

New Insights to Zinc Biofortification of Wheat: Opportunities to Fine-tune Zinc Uptake, Remobilization and Grain Loading

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Abstract

Wheat contains low grain zinc (Zn) due to its genetics and the physiochemical properties of the soil in which it is grown. Consequently, where wheat forms a major part of the human diet, bioavailable Zn is below dietary requirements. Understanding the regulation of genes responsible for cellular Zn-transport, particularly those responsible for the control of the biosynthesis pathway of nicotianamine, provides an opportunity to increase Zn loading into the grain. Decreasing the levels of phytic acid, an inhibitor of Zn absorption in humans, provides another opportunity to increase the bioavailability of grain Zn. Synchrotron X-ray fluorescence microscopy clearly demonstrated that the crease region of the wheat grain is a major bottleneck to Zn loading in the endosperm. Higher expression of Zn transporter families, particularly metal tolerance proteins and yellow stripe like transporter families in the aleurone layer are also likely to play a major role in determining grain Zn content. Finally, anatomical barriers in the vascular region at the base of the wheat grain are a major limitation to Zn loading. Modification of any of these traits through traditional plant breeding or gene editing provides an opportunity to increase the Zn concentration in wheat grain.

Abstract

Wheat contains low grain zinc (Zn) due to its genetics and the physiochemical properties of the soil in which it is grown. Consequently, where wheat forms a major part of the human diet, bioavailable Zn is below dietary requirements. Understanding the regulation of genes responsible for cellular Zn-transport, particularly those responsible for the control of the biosynthesis pathway of nicotianamine, provides an opportunity to increase Zn loading into the grain. Decreasing the levels of phytic acid, an inhibitor of Zn absorption in humans, provides another opportunity to increase the bioavailability of grain Zn. Synchrotron X-ray fluorescence microscopy clearly demonstrated that the crease region of the wheat grain is a major bottleneck to Zn loading in the endosperm. Higher expression of Zn transporter families, particularly metal tolerance proteins and yellow stripe like transporter families in the aleurone layer are also likely to play a major role in determining grain Zn content. Finally, anatomical barriers in the vascular region at the base of the wheat grain are a major limitation to Zn loading. Modification of any of these traits through traditional plant breeding or gene editing provides an opportunity to increase the Zn concentration in wheat grain.

Key Words : Zinc, wheat, biofortification, Zn speciation, Zn transporters, grain Zn loading

Abbreviations: (DTPA) diethylenetriaminepentaacetic acid, (DMA) 2'-deoxy mugineic, (HMA) P_{1B}-ATPases: also known as heavy metal ATPases, (MA) mugineic acid, (CDF) cation diffusion facilitators family transporters also known as (MTP) metal tolerance proteins, (NA) nicotianamine, (NAS) nicotianamine

synthase, (NAM) No apical meristem also known as GPC genes, (NAAT) nicotianamine aminotransferase, (NRAMP) natural resistance associated macrophage protein, (OsFer) ferritin gene, (PA) phytic acid, (QTL) quantitative trait loci, (SAM) S-adenosyl-L-methionine, (VIT) vacuolar iron transporters, (XFM) x-ray fluorescence microscopy, (YSL) yellow stripe like transporter family, (ZIP) Zn/Iron -regulated transporter like proteins

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1.) INTRODUCTION: OPPORTUNITIES AND CHALLENGES

Wheat (*Triticum aestivum*) is grown over more land area than any other cereal, and in 2019, world wheat production was estimated to be 760 million tonnes (FAO, 2019). Wheat grain contains 60-70% starch and is an important source of calories. It also provides approximately 20% of the world's daily dietary intake of protein, 25% of the human intake of zinc (Zn), and 44% of the iron (Fe) (Henderson et al., 2007; Shewry, 2009).

The human population is at risk of inadequate Zn intake, with 17% of the global population and 30% of the South Asian population recognized as Zn deficient (Maxfield & Crane, 2019). Low levels of bioavailable dietary Zn cause adverse physiological impacts, metabolic diseases and severe health complications (J. G. White & Zasoski, 1999). Severe deficiency symptoms are readily detectable, but subclinical deficiency is difficult to detect (McClung, 2019; Sandstead, 2012). Therefore, identifying sensitive biomarkers is needed to identify people at risk. As wheat provides a large portion of the daily calorie intake in rural communities in many developing countries (Cakmak & Kutman, 2018), human Zn deficiency could be addressed by providing access to wheat grains with higher bioavailable grain Zn content.

Soil is the primary source of Zn for the wheat plant, although the plant can also absorb foliar applied Zn (Dhaliwal, Ram, Shukla, & Mavi, 2019). Plant available soil Zn is determined by the forms of Zn present in the soil, for example, water soluble, adsorbed, exchangeable, colloidal and organic matter pools (Alloway, 2008). More than 90% of soil Zn is insoluble and consequently unavailable for plant uptake. Exchangeable Zn (Zn^{2+} and $ZnOH^{+}$) in the soil is typically in the range of 0.1-2 mg kg⁻¹, which is much less than plant requirements (Barber, 1995); therefore, plants should have evolved efficient mechanisms to acquire soil Zn. Nonetheless, half of the world's wheat is cultivated on soils whose plant available Zn is so low that wheat fails to achieve its growth potential (Cakmak, 2008), including large areas in India, Sri Lanka, Turkey, China and Australia (Alloway, 2008; Cakmak, 2008; Sillanpää, 1982). Zinc deficiency in soils decreases Zn uptake and loading into the wheat grain (Rehman, Farooq, Ozturk, Asif, & Siddique, 2018); however, detailed understanding of the effects is limited.

1.1) Biofortification

P. J. White and Broadley (2005) defined biofortification as *the process of increasing the bioavailable concentrations of an element in edible portions of crop plants through agronomic intervention or genetic selection*. Compared to physical-fortification, biofortification is considered the more practical and environmentally friendly method to improve bioavailable micronutrient density in food crops as it is a one-time investment that can easily be adopted across landscapes (Meenakshi et al., 2010; Saltzman et al., 2013; Yaseen & Hussain, 2020). Further, in the recent past, the global benefits acquired from biofortified cereals have been widely reported (Glahn et al., 2017; Kaur, Kaur, & Mavi, 2020). However, as soil Zn is gradually depleted due to crop removal, physical biofortification is inevitable.

