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Creation of Bacterial Blight Resistant Rice by Targeting Homologous Sequences of *Xa13* and *Xa25* Genes

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Abstract: Bacterial blight is a destructive disease in rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). Single resistance genes often have limitations in providing broad-spectrum resistance, as pathogens continuously evolve and vary. Breeding rice varieties with multiple disease resistance genes has proven to be an effective strategy for controlling bacterial blight. In this study, a single Cas9/gRNA construct was used to target the homologous sequences of *Xa13* and *Xa25* genes through destroying the target gene function, creating bacterial blight resistance in five rice varieties. These materials provide promising germplasm resources for the development of rice varieties with durable resistance to bacterial blight.

Keywords: bacterial blight resistant; CRISPR/Cas9; *Xa13*; *Xa25*; rice

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1. Introduction

Bacterial blight (BB) of rice, caused by the Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating bacterial diseases in rice production [1]. This pathogen infects rice plants and disrupts their normal physiological functions, particularly photosynthesis. By colonizing the vascular tissues, the bacterium obstructs the movement of water and nutrients, impairs the plant's ability to absorb sunlight, and hampers the synthesis of essential carbohydrates. As a result, infected plants exhibit characteristic symptoms such as leaf wilting, chlorosis, and necrosis, ultimately leading to significant yield reductions that can reach up to 50% [2]. Evidence from crop research has shown that plants have co-evolved resistance (*R*) genes that can specifically recognize pathogen effectors to activate effector-triggered immunity [3,4]. Therefore, breeding rice varieties with major disease resistance genes is an effective and economical strategy for controlling bacterial blight disease in rice production [5].

Genome editing, facilitated by engineered nucleases, has revolutionized basic and applied biology. This technology offers significant advantages in both fundamental research and crop improvement by enabling precise modifications at specific target sequences. The

emergence of CRISPR/Cas9 has greatly contributed to the widespread use of genome editing in plant breeding. It has been instrumental in exploring gene function and enhancing desirable traits in plants [6–9]. CRISPR/Cas9 is particularly effective in introducing mutations through non-homologous end joining of site-specific double–stranded DNA breaks [10,11]. Deletions were found to be the most common type of mutation, followed by insertions, with the majority of mutations being single base changes. Occasionally, base replacements and combined mutations were also observed at the target sequence. Notably, numerous agronomic trait-related genes have been successfully edited in rice using this technology [12,13].

To date, many genes that confer dominant or recessive host resistance to *Xoo* have been identified and some of them have been molecularly cloned, including *Xa1*, *Xa2*, *Xa3/Xa26*, *Xa5*, *Xa7*, *Xa10*, *Xa13*, *Xa21*, *Xa23*, *Xa25*, *Xa26*, *Xa27*, *Xa31(t)*, *Xa41(t)*, *Xa45(t)*, and *Xa46(t)* [4,14–18]. Of these, most are effective resistance (*R*) genes against bacterial blight which have been integrated into cultivated rice varieties for genetic improvement mainly through continuously hybridized or transgenic means. Nevertheless, crossbreeding is time-consuming and transgenesis introduces exogenous genes, resulting in limited applications. In contrast with the numerous examples of dominant *R* gene-mediated resistance, only a few susceptibility (*S*) genes, such as *Xa5*, *Xa13*, *Xa25*, and *Xa41(t)*, have been identified in effector-triggered susceptibility [4,14,19,20]. *Xa5* encodes a γ subunit of the transcription factor IIA (TFIIAγ), inhibiting the transfer of pathogens to disrupt disease progression [4,21]. Three *S* genes, *Xa13*, *Xa25*, and *Xa41(t)*, belong to the SWEET multiple gene family, leading to sucrose exportation into the xylem vessels and facilitating the pathogen's proliferation in rice [14,20,22]. Thus, *S* genes can serve as targets for genome editing to create new materials resistant to bacterial blight disease. However, single resistance genes generally have limitations in broad-spectrum resistance due to the continuous evolution and variation of pathogens. Therefore, the development of durable and broad-spectrum resistant materials would be of significant importance for research on the control of bacterial blight in rice.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Two indica varieties, YuXiangYouZhan (YXYZ) and WuXiangSiMian (WXSM), an indica restorer line, Shuhui143 (S143), an indica sterile line, ZhiNongS (ZNS), and a maintainer line, GuFengB (GFB), of rice were used as the wild type (WT) control and transformation host. Most of the WT and transgenic plants were cultivated in a standard greenhouse at Fuzhou Experimental Station (26.08° N, 119.28° E), Fujian Province, China. The growing season in this province begins in May and extends to mid-October.

