Pleiotropic Effect Analysis and Marker Development for Grain Zinc and Iron Concentrations in Spring wheat

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Abstract

Wheat (*Triticum aestivum* L.) is one of the main food crops in the world and a primary source of zinc (Zn) and iron (Fe) in the human body. The genetic mechanisms underlying related traits have been clarified, thereby providing a molecular theoretical foundation for the development of germplasm resources. In this study, 23,536 high-quality DArT markers were used to map quantitative trait loci (QTL) of grain Zn (GZn) and grain Fe (GFe) concentrations in recombinant inbred lines from Avocet/Chilero. A total of 17 QTLs located on chromosomes 1BL, 2BL, 3BL, 4AL, 4BS, 5AL, 5DL, 6AS, 6BS, 6DS, and 7AS accounted for 0.38–16.62% of the phenotypic variance. *QGZn.haust-4AL*, *QGZn.haust-7AS.1*, and *QGFe.haust-6BS* were detected on chromosomes 4AL, 6BS, and 7AS, accounting for 10.63–16.62% of the phenotypic variance. Four stable QTLs, *QGZn.haust-4AL*, *QGFe.haust-1BL*, *QGFe.haust-4AL*, and *QGFe.haust-5DL* were located on chromosomes 1BL, 4AL, and 5DL. Three pleiotropic effects locus for GZn and GFe concentrations were located on chromosomes 1BL, 4AL, and 5DL. Two high-throughput Kompetitive Allele Specific PCR markers were developed by closely linking single nucleotide polymorphisms on chromosomes 4AL and 5DL, which were validated by a germplasm panel. Therefore, it is the most important that quantitative trait loci and KASP marker for grain zinc and iron concentrations were developed for utilizing in marker-assisted breeding and biofortification of wheat grain in breeding programs.

Introduction

Wheat (*Triticum aestivum* L.) is a vital crop in the food production industry. Wheat grains contain various mineral elements, among which, zinc (Zn) and iron (Fe) have important clinical significance for global public health (Maathuis 2009; Nikwan et al. 2021). Malnutrition caused by mineral deficiencies afflict > 2 billion people worldwide (Zhang et al. 2011; Stevens et al. 2013; Shivangi et al. 2021). Statistically, Zn deficiency threatens 17.3% of the world's population and roughly 433,000 children under the age of 5 die every year due to Zn deficiency. Fe deficiency is the thirteenth greatest health risk factor in the world with 24.8% of the global population and > 65% of preschool children in Africa and Southeast Asia suffering from Fe deficiency anemia (Stevens et al. 2013; Liu et al. 2019). Many people in developing countries supplement Zn and Fe through the consumption of wheat foods. Genetic modification is the primary method by which grain Zn (GZn) and grain Fe (GFe) concentrations are increased and have become an increasingly popular area of research in crop science (Xue et al. 2012).

At the beginning of the 21st century, while GZn and GFe enrichment in wheat was being promoted, different researchers presented various findings (Ryan et al. 2010). In earlier years, researchers were focused on increasing agricultural production. Kutman et al. (2011) showed that nitrogen (N) supply improved the rhizosphere environment of wheat and changed the rhizosphere morphology, thereby facilitating Zn and Fe absorption by the root system, which is then transferred to the grains. Ryan et al. (2010) found that phosphorous (P) fertilizer application had different effects on Zn and Fe absorption when compared to N fertilizer. If the P concentration in the soil was too high, Zn transport was inhibited. Srivastava et al. (2015) showed that potassium (K) application significantly enhanced Zn absorption in wheat grains and straws. Furthermore, Jalal et al. (2020) found that GZn and GFe concentrations significantly increased after spraying Zn and Fe fertilizer on plant leaves. However, these traditional fertilization methods are costly and easily poison the soil and plants. Currently, the safest and most effective method for addressing Zn and Fe deficiencies in humans is breeding Zn- and Fe-rich wheat varieties.