In terms of human nutrition, wheat is an ideal candidate for biofortification since it is the staple cereal for more than half of the world population and provides a substantial proportion of the daily Zn requirement (R. P. Singh & Velu, 2017). Moreover, increase in grain Zn concentration is accompanied by an accumulation of other nutrients such as Fe, Mn, Ca and K (Fernando, Panozzo, Tausz, Norton, Fitzgerald, et al., 2014; Fernando et al., 2012). Notably, there are high positive correlations occur between grain Zn and Fe content

(Cakmak et al., 2004; Velu et al., 2019; Velu, Singh, Huerta-Espino, Peña-Bautista, & Ortiz-Monasterio, 2011). Further, over-expressed ferritin gene (Osfer2) facilitates increased Fe and Zn levels in the grain of basmati rice cv. Pusa Sugandh II (Paul, Ali, Gayen, Datta, & Datta, 2012). All these findings suggest that through increasing grain Zn concentration, the concentrations of Fe, Mn, and Ca may also be improved; however, a mechanism for multiple nutrient accumulation is obscure. One possibility is that divalent cations share some transporters to uptake, transport, remobilization of Zn (Caldelas & Weiss, 2017).

Bioavailability of grain Zn in the human digestive system is mainly determined by the concentration of phytic acid (PA), which strongly binds metallic ions such as Fe, Zn, K, Mg (Perera et al., 2019; Perera, Seneweera, & Hirotsu, 2018). Wild wheat populations show a large genetic variability in nutrient accumulation and bioavailability (Bilgrami et al., 2017; Rawat et al., 2009), whereas in present day cultivars, Zn accumulation is less, perhaps due to the breeding focus on yield rather than quality (Fan et al., 2008; Murphy, Reeves, & Jones, 2008).

1.1.1) Agronomic Zn biofortification

Increases in plant Zn can be achieved through fertilizer application (soil and/or foliar) and appropriate crop rotation (De Valena, Bake, Brouwer, & Giller, 2017). Green manure applications increase Zn content in wheat grain, and nutrient release follows decomposition rate (Gruter, Meister, Schulin, & Tandy, 2018). Soil applications of Zn fertilizer can raise grain Zn concentration in wheat three or four-fold (Cakmak, 2008), and one-off soil amendments give persistent benefits in many soil types. Nonetheless, there are challenges, including: the cost, and the effects of formulation, rate and time of application, and the risk of over-application creating Zn toxicity (Cakmak, 2008). Foliar Zn application can efficiently raise Zn levels in wheat grain (Ramzan et al., 2020; Xia et al., 2018) as foliar applied Zn can translocate efficiently to the endosperm (Ajiboye et al., 2015).

1.1.2) Genetic biofortification

Selective breeding, or genetic biofortification, creates new cultivars with increased levels of micronutrients (Garcia-Oliveira, Chander, Ortiz, Menkir, & Gedil, 2018). However, the genetic variability existing is claimed to be inadequate to support development of cultivars having high grain Zn concentration (HarvestPlus, 2019). However, Cu et al. (2020) and Velu et al. (2019) disagree, and genetic biofortification through screening of natural genetic variation has been identified as the most sustainable and cost-effective way to deliver increased Zn to vulnerable populations globally (Pfeiffer & McClafferty, 2007). Fortunately, (Ramzan et al., 2020) found that the relationship between grain yield and Zn concentration is not negative, indicating that Zn biofortification targets can be achieved without compromising yield. Molecular approaches such as gene identification, gene mapping and molecular markers can assist in developing micronutrient rich crop varieties (Bouis & Saltzman, 2017; Velu et al., 2018).

Genetic engineering is a promising technology, which allows for the improvement of the nutritional quality of staple crops by transferring desirable, heritable traits between unrelated plant species (De Steur, Demont, Gellynck, & Stein, 2017). It can be used to overcome the limitation of narrow genetic variation or the presence of hybridization barriers (Giri & Praveena, 2015). For instance, transgenic maize plants with reduced levels of phytate, and transgenic rice with increased numbers of aleurone cell layers, have been genetically engineered (J. Liu et al., 2018; Shukla et al., 2009).

1.1.3) Zinc in wheat grain

More than 6000 wheat genotypes have been tested for grain Zn concentration and a large genetic variation (11.7-117 mg kg⁻¹) has been reported (Velu, Ortiz-Monasterio, Cakmak, Hao, & Singh, 2014); however, much of this variation may be environmental in origin (Fernando, Panozzo, Tausz, Norton, Neumann, et al., 2014;

Velu et al., 2018). Indeed, although some breeding lines accumulate Zn in the range of 55-102 mg kg⁻¹ (Hao, Velu, Pena, Singh, & Singh, 2014; Velu, Tutus, et al., 2017), the Zn content in the grain of most wheat cultivars is 20-35 mg kg⁻¹ (Cakmak et al., 2004; Rengel, Batten, & Crowley, 1999). Zinc is heterogeneously distributed in wheat grain, with the levels being highest Zn in the aleurone layer and embryo, and lowest in the endosperm (Ozturk et al., 2006). This distribution pattern causes a significant proportion of grain Zn to be removed during milling, and the Zn concentration is further reduced in white flour, which contains 5-10 mg Zn kg⁻¹, far below human dietary requirements for a staple dietary carbohydrate (Cakmak, Pfeiffer, & McClafferty, 2010). This is particularly important as most wheat flour-based products are made from white flour (Shewry, 2009). Antinutrients such as phytate can lower the bioavailability of Zn in wheat grain; therefore, both phytate and Zn levels are important to Zn bioavailability.

2.) SPECIATION-REGULATED ZINC CELLULAR TRAFFICKING FOR BIOFORTIFICATION

Strategies to increase the bioavailability of minerals in staple crops must take their chemical speciation into account. Zinc forms chemical complexes (Zn species) with organic molecules (ligands) through the sharing of one or more electrons. The formation of low molecular weight Zn complexes effectively facilitates intra- and inter- cellular Zn transport from root to shoot and to the developing grain (Sinclair & Kramer, 2012). A significant fraction of Zn and other micronutrients taken up by plants is present as low molecular weight organic complexes (Figure 1). Zn complexes within the plant and soil exhibit different characteristic in different environments (Impa & Johnson-Beebout, 2012), but this behaviour has not been closely studied. We argue that Zn speciation should be taken into account when attempting to increase Zn content and Zn bioavailability in the wheat grain. In the following section, we summarise routes of Zn transport and accumulation in wheat plants, discussing Zn species in the xylem, phloem and grain and how Zn species influence bioaccumulation of Zn in the grain.

Zinc species facilitate plant function by maintaining intercellular acid/base equilibrium and electron transfer reactions (Alvarez-Fernandez, Diaz-Benito, Abadia, Lopez-Millan, & Abadia, 2014). A excess of free Zn²⁺ is however toxic in the plant cell, interrupting cytosolic metabolism and restricting Zn transport to sink tissues (Caldelas & Weiss, 2017; Marschner, 2012). The formation of Zn complexes enhances Zn movement within the plant and helps to maintain the free Zn²⁺ concentration within the narrow, risk-free range for effective function (Kawakami & Bhullar, 2018). In addition, dynamic changes of Zn speciation play a role in the efficiency of Zn translocation into the wheat grain (L. J. Palmer & Stangoulis, 2018). A deeper understanding of the functions of Zn species requires careful investigation to increase bioavailable grain Zn.

