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Association mapping of morpho-physiological traits in barley (*Hordeum vulgare* L.) under salinity stress

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Abstract: In this study molecular markers associated with morpho-physiological traits were identified using 14 AFLP primer combinations and 32 SSRs primer pairs across a cohort of 148 barley cultivars employing the association mapping approach. Phenotypic analysis was carried out using an alpha-lattice design with five incomplete blocks replicated twice under normal and salinity stress conditions (EC = 12 dS m^{-1}) in two growing seasons. Population genetic structure was divided into two subpopulations (K = 2). In the present association panel, the mean of D´and r^2, indicators for linkage disequilibrium (LD) were estimated at 0.25 and 0.02, respectively. The mixed linear model identified 194 significant marker-trait associations for nine studied traits under normal and salinity stress conditions. Several quantitative trait loci (QTLs) were stable for plant height, number of grains per spike, grain weight per spike, and leaf proline content traits under each of the environmental conditions, and termed stable QTLs. In addition, some stable QTLs were common to several traits and thereby enable barley breeder to undertake a concurrent selection of multiple traits to develop high-yielding cultivars. The identified markers could be useful in the implementation of marker-assisted selection in barley to improve the efficiency of selecting genotypes for salinity tolerance.

Keywords: association mapping, barley, linkage disequilibrium, mixed linear model, salinity stress, stable QTLs.

Introduction

Barley (*Hordeum vulgare* L.) belongs to the cereal group of Gramineae family. It today ranks fourth in importance after wheat, rice, and corn. Barley serves as a model cereal for studying mechanisms of salinity tolerance due to its simpler genome than wheat and its notably higher salinity tolerance compared to wheat and rice (Gharaghanipor et al., 2022). Salinity stress poses a significant threat to agricultural production worldwide, exacerbated by climate change, salt intrusion into irrigation from surface and groundwater sources, and depletion of genetic resources (Arzani and Ashraf, 2016). Ellis et al. (2000) and Kilian et al. (2006) motioned that the new barley cultivars contain only 15-40% of the alleles in the barley gene pool. Thus, a part of barley's gene pool is tapped by breeders to improve salinity tolerance.

Salinity tolerance in crops is a quantitative trait with complex genetic and physiological architectures controlled by many gene loci (Flowers, 2004; Arzani, 2008; Omrani et al., 2022). With the advent of biotechnological tools such as molecular markers and transformation techniques, the science of plant breeding has evolved into a new realm (Arzani and Ashraf, 2016). The two most common methods for identifying and locating quantitative trait locus (QTL) are linkage mapping and association mapping (Flint - Garcia et al., 2005). In association mapping, QTL identification performs in a general population instead of a specific and segregating population (Zhu et al., 2008). It has advantages over linkage mapping, including examining more alleles and saving time and money because there is no need to create two-parent populations. Another advantage of association mapping is its high accuracy; due to many recombinations during the Ancestorl pedigree, genetic mapping has a high resolution and can easily use in marker-assisted selection (Flint-Garcia et al., 2003; Moose and Mumm, 2008). Therefore, this method avoids the disadvantages and limitations associated with linkage mapping. Association mapping does by the general linear model (GLM) and mixed linear model (MLM) methods. In the MLM method, population structure (Q-matrix) and kinship relationships between individuals (K-matrix) are predicted using several markers and used as a covariate in the model. Therefore, this method minimizes the results of false marker-trait associations. Fan et al. (2016) experiment showed that 206 barley genotypes with 408 markers were genotyped and tested for salinity stress tolerance. In their study, association analysis was performed by both GLM and MLM models based on population structure and kinship relationships. Finally, 24 markers that were highly associated with traits were identified.

Irrigated agricultural lands in arid and semi-arid regions contribute to the accumulation of soluble salts and exchangeable sodium in the soil where the roots grow (Arzani and Ashraf, 2016). Salinity imposes primary stresses such as osmotic stress and specific ion toxicity (predominantly from Na^+ and Cl^-); as well as secondary stresses like nutritional disorder and oxidative stress (Arzani, 2008). These stresses ultimately impair plant growth and development. Eleuch et al. (2008) experimented in two different environments (Egypt and India) to investigate the barley's genetic diversity and association analysis of salinity tolerance. Their study evaluated traits using 22 SSR markers and 48 barley genotypes. Their results showed that some QTLs were identified as responsible for salinity tolerance in each experimental environment, but only a small number of QTLs were identified in both environments. Also, Inostroza et al. (2009), El-Denary et al. (2012), Long et al. (2013), Sbei et al. (2014), Elakhdar et al. (2016a), and Elakhdar et al. (2016b) used association mapping under salinity stress in barley.

This study aimed to analyze the population structure of barley germplasm cultivars and investigate the relationship between AFLP and SSR markers and morpho-physiological traits of barley under salinity stress conditions. Breeding stress-tolerant barley cultivars is a complex and time-consuming activity. Therefore, introducing markers associated with these traits can facilitate marker-assisted selection in barley breeding programs.

Materials and Methods

Plant material (germplasm)

This study used 148 modern European two-row spring barley cultivars (Supplementary Table 1), representing commercial germplasm used all over

North and West Europe (Kraakman et al., 2006). The seeds of the association panel were received from the Khorasan Razavi Agricultural and Natural Resources Research and Education Center.

Phenotyping

An alpha-lattice design with five incomplete blocks replicated twice was used. Each block includes 30 plots in normal (water EC 2, soil EC 3.4 dS m^{-1}) and salinity stress (water EC 12, soil EC 14 dS m^{-1}) environments at the Agriculture and Natural Resources Research Station of Yazd (31° 55′ N, 54° 16′ E, 1213 m of sea level), Iran, for the two years. Salinity treatment was applied with water. The field soil in this experiment was naturally saline. Soil salinity was measured regularly during the growth period. The soil salinity was kept constant in each plot at the desired treatment level through the amount of water used and the need for soil leaching. The studied traits include Plant height (PH), Thousand-grain weight (TGW), Harvest index (HI), number of grains per spike (NGS), Grain weight per spike (GWS), number of total tillers (NTT), Relative water content (RWC), leaf proline content (LPC), and leaf chlorophyll content (LChC). The data normality test was first performed based on the Kolmogorov-Smirnov method using SPSS software. Then, a combined analysis of variance was performed with SAS 9.1 software.

Genotyping

In this study, a genetic map of molecular markers, including 407 AFLP and SSR markers, was prepared by Kraakman et al. (2006), and Aghnoum et al. (Unpublished data) were used.

(Kraakman et al., 2006) used 14 AFLP primers (E33M54, E35M48, E35M54, E35M55, E35M61, E37M33, E38M50, E38M54, E38M55, E39M61, E42M32, E42M48, E45M49, and E45M55) for genotyping and identified 286 polymorphic markers. Then, in 2006, 11 SSR primers (Bmac0018, Bmag0009, HVM14, HVM22, HVM65, HVM74, Bmag0223, Bmac0134, HVM54, Bmac0163, and Bmac0316) were added to the genotyping map (Kraakman et al., 2006). Also, Aghnoum et al. (Unpublished data) mapped 21 SSR molecular markers (EBmac0603, GBMS035, HVM36, scssr10559, Bmag0225, Bmag0841, Bmag0606, Bmag0013, HVM40, GBM1482, GBM1015, GBMS062, Bmac0399, EBmac0560, HvHVA1, Bmag0500, GBM1021, Bmag0173, scssr07106, Bmag0357, and Bmag0222) in this population. Finally, in total, and considering all the alleles of AFLP and SSR markers, 407 polymorphic markers were used in the present population. In this study, the sites of mapped QTLs were obtained from an integrated barley genetic map consisting of 6990 molecular markers (Aghnoum et al., 2010). This integrated genetic map included 7 linkage groups, and the molecular markers density was 0.125 markers per cM.

