Identification of novel genomic regions associated with nine mineral elements in Chinese winter wheat grain

Wei Wang†, Hong Guo†, Chongning Wu, Hui Yu, Xiaokang Li, Guangfeng Chen, Jichun Tian and Zhiying Deng*

Abstract
Background: Mineral elements are important for maintaining good human health besides heavy metals. Mining genes that control mineral elements are paramount for improving their accumulation in the wheat grain. Although previous studies have reported some loci for beneficial trace elements, they have mainly focused on Zn and Fe content. However, little information is available regarding the genetic loci differences in dissecting synchronous accumulation of multiple mineral elements in wheat grains, including beneficial and heavy elements. Therefore, a genome-wide association study (GWAS) was conducted on 205 wheat accessions with 24,355 single nucleotide polymorphisms (SNPs) to identify important loci and candidate genes for controlling Ca, Fe, Zn, Se, Cu, Mn, Cd, As, and Pb accumulation in wheat grains.

Results: A total of 101 marker-trait associations (MTAs) ($P < 10^{-5}$) loci affecting the content of nine mineral elements was identified on chromosomes 1B, 1D, 2A, 2B, 3A, 3B, 3D, 4A, 4B, 5A, 5B, 5D, 6B, 7A, 7B, and 7D. Among these, 17 major MTAs loci for the nine mineral elements were located, and four MTAs loci ($P < 10^{-5}$) were found on chromosomes 1B, 6B, 7B, and 7D. Eight multi-effect MTAs loci were detected that are responsible for the control of more than one trait, mainly distributed on chromosomes 3B, 7B, and 5A. Furthermore, sixteen candidate genes controlling Ca, Fe, Zn, Se, Cd, and Pb were predicted, whose functions were primarily related to ion binding, including metals, Fe, Ca, Cu, Mg, and Zn, ATP binding, ATPase activity, DNA binding, RNA binding, and protein kinase activity.

Conclusions: Our study indicated the existence of gene interactions among mineral elements based on multi-effect MTAs loci and candidate genes. Meanwhile this study provided new insights into the genetic control of mineral element concentrations, and the important loci and genes identified may contribute to the rapid development of beneficial mineral elements and a reduced content of harmful heavy metals in wheat grain.

Keywords: Wheat grain, Nine mineral elements, Genome wide association study, Candidate gene

Background
Wheat is one of the most important crops grown globally, and as a staple food, it provides approximately 20% of the calories and 40% of the protein consumed worldwide. With the improvement in living standards, the nutritional quality of wheat grain has become increasingly important. There are many mineral elements in the wheat grain, that are important sources of trace elements in the human body. Although some important mineral
elements can be found in wheat grains, such as iron (Fe), zinc (Zn), and selenium (Se), their content is relatively low and their bioavailability is poor, which leads to the potential threat of mineral-element nutritional deficiencies in developing countries around the world [1].

More than one-third of the women and children in economically underdeveloped countries do not receive key trace elements, such as Fe, Zn, and iodine (I), in sufficient amounts to sustain a healthy condition. Specifically, more than two-thirds of the population in China suffers from Se deficiency [2]. Therefore, effectively improving the content of beneficial mineral elements in the wheat grain has become an issue of the highest priority in plant breeding programs.

In addition to some beneficial mineral elements, wheat also contains some heavy metals because of environmental pollution, such as Cd, Pb, and As, whose excessive intake may cause damage to human health, including cancer of the prostate, lungs, and testes, as well as kidney tubule damage [3, 4]. Therefore, in recent years, the heavy metal content in food has become a focus of attention for the society as a whole. The essential and toxic nature of several dietary trace elements, including Cr, Co, Cu, Fe, Mg, Mn, Se, and Zn, have been thoroughly investigated [5–10].

Micronutrient deficiencies (especially Zn and Fe), are responsible for the effects of malnutrition on a very large proportion of the world population. In developing countries, most people rely on cereal grains as their staple food, and malnutrition has been detected among children because of micronutrient deficiencies, a phenomenon that has been described as the ‘hidden hunger’ [1, 11, 12]. To solve malnutrition caused by the lack of beneficial mineral elements in food, biofortification of wheat grain has become a routine in the food industry, especially for Zn, Fe, and Se. Since 2003, scientists from various international agricultural research institutes have begun to implement the Harvest Plus project to solve this problem by cultivating mineral-rich food crops. Generally, there are two methods used to improve micronutrient content of wheat grain, which involves agronomic practices and genetic improvement. However, agronomic practices are reputedly neither economical nor environmentally friendly [13].

Alternatively, the genetic improvement for micronutrient content such as Zn, Fe, and Se in wheat grain is an important and effective approach. In previous studies, some important genes/loci for micronutrients including, Zn, Fe, and Se, were found using quantitative trait loci (QTL) mapping and genome-wide association study (GWAS) methods. Thus, using three different sets of recombinant inbred lines (RILs) [14, 15] and QTL mapping, two major QTLs for Zn content on chromosome 7B (QGZn.cimmyt-7B_1P2 and QGZn.cimmyt-7B_1P1) and one major QTL for Fe content on chromosome 4A (QGFe.cimmyt-4A_P2) were identified, and pleiotropic QTLs were also found on chromosome 3B. Additionally, through the meta-QTL (MQTL) method, some important MQTLs for Zn and Fe content were found on chromosomes 2D, 5A, 5B, 6A, and 7A [13]. However, there were only three studies reported for the QTLs of Se content on 21 chromosomes [16–18].

Using association panels, including common wheat (Triticum aestivum L.), synthetic hexaploid wheat, harvest plus association panel, European wheat varieties, and spring wheat, a total of 442 marker-trait associations (MTAs) for Zn content and 287 MATs for Fe content were identified [19–27]. Of these, two highly significant MATs for Zn content were found on chromosomes 5A and 3B, and six candidate genes on 3BS were predicted to belong to the mitogen-activated protein kinase family, which is involved in protein kinase activity, protein phosphorylation, and protein transport [13]. This is related to Zn uptake and transport. On chromosome 5AL, four candidate genes were found in the bZip family and FAR1 protein, which are related to Zn biofortification [13]. Furthermore, the Gpc-b1 gene cloned from wild wheat has been shown to increase protein, Zn, and Fe content in wheat grain [28].

Although previous studies have reported some loci for beneficial trace elements, they have mainly focused on Zn and Fe content. However, little information is available regarding the genetic loci differences in dissecting synchronous accumulation of multiple mineral elements in wheat grains, including beneficial and heavy elements. Therefore, this study used GWAS to dissect the accumulation of six beneficial elements and three harmful heavy elements using 24,355 single nucleotide polymorphisms (SNPs) genotyped from the 90 K Illumina iSelect array in a population of diverse winter wheat varieties. The objective of this study was to identify SNPs markers and candidate genes for loci associated with these traits, and improve the micronutrient content, and reduce the threat of heavy metals in the wheat grain through molecular breeding. Our results will provide the theoretical basis for improving grain micronutrient content without increasing harmful mineral elements through molecular marker-assisted selection.

