



Mapping QTLs for tolerance to salt stress at the early seedling stage in rice (*Oryza sativa* L.) using a newly identified donor ‘Madina Koyo’

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Abstract Salt stress is a menace to rice production and a threat to food security worldwide. We evaluated 308 F₄ families from Sahel 317/Madina Koyo for tolerance to salt stress at the early seedling stage. To better understand genomic regions controlling tolerance in the population, we genotyped the progenies and the two parents using single nucleotide polymorphism (SNP) markers and regressed the genotypic data

on their phenotype to detect QTLs. An average reduction of 63.4% was observed for all fitness-related traits among the F₄ families. A total of 46 progenies recorded an average salt injury score (SIS) between 1–3 and were rated as tolerant to salt stress at the early seedling stage. A high-density genetic map was constructed for the 12 rice chromosomes using 3698 SNP markers. Multiple interval mapping identified 13 QTLs for SIS, shoot length, shoot dry weight and root length on chromosomes 2, 3, 4, 6, 7, 10 and 12, with trait increasing alleles coming from both parents. Two (*qSDW2* and *qRL2.2*) and three (*qSL2*, *qRL2.1* and *qSIS2*) QTLs at different regions on chromosome 2 and another two on chromosome 7 (*qSDW7* and *qSL7*) were tightly linked. These QTLs could facilitate breeding for salt tolerance at the early seedling stage as direct selection for one, would mean indirectly selecting for the other. Fine mapping of these novel QTLs in a different genetic background is necessary to confirm their stability and usefulness in breeding for tolerance to salinity in rice.

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Introduction

Salt stress is a major constraint in the production of rice, considerably reducing grain yield while

rendering vast majority of arable lands unproductive. Resulting from natural causes and improper irrigation/drainage schemes, the problem of salt stress is likely to worsen due to the numerous effects of climate change (Ismail et al. 2008; Ismail and Tuong 2009; Wassmann et al. 2009). In rice production, salt stress is most problematic when it occurs at the early seedling stage and/or the reproductive growth stages. Though it is the reproductive stage salt stress that causes direct yield loss, early seedling stage salt stress is equally important as it dictates the actual crop stand hence grain yield. In the field, salt stress occurs heterogeneously, making evaluation of germplasm difficult. Through a hydroponic protocol developed by Gregorio et al. (1997), it is possible to efficiently evaluate larger number of progenies for tolerance to salt stress at the early seedling stage under homogenous conditions in a relatively shorter period based on visual salt injury score (SIS).

The complex and quantitative nature of salt stress has resulted in marginal progress in breeding for tolerance through phenotypic selection. Since the advent of molecular markers, several studies have employed the technology in diverse ways to complement conventional breeding including genetic diversity studies, marker assisted selection (MAS) and quantitative trait loci (QTL) studies. QTLs for complex traits including tolerance to salt stress have been mapped through regression of phenotypic data of a mapping population on its molecular data (Ismail et al. 2007; Thomson et al. 2010; Bimpong et al. 2014; De Leon et al. 2016; Bizimana et al. 2017; Rahman et al. 2017) using different mapping populations. It has become a common practice to take family mean from the F_3 population rather than individual F_2 plants as a unit of phenotypic measurement for a trait whose heritability is low (Mather and Jinks 1982; Lynch and Walsh 1998; Zhang et al. 2003; Zhang and Xu 2004). It is possible to increase the number of generations from F_3 to F_y , leading to an $F_{2;y}$, or a more general $F_{x;y}$ design in plant genetics (Austin and Lee 1996; Cockerham and Zeng 1996; Fisch et al. 1996; Jiang and Zeng 1997; Chapman et al. 2003; Zhang and Xu 2004; Kao 2006) and daughter or granddaughter design in animal genetics (Weller et al. 1990). For the purpose of QTL mapping, Zhang and Xu (2004) suggested that, it is possible to genotype plants in generation x and phenotype plants in generation y to conduct QTL mapping using a mapping population

generated through the $F_{x;y}$ design for y_x . This design has been used by Ghomi et al. (2013), Ding et al. (2011) and Chapman et al. (2003) to map QTLs by phenotyping $F_{2;4}$, $F_{2;3}$ and $F_{4;6}$ generations respectively and genotyping the different F_2 populations.

By far, the most promising QTL reported for seedling stage salt tolerance to stress in rice is *Saltol*; which was mapped within 10.7–12.2 Mb region on the short arm of chromosome 1, explaining 43% of phenotypic variation in shoot Na^+/K^+ ratio (Gregorio 1997; Bonilla et al. 2002). However, little progress has been made in breeding for and delivering salt tolerant rice varieties in farmers' fields using *Saltol* alone (Bizimana et al. 2017). Since tolerance to salt stress is a complex quantitative trait, the identification and subsequent pyramiding of several QTLs for different salt tolerance-related traits may be a better option in improving salt stress tolerance among rice varieties (Rahman et al. 2016). Jahan et al. (2020), using salt tolerant cultivar 'PA64s' as donor source, identified 38 QTLs for 6 traits including shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight under. Sun et al. (2019) mapped 23 QTLs for traits related to salt stress with cultivar 'Changbai 10' as donor parent. Similarly, Rahman et al. (2019) identified 20 QTLs for salt tolerance in rice using F_2 mapping population developed with a landrace 'Capsule' as donor parent. In this study, we evaluated progenies from a new donor source for salt tolerance; 'Madina Koyo' and mapped regions on the chromosomes controlling tolerance to salt stress at the early seedling stage.

Materials and methods

Screening for tolerance to salt stress at the early seedling stage

Altogether, 308 F_4 families developed from 'Sahel 317' (recipient parent; irrigated lowland *indica* type developed by Africa Rice Center) and 'Madina Koyo' (donor parent; a landrace with no documented pedigree but with better adaptability as demonstrated by Bimpong et al. 2011) were evaluated for tolerance to salt stress at the early seedling stage using a hydroponic system. The experiment was established in a greenhouse at the M'be Station of Africa Rice Centre in Bouake, Cote d'Ivoire between April and May

2018. Typically, the ambient temperature around this period of the year ranges from 20 to 25 °C during early parts of the day and 28–38 °C during later hours of the day. The average relative humidity varies from 35 to 95%. The same progenies were evaluated in two adjacent trials, saline (stress) and non-saline (control). Each experiment was laid out in a 5 × 62 alpha lattice design with two replications, each containing 5 blocks. Each block consisted of a plastic float of 64 lanes, with each lane made up of 7 wells/holes. Each lane of 7 wells/holes was sown to one entry. Each plastic float was occupied by 62 test entries with the remaining two slots sown to the two checks IR29 (international sensitive check) and FL478 (international tolerant check), for the purposes of assessing the effect of saline treatment and also as a guide in estimating the salt injury score. Thus, the two check entries appeared in all five blocks for each replication. Evaluation of test entries followed a method described by Gregorio et al. (1997). Four-day old pre-germinated seeds were sown in holes on plastic floats with a net bottom suspended on trays filled with distilled water for 3 days. This was to allow for the repair of any damage to radicles while transferring onto the plastic float before salinization. After the three-day recovery period, salinized Yoshida's solution (Yoshida et al. 1976), with slight modifications as per the protocol by Singh and Flowers (2010), replaced the distilled water in the stress set-up while the control set-up received Yoshida's solution with no salt. The initial salinization was maintained at an electrical conductivity (EC) of 6.0 dSm⁻¹ by adding approximately 5.13 mM of NaCl to the solution. This concentration was increased to 12.0 dSm⁻¹ after 3 days and 18 dSm⁻¹ another 3 days after. The pH of the nutrient solution was maintained at approximately 5.0 with a weekly renewal of the solution throughout the experiment. The test entries were assessed based on SIS using IRRI's standard evaluation system for rice (IRRI-SES 2014), with ratings from 1.0 (highly tolerant), 3.0 (Tolerant), 5.0 (Moderately tolerant), 7.0 (Susceptible) to 9.0 (highly susceptible). Ten days after the initial application of salt, the first assessment was done while the second and final assessment was done 9 days after the first assessment.

For each test entry, shoot length was estimated as the average length from the base to the tip of the longest leaf while root length was estimated as length of roots from the base of shoots to the tip of the deepest

root in centimeters. Subsequently, all 7 shoots and roots from each entry were bulked in an envelope and dried in an oven at 50 °C for 5 days. Shoot and root dry weights were recorded in grams after the 5th day. The concentration of salts absorbed into plant tissues was quantified as the concentration of the ions that leaked from the leaf tissue using a conductivity meter (Orion Star A222) following methods described by De Leon et al. (2015) based on the formula of Flint et al. (1967).

Stability of progenies under stress and control experiments

Comparisons of genotypes for all measured traits (except SIS) were computed as the percentage reduction of genotypic performance under stress conditions relative to their performance under control using their generated LSmeans. These were computed as below:

$$\text{Stability } (y) = 1 - \left[\frac{x - z}{x} * 100\% \right]$$

where y = trait, x = trait measurement under control setup, z = trait measurement under salinized setup.

