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Moving micronutrients from the soil to the seeds: Genes and physiological processes from a biofortification perspective

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Abstract

The micronutrients iron (Fe), zinc (Zn), and copper (Cu) are essential for plants and the humans and animals that consume plants. Increasing the micronutrient density of staple crops, or biofortification, will greatly improve human nutrition on a global scale. This review discusses the processes and genes needed to translocate micronutrients through the plant to the developing seeds, and potential strategies for developing biofortified crops.

Keywords: iron, zinc, copper, remobilization, micronutrient transporters, biofortification

Contents

1. Introduction

Seeds are major sources of human and animal foods, and also the input material for cultivating most agricultural crops. Seed quality determines suitability of seeds for both of these purposes. One component of seed quality is chemical composition, such as the concentrations of mineral elements, including micronutrients such as Fe, Zn, and Cu. The maternal plant supplies the metabolic building blocks that ultimately make up the seed. These inputs are influenced by environmental conditions and by the genetic makeup of the maternal plant. An increased understanding of control over supply of nutrients to developing seeds will allow development of improved varieties that can respond favorably to maintain seed production and quality in adverse environments. This understanding will also allow production of biofortified seeds with increased concentrations of micronutrients.

Plant derived foods provide an important source of proteins and dietary minerals. This is especially true in developing countries where plant foods are a predominant portion of the diet. The concentrations of some minerals, especially iron, zinc, iodine, and selenium, are inherently low in plants as opposed to animal derived foods. As a result, more than 3 billion people worldwide suffer from micronutrient malnutrition [1,2]. Hence, there is a need to improve the mineral concentrations of important seed crops such as rice (*Oryza sativa*), wheat (*Triticum aestivum* or *T. durum*), maize (*Zea mays*), as well as common bean (*Phaseolus vulgaris*) and other legumes. Biofortification is a recent approach aimed at increasing the bioavailable nutrients, such as Fe and Zn, in these staple crops [3] rather than using fortificants or supplements. However, breeding or developing transgenic varieties for increased micronutrient density will require the enhancement of several processes involving both membrane localized transport proteins and long distance systems. The quantities of minerals in seeds depends on uptake from the rhizosphere into the roots, translocation to the transpiring shoots in the xylem, transfer into leaves or other tissues, and finally, translocation into the seeds in the phloem. A major challenge of biofortification is an incomplete understanding of the pathways and the rate limiting steps involved in translocating minerals to the seeds.

This review will focus on the genes (summarized in fig. 1) and processes in the plant that act to supply nutrients, specifically the mineral micronutrients Fe and Zn, to developing seeds. While Cu in human diets is not limiting on a widespread basis like Fe and Zn, Cu interacts with Fe and Zn, and shares common transport proteins and mechanisms. As such, a discussion of Cu transport is also included in this review.

2. What are the sources of seed micronutrients?

Specific strategies for developing crop varieties with increased seed micronutrient concentration can be developed by knowing which maternal tissues have the greatest influence on final seed composition. With this knowledge, efforts for gene discovery and targeted overexpression strategies can be directed to the most appropriate tissues.

2.1. Which tissues supply micronutrients to seeds, and at which developmental stages?

A series of studies using radioactive Fe and Zn were carried out by nutritionists to determine the best ways to produce labeled food materials for animal feeding studies (table 1) [4–10]. These studies showed that micronutrients can be translocated to seeds from various tissues, hinting at a pathway that includes root, stem, leaf, and reproductive tissues. Later, more focused studies by plant biologists corroborated these results. In pea (*Pisum sativum*), ^{59}Fe applied to leaflets, stipules, and pod walls was translocated into seeds [11]. In wheat and rice, ^{65}Zn applied to leaf tips was translocated to grain [12,13]. Foliar Zn fertilization was highly effective at increasing Zn concentration in wheat grain, especially when applied at various times during the grain filling period [1,14]. Taken together, these results suggest that the roles of vegetative tissues in supplying micronutrients to grain, and the specific mechanisms involved, should be a continued focus of future research efforts.

2.2. Remobilization or continuous uptake?

During the senescence stage of development, remobilization (i.e., net export of stored or recycled nutrients) of some nutrients occurs from vegetative tissues, such as leaves and stems. At the end of growing seasons, mass senescence typically occurs in leaves and other tissues. These remobilized nutrients are most likely moved to developing seeds in annual crop species, providing that the senescence and seed import are synchronized to provide source-sink relationships. While it is well established that remobilized N is a major source of seed protein components for wheat and barley [15–17], data for other mineral elements is less abundant, although older studies demonstrated remobilization of Cu, Fe, and Zn in legumes [18,19] and wheat [20–22], and some newer studies have found similar results [23,24]. Remobilization of Fe and Zn increased when these micronutrients were withheld from the hydroponic solution post-anthesis, indicating that remobilization mechanisms might be upregulated under nutrient limitation [24]. In this wheat study, flag leaf and lower leaves were the major sources of remobilized micronutrients, in contrast to results from rice, where stems accounted for the major source of Zn remobilized to grain [13]. In another rice study, the authors concluded that remobilization accounted for very little grain Zn, and that uptake after anthesis supplied the majority of Zn to grain, with most Zn passing through stems, and only a small amount passing through leaves [25]. It is possible that differences in plant anatomy between rice and wheat [26] account for these contrasting observations, and highlights the importance of conducting similar experiments in a variety of plant species.

