



# Meta-QTLs, ortho-MetaQTLs and candidate genes for grain Fe and Zn contents in wheat (*Triticum aestivum* L.)

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**Abstract** Majority of cereals are deficient in essential micronutrients including grain iron (GFe) and grain zinc (GZn), which are therefore the subject of research involving biofortification. In the present study, 11 meta-QTLs (MQTLs) including nine novel MQTLs for GFe and GZn contents were identified in wheat. Eight of these 11 MQTLs controlled both GFe and GZn. The confidence intervals of the MQTLs were narrower (0.51–15.75 cM) relative to those of the corresponding QTLs (0.6 to 55.1 cM). Two ortho-MQTLs involving three cereals (wheat, rice and maize) were also identified. Results of MQTLs were also compared with the results of earlier genome wide association studies (GWAS). As many as 101 candidate genes (CGs) underlying MQTLs were also identified. Twelve of these CGs were prioritized; these CGs encoded proteins with important domains (zinc finger, RING/FYVE/PHD type, flavin adenine dinucleotide linked oxidase, etc.) that are involved in metal ion binding, heme binding, iron binding, etc. qRT-PCR analysis was conducted for four of these 12 prioritized CGs using genotypes which have differed for GFe and GZn. Significant differential expression in these genotypes was observed at 14 and 28 days after anthesis. The MQTLs/CGs identified in the present study may be utilized in marker-assisted selection (MAS) for improvement of

GFe/GZn contents and also for understanding the molecular basis of GFe/GZn homeostasis in wheat.

**Keywords** Wheat · Grain iron (GFe) · Grain zinc (GZn) · meta-QTL · Candidate genes · Ortho-meta QTL · qRT-PCR

## Introduction

Wheat is the major constituent of cereal-based diet, and is the third most important cereal after maize and rice. Wheat grain is a major staple food, and is an important source of calories for a major fraction of population in the developing world. However, majority of the modern wheat varieties are poor in essential nutrients including grain protein and micronutrients like grain iron (GFe) and grain zinc (GZn). For this reason alone, micronutrients and vitamins are sometimes also used in the form of dietary supplements, which are often out of reach for most people living in the developing world (Ward 2014; De valenca et al. 2017). Malnutrition due to deficiency for micronutrients has been particularly high among children which also cause > 45% of the deaths of children of < 5 years of age (WHO 2017). This phenomenon of malnutrition has also been described as ‘hidden hunger’ (Stein and Qaim 2007; Harding et al. 2018; Gödecke et al. 2018) reviewed by Gupta et al. (2021).

According to estimates by WHO (World Health Organization), globally > 2 billion people suffer with deficiency of Fe/Zn (Lyons et al. 2005; Alina et al. 2019). Of these two micronutrients, Fe is an essential component of haemoglobin and myoglobin and its deficiency cause severe health problems, including anemia, mental retardation, weak immune system and increased morbidity and mortality (Black 2003). The serious impact is particularly witnessed in women of reproductive age (especially pregnant women) and in children, < 5 years of age (Abbaspour et al. 2014; Roohani et al. 2013). Similarly, Zn plays a significant role in

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different metabolic processes and is an essential cofactor for many enzymes and regulatory proteins. The symptoms of deficiency for Zn intake for humans can be observed in the form of retardation of growth and development, excessive weight loss, diarrhea and depression (Ozturk et al. 2006; Kambe et al. 2014). This makes the development of biofortified wheat varieties enriched for GFe and GZn, a priority area of wheat breeding.

A pre-requisite for biofortification involving Fe and Zn is the identification of the genomic regions/genes, which control the contents of these micronutrients in the grain. The results of efforts already made in this direction in wheat include the identification of the following: (i) 168 QTLs using interval mapping, 716 marker trait associations (MTAs) using LD-based association mapping and a number of gene families involved in GFe and GZn homeostasis (Singh et al. 2021) (ii) A large number of cereal proteins (too many to be listed here) (iii) Seven MQTLs for GFe/GZn content and other associated yield and quality traits utilizing 50 QTLs reported earlier, and identification of 1,336 candidate genes (CGs) associated with these 7 MQTLs (Shariatipour et al. 2021a).

The QTLs and MTAs, which have already been known for a variety of traits in wheat are often not robust enough to be used for marker-assisted selection (MAS) in wheat breeding in any significant measure. Therefore, in recent years, MQTL analysis has been conducted for almost all important traits in wheat (Kumar et al. 2020; Jan et al. 2021). However, for GFe and GZn, only a solitary MQTL study involving a limited number of QTLs is available warranting a fresh study involving all known QTLs.

Keeping the above in view, the present study on GFe and GZn was planned for identification of MQTLs and candidate genes (CGs) in wheat and ortho-MQTL involving three cereals (wheat, maize and rice). MQTLs identified in the present study were also compared with MTAs reported in genome wide association studies (GWAS), leading to identification of genomic regions showing co-localization of MTAs and the MQTLs. qRT-PCR was also conducted for validation of four prioritized CGs underlying the MQTL regions. It has been proposed that the results of the present study may be proved useful in wheat breeding and will also help in developing a better understanding of the genetic architecture underlying GFe and GZn contents.

## Materials and methods

### Bibliographic review and retrieval of GFe and GZn QTL information

For conducting MQTL analysis, information on known QTLs for GFe and GZn from published literature (till 2019)

on interval mapping was retrieved by search of Google Scholar using different keywords. In total, 15 such studies were available, but only 12 could be used for MQTL analysis, because the remaining three QTL studies did not carry all the required information (e.g. flanking markers, QTL position and LOD score of the QTLs). Following two types of input data text files were prepared from each study following Biomeqator v4.2 (Arcade et al. 2004; Sosnowski et al. 2012): (i) genetic map file, and (ii) QTL information [name of QTL, trait, chromosome carrying the QTLs, range and mean of the lengths of confidence interval (CI), LOD score,  $R^2$  etc.]. If the value of CI for a particular QTL was not available in the original study, it was worked out using following formulae provided by Darvasi and Soller (1997) and Guo et al. (2006): RIL:  $CI = 163/(N R^2)$ ; DH:  $CI = 287/(N R^2)$ , where N in both the equations is size of the mapping population and  $R^2$  is the phenotypic variation explained (PVE).

### Development of consensus map and meta-QTL analysis

Consensus genetic map of wheat was developed using LPmerge software (Endelman et al. 2014) using five published linkage maps (Somers et al. 2004; Marone et al. 2012; Maccaferri et al. 2014; Wang et al. 2014; Bokore et al. 2020). Markers flanking individual QTLs were also included in the consensus genetic map. The consensus map was used for projection of QTLs reported in 12 earlier studies (Table 1). Details of the procedure used for this purpose are available in BioMercator v 4.2 manual (Arcade et al. 2004; Sosnowski et al. 2012).

The original QTLs were projected onto the consensus map using the QTL projection tool (QTL Proj). QTLs which could not be projected onto the consensus map or those with mapping positions outside the consensus map were excluded. MQTLs were identified using Goffinet and Gerber algorithm (Goffinet and Gerber 2000). Model selection was based on lowest AIC, which estimates the relative amount of data lost by different statistical models (Akaike 1998).

### MQTLs overlapping MTAs from GWAS studies

The most promising wheat MQTLs were compared with known MTAs for GFe and GZn contents reported in earlier GWAS in wheat. The data on MTAs were collected from nine published GWAS and used for comparison with MQTL regions. The MTAs overlapping or in close proximity of 5 Mb region associated with a MQTL were accepted to be co-located with MQTL.

