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POOLED MAPPING OF QTLs ASSOCIATED WITH SALT TOLERANCE TRAITS AT SEEDLING STAGE IN RICE

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Abstract

Salt stress has been identified as a vital limiting factor affecting rice output across the world. In rice, the salt tolerance nature is complicated, since it is dependent on different components and is lowly heritable. Consequently, it is a key method to breed salt-tolerant varieties for improving rice output upon salt stress. To investigate the genetic foundation for salt stress tolerance of rice seedlings (*Oryza sativa* L.), bulked segregant analysis coupled with whole-genome sequencing (BSA-seq) was performed in QTL mapping on the huge F_2 population including totally 2,500 plants obtained through crossing the *indica* rice variety 1892S with the *japonica* rice variety Huaidao 5 (HD5). In BSA-seq, only extremely-sensitive (ES) and extremely-tolerant (ET) seedlings were utilized, making it not difficult for identification with no requirement of quantitative analysis. Therefore, the seedling survival state was an appropriate indicator trait in BSA-seq. HD5 in seedling stage exhibited enhanced tolerance to extended salt stress when compared with 1892S. The DNA pools prepared based on 235 ES together with 165 ET seedlings of F_2 population through block regression mapping (BRM) were analyzed, and a QTL was mapped onto chromosome 3 and termed QTL *qSLST3.1*. There were numerous rice salt tolerance-associated QTLs on chromosome 3 in seedling stage, but just one was situated in the confidence interval of *qSLST3.1*. These QTLs did not have identical positions. So *qSLST3.1* should be the new QTL. Moreover, our results can shed more lights on marker-assisted salt-resistant variety breeding and positional cloning of rice salt tolerance trait-related genes.

Soil salinity is a main environmental stress which can limit rice growth and output globally (Hoang *et al*. 2016). It has been indicated that over 1/3 of global irrigated lands experience salinization to varying degrees (Zhao *et al*. 2020). As a vital staple food and cereal crop with the highest salt sensitivity worldwide, rice feeds over 50% of global population (Munns and Tester 2008). Salt stress will result in ion toxicity, osmotic stress (Yang *et al*. 2019), as well as oxidative stress (Xu *et al*. 2018), inducing leaf injuries, decreased yield, and even death. Even though large-scale irrigation, chemical treatment and drainage strategies can improve the saline soil, such solutions can be extremely expensive (Munns and Gilliham 2015). Obviously, it is an useful and inexpensive way to develop salt-tolerant rice species for the improvement of rice output in salinized regions. Therefore, breeding varieties that have high output and salt tolerance is important to sustain rice output.

Based on some studies, numerous QTLs and genes affect the inheritance of complicated traits including salt tolerance (Rahman *et al*. 2019). Many studies are performed to determine salt stress-related QTLs and genes (Gimhani *et al*. 2016, Wang *et al*. 2017, Kong *et al*. 2021, Yuan *et al*. 2022). Salt stress is reported to influence different rice developmental stages. It has been found to impact rice in the reproductive developmental stage, while many relevant studies mainly focus on

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the function of salt stress in rice seedlings (Leon *et al*. 2016, Bizimana *et al*. 2017, Chen *et al*. 2020, Nayyeripasand *et al*. 2021, Maniruzzaman *et al*. 2022, Kim and Kim 2023). More attention should be paid due to the critical function of salt stress in rice seedlings. In the case of direct seeding, salt stress in seedling stage makes a vital impact on crop establishment, and is genetically associated with salt stress in additional developmental stages. Therefore, investigating the genetic basis for salt stress in seedling stage is vital for breeding salt-tolerant rice.

Bulked segregant analysis through deep sequencing (BSA-seq) proves to be an effective way to conduct QTL mapping. The combination of BSA-seq and conventional gene mapping approach can significantly promote QTLs/genes fine mapping (Liang *et al*. 2020). Recently, BSA-seq has been used to mine rice QTLs (Liang *et al*. 2020). Huang *et al*. (2020) put forward the new statistical method to conduct BSA-seq, which was termed Block Regression Mapping (BRM). The present study employed the BRM approach to map rice salt tolerance-related QTLs in seedling stage. Finally, one QTL on chromosome 3 was obtained. Our findings can contribute to identifying relevant markers for breeding salt-stressed rice, fine mapping, and cloning causal gene within QTLs.

