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Association Mapping For Salinity Tolerance Related Traits in a Structured Barley Population

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ASTRUCTURED barley population of 103 wild barley accession and 19 spring barley cultivars was used to identify quantitative trait loci (QTLs) for salt tolerance traits by means of an association mapping approach using 660 DArT markers. In this investigation barley accession and spring barley cultivars were employed in a two-year greenhouse project having a completely randomized design involving four irrigation water treatments having different salinities and twice replicated. Measurement parameters included grain yield per plant, straw weight, relative water content, chlorophyll content, Na^+, K^+ and salt tolerance %. Several statistical models were compared, the K-model was less spurious background associations, in this model 61 QTLs were detected under both of control and salt stress conditions (1000, 3000 and 5000 ppm NaCl of water irrigation) over whole barley genome for yield, straw weight, relative water content, chlorophyll content , Na^+, K^+ and salt tolerance %. Among of these QTLs, 21 detected under control, phenotypic variations explained by these QTLs, were ranged from 8.02 in N+ to 25.67% of the total variation in K^+. 40 QTLs were identified under saline conditions and the phenotypic variation explained by each main effect QTLs (M-QTL) ranged from 4.65 % in chlorophyll content at 3000 ppm condition to 28.13 % in ST at 5000 ppm condition. The genomic regions that harbor QTLs for Na^+, salt tolerance and related traits on chromosome 1H, 2H and 7H in our study can be used for targeting candidate gene (s) for salt tolerance of barley.

Keywords: Association mapping, Barley, QTL, Salinity, Egypt.

Barely (*Hordeum vulgare* L.) is recognized as one of the most economic and important cereals in the world. By area and production, barley is the fourth most important cultivated crop following wheat, rice and maize. It can be grown in a wide range of environmental conditions and give satisfactory yields in areas that are not suitable for growing most of the other cereals crops due to problems of abiotic and biotic stress (Mass, 1986 and Katja *et al*., 2009). Abiotic stress causes losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure. Salinity in soil and in irrigation water is among abiotic stresses, (Shilpi & Narendra, 2005). Salinity in the soil and irrigation water is an environmental serious problem and a major constraint for crop production in one third of the irrigation land and limiting the yield potential of modern cultivars. It has been estimated that salts affected nearly 950

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million hectares land in the world (Mass & Hoffman, 1977, Babu *et al*., 2007 and Taghipour & Salehi, 2008). Salt stress (NaCI) in plants influences some basic plant metabolic processes such as, photosynthesis, protein synthesis, and energy and lipid metabolism (Parida & Das, 2005 and Ozturk *et al*., 2012). NaCl has both osmotic (cell dehydration) and toxic (ion accumulation) effects on plant cells, impairing growth, ion homeostasis, photosynthesis and nitrogen fixation among other key physiological processes.

Salt stress (NaCl) in plants is a complex trait and affected by large number of mechanisms. Identification of a single criterion for ranking genotypes for their tolerance to salt stress is very difficult (Ashraf & Haris, 2004). Therefore For many years breeding for salt tolerance has been an important task to increase crop productivity under salt stress and choice of parents for crossing is considered an important step in any plant breeding program aimed to an increase in the salinity tolerance of barley which could improve the profitability of more than one billion salt affected hectares present in the world (El-Fadly *et al*., 2007). Using non-conventional approaches such as molecular marker as a strategy to obtain plants with higher performance under salt stress conditions by identify the genes and banding patterns that take place when the plant become growing under salt stress may further accelerate the progress of such breeding programs (Abd-El-Haleem *et al*., 2009 and Ehab & Metwali, 2012).

Genome-wide association studies (GWAS) are a novel tool in crop genetics for identifying significant marker trait correlations. In contrast to conventional bi-parental segregation-based mapping, which exploits allelic differences between two parental lines only, whole-genome association scans use the complete genetic variation across a wide spectrum of germplasm. This implies that many traits will vary in a GWAS, and can thus be addressed, whereas in a bi-parental population only those traits that vary between the parents can be mapped. Other advantages are the finer mapping resolution compared to classical mapping in bi-parental populations (Remington *et al*., 2001 and Matthies *et al*., 2012) and the direct use of existing genetic variation in diverse genotype collections. An alternative approach, association mapping (AM) known as LD mapping relies on existing natural populations or designed populations of plants to overcome the constraints inherent to linkage mapping. LD mapping exploits ancestral recombination events that occurred in the population and takes into account all major alleles present in the population to identify significant marker phenotype associations. Our investigation with barley aims to: 1) Establish marker-trait associations for each salt tolerance trait, 2) To evaluate genetic variation for salt tolerance and traits contributing to salt tolerance in a structured barley population and 3) To identify major genes/loci affecting salt tolerance that can be used for genetic improvement of salt tolerance.

Material and Methods

Plant material

The association panel consisted of a collection of 103 wild barley accessions (*H. vulgare* ssp. spontaneum) from the ICBB core collection (gene banks in Gatersleben and Braunschweig) and 19 spring barley cultivars representative for the breeding pool of spring barley in North Rhine Westphalia (NRW),

Germany, (Reetz & Leon, 2004). These cultivars were provided by the Institute of Crop Science and Resource Conservation (INRES), chair of plant breeding.

Field trials

A green house experiments was carried out in 2012/2013 and 2013/2014 seasons at the Research Farm of Faculty of Agriculture, Sohag University, Egypt. The seeds of 122 barley genotypes have been germinated in Petri dishes on welted tissue paper in a refrigerator at 4 °C for 7 days. Further, 10 seeds of each genotype were grown in one row inside the Wooden Boxes (100 cm x 120 cm x 30 cm, width x length x depth, respectively) filled with clay loam textured soil. The physical and chemical properties of the soils were determined according Page *et al*. (1982) and Cottenie *et al*. (1982) (Table 1). All boxes were watered with tap water (having EC of 300 ppm) for 30 days after sowing. After that, four levels of irrigation water salinity were applied viz., 300 (as a control),1000, 3000 and 5000 ppm. The salinity of irrigation water was prepared by using NaCl salt. The recommended fertilizers doses were given for each box. All treatments were replicated two times and arranged in completely randomized block design.

TABLE 1. The physical and chemical properties of the studied soil.

Property	**Before treatments**	**After treatments**		
		1	**2**	**3**
ECe (dS/m)	0.88	0.89	4.1	6.3
pH	7.5	7.8	8.1	8.3
SAR	6	5.7	9	9.5
ESP	3	4	10	10.3
Soluble cations (me/l)				
Na^+	4.3	3.8	35	56
K^+	1.1	2.0	2.5	2.3
Ca^{++}	2.3	2.1	3.6	2.9
Mg^{++}	2.1	2.4	2.8	2.3
Soluble anions(me/l)				
HCO_3^-	2.8	2.8	1.8	1.6
Cl^-	4.8	4.3	1.7	1.5
SO_4^{--}	3.5	3.1	38	60
Organic carbone(%)	0.42	0.39	0.35	0.33
Sand (%)	36.4	36.1	35.6	36.1
Silt(%)	27.8	27.6	27.4	27.5
Clay(%)	35.8	36.3	37.0	36.4
Texture	Clay loam	Clay loam	Clay loam	Clay loam
$CaCO_3$(%)	3	3.1	2.9	2.4
Total N(%)	0.7	0.7	0.66	0.65
Available P (ppm)	18	16	13	17
Available K (ppm)	210	225	228	300

For association studies, the following traits were considered: grain yield/plant (g), straw weight/plant (g), relative water content % (RWC) calculated as RWC = [(FW-DW) / FW] x100, chlorophyll content was measured

before harvesting as a SPAD index using a Minolta Chlorophyll Meter SPAD-502 (Konica Minolta, Osaka, Japan) on the second upper fully leaf of the main tiller, at a position about one quarter of the length of the leaf from the leaf tip, Na^+ and K^+ content in plant tissues were estimated by Flame photometric according to Jackson (1973) method and Salt tolerance % (ST) was calculated as the percentage of relative biomass production under saline and non-saline conditions according to the definition of Munns & James (2003). These traits are mainly affected by salt stress conditions.

