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GENOME WIDE ASSOCIATION ANALYSIS OF PLANT HEIGHT, SPIKE AND AWN LENGTH IN BARLEY (*HORDEUM VULGARE* L.) EXPOSED TO MN STRESS

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ABSTRACT

Plant productivity is influenced by environmental conditions. Abiotic stresses limit yield potential in crops. Mn toxicity is an important constraint to crop production. In this study, whole genome association mapping was used in a spring barley population with 148 diverse genotypes for morphological traits underlying Mn stress. Association analysis between markers and phenotypic traits was performed with a mixed linear model (MLM with K+Q). A total of 39 significant markers were identified under both non stress and stress conditions. These markers were found on chromosomes 1H, 2H, 3H, 4H and 5H. Many of the associated markers were located in regions where QTL had previously been identified. Three significant markers on chromosomes 3H and 5H were associated with main spike length (MSL) and awn length (AL). Thus, it can be proved that MSL and AL have the same controller genomic regions. The results indicated that Mn tolerance was quantitatively inherited, and the detected QTLs may be useful for marker-assistant selection and identification of the genes controlling Mn tolerance in barley.

Keywords: Barley, Genome Wide Association Analysis, Manganese, Morphological Traits, QTL

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INTRODUCTION

In agricultural systems, plant productivity is strongly influenced by environmental conditions. Yield potential in crops is limited due to different abiotic stresses (Holmberg and Bülow, 1998; Atienza *et al*., 2004; Fahad *et al*., 2017). Manganese (Mn) is an essential micronutrient for plants. At high concentrations, however, this element also may be toxic to plants (Alam *et al*., 2001; Millaleo *et al*., 2010). Mn participates in several metabolic processes, including photosynthesis (Goussias *et al*., 2002), and as a cofactor of several enzymes (Hebbern *et al*., 2009). The high concentrations of Mn in plant tissues can alter activities of enzymes and hormones; therefore, essential Mn-requiring processes become less active or nonfunctional (Horst, 1988; Wang *et al*., 2002).

Barley is one of the most important crops with uses ranging from food to feed production; it represents the fourth most abundant cereal in both area and harvested tonnage (Abou-Elwafa, 2016). Analysis of genetic diversity and relationships in barley germplasm will provide useful information for breeding programs (Sun *et al*., 2011). Association have been detected between genetic markers and phenotypic traits in barley plants (Wang *et al*., 2012; Pauli *et al*., 2014; Abou-Elwafa, 2016; Wójcik-Jagła *et al*., 2018). Fast and affordable genotyping and sequencing technologies have made it possible to association mapping studies in a much broader germplasm (Zhu *et al*., 2008; Long *et al*., 2013).

There have been few reports on genetic studies associated with Mn tolerance in crops. Wang *et al*. (2002) detected eight QTLs associated with Mn toxicity in rice. Kassem *et al*. (2004) found three genomic regions contain QTL for resistance to necrosis during manganese toxicity in soybean. Huang *et al*. (2018) identified four significant QTLs for plant survival and two significant QTLs controlling leaf chlorosis under Mn stress in barley. Only few studies reporting quantitative trait loci (QTL) for Mn toxicity in barley using bi-parental mapping population have been published till date. However, bi-parental populations do not cover the enormous diversity (Shrestha *et al*., 2018). Recently, association mapping based on the strength of linkage disequilibrium between markers and functional polymorphism across a set of diverse germplasm emerged as a powerful tool for gene tagging (Mackay and Powell, 2007; Zhu *et al*., 2008; Ahmadi *et al*., 2011).

The objectives of this study were (1) to evaluate genomic regions affected Mn stress and contributive morphological traits in a diverse spring barley collection, (2) to establish marker-trait associations for each trait, and (3) to identify genes/loci affecting Mn stress in spring barley that can be used for genetic improvement in breeding programs.

MATERIALS AND METHODS

Plant material: A total of 148 spring barley cultivars from European countries were used in this study (see Kraakman *et al*., 2004 and Kraakman *et al*., 2006 for more details).

Field experiments: The experiment was conducted at Zahak Agricultural Research Station (30˚15'N and 60˚15'E), Sistan and Baluchestan, Iran, in 2014-2015 and 2015-2016 growing seasons and were sampled in 2015 and 2016 respectively. The cultivars were planted in alpha-lattice design with two replications in two conditions: non stress and Mn stress. Each replicate contained 9 incomplete blocks with 17 pots. The five barley varieties used throughout the experiment were: local, 5-salinity-white cluster, NP-90-113, Nomar and Zahak. In stress conditions, the Mn was applied in the form of Oligo Manganese-EDTA 13% (500 mg L^{-1}) at three stages (Tillering, Stem elongation, Booting). Induction of Mn toxicity was attempted by spraying Mn on the canopy and soil. Accordance with standard crop management procedures diseases and weeds were controlled in all trials. Morphological traits including plant height (PH), main spike length (MSL), and awn length (AL) were measured for two years in both conditions.