In 2018, the F_1 hybrid seed was obtained through crossing the *japonica* rice variety Huaidao 5 (HD5) with the *indica* rice variety 1892S, the elite photoperiodic and thermo-sensitive male sterile (P/TGMS) line in Hangzhou. The F_2 hybrid seed was obtained in 2019 in Hainan. The F_2 population was used in QTL mapping. Our preliminary experimental results showed that 1892S in the seedling stage was more tolerant to salt stress than HD5.

There were 54 seeds within every box (9 rows × 6 seeds/row). After pre-germination, seeds of mapping population were sowed into the clean sand inside rectangular plastic turnover boxes (38 × 25 × 10 cm^3). Seedlings were grown within the greenhouse at 25°C. Tap water was sprayed to moisten the sand every day. After growing to three-leaf stage, nutrient solution (Yoshida *et al*. 1976) spraying was performed every three days. In addition, 100 seeds were sown for each parental line as the control.

Seedlings were grown within the phytotron growth chamber containing 150 mM NaCl under the 12 hrs/12 hea light(15000 LX)/dark condition, while those in the three-leaf stage showing consistent growth performance were processed. Furthermore, seedlings were under 12 hrs stress of 150 mM NaCl, and those most sensitive to salt stress (with leaf tip wilting) were selected as extremely-sensitive (ES) seedlings. While the rest were maintained for 36 h in the chamber. At the same time, leaf tip wilting or death could be observed in many seedlings, while the few normal seedlings were selected to be extremely-tolerant (ET) seedlings. Ten turnover boxes were selected for each one batch, with totally 5 batches. There were approximately 50 ES and 35 ET seedlings in each batch, occupying approximately 9% of the overall seedlings tested, respectively.

In bulked segregant analysis (BSA), 235 ES and 165 ET seedlings were selected to establish two different groups among the 2,500 valid F_2 plants. A part of each young leave of the same weight from every group was sliced (stored under -80°C in advance). Later, all parts were blended for DNA extraction using the CTAB approach. As a result, we obtained two DNA pools, which were referred to as ES and ET pools, respectively. Following standard construction instructions for paired-end 150-bp sequencing libraries, this study implemented whole-genome resequencing on DNA samples collected in HD5 and 1892S parents and two pools with Illumina HiSeq X Ten platform.

Raw reads obtained from sequencing of ES and ET pools were washed, followed by trimming with BBDuk program for BBTools (http://jgi.doe.gov/data-and-tools/bbtools/). With the use of Burrows-Wheeler Aligner, Maximal Exact Matches algorithm (BWA MEM) was used for mapping paired reads onto IRGSP-1.0 reference rice genome (http://rapdb.dna.affrc.go.jp), while

SAMTools (Kawahara *et al.* 2013) for alignment treatment. Moreover, Freebayes was employed to call SNPs and InDels according to defaults (Garrison and Marth 2012). To obtain creditable polymorphic markers, custom perl scripts were employed to filter variants (SNP or short InDel). In order to prevent severe segregation distortion, we just retained SNPs or short InDels whose allele frequency (AF) in the population was 0.3-0.7. In addition, snpEff was applied in marker annotation (Cingolani *et al*. 2012).

QTLs were mapped with the use of the marker set. Allele frequency difference (AFD) in every marker of both pools was determined before smoothing through block regression by BRM (Huang *et al*. 2020). In the meantime, block size was calculated for regression as 20 kb. To measure threshold of AFD curve at a 0.05 overall (genome-wise) significance level, theoretical allele frequency assumption (= 0.5) of F_2 population was applied. 95% confidence intervals were determined regarding significant AFD peaks (candidate QTLs).

Totally 2,500 F_2 seedlings obtained by crossing HD5 with 1892S were selected to detect their salt tolerance, showing persistent change in wilting degree upon salt stress. This demonstrates salt tolerance as the quantitative trait.

The HD5 and 1892S parents and ET and ES pools were subjected to whole-genome sequencing to generate 75.5-108.2 million reads for each parental line or pool. After filtering, 1,984,092 SNPs and 242,848 short InDels were obtained (Table 1), with the mean densities of 5.32 SNP/kb (or 1 SNP for each 188 bp) and 0.65 InDel/kb (or 1 InDel for 1,538 bp), respectively. The values were sufficient to perform QTL mapping.

