Mining Beneficial Genes for Salt Tolerance From a Core Collection of Rice Landraces at the Seedling Stage Through Genome-Wide Association Mapping

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Rice is a salt-sensitive plant. High concentration of salt will hinder the absorption of water and nutrients and ultimately affect the yield. In this study, eight seedling-stage salt-related traits within a core collection of rice landraces were evaluated under salinity stress (100 mM NaCl) and normal conditions in a growth chamber. Genome-wide association study (GWAS) was performed with the genotypic data including 2,487,353 single-nucleotide polymorphisms (SNPs) detected in the core collection. A total of 65 QTLs significantly associated with salt tolerance (ST) were identified by GWAS. Among them, a co-localization QTL qTL4 associated with the SKC, RN/K, and SNC on chromosome 6, which explained 14.38–17.94% of phenotypic variation, was selected for further analysis. According to haplotype analysis, qRT-PCR analysis, and sequence alignment, it was finally determined that 4 candidate genes (LOC_Os06g47720, LOC_Os06g47820, LOC_Os06g47850, LOC_Os06g47970) were related to ST. The results provide useful candidate genes for marker assisted selection for ST in the rice molecular breeding programs.

Keywords: rice, salinity tolerance, candidate gene, GWAS, QTL

INTRODUCTION

Soil salinization is one of the important abiotic stresses that limits agricultural food production, because it reduces crop yields and restricts land use. With the modernization of industry and the continuous deterioration of the ecological environment, the area of arable land is gradually decreasing, while the area of soil salinization continues to increase because of unreasonable irrigation to the farmland (Qadir et al., 2014). It is estimated that about 950 million hectares of arable land in the world, including 250 million hectares of irrigated land, are affected by salinization (Solis et al., 2020). Rice (Oryza sativa) is a salt-sensitive plant, and salt stress seriously affects its growth, development, and yield (Wang et al., 2018a). Therefore, it is of great significance to mine and utilize the novel salt-tolerant (ST) genes in rice and breed ST varieties (Li et al., 2014).
A large number of studies have shown that ST is a complex trait controlled by quantitative trait loci (QTL; Roy et al., 2011). In recent years, with the development of modern science and technology, sequencing technology has become more and more mature. A lot of researches have been done on QTL mapping of ST in rice, and many QTLs related to ST have been identified. Using the F₂ population derived from the parent Dianjingyou1 as the recipient parent and SR86 as the donor parent, a huge segregation analysis library was constructed and a QTL named qst1.1 related to ST was identified on chromosome 1, which can explain 62.6% of the phenotypic variation (Wu et al., 2020). Using the RIL population, 38 QTLs related to ST were identified and a new QTL affecting stem length was found on chromosome 7, which could explain 6.8% of the phenotypic variation (Jahan et al., 2020). Takehisa et al. (2004) identified 12 QTLs for stem length under salt stress on chromosomes 1, 3, and 7, explaining 12–30% of the phenotypic variation. Wang et al. (2012) used the IR26/Jucaiqing recombinant inbred line (RIL) population and found 16 QTLs related to the swelling rate and germination rate under salt stress. Prasad et al. (2000) used the DH population and identified a QTL for seed root length under salt stress on chromosome 6, which explained 18.9% of the phenotypic variation. Zeng et al. (2021) used the BC₂F₂ population and performed QTL mapping for the seed germination rate (GR) and germination index (GI). A total of 13 QTLs were identified, which explained 7.32–24.39% of the phenotypic variation.

GWAS can be used to perform association analysis on the genetic variation of complex traits at the genome-wide level (Hirschhorn and Daly, 2005). It is an effective method for in-depth understanding of the genetic structure of complex traits of crops. With GWAS, several researchers have identified many QTLs and candidate genes related to ST traits. Yuan et al. (2020) used 664 rice varieties and different statistical models to conduct GWAS for ST, and a total of 21 QTLs were identified. Using 208 rice varieties from a core collection, Naveed et al. (2018) identified 20 quantitative trait nucleotides (QTNs) for one salt related trait through GWAS, including 6 QTNs affecting ST at the germination stage and 14 QTNs at the seedling stage, and identified 22 candidate genes. Using 708 rice varieties, Liu et al. (2019) identified 7 candidate genes through GWAS, which were significantly associated with grain yield and its related traits under saline stress conditions. Zhang et al. (2017) used GWAS to identify salt-tolerant loci and favorable alleles for iron and zinc resistance and detected 60 salt-tolerant loci as well as 22 candidate genes in 10 important QTLs regions. An et al. (2020) used a core collection which consisted of 181 varieties and detected 17 loci significantly associated with dry weight ratio (DWR) for ST through GWAS.

The seedling stage is the key stage for ST in rice, and it is highly sensitive to salt stress (Nayyeripasand et al., 2021). High concentration of salt will hinder the absorption of water and nutrients in the rice seedling stage, inhibit the growth of seedlings, and ultimately reduce rice yield (Ruan et al., 2011). To a certain extent, it can be used as a reference for ST during the whole growth period. The ST identification at the seedling stage is easy to operate with short cycle and high efficiency and can be widely used in screening germplasm resources for ST and breeding selection (Walia et al., 2005).

