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# Identification and Validation a Major QTL from "Sea Rice 86" Seedlings Conferred Salt Tolerance

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**Abstract:** Saline stress severely affects rice (*Oryza sativa* L.) growth and development and reduces crop yield. Therefore, developing salt-tolerant and high-yielding rice using quantitative trait loci (QTLs) and linkage markers is a priority for molecular breeding. Here, the *indica* rice Sea Rice 86 (SR86) seedlings showed higher tolerance than ordinary rice varieties in saline soil, and a dominant effect on salinity sensitivity was demonstrated by genetic analysis. We constructed bulked segregant analysis pools using  $F_2$  populations from parents Dianjingyou 1 as the recipient and SR86 as the donor. We identified a 2.78 Mb region on chromosome 1 as the candidate region. Using simple sequence repeat markers and substitution analysis, we mapped the target region within 5.49 cM in the vicinity of markers RM8904–RM493. We speculated that this QTL, named *qST1.1*, might contribute significantly to the salt tolerance of SR86. The high salt tolerance of introgression lines obtained by marker assistant selection (MAS) confirmed that the *qST1.1* region was associated with salinity tolerance. This newly-discovered QTL will be helpful for the analysis of the salt-tolerant mechanism of rice and breeding high-quality rice varieties using MAS.

Keywords: Sea Rice 86; salt tolerance; bulked segregant analysis; QTL mapping; qST1.1

# 1. Introduction

Salinity is one of the major abiotic stresses that adversely effects plant growth and development, constraining crop yield [1,2]. At least 6% of the world's land is affected by salinity [3], and the yield of many rice varieties can be reduced by up to 50% in response to 50 mM NaCl. Hence, efforts to increase the salt tolerance of rice would aid in increasing the use of marginal saline-alkaline land and improving crop production [4,5]. Rice is considered as most susceptible to salt stress in the seedling, reproductive, and 2–3 leaf stages [6]. Previous investigations clarified that salinity influences rice growth in three phases: osmotic stress, ionic toxicity, and oxidative stress; and salt tolerance is a comprehensive quantitative trait involving both genetic characteristics and physiological responses [7–9].

Emerging new technologies, combining high-throughput sequencing technology with traditional technologies such as RNA-sequencing (RNA-seq), alternative splicing analysis, mapping by sequencing (MBS), bulked segregant analysis (BSA), whole-genome resequencing, and quantitative trait locus (QTL) mapping, have facilitated the analysis of similarly complex characters [10]. Marker assisted selection (MAS) based on QTL location is a generally accepted technique for breeding new salt-tolerant rice varieties [11]. However, it is difficult to assess salt tolerance by evaluating physiological traits. A

great number of QTLs have been reported based on various indicators. Gregorio et al. reported a QTL "Saltol" flanked by simple sequence repeat (SSR) RM1287 and RM6711 in the 10.7 to 16.4 Mb region on chromosome 1, using a population generated from a cross between susceptible variety IR29 and the tolerant landrace Pokkali. The QTL contributed to more than 70% of the variation in salt uptake and was successively transferred to other rice varieties [12]. Thomson et al. also found some QTL segments related to salt tolerance in this region [13]. Several QTLs related to survival days after salt treatment, where the logarithm of odds (LOD) value of *q1* reached 11.55, were reported by Gong et al. [14]. A specific QTL, qSKC-1 with 11.74 LOD value, was identified in populations derived from crossing Nona Bokra and Koshihikari, and the Na<sup>+</sup> transporter gene SKC1 was found [15,16]. Lee et al. mapped two QTLs (qST-1 and qST-3) that conferred salt tolerance at the young seedling stage [17]. Fourteen QTLs with a LOD value above 10, based on the traits of standard tolerance ranking, shoot dry mass, shoot  $Na^+$  content, shoot  $K^+$  content, and  $Na^+/K^+$  ratio, were reported [18]. A further study obtained three QTLs (qRRW7, qRRW10, and qRTW10) that explained more than 20% of the total phenotypic variances [19]. Gimhani et al. reported 83 QTLs by measuring the shoot Na<sup>+</sup>/K<sup>+</sup> ratio, shoot/root Na<sup>+</sup> concentration, and tissue fresh/dry weight among other parameters [20]. Similarly, 85 QTLs with 187 recombinant inbred lines (RILs) crossed from Pokkali and Bengal were reported by De Leon et al. [21]. QTLs for traits such as chlorophyll contents, shoot length, or root length, were also identified using introgression lines from crossing Nonabokra and Jupiter [11]. Across numerous reports, the first six chromosomes contributed strongly towards the salinity tolerance of rice [22].

Although extensive studies on QTL mapping for salinity tolerance of rice have been conducted, few QTLs that could be effectively applied in practical breeding were validated. It remains of great value to identify salt-tolerant QTLs or related genes in elite germplasm resources and apply them to breed new salt-tolerant varieties. SR86 is an important salt-tolerant rice, but its tolerance mechanism is still unclear and there are few reported studies of SR86. Chen et al. performed transcriptome analysis of SR86 grown under normal and highly saline conditions and identified a large number of salt-induced differentially expressed genes; they classified these genes as pentatricopeptide repeat (PPR) family, peroxidase genes, dirigent genes, multi-antimicrobial extrusion (MATE) protein family genes, glutathione S-transferases (GST) genes, NB-ARC and NBS-LRR gene families, and the kinesin motor domain containing gene family [23]. A total of 51 loci significantly correlated with salt stress were found by genome-wide correlation analysis of various traits from SR86 grown under different salt concentrations [24].