Materials and methods

Plant material
The association mapping panel of 205 wheat genotypes for GWAS comprised 77 released cultivars, 55 landraces, including two lines from Mexico and France, and 73 breeding lines from 10 provinces representing the major winter wheat-production regions in China [29]. About
thirty seeds per each of these materials were originally acquired from National Germplasm Bank, Shandong Germplasm Bank, Academy of Agricultural Sciences of different province, and wheat breeders. And then they were multiply reproduced in our research field by our Research Group of Wheat Quality breeding from Shandong Agricultural University, Shandong Province, China. The details were seen in previous published paper [29].

Growth conditions
The seeds used for the association mapping panel were planted in the 2014, 2015, 2016, and 2017 growing seasons in experimental fields at two locations: Shandong Agricultural University, Tai’an (TA, 36°57’ N 116°36E) and the Dezhou Institute of Agricultural Sciences, Dezhou (DZ, 37°45’ N 116°29E). E1, E2, E3, and E4 represented the Dezhou location in 2014 (2014 DZ), Tai’an location in 2015 (2015 TA), Tai’an location in 2016 (2016 TA), and Tai’an location in 2017 (2017 TA), respectively. All experiments were laid in a completely randomised block design with two replicates in each environment. All lines were grown in 1.3 m plots with three rows spaced 25 cm apart, and 40 seeds evenly broadcast in each row. All recommended local crop management practices were followed during all the growing seasons, and no damage attributed to lodging, disease, or pests was observed.

The soil conditions at different locations are shown in Table S1. There were no significant differences in different mineral elements between the two locations. This indicated that the soil conditions at the two locations appeared to be the same.

Phenotypic trait evaluation
Whole flour milling
Grain samples were washed three times with distilled water to remove any attached particles and then oven-dried at 80 °C. Dried grain samples (50 g) were milled using a whole flour experimental mill (Perten 3100 type mill, Perten Co., Stockholm, Sweden).

Determination of metal element content in whole flour
Whole flour (0.2 g) samples were introduced into digestion tubes for digestion with 6 mL of nitric acid (HNO₃) in a microwave digester. The digested solutions were filtered through a 0.45 μm water-based microporous membranes after dilution to a constant volume of 50 mL with deionised water. Subsequently, the concentrations of different metal elements including, Ca, Mn, Fe, Cu, Zn, Se, As, Cd, and Pb, were determined using inductively coupled plasma-atomic emission spectrometry (ICP-MS, Thermo Fisher, iCAP Qc). The standard curves for the different metal elements are shown in Fig. S1, and the correlation linear valuer [2]. was between 0.9977 and 0.9999. The recovery rates of all the elements ranged between 80 and 120%.

Statistical analysis
An analysis of variance (ANOVA) and correlations among phenotypic traits were conducted using the PROC GLM procedure of SAS 8.0 (SAS Institute Inc., Cary, NC, USA) and the statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA), respectively.

Genome-wide association analysis
SNP markers, genotyping, and the population structure of the samples have been previously reported [30]. Based on this information, significant MTAs were identified using a mixed linear model (MLM) in TASSEL3.0. The P-value was used to determine whether a QTL was associated with a marker. The $R^2$ value was used to evaluate the magnitude of the MTA effects. The genome-wide significance threshold ($P \leq 10^{-4}$) was determined. SNPs with a P-value $\leq 10^{-4}$ were considered to be significantly associated with phenotypic traits. When an MTA locus was detected in two or more environments, it was considered a site-stable association [29].

Candidate genes prediction for important MTAs loci associated with mineral elements
To identify the position of important MTAs loci and possible candidate genes on a physical map, significant markers detected in this study were used to identify putative candidate genes. A BLAST (Basic Local Alignment Search Tool) search was performed using the International Wheat Genome Sequencing Consortium database (IWGSC; http://www.wheatgenome.org/, 20th January 2021) with the sequence of the significant SNP markers identified by GWAS. When an SNP marker sequence from the IWGSC was 100% identical to any wheat contig, the sequence was extended by 2 Mb for each marker using the IWGSC BLAST results. The extended sequence was used to run a BLAST search at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov, 20th January 2021) and Ensembl Plants (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast, 20th January 2021) to confirm possible candidate genes and functions.

Results
Phenotypic variation and correlation analysis for nine mineral elements
Extensive phenotypic variations in Ca, Mn, Fe, Cu, Zn, Se, As, Cd, and Pb were observed among the 205 winter wheat accessions across the four environments (i.e. 3 years and two locations, Table 1). Continuous distributions in the population were observed for nine mineral
elements, which showed typical quantitative traits, indicating that they were genetically controlled by multiple genes.

The ANOVA showed significant differences for the content of Ca, Mn, Fe, Cu, Zn, Se, and Cd ($P < 0.0001$) among genotypes and environments, as well as G × E interactions (Table S2), which indicated that mineral element content was significantly affected by genotype, environment, and their interaction. Further, correlation analysis indicated that significant positive correlations were observed among Ca, Mn, Fe, Cu, and Zn, but not Se (Table 2).

Table 1 Phenotypic variation of beneficial and harmful mineral elements in wheat grain