Recently, after the publication of the Chinese Spring Reference Genome, several gene loci related to GZn and GFe have been mined through linkage analyses and genome-wide association studies (GWAS) (Rasheed et al. 2017; Velu et al. 2019; Tong et al. 2020). Specifically, Crespo-Herrera et al (2016). detected three main quantitative trait loci (QTL) for GZn and three for GFe on chromosomes 4B and 7D using recombinant inbred line (RIL) populations of spring wheat, which accounted for 10.7–19.6% of the phenotypic variation. Velu et al. (2016) detected two major QTLs for GZn and three for GFe on chromosomes 1B, 3A, 5B, and 6B using a total of 105 tetraploid RILs, which accounted for 10.6–16.9% of the phenotypic variation. In a separate study, eight major QTLs for GZn and four for GFe were detected on chromosomes 1B, 1D, 2B, 3A, 3D, 6B, 7A, and 7B using a total of 127 hexaploid RILs, which accounted for 10.0–31.0% of the phenotypic variation. Wang et al. (2021) detected one major QTL for GZn and one for GFe on chromosome 4D, which was a pleiotropic QTL. Liu et al. (2019) detected 1 major QTL for GZn and 2 for GFe on chromosomes 2B, 3B, and 5A, which accounted for 10.8–14.6% of the phenotypic variation. Genc et al. (2009)

detected four major QTLs for GZn on chromosomes 3D, 4B, 6B, and 7A using a doubled haploid population. Each QTL exerted little effect but the aggregation of four high Zn alleles increased GZn by 23%.

Several researches have proven very effective in increasing Zn and Fe content by wheat breeding method (Shivangi et al. 2021). Based on advancements using different populations for gene mapping, several QTLs that control GZn and GFe have been identified; however, the number of cloned genes is relatively scarce. Therefore, in this study, a genetic analysis was carried out using new populations to serve as a reference for the mining of effective candidate genes in future studies. The high-density maps were constructed to mine important QTLs and develop molecular markers that can be used for breeding are of great significance for the genetic improvement of GZn and GFe nutritional quality. Thus, we aimed to identify QTLs for GZn and GFe using a total of 164 RILs derived from Avocet/Chilero. This study provides a theoretical basis and technical support for high Fe and Zn concentrations by utilizing wheat biotechnological breeding.

Materials And Methods

Plant materials

A total of 164 F6 RILs derived from crossing Avocet/Chilero were used for the QTL mapping of GZn and GFe concentrations. The population was developed by the International Maize and Wheat Improvement Center (CIMMYT) with single-head descent approach was described (Basnet et al. 2014), and was used previously to characterize leaf rust, stripe rust resistance and fructan content (Ponce-Molina et al. 2018; Zeng et al. 2020). A germplasm panel, including 54 domestic and 97 foreign germplasm resources was used to validate QTLs for GZn and GFe. Materials were provided by the International Maize and Wheat Improvement Center (CIMMYT) and the wheat germplasm innovation and molecular breeding project of Henan University of Science and Technology, China.

Field trials

The RILs population was tested under a total of three environments in three sites. Field trials were conducted in the experimental field at the Agricultural College of Henan University of Science and Technology (E1; 34°62'N, 112°45'E) during the 2019–2020 cropping seasons, Luoning (E2; 34°38'N, 111°65'E) during the 2019–2020 cropping seasons, and Mengjin (E3; 34°49'N, 112°26'E) during the 2020–2021 cropping seasons in Henan Province, China. Each plot consisted of 2-m rows that were spaced 25 cm apart with 25 seeds in each row. The experiment was arranged in a randomized block design with three replicates. Field management was carried out in accordance with local conventional cultivation measures. There were no serious pests or diseases and lodging throughout the entire growth period.

Measuring of grain Zn and Fe concentrations

After wheat plants matured, we randomly selected normal grains weighing ~5 g, placed the samples in a paper bag, and oven-dried the grains at 80°C to a constant weight. Dried samples were crushed with a tungsten carbide ball mill (MM 400; Retsch, Haan, Germany) to obtain whole wheat flour. Whole wheat flour weighing 0.2 g was placed in a digestion tube, to which, 5 mL HNO₃ (analytical grade) and 1 mL H₂O₂ (analytical grade) were added. Samples were digested in a microwave digestion machine (Multiwave PRO; Anton Paar, Shanghai, China) until no visible tissue pieces were left in the tube. After cooling, the volume was fixed to 25 mL. An inductively coupled plasma emission spectrometer (5110 ICP-OES; Agilent, California, USA) was used to determine the GZn and GFe concentrations (Lu et al. 2010; Vijay et al. 2009). An analysis of variance (ANOVA) and normal distribution tests were performed using IciMapping v4.1 (<http://www.isbreeding.net>) and SPSS v21.0 (IBM Inc., Chicago, USA) (Meng et al. 2015; Yan et al. 2013). A Student's t-test was used to calculate whether there was significant differences between the two genotypes; the genotypes were used as the random effects (Velu et al. 2014).