Zinc forms complexes with organic compounds having N-, S- and O- electron donors (Alvarez-Fernandez et al., 2014). These compounds include non-proteinogenic amino acids such as nicotianamine (NA) [2(S),3'(S),3''(S)-N-[N(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl] azetidine-2-carboxylic acid], that are derived from NA including the mugineic acid family phytosiderophores (MA) and 2'-deoxy mugineic (DMA) amino acids (Clemens, Deinlein, Ahmadi, Horeth, & Uraguchi, 2013; Sinclair & Kramer, 2012), including histidine and cysteine (Cheah et al., 2019; Cheah, Kopittke, Scheckel, Noerpel, & Bell, 2020), organic acid-carboxylates including malate and citrate (Haydon & Cobbett, 2007), peptides (Clemens, 2019; Lemmens et al., 2019), as well as small proteins (metallothioneins) (Leszczyszyn & Blindauer, 2010) (Figure 1).

Within separate compartments of the plant, different Zn complexes exist in varying proportions (Terzano et al., 2008). Among these Zn complexes, the ligands NA and DMA play significant roles in maintaining cytoplasmic Zn²⁺ concentrations within the desirable range. Genetic composition and/or the external environment are important in determining the Zn complexes present, their quantity, and in turn their effect on Zn use efficiency of the plant (Impa & Johnson-Beebout, 2012; Moreira, Moraes, & dos Reis, 2018). For instance, within a plant compartment, Zn speciation may respond to external stress factors such as low

availability of soil Zn (Alvarez-Fernandez et al., 2014). Therefore, achieving Zn biofortification targets is likely to require deeper understanding of the compartmentalisation of Zn complexes and their environmental responses.

2.1) Nicotianamine

Nicotianamine is an intracellular metal chelator in all higher plants and the dominant Zn ligand in the cytosol, xylem and phloem, where it facilitates cellular trafficking of Zn (Rellan-Alvarez, Abadia, & Alvarez-Fernandez, 2008). Nicotianamine complexes with divalent transition metal ions such as: Zn^{2+} , Fe^{2+} , Fe^{3+} , Mn^{2+} , Ni^{2+} and Cu^{2+} (Clemens et al., 2013; Nishiyama, Kato, Nagata, Yanagisawa, & Yoneyama, 2012).

The nicotianamine synthase enzyme (NAS) regulates NA synthesis by converting S-adenosyl-L-methionine (SAM) to NA (Higuchi, Kanazawa, Nishizawa, Chino, & Mori, 1994), and expression of NAS genes in roots of *Triticum monococcum* increased under deficient Zn or Fe conditions (Du et al., 2018). Zhao, Qu, Tong, and Zou (2019) suggesting that under higher nitrogen and low Zn supply, expression of TaNAS1 and TaNAS2 genes is upregulated and the consequent increase in NA biosynthesis facilitates increased Zn uptake and root to shoot transport in wheat. Studies based on rice under Fe starved conditions, revealed that over expression of OsNAS2 and OsNAS3 genes during grain filling increased NA, Zn and Fe concentrations in the roots, shoots and grain (Lee et al., 2009; S. P. Singh, Keller, Gruissem, & Bhullar, 2017). In bread wheat, the higher expression of TaNAS genes resulted in increased biosynthesis of NA, which facilitates response to Fe deficiency (Bonneau, Baumann, Beasley, Li, & Johnson, 2016). There is a major knowledge gap in understanding the functions of the NAS gene in relation to Zn use efficiency under environmental conditions, such as variable soil Zn and pH.

2.2) Chelating compounds derived from nicotianamine

Zinc also chelates with compounds derived from NA, such as 2'-deoxy mugineic acid (DMA) and other mugineic acids (MAs) (Figure 1) that are commonly found in graminaceous plants (Diaz-Benito et al., 2018). To produce DMA, NA is converted to a 3"-keto acid by NA aminotransferase (NAAT), then to 2'-deoxy-mugineic acid, and to other MAs produced by DMA synthase (Bashir et al., 2006; Bashir & Nishizawa, 2006). Increased expression of TaNAAT2 gene in anther tissues, and TaNAAT1 in roots under Fe starved conditions, regulates DMA biosynthesis and maintains cellular Fe content at an appropriate level in wheat (Beasley, Bonneau, & Johnson, 2017). Comparable studies are required under Zn deficiency to determine the temporal regulation of these genes.

Mugineic acid is a low molecular weight, non-proteinogenic amino acid having chelating properties. The mugineic acid family of phytosiderophores are the main compounds derived from NA in gramineaceous plants, including wheat (Cakmak, Gulut, Marschner, & Graham, 1994). Zinc deficient plants produce phytosiderophores that are excreted into the rhizosphere, where they complex with Zn and increase its availability (Daneshbakhsh, Khoshgoftarmanesh, Shariatmadari, & Cakmak, 2013; Hopkins, Whitney, Lamond, & Jolley, 1998; Mitra, 2015). Therefore, it has been recognised that phytosiderophores are a promising target to improve the efficiency of Zn uptake in wheat.

3.) THE CRUCIAL REGULATORY ROUTE FOR ZN BIOFORTIFICATION

There is considerable uncertainty about the crucial regulatory routes of Zn uptake, transport, re-transport and grain loading in wheat, and filling these knowledge gaps is essential for developing cultivars having a higher level of bioavailable grain Zn. For Zn biofortification to succeed requires a greater understanding of Zn homeostasis, e.g. the facility to maintain cellular Zn levels sufficient to maintain function in the face of temporal fluctuations in the external supply (Caldelas & Weiss, 2017; Clemens, Palmgren, & Kramer, 2002). A strongly controlled network of Zn membrane transporters, regulators, Zn binding proteins and Zn speciation ensures that an adequate level of Zn reaches the sink tissues while maintaining a sufficient level of cellular Zn to maintain other regulatory functions (Kramer & Clemens, 2005; Sinclair & Kramer, 2012).

