subsequently, we evaluated rice blast resistance of these mutants with punch inoculation. The results showed that all of the four knockout mutants had smaller disease lesions than the

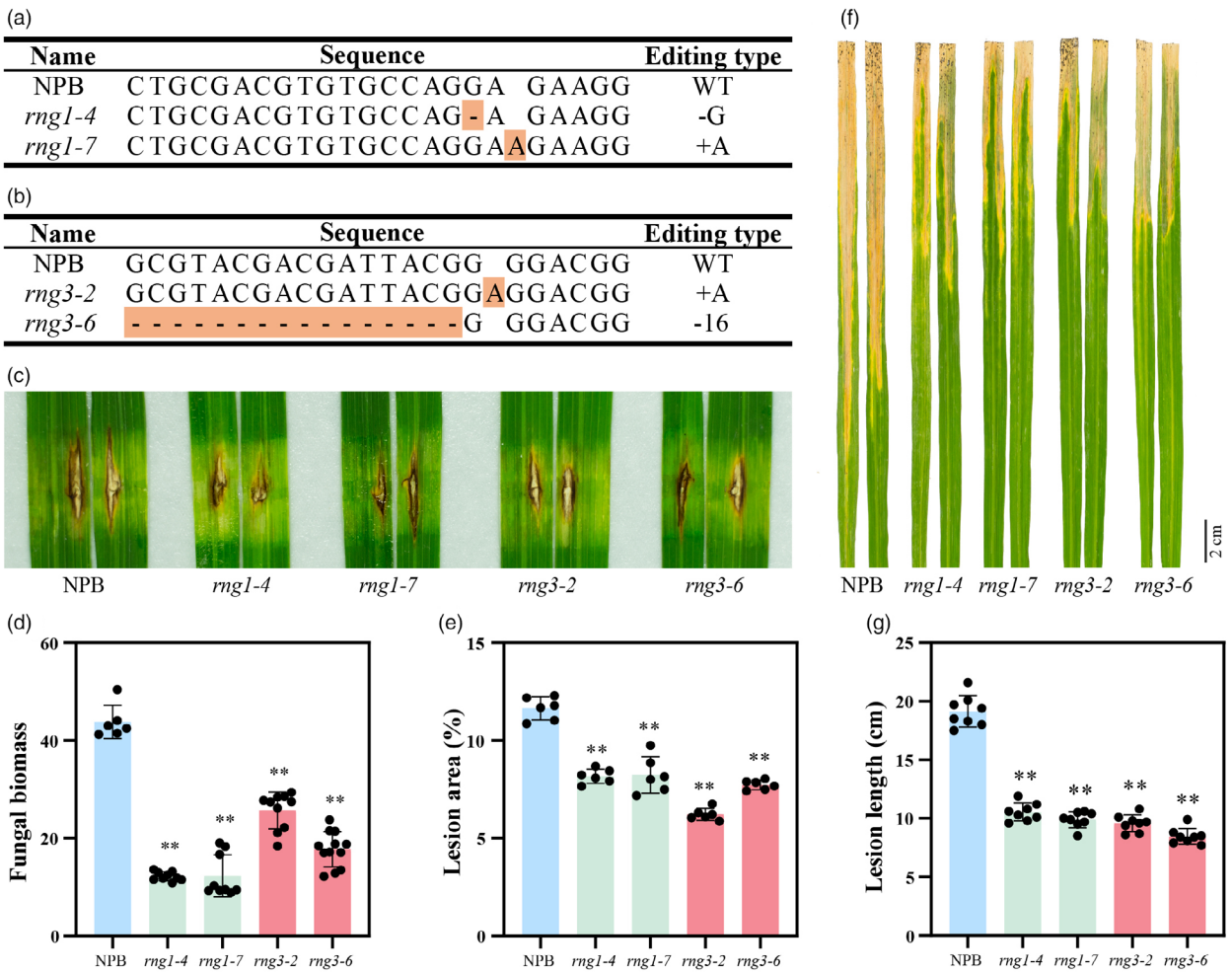


Figure 5 *RNG1* and *RNG3* knockout mutants were more resistant to *M. oryzae* and *Xoo* than the WT. (a) Two different mutation types of *RNG1* obtained from CPRSIR-Cas9 target gene editing. (b) Two different mutation types of *RNG3*. (c) Rice blast resistance evaluation of *rng1* and *rng3* mutants after 14 days post-inoculation (DPI). (d) Fungal biomass of NPB (WT control), *rng1* mutants and *rng3* mutants after 14 DPI. (e) Corresponding lesion area of NPB, *rng1* mutants and *rng3* mutants after 14 DPI. (f) Bacterial leaf blight resistance evaluation of *rng1* and *rng3* mutants after 14 DPI. The scale bar represented 2 cm. (g) Lesion length of NPB, *rng1* mutants and *rng3* mutants. Error bars represent the SEM of replications. '**' represent significant differences ($P < 0.01$).

