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QTL MAPPING FOR SALT TOLERANCE IN AN INTRA-SPECIFIC UPLAND COTTON AT SEEDLING STAGE USING SSR MARKERS

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ABSTRACT

Cotton is a moderately salt-tolerant crop, but its salt tolerance threshold is not more than 7.7 $ds \cdot m^{-1}$. The seedling stage of cotton is the highly sensitive to salinity and the effects can be quantified by measuring morphological and physiological traits. The purpose of this study was to identify QTLs related with salinity tolerance at seedling stage. Meanwhile where they were localized in the genome, investigate the relationships between the traits at seedling stage under salt and to find candidate genes related with salt tolerance. To achieve this goal, two upland cotton accessions mainly cultivated in China; CCRI-35 (Source: 132062) tolerant to salinity as female parent and Nan Dan Ba Di Da Hua: NH (Source: 130549) sensitive to salinity as male parent and their 277 offspring $F_{2:3}$ population have been used. Our experiment revealed 05 consistent QTLs found in at least two environments. Only 02 major QTLs with high phenotypic variation explained by a single QTL, R^2 (%), and high percentage of heritability, H_B (%), values were detected on chromosome Chr1 and Chr 7/ Chr16.These QTLs explained phenotypic variation from 5.7 to 60.03 %. Broad sense heritability was high for SL (83.1%) and moderate for GR (68.4). These 02 major QTLs and the 26 genes identified in this study could be used in cotton breeding program and with few obstacles.

Keywords: genes, cotton, markers, salinity, tolerance, location.

INTRODUCTION

Cotton is a dual-purpose crop, widely used for fiber and oil throughout the world. Salinity of agricultural land and poor irrigation techniques, which results into high soil pH elevation, is the most significant environmental problem which limits the growth and yield of cotton and other crops in arid and semiarid regions of the world (Thengane *et al.*, 2007). Salinity is more prevalent in irrigated agricultural and marginal lands associated with poor drainage. Currently, it is estimated that over 6% of the total agricultural land is affected by salinity (Munns, 2005). The mechanisms, by which plants cope with salt stress, are a subject of great interest globally as the problem of salinity increases. Plants in saline environments must have optimal mechanism to minimize salt uptake in order to prevent salt toxicity up the plants organs (Bhaskar and Huang, 2014). The rate of plant water loss is 50 times higher than the amount of water retention (Munns, 2005). The stresses imposed by salinity are mainly due to ion deposition in plants organs and concentration in the rhizosphere (Volkmar *et al.*, 1998). Information on inherent mechanisms involved in salt stress is essential to facilitate selection and designing for an efficient breeding program for salinity tolerant plants (Ashraf, 1994). There is a lot of diversity of plants in term of levels of salt tolerance and mechanisms. Na^+ and Cl^-ions are generally retained in the roots of barley, wheat, maize and sorghum under salt stress. However, this situation is different in upland cotton (Tester and Davenport, 2003). Cotton is a

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moderately salt-tolerant crop, but its salt tolerance threshold is not more than 7.7 $ds \cdot m^{-1}$ (Maas and Hoffman, 1977). Under salt stress, the growth of cotton is severely suppressed, especially at germination and at the seedling stage (Tester and Davenport, 2003). At the tissue level, cotton accumulates more than 95% of Na^+ in their shoots (Gouia *et al.*, 1994).

Little research has been done on determining the effect of salt stress in cotton seedling. Previous study, demonstrated and reported on association mapping for salinity stress tolerance in cotton , but the use of QTL mapping for salt tolerance in cotton has not been reported yet (Oluoch *et al.*, 2016).The seedling stage of cotton is the most sensitive to salinity and the effects can be quantified by measuring morphological and physiological traits (Munns, 2007). Identifying proper selection criteria for salinity tolerance is a major problem. The specificity of our experiment was to simulate the field condition by sowing the seeds in different salt concentrations to investigate the response of seedling to salt stress. Besides measuring parameters such as MDA, CHL, EC which acts both on biochemical and physiological levels, comparison of determinant traits such as germination and growth parameters of genotypes in salinity was also undertaken.

In this study, a genetic map consisting of 119 simple sequence repeat (SSR) were developed using 277 $F_{2:3}$ population derived from an intra-specific cross between two upland cotton accessions, mainly cultivated in China, CCRI-35 (Source: 132062) tolerant to salinity as female parent and Nan Dan Ba Di Da Hua: NH (Source: 130549) sensitive to salinity as male parent. The genetic map was used to analyze QTLs associated with salt related traits using QTL cartographer (Wang and Zeng 2007). The purpose of the study was to identify QTLs related to salinity tolerance and their position in cotton genome at seedling stage. The findings of this research may provide valuable insight for breeders to develop salt tolerant cultivars and enhance selection.

MATERIALS AND METHODS

Greenhouse Experiment: Seeds of two upland cotton (*G. hirsutum*) lines, CCRI-35(Source: 132062) salt tolerant and Nan Dan Ba Di Da Hua: NH (Source: 130549) salt sensitive were used. The salt tolerant cultivar was used as the female plant while the salt sensitive as the male plant. Sumian, sensitive and Earlistaple7, highly tolerant were used as checker. All the plant materials were obtained from the National Mid-term Gene bank of the Institute of Cotton Research, Chinese Academy of Agricultural Sciences (Du *et al.*, 2012). Only good quality seeds were selected, the seeds were then sterilized in 70% ethanol for 15 seconds, and then put in 4% sodium hypochlorite for 15 minutes. The seeds were later submerged in sterile water for 12 hours to rinse off the treatment chemicals. The seeds were sown in sterile silica sand with 0, 110 and 150mM of salt concentrations in planting boxes, measuring 60 cm by 35cm and depths of 12 cm in a greenhouse, the conditions within the green house was optimized at 28/22 °C day/night, 60–80 % relative humidity, and a 14 hours photoperiod under 450 $\mu mol \cdot m^{-2} \cdot s$ light intensity with day average temperature of. Two experimental set up were done, the first one from April to June 2015, then the second one from April to June 2016, corresponding to the seasons of cotton cultivation in the Yellow River China with different salt concentrations of 110mM and 150 mM respectively. In both experiment, the plant population size was 277 with three replicates. The two salt concentrations ,110mM and 150mM, were made based on the proportion of Hoagland (Hoagland and Arnon, 1950). Seven 07 days after sowing (D.A.S), 05 seedlings with uniform growth for each treatment were sampled for all traits. Seed germination (GR) was determined by counting the seedlings in each replication. Five fresh seedlings were weighed for fresh weight (FW) in each replicate, leaves fresh weight (LFW), stem length (SL) were all measured immediately after collection. The sampled leaves were submerged in distilled water overnight and their saturated leaves weight (SLW) obtained by weighing. The leave samples were then oven dried at60°C for 48 hours, after attaining constant mass, all were weighed to obtain dry leaves weight (DLW). Relative Water Content (RWC) was calculated using the formula described by (Barr and Weatherley, 1962).