3.1) Zinc uptake

Plants have varied, complicated and highly regulated sensing and response mechanisms to control the uptake and translocation of micronutrients (Marschner, 2012). These mechanisms are governed by many genes (Xu et al., 2012). The majority of cereal cultivars that thrive in Zn deficient soils may take up larger quantities of Zn than their less successful counterparts (Graham, 1984; Ruel & Bouis, 1998). Zinc ions in the soil solution are transported towards the roots by mass flow and diffusion (Marschner, 2012). Zinc concentration in the rhizosphere (soil-root interface) is important for the efficient influx of Zn into plant roots (Khoshgoftarmanesh, Afyuni, Norouzi, Ghiasi, & Schulz, 2018). However, Zn^{2+} cannot passively enter the root cell membranes (Alberts et al., 2008) and wheat uses two strategies to take Zn^{2+} into the root system as described in the following.

In the first, the majority of Zn^{2+} enters into the root through the cell-plasma membrane, where it is taken up by an active, specific transport system driven by photosynthetic energy (Santa-Maria & Cogliatti, 1998). The Zn/Fe-regulated transporter-like proteins (ZIPs) are proposed as the major transporter family (Colangelo & Guerinot, 2006; Eide, 2005), which is highly expressed during Zn-deficiency (Pedas, Schjoerring, & Husted, 2009; Tiong et al., 2015). With adequate nitrogen supply, expression of TaZIP3 and TaZIP7 is upregulated in roots of Zn deficient wheat plants, increasing Zn uptake and grain Zn (Nie et al., 2019).

The second uptake strategy operates under low Zn availability, when the wheat root releases phytosiderophores that complex Zn, e.g. Zn-MA, and the complex is taken up with the help of Zn transporter families (Cakmak et al., 1996). In the wheat root it remains unclear which individuals of the specific transporters families are responsible for the uptake of the Zn-MA complex. However, the yellow stripe like (YSL) transporter family, in particular TaYS1A, TaYS1B, TaYSL3, TaYSL5 and TaYSL6, are involved in the uptake of the Fe-MA complex by wheat from the soil solution (Kumar et al., 2019). In wheat, it is possible that the YSL transporter family also regulates soil Zn uptake in the form of Zn-MA complex and transports it as NA-Zn complex. Further, it is also possible that the Zn-MA complex dissociates near the root surface, buffering the free Zn^{2+} activity at a higher level than in the bulk phase, and that uptake is predominately as Zn^{2+} . The detail of this uptake mechanism still needs to be studied.

Once Zn has been taken up by the roots, radial transport moves it through a number of plant cell membranes: epidermis, cortex, endodermis and pericycle, before entering the vascular tissue (xylem or phloem). Radial Zn movement from the root epidermis to xylem and phloem is critical to avoid excessive accumulation in root cell vacuoles (Ricachenevsky, de Araujo Junior, Fett, & Sperotto, 2018). To minimise excessive accumulation of Zn, plants have evolved a chelator-dependent Zn mobilization system within roots (Cakmak et al., 1996). Chelator composition varies between plant species and in wheat roots, DMA is the predominant ligand (Puschenreiter et al., 2017). Different Zn transporter families are involved in the radial movement of Zn from the root cortex to the vascular tissue. For example, in wheat, the ZIP family transporters (Evens, Buchner, Williams, & Hawkesford, 2017), $\text{P}_{1\text{B}}$ -ATPases, also known as heavy metal ATPases (HMAs) (Tan et al., 2013), and cation diffusion facilitator family transporters (CDFs), and metal tolerance proteins (MTPs) (Kolaj-Robin, Russell, Hayes, Pembroke, & Soulimane, 2015) regulate Zn transport within the plants. On the other hand, under Fe deficiency, the YSL transporter family assists Fe uptake / transport within the wheat root system (Kumar et al., 2019). It is suggested that the YSL transporter family may also support Zn uptake / transport within wheat roots under Zn deficiency (Kumar et al., 2019). However, little is known about the relationship between Zn complexes and the function of Zn transporter families in wheat (Borrill et al., 2014).

3.2) Zinc translocation, grain Zn loading and bioavailability

3.2.1) Xylem

Long distance metal transport from root-to-shoot mainly occurs passively in the xylem with the transpiration stream (Page & Feller, 2015; Sadeghzadeh, 2013). However, Zn loading and unloading from the xylem require energy and involve a number of active transporters (Sondergaard, Schulz, & Palmgren, 2004). Information on the loading and unloading of Zn in the wheat xylem is not well-documented; nonetheless, based on

model plants and other cereals, it is suggested that HMA, ZIP and YSL family transporters regulate xylem loading. It has also been suggested that ZIP family transporters play a role in xylem unloading (Borrill et al., 2014; Curie et al., 2009; C. M. Palmer & Guerinot, 2009). Further, it may be relevant that the HMA family transporters, either OshMA2, OshMA4, or both, have been involved in root xylem loading in rice (Takahashi et al., 2012).

While Zn can move in the xylem as a free cation or as a complex, the acidic pH of xylem sap (5.5) favors Zn movement as free Zn^{2+} (Alves et al., 2004). Smaller quantities of Zn complexes have been reported within the xylem of other plant species. e.g. in rocket (*Eruca vesicaria* L. Cavaleri) and the Zn hyperaccumulator, *Sedum alfredii*, significant amounts of Zn are associated with citric acid (Lu et al., 2013; Terzano et al., 2008). In contrast, peas (*Pisum sativum*) have large amounts of Zn associated with NA (Flis et al., 2016). When the xylem is alkaline in response to flooding, drought, nutrients, bicarbonate, light, daily rhythms, diseases and change of season (Wilkinson, Corlett, Oger, & Davies, 1998), conditions are more favourable to Zn complexes. It is still not known whether Zn is transported via the wheat xylem as a free cation or as different Zn complexes, e.g. little is yet known about speciation-mediated cellular trafficking of Zn in the wheat xylem.

3.2.2) Phloem

Zinc transport in the phloem is an active process (Palmgren et al., 2008). Senescing leaves are the primary site of phloem loading during Zn remobilization (Distelfeld, Avni, & Fischer, 2014). Once Zn is moved into the phloem, it can reach developing sinks such as grain, where it is in high demand. In wheat, the xylem is discontinuous at the base of each grain, whereas the phloem provides a continuous gateway for Zn movement into the developing grain. Therefore, efficient phloem loading and unloading are required to upload Zn into the wheat grain (Pearson & Rengel, 1995).

Phloem sap is slightly alkaline (pH 7.3-8.5) (Dinant, Bonnemain, Girousse, & Kehr, 2010), and this is a major limitation on Zn^{2+} mobility (P. J. White & Broadley, 2011). The formation of complexes prevents precipitation and off-target metal binding, facilitating effective long-distance Zn transport via the phloem (Sinclair & Kramer, 2012). While, Zn-NA is the predominant Zn complex (~54%) (Harris, Sammons, & Grabiak, 2012; L. J. Palmer et al., 2014), a Zn-cysteine complex is also reported in high quantities (~41%) within the wheat phloem (L. J. Palmer & Stangoulis, 2018). Further, Zn transport in the phloem is more efficient than through the xylem due to the presence of range of Zn species (N. Gupta, Ram, & Kumar, 2016). Taken together, these results suggest that increasing Zn speciation in the wheat phloem, particularly complexation with NA and carboxylic acids, enhances Zn translocation.