2.2. Vectors Construction and Rice Transformation

A single-guide RNA (sgRNA) sequence targeting the flanking sequences of the 30 bp homologous sequences in the *Xa13* and *Xa25* alleles was designed. The oligonucleotides corresponding to the designed sgRNA sequences were synthesized (Table S2), and oligonucleotide dimers were cloned into a CRISPR/Cas9 plant expression vector VK005-01 (ViewSolid Biotech, Beijing, China) according to the manufacturer's instructions. The resulting constructs contained a *Cas9* gene driven by the maize ubiquitin promoter and a designed sgRNA sequence under control of the rice *U6* promoter.

2.3. Rice Transformation

The Cas9/sgRNA construct was transfected into *Agrobacterium tumefaciens* EHA105 by means of electroporation. Rice calli of YXYZ, WXSM, S143, ZNS and GFB were transformed with *Agrobacterium* strains harboring the Cas9/sgRNA construct. Generation of transgenic rice plants was carried out as previously described [23].

2.4. Genotype Analysis

Rice leaf samples were subjected to genomic DNA extraction using the CTAB method [24]. PCR amplification was performed to determine the genotypes of *Xa13/Xa25* alleles in YXYZ, WXSM, S143, ZNS, and GFB. The annealing temperature was 58 °C and PCR amplification was performed after 35 cycles. The primers were purchased from the Sangon Biological Engineering Technology Company (Shanghai, China). Sanger sequencing was employed to analyze the PCR products. Sequencing chromatograms were deciphered following the protocol described [25]. The DNA sequences were aligned using Clustal Omega [26]. To assess the presence or absence of the Cas9/sgRNA T-DNA, PCR amplification was conducted using specific primers targeting the hygromycin phosphotransferase *(Hpt*) gene and the *Cas9* gene.

2.5. Disease Assays

Xoo populations PXO99 were kept in a −80 °C refrigerator at the Institute of Biotechnology, Fujian Academy of Agricultural Sciences. To rejuvenate the bacteria, we incubated the strains on TSA (tryptic soy agar) plates containing appropriate antibiotics and stored them at 28 °C for 2–4 days to allow the bacteria to grow adequately. The bacteria were then collected from the TSA plates and resuspended in sterilized distilled water to form a suspension. The optical density of the suspension was measured at OD_{600} = 0.5. The scissor blades were dipped into the Xoo suspension and cut at about 2 cm from the leaf tip, and then the fully expanded leaves of rice plants (6–8 weeks old) were inoculated, with 5 leaves per plant. Three or more mutants were inoculated at a time. The length of the spots was measured 15 days after inoculation. The spots were measured on each test plant.

3. Results

3.1. Selection of the Targeted Genes and sgRNA Recognition Site

Previous studies demonstrated that knockdown of the *S* genes, *Xa13* or *Xa25*, resulted in enhanced resistance to *Xoo*. We discovered a 30 bp homologous sequence in the third exon of these two genes (Figure 1; Figure S1). Hence, we designed a single Cas9/gRNA within the shared sequence to target *Xa13* and *Xa25* genes simultaneously. To test our hypothesis, we selected an elite rice variety, YuXiangYouZhan (YXYZ), which is known for its high yield and eating quality but is susceptible to bacterial blight. Sequence analysis revealed that the YXYZ contained the same 30 bp homologous fragments as described above.