Abundant germplasm resources for ST are available in the Asian cultivated rice, especially in rice landraces. As early as in 1920–1964, a total of 7,128 accessions of rice landraces had been collected by Prof. Ying Ting, which was named as Ting’s rice collection (Li et al., 2011). They were from all over China as well as from some main rice cultivation countries. Based on 48 phenotypic data, Li et al. (2011) have constructed a rice core collection consisting of 150 accessions. The large variation within the core collection provides an important gene pool of genetic diversity and beneficial genes for rice breeding. Therefore, it is worth to perform GWAS with such a core collection for ST in rice. Population structure analysis for the core collection indicated that there existed two subgroups mainly corresponding to indica and japonica subspecies and the linkage disequilibrium (LD) decay distance was about 200–500 kb (Zhang et al., 2011; Li and Zhang, 2012; Zhao et al., 2018). Using this core collection, several researches have been performed to identify resistant QTLs for aluminum (Al) tolerance and cold tolerance. With the mixed linear model, GWAS identified a total of 30 QTLs for Al tolerant traits which explained 7.73 to 13.30% of the phenotypic variation (Zhao et al., 2018). A total of 26 QTLs were found to be significantly associated with cold tolerance, which explained 26–33% of the phenotypic variation (Song et al., 2018). These results indicated that these landraces are importance sources for stress tolerance in rice and the mapping results could provide important information to breed stress tolerant rice cultivars through marker-assisted selection. However, to our knowledge, no previous research was performed for ST at the seedling stage with the core collection. Moreover, no previous research was performed to map QTLs for ST and further identify candidate genes with the core collection. Therefore, the objectives for this study were: (1) to screen the performance of ST in the in the core collection; (2) to map the QTLs for ST through GWAS; and (3) to identify some candidate genes for ST in rice for better understanding the genetic basis of ST at the seedling stage in rice and providing new genetic resources for improvement of ST in rice cultivars.

**MATERIALS AND METHODS**

**Plant Materials**

The Ting’s rice core collection, i.e., a total of 150 accessions of rice landraces, was used to screen their salt tolerance (Supplementary Table S9). These landraces were mainly collected from 20 different provinces in China as well as from North Korea, Japan, Philippines, Brazil, Celebes, Java, Oceania, and Vietnam. These regions are distributed across the north latitude 55° to south latitude 10° and including regions with temperate, tropical, and subtropical climate. The core collection was constructed from 150 accessions of 2,262 based on a strategy of stepwise clustering and preferred sampling on adjusted Euclidean distances and weighted pair-group average method using integrated qualitative and quantitative
traits (Li et al., 2007). Of the 150 landraces, 32 were classified as japonica rice (24 were typical japonica rice, and 8 were japonica-clined rice), and 118 were classified as indica rice (16 were indica-clined rice, and 102 were typical indica rice), according to Cheng’s index criterion (Li et al., 2011).

**ST Evaluation at the Seedling Stage**
The seeds of the 150 landraces were placed in an oven at 50°C for 48 h to break dormancy. From each landrace, 40 seeds with uniform size and full rice grains were selected, and then the seeds were soaked in 75% alcohol for 15–20 min for disinfection treatment. Then, the seeds were rinsed with sterile water for three times. The rinsed seeds were put in a net bag, soaked in distilled water, and placed in a thermostat at 30°C for 48 h to incubate germination. Then, the seeds were transferred to 96-well PCR plates with cut wells and distilled water. The PCR plates were placed in a culture room with light at 28°C for cultivation and later cultivated with 12 h of light and 12 h of darkness. After 7 days, the distilled water was changed to the standard nutrition of the International Rice Research Institute (IRRI) but with only 0.5-fold concentration (Yoshida et al., 1976), while keeping the PH value of Yoshida’s solution at 5.5. The nutrient solution was changed once per 3 days. After culturing for 7 days, the cultivation was changed to 0.5-fold Yoshida’s solution and later changed to one-fold Yoshida’s solution with regularly replacing the nutrient solution. When the seedlings grew to the three-leaf stage, the samples of both the control and the salt treated were extracted in 25 ml acetic acid (100 mm) at 90°C for 2 h, and 2 ml extraction was divided into two groups for sodium and potassium, respectively. 100 mm NaCl solution was added to the nutrient solution for salt stress treatment, while the control group continued to grow in the nutrient solution. Each treatment was set with three repetitions. After 10 days of treatment, samples were taken to measure the phenotypic traits. Shoot and root Na⁺ and K⁺ concentrations (RNC, SKC, and SNC) of each sample were determined by atomic absorption spectrometry (AAS, Series 2, Thermo Electron Corporation). Concentrations of sodium and potassium in shoots and roots were expressed in millimoles per gram (mMg⁻¹; Qi et al., 2005; An et al., 2020). The K⁺/Na⁺ ratios in roots (RN/K) were calculated subsequently. A root scanner (Expression 1100XL) was used to analyze root traits. To reduce errors, 10 seedlings were scanned for each material, and each seedling was scanned 3 times, and the average values of TRSA (total root surface area), TRV (total root volume), and TRL (total root length) were calculated. Set up 3 biological replicates. RTRSA (relative total root surface area), RTRV (relative total root volume), RTRL (relative total root length), and RSN/K (relative ratio of Na⁺ to K⁺ concentrations in shoots) were calculated according to the following formula: relative trait value (%) = (trait value under salt stress) / (trait value under control) × 100.

