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Review

Harnessing the Wild Relatives and Landraces for Fe and Zn Biofortification in Wheat through Genetic Interventions—A Review

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Abstract: Micronutrient deficiencies, particularly iron (Fe) and zinc (Zn), in human diets are affecting over three billion people globally, especially in developing nations where diet is cereal-based. Wheat is one of several important cereal crops that provide food calories to nearly one-third of the population of the world. However, the bioavailability of Zn and Fe in wheat is inherently low, especially under Zn deficient soils. Although various fortification approaches are available, biofortification, i.e., development of mineral-enriched cultivars, is an efficient and sustainable approach to alleviate malnutrition. There is enormous variability in Fe and Zn in wheat germplasm, especially in wild relatives, but this is not utilized to the full extent. Grain Fe and Zn are quantitatively inherited, but high-heritability and genetic correlation at multiple locations indicate the high stability of Fe and Zn in wheat. In the last decade, pre-breeding activities have explored the potential of wild relatives to develop Fe and Zn rich wheat varieties. Furthermore, recent advances in molecular biology have improved the understanding of the uptake, storage, and bioavailability of Fe and Zn. Various transportation proteins encoding genes like *YSL 2*, *IRT 1*, *OsNAS 3*, *VIT 1*, and *VIT 2* have been identified for Fe and Zn uptake, transfer, and accumulation at different developing stages. Hence, the availability of major genomic regions for Fe and Zn content and genome editing technologies are likely to result in high-yielding Fe and Zn biofortified wheat varieties. This review covers the importance of wheat wild relatives for Fe and Zn biofortification, progress in genomics-assisted breeding, and transgenic breeding for improving Fe and Zn content in wheat.

Keywords: malnutrition; iron; zinc; wheat; wild relatives; biofortification; QTLs

1. Introduction

Wheat is an important cereal crop that provides food calories to one-third of the global population [1]. The annual global wheat production is 765.8 million tonnes with a planted area of 215.9 million hectares [2]. Therefore, wheat holds immense importance from a nutritional quality point of view given that poor nutrition can impact human health, especially children and pregnant women [3]. Micronutrient malnutrition affects more than 3 billion people worldwide, with Fe and Zn deficiencies being the common

causes [4]. Low dietary intake is the main cause of microelement deficiencies in humans [5]. Deficiencies in Fe and Zn in children result in retarded growth that leads to diseases such as diarrhea, anemia, pneumonia, and malaria [6]. Fe deficiency in women leads to pre-term birth and low birth weight [7]. However, the bioavailability of Fe and Zn in wheat is inherently low, especially under Zn deficient soils. The wheat grain Zn content varies from 10–30 mg/kg under a wide range of soil conditions globally, but this is far below the daily recommended dietary requirement of 40–60 $mgkg^{-1}$. The recommended dietary allowance (RDA) of micronutrients for young adults ranges from 8–18 mg/person/day for Fe and 8–12 mg/person/day for Zn [8]. The available range of Fe and Zn in Indian wheat varieties varies from 28–51 $mgkg^{-1}$ and 10–20 $mgkg^{-1}$, respectively [9,10]. Fe and Zn deficiencies persist despite the availability of sufficient Fe and Zn content in wheat grain because most of the micro-nutrients present in the wheat bran are removed during processing into flour. Anti-nutritional factors, for example, phytates, present in wheat germ also reduce the Fe and Zn absorption and bio-availability in the human gut [11]. Furthermore, increases in the CO_2 concentration can also result in a reduction in Fe and Zn content in wheat grain [12,13].

Various approaches to fortification such as dietary diversity, food fortification and supplementation, biofortification (e.g., agronomic and genetic approaches) are available to alleviate the problem of malnutrition. The agronomic wheat biofortification strategy is a quick, short-term process for enhancing nutrient availability by using integrated soil nutrient management (Fe and Zn fertilizers) to improve the kernel Fe and Zn concentration [14], but it is not a sustainable approach in the long run [15] and change or the adoption of different farming practices may be needed. However, developing high Fe and Zn-enriched wheat varieties using breeding and genetic engineering approaches is a sustainable way to fight "hidden hunger" in the long term, provided enough micronutrients are available in the soil [16]. Biofortified food crops not only reduce malnutrition, but also provide sustainable long-term health benefits. Hence, biofortification is a game-changing approach for alleviating hidden hunger, as indicated by recent successes in maize, rice, and wheat via the combined efforts of researchers and policymakers [17–19].

Biofortification efforts for enhancing Fe and Zn content in wheat grain can be initiated with the use of the existing natural variation in wheat wild progenitors, non-progenitors or related species [20,21]. However, the use of wild progenitors leads to the problem of linkage drag, resulting in undesirable yield losses. However, recent technological developments in genomics have resulted in better utilization of existing variation through genome-wide association studies (GWAS), thus helping to attain rapid enhancement in Fe and Zn content in wheat grain [22]. This review focuses on the impact of domestication, especially the green revolution, on wheat quality, the significance of genetic variation in wheat wild relatives, challenges in the genetic enhancement of Fe and Zn content in wheat, and utilization of genetic engineering to develop transgenic-based Fe and Zn-enriched wheat varieties.