Statistical analysis

Analysis of variance was computed using a linear mixed model with a normal distribution for shoot length, root length, shoot dry weight, root dry weight and ion leakage while a generalized linear mixed model (McCullagh and Nelder 1989) under the assumption of a multinomial distribution was used for SIS. A significant genotype × environment (G×E) interaction effect was tested, and where this was the case, a model for each environment (control, and stress) was fitted separately. Genotype, environment and G×E were considered as fixed factors with replicate nested into environment and block nested into replication and environment as random factor. Comparison of means was done by LSD for data with normal distribution. For the SIS, where a multinomial distribution was assumed, we modelled the probability levels of scores having lower ordered values. Odds ratios were computed to indicate relative differences between entries and the two parents (Sahel 317 and Madina Koyo) as well as the two international checks, IR29 or FL478 respectively. Variation due to genotype and environment (residual) as well as heritability were

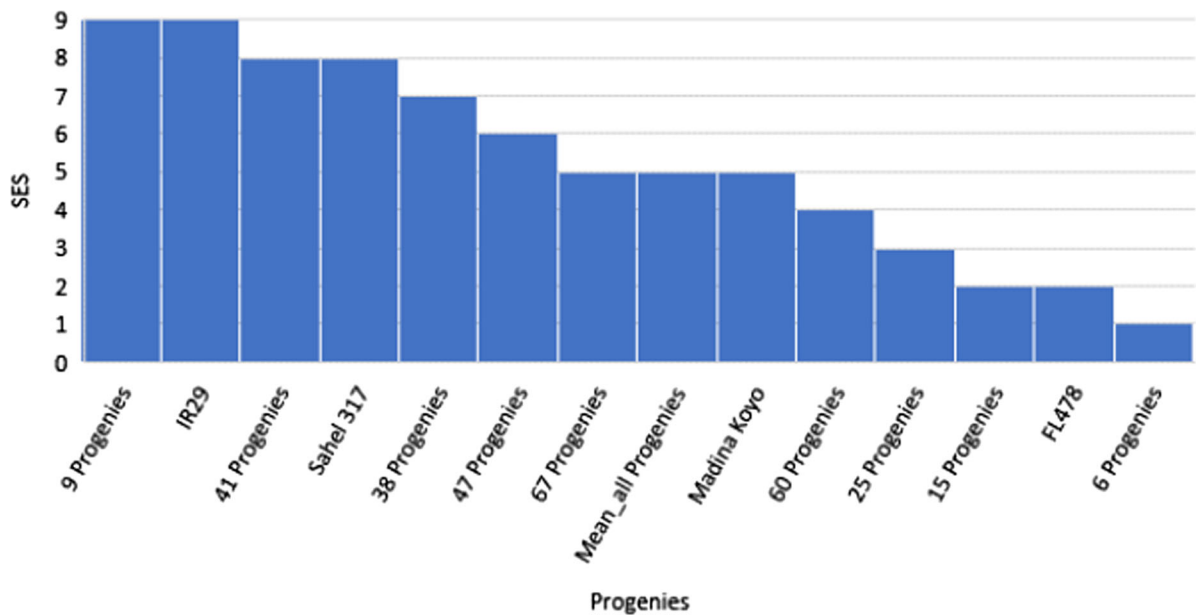


Fig. 1 Distribution of SIS among the test entries

computed to estimate the level of genotypic contribution to the phenotypic variation of measured traits. Relationship among traits was computed using genetic correlation, based on the pooled least square (LS) mean of two replications per trait. All analyses were done using R statistical package (R Core Team 2018).

Genotyping and molecular analysis

Eight leaf discs of 6 mm diameter were punched from 15-day old F_3 leaves and sampled into 96 deep well PCR plates. Prior to sampling, leaves were dried at 50 °C overnight to prevent possible mold development until the commencement of analysis. DNA extraction and genotyping were done using Genotype by sequencing (GBS) at the Integrated Genotyping Service and Support (IGSS) laboratory based in the Biosciences eastern and central Africa (BeCA) in Kenya based on DArTseq™ technology from DArT (Diversity Arrays Technology Pty Ltd) in Australia.

Detection of QTLs

A total of 3698 SNPs (out of 10,336) and 308 F_3 progenies were considered for analysis after filtering and data quality control process. Missing data were imputed using KDCCompute, an online analysis plugin

tool that runs on R scripts. Estimates for genetic distances in centimorgan (cM) were computed using cM Converter (<http://mapdisto.free.fr/cMconverter/>), a program that converts in batch physical to genetical map positions of molecular markers of rice using in silico mapping data provided by the Rice Genome Annotation project. Only traits for which the progenies showed significant differences were maintained



Fig. 2 Comparison between a tolerant progeny, the two parents and the two checks

Table 1 Analysis of variance

Source of variation	SIS	Shoot length	Root length	Shoot dry weight	Root dry weight	Ion leakage
<i>Combined</i>						
Genotype significance	–	0.0001	< 0.0001	0.5828	0.3806	0.6741
Site significance	–	0.0122	0.0181	0.0034	0.0102	0.001
Gen × site significance	–	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1873
<i>Control</i>						
Genotype significance	–	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.2986
Genotype variance	–	58.41	2.97	0.09	0.01	0
Residual variance	–	37.32	2.82	0.04	0	0
<i>Stress</i>						
Genotype significance	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1594
Genotype variance	–	7.39	0.92	0	0	0
Residual variance	–	14.99	3.24	0.01	0	0.01
<i>Trait stability</i>						
Genotype significance	–	< 0.0001	0.02745	< 0.0001	< 0.0001	0.02549

for QTL analysis. All QTL analyses were performed in R (version 3.6.3) (R Core Team 2020). A multiple QTL model that incorporated pairwise interactions was fitted for each trait using the Harley-Knott regression algorithm as implement in R/qtl (Broman et al. 2003). Prior to multiple QTL model fitting, the quality of the phenotypic data was assessed using histograms. Model search and comparison were based on forward and backward regression methods as well as a penalized LOD (pLOD) score. The pLOD score is the LOD score for the model (the log₁₀ likelihood ratio comparing the model to the null model with no QTL) with penalties on the number of QTL and QTL:QTL interactions (Manichaikul et al. 2009). The chosen model after a forward and backward regression procedure was that which optimized the pLOD score criterion, among all models visited. The effects of detected QTLs and their pairwise interactions, if present, were estimated for each trait based on the selected optimized model. Penalties and LOD thresholds for declaring significant QTLs were obtained by performing 1000 permutation tests at a significance level of 5% as implemented in R/qtl. Missing genotypic data was imputed at an error probability rate of 0.1%.

A multiple QTL model with pairwise interactions is given by the equation:

$$y = \mu + \sum_j \beta_j q_j + \sum_{j,k} \beta_{jk} q_j q_k + \varepsilon$$

where y is the observed phenotype for any individual in the mapping population, β_j = effect of putative QTL, q_j , β_{jk} = pairwise interaction effect of putative QTLs, q_j and q_k , ε = residual error.

QTLs were named following nomenclature proposed by McCouch et al. (1997). Basically, QTL names started with a lowercase “ q ”, followed by a 2–3 initials of the corresponding measured trait (in upper cases) and then followed by the chromosome number. If there were more than one QTL on any chromosome, a “.” was used to uniquely identify them. Graphical representations of QTL positions were generated using Windows QTL cartographer (Wang et al. 2012).

Results

Injury due to salt stress was visible 5–10 days after imposing stress, causing a general reduction in all measured quantitative traits (Figs. 1 and 2). Effect of salt stress on the test entries and interaction between salt stress-genotypes were significantly different for all traits except for ion leakage (Table 1). Mean SIS for all progenies was 5 with 16 progenies (5%) being superior and having better mean SIS (SIS = 1.6) than both donor parent Madina Koyo and tolerant check

Table 2 SIS, *P* values and odd ratios of entries of the top 5% progenies from the F₄ population

Genotype	Mean SIS	Progenies vs Madina Koyo		Progenies vs FL478	
		<i>P</i> value	Odds ratio	<i>P</i> value	Odds ratio
ARS1181-1-9-6-B-6	1	< 0.0001	817.61	< 0.0001	442.42
ARS1181-1-2-11-B-11	1	< 0.0001	639.84	< 0.0001	156.81
ARS1181-1-2-13-B-13	1	< 0.0001	639.84	< 0.0001	122.71
ARS1181-1-7-2-B-2	1	< 0.0001	553.94	< 0.0001	122.71
ARS1181-1-9-30-B-30	1	< 0.0001	515.21	< 0.0001	106.24
ARS1181-1-6-9-B-9	1	< 0.0001	493.8	< 0.0001	98.81
ARS1181-1-12-3-B-3	2	< 0.0001	489.24	0.01	94.71
ARS1181-1-12-14-B-14	2	< 0.0001	469.16	0.01	93.83
ARS1181-1-13-1-B-1	2	< 0.0001	371.8	0.01	71.31
ARS1181-1-13-18-B-18	2	< 0.0001	371.8	0.01	89.98
ARS1181-1-1-12-B-12	2	< 0.0001	353.48	0.01	71.31
ARS1181-1-3-17-B-17	2	< 0.0001	332.31	0.01	67.79
ARS1181-1-4-30-B-30	2	< 0.0001	332.31	0.02	63.73
ARS1181-1-4-38-B-38	2	< 0.0001	318.66	0.02	63.73
ARS1181-1-7-6-B-6	2	< 0.0001	312.2	0.02	61.12
ARS1181-1-8-26-B-26	2	< 0.0001	312.2	0.02	59.88
Mean of top 5% progenies	1.6				
Mean of all progenies	5.3				
Madina Koyo	4				
FL478	2.4				
Sahel 317	8				
IR 29	8.4				

FL478 (Table 2). Of these, 6 progenies recovered fully from salt injury and had a final score of 1 and were significantly different from FL478 and Madina Koyo with odd ratios ranging 312–817 and 60–442 respectively.

Ion leakage

Though ion leakage under both control and stress were not significant, differences in trait stability among test entries was significant. Mean stability for the entire progenies was 53.1% while that of the donor (Madina Koyo) and the recipient (Sahel 317) parents were 73.3% and 67.6% respectively (Table 3).