The electro conductivity (EC) of the sample leaves were determined by using a sliced leave of 0.5g dipped in 100 ml of ddH_20, initial measurements done at the start, while the final measurements done after 48 hours, as explained by (Madhava and Sresty 2000). MDA is an important stress indicator, we determined MDA through the method described by (Greathouse, 1938). For the chlorophyll content (CHL) estimation, we used chlorophyll meter (SPAD 502 m, Minolta, Osaka, Japan), average of five measurements on the same leaf were done.

SSR-PCR analysis: DNA of the entire population of 277 and three parents was extracted by CTAB method (Paterson *et al.*, 1993). A total of 5723 simple sequence repeats (SSRs) markers were screened for polymorphism, out of which a total of 220 showed high level of polymorphism and were used in mapping the entire population. The molecular markers used were obtained from different sources, Table 1. The PCR reaction profile, denaturation at 94°C for 2 min, 35 cycles of 30s at 94°C for denaturation, 30s at 52°C for annealing, 30s at 72°C for extension and 5 min at 72°C for final extension after the last cycle. The amplified PCR products were separated on 8% denaturing polyacrylamide gel and visualized by silver nitrate staining (Sun *et al.*, 2009).SSR marker analysis was performed and a linkage map constructed.

Table 1. Information of the SSR markers on the linkage map

Primer Name	Number of polymorphic primers	Percentage of polymorphic primers	Source
NAU	78	35	Nanjing Agricultural University, CHN
BNL	22	10	Brookhaven National Laboratory, NY
TMB	20	9	USDA-ARS, Texas
CIR	4	2	CIRAD, France
GH	15	7	Texas A&M University, USA
HAU	6	3	Huazhong Agricultural University. CHN
MGHES	4	2	USDA-ARS, Texas
MUCS	2	1	University of California Davis, USA
JESPR	6	3	Texas A&M University, USA
DPL	14	6	Monsanto Company, USA
TOTAL	220	100	-

Construction of the linkage maps: In the construction of genetic map, a total of 277 $F_{2:3}$ populations developed from two accessions of *Gossypium hirsutum*, CCRI-35, female and NH male were used for the genotyping and final construction of the map. 119 SSR markers were mapped out of 220 polymorphic primers used in our study.

Data Analysis: The greenhouse experiment data, for the two seasons, 2015 and 2016 were analyzed using a mixed model, with accession as the fixed and replicates as the random variables. The mixed procedure of SPSS was used to estimates the means. ANOVA was performed using mixed procedure of SPSS Type III, the genotypes and the environments were used as factors in order to detect the heritability, the broad-sense heritability (H), was calculated using the formula described by (Uma *et al.*, 2017).

$$H=\sigma^2G/\sigma^2G+ (\sigma^2e/r)$$

With σ^2G is the genotypic variance; σ^2e: phenotypic variance and r: replication. Linkage analysis was conducted using Join Map 4.0 (Stam and Ooijen, 1995) with a recombination frequency of 0.40 and a LOD score of 2.5 for the $F_{2:3}$ population. A LOD threshold of 5.0 was used for $F_{2:3}$ population due to severe skewed segregation ratio in $F_{2:3}$populations. The Kosambi mapping function was used to convert the recombination frequencies to map distances. Linkage groups were localized to chromosomes using previously anchored SSR markers (http://mascotton.njau.edu.cn) and the source information of SSR primers were obtained from (http: //www. cottonmarker.org/). For the two experiments, each data point represented the mean of three replications, with each treatment consisting of forty plants. The descriptive statistics and correlation between different traits were determined by IBM SPSS Statistical software (Prabha *et al.*, 2014) and R-software version 3.3.3 (Team, 2008) respectively. The values were expressed as the means ± SD. The statistical analysis was performed using two-way, analysis of variance (ANOVA) in IBM SPSS Statistical software. P-values < 0.05 were considered statistically significant. The means were compared by using Tukey HSD test. Salt tolerance related

traits, MDA, EC, GR, SL, FW, LFW, SLW, DLW, RWC and CHL were used to conduct QTL analysis. QTLs were detected using composite interval mapping (Marlon *et al.*, .2016) by WinQTL Cartographer 2.5 (Wang and Zeng 2007). In the CIM mapping method, model 6, forward–backward regression method with 1 cM walking speed, a probability into and out of the model of 0.01 and window size set at 10 cM were used. A stringent logarithm of odds (LOD) threshold value was estimated by 1000 permutation test for all traits and was used to declare the significant QTLs with a significance level of P = 0.05. However, QTLs in two or more environments with LOD threshold of at least 2.5 were considered as common QTLs based on the explanation by Lander and Kruglyak (Lander and Kruglyak, 1995). QTL nomenclature was done based on previous criteria (Liang *et al.*, 2013). Positive additive effects meant that CCRI-35 alleles increased the phenotypic trait values and negative scores indicated that NH alleles reduced these values. QTLs were defined by 2.0 LOD supporting intervals. The proportion of observed phenotypic variance explained by each QTL was estimated by the coefficient of determination R^2 (%) as a percentage. The additive and dominance effects from QTL cartographer results were used to calculate genetic effects (|d/a|). The results were used to classify the QTLs as additive (A) (0–0.20), partial dominance (PD) (0.21–0.80), dominance(D) (0.81–1.20) and over dominance (OD) >1.20 according to (Stuber *et al.*, 1987). The graphic presentation of the linkage group and QTLs marked were created by Map Chart 2.2 (Voorrips, 2002). In this paper, we presented only segments of linkage groups associated with significant QTLs detected. The detected stable QTLs were used to identify the crucial candidate genes for salinity tolerance. The genes identities were searched through the available resources (Zhang *et al.*, 2015), (http://mascotton.njau.edu.cn). Sequences of SSR flanking QTLs for salinity tolerance on Chr 1, Chr 2, Chr 3, Chr 1/Chr 15, Chr 7/Chr 16 and Chr 13/Chr 18 were subjected to e-PCR on (http://mascotton.njau.edu.cn) database. The outputs were used to retrieve putative candidate genes in the genome, (http://mascotton.njau.edu.cn) website. The function of the identified genes was determined through GO analysis.