**DNA Extraction and Candidate Gene Sequence Alignment**
DNA Quick Plant System (Tiangen Biotechnology, Beijing, China) was used to extract plant genomic DNA. The target sequence was amplified with specific primers. The amplified product was purified with EasyPure PCR purification kit (Tiangen Biotech, China) and quantified with NanoDrop 8,000 spectrophotometer (Thermo Fisher Science, Waltham, MA, United States). Sequence alignment was performed with the DNAMAN software using the genes in the Nipponbare genome as a reference.

**GWAS Analysis and Haplotype Analysis**
GWAS was performed by using the compressed MLM program in the Tassel5.0 software, where the model was as follows: Y = Xα + Qβ + Kμ + e, where Q represents the kinship, X is genotype, Y is phenotype, while Xα and Qβ are considered as fixed effects and Kμ and e as random effects. The population structure K was calculated by the Admixture software, and the kinship between samples was calculated by the SPAGEDi software. The significant SNPs were identified by p value. To obtain high-density SNPs, we encrypted the original 67,511 SNPs and obtained 2,487,353 SNPs by the following method: the user uploads input files in oxford format (.gen/sample) per chromosome. The imputation server utilizes chromosome recombination maps and the Rice Reference Panel (RICE-RP).
RESULTS

Phenotypic Variation
In order to evaluate the phenotypic variation of ST in the core collection at the seedling stage, a statistical analysis was performed on 8 ST-related traits: RNC (root Na⁺ concentration), SKC (shoot K⁺ concentration), SNC (shoot Na⁺ concentration), RN/K (ratio of Na⁺ to K⁺ concentrations in roots), RTRSA (relative total root surface area), RTRV (relative total root volume), RTRL (relative total root length), and RN/K (relative ratio of Na⁺ to K⁺ concentrations in shoots; Supplementary Table S1). The results showed that all traits showed tremendous phenotypic variation in the population. In particular, RN/K had the highest coefficient of variation, and RTRSA had the lowest coefficient of variation. Correlation analysis showed that most traits were significantly positively correlated. Significant negative correlations were observed only between SKC and RN/K, SKC, and RN/K (Supplementary Table S2). The results showed that salt stress has different degrees of influence on the 8 ST-related traits at rice seedling stage.

GWAS Results
In previous studies, the rice core collection was sequenced using the Specific-Locus Amplified Fragment Sequencing (SLAF-seq) approach and 67,511 SNPs were obtained (Zhao et al., 2018). Based on them, 2,487,353 SNPs were further obtained by the imputation method of (Wang et al., 2018b). With consideration of the population structure and kinship, the MLM (+Q+K) model and 2,487,353 SNPs were used to perform GWAS on eight traits related to ST with the high density SNPs set, and the results are presented in the form of Manhattan plots (Figure 1) and QQ plots (Supplementary Figure S1). With a significant threshold of \( p < 0.0001 \), 843 significant SNPs were detected (Supplementary Table S3), which were unevenly distributed on the 12 chromosomes. The most significant position with the largest contribution rate is located on chromosome 1, which can explain 28.11% of the phenotypic variation.

QTLs for ST at the Seedling Stage
ST-related QTLs are defined by the decay distance of linkage disequilibrium (LD). Previous studies had shown that the decay distance of LD is 500 kb (Zhao et al., 2018). Therefore, a region was considered as one QTL if it had more than one SNP with \( p < 0.0001 \) within the LD decay distance. We named salt tolerance QTLs with reference to the method proposed by Mccouch et al. (1997). In total, 65 QTLs were identified significantly associated with ST (Figure 2). There are 1–13 of QTLs on each chromosome, and each QTL contains 1–379 SNPs. These QTLs explained 13.47 to 28.11% of the phenotype variation (Supplementary Table S4).

