

# Genome-wide association studies uncover candidate genes for Zinc, Iron, and Selenium concentration in barley grains

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## Abstract

## Background

Barley (*Hordeum vulgare* L.) is one of the most important staple food crops worldwide. Mineral concentrations in cereals are important for human health; hence improving Zn, Fe and Se accumulation in grains is an imperative need. This study was designed to understand the genetic architecture of Zn, Fe and Se grain concentrations in barley grains.

## Results

We performed a genome-wide association study (GWAS) for grain Zn, Fe and Se concentrations in 216 spring barley accessions, using field data from 2 years. All the accessions were genotyped with a high-density 9K SNPs array from IlluminaTM. The mean values of estimated BLUEs for Zn, Fe and Se were 38.37, 35.56 and 39.45  $\mu\text{g g}^{-1}$  dry weight, respectively. High heritability was equaled 75.65% for Fe across the two environments, while moderate heritability values were detected for Zn and Se. Notably, wide genetic variation was found among genotypes for Zn, Fe and Se concentrations. A total of 222 SNPs associated with Zn, Fe and Se were detected on all chromosomes, where the highest significant associations is linked to Fe accumulation. Three genomic regions include newly identified putative candidate genes, which are related to Zn uptake and transport or represent Homeobox leucine zipper protein. Additionally, several significant associations were physically located inside or near genes which are potentially involved in Zn and Fe homeostasis of which two candidate genes at 5H (502,454,312–502,455,148 bp) and 7H (205,216,091–205,221,133 bp) were found to be involved in Basic helix loop helix (BHLH) family transcription factor and Squamosa promoter binding-like protein, respectively.

## Conclusions

These findings provide new insights into the genetic basis of Zn, Fe and Se concentration in barley grains that in turn may help plant breeders to select high Zn, Fe and Se-containing genotypes to improve human consumption and grain quality.

## Background

Nowadays, micronutrient deficiency, also known as hidden hunger, is one of the major challenges to human health in 21<sup>st</sup> century. More than two billion people worldwide are estimated to suffer from several disorders due to Fe and Zn deficiency (Welch and Graham 2004), which affects mostly in middle- or low-income countries where staple crops are the major food source (Sands, et al. 2009); recently, the problem was also reported in developed countries and high-risk groups are women and children (Graham 2008; Pandey, et al. 2016). Micronutrient deficiency causes 5 Million. childhood death causalities every year (Lancet 2007). Therefore, a better understanding of the genetic basis of micronutrient accumulation in barley grains improves barley quality and its value for human dietary consumption.

Zinc is an essential cofactor for many enzymes that are used in the metabolism of auxin and carbohydrates, and synthesis of regulatory proteins (Cakmak 2000; Rehman, et al. 2018). Additionally, it also plays important roles in pollen development, fertilization and chlorophyll synthesis (Pandey, et al. 2006). A major consequence of Zn

deficiency provokes health risks, such as retardation of growth, excessive weight loss, diarrhea, and depression (Kambe, et al. 2014; Krishnappa, et al. 2017).

Iron (Fe) is one of the most important micronutrients that is required for multiple important cellular and physiological processes in plants (Briat, et al. 2007; Morrissey and Guerinot 2009; Rout and Sahoo 2015). Fe is necessary for the maintenance of essential processes including photosynthesis, respiration and nitrogen fixation, participating in the electron transport chain and cytochrome, therefore, important for crop production of cultivated or natural species (Soetan, et al. 2010; TANG, et al. 1990). Fe ions also act as cofactors in many enzymatic reactions and are important for several cellular activities (Briat, et al. 1995). Fe deficiency has an adverse effect on human health, such as retarding the physical growth and affecting the motoric development, leading to fatigue and low productivity (Bouis 2003; Bouis 2002). Therefore, improving Fe and Zn concentrations in the edible part of crops has positive consequences on grain yield and nutritional status as well as human health (Alomari, et al. 2018; Alomari, et al. 2019; Graham, et al. 1999).

Another essential mineral element as a nutrient for humans and animals is selenium (Se) (Schwarz and Foltz 1957). Since then, its active role as an antioxidant, anticancer, antibacterial, antiviral activity and general immune function regulator has been highlighted, while its deficiency is linked to several diseases, including hypothyroidism and osteoarthropathy (Rayman 2000). Most of selenium dietary consumption in humans is lower than the recommended dietary intake of 50–55 µg day (Rayman 2000; Schwarz and Foltz 1957). Around 0.5–1 billion people worldwide do not consume sufficient Se and are at risk of several diseases (Haug, et al. 2007; Kumssa, et al. 2015).

Genetic biofortification of staple crops, such as grains and cereals is the most feasible strategy involving plant breeding, which offers a sustainable and long-term approach to alleviate micronutrient deficiency and develop mineral-rich crop varieties that are beneficial to human health (Rawat, et al. 2013; Singh, et al. 2016).

Since accumulation of micronutrients in grains is a genetically complex trait, genome-wide association study (GWAS) is a powerful tool to detect the genetic factors underlying the natural variation in such complex traits (Alqudah, et al. 2020b; Hamblin, et al. 2011). Several studies identified QTL associated with grain Zn and Fe concentrations and efficiency that distributed on various chromosomes of wheat and wheat relatives such as 1A, 2A, 2B, 3D, 4B, 6A, 6B, and 7A (Srinivasa, et al. 2014; Tiwari, et al. 2009; Velu, et al. 2017; Xu, et al. 2012). In barley, (Hussain, et al. 2016) mapped QTLs associated with Zn remobilization from vegetative tissues into barley grains. Additionally, Pradhan, et al. (2020) detected novel QTLs in rice namely *qFe3.3* and *qFe7.3* for grain Fe and *qZn2.2*, *qZn8.3* and *qZn12.3* for Zn content using association mapping. Interestingly, a major QTL from wild emmer wheat was identified and cloned, pinpointing the chromosomal location of Gpc-B1, a gene associated with increased grain protein, Zn, and Fe contents (Uauy et al. 2006). Recently, studies on QTL mapping of Se content in lentils (Ates, et al. 2016) and rice (Norton, et al. 2010; ZHANG, et al. 2010) have been reported. PU, et al. (2014) detected five QTLs controlling Se concentration in wheat grains, on chromosomes 3D, 4A, 4D, 5B, and 7D, using two recombinant inbred lines (RILs). However, QTL mapping studies of grain selenium content of wheat and barley are rare. Therefore, it is important to dissect the genetic basis of grain Zn, Fe and Se concentrations in the grains of barley, and their further validation and utilization in marker assisted selection schemes.

Barley (*Hordeum vulgare*) is one of the most important cereal crop worldwide, ranking fourth in grain production (Food and Agriculture Organization of the United Nations (FAOSTAT), 2018). Although barley grains are used mostly as animal feed and for brewing, this species nevertheless offers considerable potential for biofortification attempts (Tricase, et al. 2018).