Population structure (Q-matrix) and kinship relationships (K-matrix)

In association analysis studies using natural populations, it is important to avoid population structure, as its presence can hinder the attainment of reliable results. Therefore, if the effect of population structure and kinship relationships is not considered to determine the trait-marker associations in association mapping, LD increases. As a result, false-positive results occur, leading to false marker-trait associations (Breseghello and Sorrells, 2006; Yu and Buckler, 2006; Zhang et al., 2012). Therefore, to determine the population structure (Q-matrix), the Bayesian method and Structure 2.3.4 software (Pritchard et al., 2000; Falush et al., 2003) were used on genotypic data. This analysis was performed on 148 barley genotypes in the Admixture model. The length of the Burnin period was 100,000, and the number of Markov Chain Monte Carlo (MCMC) replications was 100,000. Set K from 1 to 10, and the number of iterations 10 was considered. The optimal K was determined based on the delta K method. Finally, the Q-matrix was calculated with the same software by determining the optimal K, related to the highest value of delta K. Also, using genotypic data, the kinship relationships (K-matrix) were determined by TASSEL4.3.15 software.

LD and association analysis

Associations mapping was used to identify the markers related to the studied traits under normal and salinity stress conditions. For this purpose, LD for each pair of markers was estimated by the r2 statistic for each linkage group and D′statistic with LD plot by TASSEL 4.3.15 and TASSEL 2.1 software [5]. Marker-trait associations were performed using MLM with TASSEL 4.3.15 software. In the MLM

method, in addition to genotypic data, phenotypic data, and population structure (Q-matrix), kinship relationships (K-matrix) were also used as covariates in the model (Yu et al., 2006). In association analysis, just markers with a frequency of more than 10% were used, and the p-value with 1000 permutations was estimated. Also, the selection basis of the associated marker was the existence of the lowest P-value. The distribution of markers was examined based on the determination coefficient of marker (R2) in the regression model, that R2 is the ratio of calculated phenotypic variance for QTL in each location. Finally, MapChart software was used to show the mapped gene loci.

Results

Analysis of variance

A combined analysis of variance revealed high levels of genetic variability among genotypes across all traits except for harvest index and relative water content, indicating variations among genotypes in the environment. (Supplementary Table 2). The effect of the environment was significant on all of the studied traits. Also, the year effect was significant on all traits except the number of grains per spike and grain weight per spike. The environment × year, environment × genotype, year× genotype, and environment × year× genotype were significant for some traits. G × E interaction usually affects the efficiency of phenotypic selection in breeding programs (Sallam et al., 2019).

Population structure

This study determined the population's genetic structure by the Bayesian method. This method attributes each genotype to hypothetical subpopulations with a probability that in each subpopulation, the linkage disequilibrium is minimum and the gamete equilibrium is maximum. According to Supplementary Table 3 and Figure 1, the K = 2, which corresponds to the highest value of Delta K, was determined as the optimum K. Therefore, it is the most appropriate number to use for calculating the Q-matrix. Finally, the Q-matrix was obtained by placing K = 2 in the Structure 2.3.4 software.

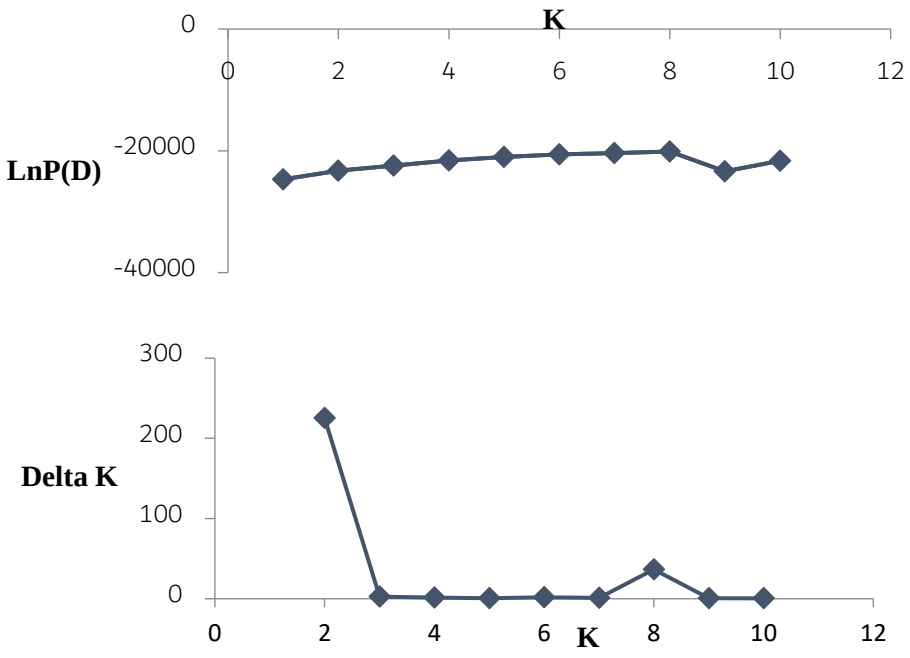

Figure 1. The two-way graphs to determine the optimum K value using 2.3.4 Structure software.

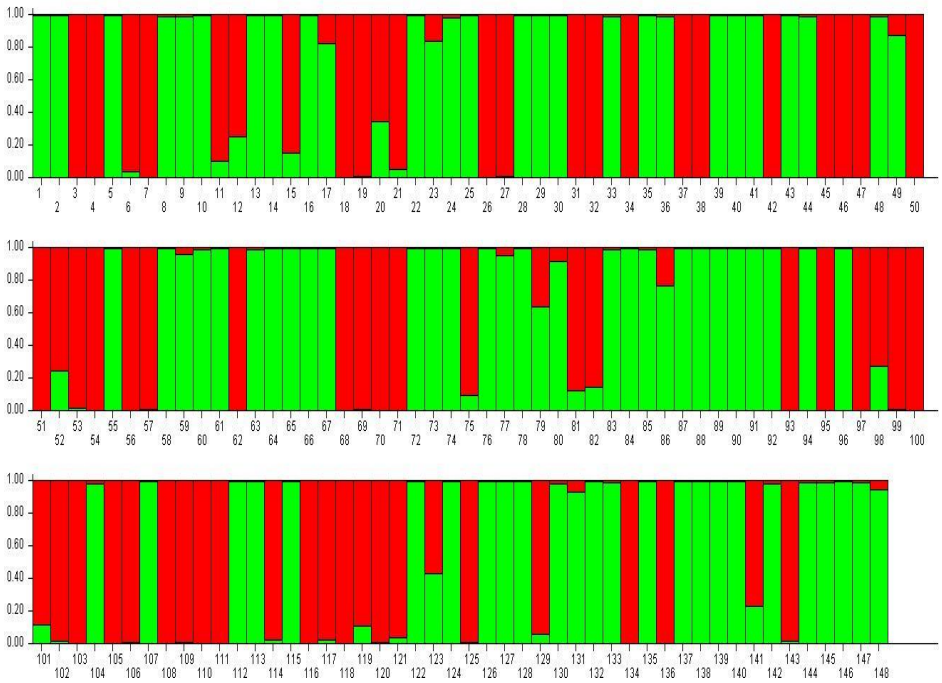

Figure 2. Bar plot generated using 407 AFLP and SSR markers by Structure 2.3.4 software. The horizontal axis represents genotypes, while the vertical axis shows the share of each genotype in each group.