3.2. Efficient CRISPR/Cas9-Mediated Targeted Mutagenesis in T_0 Transgenic Rice

Next, the Cas9/sgRNA was constructed and transformed into calli via *Agrobacterium*-mediated transformation. A total of 32 independent T_0 transgenic plants were generated from the calli of YXYZ. Subsequent genotyping of T_0 transgenic plants identified 29, 26 and 18 plants harboring *xa13*, *xa25*, and *xa13/xa25* double gene mutations, respectively (Table 1; Figure 2). Among the T_0 edited lines, most of the mutants obtained were bi-allelic and homozygous mutations (Table S1). These results indicate that the *xa13*, *xa25*, and *xa13/xa25* double mutants were successfully obtained by the CRISPR/Cas9 system relying on a single Cas9/sgRNA.

3.3. The Homozygous Lines Increased the Bacterial Blight Resistance of Rice

To characterize the bacterial blight resistance phenotype of the mutant lines, eight-week-old plants of the homozygous *xa13*, *xa25*, and *xa13/xa25* mutants (K4-#14, K4-#16) were inoculated with PXO99, which is a strain of *Xanthomonas oryzae* pv. *oryzae*. The results show that *xa13/xa25* variants had short lesions on the inoculated leaves, whereas the leaves of wild-type plants exhibited typical *Xoo* infection with longer water-soaked lesions (Figure 3). Simultaneously, the obtained *xa13* single-gene mutations of YXYZ were also inoculated with pathogens, which displayed shorter lesions on the inoculated leaves than the wild-type plants (Figure 3). These findings demonstrate that the *xa13*, *xa25*, and

xa13/*xa25* double mutants possessed enhanced bacterial blight resistance compared with wild-type plants.

