

Article

Development of Novel Rice Germplasm for Salt-Tolerance at Seedling Stage Using CRISPR-Cas9

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Abstract: Saline-alkali tolerant rice, as the first selected grain crop for improving coastal tidal land and saline-alkali land, has great potential for comprehensive utilization. In this study, an elite three-line restorer in breeding, R192, was taken as the receptor, and CRISPR/Cas9 technology was used to perform the directional editing of *OsRR22*, the main effect gene, which controls salt tolerance in rice. Eight transgenic plants of the T₀ generation with the *OsRR22* gene knockout were obtained, and the transgenic seedlings were screened by using PCR amplification and sequence comparison. The homozygous mutant lines, M16 and M18, with *OsRR22* knockout, which did not contain a transgenic vector skeleton, were identified in the T₂ generation. There were +1 bp and −20 bp in the exon regions of M16 and M18, respectively. At the three-leaf and one-heart stage, the seedlings were treated with 0.4% and 0.8% NaCl solution, and then their salt tolerance during the seedling stage was identified. The results showed that, without the salt treatment, no significant differences were found in plant height, the number of green leaves, total dry weight, and total fresh weight between the mutant lines M16 and M18 and the wildtype (WT) R192. However, after treatment with two different salt concentrations, the M16 and M18 mutants showed extremely significant differences in comparison with WT in plant height, the number of green leaves, total dry weight, and total fresh weight; between mutants and WT, there were significant differences in the number of green leaves, total fresh weight, and total dry weight after two salt treatments. Our results indicate the new germplasm with the *OsRR22* mutation induced by CRISPR/Cas9 technology could improve the salt tolerance of rice, providing a reference for the improvement of salt tolerance of rice.

Keywords: rice; salt tolerance; germplasm innovation; gene knockout; *OsRR22*



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1. Introduction

The area of saline-alkali land worldwide is around 950 million hm². Due to factors such as global warming, about 20% of irrigated farmland is salinized and alkalified. In China, the area of existing inland saline-alkali land is nearly 100 million hm², and the coastal tidal land is 2.34 million hm² [1]. Saline-alkali land is a rare reserve land resource in China, with huge potential for comprehensive utilization. Increasingly severe soil salinization and secondary salinization have lowered fertility, resulted in the destruction of the originally cultivated land resources, and caused agricultural production to face enormous risk, becoming worldwide ecological problems [2]. As arable land resources become increasingly scarce, how to transform saline-alkali land into high-yield fertile farmland has now

become one of the main research directions for agricultural scientists [3]. Rice, as the first selected grain crop for improving coastal and saline-alkali land, has tremendous potential for comprehensive utilization. Rice likes sweet soil and is a moderately salt-susceptible crop. Salt stress has detrimental effects on rice molecules, cells, as well as physiological and biochemical levels, resulting in a reduction in rice yield or death [4]. It is one of the significant actions to guarantee world food security via screening and innovating saline-alkali tolerant rice germplasm resources, studying the genetic and molecular mechanism of salt-alkali tolerance, cultivating novel rice varieties with saline-alkali-tolerant capacity, and then conducting promotion and application in large areas [5,6].

Numerous salt tolerance quantitative trait loci have been identified, and a few of them have been transferred into popular rice varieties via marker-assisted selection (MAS) [7–9]. During the past two decades, many salt-related genes (*SKC1*, *SNAC1*, *DST*, *OsRR22*, *OsSPL10*, *STRK1*, and *SOS1*) have been successfully cloned [10–16]. Currently, among the cloned salt-tolerant genes in rice, *OsRR22* is one of the most important negative regulatory genes for salt tolerance in rice; this gene encodes the B-type response regulatory protein composed of 696 amino acids, which is located on chromosome 1 of rice and is involved in cytokine signal transduction and metabolism. The deletion of this gene can lead to a significant increase in salt tolerance in rice [13]. Notwithstanding the tremendous advances that have been made in the salt-tolerant breeding of rice in China, there are still many problems to be solved, for instance, the study of salt-tolerant mechanisms in rice, few salt-tolerant genes that can be directly utilized, and the need for further breakthroughs in salt-tolerant breeding approaches and germplasm innovation research. This study expects to knock out the *OsRR22* gene using CRISPR/Cas9 technology and obtain a novel rice germplasm with higher salt tolerance, providing new materials for the cultivation of salt-tolerant rice varieties. In this study, the existing elite parents with excellent comprehensive traits were improved to create a novel salt-tolerant rice germplasm by using up-to-date gene-editing technology, which accelerates the process of salt-tolerant rice breeding.