In this study, we first used the salt-tolerant germplasm SR86 to cross with salt-sensitive *japonica* variety DJY1 to generate  $F_1$  and  $F_2$  populations, and cross with Yunnan *japonica* rice to generate introgression lines, respectively. By combining BSA analysis and QTL mapping, a QTL (*qST1.1*) was found to be significantly associated with the salt tolerance of SR86. To assess the effectiveness of the salt tolerance *qST1.1*, substitution experiments among the BC<sub>2</sub>F<sub>3</sub> populations were carried out. The results showed that the methods we used in this study were able to provide a new and effective way to select salt-tolerant rice varieties. Furthermore, our identification of *qST1.1* enabled analysis of the major salt-tolerant gene of SR86. Finally, by investigating some yield characteristics of the introgression lines (ILs), we found that the ILs not only showed high salt tolerance, but also had high yield and high quality, and thus might provide potential resources for rice breeding.

#### 2. Materials and Methods

#### 2.1. Plant Materials

Sea Rice 86 (SR86) is an *indica* rice germplasm with the ability to grow in saline-alkaline and infertile soil, and was first found at Zhanjiang, Guangdong, China in 1986 by Chen et al. [23]. Dianjingyou1 (DJY1), Yunjing 26 (YJ26), Yunjing 29 (YJ29), and Yunjing 32 (YJ32) are *japonica* rice varieties developed by the *Japonica* Rice Breeding Center of Yunnan Academy of Agricultural Sciences, and all of them are salt-sensitive. The populations  $F_1$  and  $F_2$  used in this study were derived from a cross between recipient

DJY1 and donor SR86; the overlapped segment substitution lines (BC<sub>2</sub>F<sub>3</sub>) were derived from a cross between recipient (DJY1, YJ26, YJ29, YJ32) and donor SR86. The introgression line 2018H3T22 (T22) was from BC<sub>2</sub>F<sub>5</sub> and was homozygous for the SR86 genotype of *qST1.1*. T22 was bred as follows: BC<sub>2</sub>F<sub>3</sub> plants (SR86 × YJ26) with good salt tolerance were transplanted to the field; some of the plants in BC<sub>2</sub>F<sub>4</sub> (collected from BC<sub>2</sub>F<sub>3</sub>) were tested for salt tolerance and used for genetic linkage analysis with SSR markers; the plants that had good salt tolerance and were homozygous for the SR86 genotype of *qST1.1* were transplanted; and the BC<sub>2</sub>F<sub>5</sub> plants contained the homozygous target fragment were served as the introgression line. Both hybridization and propagation were carried out at Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, located in Menglun, Jinghong, Yunnan Province, China, with planting twice per year.

#### 2.2. Phenotype Measurements

Phenotype identification of all the populations and the two parents was conducted in greenhouses (natural illumination, 25–28 °C). Seeds were germinated at 30 °C. After germination, they were grown in plastic seedling pots with 2 cm plant spacing and normal seedling management (red soil: organic matter: coconut bran = 1:2:1). When the plants grew to the trifoliate stage, the soil was dried under natural conditions (but without drought stress), then treated with 1.2% NaCl (w/v) solution and the water level was kept at 1 cm depth above the soil during the treatment. Finally, the phenotype of the populations and parents were identified when they were significantly different in appearance (after 8–10 d of salt treatment, usually). According to the salt damage degree classification standard developed by the International Rice Research Institute (IRRI) (Table S1), all the lines or individuals of populations were assigned into five grades as 1, 3, 5, 7, and 9 corresponding to resistance levels: extremely resistant, medium resistant, medium sensitive, and sensitive, respectively.

#### 2.3. DNA-BSA Analysis

Plants of the F<sub>2</sub> generation and the parents SR86 and DJY1 were treated with 1.2% NaCl solution at the trifoliate stage. The healthy leaves of 100 individuals in F<sub>2</sub> belonging to grade 7 or 9 at day 8, 100 individuals belonging to grade 1 or 3 at day 12, and the two parents were collected and quickly frozen with liquid nitrogen then stored at -80 °C. The genomic DNA of all samples was extracted using the cetyltrimethylammonium ammonium bromide (CTAB) method [25]. Four mixed DNA pools (R01–DJY1, R02–SR86, R03–salt-tolerant pool, R04–salt-sensitive pool) were constructed for bulked segregant analysis (BSA). The BSA sequence and analysis were performed by Biomarker Biotechnology Co., Ltd. (Beijing, China).

#### 2.4. Genotype Identification and QTL Analysis

Genomic DNA was extracted from fresh leaves as described above. Primers of SSR markers were selected from those reported by McCouch et al. and Ganie et al. [26,27]. A total of 36 SSR markers covering the region 7.9–12.26 Mb on chromosome 1 were used for testing parental polymorphisms and mapping genotypes; 9 markers with polymorphisms among the population were screened to proceed the QTL mapping using 186  $F_2$  (SR86 × DJY1) individuals (Table S2). PCR reactions (20 µL) contained: 10 µL 2 × Taq Master Mix (Novoprotein Scientific Inc.), 1 µL forward primer (10 µmol/µL), 1 µL reverse primer (10 µmol/µL), 2 µL DNA samples, and 6 µL ddH<sub>2</sub>O. The PCR reactions involved: 95 °C for 5 mins, and then 26 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by 72 °C for 2 mins. The PCR products were detected by polyacrylamide gel electrophoresis (PAGE) and stained with silver.