The movement of Zn complexes through the phloem is powered by several active transporter families that regulate Zn loading and unloading process (Yoneyama, Ishikawa, & Fujimaki, 2015). Based on studies with barley, the YSL transporter family assists phloem transport of Zn-NA complexes (Tauris, Borg, Gregersen, & Holm, 2009). On the other hand, the HMA family transporters located in the intercellular membranes, particularly the tonoplast and chloroplast membranes, appear to be involved in Zn remobilization by releasing Zn into the phloem from storage tissues in the mesophyll cell (Kim et al., 2009; Mikkelsen et al., 2012). It has also been demonstrated that Zn movement from xylem to phloem is mainly governed by ZIP family transporters (Borrill et al., 2014). Although, this process is not fully understood in wheat, it is likely that YSL and HMA transporter families regulate Zn movement into the phloem.

3.3) Wheat Grain Zn loading

The wheat grain can acquire Zn from two main sources during the grain filling, e.g. from storage tissues by remobilization and through the root system by direct uptake (D. Y. Liu, Liu, Zhang, Chen, & Zou, 2019). The relative contribution of each process has long been debated (Stomph, Jiang, & Struik, 2009; Waters, Uauy, Dubcovsky, & Grusak, 2009). Zn uptake and remobilization into the grain is largely dependent on soil and plant factors, e.g. length of the grain-filling period, plant nitrogen status, and the availability of soil

Zn and water post-anthesis (Kutman, Kutman, Ceylan, Ova, & Cakmak, 2012; Pearson & Rengel, 1994). When soil Zn levels are low (D. Y. Liu et al., 2019), or moisture limits uptake (Cakmak & Kutman, 2018), grain Zn concentration depends more on remobilization from storage organs (Xue et al., 2012). In contrast, under sufficient Zn supply, *de novo* root uptake predominates (D. Y. Liu et al., 2019), and under super optimal Zn supply, remobilization is barely detectable (Waters et al., 2009). Therefore, maintaining soil Zn uptake during grain-filling could increase levels of grain Zn; however, soil moisture is likely to constrain Zn uptake across most of the wheat cropping area of the world. Consequently, a greater understanding of Zn remobilization may provide greater opportunities to increase grain Zn content.

Remobilization of nutrients from senescing plant parts to the developing grain has been demonstrated with many plant species, including wheat (Uauy, Distelfeld, Fahima, Blechl, & Dubcovsky, 2006). In many cereals, grain development and plant senescence co-occur; therefore, nutrient remobilization, senescence and grain filling are intertwined with large scale transcriptional changes at the whole plant level (Cantu et al., 2011; Gregersen & Holm, 2007). During this stage, Zn remobilization into the grain occurs from multiple plant tissues including leaves, stems, peduncle, rachis and florets (Pearson & Rengel, 1994; Sharma et al., 2017).

Zinc remobilization into the grain and/or direct translocation of newly taken-up Zn into the developing grain is also highly regulated by the GPC-B1 gene (grain protein content) also called NAM (no apical meristem) (Uauy et al., 2006; Waters et al., 2009). The highest level of GPC genes is expressed in the flag leaf during grain filling (Uauy et al., 2006), which supports hypothesis that GPC plays a key role in Zn remobilization. The wheat genome consists of three NAM genes, but most of the modern bread and durum wheat varieties have a non-functional allele of NAM-B1, perhaps, gene product could cause delayed senescence thus reduce the remobilization and then loading into wheat grain (Velu, Singh, et al., 2017). Recently, an Australian germplasm screening study identified varieties with a functional copy of the NAM gene (Yang et al., 2018). The NAM-B1 genes that belong to NAC transcription factor found in wild emmer wheat (*Triticum turgidum* L. var. *dicoccoides*) have the ability to increase grain Zn, Fe and protein content more than that of modern wheat varieties (Cakmak et al., 2004; Uauy et al., 2006; Waters et al., 2009). These findings suggest that there are opportunities to use these varieties in wheat breeding programs as parental materials to increase grain Zn level. However, accelerated senescence caused by the same gene reduces the length of the grain filling period; therefore, the NAM-B1 gene may be a Damocles' Sword (Barneix, 2007). Fortunately, some wheat breeding lines have a positive correlation between the NAM-B1 gene and grain yield (Velu, Singh, et al., 2017). Though it is challenging to utilize only the positive aspects of the NAM-B1 gene, it remains a promising target for increased grain Zn. Information on other loci influencing grain Zn content is scarce. Therefore, identification of loci that are likely to be associated with higher grain Zn, together with higher grain yields are essential to achieve biofortification targets.

Zinc is an essential functional element of photosystem II, and a large amount of Zn is located in the vacuole, which is a major Zn reservoir for the developing grain. Several members of ZIP, YSL, HMA and NRAMP are located in the plasma membrane, vacuole or chloroplast, and regulate the direction of Zn remobilization from the source tissues to the developing grain in rice and maize (Li et al., 2013; Mani & Sankaranarayanan, 2018; Olsen et al., 2016; Sperotto et al., 2010). The functional role of each transporter family in the wheat chloroplast and tonoplast during grain filling is unclear; however, the HvHMA1 transporters facilitate the movement of Zn from the chloroplast during senescence in barley (Mikkelsen et al., 2012). As wheat, rice and barley are closely related, it is possible that the VIT and MTP transporter families are also involved in regulating vascular Zn transport. On the other hand, HMA transporters are likely to play a role in regulating Zn transport from the chloroplast in wheat.

Zinc stored in the leaf vacuole is usually complexed with ligands such as NA, malate, citrate, histidine, cysteine and PA (Sinclair & Kramer, 2012). In the leaf vacuole of wheat, Zn is co-localized with phytate and citrate, which resist the Zn translocation process and may lead to the development of Zn toxicity at the point of foliar fertilizer application (Doolette et al., 2018). In Zn hyperaccumulator plants, the main Zn ligands are cysteine, histidine and malate, which may tightly regulate the storage, redistribution and detoxifying process within the plant (Lu et al., 2014; Terzano et al., 2008). It has been suggested that Zn

remobilization can be improved by decreasing the less mobile Zn complexes in leaf vacuoles such as phytate and citrate.