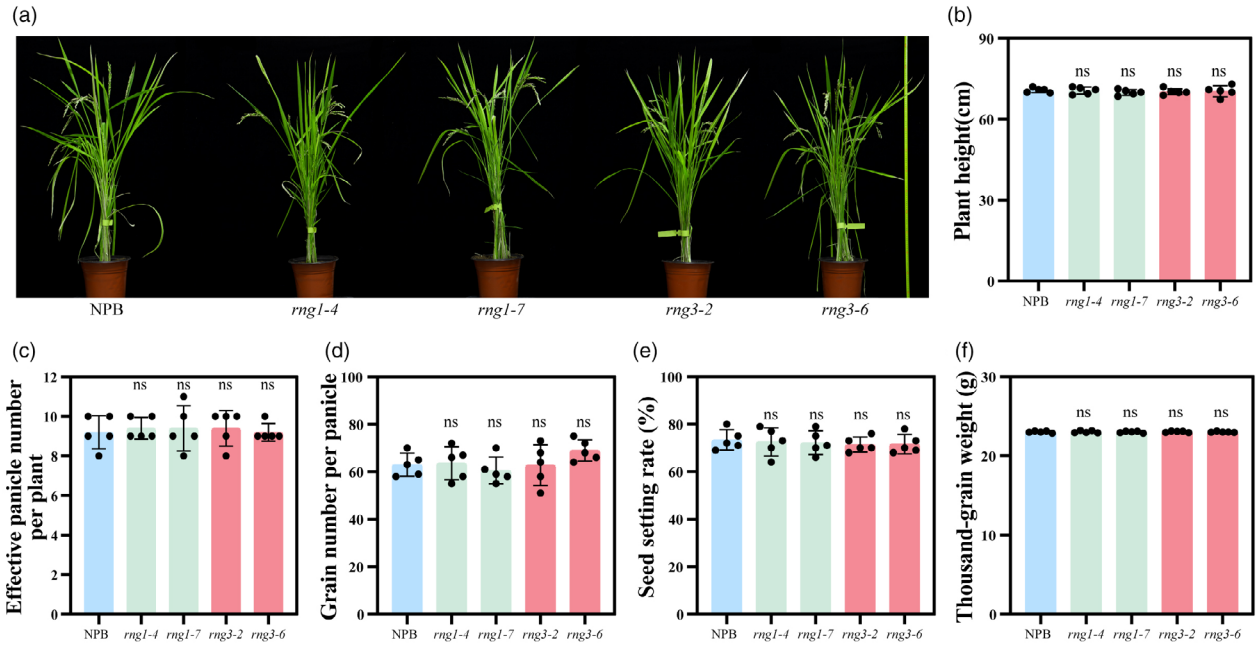


Figure 6 Evaluation of the agronomic traits of *RNG1* and *RNG3* mutants. (a) Photograph of NPB, *rng1* and *rng3*. (b–f) The measured phenotypes of plant height, effective panicle number per plant, grain number per panicle, seed setting rate and thousand-grain weight between the WT and mutants of *rng1* and *rng3*.

WT after 14 days post-inoculation (DPI) (Figure 5c). Both lesion area and fungal biomass of the mutants were also less than the WT (Figure 5d,e). In addition, we evaluated the resistance of the mutants to the rice bacterial blight pathogen *Xanthomonas oryzae pv. oryzae (Xoo)*. Surprisingly, all *rng1* and *rng3* mutants showed significantly enhanced resistance to *Xoo* than the WT of NPB (Figure 5f). The lesion length of the mutants was reduced by approximately 50% compared with WT (Figure 5g). These results suggested that knockout of *RNG1* and *RNG3* in rice led to broad-spectrum resistance to both rice pathogens *M oryzae* and *Xoo*.

Mutation of *RNG1* or *RNG3* had no effect on main agronomic traits

Enhanced rice blast resistance is always accompanied by defects in agronomic traits (Tao *et al.*, 2021). To verify whether *rng1* or *rng3* mutants had any effects on agronomic traits, we investigated five important agronomic traits, including plant height, effective panicle number, grain number per panicle, seed setting rate and thousand-grain weight. The results indicated that, for all of the above tested five agronomic traits, there was no significant difference between NPB and *rng1/rng3* mutants ($p > 0.38$) (Figure 6). These results indicated that mutation of *RNG1* or *RNG3* may not affect the main agronomic traits such as growth and yield; however, more tests are required for further confirmation in multiple fields.

RNG3 may have been artificially selected during rice domestication and modern breeding