#### 2.5. Measurements of Agronomic Traits

Normal sowing and germinating were done for seeds, and when the seedlings grew to four leaves stage, the seedlings were transplanted to the field with six rows per plot and  $25 \times 20$  cm between plants. The agronomic traits of the plants in the middle of the middle rows were measured when plants grew to maturity under normal field management. At least 10 individuals of each line were randomly

selected for measurement. Plant height (cm) (awns excluded), the panicle length(cm), number of filled grains per panicle, number of primary branches, number of secondary branches, effective tiller number, 1000-grain weight (g), grain length (mm), grain width (mm), grain length-to-width ratio, and grain yield per plant (g) were measured with units per plant and averaged. The experiments were conducted three times independently.

## 2.6. Statistical Analysis

QTL IciMapping software (version 3.2) was used to construct a genetic linkage map based on the genotypes and phenotypes. SPSS (version 18) in International Business Machines (IBM) was used to analyze data of agronomic traits in the introgression lines and two parents. Student's t-tests were used for analysis, and the threshold of p < 0.05 was considered as statistically significant, and values of p < 0.01 as highly significant.

# 3. Results

## 3.1. Phenotypes and Genetics Analysis of Salt Tolerance in SR86

SR86 is a popular variety for salt-tolerant rice breeding. We compared the salt tolerance of SR86 with four common rice varieties grown in saline soil. SR86 showed higher salt tolerance compared with DJY1, YJ26, YJ29, and YJ32 under increasing concentrations of NaCl in the soil (Table 1 and Table S1). SR86 was classified as salt resistant and graded as 1–3 with survival for more than 15 d, while the other varieties survived less than 10 d during the trifoliate stage in soil with 1%, 1.2%, and 1.5% NaCl. The significant difference of the phenotypes was clearly visible, and we chose 1.2% NaCl for further analysis. We also performed the above-mentioned experiments with the same treatments under hydroponic conditions; surprisingly, there was no difference between SR86 and the other varieties (Figure 1A and Figure S1), which might imply a different mechanism of salt tolerance between growth in soil and in hydroponic solutions.

To carry out genetic analysis of salt tolerance in SR86, we hybridized SR86 with salt-sensitive variety DJY1 to obtain an  $F_1$  population. The  $F_1$  generation showed stronger salt tolerance than DJY1 after 8 d under 1.2% NaCl treatment (Figure 1A), and we speculated that the salt tolerance of SR86 was effectively dominant.

To further assess the characteristics of phenotypical variation in salt tolerance of SR86, the phenotype classification was performed in the  $F_2$  population according to the degree of salt damage under 1.2% NaCl treatment. The  $F_2$  population showed different degrees of damage among 893 individuals after salt treatment for 8 d and there were significantly more salt-tolerant plants (448 individuals scored as grades 1–3) than salt-sensitive plants (166 individuals scored as grades 7–9). Thus, the salt tolerance showed dominance or partial dominance, which was consistent with the  $F_1$  population phenotype (Figure 1B, C and Table S3).

	S	oil Condi	tions (8 d	)				Hydropo	nic Condi	itions (4 d	.)
Variety/NaCl Concentration (%)	0	0.6	0.8	1	1.2	1.5	0	0.4	0.6	0.8	1
SR86	1	1–3	1–3	1–3	1–3	3–5	1	3–5	5–7	7–9	9
DJY1	1	1–3	5–7	7–9	9	9	1	3	5	7–9	9
YJ26	1	1–3	5–7	7–9	9	9	1	3	5	7–9	9
YJ29	1	1–3	5–7	7–9	9	9	1	3	5	7–9	9
YJ32	1	1–3	5-7	7–9	9	9	1	3	5	7–9	9

Table 1. The grades of different rice varieties under soil and hydroponic conditions.

The grade was evaluated according to the salt tolerance on the relative salt damage rate, see Table S1 for detailed grading standards.



**Figure 1.** The phenotype and genetic analysis of SR86 under salt stress. (**A**) The phenotype of SR86, DJY1, and  $F_1$  after being treated with 1.2% NaCl solution for 8 d. (**B**) The phenotype classification of  $F_2$  population according to the degree of salt damage; five grades, 1–extremely resistant, 3–resistant, 5–medium resistant, 7–medium sensitive, and 9–sensitive. (**C**) The phenotypic frequency statistics of  $F_2$  population under 1.2% NaCl treatment.

# 3.2. A New Potential QTL (qST1.1) Involved in the Salt Tolerance of SR86

We selected individuals with extreme phenotypes as two mixed pools (salt-sensitive pool and salt-tolerant pool) from the  $F_2$  population for BSA. Four mixed DNA pools (R01–parent DJY1, R02–parent SR86, R03–salt-tolerant pool, R04–salt-sensitive pool) were constructed and used for DNA sequencing. A total of 71.07 Gbp clean data were filtered from the raw data: quantification of data showed Q20  $\geq$  91.87% and Q30  $\geq$  85.7% and the GC content was 41.91%–43.43% across all four mixed samples. The reading depth of both parental pools, R01 and R02, was 13 × because of the small number of samples, while the reading depths of the two offspring pools, R03 and R04, were 72 × and 63 × , respectively (Tables S4 and S5). When the pooled samples were aligned to the reference genome, we identified respectively in the four pools (R01–R04) about 0.27 million, 2.10 million, 2.23 million, and 2.22 million single nucleotide polymorphisms (SNPs); more than 7500, 43,800, 47,300, and 47,300 indels in genome; 9228 genes, 37,395 genes, 38,923 genes, and 38,890 genes with non-synonymous SNPs; and 3023 genes, 15,223 genes, 16,225 genes, and 16,226 genes with indels (Tables S6–S8). The  $\triangle$ SNP-index

between R03 and R04 was calculated and visualized (Figure 2A and Figure S2). Two adjacent intervals were found on chromosome 1 that exceeded the threshold value 0.99 (Figure 2A and Table 2). Since the distance between the two sections was short, we considered these two sections as one candidate region at around 9.46–12.24 Mb, which contained 448 open reading frames (ORFs). This result indicated that the genes responsible for salt tolerance in SR86 might be located within this 2.78 Mb region on chromosome 1.