Anatomical barriers are also responsible for inefficient Zn loading into the wheat grain (Palmgren et al., 2008). At the base of the pericarp, thick-walled vessel elements with no pathway via the lumina (modified tracheary elements) make the xylem discontinuous: a barrier for micronutrient translocation into the grain unique to wheat (Herren & Feller, 1994; Wang, Specht, & Horst, 2011; Zee & O'Brien, 1970). Xylem discontinuity between the floral stalk and the pericarp adds an additional apoplastic barrier between the grain and stem. Therefore, additional transport steps such as xylem unloading, phloem loading and unloading are required in the wheat rachis or peduncle for Zn in the xylem to reach the developing grain (Herren & Feller, 1994; Pearson, Rengel, Jenner, & Graham, 1995) via the phloem (Wang et al., 2011; Zee & O'Brien, 1970). Pearson et al. (1995) suggest that the majority of Zn enters into the phloem with the help of a xylem-to-phloem transport system operating in the rachis. Small amounts of Zn could also be transferred from the xylem sap into the phloem in the peduncle (Herren & Feller, 1994). One approach to increase the efficiency of Zn loading into the wheat grain would be if Zn were translocated directly to the grain through the xylem and such a mechanism has been reported in rice (Zee, 1972). Identifying wheat mutants or wild relatives without these anatomical barriers could provide a major breakthrough in Zn biofortification.

Zinc loading into the endosperm of the wheat grain is the major bottleneck to increase Zn content in white flour. Zinc transported into the stem phloem of wheat enters the grain crease phloem where it transfers into the vascular bundle, and moves through the transfer cells of the nucellar projection into the endosperm cavity (Borg, Brinch-Pedersen, Tauris, & Holm, 2009; Pearson, Rengel, Jenner, & Graham, 1996). From there, Zn is taken up by cells of the modified aleurone layer before being transferred into the endosperm, embryo and aleurone layer. Consequently, a higher Zn concentration is present in the aleurone layer and embryo, but the endosperm contains relatively low amounts of Zn. The highest Zn content was reported in modified aleurone cells adjoining the crease region (De Brier et al., 2016). It has been demonstrated that constitutive expression of OsNAS2 gene in the modified aleurone layer of wheat grain is well associated with Zn concentration in the crease region (Beasley et al., 2019). In many instances, Zn concentration increases in the crease region when Zn is externally supplied, but endosperm Zn concentration increases only at a marginal rate (Ajiboye et al., 2015; Stomph, Choi, & Stangoulis, 2011). These findings clearly demonstrate tight physical and/or physiochemical control of Zn transfer from the crease region transfer cells to the inner endosperm. De Brier et al. (2016), suggesting that Zn transfer to the endosperm is inhibited and/or slowed at the transfer cell level. It is possible that the presence of specific Zn binding ligands in the transfer cell vacuole act as a crucial limiting factor for Zn transfer into the endosperm (Wang et al., 2011). Further, the presence of phytate globoids in the aleurone cells also restricts Zn mobility, causing lower Zn concentration in the endosperm (Ajiboye et al., 2015). On the other hand, in wheat endosperm, Zn is present as complexes with NA, DMA and S-containing peptides (Eagling et al., 2014; Persson et al., 2016; Xue et al., 2014). In rice, NA and DMA help to increase Zn content in the grain (Lee et al., 2011). In the embryo of wheat grain, higher levels of Zn is reported in the root and shoot primordium by making a complex with sulphur, probably thiol groups such as cysteine (De Brier et al., 2016).

Studies of foliar Zn application have suggested that Zn transfer to the inner endosperm from the rachis is relatively easy when Zn is applied during grain filling (Ajiboye et al., 2015). Under these conditions, 70-80% of the grain Zn is complexed with phosphate (Zhang et al., 2018), and a smaller proportion of endosperm Zn is complexed with phytate (Velu et al., 2014). Therefore, most of the foliar applied Zn present in the wheat endosperm is bioavailable (Ajiboye et al., 2015). These findings suggest that strategies for Zn uptake into the grain may differ when it comes to soil and foliar applications, which may create new opportunities for Zn biofortification.

Transegenic approaches have been made to over express Zn transporters in wheat grains. Vatansever, Filiz, and Eroglu (2017) showed that overexpression of MTP transporters in the aleurone layer contributes to high level of Zn in wheat grain. Kumar et al. (2019) suggested that TaYSL20 is a key candidate gene regulating mineral distribution in the wheat grain. Moreover, higher expression of TaYSL3-2A, TaYSL12-2A, TaYSL6,

and TaYSL15-3D are also reported in the seed coat and aleurone layer. The expression of these genes and grain Zn and Fe levels are well correlated across a number of wheat cultivars. However, in-depth knowledge is required to understand how Zn transporters are regulated in a site-specific manner and how they are associated with Zn localization in the wheat grain.

Detailed descriptions of the crease region and embryo are shown in Figure 2.

3.4) Anti-nutritional factors controlling the bioavailability of grain Zn

The bioavailability of Zn and other micronutrients in the human diet is lowered by the low Zn levels in wheat flour, together with the anti-nutritional factors in plant-derived foods (Perera et al., 2018). Phytic acid [(myo-inositol-1,2,3,4,5,6-hexakisphosphate) and polyphenols (Schlemmer, Frolich, Prieto, & Grases, 2009) are recognised as major determinants of the bioavailability of Zn and other essential micronutrients. Phytic acid, the main store of phosphorus in the grain, makes micronutrients less available for monogastric animals, including humans, due to its chelation characteristics (R. K. Gupta, Gangoliya, & Singh, 2015). In general, PA is abundant in the aleurone layer and pericarp (2.0-5.3%), while lesser amounts (1.1-3.9%) have been recorded in the embryo (Kasim & Edwards, 1998). Most of the micronutrients and macronutrients including Zn, Fe, Ca, Mg and K in the aleurone layer complex with PA. Further, nutrient transfer is inhibited at the aleurone layer level, which lowers both Zn concentration and its bioavailability (Raboy, 2003; Schlemmer et al., 2009; Simpson & Wise, 1990). Therefore, lowering the grain PA level is an indirect strategy to improve Zn concentration and its bioavailability in the wheat grain (Gregory et al., 2017).

Large genetic variations in grain PA levels occur among wheat cultivars (Shitre et al., 2015). This existing genetic variation could effectively be utilized to select wheat lines with less PA content for current breeding programs (Magallanes-Lopez et al., 2017). There are other opportunities to reduce PA content; for example, wheat lines with lpa1-type mutants have about 43% less PA in the grain (Guttieri, Bowen, Dorsch, Raboy, & Souza, 2004). Moreover, lower PA concentrations could be achieved by genetically transforming the plant to produce greater amounts of phytase (Brinch-Pedersen, Madsen, Dionisio, & Holm, 2017; Brinch-Pedersen, Olesen, Rasmussen, & Holm, 2000), to release phosphate and mineral residues from phytate (Brinch-Pedersen, Sørensen, & Holm, 2002). However, PA may play a role in signalling plant development and defence responses; therefore, careful consideration is needed (Sparvoli & Cominelli, 2015).