Extensive genetic variation, a sequenced genome (Consortium 2012), self-fertilization and diploidy render barley a genetically tractable model system to unravel natural variation and the underlying molecular mechanisms of grain micronutrient accumulation traits (Saisho and Takeda 2011; Sreenivasulu, et al. 2008).

Here, this study aims to identify causative alleles underlying the natural phenotypic variation of micronutrient grain concentrations such as Zn, Fe and Se for 216 worldwide spring barley accessions during two seasons (2019/2020) field experiments. Also, we detected 222 SNPs associated with Zn, Fe and Se elements. The annotation of candidate genes which control Zn, Fe and Se concentrations in barley grains showed their pivotal roles for human health.

## Material And Methods

### Plant material and field trials

In this study, we used a collection of 218 worldwide spring barley accessions. The collection includes 149 cultivars, 57 landraces, and 18 breeding lines. The origins of these accessions were from Europe (EU, 108), West Asia and North Africa (WANA, 45), East Asia (EA, 36), and the Americas (AM, 29). Field trials were conducted at the Experimental Station of the University of Fayoum during two seasons (2019/2020 and 2020/2021). A randomized complete block design with four replicates was chosen as test design for the trials. The trials were conducted following local practices regarding tillage and pest management. Each plot size was 2 m × 2 m with six rows spaced 0.20 m apart. Plants were grown in clayey loam soil with phosphorus ranges between 7.3–9.0 µg g<sup>-1</sup> and pH ≈ 7 across years. Barley Grain Samples Preparation and Milling The complete panel of genotypes was analyzed for each individual year. For each genotype, thousand kernel weight (TKW) was measured using with a digital weighing balance. Samples of grain were kept in an oven (UF1060plus Memmert, Germany) at 70 °C, and oven-dried samples were ground and weighed.

### Determination of Micronutrient Concentrations

Micronutrient concentration (Zn, Fe, and Se) was determined according to the method of Zulfiqar, et al. (2020). Samples of dried and milled barley grain flour were taken to be digested by di-acid (HClO<sub>4</sub>:HNO<sub>3</sub> at 3:10 v/v ratio) mixture and placed on a digestion plate (Heidolph, USA model, MR3003). Afterward, the atomic absorption spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan) was used to determine Zn, Fe, and Se concentrations in grain samples.

### Statistical Analysis

The broad-sense heritability ( $H^2$ ) was calculated using the equation:

$$VG / (VG + ((Ge / nE))$$

Where VG is the variance of the genotype, Ge represents the variance of the residual and nE is the number of years.

Analysis of variance (ANOVA) for each mineral among the years was calculated and the significant differences among genotypes and years was detected at a probability level of  $P \leq 0.05$ . Pearson correlation coefficient was used to assess the relationships among the measured parameters at p-value 0.05 (Wei and Simko 2017). Our data were curated to Residual Maximum Likelihood (REML) to analyze it in a mixed linear model (MLM). The mean value

of each element for each accession was calculated as Best Linear Unbiased Estimates (BLUEs) using lme4 package (Bates, et al. 2015).

### Genome-wide association scan underlying the studied traits

Our barley population was genotyped with a high-density 9K SNPs array from Illumina™. The physical positions of 9k iSelect Morex v2 (2019) were used in this study. This array assayed 7842 SNPs which are distributed over the whole genome. A population structure analysis revealed no population structure in this panel, regardless of biological status, geographic origin, or row type (Thabet, et al. 2020). Recently, the association analysis between SNP markers with the BLUE values for all the studied elements was performed using the FarmCPU model in the GAPIT R package. The significant SNPs are shown on the genetic map using PhenoGram Plot (<http://visualization.ritchielab.org/>). The procedure of GWAS analysis and validation is described by Alqudah, et al. (2020a).

### Candidate gene associated with the studied traits

The significant SNPs and SNP markers within the haploblock of linkage disequilibrium (LD) interval were used to detect candidate genes and identify the annotation of the high-confidence (HC) candidate gene using Morex v2 (Gene Models 2019) and the BARLEX database <https://apex.ipk-gatersleben.de/apex/f?p=284:10>.

## Results

### Description of Phenotypic Data

The overall mean and range of each trait measured in grains of the 216 barley accessions grown during both 2019 and 2020 are presented in **Table S1**. ANOVA showed the significant genotype by environment interactions for these mineral elements in the grain. A wide range of variation was observed for all of the mineral elements studied in each season (**Fig. 4, Table S2**). Normal distribution for each trait measured in all barley accessions was detected (**Fig. 1-3**). In the season 2019, the highest measured grain Zn, Fe and Se concentrations were 46.71, 51.38 and 50.60  $\mu\text{g g}^{-1}$  DW, respectively while in 2020 the highest Zn, Fe and Se values were around 48.45, 49.78 and 54.23  $\mu\text{g g}^{-1}$  DW, respectively (**Table S1**). The mean values of estimated BLUEs for Zn, Fe and Se were 38.37, 35.56 and 39.45  $\mu\text{g g}^{-1}$  DW, respectively (**Table S1**).

High heritability equaled 75.65% for Fe across the two environments indicating that the phenotypic values in the two years are relatively stable for all genotypes (**Table S2**). However, Zn and Se showed moderate heritability (30.81% and 58.52%), whereby a lot of the phenotypic variance was represented by the year and error variance components. Similar heritabilities were found by Herzig, et al. (2019) for grain elements in a wild barley NAM population.

The Pearson's correlation measured for all mineral traits among the growing environments and BLUEs, are presented in **Fig. 5**. The highest correlation was found between Fe\_BLUEs and Fe\_2019 and Fe\_2020 ( $r = 0.88^{***}$  and  $0.92^{***}$ ), respectively. A significant positive correlation was found between Zn\_BLUEs and Zn\_2019 and Zn\_2020 ( $r = 0.73^{***}$  and  $0.81^{***}$ ), respectively. Moreover, highly positive correlation was between Se\_BLUEs and Se\_2019 and Se\_2020 ( $r = 0.83^{***}$  and  $0.85^{***}$ ), respectively. On the other hand, moderate positive correlation was between Zn\_BLUEs and Se\_2019, Se\_2020 and Se\_BLUEs ( $r = 0.36^{**}$ ,  $0.41^{**}$  and  $0.46^{**}$ ), respectively. Interestingly, negative correlation was found between Zn\_BLUEs and Fe\_2020 and Fe\_BLUEs ( $r = -0.02$  and  $-0.01$ ), respectively.

