agronomy

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Identification of Candidate Genes for Salt Tolerance at Seedling Stage in Rice Using QTL-Seq and Chromosome Segment Substitution Line-Derived Population

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Abstract: Rice is a staple food for more than half of the world's population. However, the pervasive problem of salinity is severely undermining rice production, especially in coastal and low-lying areas where soil salinization is widespread. This stress, exacerbated by climate change, necessitates the development of salt-tolerant rice varieties to ensure food security. In this study, an $F_{2:3}$ population (n = 454) from a cross of KDML105 and its chromosome segment substitution line (CSSL) was used to identify genomic regions associated with salt tolerance at the seedling stage. Using the QTL-seq approach, a QTL significantly associated with salt tolerance was identified on chromosome 1. Annotation of candidate genes in this region revealed the potential regulators of salt tolerance, including MIKC-type MADS domain proteins, calmodulin-binding transcription factors, and NB-ARC domain-containing proteins. These and other identified genes provide insights into the genetic basis of salt tolerance. This study underscores the importance of using advanced genomics tools and CSSL populations in the study of complex traits such as salt tolerance in rice. Several candidate genes identified in this study could be used in further studies on molecular or physiological mechanisms related to the salt response and tolerance mechanism in rice. Additionally, these genes could also be utilized in plant breeding programs for salt tolerance.

Keywords: rice; salt; QTL-seq; CSSL; KDML105; whole-genome sequencing; bulk-segregant analysis

Citation: Leawtrakun, J.; Aesomnuk, W.; Khanthong, S.; Dumhai, R.; Songtoasesakul, D.; Phosuwan, S.; Nuanpirom, J.; Charoensawan, V.; Siangliw, J.L.; Ruanjaichon, V.; et al. Identification of Candidate Genes for Salt Tolerance at Seedling Stage in Rice Using QTL-Seq and Chromosome Segment Substitution Line-Derived Population. *Agronomy* **2024**, *14*, 929. https://doi.org/10.3390/agronomy14050929

Academic Editor: Jianbo Wang

Received: 18 March 2024
Revised: 24 April 2024
Accepted: 26 April 2024
Published: 28 April 2024

1. Introduction

Rice (*Oryza sativa* L.) is a staple food consumed by more than half of the world's population, especially people in East and Southeast Asia [1]. Salinity is a major abiotic stress factor that severely affects the global rice production, especially in coastal and low-lying areas where soil salinity is widespread [2]. The areas affected by salinity will increase continuously, mainly due to climate change [3]. Rice is considered a salt-sensitive species (also known as glycophyte) and is the cereal species most sensitive to salt stress [4]. It is particularly sensitive in the early seedling and reproductive stages [2]. Salt stress inhibits photosynthesis and growth, leading to biomass losses and partial sterility, which ultimately results in rice yield losses [5]. Hence, salinity tolerance of rice is critical for ensuring food security and maintaining agricultural productivity in regions affected by salt stress.

Rice has shown considerable genetic variation in its ability to tolerate salt stress [6]. Understanding the genetic basis of salt tolerance in rice is essential for targeted breeding programs aimed at developing high-yielding, salt-tolerant varieties. Salt tolerance is known to be a complex trait controlled by multiple genes (quantitative trait loci—QTL) [7,8]. A limited number of rice varieties, such as Pokkali and Nona bokra, have been used as donors in breeding programs and in the mapping of salt-tolerant QTLs because of their ability to provide more salt tolerance [9]. Currently, hundreds of QTLs have been reported to be associated with salt tolerance at different growth stages [10]. However, the majority of QTLs identified were found to be marginally effective [11]. *Saltol*, located on chromosome 1, has been identified as a major QTL explaining 43–70% of the phenotypic variation associated with salt tolerance at the seedling stage [12,13]. *SKC1* (*OsHKT1:5*), which encodes the HKT-type transporter, was cloned within the *Saltol* QTL region using a map-based cloning method [13]. This gene has been reported to play an important role in Na^+/K^+ homeostasis under salt stress [13]. The *Saltol* QTL has been transferred into several rice cultivars to improve salt tolerance at the seedling stage [14]. However, the application of this QTL in salt tolerance improvement had been reported to be limited in some genetic backgrounds [15]. Therefore, different QTLs from various donors are required to be applied in rice breeding programs for sustainable salt tolerance in rice.

With the advancement and feasibility of the next-generation sequencing (NGS), QTL-seq has emerged as a powerful genomic tool offering high-resolution and cost-effective solutions for dissecting the genetic basis of complex traits in plants [16]. This approach integrates NGS technologies with bulk-segregant analysis (BSA) in analyzing allelic variations between contrasting bulks [16]. QTL-seq has been widely applied in crop breeding programs, including rice, to identify QTLs for various agronomic traits, i.e., yield [17], quality [18], disease resistance [19,20], and abiotic stress tolerance [21,22]. Different types of segregating population, e.g., F_2, $F_{2:3}$, near-isogenic lines (NILs), and recombinant inbred lines (RILs) have been applied to identify QTLs for traits of interest using the QTL-seq approaches [23]. However, the use of chromosome segment substitution line (CSSL)-derived population in QTL-seq analysis has rarely been reported in rice.

Chromosome segment substitution lines (CSSLs) have been a crucial tool in rice genetics and breeding, offering insights into the genetic control of various genomic traits [24]. A population of 135 CSSLs with the KDML105 rice genetic background has been developed using the donors IR68586-F2-CA-31 (DH103) and IR68586-F2-CA-143 (DH212), which are double haploids of the cross of CT9993 × IR62266 [25]. These CSSLs were originally reported as carry substituted regions associated with drought tolerance; however, the chromosome substitutions in some of these CSSLs were also found to be associated with salt tolerance. Recently, a few lines of this CSSL population that are salt and drought tolerant have been characterized, and candidate genes have been proposed [26].