From wild rice to modern cultivars, hundreds of genes associated with agronomical traits have been selected during domestication (Mi *et al.*, 2020). Since the polymorphisms in 3′-UTR of *RNG1* and *RNG3* were closely associated with rice blast resistance in rice (Figures 4 and 5), to test whether they were also selected during rice domestication and modern breeding, we analysed the proportion of the *R*-type polymorphisms among wild rice, landrace rice and modern cultivars. For *RNG1*, 90% of wild rice accessions (36 of 40) had its orthologous gene, 37.5% (15 of 40) of which were *S*-type and 52.5% (21 of 40) were *R*-type (Figure 7a). Because *RNG1* was negatively associated with rice blast resistance (Figure 2 and Figure 4), we marked the other four wild rice varieties, which did not carry high similarity homologues, as *R*(-)-type. In the 3K population, there are 205 accessions belonging to modern cultivars, while most of the other 2819 accessions are landrace rice collected from different countries (Wang *et al.*, 2018). Sequence analysis results showed that 41% of the landrace rice carried *R*-type *RNG1*, indicating that the proportion of *R*-type *RNG1* significantly decreased during rice domestication from wild rice to landrace rice. However, during the breeding from landrace rice to modern cultivars, the proportion of *R*-type *RNG1* was increased from 41% to 58% (Figure 7a). For *RNG3*, 80% (32/40) of wild rice varieties were *R*-type, 15% (6 of 40) were *S*-type and 5% (2 of 40) were *R*(-)-type. The variation trend of *RNG3* was similar to that of *RNG1*. The proportion of *R*-type *RNG3* dropped to 77% during domestication from wild rice to landrace rice. It raised to 98% during the breeding from landrace rice to modern cultivars (Figure 7b). All these results suggested that the polymorphisms of *RNG1* and *RNG3* in 3′-UTR have been artificially selected during modern breeding from landrace rice to modern cultivars.

Selective sweep imprint in adjacent loci is one of the most representative features of artificial selection (Hua *et al.*, 2015; Tang *et al.*, 2019). To identify whether *RNG1* and *RNG3* loci have also been selected, we used the 3K population to further analyse the two loci. 100-kb intervals and 100-kb slide windows were used to calculate the nucleotide diversity (π) in the two 1.5-Mb target regions. For *RNG1*, the average nucleotide diversity of these loci in landrace rice is 3.9×10^{-3}, it decreased to 3.2×10^{-3} in modern cultivars, but no obvious selective sweep occurred in the surrounding loci (Figure S7). For *RNG3*, the average nucleotide diversity was significantly decreased from

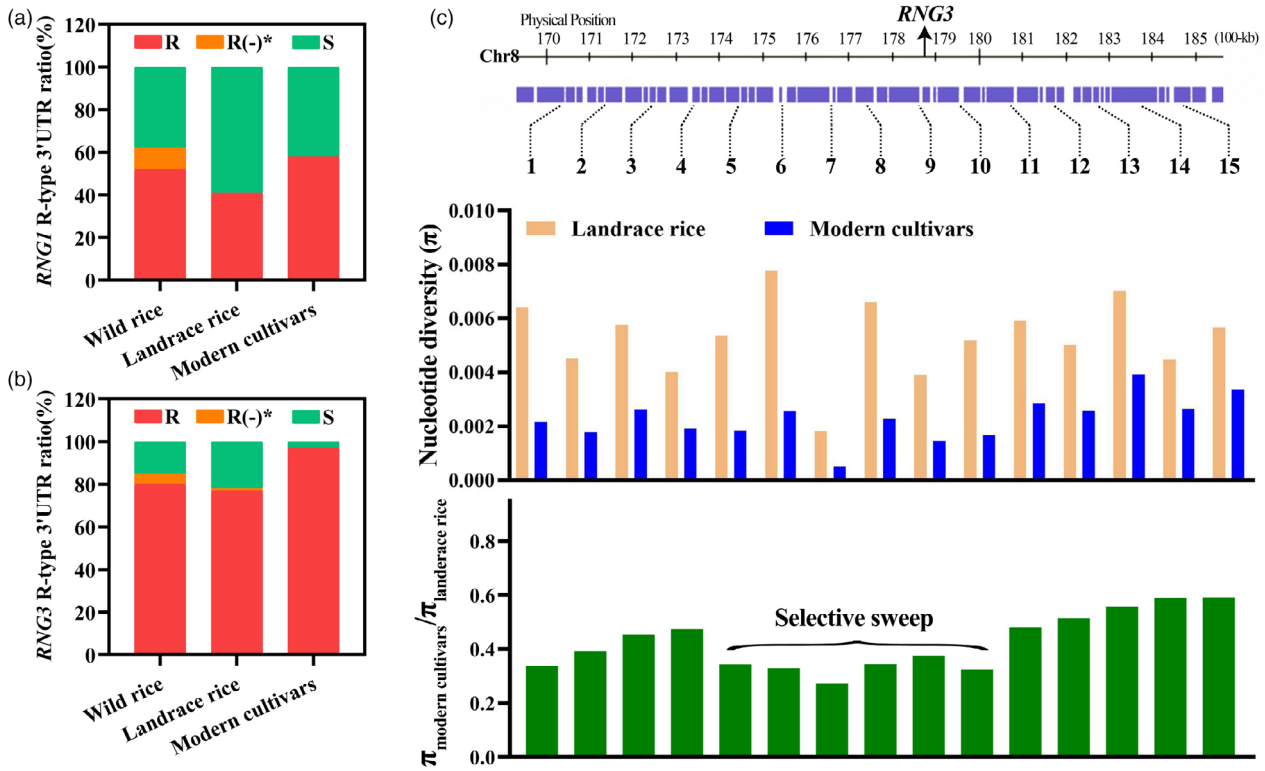


Figure 7 *R*-type 3′UTR of *RNG3* undergone human selection from landrace rice to modern cultivars during rice domestication. The proportion of different polymorphisms in 3′UTR of *RNG1* (a) and *RNG3* (b) in wild rice, landrace rice and modern cultivars. (c) Nucleotide diversity (π) of landrace rice and modern cultivars at the loci of and *RNG3*. Top panel showed the location of ~1.5-Mb genomic region flanking *RNG3*. Middle panel indicated the nucleotide diversity of landrace rice and modern cultivars in the corresponding ~1.5-Mb genomic region. Bottom panel represented the relative π ratio in modern cultivars to landrace rice in the corresponding region.