The 122 accessions were genotyped for 1081 DArT markers in the Australian lab of Diversity Arrays Technology P/L - Triticarte P/L, 1 Wilf Crane Crescent, Yarralumla ACT 600, Australia for doing the marker analysis with their hybridization based markers, Their technology involves reducing the complexity of the DNA sample by cutting the DNA with restriction enzymes and annealing adaptors. Then fragments are amplified from the adaptors. The fragments are labelled and hybridized to a microarray of variable fragments representing the diversity within the species. See the Diversity Arrays website at www.diversityarrays.com for more information. DArT markers are biallelic dominant markers. Each marker was scored for each sample as 0, 1, and x, whereas 0 stands for absent, 1 for Present, and x stands for missing data.

Association analysis

Association mapping analysis was applied using filtered DArT data (660 DArT markers) to identify favorable QTLs that related to salt tolerance. Several statistical models were used to calculate P-values for associating each marker with the trait of interest, along with accounting for population structure to avoid spurious associations by TASSEL v.4.3 (http://www. maizegenetics. net). Four models comprising both general linear models (GLM) and mixed linear models (MLM) were selected to test the marker trait-associations (MTA). Results were compared to determine the best model for our analysis. PCA was conducted with TASSEL. The first ten significant PCs explained 41% of the cumulative variance of all markers. A kinship matrix (K-matrix), the pair-wise relationship matrix which is further used for population correction in the association models was calculated with 1081 SNP DArT markers using TASSEL (Bradbury *et al*., 2007). The following models were tested: 1) Naive model: GLM without any correction for population structure; 2) P-model: GLM with PCs as correction for population structure; 3) K-model: MLM with K-matrix as correction for population structure and 4) PK-model: MLM with PCs and K-matrix as correction for population structure (Pritchard *et. al*, 2000; Yu *et al*., 2006 and Stich *et al*., 2008). The critical P-values for assessing the significance of MTAs were calculated based on a false discovery rate (FDR) separately for each trait (Benjamini & Hochberg, 1995), which was found to be highly stringent. Considering the stringency of the model used for accounting for population structure, most of the false positives were inherently controlled. The threshold level for significant MTAs, P-values can be considered as significant, which in our analysis resulted in threshold levels of FDR = 0.001 for individual traits. This rather rough estimate was obtained by arranging-log10 P-values in a

descending order, and the value at which the curve starts to flatten is determined as the threshold value.

Results

Phenotypic data

Large phenotypic variation was observed for all studied traits across the environments (Table 2), where as the highest value of grain yield/plant (34.51 g) was given by unsalted treatment and lowest one (4.62 g) was given by 5000 ppm NaCl treatment. RWC % values were ranged from (86.04) in control to (24.18) in 5000 ppm NaCl treatments. Shoot weight (SW) range was (86.04-24.18 g), chlorophyll content (CC) range was (71.55-12.50), Na concentration range was (2.1- 0.07) and K concentration range was (14 - 0.5). ST values ranged from 7.84% in the 5000 ppm conditions to up 85.55 % under 1000 ppm salt stress conditions (Fig. 1). These results are very similar with those obtained by Nguyen *et al*. (2013) who stated that, ST values of the DH population ranged from 14.7 to 61.35 %. The results showed highly significant differences between the genotypes, salt treatments and their interaction in all studied traits (data not shown). These differences in all traits due to the wide range variability in a structured barley population and the used salt treatments are fitted for QTLs identification under the salt stress by using Association mapping analysis.

TABLE 2. Estimation of mean, minimum (Min), maximum (Max) and heritabilities (h^2) of studied traits under control and salt stress conditions.

Treatment		Yield	SW	RWC	CC	Na+	K+	ST (%)
Control	Max	34.51	76.34	95.13	71.55	1.5	14	
	Min	16.56	42.32	74.23	41.65	0.05	2.25	
	Average	21.507	49.92	86.04	64.93	0.74	7.44	
1000 ppm	Max	24.72	65.31	88.53	65.9	2.1	24.72	85.55
	Min	14.21	16.43	54.87	37.75	0.35	14.21	38.82
	Average	17.1	32	65.67	52.42	0.76	17.1	64.10
3000 ppm	Max	18.83	47.83	68.51	61.85	1.4	18.83	62.65
	Min	10.52	13.44	47.76	26.22	0.07	10.52	31.76
	Average	11.84	23.21	57.6	46.11	0.75	11.84	46.49
5000 ppm	Max	11.81	25.78	66.63	32.5	1.22	11.81	33.77
	Min	2.62	3.32	24.18	12.5	0.25	2.62	7.84
	Average	5.29	7.71	38.01	22.52	0.56	5.29	15.44
Average		15.18	31.96	61.83	46.50	0.70	6.8	56.51
h^2		42.34	78.23	82.11	67.86	65.76	58.54	72.23

SW: shoot weight, RWC: relative water content, CC: chlorophyll content, Na+: sodium concentration, K+: Potassium concentration, ST: Salt tolerance, h^2: heritability % of GxE.

Marker-trait association

Different models were used to detect association between Marker and phenotypic traits. Owing to the complexity and the considerable amount of population structure present in our panel, we observed numerous spurious associations when using the naive (simple) model for association mapping (AM). Hence, we assessed the usefulness of various linear models to account

for population structure by comparing their ability to reduce the inflation of false positive associations. To fulfill this end, ranked P-values from association mapping were plotted in a cumulative way for each model by using grain yield/plant as phenotypic trait (Fig. 2). As demonstrated by Kang *et al*. (2008) the distribution of P-values ideally should follow a uniform distribution with less deviation from the expected P-values. The K and PK models showed a good fit for P-values, while the Naive and P models were characterized by the excess of small P-values which is tantamount to an abundance of spurious associations. The K-model performed similarly to the PK model in displaying a highly uniform distribution of P-values and at the same time requiring less computational time. Irrespective of the model, major marker trait associations were constantly detected. However, the more stringent the model was the less spurious background associations were detected. For all other traits only results from the K-model will be presented and discussed. A marker trait association was considered when the marker main effect was significant at 0.001 [-log10 (0.001) = 3]. Table 3 represents the detected QTLs in this study at 0.001 significance onto different chromosomes of barley genome for all traits. However, Fig. 3, 4. 5, 6, 7, 8 and 9 show the manhatten plots for significant QTLs for each trait separately under study, where as the QTLs above the threshold in each graph have been detected over whole the barley genome. In this study we found a number of trait-marker associations in different regions of the barley genome controlling salt tolerance and related traits (Table 3). Some of these candidate genes may not be specific for stressed conditions as they were identified under both control and stress conditions which might relate to developmental traits.

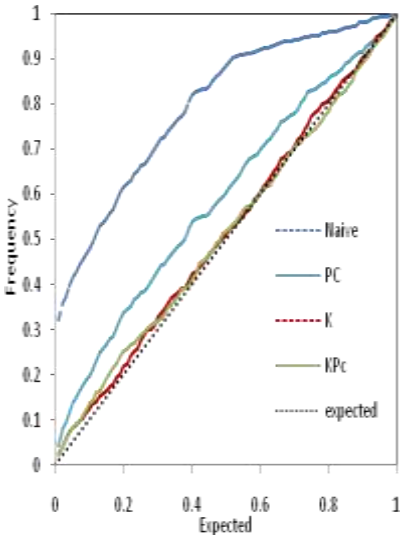

Fig. 1. The plotted P-values from AM a cumulative way for each model by using grain yield / plant as phenotypic trait.