Mineral element concentrations decrease over developmental time in *Arabidopsis thaliana* rosette leaves [27,28]. This clearly suggests remobilization of minerals from these leaves, which may be used to supply seeds. Quantitative analysis of *Arabidopsis* plants over developmental time indicated that remobilization accounted for up to 30% of the total Cu, Fe, and Zn in *Arabidopsis* seeds [28]. Future crop breeding or variety development strategies may include modifications that result in increased efficiency of remobilization to generate biofortified seeds. However, in all of the studies mentioned above, remobilized micronutrient sources did not account for the total quantities of seed micronutrients, suggesting that plants continue to take up and translocate minerals into seeds over the seed filling period. The pattern of Zn and Cd distribution in the plant were similar when *Brassica juncea* plants were simultaneously exposed to both Cd and Zn to examine the transport of Cd to seeds (most likely by Zn transporters, see below). Plants treated with Cd during seed set accumulated the highest concentrations of Cd in seeds, suggesting that uptake during seed fill is an important contributor for seed mineral content [29]. Mineral micronutrients that are supplied to seeds by uptake during seed development must pass through the plant to arrive at the seeds. Thus, understanding the route of these minerals, and the genes responsible for their translocation may lead to strategies to increase flux to seeds and result in greater seed Fe, Zn, and Cu density.

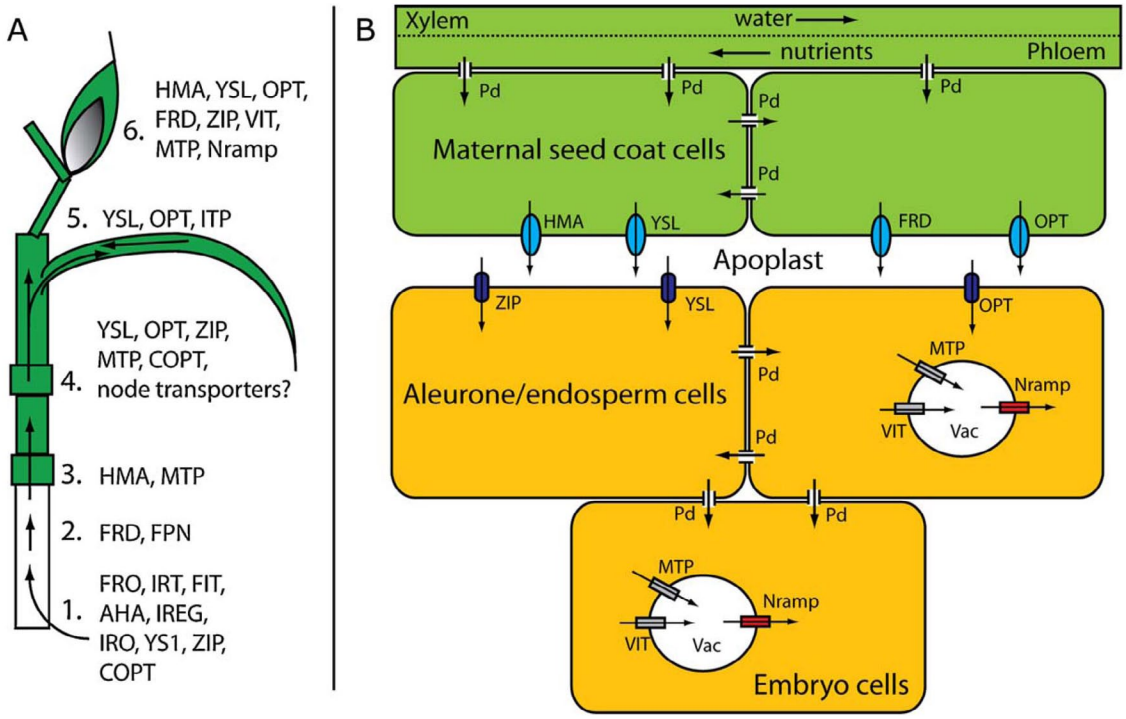

Figure 1. Model of location of Fe, Zn, and Cu uptake or transport genes discussed in this review. (A) Model of wheat plant showing the following translocation steps to the seed: 1, uptake from the rhizospere; 2, xylem loading; 3, root-to-shoot transfer; 4, distribution to the leaves or seed-covering tissues; 5, phloem loading for movement to seed; 6, loading into the seed. (B) Detail of seed loading. Gene families potentially involved in seed mineral micronutrient transport are pictured in hypothetical or known localizations. Maternal tissues are shown in green, filial tissues in gold. Efflux transporters are shown in light blue, plasma-membrane localized uptake transporters in dark blue, vacuolar uptake transporters in gray, vaculolar efflux transporters in red. Pd: plasmodesmata.

3. Defining the pathway of mineral micronutrients through the plant body

3.1. Uptake from rhizosphere

Uptake of micronutrients from the rhizosphere is the first step for accumulating micronutrients into the plant prior to translocation to seeds. Several recent reviews [30–32] have provided excellent coverage of the literature on Fe uptake processes and regulation of Fe uptake genes, such as *FIT/FER* transcription factors, *FRO* ferric-chelate reductase gens, and *IRT* ferrous-iron uptake transporters in dicots, and IREG and IRO transcription factors, phytosiderophore synthesis genes, and *YS/YSL* uptake genes in grasses [33–57] (table 2). Primary uptake of Zn is less well defined, but in *Arabidopsis* is likely carried out by Zn transporters of the *ZIP* family, some of which are Zn regulated in roots and other tissues [58]. Two transcription factors of the *bZIP* family are involved in upregulation of several *ZIP* genes in response to Zn deficiency [59]. Uptake of Cu from the rhizosphere is also not well defined, and may be carried out by transporters of the *COPT* family [60,61]. Toxic heavy metals are generally transported in plants by transporters of essential micronutrients. Cadmium and zinc have similar chemical properties and are taken up and translocated within