2. Green Revolution and Its Effect on Quality Traits

Wheat domestication started about 8000 years ago in Southwest Asia [23]. Wild, traditional and ancient food crops have always served as a good source of food diversity to meet the essential nutritional requirements of the human species. However, the increasing popularity of cereals as staple food crops shifted the focus to the development of high-yielding varieties, thus sidelining the nutritional quality of the crops [24,25]. The efforts of researchers were focused on developing short stature and fertilizer responsive varieties (compared to traditional tall wheat and rice), which catalyzed a "green revolution" that resulted in a significant jump in crop productivity [26,27]. As a result, the aim of farmers shifted to economical farming to earn more profits through the adoption of mechanization for the cultivation of dwarf wheat and rice varieties, which resulted in a dramatic improvement in food production, especially in China and India [28,29]. Consequently, the areas planted with nutritionally rich crops such as pulses, fruits, and oil crops were reduced, which led to these crops fetching higher prices relative to the low price of wheat and rice. This intensification of agricultural research and over-dependence on wheat and rice led to

a significant loss in the genetic diversity for various quality traits in wheat and rice and the extinction of some distinctive indigenous crops [30]. Food and nutritional security are interrelated; hence, the balance of economic and social sustainability through the evergreen revolution (technology development and dissemination) is the best approach to achieve holistic nutri-food security [28,30].

The achievements of the green revolution in the 1970s are highly applauded, but an analysis of grain shows a significant decrease in the mineral content of dwarf wheat cultivars (Fe, 29.7 $mgkg^{-1}$; Zn, 24.3 $mgkg^{-1}$) over tall cultivars (Fe, 38.2 $mgkg^{-1}$; Zn, 33.2 $mgkg^{-1}$) [24,31]. This dilution in Fe and Zn content was due to a negative association with grain, indicating the poor efficiency of dwarf high-yielding wheat varieties in partitioning minerals to the grain [31,32]. Although, modern wheat cultivars have sufficient genetic diversity and are adaptable to diverse ecologies [33], they are deficient in micronutrients, especially Fe and Zn content. Although improving the content of Fe and Zn was not prioritized during yield improvement, after establishing food security, there has been a tremendous increase in Fe and Zn in wheat cultivars. For example, in a major wheat producing country, India, the significant improvement in Fe and Zn content in bread wheat in the last decade is the result of the release of Fe and Zn-enriched wheat varieties (Figure 1).

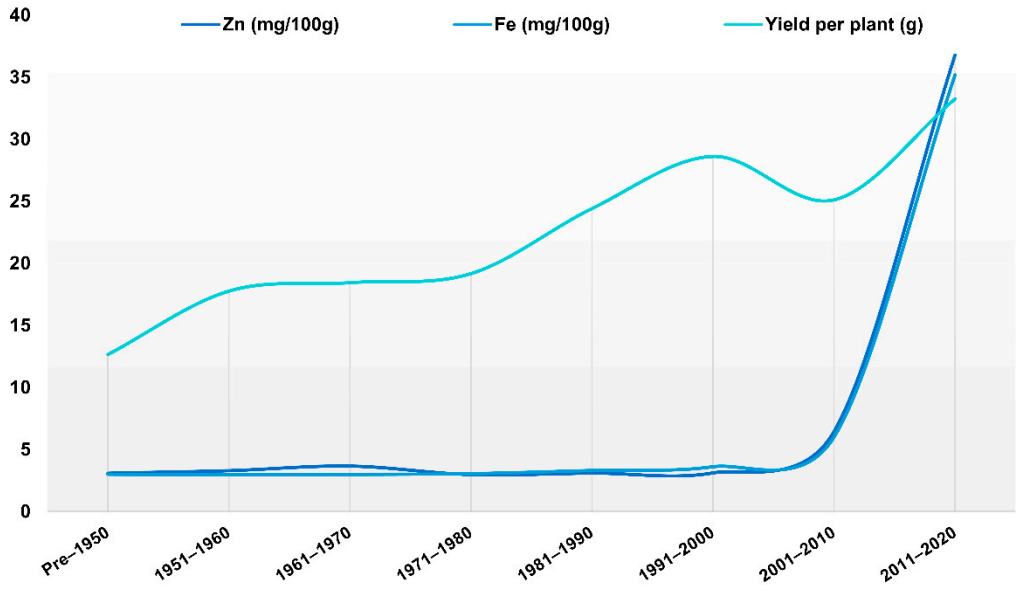


Figure 1. Fe and Zn content trends in wheat during domestication and wheat breeding in India (based on yield, Fe and Zn content of varieties released during various decades). Data source: [9,10].