**Table 1** A summary of 12 QTL studies involving 14 mapping populations used for MQTL analysis for GFe and GZn in wheat

Sn	Mapping population	Type of markers	Chromosome identity	Range of PVE%*	No. of QTLs		Reference	
					GZn	GFe		
	<b>Cross</b>	<b>Type (number)</b>						
1.	Hanxuan 10 x Lumai 14	DH (119)	SSR, AFLP	1 A, 2D, 3 A, 4 A, 4D, 5 A, 7 A, 7B	4.6–14.6	8	4	Shi et al. (2008, 2013)
2.	Tabassi x Taifun	RIL (118)	SSR	1 A, 2 A, 3D, 4 A, 4D, 7B, 7D	8.9–50.7	2	6	Roshanzamir et al. (2013)
3.	SHW L1 x Chuanmai32	RIL (171)	SSR, DArT	2B, 2D, 3D, 4D, 5D, 7D	5.4–9.5	4	4	Pu et al. (2014)
4.	Chuanmai42 x Chuannong16	RIL (127)	SSR, SRAP	3D, 4 A, 4D, 5 A, 5B	9.2–15.9	3	4	Pu et al. (2014)
5.	PBW343 x Kenya Swara	RIL (177)	SSR, DArT	1BS, 2Bc, 2D, 3AL, 4AS, 5BL, 6AL	7.0–15.0	6	-	Hao et al. (2014)
6.	<i>T. spelta</i> accession H+26 (PI348449) × <i>T. aestivum</i> cv. HUW 234	RIL (185)	SNP, DArT	1 A, 2 A, 2B, 3B, 3D, 6 A, 6B	3.5–27.1	5	5	Srinivasa et al. (2014)
7.	Berkut 9 x Krichauff	DH (138)	SSR, DArT	1B, 2B	22.2–35.9	2	1	Tiwari et al. (2016)
8.	Seri M82 x SHW CW176364	RIL (140)	SNP	2BL, 2DS, 4BS, 5AL, 5BL, 6AL, 6BL, 6DS, 7DS	7.2–19.6	6	10	Crespo-Herrera et al. (2016)
9.	Adana99 x <i>T. Sphaerococcum</i> (70,711)	RIL (127)	DArT	1B, 1D, 2 A, 2B, 3 A, 3D, 6 A, 6B, 7 A, 7B	9.0–31.0	8	3	Velu et al. (2017a, b)
10.	Saricanak98 x MM5/4 (4x wheat)	RILs (105)	DArT	1B, 3 A/3B, 5 A, 5B, 6 A, 6B	5.9–16.9	8	4	Velu et al. (2017a, b)
11.	WH542 x Synthetic derivative (PI94624)	RILs (286)	SSR	2 A, 4 A, 5 A, 7 A, 7B	2.3–14.4	7	6	Krishnappa et al. (2017)
12.	Bubo x Turtur	RILs (188)	DArT	6 A, 3 A, 1B, 4B, 5B, 7B	2.8–16.7	4	3	Crespo-Herrera et al. (2017)
13.	Louries x Batelur	RILs (188)	DArT	1 A, 1B, 2 A, 2B, 3B, 3D, 4 A, 4D, 5B, 6 A, 7B, 7D	3.3–32.8	12	7	Crespo-Herrera et al. (2017)
14.	Roelfs F 2007 x Chinese Parental Line	RILs (200)	DArT	1 A, 2 A, 1B, 2B, 3 A, 3B, 3D, 4B, 5 A, 6B, 7 A	2.10–14.6	13	6	Liu et al. (2019)

\* PVE=Phenotypic variation explained; RILs=Recombinant inbred lines; DH=Double haploid

### Ortho-meta QTL analysis and comparison

Based on the high synteny among wheat, rice and maize, the most promising wheat MQTLs were explored for identification of ortho-MQTLs for the same traits conserved across rice (Raza et al. 2019) and maize (Jin et al. 2013). The syntenic regions were identified using a set of orthologous genes within 2 Mb region for an individual MQTL of wheat using EnsemblPlants database.

### Identification of candidate genes (CGs) underlying the MQTL region

The CGs were identified using the following steps: (i) Nucleotide sequences of the markers flanking a particular MQTL were retrieved either from Gramene or Graingenes database and these sequences were utilized to identify their physical position. For this purpose, the nucleotide sequences were blasted against wheat reference genomic sequence available in EnsemblPlants database (*Triticum aestivum* IWGSC\_ensembl\_release 48) employing nucleotide blast (maximum E-value = 1E-100 and minimum 95% identity of

the sequence). If the sequence of a marker(s) was absent, then we used the sequence of the closely adjacent genetic marker. (ii) The physical interval (in Mb) for an individual MQTL was calculated using the genetic confidence interval (in cM) of the MQTL regions. For this purpose, the physical interval (in Mb, calculated from the coordinate information of the MQTL) was divided by the genetic interval (in cM) and the distance in units of bases per cM was calculated. (iii) Actual physical position of the MQTL was calculated and 1 Mb region on either side of the MQTL peak (total 2 Mb intervals) was used for identification of the putative CGs associated with the respective MQTL region. (iv) Annotation of CGs was undertaken on the basis of the domain in the corresponding protein sequences, which were obtained using InterPro database.

### qRT-PCR for some selected CGs

For expression analysis of the selected CGs, following four wheat genotypes with different levels of GZn and GFe contents were utilized: FAR4 and WB02 each with high GZn (40 ppm to ~48 ppm) and GFe (42 ppm to ~44 ppm)

relative to those in two other genotypes, namely K8027, and HD3226 (33 ppm to 36 ppm GZn and ~23 to ~35 ppm GFe). For each of these four field grown genotypes, grains at 14 and 28 days after anthesis (DAA) were collected and immediately transferred to liquid nitrogen to be stored at  $-80^{\circ}\text{C}$ . Total RNA was isolated using the TRIzol Reagent (Ambion) as per the manufacturer's specifications. cDNA synthesis was carried out using iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. The qRT-PCR was performed in optical 96 well plates with StepOnePlus Real-Time PCR system (Applied Biosystems) using SYBR Green (Applied Biosystem). Wheat  $\beta$ -actin gene (AB181991) was used as an endogenous control.

## Results and discussion

### Literature survey and original QTLs for MQTL analysis

Information about the QTLs from 12 of the 15 studies used for MQTL analysis in the present study is summarized in Table 1 (detailed information is available in supplementary Table S1). The remaining 3 studies could not be used due to inadequacy of all the information needed for MQTL analysis.

