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REVIEW

Biofortification and bioavailability of Zn, Fe and Se in wheat: present status and future prospects

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Abstract

Key message **Knowledge of genetic variation, genetics, physiology/molecular basis and breeding (including biotechnological approaches) for biofortification and bioavailability for Zn, Fe and Se will help in developing nutritionally improved wheat.**

Abstract Biofortification of wheat cultivars for micronutrients is a priority research area for wheat geneticists and breeders. It is known that during breeding of wheat cultivars for productivity and quality, a loss of grain micronutrient contents occurred, leading to decline in nutritional quality of wheat grain. Keeping this in view, major efforts have been made during the last two decades for achieving biofortification and bioavailability of wheat grain for micronutrients including Zn, Fe and Se. The studies conducted so far included evaluation of gene pools for contents of not only grain micronutrients as above, but also for phytic acid (PA) or phytate and phytase, so that, while breeding for the micronutrients, bioavailability is also improved. For this purpose, QTL interval mapping and GWAS were carried out to identify QTLs/genes and associated markers that were subsequently used for marker-assisted selection (MAS) during breeding for biofortification. Studies have also been conducted to understand the physiology and molecular basis of biofortification, which also allowed identification of genes for uptake, transport and storage of micronutrients. Transgenics using transgenes have also been produced. The breeding efforts led to the development of at least a dozen cultivars with improved contents of grain micronutrients, although land area occupied by these biofortified cultivars is still marginal. In this review, the available information on different aspects of biofortification and bioavailability of micronutrients including Zn, Fe and Se in wheat has been reviewed for the benefit of those, who plan to start work or already conducting research in this area.

Keywords Nutritional quality · Micronutrients (Zn, Fe, Se) · Biofortification/bioavailability · QTLs/genes · Breeding · MAS · Transgenics · Wheat

Introduction

Wheat is one of the most widely grown crops globally and provides ~ 20% of calories and ~ 40% of protein worldwide; in developing countries, wheat also provides 60% of daily energy intake (Wang et al. 2011). The crop has witnessed a significant progress in the improvement of productivity and production during the last 50 years. This increase in production is generally attributed to two green revolutions: the first during 1960s and the second during 1980s (Evenson and Gollin 2003; Pingali 2012; Yadav et al. 2019). Consequently, we are in a fairly comfortable situation to meet the demand and consumption of wheat, as evident from a record global wheat production of 759 mt in 2019 and a forecast of 777 mt for the year 2020 showing an increase of about 34 mt in 2019 over the production level of 2018 (735 mt; most data based on estimates by FAO; http://www.fao.org/faostat/en/#home). However, during the period of growth in grain production, improvement in nutritional quality of wheat grain has not received the desired attention; rather, it has suffered at the hands of plant breeders, because the micronutrients level in the grain of our improved cultivars is low relative to that in the land races, that were used for breeding some 100 years ago; in the published literature, this has been described as dilution effect (Murphy et al. 2008; Khoshgoftarmanesh

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et al. 2010; Amiri et al. 2015). However, in recent years (last two decades), some concern has been witnessed, and several major projects have been initiated to address the problem of malnutrition (https://sustainabledevelopment.un.org/partnership/?p=1490). An important area for improvement in nutritional quality is biofortification, which includes the contents and concentrations of grain micronutrients including Zn (zinc), Fe (iron) and Se (selenium).

It has been reported that malnutrition due to deficiency for micronutrients (particularly Zn and Fe) affects a very large proportion of the world's population. According to estimates by WHO (World Health Organization), globally > 2 billion people suffer with deficiencies for Zn/Fe and at least one billion people suffer with deficiency for Se (https://www.who.int/nutrition/topics/ida/en/; Lyons et al. 2005a; Alina et al. 2019). This has resulted in overall poor health including problems like anaemia, increased morbidity and mortality rates, and low worker productivity among those affected with deficiency of micronutrients (Hotz and Brown 2004; Welch and Graham 2004; Bouis 2007; Cakmak 2008; Salim-Ur-Rehman et al. 2010). In developing countries, where major fraction of the population relies on cereal grain as their staple food, malnutrition due to deficiency for micronutrients has been particularly high among children; the phenomenon has been described as 'hidden hunger' (Stein and Qaim 2007; Harding et al. 2018; Godecke et al. 2018).

In 2017, nearly 69% children in Asia and 27% children in Africa under the age of five suffered from malnutrition and/or disease (https://www.who.int/nutgrowthdb/2018-jme-brochure.pdf). Thus, micronutrient deficiencies are widespread in countries with low and middle per capita income; the countries with high per capita income from Europe and North America also suffer from a different kind of 'hidden hunger' due to insufficient intake of mineral, vitamins and iodine (https://foodsustainability.eiu.com/unmasking-hidden-hunger-in-the-developed-world/). There are also reports of intake of excess micronutrients in some regions of the developed countries, so that efforts have been made to address the twin problems of nutrition, namely the intake of micronutrients below the Estimated Average Requirement (EAR) and intake of above the tolerable Upper-Intake Level (UL); this subject has been discussed in some detail in a review by Bruins (2015). The micronutrients for which there is a major concern include Zn, Fe and Se because their deficiencies in diet lead to retarded growth and adverse effects not only on the immune system but also on individual's cognitive abilities. In order to address this problem of the so-called hidden hunger, biofortification is believed to be one solution aimed at reducing the incidence of micronutrient deficiencies.

The problem of biofortification can be addressed either through agronomic practices or through conventional methods of genetic manipulation. The agronomic practices involve correction of soil micronutrient deficiency through application of soil fertilizers or correction of uptake and transport through foliar spray. The genetic problem, on the other hand, involves identification of genes and their deployment for developing biofortified crops (Dhaliwal et al. 2019). The agronomic practices are neither economical nor environment-friendly, since only 20% of the applied Zn is available for plant uptake, while the remainder gets adsorbed on soil particles and is therefore rendered immobile. Therefore, improvement of cereals through genetic improvement for Zn, Fe and Se contents in the grain have been considered to be the most effective approach. In this connection, marker-assisted selection (MAS) and other marker-aided approaches like marker-assisted recurrent selection (MARS) and genomic selection (GS) provide cost-effective approaches for the selection of desirable plants in segregation populations in breeding programmes.

In order to breed cereals like wheat for biofortification using MAS, it is important to have information about the genomic regions, which control grain Zn, Fe and Se content/concentration. Keeping this in view, genetic studies in wheat have been conducted, which allowed identification of a large number of QTLs/genes affecting the contents of these micronutrients. However, it has been recognized that genetic variability for micronutrient contents in wheat germplasm is limited and is not sufficient to bring about the desired improvement in wheat grain. Keeping this in view, efforts have also been made to use secondary and tertiary gene pools involving use of synthetic hexaploid wheats (SHWs) and alien species of wheat for bringing about the desired improvement in the contents of micronutrients.

The work on genetics of micronutrients within the wheat germplasm has been regularly reviewed (White and Broadley 2005; Prasad 2010; Ludwig and Slamet-Loedin 2019; Neeraja et al. 2017; Khan et al. 2017; Riaz et al. 2017; Bouis and Saltzman 2017; Das et al. 2019; Kumar et al. 2019b; Devi et al. 2019; Saini et al. 2020). An edited volume entitled '*Biofortification of Food Crops*' has also become available, but in this volume also, all aspects have not been covered in a single chapter (Singh et al. 2016). It will be desirable to have all available relevant information at one place. The present review has been written to fulfil this need, so that future workers will have all the information at one place. The subject of agronomic biofortification will not be covered in this review, and among micronutrients, only Zn, Fe and Se will be included. The literature on bioavailability involving reduction of phytic acid (PA) or increase in phytase content for improved bioavailability will also be covered. The review consists of four major sections: the first section deals with available information and future need for additional studies on genetic variation, the second section deals with available information on genetic analysis involving identification of QTLs/genes for Zn, Fe and Se

contents using IM and GWAS (along with studies on use of associated markers for MAS and MARS), the third section deals with physiology and molecular basis of uptake, transport and storage of micronutrients including Zn, Fe and Se and the fourth section deals with breeding approaches for biofortification and bioavailability including conventional approaches, which need to be supplemented with marker-based newer approaches.

Biofortification for Zn, Fe and Se

Genetic variation for biofortification traits (*primary, secondary and tertiary gene pools*)

For any breeding program aimed at biofortification, the breeder should have germplasm that is rich in grain micronutrients. Therefore, efforts have been made to study the available genetic variation for the targeted micronutrients (Zn, Fe and Se in the present case). A number of studies have been conducted, where genetic variation was examined for micronutrients (particularly in the grain) including Zn, Fe and Se in primary, secondary and tertiary gene pools. The primary gene pool includes bread wheat cultivars, land races and related species, which have common genomes and sub-genomes [e.g. *T. aestivum* with AABBDD, *T. turgidum* AABB, *Ae. tauschii* with DD]. These species on crossing will produce fertile hybrids with chromosome pairing, so that transfer of genes is easy through meiotic recombination. The secondary gene pool includes species, which have at least one sub-genome common, and can be used for crossing with species of the primary gene pool, producing fertile or semi-sterile hybrids, so that transfer of genes can take place, but with some difficulty (e.g. *T. timopheevii*, *T. zhukovskii*, *Ae. speltoides*). The tertiary gene pool includes alien species, which have no common sub-genomes and can be used for crossing with members of primary and secondary gene pools only with the help of techniques like embryo rescue, and produce sterile hybrids, so that transfer of genes from tertiary gene pool is not possible through normal meiotic pairing, and special techniques like irradiation, induced meiotic pairing or genome editing are utilized (e.g. *Secale cereale* and several species of the genera like *Aegilops*, *Thinopyrum*, *Elymus,* etc.).

It has been demonstrated that a relatively high level of micronutrients is available in land races, SHWs and alien species, relative to that in high-yielding wheat cultivars. Some details of the genetic variation recorded in the different gene pools during the last two decades will be presented in this section. Since many more studies are available on genetic variation for Zn and Fe, these will be described first, which will be followed by an account of genetic variation for Se.

Genetic variation for Zn and Fe contents

Naturally occurring genetic variation for Zn and Fe to be used for biofortification of wheat cultivars has been studied in the following available resources of plant material: (i) wheat cultivars and land races (primary gene pool); (ii) synthetic hexaploid wheats (SHWs) and progenitors of hexaploid wheat (primary and secondary gene pools); (iii) alien species, which constitute the tertiary gene pool; and (iv) amphiploids, each carrying an entire genone of an alien species and alien addition/substitution lines, each carrying an individual alien chromosome. The genetic variation available and reported in these resources will be discussed separately. In the published literature, the grain Zn and Fe have been expressed as content (mg/kg dry mass) or concentration (ppm), respectively. For the purpose of uniformity, wherever necessary, we converted the available values of concentration (expressed in ppm) into contents (expressed as mg/kg).

Variability in wheat germplasm (including landraces)

During the last two decades, a number of studies have been conducted to examine the variability for grain Zn and Fe contents in wheat varieties and the landraces (Cakmak et al. 2000; Ortiz-Monasterio and Graham 2000; Ortiz-Monasterio et al. 2007; Morgounov et al. 2007; Ficco et al. 2009; Rawat et al. 2009a, b; Zhang et al. 2014; Amiri et al. 2015; Heidari et al. 2016; Goel et al. 2018). These studies suggested a wide range of variation for these micronutrients in cultivars and the landraces (Fig. 1). However, relative to cultivars, the landraces were found to be rich in both Zn and Fe, with levels as high as 87.29 mg/kg Zn (up to 53.3 mg/kg in wheat cultivars) and up to 122.20 mg/kg Fe (up to 56.5 mg/kg in wheat cultivars). This knowledge base vindicated the

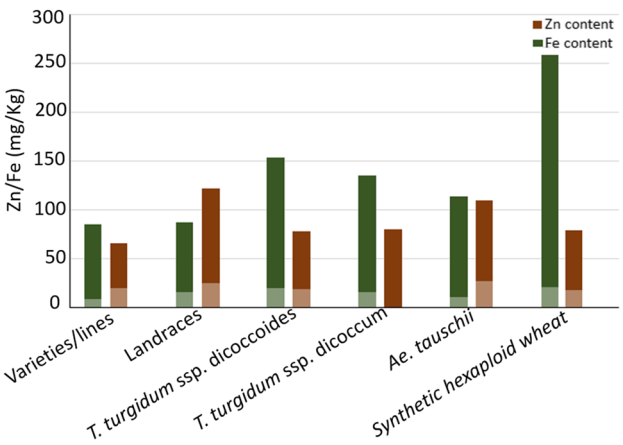

Fig. 1 Zn/Fe contents in grain of wheat and its related species reported in the literature. In each bar, the light colour indicates the minimum value of Zn/Fe content and the entire bar with light and dark colour together indicate the maximum value of Zn/Fe content

utility of landraces in micronutrient biofortification programmes (Graham et al. 1999).

It was also shown that during breeding for high-yielding semi-dwarf wheat cultivars following the green revolutions of 1960s and 1980s, the levels of grain Zn and Fe suffered a decline at the cost of higher grain yield. In a study involving the analysis of genetic diversity in 80 wheat genotypes, it was concluded that during the last 70 years, Zn and Fe contents were reduced by 0.13 mg/kg/year in absolute terms and 0.3% reduction/year in relative terms (Ortiz-Monasterio and Graham 2000; Amiri et al. 2015). This decline, popularly termed as 'dilution effect', has been attributed to the accumulation of disproportionately more starch in the endosperm as a result of breeding for higher yield. Therefore, generally, the high-yielding lines have low levels of Zn and Fe and the lines with high levels of these micronutrients have low yield (Ortiz-Monasterio et al. 2007). A brief account of overall variation for grain Zn and Fe levels reported in different studies will be presented in this section, although a part of this variation may be due to environment and G x E interactions.

In a study involving analysis of micronutrients and GPC (grain protein content) in 50 landraces and 10 cultivars, it was reported that landraces had higher contents of Fe^{2+} (24.93 to 66.51 mg/kg dry weight) and Zn^{2+} (18.68 to 38.66 mg/kg dry weight) relative to those in cultivars (Heidari et al. 2016). In another study involving 269 wheat landraces from Afghanistan were found to have much wider range of variation for the contents of Zn (15.56 to 87.29 mg/kg dry weight) and Fe (55.14 to 122.2 mg/kg dry weight) relative to those reported for 132 cultivars screened at CIMMYT (Zn, 25.2 to 53.3 mg/kg; Fe, 28.8 to 56.5 mg/kg; Graham et al. 1999). A representative set of landraces from Mexico and Iran were also tested under Zn-enriched soil conditions in Obregon, Mexico, which showed more than twofold variation for Zn (40–96 mg/kg) and Fe (27–56 mg/kg).

Genetic variation in progenitors of hexaploid wheats and in SHWs

Wide range of variation for Zn and Fe was also observed in diploid (2*x*) and tetraploid (4*x*) progenitors of hexaploid wheat and also in SHWs. These included the wild and domesticated tetraploid wheats (*T. turgidum* ssp. durum and *T. turgidum* ssp. dicoccoides), the D sub-genome progenitor, *Ae. tauschii* and SHWs developed at CIMMYT. It is apparent that the maximum values of Zn as well as Fe in progenitors and SHWs were far greater than those in the wheat cultivars (Cakmak et al. 2000; Ortiz-Monasterio and Graham 2000; Rawat et al. 2009a, b; Chatzav et al. 2010; Chhuneja et al. 2006; Zhang et al. 2014; Arora et al. 2019). From data presented in Fig. 1, it is also apparent that the development of SHWs was quite rewarding not only in terms of the study of the genetics of Zn and Fe levels in wheat germplasm, but also for their use in breeding biofortified wheat cultivars (Calderini and Ortiz-Monasterio 2003; Gorafi et al. 2016; Velu et al. 2018; Morgounov et al. 2007; Murphy et al. 2008; Guttieri et al. 2015 Velu et al. 2011, 2014; Singh and Velu 2017). Therefore, the landraces, the immediate progenitors (2*x* and 4*x*) of bread wheat and the SHWs constitute valuable genetic resources for developing biofortified wheat varieties. Under the HarvestPlus programme at CIMMYT also, the SHWs were extensively used in breeding for high-yielding biofortified wheat lines; some of these improved lines were eventually released as biofortified cultivars in different countries (Velu et al. 2012, 2014; for more details, see later).