Table 1. Quantitative isotope labeling of seeds of crop plants

Label	Crop species	Application method	Stage of application	% of label in seed	Reference
A. Radioisotopes					
$^{59}FeCl_3$	Wheat	Hydroponic	Prior to anthesis	8	[5]
$^{65}ZnCl_2$	Maize	Hydroponic	Prior to anthesis	27.6	[4]
$^{65}ZnCl_2$	Soybean	Hydroponic	5 weeks to maturity	21.3	[6]
$^{65}ZnCl_2$	Soybean	Hydroponic	Anthesis to maturity	27.6	[6]
$^{65}ZnCl_2$	Soybean	Hydroponic	Germination to maturity	21.6	[7]
$^{65}ZnCl_2$	Soybean	Hydroponic	4 weeks prior to anthesis	28.3	[7]
$^{65}ZnCl_2$	Soybean	Hydroponic	Anthesis to maturity	16.9	[7]
$^{65}ZnCl_2$	Soybean	Hydroponic	At anthesis for one week	23.4	[10]
$^{65}ZnCl_2$	Soybean	Foliar application	At anthesis	37.5	[10]
$^{65}ZnCl_2$	Soybean	Stem injection	At anthesis	64.5	[10]
$^{65}ZnCl_2$	Wheat	Hydroponic	At anthesis	2.3	[8]
$^{65}ZnCl_2$	Wheat	Foliar application	At anthesis	57.5	[8]
$^{65}ZnCl_2$	Wheat	Stem injection	At anthesis	62.6	[8]
Label	Crop species	Application method	Stage of application	Concentration in seed ($\mu g g^{-1}$)	Reference
B. Stable isotope enrichment					
$^{65}CuCl_2$	Wheat	None (control)	Germination to maturity	8.1	[9]
$^{65}CuCl_2$	Wheat	Foliar application	At anthesis	12.9	[9]
$^{65}CuCl_2$	Wheat	Stem injection	At anthesis	22.1	[9]

Table 2. Genes implicated in uptake of Fe

Species	Gene	Description	References
A. Dicots			
Solanaceae lycopersicum	*FER*	Transcription factor – regulates Fe uptake genes	[37]
Arabidopsis thaliana	*FIT*	Transcription factor – regulates Fe uptake genes	[42,43]
Arabidopsis thaliana	*bHLH38*	Transcription factor – interacts with FIT	[51,54]
Arabidopsis thaliana	*bHLH39*	Transcription factor – interacts with FIT	[51,54]
Arabidopsis thaliana	*bHLH100*	Transcription factor – unknown targets	[51]
Arabidopsis thaliana	*bHLH101*	Transcription factor – unknown targets	[51]
Arabidopsis thaliana	*FRO2*	Ferric-chelate reductase	[33]
Pisum sativum	*FRO1*	Ferric-chelate reductase	[40]
Cucumis sativus	*FRO1*	Ferric-chelate reductase	[52]
Solanaceae lycopersicum	*FRO1*	Ferric-chelate reductase	[44]
Arabidopsis thaliana	*IRT1*	Iron transporter	[36,38,39]
Pisum sativum	*RIT1*	Iron transporter	[41]
Cucumis sativus	*IRT1*	Iron transporter	[52]
Solanaceae lycopersicum	*IRT1*	Iron transporter	[35]
Arabidopsis thaliana	*AHA2*	Proton ATPase	[57]
Cucumis sativus	*HA1*	Proton ATPase	[45]
B. Grass			
Oryza sativa	*IDEF1*	Transcription factor – regulates *IRO2*	[49]
Oryza sativa	*IDEF2*	Transcription factor – regulates *IRO2*	[53]
Oryza sativa	*IRO2*	Transcription factor – regulates Fe uptake genes	[48,50]
Oryza sativa	*IRT1*	Iron transporter	[46]
Oryza sativa	*YSL15*	Iron-phytosiderophore transporter	[55,56]
Zea mays	*YS1*	Iron-phytosiderophore transporter	[34]
Hordeum vulgare	*YS1*	Iron-phytosiderophore transporter	[47]

plants by similar pathways [62,63]. Therefore, tracing the movement of Cd could be helpful to understand the physiology and the transport of Zn. Several physiological studies with Cd and Zn in plants have demonstrated that Zn competitively inhibits Cd uptake in roots, suggesting a common transport mechanism [64–66]. It should be noted that many ZIP family proteins can transport several micronutrients, including Mn, Zn, Cu and Cd [35,41,46,52,58,59,67–87] (table 3). Since mutations in transporters can modify metal specificity [82], selecting for or engineering alleles that encode for Fe or Zn transporters that do not take up Cd presents a possible means to exclude Cd uptake into crop plants. However, some Cd can enter the xylem at the root tips where the Casparian strip is not yet formed or where it is disrupted by emerging lateral roots [88].

