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Identification of QTLs by Genome-Wide Association Study in Rice for Salt Tolerance

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Abstract

A genome-wide association study (GWAS) was performed to identify potential QTLs associated with salt stress tolerance in rice. The correlation between the genotyping data set and the phenotypic expression of 213 diverse rice accessions for 11 biochemical and agronomic traits was assessed. GWAS was run using a mixed linear model and population parameters previously defined in Tassel 5.0 to predict genomic regions associated with traits for the Japonica and Indica subpopulations. GWAS resulted in the detection of numerous SNP markers scattered over the rice genome that were associated with various salt tolerance traits. A QTL region on chromosome 3 was found to contribute to the variation in salt tolerance in the Indica subpopulation and related to the two traits of sheath Ca and sheath Mg contents. Three QTL regions on chromosomes 2, 4, and 5 were found to contribute to the variation in salt tolerance in the Japonica subpopulation and related to the traits of sheath Na content, sheath Mg content, the sheath Na/K ratio, leaf Na content, and the leaf Na/K ratio.

Keywords

GWAS, QTLs, rice, salt tolerance

Introduction

The global population is estimated to increase to more than nine billion people by 2050 (United Nations, 2025). To meet this world demand, global agricultural production may have to increase by 70-110% (Wu *et al.*, 2018). Moreover, 20% of the total cultivated and 33% of irrigated agricultural lands worldwide (corresponding to one-third of the world's food production) are afflicted by high salinity, which is considered to be the most severe abiotic stress on crops (Tuteja, 2007). Breeding salt-tolerant rice varieties has been an effective strategy to ensure rice productivity in saline lands. One of the important steps for improving salt tolerance through breeding is the evaluation of variation of genetic sources and the identification of molecular markers associated with QTLs or genes conferring

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tolerance to salt stress. Salinity tolerance in rice is regulated by multiple genes (Xu et al., 2023), and expression of salinity tolerance is the result of many physiological and biochemical activities in plants. Therefore, determining genome-wide molecular signals associated with the representative traits of salinity tolerance is essential.

Quantitative trait locus (QTL) (Pandit *et al.*, 2010) analyses typically point to specific chromosomal sub-regions, whereas the more recently developed genome-wide association studies (GWAS) can precisely identify chromosomal locations with the resolution to define specific genes or even polymorphisms within coding regions (Korte & Farlow, 2013; Si *et al.*, 2016). A GWAS can be used to detect many natural allelic variations simultaneously in a single study (Yano *et al.*, 2016). These approaches employ statistical formalisms to determine the strength of the association between a genotype and phenotype, thus, supporting breeding by providing molecular markers or by identifying genes and alleles that contribute to specific traits (Zhang *et al.*, 2022). Generally, rice is sensitive to salt stress. However, salt-tolerant cultivars may provide opportunities to improve the salinity tolerance of rice through breeding. GWAS has identified genes that have previously been shown to play a role in salt tolerance but has also discovered many new genes. The outcomes will support our understanding of the physiological basis of salinity tolerance and provide suitable markers for future breeding efforts. The purpose of this study was to identify QTLs conferring mineral contents for salt tolerance in rice.

Materials and Methods

Plant materials

A diverse set of 213 rice varieties was used. The seeds were supplied by the Plant Breeding Laboratory, Faculty of Agriculture, Kyushu University.

Hydroponic system, plant growth conditions, and screening procedures

A hydroponic system was used to screen rice seedlings for salt tolerance. The experiment, consisting of a salt stress treatment and a control following a completely randomized design, was conducted with three replications. The germinated seeds were transplanted into trays of seedbed soil (Kokuryu Baido, Seisin Sangyo Co., Kitakyushu, Japan) with tap water. Rice seedlings were grown uniformly with tap water in the first week, and then with Yoshida (Y) solution (Yoshida *et al.*, 1976) in the second and third weeks. In the control, the Y solution was used continuously during the experiment. In the salt treatment, seedlings were grown in a 12 dS m^{-1} electrical conductivity (EC) solution, which was made by adding artificial seawater (ASW) to the Y solution (ASW-Y solution) during the fourth and fifth weeks. The EC of the solution was measured by an EC meter (Hand Held Conductivity Meter, Model CM-31P, DKK-TOA Corporation, Tokyo, Japan). The NaCl, Na_2SO_4, $MgCl_2$, and $CaCl_2$ contents of the ASW-Y solution were 87.48mM, 5.76mM, 11.19mM, and 2.16mM, respectively. The solution was changed two times each week. The pH was measured by a pH meter (pH Meter HM-10P, DKK-TOA Corporation, Tokyo, Japan) and adjusted to 5.0. The hydroponic system was placed in a phytotron with a constant temperature of 30°C and a humidity of 70%.

Phenotypic evaluation of rice germplasm

Assessments of all seedlings were completed using the standard evaluation score (SES) system to visually rate salt injuries and categorize salt tolerance after two weeks of salt stress following the methods described by Gregorio *et al.* (1997). After the cleaned seedlings were oven dried at 70°C for 24h, the sheaths and leaves were separated from the shoots and cut into small pieces. The total K, Ca, Mg, and Na contents in each plant part were analyzed by the nitric acid digestion method (Niazi *et al.*, 1993) and measured by atomic absorption spectrophotometer (Z5300 Polarized Zeeman Atomic Absorption Spectrophotometer, Hitachi, Tokyo, Japan).

Statistical analysis

Frequency distribution and statistical analysis of the collected data were conducted

using Microsoft Excel (Microsoft Office Professional Plus 2010) and Statistix 8 (Analytical Software, Tallahassee, FL, USA).

Genome wide association study

GWAS was conducted by using a mixed linear model (MLM) and previously defined population parameters (P3D) in Tassel 5.0 to predict the genomic regions associated with traits for the Japonica subpopulation and Indica subpopulation. To analyze the single nucleotide polymorphism (SNP) calls, a high-density rice array of 700,000 SNPs derived from sequencing wild and domesticated rice accessions was used (Mccouch *et al.,* 2016). The kinship matrix was estimated from the SNP genotyping data. Association signals at the same QTL block had their positions confirmed on the chromosome (Chr) using gPlink-2.050 and HaploView version 4.2. For each association signal, a genome area of 500 kb around the SNP with the highest $-\log_{10}$ (*P*-value*)* value was defined.