Genetic variation in alien species from tertiary gene pool

A number of alien species of wheat including some wild species from the tribe Triticeae have also been examined for genetic variation for Zn, Fe and Se contents (Tiwari et al. 2010; Rawat et al. 2009b; Wang et al. 2011). Estimations of Zn and Fe in alien species suggested that the grain of wild relatives of wheat has up to 2–3 times Zn and Fe relative to that in modern hexaploid wheat cultivars. These alien species belonging to the tertiary gene pool and examined for Zn and Fe contents mainly included the following species and were utilized for biofortification of bread wheat: *Ae. searsii*, *Ae. umbellulata*, *Ae. caudata*, *Ae. geniculata*, *Ae. longissima, Ae. peregrina* and *Ae. kotschyi* (Cakmak et al. 2000; Chhuneja et al. 2006; Ortiz-Monasterio et al. 2007; Rawat et al. 2009b; Neelam et al. 2011; Wang et al. 2011).

In an initial study in India, HS Dhaliwal and his group evaluated 80 accessions of wheat and alien species for grain Zn and Fe contents. It was observed that the related wild species with S, U and M genomes of wheat had up to threefold–fourfold higher Zn and Fe contents relative to bread wheat and durum wheat (Fig. 2). In particular, two accessions of *Ae. kotschyi* had more than 60% higher Zn and 75% higher Fe over the wheat cultivars that were used as controls (Rawat et al. 2009b).

Amphiploids and alien addition/substitution lines. It is known that extensive backcrossing is required for direct transfer of genes from alien species to wheat. This can be avoided by utilizing synthetic amphiploids and alien addition/substitution lines (Rawat et al. 2011; Tiwari et al. 2010). Therefore, a number of species were utilized for the development of amphiploids, followed by development of alien addition/substitution lines. In an initial study conducted by HS Dhaliwal and his group, decaploid amphiploids [6x wheat (AABBDD) + 4x *Ae kotschyi* (UUS^lS^l) = $AABBDDUUS^lS^l$] were found to have seeds as large as those of wheat cultivars. These amphiploids had higher Zn and Fe contents not only in the grain, but also in flag leaf and grain ash relative to

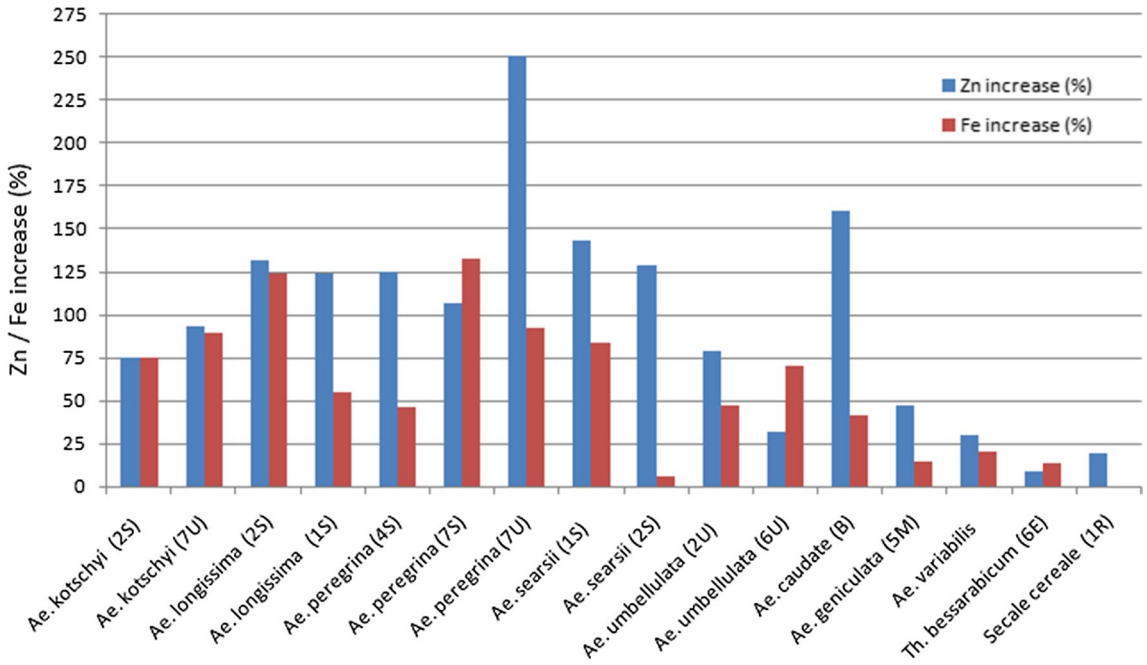

Fig. 2 Percent increase of Zn and Fe contents in different *Aegilops* species over wheat cv. Chinese Spring (CS) or any other wheat cultivar used in a study (associated chromosomes are shown in parentheses with the name of species) (for references, see Gupta et al. 2020)

those in the parent alien species *Ae. kotschyi*. This suggested that *Ae. kotschyi* carried a genetic system for micronutrient uptake, translocation and sequestration that is distinct from that in wheat cultivars (Rawat et al. 2009a). In another study, *Ae. longissima* (S^lS^l) and *T. turgidum* (AABB) were crossed for developing amphiploids, $AABBS^lS^l$. These amphiploids also carried bold seed associated with high Zn and Fe content, associated with higher grain ash content relative to durum wheat cultivars that were involved in the constitution of the amphiploids. This suggested that *Ae. longissima* also carried a better genetic system(s) for uptake and sequestration of Zn and Fe. Efforts were also made to transfer these features of *Ae. longissima* to elite durum and bread wheat cultivars (Tiwari et al. 2008).

The above amphiploids were later utilized for production of alien addition and substitution lines in a systematic manner, although these alien addition/substitution lines could also be derived by other interventions. In one such study, 47 alien chromosome addition lines derived from six species of *Aegilops* were found to have significantly higher grain Zn and Fe contents, relative to those in wheat cv. Chinese Spring (CS). Alien addition lines, each with following individual alien chromosomes, had 50% to 248% higher Zn and Fe contents in the grain relative to CS: (i) 2U and 6U of *Ae. umbellulata*, (ii) $1S^l$ and $2S^l$ of *Ae. longissima*, (iii) $1S^s$ and $2S^s$ of *Ae. searsii*, (iv) $4S^P$ of *Ae. peregrina*, (v) chromosome B of *Ae. caudata* and (vi) 5 M^g of *Ae. geniculata*. There are also other studies which suggested that wheat-*Aegilops* alien addition lines offered great potential for biofortification of wheat grain to enhance the contents of Zn and Fe (Wang et al. 2011).

Genetic variation in *T. timopheevii*

Among other species of *Triticum,* which are not the progenitors of hexaploid wheat, *T. timopheevii* is also a good source of grain Zn and Fe. In a study conducted using 12 accessions of *T. timopheevii* (Zhuk.) Zhuk. ssp. *timopheevii* (AAGG), it was observed that the grains of *T. timopheevii* had significantly higher content of Fe and Zn relative to those in hexaploid cv. Chinese Spring (CS) and tetraploid cv. Langdon (LDN), which were used as controls. The average genetic variabiltiy among *T. timopheevii* ssp. *timopheevii* accessions for Zn content ranged from 30.05 to 65.91 mg/kg, while that for Fe content ranged from 47.06 to 90.26 mg/kg. The contents of the two micronutrients could not be explained by either the seed size/weight or the presence of the gene *NAM-G1* (a domestication gene for protein and micronutrient content), implying that there must be some other genetic factors controlling Zn and Fe contents in the grain. These results thus demonstrate that *T. timopheevii* ssp. *timopheevii* might also be a promising genetic resource for biofortification of popular high-yielding wheat cultivars.

Genetic variation for selenium (Se) content

Selenium (Se) is also a nutritionally important micronutrient, and according to some estimates, more than one

billion people worldwide suffer with Se deficiency (Lyons et al. 2003). Besides other roles, Se is particularly known for its protective role against oxidative stress (Brenneisen et al. 2005). A dietary intake of < 40 µg Se day^{-1} is insufficient for human needs, whereas > 400 µg Se day^{-1} can be toxic (Kabata-Pendias and Mukherjee 2007; Fordyce 2013). According to USDA, the need for daily Se intake for humans is 55 to 200 µg/day/person (Wu et al. 2015). Among developed countries like USA and Australia, although no deficiency for Se has been noticed, the daily intake has gone down in recent years (after 1970s), as shown in a report from South Australia (Lyons et al. 2003, 2005a). In developing countries in Asia and Africa also, the daily intake is much below the recommended dose, thus causing deficiency of this element associated with severe and adverse effects on general human health. Several diseases caused due to Se deficiency include the following (Boldrin et al. 2018): (i) some cardiovascular diseases; (ii) Keshan disease involving development of abnormal heart muscles leading to premature death of children in China and Brazil; (iii) Kashin-Beck disease which is a joint and bone disease; (iv) Myxedematous endemic cretinism causing intellectual disability; (v) gastrointestinal disorders leading to Crohn's disease; and (vi) selenosis disease involving hair loss, nail deformation, mild nerve damage, etc.

It has also been shown that an increase in yield potential in wheat during the last few decades was accompanied with a decrease in grain Se also (Garvin et al. 2006), which means that there is a need for breeding wheat cultivars with improved Se. Efforts were also made to study the genetic variation for grain Se, although these efforts are not comparable to the efforts made for the study of genetic variation for Zn and Fe contents. Genetic variation in grain Se content has been reported for several cereals including bread wheat (Garvin et al. 2006; Murphy et al. 2008; Rodríguez et al. 2011; Pu et al. 2014; Souza et al. 2014; Lyons et al. 2005b) and durum wheat (Rodríguez et al. 2011; Yang et al. 2013). However, relative to Zn and Fe, limited germplasm has been screened for Se. Variation also occurs in the content of Se and its different chemical forms, which are absorbed and metabolized differently. The content of Se in different food sources varies from 4.4 mg/kg (0.00044%) fresh weight in biofortified wheat flour to 8.3 mg/kg (0.00083%) in biofortified wheat biscuits and 8.5 mg/kg (0.00085%) in unfortified wheat. This Se is available in different forms including SeMet (76–85% in biofortified wheat) and SeMet selenoxide. Thus, it is apparent that Se content in wheat grain is relatively very low relative to the contents of Zn and Fe. (Perhaps Se is also required for human body in a relatively very low concentration, relative to Zn and Fe.) In many cases, this variation was actually attributed to weather conditions, crop husbandry and selenium fertilization (Eurola et al. 2004; Lyons et al. 2005b; Garvin et al. 2006). For example, in a study, same wheat genotypes when grown in North America were tenfold richer in Se relative to those grown in Europe (for a review, see Hawkesford and Zhao 2007). Even within USA, variation in grain Se for the same 14 hard red wheats at two locations differed widely, being in the range of 0.20–0.48 µg/kg (mean 0.36 µg/kg) at one location and 0.030–0.055 µg/kg (mean 0.045 µg/kg) at another location (Garvin et al. 2006).

In a major study conducted in Australia in collaboration with CIMMYT (Australia and Mexico), grain Se content was examined in bread wheats, landraces, cultivars, pre-breeding and advanced lines, SHWs, hybrids, emmer wheats and *Ae. tauschii*. In this study, the grain Se content had a wide range (5 µg/kg to 720 µg/kg), although much of this variation was correlated with spatial variation in soil Se; only a part of this variation was found to be genetic in nature (Lyons et al. 2005b). The genetic variation for Se in *Ae. tauschii* was found to be 42% higher (173.0–360 µg/kg) (in rye it was 35% higher), relative to the values for SHWs (116.5 µg/kg-206.4 µg/kg) and tetraploid wheats (114.7–349.6 µg/kg). Higher Se in *Ae. tauschii* was also reported in another study conducted by Zhao et al. (2017). Similarly, higher Se was also reported in a solitary accession of SHW (80.0 µg/kg) relative to three bread wheat lines (30.0–50.0 µg/kg) (Pu et al. 2014). Grain Se in spelt wheat (19.0–58.0 µg/kg; mean 39.0 µg/kg) was also found to be relatively higher than that in emmer wheats (18–35 µg/kg; mean 28 µg/kg) (Piergiovanni et al. 1997). Contrary to expectation, the richness of Se in *Ae. tauschii* was not reflected in corresponding SHWs that were utilized for biofortification of wheat involving Zn and Fe. The reported richness of Se in *Ae. tauschii* can certainly be exploited for biofortification. If, in some genotypes, high Se occurs in association with high Zn and high Fe, such genotypes of *Ae. tauschii* may be the most desirable genetic resource for biofortification. Even if high Se and high Zn and Fe occur in different *Ae. tauschii* genotypes, this variation could be combined into a single wheat genotype for biofortification using a suitable breeding strategy.

Induced mutations for novel genetic variation (Zn, Fe, Se)

In the absence of genetic variability for a trait in the available germplasm, mutagenesis is always a powerful strategy to create and broaden the genetic variation. Mutagenesis is especially valuable for inducing novel genetic variation in any crop for traits that have limited natural genetic variability (Parry et al. 2009). It has already been used for improvement of a variety of traits including yield and tolerance to biotic and abiotic stresses in a number of crops, as evident from the fact that globally 3320 cultivars have so far been released through the use of induced mutations involving 214 plant species (see FAO/IAEA Mutant Variety Database;

https://mvd.iaea.org/). However, for improvement of nutritional quality of grain in wheat and other cereals, there are very few reports of mutants with improved contents of grain Zn, Fe or Se.

In order to broaden the genetic variation for micronutrients, a major activity was witnessed at Al-Farabi Kazakh National University, Almaty, Kazakhstan, in collaboration with institutions from some other countries. They published the results of three major studies during 2017–2019, where improvement in grain micronutrients like Ca, Zn and Fe and the bioavailability factor phytic acid (PA) were reported (Kenzhebayeva et al. 2017, 2018, 2019). In all the three studies, promising stable mutants were obtained for improving grain parameters such as grain area, length, width as well as quality. The data presented showed how the genetic variation for micronutrients can be generated and examined for its association with other important grain attributes.

The first study of the above three multi-institutional studies was conducted jointly by the following institutions from different countries: (i) John Innes Centre, Norwich (UK), (ii) FAO/IAEA Division, Vienna (Austria) and (iii) Tel Aviv University, Tel Aviv (Israel). In this study, the seed of spring wheat cv. Almaken was irradiated with different doses of gamma rays, and M_5 population was screened for mutants. Some of the mutant lines, obtained due to an irradiation dose of 200-Gy, had 2–4 times higher grain Fe and Zn and 7–11% higher GPC relative to the parent line.

In the second study conducted with Zhejiang University (China), seeds of cv. Eritrospermum-35 were irradiated with 100 and 200 Gy using ^{60}Co, and the mutagenized populations were grown up to M_7 generation with successive selection for high yield in each generation. Selected lines were evaluated for yield and grain quality traits including grain Fe, Zn and PA contents. Some of the mutant lines had 2–3 times more Fe and Zn, lower PA (1.1–3.5 times) and higher GPC (11.2–12.4%) relative to the parent genotype. Some of the M_7 lines exhibited significantly larger grain weight (1.3–1.5 times) and improved number of spikelets per spike (2.0–2.1 times) relative to the parent used for mutagenesis. The GPC, Zn and Fe and grain weight per spike (mainly in 100 Gy-dose lines) had significant positive correlation, while PA had a negative correlation with grain yield. The similarity of nucleotide sequences of cDNA for myo-inositol hexakisphosphate phosphohydrolases (MINPP; synthesized using TaPhyllc primers for PCR) varied from 28.2% and 30.2% in parents to 45.7% and 56.5% in the M_7 lines. The results clearly indicated that variability in grain micronutrients and bioavailability (low PA) in mutant lines constituted a potential resource for improvement of nutritional quality of wheat.

In the third joint program undertaken with Zhejiang University, Zijingang Campus, China, and the Joint FAO/IAEA Division, IAEA. Vienna, Austria, stable M_7 mutant lines were produced by treating seed of spring wheat cv. Zhenis with irradiation dose of 100 or 200 Gy. The mutant lines were screened for contents and bioavailability of nutritionally important micronutrients (Zn, Fe and Ca) and GPC; the range for Zn was 22.2–89.6 mg/kg and that for Fe was 40.9–89.0 mg/kg. The highest values for grain Zn and Fe were higher relative to the values for the parent cv. Zhenis. Some mutant lines (mostly in the 100 Gy-derived germplasm) had more than twofold higher Zn and Fe, associated with desirable low PA concentration (1.4–2.1-fold), and 6.5–7.0% higher GPC relative to the parent.

Genetics of micronutrients

Studies on genetics of grain micronutrients (including Zn, Fe and Se) in cereals have been conducted using two major approaches, which include linkage-based interval mapping (IM) and LD-based GWAS. The results of these studies have also been utilized for identification of candidate genes for micronutrients. Some details of these studies will be briefly described in this section.

QTL interval mapping

A number of QTL interval mapping studies have been conducted exclusively for Zn and Fe. Separate studies were also conducted for Se. Since different mapping populations were used for Zn/Fe and Se, these will be described separately.

