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REVIEW PAPER

Zinc deficiency responses: bridging the gap between Arabidopsis and dicotyledonous crops

Noémie Thiébaut and Marc Hanikenne*

InBioS - PhytoSystems, Translational Plant Biology, University of Liège, 4000 Liège, Belgium

*Correspondence: marc.hanikenne@uliege.be

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Abstract

Zinc (Zn) deficiency is a widespread phenomenon in agricultural soils worldwide and has a major impact on crop yield and quality, and hence on human nutrition and health. Although dicotyledonous crops represent >30% of human plant-based nutrition, relatively few efforts have been dedicated to the investigation of Zn deficiency response mechanisms in dicotyledonous, in contrast to monocotyledonous crops, such as rice or barley. Here, we describe the Zn requirement and impact of Zn deficiency in several economically important dicotyledonous crops, *Phaseolus vulgaris*, *Glycine max*, *Brassica oleracea*, and *Solanum lycopersicum*. We briefly review our current knowledge of the Zn deficiency response in Arabidopsis and outline how this knowledge is translated in dicotyledonous crops. We highlight commonalities and differences between dicotyledonous species (and with monocotyledonous species) regarding the function and regulation of Zn transporters and chelators, as well as the Zn-sensing mechanisms and the role of hormones in the Zn deficiency response. Moreover, we show how the Zn homeostatic network intimately interacts with other nutrients, such as iron or phosphate. Finally, we outline how variation in Zn deficiency tolerance and Zn use efficiency among cultivars of dicotyledonous species can be leveraged for the design of Zn biofortification strategies.

Keywords: Arabidopsis, bean, biofortification, Brassica, dicotyledonous crop, sensing, soybean, tomato, transport, zinc deficiency.

Introduction

Zinc (Zn) is a micronutrient essential for plant growth and development, but also for prokaryotic and eukaryotic organisms, including humans and cattle (Maret, 2013; Kaur *et al.*, 2014). It acts as a cofactor of many enzymes [e.g. carbonic anhydrase or copper/zinc superoxide dismutase (Cu/ZnSOD)], and plays a structural role in many molecules [e.g. zinc-finger transcription factors (TFs)] (Clemens, 2010; Marschner, 2012). Zn deficiency impacts different health aspects, especially in children and pregnant women (Poskitt, 2005; Marschner, 2012). The first symptom in children is often diarrhea, which can still be lethal nowadays if Zn supplementation is not provided (Poskitt, 2005; Walker *et al.*, 2013). Moreover, suffering from Zn deficiency is also an aggravating factor of many other health problems and diseases (Beisel, 1998). Zn is indeed an essential cofactor of several antiviral molecules and regulators of the immune system, and is also needed for correct hematopoiesis (Beisel, 1998). For instance, a lower Zn serum level is associated with higher susceptibility to parasitic infections [e.g. visceral leishmaniasis

(Mishra *et al.*, 2010), https://www.who.int/news-room/fact-sheets/detail/leishmaniasis], and Zn malnutrition has been identified as a possible aggravating factor in COVID-19 patients (Jothimani *et al.*, 2020; Wessels *et al.*, 2021).

Zn malnutrition is still harming human populations in many countries worldwide. Zn deficiency prevalence even reaches 80% in children in some low- and middle-income countries (LMICs) (Wessells and Brown, 2012; Gupta *et al.*, 2020). Even though Zn supplementation programs, which can take different forms with varying efficacy and cost, are in place in many countries, they are often therapeutic and not preventive, and thus do not fully reduce the health impact of Zn deficiency (Maxfield and Crane, 2021). Zn supplementation is usually achieved by administering tablets or via enrichment of flour (World Health Organization, 2009) and it may be advantageous to tackle the issue by producing agronomically or genetically Zn-fortified crops.

Indeed, plants are at the base of the food pyramid, and appropriate Zn supply from plant products is therefore key, especially in countries where plant-based food is predominant in the diet. Zn availability in soils (i.e. soluble in the soil solution) is determined by multiple factors, for instance soil type and composition, soil pH, and moisture (Broadley *et al.*, 2007; Alloway, 2009). In many areas worldwide, Zn availability in soils is low, causing loss of productivity and reduced quality in crops (Alloway, 2009; Noulas *et al.*, 2018). Major efforts of Zn biofortification have been directed towards monocotyledonous staple crops, such as rice, wheat, or maize, as those cereals are essential to feed the world's population (Maqbool and Beshir, 2019; Bhatt *et al.*, 2020; Rehman *et al.*, 2020; Sanjeeva Rao *et al.*, 2020) (https://www.harvestplus.org/what-we-do/crops) (Box 1). Nevertheless, the 2019 Food and Agriculture Organization (FOA) database indicates that a significant portion of human (33%) and cattle (30%) nutrition is provided by dicotyledonous crops (Fig. 1A–C) (Food and Agriculture Organization of the United Nations, 2019).

In dicotyledonous plants, most of our molecular understanding of Zn homeostasis, its regulation and its interactions with other nutrients, has been acquired in the model plant Arabidopsis (*Arabidopsis thaliana*, *At*) in the last 20 years. Hundreds of studies have examined the function of Zn transporters and chelators, as well as transcriptional regulators in this species. Even if major gaps in our knowledge in Arabidopsis endure, this massive work enabled building a model of the Zn homeostatic network relying on Zn mobilization and uptake, radial transport and storage in roots, translocation to the shoot, and distribution to vegetative and reproductive tissues (see for extensive reviews: Sinclair and Krämer, 2012; Ricachenevsky *et al.*, 2015; Andresen *et al.*, 2018; Bouain *et al.*, 2019; Clemens, 2019; Hanikenne *et al.*, 2021; Zlobin, 2021). In that respect, the characterization of Zn hyperaccumulator plants, such as *Arabidopsis halleri* and *Noccaea caerulescens*, has been instrumental for identifying the key nodes of this network that are controlling the Zn flux in the plant (Krämer, 2010; Merlot *et al.*, 2021). In contrast, translation of this knowledge to dicotyledonous crops is lagging behind.

Here, we will briefly describe Zn homeostasis and Zn deficiency response mechanisms in Arabidopsis, then mostly focus on important dicotyledonous crops, such as soybean (*Glycine max*, *Gm*), tomato (*Solanum lycopersicum*, *Sl*), common bean (*Phaseolus vulgaris*, *Pv*), cabbage (*Brassica oleracea* L. var. *capitata*, *Boc*), and broccoli (*B. oleracea* L. var. *italica*, *Boi*) (*Bo*). These species have been selected for their economic importance, and their contribution to food production and to a diversified nutritional intake (Fig. 1D) (Burton-Freeman and Reimers, 2011; Anwar *et al.*, 2019; Da Silveira Vasconcelos *et al.*, 2019; Nanjundan *et al.*, 2020; Żyła *et al.*, 2021) but also based on the availability of Zn-related research reports in the literature. Oilseed rape (*Brassica napus*, *Bn*), another very important dicotyledonous crop, will not be covered as, to our knowledge, the Zn deficiency response has not been dissected at the molecular level in this species (Billard *et al.*, 2015) and only a couple of Zn transporters were recently examined (Gu *et al.*, 2021; Jiao *et al.*, 2021). We will present what is known and remains unknown about Zn homeostasis in these species and highlight (dis)similarities with Arabidopsis.

Zinc deficiency symptoms in dicotyledonous plants

Essentially due to high soil pH, poor Zn availability in soils is highly prevalent worldwide, including substantial proportions of cultivated soils in India or China. Consequently, Zn deficiency is the most common micronutrient deficiency in crops. Typically, Zn deficiency symptoms in plants appear when the leaf Zn concentration drops below 15–20 μg Zn g^{-1} DW (Marschner, 2012; Noulas *et al.*, 2018). Those symptoms include biomass and fertility reduction (and thus yield reduction in crops), leaf chlorosis (and thus an impact on photosynthesis), increased iron (Fe) accumulation, and increased oxidative stress resulting from impaired Cu/Zn SOD activity (Sinclair and Krämer, 2012; Marschner, 2012). Table 1 summarizes the main physiological impact of Zn deficiency in Arabidopsis and selected dicotyledonous crops.

