

Utilization of SNP-based Highly Saturated Molecular Map of a RIL Population for the Detection of QTLs and Mining of Candidate Genes for Salinity Tolerance in Rice

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Date Received: 17th January 2020 / Date Accepted: 7th June 2020

ABSTRACT

Purpose : Previously, QTL hotspots were identified for salt tolerance from a RIL population of At354 x Bg352, under a temperature-controlled environment at the International Rice Research Institute, Philippine. However, as the rice-growing environment in Sri Lanka experiences salinity stress exaggerated with high temperature, the importance of revealing QTLs under such environment of a tropical region was realized. Therefore, the present study was focused to examine QTLs under such environment, deploying SNP-based molecular map, and retrieving potential candidate genes underlying the QTLs.

Research Method : RIL population was assessed at 12 dSm⁻¹ electrical conductivity coupled with average temperature ranged from 38 to 32 °C, day and night, respectively. QTLs were mapped using SNP markers. Potential candidate genes were identified using NGS-based high-throughput QTL-seq strategy employing whole-genome re-sequencing data of At354 and Bg352 and Gene Ontology approach.

Findings : The results revealed a broad spectrum of phenotypic variation and a significant coefficient of correlation among the morpho-physiological traits in the RIL population. Four QTLs were revealed on chromosome 7, 9, and 11 for shoot Na⁺ concentration (qSNC7), shoot K⁺ concentration (qSKC9), shoot Na⁺/K⁺ ratio (qSNK9) and root length (qRL11). Five genes, Os07g0635900, Os07g0637300, Os09g0330000, Os11g0514500, and Os11g0523800 within novel QTLs with polymorphic variants between At354 and Bg352, were recognized as potential candidate genes regulating salinity stress.

Originality/Value : The putative candidate genes have been reported to be involved in cellular transmembrane and growth modulating functions under stress, indicating their usefulness to be further researched.

Keywords: Abiotic stress, Candidate genes, Quantitative Trait Loci, Rice, Salinity tolerance, SNPs

INTRODUCTION

Rice is the second most widely grown cereal crop and considered as a model plant due to its smaller genome size, availability of vast germplasm collection, and a wide range of molecular genetic resources (Bruno *et al.*, 2017). Although the demand for rice is high with the increasing global population, the possibility of simultaneous expansion of the lands accessible for rice production remains limited due to various challenges such as scarcity of water, land degradation and the climate change as a result of global warming (Wang *et al.*, 2013).

According to Wang *et al.*, (2013), the most important factor which limits the rice yield in agricultural lands is drought, while the second

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most important factor is salinity stress. Often soil salinity is closely associated with drought causing secondary salinization in the areas where freshwater is reduced due to evaporation. Moreover, it is expected that soil salinization would be continuously increased due to the consequences of many irrigational practices and climate change (Reddy *et al.*, 2017).

Salinity tolerance in rice varies at various growth stages. Even though rice exhibits tolerance to salinity, during the vegetative growth stage, it is highly sensitive to salinity during the early seedling stage and reproductive stage which are the two most critical stages in the determination of final yield (Zeng and Shannon, 1998; Rahman *et al.*, 2016). Accumulation of excess salt, adversely affects all metabolic activities in rice, leading to a significant reduction in growth performances and productivity (Turan *et al.*, 2012). Salinity tolerance in rice is a polygenic/quantitative trait, which is governed by the cumulative effect of many genes (Thomson *et al.*, 2010; Horie *et al.*, 2012). Therefore, fine analysis of quantitative trait loci (QTLs) and their underlying genes can be effectively employed to improve rice for marker-assisted breeding. The genetic background of salt tolerance in rice has been studied for many years. Several QTLs associated with salinity tolerance have been reported through several mapping studies using different salt-tolerant donors (Koyama *et al.*, 2001; Ahmadi *et al.*, Wang *et al.*, 2012; Gimhani *et al.*, 2016; Rahman *et al.*, 2016; Tiwari *et al.*, 2016; De Leon *et al.*, Quan *et al.*, 2018).

A major QTL for salt tolerance at the seedling stage, *Saltol* was identified on chromosome 1 by using recombinant inbred lines (F_8) from the Pokkali x IR29 cross, explaining 43% of phenotypic variation in shoot Na^+/K^+ ratio (Bonilla *et al.*, 2002; Thomson *et al.*, 2010). Another QTL, *SKC1*, was mapped as a major QTL for shoot K^+ content on rice chromosome 1 (Lin *et al.*, 2004). Koyama *et al.*, (2001) identified 11 QTLs on four different chromosomes 1, 4, 6 and 9, for different component traits related to salinity including Na^+ uptake, K^+ uptake, Na^+ concentration, K^+ concentration, and Na^+/K^+ ratio.

Recent advances in high-throughput genotyping have enabled the use of single nucleotide polymorphism (SNP) markers over traditionally used SSR markers as SNP markers provide sufficiently dense genome coverage for candidate gene discovery of complex traits (Thomson, 2014). Thomson *et al.*, (2017) have been able to design an Illumina Infinium SNP chip for rice called Cornell_6K_array_Infinium_Rice (C6AIR) chip containing about 6000 SNPs. The C6AIR chip is being widely used in genetic diversity studies and QTL mapping analysis due to its ease of use and cost-effectiveness. In our previous study, it was able to produce the SNP-based highly dense and saturated molecular map for a Recombinant Inbred Line (RIL) population derived from the cross between At354 (salinity tolerant) x Bg352 (salinity susceptible) using C6AIR chip. The map consisted of 1135 polymorphic SNP markers covering 1460.81 cM of the rice genome with an average interval of 1.29 cM between marker loci (Gimhani *et al.*, 2016). Moreover, in this study, it was able to identify 14 QTL hotspots associated with salinity tolerance under controlled environmental conditions (29/21 °C day/night temperature and natural daylight) provided by the phytotron at the International Rice Research Institute (IRRI), Philippines. However, the genetic consistency of QTLs could be varied depending on different environmental parameters and genetic backgrounds (Gelli *et al.*, 2017). Therefore, as quantitative traits are highly varied due to genotype x environment interactions, there is a high possibility of detecting different QTLs for the same trait in different locations even within the same mapping population (Zou *et al.*, 2012). Therefore, there is a necessity for understanding the variability of expression of QTLs under a different environment to utilize them effectively in future Marker Assisted Selection (MAS). In Sri Lanka, rice-growing areas are mainly located in the coastal region and the inland dry zone where soil salinity condition is usually coupled with extremely high temperature ranging from 35 °C to 40 °C. Hence, in such areas, rice cultivation is experiencing comparatively higher salinity stress under field conditions. Therefore, the present study was focused to identify putative salinity responsive QTLs under the environment, which experiences both salinity stress and high