Nutritional Composition Status and Fe and Zn in Wheat

Wheat is one of the most popular staple food crops not only because of its highly productive varieties, but because of its varied food products. It is used to make flours, bread, and other products such as pasta, macaroni, noodles, semolina, bulgur, couscous, biscuit, chapatti, and bread, supplying calories and nutrients to most of the world's population [34]. The wheat grain consists of endospermic or aluminous layers (aleurone cell layer and starchy endosperm, which represents 80 to 85% of the grain), 2–3% germ and 13–17% bran (outer layer of wheat grain) [35], and it contains water-insoluble fiber and B vitamins and ~7.2% minerals, proteins and carbohydrates. Besides carbohydrates, endosperm comprises fats (~1.5%) and proteins (~13%). Wheat germ is a good source of vitamin E and also has appreciable levels of amino acids such as alanine, arginine, glycine, and lysine [36]. The germ portion of wheat is also abundant in proteins (~25%), lipids (upto 13%), and minerals (4.5%). However, wheat contains suboptimal quantities of micronutrients, especially Fe and Zn [37]. Wheat contains Fe in the range of 29–73 $mgkg^{-1}$ and Zn in the range of 7–85 $mgkg^{-1}$ [32,38]. In the whole grain, the maximum amount of Fe (~80%) is present in

the form of ferric ions, which form a spherical crystalline inclusion in a protein body of a nucellar part called globoids, and remain present in the form of ferrous ion, which is mainly present in the scutellum as ferrous sulphate. Wheat-based diets are insufficient to meet Fe requirements in children, women, and men [39]. To meet human health requirements, average levels of Fe and Zn content in the grain must be around 40 and 60 $mgkg^{-1}$, respectively [40]. The assessment of the variation in Fe and Zn content in cultivated genotypes and wild relatives at the genetic level is the first step of any biofortification program.

3. Genetic Variability, Heritability and Gene Action for Fe and Zn

Wheat germplasm has been extensively screened at the International Maize and Wheat Improvement Centre (CIMMYT)for Fe and Zn content variation in the grain and stability in different growing environments. Grain Fe and Zn content showed a positive correlation with moderate heritability [41,42]. In another study, an assessment of a set of global germplasm, elite breeding lines, and modern cultivars (a total of 243 lines) evaluated in multiple locations in field conditions, revealed that the variability in Fe and Zn content was in the range of 20–88 ppm and 15–43 ppm, respectively [43].Later, screening of 22 wild emmer wheat accessions (drought-tolerant) resulted in the identification of lines with high concentrations of Fe (85 $mgkg^{-1}$) and Zn (125 $mgkg^{-1}$)in grain under Zn deficient soil [44]. Thus, grain Fe and Zn content was found to increase many times, indicating that the conservation of Fe and Zn was a genetic treasure in CIMMYT's wild wheat germplasm that could be introduced into modern varieties. Furthermore, research on bread wheat at CIMMYT revealed the absence of a negative linkage between grain yield and Fe and Zn density in the grain, i.e., no or little dilution was observed and relatively small portions of genotype effects contributed towards overall variations in grain Fe and Zn content [45]. In another study on 825 wild emmer accessions in field conditions, the Fe concentration of wheat kernel varied from 15–109 $mgkg^{-1}$ with an average of 46 $mgkg^{-1}$ and Zn concentration varied from 30–118 $mgkg^{-1}$ with an average of 61 $mgkg^{-1}$ [46]. Accessions with high seed weight (seed size) possessed the highest amount of Fe (>2.4 µg Fe per seed) and Zn (>5 µg Zn per seed). Cultivated wheat is characterized by less variability and lower Fe and Zn content as compared to the wild relatives [47].

The Zn content of wheat grain is positively correlated with Fe content [41,42,48], indicating the feasibility of simultaneous improvement in Fe and Zn [49,50]. A positive correlation between protein content and Fe/Zn content in wheat grain was also found under field conditions in Kazakhstan. However, Zn concentration showed a positive relationship with protein content but a negative correlation with grain yield [51,52]. The significant correlation between Fe and Zn content in wheat grain indicates common genetic factors or co-segregation of genetic regions governing the accumulation of Fe and Zn in mature grain [46]. Therefore, increasing seed-associated Fe content in wheat can simultaneously increase Zn content or vice versa. The Fe and Zn content in wheat grain is strongly correlated to each other and it is affected by several factors [53]. The highest amount of Zn in wheat seed is observed in the initial stage of seed development (early milk stage); then Zn concentration gradually decreases up to maturity [54]. The presence of significant differences in Fe and Zn content among bread wheat lines indicates the influence of soil fertilization [55]. Unlike Zn, soluble Fe^{+2} fertilizers are ineffective in increasing the Fe concentration in plants, especially in the grain. Thus, the foliar application of $FeSO_4$ was not effective for increasing Fe content in wheat grains [56]. However, Zn concentration was efficiently increased by foliar application of Zn only or mixed with Fe.

The heritability of the trait is an important factor to determine the chances of achieving better genetic gains. Broad sense heritability of grain Fe and Zn content was observed to be in the range of moderate (4–6) to high (above 6) [45,49,57,58] thus, indicating the quantitative nature of grain Fe and Zn [58]. However, [59] observed low heritability for Fe and Zn; this may be due to the relatively higher influence of the environment. The authors of [42] revealed that Fe and Zn uptake efficiency is governed by additive as well as non-

additive gene actions. However, in [60], it was reported that Fe content is mainly governed by non-additive gene action. Hence, the selection for Fe and Zn content seems tricky, i.e., if the trait is governed majorly through additive gene action, early generation selection is effective whereas if non-additive gene action is prominent then the selection is effective at later generations. Prominent non-additive gene action also indicates the utilization of a hybrid breeding approach for developing Fe and Zn-enriched wheat hybrids.