**Figure 2.** The results of quantitative trait locus (QTL) mapping and bulked segregant analysis (BSA). (**A**) The results of BSA. (**B**) The results of QTL mapping in F<sub>2</sub> population. (**C**) Verification of homozygous recombinants in introgression lines (ILs). &, \*, and # represent the plant number, genetic position (cM) of markers on the chromosome, and phenotype, respectively. W386 was crossed from SR86 and DJY1; W401 was crossed from SR86 and YJ26; W402 was crossed from SR86 and YJ29; W404 was crossed from SR86 and YJ26; SR86 was the donor.

Chromosome ID	Start	End	Size (Mb)	Gene Number
Chr1	9,459,487	12,134,056	2.67	432
Chr1	12,211,515	12,244,801	0.03	5
Total	-	-	2.70	437

Table 2. Results of associated region by BSA.

Start: the start position of the associated region; End: the end position of the associated region; Size: the size of associated region (Mb); Gene number: the number of genes in the associated region.

To further narrow the candidate region, 36 SSR markers were selected to screen for polymorphism in the region of 7.45–12.39 Mb. Among them, nine SSR markers that showed polymorphism were used to perform genetic linkage analysis and QTL mapping of 186 individuals in the  $F_2$  population, with the following parameters: SR86 was R (salt-resistant), DJY1 was S (salt-sensitive); grades 1 and 3 were assigned as R, grades 7 and 9 were assigned as S. From the analysis, a QTL was identified within a 5.49 cM region flanked by markers RM8094 and RM493 and extending from 11.24 to 12.26 Mb on chromosome 1. The salt tolerant-related region was designated as *qST1.1* and showed a LOD score of 14.8014 (Figure 2B); the phenotypic variance explained (PVE) by this QTL was 62.6%, which suggested that *qST1.1* might play a key role in salt tolerance in SR86. Additionally, the additive effect value and dominant effect value of the enhancing alleles for salt tolerance were derived from SR86 (Table S9) indicating that *qST1.1* had a positive effect on increasing the salt tolerance of rice. Furthermore, *qST1.1* was located in the region of *Saltol*, where there was a key salt-resistant gene *SKC1* [12,16]. However, whether *SKC1* was important in determining salt tolerance of SR86 remains to be verified.

## 3.3. qST1.1 Validation by Substitution Experiments and Introgression Line T22

To evaluate the accuracy and precision of the target region *qST1.1* that we identified by QTL mapping, segment substitution lines were constructed and used to analyze the salt tolerance related regions within *qST1.1*. Four BC<sub>2</sub>F<sub>3</sub> populations (W386, W401, W402, and W404) generated using the donor SR86 and recipients DJY1, YJ26, YJ29, and YJ32, respectively, were screened for recombined homozygotes with or without the *qST1.1* region. From the 400 introgression lines, 15 homozygous substitution lines with different length overlapping segments were screened for phenotype investigation (Figure 2C). The phenotypes showed that the lines harbored with the introgression region between RM8094 and RM493 conferred salt tolerance; in contrast, the other recombined lines that lacked the RM8094–RM493 region were sensitive to salt. By the substitution mapping, we narrowed down and mapped *qST1.1* to the interval between flanking markers RM8094 and RM493 with a length of 5.49 cM (Figure 2B). This substitution experiment confirmed the consistency and accuracy of our QTL mapping, and so we inferred that *qST1.1* was likely associated with the salt tolerance of SR86 in saline soil conditions.

We aimed to utilize the introgression lines carrying qST1.1 from SR86 in breeding practice for salt tolerance. The BC<sub>2</sub> introgression line T22, derived from crossing SR86 with YJ26, was evaluated for 12 agronomic traits including plant height, yield per plant, and seed setting rate, etc. (Table 3). It was notable that the grain number per panicle and the yield per plant of T22 were significantly higher than those of the parents, although the seed setting rate and 1000-grain weight of T22 were less than YJ26. Notably, the lower plant height of T22 (100.52 cm) was suitable for lodging resistance compared with SR86 (163.22 cm), and this feature might contribute to the increased yield in T22. Importantly, the salt resistance of T22 was similar to, or even better than, SR86 (Figure 3). These data proved that T22 did have a better performance than its parents and offered the prospect of being useful for breeding new rice varieties with improved salt tolerance. We developed further salt-tolerant QTL introgression lines in four different genetic backgrounds (DJY1, YJ26, YJ29, YJ32 as recipients); seven recombinants harboring *qST1.1* showed salt resistance (Figure 2B and Table S10). These salt-tolerant QTL introgression lines might also be useful in breeding for salt tolerance with evaluation under saline soil conditions.