temperature utilizing the same RIL population and SNP-based high density saturated molecular map, which was previously developed. Also, usually, SNP-based high-density molecular maps provide the opportunity to directly identify candidate genes if NGS based high-throughput QTL-seq approach is employed (Das *et al.*, 2015; Srivastava *et al.*, 2017; Gudys *et al.*, 2018; Su *et al.*, 2019; Abhayawickrama *et al.*, 2020; Liu *et al.*, 2020). Accordingly, this study was extended to mine the candidate genes underlying the salinity responsive QTLs detected under salt stress coupled with the high-temperature environment by employing whole-genomic data of At354 and Bg352 parents re-sequenced from our previous study (Abhayawickrama *et al.*, 2020) and Gene Ontology (GO) enrichment approach.

MATERIALS AND METHODS

Plant material

We used 92 RILs ($F_{6,7}$) derived from the At354 x Bg352 cross developed by Gimhani *et al.*, (2014). This population has been developed for mapping genes for salt tolerance and their parents, At354 (salt-tolerant) had the pedigree of Pokkali and Bg94-1 while Bg352 (salt susceptible) had the pedigree of Bg380 and Bg367-4. The 92 RILs consisted of extremely salinity tolerant and extremely salinity susceptible lines, selected based on the growth performance of the RILs of the whole population [281 RILs ($F_{5,6}$)] under salinity stress at controlled environment conditions in IRRI (Gimhani *et al.*, 2016). The rice seeds were surface sterilized, washed thoroughly with distilled water, placed on filter papers soaked with water, and incubated for 3 days at 30 °C for germination.

Establishment of seedlings under hydroponics system

A hydroponics system was established according to the protocol described by Gregorio, (1997) to phenotypically evaluate the seedling stage salinity tolerance of the 92 RILs along with At354

and Bg352 parents. The hydroponic solution was prepared using Peter's nutrient solution (Peter's 1 g/L) (Scotts Peters 20-20-20, Professional water-soluble fertilizer) containing $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (300 mg/L).

The experiment was conducted in a plant house where the average day/night temperature ranged from 38 to 32 °C, and 100 % natural daylight penetration, situated in the Low Country Intermediate Zone (7° 32' 0" North, 79° 98' 0" East), Sri Lanka. Two blocks were maintained according to the Randomized Complete Block Design (RCBD) containing four individual plants from each line per block (*i.e.* eight replicates per RIL) in the hydroponic system.

Pre-germinated seeds with emerging radicals were carefully transferred to the holes of styrofoam seedling floats with a nylon mesh bottom, suspended in water-filled trays. After four days, when the seedlings were established in the hydroponic system, water was replaced with the nutrient solution. The appropriate amount of analytical grade NaCl was added to the nutrients solution to adjust the salinity level up to 6 dSm^{-1} of electrical conductivity (EC). After two days, salinity stress was imposed upon the seedlings by increasing the EC level up to 12 dSm^{-1} (100 mM) and continued for 21 days for screening. During this period, the pH of the nutrient solution was maintained at 5.0.

Evaluation of morphological traits of the RILs

The mapping population was assessed by the salinity responsive eight morpho-physiological traits namely shoot length (SL), root length (RL), shoot dry weight (SDW) and root dry weight (RDW), salinity survival index (SSI), shoot Na^+ concentration (SNC), shoot K^+ concentration (SKC) and shoot Na^+/K^+ ratio (SNK).

A quantitative parameter called salinity survival index (SSI) was used to evaluate the survival ability of seedlings under salinity stress. The survival percentage of each RIL was calculated once in every three days after imposing salinity till the end of 21 days of salinization (DAS). The seedling survival percentages were converted

into quantitative data by expressing them as a weighted salinity survival index by giving the maximum weight to the seedlings survived throughout the experimental period (SSI=1.00) while giving minimum weight to the seedlings died at the beginning of the experiment (SSI=0). SSI was calculated using the following formula,

$$SSI = \frac{\sum_{k=1}^n D_k S_k}{(\sum_{k=1}^n D_k)} 100$$

where D is the DAS, S is the survival percentage observed on a particular day, n is the total period of DAS, D_k is the DAS at k^{th} data collection, S_k is the seedling survival percentage at k^{th} data collection and $k = 1, 2, 3, \dots, n$ (Wijerathna *et al.*, 2014).

After 21 days of salinity stress, seedlings were collected without damaging the root system, and growth parameters viz., SL, and RL of each RIL were measured. Shoots and roots of the plant materials were separated and oven-dried at 70 °C for 3 days. SDW and RDW of the samples were measured at the end of the drying period.

Evaluation of Na⁺ and K⁺ ions of the shoot

The separated shoot materials which were oven-dried at 70 °C for 3 days, were ground to a fine powder. The extractions for the Na⁺ and K⁺ analysis were prepared according to the procedure described by Thomson *et al.*, (2010). Na⁺ and K⁺ concentrations (SNC and SNK respectively) of the samples were measured by atomic absorption spectrophotometer (Agilent Technologies 200 series AA, United States) and the SNK ratio of each RIL was calculated.