4. Genetic Biofortification via Conventional Breeding

Genetic biofortification involves the genetic improvement of crops for quality traits through characterization of germplasm for genetic variation, gene discovery, and introgression (conventionally/marker-assisted) of favorable genes/alleles [52]. Different breeding strategies can be used for the improvement of Fe and Zn content in wheat as shown in Figure 2. In the past several decades, the Harvest-Plus project and CIMMYT have initiated research to assess the genetic variation in grain Fe and Zn content in a wide range of germplasm in the genus Triticum. Based on studies, some accessions of wild wheat relatives (*Triticum dicoccom* PI254187, PI94677), wild varieties (MIAN YANG #11, VEE/MYNA, MRGN), landraces (TXL92.3.2.2, 84TK536-003.01-728, OAXACA92.5.17) and derived lines from the pre-breeding program (CMH84.3595, RICARDO E211#1) contained higher grain Fe and Zn than the popular modern cultivars/advanced lines at CIMMYT [43,52]. Hence, at CIMMYT, the Harvest Plus Yield Trial (HPYT) focused on the transfer of Zn content from *T. aestivum* ssp. *spelta* and *T. turgidum* ssp. derived synthetics and wheat landraces to elite wheat backgrounds. Subsequently, 6–7 superior lines (with grain Zn 75–150% higher than checks) were identified in the second HPYT (which consisted of 50 biofortified wheat lines). The identified superior lines were also found to be high-yielders and rust-resistant. This demonstrates the dedicated efforts made over the years that have resulted in the development of candidate varieties with higher grain Fe and Zn and other important agronomic traits.

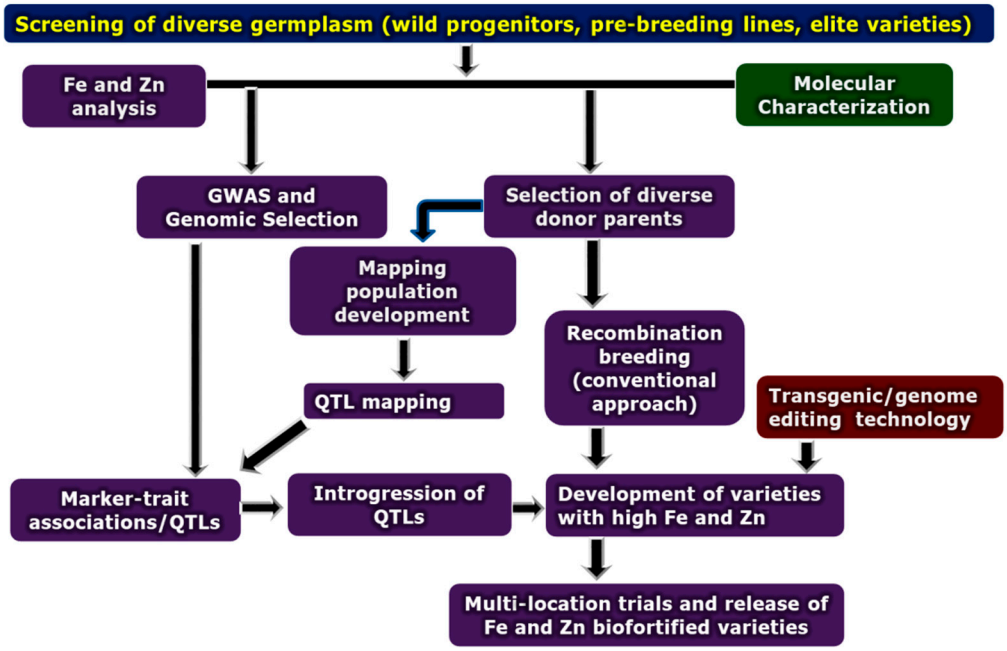


Figure 2. Different genetic interventions and approaches for Fe and Zn biofortification in wheat.

Modern wheat varieties are characterized by limited variation in Fe and Zn content [47], whilst wheat's wild relatives and progenitors, i.e., einkorn wheat (*Triticum monococcum*), wild emmer wheat (*T. dicoccoides*), diploid progenitors of bread wheat (*T. spelta*, *T. polonicum*) and landraces of bread wheat have high grain Fe and Zn content. Hence, non-progenitor *Aegilops* species have been explored to transfer the grain micronutrients to

