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Genome wide association studies (GWAS) of element contents in grain with a special focus on zinc and iron in a world collection of barley (*Hordeum vulgare* L.)

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ABSTRACT

Genome wide association studies (GWAS) were carried out to map Quantitative Trait Loci (QTL) associated with element contents in the grain using 336 spring barley. Of the elements analyzed, Fe content ranged from 21.9 to 91.0 mg kg^{-1}, Zn from 10.4 to 54.5 mg kg^{-1}, Ba from 0.2 to 8.9, Ca from 186.4 to 977.5, Cu from 1.5 to 9.8, K from 353.2 to 7721.5, Mg from 1049.8 to 2024.2, Mn from 8.1 to 22.9, Na from 55.9 to 627.9, P from 2272.9 to 5428.8, S from 880.7 to 1898.0, Si from 19.1 to 663.2, and Sr from 0.35 to 2.62 mg kg^{-1}. GWAS were carried out using 6519 SNP markers and multiple elements in MLM:PCA + K model in TASSEL software. Population analyses showed two sub-populations, primarily based on row types. GWAS for row types showed association with *INTERMEDIUM-C*, a modifier gene for lateral spikelet fertility in the 4H chromosome, validating current GWAS approach. GWAS also showed that 2 QTL for Ba, 2 for Ca, 4 for Cu, 11 for Fe, 2 for K, 3 for Mg, 6 for Mn, 4 for Na, 3 for S, 5 for Si, and 3 for Zn were mapped in barley chromosomes. The QTL identified in the current study are valuable for breeding nutrient dense barley cultivars in the future, especially Zn and Fe.

1. Introduction

Three billion people around the world suffer from element deficiencies and malnutrition (Welch and Graham, 2004). Deficiencies of elements, especially iron (Fe), zinc (Zn) magnesium (Mg), phosphorous (P), potassium (K), selenium (Se), and copper (C) are the major causes of over 65% of childhood deaths worldwide (Welch and Graham, 2004). Fe and Zn are particularly important for all living organisms, including humans, and cause several deficiencies related human diseases (Marler and Wallin, 2006). Fe deficiency affects two billion people globally (Stoltzfus and Dreyfuss, 1998). Similarly, Zn deficiency affects one third of world population (Sandstead, 1991). Both Fe and Zn deficiencies pose a particular threat to human health in developing countries (WHO, 2002). Fe is the major component of hemoglobin responsible for oxygen and carbon dioxide transport during respiration, maintenance of blood acid-base ratio, the metabolism of body energy, and contributes to several immunological functions. Likewise, Zn has important roles in several metabolic functions including accelerated growth, body's immune responses and synthesis of several enzymes such as DNA polymerases, carbonic anhydrases and alkaline phosphatases (Prasad, 1991). Rapid changes in food habits and nutritive values of modern food in terms of micronutrients have contributed to element deficiency related problems. A comparison of historic genotypes of food crops with modern cultivars suggests that their nutritional value has drastically changed, with reduced mineral contents in modern small grains and vegetables

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compared to food produced 70 years ago (Marler and Wallin, 2006). Monasterio and Graham (2000) reported that the Fe and Zn contents declined gradually in wheat germplasm as an effect of modern plant breeding approaches during 1945–1999. Similarly, the development of soft white wheat has led to a decrease in element contents in this wheat class (Murphy et al., 2008). Declines in mineral contents in modern food have resulted in an increase of mineral deficiency associated diseases, such as heart conditions, chronic bronchitis, asthma, tinnitus, and bone deformities (Marler and Wallin, 2006).

Barley (*Hordeum vulgare* L.) is one of the major staple foods in several regions of the world, including the highlands of Asia, North and East Africa, the Andean countries, and the Baltic States (El-Haramein and Grando, 2010). Barley is one of the major cereals after rice, wheat, and maize by area and production (FAOStat, 2014). Around 3.7% of total barley production is used as human food annually worldwide, but in some countries, like in Morocco, Ethiopia and Eritrea, barley comprise as high as 60% of total food production (Newman and Newman, 2006).

Of the three strategies for resolving nutritional deficiencies in food crops: biofortification, food fortification, and mineral supplement, biofortification is the cheapest and most accessible (Welch and Graham, 2004). Barley grain contains about 65–68% starch, 10–17% protein, 4–9% β-glucan, 2–3% free lipids, and 1.5–2.5% minerals (Quinde et al., 2004). Barley is less sensitive to both Fe and Zn deficiencies, because it has genes involved in the mobilization and uptake of Fe and Zn. Other mega crops, such as rice, lack that capability (Nakanishi et al., 2000; Takahashi et al., 1999). The genes, including *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, *HvDMAS1*, *IDS2*, and *IDS3*, are up-regulated in Fe-deficient barley roots (Nakanishi et al., 2000; Takahashi et al., 1999). These observations suggest that there is a tremendous opportunity to improve Fe and Zn contents in barley grains using biofortification, and potentially also in other crops, such as rice (Masuda et al., 2008). The first step towards the exploitation of biofortification of elements in barley is to assess the status of element concentrations in the kernel and map Quantitative Trait Loci (QTL) associated with element uptake in plants. Mapping QTL associated with elements in barley is just beginning. Lonergan et al. (2009) mapped QTL for Zn uptake in 1H, 2H and 5H barley chromosomes using double haploid (DH) population. Sadeghzadeh et al. (2010) mapped Zn accumulating QTL on the 2H, 3H and 4H barley chromosomes using a DH population. A few studies showed genetic variation in Fe uptake in barley, however QTL for Fe uptake and other elements are scant. Mamo et al. (2014) mapped QTL for Zn and Fe uptakes in barley using the genome-wide association studies (GWAS) approach. ICARDA's barley program aims to improve barley genetic resources on a global scale. The objectives of the current study were to (1) assess variability of element uptake in a world collection of cultivated barley germplasms at ICARDA and to (2) map QTL associated with element uptake in barley grain using the GWAS approach.

2. Materials and methods

2.1. Plant materials

The Genome Wide Association Study reported here, which we named Association Mapping panel 2014 (AM-2014) comprised a total of 336 genotypes. Out of these, 230 genotypes were collected from the low input barley breeding program (genotypes adapted for biotic and abiotic stress tolerances) of ICARDA, 82 from the high input barley breeding program (genotypes adapted to favorable conditions) and the remaining 26 genotypes were frequently used in both programs (Supplement Table S1). Based on grain types, 276 genotypes in the AM-2014 were hulled and 60 were hull-less barley. In terms of row type, 136 genotypes were two-rowed and 200 were six-rowed. The majority of the barley genotypes was collected from barley breeding programs of ICARDA (advanced breeding lines), but also represented genotypes from different sources, including the Genetic Resource Unit (Gene Bank) of ICARDA and barley breeding programs from India, Australia, USA, Canada, and Morocco. The majority of the genotypes of AM-2014 were advanced breeding lines and release cultivars; a few were landraces. The AM-2014 panel was described in detail at the molecular and phenotypic level by Amezrou et al. (2017). The passport data of AM-2014 are presented in Supplement Table S1.