The Zn requirement varies considerably with the developmental stage of the plants, between species, and among cultivars of a species. Indeed, among species listed in Table 1, the sensitivity to Zn deficiency varies substantially (Alloway, 2008*a*; Noulas *et al.*, 2018). Common bean, the third most produced grain legume worldwide, has higher Zn and Fe grain content than cereals (Beebe *et al.*, 2000; Myers and Kmiecik, 2017). This crop is, however, highly susceptible to Zn deficiency (Alloway, 2008*a*, 2009). Under these conditions, shoot protein content is reduced, and maturity is delayed, which impacts yield (Alloway, 2008*a*; Gurmani *et al.*, 2012; Singh *et al.*, 2017). The effect of Zn deficiency is also important in *B. oleracea*, the ninth most produced dicotyledonous crop in Asia and the 16th worldwide (Fig. 1D): the cabbage shoot biomass and Zn content are affected, as well as the plant and floret weight in broccoli (Navarro-León *et al.*, 2016; Rivera-Martin *et al.*, 2020). *Brassica*

Box 1. Zn biofortification

The term biofortification refers to strategies that are implemented to counteract Zn deficiency symptoms, and increase the yield and nutritional value of crops. It can take several forms: (i) agronomic means such as Zn fertilization of soils or Zn foliar application or (ii) genetic means, via varietal selection (i.e. plant breeding) or via the creation of transgenic crops with modified Zn pathways (Palmgren *et al.*, 2008; Andersson, 2017; Cakmak and Kutman, 2018; Gupta *et al.*, 2021). A large array of studies, mostly dedicated to cereal crops, have explored agronomic Zn biofortification in the field, with major success (see Cakmak and Kutman, 2018, and references therein), the biggest limitation remaining the cost of fertilizers in LMICs (Nielsen, 2012). Zn-biofortified cultivars of major crops are also developed, which not only have higher Zn content in seeds, but also optimally have assimilable Zn in seeds (i.e. with low phytate). These breeding approaches exploit natural variation of Zn homeostasis among cultivars of a species (e.g. Andersson, 2017; Sanjeeva Rao *et al.*, 2020). Engineering transgenic crops with higher Zn content has often been less successful, as most of the time the (over-)expression of a single gene in the plant is not sufficient to achieve a large increase of Zn content in seeds, reflecting the complexity of the Zn homeostasis network (Kumar *et al.*, 2019; Gupta *et al.*, 2021). It may, however, be required in species with low genetic diversity. Gene stacking approaches, combining homeostasis of several metals into a transgenic line, is likely to yield better results (e.g. Narayanan *et al.*, 2019), but requires a deep prior knowledge of Zn homeostasis mechanisms. Such knowledge is also valuable for an educated design of marker-assisted selection of new cultivars. Note that in several cases, both Zn and Fe biofortification are objectives of the developed approaches.

Biofortification strategies also have to depend on the edible part of the plant—root/tuber, leaf, or fruit/seeds. For instance, Zn reaches flowers, seeds, and fruits via phloem transport from the leaves. This step constitutes a bottleneck for Zn biofortification in both monocotyledonous (Cakmak and Kutman, 2018) and dicotyledonous plants (Xie et al., 2020, 2021). In *Solanum lycopersicum* and other species, <20% of Zn foliar application was shown to be actually redistributed via the phloem (Du *et al.*, 2015). Similarly, foliar Zn application has a distinct impact on Zn content of the edible part of cabbage (i.e. leaves) and broccoli (i.e. florets) (Rivera-Martin *et al.*, 2020). In the related oilseed rape, Zn is poorly remobilized from leaves upon Zn deficiency (Billard *et al.*, 2015). In beans, depending on the cultivar, either the seeds or the complete pod are consumed, and thus this difference in edible structure has to be taken into account in biofortification strategies (Olsen and Palmgren, 2014).

Some of the biofortification strategies briefly outlined here have been applied in dicotyledonous crops. For instance, improved tomato fruit production was reported upon Zn supplementation of a Zn-deficient Pakistani soil (Rehman *et al.*, 2020), and several trials have described how the timing of application and the Zn form determine the efficiency of biofortification in soybean (Heidarian *et al.*, 2010; de Oliveira *et al.*, 2019; Gomes *et al.*, 2020; Vaghar *et al.*, 2020; Yu *et al.*, 2020), or the pertinence of using ZnO nanoparticles in bean and its influence on known Zn transporters (da Cruz *et al.*, 2019). Expression of *AtHMA4* in tomato (Kendziorek *et al.*, 2014) or of a *NAS* gene from the tree *Malus xiaojinensis* in tobacco (Zhang *et al.*, 2009) both permitted enhancement of the Zn shoot concentration. Finally, natural variation in Zn deficiency tolerance and responses has been reported in Arabidopsis, which is important to rapidly identify (some of) the underlying mechanisms (Campos *et al.*, 2017; Bouain *et al.*, 2018; Chen and Ludewig, 2018), and also in dicotyledonous crops (Castro-Guerrero *et al.*, 2016).

oleracea is, however, a promising crop to reduce Zn deficiency prevalence in some countries. Indeed, its shoot phytate content, which decreases Zn absorption in the intestine, is quite low (Wessells *et al.*, 2012; White *et al.*, 2018) . Tomato and soybean, both in the top 10 of produced dicotyledonous crops (Fig. 1D), also display intermediate sensitivity to Zn deficiency (Alloway, 2008*a*, 2009).

Two types of criteria can be used to determine the performance of crops and model plants when exposed to Zn deficiency, reflecting the diversity of Zn homeostasis mechanisms between and within species: (i) Zn deficiency tolerance, which is the efficacy of the Zn deficiency response and mechanisms, including its impact on Zn uptake, remobilization, and trafficking; and (ii) Zn efficiency [or Zn use efficiency (ZUE)], which is the overall performance of the plant at a given Zn content; in other words, its capacity to sustain growth and thus producing nutrient-rich edible parts. Assuming that a Zn deficiency-tolerant plant will also be Zn efficient appears straightforward. However, the link between these two types of criterion is not always made in the literature. Although variation in Zn deficiency tolerance and in ZUE has been reported in several dicotyledonous species (Hacisalihoglu *et al.*, 2004; Sadeghzadeh, 2013; Campos *et al.*, 2017; González *et al.*, 2017; Hacisalihoglu, 2020), little is still known about the molecular mechanisms underlying those processes (Sinclair and Krämer, 2012). In Arabidopsis, higher tolerance to Zn deficiency among accessions has been associated with higher expression of two Zn status marker genes (*AtZIP4* and *AtIRT3*)