Statistical analysis of data

Morpho-physiological data of the RILs and parents were statistically analyzed using Minitab (V 18.0). All the measured parameters of At354 and Bg352 were compared using the student's t-test. Significant differences among RILs for the morpho-physiological parameters viz., SSI, SL, RL, SDW, RDW, SNC, SKC, and SNK ratio were analyzed by analysis of variants (ANOVA).

Frequency distributions were drawn for each of the traits, to identify the patterns of variation within the population. Correlation coefficients were also calculated between pairs of traits using the Pearson correlation coefficient.

QTL analysis

QTL analysis was performed using the salinity responsive morpho-physiological data resulted in the present study and the SNP-based highly saturated genetic map developed using the same mapping population in our previous study (Gimhani *et al.*, 2016). QTLs for eight traits were mapped to the previous genetic map by composite interval mapping (CIM) approach (standard model with a walk speed of 2 cM and forward cofactor selection) using Qgene version 4.4.0 (Joehanes and Nelson, 2008). The marker distances were approximately estimated in centiMorgan (cM) by multiplying the physical position (Mb) of DNA markers by a factor of 4 assuming that one million bases on a rice chromosome are equivalent to approximately 3.92 cM (Thomson *et al.*, 2010; Ye *et al.*, 2012; Sandhu *et al.*, 2014). Experiment-wide LOD threshold values at the 0.05 significance level for each trait were estimated based on the 1000 times permutation test (Doerge and Churchill, 1996). The coefficient of determination (R^2) was estimated to detect the proportion of the observed phenotypic variance explained by each QTL. The QTL names for novel QTLs were designated following the standard rice QTL nomenclature (McCouch, 2008).

Screening candidate genes for salt tolerance based on QTLs

To detect the candidate genes responsible for salinity tolerance, located within the novel QTLs identified in this study, we used whole-genome data of At354 and Bg352 re-sequenced with reference to Nipponbare genome obtained from our previous study (Abhayawickrama *et al.*, 2020). The total number of genes was first examined separately within each QTL-enriched region and out of them, genes related to abiotic

stress were identified based on Gramene (<http://www.gramene.org/>) and NCBI GenBank Databases (<https://www.ncbi.nlm.nih.gov/genbank/>). Corresponding gene sequences were retrieved from At354 and Bg352 whole genomes and screened for SNPs and InDels variants. The genes that had polymorphic variants between At354 and Bg352 were considered as candidate genes associated with abiotic stress, possibly related to salinity tolerance or susceptibility. Gene Ontology (GO) analysis was carried out for the sequences of the identified candidate genes by annotating them using Blast2GO software through the CloudBlast Database and Blastn algorithm options to determine the causative function of candidate genes in terms of molecular, biological and cellular level associated with the genes and gene products (Conesa and Gotz, 2008).

RESULTS AND DISCUSSION

Salinity responsive phenotypic variation of the RIL population

According to the mean performances of the two parents At354 and Bg 352, under salinity stress coupled with high temperature, all the studied parameters were significantly different in two parental genotypes indicating their divergent performances under the target environment (Table

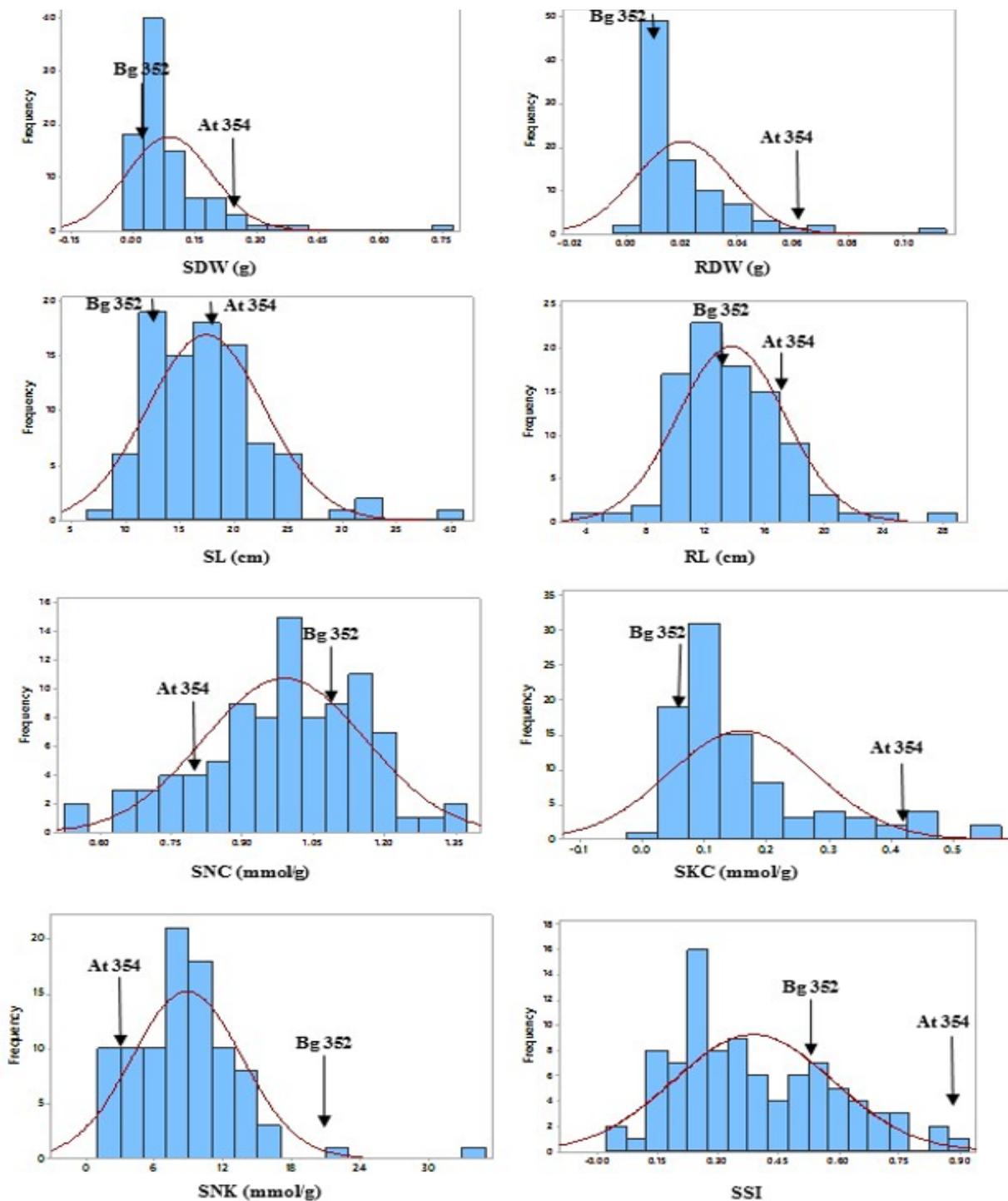
01). It was noted that the survival potential of two parents was lower in the present environment compared to the previous study where salinity stress coupled with the controlled environment at IRRI. For example, mean survival potential measured by SSI showed a comparatively lower survival rate indicating experience of higher salinity stress condition (Table 01), and probably due to high temperature of the plant house. According to the frequency distributions, the RILs have shown continuous and normal distribution, indicating the quantitative nature of the salinity responsive parameters (Figure 01). A comparatively higher coefficient of variation (CV) also resulted in each trait ranging from 17.27% to 117.8% indicates the occurrence of a substantial number of recombinant events to enable the QTL mapping for salinity tolerance (Figure 01; Table 01). Transgressive segregants were also observed in both directions for all the traits indicating superior or inferior performances of certain RILs beyond the salinity tolerant or susceptible parent (Figure 01). The transgressive segregants performing beyond the favorable parent imply the possibility of using them in varietal improvement for salt tolerance. According to the correlation analysis of the traits, all the salinity responsive morpho-physiological indices except for the relationship of SNC, with SKC and SNK, significantly correlated with each other showing their co-dependence in response to the saline condition in the target environment (Table 02).