Traits	SR86	T22	YJ26
Plant height (cm)	$163.22 \pm 6.10$	100.52 ± 3.44 *	$95.56 \pm 5.63$
Number of filled grains per panicle	$71.07 \pm 15.74$	110.70 ± 8.90 **	$83.33 \pm 22.32$
Seed setting rate	$0.74 \pm 0.10$	$0.77 \pm 0.04$ **	$0.86 \pm 0.04$
Effective tiller	$11.78 \pm 2.33$	$10.11 \pm 1.21$	$8.77 \pm 2.33$
1000-grain weight (g)	$25.59 \pm 0.85$	26.77 ± 0.72 **	$28.44 \pm 0.52$
Panicle length (cm)	$22.68 \pm 0.75$	$23.50 \pm 0.90 *$	$17.60 \pm 0.93$
Primary branching	$9.82 \pm 0.16$	10.3 1± 0.63 *	$8.96 \pm 0.54$
Secondary branching	$27.00 \pm 2.75$	26.33 ± 3.05 **	$18.05 \pm 2.56$
Grain length (mm)	$7.19 \pm 0.24$	$7.81 \pm 0.07$ **	$7.12 \pm 0.05$
Grain width (mm)	$2.76\pm0.08$	2.83 ± 0.36 **	$3.07\pm0.06$
Grain length-to-width ratio	$2.64\pm0.50$	2.82 ± 0.03 **	$2.36 \pm 0.05$
Grain yield per plant (g)	$22.21 \pm 6.51$	29.32 ± 3.03 **	$19.88 \pm 2.43$

Table 3. Results of agronomic traits in infiltrated lines and two parents under normal field conditions.

The asterisk \* and \*\* indicate significant difference from control at p < 0.05 and p < 0.01, respectively; the T22 column was marked as the comparison result with YJ26.



**Figure 3.** Identification of salt tolerance in IL-T22. It shows the salt resistance of T22 was similar or even better than SR86 compared to YJ26 after being treated with 1.2% NaCl solution for 8 d.

## 4. Discussion

Improving the salt tolerance of rice is valuable for increasing crop yield. Salt tolerance is a quantitative trait, and a complex set of genetic and physiological mechanisms have been reported as related to salt tolerance in rice, including regulation of osmotic potential, ion transport capacity, antioxidant ability, and hormone-related pathways [22]. Various kinds of genes and QTLs have been shown to contribute to resisting salt stress under experimental conditions. However, few of them work in practice to improve the salt tolerance of rice in the field, and there remains a need to explore QTLs and genes that might make a greater contribution under saline conditions [28,29].

SR86, an ancient *indica* subspecies that is phylogenetically close to the divergence point of the major rice varietals, has been shown to have strong salt tolerance [23]. Surprisingly, only a few studies on SR86 have been conducted, and QTL mapping or analysis of the salt tolerance mechanism in SR86 has not been reported. Most of the previous studies on rice salt tolerance were conducted in hydroponic solutions to ensure a homogeneous medium for the salt treatment; however, in the current study, the salt-response differences between SR86 and salt-sensitive varieties were not observed under hydroponic conditions (Figure S1). We simulated saline soil conditions based on the natural growth environment of SR86, and observed a different phenotype between SR86 and salt-sensitive varieties. In this study, SR86 seedlings were treated with 1.2% NaCl solutions applied to the soil. The electrical conductivity of the water in the soil was measured and showed a relatively high NaCl content at

about 0.95% to 1.12% (Figure S3), which indicated that SR86 has a high salt tolerance compared with other rice varieties. The different performance of SR86 between soil and hydroponic conditions might be attributed to the complex soil environment, such as the soil microorganisms, root exudates, and interaction between the plants and soil microenvironment [30,31].

The identified QTL was mapped using BSA and found to be located within two candidate regions, one around 9.46–12.13 Mb and the other one around 12.21–12.24 Mb. The QTL, which was named as qST1.1, was located between the markers RM8094 and RM493 in the 11.24–12.26 Mb regions on chromosome 1, with an interval of 5.49 cM between flanking markers. This *qST1.1* was located within the QTL "Saltol", which was reported as contributing more than 70% of the variation in salt uptake in a population, with an LOD >14.5 [32]. "Saltol" has proven to be an effective salt tolerance region and was successfully applied in breeding. Field experiments proved that "Saltol" significantly improved the salt tolerance of rice at the seedling stage and maintained stable yield under salt stress [28,33]. Thomson et al. also found some QTL segments related to salt tolerance at the seedling stage in this chromosome region, and some segments overlapped with SKC1, possibly encoding the same gene (OsHKT1;5) [13]. *SKC1*, as a major member of the high-affinity K<sup>+</sup> transporter (HKT), functions as a Na<sup>+</sup>-selective transporter and was mapped using populations derived from a cross between a salt-tolerant indica variety, Nona Bokra, and a susceptible elite *japonica* variety, Koshihikari [16]. Coincidentally, SKC1 was also located in *qST1.1*, and SR86 had the same amino acid sequence as Nona Bokra (Figure S4). The balance of cations, such as Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup>, and their transport ability played key roles in the salt tolerance response of rice [31,34,35]. Whether the ions transport affects salt tolerance in SR86 remains to be further explored.