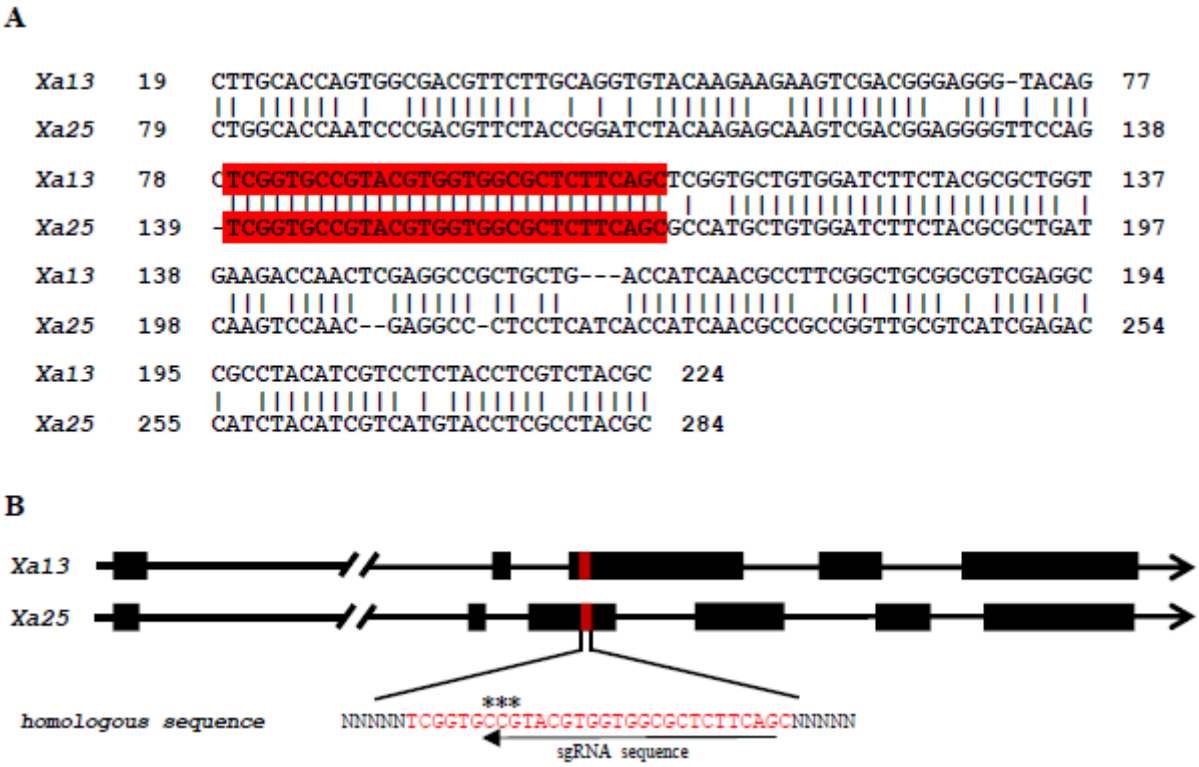

Figure 1. Homology alignment of genes *Xa13* and *Xa25*. (**A**) Sequence alignment revealed a 30 bp homologous sequence containing a gRNA recognition site. (**B**) Gene structures of *Xa13* and *Xa25*. The 30 bp homologous sequence and protospacer adjacent motif (PAM) in *Xa13* and *Xa25* are indicated by red letters and asterisks, respectively.

Table 1. T_0 plants transformed with Cas9/sgRNA constructs targeting the homologous sequences of *Xa13* and *Xa25* genes.

Rice Variety	No. of Transgenic Plants	No. of Plants with Mutations	No. of Plants with Single Gene Mutations		No. of Plants with *xa13*/*xa25* Double Gene Mutations
			Mutations of *xa13* (%)	Mutations of *xa25*	
YXYZ	55	32	29	26	18
WXSM	63	33	27	23	16
S143	43	26	19	19	12
ZNS	34	20	13	15	7
GFB	61	35	29	24	16
Total	256	146	127	107	69

3.4. Putative Off-Target Analysis

To identify whether the gRNA would edit a non-matching genomic sequence, the bi-allelic and homozygous *xa13*/*xa25* mutants YXYZ-#14, YXYZ-#16, YXYZ-#16, and YXYZ-#26 were evaluated for potential off-target effects. Considering that the greater number of mismatched bases and those closer to the PAM region are more likely to interfere with gRNA recognition, we selected possible off-target sequences based on the following two criteria: firstly, there is at least one base mismatch near the PAM region, and the total number of mismatched bases is between 1 and 5 bp. Secondly, seven groups of Cas9/sgRNA candidate targets were selected using CRISPR-P (http://skl.scau.edu.cn/targetdesign/, accessed on 14 February 2023) and BLASTN online tools. As a result, a 21 bp target point sequence was obtained (Table 2; Table S2). No obvious off-target events were found in the transgenic T_0 mutants (Table 2).

