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Research article

Screening and analysis of candidate genes conferring alkalinity tolerance in rice (*Oryza sativa* L.) at the bud burst stage based on QTL-seq and RNA-seq ☆

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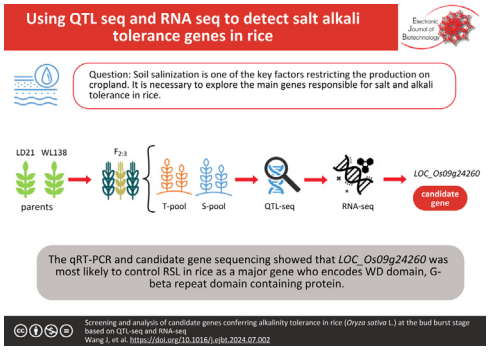
GRAPHICAL ABSTRACT



Using QTL seq and RNA seq to detect salt alkali tolerance genes in rice.

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ABSTRACT

Background: Soil salinization is one of the key factors restricting the production of cropland. Once rice is subjected to alkali stress at the bud burst stage, the yield will suffer irreparable serious loss. Compared with salt tolerance, studies on QTL mapping and candidate gene analysis of rice alkali tolerance are limited.

Results: In this study, we used the $F_{2:3}$ population derived from the alkali-tolerant cultivar LD21 and the alkali-sensitive cultivar WL138 to construct an alkali-tolerant DNA mixing pool, and the BSA (Bulked Segregation Analysis) method was used for re-sequencing. The main QTL *qRSLB9* controlling the relative shoot length of rice under alkali stress was mapped by QTL-seq. The candidate interval was narrowed to 346.5 kb by regional linkage mapping, which containing 6 DEGs screened through transcriptome

Abbreviations: BSA, Bulked segregant analysis; DEGs, Differentially expressed genes; ED, Euclidean distance; GO, Gene ontology; InDel, Insertion deletion; KASP, Kompetitive allele-specific PCR; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, Real time quantitative polymerase chain reaction; QTL, Quantitative trait locus; RIL, Recombinant inbred line; RSL, Relative shoot length; SL, Shoot length; SLS, Shoot length under alkali stress; SNK, Na^+ /K^+ ratio of shoots; SNPs, Single nucleotide polymorphisms; S-pool, Sensitive pool; T-pool, Tolerant pool.

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Oryza sativa L.
QTL-seq
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RNA-seq
Soil salinization

sequencing. The qRT-PCR and candidate gene sequencing showed that *LOC_Os09g24260* was most likely to control relative shoot length (RSL) in rice as a major gene who encodes the WD domain, G-beta repeat domain-containing protein.
Conclusions: Based on these results, *LOC_Os09g24260* was the candidate gene of *qRSLB9* conferring alkalinity tolerance to rice at the bud burst stage. Our study provides valuable genetic information and materials for breeding new rice varieties with alkalinity tolerance.
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1. Introduction

There is no doubt that rice plays an important role in the world's food crops. More than 2 billion people around the world live on rice [1]. Among the environmental factors that reduce rice yield, soil salt-alkali damage is to blame [2]. According to incomplete statistics of UNESCO and FAO, the area of salt-alkali land in the world is 954.38 million hectares [3], which is an important strategic reserve farmland. Rice is the preferred food crop for improving salt-alkali soil. It could improve soil structure and the microclimate of salt-alkali land, reduce surface water evaporation, and inhibit salt return by planting salt-tolerant rice [4]. While conventional rice itself is a salt-alkali sensitive freshwater crop, which has different performance under salt-alkali stress in different growth stages, especially in the early nutritional growth stage such as bud burst stage. Once rice is subjected to alkali stress at the bud stage, the yield will suffer irreparable serious loss [5], reflecting the necessity of studying alkaline tolerance during the bud stage. At present, neutral salt (NaCl) is the research object of most plant salt-alkali stress research, but there is indeed very little research on the alkali tolerance. Studies have shown that alkali damage caused by $NaHCO_3$ and Na_2CO_3 may be more serious than that caused by neutral salts such as NaCl and Na_2SO_4 [6]. Therefore, the breeding of new rice varieties with alkali tolerance and the mining of candidate genes for alkali tolerance in rice bud burst stage is particularly important.

The research on the mechanism of rice salt tolerance has never stopped, because of its complexity and polygenicity. Studies on QTLs mapping and candidate gene mining of salt tolerance in rice have been carried out in large numbers. In recent years, hundreds of salt tolerance QTLs at different growth stages have been reported, such as germination stage [7], bud stage [8], seedling stage [9], grain filling stage [10], and reproductive stage [11]. In general, compared to neutral salts, researches on alkaline salt stress are insufficient, and the number of major genes that can be applied to breeding practice is very limited. Li et al. [12] used the RIL population to detect the significant association site Chr11_21,999,659 between root length and relative root length under alkali stress through linkage analysis and GWAS, and screened the candidate gene *LOC_Os11g37320* through haplotype analysis. Liang et al. [13] found that there was no significant correlation between the concentration of Na^+ and K^+ under salt stress and the concentration of Na^+ and K^+ under alkali stress when conducting QTL analysis of salt and alkali tolerance in rice under NaCl and Na_2CO_3 stress, and the QTLs detected under the two stresses were located on different chromosomes. That is consistent with the results of our laboratory research [9], indicating that the salt tolerance and alkali tolerance of rice have different molecular mechanisms and should be studied separately.

Bulked Segregation Analysis (BSA) is a genetic marker technology for quickly identifying mutation phenotypes. Combined with BSA and the Next-Generation Sequencing Technology (NGS), QTL-seq [14] becomes more efficient, and more accurate in mapping QTL. Combining BSA to conduct QTL-seq and mine candidate genes of abiotic stress resistance have been reported in rice [15], corn [16] and soybean [17], but there are few studies on mining candidate genes for alkali tolerance in rice. Ten salt sensitivity index intervals were found for bulks constructed by grouping two RIL populations based on CSR 11/MI48 and CSR 27/MI48 as parents [11]. *Os02g39884* was screened out containing non-synonymous mutation SNP from 18 candidate genes in the 116 kb candidate interval narrowed by BSA of QTL *qATGW2-2* form linkage analysis [18].