| Trait | Environment | Minimum (µg/mL) | Maximum (µg/mL) | Mean (µg/mL) | Standard deviation | Kurtosis | Skewness |
|-------|-------------|----------------|----------------|--------------|--------------------|----------|----------|
| Ca    | E1          | 1.305.341 2    | 3.494.334 9    | 2.057.703 3 aA | 131.193 6          | 0.342 9  | 0.487 9  |
|       | E2          | 943.268 1      | 3.851.026 7    | 1.671.181 2 cC | 135.305 9          | 4.233 2  | 1.429 6  |
|       | E3          | 1.041.368 2    | 3.571.288 4    | 1.904.142 4 bB | 142.117 6          | 0.911 6  | 0.832 5  |
|       | E4          | 745.555 5      | 4.337.089 3    | 1.635.239 5 cC | 155.169 3          | 8.360 6  | 2.174 4  |
| Mn    | E1          | 72.392 9       | 177.832 1      | 114.308 9 bAb | 7.170 7            | 0.411 3  | 0.780 9  |
|       | E2          | 64.679 8       | 233.943 3      | 110.938 3 bB  | 7.112 4            | 7.099 9  | 1.644 4  |
|       | E3          | 71.567 9       | 170.755 6      | 110.751 4 bB  | 6.072 6            | 0.320 1  | 0.443 5  |
|       | E4          | 78.885 2       | 177.475 3      | 117.476 9 aA  | 6.159 6            | 0.151 3  | 0.603 5  |
| Fe    | E1          | 49.433 0       | 275.962 4      | 122.362 9 cC  | 14.895 5           | -0.377 8 | 0.528 8  |
|       | E2          | 44.191 4       | 1.276.636 9    | 160.493 0 bB  | 55.525 0           | 15.723 5 | 3.650 2  |
|       | E3          | 60.090 6       | 1.226.409 9    | 150.083 0 aA  | 72.516 1           | 9.590 7  | 3.047 8  |
|       | E4          | 41.778 4       | 810.932 2      | 137.228 4 bCc | 34.887 9           | 19.821 1 | 4.036 5  |
| Cu    | E1          | 8.789 5        | 20.809 0       | 14.514 1 aA   | 0.854 3            | -0.537 2 | 0.230 7  |
|       | E2          | 6.594 2        | 38.289 7       | 11.147 0 dD   | 0.905 9            | 49.930 1 | 5.185 5  |
|       | E3          | 6.452 0        | 19.370 1       | 12.105 5 cC   | 0.718 3            | 0.905 7  | 0.496 5  |
|       | E4          | 8.134 2        | 31.327 0       | 13.098 8 bB   | 0.879 0            | 10.945 2 | 1.887 1  |
| Zn    | E1          | 58.830 3       | 391.240 3      | 104.609 1 aA  | 9.345 3            | 53.810 6 | 5.514 4  |
|       | E2          | 40.104 5       | 166.760 3      | 81.535 0 dC   | 7.031 0            | 3.050 4  | 1.448 9  |
|       | E3          | 52.568 7       | 156.971 2      | 91.933 2 cB   | 6.348 7            | 1.122 6  | 0.796 9  |
|       | E4          | 61.455 9       | 275.946 8      | 99.299 3 bA   | 8.670 5            | 19.452 2 | 3.620 9  |
| Se    | E1          | 0              | 0.004 5        | 0.001 6 aA    | 0.000 9            | 0.501 0  | 0.069 0  |
|       | E2          | 0              | 0.004 5        | 0.000 7 cB    | 0.000 9            | 1.426 0  | 1.729 0  |
|       | E3          | 0              | 0.005 1        | 0.000 9 bB    | 0.000 9            | 1.299 0  | 1.968 0  |
|       | E4          | 0              | 0.003 6        | 0.000 7 cB    | 0.000 8            | 1.165 0  | 0.767 0  |
| As    | E1          | 0              | 0.002 2        | 0.000 1 cC    | 0.000 3            | 3.959 0  | 16.506 0 |
|       | E2          | 0              | 0.010 2        | 0.000 8 bB    | 0.001 5            | 3.397 0  | 15.058 0 |
|       | E3          | 0              | 0.004 5        | 0.000 2 cC    | 0.000 5            | 5.503 0  | 37.033 0 |
|       | E4          | 0              | 0.008 2        | 0.001 9 aA    | 0.001 8            | 1.156 0  | 1.248 0  |
| Cd    | E1          | 0              | 0.017 9        | 0.001 3 aA    | 0.001 4            | 102.407 0 | 8.848 0 |
|       | E2          | 0              | 0.020 3        | 0.000 4 cC    | 0.001 6            | 135.468 0 | 10.852 0 |
|       | E3          | 0              | 0.014 4        | 0.000 8 bB    | 0.001 2            | 73.888 0 | 7.348 0  |
|       | E4          | 0              | 0.016 1        | 0.0005 cBC    | 0.001 4            | 86.977 0 | 8.520 0  |
| Pb    | E1          | 0              | 0.213 3        | -             | -                   | -        | -        |
|       | E2          | 0              | 0.098 8        | -             | -                   | -        | -        |
|       | E3          | 0              | 0.214 8        | -             | -                   | -        | -        |
|       | E4          | 0              | 0.692 6        | -             | -                   | -        | -        |

E1: 2014DZ; E2: 2015TA; E3: 2016TA; E4: 2017TA

Marker–trait associations (MTAs) of beneficial mineral elements
A total of 64 MTAs ($P < 10^{-4}$) for Ca content in wheat grains were detected, mainly distributed on chromosomes 1A, 2A, 2B, 3A, 3B, 5A, 5B, 5D, 6B, 7A, and 7B in the four experimental environments (Table S3). Some MTA clusters were found on chromosomes 1A, 2B, 3B,
Four MTAs were identified ($P < 10^{-5}$) on chromosomes 3B and 5A (Table 3 and Fig. S2), and the Kukri_c41797_393 locus on chromosome 5A contributed to the phenotypic variation with 10.97%, as did the RFL_Contig2187_1025 locus.

For Mn content of the wheat grain, a total of 66 MTAs loci ($P < 10^{-4}$) were identified on chromosomes 1B, 2A, 2B, 3A, 4A, 4B, 5A, 5B, and 7B in three environments (Table S3). Three MTAs clusters were found on chromosomes 1B, 5A, and 7B. Of these, 14 MTAs loci at a genetic position of 75 cM of chromosome 1B were found at $P < 10^{-5}$ levels in E2 (Table 3).

In turn, 77 MTAs loci ($P < 10^{-4}$) were identified to be associated with Fe content on 13 chromosomes (1A, 1D, 3A, 4A, 4B, 4D, 5A, 5B, 6B, 7A, 7B and 7D) in the four experimental environments (Table S3). MTAs clusters were found on chromosomes 1B, 5A, and 7B. Of these, 14 MTAs loci at a genetic position of 75 cM of chromosome 1B were found at $P < 10^{-5}$ levels in E2 (Table 3).

In turn, 77 MTAs loci ($P < 10^{-4}$) were identified to be associated with Fe content on 13 chromosomes (1A, 1D, 3A, 4A, 4B, 4D, 5A, 5B, 6B, 7A, 7B and 7D) in the four experimental environments (Table S3). MTAs clusters were found on chromosomes 1B, 5A, and 7B. Of these, 14 MTAs loci at a genetic position of 75 cM of chromosome 1B were found at $P < 10^{-5}$ levels in E2 (Table 3).

Table 2  Correlation analysis of beneficial mineral elements

|        | Ca    | Mn    | Fe    | Cu    | Zn    | Se    |
|--------|-------|-------|-------|-------|-------|-------|
| Ca     | 1     |       |       |       |       |       |
| Mn     | 0.344* | 1     |       |       |       |       |
| Fe     | 0.237* | 0.277*| 1     |       |       |       |
| Cu     | 0.359* | 0.575*| 0.436*| 1     |       |       |
| Zn     | 0.484* | 0.485*| 0.202*| 0.537*| 1     |       |
| Se     | -0.085| -0.020| -0.024| 0.022 | -0.099| 1     |

* The correlation coefficient was very significant at $P < 0.01$ level
** Correlation is significant at $P < 0.01$ level

For the As content in wheat grain, 67 MTAs loci ($P < 10^{-4}$) were identified on 14 chromosomes (1A, 1B, 2A, 2B, 3B, 3D, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B and 7D) in the four experimental environments (Table S4). Some MTAs clusters were found on chromosomes 1B, 3B, and 7B. However, at $P < 10^{-5}$ level, no loci were found.

In turn, there were 159 MTAs loci ($P < 10^{-4}$) associated with Cd content in wheat grain on 19 chromosomes, except for chromosomes 2D and 7D in the four experimental environments (Table S4). There were MTAs clusters on chromosomes 1B, 1D, 2B, 3B, and 5B; further, 22 loci were found at the $P < 10^{-5}$ level in E2, E3, and E4 (Table 4). Six loci explained more than 10% of phenotypic variation on chromosomes 1B, 3B, 5A, 5B, 6B, and 7B. Of these, 18 loci were identified at the $P < 10^{-5}$ level, which involved chromosomes 1B, 2B, 3A, 5A, 6B, and 7B.