2. Materials and Methods

2.1. Plant Growth Conditions

The elite indica rice cultivar R192 was bred in our laboratory. All transgenic plants and R192 (wildtype, WT) were grown in the greenhouse at 28–35 °C or in fields at the station of the Hubei Academy of Agricultural Sciences in Wuchang under normal growth conditions. For salinity stress at the seedling stage, seedlings of rice were cultivated in normal nutrient solution for two weeks after germination on a 96-well plate. They were placed in a growth chamber (14 h of daytime at 30 °C and 10 h at night at 25 °C with 70% relative humidity) [13].

2.2. Vector Construction

The Cas9 plant expression vector (pYLCRISPR/Cas9Pubi-H) and the sgRNA expression vector (pYLgRNA) were provided by Prof. Yao-Guang Liu of South China Agricultural University (Figure 1). The Cas9-*OsRR22*-gRNA expressing vector was constructed following the previously described protocol [17]. Briefly, according to the design principles of the target sequences of the CRISPR/Cas9 system, 19 to 20 bases upstream of the protospacer adjacent motif (PAM) were selected as a candidate target sequence (Figure 1).

In this study, *Agrobacterium* was used to infect the callus of mature rice embryos, with hygromycin as the selection marker. The green regenerated plants were obtained from transformed callus grown on the induction medium. Then, further analysis was conducted.

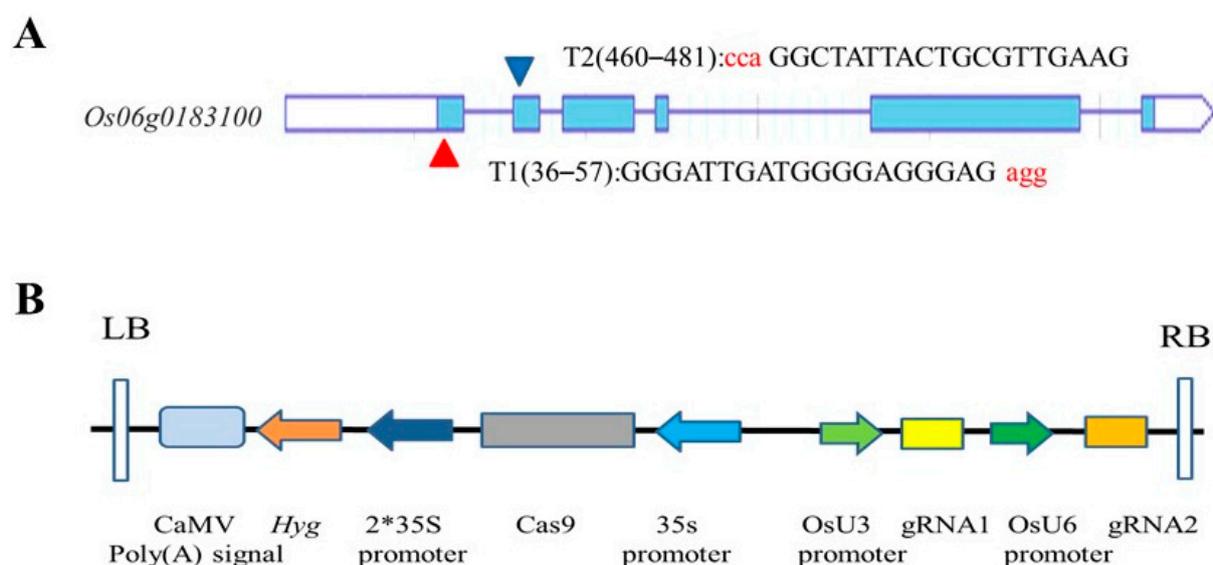


Figure 1. The site of the target sequence and schematic diagram of the cas9-OsRR22 vector. (A). Schematic diagram of the *OsRR22* (Os06g0183100) gene structure and gRNA targets. (B). Schematic diagram of the cas9-OsRR22 expression vector. LB, vector left border; RB, vector right border; Hyg, the resistance gene of hygromycin; OsU3 promoter, rice U3 promoter; OsU6 promoter, rice U6 promoter; 2*35S promoter, two CaMV35S promoter; gRNA scaffold, guide RNA scaffold; Cas9, the gene of Cas9.