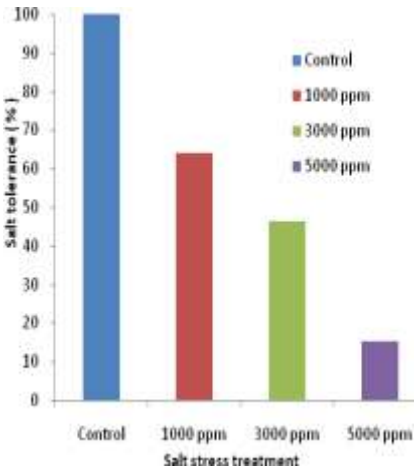

Fig. 2. Salt tolerance as the percentage of Shoot biomass under salt stress conditions and shoot biomass under control.

TABLE 3: Significant marker traits associations on the barley genome identified under salt stress conditions.

Trait	Treat.	Marker	chr	Position	P_value	-Log_{10}(p)	FDR	R^2%	Effect
yield	T1	bPb-1959	1H	133.12	8.33E-06	5.08	0.004705	17.84	-60.24
		bPb-7695	3H	115.5	7.62E-04	3.12	0.015065	11.36	-32.5
		bPb-5379	5H	137.79	2.83E-06	5.55	0.002238	19.32	12.45
		bPb-5379	6H	65.61	2.83E-06	5.55	0.002238	19.32	-4.19
	T2	bPb-1959	1H	133.12	1.84E-04	3.74	0.016271	11.76	-18.7
		bPb-5900	2H	13.94	9.42E-05	4.02	0.016271	11.3	6.27
		bPb-8978	3H	133.53	2.20E-04	5.65	0.001627	11.09	-4.22
	T3	bPb-9423	1H	48.95	3.83E-04	5.42	0.011649	12.39	-5.05
		bPb-0040	3H	72.18	4.00E-04	4.37	0.034175	8.78	4.32
		bPb-8978	3H	133.53	2.00E-04	6.61	0.003523	9.61	-2.96
	T4	bPb-7407	5H	16.91	3.00E-04	6.59	0.001234	5.98	3.65
		bPb-2188	7H	87.11	8.00E-04	5.07	0.021647	4.66	-2.81
S.W	T1	bPb-4614	1H	67.88	6.33E-04	4.2	0.014713	11.64	-3.22
		bPb-0068	3H	66.5	6.73E-04	8.17	0.014713	11.55	6.27
		bPb-6363	5H	36.1	9.90E-04	6	0.01864	10.97	33.11
	T2	bPb-6822	2H	114.4	8.48E-04	4.07	0.001627	11.21	8.79
		bPb-2006	5H	140.7	2.66E-04	5.57	0.001331	12.92	12.85
		bPb-5597	7H	89.81	3.00E-04	4.52	0.002573	9.3	13.52
	T3	bPb-6822	2H	114.4	1.02E-04	4.99	0.019574	14	8.45
		bPb-1661	5H	125.18	3.31E-05	7.48	0.000654	16	16.65
	T4	bPb-6822	2H	114.4	2.07E-04	6.68	0.001608	13.28	9.04
		bPb-1661	5H	125.18	6.59E-04	3.18	0.017645	11.58	13.43
		bPb-7915	7H	87.55	9.20E-04	5.04	0.011791	11.09	-24.55
RWC	T1	bPb-1959	1H	133.12	6.47E-06	5.19	0.004264	18.19	56.86
		bPb-1609	3H	140.29	4.10E-07	6.39	0.00054	21.9	34.18
		bPb-6264	6H	98.71	7.07E-04	3.15	0.014713	11.48	-7.91
	T2	bPb-4261	2H	44.79	5.60E-04	3.25	0.016271	11.82	-27.71
		bPb-0870	3H	1.48	8.53E-04	6.07	0.016271	11.2	-12.02
		bPb-4125	6H	84.81	2.75E-04	3.56	0.013312	12.87	-21.87
	T3	bPb-3412	5H	45.58	2.01E-04	3.7	0.028306	13.33	8.52
	T4	bPb-9104	7H	127.4	4.64E-04	3.33	0.031764	12.1	11.55
CC	T1	bPb-9945	3H	10.2	2.88E-04	3.54	0.027132	9.36	7.28
		bPb-7695	3H	115.5	2.47E-04	3.61	0.027132	9.6	-7.62
		bPb-5129	3H	146.78	3.93E-04	3.41	0.033084	8.89	3.16
		bPb-6477	6H	107.69	1.95E-04	3.71	0.024903	9.95	-9.67
	T2	bPb-9945	3H	10.2	2.00E-04	5.62	0.022892	9.66	6.54
		bPb-9907	4H	72.21	9.00E-04	4.07	0.015092	7.69	4.61
		bPb-3246	5H	81.39	7.00E-04	5.18	0.028526	8.09	-8.11
		bPb-5778	6H	84.64	5.05E-04	3.29	0.016271	11.98	11.97
	T3	bPb-4590	1H	67.88	7.00E-04	6.17	0.000246	8.08	-6.98
		bPb-6640	4H	60.55	9.00E-04	4.05	0.002378	4.65	-0.89
		bPb-7113	5H	81.39	4.00E-04	4.38	0.024174	8.8	-5.04
	T4	bPb-4531	1H	60.21	3.93E-04	3.41	0.017645	12.34	17.82
		bPb-2394	3H	68.01	3.06E-04	6.51	0.001728	12.71	-12.47

T1, T2, T3 and T4: control, 1000 ppm, 3000 ppm and 5000 ppm NaCl of water irrigation; FDR: false discovery rate.

TABLE 3 Cont.

Trait	Treat.	Marker	chr	Position	P_value	-Log$_{10}$(p)	FDR	R^2%	Effect
Na+	T1	bPb-1986	2H	147.61	2.00E-04	3.76	0.023558	10.14	0.26
		bPb-3030	3H	35.93	3.00E-04	8.46	0.003031	9.09	0.18
		bPb-0716	3H	128.64	5.00E-04	3.32	0.034138	8.58	0.14
		bPb-5252	6H	28.84	6.00E-04	6.24	0.003522	8.32	0.06
		bPb-1466	6H	70.57	7.00E-04	4.16	0.039462	8.02	-0.11
	T2	bPb-8779	2H	77.41	5.00E-04	4.28	0.035986	8.44	-0.1
		bPb-9587	2H	156.85	5.18E-04	5.29	0.016271	11.94	0.34
		bPb-8956	7H	82.61	8.73E-04	4.06	0.016271	11.16	-0.24
	T3	bPb-7938	3H	51.44	6.00E-04	4.26	0.024457	8.37	-0.24
		bPb-8382	6H	136.73	6.00E-04	3.21	0.024682	8.22	0.32
	T4	bPb-1681	3H	87.77	7.62E-04	6.12	0.001772	11.37	0.11
		bPb-2410	6H	143.98	9.51E-04	5.02	0.019791	11.04	0.19
K+	T1	bPb-5201	1H	141.29	4.62E-04	3.33	0.012178	12.11	1.35
		bPb-6765	3H	84.89	2.51E-07	6.6	0.000496	22.54	-7.79
		bPb-2940	6H	137.67	1.80E-04	3.75	0.04931	13.49	2.48
		bPb-4389	7H	125.4	2.17E-08	7.66	0.00858	25.67	-6.81
	T2	bPb-9909	2H	161.12	1.00E-04	3.93	0.016549	10.71	-1.85
		bPb-0098	4H	86.69	5.00E-04	3.31	0.028342	8.56	-1.32
		bPb-0889	7H	140.94	8.00E-04	3.08	0.025092	5.66	0.78
	T3	bPb-0775	2H	140.87	1.54E-04	3.81	0.029574	13.71	2.13
		bPb-2593	6H	68.22	7.90E-04	3.1	0.018374	11.31	-2.35
	T4	bPb-2055	1H	12.96	6.76E-04	3.17	0.017645	9.22	1
		bPb-1893	3H	167.34	5.12E-04	8.29	0.000176	11.96	0.52
		bPb-8382	6H	136.73	2.44E-04	3.61	0.01608	13.05	-1.76
ST	T2	bPb-0716	3H	128.64	3.60E-05	4.44	0.001	5.21	1.56
	T3	bPb-9423	1H	48.95	3.83E-06	5.42	0.0116	4.86	-2.78
	T4	bPb-2188	7H	87.11	8.42E-06	5.07	0.0086	6.98	-4.26
	T3&T4	bPb-1661	5H	125.18	3.20E-05	4.49	0.0091	8.42	13.54
	T2, T3 & T4	bPb-4531	1H	60.21	0.0002685	3.57	0.0146	28.12	18.12
		bPb-4614	1H	67.88	0.0003861	3.41	0.0219	16.52	-2.4
		bPb-3246	5H	81.39	0.0007826	3.11	0.0252	11.32	-0.68
		bPb-5252	6H	28.84	1.791E-05	4.75	0.0009	13.11	1.52
		bPb-8956	7H	82.61	0.000427	3.37	0.0196	9.81	-2.44

T1, T2, T3 and T4: control, 1000 ppm, 3000 ppm and 5000 ppm NaCl of water irrigation; FDR: false discovery rate.