5.3×10^{-3} in landrace rice to 2.2×10^{-3} in modern cultivars. A noticeable selective sweep feature was observed in the ~600-kb interval flanking *RNG3*. The average π ratio ($\pi_{\text{modern cultivars}}/\pi_{\text{landrace rice}}$) in this interval is 0.33, which is significantly lower than that in other loci (0.49) (Figure 7c). These results indicated that *RNG3* had been artificially selected during modern rice breeding from landrace rice to modern cultivars. Based on the above results, we proposed that further functional analysis of those genes, whose expression levels were negatively associated with rice blast resistance, would open a new avenue for revealing the new resistant mechanism and developing new methods for rice disease control.

Discussion

Following large-area cultivation, the rice blast resistance conferred by a single *R* gene could be frequently overcome by *M. oryzae* (Xu *et al*., 2017). *S* gene is another option in current disease resistance breeding. With the development of gene-editing technology, the application of *S* genes in crop breeding is more promising. Disruption of the transcription activator-like effectors-binding elements of *OsSWEET11* and *OsSWEET14* by CRISPR/Cas9 technology led to broad-spectrum resistance to *Xoo* in rice (Xu *et al*., 2019). Tao *et al*. (2021) created a broad-spectrum disease-resistant rice by editing multiple *S* genes, including *Pi21*, *Bsr-d1* and *Xa5*. In this study, we identified two *S* genes, *RNG1* and *RNG3*, which were highly expressed in susceptible accessions after inoculation of the blast pathogen (Figure 1). The polymorphisms in 3′-UTR were tightly associated with the expression level of *RNG1* and *RNG3* (Figure 2). The spray inoculation results of different rice populations further supported the negative relationships between rice blast resistance and the 3′-UTR polymorphisms (Figure S4 and Figure 3). Suppressing the expression level or knocking out either *RNG1* or *RNG3* through gene-editing technology showed a stronger rice blast resistance (Figures 4 and 5).

Editing the 3′-UTR of genes could impact expression levels by disrupting microRNA binding sites or altering mRNA stability. Modifying the microRNA156 recognition element in 3′-UTR of *TaSPL13* by CRISPR/Cas9 led to an approximately twofold increase in transcripts (Gupta *et al*., 2023). Editing the 3′-UTR of *CXCL1/6/8* by CRISPR system reduced the stability of mRNA and decreased the expression of these genes (Zhao *et al*., 2017). In this study, we edited the 3′-UTR of *RNG1* and *RNG3* and generated mutants with various editing types (Figure 4a,d). As demonstrated in Figure 4b,e, observations indicate varying changes in gene expression, with some mutants exhibiting increases, decreases or no alteration. These changes could be attributed to modifications in microRNA binding to 3′-UTR or mRNA stability. However, further research is required to fully elucidate the underlying mechanisms.

RNG1 encodes a zinc finger protein with a B-box domain, which could enhance abiotic resistance by suppressing ROS burst when heterogeneously expressed in Arabidopsis (Huang *et al*., 2012). Higher ROS burst could provide rice with stronger resistance to rice blast (Park *et al*., 2012); thus, the negative

impact of *RNG1* on rice blast resistance may be caused by inhibiting ROS burst. *RNG3* encodes a dehydrogenase whose function has not been reported in rice. *RNG3*'s orthologous gene in Arabidopsis, *AtAOR*, is involved in detoxifying α,β-unsaturated carbonyl of reactive carbonyl species (RCS) (Takagi *et al*., 2016). Overexpressing *AtAOR* inhibited hydrogen peroxide and reduced cell death in Arabidopsis (Biswas and Mano, 2015). As cell death plays a vital role in plant immune and defence response (Fang *et al*., 2021), *RNG3* might inhibit cell death, resulting in suppressed resistance against rice blast.

S genes always function quantitatively in disease resistance. Simultaneous knockout of *TMS5* and *Pi21* in rice resulted in stronger rice blast resistance than the single mutants of these two genes (Li *et al*., 2019). Tao *et al*. (2021) obtained similar results with the knockout of *Pi21* and *Bsr-d1*. In this study, 16 accessions with different genotypes of *RNG1* and *RNG3* from the 3K population were selected. SS accessions (accessions with *S*-type *RNG1* and *S*-type *RNG3*) were susceptible to rice blast (Figure 3a,b). SR, RS and RR accessions showed stronger resistance to rice blast, although there were no significant differences in the statistical results of lesion area between RR, SR and RS types of accessions (Figure 3c,d). However, the average lesion area of the RR accessions was slightly smaller than that of SR or RS accessions. To explore whether *RNG1* and *RNG3* have additive effects on rice blast resistance, more in-depth studies are required, such as knockout of both genes to evaluate the rice blast resistance, and to further investigate the mechanism.