Table 01: Salinity responsive phenotypic variation of the RIL population.

Trait ^a	Parents			RILs ^b				
	At 354	Bg 352	P value	Mean	Min	Max	SD ^d	CV% ^e
SSI	0.865	0.5295	0.000***	0.388	0.061	0.9107	0.198	50.99
SDW	0.2477	0.0293	0.000***	0.088	0.0089	0.7307	0.1036	117.8
RDW	0.0612	0.00945	0.000***	0.02	0.00387	0.113	0.0172	85.05
SL	18.7	12.85	0.002**	17.392	7.8	38.95	5.419	31.16
RL	17.37	13.39	0.003**	13.742	4.525	27.95	3.62	26.34
SNC	0.795	1.087	0.039**	0.989	0.554	1.347	0.169	17.27
SKC	0.409	0.071	0.045**	0.160	0.021	0.544	0.117	73.51
SNK	3.01	20.2	0.096*	8.829	1.189	33.911	4.812	54.5

* Significant at $P < 0.1$, ** Significant at $P < 0.05$, *** Significant at $P < 0.001$ according to Student's *t* test

^a Salt responsive morpho-physiological indices - Shoot Na^+/K^+ ratio (SNK ratio), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC), Salinity survival index (SSI), shoot length (SL), root length (RL), shoot dry weight (SDW), and root dry weight (RDW); ^b Recombinant Inbred Lines; ^d Standard Deviation; ^e Coefficient of variance



Shoot Na⁺/K⁺ ratio (SNK ratio), Shoot Na⁺ concentration (SNC), Shoot K⁺ concentration (SKC), Salinity survival index (SSI), shoot length (SL), root length (RL), shoot dry weight (SDW), and root dry weight (RDW). The mean values of At354 and Bg352 parents are indicated by arrows.

Figure 01: Frequency distributions of eight morpho-physiological salinity responsive traits assessed in the mapping population under salinity stress coupled with the high-temperature environment.

Mapping of QTLs under salinity stress coupled with the high-temperature environment

The results revealed four novel QTLs on chromosome 7, 9, and 11 (Table 03, Figure 02)

indicating probable genomic regions, which could be associated with adverse saline stress coupled with high temperature.

Table 02: Correlation among the morpho-physiological traits.

Trait ^a	SSI	SDW	RDW	SL	RL	SNC	SKC
SDW	0.667*						
RDW	0.543*	0.816*					
SL	0.775*	0.856*	0.691*				
RL	0.734*	0.726*	0.572*	0.811*			
SNC	0.359*	0.217**	0.152 ^{ns}	0.358*	0.339**		
SKC	0.51*	0.672*	0.526*	0.643*	0.514*	0.13 ^{ns}	
SNK	-0.412*	-0.48*	-0.395*	-0.47*	-0.45*	-0.19 ^{ns}	-0.76*

* Significant at $P < 0.05$ ** Significant at $P < 0.001$

^a Salt responsive morpho-physiological indices - Shoot Na^+/K^+ ratio (SNK ratio), Shoot Na^+ concentration (SNC), Shoot K^+ concentration (SKC), Salinity survival index (SSI), shoot length (SL), root length (RL), shoot dry weight (SDW), and root dry weight (RDW).

Table 03: Identified putative QTLs for salinity responsive morpho-physiological traits assessed in the F6;7 RIL population under salinity stress coupled with high temperature.