3.2. Xylem loading and transfer from root to shoot

Transfer of micronutrients from the root system into the shoot system has been considered a rate-limiting step for translocation to seeds [89]. Since Cd and Zn are loaded into the xylem by similar transport systems and the transfer of Cd from root to shoots (as opposed to Cd uptake at the roots) is the major physiological difference between high grain Cd and low grain Cd wheat and rice [90,91], this potentially offers another opportunity for allele selection or engineering to prevent Cd from entering the food supply. Several genes have been implicated in translocation of metal micronutrients into xylem or across the root-shoot junction (table 4), including *FRD3*, *FPN1*, *HMA2*, *HMA4*, *HMA5*, and *MTP3* [92–103]. *HMA* genes are members of the P_{1B} ATPase family [104], and some of these genes have been implicated in Zn and Cd xylem loading by pumping the ions from the pericycle to the xylem vessels [103]. Multiple copies of *HMA4* are found in the Zn hyperaccumulator *Arabidopsis halleri*, leading to higher *HMA4* expression and a mechanism for high Zn translocation rates to shoots [97]. Although much is to be learned, modification or overexpression of these genes offers potential for increasing movement of micronutrients into the shoot systems. Increased flux into shoots could possibly provide additional micronutrients for seed biofortification, and may activate native homeostasis mechanisms that increase root uptake capacity to keep pace.

Table 3. ZIP protein uptake specificities

Species	Protein	Uptake				Sensitivity	
		Zn	Fe	Mn	Cu	Cd	References
Arabidopsis halleri	IRT3	Y	Y	N[a]		N	[86]
Arabidopsis thaliana	IRT1	Y	Y	Y	N	Y	[68,70,82]
Arabidopsis thaliana	IRT2	Y	Y	N		N	[83]
Arabidopsis thaliana	IRT3	Y	Y	N		N	[86]
Arabidopsis thaliana	ZIP1	Y					[58]
Arabidopsis thaliana	ZIP2	Y			Y		[58,84]
Arabidopsis thaliana	ZIP3	Y					[58]
Arabidopsis thaliana	ZIP4	Y			Y		[59,84]
Cucumis sativus	IRT1		Y				[52]
Glycine max	ZIP1	Y	N	N	N	Y	[76]
Hordeum vulgare	IRT1	Y	Y	Y	Y		[78]
Hordeum vulgare	ZIP3	Y	N	N			[77]
Hordeum vulgare	ZIP5	Y	N	N			[77]
Hordeum vulgare	ZIP8	Y	N	N			[77]
Solanaceae lycopersicum[b]	IRT1	Y	Y	Y	Y		[35]
Solanaceae lycopersicum	IRT2	Y	Y	Y	Y		[35]
Malus xiaojinensis	IRT1		Y				[85]
Medicago truncatula	ZIP1	Y	N	N	N		[74]
Medicago truncatula	ZIP3	N	Y	N	N		[74]
Medicago truncatula	ZIP4	N	N	Y	N		[74]
Medicago truncatula	ZIP5	Y	Y	N	N		[74]
Medicago truncatula	ZIP6	Y	Y	N	N		[74]
Medicago truncatula	ZIP7	N	N	Y	N		[74]
Oryza sativa	IRT1		Y				[46,67]
Oryza sativa	IRT2		Y				[46]
Oryza sativa	ZIP1	Y				Y	[81]
Oryza sativa	ZIP3	Y					[81]
Oryza sativa	ZIP4	Y	Y				[69,72]
Oryza sativa	ZIP5	Y					[72]
Oryza sativa	ZIP7[a]	Y					[87]
Oryza sativa	ZIP8	Y	Y				[73]
Pisum sativum	RIT1	Y	Y			Y	[41]
Noccaeae caerulescens[c]	IRT1		Y			N	[80]
Noccaeae caerulescens	Znt1	Y				Y	[71,79]
Thlaspi japonicum	Znt1	Y		Y		Y	[75]
Thlaspi japonicum	Znt2			Y		Y	[75]

a. N = tested with negative results b. Formerly *Lycopersicon esculentum*
c. Formerly *Thalspi caerulescens*

Table 4. Genes implicated in the transfer of metal micronutrients (or Cd) from roots to shoots

Gene	Mineral	References
FRD3	Fe	[95,96]
FPN1	Fe	[100]
HMA2	Zn	[98]
HMA4	Zn, Cd	[94,97,98,101–103]
HMA5	Cu	[92,99]
MTP3	Zn	[93]

3.3. Translocation to leaf or seed covering tissues

Once in the shoot xylem, transpirational tension can carry micronutrients to leaves, where they may be taken into the symplast once again. Siliques have stomata [105] and carry out photosynthesis [106], and apparently transpire [107], as do glumes of wheat [108,109]. Thus, the potential for xylem transport of micronutrients directly to these seed covering tissues exists. Xylem unloading, or uptake from leaf or other xylem parenchyma apoplastic spaces into live cells, is not well characterized. For Fe, the process may be similar to primary uptake from the rhizosphere at the root surface, utilizing leaf expressed ferric chelate reductase [40,44,110,111] and Fe(II) transporters [52]. Several Zn regulated *ZIP* genes are expressed in leaves [84], as are *COPT* genes [60,61]. Studies in hyperaccumulator species have highlighted the involvement of *ZIP* genes in shoot Zn distribution. In *Thlaspi caerulescens*, the ZIP genes *Znt1* and *Znt5* are highly expressed compared to the nonaccumulator, *T. arvense* [79,112,113]. *T. caerulescens* accumulates Zn in epidermal storage cells of leaves to several-fold higher than in mesophyll cells [114]. Quantitative *in situ* hybridization indicated that Znt5, rather than Znt1 was expressed in the Zn accumulating cells [115,116]. *MTP1* is a member of the cation diffusion facilitator (CDF) family, and is has been associated with tolerance to heavy metals in shoots. *Arabidopsis MTP1* plays a role in Zn homeostasis [117,118] by Zn sequestration in the vacuole. In *T. caerulescens* and *A. halleri*, *MTP1* is highly expressed in the shoot tissues [112,116,119].