During rice domestication, hundreds of genes have been artificially selected (Hua *et al*., 2015; Konishi *et al*., 2006; Li, 2006). From wild rice to landrace rice, the proportion of *R*-type 3′-UTR in *RNG1* and *RNG3* was decreased (Figure 7a,b); it is possible that *R*-type genes were partially lost during rice domestication. However, compared with landrace rice, the frequency of *R*-type of these two genes was significantly increased in modern cultivars, the *R*-type of *RNG3* even increased to 97%, which indicated that the *R*-type of these two genes was positively selected in the process of modern rice breeding. Unlike *RNG3*, which showed a strong selective sweep, the nucleotide diversity of *RNG1* locus has just slightly reduced in modern cultivars (Figure 7c), indicating a relatively weak selection of *RNG1* has occurred during rice breeding from landrace rice to modern cultivars.

Conclusion

We identified two genes, *RNG1* and *RNG3*, whose expression levels were associated with rice blast susceptibility. qRT-PCR and sequence analysis results showed that the polymorphisms in 3′-UTR of *RNG1* and *RNG3* were tightly associated with their expression levels. We confirmed these results in both RDP2 and 3K rice populations. Editing 3′-UTR altered the expression level of *RNG1* and *RNG3*. The changed expression level was positively related to rice blast susceptibility. Knock out either *RNG1* or *RNG3* in rice could enhance resistance to rice blast, without impact in agronomic traits. Further population genomics analyses revealed the frequencies of the resistance genotypes of these two genes were decreased during rice domestication from wild rice to landrace rice, and were increased during modern rice breeding from landrace rice to modern cultivars. Nucleotide diversity analysis found an obvious selective sweep in the surrounding loci of *RNG3*, indicating that *RNG3* has been artificially selected. The results obtained in this study provide new targets for further gene functional analysis as well as genome editing-based rice blast resistance breeding.

Materials and methods

GWAS and transcriptome data sets

All GWAS data used in this study are publicly available (Liu *et al*., 2020). Transcriptome data sets used in this study were from NCBI (Accession number: DRX001418) (Kawahara *et al*., 2012).

Plant materials

A total of 586 rice accessions in the RPD2 were collected from International Rice Research Institute (IRRI) and publicly available (McCouch *et al*., 2016). 430 rice cultivars in the 3K population were publicly available (Wang *et al*., 2018).

Construction of CRISPR/Cas9 vector and genetic transformation

All the mutants were generated using the *Agrobacterium*-mediated method published previously (Wang *et al*., 2021). CRISPR/Cas9 vectors were constructed as following steps: The target of CRISPR/Cas9 was designed using online tool (He *et al*., 2021), the primers were synthesized, and the positive and negative primers were formed into double chains by annealing. The product was connected to the plasmid pEntryA linearized by Bas I. Both the correctly sequenced vector and pRHCas9 vector were double digested with Pst I and Spe I and then recover the target fragments for connection and transformation. The vector with correct sequencing was transformed into *Agrobacterium* EHA105 and incubated at 30 °C for 2 days. The correct clones identified by colony PCR could be used for subsequent rice transformation.

Rice blast strain and blast inoculation

The isolates YN716 and RO1-1 were cultured in the oatmeal agar, 3 days in dark and 11 days in light under 25 °C. Soak the seeds in an incubator at 37 °C for 3 days to accelerate germination. Three-week-old seedlings were used to perform the spray inoculation. Spore suspension was diluted with water (0.05% Tween-20) to 3×10^5/mL and then sprayed onto the surface of leaves to form a water film and keep dark for 24 h. Disease index was counted 7 days after inoculation (Kang *et al*., 2016). Two-month-old rice plants were used for punch inoculation. The second leaves from top were wounded with a hole punch and then dropped *M. oryzae* spore suspension to the injured area. Two weeks later, measured the lesion area and fungal biomass. YN716 and RO1-1 were used for expression validation and spray inoculation of accessions in the 3K population. RO1-1 was used for spray inoculation of accessions in RDP2 and punch inoculation.

DNA, RNA isolation and qRT-PCR

The DNA was extracted from 10-day-old leaves, which were seeded in the 1/2 MS medium, with the 2% cetyltrimethyl ammonium bromide (CTAB) method. The RNA was extracted from rice leaves which were inoculated with the isolate YN716 or RO1-1 at different time points. The method referenced the nature magazine protocol (Chan *et al*., 2007). Primers for qRT-PCR were designed using Primer Premier 6 software. The qRT-PCR was performed with Top Green qPCR SuperMix (Trans-Gen). The rice gene *Osubiquitin* was used as internal control,

and the $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels (Livak and Schmittgen, 2001). All primers were showed in Table S5.

Candidate gene sequencing and sequence analysis

The full-length sequence of the candidate genes contains from ~2000-bp upstream of initiation to 500-bp downstream of termination codon. DNA sequencing of the cloned genes was finished by the Beijing Tsingke Biotech Co., Ltd. Sequence alignment was performed with MEGA5. All primers used in the study were designed from NCBI and listed in Table S5.

