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QTL-seq Identifies Pokkali-Derived QTLs and Candidate Genes for Salt Tolerance at Seedling Stage in Rice (*Oryza sativa* L.)

Decha Songtoasesakul [1,2], Wanchana Aesomnuk [3], Sarinthip Pannak [3], Jonaliza Lanceras Siangliw [4], Meechai Siangliw [4], Theerayut Toojinda [4], Samart Wanchana [4,*] and Siwaret Arikit [3,5,*]

[1] Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand; decha.s@ku.th
[2] Center of Excellence on Agricultural Biotechnology: (AG-BIO/MHESI), Bangkok 10900, Thailand
[3] Rice Science Center, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand; waesomnuk@gmail.com (W.A.); srth.pn@gmail.com (S.P.)
[4] National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Pahonyothin Road, Khlong Luang, Pathum Thani 12120, Thailand; jonaliza.sia@biotec.or.th (J.L.S.); meechai@biotec.or.th (M.S.); theerayut@biotec.or.th (T.T.)
[5] Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
* Correspondence: samart.wan@biotec.or.th (S.W.); siwaret.a@ku.th (S.A.)

Abstract: Rice is a staple food crop that plays a pivotal role in global food security, feeding more than half of the world's population. Soil salinity is one of the most important global problems affecting rice productivity. Salt stress at the seedling stage inhibits root growth, impairs nutrient and water uptake, and affects overall plant vigor, resulting in poor establishment and reduced growth. Therefore, acquiring salt tolerance, especially at the seedling stage, is critical for successful rice production in salinity-affected areas. In this study, 160 RILs derived from a cross between Pokkali and KDML105 were evaluated for their salt tolerance at the seedling stage. QTL-seq analysis with this population identified nine QTLs associated with salt tolerance. Through a comprehensive examination of the effects of coding sequence variants of the 360 annotated genes within the QTLs and gene expression under salt stress, 47 candidate genes were prioritized. In particular, Os01g0200700 (metallothionein-like protein) and Os12g0625000 (O-acetylserine (thiol)lyase) were suggested as potential candidates based on annotated functions and expression data. The results provide valuable insights for improving rice productivity and resistance under salt stress conditions during the critical seedling stage.

Keywords: *Oryza sativa*; rice; salt stress; soil salinity; QTL-seq; Pokkali; KDML105

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1. Introduction

Rice is a staple food crop that plays a pivotal role in global food security, feeding a large proportion of the world's population [1]. However, rice cultivation faces significant challenges, and in many rice-growing areas, salinity is one of the major problems after drought [2]. Soil salinity currently affects about 33% of irrigated agricultural land and 20% of all cultivated land globally, which will increase further by 2050 [3]. Salinity, caused by the accumulation of salts in soil and irrigation water, affects rice growth and productivity [4]. Salinity stress can cause annual losses of 30–50% in rice production [5,6]. Typically, there is an average yield loss of approximately 12% in rice for every increase of one dS m^{-1} in soil EC value, although these effects may not be consistent across all cases [7]. The trait of salt tolerance has become an important rice breeding target in many Asian countries where large salinized rice fields occur [8,9].

Rice is a relatively highly salt-sensitive crop [10]. Salt stress adversely affects various stages of rice growth and development [11]. Among these stages, the seedling stage is particularly susceptible to salt stress [12]. Salt stress at the seedling stage inhibits root growth, impairs nutrient and water uptake, and affects overall plant vigor, resulting in poor establishment and reduced growth [13]. Therefore, acquiring salt tolerance, especially at the seedling stage, is critical for successful rice production in salinity-affected areas. Salt tolerance in plants is the ultimate manifestation of several physiological processes, including Na^+ uptake and exclusion, ionic balance (especially Na^+/K^+ ratio), and distribution [14]. Salt tolerance in rice is controlled by multiple genes with a complex genetic mechanism [15,16]. In addition, salt tolerance is controlled by complex and interacting genetic, molecular, and physiological mechanisms, such that numerous gene families and interaction networks are involved in regulating the response of rice to salinity [17,18].

Efforts have been made to understand the genetic basis of salt tolerance in rice by identifying quantitative trait loci (QTLs). To date, hundreds of QTLs for salt response have been identified using segregating populations derived from crosses between salt-tolerant and salt-sensitive varieties, and a number of candidate genes have been characterized [8,18–25]. A notable QTL, *Saltol*, associated with K^+/Na^+ homeostasis in the shoot was mapped to chromosome 1 using recombinant inbred lines (RILs) derived from a cross between IR29 and Pokkali [26]. The *SKC1* gene, which encodes a member of the HKT transporter, is located within the *Saltol* QTL region. *SKC1* in salt-tolerant rice varieties, e.g., Pokkali and Nona bokra, plays a critical role in potassium transport, an important physiological process affected by salt stress. Inclusion of *SKC1* in rice breeding programs has shown promising results in improving salt tolerance [27]. However, it is important to consider the limitations of *SKC1* in specific populations, as its efficacy may vary due to genetic variation or interactions with other genetic factors [26].

QTL mapping allows the discovery and characterization of genomic regions associated with specific traits and provides valuable insights into underlying genetic factors [28]. QTL mapping has traditionally relied on bi-parental mapping populations and molecular markers. However, recent advances in genomics and sequencing technologies have paved the way for more efficient and rapid methods for QTL identification. QTL-seq, a high-throughput sequencing-based approach, enables the identification of QTLs by comparing the genome sequences of two groups of individuals with contrasting phenotypes from segregating populations [29]. The method aims to identify genomic regions showing variations in the single nucleotide polymorphism (SNP) index between two pooled samples. QTL-seq has been used to rapidly identify genes or QTLs in various crops such as chickpea [30], peanut [31,32], barley [33], soybean [34], canola [35], cucumber [36–39], sesame [38], squash [40], and rice [29,41–49]. In this study, we used the QTL-seq approach to identify genomic regions associated with salt tolerance at the seedling stage of rice in an RIL population. RNA-seq was also used to prioritize candidate genes for the discovered QTL regions. The differentially expressed genes (DEGs) and their variants in the QTL regions for salt tolerance were identified for both validation and use in molecular breeding to improve salt tolerance in the future. The results of this study will contribute to the development of improved salt-tolerant rice varieties and facilitate sustainable rice production in salt-stressed regions.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions and SKC1 Genotyping

The mapping population used for identification of QTLs associated with salt tolerance using QTL-seq analysis composed of 160 recombinant inbred lines (RILs: F_7) derived from a cross of Pokkali (salt tolerant) and KDML105 (salt sensitive). The experiment was carried out in a greenhouse at the Rice Science Center, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand. The design of the experiment was randomized complete block design (RCBD) with four replications under normal condition and salt-stressed treatment (150 mM NaCl). Seeds of each RIL were germinated in a petri dish for three

days. Germinated seeds were then transferred to seedling hole-trays (200 holes per tray) containing clay soil from the rice field. In each replication, five seeds per line were grown in a row, one seed per hole, in the germinating trays. The plants were allowed to grow in the greenhouse for 14 days.