QTLs for Zn and Fe

A number of biparental mapping populations have been utilized for interval mapping. While, in most cases, the parents for development of biparental populations were bread wheat cultivars, in some studies, the parents were either SHWs or *Triticum spelta* or both. These studies were conducted in at least eight different countries distributed in Asia, Middle East, Australia, Europe and South America; in many cases, phenotypic data were collected over locations and years to study the variation due to environmental conditions. In two of these studies, 20–25 kg ha^{-}$ZnSO_4$ was applied either at the time of the experiment or in the preceding season as a source of Zn in the soil (Hao et al. 2014; Srinivasa et al. 2014).

The results of interval mapping studies conducted for identification of QTLs for Zn and Fe are summarized in Table 1. Among these studies, an important study was conducted at CIMMYT, Mexico, using three different sets of RILs (Crespo-Herrera et al. 2016, 2017), where a number of QTLs were identified. Among these QTLs, the following two important QTLs deserve attention: (i) *QGZn.cimmyt-7B_1P2* on chromosome 7B, which explained the largest proportion of phenotypic variance (PVE 32.7%) for Zn;

Table 1 Mapping populations and QTLs for grain Zn and Fe contents in bread wheat and related species

S.N.	Mapping population		No. of QTL		References
	Cross	Type (number)	Zn	Fe	
(a)	*Diploid wheat (2x)*				
1.	T. *boeoticum* (pau5088) × *T. monococcum* (pau14087)	RIL (93)	1	2	Tiwari et al. (2009)
(b)	*Tetraploid wheat (4x)*				
2.	Langdon × Accession #G18-16	RIL (152)	6	11	Peleg et al. (2009)
3.	Saricanak98 × MM5/4 (4× wheat)	RIL (105)	8	4	Velu et al. (2017)
(c)	*Hexaploid wheat (6x)*				
4.	W7984 × Opata85	RIL (114)	1	2	Balint et al. (2007)
5.	Hanxuan l0 x Lumai 14	DH (119)	11[1]	4	Shi et al. (2008, 2013)
6.	RAC875-2 × Cascades	DH (90)	4	1	Genc et al. (2009)
7.	Xiaoyan 54 × Jing 411	RIL (182)	2 + 1[2]	2 + 1[2]	Xu et al. (2012)
8.	Tabassi × Taifun	RIL (118)	2	6	Roshanzamir et al. (2013)
9.	Berkut 9 × Krichauff	DH (–)	–	4	Yasmin et al. (2014)
10.	SHW L1 × Chuanmai32	RIL (171)	4	4	Pu et al. (2014)
11.	Chuanmai32 × Chuannong16	RIL (127)	3	4	Pu et al. (2014)
12.	PBW343 × Kenya Swara	RIL (177)	12	–	Hao et al. (2014)
13.	*T. spelta* accession H + 26 (PI348449) × HUW 234	RIL (185)	5	5	Srinivasa et al. (2014)
14.	Berkut 9 × Krichauff	DH (138)	2	1	Tiwari et al. (2016)
15.	Seri M82 × SHW CWI76364	RIL (140)	6	10	Crespo-Herrera et al. (2016)
16.	Adana99 × *T. Sphaerococcum* (70.711)	RIL (127)	10	7	Velu et al. (2017)
17.	WH542 × Synthetic derivative (PI94624)	RIL (286)	7	6	Krishnappa et al. (2017)
18.	Bubo × Turtur	RIL (188)	5	3	Crespo-Herrera et al. (2017)
19.	Louries × Batelur	RIL (188)	11	7	Crespo-Herrera et al. (2017)
20.	Roelfs F 2007 × Chinese Parental Line	RIL (200)	10	9	Liu et al. (2019)
Total number of QTLs			111	93	

Zn = zinc content, Fe = iron content, RIL = recombinant inbred lines, DH = doubled haploid, [1]Zn content + concentration, [2]Pair of QTLs involved in additive x additive epistatic interaction

this QTL was closest to the QTL *QGZn.cimmyt-7B_1P1* identified using another mapping population and (ii) *QGFe.cimmyt-4A_P2* on chromosome 4A with the largest PVE (21.14%) for Fe. Pleiotropic or tightly linked QTLs were also found on chromosome 3B (Crespo-Herrera et al. 2017); these tightly linked/pleiotropic QTLs, however, had minor effects, the PVE ranging from 4.3 to 10.9%.

In order to find common QTLs across different studies involving different mapping populations, we compared the genetic position (in cM) and the physical (Mb) position of the different QTLs. However, common QTLs were not available (results not presented). Therefore, we conducted meta-QTL (MQTL) analysis to find more robust QTL (for both the Zn and Fe contents) and the closely linked markers for use in MAS. As a result, 15 MQTLs representing 148 of the 159 original QTLs reported in 12 earlier studies could be identified (manuscript under preparation). These MQTLs were distributed on five different chromosomes (2D, 5A, 5B, 6A and 7A) and included 5 MQTLs for Zn only, 3 MQTLs for Fe only and 7MQTLs for both Zn and Fe. The confidence intervals (CIs) of the MQTLs were narrow (0.51 cM to 15.75 cM) relative to those for the original QTLs, suggesting that they are more precisely mapped and have closely linked markers. Three of these MQTLs, one on 5A (R^2 value = 12.3%) and two on 7A (R^2 value = 7.4% and 15%), have the potential for use in MAS for improvement of grain Zn and Fe.

QTLs for Se content

Few QTL interval mapping studies were also conducted for identification of QTLs for Se accumulation in tetraploid and hexaploid wheats. The number of QTLs reported in individual studies ranged from 3 to 19 (including those for Zn and Fe), which were distributed on all the 21 chromosomes (Table 2).

GWAS for Zn and Fe

Genome-wide association mapping studies (GWAS) have also been conducted for identification of markers associated with relative abundance of Zn and Fe in grain (Table 3).

Table 2 A summary of the results from studies conducted for identification of QTLs for selenium (Se) content in 4*x* and 6*x* wheats

S.No.	Cross	Mapping population	No. of QTL & chromosomes carrying these QTL	References
(a)	*Tetraploid wheat (4x)*			
1.	LDN × G18-16	RIL (152)	15 (1A, 1B, 2B, 3A, 4B, 5A, 6A, 7A, 7B)	Yang et al. (2013)
(b)	*Hexaploid wheat (6x)*			
2.	SHW-L1 × Chuanmai 32	RIL (171)	39 (1B, 1D, 2A, 2D, 3A, 3B, 3D, 4A, 4B, 4D, 5A, 5B, 6A, 6B, 6D, 7B, 7D)	Pu et al. (2014)
3.	Tianong18 × Limmai6	RIL (184)	16 (1B, 2B, 4B, 5A, 5B, 5D, 6A, 7D),	Wang et al. (2017)

RIL = recombinant inbred line

Table 3 MTAs identified in *Triticum aestivum* and *Ae. tauschii* using GWAS

S No.	Assoc Panel (size)	Location(s)	No. of env.	Zn, Fe analysis	No. of markers	No. of MTAs		References
						Zn	Fe	
1	HPAM Panel (330)	India, Mexico	6	EDXRF	14,273	39	–	Velu et al. (2018)
2	HPAM Panel (330)	Mexico	2	ICP-MS	28,074	72	65	Cu et al. (2020)
3	EuWV Panel (369)	Germany	3	ICP-OES	15,523	40	41	Alomari et al. (2018, 2019)
	Sub-panel (183)		3	ICP-OES	$28{,}710_{(Zn)}$ $44{,}233_{(Fe)}$	161 –	– 137	
4	SWRS (246)	India	2	EDXRF		94	33	Kumar et al. (2018)
5	SHW (Longdon × 47 *Ae tauschii*) (47)	Japan	2	ICP-AES	70 (SSRs)	03	03	Gorafi et al. (2016)
6	SHW (123)	Turkey	2	ICP-MS	35,648	13	03	Bhatta et al. (2018)
7	*Ae tauschii* panel (114)	India	3	ICP-OES	5249	4	5	Arora et al. (2019)

HPAM = Harvest Plus Association Panel; SWRS = Spring Wheat Reference Set; EuWV = European Wheat Varieties; EDXRF = Energy-dispersive X-ray fluorescence spectrometry; ICP-MS = Inductively coupled plasma mass spectrometry; ICP-OES = Inductively coupled plasma optical emission spectrometry; ICP-AES = Inductively coupled plasma atomic emission spectroscopy

These studies were conducted in five different countries located in Asia, Europe and South America. The experiments were either repeated over years or over locations comprising 2–6 environments. In some studies, $ZnSO_4$ was also applied (@25 kg ha^{-1}) as a fertilizer, while growing the genotypes comprising the association mapping panels (Velu et al. 2018; Cu et al. 2020). The phenotyping for Zn and Fe in grains of the association mapping panels was carried out in varying environments, so that the expression of grain Zn and Fe contents might have been influenced by the environmental factors also to varying degrees in the different studies. Also the estimation of the Zn and Fe in grain was conducted using equipments operating on different principles introducing another variable, thus making a comparison of the results from different studies difficult.

In the above GWA studies for Zn and Fe, the association panels differed; the genotypes included SHWs (47 and 123), *Ae tauschii* accessions (114) and wheat cultivars (369). Two most important association panels included the HPAM panel comprising 330 accessions and assembled at CIMMYT and the panel of 369 European wheat varieties assembled and used by Alomari et al. (2018, 2009; see Table 3). The markers used for genotyping ranged from 70 SSRs to > 35,000 SNPs, so that the resolution in different studies differed. Also, due to the use of different markers, it was not possible to identify common MTAs in different studies, although some of the reported MTAs were located on specific positions on individual chromosomes that were earlier reported to carry QTLs for Zn/Fe. Also, no haplotyping was used in these studies, although haplotyping-based GWAS is considered to be more efficient.

Most GWA studies (GWAS) involved use of general linear model (GLM) and mixed linear model (MLM) for single-locus single-trait analysis utilizing TASSEL (Yu et al. 2006) or GAPIT in R (Lipka et al. 2012). However, several improved models have now become available, which are yet to be employed for GWAS involving biofortification traits. For instance, an improved single-locus model named 'Settlement of MLM Under Progressively Exclusive Relationship' (SUPER), proposed by Wang et al. (2014), has become popular. However, all these single-locus models suffer with the problem of 'multiple testing', which leads to many false positives. In order to overcome this problem, often either Bonferroni correction (Holm 1979) or false discovery rate

(FDR) (Benjamini and Hochberg 1995) is used; these corrections following single-locus single-trait analysis, however, make use of overly conservative thresholds, thus resulting in many false negatives. Therefore, these single-locus models have largely been replaced by multi-locus models, including the following: FASTmrMLM, FASTmrEMMA and ISIS EM-BLASSO (Cui et al. 2018). A more popular model used in several studies is the model named 'fixed and random model circulating probability unification' (FarmCPU; Liu et al. 2016). In a recent study carried out by Cu et al. (2020), multi-locus mixed models (MLMM) were utilized, which involved the following two steps using the mrMLM.GUI software. (i) A single-locus GWAS to identify MTAs with low confidence level; (ii) a multiple-locus GWAS involving markers identified in the first step. Most of the available improved multi-locus and multi-trait approaches for GWAS have yet to be utilized for biofortification traits.

Candidate genes involved in biofortification

Efforts have also been made to identify genes (or candidate genes) responsible for high Zn and Fe concentration (Table 3). In the study involving complete panel of 369 genotypes and its sub-panel (Alomari et al. (2018), 201 MTAs (40 on complete panel +161 on sub-panel) for Zn were identified, of which highly significant MTAs were found to be present on chromosomes 5A and 3B (Table 4; Fig. 3). QTLs on these two wheat chromosomes were reported in other studies also (Crespo-Herrera et al. 2017; Peleg et al. 2009). Using bioinformatics, Alomari et al. (2018) also identified the physical regions, which corresponded to the SNPs associated with Zn content: these genomic regions included a region on 3BS (462,763,758 to 468,582,184; Fig. 3) and another region on 5AL (723,504,241 to 723,611,488). These genomic regions were then looked for important genes encoding proteins related to Zn content. This information could then be utilized for identification of putative candidate genes, which included six genes on 3BS and four genes on 5AL (Table 4).

As shown in Table 4, five of the six genes on 3BS were found to belong to MAPK (mitogen-activated protein kinase) family of genes involved in kinase activity leading to protein phosphorylation that will help performing the desired molecular function in biological processes. It is well documented that Zn uptake and transport make use of MAPKs through their involvement in a number of signalling pathways. The sixth gene (mRNA32.1) encodes SWAP/surp domain (SWAP = Suppressor of White Apricot) containing protein, which has been shown to be associated with Zn concentration in seed of chickpea (Upadhyaya et al. 2016). The protein is a RNA-binding protein involved in the biological process involving RNA processing. The four genes on chromosome arm 5AL (descriptions shown in Table 4) encode proteins named TaMTPs, which include TFs belonging to bZIP family, and FAR1 protein, which are known to be directly or indirectly involved in Zn biofortification (see Alomari et al. 2018 for details of relevant references).

The details of the four candidate genes on 5AL are also available in Table 4. The major functions of these genes include DNA binding (transcription factor), Zn/Fe binding and protein dimerization. The rice chromosome 12, which is syntenous with wheat chromosome 5A (Salse et al. 2009),

Table 4 Putative candidate genes for Zn grain content in wheat (modified from Alomari et al. 2018)

Gene number	Description	GO ID	GO term	GO category
1. *Chromosome arm 3BS (six genes)*				
mRNA_2.1	Mitogen-activated protein kinase kinase kinase (MAPKKK)	GO:0004672	Protein kinase activity, protein phosphorylation, protein transport	Molecular function, biological process
mRNA_3.1		GO:0006468		
mRNA_10.1		GO:0015031		
mRNA_23.1				
mRNA_24.1				
mRNA_32.1	SWAP*/surp*	GO:0003723	RNA binding	Molecular function
2. *Chromosome arm 5AL (four genes)*				
mRNA_11.1	Homeobox-leucine zipper protein HOX4	GO:0003677/ GO:0003700	DNA binding/DNA binding transcription factor activity	Molecular function
mRNA_34.1	Protein FRS11#)#	GO:0008270	Zinc ion binding	
mRNA_42.1	BZIP protein	GO:0003700	DNA-binding TF activity	
mRNA_44.1	Transcription factor bHLH76	GO:0046983	Protein dimerization activity	

*SWAP/surp = Suppressor of White Apricot/surp domain containing protein; #FRS11 = FAR1-Related SEQUENCE11

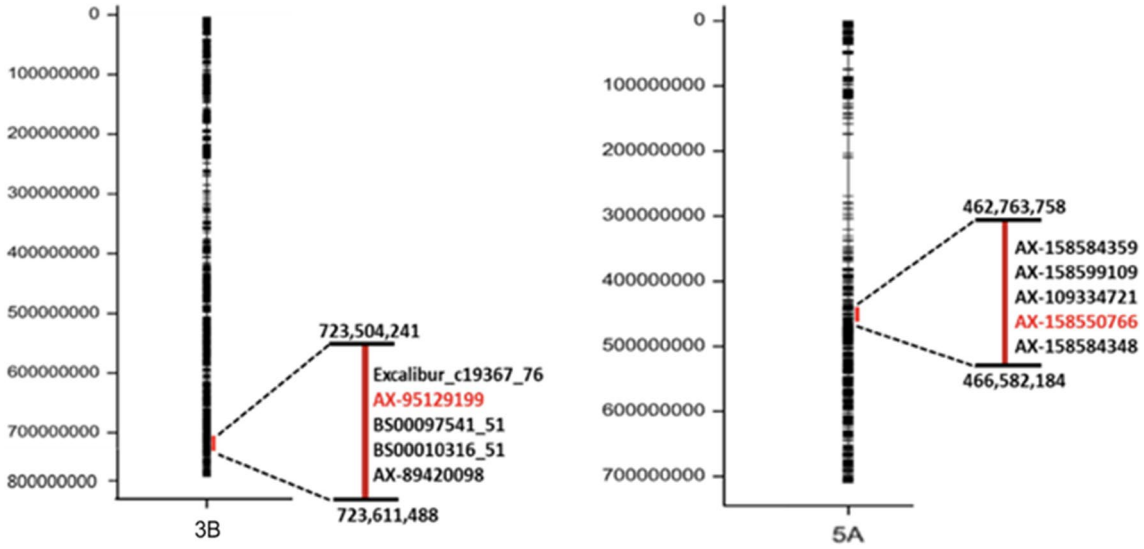

Fig. 3 Alignment of the significant SNP markers (in black) to chromosome 3BS and 5AL. The most significant SNP (in red) with –log (*P*) value equalling 5.84. (Alomari et al. 2018)

has also been reported to carry many QTLs for Zn (Swamy et al. 2016) on corresponding genomic regions. This suggests that the genomic regions on chromosome arms 5AL and 3BS of wheat-carrying genes for Zn may be found in other cereals also and are therefore suitable candidates for further molecular genetic studies.