RESULTS

Phenotypic variation between parents: There was a wide range of phenotypic variation among the $F_{2:3}$ population, with respects to the following measured traits; MDA; EC; GR; FW; SL; LFW; SLW; DLW; RWC and CHL. In all the environments as shown in Figure 1 and in Table 2, all the traits exhibited normal segregation pattern, with equal distribution Supplementary Figure S1 and S2.

In control, there was no significance difference observed between the parents, except variation was noted in chlorophyll content (CHL) (Figure1). But, under salt treatment in all the environments, 110mM and 150 mM, all the traits were significantly reduced compared to the resistant parent; MDA, LFW, SLW, GR and SL showed a significant difference. However, in MDA, higher concentration denotes sensitivity; its concentration in resistant accession was significantly lower than the sensitive accession (Figure 1).

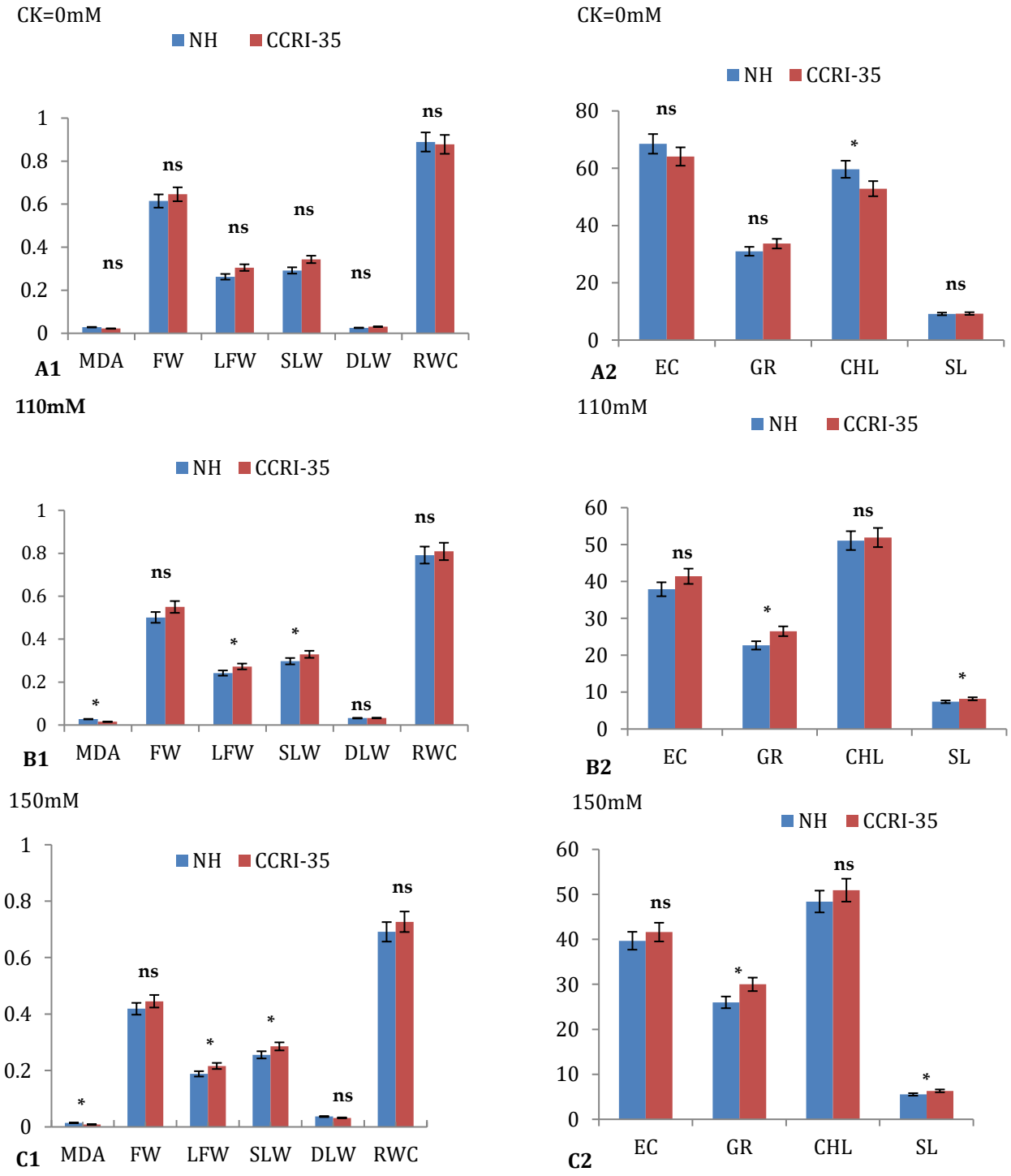

Figure 1. Phenotypic analysis of salt tolerance at seedling stage of the two parents.

Figure 1: Phenotypic analysis of salt tolerance at seedling stage of the two parents (CCRI-35: Tolerant, NH: Sensitive, F1: cross hybrid); MDA (μM/g.FW); EC (%); GR(number/40seeds); FW(g/seedling); SL(cm); LFW(g/seedling); SLW(g/seedling); DLW(g/seedling); RWC(%) and CHL(mg/g.FW). Asterisk indicates significant difference between CCRI-35 and NH with and without NaCl treatment by using Tukey HSD test; ns means not significant. For traits meanings see Table 1.