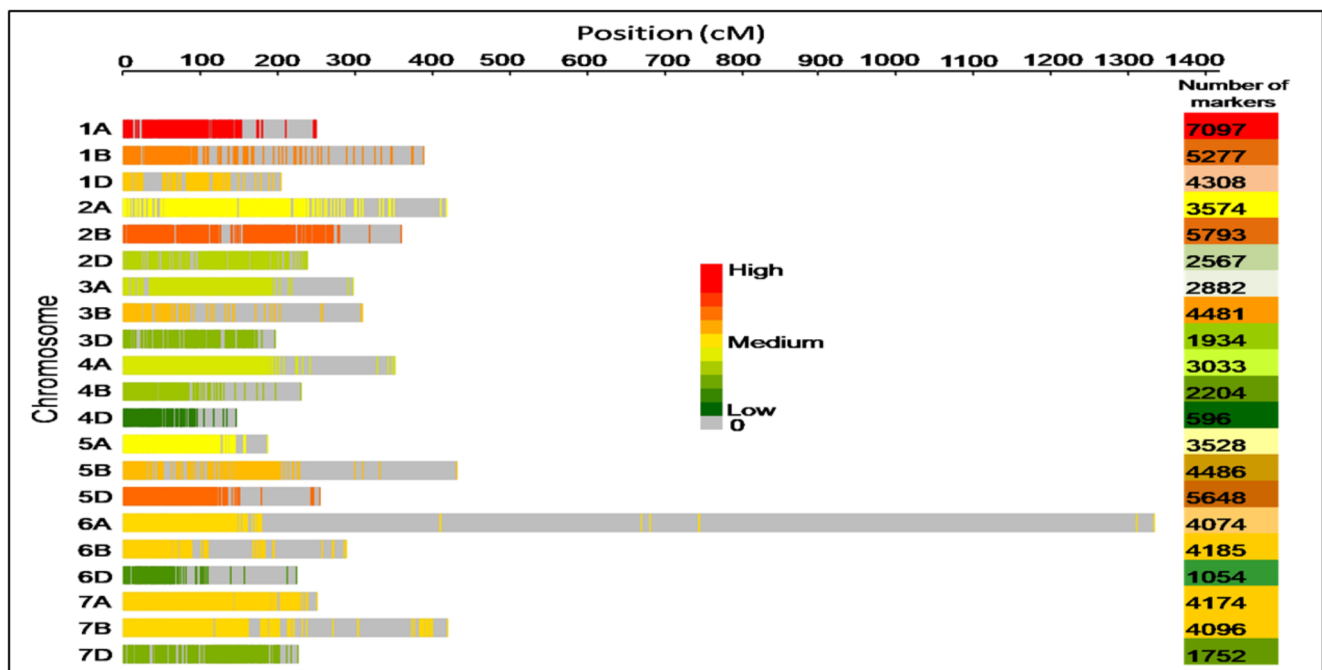
The above 12 studies involved 14 different DH/RIL mapping populations (size range: 118 to 286 lines) genotyped

with SSR, SNP, DArT, and several other marker systems. In two recent studies, seven mapping populations were used for MQTL analysis for GFe and GZn (Shariatipour et al. 2021a, b). Out of the seven populations used in these two earlier studies, results from six mapping populations were also included in the present study; the results from the remaining eight mapping populations were used in the present MQTL study for the first time.

### High resolution consensus map of wheat

A high-resolution consensus genetic map of wheat was prepared using LPmerge software (Endelman and Plomion 2014), which is superior to other available software like Bio-mercator, LPmerge integrates a very large number of markers and has the power to resolve inconsistencies in marker order between different linkage maps. For these reasons, the software LPMerge has also been used in some recent studies on MQTL analysis in wheat (Venske et al. 2019; Liu et al. 2020a, b; Jan et al. 2021; Kumar et al. 2021; Saini et al. 2021; Yang et al. 2021).

The consensus genetic map prepared during the present study had 76,743 markers (with an average of 3,655 markers per chromosome) with a genetic distance of 7184.77 cM (Fig. 1). The available marker types included the following: SNPs (65,459), SSRs (3,965), DArTs (3,526), and a variety of other markers (3,793) including AFLP, and STS markers. The consensus map carried many more markers



**Fig. 1** Marker densities (high to low) on each of 21 chromosomes of the consensus genetic map used for meta-QTL analysis. Red to green colors shows high to low densities of markers within each individual chromosome

relative to consensus maps used in some earlier MQTL studies in wheat, where the number of markers used ranged from 558 to 57,112 (Zheng et al. 2021; Liu et al. 2020a, b; Shariatipour et al. 2021a, b; Venske et al. 2019; Yang et al. 2021; Quraishi et al. 2017; Saini et al. 2021). The high marker density in our consensus map allowed us to identify markers that were closely associated with corresponding MQTLs. The maximum number of markers were available in the B sub-genome (30,522) followed by A sub-genome (28,362) and D sub-genome (17,859). The size of the 21 individual linkage groups ranged from 163.7 cM (4D) to 1365.8 cM (6A) (Fig. 1). The marker densities ranged from 2.98 markers per cM for 6A to 27.77 markers per cM for 1A. In individual sub-genomes, the average marker densities were lowest for D sub-genome (11.17 markers per cM) followed by B sub-genome (12.33 markers per cM) and A sub-genome (13.10 markers per cM).

### MQTL analysis

As mentioned earlier, only 148 of the 151 available QTLs could be used for MQTL analysis (see Material and Methods). The original QTLs did not follow any specific pattern of distribution on the 21 wheat chromosomes, but the distribution of QTLs on the basis of values of LOD scores and

PVE (%) did follow a pattern (unimodal skewed distribution), where majority of QTLs have low LOD score and low PVE value, with only a small fraction with high LOD score and high PVE (Fig. 2).

Using the lowest AIC value as the basis for selection of major QTLs for projection, only 32 (~20%) of the 148 available QTLs (PVE, 21–50.8%; CIs, 0.6 to 55.1 cM) could be projected on the consensus map. In a number of previous studies on MQTL analysis also, not all QTLs for an individual trait could be projected (Quraishi et al. 2017; Saini et al. 2021; Kumar et al. 2021; Shariatipour et al. 2021a, b).

### MQTLs for GFe and/or GZn content

Only 11 MQTLs, each based on 2–4 QTLs, were derived from 32 QTLs which were used for projection (Table 2; Fig. 3). This indicated roughly three times reduction in the number of genomic regions that are involved in controlling GFe and GZn contents in wheat. These results are not very different from the results of an earlier recent study, where 7 MQTLs were obtained using 21 original QTLs (Shariatipour et al. 2021a). In contrast, in another major study involving a variety of traits including GFe and GZn, 100