In this study, we sought to identify genomic regions associated with salt tolerance at the seedling stage of rice using the QTL-seq approach with an $F_{2:3}$ population derived from a cross of KDML105 and one of its CSSL lines. The results of our study shed light on a QTL for salt tolerance that can be used in breeding programs for salt tolerance. In addition,

several novel and known genes from this QTL have been proposed as candidate genes for salt tolerance at seedling stage of rice.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

A set of 454 $F_{2:3}$ from a cross of KDML105 and KD-CSSL106 was used to evaluate salinity tolerance (150 mM NaCl) at the seedling stage (Figure S1). KDML105 was used as a salt-sensitive parent, and KD-CSSL106 was used as a salt-tolerant parent. KDML105 is a high-value aromatic rice variety from Thailand but is sensitive to various stress factors such as salt. KD-CSSL106 is a near-isogenic line (NIL, BC_5F_5) derived from a cross between KDML105 and IR68586-F2-CA-31 (DH103), with KDML105 serving as the recurrent parent (Figure S1). KD-CSSL106 exhibits moderate tolerance to salt stress at seedling stage. The experiment was conducted in a greenhouse at the Rice Science Center, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand. For each $F_{2:3}$ family, a total of 15 seeds were germinated at room temperature for 5 days and then transplanted into clay soil from a rice field and placed in a plastic tray with 200 wells (52 cm × 26 cm × 4 cm). For each $F_{2:3}$ family, the plants were planted in three rows with five plants per row. The seedling trays were placed in a cement tank (900 cm × 900 cm × 50 cm) containing a nutrient solution (1:100) according to the instructions of Bangsai Agricultural Center Co. Ltd., Bangkok, Thailand. The details of the solution were described in the study by Phosuwan et al. [27]. The seedlings were allowed to grow for 21 days before being used for the salt stress experiment.

2.2. Salt Stress Experiment and Evaluation of Salt Tolerance

The experimental design for the salt stress experiment was a randomized complete block design (RCBD) with three replicates. The solution used for growing the seedlings was drained, and the tank was filled with a salt treatment solution (150 mM NaCl). This was the same nutrient solution to which 150 mM NaCl (EC 16–17 dS/m) was added. Evaluation of salt tolerance of the $F_{2:3}$ progeny as well as the parental lines KDML105 and KD-CSSL106 and the reference variety, Pokkali (salt tolerant), was performed 12 days after treatment or when the susceptible parent showed a clear symptom of salt injury, using IRRI's standard evaluation system (SES) for Salt Injury Score (SIS) of 1–9 [28] (Table 1). The mean SIS scores of fifteen plants in each $F_{2:3}$ family were reported.

Table 1. Modified standard evaluation score of visual salt injury at seedling stage [29].

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves are whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongated	Moderately tolerant
7	Complete cessation of growth; most leaves are dry; some plants are dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

2.3. Bulk Construction, DNA Extraction, Whole-Genome Sequencing, and RNA-Seq

Twenty $F_{2:3}$ families with the highest SIS values and another 20 $F_{2:3}$ families with the lowest SIS values were selected to form the tolerant bulk (T-bulk) and the sensitive bulk (S-bulk), respectively. Leaf samples were pooled from 15 plants of each of the 40 selected $F_{2:3}$ families (samples were taken before salt treatment) and the parental lines KDML105 and KD-CSSL106. The pooled leaf samples of each $F_{2:3}$ family together with the parental lines were used for DNA extraction using the DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany). The quality and quantity of DNA were determined using Nanodrop 8000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA was then collected in equal

amounts (50 ng/μL, 80 μL) from individual $F_{2:3}$ families in the salt-tolerant group and the salt-sensitive group, and the DNA from each group was pooled into a tolerant bulk (T-bulk) and a sensitive bulk (S-bulk), respectively. The DNA samples from the two bulks and the two parents were sent to Beijing Genome Institute, BGI (Shenzhen, China) for whole-genome sequencing using Illumina Hiseq 2500 sequencing platform.

For transcriptomic analyses of DH103 and KDML105, the growth of rice and salt stress experiments were performed as described in our previous study [27], and the RNA-seq analyses were performed as described by Sriden and Charoensawan [30], except that Nipponbare-IRGSP1.0 was used as the reference genome. Briefly, raw sequence reads were quality-checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc, accessed on 1 April 2024) and preprocessed using Trimmomatic version 0.36 [31]. Cleaned reads were then mapped to the Nipponbare-IRGSP1.0 reference genome using HISAT version 2.2.1 [32] by allowing each read to align to one genomic region. Default mapping parameters were used unless stated otherwise. Post-mapping processing including sorting and removal of duplicated artefact reads were performed using Samtools version 1.3.1 [33] and Picard MarkDuplicates version 1.139 (https://broadinstitute.github.io/picard, accessed on 1 April 2024), respectively. Quantification of reads was then performed using HTSeq version 2.0.4 [34] with no strand specified (-s no) and the relevant mRNA information used from Nipponbare-IRGSP1.0 GFF3 file (-t mRNA -i Parent) to obtain raw and transcript per million (TPM) count matrices. Notably, gene expression analyses of a number of candidate genes presented here are a part of the complete transcriptomic dataset, which will soon be presented in a separate publication, together with the comprehensive analyses of the transcriptomes.

2.4. QTL-Seq Analysis and Candidate Gene Determination

Raw paired-end reads from each sample were filtered out of low-quality sequences using Trimmomatic software version 0.30 [31] to obtain clean reads. QTL-seq analysis was performed using the QTL-seq pipeline version 2.2.3 [35]. The parent KDML105 was used to generate the pseudo-reference genome according to [16,35]. Briefly, the clean reads of KDML105 were aligned to the publicly available reference genome (Nipponbare-IRGSP1.0), then the genome of Nipponbare was replaced by the variants of the KDML105 parents. The SNP index for each SNP position was calculated using the following formula: SNP index = (number of alternative bases)/(number of total aligned reads) [16]. The SNP positions with an SNP index of <0.3 and those with a read depth of <8 in both bulks were filtered out. Δ(SNP index) for each SNP position was calculated by subtracting the SNP index of the T-bulk from the SNP index of the S-bulk. A sliding window analysis was performed based on Δ(SNP index) with a window size of 750 kb and 10 kb increments. The cutoff for the minimum read depth to obtain SNPs was set to 12 reads. The genomic regions with the highest average ΔSNP index in the sliding windows were considered as the associated regions.