elite wheat cultivars [22]. The amphiploids developed from *Ae. longissimi* and *T. durum* exhibited higher grain ash iron and zinc content along with bolder grains, conferred by the superior genetic make-up of *Ae. longissimi* for the uptake and sequestration of micronutrients. The introgression of 7UP/7SP and 4SP chromosomes from *Ae. peregrina* grain increased Fe and Zn density by 100–200% in the backcrossed derivatives relative to elite wheat cultivars. Similarly, the introgression of 2S and 7U chromosomes of *Ae. kotschyi* also increased grain Fe and Zn content in backcross derivatives [61]. Studies on disomic wheat-rye addition lines and an octoploid triticale line revealed higher Zn efficiency in addition lines, enhanced shoot Zn concentration, and grain Zn content over wheat parents, indicating the presence of Zn efficiency genes in the rye, especially 1R (highest Zn efficiency~80%), 2R, and 7R [62]. Hexaploid wheat has the highest Zn efficiency (64%) followed by diploid (60%) and tetraploid (36%). This variation is due to the high Zn efficiency of A and D genomes as evident from the transfer of DD genome (*Ae. tauschii*) or AA genome (*T. monococcum*) to tetraploid wheat (*T. turgidum*) to develop synthetic hexaploid wheat (AABBDD). Developed synthetics, exhibited an improved amount of Zn content under Zn deficient as well as Zn-sufficient soil conditions [62,63]. Thus, Zn efficiency, grain Zn concentration and the total amount of Zn per shoot were more associated with triticale and rye parent cultivars than wheat parental lines [62]. Similarly, in another study, synthetic hexaploid lines exhibited higher grain Fe and Zn content than the cultivated wheat, with nearly equivalent yield. This indicates that synthetic wheat has higher Zn efficiency and can be used as a donor for Zn improvement without a dilution effect (linkage drag) [64]. Successful examples of harnessing wheat wild relatives for developing high Zn varieties in India include "Zinc Shakti" (Chitra), "WB 02" and "HPBW 01" [65] and the Fe-rich variety, "HD 3171" [66]. The impact of these varieties in improving Fe and Zn content has been witnessed over the last decade (Figure 1). Such wheat varieties with improved grain Zn density and high Zn efficiency can be recommended for cultivation in severely Zn-deficient targeted regions and can help to alleviate malnutrition in developing countries [63,67].

Genetic biofortification for Fe and Zn content is challenged by growth conditions, particularly soil type, fertility level, and environmental interactions [45,68]. There are reports of significant effects of the environment on grain Fe and Zn content in wheat [45,49,69]. Based on research, the genotype × environment (G × E) interaction was found over a number of sites and years for Zn content in some genotypes; therefore, genotypes need to be evaluated precisely over multiple years at different locations [45,68]. Significant G × E interactions have been found for grain Fe and Zn content in wild relatives and modern wheat cultivars [16,21,28]. However, the testing of biofortified wheat lines at multiple sites revealed the presence of high-heritability and a good correlation between testing sites for grain Zn [70].

5. Marker-Assisted Selection for Fe and Zn Biofortification

Genetic biofortification for complex traits such as Fe and Zn using classical breeding is time-consuming and less effective compared to marker-aided breeding. DNA-based markers that are tightly linked to the target trait (Fe and Zn content) enable rapid development of biofortified varieties via the indirect selection of favorable genotypes, irrespective of the stage of expression. This helps to overcome the physiological complexity and slow progress of phenotypic selection. Fe and Zn content are quantitative traits governed by many genomic regions called quantitative trait loci (QTL), which are located over different chromosomes and govern different phenotypic expressions [67,71–74]. For example, three QTL have been identified for Zn concentration (*qZN-5*, *qZN-7* and *qZN-11*) on chromosomes 5, 7, and 11, respectively, in rice [75]. Numerous QTLs for grain Fe and Zn have been identified in wheat (Table 1). For example, wild emmer wheat possesses a major QTL *GPC-B1* for Fe and Zn content [76]. The locus, *GPC-B1* encodes NAC transcription factor (*NAM-B1*) that enhances the remobilization of nutrients from plant parts to grains was

later cloned [76,77]. Wild emmer also contributed the positive alleles for 82 QTLs for ten minerals at homoeologous positions of A and B genome chromosomes.

Table 1. List of major QTLs identified for Fe and Zn concentration in wheat.