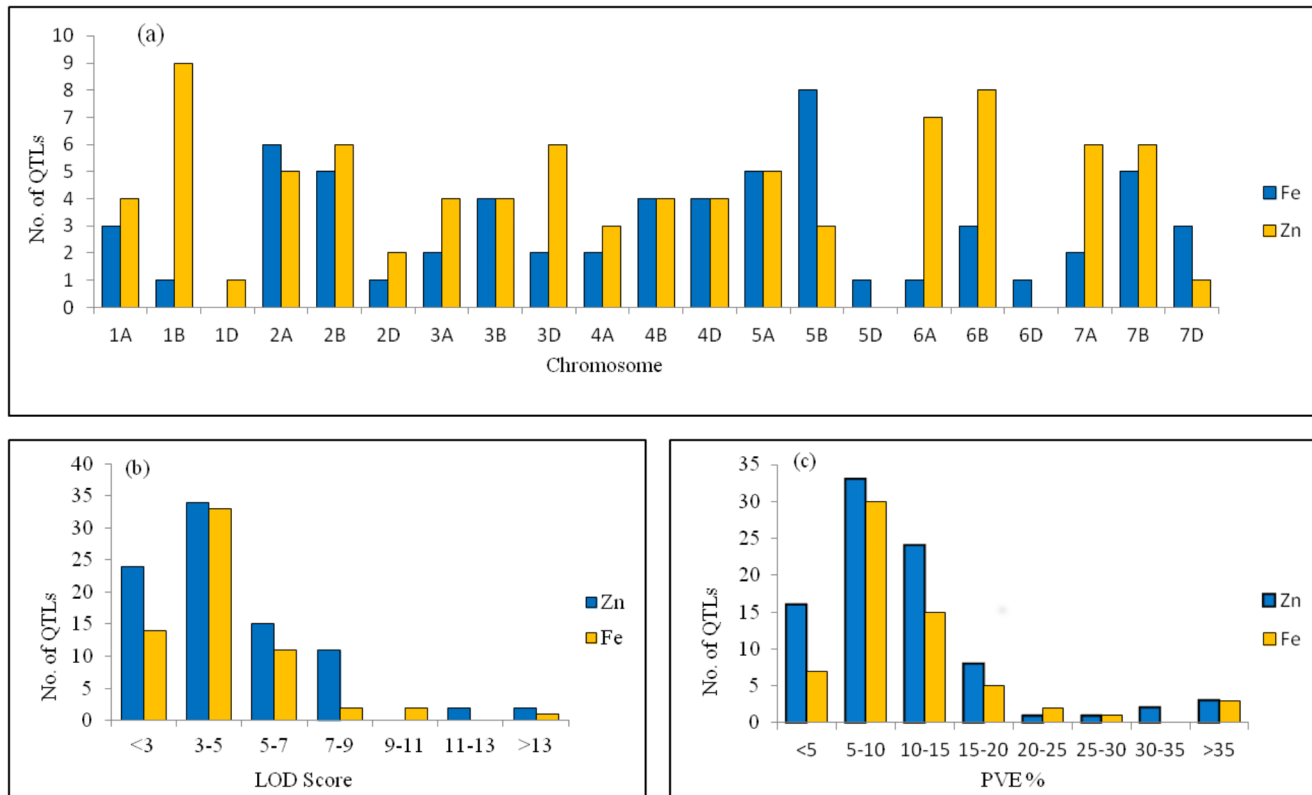
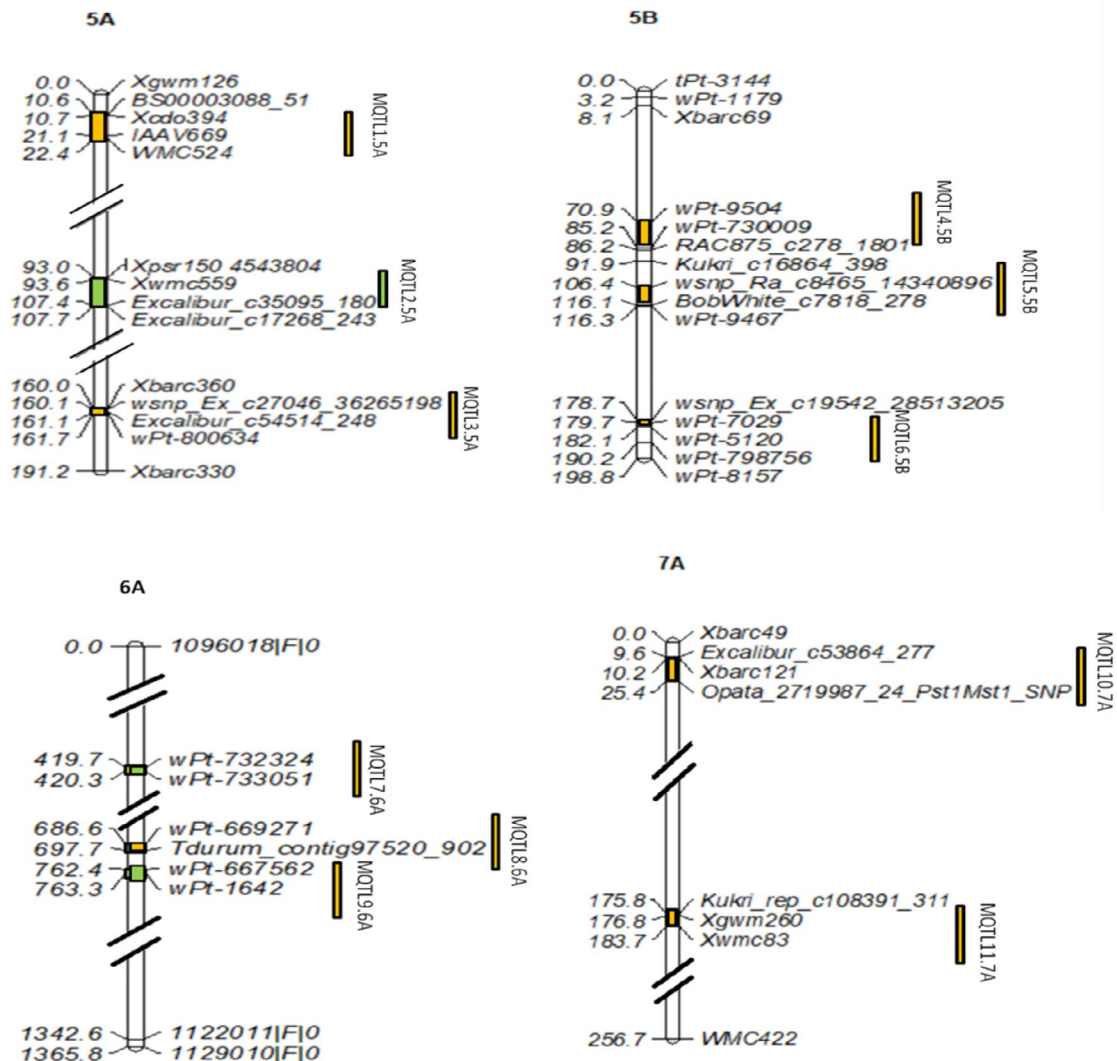


Fig. 2 Distribution of QTLs (a) on individual chromosomes; (b) based on LOD Score; (c) based on PVE%

**Table 2** List of meta-QTLs (MQTLs) for GFe and GZn contents discovered during the present study in wheat

Sn	Meta-QTL	Flanking marker	Chr. no.	CI (cM)	Position cM/Mb	R <sup>2</sup>	No. of QTLs (trait)
1.	MQTL1.5 A	Xcda394 - IAAV669	5 A	9.52	16.32/-	4.2	2 (1-Fe, 1-Zn)
2.	MQTL2.5 A	Xwmc559 - Excalibur-c35095-180	5 A	14.26	100.6/571.16	9.15	2 (Zn)
3.	MQTL3.5 A	wsnp_Ex_c27046_36265198 - Excalibur_c54514_248	5 A	1.04	160.61/538.66	12.3	4 (2-Fe, 2-Zn)
4.	MQTL4.5B	wPt-9504 - wPt-730,009	5B	14.18	71.4/643.66	14.9	2 (1-Fe, 1-Zn)
5.	MQTL5.5B	wsnp_Ra_c8465_14340896 - Bobwhite c7818_278	5B	9.47	111.25/533.86	11.5	2 (1-Fe, 1-Zn)
6.	MQTL6.5B	wPt-7029 - wPt-5120	5B	1.7	180/287.30	7.7	4 (3-Fe, 1-Zn)
7.	MQTL7.6 A	wPt-732,324 - wPt-733,051	6 A	0.6	419.97/-	6.5	2 (Zn)
8.	MQTL8.6 A	wPt-669,271 - Tduram_contig97520_902	6 A	2.36	694.77/-	9.6	3 (1-Fe, 2-Zn)
9.	MQTL9.6 A	wPt-667,562 - wPt-1642	6 A	0.88	762.82/-	8.76	2 (Zn)
10.	MQTL10.7 A	Excalibur_c53864_277 - Opata_2719987_24_Pst1Mst1_SNP	7 A	15.31	17.78/477.47	3.75	2 (1-Fe, 1-Zn)
11.	MQTL11.7 A	Xgwm260 - Xwmc83	7 A	2.46	178.06/169.04	7.4	3 (1-Fe, 2 -Zn)

Chr, chromosome; CI, confidence interval

**Fig. 3** Distribution of 11 MQTLs on four different chromosomes of wheat. The colored blocks inside each chromosome indicate MQTL regions. Green colored bars indicate MQTLs only for GZn and yellow colored bar indicate MQTLs for both GFe and GZn. Only the flanking markers closely associated with MQTLs are shown

MQTLs (including 19 MQTLs for GFe and 12 MQTLs for GZn) were identified using 449 QTLs (Shariatipour et al. 2021b).