The genes located within the associated regions were determined based on the Rice Genome Annotation Project database (http://rice.uga.edu, accessed on 1 April 2024). The genes with an annotated function relevant to salt response or salt tolerance were identified, and the SNPs in these genes were compared with the SNP index between T-bulk and S-bulk. In addition, SNPs in each annotated gene were inferred for potential effects using SnpEff version 3.0 [36]. Overall, genes with the relevant functions and/or those containing SNPs with moderate or high effects, i.e., causing an amino acid change, were considered as possible candidate genes.

2.5. KASP Marker Genotyping and Marker–Trait Association Analysis

The KASP markers developed for the SNPs within the discovered regions were used to validate the QTL using individual $F_{2:3}$ families. These markers were courtesy of the Innovative Plant Biotechnology and Precision Agriculture Research Team, National Center for Genetic Engineering and Biotechnology, Thailand. The KASP reaction (LGC Biosearch,

Hoddesdon, UK) contained 2× Master Mix 2.5 µL, KASP primer 0.075 µL, sample DNA 2 µL, and ddH_2O to a final volume of 5 µL. The amplification process started at 94 °C for 5 min. It went through 10 cycles: 94 °C for 20 s, followed by 61 °C for 60 s, using a touchdown method with a gradual temperature decrease of 0.6 °C per cycle. This was followed by 27 cycles at 94 °C for 20 s and 55 °C for 30 s, with a subsequent rest period at 37 °C for 1 min. The fluorescence signals of the resulting PCR products were analyzed using the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The analysis of marker–trait association was performed with a simple regression method using the lm() function in R (http://www.r-project.org, accessed on 1 April 2024).

2.6. Statistical Analysis

Data analyses were performed using the package "Agricolae" version 1.3–5 [37] available through R studio (R 4.3.3) for Windows. Graphs and plots were performed using the following R packages: "ggplot2" version 3.3.6 [38], "ggpubr" [39], "ggsignif" [40] and "RColorBrewer" [41], and "karyoploteR" [42].

3. Results

3.1. Evaluation of Salt Tolerance and Construction of the Two Extreme Bulks

Salt tolerance at the seedling stage (21 days old) was evaluated using the 454 $F_{2:3}$ families together with the two parents, KDML105 and KD-CSSL106, 12 days after salt stress treatment (150 mM NaCl). Pokkali was used as a salt-tolerant reference cultivar. The salt injury phenotype of rice seedlings under salt stress was observed as leaf senescence from older to younger leaves. The average salt injury scores (SISs) of KDML105, KD-CSSL106, and Pokkali were 7.85, 6.13, and 4.99, respectively (Figure S2). Based on the SIS values, KD-CSSL106 was found to be moderately salt tolerant compared to Pokkali. The salt injury scores (SISs) determined in the $F_{2:3}$ families ranged from 4.6 to 9.0, with an average of 6.77 (Figure 1). To construct the salt-tolerant and salt-sensitive bulks, we selected 20 $F_{2:3}$ families with the highest salt tolerance (SIS values between 4.60 and 5.57) and another 20 $F_{2:3}$ families with the highest salt sensitivity (SIS values between 7.80 and 9.00) to sequence the entire genome along with the two parents, KDML105 and KD-CSSL106.

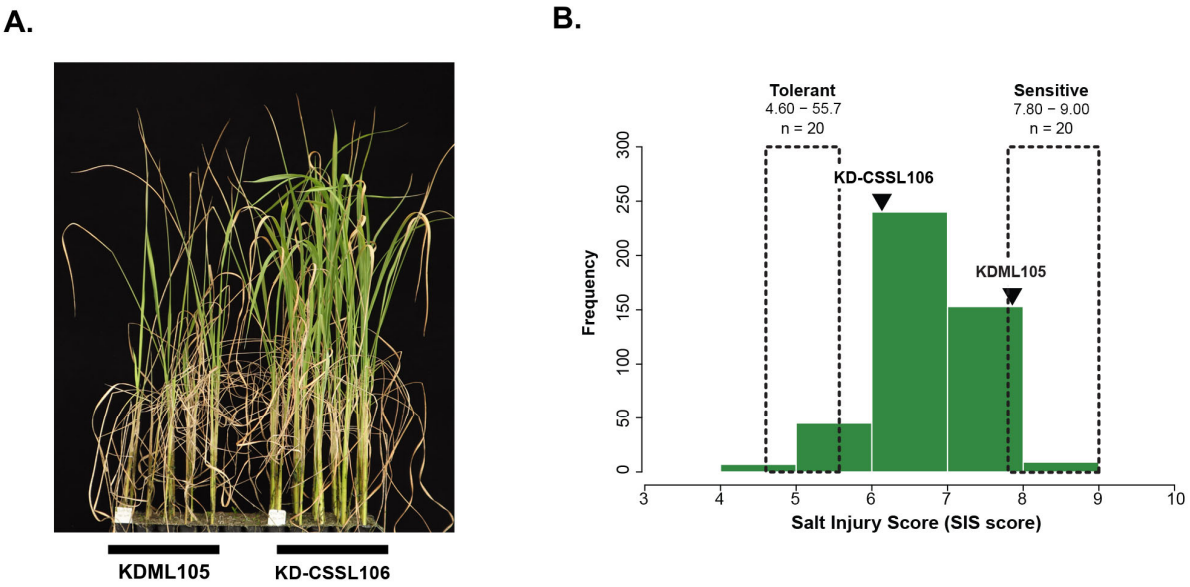

Figure 1. Phenotypic variation (salt injury score: SIS) in the parents and $F_{2:3}$ population. (**A**) The difference of salt injury phenotypes between the two parents, KDML105 and KD-CSSL106 (CSSL106). (**B**) The distribution of SIS values in the $F_{2:3}$ population. The SIS values of KDML105 and KD-CSSL106 were denoted by a black triangle. The selected plants for the QTL-seq analysis were those in the dotted rectangular.