Cross	QTLs	Chr.	Position (cM)	Flanking Markers	Confidence Interval (cM)	PVE (%)	Ref.
Iron (Fe)							
T. durumSaricanak98 × ***T. dicoccon* MM 5/4**	*QGFe.sar_5B$_{MCO}$*	5B	97	*wPt-7400–wPt-8449*	94.1–108.6	16.9	[41]
***T. durum* Saricanak98** × *T. dicoccon* MM 5/4	*QGFe.sar_5B$_{TKM}$*	5B	28	*wPt-81.25–wPt-9504*	27.5–40.2	~15	
T. aestivum Adana 99 × ***T. sphaerococum* 70711**	*QGFe.ada-7B*	7B	35.7	*wPt-5922*	35.70	18.0	
	QGFe.ada-2B$_{tsk}$	2B	77.1	*wPt-9812*	77.10	17.0	
	QGFe.ada-2B$_{tkm}$	2B	52.4	*wPt-1394–wPt-7864*	50.3–53.6	17.0	
T. spelta H$^+$ 26 × ***T. aestivum* cv. HUW 234**	*QFe.bhu-3B*	3B	1022	*3022954 | F | 0–1102324 | F | 0*	1015.23–1022.28	25.9	[57]
***T. spelta* H$^+$ 26** × *T. aestivum* cv. HUW 234	*QFe.bhu-1A.3*	1A	346	*1708014 | F | 0–1000008 | F | 0*	345.76–355.02	16.5	
T. aestivum SeriM82 × ***T. dicoccoides*/*Ae. tauschii* SHW CWI76364**	*QFe.Across_7DS*	7DS	5	*TP43715–TP37547*	2.0–5.4	~15.0	[72]
Synthetic hexaploid wheat Louries × ***T. spelta***	*QGFe.cimmyt-4A_P2*	4A	199	*3385350–1211533*	198.5–199.5	21.1	[73]
Roelfs F 2007 × ***Chinese Parental Line***	*QGFe.co-3B.1*	3B	200	*1089107 1127875 | F | 0*	199.5–200.5	14.56	[78]
WH 542 × ***Synthetic derivative*** *(T.dicoccon/Ae. tauschii[409]//BCN)*	*QGFe.iari-7D.1*	7D	11	*Xwmc550–Xgwm350*	9.5–12.5	42.13	[79]
***T. aestivum* Chuanmai 42** × *T. aestivum* Chuannong 16	QTL	4D	160	*Xgwm154–Xbarc108*	160.00	19.1	[80]
Zinc (Zn)							
T. aestivum Adana 99 × ***T. sphaerococum* 70711**	*QGZn.ada-1D*	1D	80.3	*wPt-6979–wPt-730,718*	67.8–125.8	31.0	[41]
	QGZn.ada-6B	6B	65.1	*wPt-667,798–wPt-7065*	62.0–66.1	27.0	
	QGZn.ada-7B	7B	28.1	*wPt-733,112*	28.10	25.0	
	QGZn.ada-7A	7A	76.1	*wPt-2083–wPt-6083*	74.0–77.3	15.0	
***T. spelta* H$^+$ 26** × *T. aestivum* cv. HUW 234	*QZn.bhu-2B*	2B	966	*989092 | F | 0–1101425 | F | 0*	948.23–966.68	16.5	[57]
T. aestivum SeriM82 × ***T. dicoccoides*/*Ae. tauschii* SHW CWI76364**	*QZn.Y13-14_4BS*	4BS	23.2	*TP91631–TP81797*	23.1–23.7	19.6	[72]
	QZn.Across_4BS	4BS	23.2	*TP91631–TP81797*	23.1–23.7	17.3	
T. spelta × **synthetic hexaploid wheat**	*QGZn.cimmyt-7B_1P2*	7B	44	*1079651–1262676*	43.5–44.5	32.7	
T. spelta × synthesized hexaploid wheat	*QGZn.cimmyt-7B_1P1*	7B	62	*3945822 -1132640F0-5CG*	61.5–62.5	16.8	
	QGZn.cimmyt-1B_P1	1B	84	*3934172–33934936*	83.5–84.5	15.1	
Roelfs F 2007 × ***Chinese Parental Line***	QGZn.co-5A	5A	54	*1244217–1272027 | F | 0*	53.5–54.5	14.22	[78]
WH 542 × ***Synthetic derivative***	QGZn.iari-7D.2	7D	21	*Xgwm350–AX-94958668*	4.5–41.5	13.07	[79]

Table 1. *Cont.*

Cross	QTLs	Chr.	Position (cM)	Flanking Markers	Confidence Interval (cM)	PVE (%)	Ref.
***T. aestivum* Chuanmai 42** × *T. aestivum* Chuannong 16	QTL	4D	194	*Xcfa2149–Xbarc48*	194.00	15.9	[80]
T. aestivum PBW343 × ***T. aestivum* Kenya Swara**	*QGzncpk.cimmyt-3AL*	3A	79.1	*wPt-0286*	74.4–84.0	15.0	[81]
Zinc-Shakti × Kachu	QFeC-2A	2A	111	*1074973–2253877*	104.5–148.5	10.6	[82]

Note: Bold indicates QTL/favorable allele contributing parent.

