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New QTL for Salt Tolerance at the Seedling Stage in Rice var. Hasawi Using Recombinant Inbred Lines

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Novel quantitative trait loci (QTL) for seedling-stage salt tolerance were determined in Hasawi variety of rice (*Oryza sativa* L.) using 384-plex single nucleotide polymorphism (SNP) markers. The F_6 recombinant inbred lines (RILs) population, which was produced from the cross IR29 x Hasawi, generated phenotypic data for seedling length and weight, biomass, shoot sodium (Na^+) and potassium (K^+) concentration, and Na-K ratio. Genotyping analysis resulted in a linkage map length of 1379.80 cM with an average of 8 cM interval, thereby producing a total of 17 significant QTLs. Most of the QTLs detected were for seedling vigor, specifically: two for visual salt injury (*qSES1, qSES4*); three for shoot length (*qSL1, qSL9, qSL12*); two for root fresh weight (*qRFW4.1, qRFW4.2*); three for root dry weight (*qRDW12, qRDW4.1, qRDW4.2*); one for reduction in root length (*qRLRED1*); two for shoot fresh weight (*qSFW4 and qSFW12*); three for shoot dry weight (*qSDW4.1, qSDW4.2*, and *qSDW7*); and one for shoot sodium concentration (*qSNC1*). Two large-effect QTLs from chromosome 1 were found to be responsible for 37.6% of the phenotypic variation in visual salt-injury score and 41.1% of the variation in shoot length. Four QTL clusters were found in this study: one in chromosome 1 for visual salt injury and shoot sodium concentration, two in chromosome 4 responsible for seedling vigor, and one in chromosome 12, contributing to vigor as well. The results suggest that Hasawi employs a different salt-tolerance mechanism since very few studies reported QTLs in chromosomes 2, 9, and 12. The single nucleotide polymorphism (SNP) markers which co-segregrated with identified QTLs could be potential candidates for marker-aided breeding.

Key Words: Hasawi, QTL, rice, salinity, seedling stage, SNP

Abbreviations: AFLP – amplified fragment length polymorphism, CIM – complete interval mapping, EC – electrical conductivity, LOD – logarithm of odds, QTL – quantitative trait loci, RFLP – restriction fragment length polymorphism, RIL – recombinant inbred line, SES – standard evaluation scoring, SNAP – Single nutrient addition program, SNP – single nucleotide polymorphism

INTRODUCTION

Salinity stress has increasingly become an important focus of concern as a major hindrance to rice productivity worldwide. Seawater rise due to global warming has pushed more salt water to major rice-growing areas along the deltas. In Asia alone, salinity affects 21.5 million ha, of which 12 million ha are saline and 9.5 million ha are alkaline/sodic (Lafitte et al. 2004). This abiotic stress is hastened by land clearing or irrigation that causes secondary salinization, global warming, and a consequent rise in sea level and increase in storm incidences particularly in coastal areas (Wassmann et al. 2004; Rosegrant et al. 2008; Mimi and Jamous 2010).

Rice has been found to be sensitive to salinity at the seedling and reproductive stages (Flowers and Yeo 1981; Lutts et al. 1995; Grattan et al. 2002). However, rice breeders have also found some varieties with natural variability for salt tolerance (Negrao et al. 2011). The common salt-tolerant varieties, which are often used in breeding programs

to develop and improve rice varieties, are Pokkali and Nona Bokra.

More recently, Hasawi, another indica type variety from Saudi Arabia, has been found to show high levels of salt tolerance compared with Pokkali and Nona Bokra (Negrao et al. 2011). It is a landrace adapted to the climate of eastern Saudi Arabia and characterized by its strong adaptability to soil salinity and drought (AL-Mssallem et al. 2011). Hasawi has been classified to belong to the *Aus* subgroup based on allelic diversity studies using single nucleotide polymorphisms (SNPs), thereby suggesting that there could be a high level of polymorphism in offspring derived by crossing this Saudi landrace and *indica* cultivars (Thomson et al. 2010).

Salt tolerance is a complex multigenic trait (Shanon 1985; Yeo and Flowers 1986; Singh et al. 2001). The conventional methods of plant selection for this trait are not easy because of the large effects of the environment and the low narrow-sense heritability of this trait (Gregorio 1997). With the advent of DNA markers, genes underlying salt tolerance could be mapped onto different chromosomes through quantitative trait loci (QTL) analysis. The detection of QTLs could be of great help to understand the genetic control of the trait. Most studies in QTL mapping for salt tolerance have utilized amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and the widely used microsatellites or simple sequence repeat (SSR) markers. Microsatellites have been used effectively to map QTLs associated with salinity tolerance (Singh et al. 2007; Bonilla et al. 2002), but the completion of the high-quality rice genome sequence (Yu et al. 2002; International Rice Genome Sequence Project 2005) allowed the genome-wide discovery of SNPs as new markers for genetic research. SNPs are rapidly replacing SSRs as DNA markers of choice for applications in plant breeding and genetics because they are abundant in the genome, stable, amenable to automation, efficient, and increasingly cost-effective (Edwards and Batley 2010; Thomson et al. 2011).