## Genetic analysis and Genes underlying minerals in Barley

GWAS analysis for the whole panel identified a total of 222 SNPs as significantly associated with three elements, including Zn, Fe and Se considering thresholds of  $(-\text{Log}_{10}(p) \geq 3.0)$  for each individual year in addition to BLUEs (**Table S3**). The highest number of associated SNPs for all the studied traits were located on chromosomes 2H (89 SNPs), followed by 5H (36 SNPs), 4H and 7H (21 SNPs each), 1H and 6H (20 SNPs each), 3H (15 SNPs), (**Table S3**).

Of these significant SNP markers, the highest number of associated markers was detected for Se (167 SNPs), followed by Fe (43 SNPs) and Zn (13 SNPs), which were present in both environments plus BLUEs.

Exclusively, the associated genomic regions for all the studied traits were classified into three regions based on the linkage disequilibrium of multi-traits associated marker that located on chromosomes 3H, 5H and 7H. For instance, at 3H BOPA1\_6402-691 SNP (578,601,859 bp) was associated with Zn\_BLUEs, Se\_2019 and Se\_BLUEs by three SNPs. In addition, BOPA1\_2251-643 SNP at 7H (206,751,899 bp) was associated with Se and Zn\_BLUEs by two SNPs (**Table S5**).

The QQ plots for SNP results revealed that the distribution of observed association p-values were close to the distribution of expected associations (**Fig. 6-8**); that means the FarmCPU model which we implemented for GWAS was sufficiently stringent to control for false positive associations.

The gene content of the three genomic regions on chromosomes 3H, 5H and 7H harbors 394 potential candidate genes or proteins including TFs and transporter proteins that found to be highly associated with Zn, Fe and Se among the growing environments and BLUEs (**Table S6**). The first region located on chromosome 3H and harbors two important candidate genes; *HORVU.MOREX.r2.3HG0258450* that annotated as Selenium-binding protein at (578,710,262- 578,713,489 bp) and *HORVU.MOREX.r2.3HG0258460* that encodes 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (2-ODDs) at (578,715,293-578,716,369 bp). The second important region located on chromosome 5H harbors four candidate genes that regulated the variation of all the studied minerals including Zn, Fe and Se in barley grains. The most prominent multifunctional gene *HORVU.MOREX.r2.5HG0411320* at (500,037,722-500,044,141bp) annotated as An ATP-dependent zinc metalloprotease, FtsH. Interestingly, on chromosome 7H (205,216,091-205,221,133 bp), Squamosa promoter binding-like protein (*SPL*) gene family is detected that explain the variation of Zn and Se concentration in barley grains (**Table 1**).

## Discussion

Genetic fortification strategies are highly suitable for developing barley genotypes with high mineral element contents. Therefore, this study focused on investigating the natural genetic variation in 216 spring barley accessions and on identifying candidate genes contributing to mineral accumulation in barley grains. Phenotypic analysis for mineral concentrations including Zn, Fe and Se, showed a wide variation between the genotypes based on BLUEs that equaled 38.37, 35.56 and 39.45  $\mu\text{g g}^{-1}$  DW, respectively. Our findings agree with Herzig, et al. (2019) for grain elements in a wild barley. High heritability equaled 75.65% for Fe across the two environments indicating that the major part of the variability was due to genotypic effects, which is in agreement with previous studies (Garcia-Oliveira, et al. 2009; Peleg, et al. 2009; VREUGDENHIL, et al. 2004). However, Zn and Se showed moderate heritability (30.81% and 58.52%), whereby a lot of the phenotypic variance was represented by the year and error variance components. Similar heritabilities were found by Herzig, et al. (2019) for grain elements in a wild barley

NAM population. Very strong significant Pearson's correlation was detected between the two seasons indicating that the phenotypic measurements were quite stable in the different years for all the studied traits. Similar results were reported by (Alomari, et al. 2018; Alomari, et al. 2019) for Zn and Fe in wheat cultivars. The ANOVA results indicated that genotypes and environmental factors have a significant effect on mineral concentration including Zn, Fe and Se in barley grains. A similar conclusion was reached by (Alomari, et al. 2018; Alomari, et al. 2019) for grain Zn and Fe accumulation in wheat cultivars.

## Candidate Genes

Candidate genes were identified for the most effective markers that had associations with more than one trait. Based on GWAS analysis, 222 significant associations were underlying grain Zn, Fe and Se which distributed on all chromosomes. Exclusively, 394 potential candidate genes were discovered on chromosome 3H, 5H and 7H that found to be highly associated with Zn, Fe and Se among the growing environments and BLUEs. The first region located on chromosome 3H and harbors two important candidate genes that explained the variation of Zn and Se\_BLUEs. The first candidate at 3H is *HORVU.MOREX.r2.3HG0258450* that annotated as Selenium-binding protein, is a ubiquitously expressed protein that can bind selenium (Se) specifically. SBP also plays essential roles in senescence, the stress response, cellular differentiation, protein transport, and the ubiquitinating/deubiquitinating pathways (Zhao and Castonguay 2015).

The second gene is *HORVU.MOREX.r2.3HG0258460* that encodes 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (2-ODDs), are non-heme proteins that are ubiquitously distributed throughout nature (de Carolis and de Luca 1994; Martens, et al. 2010). 2-ODDs are dependent on ferrous iron as a co-factor for the binding of molecular oxygen and subsequent oxidative reactions in plant metabolism. The landmark discovery of the 2-ODDs involved in epigenetic regulation, and others catalyzing several characteristic steps in specialized metabolic pathways (Farrow and Facchini 2014). Furthermore, 2-ODDs catalyses numerous oxidative reactions including hydroxylations, halogenations, desaturations, ring closure, ring cleavage, epimerization, rearrangements, halogenation, re-arrangements, demethylations, and demethylenations (Clifton, et al. 2006; Flashman and Schofield 2007; Loenarz and Schofield 2008; Tarhonskaya, et al. 2014). This impressive list of oxidative reactions reveals that 2-ODDs are among the most versatile and important oxidizing biological catalysts (Flashman and Schofield 2007).

The second important region located on chromosome 5H harbors four candidate genes that regulated the variation of all the studied minerals including Zn, Fe and Se in barley grains. The most prominent multifunctional gene *HORVU.MOREX.r2.5HG0411320* at (500,037,722-500,044,141bp) annotated as An ATP-dependent zinc metalloprotease, FtsH, is the major thylakoid membrane protease. FtsHs in the thylakoid membranes of *Arabidopsis thaliana* form a hetero-hexameric complex consisting of two types of FtsH subunits: type A (FtsH1 and FtsH5) and type B (FtsH2 and FtsH8) (Sakamoto, et al. 2003; Yu, et al. 2005; Yu, et al. 2004). A plethora of studies have identified the critical roles of FtsH in the biogenesis of thylakoid membranes and quality control in the photosystem II repair cycle. Furthermore, the involvement of FtsH in the degradation and assembly of several protein complexes in the photosynthetic electron-transport pathways (Kato and Sakamoto 2018). Substantial proportions of the cellular quota of the micronutrient metals copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn), are allocated to proteins acting in plant photosynthesis as well as the mechanisms involved in their homeostasis within chloroplasts, which places these metals at the core of plant energy metabolism and highlights their importance for plant-specific biochemistry (Yruela 2013).