Table 2. Phenotypic analysis of salt tolerance traits at seedling stage

Trait[a]	Env[b]	Parents		$F_{2:3}$							
		NH	CCRI35	F1	Mean	Var	SD	Min	Max	Skew	Kurt
MDA	CK	0.028	0.022	0.034	0.042	0.000	0.011	0.000	0.085	0.052	0.753
	1	0.027	0.015	0.041	0.037	0.000	0.011	0.004	0.066	-0.050	0.159
	2	0.014	0.009	0.000	0.036	0.000	0.016	0.000	0.098	0.198	0.081
EC	CK	68.505	64.085	57.257	45.469	109.207	10.450	22.754	92.320	0.720	0.762
	1	37.882	41.406	40.418	35.448	41.403	6.435	18.824	59.902	0.630	0.645
	2	39.695	41.617	40.642	34.601	75.858	8.710	16.713	67.505	0.966	0.883
GR	CK	31.000	33.667	37.000	32.922	33.583	5.795	7.000	40.000	-1.170	1.364
	1	22.667	26.500	18.000	26.677	61.173	7.821	6.000	40.000	-0.533	-0.535
	2	26.000	30.000	20.333	28.355	54.829	7.405	4.000	40.000	-0.774	0.058
FW	CK	0.615	0.646	0.672	0.668	0.007	0.086	0.412	0.894	-0.055	-0.394
	1	0.501	0.550	0.482	0.513	0.005	0.071	0.318	0.700	0.053	-0.343
	2	0.419	0.445	0.449	0.472	0.007	0.085	0.284	0.790	0.444	-0.029
SL	CK	9.140	9.267	9.780	8.082	0.512	0.716	5.460	10.540	-0.269	0.407
	1	7.380	8.187	7.060	7.060	0.882	0.939	4.120	10.440	0.107	0.194
	2	5.533	6.320	5.327	5.852	1.575	1.255	2.900	9.580	0.172	-0.388
LFW	CK	0.263	0.305	0.298	0.292	0.002	0.042	0.168	0.416	-0.019	-0.210
	1	0.242	0.273	0.225	0.258	0.002	0.042	0.138	0.396	0.205	0.064
	2	0.188	0.216	0.205	0.217	0.002	0.046	0.062	0.390	0.449	0.385
SLW	CK	0.292	0.343	0.329	0.311	0.002	0.044	0.178	0.432	-0.035	-0.152
	1	0.297	0.329	0.276	0.319	0.002	0.049	0.180	0.494	0.147	0.037
	2	0.255	0.285	0.275	0.260	0.003	0.050	0.076	0.478	0.398	0.657
DLW	CK	0.025	0.031	0.031	0.032	0.000	0.005	0.016	0.046	-0.015	-0.053
	1	0.032	0.033	0.032	0.034	0.000	0.005	0.018	0.050	0.044	0.113
	2	0.037	0.032	0.037	0.034	0.000	0.005	0.018	0.052	0.145	0.020
RWC	CK	0.889	0.878	0.895	0.934	0.002	0.042	0.524	0.994	-0.357	0.222
	1	0.792	0.809	0.789	0.786	0.001	0.038	0.608	0.941	-0.138	1.348
	2	0.691	0.727	0.709	0.807	0.003	0.057	0.615	0.987	-0.141	0.969
CHL	CK	59.633	52.860	51.980	54.091	15.969	3.996	39.580	65.820	-0.173	0.345
	1	51.073	51.907	54.260	57.583	14.450	3.801	41.440	66.780	-0.486	0.467
	2	48.413	50.940	49.547	48.721	38.069	6.170	29.740	63.460	-0.530	0.126

CK=0mM; 1=110mM; 2=150mM; MDA: Malondialdehyde; EC: Electric Conductivity; GR: Germination Rate; FW: Fresh Weight; SL: Stem Length; LFW: Leaves Fresh Weight; SLW: Saturated Leaves Weight; DLW: Dry Leaves Weight; RWC: Related Water Content and CHL: Chlorophyll. For the traits units see Figure1, Var: Variance, SD: Standard deviation, Min: Minimum, Max: Maximum, Skew: Skewness, Kurt: Kurtosis. For traits units see Figure1.

Table3. Pearson's correlation of the 10 traits for the $F_{2:3}$in two environments.

Trait[a]	Env[b]	MDA	EC	GR	FW	SL	LFW	SLW	DLW	RWC	CHL
MDA		1									
EC	1	-0.065									
	2	-0.059									
GR	1	-0.071	0.213**								
	2	-0.100*	-0.077								
FW	1	0.021	-0.048	0.158*							
	2	-0.239**	0.046	0.212**							
SL	1	-0.042	0.131*	0.259**	0.604**						
	2	-0.132*	0.132*	0.131*	0.759**						
LFW	1	0.037	-0.012	0.183*	0.898**	0.384**					
	2	-0.263**	0.069	0.246**	0.908**	0.657**					
SLW	1	0.046	-0.012	0.180*	0.855**	0.336**	0.965**				
	2	-0.256**	0.087	0.266**	0.825**	0.569**	0.959**				
DLW	1	0.063	-0.127*	0.096	0.693**	0.095	0.793**	0.843**			
	2	-0.153*	-0.143*	0.197*	0.417**	-0.022	0.533**	0.595**			
RWC	1	-0.029	0.005	0.049	0.324**	0.271**	0.314**	0.059	-0.055		
	2	-0.096	-0.016	-0.004	0.542**	0.516**	0.428**	0.161*	-0.093		
CHL	1	-0.002	0.145*	0.007	0.036	0.149*	0.084	0.117*	0.163*	-0.096	
	2	-0.179*	0.021	0.242**	0.384**	0.368**	0.442**	0.428**	0.144*	0.206**	1

[a,b]For abbreviations see Table 2; *; ** Correlation is significant at 0.05 and 0.01 probability levels, respectively.

Correlation analysis: Correlation analysis was carried out using the mean values of the five (05) seedlings in each replicate for all the environments (Table 3). Most of the traits were positively correlated. However, MDA was negatively correlated except for environment 1 (110mM) for the following traits FW, LFW, SLW and DLW. A negative correlation was also observed between EC and DLW for all the environments. .GR, FW, SL, LFW and SLW had positive correlation with all the 10 traits. However, no correlation was noted between RWC& GR, and DLW & SL.

Analysis of Variance and heritability of the different traits in $F_{2:3}$ population: All the data collected were analyzed for ANOVA using mixed model analysis. The ANOVA results revealed significant differences between the genotype, environment and their interactions in all the traits (Table 4). The measured traits heritability percentage, were much higher on the morphological traits as opposed to physiological traits. The highest percentage in heritability was achieved in fresh weight (FW), with 87.4% while the lowest level of heritability was noted in relative water content (RWC) with 42.3%.

Table4. ANOVA and broad sense heritability for the 10 traits for salt tolerance.