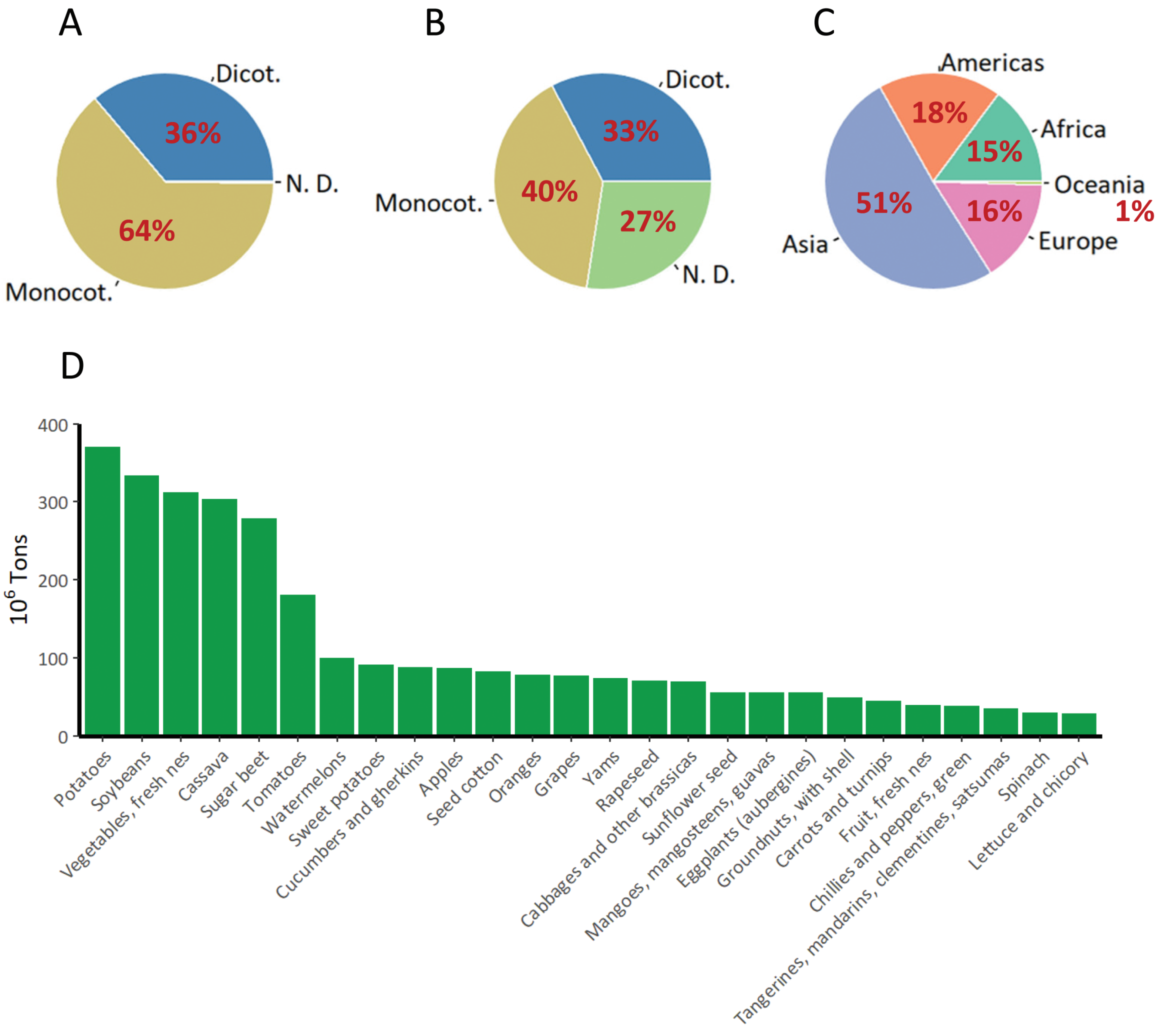

Fig. 1. Repartition of mono- and dicotyledonous plant world production and consumption. (A) World crop production. (B) World food supply. (C) Dicotyledonous species production per continent. (D) World production of the 25 most produced dicotyledonous crops. The representations are based on 2019 data from the Food and Agriculture Organization of the United Nations database (FOAStat, http://www.fao.org/faostat/en/#data/QC). N.D., not determined; nes, not elsewhere specified.

upon Zn deficiency (Campos *et al.*, 2017) or to allelic variation in the immune system gene *AZI1* contributing to primary root growth at low Zn supply (Bouain *et al.*, 2018). Moreover, two different Zn deficiency responses were observed among Arabidopsis accessions when it comes to reproduction and seed production (Chen and Ludewig, 2018): (i) reduced vegetative growth and early flowering permitting the plant to use its nutrients in seed production or (ii) delayed flowering permitting the plant to acquire a sufficient vegetative rosette and thus produce more seeds. The latter concerns accessions with early flowering phenotypes in control conditions, in which Zn deficiency leads to a repression of the expression of the regulator FLOWERING LOCUS T (Corbesier *et al.*, 2007; Chen and Ludewig, 2018). Both strategies have their own ecological and evolutionary advantages, and would be interesting to consider in the frame of crop production on Zn-deficient soils. However, determining how both strategies impact seed production yield and quality in Arabidopsis is required before extrapolating to crop species. In other dicotyledonous species, higher tolerance to Zn deficiency has also been associated with differences in Zn homeostasis gene expression, in hormone profiles, and/or reactive oxygen species (ROS) detoxification capacity (see below). For instance, the capacity to conserve Cu/ZnSOD activity at low Zn supply distinguishes Zn deficiency-tolerant and -sensitive cultivars in tomato (Akther *et al.*, 2020). Similarly, in soybean, the transcriptional induction or inhibition of ROS-scavenging genes upon Zn deficiency suggests their implication in Zn deficiency tolerance (Zeng *et al.*, 2019).

Zinc deficiency response and Zn transport

The release of the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000) enabled the identification of putative Zn transporters in this species (Axelsen and Palmgren, 2001;

Table 1. Summary of the phenotypic responses to Zn deficiency in selected dicotyledonous crops.

Species	Common name	ZnD phenotype	Hormone shoot content and regulation	ZnD sensitivity [3, 17]	Identification of ZnD-tolerant and or -sensitive cultivars	ZnD tolerance strategies
Arabidopsis thaliana	Arabidopsis Thale cress	Shoot biomass ↓ [1] Chlorosis and ROS [2-3] Root growth ↓ or ↑ [4]	IAA ↓ [14]	/	Flowering strategy [18]	Zn homeostasis genes [23] Flowering time modulation [18]
Brassica oleracea	Cabbage, Cauliflower, Broccoli (non-exhaustive)	Shoot DW ↓ [5, 6] Floret weight ↓ [5, 7] Variation in P content [8]	[6] IAA stable Total CK stable Specific CK (I-Ade) ↑ GA ↑ ACC↑ SA ↓ JA ↓	/	Different Zn/P crosstalk [19]	Hypothesis on a role for auxin [6]
Glycine max	Soybean	Nodulation ↓ [9] N fixation ↓ [9]	[15] Tr ↑ CK degradation Tr ↑ GA inactivation Tr ↓ Ethylene synthesis	Medium	Different Zn/Fe seed content [20]	ROS scavenging [15]
Phaseolus vulgaris	Common bean	Interveinal chlorosis [3,10] Brown veins on leaves [3,10] Whirling [10] Chlorophyll [11] Protein (shoot) ↓ [11] Amino acid ↑ [11]	[11] IAA ↓ ABA ↓ Some CK (I-Ade) ↓ Some CK (I-Ado) ↓	High	[21, 22]	
Solanum lycopersicum	Tomato	Shoot DW ↓ [12] Chlorosis [3,10] PSII activity↓ [12] Electrolyte leakage ↑ [12] H_2O_2 ↑ [12] Yield ↓ [13]	[16] IAA ↓	Medium	[12]	SOD activity *ZIP* genes induction [12]

↑ and ↓ indicate an increase or decrease of content or intensity. Tr↑ and Tr↓ indicate a transcriptomic regulation of a process. '/' indicates absence of data. ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; CK, cytokinins; GA, gibberellic acid; IAA, indole-3-acetic acid; I-Ade, isopentenyl adenine; I-Ado, isopentenyl adenoside; JA, jasmonic acid; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase; ZnD, zinc deficiency. References: [1] (Assunção *et al.*, 2010), [2] (Shinozaki *et al.*, 2020), [3] (Noulas *et al.*, 2018), [4] (Bouain *et al.*, 2018), [5] (White *et al.*, 2018), [6] (Navarro-León *et al.*, 2016), [7] (Rivera-Martin *et al.*, 2020), [8] (Broadley *et al.*, 2010), [9] (Zhang *et al.*, 2020), [10] (Alloway, 2008*b*), [11] (Cakmak *et al.*, 1989), [12] (Akther *et al.*, 2020), [13] (Gurmani *et al.*, 2012), [14] (Wang *et al.*, 2021), [15] (Zeng *et al.*, 2019), [16] (Skoog, 1940), [17] (Alloway, 2009), [18] (Chen and Ludewig, 2018), [19] (Pongrac *et al.*, 2020b), [20] (King *et al.*, 2013), [21] (Singh and Westermann, 2002), [22] (Moraghan and Grafton, 1999), [23] (Wintz *et al.*, 2003; Talke *et al.*, 2006; Van De Mortel *et al.*, 2006*a*; Assunção *et al.*, 2010; Sinclair *et al.*, 2018a).