Transcriptome is a complete collection of all transcripts at a specific developmental stage or under a specific physiological condition during cell development. RNA-seq determines all RNA transcribed by cells under specific stress conditions to study the difference in gene expression level caused by abiotic stress [19], thus identifying a large number of DEGs significantly related to stress resistance, and revealing the molecular mechanism of resistance formation of the research object. With the maturity and development of high-throughput sequencing technology, there are many reports on the genetic dissection of abiotic tolerance of rice [20], corn [21], soybean [22] and other crops by RNA-seq. Benefiting its high efficiency and accuracy, combining QTL-seq and RNA-seq to discover rice salt-alkali tolerance QTL (candidate genes) has become very valuable. Sun et al. [23] used the extreme bulks constructed by CMG (salt tolerant) and Zhefu 802 (salt sensitive) to conduct QTL-seq and RNA-seq of CMG at the germination and seedling stages. Six salt tolerance candidate regions were mapped on chromosome 1, and a rice salt tolerance candidate gene *OsPP2C8* was identified based on ORF region polymorphism, differentially expressed genes (DEGs) and KEGG analysis.

In this study, we used the $F_{2:3}$ population derived from the alkali-tolerant cultivar LD21 and the alkali-sensitive cultivar WL138 to construct an alkali-tolerant DNA mixing pool, and the BSA method was used for re-sequencing. The main QTL *qRSLB9* controlling the relative shoot length of rice under alkali stress was mapped by QTL-seq. The candidate interval was narrowed to 346.5 kb by regional linkage mapping, which contained 6 DEGs screened through transcriptome sequencing. The qRT-PCR and candidate gene sequencing showed that *LOC_Os09g24260* was most likely to control RSL in rice as a major gene.

2. Methods

2.1. Parents and populations

Five hundred and forty-five $F_{2:3}$ population was constructed by crossing the strong alkali tolerant Japonica variety LD21 (male) and alkali-sensitive Japonica variety WL138 (female) preserved in our laboratory. All of the experimental materials were obtained from Heilongjiang Academy of Agricultural Sciences in northeast China.

2.2. Evaluation of alkali-tolerance

The seeds of each $F_{2:3}$ line and two parents were dried naturally, break the seed dormancy at 50°C for 3 d. The seeds were soaked in 3% NaClO solution for 30 min and then rinsed thrice with sterile water. Then, put the hydroponic seeds into a 30°C incubator for germination for 3 d. When most buds were greater than or equal to half the length of the seeds, we randomly selected 120 well-germinated seeds in each line and divided them into 2 parts on average, for alkali stress treatment and control portions. The germinated seeds were transferred to 96-well PCR plates, and single seed was sown per well. The seeds were cultured in an artificial climate chamber under 14 h light /10 h darkness photoperiod (27/25°C). 0.15% Na_2CO_3 was (pH = 8.8) used for alkali treatment [24] and distilled water for control (the culture medium was changed every 2 d). The experiment was repeated three times. After 7 d of alkali stress/control treatment, 5 seedlings were randomly selected to measure Shoot length (SL), Shoot length under alkali stress (SLS) and their relative value Relative shoot length (RSL) to evaluate alkali damage. The measurement of SL, SLS, and their relative values of 545 $F_{2:3}$ individuals refer to the operation of Lei et al. [8]. Select the above-ground part of the individual in the mixing pools and dry it at 70°C for 2 d. Transfer 0.1 g of dry sample powder to a glass tube containing 10 ml of 0.1 N Nitric acid solution, and conduct a 70°C water bath for 8 h until digested [25]. The concentrations of Na^+, K^+ of mixing pools' shoot were analyzed by an atomic absorption spectrometer (PerkinElmer, Massachusetts, USA) to calculate the Na^+, K^+ ratio of shoots (SNK = Na^+ of shoots/ K^+ of shoots).

2.3. Construction of segregating pools

On the basis of RSL results, 30 extremely alkali-tolerant and 30 extremely alkali-sensitivity individuals of $F_{2:3}$ population were selected to construct bulked pools. The alkali-resistant pool was named as T-pool, and the alkali-sensitive pool was named as S-pool. The DNA of two bulked pools and their parents was extracted with DNA Extraction Kit (CWBIO cw0553). After quantified by Nanodrop 2000 spectrophotometer (Thermo Scientific, Fremont, CA, United States), the individual DNA was diluted to the same concentration and then mixed in the same amount.

2.4. QTL-seq analysis

At least 3 μg bulked genomic DNA was extracted from 2 bulked pools, and a 500 bp insert size paired-end libraries was constructed by Paired-End DNA Sample Prep kit (Illumina Inc., San Diego, CA, USA). Libraries sequencing was implemented by Genedenovo (Guangzhou, China) using HiSeq X10 (Illumina Inc., San Diego, CA, United States) NGS platform.

In terms of data quality trimming, we process rough reads into high-quality reads according to the following principles: 1) removing reads with $\geq$ 10% unidentified nucleotides (N); 2) removing reads with > 50% bases having phred quality scores of $\leq$ 20; 3) removing reads aligned to the barcode adapter.

To identify SNPs and InDels, filtered reads were aligned to the Gap-free Reference Genome of ZS97 [26] using Burrows-Wheeler Aligner (BWA, v 0.7.16a-r1181) with parameter "mem -M"; -M is an option used to mark shorter split alignment hits as secondary alignments [27]. Variant calling was carried out using GATK Unified Genotyper (v3.5). SNPs and InDels were filtered by the standards of -Window 4, -filter "QD < 4.0 || FS > 60.0 || MQ < 40.0 ", -G_filter "GQ < 20" through GATK Variant Filtration function. ANNOVAR was used to annotate genes and genomic regions. SNP-index, Δ(SNP-Index) [28], Euclidean Distance [29], G′ value [30] and two-tailed Fisher exact test [31] were adopted for association analysis of SNPs. The average Δ(SNP-index) and SNP-index, G value, ED value, and q value were calculated using a 2000 kb sliding window with a step size of 20 kb, windows with < 10 SNP/Indel were discarded. The overlapped regions based on the above four methods were considered candidate regions for alkali tolerance.

2.5. Regional linkage mapping analysis

Using the SNPs near the candidate interval obtained by QTL-seq, 26 KASP markers (**Table S1**) were designed by Primer 5.0 to conduct regional linkage mapping to further screen the association analysis results of the 4 QTL-seq statistical methods. KASP assays followed the principle of LGC Genomics (LGC, Middlesex, UK), as described in Dangthaisong et al.'s research [32]. The setting of the KASP reaction mixture system was the same as Liu et al. [33]. The SNP genotyping of the 545 $F_{2:3}$ individuals was carried out according to the operating procedures of Lei et al. [8]. Linkage map construction and linkage analysis carried out by IciMapping 4.22 (https://www.isbreeding.net), setting the threshold LOD $\geq$ 3.0, $p < 0.01$ to locate significant QTLs.