Dual-luciferase assay

First, promoters and UTRs were cloned to the front and back ends of LUC on the pGREEN-0800 vector by enzyme-ligand. After that, rice protoplasts were prepared, and the vectors were transformed into protoplasts and cultured for 24 h. Follow the instruction of Duo-Lite Luciferase Assay System (Vazyme, DD1205) for subsequent steps. In brief, the protoplasts were gently mixed upside down. 75 μL of protoplast and Duo-Lite Luciferase detection reagent were added into a 96-well plate. After mixing, placed it at room temperature for 10 min. The fluorescence value of firefly luciferase was detected. Afterwards, 75 μL Duo-Lite Stop & Lite detection reagent was added and mixed to detect the luciferase fluorescence value of sea kidney. Each reaction was repeated three times. Finally, the fluorescence value of firefly luciferase was divided by that of sea kidney luciferase, and the ratio was the transcriptional activity of the UTR.

Bacteria strain and inoculation

Rice bacterial blight pathogen *Xoo*, PXO99A, was grown on the TSA plates for 2 days. The bacteria on the plates were resuspended with liquid TSA medium and OD_{600} was adjusted to 0.5. The scissor was sterilized using 75% alcohol and washed with sterile water. The scissor was dipped in the bacterial solution and quickly used to cut the leaves at a position, 5 cm below the tip, of the second leaf. The rice plants were then placed in a greenhouse and the disease phenotypes were investigated after 14 days.

3K rice population analysis

SNPs in 3K population were downloaded from http://iric.irri.org/ (Alexandrov *et al*., 2015). Nucleotide diversity and π ratio were calculated using DnaSP, version 5.0 (Hua *et al*., 2015). Raw sequences of the 430 accessions with high coverage of re-sequencing (depths > 20×) were downloaded from 3K rice database in NCBI GenBank (Project accession PRJEB6180). The Bowtie2 alignment tool (Version 2.3.5.1) was used to find out homologous genes in different rice cultivars (Langmead and Salzberg, 2012), the samtools (Version 1.9) was used for sequence alignments. Perl scripts were used for SNP calling and further sequence analysis. The phylogenetic tree was constructed using MEGA (Version 5.0).

Accession numbers

The gene sequences have been deposited in the NCBI GenBank, the accession numbers of *RNG1* alleles in 14 rice accessions are from ON854866 to ON854879, and the accession numbers of *RNG3* alleles in 21 rice accessions are from ON854880 to ON854900.

Acknowledgements

We appreciate the initiative of the International Rice Research Institute for the establishment of the RDP2 rice variety pool and thank Drs. Bin Liu and Junliang Zhao at the Rice Research Institute, Guangdong Academy of Agricultural Sciences, China, for providing the RDP2 seeds used in this study.

Funding

This research was supported by the National Natural Science Foundation of China to Houxiang Kang (32261143468) and the National Natural Science Foundation of China to Lijun Gao (31860370), and the International Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences (CAASTIP) (CAAS-ZDRW202108).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HK designed and initiated this project and supervised the experiments. YX, LB, ML, HT, YL, SP, PH, DW, QL, SY, LG, XW, YN, WX, SZ and WZ performed experiments. HK, YX and YL analysed the data. HK, G-LW, ML, SL and YX composed the manuscript. All authors have discussed the results and commented on the manuscript. All authors have read and approved the final manuscript.

Ethics approval

Not applicable.

Consent for publication

All authors reviewed the manuscript and agreed to publish it.

Data availability statement

All data generated or analysed during this study are included in this published article and its supplementary information files.

References

Alexandrov, N., Tai, S., Wang, W., Mansueto, L., Palis, K., Fuentes, R.R., Ulat, V.J. *et al*. (2015) SNP-Seek database of SNPs derived from 3000 rice genomes. *Nucleic Acids Res.* **43**(D1), D1023–D1027.

Biswas, M.S. and Mano, J.I. (2015) Lipid peroxide-derived short-chain carbonyls mediate hydrogen peroxide-induced and salt-induced programmed cell death in plants [OPEN]. *Plant Physiol. (Bethesda)* **168**, 885–898.

Chan, K.-L., Ho, C.-L., Namasivayam, P. and Napis, S. (2007) A simple and rapid method for RNA isolation from plant tissues with high phenolic compounds and polysaccharides. *Protocol Exchange*.

Cruz, C.D. and Valent, B. (2017) Wheat blast disease: danger on the move. *Trop. Plant Pathol.* **42**, 210–222.

Eckardt, N.A. (2002) Plant disease susceptibility genes? *Plant Cell*, **14**, 1983–1986.

Fang, H., Shen, S., Wang, D., Zhang, F., Zhang, C., Wang, Z., Zhou, Q. *et al*. (2021) A monocot-specific hydroxycinnamoylputrescine gene cluster contributes to immunity and cell death in rice. *Sci. Bull.* **66**, 2381–2393.