Physiology and molecular basis of uptake of Zn, Fe and Se (including specific genes and proteins involved)

A complete understanding of the physiology and molecular basis of uptake, transport and storage of Zn, Fe and Se is a pre-requisite for biofortification in different crops including wheat. The uptake includes acquisition of these micronutrients from the rhizosphere, followed by their radial transfer to the xylem in the central cylinder of the root. From the root xylem, the micronutrients are transported to the shoot followed by their transfer to the grain during grain filling period (Zhao and McGrath 2009; Carvalho and Vasconcelos 2013).

A number of classical genes, which play each an important role in biofortification, have been characterized in cereals (e.g. rice, barley, wheat and maize) and in the model dicot species, *Arabidopsis thaliana*. However, all these genes have not been characterized in wheat, so that the genes from cereals like rice, barley and maize and those discovered in Arabidopsis are also being used for identification and characterization of corresponding genes in wheat using in silico approaches. Once characterized in wheat, these other genes can also be utilized either for (i) development of gene-based functional markers, (ii) production of transgenics or (iii) gene/base editing (these are some novel approaches, but have not been fully utilized so far for biofortification, particularly in wheat).

Different aspects of physiology and molecular basis of biofortification, including uptake, translocation and storage of micronutrients, are largely depicted in Fig. 4 and relevant information is summarized in Tables 5, 6 and 7. Since physiological processes and the corresponding genes for biofortification of Zn/Fe in cereals differ from those involved in biofortification of Se, we will first discuss these processes and the corresponding genes for Zn and Fe. This will be followed by a brief account of similar information for Se content in wheat grain.

Uptake, transport and storage of Zn and Fe

The mechanism of Zn/Fe acquisition has been shown to follow one of the following two strategies (this classification in two strategies is mainly based on work done on Fe, although holds good for Zn also): Strategy I (also described as 'reduction-based strategy') and Strategy II (also described as 'chelation-based strategy'). Strategy I involves reduction of insoluble ferric (F^{3+}) into soluble ferrous (Fe^{2+}) compounds through ferric chelate reductase activity (PRO2/FRO) in the root plasma membrane, followed by uptake of Fe^{2+} ions or compounds across the root plasma membrane. Strategy II involves synthesis and use of phytosiderophores (PS), namely nicotianamine = NA; mugineic acid = MA or avenic acid (AA) (siderophore = Greek 'iron carrier'); the PSs are effluxed into the rhizosphere through membrane transporter TOM1 (TOM stands for transport of MAs, which are important

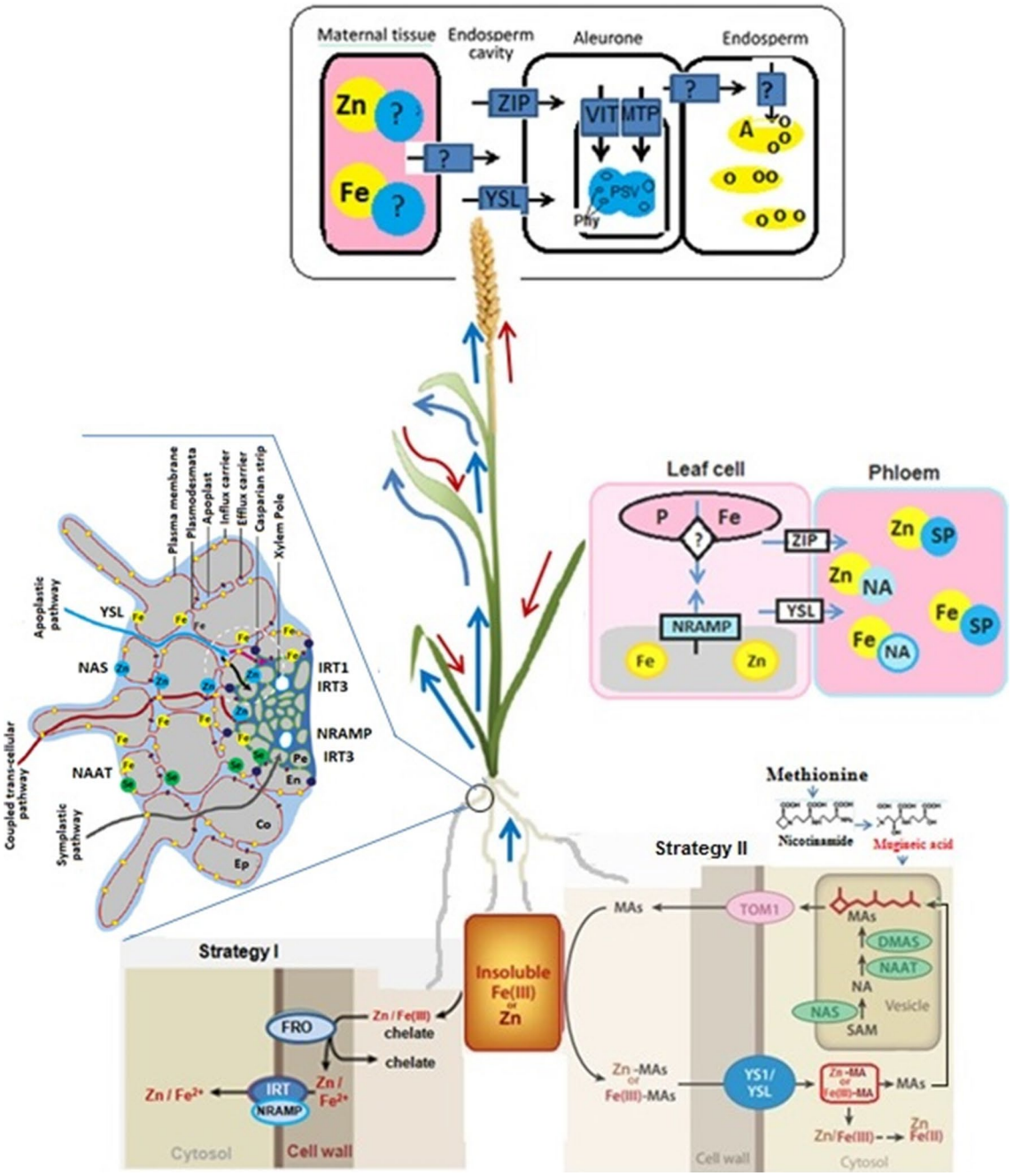

Fig. 4 Physiological processes and genes involved in uptake, transport and storage of Zn and Fe in strategy I and strategy II for biofortification (some of these transporters take part only under conditions of Fe deficiency; see text for details). Free Zn^{2+} and phytosiderophore (PS)-bound Zn/Fe are acquired from the soil (rhizosphere) by the root epidermal cells. Zn and Fe move via the apoplast and symplast to the pericycle, via cortex and endodermis, but may be sequestered en route in vacuoles (V) in endodermis cells. Zn and Fe are loaded into the xylem and transferred into the phloem in the root. Later, these are transferred to basal shoot or leaf tissues (not shown). Zn and Fe from leaf cell plastids (P) and vacuoles (V) are remobilised and loaded into the phloem for transport to the ear. After uptake into the aleurone layer, most Zn and Fe are sequestered in protein storage vacuoles (PSVs) bound to phytate (Phy). A small proportion of Zn and Fe may enter the endosperm and be stored there with ferritin (Fer) in amyloplasts (A). ZIP = ZRT-IRT-like protein, YSL = yellow-stripe-like transporter, MFS = major facilitator superfamily transporter, MTP = metal tolerance protein, HMA = heavy metal ATPase, FPN = ferroportin, NRAMP = natural resistance-associated macrophage protein, VIT = vacuolar iron transporter, NAS = nicotianamine synthase; NAAT = nicotianamine aminotransferase; FRO = ferric chelate reductases; TOM = transporter of mugineic acid; IRT = iron-regulated transporter; NA = nicotianamine, Cit = citrate, SP = small proteins, Co = Cortex; En = endodermis; Ep = epidermis; Pe = pericycle

Table 5 Different transporters with corresponding genes known to be involved in uptake, transport and storage of micronutrients in wheat

Transporter	Gene (number of genes)	Metal/PS	References
VIT	*TaVIT2* (8)	Fe +Mn	Connorton et al. (2017), Wang et al. (2019), Sharma et al. (2020)
VIT	*TaVTL* (23)	Heavy metals	Sharma et al. (2020)
DMAS	*TaDMAS1* (1)	Fe	Bashir and Nishizawa (2006)
HMA	*TaHMA2* (1)	Zn +Cd	Tan et al. (2013)
ZIFL	*TaZIFL1* to *TaZIFL7* (15)	Zn +Fe	Sharma et al. (2019)
YS/YSL	*TaYSIA-6A to TaYSL23-5A*(66/67)	Zn + Fe	Kumar et al. (2019a), Wang et al. (2019)
ZRT/IRT-like	*ZIP* (1)	Zn +Fe	Wang et al. (2019)
NRAMP	*TaNRAMP* (24)	Fe	Borrill et al. (2014), Wang et al. (2019)
FRO	*FRO* (1)	Fe	Wang et al. (2019)
MTP	*TaMTP1A* (15)	Zn/Fe/Mn	Vatansever et al. (2017)
TOM*	*TOM* (1)	DMA/MA	Wang et al. (2019)
IREG/FPN	*IREG/FPN* (1)	Fe	Wang et al. (2019)
Fer	*TaFer1* and *2* (2)	Fe	Borg et al. (2012)
IDS3	*TaIDS3* (1)	Fe	Mathpal et al. (2018)

* Transporter involved in efflux; all other transporters are involved in influx

VIT = vacuolar iron transporter; DMAS = deoxymugineic acid synthase; HMA = heavy metal ATPases; ZIFL = Zinc–induced facilitator like; YS/YSL = yellow stripe or yellow-stripe-like; ZRT/IRT = zinc-regulated transporter/iron-regulated transporter-like protein; NRAMP = natural resistance-associated macrophage protein;FRO = ferric chelate reductases; MTP = metal tolerance protein; TOM = transporter of mugineic acid; IREG/FPN = iron-regulated/ferroportin; Fer = ferritin; IDS3 = iron-deficiency-specific clone 3

Table 6 Transporters and their genes involved in uptake, transport and storage of micronutrients reported in rice and barley but are yet to be discovered in wheat

Transporter	Gene	Location	Zn/Fe	Function	References
NAS	*HvNas1-6*	Root	Fe	Synthesis of NA	Higuchi et al. (1999)
NAAT	*HvNaat-A, -B*	Root	Fe	Synthesis of DMAs	Takahashi et al. (1999)
MAF	*HvIDS2, 3*	Root	Zn	Uptake of Zn	Suzuki et al. (2006)
ENA	*OsENA1, 2*	Root	Fe	Uptake of Fe	Nozoye et al. (2011)
MFS	*OsVMT/OsZIFL12*	Rice node	Zn + Fe	Node to grain	Che et al. (2019)

NAS = nicotianamine synthase; NAAT = nicotianamine aminotransferase; MAF = mugineic acid family

E NA = efflux transporter of NA (nicotianamine, a phytosiderophore); MFS = major facilitator superfamily

HvIDS = *Hordeum vulgare* iron-deficiency-specific; *OsVMT* = *Oryza sativa* vacuolar mugineic acid transporter/*OsZIFL12* = *Oryza sativa* zinc-induced facilitator

PSs). In the rhizosphere, PSs form complexes with insoluble Zn^{3+}/Fe^{3+} and facilitate uptake by roots (Lindsay and Schwab 1982).

Uptake of Zn and Fe by the root

In wheat and other graminaceous plants, although mainly Strategy II is utilized, there is a reason to believe that some features of Strategy I (commonly used in non-graminaceous crops) also operate in these cereal crops (Borrill et al. 2014; Wairich et al. 2019; Kaur et al. 2019; see later for some details). We will describe Strategy I only in brief, since it is only sparingly used by cereals including wheat, but will describe Strategy II in much greater detail, since this strategy is predominantly used by cereals including wheat,

(i) Strategy I (*Reduction-based strategy for uptake of Fe in reduced state*). The two major classes of transporters used for uptake and transport of reduced form of iron (Fe^{2+}) include transporters of ZIP family (including IRT1 and ZRT) and NRAMP1 (ZIP stands for ZRT-IRT–like proteins). Functional homologs of genes encoding these two classes of transporters have been identified in wheat and rice, suggesting that some features of Strategy I are also used by cereals, although Strategy II may be the predominant mode of uptake in cereals (as mentioned above). Such a conclusion is based on the results of some recent transcriptome studies under iron starvation, where high expression of *TaIRT1* and *TaNRAMP1*, *2, 3* was confirmed in wheat (Borrill et al. 2014; Kaur et al. 2019). This also suggests that genes encoding

Table 7 A summary of transporter genes along with the genes for transcription factors (TFs), which regulate their expression in cereals

Downstream transporter genes	Transcription factor genes	Expression tissue	References
FRO2, IRT1	FIT1; bHLH38, 39, 100, 101	Root and other tissues	Colangelo and Guerinot (2004), Jakoby et al. (2004), Yuan et al. (2005, 2008), Wang et al. (2019)
YSL15, YSL2, NAS1,NAS2, NAAT1, DMAS1, TOM1	IRO2 (bHLH), OsbHLH058	Roots, shoots, leaves, flowers and developing seeds	Ogo et al. (2007, 2011), Wang et al. (2019), Kobayashi et al. (2019)
YSL2	IDEF2	Roots; leaves (vasculature)	Ogo et al. (2008), Kobayashi et al. (2010)
IRT1, IRO2, YSL15, YSL2, NAS1, NAS2, NAS3, DMAS1	IDEF1	Roots and shoots	Kobayashi et al. (2009, 2010)
NAS4, ZIF1, FRO3	PYE	Roots; shoots	Long et al. (2010)
NAS4, ZIF1, FRO3	PYE	Roots; shoots	Long et al. (2010)
NAS1, NAS2, IRO2	IRO3	Roots and shoots	Zheng et al. (2010)
NAS4, BGLU42	MYB10, MYB 72	Roots	Palmer et al. (2013), Zamioudis et al. (2014)
ZIP, YSL	NAM-B1	Flag leaves	Pearce et al. (2014)
VITL, NAS2	WRKY46	Root	Yan et al. (2016)
TaZIPs	TabZIP	Roots and shoots	Evens et al. (2017)
ZIFL	IDE1	Roots and shoots	Sharma et al. (2019)

IRT and NRAMP transporters might be conserved among plant species including both dicots and monocots (including cereals). The transporters IRT and NRAMP used for uptake of Fe in the root also take part in the transport of Fe in shoot and in developing grain (Fig. 3). The ZRT transporters (for Zn) that are analogous to IRTs for Fe transporters have also been described and their genes identified in Arabidopsis and some other plant species.

(ii) *Strategy II* (*Chelation-based strategy* for *uptake of Zn/Fe in oxidized ferric state*). In this strategy, acquisition of Zn/Fe makes use of a variety of PSs including NA (nicotinamine), DMA (deoxymugineic acid) and other MAs (mugineic acids) that are synthesized within the root. The pathway used for synthesis of these PSs is depicted in Fig. 5. After synthesis, these PSs are effluxed from the roots into the rhizosphere, where they function as chelators and form Zn^{3+}/Fe^{3+}-PS complexes, which facilitate uptake of Zn and FE (von Wirén et al. 1999; Takahashi et al. 2003; von Wirén et al. 1996; Kobayashi and Nishizawa 2012). After PS-Zn/Fe complexes are transported into the cytoplasm of the epidermal cells of the root, PSs are released so that these PSs are recycled back to the rhizosphere through TOM transporter and are utilized once again for chelation of Zn^{3+}/Fe^{3+} molecules (Fig. 4).