Results

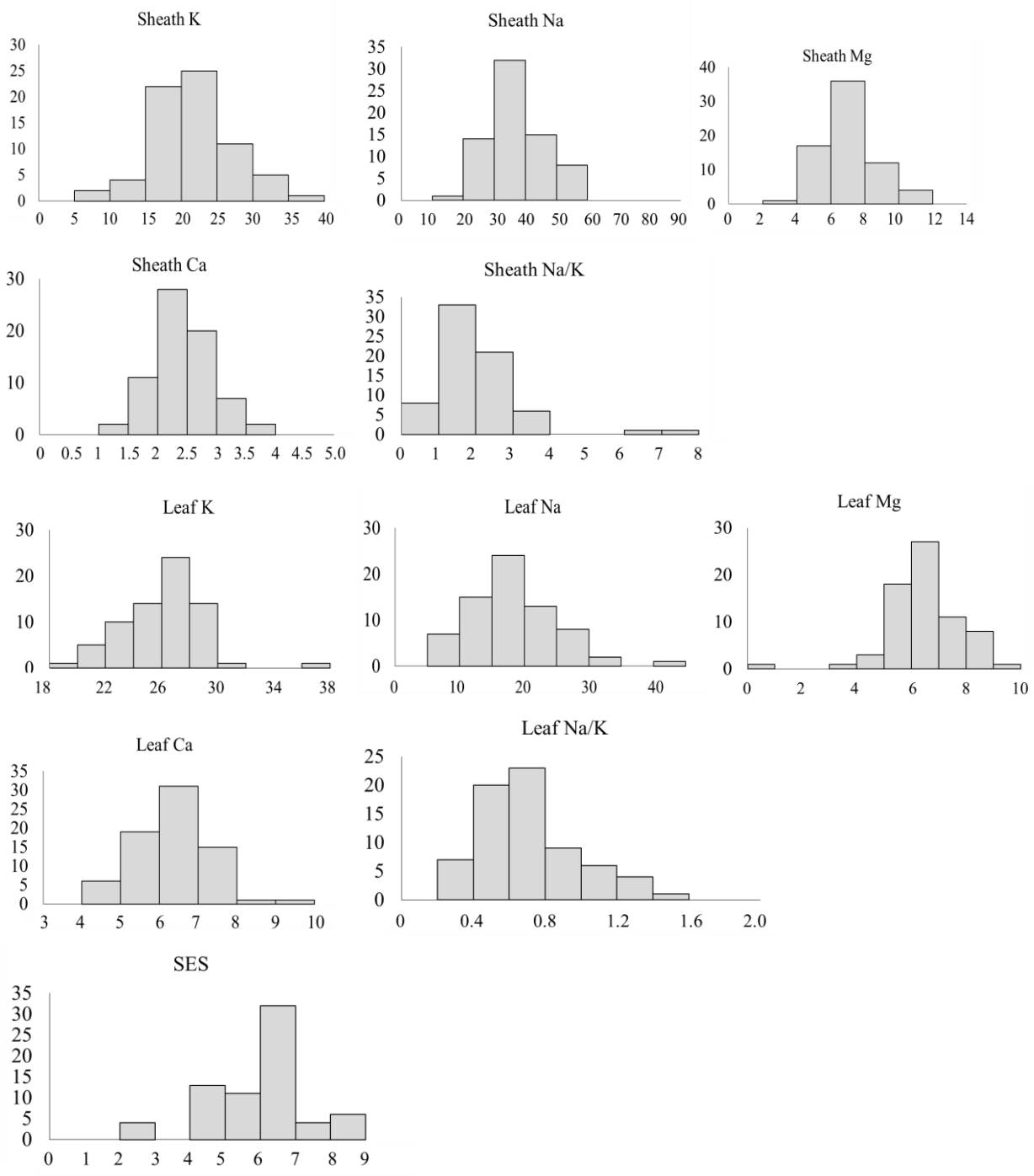
Phenotypic variation among accessions for salinity tolerance traits

The assessment of the salt-tolerant ability of rice seedlings was based on SES (Gregorio *et al.*,1997) under salt stress conditions. A rice variety is categorized as salt-tolerant if its SES is in the 3.0-5.0 range. Pokkali and FL478 are highly tolerant varieties to salt stress (the salt stress screening condition was 12 dS m^{-1} EC) with SES values of 3.0 (De Leon *et al.*, 2015; Rahman *et al.*, 2016). In this study, salt tolerance was highly variable among the 70 Indica varieties and 143 Japonica varieties. SES ranged from a score of 3.0 to a score of 9.0 in both groups. According to the SES results, there were four Indica varieties categorized as salt tolerant (NSFTV 525, 284, 313, and 643 with SES values of 3.0) and three Japonica varieties categorized as salt tolerant (NSFTV 306, 256, and 264 with SES values of 3.0). In the moderately salt-tolerant category, there were 24 Indica varieties and 26 Japonica varieties. In the salt susceptible category, there were 36 Indica varieties and 95 Japonica varieties. In the high salt susceptible group category, there were six Indica varieties and 19 Japonica varieties.

Regarding mineral contents, the results of the Indica group showed that the leaf Na contents ranged from 5.83 (NSFTV 284) to 42.76 mg g^{-1} DW (NSFTV 178). The leaf K contents ranged from 19.8 (NSFTV 620) to 37.72 mg g^{-1} DW (NSFTV269). The leaf Mg contents ranged from 0.69 (NSFTV 325) to 9.06 mg g^{-1} DW (NSFTV200). The leaf Ca contents ranged from 4.64 (NSFTV 284) to 9.23 mg g^{-1} DW (NSFTV293). The leaf Na/K ratio ranged from 0.24 (NSFTV 284) to 1.51 (NSFTV178). The sheath Na contents ranged from 19.22 (NSFTV 385) to 58 mg g^{-1} DW (NSFTV 299). The sheath K contents ranged from 6.58 (NSFTV 178) to 35.10 mg g^{-1} DW (NSFTV385). The sheath Mg contents ranged from 3.97 (NSFTV 252) to 11.79 mg g^{-1} DW (NSFTV178). The sheath Ca contents ranged from 1.42 (NSFTV 252) to 4.00 mg g^{-1} DW (NSFTV357). The sheath Na/K ratio ranged from 0.55 (NSFTV 385) to 7.40 (NSFTV178).

The results of the mineral contents of the Japonica group showed that the leaf Na contents ranged from 3.29 (NSFTV 306) to 44.47 mg g^{-1} DW (NSFTV 69). The leaf K contents ranged from 16.14 (NSFTV 187) to 34.42 mg g^{-1} DW (NSFTV 41). The leaf Mg contents ranged from 4.05 (NSFTV 113) to 9.54 mg g^{-1} DW (NSFTV 201). The leaf Ca contents ranged from 3.75 (NSFTV 113) to 9.91 mg g^{-1} DW (NSFTV 41). The leaf Na/K ratio ranged from 0.12 (NSFTV 306) to 1.86 (NSFTV 242). The sheath Na contents ranged from 5.04 (NSFTV 32) to 80.51 mg g^{-1} DW (NSFTV 69). The sheath K contents ranged from 1.13 (NSFTV 32) to 33.13 mg g^{-1} DW (NSFTV169). The sheath Mg contents ranged from 0.86 (NSFTV 32) to 13.74 mg g^{-1} DW (NSFTV624). The sheath Ca contents ranged from 0.30 (NSFTV 32) to 4.96 mg g^{-1} DW (NSFTV54). The sheath Na/K ratio ranged from 0.78 (NSFTV 244) to 6.98 (NSFTV149).