Finally, 99 MTAs ($P < 10^{-4}$) associated with Pb content were found on 16 chromosomes (1A, 1B, 1D, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B, and 7D) in the four experimental environments (Table S4). MTAs clusters were detected mainly on chromosomes 1B, 3B, 5B, 6B, and 7B. Of these, 16 MTAs loci were found at the $P < 10^{-5}$ level in E1, E3, and E4 (Table 3). These three explained more than 10% of the phenotypic variation on chromosomes 3B and 4B. The BS00057451_51 locus exhibited the maximum (13.25%) phenotypic variation observed.

With respect to Se, 57 MTAs loci ($P < 10^{-4}$) controlling Se content in wheat grain were identified on chromosomes 1A, 2B, 3B, 4A, 4B, 5A, 6B, 7A, 7B, and 7D in the four experimental environments (Table S4). Some MTAs clusters were found on chromosomes 5A, 6B, and 7B, and six MATs loci were detected at the $P < 10^{-5}$ level (Table 4), but only one major MAT with 10.06% phenotypic variation was detected on chromosome 3D.
| Trait | Env | SNP marker                          | Chr. | Position | P      | R^2 (%) |
|-------|-----|-------------------------------------|------|----------|--------|---------|
| Ca    | E4  | Excalibur_c41752_392                | 3B   | 67       | 4.70E-05 | 8.92    |
|       | E4  | BS00057451_51                       | 3B   | 67       | 4.70E-05 | 8.92    |
|       | E4  | Kukri_c41797_393                    | 5A   | 53       | 7.13E-06 | 10.97   |
|       | E4  | RFL_Contig2187_1025                 | 5A   | 53       | 7.13E-06 | 10.97   |
| Mn    | E2  | wsnp_Ex_c4561_8184576               | 1B   | 75       | 6.26E-05 | 8.33    |
|       | E2  | wsnp_BF478690B_Ta_2_1               | 1B   | 75       | 6.72E-05 | 8.28    |
|       | E2  | Excalibur_c5218_75                  | 1B   | 75       | 7.28E-05 | 8.2     |
|       | E2  | IAAV2125                            | 1B   | 75       | 6.72E-05 | 8.28    |
|       | E2  | IAAV6731                            | 1B   | 75       | 6.72E-05 | 8.28    |
|       | E2  | JD_c3116_778                        | 1B   | 75       | 6.26E-05 | 8.33    |
|       | E2  | Ja_c37969_549                       | 1B   | 75       | 8.25E-05 | 8.05    |
|       | E2  | RAC875_c19014_725                   | 1B   | 75       | 6.26E-05 | 8.33    |
|       | E2  | RAC875_rep_c112555_200              | 1B   | 75       | 6.72E-05 | 8.28    |
|       | E2  | RAC875_rep_c119728_146              | 1B   | 75       | 8.92E-05 | 7.97    |
|       | E2  | TA003725-0553                       | 1B   | 75       | 8.92E-05 | 7.97    |
|       | E2  | BS00022619_51                       | 1B   | 75       | 7.72E-05 | 8.14    |
|       | E2  | BS00022920_51                       | 1B   | 75       | 6.72E-05 | 8.28    |
|       | E2  | Tdurum_contig81102_102              | 1B   | 75       | 8.92E-05 | 7.97    |
| Fe    | E2  | wsnp_Ex_c11913_19105189             | 5A   | 16       | 5.47E-05 | 8.75    |
|       | E2  | RAC875_rep_c112368_118              | 5A   | 16       | 7.57E-05 | 8.77    |
|       | E2  | Excalibur_c6326_77                  | 6B   | 20       | 1.46E-05 | 10.19   |
|       | E2  | RAC875_s114363_172                  | 6B   | 22       | 5.14E-05 | 8.87    |
|       | E3  | wsnp_Ex_c65899_64135487              | 7D   | 26       | 8.79E-05 | 8.12    |
|       | E3  | wsnp_Ra_c8297_14095831               | 7D   | 26       | 9.09E-05 | 8.07    |
|       | E3  | D_contig11494_202                    | 7D   | 26       | 7.70E-05 | 8.25    |
|       | E3  | D_FSXZDLF01ASSE2_190                 | 7D   | 26       | 7.70E-05 | 8.25    |
|       | E3  | Ex_c25027_535                        | 7D   | 26       | 7.70E-05 | 8.25    |
|       | E3  | Excalibur_c833_1405                  | 7D   | 26       | 8.85E-05 | 8.1     |
|       | E3  | Kukri_rep_c103404_314                | 7D   | 26       | 7.70E-05 | 8.25    |
|       | E3  | BS00022449_51                       | 7D   | 26       | 7.82E-05 | 8.25    |
|       | E3  | BS001110124_51                      | 7D   | 27       | 7.70E-05 | 8.25    |
|       | E3  | BS00110642_51                       | 7D   | 27       | 7.70E-05 | 8.25    |
|       | E3  | D_GBSYY7A02IDDAA9_183                | 7D   | 30       | 7.70E-05 | 8.25    |
|       | E3  | TA005377-1076                       | 7D   | 32       | 6.50E-05 | 8.43    |
|       | E4  | Excalibur_c19455_3496                | 7B   | 163      | 8.12E-08 | 16.23   |
|       | E4  | Excalibur_c11062_582                 | 7B   | 171      | 1.44E-05 | 10.26   |
|       | E4  | Excalibur_c25090_830                 | 7B   | 171      | 2.81E-06 | 12.07   |
|       | E4  | RAC875_rep_c110526_229               | 7B   | 171      | 1.44E-05 | 10.26   |
| Cu    | E2  | Excalibur_c29255_366                 | 4B   | 104      | 8.12E-05 | 8.91    |
Stable MTAs loci at the Tai'an locations over 3 years
A total of 66 stable MTAs loci ($P<10^{-4}$) were found for Ca, Mn, Cu, Zn, Se, Cd, and Pb content in two or more of the tested environments (Table 5). Of these, five loci were detected for Ca content on chromosome 5A, and two major MATs explained more than 10% of the phenotypic variation. Eighteen stable MTAs for Mn content were identified on chromosomes 7A and 7B. Most were concentrated at the genetic position 75 cM on chromosome 7B. A stable locus was found for Cu and Zn content on chromosomes 1A and 3B, and the BS00057451_51 locus on chromosome 3B explained 13.25% of the phenotypic variation. Two stable loci on chromosomes 4B and 7A were detected for Se content. The residue of stable MTAs loci was identified for Cd and Pb content. Five major loci were identified for Pb content.

Multi-effect MTAs loci of mineral elements
There were eight multi-effect MTAs loci for controlling more than one trait, mainly distributed on chromosomes 3B, 7B, and 5A (Table 6). There were two loci, BS00057451_51 and Excalibur_c41752_392, concurrently associated with Ca and Zn content on chromosome 3B. These two loci contributed to 13.25% of the variation in Zn content. One locus, Excalibur_c11062_582, simultaneously controlled Fe and Cd content on chromosome 7B, but the contribution to Fe content of this locus exhibited more than 10%. Two loci, RAC875_rep_c110526_229 and Excalibur_c25090_830, were simultaneously associated with Fe, Cd, and Pb content on chromosome 7B. These two loci explained more than 10% of the variation in Fe and Pb content. The three loci were concentrated on the genetic position 171 cM on chromosome 7B. There was one locus, Excalibur_c19455_3496, concurrently controlling Fe and Pb content, which accounted for more than 10% of the phenotypic variation on chromosome 7B. Two loci were identified for Cd and Pb content simultaneously on chromosome 5A, accounting for more than 10% phenotypic variation.