Shoot and root parameters

Mean shoot and root lengths for the progenies were higher than parents and checks with the top 5% for SIS being superior also for shoot and root lengths (Table 4). Mean progeny stability was higher than

the two parents for shoot length but lower in the case of root length. A similar trend was observed for dry shoot and root weights (Table 5). Heritability ranged from 68 to 82% under control conditions while under stress, the range was 36–50% (Tables 3, 4, 5). Generally, heritability estimates under control conditions were higher than estimates under stress conditions. Heritability estimates for trait stability ranged from 25 to 61%.

Correlation among traits

Correlation among traits measured under control conditions were significant (Table 6). Strong and positive association were observed between shoot and root dry weights, shoot dry weight and shoot length and between root dry weight and shoot length. Strong but negative association were observed between shoot and root lengths, shoot dry weight and root length and between root dry weight and root length. Except for a positive correlation with shoot

Table 3 Summary statistics for ion leakage under control and stress conditions from the F₄ population

Genotype	Control			Stress			Stability		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
All progenies	0.168	0.16	0.17	0.64	0.615	0.665	53.1	23.4	88.9
Top 5% progenies	0.168	0.165	0.17	0.64	0.625	0.655	52	34.2	79.2
Madina Koyo (donor parent)	0.166	0.15	0.18	0.651	0.6	0.78	73.3	64.5	70.6
Sahel 317 (recipient parent)	0.17	0.17	0.19	0.656	0.71	0.76	67.6	72.8	73.8
FL478 (tolerant check)	0.169	0.17	0.18	0.615	0.52	0.61	47.9	42.3	53.5
IR 29 (susceptible check)	0.172	0.17	0.18	0.654	0.66	0.67	59.3	58.7	59.9
SD	0.002			0.009			22.75		
LSD	NS			NS			47.05		
Heritability	0.1			0.11			0.62		

Table 4 Summary statistics for shoot and root lengths under control and stress conditions from the F₄ population

Trait	Genotype	Control			Stress			Stability
		Mean	Min	Max	Mean	Min	Max	Mean
Shoot length	All progenies	43.3	28.6	59	24.5	20.3	30.5	56.6
	Top 5% progenies	40.9	29	54.4	25.5	22.3	30.5	63.3
	Madina Koyo (donor parent)	44.5	40.2	49.6	22.6	17.4	22.3	50.8
	Sahel 317 (recipient parent)	38.1	35.7	37.1	21.1	12.4	21.2	55.4
	FL478 (tolerant check)	35.6	21.2	42.8	23.3	18.5	26.1	65.4
	IR29 (susceptible check)	37.2	26.6	51.5	20	13.7	23.8	53.8
	SD	6.67			1.42			12.6
	LSD	7.37			1.92			21.3
	Heritability	0.76			0.5			0.25
Root length	All progenies	12.8	9.6	18.9	10	8.3	11.6	78.1
	Top 5% progenies	13.6	11.8	16.8	10.5	9.4	11.4	78
	Madina Koyo (donor parent)	11.1	8.5	11.9	10.3	10.6	10.7	92.8
	Sahel 317 (recipient parent)	11.2	9.6	11.3	8.9	5.1	8.4	79.5
	FL478 (tolerant check)	10.9	8.9	12.8	8.3	3.9	10.8	76.1
	IR29 (susceptible check)	11.6	8.9	17.4	7.6	4.3	10	65.5
	SD	0.27			0.07			11.4
	LSD	0.24			0.07			24.2
	Heritability	0.68			0.36			0.48

length, ion leakage demonstrated a weak and negative correlation with all other traits. Under stress, all correlation among traits were significant except between SIS and shoot length as well as correlations between ion leakage and every other trait but SIS. Very strong and positive correlations were observed between SIS and ion leakage and between shoot and

root dry weights under stress. Shoot length associated positively with root length, shoot dry weight and root dry weight while SIS associated negatively with these three traits. While root length associated negatively with shoot length, shoot dry weight, root dry weight and ion leakage under control, association among these traits were positive under stress.

Table 5 Summary statistics for Shoot and Root dry weights under control and stress conditions from the F₄ population

Trait	Genotype	Control			Stress			Stability Mean
		Mean	Min	Max	Mean	Min	Max	
Shoot dry weight	All progenies	0.79	0.34	1.38	0.28	0.2	0.38	49
	Top 5% progenies	0.76	0.38	1.37	0.3	0.26	0.38	45
	Madina Koyo (donor parent)	0.91	0.81	1.05	0.27	0.15	0.35	30
	Sahel 317 (recipient parent)	0.62	0.55	0.62	0.23	0.09	0.19	36.4
	FL478 (tolerant check)	0.74	0.28	1.24	0.27	0.04	0.35	35.8
	IR29 (susceptible check)	0.64	0.19	1.31	0.23	0.09	0.39	35.4
	SD	0.26			0.01			17.4
	LSD	3.8			0.02			24.9
Root dry weight	Heritability	0.82			0.42			0.39
	All progenies	0.2	0.09	0.435	0.07	0.05	0.1	34.5
	Top 5% progenies	0.21	0.11	0.435	0.08	0.06	0.1	41
	Madina Koyo (donor parent)	0.22	0.1	0.29	0.09	0.08	0.09	38.7
	Sahel 317 (recipient parent)	0.16	0.14	0.15	0.06	0.03	0.05	36.3
	FL478 (tolerant check)	0.18	0.17	0.36	0.06	0.04	0.1	32.2
	IR29 (susceptible check)	0.18	0.12	0.32	0.05	0.03	0.07	30.3
	SD	0.07			0.01			16.3
LSD	0.06			0.02			24.6	
Heritability	0.76			0.42			0.61	

Table 6 Genetic correlation with *P* values for measured traits under control and stress conditions from the F₄ population

SIS salt injury score, *SL* shoot length, *RL* root length, *SDW* shoot dry weight, *RDW* root dry weight, *IL* ion leakage
 ***Highly significant at < 0.0001; **significant at 1%; *significant at 5%;
^{ns}Not significant

Genetic correlations					
Traits	SIS	SL	RL	SDW	RDW
<i>Control conditions</i>					
SL	–				
RL	–	– 0.54***			
SDW	–	0.71***	– 0.45***		
RDW	–	0.52***	– 0.36***	0.93***	
IL	–	0.14**	– 0.15**	– 0.13*	– 0.26***
<i>Stress conditions</i>					
SL	0.07 ^{ns}				
RL	– 0.22***	0.47***			
SDW	– 0.53***	0.59***	0.5***		
RDW	– 0.56***	0.44***	0.54***	0.92***	
IL	0.92***	– 0.07 ^{ns}	0.04 ^{ns}	0.02 ^{ns}	– 0.1 ^{ns}

QTL mapping

All phenotypic data were normally distributed (Fig. 3); ion leakage was not considered for QTL analysis because performance among progenies was not significantly different (Table 1). Genotypic data

from 3698 SNP markers from all 12 gametic rice chromosomes, spanning a total length of 1617.1 cM (369,131,487 bp) at an average marker interval of 0.46 cM (Table 7) was considered in the analysis. Chromosome 1 was the longest (203.9 cM) and had the most markers (532 markers; average interval of

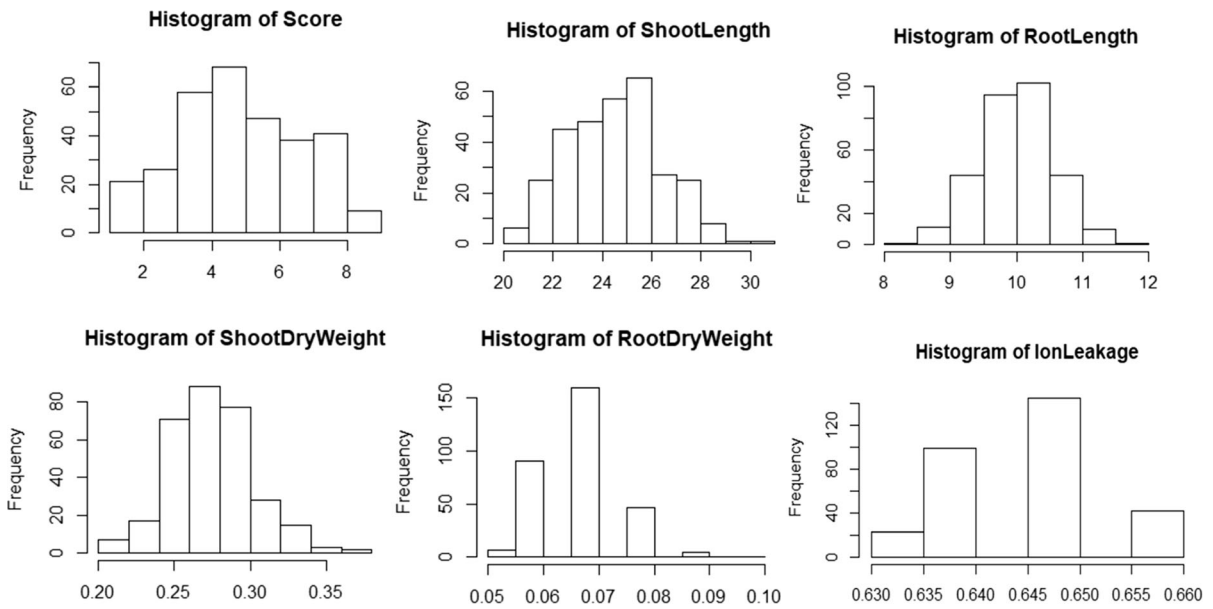


Fig. 3 Frequency distribution of traits used in QTL mapping

Table 7 Summary of genetic linkage groups for the 3698 SNP markers from the F₃ population