Trait[a]	Source	Df	Mean square	F Value	Pr> F	H_B (%)
MDA	e	2	0.009	199.712	<.0001	73.4
	g	276	0.001	14.401	<.0001	
	g*e	543	0.0003	6.11	<.0001	
EC	e	2	29102.173	978.452	<.0001	57.5
	g	276	246.072	8.273	<.0001	
	g*e	543	125.338	4.214	<.0001	
GR	e	2	8313.01	427.302	<.0001	68.4
	g	276	191.801	9.859	<.0001	
	g*e	543	65.441	3.364	<.0001	
FW	e	2	8.543	7435	<.0001	87.4
	g	276	0.033	28.453	<.0001	
	g*e	543	0.009	7.621	<.0001	
SL	e	2	971.272	6639.292	<.0001	83.1
	g	276	3.988	27.261	<.0001	
	g*e	543	1.833	12.526	<.0001	
LFW	e	2	1.103	3127.666	<.0001	87.3
	g	276	0.01	27.156	<.0001	
	g*e	543	0.002	6.484	<.0001	
SLW	e	2	0.778	1641.264	<.0001	86.8
	g	276	0.012	25.249	<.0001	
	g*e	543	0.003	5.455	<.0001	
DLW	e	2	0.001	86.178	<.0001	82.6
	g	276	0.0001	17.117	<.0001	
	g*e	543	2.18E-05	2.826	<.0001	
RWC	e	2	5.091	7581.421	<.0001	42.3
	g	276	0.006	8.758	<.0001	
	g*e	543	0.004	6.559	<.0001	
CHL	e	2	15495.369	2024.514	<.0001	51
	g	276	67.297	8.793	<.0001	
	g*e	543	43.419	5.673	<.0001	

[a]For trait abbreviations see Table 2, H_B (%) is broad sense heritability, e: is environment and g: is genotype.

Construction of the linkage maps: In total, 220 of the 5723 SSR primer pairs (3.8%) showed polymorphism between the CCRI-35, NH and F1 and produced 229 loci across the cotton genome. The number of polymorphic markers from NAU, BNL, TMB, CIR, GH, HAU, MGHES, MUCS, JESPR, DPL and other sources was 81, 22, 21, 04, 15, 07, 04, 02, 07,16 and 50 respectively in the $F_{2:3}$ population. Of these, 119 loci were assigned to 32 linkage groups (Table S4, Supplementary Figure S3) with a total map distance of 543.327cM covering approximately 12.21% of the total recombination length of the cotton genome recombination length of the cotton genome(Lacape *et al.*, 2005). The other markers were discarded because of the high segregation distortion.

The average distance between adjacent markers is 4.56 cM. The A_t sub-genome spanned 108.095 cM, consisted of 31 markers on 13 linkage groups, and with an average distance of 3.487 cM between adjacent markers. 19 groups were assigned to the D_t sub-genome and comprised 88 markers spanning 435.232cM, with an average of 4.946 cM between adjacent loci (Table S4). Chromosomes: Chr 14, Chr 15, Chr 16, Chr 18 and Chr 23 had more markers compared to the other chromosomes. Among these, Chr 18 had Chr 13 loci that encompassed95.105cM, with an average distance of 7.316 cM between two adjacent markers. The smallest linkage groups, Chr 21 and Chr 26, both had 2 markers, and a total length of 23.877 and 9.528 cM respectively. In this study, the number of markers and genome coverage was lower due to the extremely low genetic diversity within upland cotton. But this did not influence the objective of this experiment since we mainly focused on understanding the salinity tolerance gene, and where addressing the fundamental issues related to salinity gene location in cotton genome.

Identification of consistent QTLs for salt tolerance: A total of 32 QTLs were identified, but only 05 QTLs were consistent in all the environments (Table 5). The five consistent QTLs were allocated in different chromosomes, Chr 1, Chr 13/ Chr 18 and Chr 7/Chr 16, the later are homologous. The distribution of the QTLs within the identified chromosomes, exhibited multiple position as illustrated in Table5.

The 05 consistent QTLs identified could be playing a major role in salt tolerance. The contributions of the parents toward the QTLs, majority were contributed by the sensitive parent (NH), while only 06 QTLs were contributed by resistant parent (CCRI-35). Only three chromosomes out of 26 were found to harbor consistent QTLs for traits related to salt tolerance (Table 5). Four types of gene actions were revealed by the genetic effects of which 02 genes exhibited dominant effects (D), 04 partial dominance (PD), 09 over dominance (OD) and 01 had additive effect (A). Over dominance (OD) was observed for most of the traits in response to salt tolerance, as shown in (Table 5, supplementary Table S1).

"QTL Hotspots" for salt tolerance: QTL hotspots are regions of the genome which several QTLs are clustered in or localized (Uma *et al.*, 2017). The highest number of QTLs mapped was fourteen (14), all was identified in the marker interval of BNL 3649-NAU 6627 on Chr1, this region was designated as "QTL hotspot A" with the length of 86 Mb. The QTLs hotspot A harbored QTLs for EC, FW, SL, RWC and GR, with the following proportions, 04, 02, 03,02 and 03 respectively. The second hot spot was found in Chr 7/Chr 16, with seven (07) QTLs, in the marker interval of HAU 878 - NAU 2931 (B), this region was named "QTL hotspot B" with a length of 21 Mb. It harbored QTLs for EC, DLW, CHL and GR, with proportions of 04, 01, 01 and 01 respectively (Figure 2, Supplementary. Figure S3).

Identification of putative candidate genes: Only consistent QTLs which were located in the two "hotspots" were used for gene identification. QTL region flanking SSR markers BNL 3649-NAU 6627 on Chr 1 and HAU 878-NAU 2931 (B) on Chr 7/Chr 16 were considered. The single consistent QTL identified in chromosome Chr13/Chr18 was not included because of its low PVE value. A total of 26 genes were found in the QTL regions identified on Chr1 and Chr7/Chr 16, as illustrated in (Figure 3, supplementary Table S2).

The genes distribution on the two sets of chromosomes, only two were found in Chr 1 and the rest were located in Chr 7/Chr 16. The sets of genes in Chr 1, Gh_A01G1119 and Gh_A01G1094, are calcium-dependent protein kinase. The identity of the 24 genes in Chr 7/Chr 16 are described in supplementary Table S2.

Table 5. Consistent QTLs for salt tolerance traits identified by using CIM.