2.2. Phenotyping

Two sets each of barley seed samples were obtained from two field experiments that were conducted in Marchouch (MCH) (33°33′38.2″N 6°41′24.7″W), and Jemma-Shaim (JS) (32°21′09.3″N 8°50′32.0″W) Research Stations in Morocco. The experiments were conducted in an alpha lattice design, replicated twice with 10 genotypes in each block. In 2015, the 336 barley genotypes were evaluated for various phenotypic, agronomic traits in the field. From each genotype, three spikes were harvested at maturity, kept in labelled envelopes, and sun dried for two days. To eliminate any contamination of metals, grains were harvested and cleaned manually by hand, and stored in paper bags.

2.3. Element analysis

Analysis of multiple elements at the same time in the same samples, using equipment such as Inductively Coupled Plasma spectrometry (ICP), can provide information about elements in addition to those of immediate interest, and about their interactions (Markert, 1992; Otte and Jacob, 2005). For example, a higher content of one essential nutrient may coincide with a lower content of another essential nutrient. With a view to get some insight into such interactions, we wanted to analyze at least a subset of the samples for more than just Fe and Zn. However, because of the large number of samples and the amount of funding available, we could not do this for all samples. We therefore decided to use the samples from JS for analysis by ICP (NDSU, Fargo, ND, USA), and the samples from MCH for analysis for Fe and Zn only by Atomic Absorption Spectrometry AAS (INRA, Morocco).

Grain samples from the experiment in Jemma Shiam (JS) were used for multi-element analysis by Inductively Coupled Plasma (ICP) analysis in an augmented design with checks repeated multiple times. Among the checks, Rihane-03 was repeated six times while Tichedrette, Alanda-01, WI2291, Harmal, Nawair-01, Petunia 1, Jyoti, Lakhan (K226), Geetanjali (K1149), DWR28, RD2668, DWRUB52, RD2668_1, and Furat-1 each were repeated three times. The element analyses were conducted in the Department of Biological Sciences, North Dakota State University (NDSU), Fargo, ND. Barley grain samples were randomized, powdered, and homogenized using mortar, pestle, and liquid nitrogen. Samples were dissolved using a CEM Xpress microwave digester NC, USA and 55 ml PFA venting vessels with the following method: 250 mg, 1:1 nitric acid and DI water solution (10 total ml), overnight pre-digestion, then microwaved at 200 °C for 25 min after 20 min ramping from ambient temperature. Digested samples and blanks were analyzed using Spectro Genesis SOP ICP-OES (Spectro Analytical Instrument GmbH, Germany) with SmartAnalyzer Vision software and Optimist nebulizer with a cyclonic spray chamber. Quality control included digestion and analysis of certified reference material (Maize GBW10012), the ICALization internal standard procedure, analysis of an external standard, control calibration verification, and analysis of duplicate samples. Barley samples from

JS were analyzed for 31 elements in the grain, but only Ba, Ca, Cu, Fe, Mg, Mn, Na, P, S, Si, Sr, and Zn were above the method detection limit (MDL) in all 336 barley genotypes, therefore data on these elements were further used for GWAS. The MDL was calculated by using the following formula:

$$MDL = \frac{3\ x\ stdev\ (calibration\ water\ blank)\ x\ extraction\ volume\ x\ dilution\ factor}{mean\ dry\ weight}$$

Grain samples from the experiment conducted in Marchouch (MCH) station were analyzed only for Fe and Zn. The design of the field experiment and grain sampling were the same as for Jemma Shiam (JS) used for multiple element analysis described above. Sample preparation and analysis were conducted at the Institut National de la Researche Agronomique-Maroc (INRA-Maroc) following Miller and Rutzke (2003), as follows. Grain samples were dried in an oven for about 16 h (overnight) at 105 °C then at 200 °C, after which the temperature was gradually increased to 450 °C for 16 h. The remaining ash was then digested in 4 ml of nitric acid to decompose the organic matter, and the remaining inorganic residue dissolved in 5 ml of diluted hydrochloric acid (9.25%) following the protocol of Heath Canada (1985). Internationally certified reference material and black checks were used to maintain analytical quality assurance.

2.4. Statistical analysis elements

The descriptive analysis (mean, standard deviation and range) were estimated in SAS v.10 (SAS Institute, 1999) statistical software package using PROC SUMMARY command. The element content of barley grains was subjected to ANOVA using augmented block design. The ANOVA was performed using PROC GLM of the SAS. The element contents of test genotypes were differentiated by Fisher's least significant difference (LSD) ($P = 0.05$) based on the standard error of the mean difference of repeated checks, that were used in the experiment.

2.5. Single Nucleotide Polymorphism (SNP) genotyping

SNP genotyping of AM-2014 was reported by Amezrou et al. (2017). In brief, single barley plants of each line were grown in a greenhouse and the leaf tissue was lyophilized. Genomic DNA was extracted using the method described in Slotta et al. (2008). The lines were genotyped using the 9K iSelect SNP array based on Illumina's Inifinium Assay (Illumina, San Diego, CA, USA) at Cereal Crop Research Unit, USDA-ARS, Fargo, ND. The obtained SNP data were further filtered for (a) a minor allele frequency of 0.05, (b) rate of missing values above 10%.

2.6. Population structure

In order to account for false positives in GWAS, population structure was investigated in the panel using the program STRUCTURE (version 2.3.4) (Pritchard et al., 2000) Membership probability for each barley genotype to a number of hypothetical subpopulations (k) was estimated using SNP markers. Subpopulations 1–7 were tested using an admixture model. The program was first run for a burn-in period of 100,000 while the posterior probabilities were estimated using the Markov Chain Monte Carlo (MCMC) method with 100,000 repetitions. The most likely number of sub populations was determined using the Δk (Evanno et al., 2005). The population structure was further investigated using principal coordinate analysis. The kinship matrix (K) was estimated in TASSEL and used as covariate to control false positives in the marker trait analysis.

2.7. SNP-element association analysis

Marker-trait association studies were carried out in TASSEL version 4 (Bradbury et al., 2007). In TASSEL, models: naive GLM, GLM + Q, GLM + PCA, MLM + Q + K, MLM + PCA + K were tested for discovering true marker-trait associations and effectiveness in controlling false positives. Results showed that GLM models were not good enough to control the false positives (data not presented) while both MLM + Q + K and MLM + PCA + K were found effective for finding true associations. Further, the results of both MLM models were similar, therefore MLM + PCA + K was used in the current study. SNP alleles <0.05 frequency were treated as minor alleles, therefore such rare variants having minor alleles were removed from the analysis. Variants with >10% missing SNPs were also discarded from the analysis. The pFDR ($q < 0.05$) was applied in order to test the statistical significance of multiple comparisons of all detected markers according to Storey (2002). The output data included R^2 (% phenotypic variation explained by significant markers), the additive effects where a positive value indicates the presence of alleles that enhance the uptake of elements and a negative value indicated presence of alleles that reduce the elements in the grain. The sequence of significant SNPs were subjected to BLAST searches, in order to annotate predicted genes.