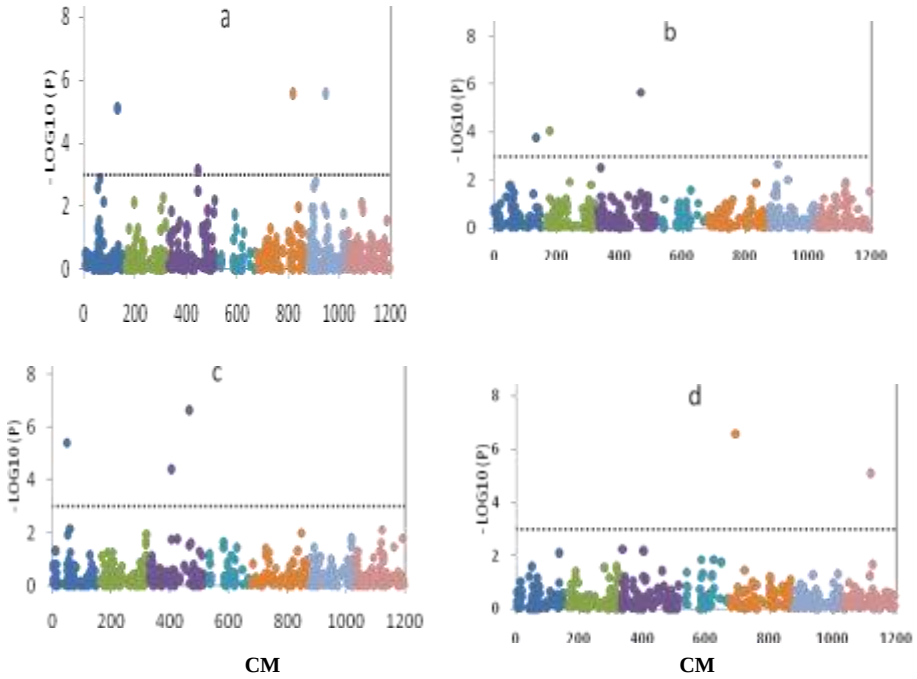

Fig. 3 . The pattern of Manhattan plot for detected QTLs of yield under a: control, b: 1000 ppm, c: 3000 ppm and d: 5000 ppm condition.

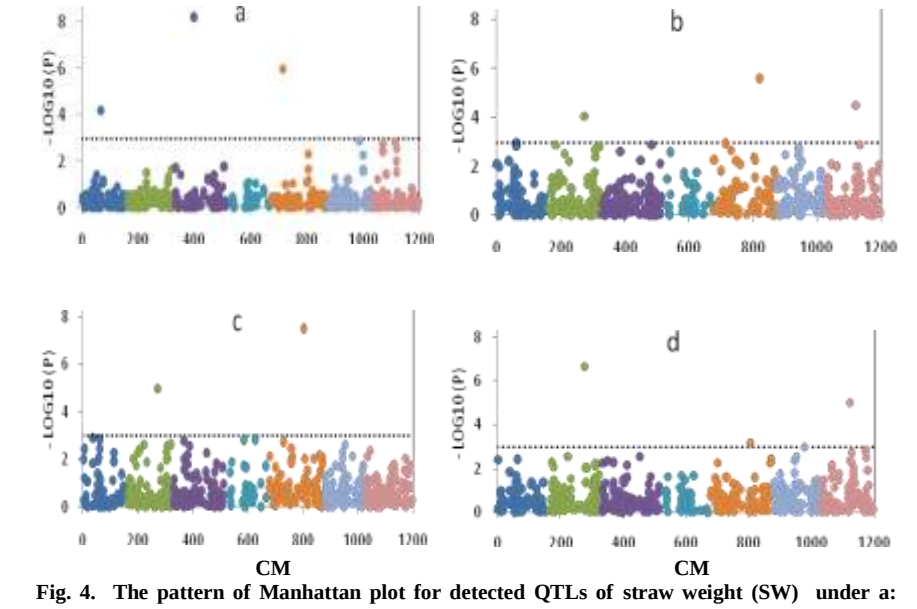

Fig. 4. The pattern of Manhattan plot for detected QTLs of straw weight (SW) under a: control, b: 1000 ppm, c: 3000 ppm and d: 5000 ppm condition.

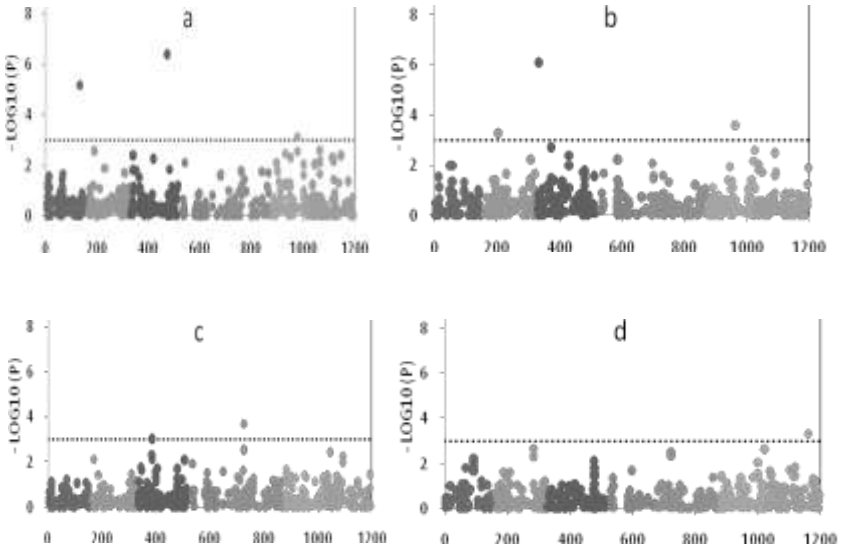

Fig. 5 . The pattern of Manhattan plot for detected QTLs of relative water content (RWC) under a: control, b: 1000 ppm, c: 3000 ppm and d: 5000 ppm condition.