Several QTLs for rice seedling traits conferring salt tolerance have already been identified. Some of these are QTLs associated with shoot Na^+ content (Sabouri et al. 2009), Na-K ratio (Koyama et al. 2001) and also major QTLs such as the *Saltol* for Na-K uptake ratio (Gregorio et al. 1997; Bonilla et al. 2002) and *SKC1* for shoot K content (Lin et al. 2004). QTLs are often mapped or detected in chromosomes 1, 4, 6, and 7, while no QTLs have been found in chromosomes 8 and 11 and very few in chromosomes 2, 3, 5, 9, 10 and 12 (Negrao et al. 2011). The major QTL, *Saltol* gene from Pokkali, when transferred to other varieties, can only confer medium salt tolerance to the recipient variety, thus the need to further look for genes with high levels of salt tolerance. Bimpong et al. (2014), in a study conducted in Burundi and Senegal, reported seven QTLs associated with salt tolerance at the seedling stage responsible for rice vigor from F_5 Recombinant Inbred Lines (RILs) population from IR29 by Hasawi parents.

Hence, this study aimed (1) to identify additional novel QTLs associated with different parameters of salinity tolerance from Hasawi using SNP markers at the seedling stage, and (2) to report QTLs derived from a more advanced population, F_6 RILs with larger sample size used and more robust screening technique done under controlled conditions at the International Rice Research Institute (IRRI), Philippines. SNP markers closely associated with major QTLs for salt tolerance might then be used for breeding programs in rice using marker-assisted selection.

MATERIALS AND METHODS

Plant Materials

A total of 686 RILs in F_5 generation, which were produced from the cross IR29 x Hasawi, designated as IR91477, were advanced to F_6 generation (96.9% homozygous) and mass-screened under salt stress for the selection of mapping population. The RILs were generated at the Plant Breeding, Genetics and Biotechnology (PBGB) Division of the International Rice Research Institute (IRRI) by using the single-seed descent method (SSD).

Screening of F_6 RILs at Seedling Stage

The F_6 RILs and the parents were screened for salinity tolerance under controlled environmental conditions at IRRI, Phytotron, Los Baños, Laguna, Philippines. The Phytotron plant growth facility was maintained at temperatures of 29° C during the day and 21 °C at night, with a minimum relative humidity of 70% at daytime in natural daylight. The genotypes were screened for salt tolerance at

seedling stage in a hydroponic system following the IRRI standard protocol (Gregorio et al. 1997). An initial screening of the 600 F_6 lines at seedling stage was done under an electrical conductivity (EC) of 12 dS/m using the Simple Nutrient Addition Program (SNAP) culture solution, developed at the Institute of Plant Breeding, University of the Philippines Los Baños (Santos and Ocampo 2005). The first screening was done to identify 200 lines with extreme salt-stress scores for both tolerant (100) and susceptible (100) which served as the mapping population. The second screening for the 200 extreme genotypes for salt tolerance was performed using hydroponics in a randomized complete block design with three replicates for salinized set-ups and one non-salinized set-up (control).

Salt stress was applied 7 d after sowing in a seedling float. Salinization of the SNAP culture solution was achieved by adding laboratory grade salt (NaCl) to the solution to make the electrical conductivity (EC) 12 dS/m. The culture solutions of the experimental set-ups were adjusted to a pH of 5.0 every after 2 d and the EC was maintained at 12 dS/m. Initial visual symptom evaluation of the RILs was done 2 wk after salinization and final scoring at 3 wk after salinization. Scoring was based on the modified IRRI standard evaluation scoring (SES) system for visual symptoms of saline injury at the seedling stage of rice (IRRI 1996).

The morphological data gathered for salinized and non-salinized conditions 21 d after salinization were root length (RL), shoot length (SL), root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW), root biomass (RBIOM), shoot biomass (SBIOM), percent reduction in root and shoot length, and percent reduction in root and shoot biomass. Phenotypic measurements were taken from three plants in each replication excluding the border plants.

For root and shoot length, measurements were taken from the seedling crown to the tip of the longest root and to the tip of the tallest leaf, which were expressed in centimeters. For root and shoot dry weight, root and shoot samples were oven-dried for 72 h at 70 °C and the dry weights were recorded in grams.

Shoot sample collection for Na-K analysis was done 14 d after salinization when the susceptible parent (IR29) from all replications scored an SES of almost 7. Shoot samples were washed in RO water three times and oven-dried at 70 °C for 3 d. Dried shoot samples were cut finely with surgical scissors until almost in powder form. A 10 mg powder was taken from each sample in all three salinized replications and the normal set-up. The protocol by Platten et al. (2011) for sodium-potassium analysis was followed. Shoot sodium and potassium concentration were read using flame photometer (Model 420, Sherwood, UK) and the corresponding shoot Na-K ratio for the lines were then computed.

Genotyping of F_6 RILs Using SNP Markers

Two- to 3-cm leaf samples were collected from 21-d-old seedlings of all RILs including the parents IR29 and Hasawi. DNA extraction was done at the Molecular Marker Applications Laboratory (MMAL), IRRI using the CTAB mini-preparation method (Murray and Thompson 1980). The quality of the isolated DNA was checked using 1% agarose gel. Quantification and purity of the isolated DNA were measured using Spectrophotometer (NanodropR, ND-1000, USA). DNA samples were all diluted to 50 ng/μL working concentration, and were submitted to the SNP Genotyping Service of the Molecular Marker Applications Laboratory, IRRI.