Chr ^a	QTL	Flanking region		Flanking markers	Additive effect ^b	salt-tolerant allele donor ^c	R ²	LOD	LOD Threshold value	Salinity stress-related genes within the region
		cM	Mb							
7	<i>qSNC7</i>	103.9-107.5	25.97-26.87	id7004940-7910596	0.737	Bg352	14.5	3.118	2.099	<i>Os07g0635900</i> <i>Os07g0637300</i>
9	<i>qSKC9</i>	39.1-43.0	9.77-10.75	9464502-9507290	0.437	At354	12.3	2.630	2.251	<i>Os09g0330000</i>
	<i>qSNK9</i>				-1.817		12.8	2.732	1.981	
11	<i>qRL11</i>	73.5-77.9	18.3-19.47	11522175-11571856	-1.562	Bg352	13.8	2.970	2.299	<i>Os11g0514500</i> <i>Os11g0523800</i>

^a Chromosome on which QTL was located; ^b Positive value means At354 alleles contributed to increasing the effect of respective trait, a negative value means Bg352 contributed to increasing the effect of respective trait; ^c Allele donor in favor of salt tolerance

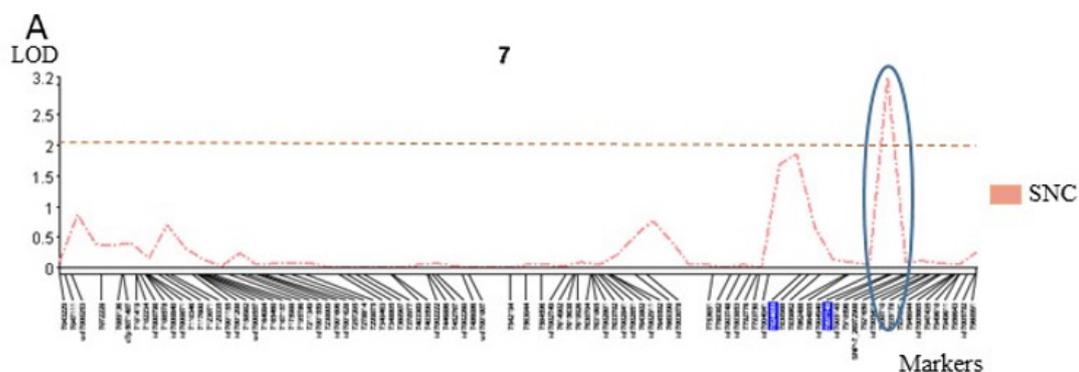


Figure 02: QTL likelihood curves of the LOD score showing the putative QTLs detected on chromosome 7, 9, and 11 under the salinity stress coupled with the high-temperature environment.

(A) QTL likelihood curve of the LOD score of Shoot Na^+ concentration (SNC) on chromosome 7; (B) Co-localization of QTL likelihood curves of the LOD score of Shoot Na^+/K^+ ratio (SNK) and Shoot K^+ concentration (SKC) on chromosome 9; (C) QTL likelihood curve of the LOD score of root length (RL) on chromosome 11.

Dotted lines indicate the experiment-wide LOD threshold values at the 0.05 significance level for each trait estimated based on the 1000 times permutation test.

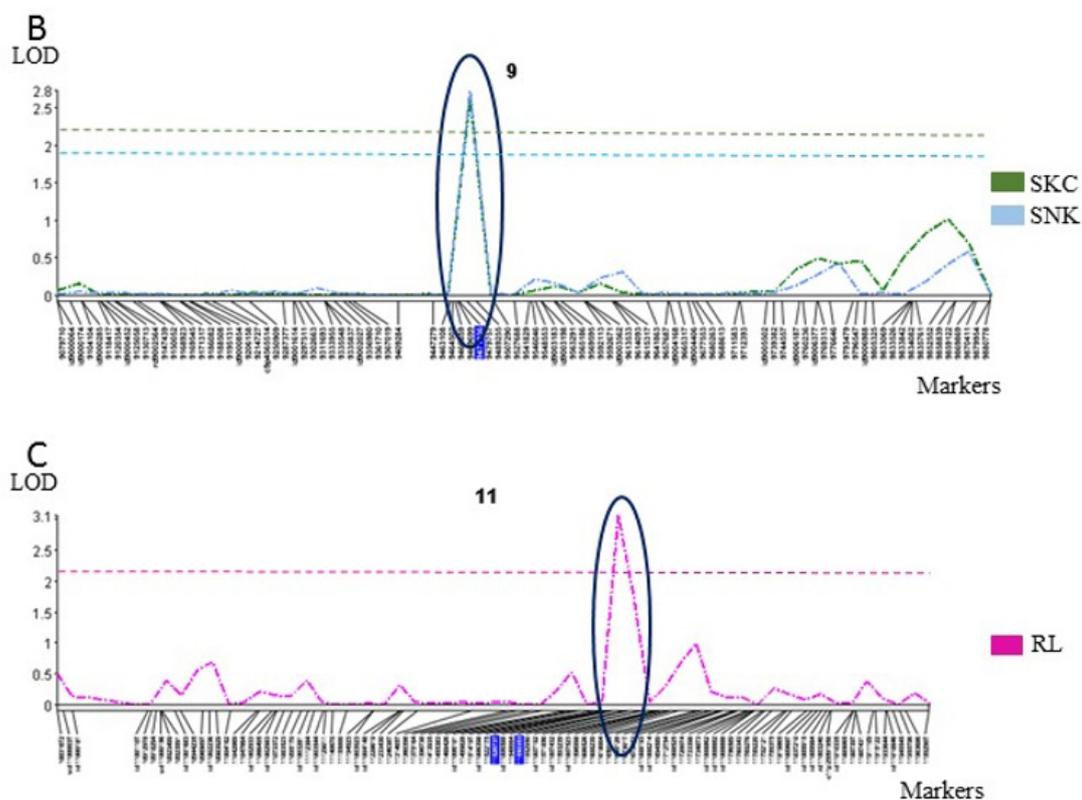


Figure 02 (cont.): QTL likelihood curves of the LOD score showing the putative QTLs detected on chromosome 7, 9, and 11 under the salinity stress coupled with the high-temperature environment.