For RNC, we detected 8 QTLs located on chromosomes 1, 3, 5, 8, 9, 10, and 12 under salt stress, which could explain 14.83–23.96% of the phenotypic variation. Under salt stress, we detected 14 QTLs for SNC on chromosomes 1, 2, 3, 5, 6, 7, 9, 11, and 12, which could explain 13.91–23.61% of the phenotypic variation. For RN/K under salt stress, ten QTLs were detected on chromosomes 1, 3, 5, 6, 8, 9, 10, and 11, which could explain 13.75–27.74% of the phenotypic variation. For RTR under salt stress, 8 QTLs were detected on chromosomes 3, 6, 8, 9, and 11, which could explain 13.47–20.52% of the phenotypic variation. Under salt stress, we detected 13 QTLs for SN/K on chromosomes 1, 2, 3, 4, 5, 8, and 9, which could explain 13.67–21.14% of the phenotypic variation. For SKC under salt stress, two QTLs were detected on chromosomes 6 and 8, which could explain 14.38–17.94% of the phenotypic variation. Under salt stress, we detected 5 QTLs for SKC on chromosomes 1 and 12, which can explain 14.46–28.11% of the phenotypic variation. For TRSA under salt stress, 5 QTLs were detected on chromosomes 1, 7, 9, 10, and 11, which can explain 14.33–23.10% of the phenotypic variation.

Co-localization of QTLs Under Salinity Stress Conditions
Through association mapping analysis, there are 6 genomic regions containing co-localization QTLs. We defined co-localization QTLs as \( qTL1 \), \( qTL2 \), \( qTL3 \), \( qTL4 \), \( qTL5 \), and \( qTL6 \), respectively (Table 1). The most complicated is the co-localization region of chromosome 6, i.e., \( qTL4 \), which is composed of 3 QTLs and has a large overlap region. The remaining 5 co-localization regions are all composed of 2 QTLs.

Identification of Candidate Genes for ST
Since \( qTL4 \) is composed of 3 QTLs with 14 significant SNPs, it was chosen for further analysis. The \( qTL4 \) contains the highest peak SNP (Chr6_28930159) at approximately 28.93Mbp, which can explain 14.38–17.94% of the phenotypic variation. According to the LD decay analysis of the population, the 250kb upstream and downstream around the peak SNP were designated as the searching range of candidate genes as shown by the LD heat map (Figure 3). Through the Rice Annotation
Project database, ninety-four genes were found in this region (Supplementary Table S5). It includes 50 functional annotation genes, 24 expressed proteins, 17 transposon proteins, and 3 hypothetical proteins. Among them, a known salt tolerant gene (OsSIDP366) was found in this region, which was located in the interval between qSKC.6 and qSNC.6. Besides it, qRN/K1.1 and qRSN/K1.1 also were co-localization, which were detected in the interval of qTL6 and related to ratio of Na$^+$ to K$^+$ concentrations in shoots.
K⁺ concentrations. The qTL6 contains the highest peak SNP (Chr9_19518843) at approximately 19.51 Mbp, which can explain 13.75–16.92% of the phenotypic variation. In addition, we also found a SNP Chr6_28822519 in the qRN/K.6 interval with a significant threshold of $p = 0.000069293$, which could explain 17.49% of the phenotypic variation, indicating that there may...
be new salt resistance-related genes in this interval. Based on gene functional annotation and GO enrichment analysis\(^1\) (Supplementary Table S6), we screened and found 11 out of 50 functional genes which are related to stress response or metabolism process-related genes were salt tolerance-related candidate genes (Supplementary Table S7).

**Haplotype Analysis of Candidate Genes**

We further performed haplotype analysis on the non-synonymous mutant SNPs and the promoter SNPs in the exon region of the 11 candidate genes and detected significant differences among different major haplotypes based on the haplotypes with all non-synonymous SNPs. It was found that the haplotypes of the 4 candidate genes had significant differences (Table 2), and each candidate gene had a different number of non-synonymous SNPs (Supplementary Table S8). The haplotype analysis showed that the four candidate genes were significantly associated with SKC (shoot K\(^+\) concentration), and the SKC of different haplotypes were also significantly different (Figure 4). These findings suggest that the four candidate genes (LOC_Os06g47720, LOC_Os06g47820, LOC_Os06g47850, and LOC_Os06g47970) may be involved in the regulation of salt tolerance at seedling stage in rice.

**Identification of Candidate Genes by Gene Expression**

To further determine whether the aforementioned 4 candidate genes are related to ST, gene expression assays for the 4 candidate genes were conducted in salt tolerant (S125) and sensitive (S87) rice landraces. We performed a statistical analysis on the dead seedling rates in S87 and S125 under salt stress to further verify their differences in salt tolerance (Figure 5). There were significant differences in the dead seedling rates of S87 and S125 under the two salt stress concentrations, indicating that they had significant differences in salt tolerance. Therefore, S87 and S125 were selected for subsequent analysis of candidate genes expression. The gene expression level of LOC_Os06g47720 in S87 showed a continuous increase trend from 0–12 h and was higher than S125 for all tested time points. The gene expression level of LOC_Os06g47720 in S87 was nearly 10-fold higher than that in S125 after 12 h of salt stress treatment. The opposite expression pattern was observed for LOC_Os06g47820, LOC_Os06g47850, and LOC_Os06g47970, where the gene expression levels in S125 were nearly 10-fold higher than those in S87 after 12 h of salt stress treatment.