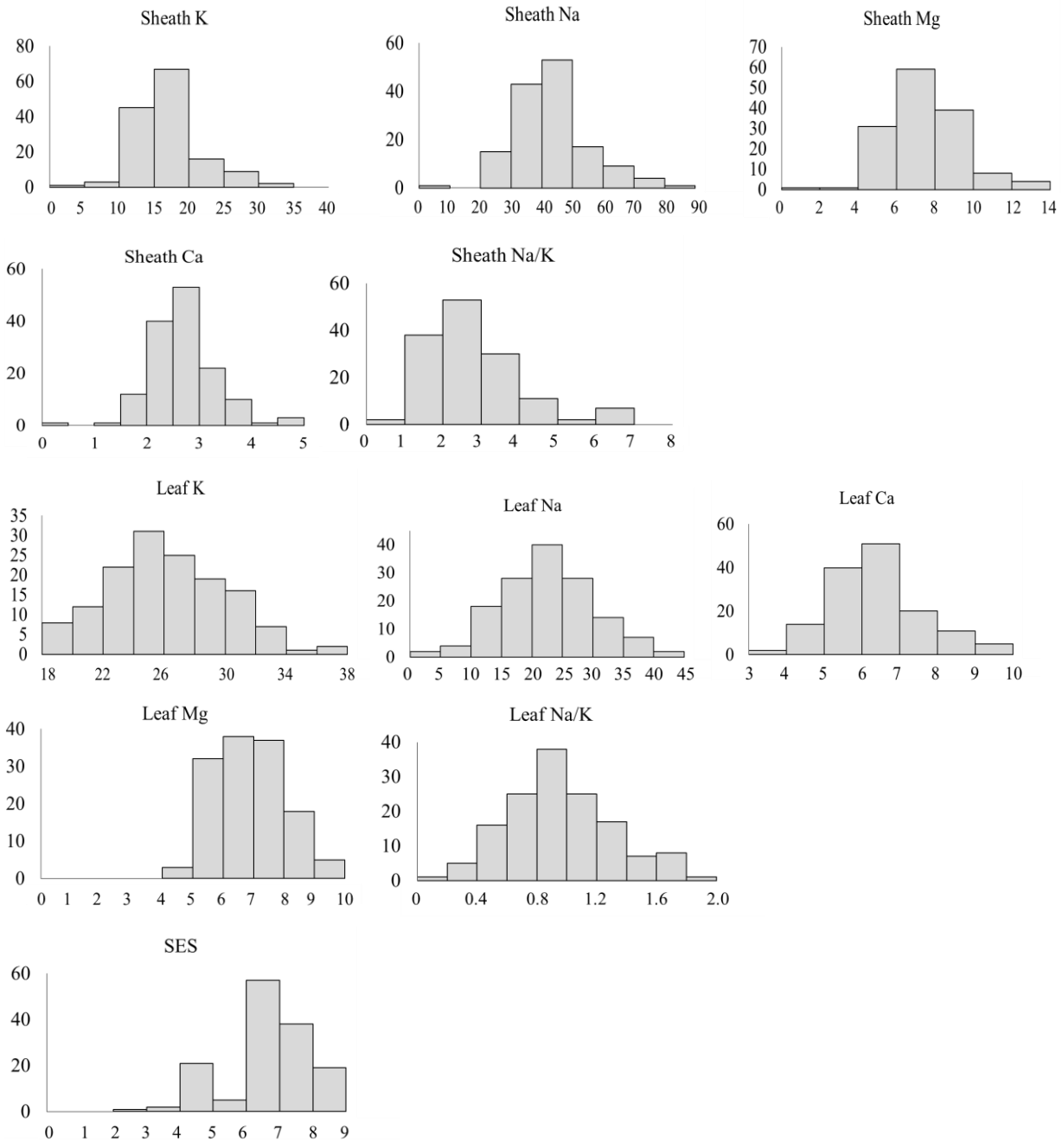
The frequency distributions of the traits observed under salt stress conditions of the Indica group and Japonica group are presented in **Figure 1** and **Figure 2**, respectively. Differences in frequency distributions between the Indica and Japonica groups were observed in the traits of sheath Na content, sheath Na/K ratio, leaf K content, leaf Na content, and leaf Na/K ratio.



Note: The vertical axes show the number of individuals. The horizontal axes show the mineral contents (mg g^{-1} dry weight). Total number of individuals is 70.

Figure 1. Frequency distributions for mineral contents, SES, and Na/K ratios of Indica rice under the salt stress treatment

The relationships among all the parameters were analyzed to understand the physiological traits that characterize salinity tolerance of the Japonica and Indica groups In the Indica group, SES was highly positively correlated with sheath Na, leaf Na, sheath Mg, and the leaf Na/K ratio with Pearson correlation index (PCI) values of 0.6, 0.65, 0.54, and 0.56, respectively. SES was negatively correlated with sheath K (PCI, -0.32). Sheath Na was highly positively correlated with sheath Mg (PCI, 0.8), sheath Na/K (PCI, 0.75), sheath Ca (PCI, 0.5), leaf Na (PCI, 0.74), and leaf



Note: The vertical axes show the number of individuals. The horizontal axes show the mineral contents (mg g^{-1} dry weight). Total number of individuals is 143.

Figure 2. Frequency distributions for mineral contents, SES, and Na/K ratios of Japonica rice under the salt stress treatment

Na/K (PCI, 0.66). Additionally, sheath K was highly negatively correlated with sheath Na (PCI, -0.60), leaf Na (PCI, -0.65), sheath Na/K (PCI, -0.82), and leaf Na/K ratio (PCI, -0.66). Sheath Mg was highly and positively correlated with the sheath Na/K ratio (PCI, 0.74), sheath Ca (PCI, 0.57), leaf Na (PCI, 0.65), and leaf Na/K (PCI, 0.58), and negatively correlated with sheath K (PCI, -0.50).

In the Japonica group, SES was positively and highly correlated with sheath Na (PCI, 0.62), leaf Na (PCI, 0.67), sheath Mg (PCI, 0.52), sheath Na/K (PCI, 0.59), and the leaf Na/K ratio (PCI, 0.60). SES was negatively correlated with

sheath K (PCI, -0.48). Sheath Na was positively and highly correlated with sheath Mg (PCI, 0.87), sheath Na/K (PCI, 0.81), sheath Ca (PCI, 0.74), leaf Na (PCI, 0.73), and leaf Na/K (PCI, 0.61). In addition, sheath K was negatively and highly correlated with leaf Na (PCI, -0.59), and the sheath and leaf Na/K ratios (PCI, -0.79 and -0.56, respectively). Sheath Mg was highly and positively correlated with the sheath Na/K ratio (PCI, 0.73), sheath Ca (PCI, 0.69), leaf Na (PCI, 0.61), and leaf Na/K (PCI, 0.59).