3. Results

3.1. Element analyses including Zn and Fe

The results of ANOVA of element contents by 336 barley genotypes are presented in Table 1. Except for Mg and Si, genotypes showed highly significant differences in element contents. The passport data and element content of individual barley genotypes are presented in Supplement Table S1. The summary statistics on element contents by 336 barley genotypes from JS and MCH in 2015 are presented in Supplement Table S2. Likewise, the summary statistics of repeated checks are presented in Supplement Table S3. The box and whisker plots of element contents are also shown in Fig. 1A–D. Higher Fe and Zn uptake were observed in MCH compared to JS locations, however the range of both Zn and Fe was higher in JS (Fig. 1A).

3.2. Single Nucleotide Polymorphism (SNP) and linkage disequilibrium (LD) in AM-2014 panel

The details of SNP markers used in the current study were described by Amezrou et al. (2017). In brief, of 9K SNP markers genotyped in 336 barley genotypes, a total of 6940 genome wide SNPs, were of high quality. Further filtering for >10% missing data and <0.05 minor allele frequency resulted into 6519 SNPs which were used in further analysis (PCA and GWAS). The genome wide LD was estimated at 3.58 cM in the current AM-2014 panel, while

Table 1
Analysis of variance of element contents of barley grains in a world collection of 336 barley genotypes by ICARDA in 2015 from Jemma Shiam, Morocco.

Source of variation	df	Ba	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Si	Sr	Zn
		P-Value												
Block	16	0.109	0.013	0.002	0.32	<0.001	0.725	0.490	0.01	0.186	0.494	0.458	<0.001	0.739
Genotypes	341	<0.001	0.003	<0.001	0.03	<0.001	0.102	0.002	<0.001	<0.001	0.002	0.155	<0.001	0.047

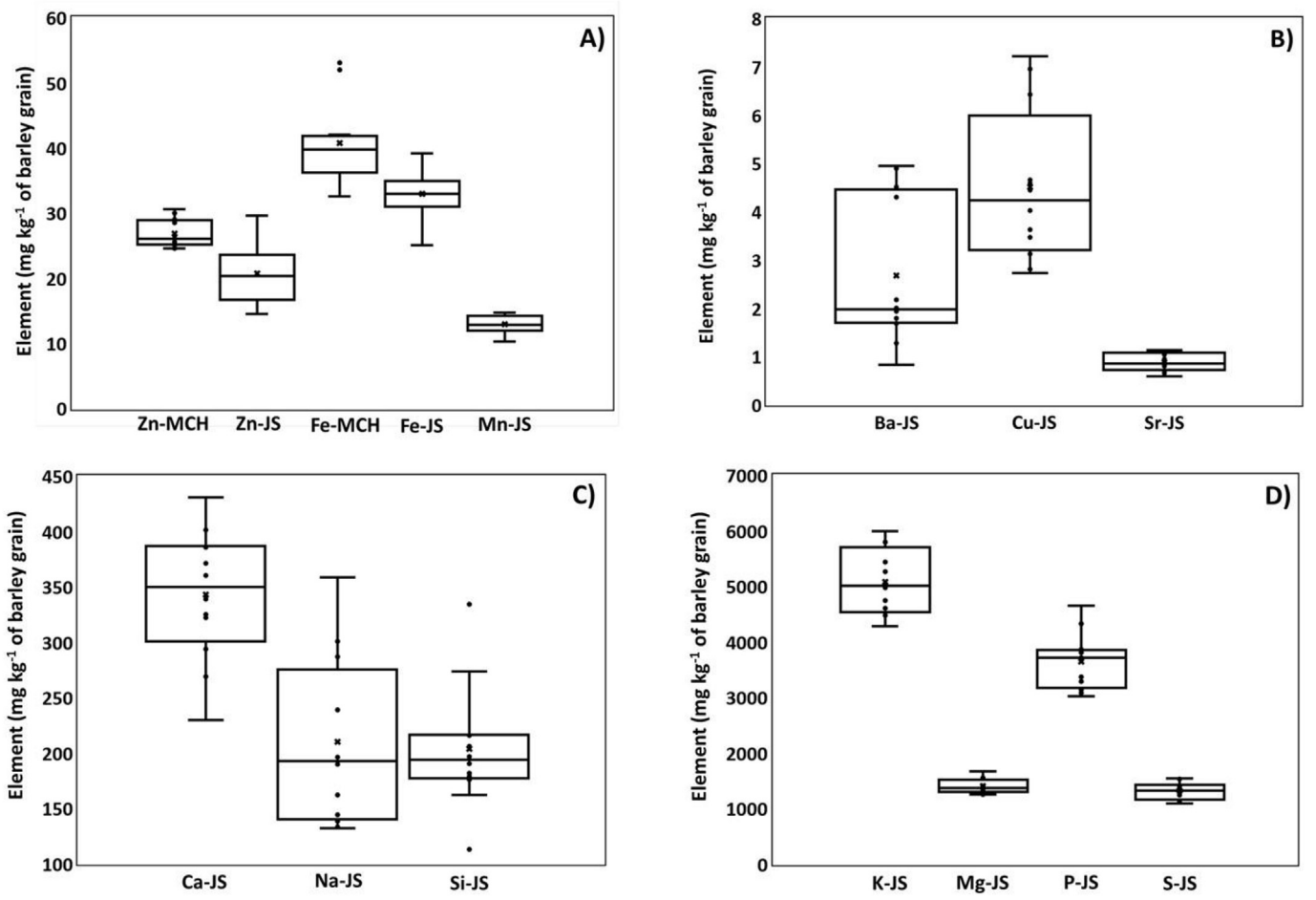

Fig. 1. Box and whisker plots of element contents in 336 barley grains collected from Marchouch and Jemma Shiam in Morocco, A) Zn and Fe uptake in Marchouch (MCH) and Jemma Shiam (JS), and Mn in Jemma Shiam, B) Ba, Cu, and Sr uptake in Jemma Shiam, C) Ca, Na, and Si uptake in Jemma Shiam, D) K, Mg, P and S uptake in Jemma Shiam.

LD decay varied between two-and six-rowed subpopulations. LD decay was recorded as 3.91 cM for the two-rowed sub-populations and 2.26 cM for the six-rowed sub-populations.

3.3. Population structure

Principal Coordinate Analysis (PCoA) and Population Structure were implemented to investigate the subpopulation in AM-2014. Both approaches showed two sub-populations, primarily based on row types in the current mapping panel (Figs. 2 and 3). Based on a 0.2 probability threshold in structure analysis, nearly 36% of barley genotypes showed admixture (Fig. 2). Similarly, PCoA clearly showed two distinct sub-populations in the current mapping panel based on row types, however, some overlap between the two-rowed and six-rowed clusters was also apparent (Fig. 3).