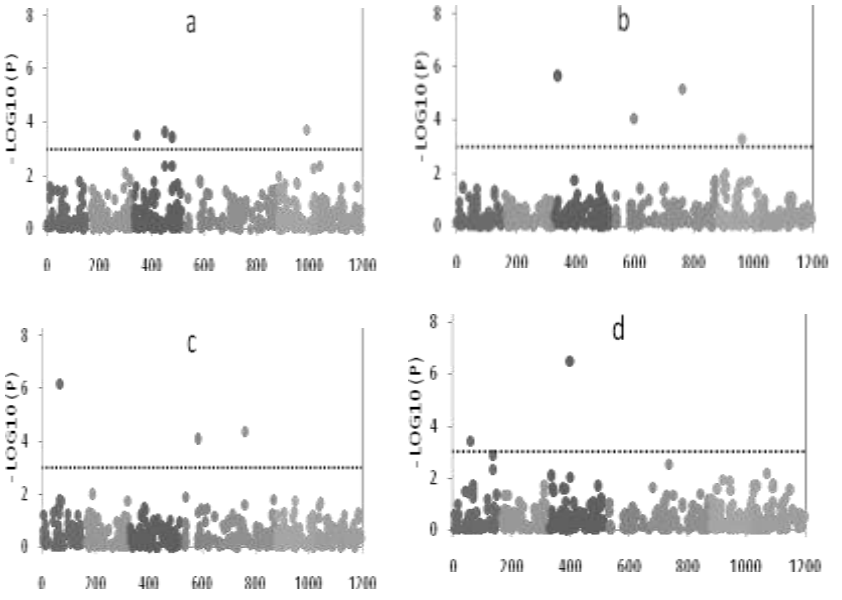

Fig. 6. The pattern of Manhattan plot for detected QTLs of chlorophyll content (CC) under a: control, b: 1000 ppm, c: 3000 ppm and d: 5000 ppm condition.

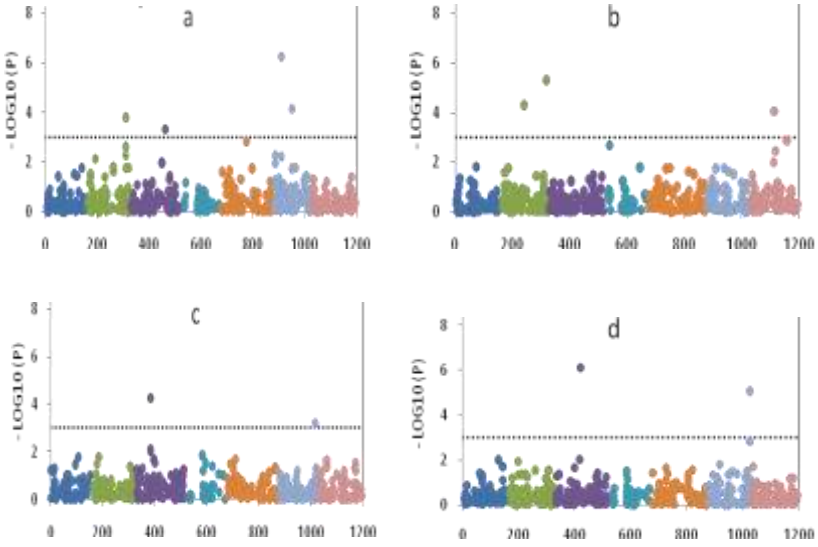

Fig. 7. The pattern of Manhattan plot for detected QTLs of Na+ under a: control, b: 1000 ppm, c: 3000 ppm and d: 5000 ppm condition.

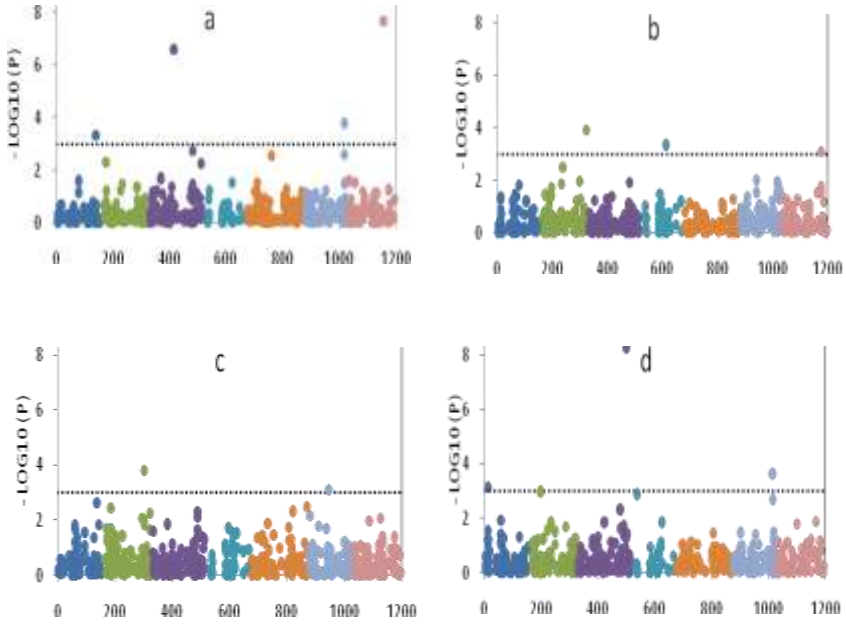

Fig. 8 . The pattern of Manhattan plot for detected QTLs of K+ under a: control, b: 1000 ppm, c: 3000 ppm and d: 5000 ppm condition.

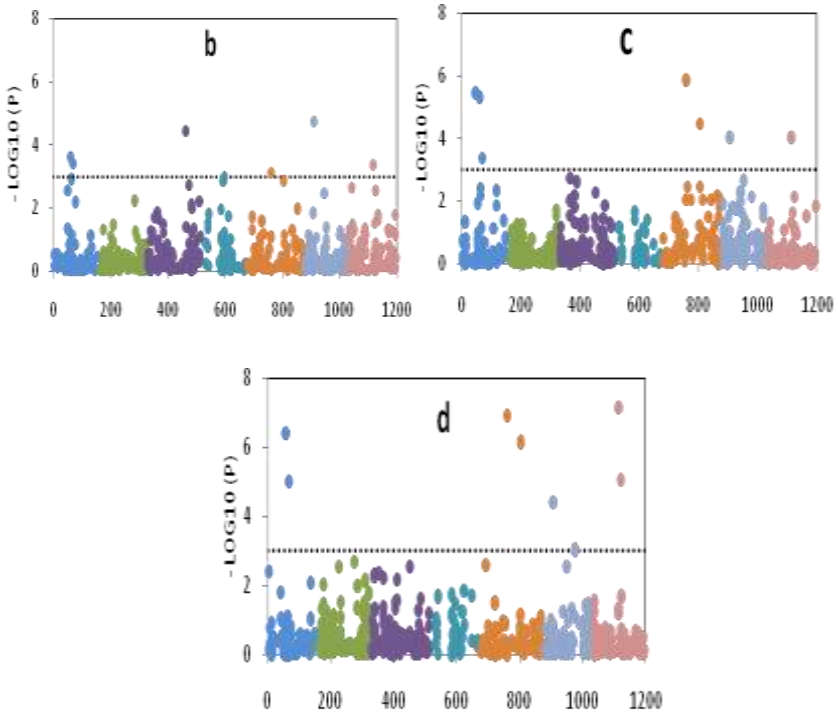

Fig. 9 . The pattern of Manhattan plot for detected QTLs of ST under b: 1000 ppm, c: 3000 ppm and d: 5000 ppm condition.

Grain yield/plant

A total of 12 DArT markers are significantly associated with the grain yield/plant trait by using the K-model (Table 3). Four of them (bPb-1959, bPb-7695, bPb-5379 and bPb-5379) are associated in the unsalted treatment by chromosomes 1H (133.12), 3H (115.50), 5H (137.79) and 6H (65.61), respectively (Fig. 3 a). Three markers (bPb-1959, bPb-5900 and bPb-8978) associated with yield in the second treatments (1000 ppm) in chromosomes 1H (133.12), 2H (13.94) and 3H (133.53) (Fig. 3 b), three markers (bPb-9423, bPb-0040 and bPb-8978) were associated with this trait in the third treatment (3000 ppm) by chromosomes 1H (48.95 cM) and 3H (72.18 and 133.53 cM) (Fig. 3 c), while two marker (bPb-7407 and bPb-2188) associated with yield for the fourth treatments (5000 ppm) in chromosomes 5H (16.91) and 7H (87.11). (Fig. 3 d).