3.2. Whole-Genome Sequencing and Variant Detection

Illumina sequencing generated 150 bp paired-end (PE) reads in a total of 53.40 million and 58.12 million of salt-tolerant bulk (T-bulk) and salt-sensitive bulk (S-bulk), respectively. In addition, a total of 58.12 million reads and 54.72 million reads were generated for the parents KD-CSSL106 and KDML105. After filtering out and trimming low-quality sequences, a total of 29.82 million reads, 33.48 million reads, 31.80 million reads, and 35.65 million reads were obtained for T-bulk, S-bulk, KD-CSSL106, and KDML105, respectively (Table 2). Based on the alignment of the clean reads of each sample to the Nipponbare reference genome (IRGSP 1.0), 93.07%, 93.04%, 93.26%, and 96.75% of the clean data were mapped for T-bulk, S-bulk, KD-CSSL106, and KDML105, respectively. These covered 97.90%, 98.00%, 97.85%, and 91.09% of the reference genome, respectively. The average depth of aligned reads for T-bulk, S-bulk, KD-CSSL106, and KDML105 was 17.26×, 18.88×, 18.72×, and 17.54× genome coverage, respectively (Table 2).

Table 2. Summary of whole-genome sequencing results for tolerant and susceptible bulks, as well as the parental lines.

Sample	Raw Reads (Million)	Cleaned Reads (Million)	Clean Base (Gb)	Read Alignment (%)	Genome Coverage (%)	Average Depth
Tolerance bulk	53.40	29.82	4.25	93.07	97.90	17.26
Susceptible bulk	58.12	33.48	4.77	93.04	98.00	18.88
KD-CSSL106	58.12	31.80	4.52	93.26	97.85	18.72
KD	54.72	35.65	5.30	96.75	91.09	17.54

After mapping the cleaned reads from T-bulk and S-bulk to the KDML105 genome reference, 15,667 genome-wide SNPs and 11,935 InDels were detected with a read support of at least eight reads (Table 3). To obtain robust results, the common SNPs in the total of 8058 SNPs identified in T-bulk and S-bulk with a read support of at least 12 reads were used to perform the QTL-seq analysis (Table 3).

Table 3. Chromosome-wise distribution of common single nucleotide polymorphisms (SNPs) and insertion–deletion polymorphisms (InDels) identified in tolerant and susceptible bulks.

Chromosome	Length (bp)	All Variants (Read Depths > 8)		Filtered Variants (Read Depths > 12)	
		SNPs	InDels	SNPs	InDels
1	43,270,923	1579	1655	839	800
2	35,937,250	849	969	462	441
3	36,413,819	183	831	66	343
4	35,502,694	1668	1435	939	728
5	29,958,434	3985	1287	1847	586
6	31,248,787	460	719	235	307
7	29,697,621	1272	1006	661	469
8	28,443,022	3744	1346	2036	656
9	23,012,720	206	571	103	242
10	23,207,287	893	622	484	275
11	29,021,106	682	1121	330	529
12	27,531,856	146	373	56	133
Total	373,245,519	15,667	11,935	8058	5509

3.3. QTL-Seq Analysis for Salt Tolerance at Seedling Stage

To identify genomic regions associated with salt tolerance using the QTL-seq approach, we calculated the SNP index of T-bulk and S-bulk for each SNP identified in the genome. The ΔSNP index was also calculated using the formula: ΔSNP = (SNP index of T-bulk)

− (SNP index of S-bulk). The SNP index of T-bulk and S-bulk and the ΔSNP index were then plotted over 12 rice chromosomes. The averaged SNP index and averaged ΔSNP index were also calculated using sliding windows within a 750 kb region with a step size of 10 kb and plotted on the graphs to identify candidate regions (Figure 2). Because the population used in the study descended from KDML105 and a CSSL line of KDML105, we found from the alignments at the KDML105 genome reference that most regions in each chromosome of the progeny were nearly identical to those of KML105, except for the introgression regions of the KD-CSSL. These included the regions on chromosomes 1, 2, 4, 5, 7, 8, and 10 (Figure 2). According to the plots of the SNP index of T-bulk and S-bulk and the ΔSNP index, a 920 kb region toward the end of chromosome 1 (40.20–41.12 Mb) was identified as the region most strongly associated with salt tolerance in this population, with the highest average ΔSNP index of 0.44 (Figure 3). We also verified the QTL region by single marker analysis using the KASP markers developed in the region and individual $F_{2:3}$ families to confirm the QTL-seq results using bulked samples. A total of 28 KASP markers were developed covering the region between 40.20 and 41.12 Mb on chromosome 1. These markers were used to genotype the 40 individual $F_{2:3}$ families. The result of the single marker analysis showed that the markers in this region could explain between 2.76 and 11.49% of the phenotypic variance (PVE) in the tested population. The markers with the highest PVE value were located at 40.50 Mb (Table S1).