Mäser *et al.*, 2001), which opened the way to their detailed characterization, and for some of them uncovered their roles in Zn cellular import/export and intracellular trafficking. Most of these transporters belong to three families: ZIP [Zn-Regulated Transporter (ZRT), Iron-Regulated Transporter (IRT)-Like Protein (ZIP)], MTP [Metal Tolerance Protein, with the name Cation Diffusion Facilitators (CDFs) in plants], and HMA (Heavy Metal ATPase). Other families such as NRAMP (Natural Resistance-Associated Macrophage Protein), VIT (Vacuolar Iron Transporter), or PCR (Plant Cadmium Resistance) also contribute to Zn homeostasis (Sinclair and Krämer, 2012; Ricachenevsky *et al.*, 2015; Andresen *et al.*, 2018).

ZIPs transport divalent metal cations, including Zn, from outside the cell or from an organelle to the cytoplasm (Guerinot, 2000). Out of 15 *ZIP* genes present in the Arabidopsis genome, eight are induced by Zn deficiency, some being classically used as markers of the Zn nutritional status of the plant (Wintz *et al.*, 2003; Talke *et al.*, 2006; Van De Mortel *et al.*, 2006; Assunção *et al.*, 2010; Nouet *et al.*, 2015), while many were suggested to transport Zn and other metals based on yeast complementation assays (Milner *et al.*, 2013). Most probably because of functional redundancy, detailed elucidation of the function of individual ZIP transporters in Zn homeostasis and that of other metals is still lacking in Arabidopsis (Wu *et al.*, 2009; Milner *et al.*, 2013; Ricachenevsky *et al.*, 2015).

In contrast to ZIPs, MTPs and HMAs transport metals out of the cytoplasm, either outside the cell or into an intracellular compartment. AtMTP1, AtMTP3, and AtHMA3 were shown to transport Zn into the vacuole, modulating Zn radial transport in roots and providing Zn tolerance by storing excess Zn in roots and shoot (Desbrosses-Fonrouge *et al.*, 2005; Arrivault *et al.*, 2006; Morel *et al.*, 2009; Chao *et al.*, 2012). AtHMA2 and AtHMA4 export Zn out of pericycle cells into the root xylem and are key actors for root to shoot Zn translocation (Hussain *et al.*, 2004). AtHMA4 is also essential to transfer Zn from maternal to seed tissues (Olsen *et al.*, 2016). By transporting Zn into the endoplasmic reticulum (ER) for symplastic movement, AtMTP2 also participates in Zn partitioning between roots and shoot (Sinclair *et al.*, 2018). Among those, only the *AtMTP2* and *AtHMA2* genes are induced by Zn deficiency, and in contrast to *AtZIP* genes which are controlled by the local Zn status, their induction upon Zn deficiency in roots is dependent on an as yet unidentified shoot-borne signal (Sinclair *et al.*, 2018). Several of these ZIP, MTP, and HMA transporter genes are constitutively overexpressed in the Zn hyperaccumulators *A. halleri* and *N. caerulescens*, and have (sometimes still putative) key functions in the Zn hyperaccumulation syndrome (Talke *et al.*, 2006; Van De Mortel *et al.*, 2006; Merlot *et al.*, 2021).

In addition to transporters, several chelators (i.e. molecules able to bind metals) are essential for Zn homeostasis, such as nicotianamine (NA), free histidine, glutathione (GSH), or citrate. They are important to (i) buffer the intracellular free Zn concentration, which is paramount to avoid Zn toxicity; and (ii) ensure intra- and extracellular Zn mobility (recently reviewed by Clemens, 2019). Among those, NA is synthesized from *S*-adenosylmethionine by NAS (NA synthase) enzymes and contributes to Zn radial movement in roots, and several pieces of evidence suggests that NA–Zn complexes facilitate xylem and phloem Zn movements (Clemens, 2019, and reference therein). ZIFL [Zn-Induced Facilitator 1 (ZIF1)-Like] and YSL (Yellow Stripe-Like) proteins acts as NA or NA–metal transporters, controlling Zn radial transport in roots (e.g. AtZIF1) or long-distance transport in the plant (e.g. several AtYSLs) (Curie *et al.*, 2009; Haydon *et al.*, 2012; Conte and Walker, 2012). Among those genes, *AtNAS2*, *AtNAS4*, *AtYSL2*, and *AtYSL3* are up-regulated upon Zn deficiency (Van De Mortel *et al.*, 2006; Assunção *et al.*, 2010). Metallothioneins (MTs) are metal chelator proteins hypothesized to play a key role to deliver Zn to target proteins, specifically during embryogenesis and seed germination in low Zn conditions. However, MTs were shown to bind Zn and to be able to deliver it to an acceptor *in vitro* only (Sinclair and Krämer, 2012; Imam and Blindauer, 2018).

Several of these transporters or chelators are able to transport or bind other metal cations in addition to Zn (e.g. Fe, manganese, or copper), directly contributing to interactions of Zn with the homeostatic networks of these other micronutrients (Hanikenne *et al.*, 2021; Kumar *et al.*, 2021). For instance, an Arabidopsis quadruple *nas4x-1* mutant displays Fe- but also Zn-related phenotypes, with significantly less Zn in rosettes during the vegetative stage, and in flowers, and in siliques during reproductive stages (Klatte *et al.*, 2009). In this mutant, long-distance transport of several cations, specifically Zn xylem loading, is extremely impaired, impacting shoot accumulation (Persson *et al.*, 2016).

Several complementary approaches are used to identify transporters with similar functions, as well as to describe their contribution to Zn deficiency responses, in dicotyledonous species other than Arabidopsis (Fig. 2; Table 2). First, sequence similarity-based *in silico* searches and phylogenetic studies are most commonly conducted to identify homologous genes in these species (e.g. Astudillo *et al.*, 2013; Aslam *et al.*, 2020). However, in many cases, dicotyledonous species are used together with Arabidopsis to provide outgroups, while the primary focus of the analyses is on economically important monocotyledonous species such as rice, wheat, or maize (e.g. Evens *et al.*, 2017; Ajeesh Krishna *et al.*, 2020). The success and pertinence of these approaches are very dependent on the quality and completeness of the available genome sequences. It is only since the mid 2010s that good quality genomes, with consolidated annotations, have gradually become available for a number of dicotyledonous crops (see the Phytozome database for instance, http://phytozome.jgi.doe.gov/; Bellucci *et al.*, 2014; Parkin *et al.*, 2014; Yu *et al.*, 2016; Guo *et al.*, 2021). Earlier studies therefore often present fragmentary information and suffer from incompatibilities between consecutive annotation versions of genomes (see Astudillo *et al.*, 2013; Astudillo-Reyes *et al.*, 2015; Ajeesh Krishna *et al.*, 2020). Second, these *in silico* analyses are completed, often in independent studies, by the identification of Zn deficiency-responsive genes among transporter family members (e.g Astudillo-Reyes *et al.*, 2015; Pavithra *et al.*, 2016; Zeng *et al.*, 2019). Third, the natural variation of Zn deficiency tolerance and/or ZUE among cultivars is exploited to identify genetic loci underlying this variation through QTL (quantitative trait locus) mapping and candidate gene approaches (King *et al.*, 2013; Kumawat *et al.*, 2016; González *et al.*, 2017; Izquierdo *et al.*, 2018; Dissanayaka *et al.*, 2020).