Association mapping (association signals and causative single nucleotide polymorphisms)

GWAS produced association signals for various traits under salt stress conditions in both the Indica and Japonica groups. An association signal was considered as a QTL if that association signal presented at least 3 SNPs (with a $-\log_{10}$ (*P*-value) value of more than 3.5) located within a physical distance of 500Kbp. The associated loci in this study for the different traits were interspersed on all the chromosomes except Chr.7. The results of the GWAS scans are summarized in **Table 1** to **Table 6.**

In the Indica group, under stress conditions, association signals were recorded for SES on Chr.2, 5, 6, and 12, leaf K content on Chr.1 and 6, leaf Na content on Chr.9, leaf Mg content on Chr.2, the leaf Na/K ratio on Chr.2, sheath Ca on Chr.3, sheath Mg on Chr.3, and sheath K on Chr.10 (**Table 1**). Among the association signals, we found that two signals (IB3 block of QTLs in **Figure 3**) were at the same location on Chr.3 with a physical distance from 26.45Mbp to 27.20Mbp. This block of QTLs was related to the two traits of sheath Ca content and sheath Mg content. **Figure 3** shows the local Manhattan plot for these two association signals. **Table 2** indicates the SNPs association, which had $-\log_{10}$ (P-value) values more than 3.5, of the block of QTLs IB3, and the 8 SNPs closely located in this QTLs region.

In the Japonica group, under stress conditions, association signals were detected for SES on Chr.1, 3, 4, 6, 8, and 9, leaf K content on Chr.12, leaf Na content on Chr.4, leaf Ca content on Chr.11, the leaf Na/K ratio on Chr.4, sheath Mg on Chr.1, 2, 5, and 6, sheath Na content on Chr.1, 2, and 5, and the sheath Na/K ratio on Chr.1, 2, 4, and 5 (**Table 3**). Among the association signals, we found three blocks of QTLs regions that included two or three association signals of traits at the same position located on Chr.2 (JB2 block of QTLs), Chr.4 (JB4 block of QTLs), and Chr.5 (JB5 block of QTLs) (**Figures 4, 5, and 6**). The JB2 block of QTLs related the three traits of sheath Na content, sheath Mg content, and sheath Na/K ratio. **Figure 4** shows the local Manhattan plot for these three association signals. **Table 4** indicates the SNPs association, which have –

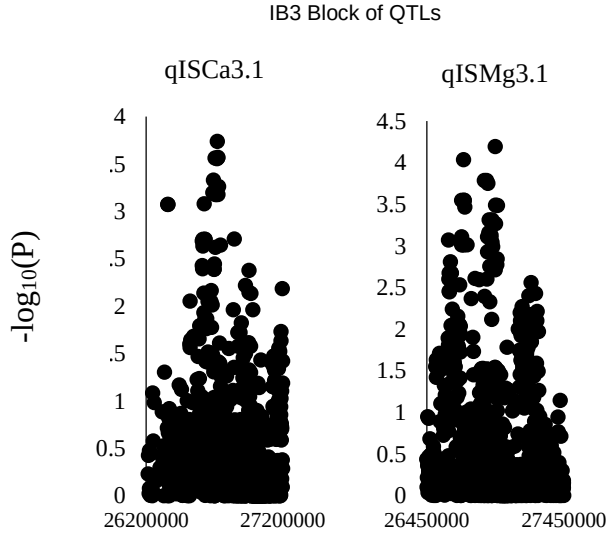


Figure 3. Local Manhattan plots show an association signal at Chr. 3 from 26.20Mb-27.20Mb for the sheath Ca content and from 26.45Mb to 27.45Mb for the sheath Mg content of the Indica group. I, Indica; B, Block

Table 1. QTLs position and markers in response to salt stress in the Indica rice subpopulation in GWAS

QTL name	Chr.	QTL region (Mbp)			Peak position (bp)	Peak marker name	-log10(P)	Trait	Allele	Effect	Obs	Allele	Effect	Obs
qILK1.1	1	2.51	-	3.51	3,013,322	SNP-1.3012321	3.74	Leaf K	C	0	26	A	4.68	37
qILMg1.1	1	3.04	-	4.04	3,540,792	SNP-1.3539791	3.67	Leaf Mg	A	0	1	G	5.44	69
qILMg2.1	2	7.46	-	8.46	7,969,755	SNP-2.7969753	3.87	Leaf Mg	T	0	54	C	5.48	1
qILNaK2.1	2	8.25	-	9.25	8,758,578	SNP-2.8758576	3.61	Leaf Na/K	G	0	49	C	0.23	21
qISES2.1	2	15.89	-	16.89	16,396,591	SNP-2.16390720	3.72	SES	C	0	3	T	3.81	67
qISCa3.1	3	26.21	-	27.21	26,719,486	SNP-3.26712539	3.74	Sheath Ca	C	0	52	T	0.67	16
qISMg3.1	3	26.45	-	27.45	26,950,344	SNP-3.26943396	4.19	Sheath Mg	T	0	60	C	2.27	9
qISES5.1	5	17.15	-	18.15	1,755,248	SNP-5.1755230	3.99	SES	A	0	5	G	2.90	62
qILK6.1	6	1.48	-	2.48	1,989,360	SNP-6.1988360	4.09	Leaf K	A	0	24	G	3.26	41
qISES6.1	6	22.16	-	23.16	22,666,120	SNP-6.22665122	3.16	SES	T	0	5	A	2.59	65
qISK10.1	10	20.94	-	21.94	21,448,855	SNP-10.21377331	3.83	Sheath K	A	0	57	G	6.67	13
qILMg12.1	12	25.85	-	26.85	26,359,117	SNP-12.26325510	3.66	Leaf Mg	C	0	1	T	5.44	66

Table 2. List of SNPs with –log10 (P)>3.5 in the IB3 block of QTLs

QTL name	Trait	Marker name	Chr	Position	-log10(P)
qISCa3.1	SheathCa	SNP-3.26712539.	3	26719486	3.74
	SheathCa	SNP-3.26717637.	3	26724584	3.57
	SheathCa	SNP-3.26697811.	3	26704758	3.56
	SheathCa	SNP-3.26716157.	3	26723104	3.56
	SheathCa	SNP-3.26711865.	3	26718812	3.56
qISMg3.1	SheathMg	SNP-3.26943396.	3	26950344	4.19
	SheathMg	SNP-3.26712539.	3	26719486	4.03
	SheathMg	SNP-3.26864306.	3	26871254	3.79
	SheathMg	SNP-3.26879551.	3	26886499	3.79
	SheathMg	SNP-3.26888983.	3	26895931	3.75
	SheathMg	SNP-3.26717637.	3	26724584	3.55
	SheathMg	SNP-3.26697811.	3	26704758	3.55
	SheathMg	SNP-3.26716157.	3	26723104	3.55
	SheathMg	SNP-3.26711865.	3	26718812	3.53