3.4. Genome wide association studies (GWAS) of row types

The results of GWAS of row type, SNP state, significant SNPs, marker positions, R^2, additive effects, allele frequency and predicted gene annotations are presented in Table 2. Multiple SNPs were highly significant ($q < 0.05$) in 1H, 3H, 4H, 6H, and 7H chromosomes. The R^2 of these significant SNPs varied from 3.3 to 8.0%. The gene annotation using the BLAST search resulted in MATH domain-containing proteins (SNP: SCRI_RS_171501), Phytanoyl-CoA dioxygenase 1(SNP: 11_20606), Glutathione S-transferase 3(SNP: 11_20422), Phytochrome A (SNP:12_30864), KNOTTED-1-like homeobox protein d (*knox1d*) (SNP: SCRI_RS_145412), and IQ-Domain-1(SNP: 12_20803).

3.5. Genome wide association studies (GWAS) of multiple elements

The results, SNP state, significant SNPs, marker positions, R^2,

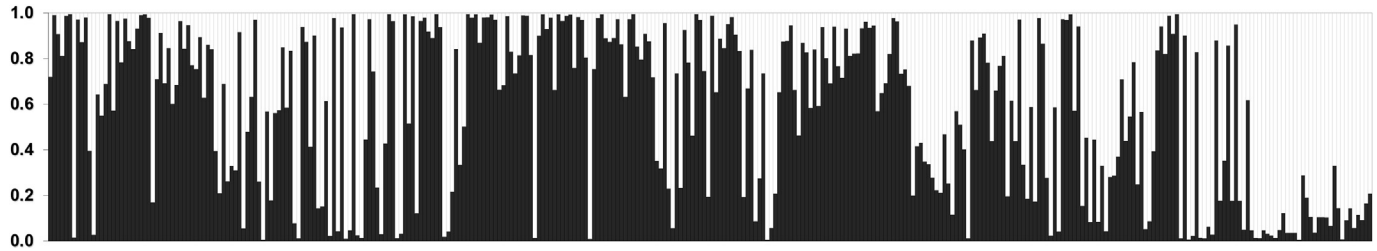

Fig. 2. Probabilities of 336 barley genotypes belonging to one of the two subpopulations indicated as the relative height of light bars (two-rowed barley) and dark bars (six-rowed barley).

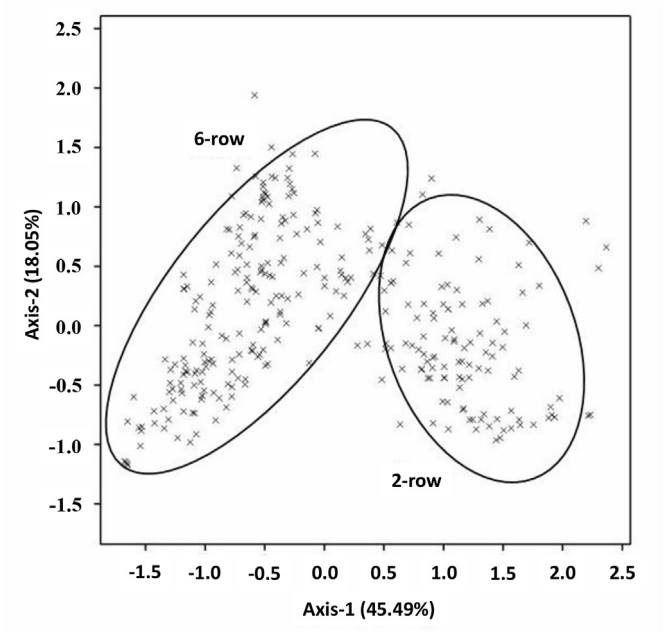

Fig. 3. Principal Coordinate Analysis (PCoA) based on genetic distance computed for 336 individuals (6-row = 6-rowed barley, 2-row = 2-rowed barley).

additive effects, allele frequency and predicted gene annotations, of GWAS of 13 elements (including Zn and Fe) in JS and Zn and Fe only in MCH locations are presented in Table 3. Some SNPs were significant for multiple elements while other SNPs were significant for specific element uptake only. In the case where a single SNP was significant for multiple elements, QTL was designated as E-CH-SNP Position, where E-represented uptake of multiple elements, CH-represented barley chromosome number followed by a number that represented SNP position in given chromosome. For example, *E-3H-66.15* (SNP: 11_10813) was significant for Cu, Mg, Na, S, and Zn in JS, while *Fe-1H-57.85* (SNP: SCRI_RS_237999) was significant only for Fe in MCH (Table 3).

Two QTL, *Ba-3H-15.52* and *Ba-5H-90.22*, were in the 3H and 5H chromosomes from JS, (Table 3). In 5H, three SNPs were significant at 90.22 cM which were highly significant ($q < 0.01$) while five SNPs with unknown positions were also significant in 5H, 6H, and 7H chromosomes. The R^2 and additive effects of these QTL ranged from 3.6 to 7.7% and −1.06–0.73 mg kg^{-1}. Two QTL, *Ca-2H-126.56* and *E-3H-153.39* were significant for Ca uptake, while QTL *E-3H-153.39* was also significant for K. The variation explained by these QTL was 3.5%, while the additive effects ranged from −42.17–40.99 mg kg^{-1}. Four QTL, *E-3H-66.15*, *Cu-6H-50.61*, *Cu-6H-52.2*, and *Cu-6H-58.91*, were significant for Cu. *E-3H-66.15* detected in the 3H chromosome; the remaining in the 6H chromosome. *E-3H-66.15* was significant for Cu, Mg, Na, S, and Zn uptake from JS. The R^2 of these QTL ranged from 3.6 to 3.9% and additive effects ranged from −0.77 to 0.70 mg kg^{-1}. Two QTL, *E-3H-153.39* and *K-5H-95.65,* were significant for K, while 3 SNP markers with unknown positions in 4H, 5H, and 7H were also significant for this element. The R^2 of these QTL ranged from 3.6 to 8.1% and additive effects ranged from −266.63 to −424.11 mg kg^{-1}. Three QTL, *Mg-2H-66.61*, *E-3H-66.15*, and *E-7H-95.02* were significant for Mg. Of these, *E-3H-66.61* (Cu, Mg, Na, S, and Zn) and *E-7H-95.02* (Mg, Mn, and Si) were significant for multiple elements. Five QTL, *Mn-3H-74.41*, *Mn-4H-60.70*, *Mn-7H-82.16*, *Mn-7H-91.73*, and *E-7H-91.73* were significant for Mn while one SNP with unknown marker position was also significant for Mn. The R^2 of these QTL ranged from 3.5 to 5.2% and additive effects ranged from −2.03–1.13 mg kg^{-1}. For Na, four QTL, *E-3H-66.15*, *Na-5H-1.12*, *E-5H-44.99*, and *Na-7H-82.89* were in 3H, 5H, and 7H chromosomes. One SNP with unknown marker position, SCRI_RS_185604, was significant for Na too. The R^2 of these QTL ranged from 3.7 to 4.8% and additive effects from 45.91 to 333.53 mg kg^{-1}. Only one SNP marker, SCRI_RS_140936 was significant for Se, but the marker position of this SNP was unknown. Five QTL, namely *Si-4H-88.81*, *Si-4H-103.38*, *Si-5H-185.05*, *Si-7H-81.7*, and *E-7H-95.09*, were significant in 4H, 5H, and 7H chromosomes for Si while three SNPs with unknown marker positions were also significant for Si. The R^2 of these QTL ranged from 3.4 to 5.7% and additive effects from −78.14–98.91 mg kg^{-1}. Three QTL, *S-1H-50*, *E-3H-66.15*, and *S-4H-117.13* were significant in the 1H, 3H, and 4H chromosomes for S. Three additional SNPs were also significant for S but their marker positions were unknown. The R^2 of these QTL ranged from 3.5 to 4.1% and additive effects from −82.0–117.91 mg kg^{-1}.