Table 1. SNPs and short InDels detected on diverse chromosomes.

Chr.	Length (bp)	SNP		Short InDel	
		Number	Density (per kb)	Number	Density (per kb)
1	43,270,923	234,218	5.42	29,969	0.69
2	35,937,250	228,470	6.31	29,381	0.81
3	36,413,819	228,949	6.27	29,433	0.81
4	35,502,694	72,265	2.02	10,316	0.34
5	29,958,434	160,950	5.34	20,314	0.68
6	31,248,787	168,486	5.35	19,386	0.62
7	29,697,621	182,242	6.11	21,256	0.72
8	28,443,022	162,385	5.69	19,278	0.68
9	23,012,720	95,502	4.11	12,514	0.54
10	23,207,287	121,606	5.16	13,603	0.58
11	29,021,106	195,618	6.72	21,854	0.75
12	27,531,856	133,401	4.77	15,439	0.55
Total	373,245,519	1,984,092	5.32	242,743	0.65

With the use of BRM approach, there was an obvious AFD peak on chromosome 3 under the 0.05 overall (genome-wise) significance level (Fig. 1), suggesting that there existed one QTL.

This QTL was denoted as *qSLST3.1*. It was most probably located at ~29.83 Mb (the peak apex), and the 95% confidence interval was 27.12–33.05 Mb. Its positive AFD peak suggested that its allele of HD5 parental line enhanced salt tolerance.

Table 2. Salt stress tolerance-related QTLs on chromosome 3 in rice seedling stage.

Tolerant/sensitive parents	Population	Appraisal period	QTL name	Position (Mb)	References
ST Pokkali/SS Bengal	F_6-RILs	at seedling stage	*qCHL3.26*	26.705-26.709	Leon *et al.* 2016
			qSHL3.34	34.72-35.06	
			qRTL3.6	6.01-6.02	
			qRTL3.7	7.13-7.20	
			qRTL3.10	10.11-10.13	
			qSRR3.8	8.32-8.35	
			qSRR3.11	11.84-11.86	
ST At354/SS Bg352	RILs	at seedling stage	*qSNK3*	35.1	Gimhani *et al.* 2016
			qSKC3	28.6	
			qSSI3	35.6	
			qSFW3.1	21.1	
ST Capsule/SS BR29	F_2	at seedling stage	*qSES3.1*	24.86	Rahman *et al.* 2019
-	-	at seedling stage	-	1.40-1.46	Chen *et al.* 2020
			-	18.79-18.79	
-	-	at seedling stage	-	6.9	Yang *et al.* 2020
			-	8.06	
ST RPY geng/SS Luohui 9	F_{15}-RILs	at seedling stage	*qST-3.1*	16.99-17.30	Kong *et al.* 2021
ST Akundi/SS BRRI dhan49	$F_{2:3}$	at seedling stage	*qSES3*	2.88	Maniruzzaman *et al.* 2022
ST *Oryza longistaminata*/SS 9311	BC_2F_{20}-BILs	at seedling stage	*qRSL3*	14.60-14.63	Yuan *et al.* 2022

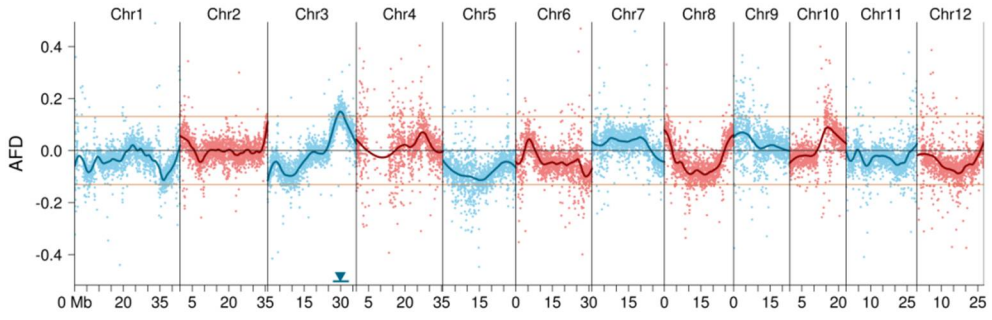

Fig. 1. Block regression mapping of QTLs that conferred salt stress tolerance in rice. The horizontal orange lines represent AFD threshold (± 0.131) at a 0.05 overall (genome-wise) significance level. Those predicted QTL locations are denoted as filled triangles. The AF of 1892S parental line of ES pool was subtracted from that of ET pool to measure the AFD of a marker.