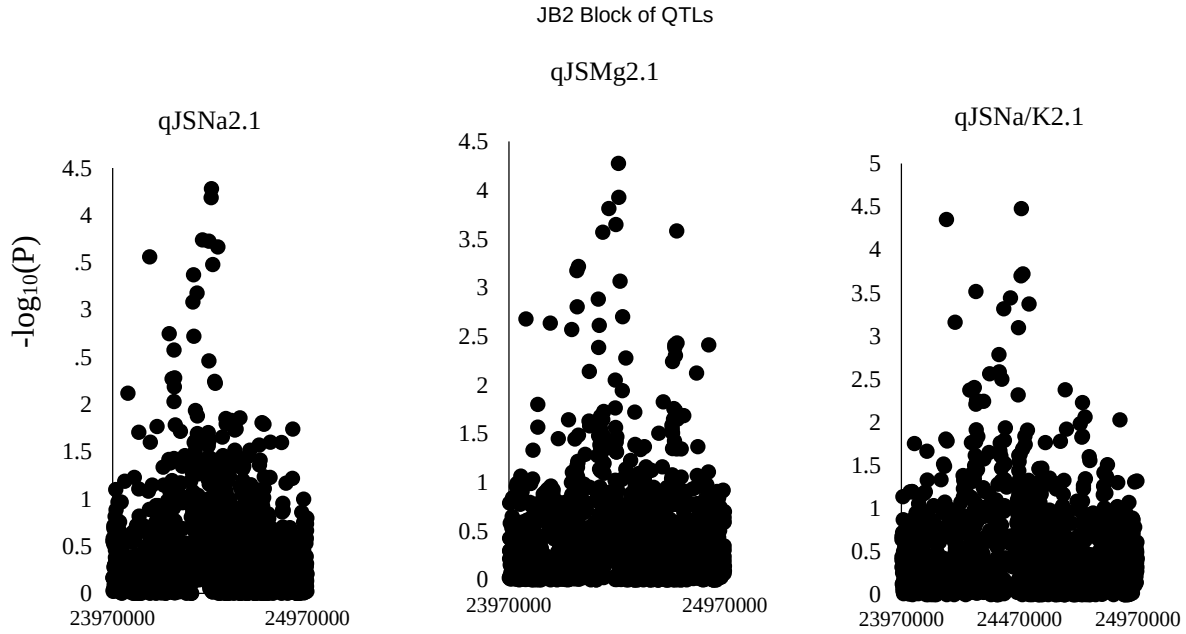


Figure 4. The local Manhattan plots show an association signal at Chr. 2 from 23.97Mb to 24.97Mb for the sheath Na content, sheath Na/K ratio, and sheath Mg content of the Japonica group

log_{10} (P-value) values more than 3.5, of the JB2 block of QTLs, and the 5 SNPs closely located in this QTLs region. The JB4 block of QTLs related the two traits of leaf Na content and leaf Na/K ratio. **Figure 5** shows the local Manhattan plots for these two association signals. **Table 5** indicates the SNPs association, which have – log_{10} (P-value) values more than 3.5, of the JB4 block of QTLs, and the 11 SNPs closely located in this QTLs region. The JB5 block of QTLs related the three traits of sheath Na content, sheath Mg content and sheath Na/K ratio. **Figure 6** shows the local Manhattan plots for these two association signals. **Table 6** indicates the SNPs association, which have – log_{10} (P-value) values more than 3.5, of the JB5 block of QTLs, and the 11 SNPs closely located in this QTLs region.

Table 3. QTLs position and markers in response to salt stress in the Japonica rice subpopulation in GWAS

QTL name	Chr.	QTL region (Mbp)			Peak position (bp)	Peak marker name	-log$_{10}$(P)	Trait	Allele	Effect	Obs	Allele	Effect	Obs
qJSES1.1	1	1.76	-	2.76	2,255,779	SNP-1.2254778	4.48	SES	T	0	34	C	1.10	101
qJSMg1.2	1	4.92	-	5.92	5,422,589	SNP-1.5421588	4.60	Sheath-Mg	A	0	122	T	2.45	8
qJSNa1.1	1	18.3	-	19.33	18,834,987	SNP-1.18833941	3.67	Sheath Na	G	0	77	A	7.29	64
qJSNa/K1.1	1	38.96	-	39.96	39,461,822	SNP-1.39460778	4.29	Sheath Na/K	C	0	123	T	1.86	9
qJSMg2.1	2	23.97	-	24.97	24,477,185	SNP-2.24471315	4.27	Sheath Mg	C	0	131	T	2.16	12
qJSNa/K2.1	2	23.97	-	24.97	24,478,886	SNP-2.24473016	4.48	Sheath Na/K	C	0	128	T	1.80	10
qJSNa2.1	2	23.97	-	24.97	24,478,886	SNP-2.24473016	4.28	Sheath Na	C	0	128	T	16.12	10
qJSES3.1	3	4.41	-	5.41	4,919,202	SNP-3.4918203	6.44	SES	C	0	23	T	1.53	120
qJSES4.1	4	30.52	-	31.52	31,026,188	SNP-4.30841068	3.81	SES	T	0	27	A	0.76	115
qJLNa/K4.1	4	32.57	-	33.57	33,073,441	SNP-4.32888329	3.96	Leaf Na/K	G	0	98	A	0.27	39
qJLNa4.1	4	32.60	-	33.60	33,104,100	SNP-4.32918988	3.66	Leaf Na	G	0	86	A	7.11	42
qJSMg5.1	5	14.14	-	15.14	14,646,033	SNP-5.14588575	4.47	Sheath Mg	C	0	125	T	2.01	17
qJSNa5.1	5	14.14	-	14.14	14,646,033	SNP-5.14588575	4.54	Sheath Na	C	0	125	T	13.60	17
qJSNa/K5.1	5	14.28	-	15.28	14,781,808	SNP-5.14724350	4.38	Sheath Na/K	C	0	124	G	1.97	12
qJSMg6.1	6	3.63	-	4.63	4,127,047	SNP-6.4126047	3.96	Sheath-Mg	T	0	123	C	1.38	12
qJSES6.1	6	9.07	-	10.07	9,571,718	SNP-6.9570718	4.50	SES	G	0	104	A	1.15	32
qJSES8.1	8	2.76	-	3.76	3,262,045	SNP-8.3261047	4.87	SES	A	0	28	C	1.20	109
qJSES9.1	9	14.91	-	15.91	15,416,334	SNP-9.15415332	4.83	SES	C	0	107	T	1.16	34
qJLCa11.1	11	6.64	-	7.60	7,144,224	SNP-11.7139968	4.83	Leaf Ca	A	0	111	C	1.66	19
qJLK12.1	12	18.34	-	19.34	18,848,241	SNP-12.18819675	4.09	Leaf K	T	0	64	A	2.94	70

Table 4. List of SNPs with $-\log_{10}(P)>3.5$ in the JB2 block of QTLs

QTL name	Trait	Marker	Chr	Pos	$-\log_{10}(P)$
qJSMg2.1	SheathMg	SNP-2.24471315.	2	24477185	4.274897
	SheathMg	SNP-2.24473016.	2	24478886	3.925989
	SheathMg	SNP-2.24426414.	2	24432284	3.80936
	SheathMg	SNP-2.24460059.	2	24465929	3.645373
	SheathMg	SNP-2.24741940.	2	24747810	3.581699
	SheathMg	SNP-2.24398574.	2	24404444	3.568266
qJSNa2.1	SheathNa	SNP-2.24473016.	2	24478886	4.278709
	SheathNa	SNP-2.24471315.	2	24477185	4.185746
	SheathNa	SNP-2.24426414.	2	24432284	3.739714
	SheathNa	SNP-2.24460059.	2	24465929	3.72462
	SheathNa	SNP-2.24505135.	2	24511005	3.665586
	SheathNa	SNP-2.24155577.	2	24161447	3.558069
qJSNa/K2.1	SheathNaK	SNP-2.24473016.	2	24478886	4.478953
	SheathNaK	SNP-2.24155577.	2	24161447	4.352911
	SheathNaK	SNP-2.24479576.	2	24485446	3.719627
	SheathNaK	SNP-2.24471315.	2	24477185	3.696523
	SheathNaK	SNP-2.24280809.	2	24286679	3.514989