Straw weight (SW)

Eleven markers were associated significantly with straw weight trait. Three out of these markers (bPb-4614, bPb-0068 and bPb-6363) located in chromosomes 1H (67.88 cM), 3H (66.50 cM) and 5H (36.10 cM), and associated under the control treatment (Fig. 4 a). Three markers (bPb-6822, bPb-2006 and bPb-5597) located on chromosomes 2H (114.40 cM), 5H (140.70

cM) and 7H (89.81cM), respectively and associated in the second treatment 1000 ppm (Fig. 4 b). The markers bPb-6822 on 2H (114.40 cM) and bPb-1661 on 5H (125.18 cM) were associated significantly with SW in the third treatment 3000 ppm (Fig. 4 c). Also, the markers (bPb-6822, bPb-1661 and bPb-7915) which Located on chromosomes 2H (114.40 cM), 5H (125.18 cM) and 7H (87.55 cM) were associated significantly with SW under 5000 ppm condition (Fig. 4 d).

Relative water content (RWC)

Eight markers were associated significantly with relative water content (RWC) and three out of them (bPb-1959, bPb-1609 and bPb-6264) were detected under control and located in 1H (133.12 cM), 3H (140.29 cM) and 6H (98.71 cM) (Fig. 5 a). The markers (bPb-4261, bPb-0870 and bPb-4125), which located in 2H (44.79 cM), 3H (1.48 cM) and 6H (84.81 cM) were significantly associated with RWC under salt treatment 1000 ppm condition (Fig. 5 b), while the markers bPb-3412 in chromosome 5H (45.58 cM) and bPb-9104 in 7H (127.40 cM) were associated significantly with RWC under the 3000 ppm and 5000 ppm treatments, respectively (Fig. 5c, d).

Chlorophyll content (CC)

Thirteen markers were associated significantly with chlorophyll content across 4 salinity stress conditions, three (bPb-9945, bPb-7695 and bPb-5129) in chromosome 3H (10.20, 115.50, 146.78 cM) and marker bPb-6477 in 6H (107.69 cM) were detected under unsalted treatment (Fig. 6 a). Four markers (bPb-9945, bPb-9907, bPb-3246 and bPb-5778), which located in 3H (10.20 cM), 4H (72.21 cM), 5H (81.39 cM) and 6H (84.64 cM) significantly associated under 1000 ppm treatment (Fig. 6 b). Three markers (bPb-4590, bPb-6640 and bPb-7113) were detected in chromosomes 1H (67.88 cM), 4H (60.55 cM) and 5H (81.39 cM) associated with CC in 3000 ppm treatment (Fig. 6 c). In addition the markers (bPb-4531 and bPb-2394) located in 1H (60.21 cM) and 3H (68.01 cM) were detected under 5000 ppm treatment (Fig. 6 d).

Sodium ion (Na^+)

Table 3 showed that twelve markers significantly associated with Na^+ over all the studied salinity stress treatments. Five markers (bPb-1986, bPb-3030, bPb-0716, bPb-5252 and bPb-1466) were significantly associated under unsalted treatment in chromosomes 2H (147.61 cM), 3H (35.93 and 128.64 cM) and 6H (28.84 and 70.57 cM), respectively (Fig.7 a). Three markers (bPb-8779, bPb-9587 and bPb-8956) located in 2H (77.41 and 156.85 cM) and 7H (82.61 cM) associated with Na^+ under the second salt treatment 2000 ppm (Fig. 7 b), while two markers out of these detected under 3000 ppm treatment, (bPb-7938 and bPb-8382) located in 3H (51.44 cM) and 6H (136.73 cM) (Fig. 7 c) and the two markers, bPb-1681 in 3H (87.77 cM) and bPb-2410 in 6H (143.98 cM) were associated under 5000 ppm treatment (Fig. 7 d).

Potassium ion (K^+)

Twelve markers distributed over whole genome shown in Fig. 8 and Table 3 were associated with K^+ across the studied salt stress treatments. Four markers (bPb-5201, bPb-6765, bPb-2940 and bPb-4389) located in 1H (141.29 cM), 3H

(84.89 cM), 6H (137.67 cM) and 7H (125.40 cM) were associated with K^+ under unsalted stress treatment (Fig. 8 a). Three markers (bPb-9909, bPb-0098 and bPb-0889) in chromosomes 2H (161.12 cM), 4H (86.69 cM) and 7H (140.94 cM) were associated significantly with K^+ under 1000 ppm salt treatment (Fig.8 b). The markers bPb-0775 and bPb-2593 located in 2H (140.87 cM) and 6H (68.22 cM) were associated with K^+ under 3000 ppm treatment (Fig.8 c). Three markers (bPb-2055, bPb-1893 and bPb-8382) in chromosomes 1H (12.96 cM), 3H (167.34 cM) and 6H (136.73 cM) were associated with K+ under 5000 ppm salt treatments (Fig. 8 d).

Salt tolerance (ST)

Nine markers (Table 3 and Fig. 9 a, b and c) were associated significantly with salt tolerance; three of these located in chromosome 1H (48.95, 60.21 and 67.88 cM), one marker in 3 H (128.64 cM); two markers located in 5H (81.39 and 125.18 cM); one marker at 28.84 cM in 6H and two markers located at 82.61 and 87.11 cM in 7H. The markers bPb-4531 and bPb-4614 on 1H, bPb-3246, bPb-5252 and bPb-8956 located in 5H, 6H and 7H, respectively, were associated with salt tolerance under 1000, 3000 and 5000 ppm conditions. The marker bPb-1661 in 5H (125.18 cM) was associated with this trait under both of 3000 and 5000 ppm conditions, while the markers bPb-0716, bPb-9423 and bPb-2188 located in 3H (128.64 cM), 1H (48.95 cM) and 7H (87.11 cM) were associated with salt tolerance under 1000 , 3000 and 5000 ppm salt stress treatments, respectively.

Discussions

Crop salt tolerance is a complex trait affected by numerous genetic and non-genetic factors, and its improvement via conventional breeding is slow. Recent advancements in biotechnology have led to the development of more efficient selection tools to substitute phenotype based selection systems. Molecular markers associated with genes or quantitative trait loci (QTLs) affecting important traits are identified, which could be used as indirect selection criteria to improve breeding efficiency via marker-assisted selection (MAS).

In the current study we describe the application of whole genome association mapping in a panel of 123 accessions of a structured barley population for some traits (grain yield/plant, straw weight, relative water content, chlorophyll content, Na^+, K^+ and salt tolerance). We identified strong QTLs affecting ST and related traits using the MLM with K-matrix as correction for population structure. Meanwhile, the relatively high heritability values for ST and related traits as well as the higher QTL -log10(P) values and effects under stress conditions compared to control indicate that traits phenotyped under saline conditions are the strongest indicators for salt tolerance selection. Whereas the heritabilities (h^2 %) for all traits ranged from 42.34% in grain yield/plant up to 82.11% in RWC. The phenotypic means reflected a broad variation (Table 2). Reducing Sodium (Na^+), while Potassium (K^+) uptake is maintained, would aid in salinity tolerance (Ahmadi & Fotokian, 2011). Fan *et al*. (2014) stated that, the tolerant variety showed consistently lower Na^+

contents and lower Na^+ / K^+ ratios, and higher contents of Na^+ and Na^+ / K^+ ratios could be seen in salt-sensitive genotypes except for Gairdner variety.

