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Genome-Wide Association Study Reveals Genetic Basis of Trace Elements Accumulation in Maize Kernels

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Abstract: Clarifying the genetic basis of trace element accumulation is of great significance to breed new maize varieties with high quality. In this study, an integrated variant map with 1.25 million (M) SNPs and 489 inbred lines was used for a genome-wide association study on the accumulation of iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), cadmium (Cd) and Arsenic (As) in maize kernels. Seventeen SNPs linked with six genes were overlapped by different trace elements. After further analysis, 65 SNPs located in 28 genes with a p-value lower than 10^{-10} were associated with Cd content by genome-wide association studies (GWAS). There was a 3.1-fold difference in Cd content between different groups, which was divided by SNP haplotype in *chr2.S_160782359*, *chr2.S_161045498* and *chr2.S_161273716*. The amino acid sequences of *GRMZM2G150608* and *GRMZM2G051367* only shared 68.85% and 88.16% similarity between B73 and Mo17, and the Cd content of Mo17 was 2.2-fold that of B73. In addition, 19 lines with higher contents of Fe, Zn, Cu, Mn and fewer contents of As and Cd were screened from GWAS associated populations. This study will lay a foundation for revealing the molecular mechanism of trace element accumulation in maize kernels and provide candidate genes for breeding new maize varieties with high nutritional quality.

Keywords: maize (*Zea mays* L.); trace elements; genome-wide association studies (GWAS); kernel

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1. Introduction

More than one-third of the world's population suffers from nutritional trace element deficiency [1]. Meanwhile, heavy metal elements are enriched in the human body, which leads to various diseases [2]. Micronutrient malnutrition, referenced as hidden hunger, is a serious threat to people's health in developing countries, by which about 40% of the world's population is affected [3]. Among mineral nutrients, Zn (zinc) and Fe (iron) deficiency are the most prevalent. It was recently found that 27% of children under five years old and 41% of pregnant women could not absorb enough Fe from food, while 89% of children could not absorb enough Zn from their diet. In plants, Fe and Zn deficiency leads to the abnormal development of young leaves and stunted plant growth, while Cu (copper) deficiency leads to dark greenish leaves and Mn (manganese) deficiency leads to defects in glycolysis and the tricarboxylic acid cycle. As for toxicity metals, Cd (cadmium) and As (arsenic) are difficult to decompose, and they could damage human health by enrichment in the food chain. It was reported that 70% of Cd intake in humans was from crops. During plant development, the toxic phenotype, such as root growth retardation and water and nutrients absorption deficiency, was presented when Cd excessive accumulations [4]. Therefore, high-quality maize varieties with highly beneficial elements and less harmful elements will be necessary for human dietary safety.

Maize is the crop with the largest planting area and the highest yield, and it is also widely used as the staple food in the world [5]. The absorptions of beneficial trace elements, such as Fe, Zn, Mn, Cd and Cu, in maize varied largely in different varieties [6]. In addition, the distribution of harmful element pollution in China is highly consistent with that in maize planting areas [7]. The contents of Cd and As in maize kernels not only affect the quality of maize but also human health. Furthermore, the absorption and accumulation of Cd and As in different cultivars are greatly different [8]. Therefore, maize may be the ideal material that can reduce food risk and supply nutrients by increasing the contents of beneficial trace elements meanwhile reducing harmful ones [5]. In previous studies, the key genes controlling the absorption of trace elements were widely located in the plant. In rice, a larger number of QTLs controlling mineral concentration were identified using biparental populations [9–14]. There are hundreds of genes identified in many different plant species as key regulators of metal ion content [15–21]. In maize, one, two and two QTL were identified for the Cu, Fe and Mg concentration of ear-leaves in an intermated B73 × Mo17 maize population (IBM) [22]. Due to the limitation of materials and technology, only a few key genes regulating metal ion content were studied, in which *Os-HKT1*;5 (or *SKC1*) for sodium (Na), *Os-HMA3* for Cd and *Os-HMA4* for copper (Cu) were included [23–27].

Genome-wide association study (GWAS), in which the natural populations were utilized, was widely used for the detection of variants associated with complex quantitative traits in different plants [6,28]. In the past decade, the loci related to different mineral accumulation was detected by GWAS. In *Arabidopsis*, several loci/genes related to mineral accumulation were identified, such as *HAC1* for As [29], *HMA3* for Cd [30] and *MOLYBDATE TRANSPORTER1* (*MOT1*) for molybdenum (Mo) [31,32]. In rice, GWAS was used to analyze the accumulation of As, Cu, Mo and Zn in grain [33] and Zn, Fe, Mn, Cu, P, Ca, K and Mg in brown rice [34]. In other studies, 72 loci associated with 17 mineral elements were identified by GWAS in rice, and the function of three candidate genes, such as *Os-HKT1*;5 for sodium, *Os-MOLYBDATE TRANSPORTER1*;1 for Mo and *heading date7* for nitrogen (N), were studied [35]. In maize leaves, *qLCd2* and *GRMZM2G175576* were identified by combination analysis between GWAS and QTL mapping [6]. In maize kernels, 11 (for Zn) and 11 (for Fe) SNPs were found by GWAS using 923 lines constructed by bi-parent, but the key genes for controlling the accumulation of Fe and Zn were not fully mined [15]. Limited by the divergence of populations and SNPs density, the regulatory network of the accumulation of trace metal ions in maize kernels was not fully uncovered.

In maize, the integrated variant map with 1.25 M SNPs and 489 inbred lines reconstructed by Yan's lab was applied in re-mapping the eQTL landscape for maize kernels [36]. In this study, the genetic map created by Yan's lab was used for the genome-wide association study of Fe, Mn, Cu, Zn, Cd and As accumulation in maize kernels. These results will lay a foundation for revealing the genetic basis of trace element accumulation and provide key genes for breeding new maize varieties beneficial to human dietary health.