3.6. Genome wide association studies (GWAS) of Zn and Fe

The GWAS results of Zn and Fe from JS and MCH locations are presented in Table 3. Three QTL regions were significant for Zn, of which two were from JS and the other from MCH. *E-3H-66.15* and *E-5H-44.99* were significant in the 3H and 5H chromosomes from JS while *Zn-2H-87.34* was significant in the 2H chromosome of MCH. Of these QTL, *E-3H-66.15* and *E-5H-44.99* were also significant for other elements, including Cu, Mg, Na, S, and Na. The R^2 of these QTL ranged from 3.5 to 4.9% and additive effects from −3.5–2.38 mg kg^{-1}. Ten QTL were significant for Fe from JS and MCH. In JS, three QTL, namely Fe-2H-84.74, *Fe-2H-139.62*, and *Fe-4H-67.91* were significant in the 2H and 4H chromosomes. In contrast, seven QTL, namely *Fe-1H-54.5*, *Fe-1H-57.85*, *Fe-1H-90.04*, *Fe-2H-84.74*, *Fe-4H-53.87*, *E-4H-54.95*, *Fe-6H-102.03*, and *Fe-7H-17.62* were significant in the 1H, 2H, 4H, 6H, and 7H chromosomes in MCH. Of these, QTL *Fe-2H-84.74* was significant in both JS and MCH locations. Five SNPs with the unknown marker position were also significant for Fe. The R^2 of these QTL ranged from 3.3 to 5.0% and additive effects from −12.9–6.15 mg kg^{-1}. The predicted gene

Table 2
Genome wide association studies of row types 336 genotypes of barley, and gene annotation of significant SNP markers using BLAST search.

SNP ID	SNP	[a] CH	cM	[b] *P*-value	R^2	Additive effect	Allele Frequency	[c] E-value	Gene annotation using BLAST search
SCRI_RS_6913	A/C	1H	100.45	4.1E-07**	8.0	1.030	A = 314	[e] -	–
SCRI_RS_171501	T/C	1H	101.05	1.4E-04*	4.4	0.689	C = 304	4.0E-24	MATH domain-containing protein
SCRI_RS_66669	A/C	1H	[d]518942719-518942839	4.1E-07**	8.0	1.039	A = 314	–	–
SCRI_RS_202425	A/G	3H	108.07	5.7E-05**	5.0	0.623	A = 268	–	–
SCRI_RS_826	T/C	3H	82.19	6.2E-04*	3.6	0.570	C = 199	–	–
SCRI_RS_228486	A/G	3H	93.23	7.6E-04*	3.4	−0.326	A = 149	–	–
SCRI_RS_7704	T/C	4H	26.35	1.0E-04*	4.6	0.573	C = 266	–	–
11_20606	C/G	4H	31.14	5.5E-05**	5.1	0.807	G = 207	5.0E-76	Phytanoyl-CoA dioxygenase 1
11_21070	A/C	4H	31.14	1.8E-04*	4.4	−0.681	A = 134	–	–
11_20422	C/G	4H	31.45	2.3E-06**	7.1	−0.882	C = 139	5.0E-91	Glutathione S-transferase 3
12_30864	A/G	4H	38.44	2.6E-04*	4.1	0.521	G = 217	1.0E-53	Phytochrome A
SCRI_RS_145412	T/C	4H	42.89	1.6E-05**	6.4	0.638	T = 236	1.0E-53	KNOTTED-1-like homeobox protein d (*knox1d*)
12_11063	A/C	4H	53.87	7.8E-04*	3.4	−0.725	C = 314	–	–
SCRI_RS_237565	A/C	6H	1.4	9.9E-04	3.3	−0.382	C = 262	–	–
12_20803	A/G	7H	28.98	2.4E-04*	4.1	−0.628	A = 285	4.0E-14	IQ-Domain-1

[a] CH-Chromosome.
[b] *, ** are significant SNP markers after FDR correction at 0.05 and 0.01 probability levels.
[c] E-value is the expected hits to find homology of SNP sequence to the given gene(s) in BLAST search.
[d] Physical position of the marker.
[e] Blast search either resulted into predicted protein or did not find any significant alignment.

annotation using SNP sequences in BLAST search was presented in Table S4. A diverse set of genes with known functions that were homologous to SNP sequences were reported while many of others SNP sequences had homology to several predicted proteins of unknown function.

4. Discussion

Among the graminaceous cereals, barley hosts several genes involved in the mobilization and uptake of Fe and Zn (*NAS*, *NAAT*, *DMAS*, *IDS2*, and *IDS3*), which other mega crops such as rice lack (Nakanishi et al., 2000; Takahashi et al., 1999). Higher levels of Zn and Fe were reported in barley landraces in a collection of ICARDA germplasm by El-Haramein and Grando (2010). In the study presented here, 336 barley genotypes of diverse origins, representing advanced lines, released cultivars, and landraces, were evaluated for the contents of Fe and Zn and 11 other elements from two large experiments in Morocco. We found Zn contents to range from 19.8 to 54.5 mg kg^{-1}, and Fe contents from 37.0 to 90.0 mg kg^{-1}. Our observations are in agreement with Ma et al. (2004), who reported Fe contents in barley grains of 24.6–63.3 mg kg^{-1} in Japanese barley and 21.0–83.0 mg kg^{-1} in American barley. Dick et al. (1985) reported a similar range of Fe (32.5–90.0 mg kg^{-1}) and Zn (19.2–53.5 mg kg^{-1}) concentration in the grains of Canadian barley cultivars which is no departure from this study. However, Wu and Zhang (2002) reported higher Fe (50.05–114.53 mg kg^{-1}) and Zn (35.76–76.10 mg kg^{-1}) concentrations in the barley grains of eight cultivars in China. Higher Zn concentrations in the barley grains were reported by Hussain et al. (2016) and Sadeghzadeh et al. (2015) in a double haploid population of Clipper x Sahara. Higher variability of Fe and Zn concentrations in barley grains of landrace and wild barley are expected. Mamo et al. (2014) found higher variability in Fe (27.26-109.60 mg kg^{-1}) and Zn (19.69–87.42 mg kg^{-1}) concentrations in the barley grains of landraces originated from Ethiopia. In contrast, higher variabilities in Fe and Zn contents were reported by Yan et al. (2012), who found that Fe ranged from 10.8 to 329.1 mg kg^{-1} and Zn from 66.3 to 493.9 mg kg^{-1} among 92 wild genotypes (*H. spontaneum*) originating from Israel.