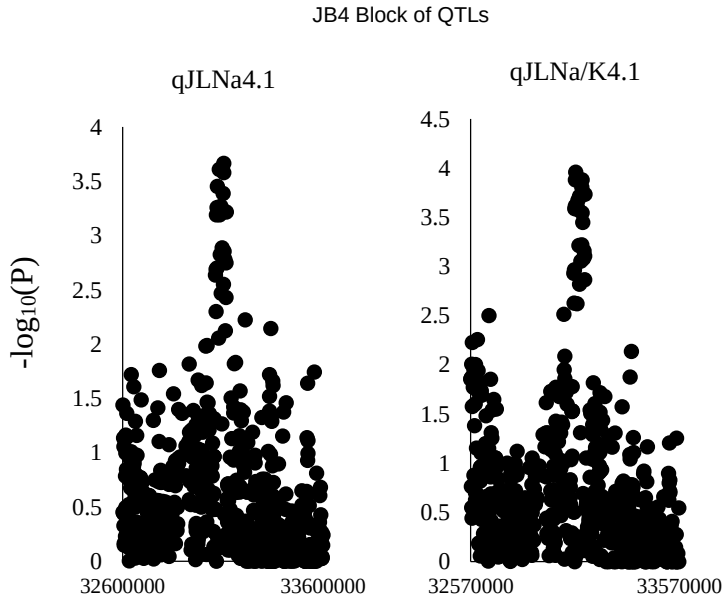


Figure 5. The local Manhattan plots show an association signal at Chr. 4 from 32.60Mb to 33.60Mb for the leaf Na content and leaf Na/K ratio of the Japonica group

Discussion

Based on the multiple correlation analysis and GWAS, it was found that the results of the blocks of QTLs were related to the multiple correlation analysis in this study. Using PCI with values of more than 0.4, the **Figure 7** and **Figure 8** showed the correlations among all 11 traits for both the Indica and Japonica groups. In the Indica group, the IB3 block of QTLs related to the two traits of sheath Ca content and sheath Mg content. Additionally, multiple correlation

Table 5. List of SNPs with $-\log_{10}$ (P)>3.5 in the JB4 block of QTLs

QTL name	Trait	Marker	Chr	Pos	$-\log_{10}$(P)
qJLNa4.1	LeafNa	SNP-4.32918988.	4	33104100	3.663841
	LeafNa	SNP-4.32897774.	4	33082886	3.608906
	LeafNa	SNP-4.32919168.	4	33104280	3.579038
qJLNa/K4.1	LeafNa/K	SNP-4.32888329.	4	33073441	3.960824
	LeafNa/K	SNP-4.32918988.	4	33104100	3.882099
	LeafNa/K	SNP-4.32886202.	4	33071314	3.881669
	LeafNa/K	SNP-4.32916825.	4	33101937	3.8076
	LeafNa/K	SNP-4.32932199.	4	33117311	3.733957
	LeafNa/K	SNP-4.32905736.	4	33090848	3.714465
	LeafNa/K	SNP-4.32897774.	4	33082886	3.673787
	LeafNa/K	SNP-4.32886261.	4	33071373	3.618831
	LeafNa/K	SNP-4.32883742.	4	33068853	3.589763
	LeafNa/K	SNP-4.32899090.	4	33084202	3.589763
	LeafNa/K	SNP-4.32895170.	4	33080282	3.582296
	LeafNa/K	SNP-4.32919168.	4	33104280	3.545353

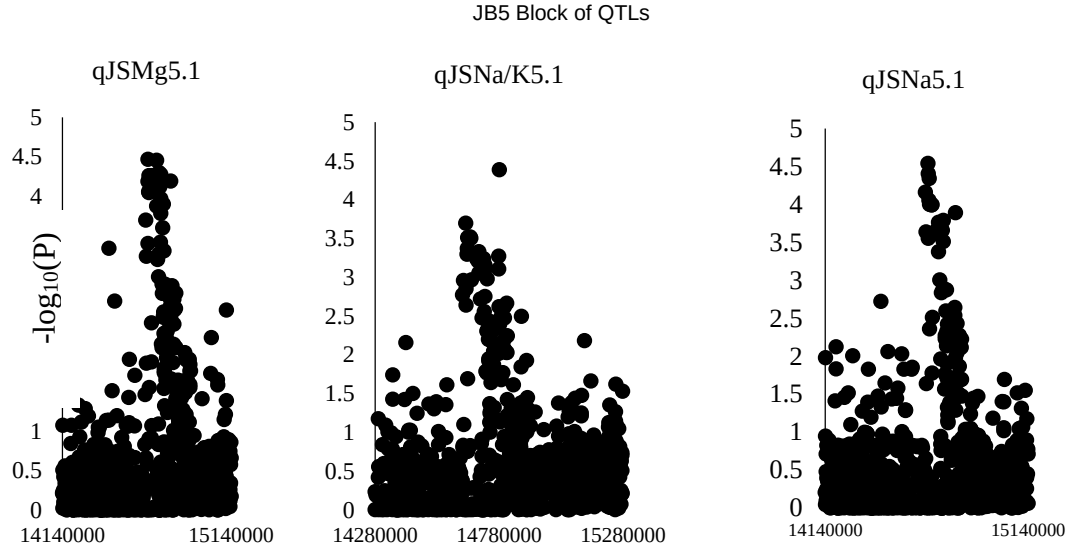


Figure 6. The local Manhattan plots show an association signal at Chr. 5 from 14.14Mb to 15.28Mb for the sheath Na content, sheath Na/K ratio, and sheath Mg content of the Japonica group

analysis showed a high correlation between these traits (PCI, 0.69). In the Japonica group, the correlation analysis showed that the three traits of sheath Na content, sheath Na/K ratio, and sheath Mg content were highly correlated. The PCI between the sheath Na content and sheath Na/K, sheath Mg content were 0.81 and 0.87, and between the sheath Mg content and sheath Na/K ratio was 0.73. Interestingly, this combination of three traits was found on both Chr.2 (JB2 block of QTLs) and Chr.5 (JB5 block of QTLs). Therefore, these results might help to confirm the reliability of the QTLs identified in this study. On the other hand, at each block of QTLs, it can be seen that almost all the SNPs with $-\log_{10}$ (*P-value*) values of more than 3.5 of the association signals for the two traits (IB3, JB4) or three traits (JB2, JB5) were the same. This indicates that these locations on chromosomes 2, 3, 4 and 5 may present multiple novelty genes or may have a gene