Linkage group	Physical length (bp)	Genetic length (cM)	Number of SNPs	Average physical distance (bp)	Average linkage distance (cM)
1	43,229,577	203.9	532	81,259	0.38
2	35,890,520	175.1	292	122,913	0.60
3	36,362,027	194.4	354	102,718	0.55
4	34,980,319	130.7	473	73,954	0.28
5	29,270,709	132.4	207	141,404	0.64
6	30,371,849	134.5	270	112,488	0.50
7	28,924,473	151.0	328	88,184	0.46
8	28,361,417	117.3	250	113,446	0.47
9	22,614,057	92.0	204	110,853	0.45
10	23,107,718	93.0	188	122,913	0.49
11	28,912,694	94.3	326	88,689	0.29
12	27,106,127	98.7	274	98,927	0.36
Total/ average	369,131,487	1617.1	3698	1,257,748	0.46

0.38 cM) but no QTL was mapped to this chromosome. Chromosome 4 with a length of 130.7 cM and 473 markers had the best coverage with a marker at every 0.28 cM but harbored only one QTL. Chromosome 2, with a length of 175.1 cM, 292 markers and density of one SNP per every 0.6 cM, harbored the most QTLs (5 QTLs for 4 traits). A total of 13 significant QTLs were identified for all studied traits,

except root dry weight, on 7 (chromosomes 2, 3, 4, 6, 7, 10 and 12) out of the 12 chromosomes (Table 8; Figs. 4A–D, 5A–G). Seven out of the 13 QTLs were for shoot related traits while 3 QTLs were mapped for root related traits (Table 8). Three QTLs were identified for SIS explaining 6.6–11.9% of the phenotypic variance. Among these three, *qSIS10* on chromosome 10, spanning 27–35.7 cM with a peak marker of

Table 8 Summary of QTLs detected for studied traits under salt stress conditions in rice using an F₃ mapping population

Trait	QTL Name	Chr.	Position (cM)	Interval (cM)	LOD	R ² (%)	P value	Peak	Left	Right	Source of increasing allele
Salt Injury Score	<i>qSIS2</i>	2	54.5	54.3–54.6	5.3	6.6	8.41E–6	3,439,917IF10-18:A > G-18:A > G	3,050,551IF10-31:T > C-31:T > C	3,059,821IF10-67:A > G-67:A > G	Sahel 317
		4	9.8	8.3–16.4	7.9	10.1	3.53E–6	3,059,794IF10-26:G > T-26:G > T	3,443,580IF10-68:A > T-68:A > T	5,403,903IF10-60:A > C-60:A > C	Madina Koyo
		10	32	27.0–35.7	9.3	11.9	2.38E–7	9,749,084IF10-12:C > T-12:C > T	3,054,302IF10-5:T > C-5:T > C	9,752,074IF10-20:G > C-20:G > C	Sahel 317
Shoot length	<i>qSL2</i>	2	42	40–54.6	7.6	8.7	4.20E–8	100,110,606IF10-63:G > T-63:G > T	3,059,697IF10-30:A > G-30:A > G	5,142,709IF10-32:A > G-32:A > G	Sahel 317
		3	161	156–161.9	6.1	6.9	1.28E–6	3,057,452IF10-55:C > G-55:C > G	100,111,889IF10-44:A > C-44:A > C	6,996,922IF10-18:A > G-18:A > G	Madina Koyo
		7	150.9	142.3–151.0	4.1	4.6	9.73E–5	3,438,968IF10-41:G > A-41:G > A	3,054,649IF10-46:T > A-46:T > A	3,763,276IF10-7:G > A-7:G > A	Sahel 317
Shoot dry weight	<i>qSL12</i>	12	0	0.0–0.9	8.2	9.5	9.84E–9	4,391,847IF10-46:G > C-46:G > C	4,391,847IF10-46:G > C-46:G > C	4,393,195IF10-7:C > A-7:C > A	Sahel 317
		2	113	108.2–117.2	5.2	6.9	6.73E–6	3,049,606IF10-62:C > T-62:C > T	5,142,245IF10-19:T > C-19:T > C	3,055,769IF10-50:A > C-50:A > C	Madina Koyo
		3	190	189.1–194.3	4.9	6.4	1.51E–5	3,453,763IF10-44:A > G-44:A > G	3,055,923IF10-47:C > A-47:C > A	3,446,472IF10-30:T > A-30:T > A	Madina Koyo
Root length	<i>qSDW7</i>	7	150.3	147.0–151.0	4.7	5.6	5.55E–5	3,452,175IF10-68:T > C-68:T > C	3,051,516IF10-13:C > T-13:C > T	3,763,276IF10-7:G > A-7:G > A	Sahel 317
		2	53	52.3–54.0	6.7	8.5	2.96E–7	3,062,378IF10-59:T > C-59:T > C	100,047,180IF10-32:T > G-32:T > G	3,051,615IF10-47:G > A-47:G > A	Madina Koyo
		2	108.7	104.0–116.0	6.1	7.7	1.13E–6	3,443,642IF10-47:T > G-47:T > G	100,134,950IF10-51:G > A-51:G > A	6,996,219IF10-19:T > C-19:T > C	Madina Koyo
	<i>qRL6</i>	6	4.9	2.7–5.7	5.9	7.5	1.69E–6	3,057,539IF10-12:C > A-12:C > A	3,443,128IF10-67:C > T-67:C > T	3,053,691IF10-23:C > T-23:C > T	Sahel 317

qSIS4 and *qSIS10* were identified to have a significant (2.6E–10) interaction with R² = 6.5%

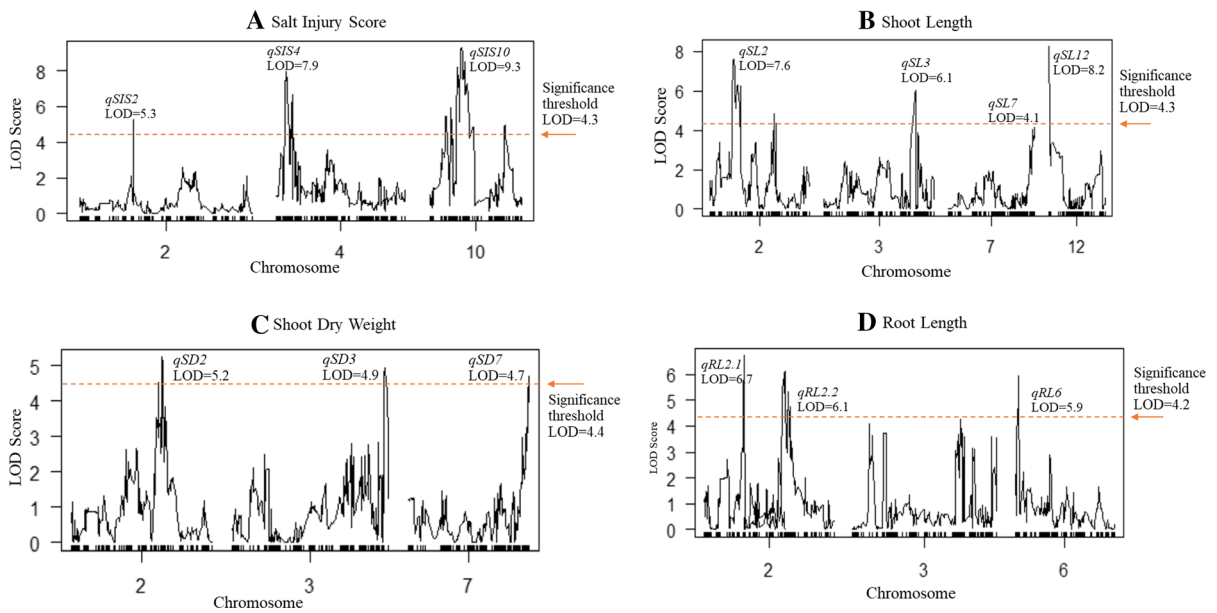


Fig. 4 QTL graphs for the various traits showing significance threshold LOD scores

9,749,084|F10-12:C > T-12:C > T contributed the most to the variation in SIS ($R^2 = 11.9\%$) at LOD of 9.3, with trait-increasing alleles coming from the female parent Sahel 317 (Table 8; Fig. 5F). This *qSIS10* (trait increasing alleles from Sahel 317) and *qSIS4* (trait increasing alleles from Madina Koyo) had a significant epistatic interaction with a phenotypic contribution of 6.5% (Table 8). Four QTLs were mapped for shoot length on chromosomes 2, 3, 7 and 12. The *qSL12* on chromosome 12 (Fig. 5G) between 0 and 0.9 cM at peak marker 4391847|F10-46:G > C-46:G > C and LOD 8.2 contributed the most to the variation observed in shoot length ($R^2 = 9.5$). Three QTLs were identified for shoot dry weight on chromosomes 2 (108.2–117.2 cM), 3 (189.1–194.3 cM) and 7 (147.0–151.0 cM) contributing 6.9%, 6.4% and 5.6% respectively to the phenotypic variation (Table 8). The *qSDW7* (147.0–151.0 cM) was mapped within the region of *qSL7* (142.3–151.0 cM) on chromosome 7 (Fig. 5E) with Sahel 317 providing alleles that increase both traits. A region spanning 14.6 cM (40.0–54.6 cM) on chromosome 2 harbored three QTLs (*qSL2*, *qSIS2* and *qRL2.1*; Fig. 5A). Two QTLs, *qRL2.1* (at 53 cM; interval = 52.3–54.0 cM) and *qSIS2* (at 54.5 cM; interval = 54.3–54.6 cM) were very close to each other with 1.5 cM separating them on chromosome 2. Alleles from the male parent

Madina Koyo increased *qRL2.1* while the female parent Sahel 317 contributed alleles that increase *qSIS2*. A similar colocalization of QTLs was observed on chromosome 7 for *qSDW7* and *qSL7* (Fig. 5E).