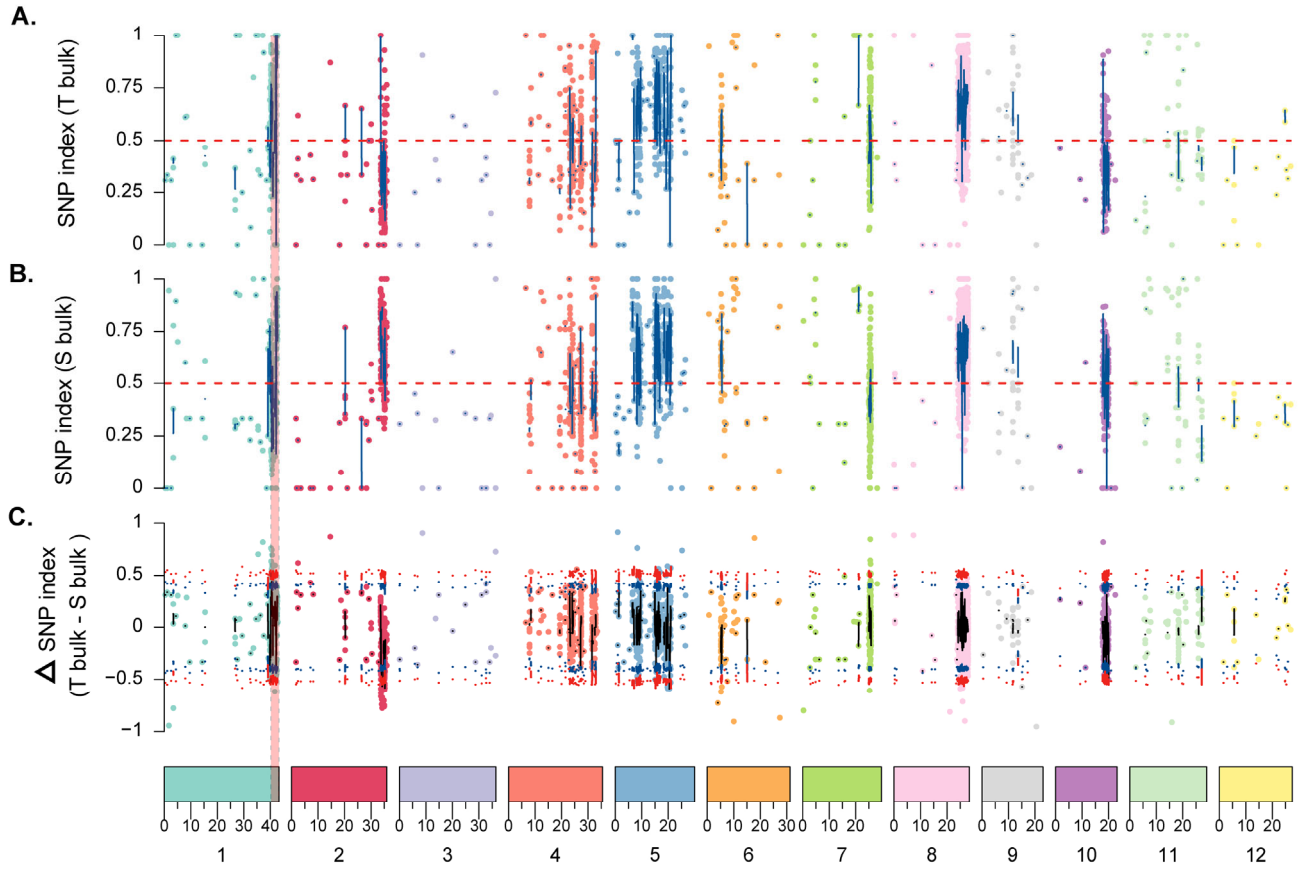

Figure 2. Diagrams illustrating the SNP index of T-bulk and S-bulk, along with the Δ(SNP index) across 12 rice chromosomes. (**A**) The SNP index diagrams for the salt-sensitive bulk (S-bulk). (**B**) The SNP index diagrams for the salt-tolerant bulk (T-bulk). (**C**) The Δ(SNP index) diagram. The plots depict the moving averages of the SNP index with a window size of 750 kb and 10 kb steps, presented as blue lines in (**A**,**B**) and as a black line in (**C**). The blue and red paired dots in (**C**) correspond to the 95% and 99% confidence intervals, respectively. Additionally, a red vertical bar in the figure denotes the candidate region on chromosome 1.