(A) QTL likelihood curve of the LOD score of Shoot Na^+ concentration (SNC) on chromosome 7; (B) Co-localization of QTL likelihood curves of the LOD score of Shoot Na^+/K^+ ratio (SNK) and Shoot K^+ concentration (SKC) on chromosome 9; (C) QTL likelihood curve of the LOD score of root length (RL) on chromosome 11.

Dotted lines indicate the experiment-wide LOD threshold values at the 0.05 significance level for each trait estimated based on the 1000 times permutation test.

On chromosome 7, a solitary QTL was observed at 25.97 – 26.87 Mb (103.9-107.5 cM) for shoot Na^+ concentration (*qSNC7*) with a LOD score of 3.118, explaining 14.5% of phenotypic variation. According to the additive effect, the particular QTL has the impact of Bg352, susceptible parent in favor of salinity tolerance. Two more novel QTLs, responsible for shoot K^+ concentration (*qSKC9*) and shoot Na^+/K^+ ratio (*qSNK9*) were detected on chromosome 9, co-localized within the flanking region of 9.77 - 10.75 Mb (39.1-43.0 cM) indicating the possibility of the existing same set of candidate genes regulating both traits. The *qSKC9* accounted for 12.3 % of phenotypic variation while *qSNK9* accounted for 12.8 % of phenotypic variation and both QTLs favor salinity tolerance *via* the At354 allele. Solitary QTL for root length (*qRL11*) was also revealed

on chromosome 11 within the 18.3-19.47 Mb region explaining 13.8% phenotypic variation. Bg352 susceptible parent allele contribution was noted at this QTL in favor of salt tolerance (Table 03, Figure 02).

Under the present environment which experienced salinity stress together with high temperature, a prominent effect of previously identified QTLs was not observed as that of the controlled environment at IRRI, but minor QTL peaks with low LOD values were colocalized at the same corresponding regions of previously reported salinity responsive QTL hotspots. Hence, this study did not account for those minor peaks for the contribution of the total phenotypic variation because they were not significant, and it could be probably due to altered environmental exposure generated with high temperature.

Salinity responsive candidate gene mining within novel QTLs

In the present study, using high density SNP-based saturated molecular map, it was able to narrow down the QTL regions to the most probable locations of the genes underlying the QTLs. Accordingly, salinity stress-related candidate genes were detected in all three novel QTL enriched regions, on chromosome 7 (25.97- 26.87 Mb), 9 (9.77- 10.75 Mb), and 11 (18.3-19.47 Mb) by screening the SNPs and InDels polymorphic between At354 and Bg352 (Table 03; Table 04). Using GO analysis, the

molecular functions, biological processes, and cellular components associated with the identified candidate genes were retrieved to understand their functional behavior with respect to salinity stress conditions (Table 05). It was reported that usually candidate genes involved in salinity tolerance could be categorized into three groups as the genes controlling salt uptake and transport, genes that are associated with osmotic or protective function, and genes which are responsible for fast growth of plant under saline condition (Munns, 2005).

Table 04: Genes detected with the polymorphic variants present between At354 and Bg352.

Gene	Variant	Total variants*		Polymorphic variants**	
		At354	Bg352	At354	Bg352
<i>Os07g0635900</i>	SNPs	16	18	0	2SNPs
	InDels	5	5		
<i>Os07g0637300</i>	SNPs	13	17	0	4 SNPs
	InDels	2	2		
<i>Os09g0330000</i>	SNPs	53	60	1 SNP	7 SNPs
	InDels	1	1		
<i>Os11g0514500</i>	SNPs	1	4	1 SNP	4 SNPs
	InDels	0	0		
<i>Os11g0523800</i>	SNPs	23	19	10 SNPs	8 SNPs
	InDels	2	3	1 InDel	1 InDel

*With reference to Nipponbare. **The variation is not present in the respective

Table 05: Functional characterization of potential candidate genes predicated within the identified novel QTLs regions based on GO analysis, gene annotation, and available literature.

Gene	Protein*	GO terms**	Evidence for the functional relationship with salinity stress
<i>Os07g0635900</i> (<i>OsLti6a</i>)	Low temperature-induced transmembrane protein 6A (Hydrophobic protein LTI6A)	Molecular function: Protein Binding (GO:0005515)	Involved in Na ⁺ excess entry in plant cells and maintaining membrane stability under abiotic stress conditions (Morsy <i>et al.</i> , 2005; Cui <i>et al.</i> , 2016; Chidambaram <i>et al.</i> , 2015; Cui <i>et al.</i> , 2016) The close relationship to <i>RCI2B</i> , <i>RCI2A</i> in <i>Arabidopsis</i> induce under ABA, cold, osmotic and salt stress in shoot meristem and <i>BLT101</i> gene from Barley induces under low temperature, dehydration and salt stress conditions (Medina <i>et al.</i> , 2001; Goddard <i>et al.</i> , 1993) <i>OsLti6a</i> promoter contains motifs - functioned as CRT/DRE and ABRE cis-elements usually present in many Cold -Regulated and Responsive to Dehydration genes (Shinozaki and Yamaguchi-Shinozaki, 2000)