Genes encoding enzymes involved in synthesis of PSs have also been identified. For instance, 26 *TaNAS* genes, 6 *TaNAAT* and 3 *TaDMAS* genes were identified in wheat (Beasley et al. 2019). The activity of these genes is differentially regulated depending upon relative abundance of Fe^{3+} and Fe^{2+} in the rhizosphere. In a recent study, the combined overexpression of NAS 1 and NAAT in rice was shown to give a 29-fold increase in DMA and four-fold increase in iron concentration (Banakar et al. 2017). Increased level of NA was also shown to give a higher zinc and manganese concentrations in the grain (Curie et al. 2009; Clemens et al. 2013). Interestingly, NA also improves the bioavailability of iron (see later in section dealing with bioavailability). It is known that the release of PSs is more pronounced (by a factor of 10–20) under Zn deficiency, relative to that under Fe deficiency, as shown in several crops including wheat, barley and rye (Römheld 1991; Neelam et al. 2010). This aspect has been studied in more detail in Fe, where it has been shown that the release of PSs and subsequent uptake of Fe^{3+}-PS are

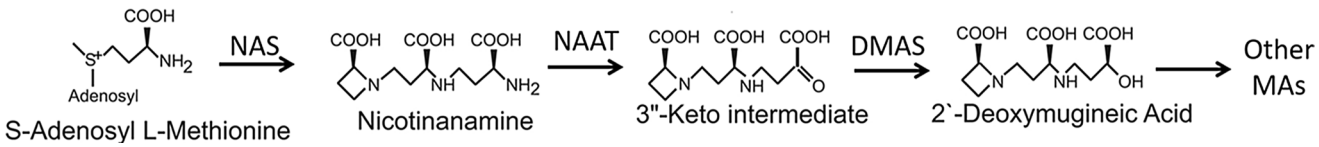

Fig. 5 Biosynthetic pathway involved in synthesis of phytosiderophores (PSs)

under different genetic controls, such that Fe acquisition has two main components, one is Fe-deficiency-induced PS synthesis and release and the other is efficient even under normal conditions, but further activated under Fe deficiency. Zn solubilization in the rhizosphere is also achieved via plant-mediated acidification and secretion of low molecular weight organic chelators (Sinclair and Krämer 2012). Thus, modification of rhizosphere chemistry through secretion of more root exudates alters soil pH and represents the first promising strategy for improving Zn and Fe acquisition in roots of cereals including wheat.

A number of transporters have also been identified for efflux of PSs and for uptake of chelated metal ions through root epidermis; the genes encoding these transporters have also been identified (Table 5). For instance, PSs are effluxed through the transporter TOM1 (so named after MAs which are the most common PSs) and take part in the formation of Zn^{3+}/Fe^{3+}-PS complexes in the rhizosphere (Roberts et al. 2004). These complexes are taken up into root cells through transporters called yellow stripe (YS) and yellow-stripe-like proteins (YSLs), which also function in shoots (Fig. 4), thus facilitating long-distance transport from leaves to grain through both xylem and phloem. Among YSL proteins, the role of YSL15 has been confirmed in rice (Curie et al. 2001; Inoue et al. 2009; Lee et al. 2009; Kumar et al. 2019a). In wheat also, 67 YSL genes have been identified using transcriptome analysis (Kaur et al. 2019; Kumar et al. 2019a).

Another important class of transporters is zinc-induced facilitator-like (ZIFL) transporters, which play an important role under conditions of different stresses including Zn stress. These transporters were studied earlier in Arabidopsis, rice and maize. Rice ZIFL transporters were also functionally characterized and were found to be TOM transporters mentioned above. The expression of these transporters has been shown to be tissue-specific, restricted to roots and root-shoot junctions. Recently, 35 ZIFL genes (located on homoeologous groups 3,4,5) were identified in wheat (Sharma et al. 2019). It was also observed that these transporters generally express under Fe-deficient and Zn-excess conditions and therefore can be modulated for improved uptake of Zn and Fe.

The cereal genes encoding transporters for uptake have sometimes been studied only in rice and not in wheat. For instance, the gene *OsYSL15* encoding the transporter YSL15 has been shown to be induced under conditions of Fe deficiency to facilitate transport of chelated Fe in the form of Fe^{3+}-DMA and Fe^{3+}-NA in rice only (Inoue et al.2008; Lee et al. 2009). Besides YSL15, there are also a number of other YSL transporters, which are used for transport of Fe and have been studied in several crops, including maize, rice, barley and wheat (Curie et al. 2001; Koike et al. 2004; Murata et al. 2006; Aoyama et al. 2009; Ishimaru et al. 2010; Kakei et al. 2012).

As mentioned earlier, ZIP-like transporters and IRT transporters primarily meant for strategy I have also been identified in cereals like rice (Ramesh et al. 2003). IRT1 (ZIP for Zn) has been shown to increase the iron concentration by 1.7-fold in rice leaves and 1.1-fold in grains (Lee and An 2009). On overexpression of IRT1 and PvFER1 in the endosperm, the Fe concentration was found to increase four-fold in rice grain (Boonyaves et al. 2017). The transporters YS1 in maize and YSL15 in rice were shown to help in the transport of Fe-chelator complexes into the cell (Curie et al. 2001, 2009). However, an increase in DMA or YSL15 (due to overexpression of corresponding genes) gave only marginal increase in the Fe concentration in rice grains (Masuda et al. 2008; Lee et al. 2009), suggesting that these are not limiting factors for uptake of micronutrients in the roots.

In a study conducted in Arabidopsis, it was shown that the mechanism of Fe uptake and radial transport within the root depends on the status of Fe in the soil or rhizosphere; for instance, the soil may be deficient for Fe, may have normal concentration of Fe or there may be abundance of Fe in the soil (Castaings et al. 2016). Fe uptake under these three different conditions of Fe in the soil has been studied, and the following results were obtained: (i) under Fe deficiency (< 1 µM), IRT1 transporter mediates Fe uptake into epidermis and cortex; Fe deficiency symptoms appear in genotypes, where IRT1 function is impaired. (ii) Under normal condition of Fe (25 µM), both IRT1 and NRAMP1 help in Fe uptake by the epidermis, followed by transport to the cortex and then to the xylem for further transport to the shoot. After passage through apoplast of root outside the casparian strip (CS) of endodermis, either IRT1 or NRAMP1 transporters located in the endodermis region of the root are utilized for further radial transport of Fe to the xylem. (iii) When Fe is abundant (> 500 µM), the plant can tolerate absence of both IRT1 and NRAMP1, as evident from the observation that *irt1 nramp1* double mutants can be rescued through supply of > 500 µM of Fe. In a recent study in wheat also, *TaYSL9*, *TaYSL1A and TaNRAMP* genes were found to exhibit high expression at the transcription level under Fe starvation (Kaur et al. 2019).

A number of transporters are also known, which are unique to apoplastic and symplastic transport and for several layers of root including endodermis (Bao et al. 2019). The endodermis carries a casparian strip (CS) and is also covered by suberin lamellae, which completely block the entry of micronutrients. Thus, endodermis functions as a checkpoint and a gateway, which will determine the nature of ions or compounds to be allowed to cross the endodermis (Geldner 2013; Barberon et al. 2016). Among these transporters, two important endodermis transporters include IRT and NRAMP, which provide a symplastic route for radial transport of specific ions to reach the xylem. These IRT and NRAMP transporters were initially

discovered only in dicots as a part of Strategy I for uptake of micronutrients, but later, these were also discovered in a number of monocots including rice, barley, maize and even wheat.

Transcription factors (TFs) have also been identified, which regulate the expression of genes involved in the synthesis of PSs and transporters which participate in the uptake of Fe and Zn in cereals (Table 7). The most important class of these transcription factors seem to be the bHLH, which regulate the expression of genes encoding a variety of transporters (Table 7). An important TF is NAM-B1 (TF of NAC family), which is non-functional in the current wheat cultivars (therefore not included in Table 7), but its allele in ancestral wheats (including the progenitors of modern wheats) is responsible for accelerated senescence leading to increase in nutrient remobilization from leaves to developing grains. The role of NAM-B1 in biofortification was validated using RNAi approach of gene silencing (Uauy et al. 2006). Similar studies can be conducted for a variety of TFs listed in Table 7. A number of genes encoding transcription factors associated with influx and efflux transporters have also been discovered and characterized in plants, including cereals (Kobayashi and Nishizawa 2012; Ricachenevsky et al. 2015; Vasconcelos et al. 2017).

Translocation of Fe and Zn to the shoot through xylem

Once Zn/Fe have reached the xylem cylinder in the root through redial translocation within the root, it is translocated to the shoot. This may or may not involve association with chelators like NA and DMA. It has been shown that Zn/Fe is not directly transported to seed, but makes a detour through leaves, where it is often first transported from dead xylem tissue to living xylem parenchyma with the help of some specific transporters and later transported from xylem parenchyma to the phloem for its transport to the developing seed. Transporters IRT3 and ZIP4 may be involved in xylem unloading and phloem uploading.

The movement of micronutrients from the root to the shoot depends on several factors including the following: (i) optimization of N supply; (ii) root: shoot ratio for Zn/Fe concentration; (iii) application of Zn/Fe fertilizer in the soil, etc. The manner in which these factors influence root-to-shoot transport is not fully understood, but it is known that in cereals like wheat, translocation from the root to the shoot continues during pre-anthesis and post-anthesis periods. It is also known that in the leaves, Zn can move as free ions or complexed with organic acids, while Fe is chelated to organic compounds before it is translocated into the xylem and later to the phloem in the leaves.

Sink tissue for Fe and Zn (storage)

In plants, leaves represent the most important sink tissue for micronutrients like Zn and Fe. In the leaf, these micronutrients are also transported to plastids and mitochondria, where they are needed for the function of a variety of enzymes that are essential for a variety of physiological/metabolic processes that occur in these organelles (Gupta et al. 2015). Remobilization of micronutrients from the leaves to the grain takes place even during uptake of these micronutrients from the root to the shoot. It has also been shown that these micronutrients make use of xylem as well as phloem for their transport to not only the grain, but also to other vegetative parts of the plant (Rellan-Alvarez et al. 2010; Lu et al. 2013). In leaves, the transport of Fe is also facilitated by P(1B)-ATPases (also known as HMAs = Heavy-Metal ATPases), which belong to FRD3 family of proteins that were studied in Arabidopsis for their role in Fe uptake (Green and Rogers 2004).

Within the seed, the micronutrients are deposited in different parts including maternal tissue (seed coat), endosperm and the embryo. An improvement in the efficient distribution of micronutrients in these different parts can be achieved through biofortification using the knowledge of the molecular aspects of the storage of Zn and Fe in different parts of the seed (Persson et al. 2016; Cakmak et al. 2010). For instance, in rice, Fe transport from the phloem into the developing seeds could be improved through overexpression of the genes encoding YSL2 transporter, through the use of high-expression *OsSUT1* promoter (*SUT1* promoter is known for tissue-specific expression of sucrose transporter in phloem companion cells and immature seeds).

Zn and Fe also differ with respect to their association with other molecules within the seed and also with respect to their spatial distribution within the seed and other parts of the plant. It is known that Cu and Mn are deposited primarily in the aleurone and embryo relative to their concentration in the endosperm, except in rice and barley, where mainly Fe rather than Zn is strictly confined to the aleurone (Persson et al. 2009). Thus, Zn and Fe occur in different forms and are localized in different parts of the seed (Persson et al. 2009; Kutman et al. 2010).

Within the seed, two important locations for deposition of micronutrients include ferritin for Fe and vacuole for both Zn and Fe. Ferritin is a ubiquitous protein that is largely used for storage of Fe, when it is abundant, so that in the event of short supply, Fe is released from ferritin (Briat et al. 2010a). Ferritin thus plays a significant role in Fe homoeostasis in plants including cereals like wheat, because Fe in free state (if not stored in ferritin) can lead to the formation of free radicals, thus causing damage to the plant. It has also been shown that despite the presence of ferritin for the storage of Fe, only ~ 5% of the total iron is stored via ferritin; most of

the remaining iron (95%) is stored in vacuoles. Both in ferritin and aleurone layers, Fe forms a complex with phytate, thus making it unavailable to the consumers. Therefore, the main function of ferritin and aleurone in seeds does not appear to be storage, but rather protection of the plant from oxidative stress (Briat et al. 2010b; Raboy 2009). However, only Fe is largely chelated by phytic acid, thus making Fe unavailable; Zn remains associated with proteins. It has actually been shown in rice that it is possible to improve Fe concentration in endosperm through endosperm-specific overexpression of ferritin cDNA derived from soybean. In such studies, the iron content in the grain could be increased by a factor of two to three (Goto et al. 1999; Lucca et al. 2001; Qu et al. 2005; Vasconcelos et al. 2003). However, similar increase in concentration of Fe in leaves through excess transport from the root was not successful, when leaves appeared to be exhausted of Fe due to its transport to seed (Qu et al. 2005).

Wheat genes for ferritin, vacuole transporter VITs and transporter-like protein VTLs have been studied in some detail. The ferritin genes *TaFer1* (homoeologous group 5) and *TaFer2* (homoeologous groups 4) are differentially regulated and expressed (Borg et al. 2012). *TaFer1* (but not *TaFer2*) was also shown to carry Fe- and ABA-responsive elements in their promoter sequences for high expression in tissues other than endosperm (including leaves, embryo and aleurone) under high levels of Fe and ABA. Endosperm-targeted overexpression of *TaFer1-A* using 1Dx5 promoter for endosperm-specific expression also resulted in an increase in iron content of the wheat grain by 50–80%; this is an example of intragenic transfer of a gene (gene of same plant introduced with a different promoter), as against transgenic or cisgenic transfer of a gene (Borg et al. 2012).

The vacuole transporter VIT genes (*TaVIT1*, *TaVIT2* and *TaVIT3*; homoeologous group 5, 2, 7) and vacuolar transport-like VTL genes (Ta*VTL1, TaVTL2, TaVTL4* and *TaVTL5*; homoeologous groups 2,4,6) were also reported in wheat (Connorton et al. 2017; Sharma et al. 2020). The vacuolar transporters encoded by these genes are important because 95% of Fe is stored in vacuoles only, as shown in Arabidopsis (Kim et al. 2006). The *TaVIT2* genes had higher expression relative to that of *TaVIT1* genes. Also, all VIT genes had high level of expression in aleurone and low expression in endosperm, showing high correlation with abundance of iron in aleurone. The three sets of VIT genes seem to have different functions. For instance, *TaVIT2*, (but not *TaVIT1*) could also rescue and complement mutants of yeast that were defective for vacuolar transport. A twofold increase in Fe with no associated increase in phytate (having adverse effect on bioavailability) was also observed in transgenics for *TaVIT2*, when used with an endosperm-specific strong promoter, GLUID-1. The transgenics with *TaVIT2*, carrying the promoter *GLU-1D-1* had more than twofold increase of Fe in the white flour that is derived from the endosperm excluding aleurone (Connorton et al. 2017).

The above information about VITs and VLTs suggests that it may be more effective to improve vacuolar storage then ferritin storage, because vacuolar storage relative to iron storage is the typical normal mode of storage in cereal grains (unlike ferritin genes, which do not express in the endosperm). This is also important because aleurone is often discarded, when flour is obtained from wheat grain, thus reducing the nutritional quality of the product used by humans. The *vit* mutant lines were also found to have relatively low concentration of Fe in roots and shoots relative to wild type. Taken together, it is apparent that VIT and VTL genes are more important than the ferritin genes for increasing the storage capacity of the wheat grain.

Another wheat gene relevant to biofortification is the major grain protein gene *Gpc1*, which also affects Zn and Fe content in the grain. The gene has been derived from tetraploid wheat and was mapped on chromosome 6B. It encodes a NAC transcription factor (NAM-B1), which as mentioned above, accelerates senescence and increases nutrient remobilization from leaves to grain (Uauy et al. 2006; Distelfeld et al. 2007). In a number of studies in wheat, it has been shown that introgression of *Gpc-B1* also improves Zn and Fe content in the grain along with improvement in GPC (for a review, see Tabbita et al. 2017). The *Gpc-B1* gene has also been shown to regulate the expression of a number of genes including transporters of the ZIP and YSL categories in early senescence stage, which are eventually responsible for the export of Zn and Fe from the cytoplasm into the phloem and also for the biosynthesis of chelators that facilitate the phloem-based transport of Zn and Fe to the grains (Pearce et al. 2014). Therefore, the gene *Gpc-B1* may also be exploited for grain Zn and Fe biofortification in wheat.

Uptake, transport and storage of Se

Se occurs in the soil in four different states including Se^{6+} (selenate), Se^{4+} (selenite), Se^{2-} (selenide) and Se° (elemental Se) (Pyrzyńska 2002; Fellowes et al. 2013). It also occurs in the form of organochemical compounds like selenocysteine (SeCys) and selenomethionine (SeMet). Plants can take up Se only in any of the following forms: selenate (Se^{6+}) and selenite (Se^{4+}), SeCys or SeMet, but cannot take up Se^{2-} or Se° (Zhao et al. Zhao et al. 2005; Bañuelos and Lin 2005). The occurrence of high-affinity sulphate transporters (HASTs; Terry et al. 2000; White et al. 2004, 2007a, b; Sors et al. 2005; Broadley et al. 2006; Hawkesford and Zhao 2007) and phosphate transporters in the plasma membrane in the root cells facilitate uptake of Se^{6+} or Se^{4+} (Li et al. 2008). In the root, selenite (Se^{4+}) is rapidly converted into organoselenium compounds like selenocysteine (SeCys) and selenomethionine (SeMet), but selenate is converted into

these organoselenium compounds only after its transport to the shoot through xylem. It has also been shown that Se resembles S in its assimilation pathway and also in its further redistribution of organoselenium compounds to different organs of the plant (Terry et al. 2000; Broadley et al. 2006; Sors et al. 2005; White et al. 2007a, b; Hawkesford and Zhao 2007; Li et al. 2008).