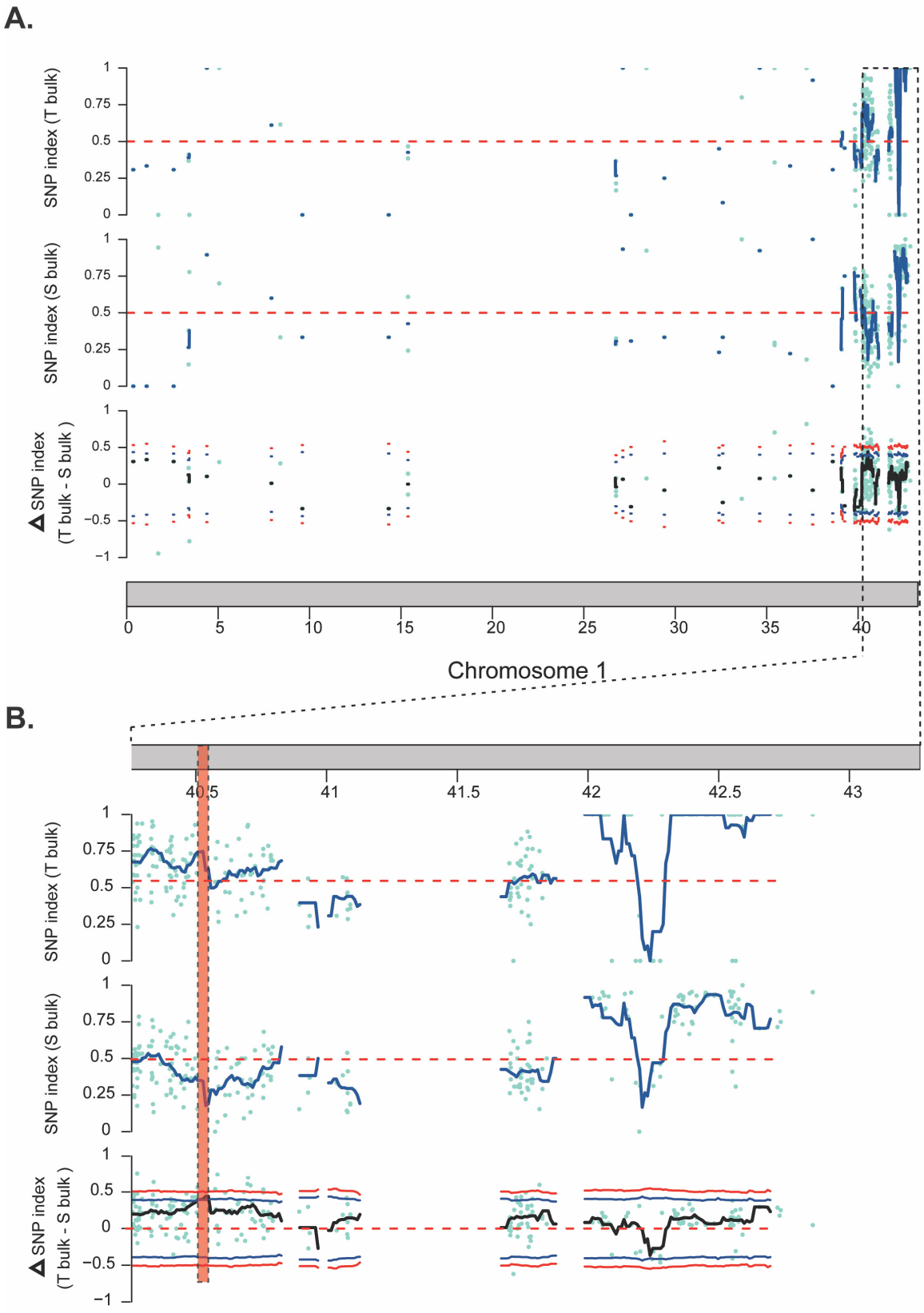

Figure 3. The extent of the QTL region detected on chromosome 1. (**A**) Diagrams illustrating the SNP index of T-bulk and S-bulk together with the Δ(SNP index) on chromosome 1. (**B**) The magnified region containing the significant SNPs. In addition, a red vertical bar in the figure indicates the top of the region.

3.4. Candidate Gene Annotation and Prioritization

A total of 124 genes were annotated within the QTL region based on the Rice Genome Annotation Project database (Table S2). Among these, there were genes previously proposed as candidate genes for salt tolerance, including potassium transporters: OsHAK5, OsHAK6, and OsHAK2. According to our preliminary analyses of the transcriptomic profiles of KDML105 and DH103 (the donor of the introgression region in chromosome 1 of KD-CSSL106) under salt stress at the seedling stage (the complete transcriptomic dataset and comprehensive analyses will be presented in a separate publication), at 1 day after salt stress, 16 genes appeared to be differentially expressed (Table S2; Figures S3 and S4). These include genes encoding a heparan-alpha-glucosaminide N-acetyltransferase, OsFBX32, BTBM1, a histidine kinase, a spermatogenesis-associated protein 20, OsMADS65, receptor-like protein kinase 2, a prefoldin subunit family protein, a histone-lysine N-methyltransferase, a WD domain G-beta repeat domain containing protein, a mitochondrial import receptor subunit TOM20, a DEK C terminal domain containing protein, a ribosomal protein L7Ae, and two expressed proteins. At 5 days after salt stress, only one gene was upregulated in DH103—*OsMADS65*, and another gene was downregulated in DH103—ribosomal protein *L7Ae*. Notably, both *OsMADS65* and ribosomal protein *L7Ae* were found to be differentially expressed at both day 1 and day 5.

In addition to this, we prioritized the number of candidate genes based on the variants present in the genes and their SNP index in the comparison between the two bulks. After filtering out the genes that did not contain a functional SNP or indel with a contrasting SNP index in two bulks (ΔSNP index > 3.0), we were able to prioritize 13 candidate genes (Table 4). These include genes encoding a MIKC-type MADS-domain protein (OsMADS65), a calmodulin-binding transcription activator, a ribosomal protein, a NB-ARC domain containing protein, an enoyl-CoA hydratase/isomerase, a zinc finger DHHC domain-containing protein, a no apical meristem (NAM), an exostosin family domain containing protein, a histone-lysine N-methyltransferase, an aspartokinase, an inducer of CBF expression, and an oxidoreductase, short chain dehydrogenase/reductase, and an expressed protein. The SNP index of the functional SNPs/Indels in these genes ranged between 0.63 and 0.91 in the S-bulk and between 0.17 and 0.50 in the T-bulk. ΔSNP index of those SNPs ranged between 0.33 and 0.63 (Table 4). Taken together, the genes that have a functional variant in the gene with contrasting SNPs and expressed differentially under salt stress in the tolerance donor, and the intolerant parents were OsMADS65 and histone-lysine N-methyltransferase.

Table 4. List of candidate genes containing SNPs/Indels with an effect contrasting the SNP index.

Chr	Pos	Effect	Gene Name	Description	KD	KD-CSSL106	SNP Index(S)	SNP Index(T)	ΔSNP Index
1	40344634	missense	LOC_Os01g69850	OsMADS65	C	T	0.71	0.17	0.55
1	40362776	missense	LOC_Os01g69850	OsMADS65	A	C	0.80	0.33	0.47
1	40401258	missense	LOC_Os01g69910	calmodulin-binding transcription activator	A	G	0.82	0.38	0.44
1	40402065	missense	LOC_Os01g69910	calmodulin-binding transcription activator	G	A	0.78	0.31	0.47
1	40402297	missense	LOC_Os01g69910	calmodulin-binding transcription activator	T	C	0.86	0.50	0.36
1	40402429	missense	LOC_Os01g69910	calmodulin-binding transcription activator	G	A	0.82	0.25	0.57