Trait[a]	QTLs	Env[b]	Marker interval	Position (cM)	Position of flanking markers(cM)	LOD	A_e	D_e	d/a =D_e/A_e	d/a	R^2 (%)	DPE
EC	qEC-C1-1	2	CCRI196-NAU6627	16.01	0-42.309	9.659066	-10.1064	-13.4001	1.326	OD	1.05	NH
	qEC-C1-2	2	CGR6528(A)-NAU990	20.01	0-29.894	7.806732	-9.8935	-11.3655	1.149	OD	0.2	NH
	qEC-C1-1	1	BNL3649-NAU2600	4.01	0-9.152	2.644951	1.7388	-2.8748	-1.653	OD	6.75	CCRI-35
	qEC-C1-2	1	CGR6528(A)-NAU990	15.01	0-29.894	7.061889	-7.8259	-4.3395	0.555	PD	13.48	NH
	qEC-C7/C16-1	2	HAU878-MUSS551	0.01	0-5.356	9.337676	8.5569	-12.388	-1.448	OD	0.03	CCRI-35
	qEC-C7/C16-2	2	NAU848-NAU2931(B)	22.61	19.622-23.406	3.307275	1.0034	12.4364	12.394	OD	0.17	CCRI-35
	qEC-C7/C16-1	1	HAU878-NAU802	4.31	0-5.539	2.948969	-3.8369	6.3147	-1.646	OD	9.47	NH
	qEC-C7/C16-2	1	MUCS80-NAU848	10.51	8.492-19.622	3.081433	-7.6865	-3.0422	0.396	PD	3.52	NH
	qEC-C13/C18-1	2	NAU3017-NAU2312	87.31	86.291-95.105	3.887079	-11.4487	-11.0273	0.963	D	1.04	NH
	qEC-C13/C18-2	1	NAU3017-NAU2312	90.31	86.291-95.105	3.279045	-4.8253	-4.5803	0.949	D	2.94	NH
GR	qGR-C1**	2	CCRI196-NAU6627	32.01	0-42.309	6.994571	0.4618	-13.3179	-28.839	OD	59.24	CCRI-35
	qGR-C1-1**	1	CGR6528(A)-NAU990	9.01	0-29.894	5.908795	5.2284	10.0926	1.930	OD	5.71	CCRI-35
	qGR-C1-2**	1	CGR6528(A)-NAU990	27.01	0-29.894	2.985885	-5.6987	4.4917	-0.788	PD	60.03	NH
SL	qSL-C1-1**	2	CGR6528(A)-NAU990	0.01	0-29.894	3.124864	0.3384	-0.0002	-0.001	A	6.18	CCRI-35
	qSL-C1-2**	2	CGR6528(A)-NAU990	24.01	0-29.894	3.863192	-0.3522	-1.4385	4.084	OD	26.37	NH
	qSL-C1**	1	CGR6528(A)-NAU990	15.01	0-29.894	3.711183	-0.8484	-0.4483	0.528	PD	10.03	NH

[a,b]For trait abbreviations see Table1; LOD: for the detected QTLs; A_e: additive effect; D_e: dominant effect; d/a: estimation of gene effect;0<A(additive effect) <0.20; 0.21<PD (partial dominance) <0.80; 0.81<D(dominance)<1.20;OD(over dominance)>1.20;PVE= R^2(%):phenotypic variation explained by a single QTL; DPE: direction of phenotypic explanation, CIM: Composite Interval Mapping, **:Indicated consistent and major QTLs.

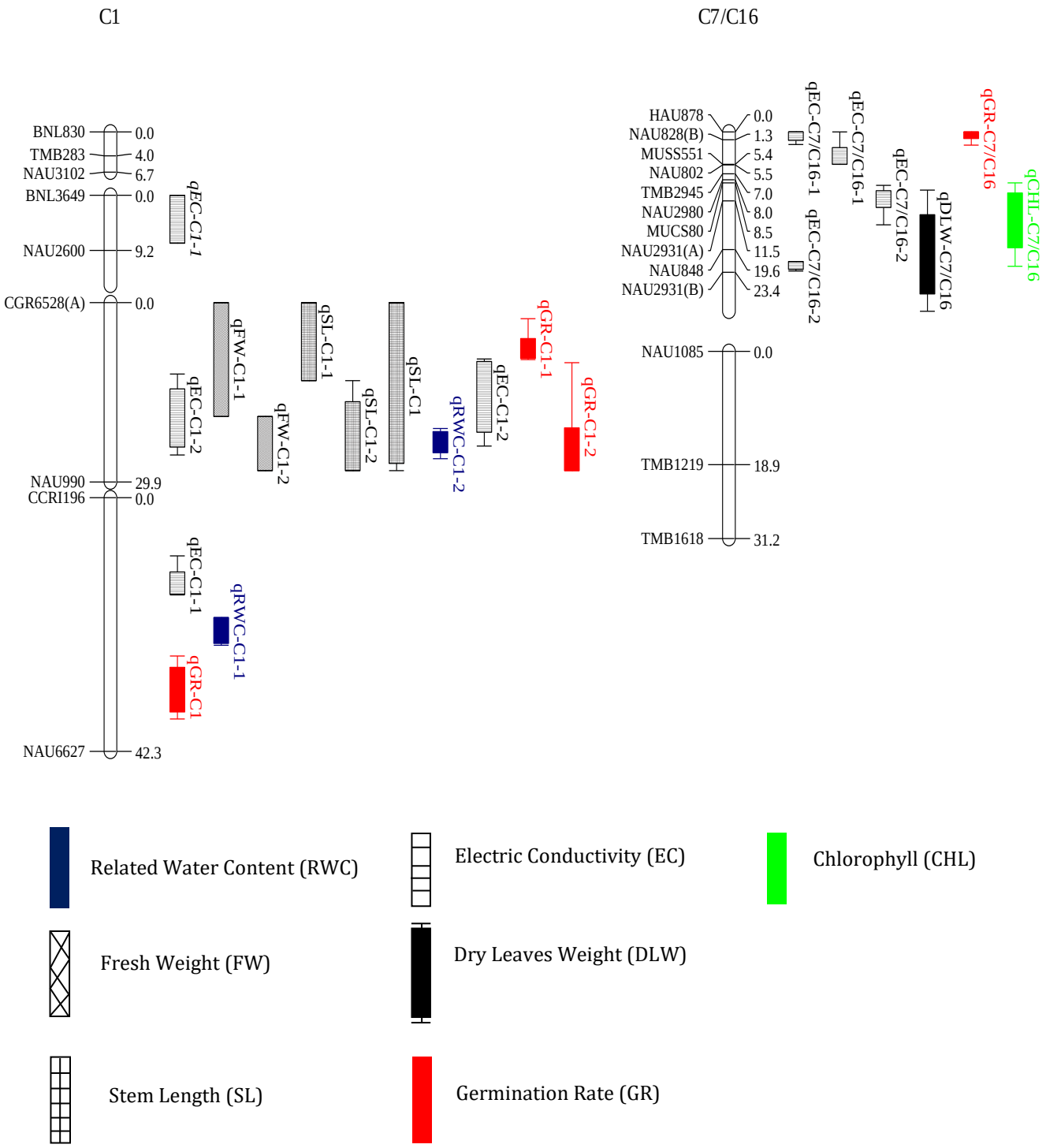

Figure 2. “Hotspot A” and “Hotspot B” respectively on Chr 1 and Chr 7/Chr 16, QTLs identified for salt tolerance trait. Bars and lines on the right-hand side of the linkage groups show the QTL likelihood intervals. Map distances in centimorgan (cM) are indicated on the left-hand side of the linkage groups.