It is known that within plant cells, extensive gene families occur, which encode enzymes of the assimilation pathway, which are common for Se and S. The products of these pathways are distributed to different compartments within the cell (Hawkesford and De Kok 2006). For instance, as in S assimilation pathway, using the enzyme ATP sulphurylase (ATPS), selenate forms APSe (adenosine 5′-phosphoselenate), which is then reduced to selenite (like sulphite) by APS reductase (APR), a major enzyme of Se/S assimilation pathway (Vauclare et al. 2002). Selenite is then further reduced to selenide by a sulphite reductase located in the chloroplast. Selenide thus produced takes part in the synthesis of SeCys using serine and the enzyme cysteine synthase, whose activity is regulated by the concentration of *O*-acetylserine. SeCys is also the precursor of SeMet, which is synthesized from SeCys and *O*-phosphohomoserine (*O*PHS) through sequential actions of the following three enzymes: cystathionine β-synthase, cystathionine β-lyase, and selenomethionine synthase. Both SeCys and SeMet are utilized for the synthesis of other Se assimilation products including Se-methylselenocysteine (SeMSeCys), γ-glutamyl-SeMSeCys and Se-methylselenomethionine, which have been characterized in the genera *Allium* and *Brassica*, but yet to be identified and characterized in cereals (Broadley et al. 2006; White et al. 2007b). Se concentrations in shoot tend to increase to a maximum during seedling growth, then decline before, or during flowering (Rosenfeld and Beath 1964; Xue et al. 2001; Turakainen et al. 2004; White et al. 2007b).

Bioavailability of micronutrients

Bioavailability of Zn and Fe

Besides the concentration/content of micronutrients like Zn and Fe, the amounts that are available for absorption is also an important criterion for increasing their intake from wheat (Frontela et al. 2009; Magallanes-López et al. 2017). It is known that Zn and Fe are available in wheat grain in forms, which cannot be fully utilized by human beings directly or indirectly. In particular, infants and pregnant/lactating women, who depend largely on cereal-based food, suffer the most due to low utilization of micronutrients (Chan et al. 2007; Al Hasan et al. 2016). Therefore, this group needs to consume biofortified cereal food, which allow higher absorption of micronutrients. Considering the importance of Zn for human health, the problem of Zn bioavailability has also been a subject of interest for International Zinc Nutrition Consultative Group (IZiNCG), which is a non-profit organization located in Oakland, California and was established to address the problem of Zn deficiency in human subjects (Brown et al. 2004).

Estimates have been made of the proportion of individual micronutrients that are generally utilized by human body from the total intake of the micronutrient in the diet. Such an estimate is described as bioavailability of the micronutrient. The estimates of Zn and Fe bioavailability vary in different reports, which include the following: (i) 25% bioavailability for Zn and 5% for Fe (Kenzhebayeva et al. 2019), (ii) 16–50%, bioavailability for Zn, which is inversely related to oral zinc intake (Maares and Haase 2020), (iii) 26–34% bioavailability for Zn for a mixed or vegetarian diet based on refined cereal grains and 18–26% for unrefined cereal-based diet (Brown et al. 2004). Absolute amount of bioavailable Zn in flour of different wheat varieties also vary and has been reported to range from 1.52 to 2.7 mg per 300 g of flour (Rosado et al. 2009; Hussain et al. 2012).

Bioavailability depends on several intrinsic and extrinsic factors including the following: (i) Zn content in the consumed diet; (ii) Zn species in the intestine (five stable isotopes of Zn are known, namely ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70Z}Zn; only some of these species are available in human diet and their relative proportion may differ); (iii) intestinal zinc bioaccessibility (bioaccessibility is also a component of bioavailability); (iv) molar phytate:Zn ratio; (v) method of estimation of bioavailability. For instance, it has been shown that with increased intake, bioavailability goes down. It has also been shown that Zn absorption is reduced from 21% in the absence of phytate to 11–16% at a phytate:Zn molar ratio of 5–15 and even lower to 4–11% at molar ratios > 15. Additionally, these phytate-Zn complexes are stronger in the presence of Ca, suggesting that the inhibitory effect of phytate may be further aggravated in the presence of calcium, although such inhibition has not been reported in several human dietary studies. Other than phytate, fibres such as cellulose seem to have no significant impact on Zn absorption. However, bioavailability has been shown to improve through biofortification (Rosado et al. 2009).

Measurement of bioavailability

Estimations of bioavailability have been made using both in vitro cell cultures (Caco-2 cell model) and in vivo rat and human subjects, and the results differ (for details and original references, consult the recent review by Maares and Haase 2020). Since a small fraction of Fe is stored in ferritin, and a large proportion remains unavailable due to association with PA, methods have been designed, where bioavailability of Fe can be measured through estimates

of ferritin production in cell cultures (Caco 2 cell line). In this method, the food is subjected to simulated gastrointestinal digestion and the digests applied to Caco 2 cells (Caco-2 cells exhibit a wide range of characteristics of intestinal epithelia with respect to uptake of Fe and other nutrients; this makes Caco-2 cells a good model system for estimation of bioavailability); ferritin formation in Caco 2 cells is then used as a measure of bioavailability (Glahn et al. 1998). Utilizing this approach, it could be demonstrated that bioavailability of Fe from whole grain and white flour differs and that a wheat genotype with relatively low grain Fe content may have higher bioavailability. Inhibitory effect of PA has also been demonstrated using this approach.

Since estimation of bioavailability of Fe using Caco 2 cells is based on formation of ferritin, it can be used for estimation of bioavailability of only Fe (not Zn). Therefore, alternative methods were developed, which could be used for estimation of bioavailability of any micronutrient. Such a modified method was developed and used for transgenic cassava by Narayanan et al. (2019); it was a modification of the method used by Glahn et al. (1998). In this modified method, a model for simulation of digestion independently in mouth, stomach and small intestine was developed and utilized for in vitro digestion of food; this was followed by biophysical estimation of mineral concentration using ICP–OES (Ciros ICP- FCE12), which can be used for estimation of bioavailability of any micronutrient including Zn and Fe.

Phytic acid (PA) and its role in bioavailability of micronutrients

Since phytic acid (PA) chelates Fe in the seed of cereals, it reduces the bioavailability of Fe. It will, therefore, be useful to describe its structure showing its negative role in bioavailability of Fe.

Structure of phytic acid (PA)

The molecular formula of PA is $C_6H_{18}O_{24}P_6$, which is a dihydrogen-hexaphosphate of *myo*-inositol (InsP6; Fig. 6). In other words, it is a form of organic phosphorus. When PA is bound to mineral element like Fe in the seed, it is described as a phytate, which ionizes at a particular physiological pH, generating partially ionized phosphates, and a phytate anion. The formation of phytates in the cereal seed make the micronutrient (particularly Fe) unavailable during digestion. PA may also degrade and produce other lower inositol esters with fewer phosphate molecules, which include inositol pentaphosphate (IP5), inositol tetraphosphate (IP4) and inositol triphosphate (IP3), which are not important for a discussion on bioavailability.

Phytic acid (PA) and bioavailability of micronutrients in wheat

In cereals, 85% of the total P is stored in the form of PA in the grains and other organs of the plant (Raboy 2000; Guttieri et al. 2004; Perera et al. 2018; Cominelli et al. 2020). The remaining 15% of P within living cells occurs either in the form of soluble inorganic phosphate (Pi: ~ 5%) or as phosphorylated molecules within the cell (~ 10 to 20%), which is utilized for a variety of functions including regulation of different cell signalling and plant processes (Sparvoli and Cominelli 2015). For instance, reversible phosphorylation of many proteins/enzymes regulates the activity of these proteins including kinases. P is also involved in the formation of the backbone of nucleic acids and also occurs as component of lipids and sugars (Larson et al. 2000).

Due to its negative charge, PA can chelate a number of cations, which also include micronutrients like Zn^{2+} and

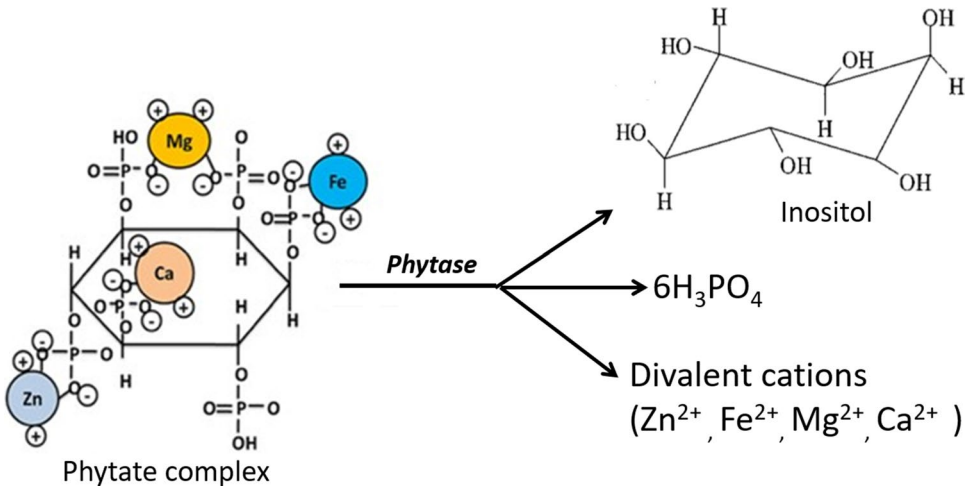

Fig. 6 Schematic representation of the structure of phytate complex with its six phosphate groups and its hydrolysis using phytase

Fe^{2+}, which generate iron phytate or zinc phytate (Perera et al. 2018; Kishor et al. 2019). Since phytates work as highly negative charged ions at a broad range of pH, it has a negative impact on the bioavailability of a variety of micronutrients, which also include Zn^{2+} and $Fe^{2+/3+}$ (Wu et al. 2009). In humans, the diets with high phytate are associated with low bioavailability of micronutrients like Zn (Guttieri et al. 2004), Fe (Hurrell et al. 2003) and Mg (Ranhotra 1983; Bohn et al. 2004). Among these, low bioavailability of Zn and Fe due to high PA intake is the most important feature from the point of view of biofortification (Al Hasan et al. 2016).

Molar ratios PA:Zn and PA:Fe have also been used for estimation of potential bioavailability of Zn and Fe. Bioavailability is considered to be high with PA:Zn molar ratio of < 5, moderate with molar ratio 5–15, and low if molar ratio is > 15 (Gibson, 2006; Magallanes-López et al. 2017). In order to significantly improve Fe absorption, the molar ratio of PA:Fe should be < 1 (preferably < 0.4) in plain cereal or legume-based meals devoid of any enhancers of Fe absorption. In composite meals with certain vegetables containing ascorbic acid and meat as enhancers, a molar ratio of < 6 may be acceptable (Hurrell and Egli 2010; Magallanes-López et al. 2017). PA:Zn ratio in wheat may also depend on planting season; in one such study, Phy:Zn molar ratio has been reported to be 1.88−4.17 for autumn planting and 2.10−4.05 for spring planting (Bilgrami et al. 2018).

Bioavailability depends on Fe^{3+}/Fe^{2+} state involved in chelation by phytic acid (PA)

Bioavailability of Fe due to chelation with PA also depends on the oxidized or reduced state of iron (Fe^{3+} or Fe^{2+}), which are the common forms involved in chelation. Iron state (Fe^{3+} and Fe^{2+}), however, can be altered due to method of food preparation. For instance, ferritin releases iron either as Fe^{3+} or as Fe^{2+} on heating: ($Fe^{3+} \leftrightarrow Fe^{2+}$) (Hoppler et al. 2008), but generally Fe^{3+} gets chelated by PA, thus reducing its bioavailability (Moore et al. 2018; Perfecto et al. 2018).

Beneficial effects of PA

Phytic acid (PA) also has a variety of essential functions, thus making it useful for human health. Therefore, one should not consider it entirely anti-nutrient and reduce its content without keeping in mind its positive roles (Rhou and Erdman 1995). For instance, PA also binds to different proteins like selectin (selectin is a cell adhesion molecule or CAM, involved in inflammation and progression of cancer) and thus functions as an anti-inflammatory and anti-cancer molecule (Jenab and Thompson 1998). PA is also an antioxidant and acts as a protector against abiotic stresses like oxidative stress (Doria et al. 2009). Therefore, it is important to design suitable strategy that can reduce PA content without compromising its beneficial effects (see later for some details).

Genetic variation for PA and phytase in wheat

Since an assessment of genetic variation is a prerequisite for a breeding program, studies were also conducted to estimate level of genetic variation for PA (Ram et al. 2010; Ram et al. 2019; Ram and Govindan 2020). With the availability of genetic variation, PA can be reduced through breeding for improvement of bioavailability of Zn and Fe (see later). It is, however, recognized that since PA also has some important beneficial effects, we should not try to reduce PA beyond a certain threshold (Ram and Govindan 2020). However, no reports are available about the minimum PA, which may be used as a threshold, while decreasing the content of PA for improving bioavailability of Zn/Fe. In a breeding programme at IIWBR (Indian Institute of Wheat and Barley Research, India), however, efforts are underway to achieve a target of the minimum of 1% PA in the grains in order to improve the bioavailability of Zn and Fe, although genotypes with less than 1% of PA are also available (Sewa Ram, personal communication).

Natural variation in PA

Considerable variation for PA content is known to be available in wheat gene pool, including released varieties, SHWs, durm wheat, advanced breeding lines, etc. In a study conducted in India, ~ 400 wheat genotypes, including released Indian varieties, advanced lines and SHWs, were evaluated for PA and phytase contents. The variation for PA ranged from 4.6 to 73.0 mg/g (Table 8), which is translated into 0.46% to 7.3% PA. For phytase levels, 3.4-fold variation among varieties and 5.9-fold variation among SHWs were detected (Ram et al. 2010; Ram and Govindan 2020). Genotype and environment each had a significant effect on phytate and phytase levels; the heritability of phytate was 0.98 and that of phytase was 0.82, suggesting only marginal effect of the environment on genetic variation for PA and phytase.

In a study conducted in India, some wheat-*Aegilops* derivatives and low PA mutants of *T. monococcum* with varying Zn concentrations (19–65 mg/kg) were also studied, where the bioavailability of Zn could be improved 1.2–2.0-fold along with 1.8–3.3-fold increases in the Zn content over that for WL711-cultivar and *T. monococcum* that were used as controls. Wheat genotypes with low phytate:Zn molar ratios and enhanced Zn bioavailability were also identified (Salunke et al. 2012).

Table 8 Variation in phytic acid and phytate (values available in % converted into mg/g) in wheat and related species

Wheat material used	PA content; phytate (mg/g)	References
Wheat bran	21.0–73.0	Harland and Oberleas (1986), Wise (1983)*
Wheat germ	11.4–39.1	Wise (1983)*
Released varieties	11.65–19.30	Ram et al. (2010)
Synthetic hexaploid wheat (SHWs)	11.07–24.41	Ram et al. (2010)
Wheat genotypes (93)	5.9–20.8	Gupta et al. (2015)
WL711 × IITR19 (48 F_2 lines)	5.8–20.1	Gupta et al. (2015)
Advanced breeding lines (100)	4.9–15.0	Shitre et al. (2015)
Five Triticeae species*	6.1−20.2	Bilgrami et al. (2018)
Durum wheats (84)	4.6–7.6	Ficco et al. (2009)
Durum wheats (46)	4.6–9.5	Magallanes-López et al. (2017)
Ploidy level and amphiploids	9.7–20.2 (spring season) 9.7–20.2 (autumn season)	Bilgrami et al. (2018)
Indian and synthetic wheats	9.0–26.5	Ram and Govindan (2020)

**Triticum monococcum* (6), *T. durum* (9), *T. aestivum* (9), *X Triticosecale* (2), X *Tritipyrum* (1); *cited from

Gupta et al. 2013, original papers were not available

Induced variation in PA

Mutagenesis (radiation) has also been used to broaden the genetic variation for improvement of grain micronutrients in wheat. In a study conducted at PAU, Ludhiana, India, seeds of *T. monococcum* were treated with EMS and a set of 76 mutant lines were isolated, which also included some low PA (*lpa*) lines (lpa = low phytic acid). In this study, two putative desirable mutants (MM169 and MM225) were selected for further characterization and bioavailability studies (Salunke et al. 2012). In another recent collaborative study conducted in Kazakhstan for inducing genetic variability both for the contents and bioavailability of Fe and Zn (see earlier in this review), genetically stable spring wheat mutant lines (M_7 generation) were produced using 100 or 200 Gy gamma irradiation (Kenzhebayeva et al. 2019). When compared with parental line, some mutant lines had 1.4–2.1-fold decrease in PA concentration.