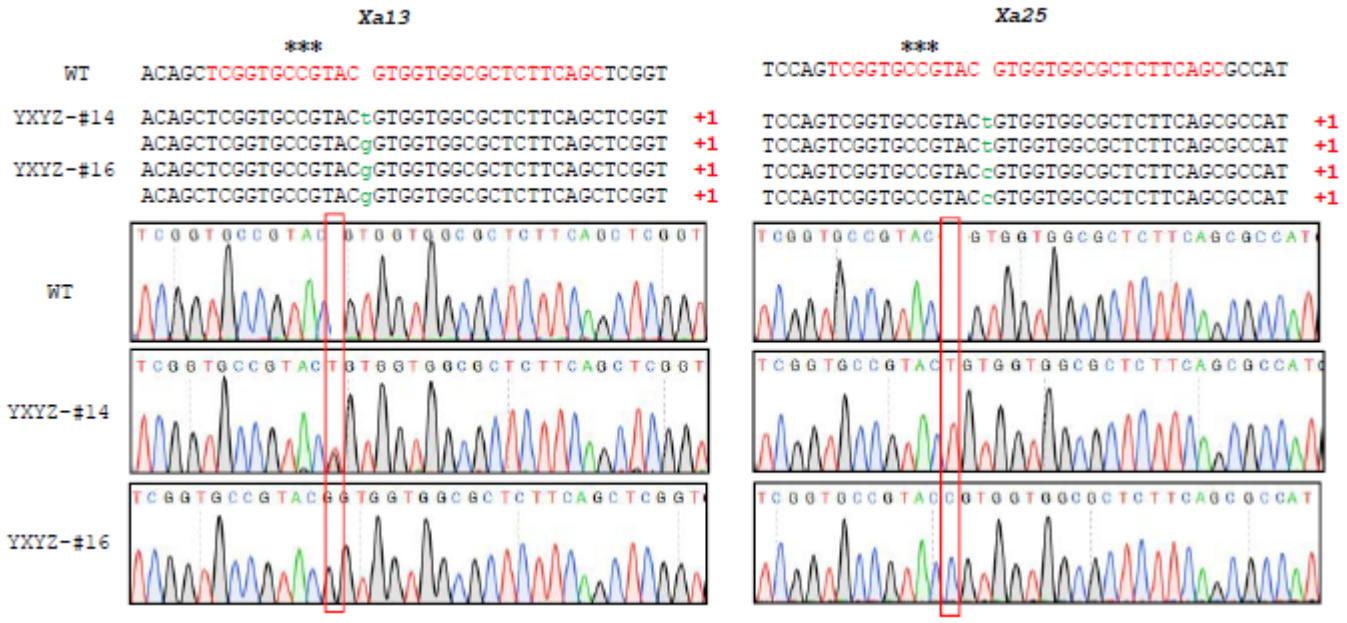

Figure 2. Mutations and corresponding sequencing chromatograms of *xa13*/*xa25* T_0 double mutants in the YXYZ. The insertions are shown in green letters. The sgRNAs are indicated by asterisks. Numbers on the right indicate the insertion length compared with *Xa13* and *Xa25*. YXYZ-#14 and YXYZ-#16 were xa13/xa25 double mutants in the YXYZ.

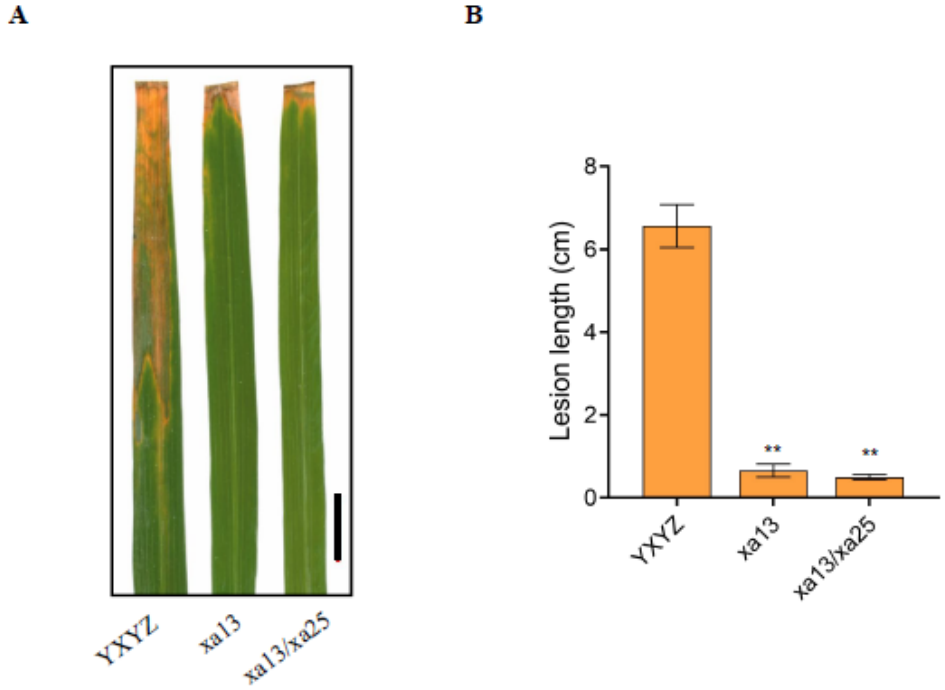

Figure 3. Resistance identification of wild-type and double-gene knockout lines. (**A**) Phenotypic characteristics of double-gene knockout lines *xa13*/*xa25*, the single-gene mutants *xa13* and *xa25*, and the corresponding wild-type YXYZ after inoculation with PXO99. Scale bar, 1 cm. (**B**) Statistical analysis of associated lesion lengths (n = 5 leaves). p values were generated by means of Student's t test. Error bars, SEM (** $p < 0.01$).