Single nucleotide polymorphism (SNP) genotyping analysis

DArT markers covering the whole wheat genome with a total of 23,536 detection probes were used in this study, which were measured by Tritacarte Co., Ltd. (Canberra, Australia). The molecular marker data was provided by the Ravi P. Singh team of

QTL mapping

Statistical processing of the markers was performed using Excel 2010 (Microsoft, Washington, USA) to screen polymorphic markers with deletion rates < 10% and minimum alleles > 30%. IciMapping v4.1 software was used to construct the linkage maps and conduct the QTL analysis (Li et al. 2010; Wang 2009). JoinMap v4.0 (<https://joinmap.software.informer.com>) was used to calibrate the genetic lengths and obtain the final genetic map (Zeng et al. 2020). QTL mapping was performed by inclusive composite interval mapping (ICIM). The linkage marker sequence was compared to the Chinese Spring Reference Genome to obtain the physical location of the marker. Physical maps for the positional comparisons of GZn and GFe QTLs with previous reports were visualized using MapChart v2.3 (<https://mapchart.net>) (Velu et al. 2016).

Conversion of SNPs to Kompetitive Allele Specific PCR (KASP) markers

SNPs closely linked to the target QTLs were transformed into KASP markers. PolyMarker (<http://www.polymarker.info/>) was used to design KASP primers (Wang et al. 2021). A fluorescent quantitative real-time (qRT)-PCR instrument (C1000 Touch; Bio-Rad, California, USA) was used for gel-free fluorescent signal scanning and allele separation (Chandra et al. 2017). KASP reaction procedures were conducted following previously described methods (Rasheed et al. 2016).

Results

Phenotypic evaluation

Concentrations and variation ranges of GZn and GFe in RILs population and the parental lines are provided in Table 1. There was a significant difference between the GFe of Avocet and Chilero, where the GFe of Avocet was higher than Chilero in three environments and there was significant differences between the GFe of the RILs. There was no significant differences between the GZn of Avocet and Chilero in three environments, but the GZn among the RILs differed considerably. Thus, it was inferred that GZn is a quantitative trait controlled by polygenes. GZn and GFe concentrations in RILs population had a continuous normal distribution (Fig. 1). Results showed that genotype, environment, and their interactions had significant effects on the GZn and GFe in the RILs population. Both GZn and GFe had a heritability of 55% (Table 2).

Table 1
Mean and range of GZn and GFe (mg kg⁻¹) in the Avocet/Chilero population among three environments

Trait	Environment	Parents		RILs		
		Avocet	Chilero	Range	Mean ± SD	CV (%)
GZn(mg kg ⁻¹)	E1	28.68	28.28	21.47-39.28	30.16±3.91	12.96
	E2	18.80	19.53	13.62-36.77	19.14±3.02	15.78
	E3	19.15	19.33	17.53-40.74	24.21±3.77	15.57
GFe(mg kg ⁻¹)	E1	36.74	30.70	26.08-69.77	38.47±8.81	22.90
	E2	57.91	49.59	18.00-78.71	41.50±8.32	20.05
	E3	44.78	37.01	26.88-63.92	38.67±7.28	18.83

Table 2
Analysis of variance of GZn and GFe in the Avocet/Chilero RILs population

Trait	Source of variation	DF	SS	MS	Fvalue	Pvalue	<i>h</i> ²
GZn	Genotype (G)	163	12197.96	74.83	6.74	0.00**	55%
	Environment (E)	2	132457.80	66228.90	5965.05	0.00**	
	Replicate	2	52.74	17.58	1.58	0.19	
	G × E	308	16053.00	52.12	4.69	0.00**	
GFe	Genotype	163	72524.34	444.93	15.37	0.00**	55%
	Environment	2	245213.91	122606.95	4235.14	0.00**	
	Replicate	2	261.18	87.06	3.01	0.03*	
	G × E	308	103876.50	341.70	11.80	0.00**	
* and ** Significant at <i>P</i> <0.05 and 0.01							

Linkage map construction

Of the 23,536 DArT markers (genotypic data is available for the RILs), 3,290 polymorphic molecular markers were used to construct the genetic linkage map. The map covered 15,281.85 cM, and the average genetic distance was 4.64 cM. Except for group 4 consisting of homoeologous chromosomes, the number of markers for other groups was roughly the same. Chromosome 4D had the least label coverage with 41 linkage markers and chromosome 6B had the most with 266 linkage markers. Chromosome 4D had the longest average genetic distance of 8.68 cM and chromosome 3B had the shortest at 3.71 cM (Table 3).