Based on the cited studies, a schematic model of speciation-mediated Zn transport in the wheat plant is presented in Figure 3.

4.) CAPITALIZING ON GENETIC DIVERSITY FOR Zn BIOFORTIFICATION IN WHEAT

A number of genetic traits can be used for enhancement of grain Zn concentration and its bioavailability. Genetic variation in wild relatives can be used to develop bread wheat, e.g. crossing hexaploid bread wheat with tetraploid durum has helped to increase grain Zn concentration. Further, wheat wild relatives *Aegilops kotschyi* and *Aegilops tauschii* also contain appreciably higher concentrations of grain Zn. Identifying and mapping molecular markers for quantitative trait loci (QTL) for grain Zn can be used to select for increased grain Zn concentration (Rawat et al., 2009; Tiwari et al., 2010). Inter-specific crosses can be made in wheat breeding programs and these traits can be easily captured using marker assisted selection (Rawat et al., 2009; Tiwari et al., 2010). However, for genetic biofortification to be quickly implemented, it is preferable to have high variability within adapted, high quality and high yielding bread wheat lines (Pascoalino et al., 2018). In this scenario, preference should be given to wheat genotypes that grow well in soils low in available Zn (Gomez-Coronado, Poblaciones, Almeida, & Cakmak, 2016). However, this is a temporary solution as the soil Zn resource is finite.

Compared to many other agriculturally important cereals, wheat is a relatively difficult breeding target. The wheat genome is large and complex (16,000 Mb), allohexaploid ($2n = 6x = 42$) comprising AABBDD

genomes, and exhibits a low transformation frequency, high proportion of repeat DNA and low regeneration after transformation (Bhalla, 2006; Sahrawat, Becker, Lütticke, & Lörz, 2003). Amongst all this, knowledge gaps in the complex molecular pathways, transporters, genes, and speciation involved in Zn homeostasis are bottlenecks, which may limit the success of genetic biofortification. Therefore, a detailed understanding of the mechanisms of Zn uptake, translocation, remobilization, and grain loading will provide much needed information to fast-track genetic biofortification.

5.) PRESENT KNOWLEDGE AND OPPORTUNITIES FOR FUTURE RESEARCH TO ACHIEVE WHEAT BIOFORTIFICATION TARGETS

Grain Zn concentration is strongly influenced by Zn translocation, remobilization and grain loading (Palmgren et al., 2008). Therefore, it is essential to select genetic traits with better Zn uptake together with efficient Zn translocation, remobilization and grain loading for wheat breeding to achieve Zn biofortification. Inefficient Zn loading into the grain, particularly to the endosperm, is a major bottleneck to improved Zn levels in wheat grain (Ajiboye et al., 2015). On the other hand, there are opportunities to enhance the transfer of Zn from source organs, e.g. roots, leaves, stem and peduncle, into the grain by selecting wheat cultivars having efficient remobilization (Pearson & Rengel, 1994; Waters & Sankaran, 2011). Moreover, considerable genetic variation has been detected in Zn use efficiency among wheat cultivars (Kalayci et al., 1999; Velu et al., 2011; Velu, Tutus, et al., 2017). These findings suggest that grain Zn concentration can be improved 3-5 fold if superior genetic traits are embedded during breeding.

NAM genes that are responsible for increased grain Zn content have already been mapped and major QTLs have been identified for this trait in wild emmer wheat (*Triticum turgidum* subsp. *dicoccum*) (Joppa, Du, Hart, & Hareland, 1997). These genes have now been cloned and have increased Zn, Fe and protein content by 12%, 18% and 38%, respectively (Distelfeld et al., 2007; Uauy et al., 2006). *Aegilops* genes responsible for higher grain Zn and Fe content have also been transferred from wheat-*Aegilops* substitution lines into modern wheat cultivars to increase the grain Zn content (Sharma et al., 2018). A problem with all of these wild crosses is linkage drag and the need to reduce the size of the introduced chromosome segment to minimize the deleterious effect on wheat morphology, yield quality or disease resistance.

Increasing the total Zn content in wheat grain is not the only focus of wheat biofortification, as absorption in the human digestive tract depends on the presence of anti-nutritional factors, notably PA (Ciccolini et al., 2017). Enhancing bioavailability of Zn in the grain by lowering PA levels is another approach (Chugh & Dhaliwal, 2013). The wheat genes responsible for PA biosynthesis and transport have been identified and can be manipulated to minimise PA biosynthesis (Sparvoli & Cominelli, 2015). In addition, QTLs for PA have been identified in wheat and moved into breeding programmes to reduce PA levels in the grain (Raboy, Young, Dorsch, & Cook, 2001). At present, grains with low PA content are being developed either through low-PA mutants produced by chemical, or irradiation-induced mutagenesis, or by transgenic *Aspergillus niger* (*phy A*) phytase gene transformed into wheat (Ahn, Kim, Jo, Kim, & Byun, 2004; Venegas et al., 2018). Combining two genes (*lpa1-1* – low grain PA and *Gpc-B1* – grain protein content) has been shown to increase the grain Zn content while maintaining a higher grain yield (Venegas et al., 2018).

Development of genome editing techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein 9 (Cas9) allows for the production of transgene-free improved varieties. Wheat grains with higher endosperm Zn content, reduced PA level, increased endosperm NA and thicker aleurone cell layer can be engineered in future by targeting multiple genes in a pathway level via the CRISPR/Cas 9 technique and can be effectively adapted to improve micronutrient assimilation into the grain. A fundamental understanding of the molecular mechanisms involved in Zn homeostasis in the wheat plant, including the pathways for Zn uptake, transport, remobilization and grain Zn loading, will in the future, help to develop Zn biofortified wheat.