Table 6. List of SNPs with $-\log_{10}(P)>3.5$ in the JB5 block of QTLs

QTL name	Trait	Marker	Chr	Pos	$-\log_{10}(P)$
qJSMg5.1	SheathMg	SNP-5.14588575.	5	14646033	4.468304
	SheathMg	SNP-5.14640621.	5	14698079	4.455312
	SheathMg	SNP-5.14654512.	5	14711970	4.300596
	SheathMg	SNP-5.14665153.	5	14722611	4.274595
	SheathMg	SNP-5.14595789.	5	14653247	4.259787
	SheathMg	SNP-5.14608841.	5	14666299	4.259653
	SheathMg	SNP-5.14605845.	5	14663303	4.256804
	SheathMg	SNP-5.14724350.	5	14781808	4.188915
	SheathMg	SNP-5.14589954.	5	14647412	4.186039
	SheathMg	SNP-5.14631784.	5	14689242	4.115148
	SheathMg	SNP-5.14660003.	5	14717461	4.115148
	SheathMg	SNP-5.14592987.	5	14650445	4.057461
	SheathMg	SNP-5.14638691.	5	14696149	4.054788
	SheathMg	SNP-5.14594257.	5	14651715	4.043817
	SheathMg	SNP-5.14664785.	5	14722243	3.96465
	SheathMg	SNP-5.14680114.	5	14737572	3.896607
	SheathMg	SNP-5.14640887.	5	14698345	3.871019
	SheathMg	SNP-5.14665596.	5	14723054	3.776322
	SheathMg	SNP-5.14575374.	5	14632832	3.690902
	SheathMg	SNP-5.14676855.	5	14734313	3.594346
qJSNa5.1	SheathNa	SNP-5.14588575.	5	14646033	4.541121
	SheathNa	SNP-5.14589954.	5	14647412	4.407468
	SheathNa	SNP-5.14594257.	5	14651715	4.34259
	SheathNa	SNP-5.14575374.	5	14632832	4.161296
	SheathNa	SNP-5.14592987.	5	14650445	4.057655
	SheathNa	SNP-5.14595789.	5	14653247	3.999002
	SheathNa	SNP-5.14608841.	5	14666299	3.998526
	SheathNa	SNP-5.14605845.	5	14663303	3.994648
	SheathNa	SNP-5.14724350.	5	14781808	3.891841
	SheathNa	SNP-5.14665153.	5	14722611	3.788026
	SheathNa	SNP-5.14640621.	5	14698079	3.764977
	SheathNa	SNP-5.14654512.	5	14711970	3.674033
	SheathNa	SNP-5.14631784.	5	14689242	3.657163
	SheathNa	SNP-5.14660003.	5	14717461	3.657163
	SheathNa	SNP-5.14579619.	5	14637077	3.637781
	SheathNa	SNP-5.14638691.	5	14696149	3.604342
	SheathNa	SNP-5.14589924.	5	14647382	3.553649
	SheathNa	SNP-5.14664785.	5	14722243	3.51248
qJSNaK5.1	SheathNa/K	SNP-5.14724350.	5	14781808	4.384713
	SheathNa/K	SNP-5.14588575.	5	14646033	3.693768
	SheathNa/K	SNP-5.14605845.	5	14663303	3.51893
	SheathNa/K	SNP-5.14595789.	5	14653247	3.509831
	SheathNa/K	SNP-5.14608841.	5	14666299	3.50067

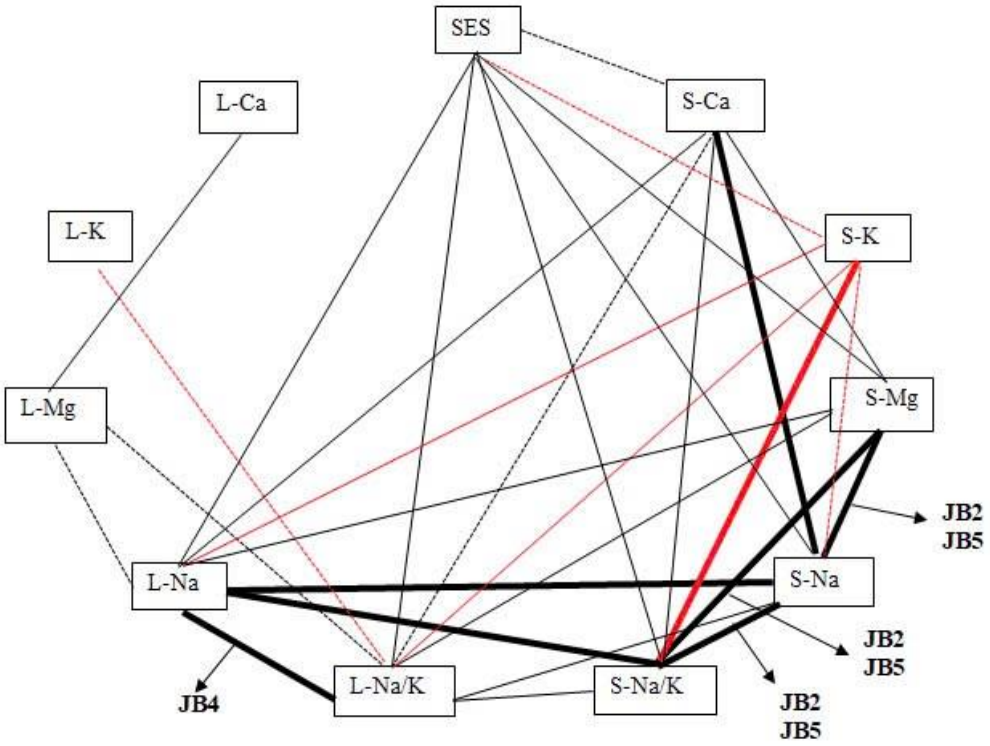


Figure 7. Pearson correlation matrix for the mineral contents for the Japonica subpopulation in response to salt stress

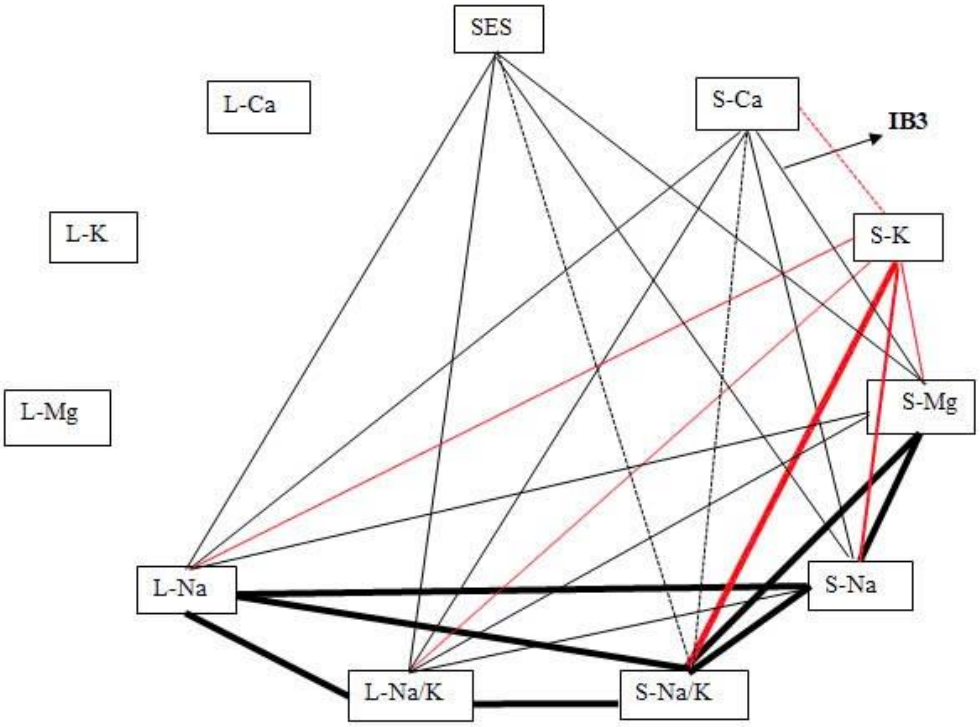


Figure 8. Pearson correlation matrix for the mineral contents for the Indica subpopulation in response to salt stress.