The bar plot provided by Structure 2.3.4 software for 148 barley genotypes (Figure 2) also confirms the optimum K value. The horizontal axis is related to genotypes, and the vertical axis shows the share of each genotype in each group. In this bar plot, when the percentage of genotype membership in one cluster is more than or equal to 0.7, the genotype is assigned to that cluster. If the membership percentage is less than this value, it is considered a mixed genotype (Spataro et al., 2011). Here, each group is marked with a distinct colour that two separate colours for each genotype indicate that the genotype belongs to one of the two groups or both groups. Then, the number of clusters that better represent the population structure (kinship relationships defined by the K-matrix) was determined by TASSEL4.3.15 software for use in the MLM method.

LD and association mapping

LD associated with each pair of markers was estimated by D′statistic shown in the LD plot (Figure 3) and the r^2 statistic for each linkage group. The average D′ was 0.25 and the average r^2 was 0.02. The upper part of the diameter indicates the linkage disequilibrium using the D′ statistic, and the lower part of the diameter indicates the P-value for the pair of markers. The presence of red colour in the P-value study indicates the high statistical probability of LD, and green, blue and white are at lower levels of LD statistical probability, respectively. This study used MLM by association analysis to identify associated markers with the studied traits. The results showed that 194 significant marker-trait associations ($P<0.001$) were observed under normal and salinity stress conditions (Tables 1, 2, 3, 4).

Thirty-seven DNA markers were found to be significantly associated with PH, from which 33 markers were associated with the trait in normal conditions, and 4 markers were associated with the trait in salinity stress conditions (Table 1). Two DNA markers were identified for TGW, from which 1 marker was associated with the trait in normal conditions and 1 marker associated with the trait in salinity stress conditions (Table 1). Also, 2 DNA markers were found to be significantly associated with HI; 1 associated with the trait under normal conditions and 1 marker associated with the trait under salinity stress conditions (Table 1).

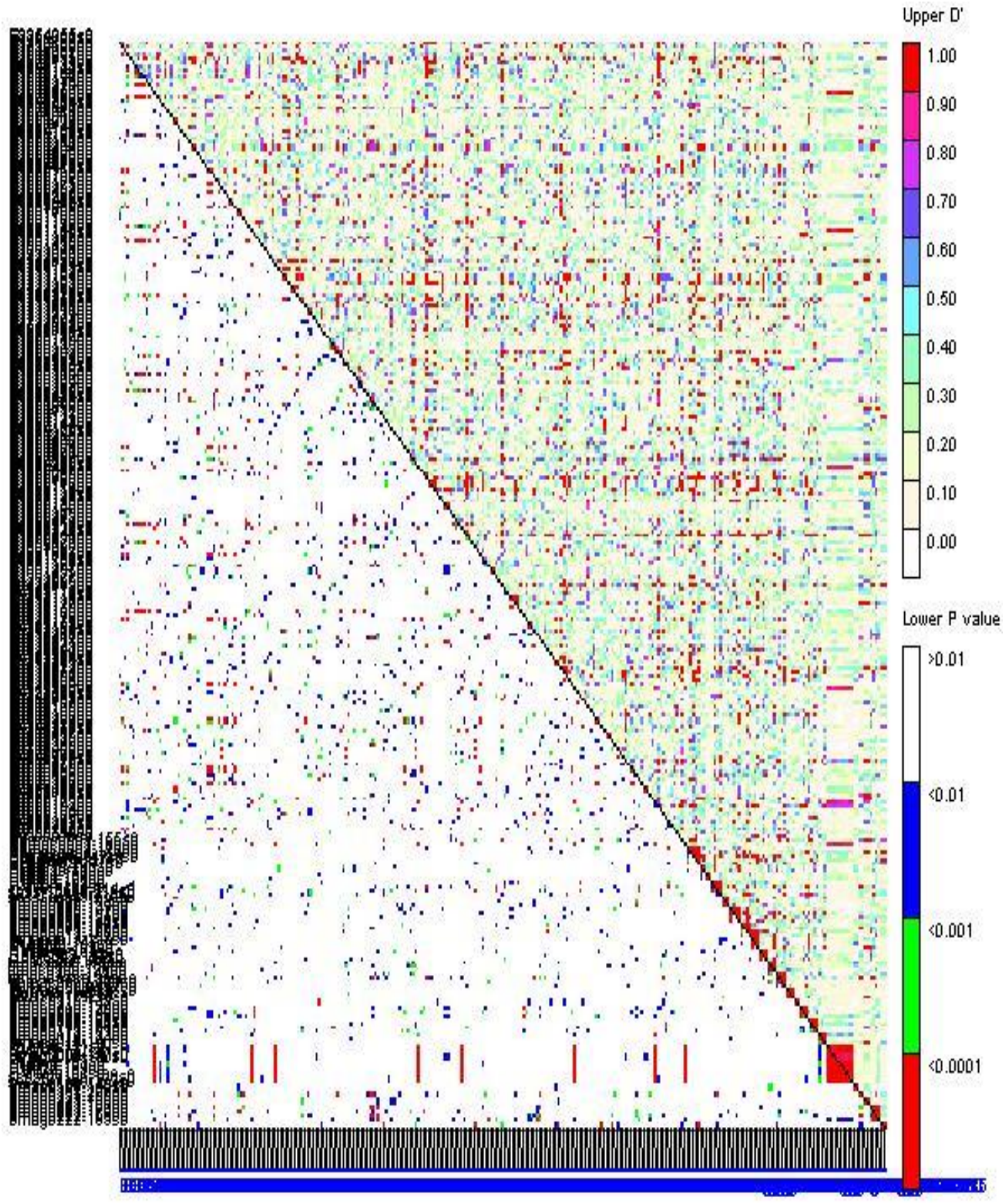

Figure 3. LD plot of barley genotypes generated using TASSEL 2.1 software. The upper part of the diameter represents the linkage disequilibrium using the D′ statistic, while the lower part of the diameter represents the corresponding *P*-value for each pair of markers.

Table 1. Markers associated with PH, TGW, HI, and NTT in barley genotypes under normal and salinity stress conditions, using the MLM model.