A 384-plex GoldenGate Veracode oligo pool assay (OPA) customized for *indica/indica* crosses designed for the Illumina BeadXpress Reader was used for parental polymorphism survey and SNP genotyping. The genotyping procedure followed the GoldenGate Genotyping Assay for VeraCode Manual Protocol (Illumina, San Diego, CA, USA). The 384-plex SNP plates were scanned using the Illumina BeadXpress to produce signal intensity read-outs. The Illumina BeadXpress scans each sample for signal intensity which is automatically converted to alleles by the GenomeStudio Software, followed by allele calling by Alchemy software. SNP markers that were polymorphic between the two parents, IR29 and Hasawi, were used in linkage map construction and QTL analysis.

QTL Mapping

Pearson correlation tests for quantitative traits and the non-parametric analysis for the visual salt-injury scores were carried out by transforming original scores into ranks and by using the ranks for analysis via SAS software (SAS Institute Inc. 2012).

In order to estimate the location and genetic effect of QTLs associated with seedling-stage

tolerance under salt stress, composite interval mapping (CIM) was carried out by using the QGene software (Joehanes and Nelson 2008). The QTLs generated from the CIM program were verified via permutation test. Permutation tests assigned logarithm of odds (LOD) threshold values for the declaration of a QTL to be significant. A linkage map was generated by using the Windows QTL Cartographer (Wang et al. 2011). Detected QTLs were named according to the QTL nomenclature suggested by McCouch and CGSNL (2008).

Comparison of QTLs

Comparison of our QTLs with the reported QTLs based on the physical positions were deduced by getting the sequences of the reported flanking simple sequence repeat (SSR) markers of reported QTLs and blasting it to the Rice Genome Annotation Project - MSU Rice Genome Annotation (Osa1) Release 7.

RESULTS

Phenotypic Variation of the Mapping Population

The F_6 lines were significantly different (p<0.05) under salt stress (EC=12 dS/m) for root length (RL), shoot length (SL), root and shoot fresh weight (RFW, SFW), and for root and shoot dry weight (RDW, SDW), except for the root and shoot biomass (RBIOM, SBIOM). Salinity stress caused a significant reduction in both root and shoot length and root and shoot biomass. Non-parametric analysis of the SES scores indicated that the lines were significantly different for visual salt-injury scores (see Table 1).

The mean values of the progenies were higher than the mid-parent value for the traits shoot length (SL) (41.20 cm vs. 34.9 cm.), shoot sodium concentration (SNC) (0.73 vs. 0.68.), and shoot potassium concentration (SKC) (0.67 vs. 0.50) (Table 2). The progenies had a lower mean for Na-K ratio (1.21 vs. 2.17) and root fresh weight (RFW) (0.28 g vs. 0.37) compared with the midparent value. All examined traits showed continuous distribution, thereby implicating quantitative character (Fig. 1). The distributions for the traits showed positive skewness for root and shoot length, freshweight and dryweight, and shoot sodium concentration. The distribution for the traits showed positive skewness for RL, SL, RFW, SFW, RDW, SDW, SNC and SBRED. Such results indicate that, for example in RL (skewness = 0.36, kurtosis = 0.00), the population was slightly skewed toward lower values in root length. Transgressive segregation was observed for the phenotypic traits (Fig. 1), wherein progenies attained values that were extremes relative to the parental lines except for Na-K ratio.

Table 1. Non-parametric ANOVA F for standard evaluation scores (SES).

SES	Num DF	Den DF	Value	Pr > F (DDF)	Pr > F (infty)
Lines	49.2	98.4	6.16	<.0001*	<.0001*

* indicates significance at p<0.05

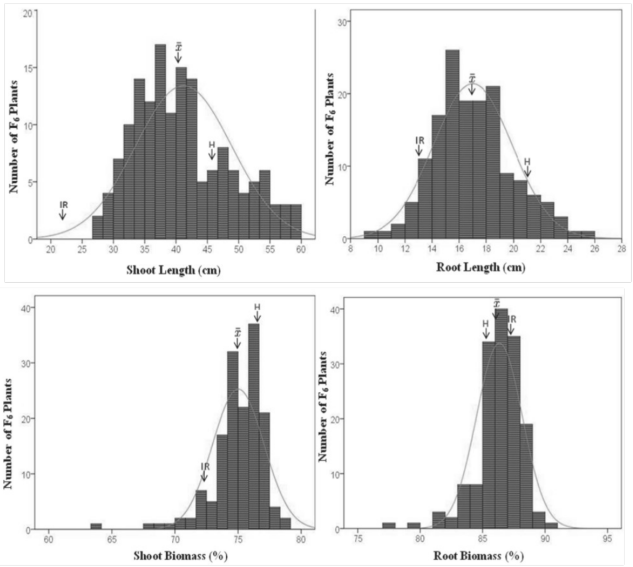

Fig. 1. Frequency distribution of phenotypic data in F_6 recombinant inbred lines (RILs) population of rice (*Oryza sativa* L.) under salt stress (electrical conductivity, EC = 12 dS.m^{-1}) from the cross IR29 x Hasawi. Arrows indicate the mean of the traits for the two parents (IR and H) and the population. (I – IR29, H – Hasawi).

Correlation among Traits

Correlation analyses demonstrated a strong negative linear association between salt injury score and traits such as root and shoot fresh weights (r= −0.637; −0.690) and root and shoot dry weights (r= −0.660; −0.636) (Table 3). A higher salt injury score means a decrease in both fresh and dry weights in root and shoot. The significant positive linear correlation between visual salt-injury score and shoot Na concentration (r = 0.601) suggests that, the higher the salt injury, the more Na was accumulated in the shoots of the plant. Salt injury score and shoot K concentration have a moderately negative linear association (r = −0.486).