Similarly, using *Ae. kotschyi* and *Ae. peregrine*, one QTL (*QFe.pau-2A*) for Fe and two QTLs and one each for Fe (*QFe.pau-7A*) and Zn (*QZn.pau-7A*) were mapped [83]. Generally, chromosome 5A is used for mapping QTLs for Fe, Zn, and protein, possibly indicating an overlapping common genetic basis for these traits. The marker, *Xgwm154* is co-located with the QTLs for shared traits, and hence can be targeted for multi-trait improvement. The finely mapped major QTLs can be further characterized for understanding the molecular basis of grain Fe and Zn content synthesis and regulation in wheat. The gene *GPC-B1*, which regulates senescence was cloned and found to improve grain protein, Fe, and Zn content by 38%, 18%, and 12%, respectively [84]. The authors of [83] also mapped a major QTL on 7A that governed 18.8% expression for grain Zn concentration. Additionally, major QTL (*QGZn.ada-7B*, *QGZn.cimmyt-7B*_1P2, and *QGZn.ada-1D*) for grain zinc content were mapped on chromosome 1D and 7B [73,83]. The authors of [49] conducted the GWAS study using the HarvestPlus Association Mapping panel at multiple location trials in India and Mexico, and mapped two major QTLs, namely, *QZn2A* and *QZn7B* for Zn content; however, these only have a phenotypic variation of around 12%. In [78], a RIL population derived from the Chinese parental line (with high Zn) and Roelfs F2007 was characterized to map 9 and 10 QTLs for kernel Fe and Zn content, respectively. In addition, the study identified promising candidate genes such as cytochrome P450, leucine-rich repeat receptor-like protein kinase, protein kinase family protein, etc. Recently, the authors of [79] used WH542 and synthetic derivative-based RILs (163) to map three QTLs for Fe and six QTLs for Zn, of which *QGFe.iari-7D.1*, *QGFe.iari-7D.2* were novel and stable. Although several QTLs have been identified, most are of minor effect except for a limited number of major QTLs [47,72,83]. Furthermore, the existence of QTL×environment interaction demands the identification of consistent or stable QTLs to develop biofortified varieties. Meta-QTL analysis is an efficient approach that uses the information of mapped QTLs across different independent studies to identify more robust, reliable QTLs and highlight the common and important genetic regions for target traits [85]. The metaQTL studies are limited for Fe and Zn content in wheat. A previous study [86] used QTL information from seven independent studies to map seven metaQTLs on six different chromosomes, with most of Fe and Zn content-associated QTLs being co-located, which indicates the possibility of simultaneous improvement of Fe and Zn content in wheat grain. The meta-QTL region contained the genes for Fe and Zn homeostasis.

MAS is quite useful for introgression of major QTLs; however, it has limited use in the case of complex traits such as Fe and Zn where the existence of minor QTLs is common. To capture the effect of minor QTLs and genotype–environment interaction effects, genomic selection is a better choice [87]. The authors of [88] conducted multi-location trials based on genomic prediction on association panels (330 diverse wheat lines) and found moderate (>3 to <5) to high (>5) prediction accuracies for Fe (0.32–0.73) and Zn (0.33–0.69), respectively. Similarly, genomic prediction was conducted in [59] on 269 Afghan wheat landraces and moderate prediction accuracies for Fe and Zn were found. Recently, the authors of [58] also obtained low to moderate prediction accuracy for grain Fe using 369 European wheat varieties. Hence, the integrated use of recurrent selection schemes with genomic selection can be vital to capture the genetic variation in major and minor QTLs for the development of Fe and Zn-rich wheat varieties.

6. Promoters and Inhibitors in Fe and Zn Biofortification and Transgenic Approaches

Different strategies have been reported for improving the uptake, transportation, and deposition of Fe and Zn in different tissues of wheat [89]. Promoters and inhibitors play a major role in micronutrient bioavailability. Insulin type-fructans are potential enhancers of the intestinal absorption of micronutrients in humans and animals, as evident from studies in wheat landraces and CIMMYT wheat lines. Significant genotypic variation was found for the content of fructans in with grain dry weights ranging from 0.7 to 2.9%, without strong G×E interaction [90]. Higher levels of phytases (myo-inositol hexakisphosphate phosphorylase) are catalyzed into inorganic phosphate and special myo-inositol phosphate that improve bioavailability [91]. Improvement of plants with a higher phytate-degrading activity helps to improve mineral bioavailability in the human stomach by improved degradation of phytate [92]. A 3.4 to 5.9-fold variation in phytase levels has been reported in wheat varieties and synthetic hexaploids [93]. The greater variation in phytase levels exhibited significant positive correlations with enzyme diversity, phosphorus utilization, and micronutrient bioavailability. Further, research is required to construct efficient working enzymes with preferred characteristics [91]. Improving wheat phytases helps to enhance the nutritional quality of the grain, increase consumer's acceptance and lower the risk of allergy [92]. Transgenic approaches have proven vital for eliminating the inhibitors of Fe uptake, absorption, and availability.

Conventional plant breeding approaches alone cannot help to realize the recommended targets of Fe and Zn in cereal crops due to the presence of a negative correlation (linkage drag) between grain yield and Fe and Zn, as evident in polished rice and wheat [93]. The transgenic approach is the only way forward when conventional breeding is not helpful or becomes ineffective, e.g., improvement in grain Se content in wheat [94]. For example, rice leaves have an entire functional pathway for β-carotene synthesis, but it is non-functional in the grain. Conventional breeding failed to switch on the pathway but the transgenic approach helped to turn on the pathway by the addition of two key genes, plant phytoene syntheses and bacterial phytoene desaturase. Pearl millet and bean varieties with high Fe content have been developed using transgenic approaches [95]. Moreover, genetic engineering has advanced to more precision via genome editing approaches such as CRISPR-cas9 (clustered regularly interspaced short palindromic repeats—CRISPR-associated) [96,97]. These advanced genome editing approaches can be used for targeted gene editing of multiple genes without altering any other part of the genome to develop multi-nutrient wheat. Various genetic engineering models are available to improve micronutrients [98]. Endosperm-specific expression of soybean ferritin, *TaFer1* and *TaFer2* in wheat, enhanced the grain Fe content by 1.5–1.9 and 1.1–1.6 times, respectively [92]. Besides, enhancing phytase activity (*phyA*) significantly reduced phytic acid in seeds [99]. Fe and Zn are also significantly accumulated in wheat grain from the root, soil, and different plant parts through increasing transportation by expression (rice *OsNAS2*, bean *PvFERRITIN*, and *TaVIT2* genes) in wheat [65,99]. The manipulation of functional transporter genes like *ABCC13* in wheat can reduce phytic acid synthesis [100]. In recent studies [101], RNA-interference was used to prove the role of *inositol pentakisphosphate kinase* (*TaIPK1*) in lowering the phytic acid synthesis and increasing the Fe and Zn content in wheat grain. A list of various approaches for transgenic-based biofortification in wheat is reported in Table 2. Thus, it can be concluded that transgenic and genome editing approaches are vital for improving the grain Fe and Zn in wheat.