Trait	Conditions	Year	Marker	R^2	P-value	Position (cM)	chromosome
PH	Normal	1	EBmac0603-157	0.10	0.0007	38.3	7H
			EBmac0603-183	0.10	0.001	38.3	7H
			EBmac0603-178	0.11	0.0007	38.3	7H
			GBMS035-147	0.16	0.00002	49	7H
			GBMS035-137	0.16	0.00002	49	7H
			Bmag0606-151	0.12	0.0003	112.5	3H
			Bmag0606-138	0.11	0.0005	112.5	3H
			Bmag0606-126	0.11	0.00046	112.5	3H
			Bmag0606-147	0.14	0.00006	112.5	3H
			Bmag0606-118	0.11	0.0004	112.5	3H
			Bmag0606-122	0.11	0.00037	112.5	3H
			Bmag0606-269	0.11	0.0001	112.5	3H
			HVM40-144	0.20	0.000002	32.3	4H
			HVM40-147	0.20	0.000001	32.3	4H
			HVM40-152	0.20	0.000002	32.3	4H
			HVM40-162	0.21	0.000001	32.3	4H
			Bmag0500-110	0.10	0.0009	29.2	6H
			Bmag0500-146	0.11	0.0005	29.2	6H
			Bmag0500-166	0.10	0.001	29.2	6H
			Bmag0500-181	0.10	0.001	29.2	6H
			Bmag0500-192	0.10	0.0009	29.2	6H
			Bmag0500-194	0.10	0.00085	29.2	6H
			scssr07106-168	0.15	0.00004	23.9	5H
			scssr07106-172	0.15	0.00004	23.9	5H
			E42M48-087	0.11	0.0005	-	unmapped
		2	EBmac0603-183	0.11	0.0007	38.3	7H
			EBmac0603-143	0.10	0.001	38.3	7H
			GBMS035-147	0.11	0.0005	49	7H
			GBMS035-137	0.13	0.00018	49	7H
			HVM40-144	0.13	0.00016	32.3	4H
			HVM40-147	0.14	0.00006	32.3	4H
			HVM40-152	0.13	0.00013	32.3	4H
			HVM40-162	0.14	0.00006	32.3	4H
	Salinity	1	-	-	-	-	-
		2	HVM40-144	0.16	0.00002	32.3	4H
			HVM40-147	0.16	0.00002	32.3	4H
			HVM40-152	0.16	0.00002	32.3	4H
			HVM40-162	0.17	0.00001	32.3	4H
TGW	Normal	1	E33M54-230	0.10	0.001	131	2H
		2	-	-	-	-	-
	Salinity	1	-	-	-	-	-
		2	E45M55-103	0.12	0.00026	-	unmapped
HI	Normal	1					
		2	E33M54-214	0.54	0.000000	83.4	7H
	Salinity	1					
		2	Bmag0606-118	0.1	0.000879	112.5	3H
NTT	Normal	1	-	-	-	-	-
		2	E35M55-434	0.10	0.00085	-	unmapped
	Salinity	1	E35M54-265	0.11	0.0004	-	unmapped
			E35M61-162	0.11	0.0005	-	unmapped
			E45M55-262	0.11	0.0005	61.2	6H
		2	E35M55-434	0.11	0.0005	-	unmapped

PH: Plant height, TGW: Thousand-grain weight, HI: Harvest index, NTT: Number of total tillers, R2: Coefficient of determination, cM: Centimorgan.

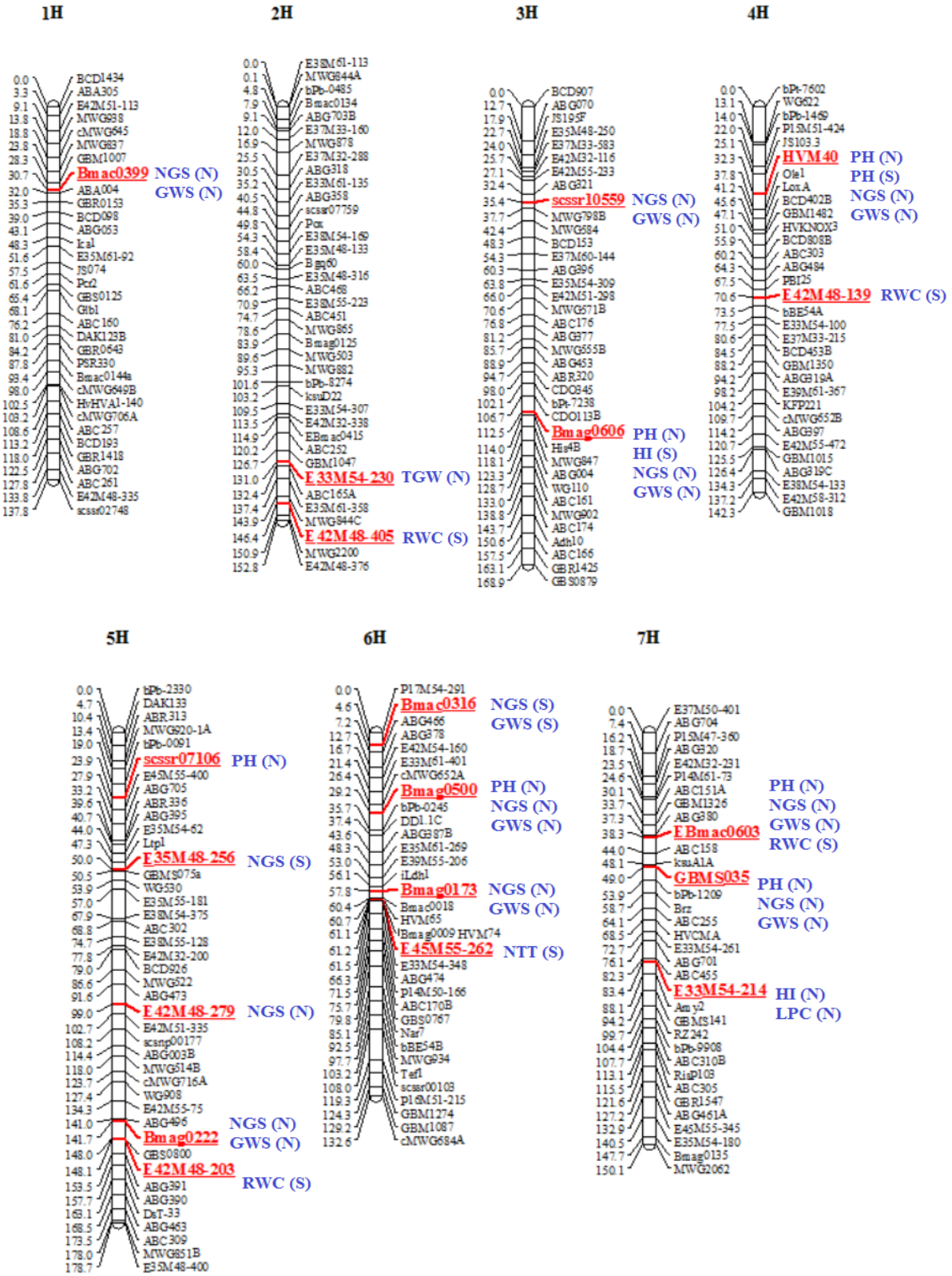

Figure 4. The genetic map of SSR and AFLP markers and genomic location of significant associated markers with studied traits in the barley (refer to Materials and Methods for the abbreviation of the traits used here; S: salinity stress, N: normal).

Table 2. Markers associated with NGS in barley genotypes using the MLM model under normal and salinity stress conditions.