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Table 3 (continued)

| Trait | Env | SNP marker         | Chr. | Position | P        | $R^2$ (%) |
|-------|-----|--------------------|------|----------|----------|-----------|
| Zn    | E1  | BS00012036_51      | 2B   | 108      | 8.49E-05 | 8         |
|       | E1  | BS00062691_51      | 4B   | 62       | 7.05E-06 | 12.12     |
|       | E1  | CAP8_rep_c6942_227 | 7A   | 148      | 8.98E-05 | 7.94      |
|       | E1  | BS00072941_51      | 7B   | 71       | 2.42E-05 | 9.29      |
|       | E1  | BobWhite_c7907_657 | 7B   | 71       | 2.42E-05 | 9.29      |
|       | E1  | Kukri_c78330_327   | 7B   | 71       | 3.23E-05 | 8.99      |
|       | E1  | RFL_Contig2540_306 | 7B   | 71       | 2.42E-05 | 9.29      |
|       | E1  | TA003961-0636      | 7B   | 71       | 2.42E-05 | 9.29      |
|       | E1  | BS00095819_51      | 7B   | 72       | 6.62E-05 | 8.25      |
|       | E1  | Tdurum_contig75931_1967 | 7B   | 72       | 6.62E-05 | 8.25      |
|       | E1  | BobWhite_c40042_842 | 7B   | 101      | 8.98E-05 | 7.94      |
|       | E3  | Ra_c19225_591      | 2B   | 130      | 4.19E-05 | 8.19      |
|       | E3  | wsnp_Ex_c9428_15641609 | 7A   | 159      | 3.63E-05 | 8.42      |
|       | E3  | wsnp_Ex_c9428_15641639 | 7A   | 159      | 1.91E-05 | 8.98      |
|       | E4  | Excalibur_c41752_392 | 3B   | 67       | 7.87E-07 | 13.25     |
|       | E4  | BS00057451_51      | 3B   | 67       | 7.87E-07 | 13.25     |
| Se    | E1  | Excalibur_rep_c93332_58 | 3D   | 107      | 1.07E-05 | 10.06     |
|       | E1  | BobWhite_c9622_723 | 3D   | 113      | 3.93E-05 | 8.99      |
|       | E1  | wsnp_Ku_c21275_31007309 | 5A   | 83       | 3.46E-05 | 8.77      |
|       | E2  | Tdurum_contig4974_355 | 4B   | 61       | 8.65E-05 | 8.7       |
|       | E2  | wsnp_Ex_c14654_22713386 | 7A   | 42       | 2.66E-05 | 9.68      |
|       | E2  | D_contig06359_118  | 7D   | 56       | 2.80E-05 | 9.52      |

E1, E2, E3 and E4 were same as the Table 1
Identification of stable MTAs and its alleles analysis in wheat accessions

By screening the results (Tables S3 and S4), there were thirteen SNP markers identified for stable MTAs associated with Ca, Mn, Zn, Se and Pb content (Table 7 and Fig. 1). Of which, the phenotypic value of Ca content associated with Kukri_c41797_393- TT on chromosome 5A was significantly higher than that associated with Kukri_c41797_393- CC across all four environments, which indicated that the contribution of Kukri_c41797_393-TT locus to Ca content was better than that of Kukri_c41797_393- CC locus, so did

Table 4  Genome-wide Association Mapping results of heavy metal elements ($P < 10^{-5}$)

| Trait | Env | SNP marker                | Chr. | Position | P       | $R^2$ (%) |
|-------|-----|---------------------------|------|----------|---------|-----------|
| Cd    | E3  | Tdurum_contig44851_927    | 1B   | 162      | 3.23E-06| 11.77     |
|       | E2  | BS00083531_51             | 1D   | 72       | 6.82E-05| 8.51      |
|       | E3  | Tdurum_contig44851_593    | 1D   | 164      | 3.23E-06| 11.77     |
|       | E4  | RAC875_c9594_1289         | 1D   | 65       | 9.63E-05| 8.20      |
|       | E4  | wsnp_Exc_rep_c108004_91402649 | 2A | 168   | 4.77E-05| 8.87      |
|       | E4  | GENE-0762_808             | 2A   | 168      | 4.64E-05| 8.90      |
|       | E2  | CAP12_rep_c6956_169       | 2B   | 115      | 7.91E-05| 8.32      |
|       | E2  | wsnp_Exc_17538_26261053   | 2B   | 100      | 8.63E-05| 8.25      |
|       | E2  | RAC875_c59545_122         | 2B   | 104      | 7.26E-05| 8.41      |
|       | E2  | Excalibur_rep_c66577_159  | 2B   | 107      | 5.00E-05| 8.82      |
|       | E3  | Tdurum_contig13489_292    | 4A   | 75       | 3.65E-06| 11.65     |
|       | E3  | Kukri_c59197_207          | 4B   | 6        | 7.70E-06| 11.05     |
|       | E3  | RAC875_c9572_588          | 4B   | 63       | 8.79E-05| 8.22      |
|       | E4  | wsnp_Exc_c28908_37989067  | 5A   | 27       | 4.85E-06| 11.34     |
|       | E4  | wsnp_Ku_c1254_2498515     | 5A   | 27       | 1.43E-05| 10.16     |
|       | E4  | BS00033185_51             | 5B   | 174      | 9.69E-05| 8.14      |
|       | E3  | RAC875_rep_c69613_547     | 5D   | 56       | 6.85E-05| 9.10      |
|       | E3  | wsnp_Exc_c7713_13153321   | 6B   | 92       | 4.09E-05| 9.03      |
|       | E3  | Excalibur_c7713_272       | 7A   | 92       | 8.57E-05| 8.24      |
|       | E4  | Excalibur_c11062_582      | 7B   | 171      | 5.52E-05| 8.72      |
|       | E4  | Excalibur_c25090_830      | 7B   | 171      | 2.68E-05| 9.49      |
|       | E4  | RAC875_rep_c110526_229    | 7B   | 171      | 5.52E-05| 8.72      |
| Pb    | E3  | Ex_c4206_502              | 1B   | 108      | 6.88E-05| 8.48      |
|       | E2  | BS00083626_51             | 2B   | 173      | 9.90E-05| 7.94      |
|       | E4  | BS00022424_51             | 3A   | 141      | 5.65E-05| 8.76      |
|       | E3  | Tdurum_contig48760_112    | 5A   | 69       | 7.77E-05| 8.37      |
|       | E4  | wsnp_Exc_c28908_37989067  | 5A   | 27       | 1.10E-05| 10.53     |
|       | E4  | wsnp_Ku_c1254_2498515     | 5A   | 27       | 3.89E-05| 9.16      |
|       | E1  | Excalibur_c16961_85       | 6B   | 64       | 2.15E-05| 9.44      |
|       | E1  | BobWhite_c73718_380      | 6B   | 67       | 6.06E-05| 8.35      |
|       | E1  | Excalibur_c6416_1712      | 6B   | 67       | 6.07E-05| 8.35      |
|       | E1  | BobWhite_c36415_378      | 6B   | 67       | 9.21E-05| 7.90      |
|       | E1  | IACX203                   | 6B   | 67       | 3.00E-05| 9.12      |
|       | E3  | Excalibur_c1215_334       | 7A   | 127      | 5.29E-05| 8.88      |
|       | E4  | RAC875_c57326_85          | 7B   | 134      | 8.71E-05| 8.30      |
|       | E4  | wsnp_Exc_c8400_14157060   | 7B   | 134      | 8.71E-05| 8.30      |
|       | E4  | Excalibur_c19455_3496     | 7B   | 163      | 9.11E-06| 10.81     |
|       | E4  | Excalibur_c11062_582      | 7B   | 171      | 1.90E-06| 12.47     |
|       | E4  | Excalibur_c25090_830      | 7B   | 171      | 8.11E-07| 13.43     |
|       | E4  | RAC875_rep_c110526_229    | 7B   | 171      | 1.90E-06| 12.47     |