**Sequence Analysis of the Candidate Genes**

To determine the association between candidate genes and ST phenotypes, four candidate genes were sequenced for the two rice landraces S125 and S87 (Supplementary Figure S1) and the sequence alignment was generated using DNAMAN.

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\(^1\)http://www.bioinformatics.com.cn
LOC_Os06g47720 has 29 mutation sites in the two ST extreme genotypes, which lead to 11 amino acids changed in the S125, while translation was terminated early in the S125. For LOC_Os06g47820, it showed that the two genotypes had 17 mutation sites and 14 amino acids changed. LOC_Os06g47970 has a pair of base substitutions in S125, which caused the glycine to change into the asparagine. LOC_Os06g47850 has no sequence difference between the two genotypes.

**DISCUSSION**

**Phenotypic Variation and Prospects of the Core Collection of Rice Landraces**

Artificial directional domestication and selection have led to the reduction of genetic diversity in many crops such as rice, and many excellent resistance gene resources have been gradually lost in common cultivated rice. Some landraces are less affected by artificial selection and contain more genetic diversity and resistance genes. The core collection of rice landraces was constructed from a total of 7,128 accessions of rice landraces from the Ting’s rice collection (Li et al., 2011) and came from all over China as well as some main rice cultivation countries. It provides an important gene pool of genetic diversity and beneficial genes for rice breeding. For example, several QTLs for aluminum tolerance and cold tolerance were identified in the core collection (Song et al., 2018; Zhao et al., 2018). In this study, 8 traits related to ST showed tremendous phenotypic variation in the core collection, which suggested that salt stress has different degrees of influence on the ST-related traits at the seedling stage, and these phenotypes could be used for GWAS. Furthermore, a total of 65 QTLs significantly associated with salt tolerance were identified by GWAS. These results further verified that the core collection of rice landraces contains abundant resistance gene resources to biotic stress and abiotic stress and is a precious germplasm resource with great prospect for mining and utilization of beneficial genes for rice breeding.

**Comparison of QTLs With Previous Studies**

GWAS is a promising method for QTL fine-mapping in plants in response to abiotic stress. At present, many QTLs related to salt stress have been identified. We compared the previously reported QTLs and found that a total of 12 previously reported QTLs for ST in this study were overlapped or close to the range of previously mapped QTLs for ST.

For example, under stress conditions 151 trait–marker associations were identified that were scattered in 29 genomic regions on 10 chromosomes of rice (Nayeripasand et al., 2021). Some of the QTLs are consistent with or close to our mapped QTL regions. The QTL qSNC1.1 on chromosome 1 coincides with the reported QTL qRDWn1.1 (root dry weight);
the QTL qRNC1 is located in the region of the reported QTL qRDWs1.1 (root dry weight). The QTLs qRTRL12.1 and qRTRL12.2 on chromosome 12 are located in the region of the reported QTL qSLn12.1 (shoot length). The QTL qRTRL12.4 is 320kbp away from the previously reported QTL qRDWs12.1 (root dry weight), and the QTL qRNC12.1 (root fresh weight) is 140 kb apart from the previously reported QTL qRFWs12.1. The QTL qRN/K8 on chromosome 8 is located in the previously reported QTL qRFWn8.1 (root fresh weight) interval. Chen et al. (2020) identified 23 QTLs with different salt tolerance indexes through GWAS. Some of the QTLs coincide with our regions. The QTL qRSN/K1.2 is located in the region of previously reported QTL qSIS1 (salt injury score) and qSNaC1.2 (shoot Na+ concentration), while the QTL qSNC2 on chromosome 2 is within the interval of previously reported qRRDW2 (relative root dry weight). The QTL qSKC8 is located in the interval of previously reported qRSKC8 (relative shoot K+ concentration) and qRNAc8 (root Na+ concentration). Zhang et al. (2020) used the 55 K rice SNP array to genotype the entire population and four parents, and a total of 7 salt-tolerant QTLs were detected. Some of the QTLs coincide with or close to our regions. The QTL qRSN/K1.5 is about 174kbp away from the previously reported QTL qSLST1 (shoot length under salt stress treatment) and is located in the intervals of previously reported QTL qRDSW1 (relative dry shoot weight) and qRBI (relative bio-mass). The QTL qSNC9 on chromosome 9 is 430 kb away.

**FIGURE 5 | (A)** Phenotypes of S87 (salt sensitive variety) and S125 (salt tolerant variety) at different salt concentrations. **(B)** Statistical analysis of S87 and S125 for dead seedling rate, where the a and b are ranked by Duncan’s test at p < 0.05. **(C)** changes in relative gene expression level of four candidate genes at different times (0, 3h, 6h, 12h) after salinity stress in different rice cultivars, where the a, b, c, d, and e are ranked by Duncan’s test at p < 0.05.
from the previously reported QTL qRL-R9.1 (root length), and the QTL qRSNC9 is 430kb away from the previously reported QTL qDSW9 (dry shoot weight) and qBST9 (biomass under salt treatment). It can be seen that some of the QTLs we mapped by GWAS were overlapped with or were close to the previously reported QTLs, which demonstrates that our GWAS mapping results are rather accurate.