Out of the 11 MQTLs identified in the present study, 8 MQTLs were localized on the A sub-genome and 3 on the B sub-genome. On the D sub-genome, two singleton MQTLs (each based on solitary QTL) were available, but being singletons, these were not accepted as MQTLs. In a previous study also, no MQTLs for GFe and GZn were available in the D sub-genome (Shariatipour et al. 2021a). Out of the above 11 MQTLs, six QTLs located on chromosomes 5B and 6 A and three MQTLs located on 5 and 7 A are completely novel, since no such MQTL was reported in two recent studies (Shariatipour et al. 2021a, b).

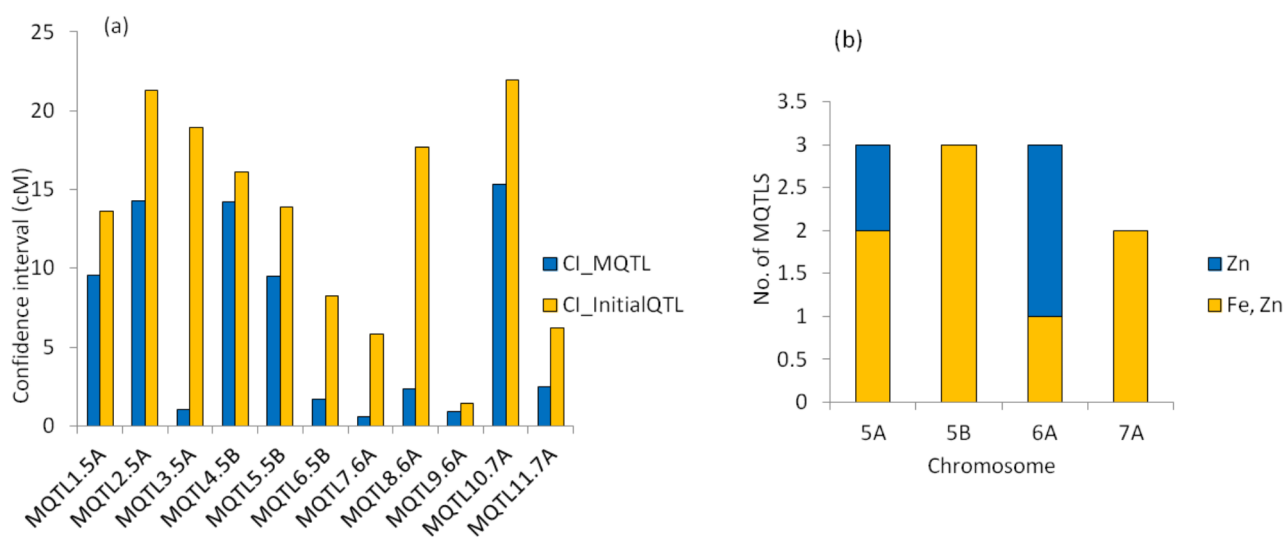
The CIs for these 11 MQTLs were narrower relative to CIs of the original QTLs, and it ranged from 0.6 cM (equivalent to 169.04 Mb) for MQTL11.7 A to 15.31 cM (equivalent to 643.66 Mb) for MQTL4.5B (Fig. 4a). This represents three-fold reduction in the length of CI. These results are not very different from those reported in two recent studies, one of them reporting 2.4 fold reduction and the other reporting 3 fold reduction (Shariatipour et al. 2021a, b). In contrast to this level of reduction on the length of CI, in a recent MQTL study in *Arabidopsis thaliana*, eight fold reduction in CI was reported (Shariatipour et al. 2021c).

The 11 MQTLs identified in the present study included 8 MQTL for both GFe and GZn, 3 MQTLs for GZn alone and none for GFe (Fig. 4b). This is in agreement with recent studies on MQTL analysis, where a high frequency of co-localization of MQTLs for GFe and GZn was observed (Shariatipour et al. 2021a, b). In another recent study in *Arabidopsis thaliana* also, strong co-localization of genes/

QTLs controlling ionic traits was reported (Shariatipour et al. 2021c). In a study in rice, 44 out of 48 MQTLs were reported to control both GFe and GZn contents (Raza et al. 2019). Similar results on co-localization of MQTLs responsible for several grain yield components under drought were reported by Kumar et al. (2020). This co-localization of QTLs for nutrient elements may be due to one of the following reasons: (i) tight linkage of distinct genes or pleiotropism, (ii) physiological association of micronutrient accumulation, suggesting a relationship at the molecular level among these traits and (iii) a correlation between phenotypic traits involving GFe and GZn contents. Such MQTLs controlling both GFe and GZn contents would be helpful in crop improvement with enhanced levels of GFe and GZn contents because the tightly correlated traits could be selected simultaneously through marker-assisted selection (MAS) (Mallimar et al. 2016).

### Comparison of MQTLs with GWAS-based MTAs

The 11 MQTLs that were identified during the present study were compared with GWAS-MTAs (Table 3). This comparison showed that only a solitary MQTL (MQTL3.5 A) controlling both GFe and GZn content was co-located with 32 known MTAs (Alomari et al. 2018, 2019). Co-location of the remaining MQTLs with MTAs reported in GWAS may be discovered as more LD-based GWAS studies on GFe and GZn become available. In several other studies, much higher proportion of co-localized MQTL genomic region was reported. For instance, Saini et al. (2021) reported co-location of 412 MTAs with 88 MQTL regions, which were associated with nitrogen use efficiency/ root system



**Fig. 4** Basic information of MQTLs. (a) The reduction in CI after meta-QTL analysis. (b) Distribution of MQTLs on different chromosomes

**Table 3** Summary of nine genome-wide association studies (GWAS) reporting marker trait associations (MTAs) for GFe and GZn contents in wheat

Sn	Asso. panel (size)	Country	Method of analysis of GFe and GZn	No. of markers	Model used	Chromosomes	No. of MTAs		No. of environments	Reference
							Zn	Fe		
1.	HPAM Panel (330)	India, Mexico	EDXRF	(14,273) SNP	MLM	1 A, 1D, 2 A, 2B, 2D, 5 A, 6B, 6D, 7B	39	-	6	Velu et al. (2018)
2.	HPAM Panel (330)	Mexico	ICP-MS	(28,074) SNP	GLM, MLM, MMLM	1B, 2 A, 2B, 2D 3 A, 3B, 4 A, 4B, 5B, 6 A, 7 A, 7B	72	65	2	Cu et al. (2020)
3.	European wheat varieties (369)	Germany	ICP-OES	(15,523) SNP	MLM	2 A, 3 A, 3B, 4 A, 4D, 5 A, 5B, 5D, 6D, 7 A, 7B, 7D	40	-	3	Alomari et al. (2018)
	Sub-panel (183)			(28,710) SNP		3B, 5 A	161	-	3	
4.	European wheat varieties (369)	Germany	ICP-OES	(15,523) SNP	MLM	2 A, 3B, 5 A	-	41	3	Alomari et al. (2019)
	Sub-panel (183)			(44,233) SNP		2 A, 5 A	-	137	3	
5.	SWRS (246)	India	EDXRF	-	-	1B, 2B, 3 A, 3B, 5B, 5D, 6 A, 6B, 7 A, 7D	94	33	2	Kumar et al. (2018)
6.	SHW (Longdon x 47 Ae tauschii) (47)	Japan	ICP-AES	70 (SSRs)	-	1D, 2D, 3D, 4D, 5D, 6D, 7D	3	3	2	Gorafi et al. (2018)
7.	SHW (123)	Turkey	ICP-MS	35,648 (SNP)	MLM	1 A, 2 A, 3 A, 3B, 4 A, 4B, 5 A, 6B	13	3	2	Bhatta et al. (2018)
8.	Chinese wheat mini core collection (246)	China	ICP-OES	545 (SSR)	MLM	1B, 2B, 2D, 3 A, 3D, 4 A, 4B, 5 A, 5D, 6B, 7D	16	-	2	Liu et al. (2021a, b)
9.	Advanced lines (161)	China	-	13,116 (DArT)	GLM, MLM	1B, 3B, 4A, 4B, 5A, 7B	3		6	Liu et al. (2021a, b)