Gupta, A., Hua, L., Zhang, Z., Yang, B. and Li, W. (2023) CRISPR-induced miRNA156-recognition element mutations in *TaSPL13* improve multiple agronomic traits in wheat. *Plant Biotechnol. J.* **21**, 536–548.
He, C., Liu, H., Chen, D., Xie, W.Z., Wang, M., Li, Y., Gong, X. *et al*. (2021) CRISPR-Cereal: a guide RNA design tool integrating regulome and genomic variation for wheat, maize and rice. *Plant Biotechnol. J.* **19**, 2141–2143.
Hua, L., Wang, D.R., Tan, L., Fu, Y., Liu, F., Xiao, L., Zhu, Z. *et al*. (2015) *LABA1*, a domestication gene associated with long, barbed awns in wild rice. *Plant Cell*, **27**, 1875–1888.
Huang, J., Zhao, X., Weng, X., Wang, L. and Xie, W. (2012) The rice B-box zinc finger gene family: genomic identification, characterization, expression profiling and diurnal analysis. *PLoS ONE*, **7**, e48242.
Jiang, S., Wang, D., Yan, S., Liu, S., Liu, B., Kang, H. and Wang, G.L. (2019) Dissection of the genetic architecture of rice Tillering using a genome-wide association study. *Rice (N.Y.)* **12**, 43.
Kang, H., Zhu, D., Lin, R., Opiyo, S.O., Jiang, N., Shiu, S.H. and Wang, G.L. (2016) A novel method for identifying polymorphic transposable elements via scanning of high-throughput short reads. *DNA Res.* **23**, 241–251.
Kang, H., Wang, Y., Peng, S., Zhang, Y., Xiao, Y., Wang, D., Qu, S. *et al*. (2016) Dissection of the genetic architecture of rice resistance to the blast fungus *Magnaporthe oryzae*. *Mol. Plant Pathol.* **17**, 959–972.
Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., Minami, E. and Lee, Y. (2012) Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PLoS ONE*, **7**, e49423.
Konishi, S., Izawa, T., Lin, S.Y., Ebana, K., Fukuta, Y., Sasaki, T. and Yano, M. (2006) An SNP caused loss of seed shattering during rice domestication. *Science*, **312**, 1392–1396.
Langmead, B. and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat. Methods*, **9**, 357–359.
Li, C. (2006) Rice domestication by reducing shattering. *Science*, **311**, 1936–1939.
Li, T., Liu, B., Spalding, M.H., Weeks, D.P. and Yang, B. (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* **30**, 390–392.
Li, W., Zhu, Z., Chern, M., Yin, J., Yang, C., Ran, L., Cheng, M. *et al*. (2017) A natural allele of a transcription factor in rice confers broad-spectrum blast resistance. *Cell*, **170**, 114–126.
Li, S., Shen, L., Hu, P., Liu, Q., Zhu, X., Qian, Q., Wang, K. *et al*. (2019) Developing disease-resistant thermosensitive male sterile rice by multiplex gene editing. *J. Integr. Plant Biol.* **61**, 1201–1205.
Li, M., Dong, L., Zhu, K., Wu, Q., Chen, Y., Lu, P., Guo, G. *et al*. (2022) Provoking a silent R gene in wheat genome confers resistance to powdery mildew. *Plant Biotechnol. J.* **20**, 2039–2041.
Li, S., Lin, D., Zhang, Y., Deng, M., Chen, Y., Lv, B., Li, B. *et al*. (2022) Genome-edited powdery mildew resistance in wheat without growth penalties. *Nature*, **602**, 455–460.
Liu, M.H., Kang, H., Xu, Y., Peng, Y., Wang, D., Gao, L., Wang, X. *et al*. (2020) Genome-wide association study identifies an NLR gene that confers partial resistance to Magnaporthe oryzae in rice. *Plant Biotechnol. J.* **18**, 1376–1383.
Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402–408.
Lu, J., Ju, H., Zhou, G., Zhu, C., Erb, M., Wang, X., Wang, P. *et al*. (2011) An EAR-motif-containing ERF transcription factor affects herbivore-induced signaling, defense and resistance in rice. *Plant J.* **68**, 583–596.
McCouch, S.R., Wright, M.H., Tung, C., Maron, L.G., McNally, K.L., Fitzgerald, M., Singh, N. *et al*. (2016) Open access resources for genome-wide association mapping in rice. *Nat. Commun.* **7**, 10532. https://doi.org/10.1038/ncomms10532
Mi, J., Li, G., Xu, C., Yang, J., Yu, H., Wang, G., Li, X. *et al*. (2020) Artificial selection in domestication and breeding prevents speciation in rice. *Mol. Plant*, **13**, 650–657.
Park, C., Chen, S., Shirsekar, G., Zhou, B., Khang, C.H., Songkumarn, P., Afzal, A.J. *et al*. (2012) The *Magnaporthe oryzae* effector AvrPiz-t targets the RING E3 ubiquitin ligase APIP6 to suppress pathogen-associated molecular pattern triggered immunity in rice. *Plant Cell*, **24**, 4748–4762.
Pennisi, E. (2010) Armed and dangerous. *Science*, **327**, 804–805.
Schulze-Lefert, P. and Vogel, J. (2000) Closing the ranks to attack by powdery mildew. *Trends Plant Sci.* **5**, 343–348.