E1, E2, E3 and E4 were same as the Table 1
the contribution of RFL_Contig2187_1025 locus to Ca content.

For Mn content, five SNP makers were detected on chromosome 1B (Table 7). The contribution of AA allele to Mn content was significantly higher than that of GG allele for BS00022619_51 and BS00022920_51 markers, but for Excalibur_c5218_75 marker, the GG allele was better than the AA allele for improving the Mn content. The contribution of CC allele of IAAV2125 and IAAV6731 to Mn content showed better than that of AA allele.

Only one stable marker BS00057451_51 was found on chromosome 3B for Zn content, and the contribution of

### Table 5

Stable marker loci at Taian location of different years ($P < 10^{-4}$)

| Trait | SNP marker                  | Chr. | Position | $P$     | $R^2$ (%) |
|-------|-----------------------------|------|----------|---------|-----------|
| Ca    | Excalibur_c15014_1170       | 5A   | 50       | 3.71E-04| 6.76      |
|       | GENE-3167_70                | 5A   | 50       | 3.71E-04| 6.76      |
|       | Kukri_c2781_719             | 5A   | 50       | 3.71E-04| 6.76      |
|       | Kukri_c47197_393            | 5A   | 53       | 7.13E-06| 10.97     |
|       | RFL_Contig2187_1025         | 5A   | 53       | 7.13E-06| 10.97     |
| Mn    | BobWhite_rep_c66032_270     | 1B   | 71       | 2.38E-04| 6.99      |
|       | wsnp_BE443332B_Ta_2_2       | 1B   | 71       | 2.21E-04| 7.05      |
|       | wsnp_BE443930B_Ta_2_2       | 1B   | 71       | 2.82E-04| 6.81      |
|       | BS00022619_51               | 1B   | 75       | 7.72E-05| 8.14      |
|       | BS00022920_51               | 1B   | 75       | 6.72E-05| 8.28      |
|       | Excalibur_c5218_75          | 1B   | 75       | 7.28E-05| 8.20      |
|       | IAAV2125                    | 1B   | 75       | 6.72E-05| 8.28      |
|       | IAAV6731                    | 1B   | 75       | 6.72E-05| 8.28      |
|       | IAAV9005                    | 1B   | 75       | 1.12E-04| 7.74      |
|       | JD_c3116_778                | 1B   | 75       | 6.26E-05| 8.33      |
|       | Ra_c37969_549               | 1B   | 75       | 8.25E-05| 8.05      |
|       | RAC875_c19014_725           | 1B   | 75       | 6.26E-05| 8.33      |
|       | RAC875_rep_c112555_200      | 1B   | 75       | 6.72E-05| 8.28      |
|       | RAC875_rep_c119728_146      | 1B   | 75       | 8.92E-05| 7.97      |
|       | TA003725-0553               | 1B   | 75       | 8.92E-05| 7.97      |
|       | Tdurum_contig81102_102      | 1B   | 75       | 8.92E-05| 7.97      |
|       | wsnp_BF478690B_Ta_2_1       | 1B   | 75       | 6.72E-05| 8.28      |
|       | wsnp_Ex_c4561_8184576       | 1B   | 75       | 6.26E-05| 8.33      |
|       | Cu                           | 1A   | 77       | 6.31E-04| 6.28      |
|       | Zn                           | 3B   | 67       | 7.87E-07| 13.25     |
|       | Se                           | 2A   | 167      | 5.30E-04| 6.41      |
|       | Cd                           | 2B   | 85       | 3.44E-04| 7.49      |
|       | Pb                           | 5A   | 83       | 8.35E-04| 5.92      |
GG allele was significantly better than that of AA allele (Table 7 and Fig. 1).

For Se content, two markers, Tdurum_contig4974_355 and wsnp_Ex_c14654_22713386, were found on chromosome 4B and 7A, respectively (Table 7 and Fig. 1). The contribution of Tdurum_contig4974_355-TT allele showed significantly better than that of Tdurum_contig4974_355-CC for Se content, but for wsnp_Ex_c14654_22713386 marker, the CC allele was better than the TT allele.

Three stable SNP markers were identified on chromosome 7B for heavy metal Pb content (Table 7 and Fig. 1). The contributions of the alleles GG, CC and CC showed better than that of the alleles AA, TT and AA for Excalibur_c19455_3496, Excalibur_c25090_830 and Excalibur_c11062_582 to reduce the Pb content, respectively. Most interestingly, these three SNP markers were also associated with Fe content.

So a pyramid analysis of the alleles of these different stable SNP markers were further studied in wheat accesses, seven accesses were found with high beneficial-mineral-element contents and low heavy-metal-element contents (Table 8). Of which, five accesses (B111, B117,
Fig. 1 The position of major stable MTAs in the chromosome maps
### Table 8  A pyramid analysis of different QTL alleles in certain wheat accessions