2.3. Detection and Identification of Transgenic Plants

To identify T-DNA-free plants from T₁, the plants were analyzed via PCR using *OsRR22*-specific and Cas9-specific primers (Table 1) in combination with agarose gel electrophoresis. The pYLCRISPR/Cas9Pubi-H plasmids and the T₀ transgenic plants were selected as positive controls, and R192 DNA and H₂O were used as negative controls. HygR- and Cas9-negative plants were considered the T-DNA-free plants.

Table 1. Primers used in this study.

Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Purpose
RR22-Y	cagtGGTCTCaggcagggattgatggggagggag	cagtGGTCTCaaaacctccctcccatcaatccc	Vector construct
RR22-B	cagtGGTCTCatgtgcttcaacgcagtaaatgcc	cagtGGTCTCaaaacgcttactgctggtgaag	Vector construct
RR22-1	TCTTGCTGAGAAATGGCTCGAT	AGTTGAGCATCATGGATAGCTGA	Target site sequencing
RR22-2	TGCTTCTGCTTGCAATTGTTCA	TCTGAACTACTGATGACTGGGAGA	Target site sequencing
Cas9	GAACGGTCGTAAGAGGATGCTG	GGTGATGGACTGGTGGATGAGA	Transgenic analysis
HPT	GCTCCATACAAGCCAACCACG	CCTGCTGAAACCGAACTGC	Transgenic analysis

A pair of dominant specific primers with a length of 449 bp was designed in accordance with the known Cas9 sequence, and the agarose detection was performed after PCR; two pairs of specific primers were designed for the two target sites. The primer sequences are shown in Table 1. Sequencing for the samples was performed after PCR amplification. PCR amplification system: The 15 µL reaction system selected for molecular marker amplification included 11.5 µL ddH₂O, 1.5 µL 10 × PCR Buffer (containing Mg²⁺), 0.4 µL (10 mM) dNTP, 0.2 µL (50 µM) of each forward and reverse primer, 0.2 µL Taq enzyme, and 1 µL DNA template (50 ng/µL). The amplification process was as follows: predenaturation at 95 °C for 33 min; denaturation at 94 °C for 30 s, denaturation at 55 °C for 30 s, and extension at 72 °C for 40 s, with 35 cycles in total.

2.4. Identification and Evaluation of Salt Tolerance for Mutant Plants

To evaluate the salt tolerance of plants at the seedling stage, we carried out a salt stress experiment according to the approach presented in Takagi et al. (2015). Seedlings were placed in an artificial climate box to grow. We compared the salt tolerance of two-week-old WT and homozygous mutant plants. Fresh groundwater, 0.4% NaCl, and 0.8% NaCl (pH = 7) were used to treat the two-week-old plants, respectively. After the two-week treatment, the salt tolerance was tested by measuring the plant height, fresh weight per plant, and dry weight per plant. Ten plants were selected from each material. The experiment was repeated five times.

2.5. Data Analysis

Excel and SPSS statistical analysis software were used to analyze all data.

3. Results and Analysis

3.1. Construction of the Recombinant Expression Vector of the *OsRR22* Gene in Rice

The complete nucleotide sequences of the *OsRR22* gene (Os06g0183100) were found via the NCBI database, and the two target sites for specific primers were designed in the exon region (Figure 1). The kit was used to quickly and easily insert the gRNA target sequence into the Cas9/gRNA plasmid. The constructed Cas9/gRNA plasmid could simultaneously express the plant codon-optimized Cas9 protein and gRNA. The knockout and editing of the target gene were performed using CRISPR technology. The information of the constructed vector and specific insertion position is shown in Figure 1 (Wuhan Biorun Biotechnology Co., Ltd., Wuhan, China).

3.2. Acquisition of Transgenic Seedlings and Identification of Homozygotes

In the T₀ generation, eight individual plants with editing positive at the target site were obtained (Figure 2). In the spring of 2019, the T₁ generation lines were planted in Hainan. PCR detection and sequencing were carried out. In total, 104 individuals without transgenic vector sequences (T-DNA free) were screened from 349 T₁ plants, which were derived from seven T₀ families (one T₀ individual plant was sterile). Then, homozygous individual plants were screened by target site examination. The results showed that the mutations were generated at the target site 1 of all eight lines. The analysis of mutation types and mutation genotypes of the T₀ generation was as follows: from the mutation genotypes, heterozygous mutations, homozygous mutations, and bivalent mutations accounted for 50%, 12.5%, and 37.5%, respectively; from the mutation types, the proportion of deletion and insertion were 62.5% and 12.5%, respectively (see Table 2).