Pearson correlation coefficients for the quantitative traits showed that shoot sodium

Table 2. Mean values of traits from F_6 recombinant inbred lines (RILs) population of rice (*Oryza sativa* L.) from the cross IR29 x Hasawi at seedling stage under salt stress (electrical conductivity, EC = 12 dS m^{-1}).

TRAITS	PARENTS			F_6 PROGENIES			
	IR29	Hasawi	MPV	Mean	SD	Min	Max
SES	6.83	5.00	5.92	5.47	1.19	3.00	9.00
RL	13.56	21.12	17.34	16.99	2.90	4.93	36.83
SL	23.14	46.68	34.91	41.20	7.71	16.87	63.10
RFW	0.21	0.52	0.37	0.28	0.13	0.05	0.98
SFW	0.44	1.03	0.73	0.77	0.26	0.16	1.67
RDW	0.02	0.07	0.05	0.04	0.02	0.01	0.13
SDW	0.10	0.24	0.17	0.19	0.06	0.05	0.46
RBIOM	87.97	85.78	86.88	86.34	2.32	55.31	94.68
SBIOM	72.59	77.12	74.86	75.02	3.12	50.05	84.10
SNC	1.10	0.26	0.68	0.73	0.33	0.09	2.18
SKC	0.28	0.72	0.50	0.67	0.12	0.18	1.41
Na/K Ratio	3.98	0.37	2.17	1.21	0.74	0.20	3.56
RLRED	29.42	23.15	26.28	8.90	21.70	-130.69	70.58
SLRED	45.56	24.14	34.85	34.30	10.12	-25.00	70.46
RBRED	-1.18	0.85	-0.17	0.07	8.83	-105.60	36.53
SBRED	8.63	8.12	8.37	8.62	4.11	-5.53	38.05

MPV – midparent value, SES – standard evaluation score, RL – root length, SL – shoot length, RFW – root fresh weight, SFW – shoot fresh weight, RDW – root dry weight, SDW – shoot dry weight, RBIOM – root biomass, SBIOM – shoot biomass, SNC – shoot sodium concentration, SKC – shoot potassium concentration, RLRED and SLRED – percent reduction in root and shoot length, RBRED and SBRED – percent reduction in root and shoot biomass

Table 3. Spearman correlation coefficients between salt injury scores (SES) and other traits of F_6 recombinant inbred lines (RILs) population of rice (*Oryza sativa* L.) from the cross IR29 x Hasawi under salt stress (electrical conductivity, EC = 12 dS/m) at seedling stage.

Trait	RL	SL	RFW	SFW	RDW	SDW	RBIOM	SBIOM	SNC	SKC	RLRED	SLRED	RBRED	SBRED
SES	−0.424*	0.056 ^{ns}	−0.637*	−0.690*	−0.660*	−0.636*	0.083 ^{ns}	−0.235*	**0.601***	**−0.486***	0.347*	0.375*	−0.153*	0.269*

(*) Indicates significance at $p \leq 0.05$; (ns) Indicates non-significance.
RL – root length, SL – shoot length, RFW – root fresh weight, SFW – shoot fresh weight, RDW – root dry weight, SDW – shoot dry weight, RBIOM – root biomass, SBIOM – shoot biomass, SNC – shoot sodium concentration, SKC – shoot potassium concentration, RLRED and SLRED – percent reduction in root and shoot length, RBRED and SBRED – percent reduction in root and shoot biomass

Table 4. Pearson correlation coefficients for morphological and physiological parameters of F_6 RILs population of rice (*Oryza sativa* L.) from the cross IR29 x Hasawi under salt stress (electrical conductivity, EC = 12 dS/m) at seedling stage (n = 200).

Traits	RL	SL	RFW	SFW	RDW	SDW	RBIOM	SBIOM	SNC	SKC	RLRED	SLRED
RL	**1.000**											
SL	−0.030 ^{ns}	**1.000**										
RFW	0.346***	0.212***	**1.000**									
SFW	0.358***	0.451***	0.792***	**1.000**								
RDW	0.347***	0.198***	0.936***	0.821***	**1.000**							
SDW	0.321***	0.379***	0.759***	0.934***	0.864***	**1.0000**						
RBIOM	0.048 ^{ns}	0.041 ^{ns}	0.201***	−0.003 ^{ns}	−0.103*	−0.199***	**1.000**					
SBIOM	0.207***	0.248***	0.222***	0.320***	0.046 ^{ns}	0.003 ^{ns}	0.551***	**1.000**				
SNC	−0.325***	−0.091 ^{ns}	−0.406***	0.469***	−0.396***	−0.410***	−0.044	−0.294***	**1.000**			
SKC	0.267***	0.136**	0.335***	0.425***	0.318***	0.359***	0.080 ^{ns}	0.256***	−0.579***	**1.0000**		
RLRED	−0.634***	0.107*	−0.182***	−0.188***	−0.163***	−0.151**	−0.073 ^{ns}	−0.167***	0.265***	−0.234***	1.000	
SLRED	−0.171***	−0.483***	−0.358***	−0.467***	−0.333***	−0.395***	−0.093*	−0.288***	0.332***	−0.336***	0.127**	1.000