Genotyping and marker analysis: The cultivars were genotyped with 14 AFLP primer combinations and 11 microsatellites (SSRs) (see Kraakman *et al*., 2004 and Kraakman *et al*., 2006 for more details). Aghnoum *et al.* (2010) added 21 new SSRs to this population (unpublished data). Genetic diversity was analyzed in 148 cultivars using 407 polymorphic markers over the barley genome. Markers with missing data more than 10% and minor allele frequency less than 5% were excluded. The final set of 218 good quality markers was used for population structure and association analysis.

Population structure analysis: Population structure was estimated using a model based (Bayesian) clustering algorithm in software package $\text{STRUCTURE}_{v2.3}$ (Pritchard *et al*., 2000; Pritchard *et al*., 2010). The admixture model was chosen with a burn-in of 100000 iterations and 100000 Markov Chain Monte Carlo (MCMC) iterations. The test was conducted ten times for several subpopulations (K= 2-10). The population structure matrix (Q) was defined by ΔK index, the highest value was obtained at K=2, which indicates that there are two sub-groups in this population. The Q-Matrix was extracted based on K value (Falush *et al*., 2003; Kraakman *et al*., 2004; Jabbari *et al*., 2018).

Statistical analysis of phenotypic data and Association analysis: Variance components of phenotypic data were estimated using GenStat_{v15} (Payne *et al*., 2012). Heritabilities and correlations of all the traits were calculated using $\text{SAS}_{v9.1}$ software. Best linear unbiased predictors (BLUPs) for each one of the traits in each environment were estimated. The BLUPs were used in association analysis.

Association analysis between markers and phenotypic traits was performed with a mixed linear model (MLM) using the TASSEL_{v3} software. It was applied by MLM with K (genetic relatedness) + Q (individual membership in the population) (Yu *et al*., 2006; Bradbury *et al*., 2007). Kinship was calculated with TASSEL_{v3}. The significant threshold of P-value for assessing marker-trait-association were calculated. The threshold p-value of 0.03 was used for all traits (Chen *et al*., 2010; Pasam *et al*., 2012; Jabbari *et al*., 2018).

RESULTS

Phenotypic data: The variance analysis of traits is shown in Table 1. All traits and environments showed highly significant differences. Genotype and environment interactions were also significant for all the traits. In non stress conditions, PH showed highly heritability, moderately heritabilities were observed for MSL and AL (Table 2). AL showed poorly heritability in stress conditions. Plant height showed significant correlation with awn length in both conditions (Table 3). A significant correlation was observed between PH and MSL under non stress conditions. Significant positive correlation between main spike length and awn length was detected in both conditions.

Association analysis: In this study, a total of 39 significant markers (20 mapped and 19 unmapped) for important morphological traits in barley were identified in both non stress and stress conditions (Tables 4 & 5). Of these, nineteen markers (10 mapped and 9 unmapped) were detected under non stress conditions and twenty markers (10 mapped and 10 unmapped) were detected under Mn stress conditions. Some significant markers associated with two traits. Ten markers (5 mapped and 5 unmapped) displayed significant associations with plant height (PH). Eleven markers (8 mapped and 3 unmapped) displayed significant associations with main spike length (MSL). For awn length (AL), twenty-one markers (10 mapped and 11 unmapped) were found.

Marker E37M33-153 on chromosome 4H showed significant association with PH under stress conditions, explaining 5.34% of trait variation. The SSR marker Bmag0223-160 on chromosome 5H was associated with AL under non stress conditions, which explained 5.75% of trait variation. Marker E35M48-256 on chromosome 5H showed significant association with AL under stress conditions, explaining 5.96% of trait variation.

Table 1. REML analysis for morphological traits, sum of squares was shown.

Trait	Wald statistic						
	Genotype	Environment	Year	G × E	G × Y	E × Y	G × E × Y
PH	65.55**	11.14**	229.02**	20.10**	12.99**	7.27**	9.53**
MSL	28.43**	26.25**	0.56$^{n.s}$	15.41**	14.58**	12.20**	8.08**
AL	30.85**	27.00**	14.67**	22.41**	15.03**	16.71**	10.02**

** Significant at $p < 0.01$, * significant at $p < 0.05$, n.s - not significant

Plant height (PH), main spike length (MSL), awn length (AL)

Table 2. Estimation of mean, minimum, maximum and heritabilities of morphological traits for two years.