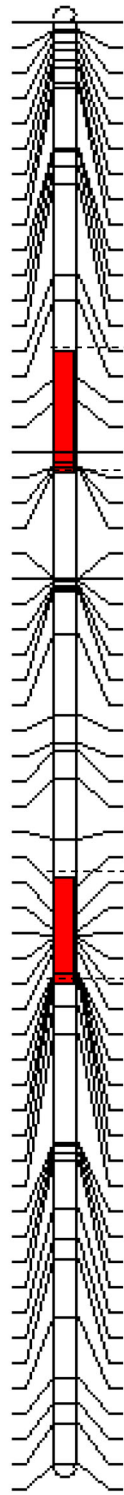
Discussion

Salt stress caused a general reduction in growth among the genotypes studied. This is a common phenomenon in most crops including rice (Bimpong et al. 2016), barley (Yousofinia et al. 2012), wheat (Sharma 2015) and sorghum (Almodares et al. 2014). However, some progenies performed better than the two parents for all traits, suggesting that segregation was transgressive. Transgressive segregation has been suggested to be common among morphological traits in plants and its occurrence has been attributed to the complementary gene action of additive alleles that are dispersed between the parental lines (Rieseberg et al. 1999). In rice, transgressive segregation under saline conditions has been reported in different populations such as F_2 (Sabouri et al. 2009), F_3 (Kaushik et al. 2003), F_6 (Bizimana et al. 2017) and RIL (Wang et al. 2012; Rahman et al. 2017) populations. Some 16 progenies recorded mean SIS between 1–2 and were classified to have significantly better tolerance than the donor

cM Chromosome 2

A

- 0.0
- 1.0
- 1.4
- 2.7
- 3.5
- 4.8
- 5.8
- 7.6
- 8.2
- 15.6
- 15.9
- 17.7
- 19.8
- 30.8
- 33.9
- 43.5
- 46.1
- 52.3
- 54.2
- 54.3
- 54.6
- 67.7
- 67.9
- 68.1
- 68.7
- 69.0
- 69.3
- 74.4
- 84.3
- 87.9
- 88.7
- 92.1
- 99.5
- 104.9
- 108.3
- 110.6
- 111.0
- 111.3
- 111.9
- 113.2
- 115.6
- 115.9
- 116.3
- 116.8
- 116.9
- 119.6
- 122.9
- 136.2
- 136.5
- 137.5
- 138.6
- 144.1
- 148.1
- 150.3
- 157.5
- 164.9
- 167.8
- 170.4
- 175.1



qSL2 (40.0-54.6 cM)

39.8	3059897 F 0-30:A>G-30:A>G
43.5	100110606 F 0-63:G>T-63:G>T
44.7	3051482 F 0-25:C>T-25:C>T
45.9	3452415 F 0-7:A>G-7:A>G
46.1	3060184 F 0-46:T>C-46:T>C
46.1	3056174 F 0-68:C>T-68:C>T
46.1	3453354 F 0-80:G>C-80:G>C
46.1	3453354 F 0-18:A>T-18:A>T
46.9	5407353 F 0-26:T>C-26:T>C
52.1	3052931 F 0-16:T>A-16:T>A
52.3	100047180 F 0-32:T>G-32:T>G
52.3	3062378 F 0-59:T>C-59:T>C
54.1	3447565 F 0-6:G>T-6:G>T
54.1	3051615 F 0-47:G>A-47:G>A
54.2	3990520 F 0-49:C>A-49:C>A
54.2	3056917 F 0-41:A>G-41:A>G
54.2	3752374 F 0-7:T>A-7:T>A
54.3	9751462 F 0-10:G>A-10:G>A
54.3	3443946 F 0-36:T>C-36:T>C
54.3	3063629 F 0-51:C>G-51:C>G
54.3	3051912 F 0-48:A>T-48:A>T
54.3	3050551 F 0-31:T>C-31:T>C
54.4	3056318 F 0-54:A>G-54:A>G
54.4	4388956 F 0-12:C>T-12:C>T
54.5	3439917 F 0-18:A>G-18:A>G
54.6	3059821 F 0-67:A>G-67:A>G
54.6	5142709 F 0-32:A>G-32:A>G

qRL2.1 (52.3-54.0 cM)

qSIS2 (54.3-54.6 cM)

103.5	100134950 F 0-51:G>A-51:G>A
104.9	3050849 F 0-58:A>T-58:A>T
104.9	3453969 F 0-49:A>G-49:A>G
105.1	3449994 F 0-19:G>T-19:G>T
108.2	10271446 F 0-11:T>C-11:T>C
108.2	5142245 F 0-19:T>C-19:T>C
108.3	3050647 F 0-44:G>A-44:G>A
108.7	3443642 F 0-47:T>G-47:T>G
108.8	12388262 F 0-35:G>A-35:G>A
108.9	5400212 F 0-6:T>C-6:T>C
110.4	3063650 F 0-10:A>G-10:A>G
110.6	6995938 F 0-25:A>G-25:A>G
110.6	9742895 F 0-58:C>T-58:C>T
110.7	3049768 F 0-38:T>G-38:T>G
110.9	100147611 F 0-22:G>A-22:G>A
111.0	5148291 F 0-53:T>C-53:T>C
111.0	100112271 F 0-7:C>G-7:C>G
111.1	3446453 F 0-17:C>T-17:C>T
111.3	3449744 F 0-48:A>G-48:A>G
111.3	3055418 F 0-35:T>G-35:T>G
111.6	3448204 F 0-14:A>G-14:A>G
111.7	3450921 F 0-43:T>G-43:T>G
111.8	3048719 F 0-56:C>T-56:C>T
111.9	3049319 F 0-48:G>A-48:G>A
112.2	3053101 F 0-15:C>T-15:C>T
112.3	3054298 F 0-65:T>C-65:T>C
112.5	3061514 F 0-11:G>C-11:G>C
112.8	4388527 F 0-60:T>C-60:T>C
113.2	3049606 F 0-62:C>T-62:C>T
113.2	100147799 F 0-21:T>A-21:T>A
113.7	9749803 F 0-64:C>T-64:C>T
114.4	3050175 F 0-29:C>T-29:C>T
114.6	4393414 F 0-7:C>G-7:C>G
115.6	3054917 F 0-24:G>A-24:G>A
115.8	9760917 F 0-5:A>G-5:A>G
115.9	100134710 F 0-19:C>T-19:C>T
115.9	6996219 F 0-19:T>C-19:T>C
116.1	100052583 F 0-60:C>G-60:C>G
116.1	100110988 F 0-33:G>A-33:G>A
116.3	4393362 F 0-61:G>C-61:G>C
116.7	9761782 F 0-11:C>T-11:C>T
116.7	3055502 F 0-39:A>C-39:A>C
116.7	3441590 F 0-10:T>C-10:T>C
116.8	3437969 F 0-22:A>G-22:A>G
116.8	100049211 F 0-15:C>A-15:C>A
116.8	3438063 F 0-15:C>T-15:C>T
116.9	3061022 F 0-57:C>G-57:C>G
116.9	3063473 F 0-29:A>G-29:A>G
117.2	3055769 F 0-50:A>C-50:A>C

qRL2.2 (104.0-116.0 cM)

qSDW2 (108.2-117.2 cM)

◀ **Fig. 5** **A** Chromosome 2 showing QTL positions in cM. **B** Chromosome 3 showing QTL positions in cM. **C** Chromosome 4 showing QTL positions in cM. **D** Chromosome 6 showing QTL positions in cM. **E** Chromosome 7 showing QTL positions in cM. **F** Chromosome 10 showing QTL positions in cM. **G** Chromosome 12 showing QTL positions in cM

parent (Madina Koyo) which had SIS of 4. Contrary to some progenies having better SIS than FL478 in this study, De Leon et al. (2016) found that, none of the F₆ Bengal/Pokkali progeny registered a better SIS than Pokkali, the donor source (parent) of FL478. These contrasting results could be due to the different populations used by the different studies.

Heritability estimates were lower for all traits measured under stress conditions compared to control, suggesting that salt stress influenced the expression of traits (Bhadru et al. 2012). On the contrary, Salam et al. (2011) observed high heritability estimates under stress conditions compared to control. Heritability for traits associated with fitness in natural populations varies between 10 and 20% (Visscher et al. 2008). In this study, heritability estimates for fitness traits (shoot and root lengths and their dry weights) ranged from 36 to 50%, confirming that heritability for the measured traits were high in this study. High heritability observed under stress in this study suggests that a greater proportion of the phenotypic variation is due to genetic variation which provides opportunities for genetic improvement through selection based on these traits.