We validated the GWAS approach by mapping loci responsible for row type (fertility of lateral spikelets and tillering) in barley. Cuesta-Marcos et al. (2010) and Ramsay et al. (2011) reported that the *INTERMEDIUM-C* (*INT-C*) gene mapped in the 4HS chromosome plays vital role in fertility of lateral spikelets on the inflorescence and tillering in barley. Ramsay et al. (2011) reported that *INT-C*, which is an ortholog of the maize domestication gene *TEOSINTE BRANCHED 1 (TB1)*, modulated the phenotypes imposed by *VRS1* alleles in barley. They reported that two SNPs 11_20606 and 11_20422 at 4HS significantly correlated with INT-C in barley. Mamo et al. (2014) also reported at least one of the SNPs, 11_20606, correlated significantly with row types in GWAS. In the current study, 3 SNPs, 11_20606, 11_21070, and 11_20422, were significantly ($q < 0.05$) correlated with row types (fertility of lateral spikelets), including three other SNPs in the 4HS chromosome at the span of 38.44–53.87 cM. Likewise, Cuesta-Marcos et al. (2010) reported that row types in barley were modulated by the interactions in *VRS1* and several alleles present in the 1H, 2H, 4H, and 5H chromosomes. Among them, we found at least two alleles reported by Cuesta-Marcos et al. (2010) in 1HL and 4HS to be highly correlated with row types in our study. The gene annotation using the BLAST search (Table 2) revealed the four most significant SNPs: 11_20606, 11_20422, 12_30864, and SCRI_RS_145412 are genes orthologous to rice LOC_Os03g50040 (Phytanoyl-CoA dioxygenase), LOC_Os03g50130 (microsomal glutathione S-transferase 3) (Ramsay et al., 2011), a gene orthologous to wheat and *Brachypodium distachyon* LOC100836209 (Phytochrome A), and a gene orthologous to wheat KNOTTED-1-like homeobox protein d (*knox1d*). These results validate that the GWAS approach employed for row types and element contents in the grain of barley was effective in mapping QTL. An additional locus in the 1H chromosome at 100.45 cM (SCRI_RS_6913) was detected on the same map location of SCRI_RS_171501 and two loci were found on the 3H chromosome at 82.19 (SCRI_RS_826) and 93.23 cM (SCRI_RS_93.23). In 1H, SCRI_RS_171501 was an orthologous to *Brachypodium distachyon* LOC100846300 (MATH domain-containing protein like At5g43560). These additional loci may also play important roles in modulating the fertility of lateral spikelets in barley.

In this study, 45 QTL were mapped in barley chromosomes for 13 element contents in barley grains. Our study is much more

Table 3
Significant SNP-element content association analyzed for barley grain in Jemma Shiam and Marchouch stations in Morocco.