2.6. Transcriptome sequencing and analysis

The buds of the two parents of $F_{2:3}$ treated with 0.15% Na_2CO_3 at the bud burst stage for 36 h and the control were sampled (3 repeats) and immediately frozen with liquid nitrogen after sampling. Tranzol up RNA Kit (TransGen Biotech) was used to extract the total RNA of the sample and then to eliminate DNA contamination in the total RNA with DNase I. The RNA purity was checked with the Nano Photometer (IMPLEN, CA, USA) spectrophotometer and quantified by Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). The sequencing library was constructed with NEB Next® Ultra™ RNA Library Prep Kit for Illumina (NEB, USA), and Illumina HiSeq sequencing was performed after the quality of the library was evaluated on the Agilent Bioanalyzer 2100 system. After raw reads were filtered (removing adaptor sequences and low-quality sequences), TopHat v2.0.12 was used to compare with the Nipponbare reference genome (IRGSP-1.0). We named the transcriptome sequencing sample as LD (LD21 CK), TLD (LD21 under alkali stress), WL (WL138 CK), and TWL (WL138 under alkali stress). The DESeq package in R 4.0.4 was used to analyze the differential expression of genes between samples. Gene Ontology (GO) enrichment analysis of DEGs was implemented by GO seq in R 4.0.4. Using KEGG database (https://www.genome.jp/kegg/) and KOBAS 2.0 to conduct pathway enrichment analyses on DEGs.

2.7. RNA extraction and qRT-PCR analysis

According to the RSL under alkali stress, we selected 5 alkali tolerant, sensitive and intermediate individuals in $F_{2:3}$ and their parents to analyze the expression of the 6 candidate genes. The experimental operation method and alkali treatment method are consistent with the previous experiments. In the treatment group, the buds (3 repeats) were taken at the same time as the control group after 36 h 0.15% Na_2CO_3 stress at the bud stage and were rapidly frozen with liquid nitrogen. TranZol Up RNA kit (TransGen Biotech) kit was used to extract the whole RNA from the buds, and then DNase I (TransGen Biotech) was used to eliminate DNA contamination. cDNA was synthesized from total RNA using HiFiScript cDNA Synthesis Kit (CoWin Biosciences, Beijing, China). **Table S2** shows the gene accessions and primer sequences for qRT-PCR, and 2 × Fast qPCR Master Mixture (DINING, Beijing, China) on the Analytik Jena qTOWER system (German) was used to perform qRT-PCR. *Actin1* (*LOC_Os05g36290*) was used as the internal control [34].

2.8. Candidate gene sequencing and sequence variation analysis

Six candidate genes were cloned by PCR and sequenced in LD21 and WL138. Sequence alignment was implemented with DNAMAN software and takes Nipponbare genome (IRGSP-1.0) as the reference genome. In order to further analyze the 1 bp insertion sequence difference between the candidate genes in two parents for *LOC_Os09g24260*, we designed it as an Indel marker (forward primer: TAATCTCAAAGCAGTAGCCACA; reverse primer: GCAGAAAATATGCGATCAACCT). This Indel marker was used to genotype 200 individuals including the 60 extreme bulks and 138 lines randomly selected from the $F_{2:3}$, and its two parents. The KASP assay method was consistent with regional linkage mapping.

3. Results

3.1. Evaluation of alkali resistance-related phenotype of two mixed pools

In order to study the alkali tolerance in rice bud stage, we constructed an F_2 population by crossing alkali-tolerant Japonica variety LD21 and alkali-sensitive Japonica variety WL138 in 2020, and then developed the $F_{2:3}$ lines.

The shoot length (SL), shoot length under alkali stress (SLS), relative shoot length (RSL) of 545 individuals and their parents were evaluated under alkali stress and control conditions at the bud stage. The statistical results of phenotypic traits are shown in **Table S3**. The difference between the average values of SL and SLS reflects the effect of alkali stress on rice bud development. The RSL of $F_{2:3}$ parent WL138 (0.39) was significantly lower than that of LD21(0.73), reflecting their differences in alkali tolerance (Fig. 1). Since SL is one of the key characters reflecting alkali stress, the relative value RSL reflects the degree of alkali stress [35] and shows a normal distribution (Fig. 2A), we chose RSL as a representative character to research and screen alkali-resistant (sensitive) extreme bulks.

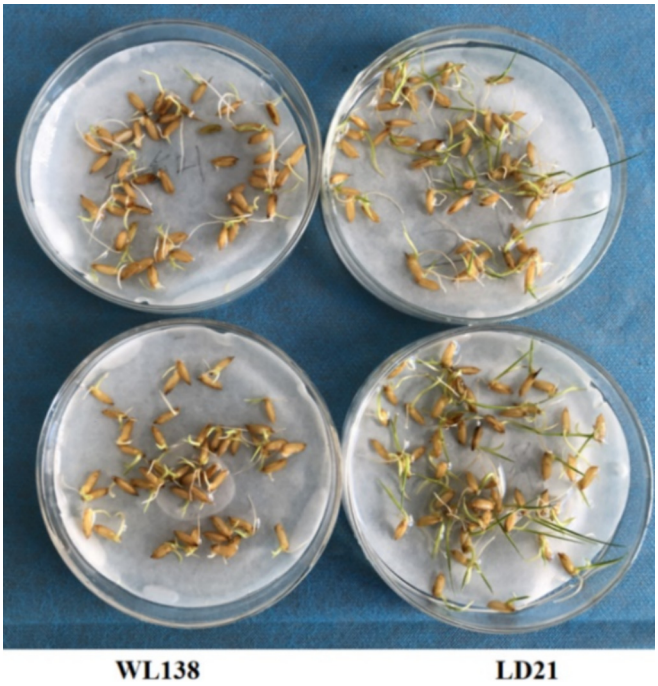

Fig. 1. Phenotypes of WL138 and LD21 after 5 d of 0.15% Na_2CO_3 treatment.

Thirty alkali-tolerant and 30 alkali -sensitive lines were screened out from the $F_{2:3}$ population composing the tolerant pool (T-pool, RSL range between 0.74 and 0.96) and sensitive pool (S-pool, RSL range between 0.16 and 0.26) respectively, which were then used for DNA re-sequencing. The RSL phenotypes of 2 extreme bulks are shown in Fig. 2B. In order to verify the tolerant difference of extreme lines selected for the mixing pool, SNK (Na^+ / K^+ ratio of shoots), the important indicator to ensure the survival of rice under alkali stress [36] has also been measured. The SNK phenotypes are shown in Fig. 2B, and the accuracy of extreme line selection is proved.