In addition, 52 newly identified QTLs for ST were also found in this study. Among them, qRN/K1.1 and qRSN/K1.1 were located on chromosome 1 between 12.98–13.34Mb and can explain 13.75–16.92% of the phenotypic variation. The QTL qRTRV9 and qRTRSA9 between 15.43–15.53Mb on chromosome 9 can explain 15.12–15.18% of the phenotypic variation. The QTL qRSN/K9.1 and qRSN/K9.3 between 19.50 and 19.54Mb on chromosome 9 can explain 16.65–21.14% of the phenotypic variation. These QTLs are newly discovered and need to be further examined in the future.

Moreover, we also compared the cold-tolerant and aluminum-tolerant QTLs previously mapped using the same core collection. Some QTLs are overlapped or are similar to the QTL regions for ST in this study. Compared with the previously located QTLs for cold tolerance, the QTL qRSN/K4 in this study on chromosome 4 is about 200kb away from the previously located cold-tolerant SNP (Chr4_20440388). The QTL qRTRV6.1 on chromosome 6 contains the previously located cold-tolerant SNP (Chr6_1320300). The QTL qRNC8.1 on chromosome 8 is about 151kb away from the previously located cold-tolerant SNP (Chr8_374729). The QTL qRN/K10 on chromosome 10 is about 18kb away from the previously located cold-tolerant SNP (Chr10_3365663; Song et al., 2018). Compared with the previously located QTLs for aluminum tolerance, the QTL qRN/K1.1 and qRSN/K1.1 on chromosome 1 are about 330kb away from the previously located QTL qALT1.3. The QTL qRNC1 and qRSN/K1.3 on chromosome 1 are about 400kb away from the previously located QTL qALT1.5. The QTL qRTRSA7 on chromosome 7 is about 84kb away from the previously located QTL qALT7.2. The QTL region for qRSN/K9.2 contains the previously located QTL qALT9.1 (Zhao et al., 2018). These results indicate that the candidate genes in these intervals may have pleiotropic effects. It also indicates that the core collection contains a wealth of excellent resistance genes to biotic and abiotic stress.

**Comparison of the QTL Locations With the ST Genes**

We also compared the QTL locations with the genes known to be related to ST.² Twenty-three ST genes were found to co-localize with our QTLs (Table 3). Three ST genes were found close to the mapped QTLs related to RNC. One ST gene was found in the QTL interval related to RN/K. Three ST genes were found close to the QTLs related to RTRV. Four ST genes were found close to the mapped QTLs related to SKC. One ST gene was found close to the QTLs related to RTRL. One ST gene was found close to the QTLs related to RTRAS. In short, these ST genes are located in or close to the relevant QTLs interval (with a searching range of +/–250kb). Among them, the QTL qTL4 region is composed of qSKC.6, qRN/K.6, and qSNC.6 and the

²http://qtaro.abr.affrc.go.jp

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**Table 3 | Known ST genes surrounding the mapped QTLs.**

| Related traits | QTLs          | Chr | Position (Mbp) | Known genes               |
|----------------|---------------|-----|----------------|--------------------------|
| RNC            | qRNc5         | 5   | 7.13           | OsCyp51G3 Xia et al. 2015 |
|                | qRNc9         | 9   | 7.44           | OsMyb6c Wang et al. 2015  |
| SNC            | qSNC1.1       | 1   | 2.07           | OsRav2 Duan et al. 2016  |
|                | qSNc2         | 2   | 6.18–6.21      | HSPT100 Ham et al. 2013   |
|                | qSNc3.1       | 3   | 6.88           | Dsm3 Du et al. 2011      |
|                | qSNc9         | 9   | 15.83–15.86    | OsDsg1 Park et al. 2010  |
| RNC/R             | qSNc11.2      | 11  | 26.92–27.49    | OsJamyb Yang et al. 2020 |
| RTRV            | qRTRV3.1      | 3   | 2.06           | ONAC022 Hong et al. 2016 |
|                 | qRTRV3.2      | 3   | 2.66–2.67      | OsAP23 Zhuang et al. 2013 |
|                 | qRTRV6.1      | 6   | 1.16–1.58      | OsSiK1 Ouyang et al. 2010 |
| RSN/K           | qRSN/K1.4     | 1   | 30.84          | OsHaM1c1b Schmidt et al. |
|                 | qRSN/K1.5     | 1   | 38.43          | OsNAC6 Chung et al. 2009 |
|                 | qRSN/K2       | 2   | 35.52–35.83    | OsEreBP7 Jisha et al. 2015 |
|                 | qRSN/K3       | 3   | 4.15           | OsJAZ9 Wu et al. 2015    |
| SKC             | qSKC8         | 6   | 28.76–29.01    | OsSIDP266 Gu et al. 2016 |
|                 | qSKC9         | 8   | 25.12          | OsDøGB Giri et al. 2011  |
| RTRL            | qTRRL1.1      | 1   | 18.01–18.26    | OsMek1 Wen et al. 2002   |
| RTRAS           | qTRAS7        | 7   | 25.30–25.80    | OsSapK2 Xu et al. 2013   |
overlapping regions are dense. A known ST gene OsSIDP366 was found in the region of qTL4, where a candidate gene and OsSIDP366 both contain the DUF domain. These findings support the reliability of the mapping results in this study.