*Significance at $p \leq 0.05$; **Significance at $p \leq 0.01$; ***Significance at $p \leq 0.005$; ^{ns}Non-significance
RL – root length, SL – shoot length, RFW – root fresh weight, SFW – shoot fresh weight, RDW – root dry weight, SDW – shoot dry weight, RBIOM – root biomass, SBIOM – shoot biomass, SNC – shoot sodium concentration, SKC – shoot potassium concentration, RLRED and SLRED – percent reduction in root and shoot length

concentration had a negative moderate linear association with RFW, SFW and SDW (Table 4). Salinity caused a significant reduction in both length and biomass of the root and shoot. Shoot K concentration had a positive moderate association with SFW (r = 0.425) and also a negative moderate linear association with SNC (r = −0.579). Shoot biomass was negatively correlated with SNC (r = −0.294) and positively correlated with SKC (r = 0.256). Moreover, the general trend with respect to visual salt injury score and Na-K ratio was that lines with higher SES had Na-K ratio value greater than one, and vice versa.

QTL Analysis of Seedling Salt Tolerance Traits

Based on the 384 markers used in the parental survey, 196 or 51.04% exhibited polymorphism between IR29 and Hasawi parents. Linkage map analysis produced 1379.80 cM total length with an average interval size of 8 cM. Interval size for the markers ranged from the smallest interval of 0.15 cM in chromosome 7 to the largest interval of 40.2 cM in chromosome 2.

Composite interval mapping (CIM) analysis

Table 5. Quantitative trait loci (QTL) identified for seedling traits of the F_6 recombinant inbred lines (RILs) population of rice (*Oryza sativa* L.) derived from IR29 and Hasawi parents.

Trait	QTL	Chromo-some No.	Peak Marker Position (cM)	Peak SNP Marker	Peak LOD CIM	Marker Interval	PVE[a]	Additive Effect	DPE[b]
Visual salt injury	*qSES1*	1	158.2	id1023892	15.875	id1020828-id1024836	37.6	0.57	H
	qSES4	4	99.16	id4008092	4.104	id4007444-id4008522	11.5	0.29	IR
Shoot length	*qSL1*	1	163.57	id1024836	17.845	id1023892-id1024972	41.1	5.81	H
	qSL9	9	23.49	id9001352	3.924	id9000881-id9001614	11.0	7.80	H
	qSL12	12	93.97	id12007988	4.784	id12005823-id12007988	13.2	2.76	H
Root fresh weight	*qRFW4.1*	4	87.35	id4007105	4.897	id4006135-id4007444	13.5	0.08	H
	qRFW4.2	4	104.38	id4008092	7.46	id4007444-id4008522	19.9	0.06	H
Root dry weight	*qRDW4.1*	4	87.35	id4007105	4.942	id4006135-id4007444	13.7	0.01	H
	qRDW4.2	4	99.16	id4008092	7.613	id4007444-id4008522	20.2	0.01	H
	qRDW12	12	58.55	id12005205	4.336	id12004491-id2005501	12.1	0.01	H
% Root length reduction	*qRLRED1*	1	158.18	id1023892	3.674	id1020828-id1024836	10.3	7.69	H
Shoot fresh weight	*qSFW4*	4	87.35	id4007105	5.174	id4006135-id4007444	14.3	0.15	H
	qSFW12	12	58.55	id12005205	4.503	id12004491-id2005501	12.5	0.08	H
Shoot dry weight	*qSDW4.1*	4	39.96	id4003259	4.128	id4002913-id4003727	11.5	0.02	H
	qSDW4.2	4	87.35	id4007105	4.912	id4006135-id4007444	13.6	0.03	H
	qSDW7	7	10.69	id7000461	3.945	ud7000066-id7000519	11.1	0.02	H
Shoot sodium concentration	*qSNC1*	1	158.18	id1023892	5.875	id1020828-id1024836	16.0	0.13	H

[a] PVE – Percentage of total phenotypic variance explained by the QTL
[b] DPE – Direction of phenotypic effect; IR – IR29, H – Hasawi
CIM – composite interval mapping, cM – centiMorgan, LOD – logarithm of odds, SNP – single nucleotide polymorphism

produced a total of 31 QTLs which have LOD scores greater than 3.0 (Table 5). A total of 17 out of 31 QTLs that had significant effects associated with salt tolerance were identified in this study and confirmed by permutation analysis. A permutation test (number of iterations = 1000 at $\alpha = 0.05$) was used to determine the threshold value for significance testing of the existence of a QTL effect tailored to the experimental data (Churchill and Doerge 1994).

Two QTLs (*qSES1* and *qSES4*) were mapped in chromosomes 1 and 4 (Table 5). In chromosome 1, a high LOD = 15.875 was recorded with *qSES1* explaining 37.6% of the phenotypic variation and a 0.57 additive effect from the Hasawi parent. This result indicates that alleles from Hasawi contributed to the trait under saline stress. In chromosome 4, however, IR29 had an additive effect of 0.29, and *qSES4* explained 11.5% of the phenotypic variance.

Three QTLs, designated as *qSL1*, *qSL9* and *qSL12*, were located in chromosomes 1, 9, and 12, respectively. QTL in chromosome 1 explained 41.1% of the total phenotypic variance with the highest LOD score of 17.845 that was recorded for shoot length with peak marker positioned at 163.57 cM. The two QTLs in chromosomes 9 and 12 explained 11% and 13.2% of the variation, respectively, with the additive effect all attributed to Hasawi.