SKC1, encoding a member of the HKT transporters, was reported as a candidate gene in a major QTL on chromosome 1 (*Saltol*), and the *SKC1* genotype of Pokkali was associated with salt tolerance [50]. A KASP marker specific for a nonsynonymous SNP (C/T) on exon 1 at position 1:11462725 in *SKC1* with distinguishable alleles in Pokkali (allele T) and KDML105 (allele C) was used to divide RILs into two groups based on the *SKC1* alleles of the parents. DNA was extracted from young leaf of each RIL using the DNeasy Plant Mini Kit (QIAGEN, Germany) and KASP assay was performed according to the LGC Genomics manual (http://www.lgcgenomics.com, accessed on 1 July 2023). The 96-well format was utilized for the KASP reaction, with a total reaction volume of 5 µL. This reaction mixture was composed of 2 µL of DNA template, 0.075 µL of assay mix, and 2.5 µL of master mix. The amplification process was initiated at 94 °C for 5 min, followed by 10 cycles involving 94 °C for 20 s and 61 °C for 60 s, using a touchdown method with a gradual temperature reduction of 0.6 °C per cycle. Subsequently, an additional 27 cycles were conducted at 94 °C for 20 s and 55 °C for 30 s, followed by a resting period at 37 °C for 1 min. To determine the fluorescence signals, the final PCR products were analyzed using the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Watham, MA, USA). The KASP marker for *SKC1* (unpublished) was courtesy of the Innovative Plant Biotechnology and Precision Agriculture Research Team, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand.

2.2. Salinity Treatment and Phenotypic Evaluation

The seedlings aged 14 days after sowing (DAS) were used to screen for salinity tolerance following the methods described in [51]. The nutrient solution in control (normal condition) was Bangsai nutrient solution (1:100) without NaCl, whereas the salinity stress treatment was the same solution supplemented with 150 mM NaCl. The trays containing seedlings were placed in two cement tanks (900 cm $\times$ 900 cm $\times$ 50 cm) for control and salinity treatment, respectively. During the growth of the seedlings, the solution pH was set to 6.0–6.5 and the salinity was set to the desired level every day. A water pump was installed to flow the water within each tank. Twelve days after treatments, all plants were evaluated for the plant injury caused by salinity stress using the salt injury score (SIS) according to the IRRI's Standard Evaluation System [52] as follows: 1 = normal growth, no leaf symptoms (highly tolerant), 3 = nearly normal growth, but leaf tips or few leaves whitish and rolled (tolerant), 5 = growth severely retarded; most leaves rolled; only a few elongating (moderate), 7 = complete cessation of growth; most leaves dry; some plants dying (sensitive), and 9 = almost all plants dead or dying (highly sensitive). Pokkali and FL496 (a salt-tolerant RIL derived from IR29 $\times$ Pokkali) were used as salt-tolerant check varieties and KDML105 and IR29 (an indica variety referred as salt-sensitive standard) [53] were used as susceptible check varieties.

In addition to salinity injury evaluation, another set of salinity stress experiments were conducted and Na^+ and K^+ contents in 14-day-old seedling shoots were measured in 160 RILs at ten days after treatment. In order to quantify the level of Na^+ and K^+ concentrations of rice samples, shoots were harvested at 10 days after salt treatment. All samples were rinsed with tap water and washed with distilled water. The samples were dried, and the exact amount of 0.2 g was carefully weighed, dissolved in 10 mL acetic acid (100 mM), and kept at 90 °C for 2 h. The Na^+ and K^+ were determined by using inductively coupled plasma optical emission spectrometry (ICP-OES, Avio 200) with unit of mg ion per g dry weight sample.

2.3. Sample Bulking, DNA Isolation and Whole-Genome Sequencing

RILs with the lowest SIS values (SIS < 4.8) and RILs with the highest SIS values (SIS > 7.2) were selected and grouped as the salt-tolerant bulk (T-bulk) and salt-sensitive bulk (S-bulk), respectively. High-quality genomic DNA was isolated from the young leaves of each selected plant in the two bulks along with the parents (Pokkali and KDML105) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The 33 RILs in the two groups together with the parents Pokkali and KDML105 were sequenced in the whole genome individually using an MGI-seq platform at China National GeneBank (CNGB; Shenzhen, China).

2.4. QTL-seq Analysis

Raw reads were processed by Trimmomatic software version 0.30 [54] to remove adapter sequences and low-quality reads. The clean read data were used to perform QTL-seq analysis using the QTL-seq pipeline v2.2.2 (https://github.com/YuSugihara/QTL-seq, accessed on 1 July 2023). The Pokkali-based pseudo reference genome was generated by aligning clean reads of Pokkali to the publicly available reference rice genome (Nipponbare: IRGSP1.0) and replacing the genome of Nipponbare with the variants of the Pokkali parent. Clean reads were obtained from each F_2 sample in equal numbers and pooled into T and S bulks. SNP calling and SNP index calculations were performed using the QTL-seq pipeline as previously described [29]. Briefly, the SNP index of the T-bulk and S-bulk was defined as the ratio between the Pokkali's SNP alleles and the total number of reads corresponding to the SNP. The Δ(SNP index) was calculated according to the formula Δ(SNP index) = [(SNP index of S-bulk) − (SNP index of T-bulk)]. A sliding window analysis was performed by averaging the Δ(SNP index), in which the window size was set to 1 Mb, with 250 kb steps. The minimum aligned read depth cutoff for obtaining SNPs was set to 15 reads.

2.5. Candidate Gene Annotation and Prioritization

The candidate genes within the detected QTL regions were obtained from the RAP-DB (https://rapdb.dna.affrc.go.jp; accessed on 1 July 2023). The genes annotated as a hypothetical gene and genes without an annotation were excluded. The candidate genes were further filtered to obtain those containing SNPs/Indels with moderate or high effects. The SNP effects were determined using a variant effect predictor (VEP; https://www.plants.ensembl.org/Oryzasativa/Tools/VEP; accessed on 1 July 2023).