3.2. Whole-genome re-sequencing and QTL-Seq analysis

We obtained 320.72 million clean reads filtered from 322.97 million raw reads (99.30%) by Illumina high-throughput sequencing, and the two parents and two extreme bulks generated 50,31 Gb bases. GATK software was used to analyze the SNPs, the four samples generated 1,884,210 SNPs and 339,199 Indels compared with Nipponbare reference genome (**Table S4**).

Four statistical methods (SNP-index Δ(SNP-Index), Euclidean Distance, G′ value and two-tailed Fisher's exact test) were used to detect the significant correlation interval of alkali tolerance in rice bud burst stage at 95 and 99% confidence intervals (CI).

Four QTL intervals were detected on rice chromosome 3,4,9 (Fig. 3; Table 1). On chromosome 3, ΔSNP-Index and ED detected two QTL *qRSLB3-1* and *qRSLB3-2* at 99% CI, respectively. For chromosome 4, *qRSLB4* was obtained by ΔSNP-Index, ED, Fisher's exact test at the 95% significance level whos' genomic region was 12.19–13.49 Mb. The most valuable result of association interval appears on chromosome 9 that *qRSLB9* was obtained at both 95% and 99% CI and that was detected by four statistical methods simultaneously when the CI value of 95% was applied. Of note, the overlapping interval 13.62–20.43 Mb simultaneously detected by four statistical methods at the 95% CI captured the result obtained by ΔSNP-Index and ED at a 99% significance level, and reduced the interval to 14.15–16.89 Mb. This 2.74 Mb interval contains 388 genes, which we consider to be a candidate interval regulating rice RSL under alkali stress.

3.3. Regional linkage mapping of qRSLB9

In order to narrow the range of candidate genes from a large number of genes in the *qRSLB9* region, we developed 26 KASP markers based on QTL-Seq sequencing results to genotype the 545 $F_{2:3}$ lines and their parents (**Table S1**). After constructing a fine mapping linkage map using IciMapping 4.22, linkage analysis was performed on the RSL of 545 $F_{2:3}$ lines under alkali stress, and *qRSLB9* was linked to the 346.5 kb interval between MK6 SNP-14354933 and MK7 SNP-14701435 (Fig. 4). The positive allele of the QTL was contributed by LD21. After regional linkage mapping, we successfully narrowed the *qRSLB9* candidate region from 2.74 Mb to 346.5 kb, which contains 52 genes (Table 2; **Table S5**).

3.4. RNA-seq analysis

Transcriptome sequencing of the two parents LD21 and WL138 was carried out under alkali stress and control conditions at bud burst stage. In the transcriptome libraries, the average clean reads in CK of LD21 and WL138 were 42.56 million and 46.04 million respectively, while in alkali stress were 46.04 million and 43.70 million, respectively (**Table S6**). The proportion of bases with a Phred quality value greater than 30 (Q30) in all samples ranges from 93.12 to 93.91% of the total bases. The alignment results show that 97.10–97.27% of clean reads from all 12 samples could be mapped to the reference genome. Based on the calculation of Top

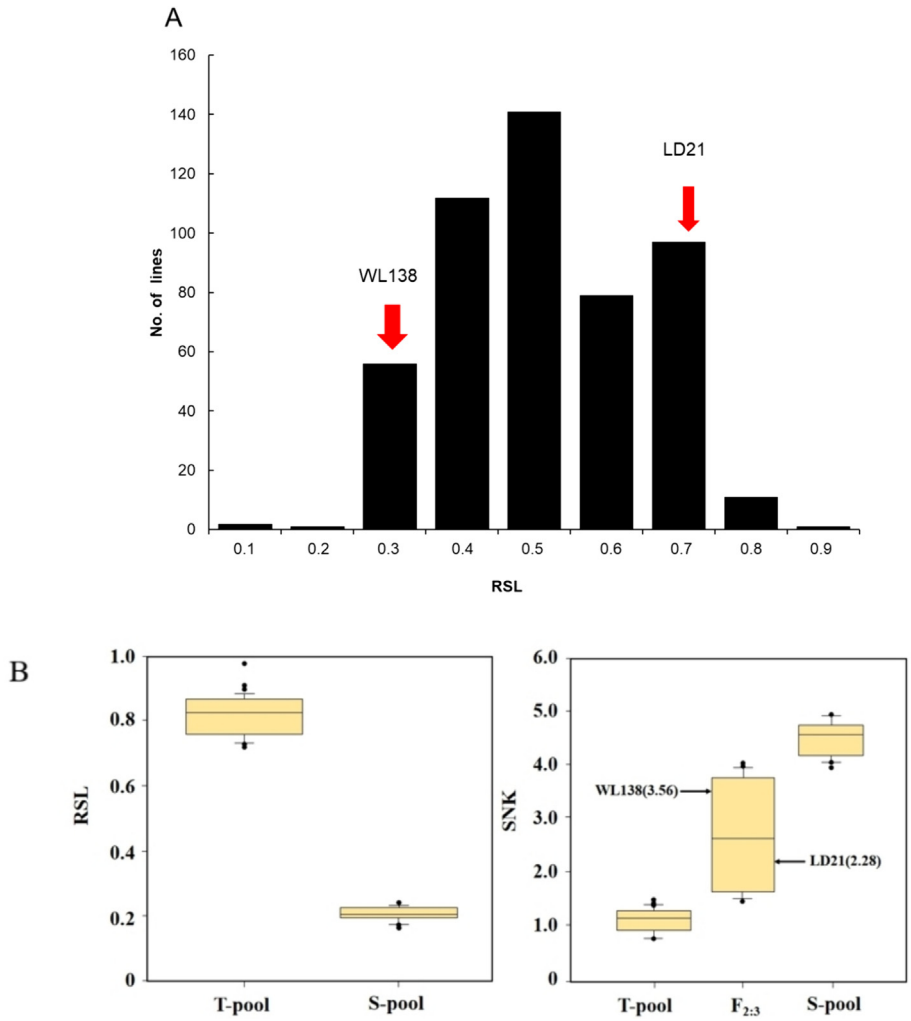

Fig. 2. (A) Phenotypic distribution of RSL in $F_{2:3}$. (B) Phenotypic statistical Box-plot of RSL and SNK in T-pool and S-pool.

Hat 2.0 software, 94.94–94.99% of reads were uniquely mapped to the reference gene, respectively (**Table S7**).