In the early 21st century, over 395 salt tolerance-associated QTLs were obtained from rice (Gimhani *et al*. 2016, Rahman *et al*. 2019, Yang *et al*. 2020, Zeng *et al*. 2021, Goto *et al*. 2022). They are mostly mapped in accordance with the performance in rice seedling stage (Chen *et al*. 2020, Kong *et al*. 2021, Kim and Kim 2023). In this study, we obtained 19 previously obtained rice salt tolerance-associated QTLs were obtained on chromosome 3 in seedling stage (Table 2), which were not located in the confidence interval of QTL *qSLST3.1*. Therefore, *qSLST3.1* located on chromosome 3 was a novel rice salt tolerance-related QTL in seedling stage.

Salt tolerance, the complex and integrative trait, are the salt stress response of plants. Many characteristics are related to rice salt tolerance, including salinity survival index (Gimhani *et al*. 2016), root length (Bizimana *et al*. 2017), and survival rate (Maniruzzaman *et al*. 2022). Nevertheless, diverse marker traits related to salt tolerance can lead to distinct QTLs (Rahman *et al*. 2019, Zeng *et al*. 2021, Yuan *et al*. 2022). Therefore, it is vital to select appropriate marker traits to map salt tolerance-related QTLs. In this study, the salt-stressed seedling survival state was utilized to represent salt tolerance. Survival state has a direct reflection of salt tolerance degree. However, it can hardly be determined in a quantitative manner. Fortunately, BSA-seq just utilizes ET and ES seedlings, which are not difficult to be identified with no requirement of quantitative analysis. In this regard, seedling survival state is an appropriate marker trait for BSA-seq, which is demonstrated to be time-saving, labor-saving and cost-effective.

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References

Bizimana JB, Luzi-Kihupi A, Murori RW and Singh RK 2017. Identification of quantitative trait loci for salinity tolerance in rice (*Oryza sativa* L.) using IR29/Hasawi mapping population. J Genet. **96**(4): 571-582.

Chen C, Norton GJ and Price AH 2020. Genome-wide association mapping for salt tolerance of rice seedlings grown in hydroponic and soil systems using the Bengal and Assam aus panel. Front Plant Sci. **11**: 576479.

Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X and Ruden DM 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin). **6**(2): 80-92.

Garrison E and Marth G 2012. Haplotype-based variant detection from short-read sequencing. Quant Biol. arXiv:1207.3907.

Gimhani DR, Gregorio GB, Kottearachchi NS and Samarasinghe WL 2016. SNP-based discovery of salinity-tolerant QTLs in a bi-parental population of rice (*Oryza sativa* L.). Mol. Genet. Genomics. **291**(6): 2081-2099.

Goto I, Neang S, Kuroki R, Reyes VP, Doi K, Skoulding NS, Taniguchi M, Yamauchi A and Mitsuya S 2022. QTL analysis for sodium removal ability in rice leaf sheaths under salinity using an IR-44595/318 F_2 population. Front Plant Sci. **13**: 1002605.

Hoang TML, Tran TN, Nguyen TKT, Williams B, Wurm P, Bellairs S and Mundree S 2016. Improvement of salinity stress tolerance in rice: Challenges and opportunities. Agronomy. **6**(4): 54.

Huang L, Tang W, Bu S and Wu W 2020. BRM: a statistical method for QTL mapping based on bulked segregant analysis by deep sequencing. Bioinformatics. **36**(7): 2150-2156.

Kawahara Y, de la Bastide M, Hamilton JP, Kanamori H, McCombie WR, Ouyang S, Schwartz DC, Tanaka T, Wu J, Zhou S, Childs KL, Davidson RM, Lin H, Quesada-Ocampo L, Vaillancourt B, Sakai H, Lee SS, Kim J, Numa H, Itoh T, Buell CR and Matsumoto T 2013. Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. Rice (NY). **6**(1): 4.