2.6. Differential Gene Expression Analysis of Candidate Genes

The expression of candidate genes within each detected QTL was analyzed by transcriptome analysis of Pokkali and KDML105 under salt treatment at 24 and 72 h. The shoot tissues of 14-day-old seedlings of Pokkali and KDML105 were collected in three replications at 24 and 72 h after salt treatment. Total RNA was extracted using the TRIzol method (Life Technologies). The RNA samples were sequenced at China National GeneBank (CNGB; Shenzhen, China) using an MGI-seq platform. Quantitative gene expression measurements based on RNA-seq data for Pokkali and KDML105 were performed using Salmon (v.0.30) [55] with the Nipponbare transcriptome as a reference (Osativa_323_v7.0: Phytozome database version 12.0). Transcript counts for all expressed genes in KDML105 and Pokkali 24 and 72 h after salt treatment in three replicates were used to perform differential gene expression analysis with DEseq2 [56]. Differentially expressed genes 24 and 72 h after salt treatment comparing Pokkali and KDML105 were determined with a p-value cutoff of $p < 0.01$ and a false discovery rate (FDR) < 0.2. Cluster analysis of differential gene expression and heatmap plots was performed using SRPLOT (https://www.bioinformatics.com.cn/en; accessed on 1 July 2023), a free online platform for data analysis and visualization.

3. Results

3.1. Phenotypic Screening of Salt Tolerance at Seedling Stage in RILs with Different SKC1 Alleles

A total of 160 recombinant inbred lines (RILs: F_7) derived from a cross of KDML105 and Pokkali were genotyped with a KASP marker specific for *SKC1* (the SNP C/T at the position 1:11,462,725) to divide the plants into two groups: (1) the group of plants containing the Pokkali allele of *SKC1* (allele T), hereafter referred to as *SKC1*$^{\mathrm{Pokkali}}$, and (2) the group of plants containing the KDML105 allele of *SKC1* (allele C), hereafter referred to as *SKC1*$^{\mathrm{KDML105}}$. As a result, 81 RILs were found to contain the *SKC1*$^{\mathrm{Pokkali}}$ allele and another 71 contained the *SKC1*$^{\mathrm{KDML105}}$ allele (Figure S1). Another eight RILs were heterozygous for *SKC1*. As a result of salt tolerance screening, the phenotype of salt injury (salt injury score: SIS) of the parents Pokkali and KDML105 differed significantly, as the mean SIS score was 4.8 in Pokkali and 7.2 in KDML105 (Figure 1A). The average SIS values in RILs containing the *SKC1*$^{\mathrm{Pokkali}}$ allele varied from 2.1 to 8.3, with an average of 5.5, and the values in RILs containing the *SKC1*$^{\mathrm{KDML105}}$ allele varied from 3.2 to 8.1, with an average of 5.8. The average SIS values in the two groups were not significantly different (Figure 1A). The results of Na^+ and K^+ content analysis showed that the shoot Na^+/K^+ ratio of Pokkali was 1.1 and that of KDML105 was 2.4 (Figure 1B). Interestingly, although the SIS values of the two RIL groups were not significantly different, the average Na^+/K^+ ratio of the RILs in the *SKC1*$^{\mathrm{POKKALI}}$ group and in the *SKC1*$^{\mathrm{KDML105}}$ group was significantly different, being 1.04 in the first group and 1.49 in the second group (Figure 1B). According to these results, it is likely that *SKC1* had little effect on salt injury and that the Na^+/K^+ ratio in this RIL population was not related to the variation of SIS. This suggests that *SKC1* has less effect on salt tolerance (determined by SIS) in this population and that the tolerance phenotype may be controlled by other genes.

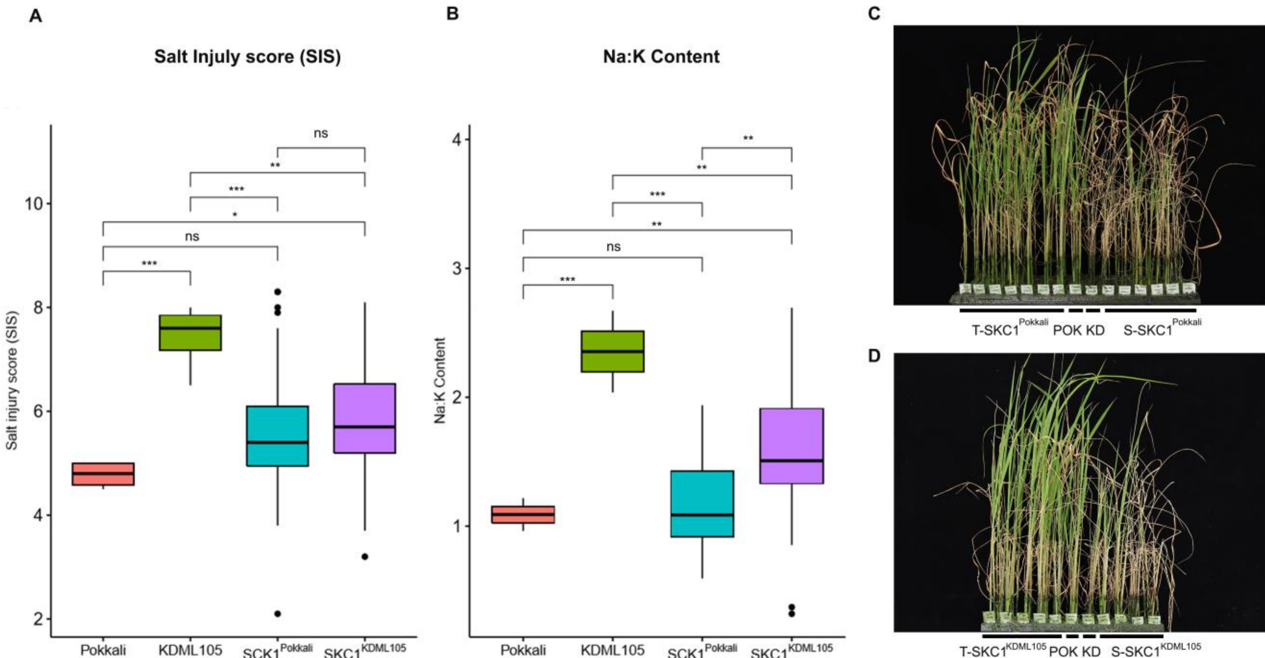

Figure 1. Evaluation of salt injury and analysis of Na^+ and K^+ content on Pokkali, KDML105, and RILs in *SKC1*$^{\mathrm{Pokkali}}$ and *SKC1*$^{\mathrm{KDML105}}$ groups. (**A**) Evaluation of salt damage and (**B**) results of analysis of Na^+/K^+ content comparing Pokkali, KDML105, RILs in *SKC1*$^{\mathrm{Pokkali}}$, and *SKC1*$^{\mathrm{KDML105}}$ groups. (**C**) The visual damage observed in the parents, Pokkali (POK) and KDML105 (KD), and representatives of tolerant RILs with Pokkali allele of *SKC1* (T-*SKC1*$^{\mathrm{Pokkali}}$) and representatives of sensitive RILs with KDML105 allele (S-*SKC1*$^{\mathrm{Pokkali}}$). (**D**) The visual damage observed in Pokkali, KDML, T-*SKC1*$^{\mathrm{KDML105}}$, and S-*SKC1*$^{\mathrm{KDML105}}$. ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$; ns, not significant.