Four comparative groups LD vs. TLD, WL vs. TWL, LD vs. WL, TLD vs. TWL were established to screen the differentially expressed genes (DEGs) of LD21 and WL138 under alkali treatment and control conditions (**Fig S1**). The total number of DEGs in WL vs. TWL, LD vs. TLD, LD vs. WL, TLD vs. TWL groups were 3217, 2225, 280, 1297, respectively, and up-regulation and down-regulation of DEGs in each comparative group were shown in **Fig. S2**.

3.5. GO KEGG pathway enrichment

We selected the comparative group DEGs under alkali stress and control for GO and KEGG annotation (**Fig. S3**, **Fig. S4**). The DEGs GO term annotations for the WL vs. TWL and LD vs. TLD comparison groups were 1682 and 1155, respectively. In biological processes, the oxidation-reduction process is significantly enriched and the situation is completely consistent in WL vs. TWL, LD vs. TLD, indicating that the oxidation-reduction process plays an important role in the alkali resistance of rice during the bud burst stage. The DEGs of WL vs. TWL, LD vs. TLD in the cellular component category are significantly enriched in plastid and Golgi apparatus, respectively. The DEGs of WL vs. TWL, LD vs. TLD in the molecular function category are significantly enriched in ion binding and oxygen reductase activity, respectively.

In the comparison group of WL vs. TWL and LD vs. TLD, there were 296 and 187 DEGs annotated into different KEGG pathways, respectively, and the metabolism pathway was the most annotated. Biosynthesis secondary metabolites and metabolic pathways accumulated the most DEGs in the two comparative groups, respectively, revealing the main regulatory pathways involved in rice response to alkali stress.

3.6. Screening of candidate genes for alkali resistance

We obtained a QTL for alkali tolerance in rice bud stage through QTL-seq and local fine mapping, named *qRSLB9*. Based on RAP-DB database (https://rapdb.dna.affrc.go.jp/index.html), there were 52 candidate genes in the 346.5 kb region of *qRSLB9* (**Table S5**). Combining the RNA-seq of WL vs. TWL and LD vs. TLD, 6 differentially expressed genes under salt stress were screened for subsequent analysis (**Table S8**). *LOC_Os09g24260* (WD domain, G-beta repeat domain-containing protein) and *LOC_Os09g24620* (uncharacterized protein *LOC4347042*) were significantly up-regulated or down-regulated in both WL vs. TWL and LD vs. TLD. In addition, *LOC_Os09g24300* (agenet domain-containing protein / bromo-adjacent homology (BAH) domain-containing protein-like) and *LOC_Os09g24430* (IAA-amino acid hydrolase *ILR1*-like 3 precursor) were significantly down-regulated in LD vs. TLD. *LOC_Os09g24320* (dihydrolipoyllysine-residue acetyltransferase component 4 of pyruvate dehydrogenase complex, chloroplastic), and *LOC_Os09g24330*

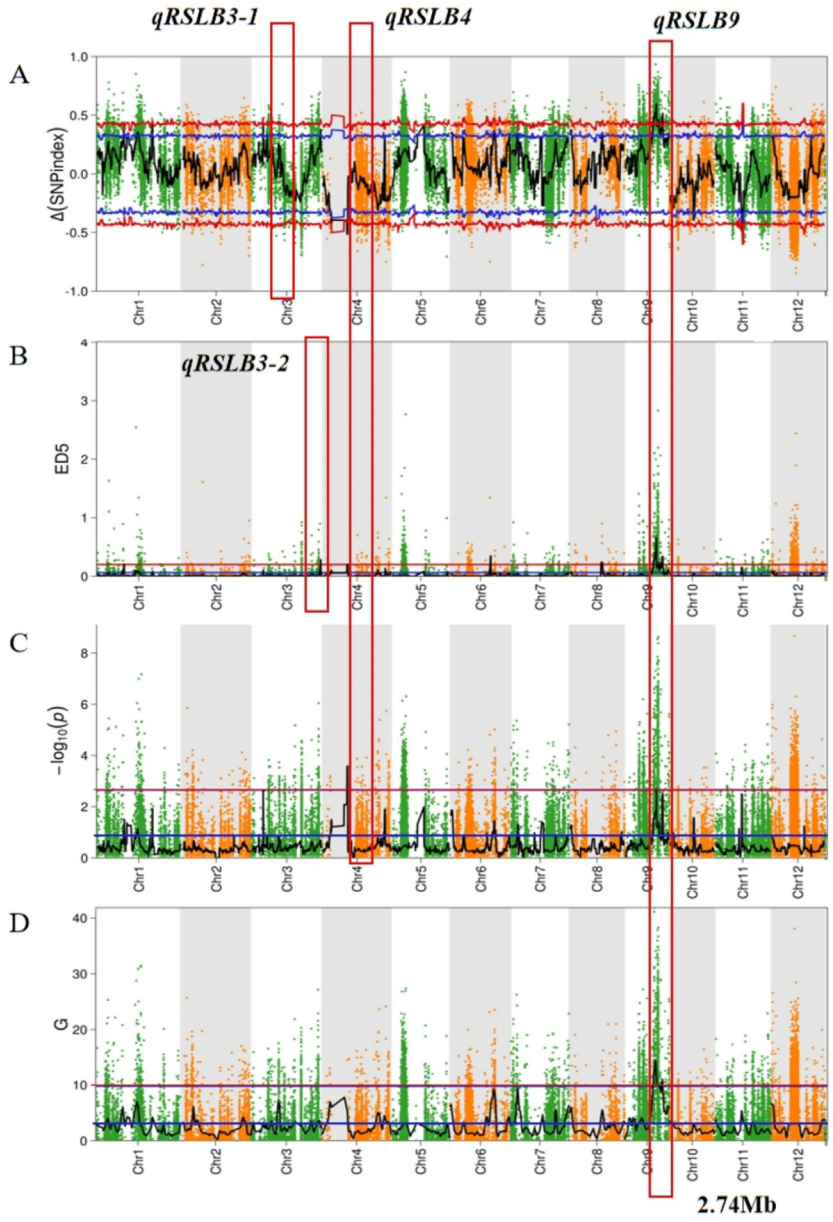

Fig. 3. QTL-seq analysis (A) Manhattan plot of SNP-index and Δ(SNP-index). (B) Manhattan plot of ED. (C) Manhattan plot of Fisher's test. (D) Manhattan plot of Gst. The blue and red lines represent 95 and 99% CI, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Alkali resistance-related QTLs detected by ΔSNP-Index, ED, G′ value and Fisher's exact test.