3.5. Xa13/xa25 Double Mutants Show Increased Resistance to Bacterial Blight Disease under the Background of Four High-Quality Varieties

In addition, we selected four other rice varieties to confirm whether *xa13*, *xa25*, and *xa13*/*xa25* double mutants displayed increased bacterial blight resistance, including an

indica variety, WuXiangSiMian (WXSM), an indica restorer line, Shuhui143 (S143), an indica sterile line, ZhiNongS (ZNS), and a maintainer line, GuFengB (GFB). The genotyping of T_0 transgenic plants identified 33, 26, 20, and 35 plants with mutations in the target site in WXSM, S143, ZNS, and GFB, respectively (Figure 4; Table S1). Among the T_0 mutant plants, the mutation rate of single genes ranged from 38.2% to 75%. Overall, 25.4% (16/63), 27.9% (12/43), 20.6% (7/34), and 26.2% (16/61) were *xa13*/*xa25* double mutants (Table S1). Likewise, the *xa13*/*xa25* double mutants in these four cultivars also displayed more resistance to bacterial blight disease than the corresponding wild-type plants after inoculation with PXO99 (Figure 5).

Table 2. Evaluation of potential off-target sites.

Target	Name of Putative Off-Target Sites	Putative Off-Target Locus	Putative Off-Target Sequence *	No. of Mismatch Bases	No. of Plants Examined	No. of Indel Mutation
Cas9/sgRNA	OFF1	ch02: 18455705	GCTGAAGAGCGTCACCACGTACGG	2	4	0
	OFF2	ch05: 15644350	GTCGAGGAGCGCCACCACGTGCGG	4	4	0
	OFF3	ch09: 13253210	GCTGAAGGCCGTCACCACGTCCGG	4	4	0
	OFF4	ch08: 25248560	GCTGAAGCACACCACCATGTACGG	4	4	0
	OFF5	ch09:14373426	GCTGAACAGCTCCCCCACGTCCGG	4	4	0
	OFF6	Ch03: 7709411	GCTGGAGAGCTCCACCACGGACGG	4	4	0
	OFF7	Ch03: 13007484	GCTCAGCAGCGCCACCGCGTACGG	5	4	0

* PAM sequence NGG is indicated in blue. Mismatch nucleotides are marked in red.