In the same genetic region, two candidate genes are coding transcription factors; *HORVU.MOREX.r2.5HG0411850* that encodes Protein FAR1-RELATED SEQUENCE 5 and *HORVU.MOREX.r2.5HG0412340* that encodes Basic helix

loop helix (BHLH). Both FAR1 and BHLH were shown to explain the variation of Zn, Fe and Se, implying that they attributed to improving grain mineral accumulation. FAR-RED ELONGATED HYPOCOTYLS3 (FHY3) and its homologue FAR-RED IMPAIRED RESPONSE1 (FAR1) encode transposase-derived transcription factors (Lin, et al. 2007; Wang and Deng 2002). Recent studies have demonstrated that FHY3 and FAR1 play multiple roles in a wide range of cellular processes, including light signal transduction (Lin, et al. 2007), circadian clock and flowering time (Li, et al. 2011), floral development (Li, et al. 2016), chloroplast division (Ouyang, et al. 2011), chlorophyll biosynthesis (Tang, et al. 2012), starch synthesis (Ma, et al. 2017), abscisic acid responses (Tang, et al. 2013), and plant immunity (Wang, et al. 2016), indicating that FHY3 and FAR1 have crucial functions in plant growth and development.

Basic helix loop helix (BHLH) family transcription factor are a superfamily of transcription factors that are important regulatory components in transcriptional networks in plants (Tanabe, et al. 2019). The bHLH transcription factor FER, a crucial regulator of iron uptake responses in root, was first identified from the analysis of the tomato *fer* mutant (Ling, et al. 2002). Furthermore, Tanabe, et al. (2019) characterized bHLH11 as a negative regulator of Fe homeostasis. The expression of FIT, a master regulator of Fe deficiency responses, was suppressed in the transgenic plants, indicating that the transcriptional repressor bHLH11 functions as a negative regulator of FIT-dependent Fe uptake and modulates Fe levels in Arabidopsis. Zheng, et al. (2010) reported that the Fe-regulated bHLH transcription factor, *OsIRO2* acts as a negative regulator of the Fe deficiency response in rice. Recent studies also reported that bHLH was found in promoter regions of all *TaMTPs* (metal tolerance proteins) in common wheat, which are involved in trace metal homeostasis and have a potential role in cereal grain biofortification with essential micronutrients including Zn (Menguier, et al. 2018; Vatansever, et al. 2017). These findings declared the potentiality of this cluster of genes in conferring mineral accumulation in barley. Besides, they are pleiotropic genes thus; a selection for the region harboring them can improve many traits at once.

The last important candidate is a homeobox-leucine zipper protein HOX4 that annotated as *HORVU.MOREX.r2.5HG0413150* at (505,319,272- 505,325,405 bp) was found to be associated with grain Zn, Fe and Se concentrations in the used panel. Similar results were found by Alomari, et al. (2018) who reported that *TaHDZIP1* was found to be associated with grain Zn concentrations in the wheat cultivars. A total of 187 *TabZIP* genes have been identified in wheat (Li, et al. 2015) and a specific group of *TabZIP* genes such as *bZIP19* and *bZIP23* were shown to regulate the adaption to Zn deficiency in roots (Assunção, et al. 2010; Inaba, et al. 2015).

Interestingly, on chromosome 7H (205,216,091-205,221,133 bp), Squamosa promoter binding-like protein (*SPL*) gene family is one of the plant-specific putative transcription factor related to plant development and each member shares a highly conserved 76 amino acid residue SBP-domain (Birkenbihl, et al. 2005; Klein, et al. 1996). The SBP domain consists of three functionally important motifs, including two Zn-finger like structures formed by conserved cysteine and histidine residues (Yamasaki, et al. 2004). In the current study *SPL* genes controlled the variation of Zn and Se content, this agrees with other studies, where Arabidopsis Squamosa promoter binding Protein-Like genes act together in the regulation of transition metal homeostasis including Cu and Zn (Schulten, et al. 2019). Furthermore, in rice, *OsSPL13* positively regulates cell size in the grain hull, resulting in enhanced grain length and yield (Si, et al. 2016). *OsSPL14* enhances shoot and panicle branching, leading to increases in grain productivity (Jiao, et al. 2010). *OsSPL16* promotes cell division and grain filling, with positive consequences for grain size, shape, and quality (Wang, et al. 2012). Cao, et al. (2019) hypothesized that the homologous gene of rice *OsSPL16* in bread wheat had functions in regulation of wheat grain size and yield. Interestingly, Thabet, et al. (2021) identified many candidate genes that were found to be linked to salinity stress tolerance during seedling developmental phase as a first time such as *Squamosa promoter-binding-like protein 6* at chromosome 5H for the

same barley population. These studies have indicated that SPL genes play crucial roles in regulation of plant development and yield-related traits in cereal crops.

Altogether, our results indicate potential genomic regions controlling Zn, Fe and Se in barley that can be used in further genetic investigations.

## Conclusion

This study discovered genetic factors controlling grain Zn, Fe and Se accumulation in barley that may establish the basis for further breeding and genetic work in cereals. A wide range of variation was observed for all of the mineral elements studied in each season. Broad-sense heritability calculation revealed high and moderate variation that could be attributed to both genetic and environmental effects. Three genomic regions harbor many putative candidate genes like HOX4 and SPL genes which are proposed as candidates conferring enhanced grain Zn, Fe and Se concentrations. Further validation and functional characterization are required to elucidate the role of these genes for mineral homeostasis in barley.

## Abbreviations

**Zn:** Zinc

**Fe:** Iron

**Se:** Selenium

**BLUEs:** Best Linear Unbiased Estimates

**GWAS:** Genome Wide Association Study

**FarmCPU:** Fixed and random model Circulating Probability Unification

## Declarations

### Availability of data and materials

All data generated during the study are interpreted in the manuscript.

### Funding

Not applicable.

### Author Contributions

SGT and AMA designed the experiment; AMA analyzed data; SGT wrote the paper. DZA helped in candidate gene analysis. SGT and AMA conceived the idea and participated in the interpretation of results. All authors read and approved the final manuscript.