Hence, the regulation of expression of a fraction of *ZIP* genes by Zn deficiency (most often up-regulation) is a conserved feature among dicotyledonous plants (Fig. 2; Table 2), as it is in monocotyledonous plants (as reviewed recently in Ajeesh Krishna *et al.*, 2020): for instance seven out 28 identified *ZIP* genes in soybean (Zeng *et al.*, 2019), seven out of 15 in tomato (Pavithra *et al.*, 2016; Akther *et al.*, 2020; Aslam *et al.*, 2020), four out of 12 in potato tubers (*Solanum tuberosum*, *St*) (Li *et al.*, 2020), or three out of 23 in common bean (Astudillo *et al.*, 2013), as well as one (Bo4g045850, a homolog of *AtZIP3*) in diverse cultivars of *B. oleracea* (Pongrac *et al.*, 2020b), are up-regulated upon Zn deficiency, in the whole plant or in specific organs, with often considerable variations between cultivars. More specifically, *SlZIP-L* (SL07g065380) and *SlZIP3* (SL02g081600) seem to be up-regulated in most tested tomato cultivars in roots and shoots in Zn deficiency conditions, with a noticeable exception in the very Zn deficiency-sensitive cultivar Ratan. In this cultivar, *SlZIP-L* and *SlZIP3* are not

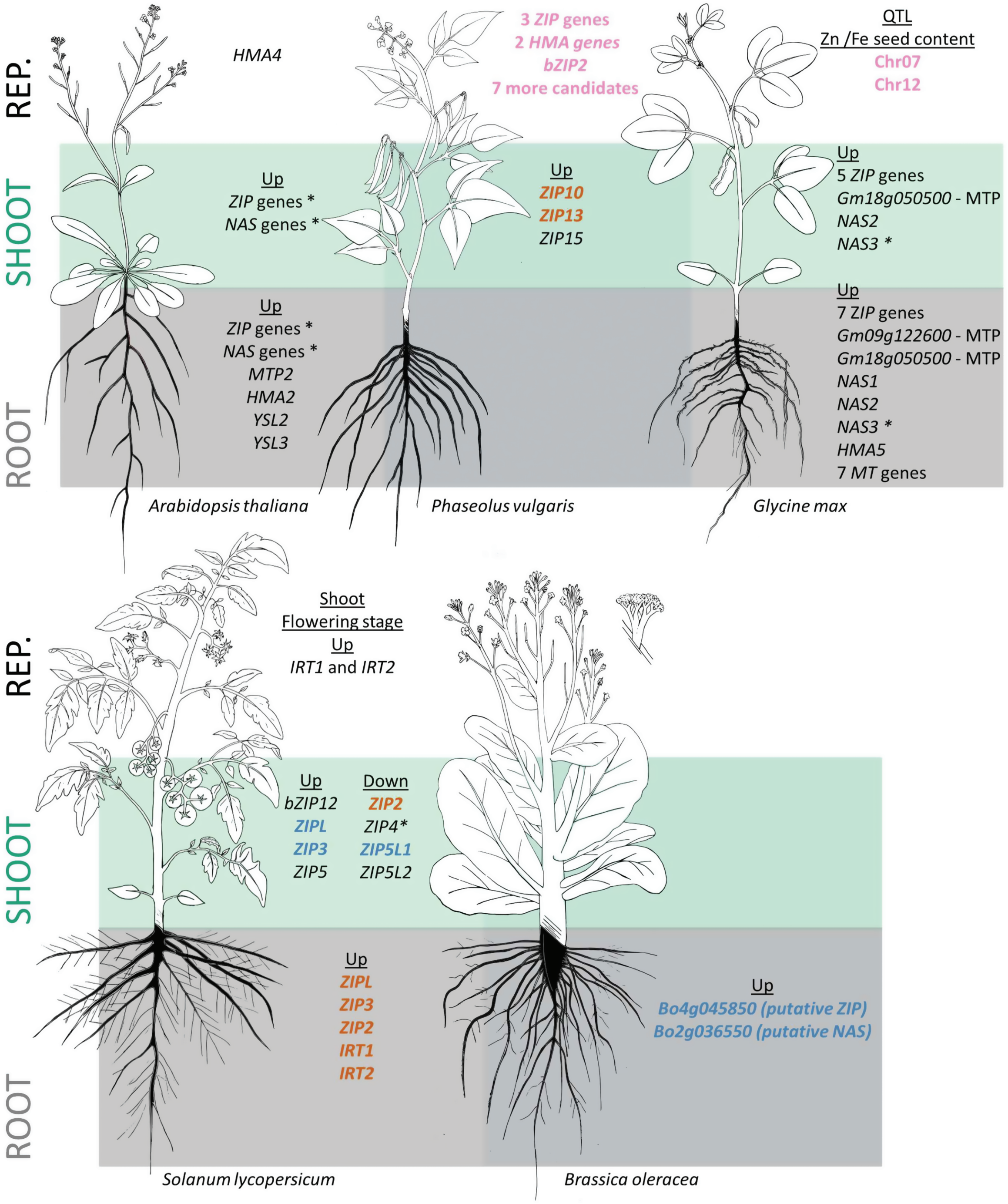

Fig. 2. Metal homeostasis gene responsive to Zn deficiency in Arabidopsis and dicotyledonous crops. The information about up- or down-regulated genes is provided for the root, the shoot, and the reproductive organs (REP.), whenever available. Annotation of genes in blue color indicates that the up-regulation upon Zn deficiency is consistent in several cultivars. The orange color indicates that the up- or down- regulation upon Zn deficiency differs among cultivars, or at different developmental stages. The pink color indicates that a putative function of the gene in Zn uptake and transport has been proposed from QTL analyses or comparisons between cultivars. Genes labeled with an asterisk (*) include (putative) ZDRE motif(s) in their promoters according to Castro *et al.* (2017) or from specific studies cited below. *Arabidopsis thaliana* panel: *ZIP* genes include *ZIP1**, *ZIP2*, *ZIP3**, *ZIP4**, *ZIP5**, *ZIP9**, *ZIP12**, and *IRT3**. *NAS* genes include *NAS2** and *NAS4** (Wintz *et al.*, 2003; Talke *et al.*, 2006; Van De Mortel *et al.*, 2006; Assunção *et al.*, 2010; Olsen *et al.*, 2016; Sinclair *et al.*, 2018b). *Phaseolus vulgaris* panel: gene annotations are based on Astudillo-Reyes *et al.* (2015) and may differ from specific publications. Astudillo-Reyes *et al.* (2015) refers to positions in https://plants.ensembl.org/ and the cultivar G19833 genome available on the NCBI, but not to positions in the most recent genome available at Phytozome. *ZIP* genes potentially implicated in Zn seed storage are *ZIP12*, *ZIP17*, and *ZIP19*. *HMA* genes potentially implicated in seed storage are *HMA3* and *HMA6*. The seven additional candidates are *NAS1*, *ZIF1*, *NRAMP1*, *NRAMP6*, *NRAMP9*, three *FRO* genes, and three *MATE* genes (Astudillo *et al.*, 2013; Astudillo-Reyes *et al.*, 2015; Izquierdo *et al.*, 2018). *Glycine max* panel: *ZIP*

up-regulated, while a reduction in biomass and photosynthetic activity as well as cell death are already detectable after 1 week of Zn deficiency (Akther *et al.*, 2020). In the closely related potato, the *ZIP* gene expression also varies between different cultivars. For example, in leaves, *StZIP4* is the only *ZIP* gene up-regulated by Zn deficiency in one cultivar (L14148-5) but down-regulated in another (Minshu1) where another *ZIP* gene (*StZIP12*) is highly induced (Li *et al.*, 2020). However, the same genes (*StZIP2*, *StZIP4*, *StZIP8*, and *StZIP7*) are induced in the economically important tubers of both cultivars during Zn deficiency (Li *et al.*, 2020). Overall, very few ZIPs were characterized further. For instance, GmZIP1 showed Zn transport activity when expressed in yeast (Moreau *et al.*, 2002).

In soybean, in addition to *ZIP* genes, one *HMA*, two *MTP*, *NAS* (3/5), and *MT* (7/9) genes are up-regulated upon Zn deficiency in roots and/or shoots, before the detection of chlorosis and growth inhibition phenotypes (Zeng *et al.*, 2019). In contrast, indole-3-acetic acid (IAA) synthesis genes in shoots as well as carbonic anhydrase and putative *VIT* genes are down-regulated in roots. In *B. oleracea*, Bo2g036550 (homolog of *AtNAS2*) is up-regulated upon Zn supplementation in roots in two cultivars (Pongrac *et al.*, 2020b). In the common bean, several studies aimed to identify transporters with a putative function in Zn loading into the seeds. This endeavor exploited the important genetic diversity found in this species. Indeed, common beans cultivated nowadays result from two distinct domestication events that took place in Mexico and in the Andes, and thus represent two diversified genome pools. Seeds from Andean origin contain more Fe, while Median-American seeds have higher Zn (Islam *et al.*, 2002).