Os07g0637300 (<i>OsPDK1</i>)	pyruvate dehydrogenase kinase (PDK) protein	<p>Molecular function: protein serine/threonine kinase activity (GO:0004674), ATP binding (GO:0005524)</p> <p>Biological process: protein phosphorylation (GO:0006468), glucose homeostasis (GO:0042593), negative regulation of pyruvate (GO:1904183)</p> <p>Cellular component: mitochondrial matrix (GO:0005759)</p>	<p>Upregulation of expression of <i>OsPDK1</i> by Gibberellin. Gibberellin plays a major role in abiotic stress tolerance, increased levels of Gibberellin contributed to plant growth favorably, on exposure to stresses including cold, salt and osmotic stress (Jan <i>et al.</i>, 2006; Colebrook <i>et al.</i>, 2014; Vishal and Kumar, 2018)</p> <p>Triggering of protein phosphorylation under salinity stress in several plants (Curran <i>et al.</i>, 2011, Latz <i>et al.</i>, 2013).</p> <p>Homeostasis of sugars and sugar partitioning plays an essential role in plants' acclimation to abiotic stress tolerance. Observed triggering of the readjustments in sugar accumulation and partitioning in <i>Arabidopsis thaliana</i> under high salinity conditions (Sellami <i>et al.</i>, 2019).</p>
Os09g0330000	UDP-glucuronosyl/UDP-glucosyltransferase family protein.	<p>Molecular function: nucleic acid binding (GO:0003676), zinc ion binding (GO:0008270)</p> <p>Biological function: DNA integration (GO:0015074)</p>	<p>UDP-glycosyltransferases are involved in "Glycosylation" and play a major role in defending plants against stress and in the storage of secondary metabolites (Ko <i>et al.</i>, 2006; including glycosylation. Glycosylation, which is mediated by UDP-glycosyltransferase (UGT Moon <i>et al.</i>, 2012).</p> <p>The glycosyltransferases of <i>Arabidopsis thaliana</i> are extensively studied and shown that they contribute to salt, cold, and drought stress tolerance (Li <i>et al.</i>, 2017).</p>
Os11g0514500	leucine-rich repeat (LRR) protein	<p>Biological function cellular process (GO:0009987)</p> <p>Cellular component: Cell membrane (GO:0016020)</p>	<p>The LRR proteins play important roles in plants by engaging in different tasks such as activating stress or defense responses, detecting pathogens, and maintaining plant growth and development (Padmanaban <i>et al.</i>, 2009).</p>
Os11g0523800	Auxin response factor containing protein (ARF1)	<p>Molecular function: DNA binding (GO:0003677)</p> <p>Biological function: regulation of transcription, DNA-templated (GO:0006355), auxin-activated signaling pathway (GO:0009734)</p> <p>Cellular component: Nucleus (GO:0005634)</p>	<p>Reviewed how the networks and pathways engaged with auxin, interact to control a wide variety of plant responses to abiotic stress conditions such as drought, salinity, heat, cold, osmotic and oxidative stresses (Bielach <i>et al.</i>, 2017).</p>

*with reference to the gene annotation using <https://www.uniprot.org/uniprot> and <https://rapdb.dna.affrc.go.jp/>

** GO- Gene Ontology

Two genes, namely *Os07g0637300* and *Os07g0635900* were identified on chromosome 7 underlying the *qSNC7* QTL (Table 03). GO analysis showed that *Os07g0637300* is associated with molecular functions such as protein serine-

threonine kinase activity and ATP binding (Table 05). The gene *Os07g0637300*, commonly known as *OsPDK1* is a pyruvate dehydrogenase kinase (PDK) protein (<https://www.uniprot.org/uniprot/A0A0P0X9C5>; <https://rapdb.dna.affrc.go.jp/>).

Research has shown that the expression of PDK had been upregulated by Gibberellin, while there was little effect from other plant hormones (Jan *et al.*, 2006). According to Colebrook *et al.*, (2014) and Vishal and Kumar (2018) Gibberellin plays a major role in abiotic stress tolerance in plants and conversely, increased levels of Gibberellin have contributed to plant growth favorably, on exposure to stresses including cold, salt and osmotic stress. Accordingly, the gene *Os07g0637300* is involved in the biological process associated with protein phosphorylation, glucose homeostasis, and negative regulation of pyruvate. Protein phosphorylation cascades are also associated with transmitting signals in the cells and controlling protein functions, thereby regulating the gene expression. Munns, (2005) has reported that candidate genes associated with growth regulation are usually involved in signaling pathways, which begins with a sensor and involves hormones, transcription factors, protein kinases, protein phosphatases, and other signaling molecules. Furthermore, Munns, (2005) has also pointed out that those genes could be common to drought stress. Therefore, it could be speculated that the *Os07g0637300* candidate gene may be responsible for controlling the growth rate under salinity stress coupled with high temperature. Genes controlling growth rate are highly essential as they could trigger the rate of producing new leaves compared to the rate of senescence of old leaves under salt stress maintaining enough photosynthesizing leaves supporting the survival of the plant under stress (Munns, 2005). Moreover, certain other research studies have also shown that protein phosphorylation is triggered by exposure to salinity stress in several plants (Curran *et al.*, 2011, Latz *et al.*, 2013). Homeostasis of sugars and sugar partitioning plays an essential role in plants' acclimation to abiotic stress tolerance. Sellami *et al.*, (2019) have demonstrated that high salinity conditions trigger the readjustments in sugar accumulation and partitioning in *Arabidopsis thaliana*. Accordingly, these shreds of evidence also supported the possibility of having a vital role in the candidate gene *Os07g0637300* under salinity stress condition.