2. Materials and Methods

2.1. Plant Materials

A set of 489 maize lines from 513 inbred lines, including temperate and tropical/subtropical elite inbred lines, were collected and sequenced as described previously [36]. The association population was planted in 2017 at three locations, Sanya, Hainan (HN, 18°14′ N, 109°31′ E), Dongyang, Zhejiang (DY, 28°59′ N, 120°05′ E) and Xinxiang, Henan (XX, 35°18′ N, 113°54′ E) of China. Approximately 185 out of 490 inbred lines are shared by the three locations; Hainan and Dongyang shared 440 inbred lines, Hainan and Xinxiang shared 193 inbred lines; Dongyang and Xinxiang shared 188 inbred lines; 13 inbred lines were unique in Hainan; 2 inbred lines were unique in Dongyang; 10 inbred lines were unique in Xinxiang. The Randomized block experiment design was used in the field, and two replicates were set. Each inbred line was planted in a single row with 17 plants in each row with a spacing of 55 cm × 40 cm. The final plant density was 45,000 plants per hectare. The field management was the same as the general style. Each maize inbred line was self-pollinated and harvested individually.

2.2. Trace Element Contents Detected in Maize Kernels and Soil

The association population was harvested at the dough stage (R4) to determine the content of trace elements in maize kernels. Fifty kernels of each inbred line, dried at 70 °C for 24 h, were ground to a powder using agate mortar without contacting any element's products. The soil samples of each location were taken from 20 cm-depth fields by an evenly distributed five-point sampling method. The samples (0.5 g dry kernel powder or soil per sample) were digested with 3 mL HNO_3 (Analytical Reagent) and 3 mL 30% (v/v) H_2O_2 on a heating block for 200 °C (kernels) or 240 °C (soil) for 8 h. The digestion solution was filtered by quantitative filter paper and diluted the volume to 50 mL. The content of Fe, Zn, Cu, Mn, As and Cd of each inbred line was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Nippon Jarrell-Ash, Tokyo, Japan). Three technical replicates were performed for each digestion.

2.3. Phenotypic and Genotypes Analysis

The *Performance Analytics* package in R software was used to calculate the correlation of traits in each location. Analysis of variance (ANOVA) and heritability (h^2) of concentrations of trace elements in leaves was performed using SPSS statistics 21.0 as per the methods described by Holland et al. [37]. The best linear unbiased prediction (BLUP) value of six trace element contents in each location was calculated using the *lem4* package (R software). The screening of inbred lines which exhibited higher beneficial elements and less harmful elements were analyzed using the online software (http://www.interactivenn.net/ (accessed on 12 December 2021)).

Four hundred eighty-nine inbred lines were genotyped using genotyping-by-sequencing (GBS). After screening with minor allele frequency (MAF) > 0.05, SNP calling rate > 5% and Heterozygosity rate < 0.05, the remaining 1,195,349 high-quality SNPs were used for subsequent analysis. In total, 1.25 M of high-density SNP type for the data was obtained from the Maizego website (http://maizego.org/Resources.html (accessed on 12 December 2021)) [38].

2.4. Genome-Wide Association Analysis

The mixed linear model (principal component analysis (PCA) + kinship (K) matrix) in the Tassel 5.0 software [39] was used for genome-wide association analysis. Kinship matrix (kinship, K) was calculated by Tassel. The PCA between inbred lines was calculated using the *SNP Relate packet* in R software. A p-value threshold less than 10^{-4} or 10^{-5} was considered to be significantly associated with different traits. Further, the SNPs associated with Cd, of which the p-value was less than 10^{-10}, was used for the next analysis. The Quantile–Quantile (Q-Q) Plots and Manhattan Plots were created using the *qqman* package (R language).

2.5. Prediction of Candidate Genes

The candidate SNPs associated with each trace elements in the maize kernels were mapped to the B73 genome (RefGen_v2) sequence, and then the candidate genes were located. The gene annotation information in the B73 genome was obtained from maizeGDB (https://chinese.maizegdb.org/ (accessed on 13 December 2021)) or the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/ (accessed on 13 December 2021)). The amino acid sequences of candidate genes in B73 and Mo17 were downloaded from the maizeGDB website. The amino sequences were compared and analyzed using DNAMAN software (version 6.3.3.99, Lynnon Corp., Vaudreuil-Dorion, QC, Canada).

2.6. Statistical Analysis

The collected data were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 19 (IBM Crop., Armonk, NY, USA). The means were separated by Tukey's test for trace element contents at different locations, while Student's *t*-test was used to determine the Cd concentration of different groups.

3. Results

3.1. Phenotypic Analysis for Trace Elements' Content of Maize Kernel in Different Locations

The characteristics and composition of mineral elements were slightly different among soil from Hainan, Dongyang and Xinxiang (Table 1). The soil sample results from Dongyang, with the contents of total Fe (14,760.52 mg/kg), Cu (24.74 mg/kg), As (19.4 mg/kg) and Cd (0.31 mg/kg), were higher than that of Hainan and Xinxiang. The Zn (29.96 mg/kg) and Mn (125.11 mg/kg) contents of Hainan soil were lower than those of Dongyang and Xinxiang (Table 1). The average contents of Fe, Zn, Cd, Cu, Mn and As in Xinxiang soil were the lowest in three detected locations, while Dongyang exhibited the highest content of Fe (24.54 mg/kg), Zn (36.38 mg/kg) and Cd (0.0121 mg/kg). The content of Cu (3.08 mg/kg), Mn (11.00 mg/kg) and As (0.13 mg/kg) of Hainan soil were highest (Figure 1). Further analysis revealed that there was no obvious correlation in the contents of trace elements between maize kernel and soil.

Table 1. The contents of Fe, Zn, Mn, Cu, As and Cd in the soil of Hainan, Dongyang and Xinxiang.