Asso. panel=Association panel; HPAM=Harvest Plus Association Panel; SWRS=Spring Wheat Reference Set; HWWAMP=Hard Winter Wheat Association Mapping Panel; EDXRF=Energy Dispersive-X-ray Fluorescence; ICP-MS=Inductively coupled plasma mass spectrometry; ICP-OES=ICP-optical emission spectrometry; GLM=General linear model; MLM; Mixed linear model; - = Unknown

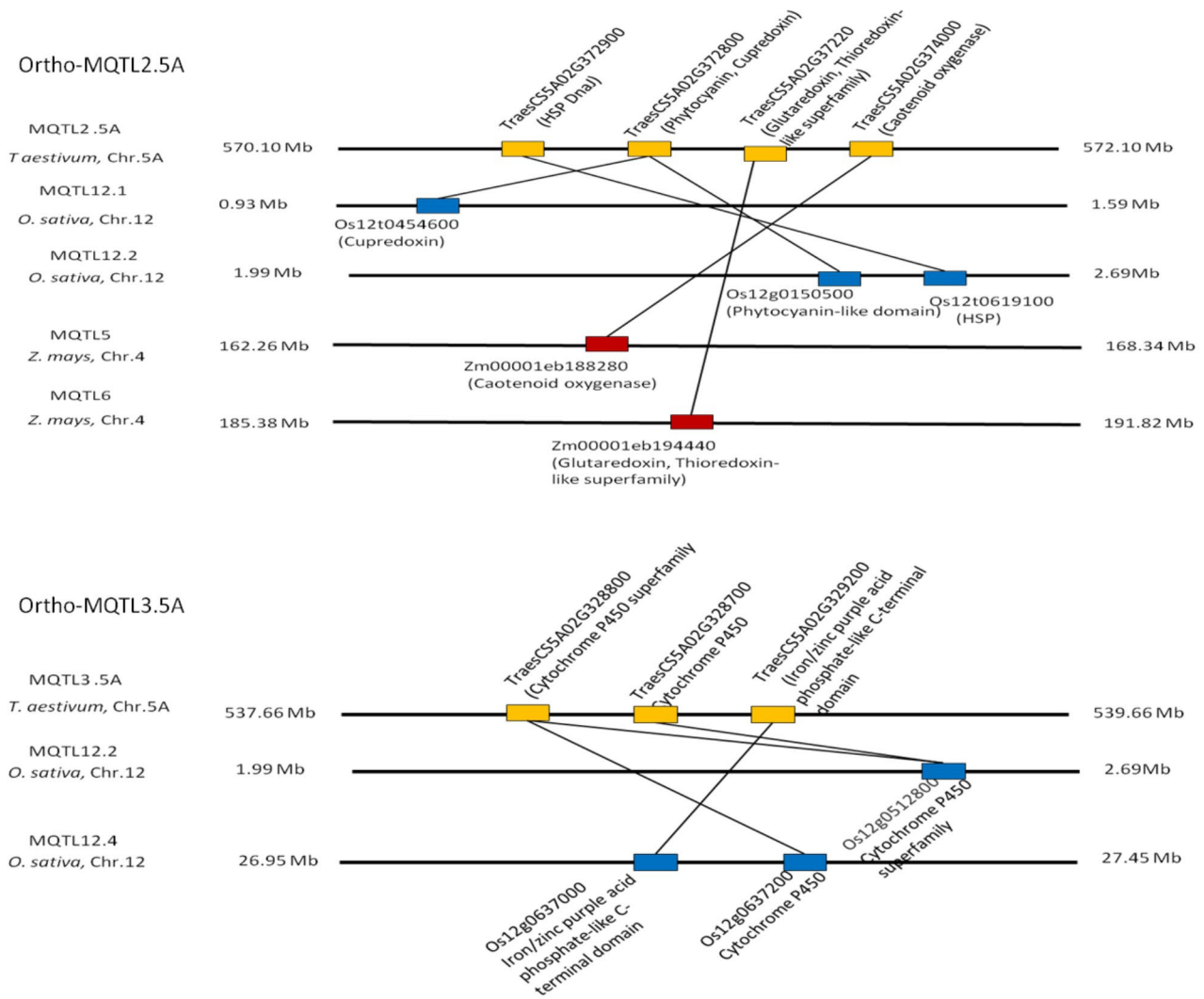
architecture in wheat. In another study, 89 MQTLs were co-located with MTAs for grain yield (Yang et al. 2021). In view of co-localization of MQTL3.5 A with GWAS-MTAs, this MQTL is recommended for use in MAS for development of biofortified wheat cultivars with higher GFe and GZn content. This validated MQTL may also be prioritized for mining CGs associated with GFe and GZn in wheat. The 32 MTAs identified in this MQTL region are perhaps important genomic regions for further studies on allele mining involving GFe and GZn.

### Ortho-MQTLs for cereals (wheat, maize and rice)

Out of 11 MQTLs, high confidence CGs were available for four MQTLs (MQTL2.5 A, MQTL3.5 A, MQTL4.5B, MQTL10.7 A). These four MQTLs were used for comparative mapping with corresponding MQTLs in rice and maize. Ortho-MQTLs in rice and maize could be identified against two of the four above mentioned wheat MQTLs, namely MQTL2.5 A and MQTL3.5 A (details

given in supplemental Table S2). Ortho-MQTLs involving wheat MQTL2.5 A were identified in syntenic regions with rMQTL12.1 and rMQTL12.2 on rice chromosome 12, and MQTL5 and MQTL6 on maize chromosome 4. Similarly, MQTL3.5 A had its corresponding syntenic MQTLs, namely rMQTL12.2 and rMQTL12.4 on rice chromosome 12 (Fig. 5). Ortho-MQTLs for GFe and GZn on chromosome 7 in rice were also reported in a recent study (Shariati-pour et al. 2021b). The identification of two ortho-MQTLs each in rice and maize against each of the two individual wheat MQTLs suggest the possibility of duplication of MQTL regions in both rice and maize or loss of one of these MQTL region in wheat. This is in line with the previous studies suggesting frequent gene duplications in both the rice and maize genomes (Gaut et al. 2000; Wang et al. 2005). Effect of semi-dwarfing *Rht* genes on micronutrients concentration (GFe and GZn) in modern wheat cultivars has also been reported, suggesting that some genes for micronutrients have been lost in modern dwarf wheat cultivars (Velu et al. 2017a, b).





**Fig. 5** Two wheat MQTLs, both on chromosome 5 A (showing four genes), with their corresponding syntenic MQTLs in rice chromosome 12 and maize chromosome 4. The chromosome number, genomic position, and common genes between the wheat, rice, and maize are indicated

The discovery of ortho-MQTLs in closely related species indicate the conservation of genomic regions controlling GFe and GZn contents across the cereal species, increases their significance and verifies their stability, as well as the confidence of association with CGs (Jin et al. 2015; Khahani et al. 2020; Yang et al. 2021). Important CGs encoding either heat shock proteins i.e. HSPs (containing DnaJ domain) or iron/zinc purple acid phosphatase-like domain involved in GFe and GZn accumulation are associated with these ortho-MQTLs (more details are discussed below). The gene-based markers developed from these CGs may be used in MAS for improvement of GFe and GZn contents across the three cereal species.