### Corresponding author

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## Ethics declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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## Tables

**Table 1 The list of candidate genes based on the linkage disequilibrium of multi-traits associated marker.**

Genomic region	Gene	Chr	Start	End	Gene length	Annotation
1	<i>HORVU.MOREX.r2.3HG0258450</i>	3	5.79E+08	5.79E+08	3228	Selenium-binding protein
1	<i>HORVU.MOREX.r2.3HG0258460</i>	3	5.79E+08	5.79E+08	1077	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
2	<i>HORVU.MOREX.r2.5HG0411320</i>	5	5E+08	5E+08	6420	ATP-dependent zinc metalloprotease FtsH
2	<i>HORVU.MOREX.r2.5HG0411850</i>	5	501,094,176	501,094,607	432	Protein FAR1-RELATED SEQUENCE 5
2	<i>HORVU.MOREX.r2.5HG0412340</i>	5	502,454,312	502,455,148	837	Basic helix loop helix (BHLH) family transcription factor
2	<i>HORVU.MOREX.r2.5HG0413150</i>	5	505,319,272	505,325,405	6134	Homeobox leucine zipper protein
3	<i>HORVU.MOREX.r2.7HG0567820</i>	7	2.05E+08	2.05E+08	5043	Squamosa promoter binding-like protein

## Figures

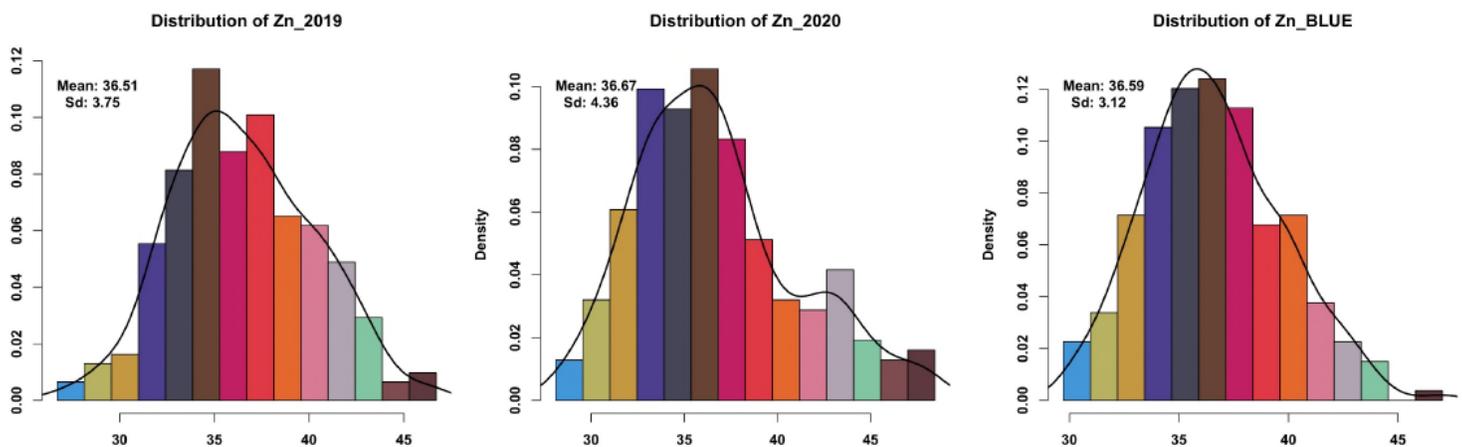
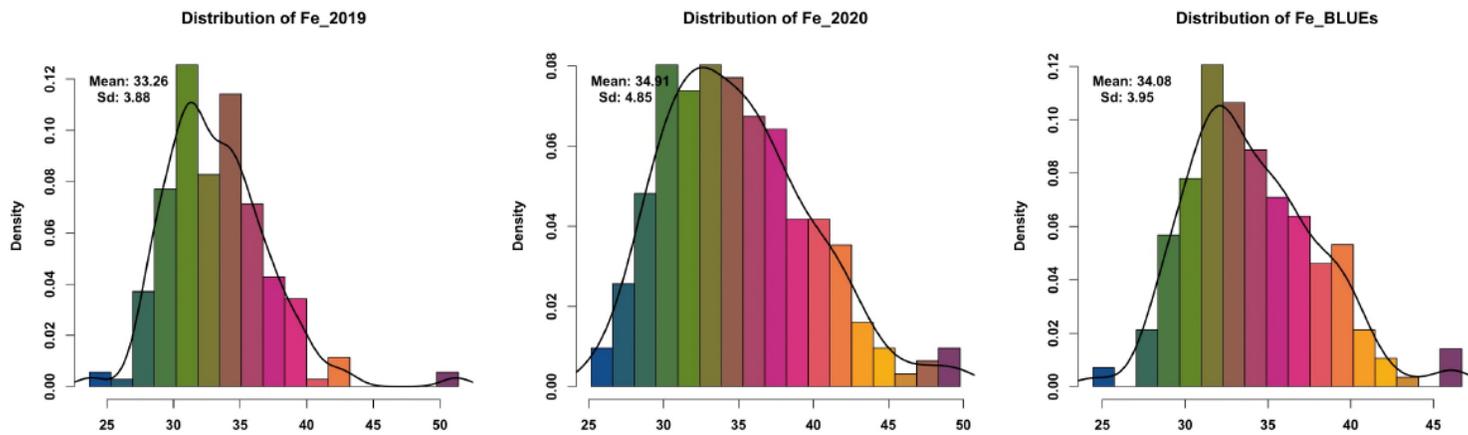


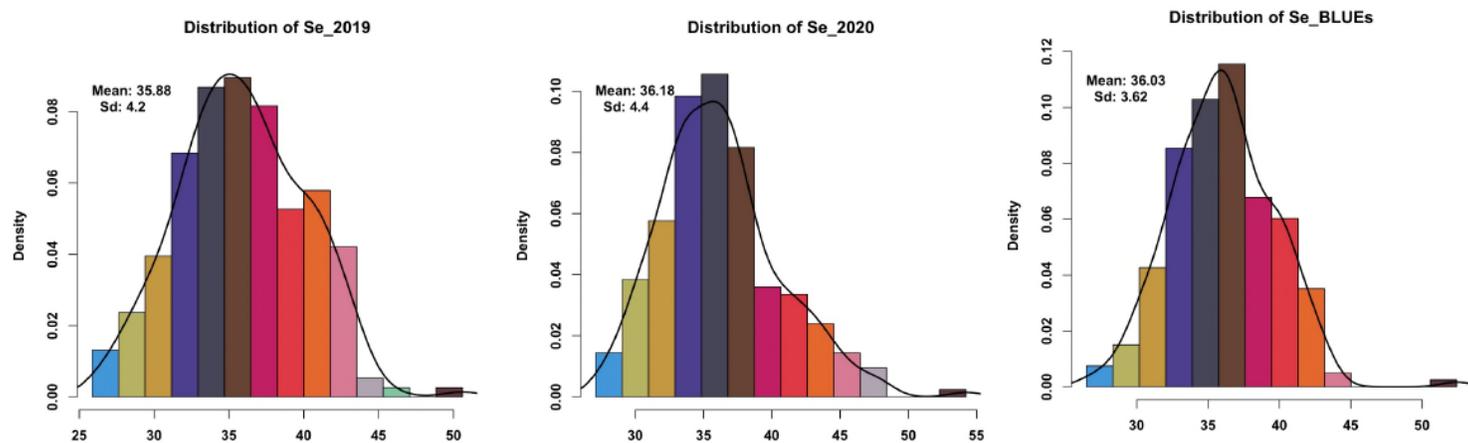
Figure 1

Distribution of genotypes for Zn concentration for each year (2019/2020) and BLUEs in spring barley.