Association mapping

In spite of the advantages of association mapping to pinpoint genetic polymorphisms underlying phenotypic traits, this approach may suffer from an inflation of false positives due to population structure (Lander *et al*., 1994; Kang *et al*., 2008 and Zhang *et al*., 2010). Several statistical models to correct for the effect of population structure have been proposed and tested in previous studies (Stich & Melchinger, 2009; Kang *et al*., 2008 and Price *et al*. 2006). Since we detected a considerable amount of structure in the present panel we used linear models to control for population structure and to reduce the false positive associations. Similar to the previous studies of comparing GWAS models in allogamous and autogamous species (Stich & Melchinger, 2009 and Kang *et al*., 2008), our results suggest that K-model and PK model performed better than others (Fig. 1). Furthermore, for the K-model computational time is faster and does not need any additional steps such as identifying appropriate population structure (Q-matrix) in the panel. Since in an exploratory analysis mostly consistent results were obtained for all two approaches, the K-model was employed in the complete analysis of all traits to avoid redundancy of data. Although, the correcting for population structure reduces the frequency of false positives, it may entail false negatives in situations where a character state is strongly correlated with population structure (Cockram *et al*., 2008). Zhou *et al*. (2012) identified five significant QTL for salinity tolerance at the vegetative stage were located on chromosomes 1H, 2H, 5H, 6H and 7H.

The Detected QTLs

Different QTLs have been detected for all studied traits, and located on the whole barley genome, these QTLs had main effects on improving or reducing the traits of interest under four salt stress conditions (unsalted stress, 1000 ppm, 3000 ppm and 5000 ppm)(Table 3 and Fig. 3,4,5,6,7,8,9). The number of markers associated with the traits and the QTLs for each trait will discuss as follow:

Grain yield/plant and straw weight

Ten QTLs were detected for grain yield per plant under control and salt stress conditions located on chromosomes 1H, 2H, 3H, 5H, 6H and 7H. Two QTLs on chromosomes 1H at 48.95 cM under 3000 ppm salt stress treatment (Fig. 3 c) and at 133.12 cM under both of control and 1000 ppm treatments (Fig. 3 a and b), one QTL in 2H (13.94 cM) identified under 1000 ppm (Fig. 3 b). Three QTLs were identified in chromosome 3H at 27.18 and 115.5 cM under 3000 ppm and unsalted treatments, respectively and 133.53 cM under both of 1000 and 3000 ppm (Fig. 3 a,b and c). The QTLs in 5H at 16.91 and 137.79 cM were detected under 5000 ppm and unsalted conditions. respectively (Fig. 3 a and d). While two QTLs located in 6H (65.61 cM) and 7H (87.11 cM) were found under control and 5000 ppm conditions, respectively (Fig. 3 a and d). The QTLs mapped on 2H (13.94 cM), 3H (72.18 cM), 5H (16.91 cM) and 5H

(137.79 cM) led to improve this trait under 1000, 3000, 5000 ppm and control conditions, respectively, where as the phenotypic variation of grain yield explained by each main effect QTLs (M-QTL) ranged from 4.66 % to 19.32 % in the region 7H (87.11 cM) and 5H (137.79 cM), respectively (Table 3).These results are similar to those obtained by von Korff *et al*. (2006) and Ellis *et al*. (2002) found a major QTL for yield under normal field conditions. The HKT1;5 gene from ancestral wheat was used recently to produce salt-tolerant durum wheat which showed increased salt tolerance with yield increases of 25 % on saline soil (Munns *et al*., 2012).

Eight QTLs (Table 3) for straw weight have been identified in chromosomes 1H (67.88 cM) and 3H (66.5 cM) under control (Fig. 4 a), 2H (114.4 cM) under the three salt treatments (Fig. 4 b,c and d). Three QTLs detected in 5H at 36.1 cM under control, 125.18 cM under 3000 and 5000 ppm and one QTL at 140.7 cM under 1000 ppm salt stress treatment (Fig. 4 a,b,c and d), while two QTLs detected in 7H at 87.55 and 89.81 cM under 5000 and 1000 ppm (Fig. 4 b and d), respectively. The proportion of the genetic variance, which is explained by the marker main effect were ranged from 9.3 % to 16%, whereas the detected QTLs had positive main effects and led to improve this trait except the QTLs located on 1H (67.88 cM) and 7H (89.81 cM) under control and 1000 ppm conditions, respectively had the main negative effects (Table 3). Long *et al*. (2013) detected several QTLs affecting straw growth and related traits under both stress and non-stress conditions. Zarre & Jafary (2013) found QTL associated with shoot length on chromosome 2H (115-140 cM). In contrast, Nguyen *et al*. (2013) found a QTL at about 78 cM on 6H contributed to shoot dry weight under both saline and control conditions and root dry weight under stress conditions.

RWC and CC

Eight QTLs were detected for relative water content. One QTL were detected for chromosomes1H (133.12 cM), 2H (44.79 cM), 5H (45.58 cM) and 7H (127.4 cM) under control, 1000, 3000 and 5000 ppm, respectively (Fig. 5 a,b,c and d). Meanwhile two QTLs were detected for chromosomes 3H (1.48 and 140.29 cM) and 6H (84.81 and 98.71 cM) under 1000 in the first one and under control in the second chromosome (Fig. 5 a and b). The main effect of QTLs explained from 11.2% at region 3H (1.48 cM) to 21.9% at 3H (140.29) of the genetic variance. Furthermore the detected QTLs on chromosomes 1H (133.12 cM), 3H (34.18 cM) Led to increase the RWC under control. The QTLs in 5H, 7H led to improve RWC under 3000 and 5000 ppm conditions, respectively, while the other QTLs decreased RWC under stress conditions (Table 3). Chen *et al*. (2010) reported QTLs for RWC on 2H barley chromosome under drought conditions. Teulat *et al*. (2003) also found QTLs for RWC on 6H chromosome of barley under dry environment. These results show the influence of environment on this trait. That's why different QTLs were detected under different environments. Ahmad *et al*. (2014) found three QTLs; QRWC. uaf. 2A. 2 at 74 cM under normal condition, QRWC. uaf. 2A.3 and QRWC. uaf. 2A. 1 at 103 and 15 cM, respectively associated with RWC on chromosome 2A of

wheat under drought stress conditions. Marker traits associations detected for RWC will help to improve our understanding about the water relations in plant under abiotic stress.

Eleven QTLs shown in Table 3 were identified for chlorophyll content and mapped on 1H (two QTLs), 3H (four QTLs), 4H (two QTLs), 5H (one QTL) and 6H (two QTLs), which are represented in Fig. 6 (a,b,c and d). The QTL on 1H (17.82 cM) had positive effect under 5000 ppm treatment. The QTLs on 3H located at 7.28 and 3.16 cM had positive effect under control and the QTL at 3H (6.54 cM) had positive effect under 1000 ppm condition. The QTLs located on 4H (4.61 cM) and 6H (11.97 cM) had positive effect under 1000 ppm conditions. These results are close to those obtained by Long *et al*. (2013) where they detected five QTLs associated with chlorophyll contents on chromosomes 1H (31.cM), 5H (6.4 cM), 6H (45.4 and 60.2 cM) and 7H (4.9 cM). In addition, they reported that genomic region on chromosome 6H strongly influenced ST as well as chlorophyll content. The other detected QTLs in this study negatively affected this trait. The phenotypic variations explained by these QTLs, ranged from 4.65 in 4H (60.55 cM) and 12.71% of the total variation in chlorophyll content in 3H (68.01 cM).