Table 2. Transgenic approaches for Fe and Zn biofortification of wheat.

Approaches/ Introduced Genes/Promoter	Cultivar/Species	Fold Increase in Fe and Zn Concentration Compared to Non-Transgenic Wheat	Remarks	Ref.
Approach 1: Improving Translocation of Fe and Zn from Root to Plant Parts				
Rice *NICOTIANAMINE SYNTHASE 2* (*OsNAS2*)	*T. aestivum* L.	Fe 2.1-fold (grains) and 2.5-fold (flour) Zn 3.7-fold (grains) and 4.2-fold (flour)	HarvestPlus recommended target level of 30% dietary estimated average requirement (EAR) for Fe, and 40% of EAR for Zn, with lines containing 93.1 µg/g of Fe and 140.6 µg/g of Zn in the grains.	[65]
Bean *FERRITIN* (*PvFERRITIN*)	*T. aestivum* L.	Fe 1.6-fold (grains) and 1.7-fold (flour) Zn 1.7-fold (grains) and 1.5-fold (flour)		
Approach 2: Holistic Approaches by Multiple Gene Family				
Both *PvFERRITIN* and *OsNAS2*	*T. aestivum* L.	-	Fe 1.7-fold (whole grains) 1.8-fold (flour)	[65]
Approach 3: Improving Sink Size for Fe and Zn				
GPC-B1	*T. turgidum* ssp. *durum* cultivar Langdon	2-fold Fe and 1.6-fold Zn	NAC transcription factor (NAM-B1) that increase senescence and nutrient remobilization	[77]
Aspergillus japonicus phytase gene (*phy*A)	*T. aestivum* L.	Significant increase	2 to 9-fold higher expression of *phyA*mRNA and 12–76% reduction in phytic acid in seed	[99]
TaFer1, *TaFer2*	Wheat cultivar Bob White	1.5 to 1.9-fold	Placed on chromosome 5 and 4	[102]
Approach 4: Improving Transportation of Fe and Zn from Soil to Different Plant Part				
TaVIT2	*T. aestivum* L.	2-fold Fe in white flour	Stable in the next generation	[103]

7. Conclusions and Future Perspectives

Wheat, being a crop of primary importance for ensuring food security (quantum of grain yield) for global populations remains deficient in Fe and Zn content (quality). However, the last decade has witnessed significant improvement in its quality owing to the availability of molecular markers for mapping major QTLs and research funding and policy support for biofortification. Wild relatives have contributed immensely to the improvement of Fe and Zn content in wheat. Hence, pre-breeding activities should always be an integral part of any breeding program. The moderate to high heritability of Fe and Zn content in wheat provides opportunities for improvement through selection. MAS has proved to be an effective approach for the development of Fe and Zn biofortified varieties through introgression of major QTLs in the background of high yielding varieties. Furthermore, reduction in phytic acid and other inhibitors in wheat will eventually enhance the Fe uptake and bioavailability. Emerging novel technologies such as oligo-directed mutagenesis, RNA-directed DNA methylation, Targeting Induced Local Lesions in Genomes (TILLING) and MutMap (mutant-based genetic map used for identification of QTLs/genes) will help to uncover novel genes or alleles in germplasm. Genome editing approaches such as CRISPR Cas9 system and Zn finger nuclease can be utilized to edit the targeted gene bases or sites for enhancing Fe and Zn content or reduce anti-nutritional factors, as evident from their success in maize [104] and rice [105]. Another promising non-transgenic approach, TILLING can help to detect novel candidate genes in wheat, and thereby can be used to unravel novel alleles for Fe and Zn content. Omics approaches such as proteomics, metabolomics, and ionomics can also provide an in-depth understanding of the molecular basis of the contributory genes for biofortification. The studies on metabolic pathways

in the past revealed the role of ferritin in the metabolic pathways of Fe transportation in isolated endosperm amyloplasts [106]. Furthermore, recent studies [107,108], have highlighted the mechanism for Fe translocation and storage in wheat, which will further open avenues of research in wheat biofortification. The recently discovered speed breeding approach and completion of the whole genome sequence of bread wheat will prove vital in progressing research on wheat biofortification programs, and hence alleviating Fe and Zn malnutrition globally. The formulation of better policies that ensure a better market support price for Fe and Zn biofortified varieties will also prove crucial in their extensive adoption and dissemination.

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