Trait	Conditions	Year	Marker	R^2	P-value	Position (cM)	chromosome
NGS	Normal	1	EBmac0603-155	0.13	0.00018	38.3	7H
			EBmac0603-180	0.13	0.00018	38.3	7H
			EBmac0603-157	0.12	0.00019	38.3	7H
			EBmac0603-159	0.12	0.00019	38.3	7H
			EBmac0603-170	0.13	0.00018	38.3	7H
			EBmac0603-183	0.13	0.00014	38.3	7H
			EBmac0603-143	0.12	0.00019	38.3	7H
			EBmac0603-178	0.12	0.00019	38.3	7H
			EBmac0603-153	0.13	0.00011	38.3	7H
			GBMS035-147	0.13	0.00014	49	H
			GBMS035-137	0.13	0.00012	49	7H
			scssr10559-210	0.10	0.00071	35.4	3H
			Bmag0606-151	0.15	0.00005	112.5	3H
			Bmag0606-138	0.15	0.00005	112.5	3H
			Bmag0606-126	0.15	0.00005	112.5	3H
			Bmag0606-147	0.16	0.00002	112.5	3H
			Bmag0606-118	0.15	0.00005	112.5	3H
			Bmag0606-122	0.15	0.00004	112.5	3H
			Bmag0606-269	0.15	0.00001	112.5	3H
			HVM40-144	0.14	0.00005	32.3	4H
			HVM40-147	0.14	0.00006	32.3	4H
			HVM40-152	0.14	0.00006	32.3	4H
			HVM40-162	0.15	0.00005	32.3	4H
			Bmac0399-130	0.10	0.00077	30.7	1H
			Bmac0399-138	0.11	0.00051	30.7	1H
			Bmac0399-152	0.10	0.00099	30.7	1H
			Bmag0500-110	0.11	0.00058	29.2	6H
			Bmag0500-146	0.11	0.0006	29.2	6H
			Bmag0500-166	0.11	0.00052	29.2	6H
			Bmag0500-181	0.11	0.00047	29.2	6H
			Bmag0500-192	0.11	0.00048	29.2	6H
			Bmag0500-194	0.11	0.00059	29.2	6H
			Bmag0173-153	0.10	0.00071	57.79	6H
			Bmag0173-156	0.14	0.00009	57.79	6H
			Bmag0222-153	0.11	0.00054	141.7	5H
			Bmag0222-185	0.11	0.00054	141.7	5H
		2	E42M48-087	0.11	0.00063	-	unmapped
			E42M48-279	0.10	0.00096	99	5H
			EBmac0603-155	0.11	0.00053	38.3	7H
			EBmac0603-180	0.11	0.00052	38.3	7H
			EBmac0603-157	0.11	0.00044	38.3	7H
			EBmac0603-159	0.11	0.00055	38.3	7H
			EBmac0603-170	0.11	0.00055	38.3	7H
			EBmac0603-183	0.11	0.00045	38.3	7H
			EBmac0603-143	0.11	0.00046	38.3	7H
			EBmac0603-178	0.11	0.00054	38.3	7H
			EBmac0603-153	0.11	0.00053	38.3	7H
			GBMS035-147	0.13	0.00014	49	7H
			GBMS035-137	0.13	0.00015	49	7H
			Bmag0606-151	0.14	0.00007	112.5	3H
			Bmag0606-138	0.14	0.00007	112.5	3H
			Bmag0606-126	0.15	0.00004	112.5	3H
			Bmag0606-147	0.17	0.00001	112.5	3H
			Bmag0606-118	0.14	0.00007	112.5	3H
			Bmag0606-122	0.14	0.00007	112.5	3H
			Bmag0606-269	0.14	0.00001	112.5	3H
			HVM40-144	0.20	0.000002	32.3	4H
			HVM40-147	0.19	0.000002	32.3	4H
			HVM40-152	0.20	0.000002	32.3	4H
			HVM40-162	0.20	0.000002	32.3	4H
			Bmac0399-138	0.10	0.00094	30.7	1H
			Bmac0399-143	0.10	0.00071	30.7	1H
			Bmag0500-110	0.11	0.00045	29.2	6H
			Bmag0500-146	0.11	0.00055	29.2	6H
			Bmag0500-166	0.11	0.00058	29.2	6H
			Bmag0500-181	0.11	0.00045	29.2	6H
			Bmag0500-192	0.11	0.0006	29.2	6H
			Bmag0500-194	0.11	0.0006	29.2	6H
			Bmag0173-156	0.11	0.00052	57.79	6H
	Salinity	1	E35M48-256	0.12	0.001	50	5H
			E35M48-408	0.12	0.0009	-	unmapped
		2	Bmac0316-168	0.18	0.00001	4.6	6H

NGS: Number of grains per spike, R2: Coefficient of determination, cM: Centimorgan.

Table 3. Markers associated with GWS in barley genotypes using the MLM model under normal and salinity stress conditions.

Trait	Conditions	Year	Marker	R^2	P-value	Position (cM)	chromosome
GWS	N	1	E42M48-087	0.10	0.00081	-	unmapped
			EBmac0603-155	0.12	0.00021	38.3	7H
			EBmac0603-180	0.12	0.00021	38.3	7H
			EBmac0603-157	0.12	0.00021	38.3	7H
			EBmac0603-159	0.13	0.00017	38.3	7H
			EBmac0603-170	0.12	0.00021	38.3	7H
			EBmac0603-183	0.13	0.00017	38.3	7H
			EBmac0603-143	0.12	0.00022	38.3	7H
			EBmac0603-178	0.12	0.00021	38.3	7H
			EBmac0603-153	0.13	0.00016	38.3	7H
			GBMS035-147	0.14	0.0001	49	7H
			GBMS035-137	0.14	0.0001	49	7H
			scssr10559-214	0.10	0.00071	35.4	3H
			scssr10559-213	0.11	0.0006	35.4	3H
			scssr10559-216	0.12	0.00034	35.4	3H
			scssr10559-210	0.11	0.0006	35.4	3H
			Bmag0606-151	0.12	0.00019	112.5	3H
			Bmag0606-138	0.12	0.00021	112.5	3H
			Bmag0606-126	0.12	0.00025	112.5	3H
			Bmag0606-147	0.15	0.00004	112.5	3H
			Bmag0606-118	0.12	0.00021	112.5	3H
			Bmag0606-122	0.12	0.00025	112.5	3H
			Bmag0606-269	0.12	0.00005	112.5	3H
			HVM40-144	0.17	0.000013	32.3	4H
			HVM40-147	0.17	0.000013	32.3	4H
			HVM40-152	0.17	0.00001	32.3	4H
			HVM40-162	0.17	0.000013	32.3	4H
			Bmac0399-138	0.11	0.00056	30.7	1H
			Bmag0500-110	0.11	0.00047	29.2	6H
			Bmag0500-146	0.11	0.00052	29.2	6H
			Bmag0500-166	0.12	0.00034	29.2	6H
			Bmag0500-181	0.11	0.00048	29.2	6H
			Bmag0500-192	0.11	0.00052	29.2	6H
			Bmag0500-194	0.11	0.00051	29.2	6H
			Bmag0173-156	0.12	0.0002	57.79	6H
			Bmag0222-153	0.10	0.001	141.7	5H
			Bmag0222-185	0.10	0.001	141.7	5H
		2	E42M48-087	0.11	0.00037	-	unmapped
			GBMS035-147	0.12	0.00025	49	7H
			GBMS035-137	0.11	0.0005	49	7H
			scssr10559-213	0.10	0.00086	35.4	3H
			scssr10559-216	0.11	0.00039	35.4	3H
			Bmag0606-151	0.13	0.0001	112.5	3H
			Bmag0606-138	0.13	0.0001	112.5	3H
			Bmag0606-126	0.15	0.00003	112.5	3H
			Bmag0606-147	0.16	0.00002	112.5	3H
			Bmag0606-118	0.13	0.0001	112.5	3H
			Bmag0606-122	0.13	0.00009	112.5	3H
			Bmag0606-269	0.13	0.00002	112.5	3H
			HVM40-144	0.18	0.00001	32.3	4H
			HVM40-147	0.17	0.00001	32.3	4H
			HVM40-152	0.17	0.00001	32.3	4H
			HVM40-162	0.17	0.00001	32.3	4H
			Bmac0399-143	0.11	0.0006	30.7	1H
			Bmag0500-110	0.11	0.00047	29.2	6H
			Bmag0500-146	0.11	0.00061	29.2	6H
			Bmag0500-166	0.11	0.00057	29.2	6H
			Bmag0500-181	0.11	0.00061	29.2	6H
			Bmag0500-192	0.11	0.00051	29.2	6H
			Bmag0500-194	0.11	0.00057	29.2	6H
			Bmag0173-156	0.10	0.00074	57.79	6H
			Bmag0222-153	0.10	0.00079	141.7	5H
			Bmag0222-185	0.10	0.00079	141.7	5H
	S	1	-	-	-	-	-
		2	Bmac0316-168	0.20	0.000002	4.6	6H