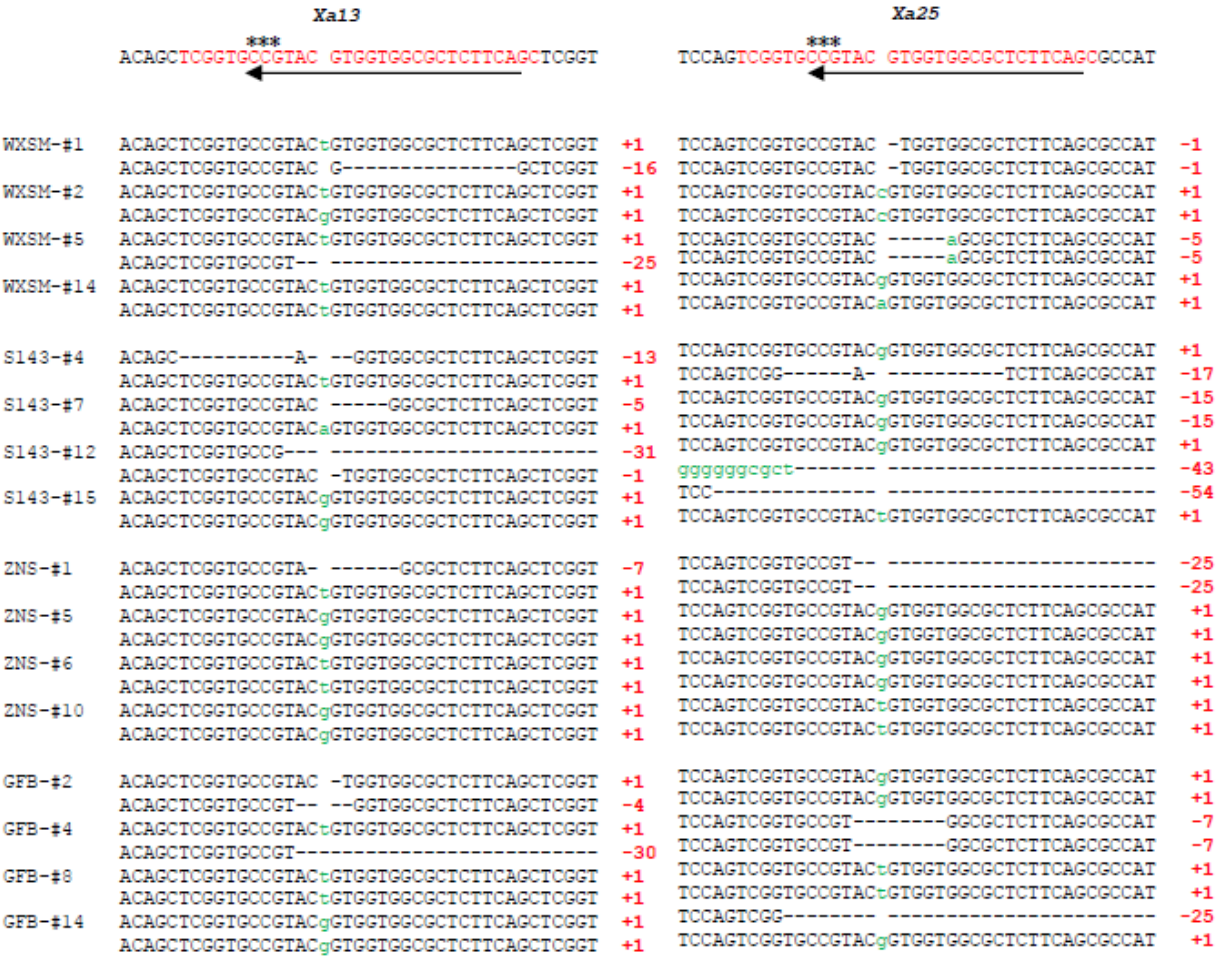

Figure 4. Mutations and corresponding sequencing chromatograms of *xa13*, *xa25*, and *xa13*/*xa25* T_0 double mutants in the WXSM, S143, ZNS and GFB plants. The insertions are shown in green letters. The sgRNAs are indicated by asterisks. Numbers on the right indicate the insertion length compared with *Xa13* and *Xa25*.