| Wheat accessions | Mineral elements | SNP marker | Allele | Content (ug/mL) |
|------------------|------------------|------------|--------|-----------------|
| B111             | Ca               | Kukri_c41797_393 | TT     | 2903.4414       |
|                  | Ca               | RFL_Contig2187_1025 | TT     |                 |
|                  | Zn               | BS00057451_51   | GG     | 139.8417        |
|                  | Pb               | Excalibur_c19455_3496 | GG     | 0               |
|                  | Pb               | Excalibur_c11062_582 | CC     |                 |
| B117             | Ca               | Kukri_c41797_393 | TT     | 2356.8505       |
|                  | Ca               | RFL_Contig2187_1025 | TT     |                 |
|                  | Fe               | Excalibur_c19455_3496 | AA     | 185.3263        |
|                  | Pb               | Excalibur_c11062_582 | CC     | 0               |
| B148             | Mn               | Excalibur_c5218_75 | GG     | 148.6161        |
|                  | Mn               | IAAV2125      | CC     |                 |
|                  | Mn               | IAAV6731      | CC     |                 |
|                  | Mn               | BS00022619_51 | AA     |                 |
|                  | Mn               | BS00022920_51 | AA     |                 |
|                  | Zn               | BS00057451_51 | GG     | 100.9967        |
|                  | Pb               | Excalibur_c19455_3496 | GG     |                 |
|                  | Pb               | Excalibur_c11062_582 | CC     | 0               |
| B46              | Mn               | Excalibur_c5218_75 | GG     | 140.9914        |
|                  | Mn               | IAAV2125      | CC     |                 |
|                  | Mn               | IAAV6731      | CC     |                 |
|                  | Mn               | BS00022619_51 | AA     |                 |
|                  | Mn               | BS00022920_51 | AA     |                 |
|                  | Zn               | BS00057451_51 | GG     | 107.564         |
|                  | Se               | Tdurum_contig4974_355 | TT     | 0.0016          |
|                  | Se               | wsnp_Ex_c14654_22713386 | CC     |                 |
|                  | Pb               | Excalibur_c19455_3496 | GG     | 0               |
|                  | Pb               | Excalibur_c11062_582 | CC     |                 |
| B67              | Fe               | Excalibur_c19455_3496 | AA     | 364.4478        |
|                  | Fe               | Excalibur_c25090_830 | CC     |                 |
|                  | Fe               | Excalibur_c11062_582 | AA     |                 |
|                  | Se               | Tdurum_contig4974_355 | TT     | 0.0016          |
|                  | Se               | wsnp_Ex_c14654_22713386 | CC     |                 |
|                  | Pb               | Excalibur_c25090_830 | CC     | 0               |
| B70              | Fe               | Excalibur_c25090_830 | TT     | 358.477         |
|                  | Se               | Tdurum_contig4974_355 | TT     | 0.0019          |
|                  | Se               | wsnp_Ex_c14654_22713386 | CC     | 0               |
|                  | Pb               | Excalibur_c19455_3496 | GG     |                 |
|                  | Pb               | Excalibur_c11062_582 | CC     |                 |
| B114             | Mn               | Excalibur_c5218_75 | GG     | 156.3802        |
|                  | Mn               | IAAV2125      | CC     |                 |
|                  | Mn               | IAAV6731      | CC     |                 |
|                  | Mn               | BS00022619_51 | AA     |                 |
|                  | Mn               | BS00022920_51 | AA     |                 |
|                  | Pb               | Excalibur_c19455_3496 | GG     | 0               |
|                  | Pb               | Excalibur_c11062_582 | CC     |                 |
B148, B46 and B67) contained more than one beneficial mineral elements with high content without Pb content. These results provided good wheat resources and elite alleles with beneficial mineral elements in wheat breeding.

**Prediction of candidate genes for important loci for mineral elements**

In all, 16 new candidate genes were predicted for 17 important loci for mineral elements (Table S5). On chromosome 3B, two candidate genes, *TraesCS3B02G307600.1* and *TraesCS3B02G307400*, were found for BS00057451_51 and Excalibur_c41752_392, respectively, which involved Zn and Ca content. Their functions were primarily involved in metal ion binding, calcium ion binding, ATP binding, ATPase activity, DNA binding, RNA binding, and protein kinase activity.

There was one candidate gene for Se content predicted on chromosome 3D, *TraesCS3D02G201900*, whose function is modification-dependent protein binding in wheat. Furthermore, metal ion binding and calcium ion binding of this gene were found in Arabidopsis and rice, respectively.

Three candidate genes, *TraesCS5A02G256700.1*, *TraesCS5A01G256800.1*, and *TraesCS5A02G257000.1*, were identified on chromosome 5A for Ca content. The gene *TraesCS5A02G256700.1* for Kukri_c41797_393 had ribosomal small subunit biogenesis and exportation from the nucleus in wheat, but metal ion binding, Ca ion binding, and Ca transmembrane activity in Arabidopsis. The *TraesCS5A02G257000.1* gene functions in Zn-ion binding, Zn-ion transmembrane transporter activity, and metal ion binding in Arabidopsis and rice.

For Zn content, there was one candidate gene *TraesCS7B02G142200* for BobWhite_c7970_657 was identified on chromosome 7B, whose function was related to DNA binding and metal ion binding in wheat.

For Fe content, two candidate genes, *TraesCS6B02G029300* and *TraesCS6B02G029200*, for Excalibur_c6326_77 were identified on chromosome 6B. The functions of these two genes are mainly related to Ca-dependent Ca-ion binding, Fe ion binding, ATP binding, and DNA binding in Arabidopsis.

On chromosome 7B, two candidate genes were identified for Fe content which were also involved in Cd and Pb content. The gene *TraesCS7B02G480300* for Excalibur_c19455_3496 was mainly related to Fe-ion binding, Fe- and Zn-ion transmembrane transporter activity, Ca-ion binding, metal ion binding, and metal-ion transmembrane transporter activity in *Hordeum vulgare*, *Oryza sativa*, and *Arabidopsis*. This gene mainly participates in the biological processes of Fe, Zn, and metal ion transport. The other gene, *TraesCS7B02G478200*, was also mainly related to Fe-ion binding, metal ion binding, Zn-ion binding, and Ca-ion binding in *Oryza*, *Arabidopsis*, and *Triticum urartu*. In *Arabidopsis*, this gene shows a biological response to Cd ions as an aspartic-type endopeptidase activity.

Five candidate genes were found for Cd content on chromosomes 1B, 1D, 4A, 4B, and 5A. The gene *TraesCS1B02G474800* for Tdurum_contig44851_927 was mainly related to Cu-ion binding and metal ion binding in *Oryza* and *Arabidopsis*. The gene mainly participates in lignin breakdown. The function of *TraesCS1D02G487800* mainly involves Fe-ion binding, Ca-ion binding, metal ion binding, and Mg-ion binding in *Arabidopsis* and *Oryza*. One of the biological processes it influences is the response to Cd ions. The gene *TraesCS4B02G004800* functions in Cu-ion binding, metal ion binding, four Fe and four S cluster binding, and the management of ion transmembrane transporter activity in *Oryza* and *Arabidopsis*. However, the gene *TraesCS5A02G014600* affected Cd and Pb content involved in Zn-ion binding, and cation- and Mn-transmembrane transporter activity, Mg-ion binding, and metal ion binding.

**Discussion**

Metal elements include beneficial and harmful elements, of which, beneficial elements are important for maintaining good human health. However, to ensure food safety, heavy metals should be avoided. In addition to the agronomic practices for biofortification of Zn, Fe, and Se, genetic improvement of these elements has become important. Previous studies have identified some QTL/gene loci for Zn, Se, and Fe content involving all 21 wheat chromosomes [13, 15–18, 27, 31–38]; however, in our study, MTAs loci for Ca, Zn, Fe, Se, Mn, and Cu content were found on 20 chromosomes, i.e. all except for chromosome 6D. Previously, the important QTLs detected for Zn, Fe, and Se were located on chromosomes 7B, 4A, 3B, 2D, 5A, 5B, 6A, and 7A [13]. In this study, chromosomes 1B, 3B, 6A, 4B, 5A, 5B, 7B, and 7D were important for MTAs loci associated with Ca, Zn, Fe, Se, Mn, and Cu content. Comparing these, common chromosomes 3B, 5A, and 7B were found to play important roles in regulating Ca, Fe, and Zn concentrations in the wheat grain, and to contain some important genes.