Table 3
Distribution of DArT markers in the linkage map on the chromosomes in the Avocet/Chilero RILs population

Chromosome	Marker number	Genetic length/cM	Average density of markers/cM	Chromosome	Marker number	Genetic length/cM	Average density of markers/cM
1A	184	785.50	4.27	6B	266	1095.01	4.12
1B	175	702.65	4.02	6D	92	641.21	6.97
1D	86	563.96	6.56	7A	214	918.82	4.29
2A	120	660.15	5.50	7B	200	833.71	4.17
2B	238	932.18	3.92	7D	121	714.42	5.90
2D	102	677.35	6.64	Group 1	445	2052.11	4.61
3A	204	773.02	3.79	Group 2	460	2269.68	4.93
3B	238	883.60	3.71	Group 3	545	2287.95	4.20
3D	103	631.33	6.13	Group 4	367	1619.49	4.41
4A	211	801.94	3.80	Group 5	467	2126.15	4.55
4B	115	461.49	4.01	Group 6	471	2459.52	5.22
4D	41	356.06	8.68	Group 7	535	2466.95	4.61
5A	210	831.55	3.96	A genome	1256	5494.28	4.37
5B	194	759.73	3.92	B genome	1426	5668.37	3.98
5D	63	534.87	8.49	D genome	608	4119.20	6.78
6A	113	723.30	6.40	Total	3290	15281.85	4.64

QTL mapping of GZn and GFe

A total of 10 QTLs of GZn were located on chromosomes 1B, 2B, 3B, 4A, 4B, 5A, 5D, 6A, and 7A, and identified in different environments (Fig. 2; Table 4). The identified QTLs were designated as *QGZn.haust-1BL*, *QGZn.haust-2BL*, *QGZn.haust-3BL*, *QGZn.haust-4AL*, *QGZn.haust-4BS*, *QGZn.haust-5AL*, *QGZn.haust-5DL*, *QGZn.haust-6AS*, *QGZn.haust-7AS.1*, and *QGZn.haust-7AS.2*, accounting for 1.07–14.67% of the phenotypic variation. The favorable alleles of *QGZn.haust-1BL*, *QGZn.haust-3BL*, *QGZn.haust-4BS*, *QGZn.haust-5AL*, *QGZn.haust-5DL*, *QGZn.haust-6AS*, and *QGZn.haust-7AS.2* originated from Avocet and the phenotypic variation of each QTL was < 10%. *QGZn.haust-2BL*, *QGZn.haust-4AL*, and *QGZn.haust-7AS.1* exhibited decreasing GZn. The phenotypic variation of *QGZn.haust-4AL*, and *QGZn.haust-7AS.1* was 11.87%, and 16.62%, respectively, indicating that they are major QTLs. *QGZn.haust-4AL* was bound to the DArT marker interval *SNP100512195–1721210* with a physical interval ranging from 720.1–731.0 Mb and was detected in E1 and E3, indicating that it is a stable major QTL for controlling GZn.

A total of 7 QTLs of GFe were located on chromosomes 1B, 2B, 4A, 5A, 5D, 6B, and 6D, and were identified in different environments (Fig. 2; Table 4). The identified QTLs were designated as *QGFe.haust-1BL*, *QGFe.haust-2BL*, *QGFe.haust-4AL*, *QGFe.haust-5AL*, *QGFe.haust-5DL*, *QGFe.haust-6BS*, and *QGFe.haust-6DS*, accounting for 0.38–10.63% of the phenotypic variation. *QGFe.haust-1BL*, *QGFe.haust-2BL*, *QGFe.haust-5AL*, *QGFe.haust-5DL*, and *QGFe.haust-6DS* exhibited increasing GFe. The favorable alleles originated from Avocet and the phenotypic variation of each QTL was < 10%. The phenotypic variation of *QGFe.haust-6BS* was 10.63%, respectively, indicating that it is a major QTL. *QGFe.haust-1BL* was bound to the DArT marker interval *2268342–SNP100598436* with a physical interval ranging from 554.7–635.1 Mb and was detected in E1 and E3. *QGFe.haust-4AL* and *QGFe.haust-5DL* were bound to the DArT marker intervals *SNP100512195–1721201* and *4440045–*

4989564 with physical intervals ranging from 720.1–731.0 and 432.8–554.8 Mb, respectively, and were detected in E1, E2, and E3. These findings indicated that the 3 loci were stable major QTLs for controlling GFe.