Numerous potential salt-responsive genes are located within *qST1.1*, which contains 198 ORFs, including 34 retrotransposon proteins, 26 transposon proteins, 57 expressed proteins, 9 hypothetical proteins, 7 peroxidase precursors, 5 transcription factors (TFs), namely *OsMADS89* (*LOC\_Os01g18440*), *OsWRKY9* (*LOC\_Os01g18584*), *OsSPL12* (*LOC\_Os01g18850*), *OsMYB78L* (*LOC\_Os01g19330*), and *OsMYBL* (*LOC\_Os01g19970*), and other genes such as PIF1 family genes, and protein kinase genes. Transcription factors play an essential role in rice development and responses to biotic and abiotic stresses [36–39]; these five TFs have been reported to respond to salt stress in rice to varying degrees [40,41]. The reactive oxygen species signaling pathway mediated by peroxidase was also critical, as it evoked a cascade of responses related to stress tolerance [42–44]. The aggregation of related genes in the same chromosome region might be a key feature underlying the salt tolerance of SR86.

## 5. Conclusions

In this study, we combined bulked segregant analysis and QTL mapping using parents SR86 (salt-tolerant), DJY1 (salt-sensitive), and their  $F_2$  population. We identified a QTL designated as *qST1.1* that played a significant role in salt tolerance in SR86 grown under soil conditions. This QTL was further validated by homozygous substitution lines and introgression lines. This *qST1.1* not only improves the ability of plants to resist salt stress, but also does not affect yield; it therefore shows potential as a genetic resource for breeding salt-tolerant rice using MAS. We further applied salt stress using high concentrations of NaCl in soil, and the saline soil environment was simulated to avoid undue disturbance of normal growth conditions. The QTL identified by this method will be more effective for future breeding practices for salt-tolerance. This study will not only help to better understand the genes and mechanisms of salt tolerance in rice, but also provides new insights that will underpin breeding strategies for salt-tolerant rice.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4395/10/3/410/s1, Figure S1: The phenotype of SR86, T22, DJY1, and YJ26 in different salt concentrations under hydroponic conditions. NaCl dissolved and diluted in 1/4 Hoagland solution, Figure S2: SNP-index distributed on chromosomes. (a) R03–salt-tolerant pool, (b) R04–salt-sensitive pool, and (c) R03–R04 pool. The black arrow points to the BSA location range, Figure S3: Linear regression graph of NaCl concentration and electrical conductivity. The blue scattered points mean different concentrations of NaCl solutions and the corresponding electrical conductivity,

and the blue dotted line is the trendline. The yellow triangles represent the sample solution, the sample was diluted 5 times and determined, Figure S4: Sequence alignment of *SKC1* (*OsHKT1;5*) protein in SR86, Nona Bokra, DJY1, and YJ26. The dark blue shows the different sites, Table S1: Grading standard of salt tolerance on the relative salt damage rate, Table S2: SSR marks tested in this study, Table S3: The phenotypic frequency statistics of F<sub>2</sub> population under 1.2% NaCl treatment, Table S4: Data quantity and quality of whole-genome resequencing, Table S5: Sequencing depth and coverage statistics of whole-genome resequencing, Table S6: SNP statistics of whole-genome resequencing, Table S7: Indel statistics of whole genome and coding regions, Table S8: Classification and statistics of differential genes produced by various mutations, Table S9: The *qST1.1* affected salt tolerance in  $F_2$  at the seedling stage, Table S10: Different length homozygous overlapping segment lines screened from BC<sub>2</sub>F<sub>3</sub>.

**Author Contributions:** P.X. designed the experiment and provided rice materials; F.W. and J.Y. performed experiments and analyzed the data; F.W. wrote the manuscript; P.X. D.Y., and J.Y. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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# References

- 1. Munns, R.; Gilliham, M. Salinity tolerance of crops-what is the cost? *New Phytol.* **2015**, *208*, 668–673. [CrossRef] [PubMed]
- Yang, Y.; Guo, Y. Elucidating the molecular mechanisms mediating plant salt-stress responses. *New Phytol.* 2018, 217, 523–539. [CrossRef] [PubMed]
- 3. Wang, J.; Zhu, J.; Zhang, Y.; Fan, F.; Li, W.; Wang, F.; Zhong, W.; Wang, C.; Yang, J. Comparative transcriptome analysis reveals molecular response to salinity stress of salt-tolerant and sensitive genotypes of *indica* rice at seedling stage. *Sci. Rep.* **2018**, *8*, 2085. [CrossRef] [PubMed]
- 4. Wang, W.C.; Lin, T.C.; Kieber, J.; Tsai, Y.C. Response regulators 9 and 10 negatively regulate salinity tolerance in rice. *Sci. Rep.* **2019**, *60*, 2549–2563. [CrossRef] [PubMed]
- 5. Radanielson, A.M.; Gaydon, D.S.; Li, T.; Angeles, O.; Roth, C.H. Modeling salinity effect on rice growth and grain yield with ORYZA v3 and APSIM-Oryza. *Eur. J. Agron.* **2018**, *100*, 44. [CrossRef]
- 6. Flowers, T.J.; Yeo, A.R. Variability in the resistance of sodium chloride salinity within rice (*Oryza sativa* L.) varieties. *New Phytol.* **1981**, *88*, 363–373. [CrossRef]
- Ghomi, K.; Rabiei, B.; Sabouri, H.; Sabouri, A. Mapping QTLs for traits related to salinity tolerance at seedling stage of rice (*Oryza sativa* L.): An agrigenomics study of an Iranian rice population. *OMICS* 2013, 17, 242–251. [CrossRef]
- 8. Ismail, A.M.; Horie, T. Genomics, Physiology, and molecular breeding approaches for improving salt tolerance. *Annu. Rev. Plant. Biol.* **2017**, *68*, 405–434. [CrossRef]
- 9. Formentin, E.; Sudiro, C.; Perin, G.; Riccadonna, S.; Barizza, E.; Baldoni, E.; Lavezzo, E.; Stevanato, P.; Sacchi, G.A.; Fontana, P.; et al. Transcriptome and cell physiological analyses in different rice cultivars provide new insights into adaptive and salinity stress responses. *Front. Plant. Sci.* **2018**, *9*, 204. [CrossRef]
- 10. Kong, W.; Zhong, H.; Gong, Z.; Fang, X.; Sun, T.; Deng, X.; Li, Y. Meta-analysis of salt stress transcriptome responses in different rice genotypes at the seedling stage. *Plants* **2019**, *8*, 64. [CrossRef]
- Puram, V.R.R.; Ontoy, J.; Linscombe, S.; Subudhi, P.K. Genetic dissection of seedling stage salinity tolerance in rice using introgression lines of a salt tolerant landrace Nona Bokra. *J. Hered.* 2017, *108*, 658–670. [CrossRef] [PubMed]
- 12. Gregorio, G.B. Tagging Salinity Tolerance Genes in Rice Using Amplified Fragment Length Polymorphism (AFLP). Ph.D. Thesis, University of the Philippines, Los Banos, Philippines, 1997.
- 13. Thomson, M.; De Ocampo, M.; Egdane, J.; Rahman, M.A.; Sajise, A.G.; Adorada, D.L.; Tumimbang-Raiz, E.; Blumwald, E.; ISeraj, Z.; Singh, R.K.; et al. Characterizing the *Saltol* quantitative trait locus for salinity tolerance in rice. *Rice* **2010**, *3*, 148–160. [CrossRef]
- 14. Gong, J.; He, P.; Qian, Q.; Shen, L.; Zhu, L.; Chen, S. Identification of salt-tolerance QTL in rice (*Oryza sativa* L.). *Chinese Sci. Bullet.* **1999**, 44, 68–71. [CrossRef]