QTL mapping for PA content

QTL interval mapping was also conducted to identify QTLs associated with the low phytate in wheat kernels and to develop molecular markers tightly linked to QTLs for PA. In one such study, a mapping population consisting of 171 RILs was derived from the cross 'Danby' (a Kansas elite variety) x 'AO2568WS-A-12-10' (a low phytate donor parent). The population was phenotyped for concentration of inorganic phosphate or Pi (a good parameter to assess phytate levels) and was also genotyped using genotyping-by-sequencing (GBS) approach; high-quality 2509 SNPs were used to construct a linkage map and QTL interval mapping was undertaken. In this study, two major QTLs were identified, one each on chromosomes 4D and 5A for the Pi trait, which accounted for 43% of the total phenotypic variation (PVE); two candidate genes were also identified. One of these candidate gene is known to synthesize a polygalacturonase-like enzyme that interferes with the synthesis of PA (Venegas et al. 2017).

QTL mapping for bioavailability

Identification of QTLs for bioavailability is not easy, due to labour-intensive and demanding method for recording phenotypic data on bioavailability using Caco 2 cells, as described earlier. Therefore, no reports on QTL analysis for bioavailability are available in wheat. However, as many as 10 QTLs for bioavailability of Fe were identified in maize (Lung'aho et al. 2011). In this study, QTL controlling total grain Fe hardly overlapped with bioavailability QTL, suggesting that PA estimates are not good estimates of bioavailability. It was also reported that a combination of three of the larger QTLs for Fe content rather than the use of QTL for bioavailability led to higher Fe bioavailability in maize. In future, QTL interval mapping and GWAS will help us to understand whether QTL for bioavailability can be utilized for improvement of bioavailability of Zn/Fe in wheat.

Genes involved in biosynthesis of PA

In order to suppress genes, involved in PA biosynthesis for reducing phytate content, it is essential first to identify the genes involved in PA accumulation during early stages of grain development and then functionally characterize them. Wheat genes involved in PA biosynthesis or their possible transport have been identified, which could

be the potential candidates for developing low PA traits (Bhati et al. 2014; Aggarwal et al. 2015). The genes that were identified for this purpose included the following: (i) genes encoding a number of inositol tetraphosphate kinases (*TaITPK1*,*TaITPK2*,*TaITPK3* and *TaITPK4*), (ii) two genes encoding inositol triphosphate kinase (*TaIPK2*) and inositol pentakisphosphate kinase (*TaIPK1*). Besides these, homologs of *Zmlpa-1* encoding an ABCC subclass multidrug resistance-associated transporter protein (*TaMRP3*), putatively involved in PA transport, were also identified. Differential expression was observed for these genes during seed development with some preferentially expressed in aleurone tissue. It is known that ABCC transporters are involved in the transport or compartmentalization of the PA after its synthesis in grains (Bhati et al. 2016). A characterization of loss-of-function mutations for ABCC transporters has shown varying levels of reduction in seed phytate (Raboy 2000; Pilu et al. 2003; Raboy 2009; Panzeri et al. 2011; Sparvoli and Cominelli 2014, 2015; Bhati et al. 2016; Nagy et al. 2009). Extensive genome-wide identification and expression characterization of ABCC-MRP transporters were also conducted in wheat, depicting critical importance of ABCC proteins in wheat improvement (Bhati et al. (2015).

Phytase: variation and role in bioavailability

The level of PA in the seed can also be reduced by increasing the level of enzyme phytase, which reduces the level of all kinds of inositol phosphates, not only during grain storage, but also during fermentation, germination, food processing and digestion in the human gut (Burbano et al. 1995; Azeke et al. 2011; Hayakawa et al. 2014). Sufficient endogenous phytase, however, is generally not available in the intestine of animals (including humans) to break down PA and release orthophosphate from the phytate molecule. To make Zn and Fe available to be absorbed (Brinch-Pedersen et al. 2002). The phytate that is not absorbed in the intestine is excreted and also contributes to phosphate pollution (Sharpley and Rekolainen 1997). In view of this, it is argued that improvement in phytase activity will not only lead to improvement of bioavailability in human intestine, but will also deal with environmental pollution that is caused due to excretion of PA. It has been reported that phytases have the potential to enhance iron absorption in the intestine from 0.6–23% to 5.5–42% (Nielsen et al. 2013). Therefore, biofortification requires low phytate and high phytase in cereals including wheat.

Variation for phytase was also studied using whole grains as well as using pearling fractions, which represented phytase content in the grain starting from the surface of the grain to the centre of the grain. The phytase activity in whole grain ranged from 640 to 1326 units/kg at one location (Nanjing) and 794 to 1496 units/kg at another location (Yanheng). In pearling, the phytase activity ranged from 1550 to 3605 units in the first five pearls and from 397 to 754 units in the 6–12 pearls at one location (Nanjing); the corresponding figures at the other location (Yancheng) were 2457 to 4178 and 357 to 803 units/kg.

Genetics of phytases (the gene PAPhy_a)

Phytase activity in the mature grains of wheat is under the control of a gene called *PAPhy_a*, so that an understanding of the function and regulation of *PAPhy_a* gene is also a component of bioavailability of micronutrients in wheat and other cereals. *PAPhys_a* gene was first reported in soybean followed by a report of its presence in several plant species including Arabidopsis and cereals including wheat. Arabidopsis genome carries as many as 25 putative *PAPhy_a* genes (Li et al. 2002). About ten cDNAs for cereal PAPhys were also obtained, which included five from wheat, three from barley and one each from maize and rice (Dionisio et al. 2011). Wheat and barley *PAPhys_a* transcripts were grouped as *PAPhy_a* and *PAPhy_b* on the basis of differences in their C-terminal amino acid. *PAPhy_a* genes are generally expressed during seed development and *PAPhy_b* genes are expressed during seed germination (for reviews, see Vashishth et al. 2017; Madsen and Brinch-Pedersen 2019). PCR-based molecular markers have also been developed for *PAPhy*s genes in wheat (Vashishth et al. 2018).

Bioavailability of Se

Literature on bioavailability of Se is also available (for reviews, see Finley 2006; Fairweather-Tait et al. 2010), and it has been shown that bioavailability of Se depends on the food source and different forms of Se. Bioavailability of Se from different sources of food and different forms of Se is fairly high, ranging from 55% to 65%, and approaching ~ 90% for SeMet and SeCys. The absorption level is 100% for selenate and 50% for selenite, but a significant fraction of selenate is lost in the urine; selenite is better retained than selenate.

For estimation of Se bioavailability, extraction of Se is often only partial, and the process of estimation can alter one form of Se (available in the food) into another form. Efforts are, therefore, needed to improve and standardize methods for selenium quantification and make these methods widely known. Similarly, although biomarkers including selenoproteins like GPX3 (Glutathione Peroxidases) and SEPP1 (Se-transporter selenoprotein P) are available for estimation of Se status, more reliable and sensitive biomarkers are required (Combs 2015). This requirement is particularly important for the assessment of bioavailability, because

functional biomarkers may respond differently to the various selenium forms, thus causing errors in estimation based on biomarkers. The bioavailability estimates made also depend on genotype, so that different estimation methods may give different values for the same genotype. Therefore, there is a need for further research on methods of extraction and estimation of different forms of Se in order to optimize the dietary recommendations (Fairweather-Tait et al. 2010).

Breeding for biofortification and bioavailability

During the past more than a decade, serious efforts have been made for breeding wheat varieties that are biofortified for Zn and Fe. Efforts were also made to improve bioavailability with limited success. These efforts involved both, the conventional breeding, which was most rewarding in terms of the release of biofortified varieties, and the marker-assisted selection (MAS), which was successful to a limited extent. Other novel approaches (including transgenics) have also been tried but have been limited to the proof-of-concept stage, and no biofortified transgenic varieties have been developed. Some details about each of these three approaches of breeding for biofortfication and bioavailability, namely conventional breeding, MAS and other novel approaches, will be briefly discussed in this section..

Conventional breeding for improvement of contents and bioavailability of micronutrients

As mentioned earlier, workers at CIMMYT developed as many as > 1500 SHWs (Rosyara et al. 2019), but many of these SHWs, particularly those developed using *T. turgidum* ssp. dicoccum/dicoccoides were tall and had poor agronomic traits, although these were rich in grain micronutrients. These were considered not suitable for direct use in wheat breeding because of the possible linkage drag. However, during implementation of biofortification programme at CIMMYT, Mexico, the derivatives of the SHWs (derived from crosses with desirable high-yielding wheat varieties/germplasm) and the landraces were utilized in crossing programme on a large scale. The crossing programme was undertaken to increase the range of genetic variation, not

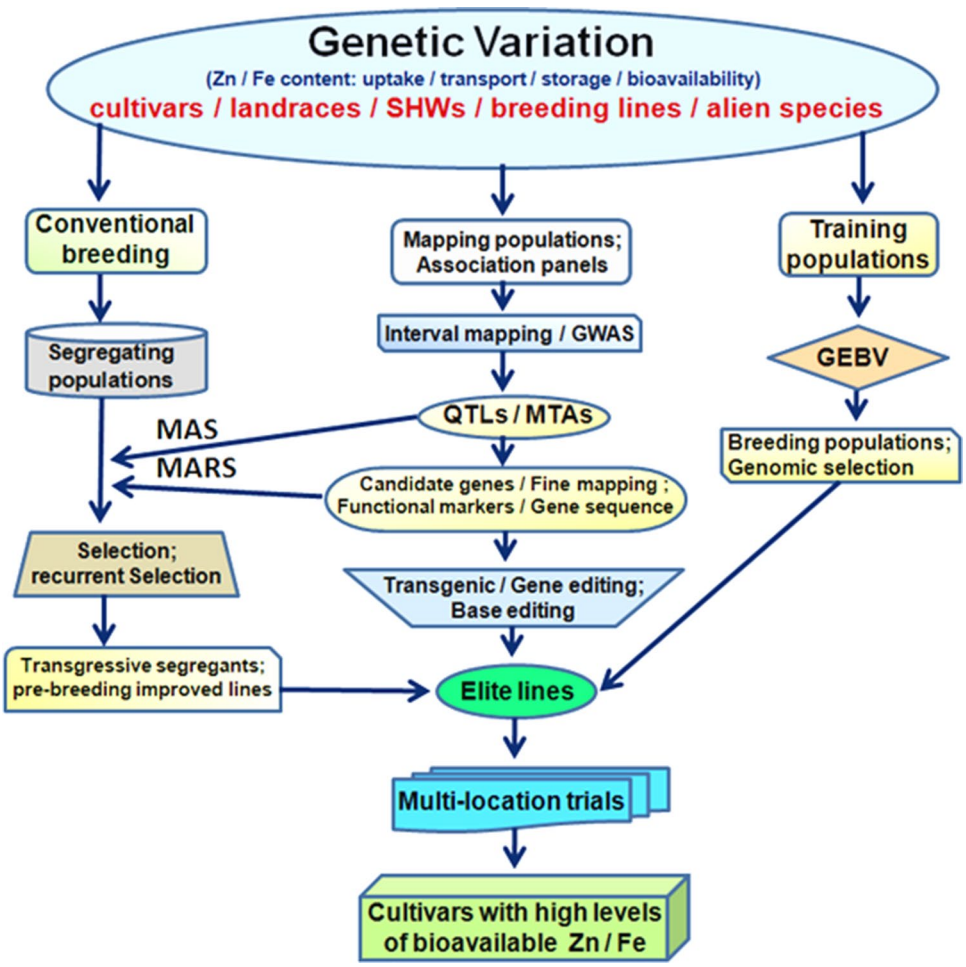

Fig. 7 Schematic representation of breeding strategies using different genetic approaches for the development of grain Zn- and Fe-biofortified cereals

only for the study of the genetics of Zn and Fe levels in wheat germplasm, but also for breeding biofortified wheat cultivars with improved bioavailability (Fig. 7; Singh and Velu 2017). The SHWs and their derivatives were particularly useful, for transfer of genes for high Zn and Fe, from diploid and tetraploid progenitors of wheat to produce high-yielding biofortified wheat lines (Fig. 8; Velu et al. 2011, 2014).

In addition to the above, the team at CIMMYT also worked in collaboration with National Agriculture Research Systems (NARS) and Agricultural Universities of several developing countries (specially India and Pakistan) for developing biofortified wheat. For this purpose, the main aim was to improve the levels of Zn and Fe (Joshi et al. 2010). However, initial focus was on improvement of Zn alone, because it was known that bioavailability of Fe was poor (Velu et al. 2014) and that there was significant and positive correlation between the Zn and Fe contents in the grain (Gomez-Becerra et al. 2010; Morgounov et al. 2007; Zhao et al. 2009; Guzman et al. 2014). After having conducted two multi-location HarvestPlus Yield Trials (HPYTs) in 2011–12, HarvestPlus programme produced 6–7 lines, each with 75–150% higher Zn levels; these lines also had high yield potential, resistance to rusts, and preferred end-use quality traits (see Velu et al. 2014). Following these initial successful efforts and subsequent testing of the improved lines for wide adaptability and stability, 11 Zn biofortified varieties (10 bread wheats + one durum wheat) were released for cultivation in different countries (Velu et al. 2012, 2015; Balocha et al. 2015; Gupta et al. 2020 for review). Following eight varieties were released in India: WB 02, HPBW 01, Pusa Tejas (HI 8759), Pusa Ujala (HI 1605), MACS 4028 (durum wheat). PBW1Zn, Zinc Shakti (Chitra), Ankur Shiva; the last two of these varieties were released with the efforts of the private seed companies like Ankur Seeds. Similarly, a Zn biofortified variety 'Zincol2016' was released in Pakistan. These varieties have up to 42 mg/kg Zn and up to 46.1 mg/kg Fe, which is 20% to 40% higher than the level of Zn in local varieties (Singh et al. 2017; Singh and Velu 2017; Saini et al. 2020 for review). Other recently released Zn biofortified wheat varieties include 'Nohely-F2018' released in Mexico for the Mexican valley of Northern Sonora region and 'BARI Gom 33' released in Bangladesh which showed 7–8 mg/kg Zn advantage; this latter variety also has resistance to wheat blast caused by *Magnaporthe oryzae*. More recently (in 2020), two more biofortified wheat varieties have been identified in India. The variety HI1633 is rich in grain protein, Fe and Zn contents whereas the variety HD3298 is rich in grain protein and Fe contents (these varieities are shortly to be notified by Central Variety Release Committee; personal communication with BS Tyagi, IIWBR, Karnal). Some of these varieties were developed using sources like alien germplasm and SHWs. For instance, 'Zinc Shakti' has genes from *Ae. tauschii*, 'Zincol2016' has genes from *T. asetivum* ssp. spelta and 'WB02′ and 'HPBW-01'have genes from *Ae. squarrosa* and *T. turgidum* ssp. dicoccum, respectively (see Singh et al. 2017; Saini et al. 2020 for some details). Overall, there has been a significant reward of using SHWs for the development of biofortified wheat varieties, and this is likely to continue in future due to the richness of SHWs in grain micronutrients, suggesting their continued importance in breeding for biofortification. In future, the efforts for improvement of Zn and Fe will also be combined with efforts for improvement of bioavailability, as discussed in the next section.

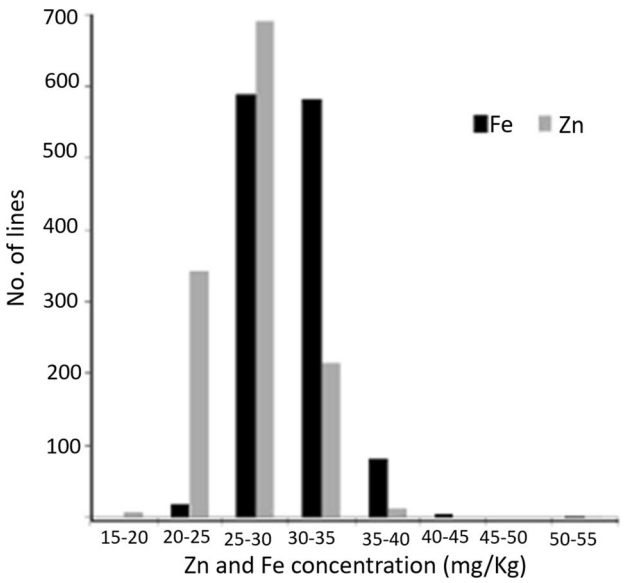

Fig. 8 Frequency distribution of grain Zn and Fe concentrations in > 1300 F_4-F_5 advanced lines grown at Obregon Mexico in 2008–2009 (from Velu et al. 2011)

Breeding for low phytic acid (lpa) for bioavailability

Low PA wheat mutant (*lpa-1*) lines have also been available for more than 10–15 years now (Guttieri et al. 2004, 2006, 2007). Significant efforts have also been made to isolate and develop genotypes with low PA (LPA), so that genotypes with 45–90% reduction in PA content became available (Sparvoli and Cominelli 2015). These efforts led to the development of biofortified winter wheats adapted to Great North American plains by incorporating low phytate mutant in lines with high contents of micronutrients and GPC (Venegas et al. 2017, 2018a, b). Although it was known that a significant decline in PA may also affect yield, it was possible to incorporate *lpa1-1* and *Gpc-B1* alleles without any adverse effect on grain yield (Venegas et al. 2018a, b), so that GPC and total grain Zn and Fe contents could be

improved in association with low PA without compromising grain yield. USDA–ARS, in cooperation with the University of Nebraska, released the following eight winter wheat lines with low PA and adapted to Great North American Plains: N16MD9012, N16MD9140, N16MD9275, N16MD9268, N16MD9046, N16MD9204, N16MD9074 and N16MD9153 (Venegas et al. 2018a, b). Six of these eight lines also contained *Lr37*/*Sr38*/*Yr17* gene combination, providing resistance for leaf, stem, and yellow/stripe rusts. No significant differences were observed for other grain traits and GPC between the LPA lines and the adapted controls. The LPA lines also had 18% improvement in Zn and 35% improvement in Fe contents over the adapted controls (Venegas et al. 2018b).