3.2. Segregation of the Salinity Tolerance Phenotype in the RIL Population and Construction of Salt-Tolerance (T) and Salt Sensitive (S) Bulks

To identify genomic regions associated with salt tolerance in this population, we performed QTL-seq analysis considering the whole population regardless of *SKC1* genotypes. We used SIS values to determine plants with different salt tolerance phenotypes. In the 160 RILs, SIS values varied from 2.1 to 8.3, with an average of 5.7 (Figure 2). The distribution of SIS values in this population was near normal distribution, indicating polygenic segregation. A total of 17 plants with high tolerance to salt stress (SIS values of 2.1–4.8) were selected as salt tolerant group and another 16 plants with high sensitivity to salt stress (SIS values of 7.2–8.3) were selected as salt sensitive group.

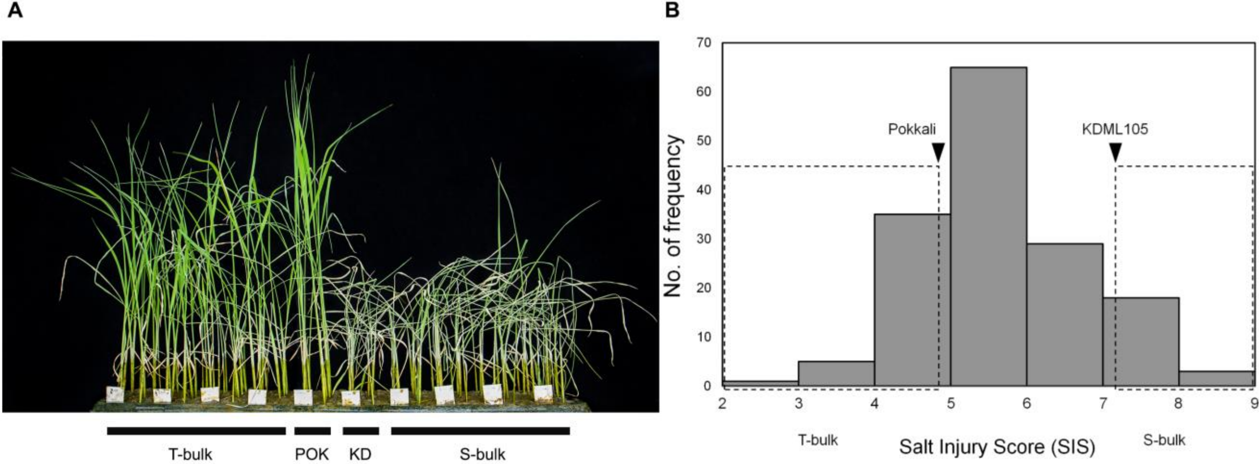

Figure 2. Salinity tolerance evaluation of two parents and the RIL (F_7) population. (**A**). Symptoms of salt damage in representative RILs in salt-tolerant bulk (T-bulk) and in salt-sensitive bulk (S-bulk), compared with the tolerant (Pokkali: POK) and susceptible parents (KDML105: KD). (**B**) Frequency distribution of the salt injury score (SIS) in the RIL (F_7) population. Dashed rectangle indicates plants selected to generate T-bulk and S-bulk.

3.3. Whole-Genome Sequencing of Parents and the Two Bulks of RILs

In the samples of Pokkali and KDML105, a total of 37.44 million reads and 41.69 million reads were obtained, respectively. This corresponded to approximately 10.38-fold and 11.44-fold coverage of the rice genome (with an approximate size of 400 Mb). Each of the 33 RIL samples yielded 20 million clean reads, providing about 5x genome coverage. The sequences of 17 RILs exhibiting salt-tolerant characteristics were combined to form the tolerant bulk (T bulk), while those of 16 RILs displaying salt-sensitive traits were combined to create the sensitive bulk (S bulk). Regarding the read alignments to the Nipponbare reference genome, the proportions of aligned reads in Pokkali, KDML105, T bulk, and S bulk were 94.21%, 94.03%, 90.61%, and 90.06%, respectively (Table 1).

Table 1. Summary of whole-genome sequencing data of parental lines and tolerant and sensitive bulks.

Sample	Cleaned Reads (Million)	Cleaned Base (Gb)	% Alignment	Average Depth Coverage (x)
Pokkali	37.44	3.74	94.21	10.38
KDML105	41.69	4.16	94.03	11.44
Tolerant bulk (17)	340.00	34.00	90.61	84
Sensitive bulk (16)	320.00	32.00	90.06	80

3.4. Variant Detection and QTL-seq Analysis for Salt Tolerance

QTL-seq analysis used the common SNP variants identified in both the tolerant (T) and sensitive (S) bulks and determined by read mapping against the Pokkali parental genome. Initially, 2,946,843 SNPs were identified in the two bulks, which required a read-support criterion of at least five reads (Table 2). However, to ensure robustness of the results, a more stringent read support criterion of 15 reads was applied to filter out the original set of SNPs. Consequently, 213,922 SNPs were detected with high confidence across the 12 chromosomes and used to calculate the SNP index (Table 2; Figure S2).

Table 2. Chromosome-wise distribution of common single nucleotide polymorphisms (SNPs) and Insertion-Deletion (InDels) between the two bulks.