Two QTLs (*qRFW4.1* and *qRFW4.2*) in chromosome 4 were responsible for the observed phenotypic variation in root fresh weight. The *qRFW4.2* found within the segment 99.16 cM – 123.17 cM is responsible for 19.9% of the variation in fresh weight. The other QTL, *qRFW4.1*, contributed 13.5% of the phenotypic variation with peak marker at 87.5 cM, which is close to *qRFW4.1*. Hasawi was responsible for an increase in fresh weight by 6% and 8% for *qRFW4.1* and *qRFW4.2*, respectively.

The same QTLs, as with root fresh weight, were detected in chromosome 4 for root dry weight with peak LOD located at 87.35 cM (*qRDW4.1*) and 99.16 cM (*qRDW4.2*). The two QTLs contributed 13.7% and 20.2% of the phenotypic variation. A third QTL for this trait was mapped in chromosome 12 (*qRDW12*) contributing 12.1% of the variation in root dry weight.

One QTL (*qRLRED1*) was mapped in chromosome 1 and was responsible for 10.3% of the phenotypic variation in root length reduction with a high additive effect (7.69) from Hasawi.

Two QTLs contributed 12.5% (*qSFW12*) and 14.3% (*qSFW4*) of the variation in shoot fresh weight. The position of *qSFW4* was found in the same region as that of the other QTLs responsible for root fresh weight and root dry weight with flanking markers id4006135–id4007444. The allelic effects for the two QTLs came from the Hasawi parent.

Of the three QTLs (*qSDW4.1*, *qSDW4.2* and *qSDW7*) detected for shoot dry weight, two were located in chromosome 4 and one in chromosome 7. The 11.5% and 13.6% of the total phenotypic variation in shoot

dry weight were attributed to *qSDW4.1* and *qSDW4.2*, respectively. The *qSDW7* in chromosome 7 explains 11.1% of the variation. All allelic effects controlling shoot dry weight came from the Hasawi parent.

Only one QTL for shoot Na concentration, *qSNC1*, was detected and mapped within 140.68 and 163.57 cM segment in chromosome 1. The markers id1020828 and id1024836 flanked the *qSNC1* spanning a 23 cM segment. The QTL explains 16% of the shoot Na variation and the additive effect is from Hasawi with peak LOD score of 5.875.

Four QTL clusters were identified in this study. The first clustering was located in chromosome 1 containing QTLs *qSES1*, *qSNC1*, and *qRLRED1* flanked by id1020828–id1024836 markers (Fig. 2). The second and third clusters were located in chromosome 4. Cluster 4-a was composed of *qRFW4.1*, *qRDW4.1*, *qSFW4* and *qSDW4.1* flanked by markers id4006135–id4007444 while cluster 4-b was flanked by markers id4007444–id4008522 with the loci *qRFW4.2*, *qSES4*, and *qRDW4.2*. The fourth cluster was found in chromosome 12 containing *qSFW12* and *qRDW12* QTLs flanked by markers id12004491–id2005501.

DISCUSSION

QTLs for Seedling Vigor

For the parameters exhibiting transgressive segregation, QTLs associated with those traits are inferred to be responsible for the appearance of transgressive individuals. Lines with high visual salt-injury scores have Na-K ratio value greater than one, and vice versa. There was a reduction in root and shoot length, and therefore biomass as observed in lines under saline stress; this reduction is attributed to high concentration of Na ions in the plants as these ions are harmful for rice growth. It is important to look at lines which have low shoot Na and high K in the leaves, since these lines can be used for varietal improvement. Rice varieties characterized to be tolerant to salinity have low shoot Na-K ratio.

The results showed that correlated traits and their corresponding QTLs cluster together in the same chromosomal region. The correlation between SES and shoot sodium concentration showed significant strong positive linear association. The correlation was translated meaningfully after QTL analysis, as *qSES1* and *qSNC1* clustered together in the lower region of chromosome 1, with their peak