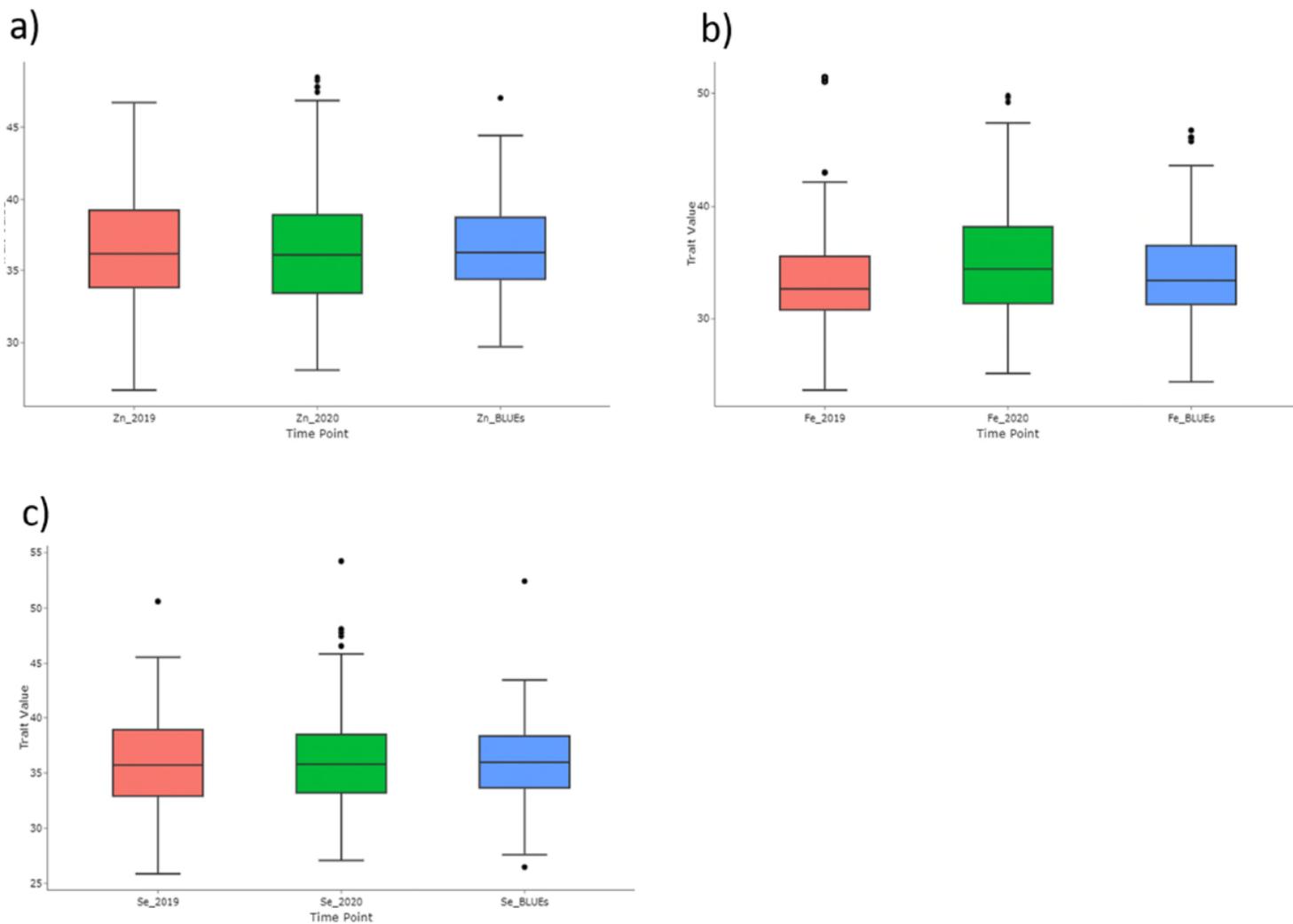
**Figure 2**

Distribution of genotypes for Fe concentration for each year (2019/2020) and BLUEs in spring barley.



**Figure 3**

Distribution of genotypes for Se concentration for each year (2019/2020) and BLUEs in spring barley.



**Figure 4**

Box plots of genotypes for each year (2019/2020) and BLUEs in spring barley; a) Zn concentration, b) Fe concentration, and c) Se concentration.