	Fe (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	As (mg/kg)	Cd (mg/kg)
HN	3706.79 ± 301.22^{b}	29.96 ± 3.49^{b}	8.16 ± 1.61^{b}	125.11 ± 7.55^{c}	5.2 ± 0.70^{c}	0.02 ± 0.01^{c}
DY	14760.52 ± 182.45^{a}	65.17 ± 2.41^{a}	24.74 ± 0.19^{a}	146.70 ± 2.89^{b}	19.4 ± 0.35^{a}	0.31 ± 0.01^{a}
XX	33428.20 ± 562.40^{b}	70.27 ± 4.15^{a}	24.51 ± 0.12^{a}	614.41 ± 12.09^{a}	11.88 ± 0.37^{b}	0.26 ± 0.01^{b}

The numbers in the table represented the average content (mg/kg) of each trace element of all inbred lines planted in different locations. HN: Hainan; DY: Dongyang; XX: Xinxiang. Different letters represent significant differences between samples.

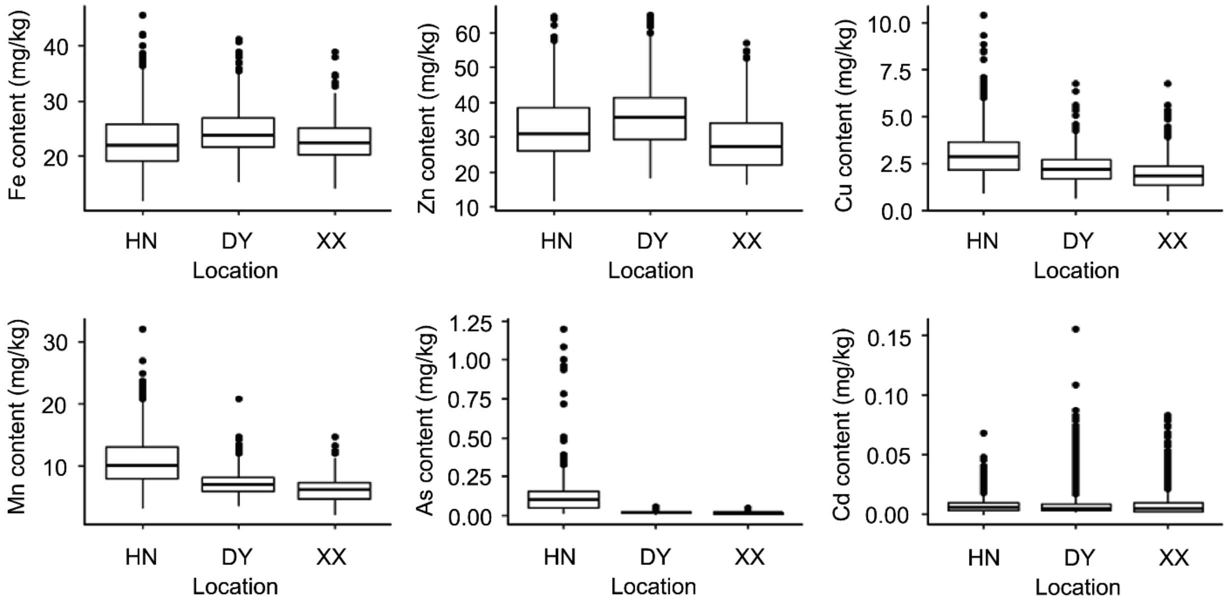

Figure 1. Contents of Fe, Zn, Cu, Mn, As and Cd in maize kernel in Hainan (HN), Dongyang (DY) and Xinxiang (XX).

The average contents of Fe, Zn, Cd, Cu, Mn and As in Xinxiang soil were the lowest among the three locations, while Dongyang exhibited the highest content of Fe (24.54 mg/kg), Zn (36.38 mg/kg) and Cd (0.0121 mg/kg). The content of Cu (3.08 mg/kg), Mn (11.00 mg/kg) and As (0.13 mg/kg) of Hainan soil were highest. The broad-sense heritability (h_2) of six mineral contents across all measured environments ranged from 0.87 to 0.99 (Table 2), which suggested the high repeatability in three locations and the key role of genetic factors in mineral accumulation. In Hainan and Dongyang, the Cu, As and Cd contents of maize kernels in the natural population had a large variation with the variation coefficient (CV) ranging from 14.32% to 61.71%, while the Fe, Zn and Mn contents exhibited a small variation with the variation coefficient (CV) only ranging from 3.75% to 7.58% (Table 2).

Table 2. Heritability and contents of Fe, Zn, Cu, Mn, As and Cd in maize kernel in three locations.

Trait	Location	Heritability	Mean [a]	Range	CV (%)
Fe	HN	0.98	22.91 a	11.74–45.47	5.41
	DY	0.95	24.54 b	15.32–41.04	5.74
	XX	0.89	21.16 a	14.09–38.89	7.58
Zn	HN	0.98	32.66 b	11.55–64.79	5.24
	DY	0.98	36.38 c	18.08–64.98	4.67
	XX	0.94	22.31 a	16.33–57.01	6.47
Cu	HN	0.87	3.08 c	0.83–10.40	25.93
	DY	0.94	2.29 b	0.61–6.78	14.32
	XX	0.94	1.58 a	0.45–6.78	16.18
Mn	HN	0.99	11.00 c	3.06–32.19	4.68
	DY	0.99	7.23 b	3.37–20.93	3.75
	XX	0.99	5.08 a	2.09–14.76	4.23
As	HN	0.95	0.13 b	0.10–1.20	37.13
	DY	0.96	0.02 a	0.0037–0.061	14.55
	XX	0.98	0.01 a	0.0031–0.05	7.55
Cd	HN	0.87	8.59×10^3 a	0–0.068	61.71
	DY	0.98	1.21×10^2 b	0.0012–0.16	28.34
	XX	0.99	7.20×10^3 ab	0.001–0.083	12.52

[a] Different lowercase letters denote significant differences ($p < 0.05$, Tukey's test).

The frequency of Fe, Zn, Cu and Mn contents of maize kernels among three locations occurred in a normal distribution (Figure 2), while the skewed distributions were presented in the frequency distribution histograms of As and Cd in Hainan, Dongyang and Xinxiang. A high correlation between Fe, Zn and Mn was detected in all three locations. Additionally, a high correlation between Cu and Cd (correlation coefficient (r) = 0.13), meanwhile a weak negative correlation between Cu and As (r = 0.15), was uniquely present in Hainan (Figure 2). In Dongyang, a significantly ($p < 0.01$) positive correlation was observed among Fe, Zn, Mn, Cu and As, while Cd only had a weakly positive correlation with Cu and As. In Xinxiang, a significantly ($p < 0.01$) positive correlation was presented among Fe, Zn, Mn and Cu, and As only had a positive correlation with Zn (r = 0.15) and Cu (r = 0.35). At the same time, there was no correlation between Cd with other trace elements (Figure 2).