QGZn.haust-1BL and *QGFe.haust-1BL* were detected on the same locus in E3 and exhibited increasing GZn and GFe.

QGZn.haust-4AL and *QGFe.haust-4AL* were detected on the same loci in E1 and E3, and exhibited decreasing GZn and GFe.

QGZn.haust-5DL and *QGFe.haust-5DL* were detected on the same locus in E3 and exhibited increasing GZn and GFe. The 3 loci were pleiotropic (Table 5).

Table 4
QTL mapping for GZn and GFe concentrations identified by inclusive composite interval mapping in the Avocet/Chilero population

Trait	QTL	Environment	Physical interval	Marker interval	LOD	PVE (%)	Favorable allele
GZn	<i>QGZn.haust-1BL</i>	E3	554.7-635.1	<i>2268342-SNP100598436</i>	5.70	3.78	Avocet
	<i>QGZn.haust-2BL</i>	E2	690.8-758.9	<i>SNP1063621-1367534</i>	3.25	3.70	Chilero
	<i>QGZn.haust-3BL</i>	E1	793.5-802.3	<i>1116594-3032800</i>	2.63	3.84	Avocet
	<i>QGZn.haust-4AL</i>	E1	720.1-731.0	<i>SNP100512195-1721210</i>	7.75	11.87	Chilero
	<i>QGZn.haust-4AL</i>	E3	720.1-731.0	<i>SNP100512195-1721210</i>	8.90	3.57	Chilero
	<i>QGZn.haust-4BS</i>	E1	44.9-89.9	<i>3955627-1124059</i>	3.75	5.44	Avocet
	<i>QGZn.haust-5AL</i>	E3	579.4-593.1	<i>4542455-4537534</i>	3.12	3.22	Avocet
	<i>QGZn.haust-5DL</i>	E3	432.8-554.8	<i>4440045-4989564</i>	3.63	3.76	Avocet
	<i>QGZn.haust-6AS</i>	E3	23.6-48.0	<i>SNP1127808-SNP1057803</i>	2.70	1.07	Avocet
	<i>QGZn.haust-7AS.1</i>	E2	50.6-51.8	<i>1102565-3940544</i>	14.67	16.62	Chilero
	<i>QGZn.haust-7AS.2</i>	E2	49.6-50.0	<i>2253164-1130512</i>	8.17	8.05	Avocet
GFe	<i>QGFe.haust-1BL</i>	E1	554.7-635.1	<i>2268342-SNP100598436</i>	4.93	3.15	Avocet
	<i>QGFe.haust-1BL</i>	E3	554.7-635.1	<i>2268342-SNP100598436</i>	6.37	2.50	Avocet
	<i>QGFe.haust-2BL</i>	E1	593.6-690.7	<i>SNP2351705-SNP1063621</i>	12.44	3.28	Avocet
	<i>QGFe.haust-4AL</i>	E1	720.1-731.0	<i>SNP100512195-1721201</i>	12.28	3.25	Chilero
	<i>QGFe.haust-4AL</i>	E2	720.1-731.0	<i>SNP100512195-1721201</i>	10.62	0.38	Chilero
	<i>QGFe.haust-4AL</i>	E3	720.1-731.0	<i>SNP100512195-1721201</i>	12.41	2.58	Chilero
	<i>QGFe.haust-5AL</i>	E3	666.9-680.4	<i>SNP1137615-4541384</i>	2.62	0.68	Avocet
	<i>QGFe.haust-5DL</i>	E1	432.8-554.8	<i>4440045-4989564</i>	6.61	3.17	Avocet
	<i>QGFe.haust-5DL</i>	E2	432.8-554.8	<i>4440045-4989564</i>	8.60	0.38	Avocet
	<i>QGFe.haust-5DL</i>	E3	432.8-554.8	<i>4440045-4989564</i>	2.59	0.82	Avocet
	<i>QGFe.haust-6BS</i>	E2	32.7-39.4	<i>1044528-986510</i>	10.52	10.63	Chilero
	<i>QGFe.haust-6DS</i>	E3	18.7-30.2	<i>3027965-4405040</i>	4.39	2.64	Avocet

Table 5
Chromosomal intervals for GZn and GFe identified by multi-trait composite interval mapping (MCIM)