Table 4. *Cont.*

Chr	Pos	Effect	Gene Name	Description	KD	KD-CSSL106	SNP Index(S)	SNP Index(T)	ΔSNP Index
1	40402459	missense	LOC_Os01g69910	calmodulin-binding transcription activator	A	G	0.75	0.36	0.39
1	40402504	missense	LOC_Os01g69910	calmodulin-binding transcription activator	G	A	0.70	0.29	0.41
1	40453979	missense	LOC_Os01g69950	ribosomal protein L27	A	C	0.91	0.33	0.58
1	40556756	frameshift	LOC_Os01g70080	NB-ARC domain containing protein	C	CA	0.71	0.36	0.36
1	40556758	frameshift	LOC_Os01g70080	NB-ARC domain containing protein	G	GC	0.67	0.31	0.36
1	40556761	frameshift	LOC_Os01g70080	NB-ARC domain containing protein	T	TC	0.78	0.42	0.36
1	40557831	missense	LOC_Os01g70080	NB-ARC domain containing protein	G	A	0.80	0.33	0.47
1	40563787	missense	LOC_Os01g70090	enoyl-CoA hydratase/isomerase family protein	T	G	0.80	0.17	0.63
1	40568751	missense	LOC_Os01g70100	zinc finger DHHC domain-containing protein	C	T	0.80	0.17	0.63
1	40572974	missense	LOC_Os01g70110	No apical meristem protein	A	G	0.73	0.33	0.39
1	40585106	missense	LOC_Os01g70120	expressed protein	CTCC TCCTCG	C	0.86	0.40	0.46
1	40617122	Inframe insertion	LOC_Os01g70180	exostosin family domain containing protein	AATCCAC	A	0.86	0.40	0.46
1	40663740	missense	LOC_Os01g70220	histone-lysine N-methyltransferase	C	T	0.67	0.31	0.35
1	40711179	missense	LOC_Os01g70300	aspartokinase 3	T	C	0.78	0.38	0.40
1	40715330	missense	LOC_Os01g70310	inducer of CBF expression 2	A	C	0.75	0.42	0.33
1	40796815	Inframe insertion	LOC_Os01g70430	oxidoreductase	CCGG CGGCGG	CCGG CGG	0.63	0.20	0.43

4. Discussion

Soil salinity is increasingly recognized as a critical environmental problem that significantly affects agricultural productivity worldwide, especially in rice-growing regions of Asia [43]. Soil salinity hinders the development of rice, especially at the seedling and reproductive stages [44], highlighting the importance of understanding and improving the salt tolerance of rice. Salt tolerance is a complex quantitative trait that is controlled by multiple genes and is also affected by external environmental factors [45]. The introgression of quantitative trait loci (QTLs) into rice varieties to improve salt tolerance is an important aspect of modern rice breeding programs. QTL mapping and gene identification for salt tolerance in rice have been advancing through various methodologies. These methods include the traditional QTL mapping using a high-density set of SNP markers [46,47], genome-wide association studies (GWAS) [48–50], QTL-seq [15,21,51], and the integra-

tion of multiple approaches [52]. To date, hundreds of QTLs associated with salt stress response and salt tolerance at various growth stages of rice have been mapped across all rice chromosomes [53,54].

While numerous QTLs have been identified at different growth stages of rice that contribute to salt tolerance, the practical application of these QTLs in breeding programs has predominantly focused on the *Saltol* QTL [5,55,56]. The *Saltol* QTL, located on chromosome 1, is known to contribute significantly to salt tolerance at the seedling stage by enhancing the plant's ability to take up and store sodium ions in the roots, thereby reducing the toxic effects of salt on plant growth and development [13]. It contributes to a large part of the phenotypic variance for salt tolerance at the seedling stage and is therefore a valuable element in breeding programs. While the *Saltol* QTL remains a cornerstone in the development of salt-tolerant rice varieties, expanding the use of other identified QTLs through advanced breeding techniques and a better understanding of their mechanisms promise to improve salt tolerance in a wider range of rice varieties. In addition to *Saltol*, *qSt1b*, located in the lower region of chromosome 1, significantly improved photosynthetic efficiency with less damage under salt stress [57].

A typical QTL mapping approach might identify a broad region of QTLs in early segregating populations such as F_2 or F_3, as there is little recombination between individuals in such mapping populations. Therefore, it is often difficult to identify candidate genes in these extended regions. In the present study, we used an $F_{2:3}$ population derived from KDML105 and one of its CSSL, KD-CSSL106, for QTL identification by QTL-seq. The main advantage of this particular population is its ability to attenuate genomic background noise [58,59]. This substantial reduction in background noise greatly increases the accuracy of detecting phenotypic effects attributable to donor segments. Consequently, it enables the more accurate identification of specific QTLs and their associated effects, solving the general challenge of annotating candidate genes within large QTL regions. The QTL region detected on chromosome 1 at 40.20–41.12 Mb overlaps with *qST1b* identified by traditional QTL mapping [60], *qST1.2* identified by QTL-seq [15], and *qDTS1-1* identified by GWAS [48].

Several candidate genes identified in this study could be used in further studies on molecular or physiological mechanisms related to the salt response and tolerance mechanism in rice and could also be used in plant breeding programs for salt tolerance. The genes encoding MIKC-type MADS domain proteins are known to play roles in plant development from vegetative growth to reproduction and to function in various stress responses [61]. The importance of *OsMADS* genes for salt tolerance in rice has been investigated in recent studies, highlighting their significant role in stress response mechanisms, particularly *OsMADS27* [62]. Calmodulin-binding transcription activator (CAMTA) is a transcription factor known to play an important role in various aspects of plant development, hormone signaling, and response to environmental stress, including salt stress [63,64]. Ribosomal proteins, including L27, are crucial for protein synthesis and are involved in various cellular processes, including responses to environmental stress [65]. Ribosomal proteins are known to play a role in stress responses, indirectly through their involvement in the translation of stress-responsive proteins. Changes in the expression of ribosomal protein genes have been observed under various stress conditions, suggesting that these proteins may be a part of the cellular machinery that helps plants cope with stress by ensuring the proper synthesis of proteins required for stress resistance [65,66]. Histone methylation is crucial in the epigenetic control of gene expression in plants, especially in response to abiotic stresses. Enzymes that either add or remove methyl groups from histones are actively involved in these stress responses. Furthermore, the relationship between changes in histone methylation and plant metabolism significantly influences how plants react to abiotic stress [67].