In order to eliminate the differences of trace element content in different places, BLUP was used for analyzing the contents of Fe, Zn, Cu, Mn, As and Cd (Supplemental Table S1). There were 219, 221, 227 and 201 lines for the content of Fe, Zn, Cu and Mn, respectively, of which the content was higher than average (Supplemental Table S1). Meanwhile, there were 311 (for As) and 358 (for Cd) lines, respectively, of which the content was lower than average (Supplemental Table S1). Further analysis revealed 19 lines with higher contents of Fe, Zn, Cu and Mn and lower contents of As and Cd (Supplemental Figure S1), such as CIMBL66, CML114, Gy462, D047, MN, 04K5702, GEMS65, CML225, CML170, CIMBL135, TX5, Gy220, GEMS61, TY9, CIMBL146, CML20, CML451, CML28 and GEMS6 (Supplemental Table S2).

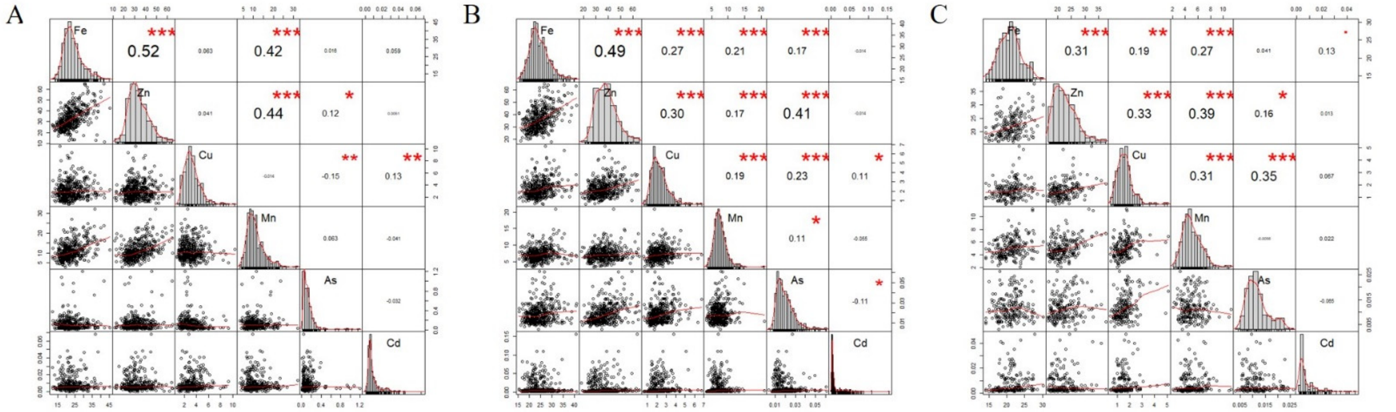

Figure 2. Frequency distributions and phenotypic correlations of Fe, Zn, Cu, Mn, As and Cd. (**A**) Hainan; (**B**) Dongyang; (**C**) Xinxiang. The *Performance Analytics* package in R software was used to calculate the correlation of traits in each location. Asterisk represents a significant difference. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test).

3.2. Genome-Wide Association Analysis of Trace Elements Accumulation in Kernels

Based on the mineral contents and SNPs data of 489 maize lines, genome-wide association analysis of Fe, Zn, Cd, Cu, Mn and As in Hainan, Dongyang and Xinxiang were performed by the mixed linear model (MLM, PCA+K). In order to mine the key genes related to mineral accumulation, $p = 10^{-5}$ was set as the threshold (Figure 3). A total of 1603 significant loci were identified at $p = 10^{-5}$, each explaining an average of 9.23% of the phenotypic variation. Seven hundred and ten loci were detected in Hainan, accounting for 9.83% of the phenotypic variation on average. Eight hundred and eleven loci were detected in Dongyang region, explaining 7.16% of the phenotypic variation on average. In Xinxiang, 82 loci were detected, explaining 12.76% of the phenotypic variation on average. Seventeen SNPs were overlapped by different elements simultaneously (Table 3). Five SNPs, *chr3.s_28354214* (*GRMZM2G375197*), *chr4.s_240465668* (*GRMZM2G384293*), *chr4.s_240465753* (*GRMZM2G384293*), *chr7.s_4960091* (*GRMZM2G039922*) and *chr10.s_110087144* (*GRMZM2G034206*) located on chromosomes 3, 4, 7 and 10 were associated with Fe and Zn, which explained a 7.07% variation of Fe content and 5.62% variation of Zn content (Table 3). Four SNPs were located on *GRMZM2G123257* of chromosome 4, *chr4.s_38571269*, *chr4.s_38571275*, *chr4.s_38571287* and *chr4.s_38571537*, were correlated with Fe and Cd content, which explained the 5.71% variation of Fe content and the 6.00% variation of Cd. Three SNPs on chromosome 3, *chr3.s_106959180*, *chr3.s_107012870* and *chr3.s_107015045*, were correlated with Zn and As content, which explained the 4.87% variation of Zn content and 5.58% variation of As content on average (Table 3). Two SNPs located on *GRMZM2G018820* of chromosome 5, *chr5.s_164728663* and *chr5.s_164728686*, were correlated with Fe and Mn content, which explains the 6.05% variation of Fe content and the 5.24% variation of Mn content on average (Table 3). *chr5.s_13803855* and *chr10.s_139994623* were correlated with As and Cd, which explained the 6.30% variation of As content and 9.55% variation of Cd content (Table 3). *chr2.s_121584444* was correlated with Zn and Cd content, explaining 6.74% of Fe content and 14.71% of Cd content (Table 3). The correlated SNPs of Fe, Zn, Cu, Mn and As in different locations presented no overlap with each other, suggesting the accumulation of these elements was greatly affected by the environment.