Chromosomes	Flanking markers	Physical position (Mb)	Traits (Environment)
1BL	2268342-SNP100598436	554.7-635.1	GZn (E3)
			GFe (E1, E3)
4AL	SNP100512195-1721201	720.1-731.0	GZn (E1, E3)
			GFe (E1, E2, E3, BLUE value)
5DL	4440045-4989564	432.8-554.8	GZn (E3)
			GFe (E1, E2, E3)

QTL pyramids and validation

The flanking SNPs of the pleiotropic loci (*QGZn.haust-4AL* and *QGFe.haust-4AL*; *QGZn.haust-5DL* and *QGFe.haust-5DL*) located on chromosomes 4AL and 5DL were converted into KASP markers, named *Kasp_4A_QGZnFe* and *Kasp_5D_QGZnFe* (Table 6). The markers were used to detect genotypes in the germplasm panels. Research indicates that both *Kasp_4A_QGZnFe* and *Kasp_5D_QGZnFe* had obvious genotypes in the germplasm panels (Fig. 3). Among the 151 germplasm panels, there was no significant difference ($P<0.05$) in GZn and GFe between the two genotypes of *Kasp_4A_QGZnFe* (Table 7). The number of germplasm panels carrying the CC (Avocet) and GG (Chilero) genotypes was 41 (27.2%) and 110 (72.8%) for *Kasp_5D_QGZnFe*, the GZn and GFe of the CC genotype were significantly higher than the GG genotype with mean differences of 3.57 and 3.01 mg kg⁻¹, respectively (Table 7). The frequency of germplasm panels carrying favorable allelic variants of CC was lower than GG allele, indicating that the pleiotropic loci (*QGZn.haust-5DL* and *QGFe.haust-5DL*) have considerable application value in breeding.

Table 6
The designed primer sequence of KASP molecular marker in this study

Chromosome	SNP name	KASP name	Primer sequence
4AL	SNP100512195	<i>Kasp_4A_QGZnFe</i>	5'-GAAGGTGACCAAGTTCATGCTTGACTAGGCTATTGCAGAAAGATT-3'
5DL	SNP1229622	<i>Kasp_5D_QGZnFe</i>	5'-GAAGGTCGGAGTCAACGGATTTGACTAGGCTATTGCAGAAAGATC-3'
			5'-TTGGTTGGAGTTGCTAGGGC-3'
			5'-GAAGGTGACCAAGTTCATGCTATGAGTCGCCACCAGGGATC-3'
			5'-GAAGGTCGGAGTCAACGGATTATGAGTCGCCACCAGGGATG-3'
			5'-GTCATTTGTGGCAGCTGCAG-3'

Table 7
Mean values of GZn and GFe for genotype classes in the germplasm panel

Trait	QTL	Marker	Genotype	Number	Mean (mg kg ⁻¹)	T value
GZn	<i>QGZn.haust-4AL</i>	<i>SNP100512195</i>	TT	89	37.72	0.83
			CC	62	37.67	
	<i>QGZn.haust-5DL</i>	<i>SNP1229622</i>	CC	41	40.40	0.007*
			GG	110	36.83	
GFe	<i>QGFe.haust-4AL</i>	<i>SNP100512195</i>	TT	89	40.39	0.15
			CC	62	40.58	
	<i>QGFe.haust-5DL</i>	<i>SNP1229622</i>	CC	41	43.38	0.047*
			GG	110	40.37	
* and ** Significant at <i>P</i> <0.05 and 0.01						

Discussion

Methods for increasing GZn and GFe concentrations

The GZn and GFe in wheat can be increased through several methods. Kutman et al. (2011) showed that the application of N fertilizer improved GZn and GFe in wheat. Srivastava et al. (Srivastava et al. 2015) showed that K fertilizer application improved GZn absorption in wheat. Furthermore, Ryan et al. (Ryan et al. 2010) found that P fertilizer inhibited the absorption of GZn and GFe in wheat. However, GZn and GFe are mainly absorbed from and accumulate in the soil, and the Zn and Fe contents in the soil that can be absorbed and transformed by plants are generally low. Therefore, only applying NPK fertilizer to improve GZn and GFe is inefficient and could easily cause soil toxicity. Jalal et al. (2020) found that the foliar application of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and FeSO_4 increased GZn and GFe in wheat. An et al. (2011) demonstrated that the combination of spraying Zn and bulk element fertilizers effectively improved crop yields and the GZn when compared to spraying Zn fertilizer alone. Although the application of Zn and Fe fertilizers increases the GZn and GFe, their cost is too high, and excessive Zn and Fe lead to toxicity that affects plants and humans (Uauy et al. 2006; Peleg et al. 2009). Thus, excavating for QTLs or genes with high Zn and Fe efficiencies to improve GZn and GFe accumulation in wheat is a safe and efficient biotechnology method. The use of genetic engineering has uncovered a new method for increasing the accumulation of GFe and GZn in wheat (Connorton et al. 2019; Kathleen et al. 2019; Henry et al. 2018).