Chr	Length (bp)	No. of SNPs (Depth > 5)	No. of InDels (Depth > 5)	SNP (Depth > 15)	InDels (Depth > 15)
1	43,270,923	338,100	74,011	26,989	8584
2	35,937,250	287,762	61,423	18,595	5970
3	36,413,819	270,715	58,493	20,410	5849
4	35,502,694	235,868	45,927	24,155	6498
5	29,958,434	225,149	45,596	22,070	5790
6	31,248,787	254,020	50,208	14,164	4220
7	29,697,621	238,007	45,750	11,104	3417
8	28,443,022	231,655	45,177	18,061	5523
9	23,012,720	187,085	36,248	10,646	2913
10	23,207,287	205,544	37,962	8752	2734
11	29,021,106	252,201	45,859	15,567	4434
12	27,531,856	220,737	41,052	23,409	6104
Total	373,245,519	2,946,843	587,706	213,922	62,036

The SNP index was calculated for the selected SNPs in both the tolerant (T) and sensitive (S) bulks, using the Pokkali parental genome as a reference. The Δ (SNP index) was then determined by subtracting the SNP index values in the T-bulk from those in the S-bulk. Moving windows were applied to calculate the average SNP indexes for SNPs located within a 1-Mb region with a 250-kb increment. To identify genomic regions associated with salt tolerance, the SNP index and Δ (SNP index) were plotted across the 12 rice chromosomes. This analysis led to the identification of nine candidate genomic regions on chromosomes 1, 2, 3, 6, 8, 10, 11, and 12, where the average Δ(SNP index) exceeded the 99% confidence interval (Figure 3; Table 3). We determined the QTL region for each QTL within a 500-kb interval surrounding the peaks. In the T-bulk, the average SNP index for these QTLs ranged from 0.10 to 0.40, while in the S-bulk, it ranged from 0.65 to 0.99. The average Δ(SNP index) for these QTLs ranged from 0.55 to 0.69 (Table 3). Interestingly, some of these QTLs, such as *qST1.2*, *qST2*, *qST3*, *qST6*, *qST8*, and *qST12*, were found to overlap with previously reported QTLs or genes associated with salt tolerance (Table 3).

Table 3. Summary of the genomic region associated with salinity tolerance in rice.

QTL	Chr.	QTL-Region (Mb)	p99 [a]	p95 [b]	SNP Index (T-Bulk)	SNP Index (S-Bulk)	Δ(SNP Index)	Reported QTLs/Genes
qST1.1	1	5.0–5.50	0.34	0.26	0.28	0.86	0.58	
qST1.2	1	41.0–41.50	0.34	0.26	0.10	0.65	0.55	*qDTS1–2* [57]; *qSIS1.41* [58]; *qSIS1* [59]; *qST1.1* [60]; *OsHAK2*, *OsHAK5*, *OsHAK6* [61]
qST2	2	27.25–27.75	0.33	0.25	0.17	0.74	0.57	*qCHL2.20*, *qRTL2.26* [58]
qST3	3	31.70–32.25	0.35	0.27	0.25	0.89	0.64	*qCHL3*, *qSES3* [26]; *qNaK3.32* [58]
qST6	6	3.5–4.0	0.34	0.26	0.21	0.79	0.58	*qSIS6.5*, *qSHL6.5*, *qDWT6.5* [58]
qST8	8	26.5–27.0	0.35	0.27	0.40	0.99	0.59	*qlogSIS8.24*, *qRTL8.27* [58]; *qRSKC8* [59]
qST10	10	17.75–18.25	0.34	0.26	0.27	0.87	0.60	
qST11	11	5.75–6.25	0.34	0.26	0.13	0.82	0.69	
qST12	12	26.50–27.00	0.34	0.26	0.16	0.75	0.58	*qSHL12.25* [59]

[a] p99 means confidence interval (99%); [b] p95 means confidence interval (95%).

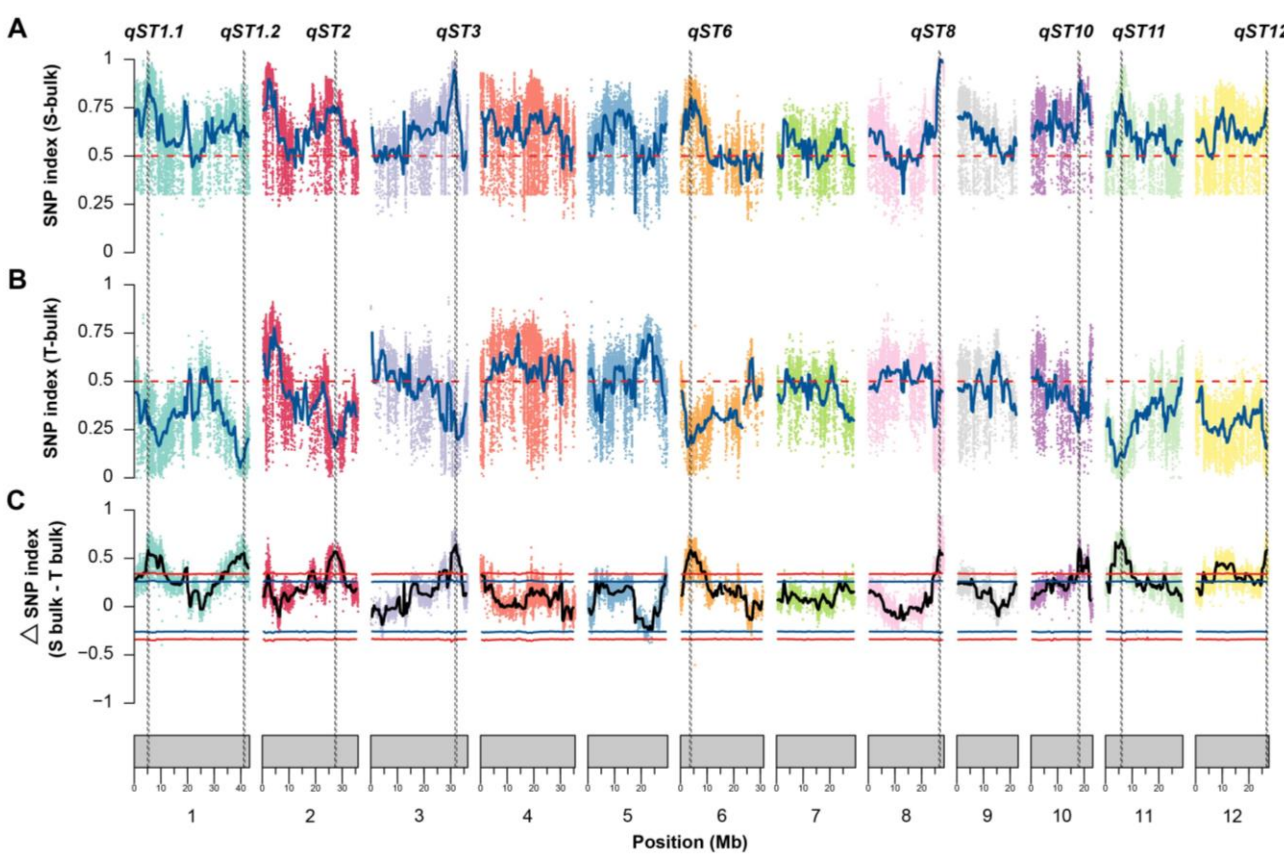

Figure 3. Diagrams showing the SNP index of the T-bulk and S-bulk and the Δ(SNP index) across 12 rice chromosomes. (**A**) The SNP index diagrams for the salt-sensitive bulk (S-bulk). (**B**) The SNP index diagrams for the salt-tolerant bulk (T-bulk). (**C**) The Δ(SNP index) diagram. The plots of the moving windows of the average SNP index with a window size of 1 Mb and 250 kb steps are shown as blue lines in (**A**,**B**) and as a black line in (**C**). The blue and red line pairs in (**C**) correspond to the 95% and 99% confidence intervals, respectively. The dashed vertical bar in the figure also indicates the peak SNPs identified in the candidate regions.