QTL	Statistical Method	CI	Chr	Interval	Peak	Overlapping region (Mb)
qRSLB3-1	ΔSNP-Index	0.99	3	5620001–6620000	0.4453793	5.62–6.62
qRSLB3-2	ED	0.99	3	35100001–36100000	0.2017549	35.10–36.10
qRSLB4	ΔSNP-Index	0.95	4	10640001–13580000	−0.513393	12.19–13.49
	ED	0.95		12190001–13490000	0.2017549	
	Fisher's exact test	0.95		12190001–13490000	−0.0268927	
qRSLB9	ΔSNP-Index	0.95	9	13620001–20430000	0.5970189	13.62–20.43
	ED	0.95		13190001–20840000	0.6617885	
	G′ value	0.95		13510001–20750000	0.0465119	
	Fisher's exact test	0.95		13090001–20670000	−0.0424411	
	ΔSNP-Index	0.99	9	14150001–16970000	0.5970189	14.15–16.89
	ED	0.99		14150001–16890000	0.6617885	

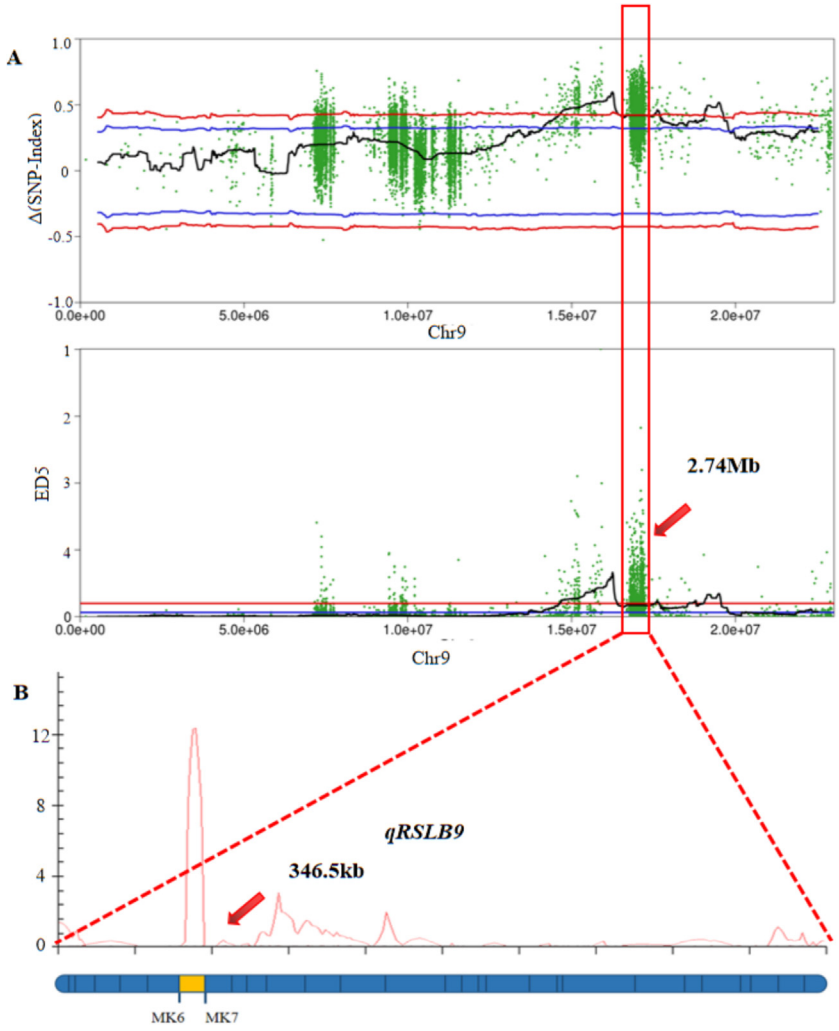

Fig. 4. Regional linkage mapping of *qRSLB9* on chromosome 9. (A) Manhattan plots of ΔSNP-index and ED on Chr9. **(B)** Linkage mapping of *qRSLB9* based on KASP markers. The blue and red lines represent 95 and 99% CI, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
qRSLB9 detected by regional linkage mapping.

Trait	Chr.	QTL	Interval	LOD	PVE (%)	Add
RSL	9	qRSLB9	MK6-MK7	12.08	19.62	1.17

(cysteine-rich receptor-like protein kinase 10) were significantly up-regulated in WL vs. TWL and LD vs. TLD, respectively.

The gene expression levels of 6 candidate genes in WL138L and LD21 under normal and salt stress were compared by qRT-PCR analysis. The analysis results are shown in Fig. 5, which confirms the accuracy and repeatability of the RNA-seq results. In the LD vs TLD comparison group, *LOC_Os09g24260*, *LOC_Os09g24320*, *LOC_Os09g24330* were up-regulated in expression, *LOC_Os09g24300*, *LOC_Os09g24430*, *LOC_Os09g24620* were down-regulated in expression. In the WL vs TWL comparison group, all candidate genes were up-regulated, except for *LOC_Os09g24620*. The overall trend of RNA-seq and candidate gene expression levels in the two comparison groups is consistent, with only slight differences in absolute expression levels.

In order to investigate the expression of these 6 genes in the $F_{2:3}$ population, we selected 5 individuals with representative high, middle, and low RSL phenotypes under alkali stress to perform qRT-PCR analysis under alkali stress/ control conditions. The results showed that *LOC_Os09g24260* was up-regulated in both high and low RSL individuals, and the expression level significantly increased in individuals with high RSL (alkali tolerance) under alkali stress, while the expression of the other 5 genes under alkali stress had no significant regularity (**Fig. S5**). The results indicate that *LOC_Os09g24260* is the most likely candidate gene for *qRSLB9*.

3.7. The verification of candidate genes

To confirm our inference of candidate genes, the 6 candidate genes were sequenced between WL138 and LD21. The sequencing results showed that except for *LOC_Os09g24260* all other genes did not differ in the cDNA and promoter regions of WL138 and LD21. *LOC_Os09g24260* had a T insertion in the CDS region (ATG start codon 2356 bp down-stream) between WL138 and LD21 (Fig. 6).