- Lin, H.X.; Zhu, M.Z.; Yano, M.; Gao, J.P.; Liang, Z.W.; Su, W.A.; Hu, X.H.; Ren, Z.; Chao, D.Y. QTLs for Na<sup>+</sup> and K<sup>+</sup> uptake of the shoots and roots controlling rice salt tolerance. *Theor. Appl. Genet.* 2004, 108, 253–260. [CrossRef]
- Ren, Z.; Gao, J.-P.; Li, L.-G.; Cai, X.-L.; Huang, W.; Chao, D.-Y.; Zhu, M.-Z.; Wang, Z.-Y.; Luan, S.; Lin, H.-X. A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat. Genet.* 2005, *37*, 1141–1146. [CrossRef]
- 17. Lee, S.Y.; Ahn, J.H.; Cha, Y.S.; Yun, D.W.; Lee, M.C.; Ko, J.C.; Lee, S.K.; Eun, M.Y. Mapping of quantitative trait loci for salt tolerance at the seedling stage in rice. *Mol. Cells* **2006**, *21*, 192–196.
- 18. Sabouri, H.; Rezai, A.M.; Moumeni, A.; Kavousi, A.; Katouzi, M.; Sabouri, A. QTLs mapping of physiological traits related to salt tolerance in young rice seedlings. *Biol. Plantarum.* **2009**, *53*, 657–662. [CrossRef]
- 19. Tian, L.; Tan, L.; Liu, F.; Cai, H.; Sun, C. Identification of quantitative trait loci associated with salt tolerance at seedling stage from *Oryza rufipogon*. *J. Genet. Genom.* **2011**, *38*, 593–601. [CrossRef]
- 20. Gimhani, D.; Gregorio, G.B.; Kottearachchi, N.; Samarasinghe, W. SNP-based discovery of salinity-tolerant QTLs in a bi-parental population of rice (*Oryza sativa* L.). *Mol. Genet. Genomics.* **2016**, *291*, 2081–2099. [CrossRef]
- 21. De Leon, T.B.; Linscombe, S.; Subudhi, P.K. Molecular dissection of seedling salinity tolerance in rice (*Oryza sativa* L.) using a high-density GBS-based SNP linkage map. *Rice* **2016**, *9*, 52. [CrossRef]
- 22. Ganie, S.A.; Molla, K.A.; Henry, R.J.; Bhat, K.V.; Mondal, T.K. Advances in understanding salt tolerance in rice. *Theor Appl Genet.* 2019, *132*, 851–870. [CrossRef]
- 23. Chen, R.; Cheng, Y.; Han, S.; Van Handel, B.; Dong, L.; Li, X.; Xie, X. Whole genome sequencing and comparative transcriptome analysis of a novel seawater adapted, salt-resistant rice cultivar-sea rice 86. *BMC Genomics.* **2017**, *18*, 655. [CrossRef]
- 24. Chen, Z.H. Salt Tolerance Evalution, Gene Discovery and Germplasm Innovation of Water-Saving and Drought Resistant Rice Core Resources. Master's Thesis, Huazhong agricultural university, Wuhan, China, 2019.
- 25. Murray, M.G.; Thompson, W.F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4325. [CrossRef]
- 26. McCouch, S.R.; Teytelman, L.; Xu, Y.; Lobos, K.B.; Clare, K.; Walton, M.; Fu, B.; Maghirang, R.; Li, Z.-K.; Xing, Y.; et al. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Res.* **2002**, *9*, 199–207. [CrossRef]
- Ganie, S.A.; Borgohain, M.J.; Kritika, K.; Talukdar, A.; Pani, D.R.; Mondal, T.K. Assessment of genetic diversity of *Saltol* QTL among the rice (*Oryza sativa* L.) genotypes. *Physiol Mol Biol Plants*. 2016, 22, 107–114. [CrossRef] [PubMed]
- Bimpong, I.K.; Manneh, B.; Sock, M.; Diaw, F.; Amoah, N.K.A.; Ismail, A.M.; Gregorio, G.B.; Singh, R.K.; Wopereis, M. Improving salt tolerance of lowland rice cultivar 'Rassi' through marker-aided backcross breeding in West Africa. *Plant. Sci.* 2016, 242, 288–299. [CrossRef] [PubMed]
- 29. Lang, N.; Buu, B.C. Enhancing and stabilizing the productivity of salt-affected areas by incorporating genes for tolerance of abiotic stresses in rice. *Omonrice* **2011**, *18*, 41–49.
- 30. Isah, T. Stress and defense responses in plant secondary metabolites production. *Biol. Res.* **2019**, *52*, 39. [CrossRef] [PubMed]
- 31. Radhakrishnan, R.; Baek, K.H. Physiological and biochemical perspectives of non-salt tolerant plants during bacterial interaction against soil salinity. *Plant. Physiol. Biochem.* **2017**, *116*, 116–126. [CrossRef] [PubMed]
- 32. Bonilla, P.; Dvorak, J.; Mackill, D.; Deal, K.; Gregorio, G. RLFP and SSLP mapping of salinity tolerance genes in chromosome 1 of rice (*Oryza sativa* L.) using recombinant inbred lines. *Philipp. Agric. Sci.* 2002, *85*, 68–76.
- 33. Singh, V.K.; Singh, B.D.; Kumar, A.; Maurya, S.; Krishnan, S.G.; Vinod, K.K.; Pal Singh, M.; Ellur, R.K.; Bhowmick, K.; Singh, A.K. Marker-assisted introgression of *Saltol* QTL enhances seedling stage salt tolerance in the rice variety "Pusa Basmati 1". *Int. J. Genom.* **2018**, *2018*, 8319879.
- 34. Chen, H.C.; Cheng, W.H.; Hong, C.Y.; Chang, Y.S.; Chang, M.C. The transcription factor *OsbHLH035* mediates seed germination and enables seedling recovery from salt stress through ABA-dependent and ABA-independent pathways, respectively. *Rice* **2018**, *11*, 50. [CrossRef] [PubMed]
- 35. Xie, T.; Chen, C.; Li, C.; Liu, J.; Liu, C.; He, Y. Genome-wide investigation of WRKY gene family in pineapple: Evolution and expression profiles during development and stress. *BMC Genomics.* **2018**, *19*, 490. [CrossRef] [PubMed]