Trait	Non stress				Stress			
	Mean	Minimum	Maximum	Heritability	Mean	Minimum	Maximum	Heritability
PH	82.434	70.175	97.375	0.735±0.043	79.722	64.300	98.850	0.745±0.041
MSL	8.097	5.975	10.375	0.469±0.086	7.741	6.325	9.850	0.499±0.081
AL	18.486	15.250	22.275	0.615±0.062	17.799	14.825	20.200	0.387±0.100

Table 3. Pearson correlation coefficients among different traits based on data from each condition for two years.

Trait	Non stress			Trait	Stress		
	PH	MSL	AL		PH	MSL	AL
PH	1			**PH**	1		
MSL	0.226**	1		**MSL**	0.106$^{n.s}$	1	
AL	0.269**	0.591**	1	**AL**	0.180*	0.564**	1

** Significant at $p < 0.01$, * significant at $p < 0.05$, n.s - not significant

Table 4. Significant markers associated with morphological traits in non stress condition.

Trait	Marker	Chromosome	Position (cM)	2015			2016		
				-log10(P)	Marker R^2 (%)	Effect	-log10(P)	Marker R^2 (%)	Effect
PH	E37M33-93	3H	126.42	-	-	-	1.55	3.47	-2.74
	E42M32-176	Unmapped	-	1.60	3.85	-2.77	-	-	-
	E45M55-164	Unmapped	-	-	-	-	2.02	4.84	2.43
MSL	E37M33-583	3H	12.00	1.66	3.97	-0.41	-	-	-
	E38M55-320	3H	40.14	1.67	4.04	-0.60	-	-	-
	Bmag0223-170	5H	86.88	1.75	4.08	0.51	-	-	-
	E35M48-400	5H	183.75	1.62	3.66	-0.54	-	-	-
	E42M32-184	5H	41.40	1.64	3.96	-0.47	-	-	-
AL	E38M54-260	1H	43.69	1.54	3.31	0.92	-	-	-
	E37M33-583	3H	12.00	-	-	-	1.81	4.40	-0.51
	E33M54-100	4H	65.92	1.69	3.89	-0.97	-	-	-
	Bmag0223-160	5H	86.88	-	-	-	2.26	5.75	0.68
	E35M48-400	5H	183.75	1.72	3.98	-1.07	-	-	-
	E35M48-410	5H	184.79	-	-	-	1.62	3.71	-0.49
	E35M48-411	Unmapped	-	-	-	-	1.62	3.71	-0.49
	E35M61-068	Unmapped	-	1.64	3.83	0.94	-	-	-
	E37M33-260	Unmapped	-	-	-	-	2.01	5.00	-0.62
	E38M55-219	Unmapped	-	1.71	3.99	1.34	-	-	-
	E38M55-226	Unmapped	-	-	-	-	1.62	3.77	-1.05
	E42M32-254	Unmapped	-	-	-	-	2.21	5.51	0.97
	E45M49-069	Unmapped	-	-	-	-	2.15	5.07	1.02

Marker R^2 (%) = percentage of phenotypic variance explained by individual marker

Table 5. Significant markers associated with morphological traits in Mn stress condition.

Trait	Marker	Chromosome	Position (cM)	2015			2016		
				-log10(P)	Marker R^2 (%)	Effect	-log10(P)	Marker R^2 (%)	Effect
PH	E35M48-146	4H	57.23	-	-	-	1.55	3.46	-3.05
	E37M33-153	4H	71.34	-	-	-	2.12	5.34	-5.47
	E42M32-179	4H	55.76	-	-	-	1.73	3.93	-3.61
	E45M55-142	4H	55.76	-	-	-	1.84	4.30	-3.19
	E35M61-355	Unmapped	-	1.53	3.87	-3.39	-	-	-
	E38M54-112	Unmapped	-	-	-	-	1.94	4.69	-3.03
	E45M49-254	Unmapped	-	-	-	-	1.79	4.07	-4.20
MSL	E35M48-133	2H	62.70	-	-	-	1.67	3.76	0.40
	E35M48-250	3H	10.67	1.66	3.76	-0.42	-	-	-
	E38M54-133	4H	125.08	2.00	4.69	-0.73	-	-	-
	E35M48-170	Unmapped	-	-	-	-	1.87	4.27	-0.30
	E42M48-195	Unmapped	-	1.53	3.69	0.46	-	-	-
	E42M48-196	Unmapped	-	1.69	4.19	0.49	-	-	-
AL	E35M48-250	3H	10.67	1.55	3.40	-0.60	-	-	-
	HVM040-162	4H	15.80	-	-	-	1.58	3.62	0.58
	E35M48-256	5H	47.95	-	-	-	2.44	5.96	0.50
	E39M61-106	5H	44.90	1.64	3.65	-0.59	-	-	-
	E35M55-434	Unmapped	-	2.16	5.23	-0.75	-	-	-
	E38M50-385	Unmapped	-	1.58	3.64	-0.61	-	-	-
	E38M50-414	Unmapped	-	-	-	-	1.53	3.49	-0.41
	E45M55-164	Unmapped	-	1.76	3.97	0.60	-	-	-