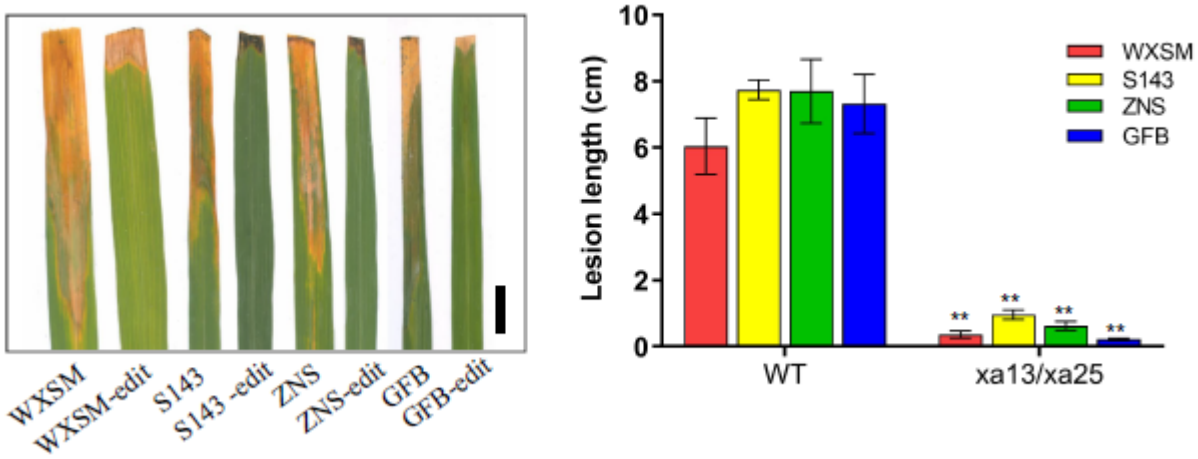

Figure 5. Phenotype of *xa13*, *xa25*, and *xa13*/*xa25* double mutants in the WXSM, S143, ZNS, and GFB plants infected with PXO99, and statistical analysis of the lesion lengths of these four cultivars after inoculation with PXO99 (n = 5 leaves). Data are presented as mean ± SD. Scale bar, 1 cm. *p* values were generated by means of Student's *t* test. Error bars, SEM (** $p < 0.01$).

4. Discussion

Rice bacterial blight is a serious disease that causes huge economic losses in global rice production. Breeding rice varieties resistant to bacterial blight is an important goal, but it faces challenges. Gene editing approaches in rice breeding for bacterial blight resistance in rice primarily rely on the utilization of single resistance genes or the knockout of a single susceptible gene [27,28]. However, single-gene resistance is susceptible to being overcome by the pathogen [29]. Therefore, combining or stacking multiple resistance genes in a rice variety is performed to enhance resistance stability and durability and reduce the pathogen's ability to adapt to resistance. This approach can generate progeny with multiple resistance genes, thereby improving resistance against bacterial blight. However, due to the complexity of genetic inheritance and gene interactions, as well as the labor-intensive nature of hybridization and stacking efforts, selecting suitable parents and implementing effective hybrid combinations for resistance remain technical challenges.

With the development of genomics and gene editing technology, opportunities have been created for the precision genetic improvement of crops. In particular, gene function research and genome editing technologies progressing rapidly. A large number of negative regulation genes involved in rice quality had been modified by genome engineering technologies. For instance, the knockout of *OsBADH2* using the TALEN technology produced fragrant rice, while a new glutinous rice variety was created by means of the targeted knockout of the *Waxy* gene using the CRISPR/Cas9 system [30]. Low-Cd-accumulating indica rice was generated by means of the CRISPR/Cas9-targeted mutagenesis of *OsNramp5* [31], while a reduction in seed chalkiness was achieved by editing *OsGS3* using CRISPR/Cas9, influencing the grain length–width ratio [32]. It is generally recognized that genome engineering technologies can be used to knock out negative regulatory genes efficiently for crop breeding.

5. Conclusions

In summary, we engineered a single Cas9/gRNA within a shared sequence to target both the *Xa13* and *Xa25* genes. Then, *xa13*, *xa25*, and *xa13*/*xa25* double mutants were simultaneously obtained in a high-quality elite rice YXYZ strain using *Agrobacterium*-mediated genetic transformation. As expected, the inoculation of leaves with PXO99 indicated that the *xa13*, *xa25*, and *xa13*/*xa25* double mutants displayed a markedly increased resistance to bacterial blight. In parallel, the mutants in the four other elite rice cultivars also displayed enhanced bacterial blight resistance. Taken together, these results provide

an efficient and potential strategy for developing improved rice varieties with bacterial blight resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14040800/s1. Figure S1. The CDS of *Xa 13* and *Xa 25* in varieties YXYZ. The 30-bp homologous sequence is indicated by Red boxes. Table S1. Genotypes of *xa13*/*xa25* T_0 mutant plants in five rice varieties. Table S2. List of primers used in this study.

Author Contributions: Original draft preparation, Y.Z. (Yiwang Zhu), X.Y. and P.L.; data curation, X.C., J.Y. and H.Q.; investigation, X.Z., F.M., M.F. and Y.F.; data curation, visualization, L.S., F.W. and Y.Z. (Yu Zhang). All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data is contained within the article or Supplementary Materials.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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