3.5. Annotation and Prioritization of Candidate Genes in QTL Regions

To determine the candidate genes for the discovered QTLs, we obtained genes within each QTL based on the RAP-DB annotation. The total number of candidate genes with an annotated function within these QTLs was 360 genes, ranging from 32 to 50 genes in each QTL (Table S1). We prioritized this number of candidate genes by filtering genes with a functional variant and a contrasting SNP index between the two bulks. As a result, only *qST1.1*, *qST1.2*, *qST6*, *qST10*, *qST11*, and *qST12* were found to contain 2, 1, 2, 1, 5, and 2 genes, respectively, with a functional variant and contrasting SNP index between the two bulks (Table 4).

Differential expression analysis was also performed to prioritize candidate genes based on transcriptome data from 14-day-old seedlings of both parent plants KDML105 and Pokkali observed 24 h and 72 h after salt stress (HAS). A total of 34 genes of all nine QTLs were found to be differentially expressed between the two parent plants (Table 5; Figure 4 and Table S2). Of these, 17 and 28 genes were differentially expressed 24 and 72 HAS, respectively. Eleven genes were differentially expressed at both 24 and 72 HAS (Table 5). These included genes encoding an esterase (Os01g0934900), a 40S ribosomal protein (Os03g0773150), a kinesin (Os03g0773600), a glucose-6-phosphate isomerase (Os03g0776000), an RNA-binding protein (Os03g0776000), a cytochrome b-c1 complex subunit 8 (Os06g0177000), two cation antiporters (Os08g0534350 and Os08g0535000), an ankyrin (Os08g0539600), various classes of plant disease resistance proteins (Os08g0539600, Os11g0212100 and Os11g0215100,

Os12g0629700, Os12g0630200), a mevalonate and galactokinase (Os11g0217300), and an O-acetylserine(thiol)lyase (Os12g0625000). Among the 17 DE genes that were strongly expressed in Pokkali at both 24 and 72 HAS were Os01g0200700 (metallothionein-like protein) from *qST1.1* and Os12g0625000 (O-acetylserine (thiol)lyase) from *qST12*.

Table 4. Candidate genes that contain nonsynonymous SNPs (missense variants) with contrasting SNP index in the two bulks.

QTL	Chr	Pos	SNP Index T-Bulk	SNP Index S-Bulk	Delta SNP Index	Pokkali	KDML105	SNP Effect	Strand	Gene ID	Description
qST1.1	1	5,288,678	0.45	0.94	0.49	G	A	GCA > GTA	-	Os01g0197900	γ-clade RNA-dependent RNA polymerase 3
	1	5,313,606	0.39	0.94	0.55	C	T	GGG > GAG	-	Os01g0198000	γ-clade RNA-dependent RNA polymerase 4
qST1.2	1	41,055,751	0.12	0.71	0.59	T	C	TAC > CAT	+	Os01g0935300	Similar to cullin-1
qST6	6	3,594,804	0.24	0.85	0.62	T	A	ATT > TTT	-	Os06g0171600	Membrane insertion protein, OxaA/YidC domain containing protein
	6	3,687,651	0.23	0.84	0.62	T	C	ATT > ACT	+	Os06g0173000	Armadillo-type fold domain containing protein
qST10	10	18,151,130	0.36	0.93	0.57	C	T	AGG > AAG	-	Os10g0481400	Similar to Zinc finger, C3HC4 type family protein
qST11	11	5,909,429	0.10	0.86	0.76	T	C	AAG > GAG	-	Os11g0213700	Leucine-rich repeat, typical subtype containing protein
	11	5,909,645	0.15	0.90	0.76	G	C	CAG > GAG	-	Os11g0213700	Leucine-rich repeat, typical subtype containing protein
	11	6,024,033	0.00	0.33	0.33	C	T	GTC > ATC	-	Os11g0215400	Peptidase aspartic, catalytic domain containing protein.
	11	6,063,875	0.16	0.93	0.77	C	T	GCG > GTG	+	Os11g0216000	Pyruvate kinase
	11	6,147,820	0.17	0.89	0.72	C	A	GTG > TTG	-	Os11g0218100	Similar to RNApol24
qST12	12	26,580,410	0.15	0.86	0.71	G	A	GAA > AAA	+	Os12g0622500	Homologue of the archaeal topoisomerase VIA
	12	26,602,781	0.14	0.87	0.74	C	T	GCA > GTA	+	Os12g0622900	Mov34/MPN/PAD-1 family protein

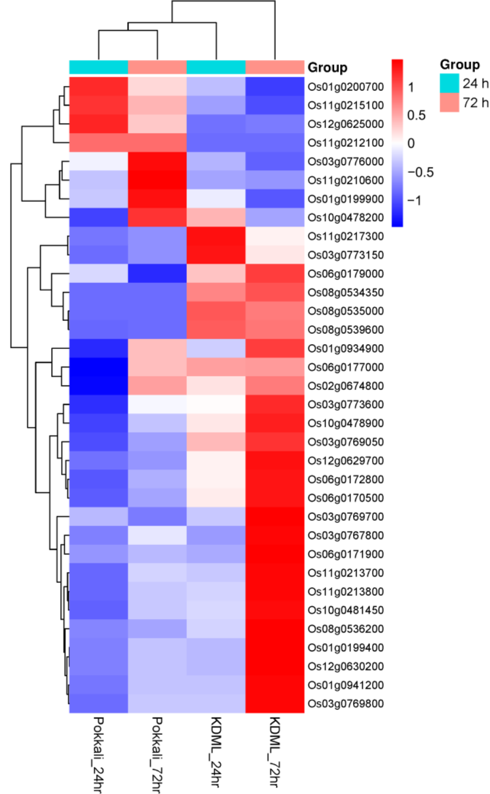

Figure 4. Cluster analysis of differential gene expression. Heatmap of 34 genes showing differential expression between Pokkali and KDML105 at 24 and 72 h after salt treatment. Expression values for each gene (row) are normalized across all samples (columns) by a Z-score. Both column and row clustering were applied.