3.3. Salt Tolerance of Rice *OsRR22* Gene Mutant Lines

To evaluate the salt tolerance phenotype of the line with the *OsRR22* knockout, two T₂ generation lines of homozygous mutants, M16 and M18, were selected in this study and treated with 0.4% and 0.8% NaCl at the two-week-old seedling stage. Compared with the wildtype (WT), the growth phenotypes of the two mutant lines under the stress of different salt concentrations were better (see Figure 3).

After being treated with 0.4% NaCl for two weeks, compared with those without NaCl treatment, the changes in plant height, the mean number of green leaves, total fresh weight, and total dry weight of WT, M16, and M18 were as follows: the plant height of WT decreased by 32.8%, while the height of M16 and M18 reduced by 29.2 and 23.1%, respectively; the mean number of green leaves of WT declined by 68.7%, whereas that of M16 and M18 dropped by 28.2 and 35.5%, respectively; the total fresh weight of WT decreased by 70.1%, yet, that of M16 and M18 decreased by 39.7 and 39.5%, respectively; the total dry weight of WT decreased by 56.1%, while that of M16 and M18 decreased by 29.6 and 26.3%, respectively (see Figure 4).

A		Sequence		
Line	Target 1		Type	
#4	Allele1:	GGAGAGGAAGGGATTGATGGGGAGG-AGAGGGATCAATTCCC	d1	
	Allele2:	GGAGAGGAAGGGATTGATGGG-AGG-AGAGGGATCAATTCCC	d2	
#5	Allele1:	GGAGAGGAAGGGATTGAT-----GGAGAGGGATCAATTCCC	d6	
	Allele2:	GGAGAGGAAGGGATTGATGGGGAGGGGAGAGGGATCAATTCCC	i1	
#6	Allele1:	GGAGAGGAAGGGATTGATGGGGAGGGAGAGGGATCAATTCCC	WT	
	Allele2:	GGAGAGGAAGGGATTGATGGGGAGG-AGAGGGATCAATTCCC	d1	
#9	Allele1:	GGAGAGGAAGGGATTGATGGGGAGGGAGAGGGATCAATTCCC	WT	
	Allele2:	GGAGAGGAAGGGATTGATGGGGAGGGGAGAGGGATCAATTCCC	i1	
#10	Allele1/2:	GGAGAGGA-----AGGGATCAATTCCC	d20	
#11	Allele1:	GGAGAGGAAGGGATTGATGGGGAGGGAGAGGGATCAATTCCC	WT	
	Allele2:	GGAGAGGAAGGGATTGAT-----	d35	
	Ref:	GGAGAGGAAGGGATTGATGGGGAGGGAGAGGGATCAATTCCC		
Line	Target 2		Type	
#13	Allele1:	TAACATCAACCAACCAGGCTA---CTGCGTTGAAGCTGCTCAG	d3	
	Allele2:	TAACATCAACCAACCAGG-----CTGCGTTGAAGCTGCTCAG	d6	
#14	Allele1:	TAACATCAACCAACCAGGCTATTACTGCGTTGAAGCTGCTCAG	WT	
	Allele2:	TAACATCAACCAACCAGGC-A-TACTGCGTTGAAGCTGCTCAG	d2	
	Ref:	TAACATCAACCAACCAGGCTATTACTGCGTTGAAGCTGCTCAG		
B		Sequence		
Line	Target1	Target2	Type	
WT	GAAGGGATTGATGGGGAGGGAGAGG...CCAGGCTATTACTGCGTTGAAG		WT	
M16	GAAGGGATTGATGGGGAGG-AGAGG...CCAGGCTATTACTGCGTTGAAG		d1	
M18	GAA-----AGG...CCAGGCTATTACTGCGTTGAAG		d20	

Figure 2. Comparison of the editing target sequences between T₀ and T₂ transgenic plants. (A) Comparison of the editing target sequences between T₀ transgenic lines and WT. (B) Comparison of the editing target sequences between T₂ homozygous transgenic lines and WT. The targeted sequences are highlighted with underline and the PAM sequences in shadow. The deleted sequences are shown by black hyphens, and the red font represents the insertion bases. i: insertion; d: deletion; WT: wildtype; #4 to #14: T₀ transgenic lines; M16, M18: T₂ homozygous knockout lines with T-DNA-free.