Comparisons with QTL mapping

The genetic basis for and QTL mapping of GZn and GFe accumulation will serve as important references for selecting suitable breeding strategies through molecular marker-assisted breeding to improve GZn and GFe in wheat. In previous studies, if the genetic loci of GZn and GFe were located on the same or similar chromosomal regions, the loci had stable genetic effects (Gupta et al. 2020; Cao et al. 2020). In this study, the variation distributions of GZn and GFe of the RILs were continuous and susceptible to environmental factors, indicating they were quantitative traits controlled by multiple genes (Fig. 1), which was consistent with the findings of Velu et al. (2016). A total of 10 QTLs were detected that controlled GZn and 7 for GFe (Fig. 2). Compared to a previous study, the physical interval of *QGZn.haust-1BL* and *QGFe.haust-1BL* was 554.7–635.1 Mb, which was close to the location of *QGZn.cimmyt-1B.5* detected by Crespo-Herrera et al. (2017). Therefore, we speculated that these 3 QTLs may be the same. The physical interval of *QGZn.haust-4AL* and *QGFe.haust-4AL* was 720.1–731.0 Mb. Crespo-Herrera et al. (2017) detected *QGFe.cimmyt-4A* and *QGZn.cimmyt-4A* at the physical positions 5.1 and 84.0 Mb, respectively. Gopalareddy et al. (2017) detected a locus for *QGZn.iari-4A* at 429.7 Mb, which was located far away from the locus detected in this study; therefore, it may be a new QTL. The physical interval of *QGZn.haust-5DL* and *QGFe.haust-5DL* was 432.8–554.8 Mb, which was located far away from *QGFe.sau-5D* in a previous study (Tong et al. 2020). However, they were similar to the loci of *GZn-*

Xwmc357 and *GFe-Xwmc357* detected by GWAS, indicating that they may be the same QTL (Gorafi et al. 2016). *QGFe.haust-6BS* is a major QTL with a physical interval of 32.7–39.4 Mb, which is located far away from *QGFe.cimmyt-6B*, *QGZn.cimmyt-6B.1*, *QGZn.cimmyt-6B.1*, *QGFe.uh-6B*, *QGZn.uh-6B*, *QGZn.ua-6B*, *QGZn.huj-6B*, and *QGFe.huj-6B* in previous studies and thus may be a new QTL. *QGZn.haust-7AS.1* is a major QTL with a physical interval of 50.6–51.8 MB, which is close to *QGZn.pau-7AS.1* and *QGFe.pau-7AS.1*, suggesting that they may be the same QTL (Tong et al. 2020).

Candidate gene analysis

There are nearly 200 Fe/Zn-related genes in *Arabidopsis* and rice, which a previous study used in a BLAST analysis against the wheat database in EnsemblPlants (Tong et al. 2020). Using the QTLs identified in this study, we compared the homologous genes controlling GZn and GFe in rice and *Arabidopsis*. *TraesCS1B02G359000* is located in the physical interval of *QGZn.haust-1BL*. *TraesCS2B02G530500*, *TraesCS2B02G543700*, and *TraesCS2B02G543800* are located in the physical interval of *QGZn.haust-2BL*. *TraesCS2B02G443800* and *TraesCS2B02G466500* are located in the physical interval of *QGFe.haust-2BL*. *TraesCS3B02G562500* is located in the physical interval of *QGZn.haust-3BL*. *TraesCS4B02G056600* and *TraesCS4B02G081200* are located in the physical interval of *QGZn.haust-4BS*. *TraesCS5A02G383400* and *TraesCS5A02G391000* are located in the physical interval of *QGZn.haust-5AL*. *TraesCS6B02G056600* and *TraesCS4B02G081200* are located in the physical interval of *QGZn.haust-5AL*. *TraesCS6B02G058700* and *TraesCS6B02G391000* are located in the physical interval of *QGZn.haust-5AL*. By editing these genes, GZn and GFe in wheat can theoretically be effectively regulated. However, this biotechnology method is rarely used for the improvement of GZn and GFe in wheat. When *OsNAS2* and *PvFERRITIN* are overexpressed in wheat, the GZn in wheat is thereby improved, thus laying a foundation for further research on Zn/Fe biofortification in wheat (Beasley et al. 2019; Singh et al. 2017).