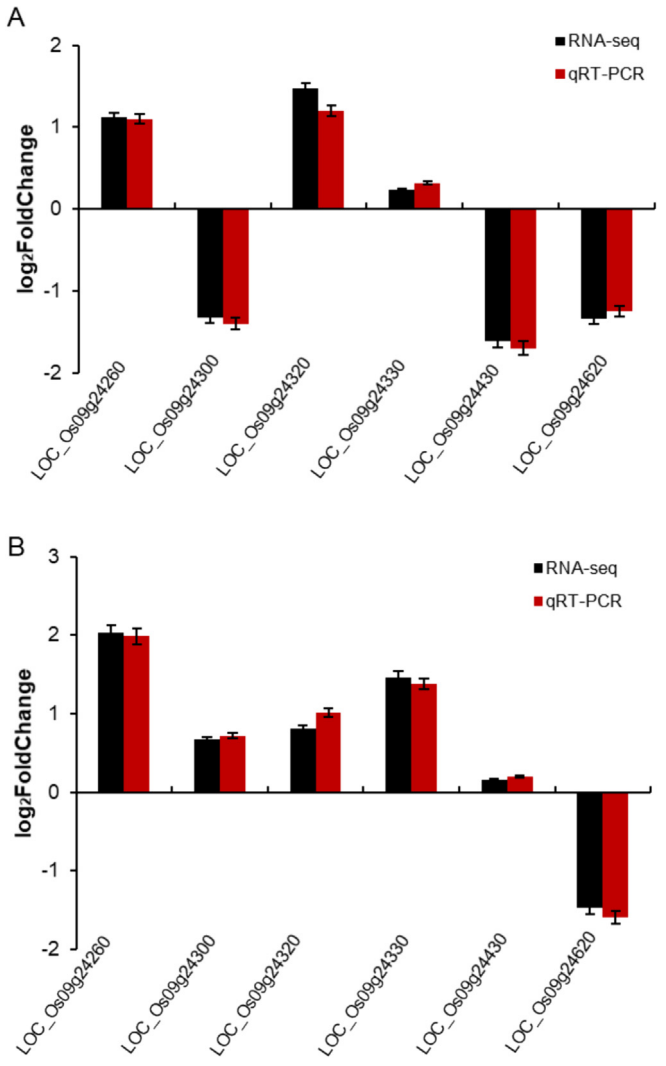

Fig. 5. Comparison of RNA-seq results and gene expression levels of 6 candidate genes. **(A)** Log_2 fold changes of candidate genes in WL vs. TWL. **(B)** Log_2 fold changes of candidate genes in LD vs. TLD. The blue column represents the RNA-seq result, while the yellow column represents the qRT-PCR result. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In order to further validate the role of candidate genes in alkali tolerance during rice bud stage, we developed a KASP marker with the 1 bp insertion to genotype 200 individuals (including the 60 extreme bulks and 138 lines randomly selected from the F2:3, and its two parents.) to reveal the sequence specificity of CDS. As shown in Fig. 7A, the tolerant genotype (T-pool) and sensitive genotype (S-pool) accounted for 93.33% and 86.67% of the two extreme bulks. There happened to be a positive correlation between the female, heterozygous, and male genotypes and their corresponding phenotypes, proving the correlation between the genotype and phenotype values determined by the KASP marker (Fig. 7B; **Table S9**). So, we believe that *LOC_Os09g24260* is the most likely gene to explain the function of *qRSLB9*.

4. Discussion

Soil salinization is one of the key factors restricting the production of cropland. Under alkaline treatment, seed germination and nutrient growth of rice will be significantly inhibited [6], leading to a significant reduction in yield and there is not much research on the mining of alkali-tolerant genes. Thanks to the significant reduction in the cost of Next-Generation Sequencing (NGS), BSA-seq has been widely used in rice abiotic stress QTL mapping and candidate gene mining in recent years. The combination of BSA and NGS (BSA-seq) accelerates the identification of closely linked markers for important traits, improving the resolution of gene identification and QTL mapping maps.

In this study, we constructed a large-scale $F_{2:3}$ population using two parents with significant differences in alkali resistance to identify the bud burst stage phenotype of their offspring. Selecting RSL as a representative trait during bud stage to construct 2 extreme differential alkali-resistant DNA library for QTL-Seq analysis. The common peak on chromosome 9 was determined to be between 14.15 and 16.89 Mb, which is *qRSLB9*, through four bioinformatics statistical methods ΔSNP-Index, ED, G' value and Fisher's exact test (99% confidence interval). By comparing previous studies, it has been found that there are QTLs/genes related to salt alkali tolerance in rice near this interval. A large number of rice alkali stress-related QTLs, such as *qAKT9.19*, *qCHL9.20*, *qSNC9.19*, *qRNC9.19*, *qRKC9.19*, *qRNK9.19* [37], *qNAUP-9a*, *qDWRO-9a* [38] were located within the interval of chromosome 9 from 14.89 to 20.52 Mb, overlapping a lot with the *qRSLB9* interval in this study, confirming the response and tolerance activity of this interval to alkali stress. In addition, there are several known genes that regulate salt and alkaline tolerance in rice near the *qRSLB9* interval. *OsWRKY62* [24] and *OsJAZ8* [39], related to jasmonic acid (JA) biosynthesis and JA signal transduction, can significantly regulate salt and alkaline tolerance of rice, just in the *qRSLB9* interval. bZIP transcription factor *OsbZIP72* in research by Wang et al. [40] regulates salt and alkaline tolerance in rice at a physical distance of 300 kb from *qRSLB9*. In addition, there are 2 genes *OsC2DP* [41], *OsAHP2* [42] that regulates salt tolerance in rice at the candidate interval of 5.92 Mb. No related genes were found on chromosome 9 that primarily regulate or affect alkaline tolerance in rice during the bud stage. Although no related genes were found on chromosome 9 that primarily regulate or affect the alkaline tolerance of rice during the bud stage, the *qRSLB9* interval contains abundant genes and loci related to salt and alkaline tolerance in rice that have been previously studied, which is sufficient to prove that *qRSLB9* can serve as an important target for searching for alkaline tolerance candidate genes.

Compared to traditional QTL fine mapping, BSA-seq can save a lot of time in population construction when mapping quantitative trait loci. Transcriptome studies gene expression at the RNA level, reflecting genes actively expressed under specific conditions, can

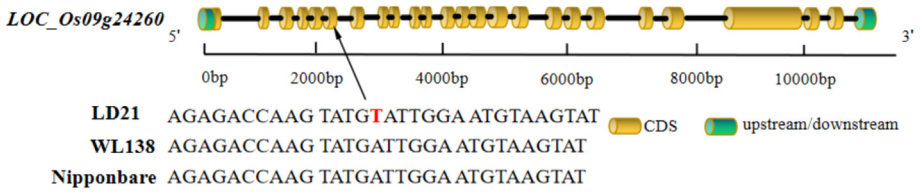

Fig. 6. The gene structure of *LOC_Os09g24260* and sequence differences between LD21 and WL138.