Table 2. Ratios of mutant genotype and mutation type at target site 1 in T₀ mutant plants.

Proportion of Mutant Genotypes			Proportion of Mutation Types	
Bivalent mutation	Heterozygote	Homozygote	Deletion	Insertion
37.5 (3/8)	50 (4/8)	12.5 (1/8)	62.5 (10/16)	12.5 (2/16)

After two weeks of treatment with 0.8% NaCl, compared with those without NaCl treatment, the plant height of the WT decreased by 32.7%, while the height of M16 and M18 decreased by 32.3 and 22.6%, respectively; the mean number of green leaves of the WT decreased by 90.7%; yet, that of M16 and M18 decreased by 51.5 and 53.9%, respectively; the total fresh weight of WT fell by 79.0%, whereas that of M16 and M18 declined by 60.0

and 48.5%, respectively; the total dry weight of WT was reduced by 59.5%, while that of M16 and M18 decreased by 36.6% and 21.1%, respectively (see Figure 4).

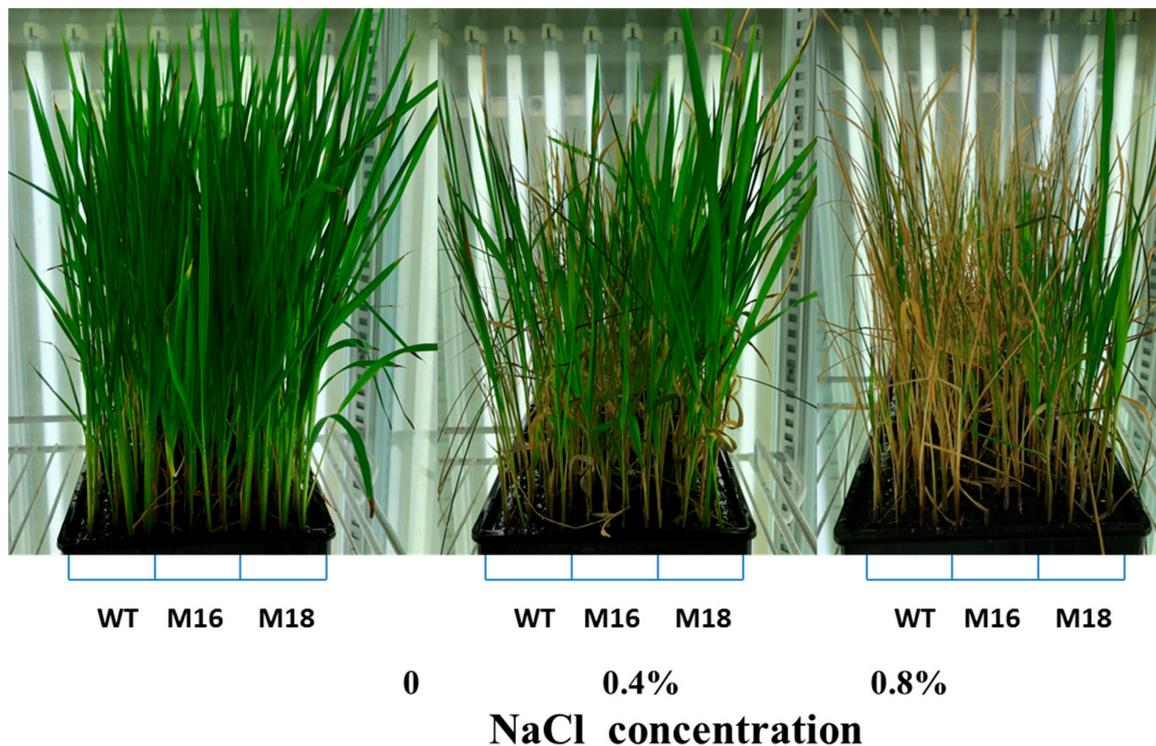


Figure 3. Phenotypes of WT, mutant M16, and mutant M18 before and after seedling salt treatment at seedling stage (Left: before treatment; Middle: after 0.4% NaCl treatment; Right: after 0.8% NaCl treatment).