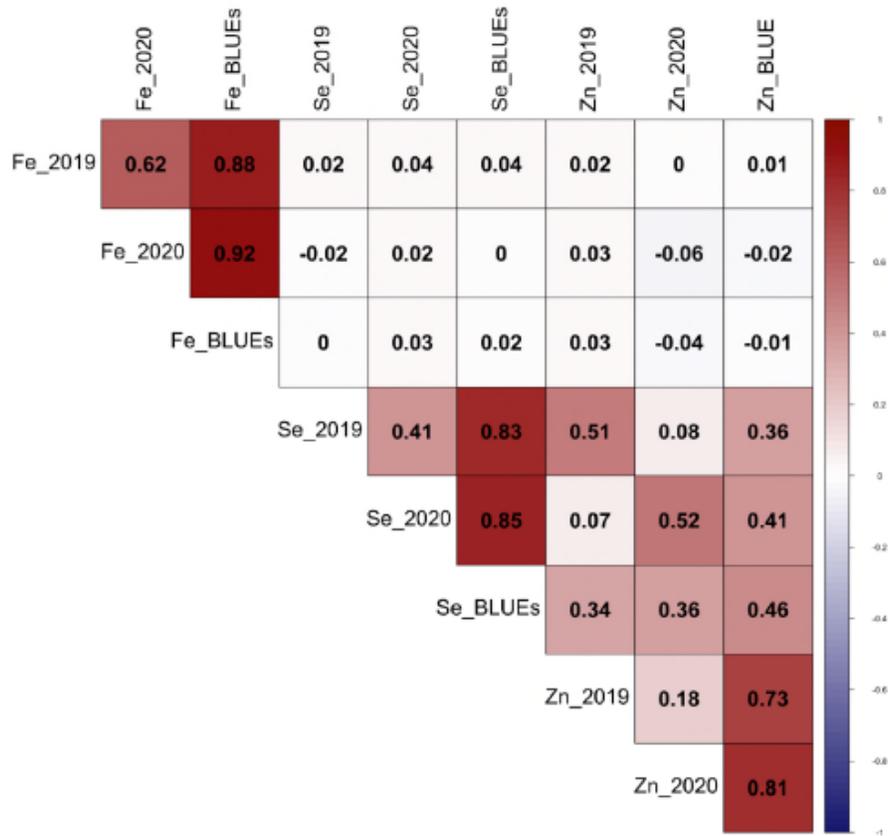
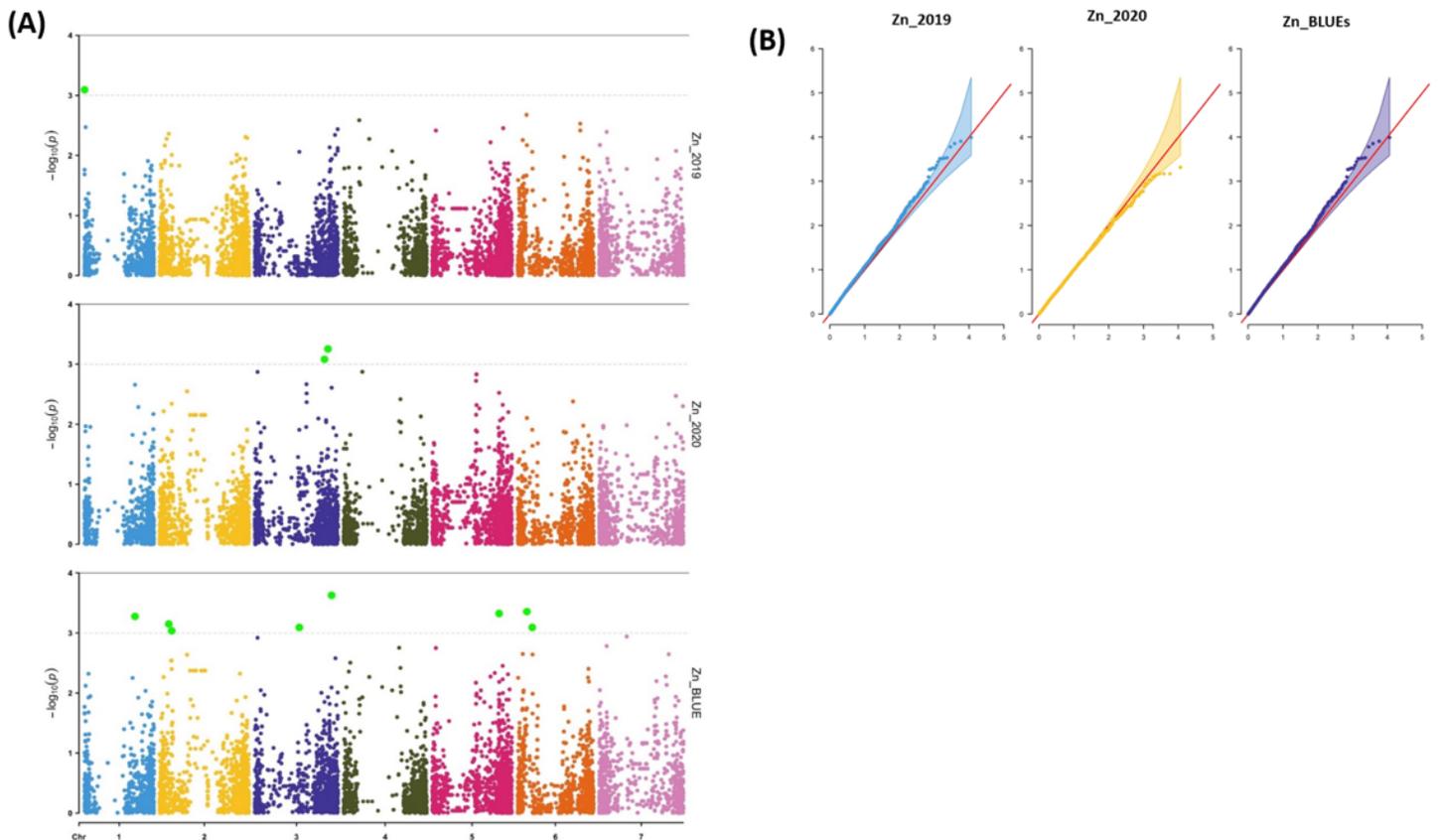