Pleiotropic analysis of GZn and GFe genes

In this study, we identified QTLs on chromosomes 1B, 4A, and 5D that simultaneously control GZn and GFe in wheat (Table 5). These locus are pleiotropic QTLs. Moreover, Gorafi et al. (2016) found a pleiotropic locus that controls GZn and GFe on chromosome 5D. Velu et al. (2016) found a pleiotropic locus on chromosome 2B that controls GZn and GFe, and demonstrated that a single locus could simultaneously increase GZn and GFe. Peleg et al. (2009) found pleiotropic QTLs that control GZn and GFe in tetraploid wheat. Therefore, it can be inferred that the accumulation of GZn and GFe has a similar genetic basis. Previous studies showed that there was a significant positive correlation between GZn and GFe in wheat (Zhao et al. 2009; Morgounov et al. 2007). The effects of the pleiotropic loci on chromosome 5DL (*QGZn.haust-5DL* and *QGFe.haust-5DL*) in the germplasm panels corroborated these findings. The frequency of germplasm panels carrying favorable allelic variants of CC was lower than GG alleles, indicating that the pleiotropic loci (*QGZn.haust-5DL* and *QGFe.haust-5DL*) have considerable application value in breeding. The development of KASP markers can be effectively used for molecular marker-assisted breeding for the enhancement of GZn and GFe in wheat. Some transporters, chelators, and genes simultaneously regulate GZn and GFe in wheat (Shanmugam et al. 2013; Tong et al. 2020). These results indicated that GZn and GFe can be improved simultaneously in breeding programs aimed at the biofortification of trace elements. Therefore, it is the most important that quantitative trait loci and KASP marker for grain zinc and iron concentrations were developed for utilizing in marker-assisted breeding and biofortification of wheat grain in breeding programs.

Conclusion

In this study, QTLs for GZn and GFe were identified using 164 RILs derived from Avocet/Chilero. Four stable QTLs, *QGZn.haust-4AL*, *QGFe.haust-1BL*, *QGFe.haust-4AL*, and *QGFe.haust-5DL* were located on chromosomes 1BL, 4AL, and 5DL. Three pleiotropic effects locus for GZn and GFe concentrations were located on chromosomes 1BL, 4AL, and 5DL. Two high-throughput Kompetitive Allele Specific PCR markers were developed by closely linking single nucleotide polymorphisms on chromosomes 4AL and 5DL, which were validated by a germplasm panel, further expanding upon the theoretical foundation for Zn and Fe biofortification and genetic improvement.

Declarations

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Author contribution

CPW conceived and supervised the complete study—including data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, Writing—review & editing. PXR performed the experiment and wrote the paper—including data curation, formal analysis, methodology, software, writing—original draft, writing—review & editing. DHZ performed the experiment—including data curation, software, validation, writing—review & editing. ZKZ, YZ and XFY participated in the field trials, trait evaluation and software analysis. CXL provided resources and extensive revision of the manuscript. All authors read the final version of the manuscript and approved its publication.

Availability of data and material

Data of biparent and 164 F_6 individuals are not publicly available due to the research on other important traits, but are available from the corresponding author on reasonable request. The other data generated or analyzed in this study are included in this manuscript and supplementary information files.

Ethics approval

We declare that these experiments complied with the ethical standards in China.

Consent to participate

Not applicable.

Consent for publication

We agree that this manuscript is published in Molecular Breeding.

Conflict of interest

All authors declare that they have no conflicts of interest.

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Figures

Figure 1

Distribution of wheat GZn and GFe concentrations in the Avocet/Chilero RILs population

Figure 2

Landscape of genetic architecture underlying Fe- and Zn-related traits in wheat. Genetic loci for Fe and Zn traits Identified from wheat orthologs of Arabidopsis and rice genes, QTL mapping, association studies, and cloned wheat genes are displayed in black, red, green, blue and purple, respectively. Yellow bars within chromosomes indicate the Physical interval of QTL, QTL associated with both Fe and Zn are underlined.

Figure 3

Genotyping of KASP molecular marker in the germplasm panel