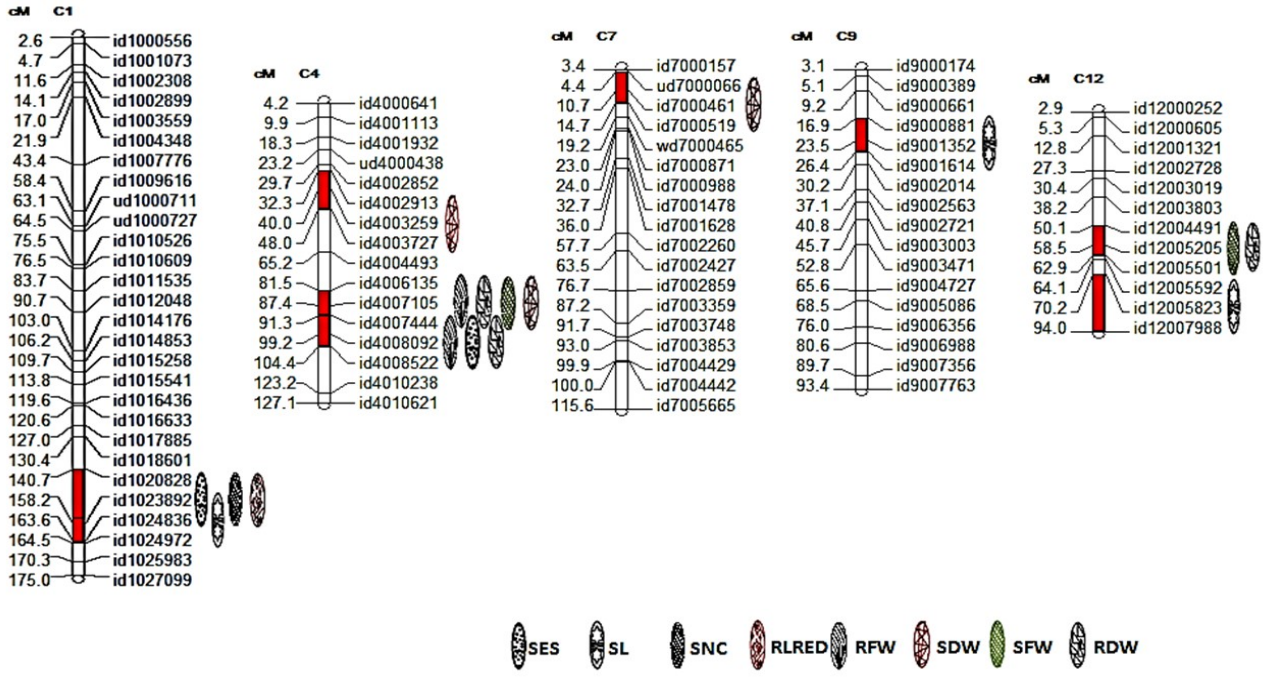

Fig. 2. Linkage map showing density of the single nucleotide polymorphism (SNP) markers and their position in centiMorgan (cM) and chromosome locations of significant quantitative trait loci (QTLs) for seedling-stage traits under salt stress produced from the cross IR29 x Hasawi. (**SES** – visual salt injury, **SL** – shoot length, **SNC** – shoot sodium concentration, **RLRED** – % root length reduction, **RFW** – root fresh weight, **SDW** – shoot dry weight, **SFW** – shoot fresh weight, **RDW** – root dry weight).

LODs positioned at 158.6 cM flanked by id1020828 and id1024836 markers (Fig. 2). Low visual salt-injury score and low shoot Na content are associated with seedling salt tolerance. Visual salt-injury score was also significantly correlated with reduction in root length and the QTLs for both approximate each other at the upper region of chromosome 1. Reduction in root length was correlated with shoot sodium concentration. Root growth inhibition is a primary response among plants subjected to salt stress (Wahid et al. 1998; Lin and Kao 2002). The *qRLRED*1 contributes to reduction in root length as a response to salinity stress. Moreover, salt stress triggers changes in root morphology, inhibiting the initiation and the elongation of lateral roots (Rubinigg et al. 2004). Root and shoot fresh weights were highly correlated and their respective QTLs clustered together in chromosome 4. Visual salt-injury score showed significant negative correlation with RFW and SFW, and their QTLs also clustered in chromosome 4 flanked by markers id4007444 and id4008522. SFW and RDW have high positive correlation and their QTLs clustered in chromosome 12. All correlated traits have additive effects in the same direction, coming from Hasawi.

The QTLs for shoot fresh weight (*qSFW4)* and shoot dry weight (*qSDW4.1)* were located in chromosome 4, explaining 14.3% and 11.5% of the total phenotypic variation, respectively. They form QTL cluster 4a flanked by markers id4006135 and id4007444. This result indicates that the genes at this region in chromosome 4 contributed to shoot biomass under saline stress conditions. QTLs for root fresh and dry weights were also located in QTL clusters 4a and 4b, suggesting that these regions are associated with increased root biomass. Most QTLs were detected in chromosome 4, implying the importance of chromosome 4 in the control of biomass and vigor at seedling stage in rice. QTLs for shoot length were distributed in chromosomes 1, 9, and 12 explaining 41.1% (large effect QTL), 11%, and 13.2%, respectively, of the shoot length variation; such loci are associated also with seedling vigor. The QTL clusters in chromosome 12 for shoot fresh weight (*qSFW12)* and root dry weight (*qRDW12),* and the QTL for shoot dry weight (*qSDW7)* in chromosome 7 all contributed to enhanced seedling vigor under saline stress.

One of the physiological bases for salt tolerance during early seedling stage is having high seedling vigor (Lafitte et al. 2004). It has been observed that most tolerant rice genotypes are the most vigorous (i.e., non-dwarfed landraces such as Pokkali and Nona Bokra) because dwarfing reduces vigor and hence the biomass into which accumulated salts can be sequestered (Singh and Flowers 2010). Although Hasawi is a tall type variety, it has a promising repertoire of genes conveying tolerance to salt stress. The results of this study showed that at seedling stage, most of the QTLs identified control seedling vigor. Majority of the genes were found in chromosomes 1 and 4.

Detecting QTLs for vigorous growth parameters at seedling stage is potentially useful for plant breeders to enhance phenotypic selection under salinity, as this stage is highly sensitive to salt stress (Ismail et al. 2007). Early seedling vigor is a desirable trait for rice to overcome high salinity levels that normally occur at the beginning of the wet season, and it could enable young plants to stand salt stress for longer duration and recover quickly after stress (Peng and Ismail 2004).