Kim TH and Kim SM 2023. Identification of candidate genes for salt tolerance at the seedling stage using integrated genome-wide association study and transcriptome analysis in rice. Plants (Basel). **12**(6): 1401.

Kong W, Zhang C, Zhang S, Qiang Y, Zhang Y, Zhong H and Li Y 2021. Uncovering the novel QTLs and candidate genes of salt tolerance in rice with linkage mapping, RTM-GWAS, and RNA-seq. Rice (N Y). **14**(1): 93.

Leon TB, Linscombe S and Subudhi PK 2016. Molecular dissection of seedling salinity tolerance in rice (*Oryza sativa* L.) using a high-density GBS-based SNP linkage map. Rice (N Y). **9**(1): 52.

Liang T, Chi W, Huang L, Qu M, Zhang S, Chen ZQ, Chen ZJ, Tian D, Gui Y, Chen X, Wang Z, Tang W and Chen S 2020. Bulked segregant analysis coupled with whole-genome sequencing (BSA-Seq) mapping identifies a novel *pi21* haplotype conferring basal resistance to rice blast disease. Int. J. Mol. Sci. **21**(6): 2162.

Maniruzzaman S, Rahman MA, Hasan M, Rasul MG, Molla AH, Khatun H and Akter S 2022. Genetic mapping to detect stringent QTLs using 1k-RiCA SNP genotyping platform from the new landrace associated with salt tolerance at the seedling stage in rice. Plants (Basel). **11**(11): 1409.

Munns R and Gilliham M 2015. Salinity tolerance of crops-what is the cost? New Phytol. **208**(3): 668-673.

Munns R and Tester M 2008. Mechanisms of salinity tolerance. Annu Rev Plant Biol. 59:651-681.

Nayyeripasand L, Garoosi GA and Ahmadikhah A. 2021. Genome-wide association study (GWAS) to identify salt-tolerance QTLs carrying novel candidate genes in rice during early vegetative stage. Rice (N Y). **14**(1): 9.

Rahman MA, Thomson MJ, De Ocampo M, Egdane JA, Salam MA, Shah-E-Alam M and Ismail AM 2019. Assessing trait contribution and mapping novel QTL for salinity tolerance using the Bangladeshi rice landrace capsule. Rice (N Y). **12**(1): 63.

Wang S, Cao M, Ma X, Chen W, Zhao J, Sun C, Tan L and Liu F 2017. Integrated RNA sequencing and QTL mapping to identify candidate genes from *Oryza rufipogon* associated with salt tolerance at the seedling stage. Front Plant Sci. **8**: 1427.

Xu N, Chu Y, Chen H, Li X, Wu Q, Jin L, Wang G and Huang J 2018. Rice transcription factor OsMADS25 modulates root growth and confers salinity tolerance via the ABA-mediated regulatory pathway and ROS scavenging. PLoS Genet. **14**(10): e1007662.

Yang L, Lei L, Liu H, Wang J, Zheng H and Zou D 2020. Whole-genome mining of abiotic stress gene loci in rice. Planta. **252**(5): 85.

Yang Z, Wang C, Xue Y, Liu X, Chen S, Song C, Yang Y and Guo Y 2019. Calcium-activated 14-3-3 proteins as a molecular switch in salt stress tolerance. Nat Commun. **10**(1): 1199.

Yoshida S, Forno D A, Cock JH and Gomez KA 1976. Laboratory manual for physiological studies of rice. International Rice Research Institute Philippines. 61-66.

Yuan L, Zhang L, Wei X, Wang R, Li N, Chen G, Fan F, Huang S, Li J and Li S 2022. Quantitative trait locus mapping of salt tolerance in wild rice *Oryza longistaminata*. Int. J. Mol. Sci. **23**(4): 2379.

Zeng P, Zhu P, Qian L, Qian X, Mi Y, Lin Z, Dong S, Aronsson H, Zhang H and Cheng J 2021. Identification and fine mapping of *qGR6.2*, a novel locus controlling rice seed germination under salt stress. BMC Plant Biol. **21**(1): 36.

Zhao C, Zhang H, Song C, Zhu JK and Shabala S 2020. Mechanisms of plant responses and adaptation to soil salinity. Innovation (Camb). **1**(1): 100017.

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