The candidate gene *Os07g0635900* underlying the same *qSNC7* QTL is also commonly known

as *OsLti6a* is responsible for producing Low temperature-induced transmembrane protein 6A (Hydrophobic protein LTI6A) (<https://www.uniprot.org/uniprot/Q8H5T6>; <https://rapdb.dna.affrc.go.jp/>). Morsy *et al.*, (2005) reported that *OsLti6a* and *OsLti6b* are mainly cold-regulated membrane protein genes present in rice, which are mainly involved, in preventing membrane injury under stress. These two genes are also homologous to rice plasma membranes protein 3 (PMP3) genes involved in Na⁺ excess entry in plant cells (Cui *et al.*, 2016). PMP3 proteins belong to the conserved hydrophobic proteins category and Chidambaram *et al.*, (2015) reported that PMP3 proteins could be involved in maintaining membrane stability under abiotic stress conditions. Furthermore, the possible involvement of these proteins to lower shoot Na⁺ levels and up-regulation of the growth performances under stress conditions has been reported in *Arabidopsis* and *Avicennia marina* by Chidambaram *et al.*, (2015). Accordingly, Cui *et al.*, (2016) demonstrated the down-regulation of *OsLti6a* and *OsLti6b* genes under salinity stress conditions. Several other research studies also emphasized the close relationship of these two genes to Rare Cold Inducible genes, *RCI2B* and *RCI2A* in *Arabidopsis* (Medina *et al.*, 2001) induce under Abscisic acid (ABA), cold, osmotic and salt stress in shoot meristem and *BLT101* gene from Barley induces under low temperature, dehydration and salt stress conditions (Goddard *et al.*, 1993). In addition, studies done by Shinozaki and Yamaguchi-Shinozaki (2000), pointed out that the *OsLti6a* promoter contains motifs that could be functioned as CRT/DRE and ABRE cis-elements usually present in many Cold-Regulated and Responsive to Dehydration genes (COR/RD genes). Therefore, based on the presence of these motifs and supportive research studies, it could be speculated that the candidate gene *Os07g0635900* (*OsLti6a*) could be a stress-inducible gene which is responsive to cold, dehydration, salt and ABA. Thus, two candidate genes, *Os07g0637300*, and *Os07g0635900* may have functional involvement to modulate the salinity stress coupled with the high temperature in the target environment of this study.

The candidate gene *Os09g0330000* was identified underlying the two coincided QTLs

i.e. *qSKC9* and *qSNK9* located on chromosome 9 (Table 03, Table 04). This gene is recognized as a member of UDP-glycosyltransferase, a superfamily of enzymes found in animals, plants, and prokaryotes (<https://rapdb.dna.affrc.go.jp/>). The gene involves molecular functions related to nucleic acid binding and zinc ion binding (Table 05). Researchers have found out that UDP-glycosyltransferases are involved in “Glycosylation” and play a major role in defending plants against stress and in the storage of secondary metabolites (Ko *et al.*, 2006; Moon *et al.*, 2012). The glycosyltransferases of *Arabidopsis thaliana* are extensively studied and shown that they contribute to salt, cold, and drought stress tolerance (Li *et al.*, 2017). Therefore, it can be speculated that the *Os09g0330000* gene has an involvement for abiotic stress tolerance and its function and expression should be further investigated to confirm its association with salinity.

Two more genes related to salinity stress tolerance were identified within the *qRL11* QTL on chromosome 11 (Table 03, Table 04). The gene *Os11g0514500* is a leucine-rich repeat (LRR) protein and the gene *Os11g0523800* which is commonly known as *ARF1* is an auxin response factor containing protein in rice (<https://rapdb.dna.affrc.go.jp/>) (Table 05). Both genes showed a different number of SNP variants between At354 and Bg352 (Table 04). The LRR domain is conserved in many proteins that are involved in maintaining the immunity of plants and animals (Ng and Xavier, 2011). The LRR proteins play important roles in plants by engaging in different tasks such as activating stress or defense responses, detecting pathogens, and maintaining plant growth and development (Padmanaban *et al.*, 2009).

Auxin has been recognized as the main phytohormone involved in regulating the plant development and its biology and underlying mechanisms are extensively studied (Waller *et al.*, 2002; Bouzroud *et al.*, 2018). Bielach *et al.*, (2017) have comprehensively reviewed how the networks and pathways engaged with auxin, interact to control a wide variety of plant responses to abiotic stress conditions such as drought, salinity, heat, cold, osmotic and

oxidative stresses. GO analysis also showed that the gene *Os11g0523800* is associated with auxin-activated signaling pathway (Table 05). Accordingly, *ARF1* being a primary auxin-responsive gene could be involved in abiotic stress-induced growth regulation in rice.

Thus, it could be speculated that all five candidate genes underlying four salinity responsive QTLs identified in the present study may have a notable functional association with salinity stress coupled with high-temperature regulation in the target environment in Sri Lanka. However, further studies on expression profiles are necessary to confirm the functional association and to elucidate the role of mutated alleles for the function.

After further comprehensive functional validation of selected genes, they could be utilized in future marker-assisted backcrossing (MAB) programs to improve the genetic architecture of rice varieties for salinity tolerance adapted to high saline conditions triggered by the high-temperature environment. Also, they could be utilized to have an in-depth understanding of the genetic regulation of rice stress-tolerant mechanism under the cumulative effect of salinity and drought stresses.

CONCLUSION

In this study, it was able to map four QTLs *viz.*, *qSNC7*, *qSKC9*, *qSNK9* and *qRL11* on chromosome 7, 9 and 11 within a narrow flanking region with less than 1 Mb interval on the SNP-based highly dense saturated molecular map which was developed using a RIL population derived from two elite *Indica* rice varieties, At354 and Bg352. All four QTLs were detected under salt (12 dSm⁻¹ EC) enriched hydroponic system coupled with high-temperature (38/32 °C day and night) conditions. By employing SNP-based high-density molecular map and NGS-based high throughput genome-wide QTL-seq approach, five genes, namely *Os07g0635900*, *Os07g0637300*, *Os09g0330000*, *Os11g0514500*, and *Os11g0523800* were identified within the novel QTLs and ascertained as possible candidate genes to regulate salinity stress. Causative genes

were also further authenticated by previous research information and GO analysis which indicated a possible association with salt stress. The findings of the present study could be utilized in future applications in MAB and deciphering the salinity tolerant mechanisms coupled with high-temperature conditions.

ACKNOWLEDGMENT

Authors thank the National Research Council of Sri Lanka for providing a research grant (NRC 16-016) and National Science Foundation for providing a one-year postgraduate scholarship (NSF/SCH/2019/01).

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