In grasses, node localized transporters can act as a sort of molecular switching station, and facilitate the xylem-to-phloem transfer of nutrients to direct translocation preferentially to flag leaves or to the spike. The transporter Lsi6 unloads silicon from xylem [120] and after panicle emergence directs silicon to the panicle [121]. Boron deficiency affected distribution of boron to the spike or flag leaf [122]. It is very possible that similar transporters for metal micronutrients exist in wheat and other species, and manipulation of these switches could direct more micronutrients toward spikes and the developing seeds, potentially improving both mineral concentrations in food and nutrient use efficiency of crops.

3.4. Phloem loading for translocation to seeds

Loading of micronutrients into the phloem is a requisite step for translocation to seeds. Even if minerals have arrived at the seed covering tissues, physical barriers between the maternal plant and developing seed and lack of transpirational tension will necessitate phloem transport to the seed tissues [126,127]. Experimental results support the idea that micronutrients pass through pods [18] or glumes [128] prior to transport to seeds. Movement of radioactive Zn through phloem from older to younger leaves, or from leaves to roots has been confirmed [129,130]. The pattern of transport and remobilization of Cd from vegetative tissues to the seeds/grains is similar to Zn [29,131]. Cadmium introduced in the cut stem of wheat was shown to be removed from the transpiration stream, loaded into the phloem in the stem, and transported to the maturing grain similarly to zinc [131,132].

Mineral micronutrients are thought to be in chelated or ligand-bound forms when not incorporated into proteins [133,134], including during phloem transport. A ligand called ITP (iron transport protein) was identified from *Ricinus communis* phloem [135], but orthologs in other species have not been described. One potential phloem chelator is nicotianamine (NA). Chemical properties of NA have been reviewed recently [136], and they include the capacity to bind Cu, Co, Fe(II) and Fe(III), Mn, Ni, and Zn. NA is a precursor to phytosiderophores in grasses [137], but NA is present in dicots as well [138]. The *chln* mutant of tomato lacks the capacity to synthesize NA [139], and has disrupted micronutrient homeostasis and accumulates abnormal micronutrient concentrations in leaves [140–142]. Transgenic tobacco that lacks NA recapitulated this phenotype and had defective floral development [143]. Conversely, when NA synthase was overexpressed, seeds had higher than normal concentrations of Fe and Zn. In *Arabidopsis*, quadruple NA synthase mutants were constructed with two sets of alleles, resulting in a sterile *chln*-like phenotype, or a less severe phenotype that could reproduce, but had seeds with low Fe concentration, was chlorotic, and accumulated excess Fe in leaves at reproductive stage [144]. This body of evidence strongly suggests that NA is a vital chelator of micronutrients for homeostasis during growth, and for translocation within vegetative parts of the plant and also in phloem transport of micronutrients to seeds.

Proteins that transport micronutrient–NA complexes have been identified in recent years. These transporters are the yellow stripe-like (YSL) proteins, which are members of the oligopeptide transport (OPT) family. YellowStripe1 (YS1) is the primary Fe(III)- mugineic acid (phytosiderophore) uptake protein in maize [34] and is also involved in internal translocation of Fe [145,146]. In nongrasses, YSL proteins seem to function in interorgan translocation rather than for primary uptake of iron at the root surface. Expression of *YSL* genes typically is localized around vascular bundles, and thus may be involved with moving micronutrients to or from the vascular tissue. *Arabidopsis* YSLs 1, 2, and 3 have been shown by yeast Fe uptake mutant complementation to transport Fe(II)-NA [147,148]. A single *ysl1* mutant had increased NA in shoots, and lower NA and Fe concentrations in seeds [149]. A double mutant of *ysl1* and *ysl3* has an even more severe phenotype, with chlorosis resembling the *chln* mutant, low pollen viability, and low seed Fe, Zn, and Cu concentrations [150], implicating nicotianamine and YSL proteins in transport of multiple micronutrients. In a study of mineral partitioning throughout the *Arabidopsis* life cycle, rosette and cauline leaves of the *ysl1ysl3* mutant plants accumulated excess Zn and Cu, while stems had lower levels, indicating that xylem transport was normal, but phloem loading was inhibited [28]. In addition to low seed Fe, Zn, and Cu, silique hulls retained these minerals, suggesting that phloem loading in this seed covering tissue is part of the pathway to import micronutrients into seeds. Grafting of inflorescence stems of *ysl1ysl3* mutants onto wild type rosettes, or the reciprocal experiment, showed that functional YSL1 and YSL3 proteins are needed in tissues of the aerial parts (i.e., flowers, cauline leaves, and silique hulls) for normal floral development and seed loading of Cu, Fe, and Mn [147]. A rice RNAi line with lowered *YSL2* expression had decreased Fe translocation to the shoots and seeds when compared to wild type, suggesting that OsYSL2 is involved in long distance transport of Fe to the seeds in rice [151].

Other members of the oligopeptide transporter family are important for transport of mineral micronutrients to seeds. The most characterized gene of the oligopeptide transporter family is *Arabidopsis OPT3*. Expression of *OPT3* was upregulated in roots and leaves of Fe-deficient *Arabidopsis* plants, and expression of this gene in yeast complemented growth defects of Cu and Fe uptake mutants [84]. Complete knockout mutants of *AtOPT3* were embryo lethal [152], but a T-DNA insertion in the promoter region resulted in an expression knockdown phenotype [153]. These mutants accumulated high Fe, Cu, Mn, and Zn concentrations in vegetative tissues, but had low Fe concentrations in seeds. Individual *OPT* family members are expressed differently from each other in various tissues [154,155], suggesting specialized functions for each gene in micronutrient transport within the plant. The substrates for plant OPTs are not well defined, but could be micronutrients chelated to NA [155], various tetra- and penta-peptides, and reduced glutathione [156].