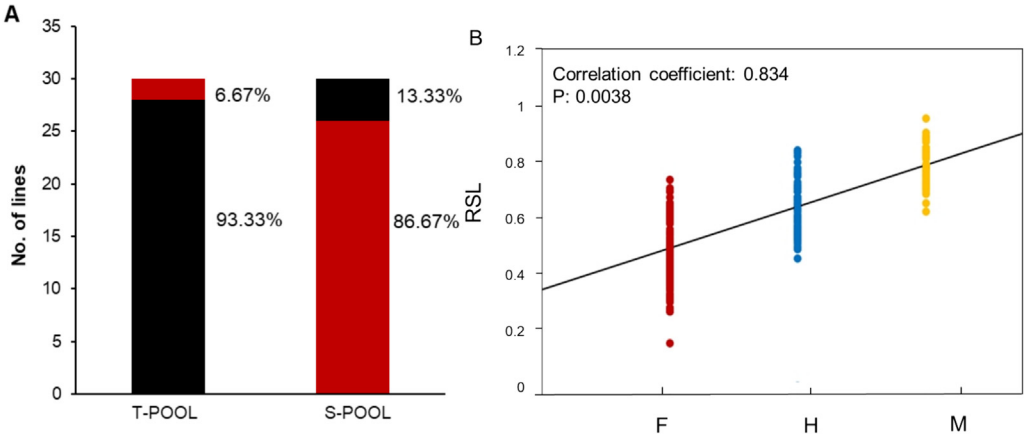

Fig. 7. Phenotypic and genotypic analysis of the KASP marker. **(A)** The distribution of tolerant, sensitive genotype, and phenotype in extreme bulks. **(B)** The correlation between genotype and RSL phenotype. F represents female parent genotype, M represents male parent genotype and H represents heterozygous genotype.

be used to characterize the response of various plant species to environmental stress [43]. The combination of two methods can quickly identify and narrow the chromosomal interval where the target QTL/gene is located. For example, Guo et al. [44] used the F_2 population derived from Longjing25 and Longjing11 to construct two mixed pools with the chill tolerance as the screening indicator for BSA-seq. Combined with transcriptome analysis, four cold tolerance candidate genes *Os09g0514200*, *Os09g0516500*, *Os09g0516600* and *Os09g0511600* were identified in two years. Our study used 26 SNPs to perform genotype scanning on all 545 strains within the range of *qRSLB9*, reducing the candidate gene range from 388 to 52. Among the 52 genes in the candidate interval, only 6 were differentially expressed in the control and alkali-treated groups of RNA-seq, which laid the foundation for the identification of candidate genes in this study. Based on qRT-PCR, candidate gene sequencing and genotype and phenotype analysis, *LOC_Os09g24260* was selected as a candidate gene for regulating alkaline tolerance in rice.

Salt and alkaline tolerance in rice is a complex trait controlled by multiple genes [33]. According to other studies on abiotic stress, the final alkali tolerance phenotype of plants should be considered as the result of favorable allelic variations from different key genes [45]. *LOC_Os09g24260* was annotated as encoding WD domain, G-beta repeat domain-containing protein. So far, research on the regulation of alkali tolerance in rice by *LOC_Os09g24260* has not been found. So, we conducted homologous gene alignment in The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/) and found a homologous gene AT3G50590. It is annotated as involving the autophagosome assembly and participating in the autophagosome membrane. Autophagy is a conservative eukaryotic mechanism that plays a role in the degradation of proteins, protein aggregates and the entire organelle [46]. It has previously been proved that Autophagy Related Gene (ATG gene) plays a role in plant abiotic stress [47]. Liu et al. [48] found that high salt and osmotic stress activate autophagy in Arabidopsis cells, while up-regulating the expression of *AtATG18a*. RNAi plants lacking *AtATG18a* autophagy exhibit high sensitivity to these two stresses. Studies have shown that the rice *atg10b* mutant is sensitive to high salt content and ROS inducer Methyl Viologen (MV) treatment, leading to an increase in the number of oxidized proteins in the seedlings of the *atg10b* mutant treated with MV [49]. Thus, we believe that *LOC_Os09g24260* is the candidate gene for *qRSLB9*.

Due to the fact that the parents LD21 and WL138 of the $F_{2:3}$ in this study were both japonica rice, we conducted the following analysis on the 1 bp Indel developed in candidate gene validation: studying the distribution of the 1 bp Indel in 66 rice accessions in pan genomic analysis (NCGR, https://db.ncgr.ac.cn/RicePanGenome/) [50]. Nine out of 66 accessions exhibit this genotype, including *O. sativa temperate japonica GP567*, *HP13-2*, *HP38*, *HP314*, *WYG7*, *IL9*; *O. sativa tropical japonica GP536*, *GP640*; *O. sativa aus GP540* (**Table S10**). No distribution was found in the group of *O. sativa indica*, *O. rufipogon* and *O. sativa aromatic*. The above results indicate that the 1 bp insertion is unique to japonica rice and is likely responsible for LD21′s alkaline tolerance during bud burst stage.

In summary, we choose *LOC_Os09g24260* as the candidate gene for controlling alkaline tolerance in rice. However, further research is needed to verify whether *LOC_Os09g24260* regulates alkaline tolerance by participating in cell autophagy through the construction of transgenic rice.

5. Conclusions

In this study, we used the $F_{2:3}$ population derived from two parents who had large differences in alkali resistance. Through QTL-seq and RNA-seq, we mapped a major QTL *qRSLB9* regulating alkalinity tolerance in rice bud burst stage and narrowed the candidate interval to 346.5 kb. The qRT-PCR and candidate gene sequencing showed that *LOC_Os09g24260* was most likely to control RSL in rice as a major gene who encodes WD domain, G-beta repeat domain-containing protein. The result provides valuable genetic information and materials for breeding new rice varieties with alkalinity tolerance.

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Conflict of interest

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Jiangxu Wang: Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Jingyang Bian:** Data curation. **Linshuai Liu:** Methodology, Data curation. **Shiwei Gao:**

Software, Funding acquisition. **Qing Liu:** Methodology, Data curation. **Yanjiang Feng:** Investigation, Funding acquisition. **Lili Shan:** Visualization, Formal analysis. **Junxiang Guo:** Resources, Investigation. **Guiling Wang:** Software, Resources. **Shichen Sun:** Validation, Funding acquisition, Conceptualization. **Hui Jiang:** Resources, Investigation. **Lei Chen:** Resources, Data curation. **Lei Lei:** Resources, Data curation. **Kai Liu:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Supplementary material

https://doi.org/10.1016/j.ejbt.2024.07.002.

Data availability

The raw sequencing data of BSA-seq were uploaded to the NCBI Sequence Read Archive database (PRJNA998745). RNA-seq data were deposited to the SRA database of NCBI (PRJNA1000797).

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