According to the analysis of the significance of the difference, without the salt stress treatment, M16 and M18 had no significant differences relative to the WT in plant height, the number of green leaves, total dry weight, and total fresh weight. However, after treatment with two different salt concentrations, M16 and M18 had highly significant differences in comparison with WT in plant height, the number of green leaves, total dry weight, and total fresh weight; the difference in plant height between the mutants and WT under two doses of salt stress was not significant, but the difference in the number of green leaves, total fresh weight, and total dry weight were significant. These results indicate that the salt tolerance of the *OsRR22* gene mutants induced by CRISPR/Cas9 technology was enhanced.

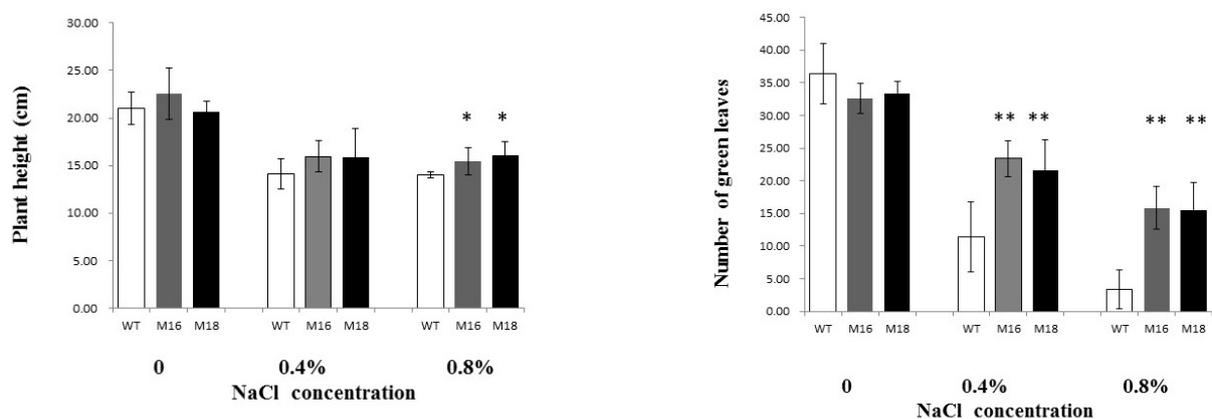


Figure 4. Cont.

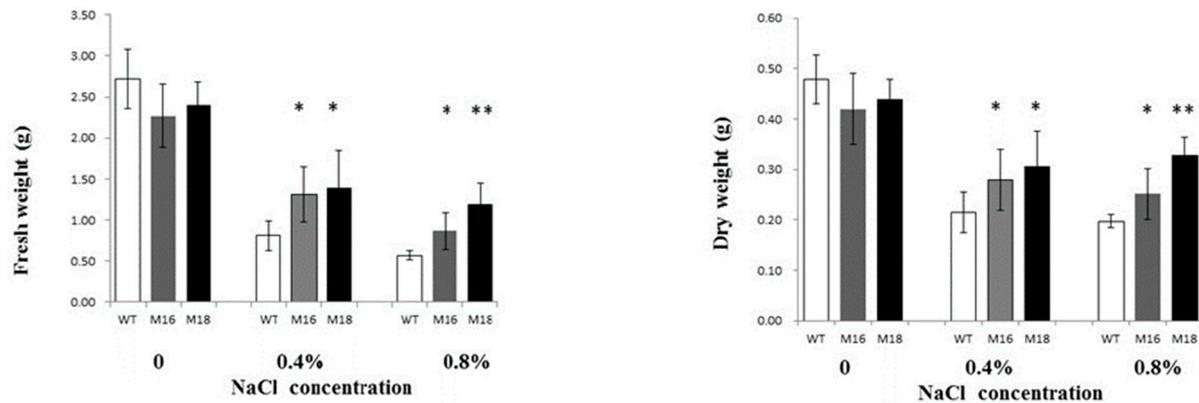


Figure 4. Comparison of plant height, the number of green leaves, total fresh weight, and total dry weight among WT, M16, and M18 after salt treatment at seedling stage. Asterisks indicate significant differences to WT (* $p < 0.01$, ** $p < 0.01$).