3.5. Gradient to the seed and co-transport with N

Movement of phloem sap occurs by bulk flow, resulting from a pressure gradient from the source (leaf, stem, etc.) tissues to the sink (seed) tissues [126]. The pressure gradient is generated by loading nutrients such as carbohydrates and potassium into phloem at source tissues to provide osmotic potential that draws in water. The unloading of these nutrients at the seed decreases the pressure and maintains a gradient. Micronutrients will not be present in high enough concentrations to create a pressure gradient on their own, and thus will move with the major nutrients—that is, K^+, Cl^-, and sugars [126,157] in the phloem. The alkaline phloem sap, at pH 7.2–8.2 [157], necessitates chelation of Fe, which decreases in solubility as pH rises [158]. As mentioned above, NA is a potential chelator of Fe in the phloem. NA can form a stable complex at alkaline pH [159], and can also chelate Cu and Zn. Other potential phloem chelators are unspecified oligopeptides and certain amino acids [134]. These compounds all contain N, suggesting that phloem transport of N and mineral micronutrients may be directly related. Additional evidence for this relationship between N and micronutrient transport to seeds comes from high correlations between N and Zn in seeds of several grass and dicot species [160]. Additionally, increasing N availability to wheat, while Zn availability remained constant, resulted in increased translocation to and concentration of Zn in wheat grain [12,161]. Thus, improved fertilization practices with N and Zn fertilizers could be a large step toward biofortification. Much of the seed N is supplied as amino acids from protein breakdown during senescence [17].Wheat lines with delayed senescence had decreased amino acid export from leaves [162] and lower grain N, Fe, and Zn concentrations [163], while an RNAi line with delayed leaf senescence had lower remobilization of N, Fe, and Zn and lower grain concentrations of these nutrients [24], suggesting possible co-transport mechanisms.

3.6. Transport into seeds and storage forms

Seeds contain filial tissues (embryo and endosperm, aleurone) surrounded by maternal tissues (seed coat). The developing seed is connected to the maternal plant by a single vascular trace [126,164]. This vascular bundle ends at the seed coat and is not symplastically connected to the endosperm or embryo. Nutrients moving to the seed are unloaded from the phloem, while excess water is thought to move in the opposite direction in this vascular strand, through the xylem or suberized cells [165]. Phloem delivered nutrients are distributed in the maternal tissue surrounding the seed, and are eventually effluxed into an apoplastic space that separates the maternal and filial tissues. Thus, specific micronutrient efflux transporters are likely to be required for this action, as well as for uptake into cells of the endosperm and/or embryo. The endosperm tissue is present in mature cereals, but is consumed by the embryo in legumes and *Arabidopsis* during seed maturation [164]. For mineral micronutrients, the transporters that are involved in phloem unloading and uptake by the filial tissues are not well defined, but likely belong to the families discussed as transporters in other parts of the plant. Indeed, laser capture microdissection of barley grain into transfer cells of the maternal vascular bundle, aluerone, endosperm, and embryo fractions, followed by microarray analysis, revealed expression of genes from the following families: HMA, ZIP, MTP, Nramp, NAS, and YSL [166]. Typically the expression was not evenly distributed among the different cell types, suggesting that, as in other tissues, certain transporters are important in specific cell types to move micronutrients through the plant.

Within the *Arabidopsis* seed, Fe is stored in vacuoles of the embryo endodermis [167]. X-ray fluorescence microtomography imaging of developing embryos revealed that Zn is not confined to the same cells, but is distributed throughout the embryo [168]. Fe is loaded into the vacuoles by the VIT1 protein during embryo development [168],while upon germination Fe is remobilized from this organelle by the Nramp3 and Nramp4 transporters [169]. In legumes, Fe is stored in ferritin protein in the embryo [170,171] but ferritin is not found in the seed coat [172]. Ferritin is degraded during seed germination [171] thus releasing stored Fe for the growing seedling. Ferritin is estimated to store 20–40% of legume seed Fe [173], and in addition to amyloplast localized ferritin, Fe is stored in epidermal cells and cells near the provascular tissue of the embryo [174]. In some varieties of soybean and common bean, a substantial percentage of total seed Fe is localized to the seed coat [175,176]. In grains, the highest concentrations of Fe and Zn co-localize with protein and free amino acids in the embryo and bran, with lower concentrations in endosperm [14,160,177]. This is problematic from a human nutrition perspective, since processing of rice, wheat, or other cereals in ways that remove these parts results in greatly reduced nutritional value. The high concentration of the phosphorus storage molecule phytate in cereal grains [178] is also problematic, as phytate can also bind Fe and Zn, and decrease absorption of these nutrients in the animal gut. Increasing wheat grain Zn levels by agronomic practices did not result in increased phytate [160], and lines of cereals with mutations that result in low phytate concentration did not consistently have altered levels of Fe or Zn in rice, maize, wheat, barley, or soybean seeds [179–183]. These results suggest that phytate can be decreased in future variety development while Fe and Zn concentrations remain at current levels or higher. Feeding trials have shown that low phytate maize had substantially increased Zn bioavailability [184,185]. Decreasing antinutrients such as phytate while simultaneously increasing micronutrients could have a synergistic effect on Fe and Zn bioavailability. Strategies for altering seed phytate levels have been reviewed recently [178].