GWS: Grain weight per spike, R2: Coefficient of determination, cM: Centimorgan, N: Normal, S: Salinity stress.

Table 4. Markers associated with RWC, LPC, and LChC in barley genotypes based on the MLM model under normal and salinity stress conditions.

Trait	Conditions	Year	Marker	R^2	P-value	Position (cM)	chromosome
RWC	Normal	1	-	-	-	-	-
		2	E38M54-091	0.08	0.0009	-	unmapped
			E45M49-339	0.11	0.00032	-	unmapped
	Salinity	1	-	-	-	-	-
		2	E35M48-111	0.13	0.00018	-	unmapped
			E42M48-139	0.15	0.00003	70.6	4H
			E42M48-195	0.1	0.00069	-	unmapped
			E42M48-196	0.1	0.00076	-	unmapped
			E42M48-203	0.1	0.00072	148.1	5H
			E42M48-405	0.15	0.00004	146.4	2H
			EBmac0603-178	0.11	0.00053	38.3	7H
LPC	Normal	1	E33M54-214	0.15	0.00003	83.4	7H
		2	E33M54-214	0.15	0.00003	83.4	7H
	Salinity	1	-	-	-	-	-
		2	-	-	-	-	-
LChC	Normal	1	E35M48-251	0.09	0.00037	-	unmapped
		2	-	-	-	-	-
	Salinity	1	-	-	-	-	-
		2	-	-	-	-	-

RWC: Relative water content, LPC: Leaf Proline content, LChC: Leaf chlorophyll content, R2: Coefficient of determination, cM: Centimorgan.

Five markers were found to be significantly associated with NTT, of which 1 marker was associated with the trait in normal conditions, while the remaining 4 markers were associated with the trait under salinity stress conditions (Table 1). Seventy-two markers were identified for NGS, from which 69 markers were linked with the trait under normal conditions, and 3 markers were associated with the trait under salinity stress conditions (Table 2). For the GWS trait, 64 DNA markers were identified, from which 63 markers were associated with the trait in normal conditions and 1 marker associated with the trait under salinity stress conditions (Table 3). Nine DNA markers were identified for RWC. Two markers were associated with this trait under normal conditions, and seven were associated under salinity stress conditions (Table 4). Totally 2 DNA markers were identified for LPC, associated with the trait in normal conditions (Table 4). For LChC, only one marker was detected in the normal conditions (Table 4). The genetic map of SSR and AFLP markers and genomic location of significant markers with studied traits showed in figure 4.

Discussion

ANOVA revealed significant genetic variation among genotypes across all traits except harvest index and relative water content, suggesting distinctions among genotypes within the environment. The environment × year, environment × genotype, year× genotype, and environment × year× genotype were significant for some traits. G × E interaction usually affects the efficiency of phenotypic selection in breeding programs [38].

In association mapping, false-positive results obtain if the population structure and kinship relatedness are not considered (Breseghello and Sorrells, 2006). Hence, estimating population structure as a prerequisite in association mapping can prevent false-positive associations between markers and traits (Pritchard and Donnelly, 2001). This study subdivided barley cultivars into two subpopulations. Some reports suggest that the

population structure of barley cultivars is related to spike morphology (two-rowed versus six-rowed cultivars) (Pasam et al., 2012). In the association mapping method, QTLs are located based on LD (Gupta et al., 2005). In the present association panel, the mean of D´and r^2, indicators for LD, were 0.25 and 0.02, respectively. According to the LD plot, LD had a significant difference between barley chromosomes, which indicates that this factor can affect the accuracy of association mapping of identified QTLs on different chromosomes.

Several studies have previously reported different rates of LD in different barley populations (Caldwell et al., 2006; Ramsay et al., 2011) and among different chromosomes Rostoks et al. (2006). Caldwell et al. (2006) reported rapid decay of LD in barley landraces compared to superior barley cultivars. Eleuch et al. (2008), Inostroza et al. (2009), El-Denary et al. (2012), Long et al. (2013), Sbei et al. (2014), Elakhdar et al. (2016a), Elakhdar et al. (2016b) and Fan et al. (2016) used association mapping under salinity stress in the barley. This study identified 194 significant marker-trait associations for nine studied morphophysiological traits under normal and salinity stress conditions.

This study detected 33 and 4 significant marker-trait associations for PH in normal and salinity stress conditions, respectively. Seven QTLs on chromosomes 3H (112.5 cM), 8 QTLs on 4H (32.3 cM), 2 QTLs on 5H (23.9 cM), 6 QTLs on 6H (29.2 cM), 5 QTLs on 7H (38.3 cM), 4 QTLs on 7H (49 cM), 1 QTL with unknown gene location in the normal experiment and 4 QTLs on chromosome 4H (32.3 cM) under salinity stress conditions were observed for PH. Elakhdar et al. (2016a), in a study on barley for mean normal and salinity stress conditions, showed that this trait had a significant association with marker EBmac0603 on chromosome 7H at 35.39 cM position, which is similar to our results. Sayed et al. (2021) reported PH on chromosome 7H, Long et al. (2013) on chromosomes 2H (59.2 cM), 6H (60.2 cM) and 7H (4.9 cM) and 7H (61.3 cM), Eleuch et al. (2008) on 1H (62 cM) and 6H (10 cM), Inostroza et al. (2009) on 2H (5, 50, and 44 cM), 4H (78 and 118 cM), 5H (66 and 126 cM), 6H (79), and 7H (80, 85 and 107 cM), El-Denary et al. (2012) on 2H, Xue et al. (2009) on 3H, Saade et al. (2020) on 6H (51.93), under salinity stress conditions in the barley. Xu et al. (2012) detected this trait on chromosome 7H under normal conditions in the barley, which is consistent with our results.This study found 1 QTL for TGW on chromosome 2H (131 cM) in normal conditions and 1 QTL with unknown gene location in salinity stress conditions. Elakhdar et al. (2016a) identified this trait on chromosomes 6H (75.42 cM), 6H (7.16 cM), and 1H (30.81 cM) for mean the normal and salinity conditions in the barley. Wang et al. (2016) observed TGW on 2H, 5H, and 7H in the barley under normal conditions.