Table 5. Differentially expressed genes within the detected QTLs in Pokkali (PK) and KDML105 (KD). Expression level is expressed in units of transcript per million (TPM).

QTL	Chr	Locus ID	24 h after Salt Stress			72 h after Salt Stress			Description
			PK (TPM)	KD (TPM)	*p*-Value	PK (TPM)	KD (TPM)	*p*-Value	
qST1.1	1	Os01g0199400	17.33	24.67	ns *	26.33	67.33	0.01	Alpha/beta hydrolase family protein
	1	Os01g0199900	15.33	16.33	ns	23.00	12.33	0.00	Phosphoribosylaminoimidazole carboxylase catalytic subunit
	1	Os01g0200700	4529.00	2433.33	ns	3203.33	1442.00	0.01	Metallothionein-like protein (Tolerance to salinity and heavy metal stresses)
qST1.2	1	Os01g0934900	3.67	19.33	0.01	30.00	42.33	ns	Esterase PIR7A
	1	Os01g0941200	3.00	11.33	ns	11.33	43.33	0.00	Glucan endo-1,3-beta-glucosidase GII precursor
qST2.1	2	Os02g0674800	13.00	34.67	0.00	40.00	42.67	ns	Homeodomain leucine zipper class IV transcriptional factor
qST3	3	Os03g0767800	128.33	138.00	ns	171.33	292.00	0.00	Cold acclimation protein WCOR413-like protein
	3	Os03g0769050	2.00	10.00	0.01	4.67	14.33	0.01	Similar to EMB2756
	3	Os03g0769700	2.33	2.67	ns	1.33	8.00	0.01	Uncharacterised conserved protein
	3	Os03g0769800	16.33	21.67	ns	21.67	39.33	0.01	Homeodomain-leucine zipper protein interfascicular fiberless 1 (Revoluta)
	3	Os03g0773150	99.00	226.00	0.01	110.00	156.33	ns	40S ribosomal protein S29 (salt tolerance (TO:0006001))
	3	Os03g0773600	32.00	72.33	0.00	70.33	113.00	0.00	Kinesin, motor region domain containing protein
	3	Os03g0776000	56.00	51.33	0.01	74.00	45.67	0.00	Glucose-6-phosphate isomerase, cytosolic A
qST6	6	Os06g0170500	38.33	111.00	0.00	66.00	197.67	0.00	RNA-binding protein-like
	6	Os06g0171900	50.67	54.67	ns	59.00	136.00	0.00	WD40 subfamily protein, Salt stress
	6	Os06g0172800	3.00	9.67	ns	6.33	18.00	0.01	Alkaline alpha galactosidase 2
	6	Os06g0177000	49.33	120.33	0.00	114.33	121.00	ns	Cytochrome b-c1 complex subunit 8
	6	Os06g0179000	36.00	42.33	ns	24.00	51.33	0.00	Glycoside hydrolase family 79
qST8	8	Os08g0534350	0.00	13.33	0.00	0.00	16.00	0.00	Cation cation antiporter
	8	Os08g0535000	0.00	5.67	0.00	0.00	5.00	0.00	Cation cation antiporter
	8	Os08g0536200	0.00	2.33	ns	1.00	11.67	0.01	trans-membrane plant subgroup domain containing protein
	8	Os08g0539600	0.00	74.33	0.00	0.67	68.67	0.00	Ankyrin repeat domain-containing protein 2
qST10	10	Os10g0478200	469.00	770.67	ns	917.00	581.67	0.00	NAD-dependent cytosolic malate dehydrogenase (CMDH)
	10	Os10g0478900	21.67	44.33	ns	35.33	65.67	0.01	Multi-organelle localized protein, Control of leaf senescence, Disease resistance, Salt tolerance
	10	Os10g0481450	15.67	23.33	ns	22.33	42.00	0.01	Zinc finger, C3HC4 type family protein
qST11	11	Os11g0210600	5.67	5.00	ns	13.00	4.67	0.01	Alcohol dehydrogenase
	11	Os11g0212100	2.33	0.00	0.01	2.33	0.00	0.01	Similar to NBS-LRR disease resistance protein family-4
	11	Os11g0213700	2.33	6.00	ns	6.33	17.33	0.01	Leucine-rich repeat, typical subtype containing protein
	11	Os11g0213800	3.67	8.67	ns	8.33	22.67	0.00	NBS-LRR disease resistance protein
	11	Os11g0215100	28.33	11.33	0.00	21.00	6.67	0.00	Plant disease resistance response protein family protein
	11	Os11g0217300	8.33	22.67	0.01	9.33	14.00	ns	Mevalonate and galactokinase family protein
qST12	12	Os12g0625000	326.67	156.00	0.00	249.67	161.00	ns	O-acetylserine(thiol)lyase, (stress trait (TO:0000164))
	12	Os12g0629700	0.67	9.00	0.00	2.67	21.00	0.00	Thaumatin-like protein precursor
	12	Os12g0630200	2.67	13.00	0.01	15.00	72.67	0.00	Thaumatin, pathogenesis-related family protein

* ns stands for "not significant".

4. Discussion

Salinity is one of the most important abiotic stresses that negatively affects various agricultural crops and can limit crop growth and productivity. For decades, efforts have been made to map genes and QTLs associated with salinity tolerance. Hundreds of QTLs associated with salt responses and salt tolerance have been reported in rice [8,18–22,24–26,50,62,63]. Among these, *Saltol* co-localized with *qSKC-1* on chromosome 1 was reported to be an important QTL associated with K^+/Na^+ homeostasis in the shoot and contributing to salt tolerance at the seedling stage [22,26]. *Saltol* has been used in rice breeding programs to develop salt-tolerant varieties [26,27]. However, since the mechanism of salt tolerance is extremely complex, not all salt tolerance can be explained by *Saltol* [64]. The *SKC1 (OsHKT1;5)* gene encoding a member of the high-affinity K^+ transporter (HKT) was cloned in the *Saltol/qSKC-1* locus [65]. It has been suggested that the function of *SKC1* is involved in the regulation of Na^+/K^+ homeostasis under salt stress [65,66]. According to

the results in our study, we confirmed that *SKC1* may affect Na^+/K^+ content in shoots of the RIL population (F_7-RILs) derived from KDML105 × Pokkali, because the plants carrying the *SKC1*$^{\text{Pokkali}}$ allele had significant lower Na^+/K^+ ratio in shoots. However, it is likely that the variation in SIS values in this population is not due to the *SKC1* or *Saltol* locus. The RIL plants with the *SKC1*$^{\text{Pokkali}}$ allele had a variable SIS score (2.1–8.3) like those with the *SKC1*$^{\text{KDML105}}$ allele (3.2–8.1), and the means of the two groups were not significantly different. Therefore, we suspected that other genes play an important role in the phenotype of salt tolerance in this population and not *SKC1*. This suggests that there is probably still a great opportunity to find more novel genes/QTLs involved in salt tolerance in rice and that it would be beneficial to obtain more genetic information on this trait.