4. Future discovery

Although some of the genes that are important for translocating Fe, Zn, and Cu to seeds have been identified, there are surely many additional genes to be discovered. Knowing the pathways taken by minerals through the plant as they are translocated to seeds will give important clues on where to focus efforts when looking for new genes or validating candidates. Major focus has been on *Arabidopsis* in recent years, as it has been the most resource rich plant species, in terms of genomic information and

mutants. Information for other plant species is quickly catching up as additional genomes are sequenced (e.g., crop species rice [186], sorghum [187], and soybean [188]) and scientific communities develop databases and technical resources. With second generation sequencing technologies, transcriptomic data can be generated from any species, even those with no previous sequence information [189,190]. Although *Arabidopsis* can be a useful species for studying many aspects of understanding mineral transport to seeds, other plants species with different characteristics may be better suited for other aspects. Knowledge may be transferred more quickly to biofortified crops if additional model species are adopted, or if the staple crop species themselves are studied directly.

4.1. Quantitative genetics

Potential methods for discovering new genes that affect translocation of micronutrients to seeds include quantitative trait locus (QTL) mapping. Two methods are commonly used for mapping QTL: linkage mapping and association mapping [191–194]. Linkage mapping requires construction of biparental populations consisting of F_2, recombinant inbred lines (RILs), or backcross progeny. Association mapping is similar to linkage mapping, except that instead of using populations specifically created for QTL mapping, any sample of individuals can be used, such as existing elite lines from breeding programs or exotic accessions from germplasm collections. In many species, high-throughput genotyping is beginning to make genome-wide association mapping feasible. If genomic information allows, candidate genes for QTLs can be proposed and testing can be initiated. If there are no obvious candidate genes in the QTL region, or if genomic information is lacking, the QT genes can be pinpointed by fine mapping and positional cloning.

One strength of using QTL or association mapping is that no prior knowledge about the new genes is required for discovery. One case in point is the discovery of *NAM-B1*, a transcription factor in bread wheat that influences timing of leaf senescence, grain protein concentration, and grain Fe and Zn concentrations [24,163]. QTL mapping of seed mineral concentration has been carried out in *Arabidopsis* [195–197], *Medicago truncatula* [198], *Lotus japonicus* [199], common bean [200–202], *Brassica napus* [203], *Brassica rapa* [204], rice [205–207], and wheat [208,209]. These studies have identified several QTL for seed mineral concentrations localized in different regions and several of these mineral QTL co-localized with each other, pointing to common transport mechanisms. Several candidate genes for the different QTLs have been identified in many of these species allowing future fine mapping of these quantitative genes aimed at developing biofortified crops.

4.2. Candidate genes by expression pattern and predicted function

Combining knowledge of when and where micronutrients are translocated to seeds with gene expression data will allow identification of candidate genes. Development of genomic resources has allowed tools such as microarrays to become commonplace. These tools allow large-scale transcriptomic profiling for various experimental treatments (i.e., Fe or Zn deficient or replete), between mutants, ecotypes or species with different micronutrient accumulation (i.e., low seed micronutrient mutants) or during development (i.e., several time points over seed filling). For example, differential expression of genes in metal micronutrient (Zn, Ni) and non-nutrient (Cd) hyperaccumulator species compared to non-hyperaccumulator species may offer clues to function of genes in micronutrient translocation to

Table 5. Metal transporter genes with higher expression in hyperaccumulator species.

Non-accumulator species			Hyperaccumulator species		
Arabidopsis thaliana	*Arabidopsis lyrata* ssp. *petraea*	*Thlaspi arvense*	*Arabidopsis halleri*	*Noccaeae caerulescensa*	References
IRT2				IRT2	[212]
IRT3		IRT3/Znt2	IRT3	IRT3/Znt2	[79,86,112,113,213,216,217]
ZIP1			ZIP1		[119,213]
ZIP3			ZIP3		[213,216]
ZIP4		Znt1	ZIP4	Znt1	[71,79,113,119,211–213]
ZIP5		Znt5	ZIP5	Znt5	[113,210,212]
ZIP6	ZIP6	ZIP6	ZIP6	Znt6	[113,119,210,211,213,214,216]
		ZIP7		ZIP7	[113]
ZIP9			ZIP9		[211,213,216]
ZIP10			ZIP10	ZIP10	[212,213]
ZIP12			ZIP12		[216]
HMA1			HMA1		[213]
HMA3		HMA3	HMA3	HMA3	[113,119,212,213,216,217]
HMA4		HMA4	HMA4	HMA4	[113,212,213,216]
HMA6			HMA6		[213]
Nramp3	Nramp3		Nramp3	Nramp3	[211–214,216]
Nramp5			Nramp5		[216]
NAS3					[119,211,213,217]
NAS2			NAS2	NAS2	[211–213]
NAS4			NAS4		[211,213]
		NAS1		NAS1	[113]
MTP1/ZAT/CDF1	MTP1		MTP1	MTP1	[119,212,213,215–217]
MTP2			MTP2		[216]
MTP3			MTP3		[216]
		MTP5		MTP5	[113]
MTP8			MTP8	MTP8	[212,213]
		MTP11		MTP11	[113,213,217]
		MTP12		MTP12	[113]
FRD3			FRD3	FRD3	[212,213]
FPN1/IREG			FPN1/IREG		[213]
YSL6			YSL6		[213]
YSL7				YSL7	[212]

a Formerly *Thalspi caerulescens*.