### Breeders' MQTLs

In the present study, the above MQTL3.5 A that was colocalized with MTA from GWAS along with two other MQTLs, namely MQTL2.5 A and MQTL4.5B, have been shortlisted for breeding purpose. These are described as breeder MQTLs. These three MQTLs are each based on 2–4 original QTLs having relatively high PVE (9–15%). For two of these MQTLs, one each for GZn (MQTL2.5 A) and GFe/GZn (MQTL3.5 A), ortho-MQTLs were also detected in maize/rice, and rice, respectively (details are presented below). Interestingly, a CG containing ‘iron/zinc purple acid phosphate-like-C-terminal domain’ was also

associated with the MQTL3.5 A. The protein corresponding to MQTL4.5B for GFe/GZn contains zinc finger domain/RING, exhibiting down-regulation under Fe deficient conditions. This latter MQTL was also validated through qRT-PCR during the present study (see below).

### CG-based genes controlling Fe/Zn content

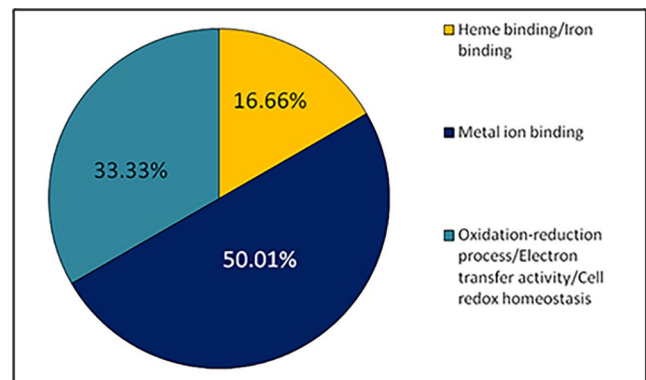
A total of 101 CGs that were found associated with 6/11 meta-QTLs, were subjected to GO analysis and based on biological functions, 12 high confidence CGs were identified that were associated with 4 MQTLs (Table 4, Supplementary Table S3). These CGs were apparently involved in metal ion homeostasis and encoded proteins containing different major domains. Six CGs involved in different biological processes encode proteins including the following: (i) proteins involved in metal ion binding, (ii) heat shock protein (HSP), (iii) metallo-dependent phosphatase-like domain, (iv) iron/zinc purple acid phosphatase-like C-terminal domain, (v) zinc finger/RING/FYVE/PHD-type domain, (vi) SMAD/forkhead-associated (FHA) domain, (vii) proteins belonging to cytochrome P450 superfamily and involved in heme binding/iron binding, (viii) four CGs encoding proteins belonging to thioredoxin-like superfamily, cupredoxin, FAD linked oxidase, and ferric reductase transmembrane, enriched with oxidation-reduction process, electron transfer activity, and cell redox homeostasis (for details see Table 4; Fig. 6). Some of these CGs were earlier reported to control GFe and GZn contents in wheat or other cereals like rice and maize (Jin et al. 2013, 2015; Raza et al. 2019).

Some of the CGs also have a role in Fe/Zn homeostasis. Following are some important examples: (i) The CG TraesCS5B02G469800 associated with MQTL4.5B encoding HRZ protein containing zinc finger/RING/FYVE/PHD-type domain plays an important role in Fe deficient condition. The iron binding regulators HRZs, major regulators of Fe homeostasis, contain three zinc (Zn) finger domains along with other domains including a RING finger (Kobayashi et al. 2011, 2013; Selote et al. 2015). In rice, the genes *OsHRZ1* and *OsHRZ2* are negative regulators of Fe deficiency response; their knockdown results in increased tolerance to Fe deficiency and Fe accumulation in rice and Arabidopsis (Kobayashi et al. 2013; Selote et al. 2015). (ii) The CG TraesCS5A02G329200 associated with the above MQTL encodes iron/zinc purple acid phosphatase (PAP)-like C-terminal (PAP) domain containing protein. PAP containing protein requires iron (Fe) for its function in diverse biological roles including Fe homeostasis. In tea (*Camellia sinensis*) also, based on conserved motifs, 19 *CsPAP* members were identified (Yin et al. 2019). (iii) The CG TraesCS5B02G470200 associated with MQTL4.5B encodes a protein containing SMAD/forkhead domain that

**Table 4** List of important proteins encoded by 12 candidate genes associated with four MQTLs for GFe and GZn contents in wheat

MQTL	Gene IDs (chromosome)	Predicted proteins
MQTL2.5 A	TraesC-S5A02G372900 (5 A)	Heat shock protein
	TraesC-S5A02G372800 (5 A)	Cupredoxin, Phytoeyanin
	TraesC-S5A02G372200 (5 A)	Glutaredoxin, Thioredoxin-like superfamily
	TraesC-S5A02G374000 (5 A)	Carotenoid oxygenase
	TraesC-S5A02G373900 (5 A)	Metallo-dependent phosphatase-like, Serine/threonine-specific protein phosphatase/bis(5-nucleosyl)-tetraphosphatase
MQTL3.5 A	TraesC-S5A02G328800 (5 A)	Cytochrome P450, E-class, group I
	TraesC-S5A02G328700 (5 A)	Cytochrome P450 superfamily
	TraesC-S5A02G329500 (5 A)	FAD linked oxidase, N-terminal,D-arabinono-1,4-lactone oxidase
	TraesC-S5A02G329200 (5 A)	Iron/zinc purple acid phosphatase-like C-terminal domain, Metallo-dependent phosphatase-like
MQTL4.5B	TraesC-S5B02G469800 (5B)	Zinc finger, RING/FYVE/PHD-type
	TraesC-S5B02G470200 (5B)	SMAD/Forkhead-associated (FHA) domain, PPM-type phosphatase domain
MQTL10.7 A	TraesCS7A02G328700 (7 A)	Ferric Rdtase_TM, CYB561/CYBRD1-like

has never been identified for Zn/Fe homeostasis in plants, although similar gene for Fe regulation has been identified in humans (Ma et al. 2019). Further analysis revealed that SMAD4 signaling selectively represses the promoters of the Fe-absorptive genes. During Fe-deficiency, the expression of SMAD3 and SMAD4 was significantly decreased