The QTL-seq method is a combination of bulk-segregant analysis (BSA) and next-generation sequencing technologies that can rapidly identify the chromosome region where the genes or QTLs of interest are located [29]. Using the QTL-seq analysis with the RIL population, we identified nine genomic regions on eight chromosomes associated with salt tolerance. Among these, six QTLs, i.e., *qST1.2*, *qST2*, *qST3*, *qST6*, *qST8*, and *qST12*, were found to overlap with the previously reported QTLs/genes associated with SIS and other physiological and morphological traits responding to salt stress (Table 3). In this study, no QTL associated with SIS was identified that overlapped with the region of *Saltol* and *SKC1* (chr.1: 11,458,955–11,463,442). This is similar to a previous report using the population derived from the same salt-tolerant donor, Pokkali [59]. This confirms that the phenotype of salt tolerance in this population is unlikely to be affected by the action of *SKC1*. Pokkali, a landrace widely known for its tolerance to salt stress, has been widely used as a salt-tolerant donor in rice breeding programs as well as in QTL mapping studies to identify QTLs associated with salt tolerance [26,58,59,67]. It was reported that *Saltol* identified from Pokkali was not highly associated with salt tolerance based on overall visual performance at the seedling stage [26], suggesting that additional QTLs associated with salt tolerance derived from Pokkali exist. Among the nine QTLs identified, *qST1.2* was located in the same genomic regions as *qSIS1*, a QTL previously identified for SIS based on conventional QTL mapping with a population of 148 RILs from a cross between IR29 and Pokkali and a set of 14,470 SNPs [59]. In addition, *qST3* was found to be co-localized with the previously reported *qSES3* associated with seedling stage salinity tolerance based on SIS values [26]. In the previous study, two additional QTLs were also identified for SIS on chromosomes 4 and 12 [68]. However, these two QTLs were not found to overlap with the QTLs identified in our study. On the other hand, *qST1.1*, *qST10*, and *qST11* identified in this study may be novel QTLs for salt tolerance at the seedling stage, which have not been identified in previous QTL mapping studies using the same salt-tolerant donor, Pokkali [26,58,67].

In a conventional QTL mapping study, candidate genes are unlikely to be proposed without further fine mapping to narrow down the region. With the QTL-seq approach, which involves bulk-segregant analysis and whole-genome sequencing, QTLs associated with traits of interest can be rapidly identified, and with the integration of transcriptome analysis, there is a greater chance that the potential candidate genes within the discovered QTLs can be suggested. In this study, a total of 360 genes within a 500-kb interval region were annotated for the nine QTLs. We used two strategies to prioritize the candidate genes: (1) filtering the genes that contain functional variants, e.g., SNPs with nonsynonymous effect, and that have contrasting SNP indices in the two bulks, and (2) filtering the genes that are differentially expressed between plants with contrasting phenotypes. A total of 13 genes were prioritized based on the identification of functional variants and 34 genes were prioritized based on the differential gene expression analysis. Based on the functional annotation from the RAP-DB, four genes contain an annotated function relevant to salt or other abiotic stresses. These include Os01g0200700 (metallothionein-like protein), Os03g0773150 (40S ribosomal protein S29), Os06g0171900 (WD40 subfamily protein), and Os12g0625000 (O-acetylserine(thiol)lyase, cysteine synthase). Among the 47 candidate genes, there was no overlap between the two groups of genes that were prioritized. According to the results in this study, we suggest that both candidate-gene prioritization methods

should be considered complementarily to obtain a more comprehensive set of potential candidate genes.

Among the 34 differentially expressed genes (DEGs), many of them were more highly expressed in KDML105 (salt-sensitive) than in Pokkali (salt-tolerant). However, there were two salt-stress or other stress-related genes that were more highly expressed in Pokkali than in KDML105, namely Os01g0200700 (metallothionein-like protein) and Os12g0625000 (O-acetylserine(thiol)lyase). Metallothionein (MT) proteins are low-molecular-weight, cysteine-rich, and metal-binding proteins that play important roles in the maintaining of metal homeostasis, detoxification, and stress response [69]. *O-acetylserine(thiol)lyase* or *OAS-TL* encodes the enzyme required for the final step of cysteine biosynthesis. It has been reported as a salt stress-induced gene in Arabidopsis and the protein has been shown to confer salt tolerance to yeast cells [70]. In addition, *OAS-TL* has been suggested to play a role in salt stress adaptation in sea daffodil (*Pancratium maritimum*) [71]. These two genes, as well as other candidate genes identified in this study, could be a good target for further studies on the molecular mechanism of salt tolerance in rice and could be useful for the rice breeding program for salt tolerance.

5. Conclusions

In this study, we identified nine QTLs associated with salt tolerance at the seedling stage of rice. The QTLs associated with SIS identified in this study included known QTLs associated with salt response and novel QTLs. It has been clearly demonstrated that salt tolerance performance in this population is unlikely to be controlled by the *Saltol* QTL. Several candidate genes have been proposed and two genes, namely Os01g0200700 (metallothionein-like protein) and Os12g0625000 (O-acetylserine(thiol)lyase), were suggested as potential candidate genes based on transcriptome analysis of the two parents. Since breeders usually make their selections based on overall visual performances, i.e., injury scores under salt stress, the QTLs and candidate genes identified in the present study for SIS will be useful for rice breeding programs for salt tolerance at the seedling stage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13081596/s1, Figure S1: Allelic discrimination plots of the PCR results of the *SKC1*-KASP marker genotyped in the RIL population; Figure S2: Distribution of SNPs on the 12 rice chromosomes.; Table S1: Annotated genes within the detected QTLs; Table S2: Gene ontology (GO) terms and annotations for the differentially expressed genes.

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