Na^+ and K^+ ion content

Na^+ exclusion and K^+ retention are considered to be key mechanisms of plant tolerance to salinity (Shabala & Cuin, 2008). Although, the Shoot Na^+ toxicity is associated with a reduction of stomatal conductance (Tavakkoli *et al*., 2011), Na^+ contents is considered important factor for salt induced damage (Hasegawa *et al*., 2000; Munns & Testers, 2008 and Teakle & Tyerman, 2010) . This is due to the toxicity effects of these ions appear to be cumulative (Tavakkoli *et al*., 2011). Twelve QTLs represented in Table 3 were detected for Na^+ concentration and located on chromosomes 2H (3 QTLs at 77.41, 147.61 and 156.85 cM), 3H (4 QTLs at 35.93, 51.44, 87.77 and 128.64 cM), 6H (4 QTLs at 28.84, 70.57, 136.73 and 143.98 cM) and 7H (one QTL at 82.61 cM) (Fig. 7 a,b,c and d). The QTLs mapped on 2H (77.41 cM) and 7H (82.61 cM) had negative effect under 1000 ppm conditions, and the QTLs mapped on 6h (70.57 cM) and 3H (51.44 cM) had negative effects under control and 3000 ppm, respectively, while the other QTLs were positively affected under different salt stress conditions. The maximum and the minimum of the explained genetic variance were found at 70.57 cM on 6H (8.02%) and 156.85 cM on 2H (11.94%). Long *et al*. (2013) identified QTL associated with Na+ on chromosome 7H (83.4 cM). This is the same region which we detected 7H (82. 61 cM). Nguyen *et al*. (2013) identified 11 chromosomal regions involved in the control of the variations observed for salt tolerance and various salt stress response traits, including Na^+, Cl^- and K^+ contents in shoots. They also found a QTL controlling shoot Na^+ content mapped on chromosome 2H under stressed conditions with a high LOD value (9.82) and explained 23 % of total genotypic variance. A similar position to the recently identified HvNax3-a locus controlling shoot sodium exclusion derived from wild barley were detected by Shavrukov *et al*. (2010).

Twelve QTLs (Table 3 and Fig. 8 a,b,c,d) were identified for K^+ on chromosomes 1H, 2H, 3H, 7H (two each), 4H (one QTL) and 6H (three QTLs). The QTLs located on 1H at 12.96 and 141.29 cM led to increase this traits under 5000 and control, and on 2H (140.87 cM), 3H (167.34 cM), 6H (137.67 cM) and 7H (140.94 cM) led to increase the K^+ concentration under 3000, 5000, control and 1000 ppm, respectively. Furthermore, the latter QTL explained 25.67% of the genetic variance in 7H (125.4 cM), while the other QTLs had negative main effect. These results are in harmony with those obtained by Long *et al*. (2013) where they detected QTLs for Na^+, K^+ content on chromosome 4H and 7H. Shavrukov *et al*. (2010) detected a QTL for Na^+, K^+ and Na^+/K^+ ratio locates near the center of chromosome 7H and this might be related to the HvNax3 locus. In contrast, this region in our investigation has tiny effects comparing with the other regions. Nguyen *et al*. (2013) identified two QTL influencing shoot K^+ content under both control and stress conditions in the same region of chromosome 2H with total genotypic variance for K^+ content explained by these two QTL was 37.8 and 18.8 %, respectively.

ST%

Nine QTLs fore salt tolerance presented in Fig. 9 and Table 3 were identified on chromosomes 1H (three QTLs), 3H (one QTL), 5H (two QTLs), 6H (one QTLs) and 7H (two QTLs). The main effect of these QTLs explained from 5.21% at 3H (128.64 cM) under 1000 ppm conditions to 28.12 % of phenotypic variance at 1H (60.21 cM) under both of three salt stress conditions. The QTLs mapped on chromosomes 1H (60.21 and 67.88 cM), 5H (81.39 cM), 6H (28.84 cM) and 7H (82.61 cM) were detected for ST under both of three salt stress conditions. The QTL on 5H (125.18 cM) identified for ST led to improve ST under 3000 and 5000 ppm conditions. The QTL identified at 128.64 cM on chromosome 3H had positive effects under 1000 ppm condition , while the detected QTLs in 1H (48.95 cM) and 7H (87.11 cM) led to decrease ST under 3000 and 5000 ppm conditions, respectively. Similarly, the detected QTLs on 1H (60.21 cM) and 6H (28.84 cM) led to improve ST under both of salt stress conditions. Long *et al*. (2013) found two QTLs under saline conditions on chromosomes 3H (126.s cM) and 6H (60.2 cM) and they concluded that the strongest QTL for ST on chromosome 6H was consistently found in all models. Similar results were obtained by Zhou *et al*. (2012) where they found QTLs for salinity tolerance identified in the DH population of YYXT 9 Franklin at the vegetative stage were located on chromosomes 1H, 2H, 5H, 6H and 7H. Witzel *et al*. (2010) also reported a QTL on 5H for salinity tolerance at germination stage and the position is similar to the QTL identified in this study.

Conclusion

The current research successfully identified many marker trait associations under normal and salt stress conditions. Association mapping identified QTLs for ion content, salt tolerance and related traits over all barley genome. Twenty one QTLs out of them were detected under control and 40 QTLs were found under saline conditions. We presented several strong QTLs: the first QTL on

chromosome 7H controlling salt tolerance, which co-located for some traits such as ST, yield and straw weight: the second QTL was identified on 1H controlling salt tolerance and chlorophyll content and the third QTL was found on 2H, which controlling the shoot weight and Na^+ content. The genomic regions that harbor QTLs for Na^+, salt tolerance and related traits on chromosome 1H, 2H and 7H in our study can be used for targeting candidate gene(s) for salt tolerance of barley. These candidate genes can be identified and their function can be characterized in the future and finally directed into salt tolerance plant breeding program.

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تحديد مواقع الصفات الكمية المرتبطة بمقاومة الاجهاد الملحي بطريقة ال Association mapping

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اجريت هذه الدراسة بالصوبة السلكية بالمزرعة البحثية بكلية الزراعة – جامعة سوهاج خلال موسمى ٢٠١٢/٢٠١٣ ، ٢٠١٣/٢٠١٤ لتحديد مواقع الصفات الكمية المرتبطة بمقاومة الملحية فى عشيرة الشعير المركبة.

فى هذه الدراسة تم استخدام ١٢٢ تركيب وراثى (١٠٣ تراكيب من الشعير البرى ، ١٩ صنف من الشعير الربيعى المنزرع) ، تم زراعة بذور هذه العشيرة فى اطباق بترى على درجة حرارة ٤ درجات مئوية لمدة ٧ ايام داخل الثلاجة بعدها تم زراعة البذور المستنبتة فى صفوف داخل احواض خشبية ذات ابعاد ١٠٠ ، ١٢٠ ، ٣٠ سم عرض ، طول ، عمق على التوالى هذه الاحواض ملئت بتربة طينية قبل الزراعة ، هذه الاحواض تم ريها بماء الصنبور (Ec= 300ppm) لمدة ٣٠ يوما ، بعدها تم تطبيق ٤ معاملات (تركيزات مختلفة من الملحية بمياه الرى) ٣٠٠ ، ١٠٠٠ ، ٣٠٠٠ ، ٥٠٠٠ جزء بالمليون NaCl . تم تنفيذ التجربة فى تصميم القطع المنشقة باستخدام مكررتين ، تم تطبيق معاملات الملحية بالقطع الرئيسية و التراكيب بالقطع المنشقة.

تم تقدير المحتوى المائى RWC بعد مرحلة طرد السنابل من الورقة اسفل ورقة العلم، المحتوى الكلى من الكلوروفيل بعد مرحلة الطرد ، محصول الحبوب للنبات بالجرام ، محصول القش للنبات بالجرام، معامل مقاومة الملحية ثم تم تقدير محتوى الأوراق من البوتاسيوم K^+ ، الصوديوم Na^+ فى تحليل الـ Association mapping تم استخدام 660 DArT markers لتحديد مواقع الصفات الكمية للصفات المدروسة. فى هذا التحليل تم استخدام Mixed linear model ببرنامج TASSEL v.4.3 ودلت النتائج على:-

باستخدام هذه الطريقة تم تحديد 61 QTLs تحت الظروف العادية و ظروف الاجهاد الملحى المختلفة من بين هذه المواقع و جد ٢١ تحت الظروف العادية ، ٤٠ تحت ظروف الاجهاد المختلفة .

الدراسة اوضحت اهم المواقع لمقاومة الاجهاد الملحى على الكروموسومات 1H, 2H, 7H . اهمية هذه الدراسة هو امكانية تحديد الـ Candidate gens المرتبطة بمقاومة الاجهاد الملحى فى الشعير .