4. Discussion

4.1. Creation of Novel Salt-Tolerant Rice Germplasm with *OsRR22* Knockout

CRISPR/Cas9 is a new targeted gene-editing technology with high efficiency and high specificity. To date, CRISPR/Cas9 has been widely applied to the genetic improvement of major crops such as rice, maize, oilseed rape, and soybeans [18]. Nevertheless, there are few studies on gene-targeted editing of elite parents in production via CRISPR/Cas9 technology. In 2020, the *Waxy* allele of 12 superior inbred lines was edited by CRISPR-Cas9 to create hybrids of waxy corn, a process that was more than a year faster than traditional trait introgression via backcross and marker-assisted selection. Field trials in 25 sites showed that CRISPR-waxy hybrids were superior to introgressed hybrids in agronomic traits, with a mean yield increase of 5.5 bushels per acre [19]. The *Badh2* gene was knocked out by CRISPR/CAS9 technology in japonica rice cultivar Zhonghua 11 to obtain the fragrant rice cultivar [20]. The *TMS5* gene was knocked out from an elite indica variety, Zhongjiazao 17, and the temperature/photoperiod sensitive male-sterile indica rice line YK17S was cultivated successfully [21].

R192, a three-line indica restorer line of WA cytoplasmic male sterility, was selected by the Food Crops Institute of Hubei Academy of Agricultural Sciences using many parents and crosses, with many advantages including superior quality, high yield, and disease resistance. This study took the parent R192, an elite restorer line, as the material, performed the targeted editing of the *OsRR22* gene related to salt tolerance by using the CRISPR/Cas9 approach, and obtained a homozygous mutant line without transgenic components. According to the experimental study that identified salt tolerance at the seedling stage, the plant height, the number of green leaves, total dry weight, and total fresh weight of two mutant lines, M16 and M18, showed highly significant differences relative to those of the WT. This study indicated that the salt tolerance of the novel *OsRR22* gene mutant germplasm induced by CRISPR/Cas9 technology was enhanced, which provided a new idea for the improvement of salt tolerance in rice.

4.2. Research on the Physiological Mechanism of Salt Tolerance in Rice

Salt stress mainly produces two kinds of stress effects on rice, namely ion stress and osmotic stress [22]. Ion stress results in the enrichment of intracellular sodium ions as well as inhibition of the uptake of potassium and calcium ions, resulting in electrolyte disorder in rice cells and retardation of rice growth [23]. The osmotic stress effect can make it difficult for rice to absorb water from the outside and cause the formation of reactive oxygen species in plant tissues [24]. Reactive oxygen species usually include singlet oxygen, hydrogen peroxide, superoxide, and other toxic substances, which can damage the cell membrane and destroy whole plant growth [25,26]. At the same time, salt stress produces the accumulation of osmotic substance proline and lipid peroxidation, which inhibits plant

growth [27,28]. It has also been reported that rice plant growth, photosynthetic pigment, and enzyme activities are affected under salt stress [29]. The physiological function of the *OsRR22* gene was studied after obtaining the *hst1* mutant of the *OsRR22* gene at an earlier stage [30]. Under salt stress, the biomass and growth rate of all rice plants decreased, but the descent degree of the *hst1* mutant was less than that of the wildtype; the K^+/Na^+ ratio of the *hst1* mutant under salt stress was higher than that of wildtype; compared with the control, the expression quantity of antioxidant genes including *OsAPX1*, *OsAPX2*, *OsCATC*, and *OsSODA* in *hst1* mutant was significantly enhanced. In terms of vegetative growth (growth rate and amount of growth), photosynthesis, antioxidant enzyme activity, water retention capacity, cell leakage, ion content, organic osmotic solute, and lipid peroxidation, the properties of the *hst1* mutant were better than the wildtype. In particular, in the steady state level of the K^+/Na^+ ratio, the *hst1* mutant showed a significant advantage, which indicates that the *hst1* mutant may improve its salt tolerance via adjusting the ion pathway.

In this study, we also identified the salt tolerance of M16 and M18 during the sprouting stage and found that there was no significant difference in salt tolerance between these two mutants and their recipient parent R192, a result agreeing with previous studies, namely, no correlation in salt tolerance between the sprouting stage and the seedling stage [31]. According to the results of previous studies, the salt tolerance of rice varieties at the seedling stage was improved by knocking out the *OsRR22* gene, which is consistent with the results of this study. For the novel saline-tolerant germplasm, M16 and M18, obtained by improving the elite restorer line, this research provides a theoretical basis and data reference for their promotion and application in saline-alkali land.

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