In the present study, one QTL was identified for HI on chromosome 7H at 83.4 cM under normal conditions and 1 QTL on chromosome 3H at 112.5 cM under salinity stress conditions. The marker E33M54-214 on chromosome 7H (83.4 cM) has a high coefficient of determination (R^2 = 0.54) with QTL controlling the HI, indicating a strong association between the marker and the trait. Under salinity stress conditions in the barley, Elakhdar et al. (2016a) on 2H and 5H and Saade et al. (2020) on 7H at 28.46 cM reported this trait.

According to the results, 1 QTL on chromosome 6H (61.2 cM), 3 QTLs with unknown gene location in salinity stress conditions, and 1 QTL with unknown gene location in normal conditions were detected for NTT. Long et al. (2013), under salinity stress conditions in the barley, found this trait on chromosomes 4H, 6H, and 7H, which were located in the positions of 79.6, 60.2, and 54.4 cM, respectively. As can be seen, our results' position of 61.2 cM is almost close to 60.2 cM in Long et al. (2013), so NTT is probably located in this gene locus. Xue et al. (2009), in a study on barley under both normal and salinity stress conditions, identified NTT on chromosome 4H, which was located in the positions of 72 cM. Long et al. (2013), under salinity stress conditions in the barley, observed this trait on chromosomes 4H, 6H, and 7H, which were located in the positions of 79.6, 60.2, and 54.4 cM, respectively.

NGS, one of the important yield components, has a major effect on the final yield. This study detected 69 and 3, a significant marker-trait association for NGS under normal conditions and salinity stress. 5 QTLs on chromosomes 1H (30.7 cM), 14 QTLs on 3H (112.5 cM), 1 QTL on 3H (35.4 cM), 8 QTLs on 4H (32.3 cM), 2 QTLs on 5H (141.7 cM), 1 QTL on 5H (99 cM), 12 QTLs on 6H (29.2 cM), 3 QTLs on 6H (57.79 cM), 18 QTLs on 7H (38.3 cM), 4 QTLs on 7H

(49 cM), and 1 QTL with unknown gene location in normal conditions were identified for NGS. Under salinity stress conditions, 1 QTL on 5H (50 cM), 1 QTL on 6H (4.6 cM), and 1 QTL with unknown gene location were observed for this trait. Xue et al. (2009), in a study on barley under both normal and salinity stress conditions, reported NGS on chromosome 2H. Elakhdar et al. (2016a) on chromosomes 1H (64.84 cM), 2H (89.83 cM), 4H (96.17 cM), 6H (7.16 cM), 7H (81.78 cM), 7H (97 cM) and Saade et al. (2020) on chromosome 7H at 128.35 cM observed this trait under salinity stress conditions in the barley. Also, Sun et al. (2011) detected NGS on chromosomes 1H, 4H, and 5H under normal conditions in the barley.

This study found for GWS 2 QTLs on chromosomes 1H (30.7 cM), 6 QTLs on 3H (35.4 cM), 14 QTL on 3H (112.5 cM), 8 QTLs on 4H (32.3 cM), 4 QTLs on 5H (141.7 cM), 12 QTLs on 6H (29.2 cM), 2 QTLs on 6H (57.79 cM), 9 QTLs on 7H (38.3 cM), 4 QTLs on 7H (49 cM), and 2 QTL with unknown gene location in normal conditions. Under salinity conditions, 1 QTL on chromosome 6H (4.6 cM) was identified with the marker for the GWS.

In the present study, 1 QTL on chromosomes 2H at 146.4 cM, 1 QTL on 4H at 70.6 cM, 1 QTL on 5H at 148.1 cM, 1 QTL on 7H at 38.3 cM, and 3 QTLs with unknown gene location was detected for RWC in salinity stress conditions. Under normal conditions, 2 QTL with unknown gene location was observed with the marker for this trait. Liu et al. (2015) on chromosomes 6H (57.8 cM), 6H (53.8 cM), and 7H (62.3 cM) reported RWC under salinity stress conditions in the barley. Mohamed et al. (2015) identified QTLs for this trait in barley under the normal conditions on chromosomes 1H, 3H, and 6H and QTLs for the trait under salt stress conditions on chromosomes 2H, 3H, 5H, 7H, and 6H. Also, Jabbari et al. (2021) observed this trait on chromosomes 2H and 7H under normal conditions.

According to the results, 2 QTLs were identified for LPC on Chromosome 7H (83.4 cM) in normal conditions. Under salinity stress conditions, no significant association was observed with the marker for the LPC. Jabbari et al. (2021), under normal conditions in the barley, detected LPC on chromosomes 2H, 4H, 5H, 6H, and 7H.

Abundant nutrition production is essential to sustain crop growth, which depends on the LChC (Yap and Harvey, 1972; Liu et al., 2015). This study found one QTL with an unknown gene location for LChC in normal conditions. Under salinity stress conditions, no significant association was observed with the markers for this trait. Elakhdar et al. (2016a) on 1H (64.84 cM), 1H (54.6 cM), 4H (58.6 cM), and 4H (96.17 cM), Elakhdar et al. (2016b) on 1H, 4H, Long et al. (2013), on 1H (31.1 cM), 5H (6.4 cM), 6H (45.4 cM), 6H (60.2 cM), 7H (4.9 cM), Liu et al. (2015) on chromosomes 2H (75.9 cM), 7H (47.5 cM), and 7H (58.9 cM) identified this trait under salinity stress conditions in the barley. Barati et al. (2017) reported two and four QTLs for LChC in barley under normal and stress conditions on chromosomes 3H, 4H, 5H, and 6H. Jabbari et al. (2021) observed QTLs for this trait on chromosomes 1H, 2H, 3H, and 4H under normal conditions.

Some identified DNA markers were common among some studied traits in this study. In normal conditions, EBmac0603-157, EBmac0603-183, EBmac0603-178, GBMS035-147, GBMS035-137, Bmag0606-151, Bmag0606-126, Bmag0606-147, Bmag0606-118, Bmag0606-122, Bmag0606-269, Bmag0606-138, HVM40-144, HVM40-147, HVM40-152, HVM40-162, Bmag0500-110, Bmag0500-146, Bmag0500-166, Bmag0500-181, Bmag0500-192, Bmag0500-194, and E42M48-087 were common for PH, GWS and NGS traits, EBmac0603-155, EBmac0603-180, EBmac0603-159, EBmac0603-170, EBmac0603-143, EBmac0603-153, Bmag0173-156 , scssr10559-210 , Bmac0399-138, Bmag0222-153, Bmag0222-185, Bmac0399-143 and E33M54-214 were common for GWS and NGS traits. Under salinity stress conditions, Bmac0316-168 was common for GWS and NGS traits. Identifying common markers is very important in plant breeding because it allows the simultaneous selection of several traits (Tuberosa et al., 2002; Hittalmani et al., 2003). The common markers among traits are helpful because they increase the efficiency of marker-assisted selection. Common markers among traits can be due to pleiotropic effects or linkage between genomic regions involved in these traits (Jun et al., 2008). Of course, the presence of common markers is valuable when they are associated with large-effect QTLs, and secondly, they are stable and can be identified by repeated testing. However, in this experiment, the value of the coefficient of determination (R^2) was negligible in most traits. Although this

phenomenon was not unexpected because the nature of QTLs is such that several positions are involved in one trait, and a high R^2 for a marker is unexpected.