The striking feature of all the 24 genes, they belong to three different protein domains, 11 NAC, 4 WRKY and 9 genes on Halo acid dehalogenase-like hydrolase (HAD) super family proteins which are known as genes for

abiotic stress, as earlier found in previous studies (Wang *et al.*, 2013)Supplementary TableS2.

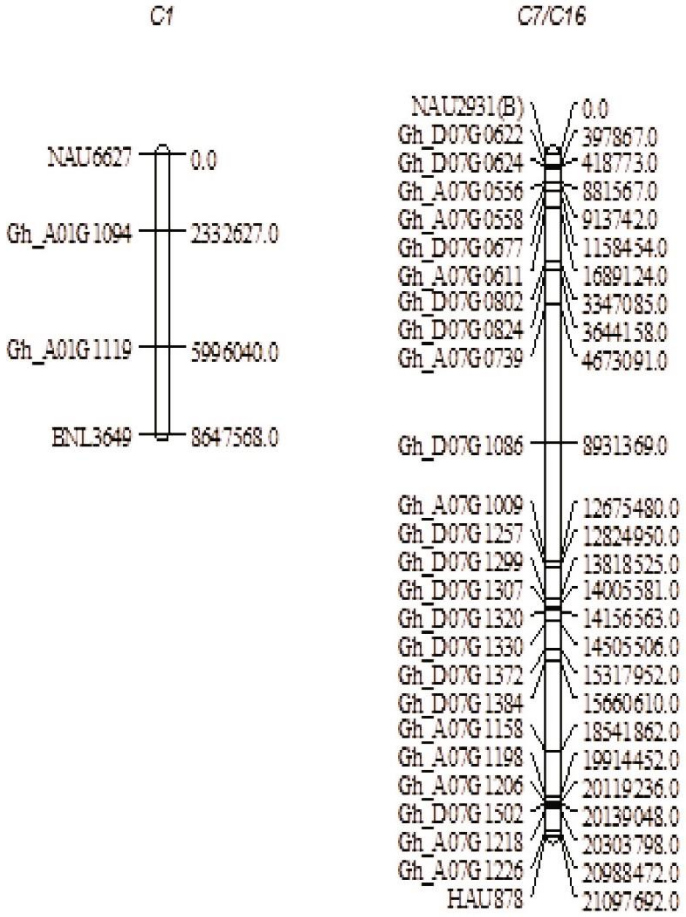

Figure 3. Candidate genes identified by syntenic analysis between two markers interval.
Map distances in base pairs (**bp**) are indicated on the left-hand side of the linkage groups.

DISCUSSION

In the two cultivars, there was a significant difference between the treated, under salt stress and the control, no salt application, in all the measured traits. Under salt treatment, all the traits exhibited significant reduction in LFW, SLW, GR, SL and MDA. In the sensitive cultivar (NH), MDA concentration was higher than in the tolerant cultivar (CCRI-35). MDA is a biochemical component of the cell; its release is triggered by exposure to stress condition. The low MDA level in the tolerant cultivar is an indication of the ability of the plant to tolerate salt stress, this result is in consistent with earlier publications (Abeer *et al.*, 2013). The low amount of MDA in tolerant cultivar can be attributed to either internal mechanism to convert the released MDA into other non-toxic compounds or minimizing the release of MDA to a minimal threshold with no major effect on the plant cell. A number of studies have shown that MDA in salt sensitive plants is more pronounced than in salt-tolerant ones(Koca *et al.*, 2007).Similar findings were found by (Azevedo-Neto *et al.*, 2006) using two maize genotypes , salt tolerant and salt sensitive genotypes. Under controlled condition (no salt stress), in all the measured traits, no significant statistical difference was noted between the tolerant parent CCRI-35 and the sensitive parent NH except for CHL concentration (Figure1). The sensitive cultivar produced more chlorophyll than the tolerant one this could be attributed to the genotype variation. Under salt stress, five traits showed significant difference, SL, GR, SLW, MDA and LFW while the rest had no significant difference. However, in chlorophyll concentration (CHL), in all the two genotypes, the tolerant cultivar (CCRI-35), showed relatively higher concentrations in chlorophyll but statistically, no significance. Similar findings, were previously reported,

salt and water stress has negative effect on chlorophyll concentration.

In their study on water stress on two grass species (Jiang and Huang, 2001), reported that chlorophyll increased during the first period of stress (6 days after stress initiation) and after which steady decline starts. The higher number of chloroplast per leaf area unit may be probably the main reason to increased chlorophyll concentration in tolerant plants under salt stress. High accumulation of sodium in plant tissues have been reported as one of the contributing factors in the reduction of photosynthetic pigments and rate of photosynthesis (Ashraf, 2004). Chlorophyll is an important factor in plant photosynthesis, long exposure to salt stress, may lead to the impairing of the photosynthetic apparatus of the plant, and in turn leads to reduction in plants performance in terms of yields and the quality of the yield of the salt sensitive cultivars.

Most of the traits were positively correlated. However, MDA was negatively correlated to all the traits. Negative correlation was also observed between EC and DLW in all the environments. This result is consistent with the previous studies on wheat under salt stress(Abeer *et al.*, 2013).

The extent of transmission of traits from the parents to the offspring is determined by levels of heritability and hence traits with higher heritability percentage are easier to manipulate(Saba *et al.*, 2001). The heritability of traits in cotton seedlings grown in saline environment for 07 days were high for FW(87.4), SL(83.1), LFW(87.3), SLW(86.8), DLW(82.6), moderate for MDA(73.4), EC(57.5), GR(68.4), and lowest in CHL(51) and RWC (42.3). These results were in agreement with previous reports which indicated that heritability estimates in cotton are moderate to high for SH, RL, SFW, RFW, SDW, and RDW under salt conditions(Hanif *et al.*, 2008). Traits with high heritability percentage are mainly regulated by genetic factors while those with low levels of heritability have got environmental influence, thus only traits with higher heritability can be selected and improved through molecular breeding. Selection done based on low heritability, may be biased towards environmental influence on the genetic make-up of the plant and thus not effective way for plants improvement (Nadarajan, 2005). In our study, a number of identified QTLs had a higher heritability percentage, though not all were consistent, the five consistent QTLs, the lowest heritability were 57.5% while the highest was 83.1%. This provides the basis for the use of these QTLs in the improvement of salt tolerance level in the sensitive cultivar (NH).