The NB-ARC domain is a common signaling motif of plant resistance proteins that play a critical role in the immune response to pathogen attack [68]. Although NB-ARC domain genes are primarily associated with plant immune responses, there is increasing evidence

for their involvement in abiotic stress responses, including salt tolerance [69]. Transcriptome analysis of the halophyte *Nitraria tangutorum* revealed several genes, including those with the NB-ARC domain, which could contribute to salt tolerance via complex regulatory mechanisms [70]. In alfalfa, a gene encoding an NB-ARC domain-containing protein was reported to be involved in salt tolerance [71]. Chen et al. reported that many NB-ARC and NBS-LRR gene families were upregulated under seawater (SW) conditions [72], suggesting that they may play a role in adaptation to salt stress in rice. The INDUCER OF CBF EXPRESSION (ICE), an MYC-type transcription factor (basic helix-loop-helix (bHLH)), activates the expression of CBFs (C-repeat Binding Factors), which in turn regulate the expression of a number of genes involved in improving cold tolerance (chilling or low temperature) in plants [73]. The gene *OsICE2* has been annotated within the QTL in this study. It has been suggested that *OsICE1* (LOC_Os11g32100) and *OsICE2* (LOC_Os01g70310) have similar functions in regulating chilling tolerance [74]. Although the function of *ICE* genes in rice for the mechanism of salt tolerance is not known, rice lines with the overexpression of Arabidopsis *ICE1* (*AtICE1*) showed an improvement in tolerance to salt and other stress factors, i.e., cold and drought [75]. In addition, an ICE1-like MYC-type transcription factor from tomato was reported to confer salt as well as cold and osmotic tolerance in transgenic tobacco [76]. The regulation of transcription is a crucial component of the plant response to abiotic stress in plants. To date, many TFs that regulate the expression of downstream target genes under stress conditions have been identified and functionally characterized [77]. In rice, the major TF families that regulate salt tolerance are well studied. These include dehydration-responsive element (DRE) binding protein (DREB), ABA-responsive element (ABRE) binding protein/factor (AREB/ABF), and NAC (NAM, ATAF1/2, CUC2) [78]. Increasing research supports the role of NAC in plant response to environmental stress conditions, including salt stress [79,80]. Several studies indicate that genetically modified rice strains with enhanced expression of stress-responsive NAC transcription factor genes show improved salt tolerance without yield loss. This indicates the possible application of these NAC-TFs in the development of salt-resistant rice varieties [81].

It Is possible that the candidate genes proposed in this study may be involved in salt tolerance based on the annotated functions; however, further study, such as gene expression analysis and gene overexpression or knock-out analysis, is required to validate the functions of these candidate genes. It is important to note that the results of this study were derived from a greenhouse experiment. Therefore, the identified QTLs and gene expressions may differ from those obtained in field experiments or other environments. The SNP/indel variations identified in the genes can be used to develop DNA markers and used in marker-assisted selection programs for salt tolerance in rice.

5. Conclusions

In this study, a QTL-seq analysis was performed on $F_{2:3}$ population derived from a chromosome segment substitution line (CSSL). A genomic region on chromosome 1 associated with salt tolerance in rice seedlings was identified. Through validation and annotation, important candidate genes were identified that are promising for breeding salt-tolerant rice varieties. However, it is important to note that the QTLs and gene expressions identified here are based on greenhouse experiments and may differ from those obtained in field experiments or other environments. The results of the present study provide insight into the genetic mechanisms of salt stress tolerance and offer a valuable resource for improving rice production in salt-affected regions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14050929/s1, Figure S1: Breeding scheme of $F_{2:3}$ population used in the study; Figure S2: Salt tolerance screening in the parental lines. (A) SIS score and (B) salt injury symptom of KDML105 (KD), KD-CSSL106 (CSSL106), and Pokkali; Figure S3: Selected genes from transcriptome data of KDML105, 1 day after salt stress; Figure S4: Selected genes from transcriptome data of DH103, 1 day after salt stress; Table S1: Single marker analysis using KASP

markers; Table S2: Candidate genes identified within the QTL region; Table S3: Candidate genes that contain SNPs or Indels with an effect.

Author Contributions: Conceptualization, S.A., T.T., M.S., V.R. and J.L.S.; methodology, M.S. and S.W.; formal analysis, J.L., W.A., R.D., D.S., S.P., J.N. and S.K.; data curation, S.W. and V.C.; writing—original draft preparation, J.L. and M.S.; writing—review and editing, S.W., M.S., V.C. and S.A.; visualization, W.A.; supervision, S.A., M.S. and S.W.; funding acquisition, T.T., V.R., J.L.S. and S.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the National Science and Technology Development Agency (NSTDA), Thailand (NSTDA Grant Numbers: P-18-51456, P-19-50205, P-23-51489) and the National Research Council of Thailand (NRCT Grant Number: NRCT-RTA/812/2563). This work was also partially financially supported by the Thailand Rice Science Research Hub of Knowledge (NRCT Grant Number: N34E670027). Also, the National Science, Research and Innovation Fund, Thailand Science Research and innovation (TSRI). J.L. was supported by Thailand Graduate Institute of Science and Technology (TGIST) Scholarship, National Science and Technology Development Agency (NSTDA), Thailand (Grant Number: TG-22-11-61-052M).

Data Availability Statement: Data presented in this study are available in the Supplementary Materials or upon request from the corresponding author.

Acknowledgments: The authors thank the Innovative Plant Biotechnology and Precision Agriculture Research Team, National Center for Genetic Engineering and Biotechnology, Thailand, for providing the plant material used in this study and the KASP markers.

Conflicts of Interest: The authors declare no conflicts of interest.

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