In the present study, some common markers were identified for a particular trait or several traits under normal and salinity stress conditions, called stable QTLs. Bmag0606-151, Bmag0606-126, Bmag0606-147, Bmag0606-118, Bmag0606-122, Bmag0606-269, Bmag0606-138, Bmag0500-110, Bmag0500-146, Bmag0500-166, Bmag0500-181, Bmag0500-192, Bmag0173-156 were common for GWS and NGS, EBmac0603-183 was common for PH and NGS, HVM40-144, HVM40-147, HVM40-152, HVM40-162 were common for PH in both normal and salinity stress conditions. GBMS035-147, GBMS035-137, HVM40-144, HVM40-147, HVM40-162 were common for PH, GWS and NGS, Bmag0606-151, Bmag0606-138, Bmag0606-126, Bmag0606-147, Bmag0606-118, Bmag0606-122, Bmag0606-269, Bmag0500-110, Bmag0500-146, Bmag0500-166, Bmag0500-181, Bmag0500-192, Bmag0500-194, GBMS035-147, GBMS035-137, HVM40-144, HVM40-147, HVM40-152, HVM40-162, Bmag0173-156 were common for GWS and NGS, EBmac0603-183, GBMS035-147, GBMS035-137 were common for PH, scssr10559-213, scssr10559-216, Bmag0222-153, Bmag0222-185, E42M48-087, were common for GWS, Bmac0399-138, EBmac0603-155, EBmac0603-180, EBmac0603-157, EBmac0603-159, EBmac0603-170, EBmac0603-183, EBmac0603-143, EBmac0603-178, EBmac0603-153 were common for NGS, E33M54-214 was common for LPC in normal conditions across two years, Which indicates the stability of these gene loci in normal environments and have no effect on the trait under stress conditions. Gene loci that act the same in different environments can be introduced as stable QTLs. Stable QTLs provide relative stability to genetic control and overcome the interaction between genotype and environment; therefore, their selection for a trait under normal conditions also improves the trait value under stress conditions. The stability of QTLs in different environments is due to the control of traits by a small number of large-effect gene loci, so marker-assisted selection efficiency is highly effective in this population. Common stable QTLs can be used in plant breeding to select several traits simultaneously

Conclusion

Salinity tolerance in crop plants is governed by a multifaceted interplay of genetic and physiological factors, with a quantitative and intricate nature influenced by numerous gene loci.. Results of the present study revealed that association mapping is a powerful tool for identifying DNA markers for morpho-physiological traits in barley. Specifically, 194 significant marker-trait associations were identified for the studied traits. Out of 194 QTLs 171 and 23 QTLs were observed for traits under normal and salinity stress conditions, respectively. Identified markers could be helpful in marker-assisted breeding programs for salinity stress tolerance in barley. It is suggested that markers with a higher determination coefficient (R^2) can use in the saturation of genetic maps. In this study, the marker E33M54-214 on chromosome 7H (83.4 cM) has a high coefficient of determination (R^2 = 0.54) with QTL controlling the HI, indicating a strong association between the marker and the trait. Several QTLs were stable for plant height, the number of grains per spike, grain weight per spike, and leaf proline content under different environmental conditions, introduced as stable QTLs. The results showed that some stable QTLs were common to several traits, providing an opportunity to improve several traits simultaneously and facilitate the development of high-yielding barley cultivars.

Supplementary Materials

The Supplementary Material for this article can be found online at: https://www.jpmb-gabit.ir/article_710678.html.

Supplementary Table 1. Genotypes used in the present study.

Supplementary Table 2. Combined analysis of variance of the studied traits in both non-stress and salinity stress conditions during two cropping years.

Supplementary Table 3. Statistics calculated for optimum K values using 2.3.4 Structure software.

Author contributions

M.Z: Conceptualization, methodology, software, formal analysis, investigation, data curation, writing - original draft preparation, Writing - review and editing; Nadali Babaeian-Jelodar: Supervision, conceptualization, Writing - review

and editing; R.A: Resources; S.A.T: Project administration; M.G.N: Data curation.

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Conflict of interest statement

The authors declare no conflict of interest.

Abbreviations

GLM: General linear model; GWS: Grain weight per spike; HI: Harvest index; LChC: Leaf chlorophyll content; LD: Linkage disequilibrium; LPC: Leaf Proline content; MLM: Mixed linear model; NGS: Number of grains per spike; NTT: Number of total tillers; PH: Plant height; QTLs: quantitative trait loci; RWC: Relative water content; TGW: Thousand-grain weight.

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نقشه‌یابی ارتباطی صفات مورفوفیزیولوژیک در جو تحت تنش شوری

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چکیده: مطالعه حاضر برای شناسایی نشانگرهای مولکولی مرتبط با صفات مورفوفیزیولوژیک، از ۱۴ ترکیب آغازگر AFLP و ۳۲ جفت آغازگر SSR در ۱۴۸ ژنوتیپ جو از روش نقشه‌یابی ارتباطی استفاده کرد. این آزمایش در قالب طرح آلفا لاتیس با پنج بلوک ناقص در دو تکرار تحت شرایط معمول و تنش شوری (EC=12 dS m^{-1}) در مزرعه مرکز تحقیقات و آموزش کشاورزی و منابع طبیعی یزد به مدت دو سال اجرا شد. ساختار ژنتیکی جمعیت به دو زیرجمعیت (K=۲) تقسیم شد. در جمعیت ارتباطی حاضر، میانگین D' و r^2 که شاخص های عدم تعادل پیوستگی هستند، به ترتیب ۰/۲۵ و ۰/۰۲ بودند. مدل خطی مخلوط ۱۹۴ ارتباط معنی‌دار نشانگر – صفت برای ۹ صفت مورد مطالعه در شرایط معمول و تنش شوری شناسایی کرد. تعدادی از جایگاه‌های ژنی کمی برای صفات ارتفاع بوته، تعداد دانه در سنبله، وزن دانه در سنبله و مقدار پرولین برگ در شرایط محیطی مختلف، پایدار بودند که به عنوان جایگاه های ژنی کمی پایدار معرفی شدند. نتایج نشان داد که تعدادی از جایگاه‌های ژنی کمی پایدار در چندین صفت مشترک بودند که می‌توان از آن‌ها در به‌نژادی گیاهان زراعی به‌منظور گزینش همزمان چند صفت استفاده کرد و بهبود ارقام جو پرمحصول را تسهیل کرد. نشانگرهای شناسایی شده را می‌توان در انتخاب به کمک نشانگر در برنامه‌های اصلاحی تحمل به تنش شوری جو استفاده نمود.

کلمات کلیدی: تنش شوری، جایگاه‌های ژنی کمی پایدار، جو، عدم تعادل پیوستگی، مدل خط مخلوط، نقشه‌یابی ارتباطی.

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