In this research, we obtained two major QTLs in all the environments (Table 5). The stable QTLs, GR and SL mapped on chromosome Chr1, the QTLs for GR were annotated as follows with their PVE values; qGR-C1(59.24), qGR-C1-1(5.71), qGR-C1-2(60.03) and SL as qSL-C1(10.03), qSL-C1-1(6.18) and qSL-C1-2(26.37). The major QTL GR on Chr1, their additive and dominance effects varied;qGR-C1and qGR-C1-1were over dominance while qGR-C1-2 had partial dominance.SL major QTL,qSL-C1 partial dominance, qSL-C1-1 additive effect and qSL-C1-2 had over dominance. This result is in disagreement with earlier studies by Oluoch et al 2016 (Oluoch *et al.*, 2016). Which stated that, partial dominance (PD) was observed for most traits in response to salt tolerance. Our findings, more of over dominance inheritance-model (OD) was observed for most traits in response to salt tolerance.

This result might explain the high heritability of some traits and their consistency, see Table 5.

A lot of publication reported that impaired seed germination is the major factor limiting the establishment of plant under salinity (Khan and Gulzar, 2003).The inhibitory effect of salinity on germination attributes of different crops has been reported earlier (Afzal, 2012; Elouaer, 2012.). The decline in germination under salinity has been attributed to combined effect of osmotic pressure and toxicity of salt or due to the effect of added chlorine ion (Almodares, 2007) which leads to osmotic stress. (Rahman, 2008)reported that salinity significantly delay germination mainly due to altered water relations caused by high salt accumulation in intercellular spaces. Root and shoot length are the most important parameters for salt stress because roots are in direct contact with soil and absorb water, nutrient from soil and shoot supply it to the rest of the plant. For this reason, root and shoot length provide an important clue to the response of the plant to salt stress (Jamil, 2004). Therefore the identification of QTLs related to GR (germination) and SL (shoot length) might be a major step towards achieving this goal. These QTLs can be used to facilitate conventional breeding of salt tolerance in cotton crop.

The genome sequence of the *G. hirsutum* provides a useful database for searching candidate genes underlying the marker loci associated with salt tolerance in cotton as they are closely related. The

BLAST search on the website (http://mascotton.njau.edu.cn), showed that 26 functional genes associated with salt tolerance were found in the QTL regions identified on Chr 1 and Chr 7/Chr 16 (Figure3, Table S2). Based on syntenic analysis, all of these genes were found to be highly homologous to genes in other plants such as in Arabidopsis. These genes play important roles in salt tolerance. Two genes, Gh_A01G1119 and Gh_A01G1094, known as calcium-dependent protein kinase, their main function is B9SDM2_RICCO Calcium-dependent protein kinase, putative OS=Ricinus communis GN=RCOM_0422070 PE=4 SV=1 and Q7XZK4_CICAR Calcium-dependent calmodulin-independent protein kinase isoform 2 OS=Cicer arietinum GN=CDPK2 PE=2 SV=2 respectively (Table S2). These two genes might be strongly related to the calmodulin family (Diego *et al.*). The CaM is a major class of calcium sensor proteins which play a role in cellular signaling cascades through the regulation of numerous target proteins (Ranty, 2006). It has been reported that calmodulin-binding motif protein encoded by a gene of osamir369c classified as a small RNA family involved in impacting growth regulation under several environmental stresses such as heat, drought and salinity in rice (Gao, 2010). The identification of calcium-dependent protein kinase in the present study supports the assumption that these regulators are important players in response to salt stress and the regulation may involve in the calcium-signaling pathway (Gao, 2010; Ranty, 2006). This may be a strong explanation of the aforementioned QTLs.

Besides the two genes found for calcium dependent, all the other 24 genes were found to be in three domains: 11NAC, 4WRKY and 9genes on Halo acid dehalogenase-like hydrolase (HAD) super family proteins which are known as genes for abiotic stress tolerance (**Figure3**, Supplementary **Table S2**).Based on previous studies, NAC genes are involved in various developmental processes in plants (Wang *et al.*, 2009). These developmental processes encompass stress responses(Satheesh *et al.*, 2014) hormone signaling, fruit ripening (Duval *et al.*, 2002),leaf senescence (Guo and Gan, 2006), organ formation and development (Hao *et al.*, 2011),and apical meristem development (Wang *et al.*, 2009).These previous reports can support our findings and these NAC genes can be used in further studies such as gene expression for better understanding of their function in salt tolerance. Moreover, WRKY proteins are plant specific transcription factors involved in various developmental and physiological processes, especially in biotic and abiotic stress resistance. Recently, studies demonstrated that WRKY proteins were involved in response to abiotic stresses, such as salt, drought, and cold (Rushton, 2012).

CONCLUSIONS

We identified 32 QTLs, of which 05 were consistent in all the environments, among the consistent QTLs, only two had high phenotypic variation (R^2) and high heritability values. All the consistent QTLs were located at the hot spot regions. A total of 26 genes were identified, this would provide a major breakthrough among the cotton breeders in the development of salt tolerant varieties. The distribution off the 26 genes were as follows, 11 NAC genes, 4 WRKY, 9 HAD and 2 calcium dependent genes. Further research on the expression levels of these genes can be conducted. Mapping of QTLs for salt tolerance has been a slow process due to the complexity of the trait and lack of proper information of the nature of the trait. Reproductive barriers and uncontrolled transfer of the traits make the conventional approach of plant breeding and genetics less desirable technique for abiotic stress tolerance in cultivars development. Other advanced techniques like genetic engineering for single gene transfer are considered more powerful to deal with this problem . These techniques must be integrated, and such approaches should be combined to effectively increase plant stress tolerance and more so salt tolerance.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XD, GWF, He, ZP, LD and RM. Performed the experiments: LD. Analyzed the data: LD. Contributed reagents/materials/analysis tools: XD, ZP. Wrote the paper: LD and RM

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