Promising Candidate Genes for ST in Rice

The salinity tolerance in rice seedling is majorly governed by root and shoots Na’/K’ ratio. The lower Na’/K’ ratio provides protection against the toxic effects of Na’, hence, tolerant to salt stress. Therefore, maintaining intracellular Na’/K’ homeostasis is a key factor in determining the survival ability of plants in response to salt stress (Yang and Yan, 2018). In this study, we found that qSKC6, qSNC6, and qRN/K.6 were co-localization and 4 candidate genes (LOC_Os06g47720, LOC_Os06g47820, LOC_Os06g47850, and LOC_Os06g47970) were detected in the interval of qTL4 which are all related to ratio of Na’ to K’ concentrations; therefore, we chose the four candidate genes for further study. Besides, qRN/K.1.1 and qRSN/K.1.1 also were co-localization and detected in the interval of qTL6 which are related to Na’ and K’, the qTL6 contains the highest peak SNP (Chr9_19518843). Therefore, searching for candidate genes in the interval of qTL6 is also the focus of our next study.

To do it, we first checked the expression profiles of these four candidate genes from the Encyclopedia of Rice Transcriptome (TENOR) database. According to the TENOR database (Supplementary Figure S2), the candidate gene LOC_Os06g47720 has a higher expression level under salt stress conditions. The gene annotation of LOC_Os06g47720 is a serine threonine protein kinase BRI1-like 2 precursor. BRI1 (protein brassinosteroid insensitive 1) is the receptor kinase of BR (brassinosteroids), located on the cell membrane, and is a leucine-rich repeat (LRR) receptor-like serine/threonine kinase on the cell surface, which is crucial in the BR signaling pathway. BR is a plant steroid hormone, which plays a key role in growth and response to abiotic and biotic stress (Zhao et al., 2019; Ma et al., 2021). The plants adapt to various environmental stresses by changing their physiological and molecular processes, which are coordinated with changes of the levels on hormones (including brassinosteroids) in plant externally and internally (Bilal et al., 2021). The response of BR under salt stress may be mediated by BRI1, inhibiting the degradation of the endoplasmic reticulum to combat salt stress (Cui et al., 2012). BR signaling also counters salt stress by signaling cascades or initiating ethylene biosynthesis (Planas-Riverola et al., 2019). In this study, the expression level of LOC_Os06g47720 in S87 is much higher than that in S125, and LOC_Os06g47720 had far more mutation sites in S87 than in S125. By comparison of amino acid sequences, it also revealed that only the amino acids in S87 were changed and the translation was terminated in S125. These findings suggest that LOC_Os06g47720 may be involved in the regulation of ST through the BR pathway.

The candidate gene LOC_Os06g47820 is receptor-like kinases, which has the highest expression level under ABA treatment conditions (Supplementary Figure S2). ABA can coordinate with hormones such as auxin, gibberellin (GA) and cytokinin (CK) to regulate the response of plants to salt stress (Yu et al., 2020). Receptor-like kinases (RLKs) are a large family of proteins that exist on the surface of plant cell membranes. Their basic function is to transmit regulatory signals on the cell surface. Plant receptor-like protein kinases occupy important metabolic positions, and rice has about 1,130 RLK genes (Quynh-Nga et al., 2015). Plant RLKs are composed of intracellular, extracellular, and transmembrane regions (Ye et al., 2017). Receptor-like protein kinase RLK is widely involved in cell signal transduction and plant response to stress (Lemmon and Schlessinger, 2010). In recent years, several researchers studied the important role of RLKs in optimizing the response of plants to salt stress and other abiotic stresses (Zhou et al., 2018; Lin et al., 2020). In this study, the expression level of LOC_Os06g47820 in S125 was found much higher than that in S87. The gene had much more mutation sites in S125 than in S87. There are more changes in amino acids in S125 than in S87. These findings indicate that LOC_Os06g47820 is a candidate gene that may be involved in the regulation of ST in rice.

The candidate gene LOC_Os06g47850 encodes a zinc finger family protein. The zinc finger protein (ZFP) family is widely distributed in eukaryotic genomes and is one of the most important transcription factors, which plays an important role in plant growth and development and abiotic stress response (Mukhopadhyay et al., 2004; Sakamoto, 2004). More than 60 transcription factor families have been reported in plants (Iuchi, 2001). LOC_Os06g47850 has the highest expression level under cold stress conditions (Supplementary Figure S2). In our study, the expression level of LOC_Os06g47820 in S125 was much higher than that in S87. DNA sequence alignment results showed that the gene has no mutation sites. Therefore, the mechanism of this candidate gene remains to be elucidated.