seeds. Hyperaccumulator species have increased expression or duplication of genes involved in metal micronutrient transport across plasma membranes (*ZIP*s), in root-to-shoot translocation (*HMA*, *FRD3*, *FPN1*), xylem or phloem transfer and chelation (*YSL*, *NAS*) and in vacuole loading/unloading (*MTP*, *Nramp*) (table 5) [71,79,86,112,113,119,210–217]. Since micronutrients pass through leaves and seed covering tissues to reach seeds, genes with increased expression in these tissues during seed fill may be important for translocation to seeds. For example, 240 transporter genes exhibited increased expression during *Arabidopsis* leaf senescence [218], including some members of *YSL*, *ZIP*, *OPT*, and *HMA* families. Knowledge of gene family transport specificity, together with expression profiling to determine which specific family members are differentially expressed in certain stages of development or more highly in hyperaccumulators may allow researchers to focus on specific members of gene families for basic research or biofortification strategies.

5. Strategies for biofortification of mineral micronutrients

To this point, this review has focused on when and where, and by which genes, micronutrients are translocated to seeds. The goal for acquiring this knowledge is the application to crop development to improve human food or animal feed supplies for micronutrient density and bioavailability. The HarvestPlus program (www.harvestplus.org) has set goals to increase Fe concentration in common bean from 50 to 94 μg/g and pearl millet (*Pennisetum glaucum*) from 47 to 77 μg/g, and to increase Zn concentration in wheat from 25 to 33 μg/g, and rice Zn from 16 to 24 μg/g. Although traditional breeding or improved crop management has great potential to improve crop quality, this section will focus on transgenic strategies for biofortification of seeds.

For successful increase of seed micronutrient concentrations, genes to be expressed differently should be targeted to the appropriate localization, at the appropriate developmental stage, and the substrates for transporters (micronutrients themselves or co-transported chelators) should be present. Constitutive expression of transporters may not adequately direct when or where the increased expression of micronutrients takes place or have other unintended consequences. Constitutively overexpressing a *ZIP* transporter in barley resulted in higher short-term Zn uptake, and plants with higher concentrations of Fe and Zn [219], however the seeds were smaller and thus yield was likely reduced. Constitutively overexpressing the rice *ZIP4* gene resulted in aberrant distribution of Zn in the plant and lower seed Zn concentrations [220]. A targeted overexpression strategy may solve these types of problems.

For wheat, increasing Zn supply seems to be adequate to increase Zn grain concentrations [1,24], implying that grain sink strength or translocation to grains are not limiting factors, and that simply increasing Zn uptake may adequately biofortify seeds of some species. However, for rice, large increases in Zn supply and Zn accumulation in the plant body translated to much smaller increases in seed Zn [221]. Similar observations have been made for Fe supply in wild-type and Fe-overaccumulating mutant pea [11], and transgenic soybean that overexpressed root ferric reductase activity [222]. These results suggest that in some species, the rate limiting steps are downstream of uptake into the plant, and that a strategy to "push" more micronutrient into the plant may not be adequate by itself.

A strategy to "pull" micronutrients into the plant by increasing sink strength could be used. In this scenario, homeostatic mechanisms would allow the plant to adjust uptake to meet the additional demand. The main "pull" strategies that have been described utilize overexpression of the soybean Fe storage protein ferritin in rice grain. This strategy resulted in variable results between transgenic events and segregating lines, with no increase in some, or others with increases of up to threefold Fe concentration [223], including in the polished grain [224]. However, the increase in Fe concentration was not proportional to increases in ferritin protein levels [225], suggesting that an upstream step in micronutrient translocation to seeds was rate limiting. Transgenic maize with soybean ferritin expressed on an endosperm specific promoter had grain Fe increases of 20–70% over the control [226].

Another strategy would be to increase the translocation capacity of micronutrients to seeds by increasing transporters or substrates of transporters. Transgenic tobacco that constitutively overexpressed *NAS* had higher seed micronutrient concentrations [143,227], and rice with higher expression of *NAS3* accumulated higher Fe, Zn, and Cu [228]. Rice *YSL2* overexpression, driven by the constitutive 35S promoter, led to lower rather than higher seed and shoot Fe concentrations, however, overexpression driven by the phloem-localized sucrose transporter *SUT1* promoter resulted in a fourfold increase in seed Fe concentration [151].

The most successful transgenic biofortification of rice Fe concentration to date used a combination of increasing translocation capacity and seed sink strength. The ferritin gene from common bean was expressed on the endosperm-specific globulin promoter, while the *Arabidopsis NAS1* gene was constitutively expressed on the 35S promoter [229]. This resulted in a five- to sixfold increase in grain Fe concentration in polished rice grains.

6. Conclusion

Seed mineral improvement or biofortification will likely require simultaneous enhancement of several physiological processes, such as uptake from the rhizosphere, translocation from roots to shoots, phloem loading, and remobilization. Although progress has been made in terms of understanding micronutrient uptake and translocation and the genes involved, there remains much to be learned. Information about phloem delivery of micronutrients to seeds in different crops is particularly lacking. Multiple genes at different steps of micronutrient movement through the plant may need to be targeted simultaneously for the development of biofortified crops. Driving expression of this set of genes by common developmentally inducible promoters may be a potential strategy, as may tissue and development specific promoters to increase uptake and translocation capacity at the same time. Perhaps QTL mapping of seed mineral concentrations will reveal a "master regulator" that transmits seed micronutrient demand to the maternal plant and homeostatically adjusts uptake and translocation processes. Ideally, manipulation of such a regulator could accomplish all of these steps at once.

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