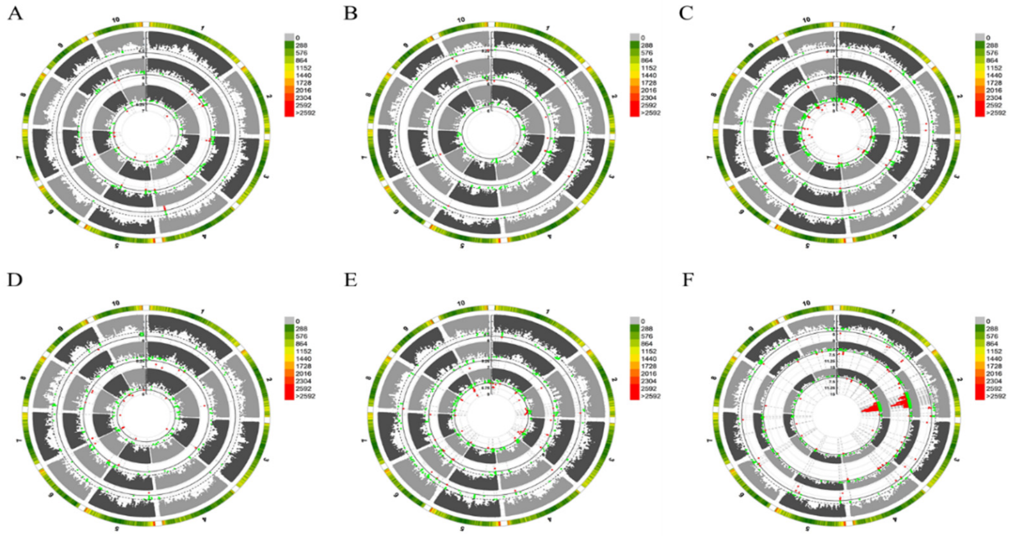

Figure 3. Manhattan plots on ten maize chromosomes for six traits in three locations. (**A**) Fe; (**B**) Zn; (**C**) Cu; (**D**) Mn; (**E**) As; (**F**) Cd. The circle from inside to outside represents HN, DY, XX and marker density. The red dotted line and black line indicate the significance at $p = 10^{-4}$ and $p = 10^{-5}$ level, respectively. The outermost circle shows the SNPs density on the 10 chromosomes. The marker density was shown in the color legend on the right side of the figure.

Table 3. Functions of the SNPs located genes associated with at least two trace elements.

SNP	Gene ID	Associated Trace Element	Annotation
chr2.s_121584444	*GRMZM2G076905*	Zn Cd	uncharacterized
chr3.s_28354214	*GRMZM2G375197*	Zn Fe	uncharacterized
chr4.s_38571269	*GRMZM2G123257*	Cd Fe	S-adenosyl-L-methionine-dependent methyltransferase superfamily protein
chr4.s_38571275	*GRMZM2G123257*	Cd Fe	S-adenosyl-L-methionine-dependent methyltransferase superfamily protein
chr4.s_38571287	*GRMZM2G123257*	Cd Fe	S-adenosyl-L-methionine-dependent methyltransferase superfamily protein
chr4.s_38571537	*GRMZM2G123257*	Cd Fe	S-adenosyl-L-methionine-dependent methyltransferase superfamily protein
chr4.s_240465668	*GRMZM2G384293*	Zn Fe	embryo defective 14
chr4.s_240465753	*GRMZM2G384293*	Zn Fe	embryo defective 14
chr5.s_164728663	*GRMZM2G018820*	Mn Fe	glycerophosphoryl phosphodiesterase
chr5.s_164728686	*GRMZM2G018820*	Mn Fe	glycerophosphoryldiester phosphodiesterase
chr7.s_4960091	*GRMZM2G039922*	Zn Fe	uncharacterized
chr10.s_110087144	*GRMZM2G034206*	Zn Fe	Beta-13-N-Acetylglucosaminyltransferase family protein

Gene functions were annotated by NCBI. A noncoding region in the table meant the SNP was not located within the coding region.

3.3. Identification of Causal Genes of Cd Accumulation

For the accumulation of Cd, the correlated SNPs in Hainan and Dongyang largely overlapped with each other. Furthermore, the Cd contents in three locations were analyzed by BLUP. Genome-wide association was also constructed by Cd-Hainan, Cd-Dongyang and Cd-BLUP (BLUP-based Cd content). At levels of 10^{-10} (*p*), 65 overlapped SNPs, which were located at 160773728 … 161276954 on Chr2, were detected among Cd-Hainan, Cd-Dongyang and Cd-BLUP (Figure 4). Additionally, 28 genes were annotated in this overlapped region (Table 4).