Correlation among all traits measured under control conditions and some associations under stress conditions were significant, confirming the existence of true relationships among these traits. Progenies with high ion leakage would have high SIS, suggesting a strong association between these two traits. Similar association between ion leakage and SIS has been previously reported (De Leon et al. 2015). We observed an inverse correlation between SIS and root length, root dry weight and shoot dry weight. The implication is that, indirect selection for long and dense root systems capable of exploring deep and wide into the soil for water (during initial osmotic stress caused by salt stress) and nutrients (in the face of deficiency or toxicity on the near surface) can be achieved through selection based on low SIS. Similar significant and negative correlation between SIS and root length, root

dry weight and shoot dry weight had earlier been reported by Barua et al. (2015) and De Leon et al. (2016). While root length associated negatively with shoot length, shoot dry weight and root dry weight under control, association among these traits were positive under stress. This suggests that increase in root length had an increasing effect on root dry weight, shoot dry weight and shoot length under stress conditions, with the reverse being true under control conditions. In the absence of salt stress, it is probable that roots did not need to grow deeper to sustain healthy supply of nutrients for proper growth, hence the negative association. However, under salt stress, roots had to grow deeper, with the anticipation that, there might be some sort of relief to the much saline conditions near the surface of the growth medium. A large increase in root length under stress compared to the control in hydroponics has been reported in tomato (Lovelli et al. 2012). In rice, root length has been frequently observed to increase under stress, especially under drought conditions (Li et al. 2017; Uga et al. 2013; Luo 2010).

A total of 13 QTLs were detected for SIS, shoot length, shoot dry weight and root length. Except for root dry weight, more than one QTL was detected for each trait with varying contributions from both parents, suggesting possible epistatic interactions across both parental genomes whereby alleles from both parents act together to express these traits. This observation goes to buttress the point that salt tolerance in rice is a complex quantitative trait hence its mitigation requires the pyramiding of QTLs for several different traits acting together in a particular cultivar. Similar observation has been reported by several studies (Bizimana et al. 2017; Rahman et al. 2017; De Leon et al. 2017).

Three QTLs, one each for shoot length *qSL2*, root length *qRL2.1* and salt injury score, *qSIS2* colocalized within a region of 14.6 cM on chromosome 2. Another two (*qRL2.2* and *qSDW2*) colocalized within 104.0–117.2 cM on the same chromosome. We also observed that, a region on chromosome 7 (around 142–151 cM) controlled QTLs for shoot dry weight *qSDW7* and shoot length *qSL7*. Though shoot length and shoot dry weight have been reported to be highly and positively correlated (Souleymane et al. 2016; Bizimana et al. 2017), it may be that, these regions either have a pleiotropic effect, or there might be the presence of several tightly linked genes acting