The candidate gene LOC_Os06g47970 encodes a DUF1517 (domains of unknown function, DUF) which are a class of proteins whose functions have not been characterized and account for about 25% of the total protein family (Mudgal et al., 2015). According to the TENOR database (Supplementary Figure S2), LOC_Os06g47970 has the highest expression level under drought stress and as we know that salinity usually occurs at the same time as drought stress (Hu et al., 2006). In recent years, an increasing number of studies were conducted on the regulation of different DUFs family genes involved in plant growth and development and plant response to stress (biotic and abiotic stress; Li et al., 2018; Lv et al., 2019). We found a DUFs gene in the qTL4, i.e., OsSIDP366, which is a stress-induced DUF1644 protein and contains a DUF1644 domain, a C2H2 and a ring finger domain. OsSIDP366 is expressed in multiple tissues. The expression is higher in young roots, mature leaves, and leaf sheaths, but lower or no expression in internodes, mature seeds, lemma and glumes. High salt and drought treatments can induce the expression of OsSIDP366. OsSIDP366 may positively regulate salt and drought resistance in rice (Guo et al., 2016). In addition, we also found that the homologous gene AT5G57345 in Arabidopsis, a single copy gene, is localized to ER and expressed in the whole plant and induced expression in response
to abiotic stress. Although the function of AT5G57345 is unclear, overexpression can lead to increased tolerance to abiotic stress and increased ascorbic acid content (Bu et al., 2016). In addition, we also found that the expression level of LOC_Os06g47970 in S125 was much higher than that in S87. Sequence analysis found that LOC_Os06g47970 only had a pair of base substitutions in S125, i.e., one amino acid was changed in S125, while no change in S87. Therefore, LOC_Os06g47970 might be involved in the regulation of ST in rice.

This study lays a foundation for the functional analysis of the candidate genes in the regulation of salt tolerance in rice and the enrichment of the rice salt tolerance regulatory network. In addition, our newly discovered QTLs also lay the foundation for further research on the ST mechanism in rice in the future. The salt tolerance-related candidate genes and QTLs would provide important resources for molecular breeding and functional analysis of the salt tolerance during the seedling stage of rice. However, the four candidate genes identified in this study which involved in the regulation of salt stress in rice need further research and verification. In addition, the mechanism and the regulation pathway for these genes under salt stress in rice still need to be clarified. In future, the function of candidate genes related to salt tolerance will be studied by Crispr-Cas9 technology, which will help to precisely uncover the mechanisms of salinity tolerance at molecular level.

CONCLUSION

Eight seedling-stage salt-related traits within a core collection of rice landraces were evaluated under salinity stress (100 mm NaCl) in a growth chamber, and abundant phenotypic variations were observed for these traits. With 2,487,353 SNPs derived from an enrichment of 67,511 SNPs from SLAF-seq, GWAS was performed for the eight traits related to ST with a mixed linear model. In total, 65 QTLs were identified significantly associated with eight ST traits. These QTLs explained 13.47 to 28.11% of the phenotype variation. There are 8 QTLs for RNC, 14 QTLs for SNC, 10 QTLs for RN/K, 8 QTLs for RTRV, 13 QTLs for SN/K, 2 QTLs for SKC, 5 QTLs for SKD, and 5 QTLs for TRSA. Several QTLs in this study were overlapped with or were close to the previously reported candidate genes or QTLs related to ST. There are 6 genome regions containing co-localization QTLs (qTL1 – qTL6). Among them, a co-localization QTL qTL4 associated with the SKC, RN/K and SNC on chromosome 6, which explained 14.38–17.94% of phenotypic variation, was selected for further analysis. According to haplotype analysis, qRT-PCR analysis, and sequence alignment, it was finally determined that 4 candidate genes (LOC_Os06g47720, LOC_Os06g47820, LOC_Os06g47850, and LOC_Os06g47970) were related to ST. The results provide useful candidate genes for marker-assisted selection for ST in the rice molecular breeding programs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

MZ and WC designed the study. MZ, XW, SG, CS, YL, and DM performed data analyses. XW and JS performed GWAS and statistical analyses. MZ, XW, and CS performed searching candidate genes/QTLs. XW wrote the paper. JL provided the rice core collection. MZ and JL revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.847863/full#supplementary-material

**Supplementary Figure S1** | QQ plots of genome-wide association studies for the eight traits related to ST. A-H: QQ plots for RNC, SKC, SNC, RN/K, RTRV, RTRSA, RTRV, and RS/N/K.

**Supplementary Figure S2** | (A–C) DNA sequence analysis and amino acid sequence analysis for three candidate genes.

**Supplementary Figure S3** | Expression profiles in rice seedling under the various environmental conditions.

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