- 36. Seifikalhor, M.; Aliniaeifard, S.; Shomali, A.; Azad, N.; Hassani, B.; Lastochkina, O.; Li, T. Calcium signaling and salt tolerance are diversely entwined in plants. *Plant. Signal. Behav.* **2019**, *14*, 1665455. [CrossRef]
- 37. Zhang, Y.; Fang, J.; Wu, X.; Dong, L. Na<sup>+</sup>/K<sup>+</sup> Balance and transport regulatory mechanisms in weedy and cultivated rice (*Oryza sativa* L.) under salt stress. *BMC Plant. Biol.* **2018**, *18*, 375. [CrossRef]
- Fang, Y.; Liao, K.; Du, H.; Xu, Y.; Song, H.; Li, X.; Xiong, L. A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. *J. Exp. Bot.* 2015, *66*, 6803–6817. [CrossRef]
- 39. Jamil, M.; Iqbal, W.; Bangash, A.; Rehman, S.U.; Imran, Q.M.; Rha, E.S. Constitutive expression of *OSC3H33*, *OSC3H50* and *OSC3H37* genes in rice under salt stress. *Pak. J. Bot.* **2010**, *42*, 4003–4009.
- 40. Nutan, K.K.; Kushwaha, H.R.; Singla-Pareek, S.L.; Pareek, A. Transcription dynamics of *Saltol* QTL localized genes encoding transcription factors, reveals their differential regulation in contrasting genotypes of rice. *Funct. Integr. Genom.* **2017**, *17*, 69–83. [CrossRef]
- 41. Buti, M.; Baldoni, E.; Formentin, E.; Milc, J.; Frugis, G.; Schiavo, F.L.; Genga, A.; Francia, E. A Meta-analysis of comparative transcriptomic data reveals a set of key genes involved in the tolerance to abiotic stresses in rice. *Int. J. Mol. Sci.* **2019**, *20*, 5662. [CrossRef]
- 42. Vannini, C.; Iriti, M.; Bracale, M.; Locatelli, F.; Faoro, F.; Croce, P.; Pirona, R.; Di Maro, A.; Coraggio, I.; Genga, A. The ectopic expression of the rice *Osmyb4* gene in Arabidopsis increases tolerance to abiotic, environmental and biotic stresses. *Physiol. Mol. Plant. Pathol.* **2006**, *69*, 26–42. [CrossRef]
- 43. Kaur, N.; Dhawan, M.; Sharma, I.; Pati, P.K. Interdependency of reactive oxygen species generating and scavenging system in salt sensitive and salt tolerant cultivars of rice. *BMC Plant. Biol.* **2016**, *16*, 131. [CrossRef]
- 44. Saini, S.; Kaur, N.; Pati, P.K. Reactive oxygen species dynamics in roots of salt sensitive and salt tolerant cultivars of rice. *Anal. Biochem.* **2018**, *550*, 99–108. [CrossRef]



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