Among multiple metal homeostasis-implicated genes basally expressed in pods (i.e. *VIT*, *NAS*, *MT*, *NRAMP*, *ZIF*, and *YSL*) (Astudillo-Reyes *et al.*, 2015), comparative transcriptomics of two navy bean cultivars (i.e. in which only seeds are consumed) with different seed Zn content, but similar Zn root, leaf, and pod content, showed a higher expression of *PvZIF1* and *PvZIP12* as well as lower expression of *PvNRAMP9* in the pods containing high Zn seeds (Astudillo-Reyes *et al.*, 2015).

Moreover, in a meta-analysis combining seven QTL studies conducted earlier within and between Andean and Median-American domestication genome pools (Blair et al., 2009, 2010, 2011, 2013; Cichy et al., 2009, 2014; Blair and Izquierdo, 2012), Izquierdo *et al.* (2018) highlighted a correlation between Zn and Fe seed contents across cultivars of all origins. Among 12 identified meta-QTLs, eight were associated with both Zn and Fe storage in seeds, and two were specific to Zn. One of these QTLs, located on chromosome 2, co-localizes with metal transporter genes, whose number and identity vary depending on the genome annotation version used in subsequent studies (Astudillo *et al.*, 2013; Astudillo-Reyes *et al.*, 2015). However, one *ZIP* and two *HMA* genes have been associated with this specific Zn QTL (Astudillo-Reyes *et al.*, 2015; Izquierdo *et al.*, 2018). Moreover, 12 metal homeostasis genes, including *ZIP* genes and *NRAMP* genes, were located in five Zn/Fe metal QTL intervals (Izquierdo *et al.*, 2018). The correlation between Fe and Zn seed contents and the high proportion of QTLs being Zn and Fe related for seed storage across all cultivars suggests the presence of a common seed loading process for both metals. In Arabidopsis, this correlation does not seem to exist, and may even be slightly negative (Chen *et al.*, 2016). Thus working with common bean is biologically interesting to address biofortification of both nutrients at once.

Overall, if it is clear that members of major Zn transporter families (such as ZIPs, MTPs, or HMA4s) are encoded in genomes, and if it is likely that these proteins play a role in Zn homeostasis and the Zn deficiency responses of dicotyledonous crops, little, if any, functional validation is currently available, beyond the ubiquitous Zn responsiveness of a number of *ZIP* genes in plants. For instance, it remains to be examined if AtHMA4-like proteins are also essential for Zn loading in seed embryos in these species (Olsen and Palmgren, 2014; Olsen *et al.*, 2016).

Regulation, sensing, and signaling

In Arabidopsis, a screen to identify *trans*-regulatory elements involved in the Zn deficiency responsiveness of *AtZIP4* identified two major TFs, AtbZIP19 and AtbZIP23, controlling Zn homeostasis in plants (Assunção *et al.*, 2010; Inaba *et al.*, 2015; Lilay *et al.*, 2019). They belong to the F-group of the large bZIP (basic leucine zipper) TF family (Castro *et al.*, 2017). An Arabidopsis *bzip19bzip23* double mutant is highly sensitive to Zn deficiency (Assunção *et al.*, 2010). Only partially redundant (Assunção *et al.*, 2010; Inaba *et al.*, 2015), AtbZIP19 and AtbZIP23 are responsible and required for the up-regulation of *ZIP* and *NAS* genes upon Zn deficiency (see above), through the binding of a *cis*-regulatory motif, named ZDRE (zinc deficiency response element) present in their promoters (Assunção *et al.*, 2010). AtbZIP19 and AtbZIP23 are in fact able to bind Zn via a specific Cys-/His-rich amino acid domain named ZSM (zinc-sensing motif) and act as sensors of the Zn status in the cells (Lilay *et al.*, 2021). At normal Zn supply, Zn binding to this motif inactivates the TFs. At low Zn supply, the TFs are activated, enabling DNA binding at the ZDRE motifs and thus the transcriptional activation of *ZIP* and *NAS* genes during the Zn deficiency response (Lilay *et al.*, 2021).

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genes up-regulated upon Zn deficiency in shoots are *ZIP1**, *ZIP2*, *ZIP3*, *ZIP4**, and *ZIP11*. *ZIP* genes up-regulated upon Zn deficiency in roots are *ZIP1**, *ZIP2*, *ZIP3*, *ZIP4**, *ZIP11*, *IRT1*, and *IRT2*. *MT* genes up-regulated upon Zn deficiency in roots are *MT-2a*, *-2b*, *-2c*, *-2d*, *-2e*, *MT-3a*, and *-3b* (Zeng *et al.*, 2019). Two QTLs for Zn and Fe content in seeds are found on chromosomes (Chr) 7 and 12 and include several candidate genes linked to metals or auxins (see King *et al.*, 2013 and details in the main text). *Solanum lycopersicum* panel: the information is from Pavithra *et al.* (2016), Akther *et al.* (2020), and Aslam *et al.* (2020). *Brassica oleracea* panel: the information is from Pongrac *et al.* (2020b).

Table 2. Evidence supporting the potential role of genes in the Zn deficiency response in the five selected species.

Gene name	Accession code	Genome	Techniques	Other names or accessions
PvZIP10	PHAVU_006G055800g	Cultivar G19833 (Andean): Ensembl: PhaVulg1_0 NCBI: *Phaseolus vulgaris* (taxid:3885) Annotation: Astudillo-Reyes *et al.*, 2015	Regulation: RT–qPCR, Astudillo *et al.*, 2013	*PvZIP12*, Astudillo *et al.*, 2013
PvZIP13	PHAVU_006G070200g			*PvZIP13*, Astudillo *et al.*, 2013
PvZIP15	PHAVU_008G2905001g			*PvZIP16*, Astudillo *et al.*, 2013
PvZIP12	PHAVU_006G003300g		Expression: RNA-Seq between cultivars and RT–qPCR confirmation, Astudillo-Reyes *et al.*, 2015	
PvNRAMP9	PHAVU_010G160800g			
PvZIF1	PHAVU_002G108300g			
PvHMA3	PHAVU_002G288300g			
PvHMA6	PHAVU_002G19000g			
PvNAS1	PHAVU_001G225000g			
PvNRAMP1	PHAVU_001G177500g		Genetic evidence: presence in QTL for Zn seed storage, Astudillo-Reyes *et al.*, 2015	Phvul.001G177500, *P. vulgaris* genome v2.1, Izquierdo *et al.*, 2018
PvNRAMP6	PHAVU_009G069700g		Genetic evidence: presence in QTL for Zn seed storage, Izquierdo *et al.*, 2018	Phvul.009G069700, *P. vulgaris* genome v2.1, Izquierdo *et al.*, 2018
PvbZIP2	PHAVU_011G035700g			Phvul.011G035700, *P. vulgaris* genome v2.1, Izquierdo *et al.*, 2018
PvZIP17	PHAVU_009G077700g			Phvul.009G077700, *P. vulgaris* genome v2.1, Izquierdo *et al.*, 2018
PvZIP19	PHAVU_011G058500g			Phvul.011G058500, *P. vulgaris* genome v2.1, Izquierdo *et al.*, 2018
GmZIP1	Gm20g063100	*Glycine max* v2 (cultivar Williams 82): Ensembl: Glycine_max_v2.0 Phytozome: Wm82.a4.v1 NCBI: *Glycine max* (taxid:3847)	Annotation: Zeng *et al.*, 2019 Regulation: Zn def. against control: RNA-Seq and RT–qPCR Zeng *et al.*, 2019	
GmZIP2	Gm08g164400			
GmZIP3	Gm15g262800			
GmZIP4	Gm13g004400			
GmZIP11	Gm06g052000			
GmIRT1	Gm07g223200			
GmIRT2	Gm20g022500			
GmNAS1	Gm03g231200			
GmNAS2	Gm19g228400			
GmNAS3	Gm09g036400			
GmHMA5	Gm16g088300			
Putative MTP	Gm09g122600			
Putative MTP	Gm18g050500			
GmMT2a	Gm07g132000			
GmMT2b	Gm17g230500			
GmMT2c	Gm14g093100			
GmMT2d	Gm03g082100			
GmMT2e	Gm18g180800			
GmMT3a	Gm06g242900	As above	As above	
GmMT3b	Gm12g154300			