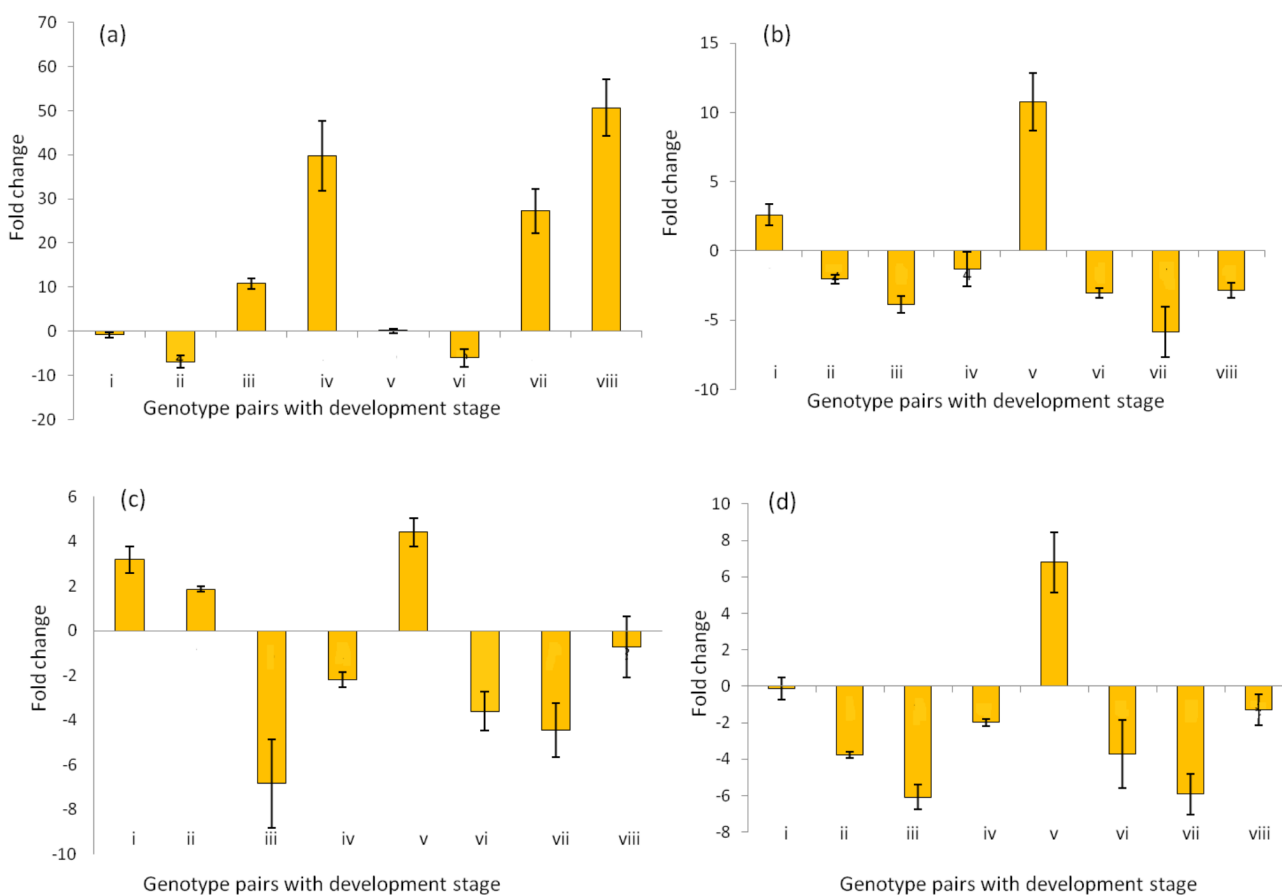
**Fig. 6** Proportions of significantly enriched GO terms associated with molecular functions of identified Fe and Zn CGs

via proteasomal degradation, allowing for repression of Fe target genes. Several Fe-regulatory genes contain a SMAD-binding element (SBE) in their proximal promoters (*DMT1*). Therefore, characterization of SMAD/forkhead domain containing gene in plants is warranted. (iv) The CG TraesCS7A02G328700 encoding for ferric reductase transmembrane protein was identified in the region of MQTL10.7 A. The major functional domain of ferric oxidoreductase (FRO) genes consisting of ferric reductase-like, transmembrane is a highly conserved protein, which is crucial for cell surface ferric reductase activity (Schagerlöf et al. 2006; Wang et al. 2013). FRO in plants is involved in Fe acquisition from soil (Gupta et al. 2021) so that in Arabidopsis, the overexpression of *fro2* gene in the roots growing under iron deficient condition led to root growth, while deletion of the *fro2* gene led to reduced growth under Fe-deficient conditions (Robinson et al. 1999). (v-vii) Among three CGs, TraesCS5A02G372900 encodes HSP protein (containing DnaJ domain), TraesCS5A02G373900 encodes

metallo-dependent phosphatase-like protein, and TraesCS5A02G372800 (associated with MQTL2.5 A) encodes protein with cupredoxin and phytocyanin domains. The HSP protein and metallo-dependent phosphatase-like protein have a role in metal ion binding, whereas, the protein with cupredoxin and phytocyanin domains has molecular function in electron transfer activity.

### qRT-PCR for validation of CGs

Using qRT-PCR, differential expression of four CGs at two grain filling stages (14 and 28 DAA) was examined in four pairs of genotypes involving two genotypes (FAR4, WB02) with high GFe and GZn contents and two other genotypes (K8027, HD3226) with low GFe and GZn. In each pair of comparison, the expression in a genotype with high GFe and GZn was compared with the other genotype with low GFe and GZn. The results are summarized in Fig. 7. All the four CGs exhibited significant differential expression



**Fig. 7** Differential expression (fold difference) of four CGs in four pairs of wheat genotypes (FAR4 and WB02 with high Fe, Zn; K8027 and HD3226 with low Fe, Zn) at two grain filling stages (14DAA and 28DAA). (a) Heat shock protein, (b) Carotenoid oxygenase (c) FAD linked oxidase, (d) HRZ containing zinc finger, RING/FYVE/PHD-type domains. (i) FAR4:K8027-14, (ii) FAR4:K8027-28, (iii) FAR4:HD3226-14, (iv) FAR4:HD3226-28, (v) WB02:K8027-14, (vi) WB02:K8027-28, (vii) WB02:HD3226-14, (viii) WB02:HD3226-28

(> 2-fold and >-2-fold) between pairs of genotypes at both the grain filling stages suggesting their possible role in accumulation of Fe and Zn in the grains. The genes encoding carotenoid oxygenase and HRZ proteins showed almost similar expression patterns involving down-regulation in pairs of genotypes with high GFe and GZn contents, with a few exceptions, whereas the remaining two genes differed in their expression in different pairs of genotypes at two grain filling stages (Fig. 7b, d).

Gene ontology analysis showed that the gene for carotenoid oxygenase is involved in metal-ion binding whereas the gene encoding for HRZ protein is a negative regulator of iron-deficiency, as stated above. The gene encoding for HSP having a role in metal-ion binding showed opposite pattern of expression in two genotypes containing high GFe and GZn contents compared with two different genotypes each containing low GFe and GZn contents (Fig. 7a). The HSP gene showed strong upregulation (10.82 fold to 50.67 fold) in both FAR4 and WB02 against HD3226 whereas, it showed down-regulation (0.05 fold to -6.89 fold) in the above two genotypes against K8027. We speculate that this contrasting expression of HSP in two genotypes in a pair is due to allelic differences in this gene. SNP based KASP markers may be developed to differentiate wheat lines, which differ for Fe and/or Zn content, presumably due to allelic differences in the gene represented by the CG. The other gene encoding for FAD linked oxidase, which is a motif present in the water soluble domain of FRO2 (Schagerl $\ddot{o}$ f et al. 2006) and also known to play an important role in photosynthesis (Johnson 2016) showed up-regulation at 14 DAA in both the high GFe and GZn containing genotypes against one genotype with low GFe and GZn contents; in the remaining combinations it showed down-regulation, suggesting its role at early grain development stages (Fig. 7c).

## Conclusions

Biofortification of crops is an important approach for overcoming micronutrient deficiency and hidden hunger, but QTLs identified so far did not prove useful. Therefore, 11 robust MQTL with reduced CI were identified in the present study, and should prove useful for a large range of populations with different genetic backgrounds and cultural conditions. These 11 robust MQTLs included nine novel MQTLs and were used for identification of a large number of CGs. Two ortho-MQTLs were also identified with their syntenic regions in rice and maize. Three breeder's QTLs and 12 CGs were prioritized to be used for further studies and use in wheat breeding programs. The markers associated with breeder's MQTLs, may be used for MAS in wheat breeding programs targeted towards development of GFe and GZn biofortified

wheat cultivars. Selected important CGs can be used to understand the genetic basis of GFe and GZn homeostasis.

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**Author contribution** PKG, HSB, SS, and RK conceived and designed the experiment. RS prepared the first draft of the MS with the help of GS. AK, IJ, RB; JK helped in the collection of literature. TG helped in qRT-PCR analysis. PKG, HSB and SS finalized the manuscript.

**Conflict of interest** There is no conflict of interest.

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