Marker R^2 (%) = percentage of phenotypic variance explained by individual marker

Two significant markers on chromosomes 3H (E37M33-583) and 5H (E35M48-400) were associated with MSL and AL under non stress conditions. One marker located on chromosome 3H (E35M48-250) was associated with MSL and AL under stress conditions.

DISCUSSION

Manganese is an essential micronutrient for plants, intervening in several metabolic pathways, mainly in photosynthesis and as an enzyme antioxidant–cofactor. However, an excess of this element is toxic for plants (Millaleo *et al*., 2010). The manganese toxicity symptoms vary widely among plant species and varieties within species, perhaps because the phytotoxic mechanisms of Mn involve different biochemical pathways in different plant genotypes (El-Jaoual and Cox, 1998).

Only few studies have addressed genetic factors with Mn tolerance in plants (Wang *et al*., 2002; Kassem *et al*., 2004; Raman *et al*., 2017; Huang *et al*., 2018; Shrestha *et al*., 2018).

In this study, whole genome association mapping was used in a spring barley population with 148 diverse genotypes for important morphological traits. Association analysis between markers and phenotypic traits was performed with a mixed linear model (MLM with K+Q). A total of 39 significant markers were identified under both non stress and Mn stress conditions.

For plant height, one marker on chromosome 3H under non stress conditions and four markers on chromosome 4H under Mn stress conditions were identified. Lakew *et al*. (2013) reported twenty marker-trait associations for plant height on all seven chromosomes for drought tolerance. Six QTLs were detected for plant height on chromosomes 3H, 4H and 6H in Mediterranean environment (von Korff *et al*., 2008). Several QTLs for plant height have been previously reported on chromosomes 3H and 4H (Talamè *et al*., 2004; Dahleen *et al*., 2005; Inostroza *et al*., 2009; Xue *et al*., 2009; Chutimanitsakun *et al*., 2011; Pasam *et al*., 2012; Locatelli *et al*., 2013; Rollins *et al*., 2013; Tondelli *et al*., 2013; Pauli *et al*., 2014; Abou-Elwafa, 2016; Maurer *et al*., 2016; Nice *et al*., 2017).

MSL was associated with five markers on chromosomes 3H and 5H under non stress conditions and three markers on chromosomes 2H, 3H and 4H under Mn stress conditions. MSL had significant associated markers with AL in both conditions. Thus, it can be proved that MSL and AL have the same controller genomic regions. In non stress conditions, one SSR marker (Bmag0223-170) on chromosome 5H showed significant association with MSL, which was previously reported for plant height (Jabbari *et al*., 2018). Wang *et al*. (2014) identified four QTLs on chromosomes 1H, 2H, 5H and 7H for spike length. Sun *et al*. (2011) detected one QTL on chromosome 2H for main spike length. Several QTLs have been previously reported on chromosomes 2H, 3H and 4H for spike length under abiotic stresses (von Korff *et al*., 2008; Varshney *et al*., 2012; Lakew *et al*., 2013; Rollins *et al*., 2013).

For awn length, six markers on chromosomes 1H, 3H, 4H and 5H under non stress conditions and four markers on chromosomes 3H, 4H and 5H under Mn stress conditions were identified. Some of these significant markers have been previously reported for internode length, flag leaf width and flag leaf sheath length (Jabbari *et al*., 2018). Liller *et al*. (2017) detected twelve significant QTLs for awn length located on all chromosomes. Wang *et al*. (2014) identified seven QTLs on chromosomes 1H, 3H, 5H and 7H for awn length. Dahleen *et al*. (2005) reported QTLs controlling awn length on chromosomes 2H, 3H, 4H, 5H and 7H.

Conclusions: The results of this study indicate that Mn tolerance in barley is a heritable character which could be selected in breeding programs. Most reports of crop plants indicate that inheritance of Mn tolerance is polygenic. (Moroni *et al*., 2013). Further investigations into the physiological mechanisms and genes controlling Mn tolerance in barley will be beneficial for our understanding of the evolutionary genetics and diversity of Mn tolerance mechanisms in barley.

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