Table 2. Continued

Gene name	Accession code	Genome	Techniques	Other names or accessions
SlIRT1	Solyc02g069200.2	Ensembl: SL3.0 NCBI: *Solanum lycopersicum* (taxid: 4081)	Regulation: RT–qPCR, Aslam *et al.*, 2020	*LeIRT1*, Aslam *et al.*, 2020
SlIRT2	Solyc02g069190.3			*LeIRT2*, Aslam *et al.*, 2020
SlZIPL	Solyc07g065380.3		Regulation: RT–qPCR, Aslam *et al.*, 2020, Akther *et al.*, 2020, and RT–PCR Pavithra *et al.*, 2016	
SlZIP2	Solyc06g005620.3		Regulation: RT–qPCR, Akther *et al.*, 2020	*SlZIP1*, Aslam *et al.*, 2020
SlZIP3	Solyc02g081600.3			*SlZIP3L*, Pavithra *et al.*, 2016 and Akther *et al.*, 2020
SlZIP4	Solyc08g065190.3		Regulation: RT–PCR, Pavithra *et al.*, 2016	
SlZIP5	Solyc05g053370.3		Regulation: RT–qPCR, Aslam *et al.*, 2020	
SlZIP5L1	Solyc07g043230.3		Regulation: RT–PCR, Pavithra *et al.*, 2016	*SlZIP5L*, Aslam *et al.*, 2020
SlZIP5L2	Solyc07g043200.2			
SlbZIP12	Solyc01g111580.3			*SlbZIP19/23L*, Pavithra *et al.*, 2016
Putative ZIP	Bo4g045850	*B. oleracea* var. *oleracea* v2.1: NCBI: *B.oleracea* var. *oleracea* (taxid:109 376)	Regulation: RNA-Seq, Pongrac *et al.*, 2020b	
Putative NAS2	Bo2g036550			

QTL, quantitative trait loci; RT–PCR, reverse transcription–PCR; RT–qPCR, reverse transcription quantitative PCR.

As their activity is directly regulated post-transcriptionally by the Zn status, the *AtbZIP19* and *AtbZIP23* transcription levels are not or only slightly up-regulated upon Zn deficiency in Arabidopsis (Assunção *et al.*, 2010; Inaba *et al.*, 2015; Arsova *et al.*, 2019, Preprint; Lilay *et al.*, 2019). If this is also the case in other dicotyledonous plants, this complicates or prevents the identification of functional homologs of AtbZIP19 and AtbZIP23 among *bZIP* genes via classical transcriptomic analyses of the Zn deficiency response. However, members of the bZIP family in dicotyledonous plants have been characterized *in silico* (e.g. a phylogenetical analysis across land plants including 13 dicotyledonous plants, among them Arabidopsis and tomato, Castro *et al.*, 2017). Among crops of focus in this review, the analyses were often conducted without a specific focus on the F-group. This led to the identification of two members of the F-group in tomato (Li *et al.*, 2015), two in soybean (Zhang *et al.*, 2018), and five in *B. oleracea* genomes (Hwang *et al.*, 2016).

In soybean, among 85 TFs that are up- or down-regulated in roots and/or shoots upon Zn deficiency, five are bZIPs (Zeng *et al.*, 2019). However, phylogenetic analysis indicates that none of those bZIPs belongs to the F-group (Zhang *et al.*, 2018), questioning their contribution to the regulation of ZDRE-containing soybean genes upon Zn deficiency (see below and Zeng *et al.*, 2019). In contrast, the two *GmbZIP* genes (*GmbZIP84* and *GmbZIP95*) that cluster with Arabidopsis F-group bZIPs (Zhang *et al.*, 2018) are not regulated by Zn deficiency (Zeng *et al.*, 2019), at least in the cultivar tested in the report.

If the low to non-induction of the expression of F-group bZIP TFs upon Zn deficiency is common to Arabidopsis and soybean, it seems to be more pronounced in tomato plants. Indeed, SlbZIP19/23-Like (LOC101266077, Solyc01g111580), also named bZIP12 and classed in the F-group (Li *et al.*, 2015), is >2-fold induced by Zn deficiency (Pavithra *et al.*, 2016). In common beans, complete characterization of the bZIP TF family is still lacking. Only two potential F-group bZIPs have been identified based on similarity to genomic sequences of *AtbZIP19* and *AtbZIP23*. One of them, *PvbZIP1*, seems more expressed in pods of plants grown in Zn deficiency conditions (Astudillo *et al.*, 2013; Astudillo-Reyes *et al.*, 2015), Moreover, the second one, *PvbZIP2*, co-locates with a meta-QTL for Zn and Fe accumulation in bean seeds (Izquierdo *et al.*, 2018).

The presence of ZDRE-binding motif(s) upstream of genes differentially expressed upon Zn deficiency has been observed in different dicotyledonous species. This is the case for example, in soybean for nine genes up-regulated upon Zn deficiency, including *GmZIP4*, *GmZIP1*, and *GmNAS3* (Zeng *et al.*, 2019). Two ZDREs are also found upstream of *SlZIP4* in the tomato genome (Castro *et al.*, 2017).

Performing complementation assays to rescue growth, an Arabidopsis *bzip19bzip23* double mutant under Zn deficiency has been instrumental to the characterization of AtbZIP19 and AtbZIP23 (e.g. Assunção *et al.*, 2010; Lilay *et al.*, 2019), as well as their functional homologs in several monocotyledonous crops, such as barley (Nazri *et al.*, 2017), wheat (Evens *et al.*, 2017), and rice (Lilay *et al.*, 2020). Ideally, the *in silico* analyses of dicotyledonous bZIPs conducted so far should be completed with such complementation assays to achieve a level of understanding and a confirmation of their function as in monocotyledonous species and Arabidopsis. However, the identification of several F-group bZIPs as well as the presence of a ZDRE motif upstream of potential Zn homeostasis genes strongly suggest a certain level of conservation of the mechanism (Castro *et al.*, 2017).

Beyond transcriptional regulation, hormone signaling has also been linked to Zn homeostasis mechanisms. In particular, a lower shoot concentration of IAA, the main form of auxin, is a frequent observation in Zn-deficient plants (Skoog, 1940; Tsui, 1948; Horák *et al.*, 1976; Cakmak *et al.*, 1989; Alloway, 2008*b*; Wang *et al.*, 2021), although it is not always the case (Domingo *et al.*, 1992; Navarro-León *et al.*, 2016). Given the key role of auxin in the control of plant development and growth (Weijers *et al.*, 2018), this may contribute to reduced growth upon Zn deficiency. That Zn deficiency causes a reduction of IAA synthesis has been presented as a fact for a long time (Skoog, 1940), but a detailed mechanistic understanding of how and with which cause(s) and/or consequence(s) it occurs is still missing. Indeed, the causality relationship—whether lower IAA is a consequence of Zn deficiency or is actively participating in the Zn deficiency response—mostly remains to be investigated in Arabidopsis and other dicotyledonous species.

Several pieces of evidence suggest, however, that IAA could play an active role in Zn deficiency tolerance. In common bean and tomato, for instance, IAA foliar application partially rescues the Zn deficiency symptoms (Skoog, 1940; Cakmak *et al.*, 1989). In Zn-deficient tomato, treatment with triiodobenzoic acid (TIBA), an inhibitor of auxin polar transport, decreases auxin effects, and increases Zn deficiency symptoms (Akther *et al.*, 2020). In soybean, transcription of putative IAA synthesis genes is repressed in the root upon Zn deficiency (Zeng *et al.*, 2019).