In above studies, it was also possible to obtain increased level of Pi (inorganic phosphorus) that can be readily absorbed by humans and other monogastric animals. Guttieri et al. (2004) identified a non-lethal wheat mutant (Js-12-LPA) selected from 562 EMS-mutagenized M_2 lines with a high inorganic phosphate (HIP) phenotype. In the seed of the above homozygous Js-12-LPA line, phytic acid phosphorus (P) accounted for only 48.2% of total seed P whereas in wild-type control (Js-12-WT), PA accounted for almost double the P (74.7%). Similarly, seed Pi increased from 9.1% in Js-12-WT to 50.1% in Js-12-LPA, with little effect on total seed P. However, Js-12 LPA mutant was described as 'agronomically unacceptable' (Guttieri et al. 2004) perhaps due to independent segregation of undesirable traits induced during mutagenesis. In order to develop LPA germplasm, backcrossing to transfer LPA trait from Js-12 LPA to non-LPA cultivars was also undertaken. This led to the development of LPA germplasm that was phenotypically similar to the non-LPA parent in all aspects other than P partitioning in the seed (Guttieri et al. 2006). In addition to the above mutant, another *lpa1-1* mutant with reduced P and increased Pi belonging to PA was also available (Guttieri et al. 2007). An evaluation of the LPA and wild-type (WT) sib-selections of hard red spring wheat families was conducted over two years for the distribution of total P, phytic acid P (PAP), and Pi in grains and in fractions obtained after milling. ICP mass spectrometry was used to determine the micronutrient (Ca, Cu, Fe, Mg, Mn, P, S, and Zn) concentrations in flour and bran fractions. Although, grains of WT and LPA sib line showed similar total P concentration, distribution of P between PA and Pi was altered: Pi in LPA grain was up to 340% of WT grain, and PA in LPA grain was reduced to as low as 65% of the concentration in WT grain. The results demonstrated that the LPA trait can provide flours with increased Pi concentration with minimal effect on bread flour functionality.

Introgression of the above *lpa* mutant allele into breeding programs was limited due to its negative pleiotropic effects, and it was observed that the reduction in PA content might affect various other traits like seed germination, plant growth, plant stress response, etc., since PA is known to participate in important regulatory roles (Raboy 2009; Sparvoli and Cominelli 2015; Cominelli et al. 2020). Therefore, appropriate strategy is needed to particularly reduce PA content in seeds without affecting plant and seed performance and also with minimal environmental impact (Cominelli et al. 2020).

Marker-assisted selection (MAS) for biofortification and bioavailability

Earlier in this review, we summarized the results of studies involving QTL interval mapping (IM) and GWAS that were used for the identification of a number of QTLs/MQTLs/MTAs or the corresponding genes, which are involved in uptake, transport, storage and bioavailability of micronutrients. The knowledge generated through these studies and the availability of markers associated with QTLs/genes can be utilized for biofortification and bioavailability (Fig. 7). Among a large number of markers, which became available through IM and GWAS, markers associated with two large effect QTLs on chromosomes of homoeologous groups 2 (2A, 2B and 2D) and 7B (Velu et al. 2018) have the potential for use in MAS after due validation for the targeted breeding material. Efforts are also being made to develop meta-QTLs and, at least three important meta-QTLs (MQTLs) were recently identified (our unpublished results; manuscript under preparation); such meta-QTLs may also be used in future for improvement of grain Fe and Zn through MAS. So far, however, only few examples are available, where the markers have been utilized in MAS for biofortification and bioavailability.

Among successful cases, an important example, where MAS was used for improvement in Zn and Fe, is the transfer of a high grain protein content gene *Gpc-B1*. Because this gene is closely associated with loci for high Zn and high Fe, a modest increase (18%) in Fe content was observed in near-isogenic lines of NAM-B1 (Distelfeld et al. 2007). The gene *Gpc-B1* has been introgressed into both tetraploid and hexaploid wheats in more than a dozen studies from the following seven countries: Argentina, Australia, Canada, India, Israel, Japan and USA (see Gupta et al. 2020). As a result, a number of hexaploid wheat and durum wheat cultivars containing *Gpc-B1* gene have been developed in USA, Canada, etc. Some of these varieties belong to hexaploid wheat (Lassik, Fernum, Lillian, Somerset, Burnside), whereas others belong to tetraploid wheat ('Westmore' and Desert King High Protein) (for more details see Balyan et al. 2013); 93% of the high GPC lines had significantly higher Zn content (on an average 11.6 mg kg^{-1} increase; Tabbita et al. 2017). Markers are also available, which can be used for MAS to reduce PA and improve bioavailability in wheat (Venegas

2017; Venegas et al. 2017). These observations suggest that indirect successful application of MAS for wheat biofortification is certainly possible. Efforts in this direction will certainly be intensified in different parts of the world.

Alien gene transfer for biofortification and bioavailability

Use of desirable genes from alien species should be a major activity in future for improvement of biofortification and bioavailability in wheat. There are two main approaches for transfer of an alien segment: (i) homoeologous pairing using the mutant gene *ph1b* or monosomic for chromosome 5B and (ii) radiation of alien addition/substitution lines to transfer small alien chromosome segments or gene(s) into wheat chromosomes. HS Dhaliwal and his group in India utilized both these approaches for transfer of alien chromosome segments carrying genes for high Zn and Fe to wheat. Before utilizing different approaches for transfer of alien chromosome segments, the status of the accumulation of micronutrients was examined in several wheat–*Aegilops* substitution lines. In most cases, the concentration of micronutrients was least in wheat cultivars and considerably improved in alien substitution lines, suggesting that the substituted alien chromosomes carried genes for higher Zn and Fe uptake and mobilization. A majority of alien substitution lines had much higher Zn and Fe content in all tissues, also demonstrating higher Zn and Fe uptake from soil followed by transport during grain filling duration. However, the Zn content in the plant tissues of all the lines was relatively low, suggesting that the *Triticeae* species capture and mobilize Fe to grains more efficiently relative to Zn (Sharma et al. 2017). However, these substitution lines were neither examined for grain yield and its relationship with Zn/Fe content and bioavailability, which could have been useful for proving the utility of these lines.

Induced homoeologous pairing (using monosomic 5B)

Homoeologous pairing between wheat chromosomes and alien chromosomes carrying genes for high Zn or high Fe and their bioavailability can be facilitated using either *ph1b* mutant/deletion or through the use of monosomics for chromosome 5B, which carries the gene *ph1b*. Although *ph1b* mutant has been utilized in the past for introgression of some desirable alien genes into wheat, no such efforts seem to have been made for biofortification and bioavailability of micronutrients like Zn, Fe and Se. However, monosomics for 5B have been used. For instance, interspecific hybrids of *T. aestivum* cv. Pavon (monosomic for chromosome 5B) with *Ae. kotschyi* ($U^kU^kS^kS^k$) and *Ae. peregrina* ($U^pU^pS^pS^p$) were used for precise introgression of alien segments carrying genes for high Fe and high Zn (Sheikh et al. 2016); the BC_2F_2 plants were found to have up to 125% increase in Fe and 158% increase in Zn over the recipient cv. PBW343, which also carried genes for leaf rust resistance (*Lr24*) and stripe rust resistance (*Yr36*). Interestingly, some of these plants showed improved harvest index relative to the elite recurrent parent wheat cv. PBW343 indicating the utility of the introgressed lines (Verma et al. 2016b).

Irradiation for transfer of alien segments

Wheat–*Ae. kotschyi* substitution lines were also evaluated in a study conducted by Dhaliwal and his group to identify alien chromosomes carrying gene(s) for high Zn and Fe contents in bran and endosperm fractions of wheat grains. The relevant alien chromosome substitution lines were irradiated using a dose of 40 krad. GISH and FISH analysis of some derivatives confirmed transfer of chromosome segments from $2S^k$ and $7U^k$ to the corresponding homoeologous chromosomes of the A sub-genome of wheat (2A and 7A) (Tiwari et al. 2010). Three BC_2F_4 wheat–*Ae. kotschyi* substitution lines were also found to have higher concentration of Zn/Fe in endosperm fraction (Kumar et al. 2016a).

In another study by the same group (as above), a comprehensive analysis revealed that there was 77% transferability of SSR markers from chromosomes of homoeologous group 7 of bread wheat to 7U/7S chromosomes of *Aegilops* species (Kumar et al. 2016b). Keeping this in view, the hybrid plants were irradiated and examined for SSR markers belonging to chromosomes of the homoeologous group 2. This allowed identification of small alien chromosome fragment(s) carrying genes for high grain Zn and Fe content. When compared with the elite wheat cultivar WL711, some of the derivatives had up to 54% increase in grain Zn and 65% increase in grain Fe contents. These lines also had better harvest index suggesting effective and compensating translocations of $2S^k$ fragments into wheat genome (Verma et al. 2016a).

Transfer of small alien chromosome segments from *Aegilops* species into wheat was also achieved through crosses, where irradiated pollen was used to obtain radiation hybrids carrying segments of alien chromosomes of homoeologous groups 2 and 7. The transferred segments of *Ae. kotschyi* were compensating and carried metal homoeostasis genes. Progenies carrying introgressed segments derived from pollen radiation hybrids (PRHs) were evaluated for Zn and Fe contents and grain yield. The introgressed lines showed > 30% increase in grain Fe and Zn content, which was associated with improved yield (up to 160%) relative to elite wheat cv. PBW343 *LrP* (Sharma et al. 2018). These results suggested that lines carrying introgressed alien segments can be utilized for improvement of Zn and Fe contents and their bioavailability.

Some novel approaches for biofortification and bioavailability

In addition to the above, several other novel approaches (including biotechnological approaches) have also been employed to enhance micronutrient enrichment and their bioavailability in crop plants, with limited success (Bhati et al. 2014). These approaches make use of the following material/strategies either in conventional breeding programme or direct use on farmers field after subjecting the developed lines to normal procedure of coordinated trials for release of cultivars: (i) production of low PA (*lpa*) mutants (mentioned earlier);, (ii) overexpression of genes encoding phytases (Lucca et al. 2001; Hong et al. 2004; Brinch-Pedersen et al. 2000, 2003, 2006), (iii) overexpression of genes encoding some proteins including ferritin (Drakakaki et al. 2005; Masuda et al. 2013) and nicotianamine synthase (NAS) (Lee et al. 2012; Zheng et al. 2010; Beasley et al. 2019; Zheng et al. 2010), (iv) post-transcriptional gene silencing (PTGS) of genes involved in PA biosynthetic pathway; such genes include *TaABCC13* and *TaIPK1*(inositol pentakisphosphate kinase); this involves use of RNAi and has been shown to result in reduction of PA by 22–34% in case of *TaABCC13* and 28–56% in case of *TaIPK1* (Bhati et al. 2016; Aggarwal et al. 2018), (v) high expression of genes like *TaFERRITIN* and *TaVIT, OsNAS2*, (vi) use of heterologous genes encoding phytase (e.g. *phyA* from *A. japonicus*; *SrPf6* from *Selenomonas ruminantium* and *appA* from *E. coli*; this has been successfully achieved in rice and wheat (Abid et al. 2017; Lucca et al. 2001; Hong et al. 2004; Brinch-Pedersen et al. 2000, 2003, 2006). (vii) Improvement in the expression of enzyme phytase; this approach has been shown to result in threefold improvement in bioavailability of Fe in maize (Drakakaki et al. 2005). (viii) Transfer of gene for phytase thermotolerance, so that phytase activity is not lost during cooking (Brinch-Pedersen et al. (2006). (ix) Targeting Fe specifically to the endosperm, to improve its concentration in endosperm, so that bioavailability of Fe may not be affected by the phytate levels (Connorton et al. 2017). However, only few of the above approaches have been applied in wheat. Although above-mentioned approaches allowed increase in total Fe content in staple crops but only marginal success was achieved in improving the bioavailability of micronutrients (Raboy 2009; Shi et al. 2007). The successful use of these approaches should be possible in future.

Conclusions and future prospects

From the literature reviewed in this article, it is apparent that during the last two decades considerable knowledge in wheat has been generated on biofortification and bioavailability of micronutrients including Zn, Fe and Se. Successful efforts have also been made in developing some wheat cultivars with improved levels of grain Zn and Fe content. But, the available information has not been fully utilized in breeding programmes. Also wheat genome sequences, which became available recently including the exome sequence is yet to be utilized. Since sequences of a number of genes involved in biofortification in wheat are now known, these genes can be utilized for development of transgenics and a variety of genetic engineering approaches that have become available. The novel breeding approaches for biofortification and bioavailability of micronutrients include the following: (i) use of tissue-specific high-expression promoters with important desirable genes for developing transgenics for biofortification and bioavailability; (ii) use of reverse genetics approaches including RNAi for functional characterization of a large number of genes encoding transporters, transcription factors, and proteins/enzymes that are important for biofortification and bioavailability; (iii) use of genome editing and base editing using a variety of CRISPR/Cas systems that have been developed during the last five years (Borisjuk et al. 2019; Gupta 2019). For instance, the CRISPR-based approach and T-DNA insertion have already been utilized for knocking down the gene *OsVIT2* in rice to achieve an increase in grain Fe (Bashir et al. 2013; Ludwig and Slamet-Loedin 2019). Similar approaches can be used in wheat also. (iv) Bioinformatics can be used for identification and characterization of new genes and the corresponding proteins, which have already been identified and characterized in other plant species including rice and Arabidopsis. Wheat genes for biofortification and bioavailability, which have already been characterized, with their sequences known, can be subjected to either overexpression (in case of desirable genes) or knocking down of genes (with a negative role).

In wheat, some success in the application of genetic manipulation to improve yield, nutritional value and health-promoting qualities, and for enhanced resistance to various biotic and abiotic stresses has already been achieved, but much more needs to be done for biofortification and bioavailability. The basic work on genetics, physiology and molecular basis of biofortification and bioavailability for generating knowledge also need to be continued. For instance, according to the present state of knowledge, wheat makes use of Strategy II (chelation-based uptake and transport), but there is some evidence at least in rice that Strategy I (reduction-based strategy) involving uptake, transport and storage of reduced form of micronutrients (Fe^{2+}) also operates. Therefore, if it is established that both strategies are used in wheat (even if Strategy II is the major approach), experiments may be designed to deal with both the strategies for uptake and transport of micronutrients.

Transcription factors, which regulate the expression of many genes encoding proteins (including transporters and

a set of enzymes), can also be targeted for improvement of biofortification and bioavailability. A search may be made for allelic variation in these genes, with an aim to identify alleles, which may improve transport of micronutrients. One such TF is NAM-B1 (a NAC transcription factor). When the activity of this TF was compared between ancestors/progenitors of wheat with the present day wheats, it was shown that the alleles in ancestors were responsible for accelerated senescence and increased remobilization of micronutrients from the leaves to the grains (Uauy et al. 2006). Similar allele mining can be undertaken for a variety of other desirable/undesirable genes.

Strategies will also have to be developed in order to respond to changing climate and agricultural practices, since these will influence adversely not only yield, but also biofortification and bioavailability of micronutrients. For instance, the high temperature and water availability may become a serious constraint. Increase in CO_2 concentration in the atmosphere and reduced fertilizer input may also have an adverse effect on the concentration of grain micronutrients. Therefore, strategies are being developed to produce more with less water and less fertilizer; biofortification and bioavailability should be an important component of all such efforts. For this purpose, we should have knowledge about the expression of genes encoding different components of the machinery used for biofortification and bioavailability. We will also have to learn, how changes in gene sequences will influence different traits including biofortification and bioavailability, so that gene/base editing approaches may be deployed for sustaining the current efforts being made to achieve desired biofortification and bioavailability.

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