cM Chromosome 3

B

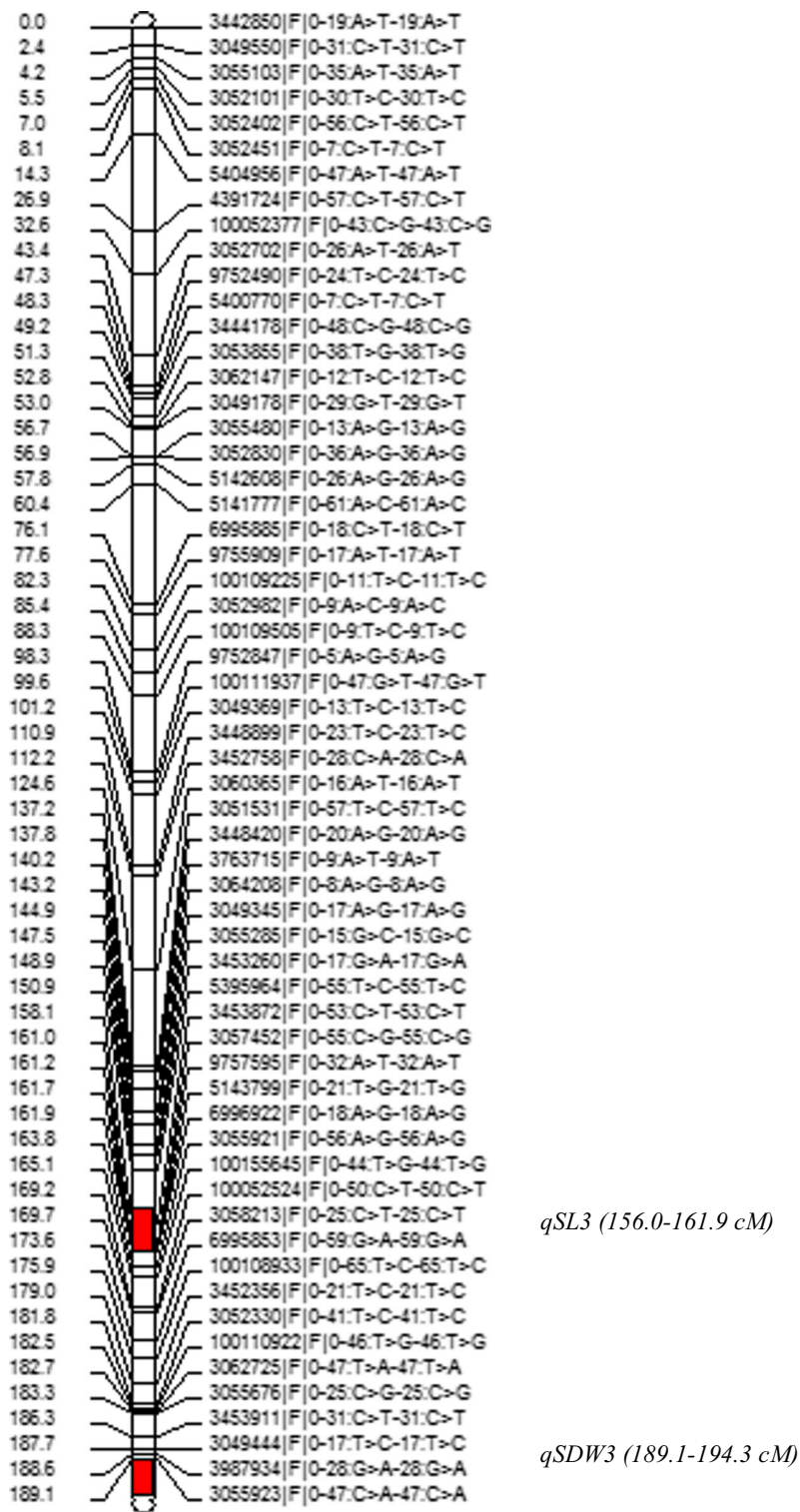


Fig. 5 continued

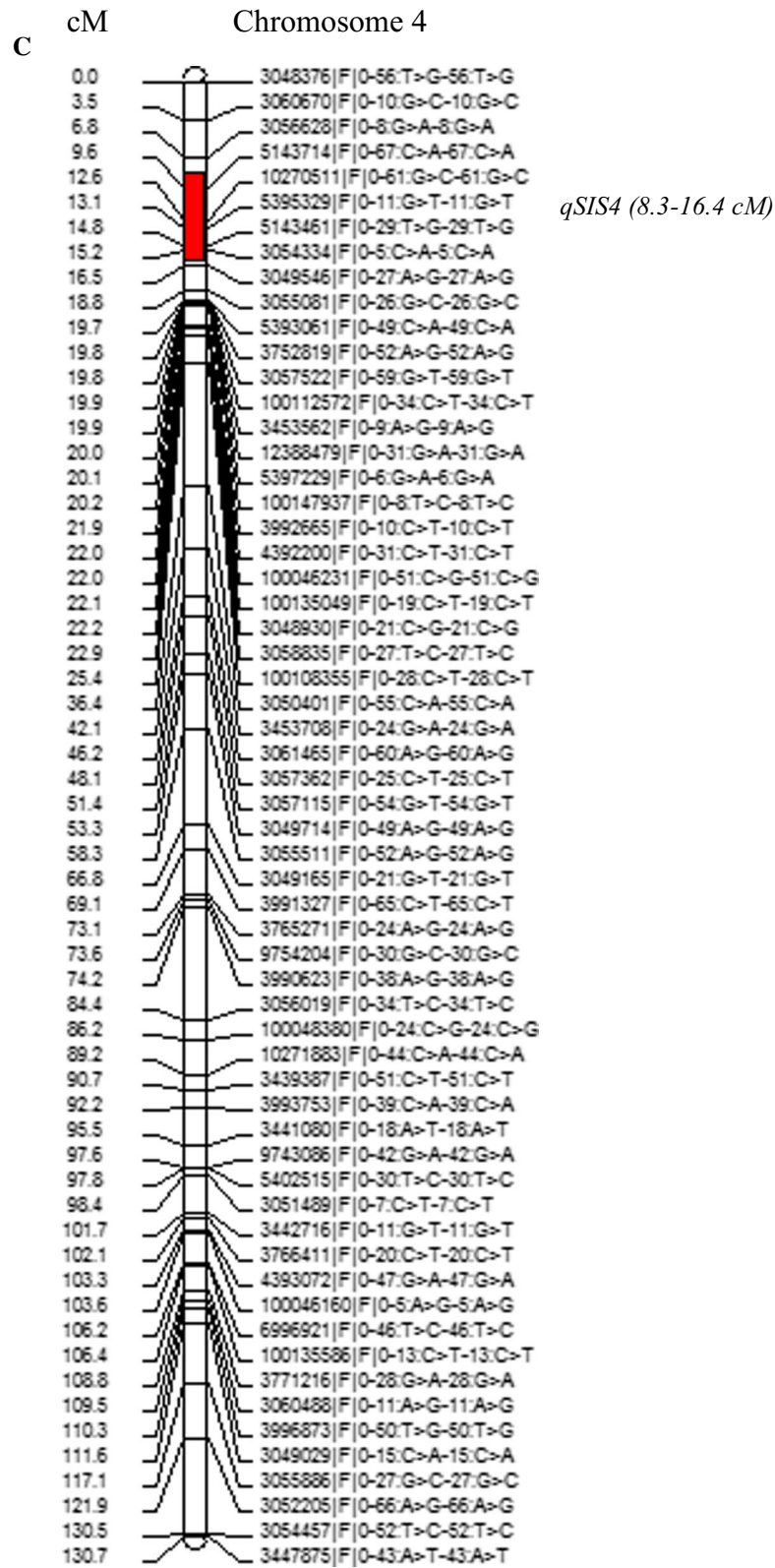


Fig. 5 continued

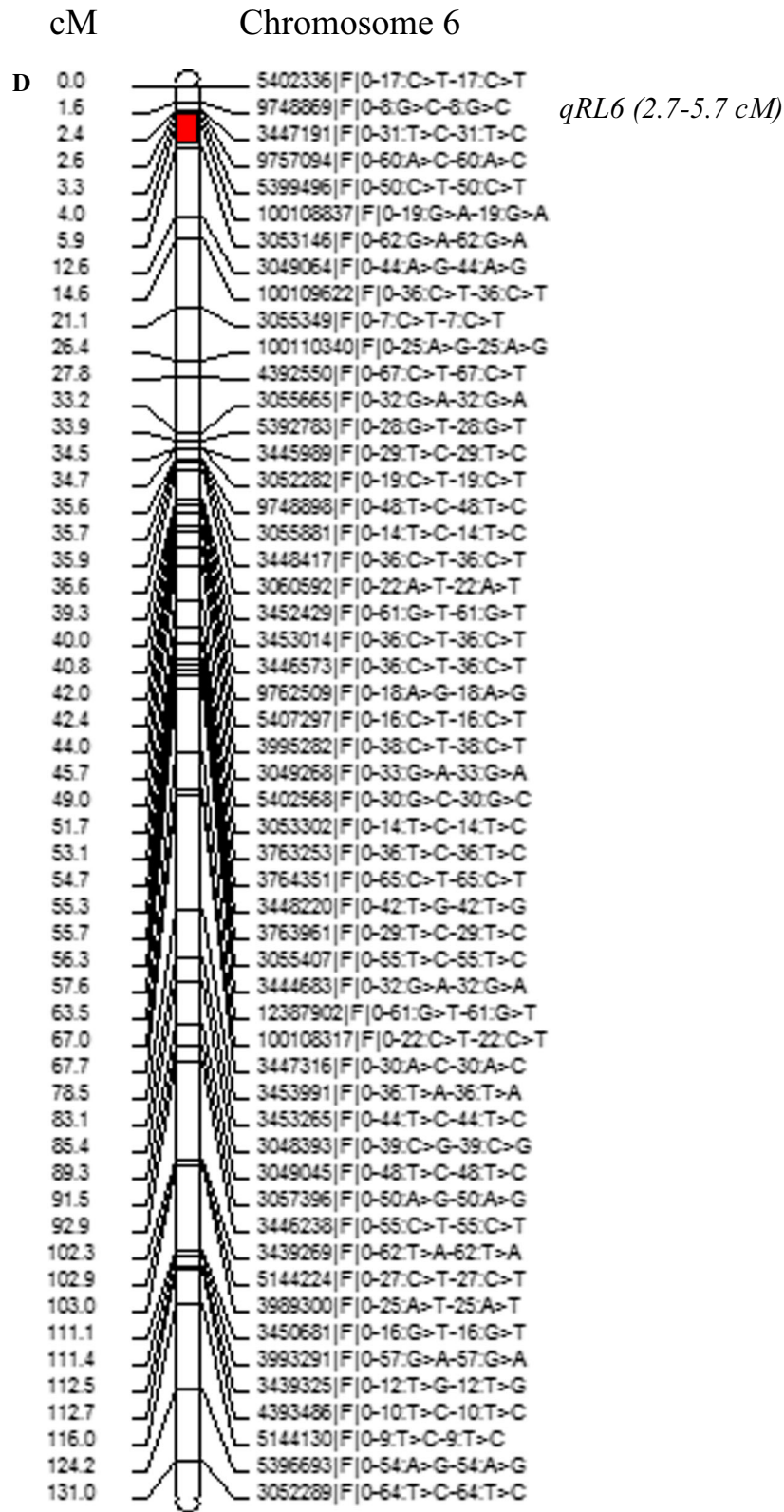


Fig. 5 continued

E cM Chromosome 7

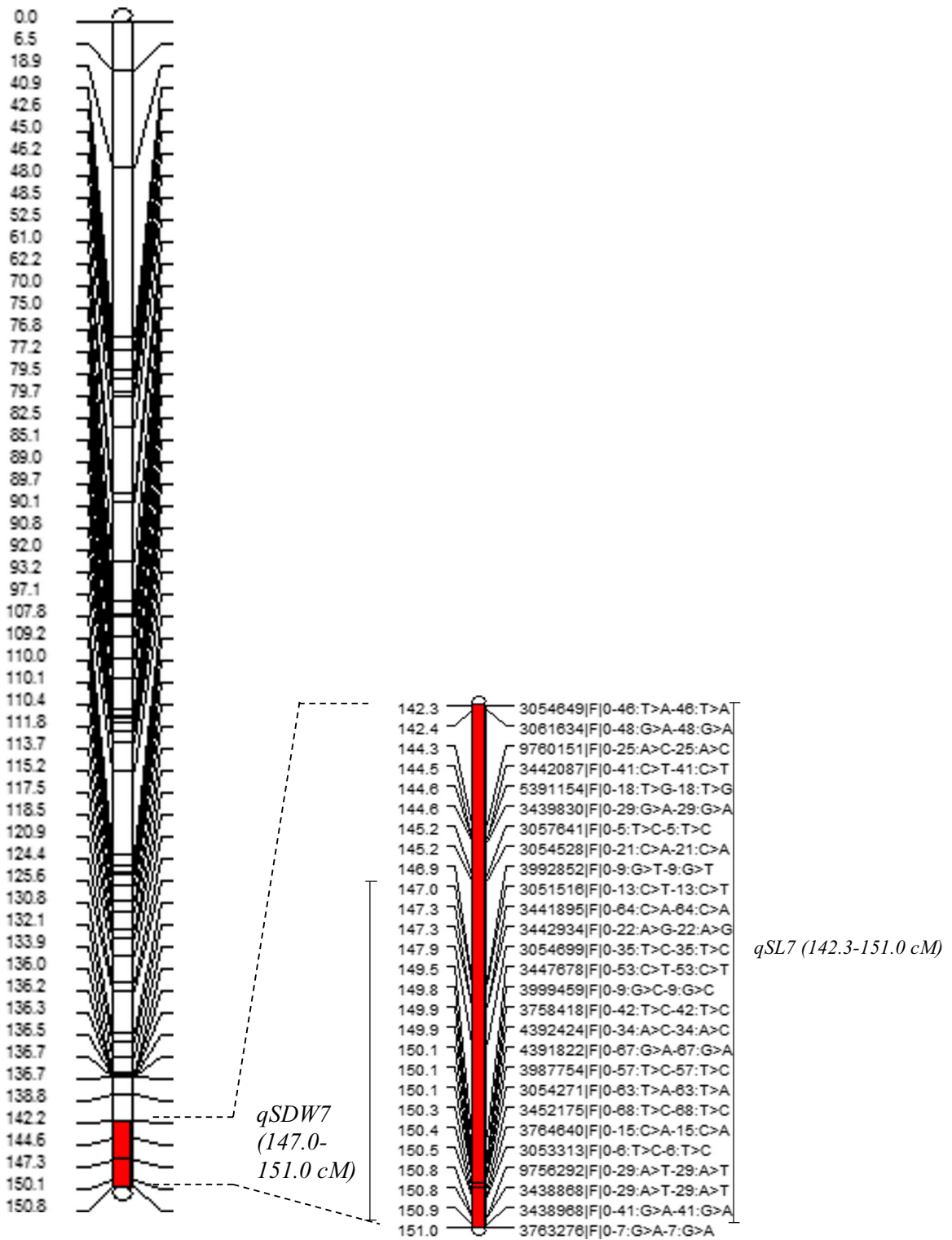


Fig. 5 continued

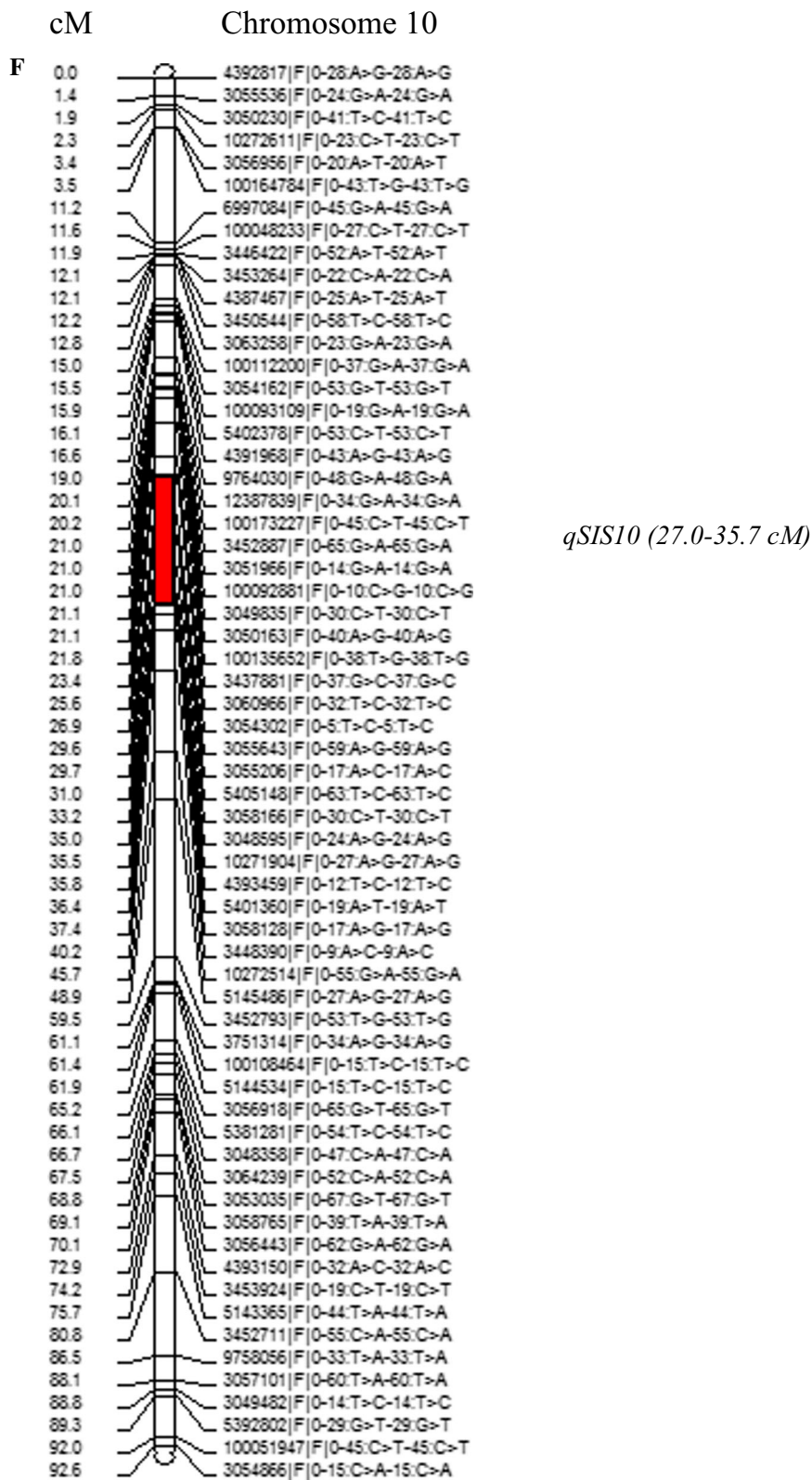


Fig. 5 continued

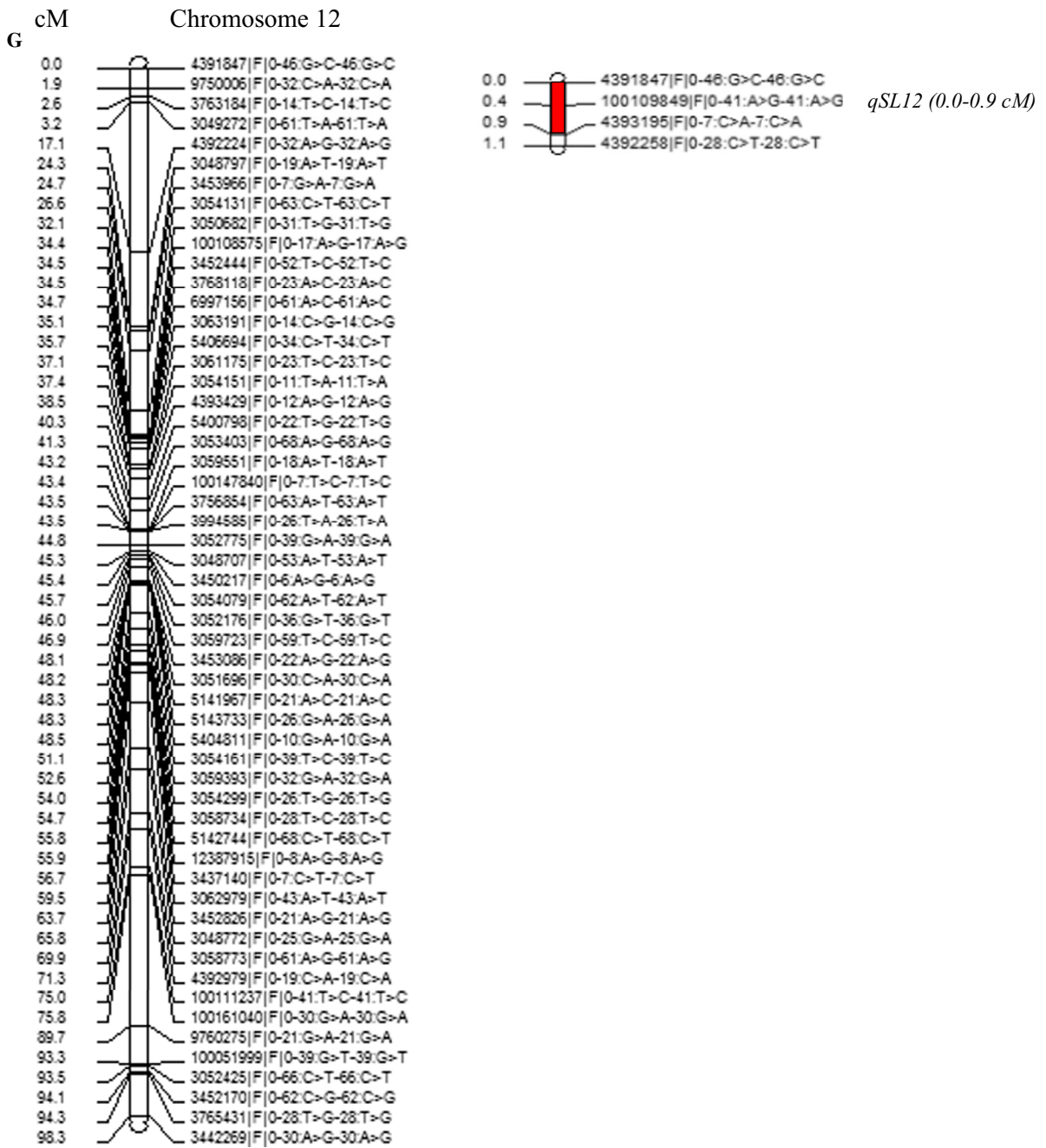


Fig. 5 continued

together to express different effects. Similar pleiotropic or tightly linked gene effect for SIS, shoot length, shoot dry weight and root length under salt tolerance has been reported by different studies on different chromosomes (Sabouri and Sabouri 2008; Bizimana et al. 2017). These QTLs could be useful in selection for tolerance as direct selection for one would indirectly select for the other.

The SIS reflects the overall plant's response to salt stress. It gives the clearest and first-hand information about the degree of tolerance for a particular genotype. Previously, a major QTL, *qSKC1* (*HKT1;5* gene or *Saltol*) controlling Na^+/K^+ ratio which is associated with SIS was mapped to 11.46 Mb region on chromosome 1 (Gregorio, 1997; Bonilla et al. 2002; Lin et al. 2004). In this study however, none of the three QTLs detected for SIS colocalized in the *Saltol* region on chromosome 1. Neither was any of the QTLs identified for the other traits mapped in this region. This indicates that tolerance genes from new donor Madina Koyo may be controlled by different QTLs acting from a different locus (or loci). Similarly, De Leon et al. (2017) did not detect any significant QTL near or around *Saltol* or *qSKC1* region in spite of dense SSR and SNP marker availability at the locus. Similar results were observed by Bizimana et al. (2017), Bimpong et al. (2014) and Thomson et al. (2010).

Majority of the QTLs identified in this study were for shoot related traits. This support reports that, under salt stress, shoot growth is more severely affected compared to roots (Läuchli and Grattan 2007; Ahmed et al. 2012; Pradheeban et al. 2015; Sakina et al. 2016; Bizimana et al. 2017). Under salt stress, the rice plant has been observed to reduce its leaf surface area as