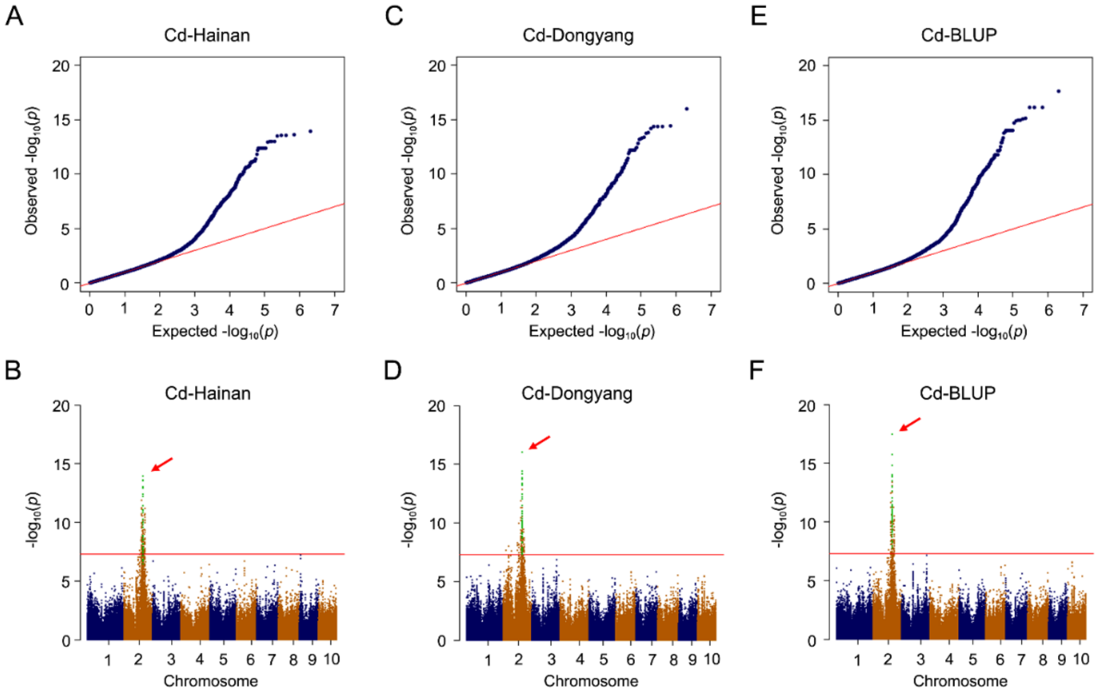

Figure 4. Presentation of a few highly reproducible and significant Q–Q Plots and Manhattan for Cd. (**A**) Quantile–quantile (Q–Q) plots showing inflation of estimated $-\log_{10}$ (p) values versus observed for traits Cd in Hainan using Q + K (MLM). Q represent 31 principal components (fixed), K represents a kinship matrix (random), GLM represents the general linear model, MLM represents the mixed linear model. (**B**) Manhattan plot from the Q + K (MLM) model for Cd in Hainan, plotted with the individual SNPs of all chromosomes on the X-axis and $-\log_{10}$ $^{(p\text{-value})}$ of each SNP in the Y-axis. The different colors indicate the 10 chromosomes of maize. The red horizontal line shows the multiple testing threshold $-\log_{10}$ $^{(p\text{-value})}$ of 7.4 for this panel. The SNPs located within the candidate genes, as identified by the GWAS of the whole population, are labeled as green dots. (**C**) Q–Q plots for GWAS of traits Cd in Dongyang. (**D**) Manhattan plot for GWAS of traits Cd. (**E**) Q–Q plots for GWAS of traits Cd-BLUP. Cd-BLUP represents the best linear unbiased prediction of Cd in Hainan, Dongyang and Xinxiang. (**F**) Manhattan plot for GWAS of traits Cd-BLUP.

Table 4. The candidate genes included in the region detected by genome-wide association study.

Gene ID	Chr	Start	End	Annotation
GRMZM2G150608	Chr2	160781682	160789474	fha7—FHA-transcription factor 7
GRMZM2G150754	Chr2	160797324	160805739	(MED33A, RFR1) REF4-related 1
GRMZM2G171370	Chr2	160956979	160963086	bzip86—bZIP-transcription factor 86
GRMZM2G100005	Chr2	161016548	161017635	Ribosomal protein S4 (RPS4A) family protein
GRMZM2G051367	Chr2	161045370	161050327	(DGL1) dolichyl-diphosphooligosaccharide-protein glycosyltransferase
GRMZM2G018241	Chr2	161123950	161130108	cesa9—cellulose synthase9
GRMZM2G319836	Chr2	161128260	161129517	cesa9—cellulose synthase9
GRMZM2G150404	Chr2	161135362	161138301	cesa9—cellulose synthase9
GRMZM2G452739	Chr2	161136453	161137710	cesa9—cellulose synthase9
GRMZM2G165133	Chr2	161197745	161199034	(FQR1) flavodoxin-like quinone reductase 1
GRMZM2G073928	Chr2	161273580	161276954	Leucine-rich repeat protein kinase family protein

Among all overlapped SNPs, only 10 SNPs were found in the exon of the candidate genes. There were three SNPs located at *GRMZM2G051367* and six SNPs located at the exon of *GRMZM2G073928* (Table 4). Among them, three SNPs, *chr2.S_160782359*, *chr2.S_161045498* and *chr2.S_161273716*, with p-values lower than 2×10^{-14} were detected, and distributed in the exon of *GRMZM2G150608*, *GRMZM2G051367* and *GRMZM2G073928*, respectively. For *chr2.S_160782359*, the maize inbred lines could be divided into two basic groups concerning allelic effects (TT or CC). The Cd content in the CC haplotype was 3.1fold that of TT (Figure 5A). B73 belonged to TT, while Mo17 belonged to CC. For *chr2.S_161045498*, the maize inbred could be divided into two groups based on allelic effects (TT or AA). The Cd content in AA was 3.1-fold that of TT (Figure 5B). B73 belonged to TT, while Mo17 belonged to CC. For *chr2.S_161273716*, the maize inbred could also be divided into two basic groups concerning allelic effects (CC or GG). The Cd content in GG was 3.1-fold that of CC (Figure 5C). B73 belonged to CC, while Mo17 belonged to GG. According to haplotype, B73 was included in the low Cd population, while Mo17 belonged to the high Cd population. Cd content of Mo17 kernel was 2.2-fold that of B73 (Figure 5D). The analysis of the amino sequence showed that there was 68.85% similarity of *GRMZM2G150608* between B73 and Mo17, 88.16% similarity of *GRMZM2G051367* and 99.89% similarity of *GRMZM2G073928* (Figure 5E–G). Additionally, large fragment deletion or replacement of *GRMZM2G150608* and *GRMZM2G051367* were present in Mo17 compared to B73, while only an amino acid replacement existed between B73 and Mo17 in the sequence of *GRMZM2G073928*. These results indicate that the differences in sequences of GRMZM2G150608 and *GRMZM2G051367* might be responsible for Cd accumulation divergences between B73 and Mo17.