QTL	[a] Element -Location	SNP ID	[b] SNP	[c] CH	cM	[d] *P*-value	R^2	[e] Additive Effect	[f] Allele Freq.
Ba-3H-15.52	Ba-JS	11_20595	A/C	3H	15.52	8.6E-04*	3.6	0.66	C = 143
Ba-3H-8.5	Ba-JS	SCRI_RS_193258	A/C	3H	8.5	4.4E-04*	4	0.64	A = 172
Ba-3H-8.5	Ba-JS	SCRI_RS_230638	T/G	3H	8.5	5.3E-04*	3.9	0.67	T = 177
Ba-4H-112.36	Ba-JS	SCRI_RS_222936	T/C	4H	112.36	3.2E-04*	4.1	0.73	C = 163
Ba-5H-90.22	Ba-JS	11_20805	A/G	5H	90.22	8.6E-05**	4.9	−0.97	A = 261
Ba-5H-90.22	Ba-JS	SCRI_RS_13960	T/C	5H	90.22	1.1E-05**	6.1	−0.89	C = 170
Ba-5H-90.22	Ba-JS	SCRI_RS_165215	T/C	5H	90.22	8.9E-07**	7.7	−1.04	C = 196
Ba-5H-95.49	Ba-JS	SCRI_RS_224346	T/C	5H	95.49	2.3E-05**	5.7	−1.06	C = 263
Ba-5H-95.9	Ba-JS	SCRI_RS_157181	C/G	5H	95.9	2.3E-05**	5.7	−1.02	G = 260
Ca-2H-126.56	Ca-JS	SCRI_RS_219799	T/C	2H	126.56	9.0E-04*	3.5	−42.17	C = 219
[g] *E-3H-153.39*	Ca-JS	12_30736	A/G	3H	153.39	7.9E-04*	3.5	40.99	A = 203
Cu-6H-50.61	Cu-JS	SCRI_RS_150028	T/C	6H	50.61	8.3E-04*	3.6	−0.66	C = 227
Cu-6H-52.2	Cu-JS	11_10244	A/T	6H	52.2	4.8E-04*	3.9	0.68	A = 94
Cu-6H-58.91	Cu-JS	12_11140	A/G	6H	58.91	9.3E-04*	3.6	−0.77	A = 83
[g] *E-3H-66.15*	Cu-JS	11_10813	A/G	3H	66.15	7.6E-04*	3.7	0.7	A = 64
[g] *Fe-2H-114.24*	Fe-JS	SCRI_RS_708	A/G	2H	114.24	8.2E-04*	3.5	3.44	G = 116
Fe-2H-117.39	Fe-JS	SCRI_RS_168451	C/G	2H	117.39	3.7E-04*	4	3.58	C = 134
Fe-2H-139.62	Fe-JS	12_30097	C/G	2H	139.62	1.6E-04*	4.5	−5.86	G = 291
[h] ***Fe-2H-84.74***	Fe-JS	12_11388	A/G	2H	84.74	2.8E-04*	4.2	−7.36	G = 291
Fe-3H-83.63	Fe-JS	SCRI_RS_234564	A/G	3H	83.63	5.1E-04*	3.8	−3.71	A = 144
Fe-4H-67.91	Fe-JS	12_20143	A/G	4H	67.91	7.6E-04*	3.6	−4.03	G = 115
E-4H-54.95	Fe-MCH	12_31462	C/G	4H	54.95	1.10E-03	3.3	−7.59	C = 224
Fe-1H-54.5	Fe-MCH	SCRI_RS_145305	A/C	1H	54.5	6.9E-04*	3.5	−7.47	A = 216
Fe-1H-57.85	Fe-MCH	SCRI_RS_237999	A/G	1H	57.85	9.7E-05**	4.6	−8.94	A = 215
Fe-1H-86.54	Fe-MCH	SCRI_RS_181300	A/G	1H	86.54	3.9E-04*	3.8	−7.17	G = 241
Fe-1H-87.87	Fe-MCH	SCRI_RS_168562	C/G	1H	87.87	2.1E-04*	4.2	−7.27	G = 229
Fe-1H-90.04	Fe-MCH	SCRI_RS_189168	A/G	1H	90.04	7.3E-04*	3.5	6.15	G = 198
[h] ***Fe-2H-84.74***	Fe-MCH	12_11388	A/G	2H	84.74	7.3E-04*	3.5	−12.9	G = 294
Fe-4H-53.87	Fe-MCH	11_10946	A/C	4H	53.87	1.9E-04*	4.2	−7.18	C = 213
Fe-4H-53.87	Fe-MCH	12_30684	A/G	4H	53.87	1.1E-04*	4.6	−7.5	A = 215
Fe-4H-53.87	Fe-MCH	SCRI_RS_157396	T/C	4H	53.87	1.9E-04*	4.2	−7.18	C = 213
Fe-4H-53.87	Fe-MCH	SCRI_RS_170494	A/C	4H	53.87	3.7E-04*	3.9	−6.57	C = 199
Fe-4H-53.87	Fe-MCH	SCRI_RS_171142	T/C	4H	53.87	1.1E-04*	4.6	−7.5	C = 214
Fe-6H-102.03	Fe-MCH	12_31115	A/G	6H	102.03	5.5E-05**	5	−7.18	G = 162
Fe-7H-17.62	Fe-MCH	SCRI_RS_222330	T/C	7H	17.62	9.7E-04*	3.3	−6.41	C = 201
[g] *E-3H-153.39*	K-JS	12_10629	A/C	3H	153.39	4.3E-04*	3.9	−424.11	A = 284
K-1H-131.94	K-JS	SCRI_RS_132472	T/G	1H	131.94	5.5E-07**	8.1	−408.43	T = 218
K-5H-95.65	K-JS	11_10477	A/G	5H	95.65	7.1E-04*	3.6	−266.63	A = 184
K-5H-97.35	K-JS	SCRI_RS_44800	A/G	5H	97.35	5.8E-04*	3.7	−270.8	G = 179
K-5H-99.93	K-JS	SCRI_RS_149479	T/C	5H	99.93	7.1E-04*	3.6	−266.75	T = 181
[g] *E-3H-66.15*	Mg-JS	11_10813	A/G	3H	66.15	4.0E-04*	4	97.07	A = 64
[g] *E-7H-1*	Mg-JS	BOPA2_12_21479	A/G	7H	[i] 58706384-58706385	3.3E-04*	4.1	−103.01	A = 258
[g] *E-7H-95.02*	Mg-JS	11_21201	A/G	7H	95.02	6.5E-04*	3.7	−91.63	A = 252
K-7H-86.56	Mg-JS	BOPA1_3568-149	A/G	7H	86.58	7.1E-04*	3.6	−110.77	A = 285
Mg-2H-66.61	Mg-JS	SCRI_RS_6792	T/C	2H	66.61	8.2E-04*	3.6	−67.06	T = 194
[g] *E-7H-1*	Mn-JS	BOPA2_12_21479	A/G	7H	[i] 58706384-58706385	1.1E-04*	4.9	−1.61	A = 258
[g] *E-7H-95.02*	Mn-JS	11_21201	A/G	7H	95.02	1.3E-04*	4.8	−1.53	A = 252
Mn-3H-74.41	Mn-JS	SCRI_RS_202772	A/G	3H	74.41	4.8E-04*	3.8	1.13	G = 186
Mn-4H-60.70	Mn-JS	11_10846	C/G	4H	60.72	8.0E-04*	3.6	−1.42	C = 271
Mn-7H-82.16	Mn-JS	11_20880	A/G	7H	82.16	1.2E-04*	4.7	−1.44	A = 232
Mn-7H-82.16	Mn-JS	12_30998	A/G	7H	82.16	1.2E-04*	4.7	−1.44	A = 232
Mn-7H-85.87	Mn-JS	12_30301	A/G	7H	85.87	8.2E-04*	3.5	−2.03	A = 293
Mn-7H-91.73	Mn-JS	12_11044	A/C	7H	91.73	6.3E-05**	5.2	−1.75	C = 273
[g] *E-1H-1*	Na-JS	SCRI_RS_185604	A/G	1H	[i] 550548357-550548566	4.8E-04*	3.8	333.53	G = 287
[g] *E-3H-66.15*	Na-JS	11_10813	A/G	3H	66.15	1.1E-04*	4.8	328.43	A = 64
[g] *E-5H-44.99*	Na-JS	11_11221	A/C	5H	44.99	9.8E-05**	4.8	312.62	A = 112
Na-5H-1.12	Na-JS	SCRI_RS_137053	T/C	5H	1.12	3.7E-04*	4	45.91	C = 251
Na-7H-82.89	Na-JS	SCRI_RS_230083	A/G	7H	82.89	6.0E-04*	3.7	50.15	A = 64
[g] *E-4H-111.97*	Se-JS	SCRI_RS_140963	T/C	4H	111.97	4.0E-04*	3.9	−0.16	C = 212
[g] *E-7H-95.02*	Si-JS	11_21201	A/G	7H	95.02	9.8E-04*	3.4	58.73	A = 252
Si-4H-103.38	Si-JS	SCRI_RS_148330	A/T	4H	103.48	2.0E-04*	4.4	−78.14	T = 279
Si-4H-103.38	Si-JS	SCRI_RS_157611	T/C	4H	103.38	2.0E-04*	4.4	−78.14	C = 279
Si-4H-88.84	Si-JS	SCRI_RS_107010	T/C	4H	88.84	5.8E-04*	3.7	−62.92	C = 256
Si-5H-185.05	Si-JS	SCRI_RS_239569	T/C	5H	185.05	2.9E-04*	4.1	−57.58	T = 129
Si-6H-55.11	Si-JS	SCRI_RS_168964	A/G	6H	55.11	5.3E-04*	3.8	55.34	G = 86
Si-7H-1	Si-JS	BOPA2_12_30790	A/G	7H	[i] 496253212-496253213	5.8E-04*	3.7	98.91	G = 298
Si-7H-81.07	Si-JS	11_10069	C/G	7H	81.07	2.7E-05**	5.6	88.79	C = 273
Si-7H-81.07	Si-JS	11_10673	A/G	7H	81.07	2.2E-05**	5.7	71.39	A = 224
Si-7H-86.58	Si-JS	BOPA1_3568-149	A/G	7H	86.58	3.5E-04*	4	77.89	A = 285
Si-7H-91.09	Si-JS	SCRI_RS_4562	A/C	7H	91.09	1.6E-04*	4.5	66.01	A = 252
[g] *E-1H-1*	S-JS	SCRI_RS_185604	A/G	1H	[i] 550548357-550548566	5.0E-04*	3.8	108.07	G = 287
[g] *E-3H-66.15*	S-JS	11_10813	A/G	3H	66.15	7.2E-04*	3.7	95.14	A = 64

Table 3 (*continued*)