opposed to their roots (Munns and Tester 2008). The authors likened this phenomenon to an adaptation mechanism where plant cells mitigate initial osmotic stress by maintaining as much water as possible. They recounted that, the osmotic imbalance caused by salt stress affects the rate at which growing leaves expand while new leaves and lateral buds emerge more slowly or in the extreme, remain quiescent. In older leaves, where no expansion occurs, dilution of accumulated salts ceases. This leads to a considerable build-up of salts in leaves and eventually results in their death. When this is not compensated for by development of new leaves, the photosynthetic capacity of plants is critically reduced, causing further reduction in shoot growth relative to roots.

Conclusion

An average reduction of 63.4% was observed for all fitness-related traits among the F_4 families. A total of 46 progenies recorded an average salt injury score (SIS) between 1–3 and were rated as tolerant to salt stress at the early seedling stage. Thirteen QTLs for all traits were identified on chromosomes 2, 3, 4, 6, 7, 10 and 12 explaining between 5.3 and 11.9% of the observed phenotypic variation. Majority of the identified QTLs were for shoot related traits which reiterates the importance of shoot related traits in salt tolerance at the early seedling stage. Alleles that increased the traits studied were contributed by both parents. Some QTLs were mapped within the same region or were very close to each other suggesting that they are tightly linked. It is also probable that, these regions contain single QTLs that control the different traits in a pleiotropic gene action. Fine mapping and/or gene annotation within these regions will bring a resolve to whether there is a pleiotropic effect or there actually exist two genes that are tightly linked.

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Authors contribution NKAA, IKB, RA and AWK conceptualized, designed the experiments, analysed data and wrote the manuscript. BM and ID helped in design, analysis and wrote sections of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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