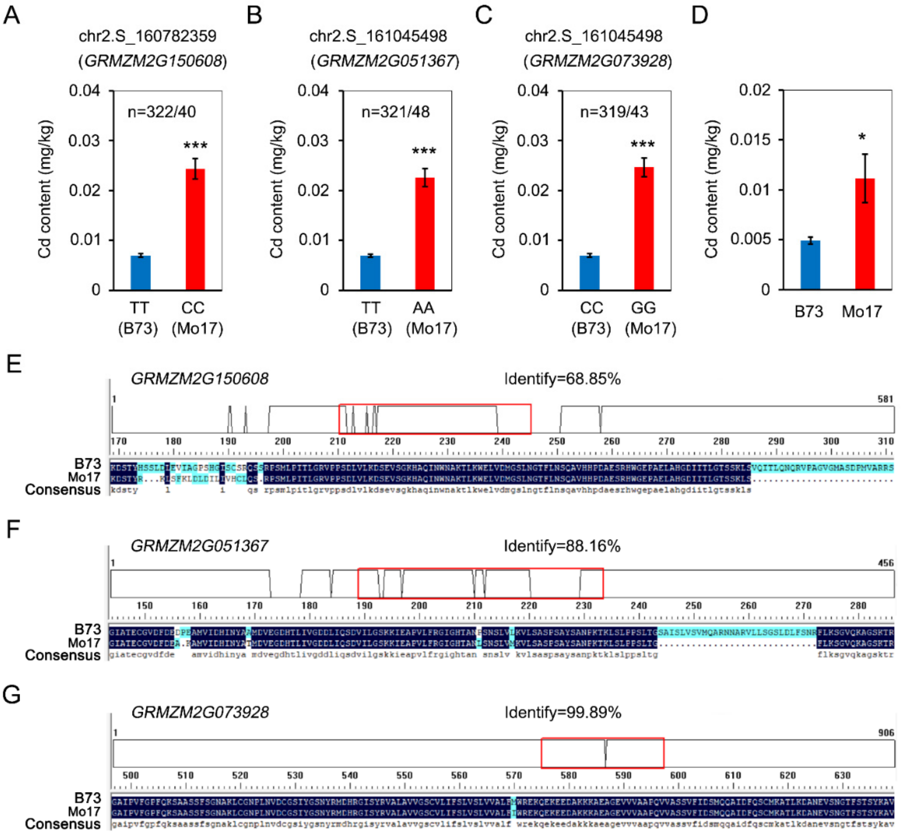

Figure 5. Phenotype analysis between the candidate genes for locus associated with Cd concentration in maize and phenotypic difference between different alleles. The number above the box represents the number of inbred lines homozygous for a determined allelic variant. (**A**–**C**) Two combinations of the minor and major between *chr2.S_160782359* (**A**), *chr2.S_161045498* (**B**) and *chr2.S_161273716* (**C**) in *GRMZM2G150608*, *GRMZM2G051367* and *GRMZM2G073928* region. B73 represent allele in B73; Mo17 represent allele in Mo17. Asterisk represents significant difference. *** $p < 0.001$ (Student's *t*-test). (**D**) Cd content in B73 or Mo17. Asterisk represents significant difference. * $p < 0.05$ (Student's *t*-test). (**E**–**G**) Amino sequence comparison of *GRMZM2G150608*, *GRMZM2G051367* and *GRMZM2G073928* between B73 and Mo17.

4. Discussion

4.1. Germplasm Resources for Cultivating New Maize Varieties with More Beneficial Trace Elements and Less Harmful Elements

This study presents a highly important way for people to survive by absorbing nutrient elements through staple food, meeting the physiological needs for the supplementation of micronutrients. As the largest planting area crop in the world, maize is the most important medium for transporting trace elements in the food chain [40]. The enrichments of beneficial or toxic trace elements in the food chain directly and indirectly affects people's health. The application of mineral elements into the soil does not necessarily lead to a significant increase of trace elements' content in maize stems, leaves and ears [41]. In our study, the accumulation of trace elements in maize kernels was mainly affected by environment and heritability (Table 2), while there was no significant correlation with the content of mineral elements in soil except As. Therefore, some researchers believed that the content of trace elements in the soil is high enough to fully meet the needs of maize growth and development, which led to the results that the content of trace elements in corn will not increase after application of trace elements [42]. Therefore, the enrichment of trace elements in maize kernels mainly depends on the genetic and physiological differences among cultivars.

In the results of the correlation analysis, the contents of Fe, Zn, Mn and Cu were highly correlated with each other, while there was a weak correlation between the content of As and Fe, Zn, Mn and Cu, and there was no correlation between Cd accumulation with other trace elements (Figure 2). This means the absorption of Fe, Zn, Mn and Cu may be regulated by the same pathway, but the accumulation of As and Cd may be independent from these beneficial trace elements. These results made it possible to cultivate high-quality maize varieties with more beneficial elements and less harmful elements. In this study, 19 lines, which had higher contents of Fe, Zn, Cu and Mn and fewer contents of As and Cd, were screened out of 489 maize lines (Supplemental Figure S1 and Table S2). These results will be helpful to cultivate high-quality maize varieties.

4.2. Candidate Genes for Cultivating New High-Quality Maize Varieties with Higher Beneficial Elements and Less Harmful Elements

In the plant, a variety of mineral elements were used in GWAS correlation analysis in one study for the sake of screening overlapped genes that regulated different mineral accumulation. Here, six trace elements were used for GWAS analysis in maize. The contents of Fe, Zn, Cu and Mn were found to be positively correlated with each other in three locations (Figure 2). Five SNPs, which were located on *GRMZM2G375197*, *GRMZM2G384293*, *GRMZM2G039922* and *GRMZM2G034206*, were overlapped in GWAS associated results of Fe and Zn (Table 3), while *chr5.s_164728663* and *chr5.s_164728686*, which were all located at *GRMZM2G018820*, were overlapped by Mn and Fe (Table 3). During correlation analysis of element contents, there was a high correlation between Fe and Zn in Hainan, Dongyang and Xinxiang (Figure 2). Fe and Mn also shared a high correlation in different locations (Figure 2). These results were consistent with the associated SNPs in Table 3. After further analysis, *GRMZm2G384293*, which was associated with Fe and Zn, was found to encode a NOS1 protein. In *Arabidopsis*, *nos1* showed Al toxicity- and Mg deficiency-insensitive phenotypes [43]. In maize, *GRMZm2G384293* (*NOS1*) may positively regulate the accumulation of Fe and Zn. In Vietnamese rice, *GLYCEROPHOSPHORYL DIESTER PHOSPHODIESTERASES* (*GDPD13*) was found by GWAS, associating with phosphate efficiency. Here, *GRMZM2G018820*, which encodes glycerophosphoryl diester phosphodiesterase1 [44], was linked with the accumulation of Fe and Mn by GWAS analysis (Table 3).