Using bioinformatics, the candidate genes (mRNA_2.1, mRNA_3.1, mRNA_10.1, mRNA_23.1, mRNA_24.1, and mRNA_32.1; mRNA_11.1, mRNA_34.1, mRNA_42.1, and mRNA_44.1) associated with Zn content were identified in the physical regions of 3BS (723,504,241 to 723,611,488) (first six genes) and 5AL (462,763,758 to 468,582,184) (last four genes) [21]. This study identified two candidate genes, *TraesCS3B02G307600.1*, with the physical region from 493,655,348 to 493,657,938
and TraesCS3B02G307400 with the physical region from 493,648,449 to 493,653,177, on chromosome 3B, which is associated with grain Ca and Zn concentrations. By comparing their physical positions, we found that these two genes are different from the above genes located upstream of previously published genes. These published 3BS genes were found to belong to the mitogen-activated protein kinase (MAPK) family of genes involved in kinase activity, leading to protein phosphorylation, which in turn assists in the desired molecular function in various biological processes [13]. MAPKs are involved in Zn uptake and transport through signalling pathways. The mRNA_32.1 gene encodes a suppressor of the white apricot protein associated with Zn concentration in chickpea seeds [39], an RNA-binding protein involved in RNA processing [13]. In our study, according to the putative functions of these two new candidate genes, they are seemingly also involved in protein kinase activity and RNA binding; although primarily they are involved in metal ion binding, Ca-ion binding, ATP binding, and ATPase activity, indicating that they are involved in Ca and Zn uptake and transport. On chromosome 5A, the last four genes encode TaMTP proteins, which are directly or indirectly involved in Zn biofortification, and their functions are mainly involved in DNA binding, Zn/Fe binding, and protein dimerisation [13, 21]. However, this study identified three candidate genes, TraesCSSA02G256700.1, TraesCSSA01G256800.1, and TraesCSSA02G257000.1 on chromosome 5A for Ca content in wheat grain; further, their physical region is from 472,274,579 to 472,347,557, which is different from the physical position of the published genes. Their functions are primarily involved in Ca-ion binding, Zn-ion binding, metal ion binding, Zn- and iron-transmembrane transporter activity, ATP binding, and protein kinase activity. Additionally, some researchers have found that chromosome 5A plays an important role in regulating grain Cu concentration [27, 34]. Therefore, these new candidate genes identified on chromosomes 3B and 5A are suitable for further molecular genetic research.

On chromosome 7B, two major QTLs for controlling Zn content were identified using DArT-seq [15]. These genes encode the kinase-like superfamily, which catalyses phosphorylation processes in which some protein structures are Zn related [15]. In this study, a new candidate gene, TraesCS7B02G142200, was predicted to be involved in DNA binding and metal ion binding in wheat. These results indicate that the genes are controlling Zn concentration on chromosome 7B.

Regarding ferritin, previous studies found genes involved in vacuolar iron transporters and transporter-like protein, such as TaFeR1 and TaFeR2 on homologous groups 5 and 4 in wheat, respectively [40]. Another wheat gene relevant to biofortification is the major grain protein gene Gpc1 on chromosome 6B, which also affects Zn and Fe concentrations in the grain [13, 41], as it can regulate the expression of several genes involved in the export and transport of Zn and Fe into the grain through the phloem [42]. This study predicted three candidate genes, TraesCS6B02G029300, TraesCS7B02G480300, and TraesCS7B02G478200, with physical regions from 17,703,175 to 17,704,083, 734,259,844 to 734,274,042, and 733,527,458 to 733,530,221, respectively, which are primarily involved in Fe- and Ca-ion binding, metal ion binding, Zn-ion binding, ATP binding, ATPase activity, and DNA binding. Thus, these genes are important for improving Fe concentration, a finding that warrants further research.

The accumulation of heavy metals (e.g. Cd, As, and Pb) is a complex quantitative trait controlled by multiple genes. Most previous studies on the mechanisms of Cd accumulation have focused on rice, maize, and A. thaliana. A major QTL was mapped to translate Cd from roots to shoots at the seedling stage in rice [43]. By means of GWAS, a single strong peak of SNPs associated with leaf Cd accumulation was identified in A. thaliana [44]. In maize, the genetic control of Cd accumulation in leaves was studied using genome-wide association analysis and QTL mapping, whereby candidate genes and favourable alleles were identified [4]. However, studies on the genetic control of Cd, As, and Pb in wheat are scarce at best. Here, we found some important MTAs loci for Cd and Pb content involving chromosomes 1B, 1D, 4A, 4B, 5A, 6B, and 7B. This indicates that some important genes need to be studied.

Previous studies have shown that heavy metal ATPases, metal tolerance proteins (MTPs), and natural resistance-associated macrophage proteins are involved in the deposition of metals in the grain [44]. Plant MTPs are transition metal transporters that catalyse the efflux of Zn, Fe, Mn, Cd, Co, or Ni ions from the cytoplasm to the outside of the cell or into subcellular compartments [45, 46]. Therefore, there seems to be a synergy between some heavy metals and some of the beneficial mineral elements or simply, between mineral elements. Once metal ions are absorbed in rice, translocation of Cd from the roots to the shoots requires loading of Cd into the xylem from the symplast in the stele, which in turn requires heavy metal ATPase [47]. The Cd-related gene GRMZM2G175576 encoding a heavy metal-transporting ATPase was identified in maize, which is homologous to the rice gene OsHMA3 [4]. However, in our study, six candidate genes associated with Cd content were predicted in wheat, two of which had multiple effects, that is, Cd and Pb content and Cd, Fe, and Pb content. Their functions are primarily involved in ion binding, including metal-, Fe-, Ca-, Cu-, Mg-, and Zn-ion binding. Therefore, there are gene
interactions among mineral elements, including some that are harmful and some that are beneficial for humans.

**Conclusions**

In brief, herein, 17 major MAT loci for nine mineral elements were identified, and 16 candidate genes were predicted. There were some MTA loci clusters found on 12 chromosomes (1A, 1B, 1D, 2B, 3B, 3D, 5A, 5B, 6B, 7A and 7D). Eight multi-effect MAT loci for controlling more than one trait were detected, mainly distributed on chromosomes 3B, 7B, and 5A. The functions of these candidate genes are primarily involved in ion binding, including metal-, Fe-, Ca-, Cu-, Mg-, and Zn-ion binding, ATP binding, ATPase activity, DNA binding, RNA binding, and protein kinase activity. There were gene interactions among some of the mineral elements under study. Therefore, this study provides important loci and gene information for improving mineral element content in the wheat grain. In the future, the candidate genes identified herein should be further studied to elucidate the molecular mechanisms for controlling the content of these mineral elements in the wheat grain.

**Abbreviations**

QTL: Quantitative trait loci; GWAS: Genome-wide association study; RILs: Recombinant inbred lines; MQTL: Meta-QTL; MTAs: Marker-trait associations; SNPs: Single nucleotide polymorphisms; DZ: Dezhou; TA: Tai’an; ANOVA: Analysis of variance; MLM: Mixed linear model; NCBI: The National Center for Biotechnology Information; MAPK: Mitogen-activated protein kinase; MTPs: Metal tolerance proteins.

**Supplementary Information**

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