Si, L., Chen, J., Huang, X., Gong, H., Luo, J., Hou, Q., Zhou, T. *et al*. (2016) OsSPL13 controls grain size in cultivated rice. *Nat. Genet.* **48**, 447–456.
Srivastava, A.K., Lu, Y., Zinta, G., Lang, Z. and Zhu, J. (2018) UTR-dependent control of gene expression in plants. *Trends Plant Sci.* **23**, 248–259.
Takagi, D., Ifuku, K., Ikeda, K., Inoue, K.I., Park, P., Tamoi, M., Inoue, H. *et al*. (2016) Suppression of Chloroplastic Alkenal/one oxidoreductase represses the carbon catabolic pathway in Arabidopsis leaves during night. *Plant Physiol.* **170**, 2024–2039.
Tang, W., Ye, J., Yao, X., Zhao, P., Xuan, W., Tian, Y., Zhang, Y. *et al*. (2019) Genome-wide associated study identifies NAC42-activated nitrate transporter conferring high nitrogen use efficiency in rice. *Nat. Commun.* **10**, 5279.
Tao, H., Shi, X., He, F., Wang, D., Xiao, N., Fang, H., Wang, R. *et al*. (2021) Engineering broad-spectrum disease-resistant rice by editing multiple susceptibility genes. *J. Integr. Plant Biol.* **63**, 1639–1648.
Van Schie, C.C. and Takken, F.L. (2014) Susceptibility genes 101: how to be a good host. *Annu. Rev. Phytopathol.* **52**, 551–581.
Wang, C., Yang, Y., Yuan, X., Xu, Q., Feng, Y., Yu, H., Wang, Y. *et al*. (2014) Genome-wide association study of blast resistance in indica rice. *BMC Plant Biol.* **14**, 311.
Wang, F., Wang, C., Liu, P., Lei, C., Hao, W., Gao, Y., Liu, Y.G. *et al*. (2016) Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene OsERF922. *PLoS ONE*, **11**, e154027.
Wang, W., Mauleon, R., Hu, Z., Chebotarov, D., Tai, S., Wu, Z., Li, M. *et al*. (2018) Genomic variation in 3,010 diverse accessions of Asian cultivated rice. *Nature*, **557**, 43–49.
Wang, J., Wang, R., Fang, H., Zhang, C., Zhang, F., Hao, Z., You, X. *et al*. (2021) Two VOZ transcription factors link an E3 ligase and an NLR immune receptor to modulate immunity in rice. *Mol. Plant*, **14**, 253–266.
Xu, G., Yuan, M., Ai, C., Liu, L., Zhuang, E., Karapetyan, S., Wang, S. *et al*. (2017) uORF-mediated translation allows engineered plant disease resistance without fitness costs. *Nature*, **545**, 491–494.
Xu, Z., Xu, X., Gong, Q., Li, Z., Li, Y., Wang, S., Yang, Y. *et al*. (2019) Engineering broad-spectrum bacterial blight resistance by simultaneously disrupting variable TALE-binding elements of multiple susceptibility genes in rice. *Mol. Plant*, **12**, 1434–1446.
Yasuda, N., Mitsunaga, T., Hayashi, K., Koizumi, S. and Fujita, Y. (2015) Effects of pyramiding quantitative resistance genes pi21, Pi34, and Pi35 on rice leaf blast disease. *Plant Dis.* **99**, 904–909.
Zhang, X., Qin, P., Peng, Y., Ma, B., Hu, J., Fan, S., Hu, B. *et al*. (2019) A single nucleotide substitution at 5′-UTR of GSN1 represses its translation and leads to an increase of grain length in rice. *J. Genet. Genomics*, **46**, 105–108.
Zhao, W., Siegel, D., Biton, A., Tonqueze, O.L., Zaitlen, N., Ahituv, N. and Erle, D.J. (2017) CRISPR-Cas9-mediated functional dissection of 3′-UTRs. *Nucleic Acids Res.* **45**, 10800–10810.
Zhu, D., Kang, H., Li, Z., Liu, M., Zhu, X., Wang, Y., Wang, D. *et al*. (2016) A genome-wide association study of field resistance to *Magnaporthe oryzae* in rice. *Rice*, **9**, 44.

Supporting information

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

Figure S1 Twenty-seven LABRs and their loci analysis.
Figure S2 qRT-PCR verification of the expression pattern of type 2 and 3 genes after inoculation with rice blast strain of YN716.
Figure S3 qRT-PCR verification of the expression pattern of type 2 and 3 genes after inoculation with rice blast strain of RO1-1.
Figure S4 Rice blast disease evaluation of accessions which have different genotypes of *RNG1* and *RNG3* in RDP2.
Figure S5 Predicted protein sequences of *RNG1* and *RNG3* in WT and knockout mutants.
Figure S6 Transcription levels of *RNG1* and *RNG3* in WT and corresponding knockout mutants.

Figure S7 Nucleotide diversity (π) of landrace rice and modern cultivars at *RNG1* locus.
Table S1 *P*-value of top SNPs of loci without NBS-LRR genes.
Table S2 Alleles or homologous of cloned *R* genes in NPB reference genome.
Table S3 Polymorphisms in 3′UTR of *RNG1* and *RNG3* in 430 accessions of 3K population.
Table S4 Genotype of *RNG1* and *RNG3* of accessions in 3K population.
Table S5 Primers for PCR and qRT-PCR.