Heavy metal ions in plant leaves will damage plant growth, and the accumulation of heavy metal ions in maize seeds will not only harm plant development but also affect human dietary safety. The CVs of As and Cd varied greatly in different locations, from 7.55% to 37.13% and from 12.52% to 61.71% (Table 2), which indicated that the absorption of As and Cd were largely dependent on the environment. Previously, the function of HMA (heavy metal ATPase), NRAMP and MFS on Cd absorption was reported in *Arabidopsis*, rice and barley [45–47]. In the leaf of maize, a region, 153.75–167.58 Mb on Chr2, was associated with Cd accumulation by GWAS analysis and QTL mapping (Table 4), of which 269 inbred lines and 43,737 SNPs were used for GWAS analysis, while 280 IBM Syn10 populations and 5955 bins for were used QTL analysis [6]. Limited by population size and marker density, the localization interval related to Cd content could not be further narrowed accurately. Recently, 513 inbred lines and 1.25 M SNPs were used for genome-wide association analysis of Cd accumulation in maize grains, and *ZmHMA3* and *ZmHMA4* were screened and verified to be related to Cd accumulation in grains [48]. *ZmHMA3* was also associated with Cd accumulation in maize leaf [49]. In this study, the same population was analyzed for Cd accumulation in maize kernels in Dongyang, Hannan and Xinxiang. Here, we found that *chr2.S_158407174* ($p = 2.71 \times 10^{-10}$), *chr2.S_158407712* ($p = 3.12 \times 10^{-13}$), *chr2.S_158408660* ($p = 4.20 \times 10^{-14}$) and *chr2.S_158410625* ($p = 9.07 \times 10^{-11}$) were located at *ZmHMA3* (Table S3). In the coding region of *ZmHMA4*, chr2.S_158388814, Cd associated SNP with p (4.31×10^{-10}) was located (Table S3) in our study. In this region, our results were consistent with previous studies. However, it is unclear whether there are other mechanisms involved in Cd absorption, and the regulation mechanism of Cd absorption is still rarely reported. In regions ranging from 160773728 to 161276954 on Chr2, 65 SNPs with p-values lower than 10^{-10} were associated with Cd content in maize kernels (Table 4), which was not determined by Tang's study [48]. Among them, three SNPs (*chr2.S_160782359*, *chr2.S_161045498* and *chr2.S_161273716*) with p-values lower than 2×10^{-14} were linked and were located at the exon of *GRMZM2G150608*, *GRMZM2G051367* and *GRMZM2G073928*, respectively (Figure 5). The amino acid sequence of *GRMZM2G150608* and *GRMZM2G051367* only shared 68.85% and 88.16% similarity between B73 and Mo17, in which Cd content was 2.2-fold that of B73 (Figure 5). In *Arabidopsis*, forkhead-associated domain 2 (*FHA2*), a plant-specific ISWI subunit, was responsible for stamen development and plant fertility [50,51]. In rice, *OsDGL1*, encoding a dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48kDa subunit, was involved in N-Glycosylation and root development in rice [52]. The function of FHA and DGL on trace element accumulation was not studied in the plant. In maize, *GRMZM2G150608* encodes an FHA transcription factor, while *GRMZM2G051367* encodes a dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48kDa subunit. The associated difference in this region between our study and Tang' report may be attributed to the environmental factors (Tang et al., 2021). In our study, the maize kernels were harvested from different latitudes, Sanya, Hainan (HN, 18°14′ N, 109°31′ E), Dongyang, Zhejiang (DY, 28°59′ N, 120°05′ E) and Xinxiang, Henan (XX, 35°18′ N, 113° 54′ E), while maize grains were collected from similarity latitudes, Zhuzhou (ZZ) (N27°49′50.88″, E113°07′41.23″) and Ningxiang (NX) (N28°16′51.16″, E112°32′47.13″) (Tang et al., 2021). This means that the materials from the diverse environment will be helpful for the excavation of specific loci.

5. Conclusions

In summary, a genetic map with 1.25 M SNPs and 489 inbred lines was used for our genome-wide association study on the accumulation of Fe, Mn, Cu, Zn, Cd and As in maize kernels. In total, 17 SNPs, with which 6 genes were linked, were overlapped by different trace elements. In further analysis, 65 SNPs, with which 28 genes were closely linked, were associated with Cd accumulation. Among these genes, *GRMZM2G150608* and *GRMZM2G051367* were further analyzed and were found to share 68.85% and 88.16% amino sequence similarity between B73 (with low content of Cd) and Mo17 (with high content of Cd). In addition, 19 lines with higher contents of Fe, Zn, Cu, Mn and fewer contents of As and Cd were screened from GWAS-associated populations. All these will lay a foundation for revealing the molecular mechanism of trace element accumulation and provide candidate genes and germplasm for breeding new maize varieties with high nutritional quality in maize.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12020262/s1, Figure S1: Overlapped lines between lines with higher contents of Fe, Zn, Cu, Mn and lines with lower contents of As and Cd.; Table S1: The BLUP-based content (mg/kg) of Fe, Zn, Cu, Mn, As, Cd in Hainan, Dongyang and Xinxiang.; Table S2: The maize inbred with higher contents of Fe, Zn, Cu, Mn and less content of As and Cd.; Table S3: SNPs associated with Cd accumulation in maize kernel, of which the p-value was less than 10^{-8}.; Table S4: Correlation of the contents of Fe, Zn, Cu, Mn, As and Cd in maize kernel compared to soil.

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