Comparison of Hasawi QTLs and other QTLs Associated with Salinity Tolerance in Rice

The QTLs for seedling traits identified here were compared with previously reported QTLs based on physical position (Table 6).

The SES scores which measure overall survival and/or vigor of the plant have been found to be good indicators of performance of the plant under salt stress (Gregorio et al. 1997). Two QTLs for visual salt-injury score, mapped in chromosomes 1 and 4, had been detected in this study. Tolerance alleles were contributed by Hasawi in chromosome 1. However, in chromosome 4, tolerance was attributed to IR29, suggesting that alleles of QTLs associated with salt tolerance may also come from the salt-sensitive parent. Yao et al. (2005) reported the presence of QTL for salinity tolerance rating in chromosomes 1, 5 and 9 in an F_2 population derived from the cross Jiucaiqing and IR36 using SSR markers. The QTL for salt injury score in chromosome 1 flanked by SNP markers id1020828–id1024836 is 2.7 Mb away from the *qSTR-1* responsible for the salinity tolerance rating identified by Yao et al. (2015).

Table 6. Comparison of quantitative trait loci (QTL) derived from IR29 x Hasawi F_6 recombinant inbred lines (RILs) population with previously reported QTL.

Trait	QTL in this Study		Reported QTL			Distance from Reported QTL	
	QTL	Physical Position	QTL	Physical Position	References	bp	Mb
Salt injury score/ Salinity tolerance rating	*qSES1*	Chr01:33414230-39136724	*qSTR-1*	Chr01:23326155-30739034	Yao et al. (2005)	2675196	**2.7**
Shoot dry weight	*qSDW7*	Chr07:1092858-3650191	*qDWSH-7*	Chr07:16199989-19258122	Sabouri and Sabouri (2008)	Located within our QTL	
			qNAUP-1a	Chr01:14627212-32783520		630710	**0.6**
Shoot sodium concentration	*qSNC1*	Chr01:33414230-39136724	*qSNC1*	Chr01:10839399-12570938	Thomson et al. (2010)	20843292	**20.8**
			Saltol	Chr01:12301656-12302017	Gregorio et al. (1997); Niones (2004)	21112213	**21.1**

Three QTLs for shoot dry weight were identified from Hasawi, two in chromosome 4 and one in chromosome 7. Only one QTL had been detected for shoot sodium concentration, which explains 16% of the phenotypic variation in shoot sodium content with peak LOD score of 5.875. Sabouri and Sabouri (2008) detected a QTL (*qDWSH-7*) for shoot dry weight also in chromosome 7 and shoot sodium uptake (*qNAUP-1a*) in chromosome 1 using SSR makers in an F2:3 population derived from the cross between Tarommahalli and Khazar. Based on the deduced physical position, *qSDWH-7* is located within *qSDW7*, which we detected, and *qNAUP-1a* is located 0.6 Mb away from *qSNC1*.

The *qSNC1* is located within the segment Chr01:33414230–39136724 bp in chromosome 1. The *Saltol* QTL, which was identified by Gregorio (1997) from the cross of IR29 and Pokkali using AFLP markers responsible for K and Na absorption and Na-K ratio, was located in the short arm of chromosome 1. Bonilla et al. (2002) further saturated this region in chromosome 1 with RFLP and SSLP markers and revealed *Saltol* QTL to be responsible for high K+ and low Na+ uptake and low Na-K ratio. QTLs for ion uptake and Na-K ratio were found in Chr01:12301656–12302017 identified by Niones et al. (2004). Thomson et al. (2010) identified a set of QTLs for survival and chlorophyll content at the lower region of chromosome 1, although the tolerance effect was derived from IR29 instead of Pokkali under higher stress conditions (EC 18) using F_8 RILs. The *Saltol* is 20.8–21.1 Mb away from the *qSNC1* we identified.

In a study conducted in Africa, using F_5 RIL population also produced from the cross IR29 x Hasawi, Bimpong et al. (2014) reported a total of seven QTLs associated with salt tolerance at seedling stage mapped in chromosomes 1, 2 and 6. The QTLs were *qDW1.1, qDW2.1, qDW2.2* and *qDW6.1* responsible for variation in shoot dry weight; *qPH1.1* and *qPH1.2* for variation in plant height; and *qF2.1* for shoot fresh weight. There was no common QTL between their study and our study. The differences in the QTLs which were identified could be ascribed to the difference in the selection of the lines which were included in the mapping population. Although we produced the mapping population using the same parents, a different representative mapping population or subpopulation was used for QTL mapping. The QTLs we identified were in chromosomes 1, 4, 7, 9 and 12 and none in chromosomes 2 and 6. Large-effect QTLs for visual salt injury and shoot length were located in chromosome 1 with LOD scores of 15.875 and 17.845, thereby explaining 37.6% and 41.1% of the phenotypic variation in the traits, respectively. This study therefore supplements the number of QTLs identified by Bimpong et al. (2014) and reveals that Hasawi provides a number of QTLs responsible for seedling-stage salinity tolerance.

CONCLUSION

The results from this study suggest a different salt tolerance mechanism employed by Hasawi since most of the QTLs identified were in chromosomes 4, 7, 9 and 12 and a large-effect QTL which is found in chromosome 1. There is a need to saturate the 12 chromosomes with more SNP markers in order to reveal additional or major-effect QTLs. Furthermore, the SNP markers which are tightly linked to the identified QTLs can be used for marker-aided breeding.

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