6.) CONCLUSIONS

With traditional breeding approaches, the complexities of the wheat genome make it difficult to predict the rate of improvement. Nonetheless, modern wheat cultivars have greater Zn uptake, but Zn loading into the endosperm remains restricted due to biochemical limitations in the crease region. Zn bioavailability is also reduced as a result of increased accumulation of PA in the grain. However, these limitations are not common among old wheat progenitors. A deeper understanding of the cumulative gene effects that regulate specific functions of Zn uptake, transport, remobilization, grain loading, Zn speciation and synthesis of PA is important to increase the level of bioavailable grain Zn.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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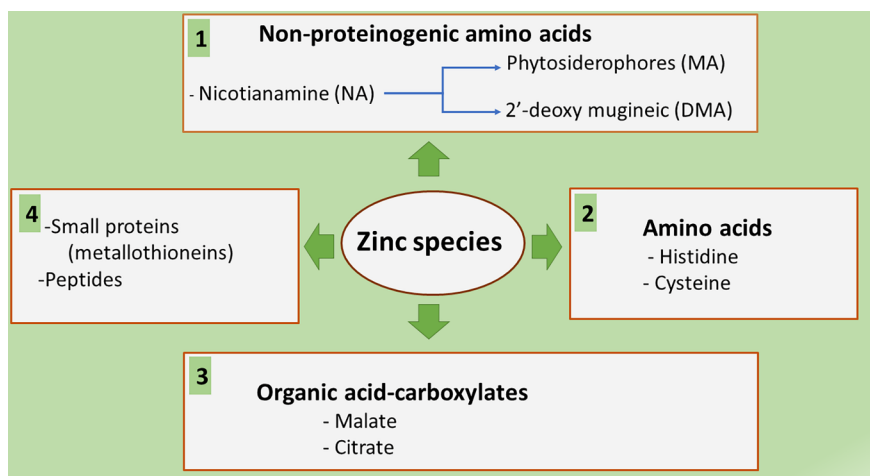


FIGURE 1. Low molecular weight Zn complexes commonly found in plants. Zinc can complex with N-, S- and O- electron donors in organic ligands. Zinc complexing agents are grouped into four main categories: 1) non-proteinogenic amino acids such as nicotianamine (NA), and chelating compounds derived from NA, including the mugineic acid family of phytosiderophores (MA) and 2'-deoxy mugineic (DMA); 2) amino acids including histidine and cysteine; and, 3) organic acid-carboxylates including malate and citrate; and 4) small proteins (metallothioneins) and peptides.

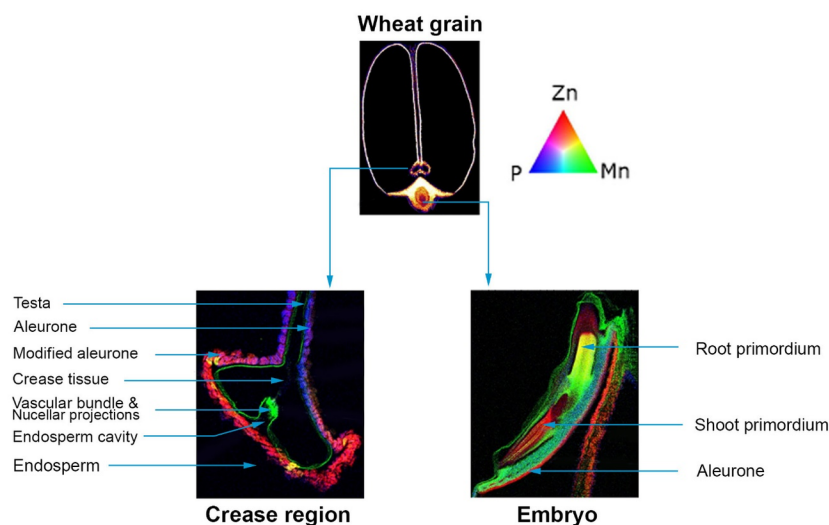


FIGURE 2. X-ray fluorescence microscopy (XFM) elemental map of transverse and longitudinal sections of wheat grain. Different locations of the crease region and the embryo of the wheat grain are elaborated. Tricolour RGB colour maps show the co-localization of Zn (red), P (blue) and Mn (green) in the embryo and crease region. Overlap (correlation) between Mn (green) and Zn (red) is shown as yellow (unpublished data).

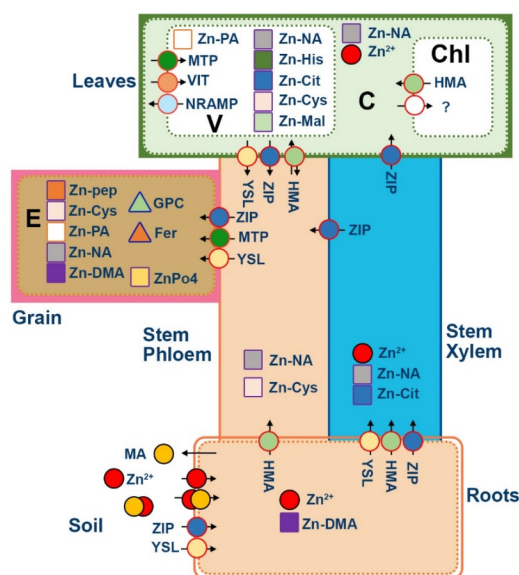


FIGURE 3. A simplified diagram of the putative mechanism of speciation-mediated Zn uptake, transport, and grain loading in wheat. Zinc in the soil solution enters roots as free Zn^{2+} or as a complex with

mugineic acid (MA), regulated by yellow stripe like (YSL) and Zn/Fe-regulated (ZIP) transporter families. Once taken up, Zn is either translocated in the form of free Zn^{2+} , or as Zn complexes, or stored as Zn complexes. In the wheat root, Zn preferentially binds with nicotianamine (NA) and 2'-deoxy mugineic (DMA) facilitating translocation and inter-cellular homeostasis. Movement of Zn is powered by different transporter families including ZIP, YSL, $\text{P}_{1\text{B}}$ -ATPases: also known as heavy metal ATPases (HMA), natural resistance associated macrophage protein (NRAMP), metal tolerance proteins (MTP) and vascular iron transporters (VIT), which are located in the plasma or organelle membranes of the cell. Different Zn speciation, and transporter families, function in different areas of the wheat plant to regulate Zn translocation. Chelated or free Zn^{2+} is sequestered into the leaf vacuole (V) and chloroplast (Chl) and remobilized when needed. MTP and VIT transporter families are involved in sequestering Zn into the vacuole while transporters involved in sequestering Zn into the chloroplast are not clear. NRAMP and HMA family transporters are responsible for liberating stored Zn from the leaf vacuole and the chloroplast back into the cytoplasm during remobilization to be loaded into the phloem. In the leaf vacuole, Zn is complexed with NA, histidine (His), citrate (Cit), cysteine (Cys), malate (Mal) and PA. Zn loading in the grain is mainly regulated by ZIP, MTP and YSL transporter families. The majority of the Zn in the wheat endosperm (E) forms complexes with PA, Cys, peptides (Pep), ZnPO_4 , NA and DMA. The ferritin gene (Fer) and grain protein genes (GPC), also known as no apical meristem (NAM) genes, also regulate endosperm grain Zn content.