: Pearson correlation index from 0.4 to 0.5

: Pearson correlation index from 0.5 to 0.7

: Pearson correlation index from 0.7 to 1.0

Red color: Negative correlation; Black color: Positive correlation; IB3: block of QTLs on chromosome 3; JB2, JB4, and JB5: blocks of QTLs on Chr.2, Chr.4, and Chr.5, respectively; L: Leaf blade; S: Leaf sheath

that performs many functions for homeostasis or transportation of Na^+, Mg^{2+}, and K^+.

Using biparental mapping populations, about 70 salt tolerance QTLs were located in rice (Zang *et al.*, 2008; Hu *et al.*, 2012), but fine mapping and narrowing down reports are limited. The Saltol QTL region (Bonilla *et al.*, 2002) has been inspected widely, and three genes (SKC1, SalT, and pectinesterase) have been annotated and functionally characterized in this region (Claes *et al.*, 1990; Ren *et al.*, 2005). The location of the Saltol QTL is on the short arm of chromosome 1 from 10.8 Mbp to 16.4 Mbp (Waziri *et al.*, 2016). A limited number of studies have applied GWAS strategies to unravel the molecular mechanism that generates tolerance (Kumar *et al.*, 2015; Patishtan *et al.*, 2017; Phan *et al.*, 2023; Xu *et al.*, 2023). Screening rice for salt tolerance at the reproductive stage, Kumar *et al.* (2015) reported that under stress conditions, significant signals were detected for yield/plant, filled grains, productive tillers, spikelet fertility, spikelet fertility SSI, Na^+ content, and the Na/K ratio. For the Na/K ratio, five GWAS peaks containing 20 SNPs were recorded on chromosomes 1, 4, 6, and 7. Only one SNP located on chromosome 1 was found significant for the Na^+ content under stress. None of the SNPs showed significant peaks for the K^+ concentration under stress conditions. Using 45 day-old rice plants, Patishtan *et al.* (2017) reported that the GWAS output based on long-term NaCl phenotypic data showed 26 association signals across all chromosomes except chromosome 9. Only after long-term treatment was there a significant enrichment in the transport of lipids and ions. Both transcriptional and post-translational regulation remained prominent in this long-term salt tress treatment. Phan *et al.* (2023) reported QTLs related to SES on chromosomes 1, 2, 3, and 10 *(qSES1.1, qSES1.3, qSES2.1, qSES3.1,* and *qSES10.1*).

The Na/K ratio is an important ion balancing parameter for salt tolerance in rice, and the GWAS mapping results identified this important region as a dense GWAS peak covering the Saltol QTL region on Chr.1, which helps control K^+ homeostasis under salinity (Ren *et al.* 2005; Kumar *et al.*, 2015). Previous studies have claimed that the Saltol QTL region controls salinity only at the seedling stage and suggested that tolerance at the seedling and reproductive stages is regulated by a different set of genes and QTLs (Moradi *et al.*, 2007). In our study, three-week-old seedlings were used for screening salt tolerance. The results did not show any association signal in the Saltol QTL region. However, the results for the Na/K ratio were found at other locations on Chr.2 (qILNaK21) in the Indica group and on Chr.1 (qJSNaK1.1), Chr.2 (qJSNaK2.1), Chr.4 (qJSNaK4.1), and Chr.5 (qJSNaK5.1) in the Japonica group. Additionally, at the same position of qJSNaK2.1 on Chr.2 and qJSNaK5.1 on Chr.5, we found two other QTLs co-localized and related to the two other traits of sheath Mg content and sheath Na content, suggesting dependency among these traits. Many QTLs for Na content, K content, and the Na/K ratio have been reported in previous studies. However, those QTLs regulated a single trait. For example, Saltol-1 is a major QTL-associated with the shoot Na/K ratio (Bonilla *et al.*, 2002; Soda *et al.*, 2016). OsHKT1.5 (positioned at 11.45 Mb) is involved in Na^+ retrieval from the xylem (Ren *et al.*, 2005). Xu *et al.* (2023) reported the lead SNP (Chr12_20864157), associated with the Na/K ratio in shoots, was detected via linkage mapping as being in qSK12. The blocks of QTLs related to the sheath Na content, sheath Na/K ratio, and sheath Mg content identified in our study have not been reported in other research in rice. Moreover, the SNPs with $-\log_{10}$ (P-value) values of more than 3.5 for these traits were found in the LD plot. This suggests that the common transporters of these three ions are present in the regions of JB2 and IB5 that may positively affect Na^+, K^+, and Mg^{2+} homeostasis and salt tolerance.

Because we were trying to unravel the genetic basis for the role of tissue cation levels concerning salt tolerance, the salty condition was created similarly to that of seawater. This was a possible reason that the resulting responses of the seedlings to salt stress were related to all Na, K, Mg, and Ca content. Therefore, the results in this study have further elucidated that salt tolerance

is the result of the synergistic actions of many processes in the plant, helping to better understand the mechanisms of salinity tolerance in rice.

Conclusions

GWAS output in this research showed 12 association signals in the Indica group and 20 association signals in the Japonica group related to salt stress tolerance. One possible block of QTLs in the Indica group conferring the two traits of sheath Ca content and sheath Mg content on chromosome 3 was found. Three possible blocks of QTLs were found in the Japonica group on chromosome 2 (related to the sheath Na content, sheath Mg content, and sheath Na/K), chromosome 4 (related to the leaf Na content and leaf Na/K ratio), and chromosome 5 (related to the sheath Na content, sheath Mg content, and sheath Na/K). The correlation analysis showed a strong relationship among the three traits of sheath Na content, sheath Mg content, and sheath Na/K ratio, and between the two traits of leaf Na content and the leaf Na/K ratio. These results may indicate that multiple novelty genes performing the functions of homeostasis or transportation of Na^+, Mg^{2+}, and K^+ are at QTL locations on chromosomes 2, 3, 4, and 5. These results have further elucidated that salt tolerance is the result of the synergistic actions of many processes in rice plants, helping to better understand the mechanism of salinity tolerance in rice.

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