QTL	[a] Element -Location	SNP ID	[b] SNP	[c] CH	cM	[d] *P*-value	R^2	[e] Additive Effect	[f] Allele Freq.
[g] *E-4H-111.97*	S-JS	SCRI_RS_140963	T/C	4H	111.97	4.3E-04*	3.9	83.19	C = 212
S-1H-50	S-JS	11_10985	A/C	1H	50	3.5E-04*	4.1	117.91	C = 233
S-4H-117.13	S-JS	SCRI_RS_192456	T/C	4H	117.13	9.1E-04*	3.5	−82	C = 235
S-4H-99.08	S-JS	SCRI_RS_226787	A/G	4H	99.08	8.6E-04*	3.5	84	A = 153
[g] *E-3H-66.15*	Zn-JS	11_10813	A/G	3H	66.15	5.8E-04*	3.7	2.38	A = 64
[g] *E-5H-44.99*	Zn-JS	11_11221	A/C	5H	44.99	7.9E-04*	3.5	2.23	A = 112
Zn-4H-1.13	Zn-JS	SCRI_RS_126417	T/G	4H	1.13	8.5E-04*	6.2	3.51	G = 151
Zn-4H-1.13	Zn-JS	SCRI_RS_236981	T/C	4H	1.13	4.5E-04*	3.9	1.87	T = 229
[g] *E-4H-54.95*	Zn-MCH	12_30839	A/C	4H	54.95	1.20E-03	3.2	−3.03	A = 288
Zn-1H-21.97	Zn-MCH	11_20371	A/G	1H	21.97	1.90E-03	3	−3.69	A = 313
Zn-2H-148.16	Zn-MCH	SCRI_RS_154153	T/C	2H	148.16	1.20E-03	3.2	−2.04	C = 205
Zn-2H-40.12	Zn-MCH	SCRI_RS_123295	T/C	2H	40.12	6.8E-04*	3.6	−2.98	C = 290
Zn-2H-86.84	Zn-MCH	SCRI_RS_139193	A/G	2H	86.84	1.10E-03	3.3	−2.92	A = 192
Zn-2H-87.34	Zn-MCH	SCRI_RS_166540	A/G	2H	87.34	1.6E-04*	4.4	−3.15	A = 204
Zn-2H-87.34	Zn-MCH	SCRI_RS_2961	T/C	2H	87.34	7.0E-05**	4.9	−3.5	T = 205

[a] Letters before - presents elements while letters after - is the location of the field experiments. Elements were analyzed in barley grain collected from Jemma Shiam (JS) and Marchouch (MCH) locations in Morocco.
[b] SNP- Single Nucleotide Polymorphism.
[c] CH-Barley Chromosome.
[d] FDR correction was performed on significant SNP markers where *, ** are FDR corrections at $q = 0.05$ and $q = 0.01$ levels.
[e] Positive additive effect indicates increased element concentration whereas negative additive effects indicates deceased element concentration in barley grain.
[f] Allele Frequency contributing additive effects.
[g] QTL affecting multiple element content in barley grain.
[h] Bold faced QTL found in both Jemma Shiam and Marchouch locations.
[i] The SNP position was aligned with Morex genome sequence data base (http://floresta.eead.csic.es/barleymap/[visited August 22, 2017]) with start and ending position of SNP given.

comprehensive compared to previous studies because QTL were mapped for thirteen elements in barley grain using GWAS, with a focus on Zn and Fe. This study will help barley breeders around the world on biofortification efforts of multiple elements. A few studies have been conducted on mapping QTL of element contents in barley grains and many of them focused on Zn uptake. Mamo et al. (2014) mapped two QTL, *Zn-qtl-6H_ SCRI_RS*_10655 at 122 cM and *Zn-qtl-6H_SCRI_RS*_10655, at 128.72 cM for Zn uptake in the barley grain. Lonergan et al. (2009) reported nine QTL for Zn uptake and translocation of Zn in various plant parts, including roots, shoots, peduncles and grain. They reported that QTL in 1HS, 2HL, and 5HL were responsible for Zn translocation in barley grain, which is confirmed by our study. We found three QTL, *Zn-1H-21.97* and *Zn-2H-87.34*, and E-5H-44.99, involved in Zn uptake in the barley grain. Sadeghzadeh et al. (2010) mapped QTL on the 2HS chromosome for Zn uptake in grain using a bi-parental mapping population (Double Haploid of 'Clipper' [low Zn] x 'Sahara 3771' [high Zn]). They mapped *SZn-R1* at 1.7 cM of the 2H chromosome, which was different than the QTL *Zn-2H-87.34* mapped in our study in the same chromosome. In addition, recently Sadeghzadeh et al. (2015) mapped additional Zn QTL in 2HL, 3H, and 4H, using the same bi-parental mapping population (Clipper x Sahara 3771). Among the QTL mapped by Sadeghzadeh et al. (2015), two QTL mapped in 3H and 4H coincided with the QTL *E-3H-66.15* and *E-4H-54.95* mapped in our study, although *E-4H-54.95* was not significant at $q < 0.05$ FDR. Likewise, Reuscher et al. (2016) reported 25, 16 and 5 QTL for Zn, Fe and Cd accumulation in barley. Our results are consistent with Lonergan et al. (2009) while QTL reported by Mamo et al. (2014) were different. The GWAS panel used by Mamo et al. (2014) included only landraces from Ethiopia and Eritrea, while our mapping panel comprised of mostly advanced breeding lines of ICARDA barley breeding program, including cultivars released in several countries, and a few landraces which originated from Nepal, India, Mongolia, Morocco, and Central Asia. A wide variety of genes were annotated using the sequences of significant SNP markers in the BLAST search. The gene annotation reported in our study is highly valuable to our understanding of complex element uptake and translocation mechanisms in plants.

5. Conclusions

The major goal of our study was to identify and map QTL involved in uptake and translocation of elements in barley grains with, a focus on identifying Zn and Fe dense barley genotypes, so that this germplasm would be available to barley researchers across the globe through ICARDA networks. Currently, several countries such as Nepal, India, Tunisa, Morocco, Ethiopia, and others have directly utilized the AM-2014 in their breeding programs. Therefore, information on barley genotypes capable of higher element uptake in the grain and the markers (SNPs) associated with the QTL is invaluable for future barley breeding programs. The utility of the GWAS panel is more successful compared to previous approaches using wild barley, because QTL mapping in barley chromosomes was achieved using advanced breeding lines and released cultivars. Therefore, less linkage drag is expected in using this germplasm for bio-fortification of important elements such as Zn and Fe. The barley germplasm used in the AM-2014 panel and the SNP markers associated with the QTL for element uptake are publicly available through the barley breeding programs of ICARDA upon request. The information generated by our GWAS is highly useful to barley breeders across the globe for marker-assisted selection and genomic selections for dense micronutrients in the barley grains. Currently, three crosses Rihane-03 x Zanbaka, Alanda-01 x Zanbaka, and Rihane-03 x Alanda-01, were made in ICARDA to study and map Zn and Fe contents in barley grains by utilizing information in genotypes identified in this study. Double haploid populations were produced from these crosses to validate QTL detected in this study. Genotyping and phenotyping of these double haploid populations are on progress in ICARDA. Barley breeding has been initiated to recombine high Fe and Zn concentrtions with higher β-Glucan accumulating genotypes obtained from the USDA.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2017.08.019.

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