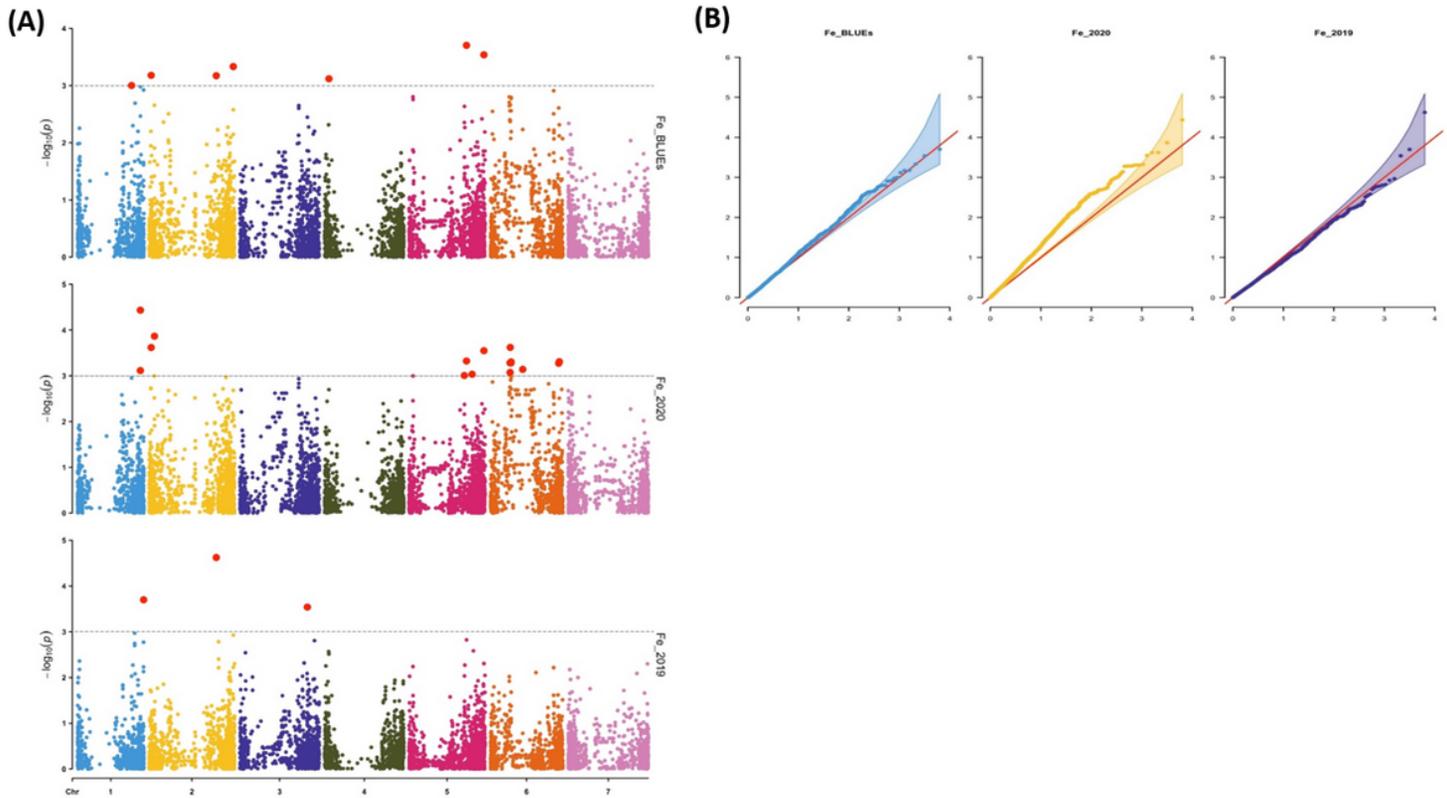
Figure 5

Correlations of all the studied traits including Zn, Fe and Se for each year (2019/2020) and BLUEs in spring barley.



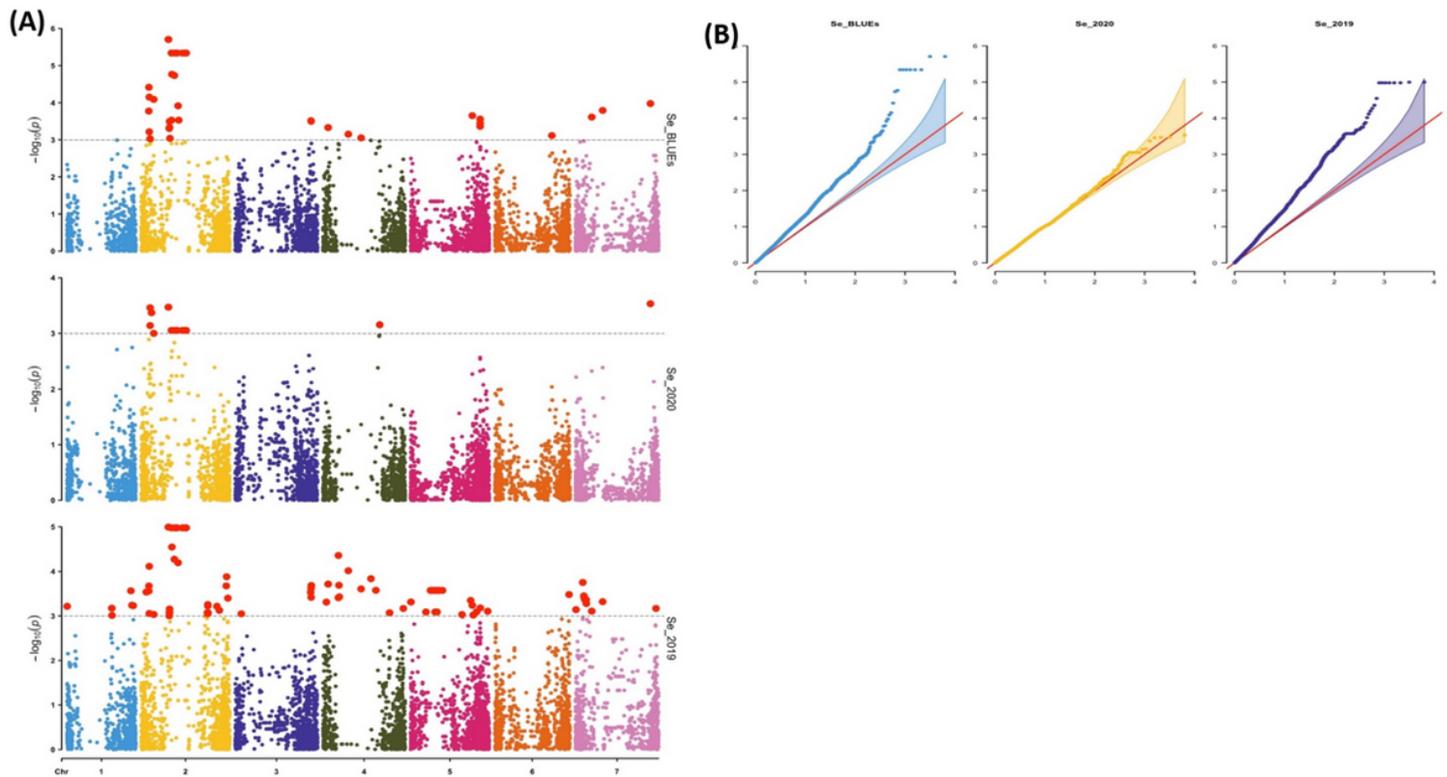
**Figure 6**

Summary of genome-wide association scans of Zn for all barley genotypes (216) which were genotyped with a high-density 9K SNPs array from Illumina™ for each year (2019/2020) and BLUEs. The horizontal red color line indicated the threshold of  $-\log_{10}(\text{p-value})$  of 3(A). Quantile-quantile scale representing expected versus observed  $-\log_{10}(\text{p-value})$  (B).



**Figure 7**

Summary of genome-wide association scans of Fe for all barley genotypes (216) which were genotyped with a high-density 9K SNPs array from Illumina™ for each year (2019/2020) and BLUEs. The horizontal red color line indicated the threshold of  $-\log_{10}(\text{p-value})$  of 3(A). Quantile-quantile scale representing expected versus observed  $-\log_{10}(\text{p-value})$  (B).



**Figure 8**

Summary of genome-wide association scans of Se for all barley genotypes (216) which were genotyped with a high-density 9K SNPs array from Illumina<sup>TM</sup> for each year (2019/2020) and BLUEs. The horizontal red color line indicated the threshold of  $-\log_{10}(\text{p-value})$  of 3(A). Quantile-quantile scale representing expected versus observed  $-\log_{10}(\text{p-value})$  (B).

## Supplementary Files

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