The best element supporting the direct implication of IAA in the Zn deficiency response is described in a highly Zn deficiency-tolerant rice cultivar (*Oryza sativa* var. Pokkali) (Begum *et al.*, 2016). This specific cultivar shows no growth reduction, nor chlorosis upon Zn deficiency: Zn deficiency tolerance is associated with strong *ZIP* gene up-regulation in roots, and higher SOD and glutathione reductase (GR) activities in Zn deficiency conditions. It also has a higher IAA shoot content (Begum *et al.*, 2016). Upon TIBA treatment, *O. sativa* var. Pokkali displays reduced root and shoot growth as well as chlorophyll content when exposed to Zn deficiency. The combination of Zn deficiency and TIBA treatment also triggered a down-regulation of *ZIP* genes as well as reduced shoot and root Zn concentrations, total protein, but also GR and SOD activities (Begum *et al.*, 2016). These data indicate that auxin signaling contributes to Zn deficiency tolerance in rice.

To our knowledge, there is so far no report of a case of higher IAA shoot content upon Zn deficiency in a dicotyledonous species. In radish and cabbage, no change in IAA shoot content was observed in response to Zn deficiency (Domingo *et al.*, 1992; Navarro-León *et al.*, 2016). When directly comparing cabbage and lettuce (*Lactuca sativa*), two leafy dicotyledonous crops, both display shoot weight reduction and a lower shoot Zn content upon Zn deficiency (Navarro-León *et al.*, 2016). However, IAA shoot content only decreases in lettuce, and not in cabbage, implying that growth reduction is not due to IAA content reduction in the latter. Moreover, the shoot concentrations of other hormones also vary between both crops: 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, and total gibberellin concentrations decrease drastically in lettuce upon Zn deficiency but increase slightly in Zn-deficient cabbage. Other hormones are affected only in one species (e.g. abscisic acid or jasmonic acid) or similarly in both (e.g. a reduction in salicylic acid) (Navarro-León *et al.*, 2016). It therefore appears that the hormonal responses to Zn deficiency are not conserved across dicotyledonous species.

Although IAA content reduction upon Zn deficiency was discovered in tomato >80 years ago (Skoog, 1940), the molecular mechanisms underlying this observation in dicotyledonous plants are however poorly addressed and resolved in the literature nowadays. Moreover, the current research on dicotyledonous crops provides information on total shoot IAA content, while local auxin gradient and distribution contribute to most of IAA regulation (Weijers *et al.*, 2018). In Arabidopsis, Zn quantity influences auxin accumulation at the root apical meristem (Wang *et al.*, 2021). Such information on local auxin gradients is still lacking for crop species. Also, there seems to be inter- and intraspecies differences in other hormonal reactions to Zn deficiency, potentially linked to tolerance. For other hormones, there is no clear consensus about their reaction to Zn deficiency, and their contributions to Zn homeostasis await clarification (Table 1).

Crosstalk with other nutrients

As the Zn and Fe absorption machineries share similar transporters and mechanisms, important interactions take place between the homeostatic networks of these two micronutrients (recently reviewed in Hanikenne *et al.*, 2021; Kumar *et al.*, 2021; see above and Box 2). An emblematic example of these interactions is the complex transcriptional and post-translational regulation of the IRT1 (Iron Regulated Transporter 1) transporter by Fe and Zn status in Arabidopsis (Connolly *et al.*, 2002; Barberon et al., 2011, 2014; Shanmugam *et al.*, 2011; Dubeaux *et al.*, 2018). If the transcriptional responses to Zn and Fe deficiencies are controlled by distinct TFs, essentially by basic helix–loop–helix TFs for Fe (Brumbarova *et al.*, 2015; Gao *et al.*, 2019; Spielmann and Vert, 2021) and bZIPs for Zn (see above), a number of genes (e.g. *AtNAS2* and *AtNAS4*; Klatte *et al.*, 2009; Assunção *et al.*, 2010; Arsova *et al.*, 2019, Preprint) are controlled by both deficiencies.

Autophagy recently emerged as a mechanism mitigating the detrimental effects of Zn/Fe interactions in Arabidopsis

(Shinozaki *et al.*, 2020, 2021; Shinozaki and Yoshimoto, 2021). Indeed, upon Zn deficiency, higher Fe uptake, and thus higher intracellular Fe concentrations, trigger oxidative stress responsible for the typical chlorosis phenotype of Zn deficiency (Cakmak, 2000). In fact, autophagy-deficient Arabidopsis mutants display stronger root growth inhibition and chlorophyll content reduction than wild-type plants upon Zn deficiency, indicating a lower capacity to deal with Fe-induced oxidative stress and to maintain a sufficient level of bioavailable Zn in cells (Shinozaki *et al.*, 2020). The authors suggest a model where autophagy actively contributes to maintain sufficient Zn intracellular concentration via remobilization of non-mobile Zn ions (e.g. Zn trapped in proteins), first from the vacuole (Shinozaki and Yoshimoto, 2021). The Fe deficiency induced by Zn excess could be resolved by similar autophagy-driven mechanisms in cells (Shinozaki and Yoshimoto, 2021; Shinozaki *et al.*, 2021). Whether similar mechanisms contribute to Zn homeostasis in general, and to Zn deficiency tolerance or ZUE in dicotyledonous crops remains to be examined.

Another well-documented crosstalk concerns Zn homeostasis and phosphate (Pi). In dico- and monocotyledonous species, the available Zn concentration influences Pi uptake, and reciprocally [e.g. in Arabidopsis (Jain *et al.*, 2013; Khan *et al.*, 2014), tomato (Parker *et al.*, 1992), soybean and maize (Yu *et al.*, 2020), cabbage (Pongrac *et al.*, 2020b), or even less studied species, such as cotton (Santos *et al.*, 2019)], Pi is also implicated in a similar interaction with Fe (reviewed in Hanikenne *et al.*, 2021). For both Fe and Zn, a low Pi level induces an Fe and Zn accumulation in shoots. Conversely, Zn or Fe deficiency induces Pi uptake (reviewed in Xie *et al.*, 2019; Yu *et al.*, 2020; Hanikenne *et al.*, 2021, and references therein). Finally, Pi-induced Zn deficiency has also been observed in a number of dico- and monocotyledonous species (Santos *et al.*, 2019; Yu *et al.*, 2020). In Arabidopsis, the PHR1 (Phosphate Response 1) TF is a major regulator of Pi homeostasis (Bustos *et al.*, 2010) and contributes to the regulation of Zn/Fe/Pi tripartite interactions (Khan *et al.*, 2014; Briat *et al.*, 2015; Xie *et al.*, 2019; Hanikenne *et al.*, 2021), which appears to be at least partly conserved in algae (Rubio *et al.*, 2001; Thiriet-Rupert *et al.*, 2021). For instance, PHR1 regulates the transcription of a ferritin gene (*AtFER1*, permitting Fe storage), as well as *AtZIP2* and *AtZIP4*. Conversely, Zn deficiency induces the expression of *AtPHT1:1*, encoding a high-affinity Pi transporter in shoots, but reduces in roots the expression of the same *AtPHT1:1*, as well as *AtPHO1:H3*, which encodes a protein permitting a better Pi translocation from roots to shoot (Khan *et al.*, 2014; Briat *et al.*, 2015). Increased *AtPH1;1* expression in Zn-deficient shoots is linked to *AtLPCAT1* (lysophosphatidylcholine acyltransferase) expression, downstream of AtbZIP23 (Kisko *et al.*, 2018). AtbZIP23 binds to a ZDRE and a ZDRE-like motif upstream of *AtLPCAT1*, which inhibits its transcription. *LPCAT1* repression consequently has a positive influence on *AtPHT1;1* mRNA levels, and thus on Pi accumulation (Kisko *et al.*, 2018). Natural variation in this regulatory cascade underlies differences in Pi accumulation under Zn deficiency in Arabidopsis (Kisko *et al.*, 2018).

In *B. oleracea*, several cultivars were studied and compared in the context of Zn and Pi deficiency. For instance, red cabbage, kale, and broccoli have all been analyzed in different combinations of Zn and Pi conditions (Pongrac et al., 2019, 2020*a*, b). Two cultivars of *B. oleracea* with distinct Pi homeostasis, Pi shoot contents, and Pi shoot to root ratios (Pongrac *et al.*, 2020*a*) also display different responses to diverse combinations of Pi and Zn supply (Pongrac *et al.*, 2020b). Both cultivars have a higher Zn translocation to shoots in low Pi than in high Pi conditions (Pongrac *et al.*, 2020b), which is in accordance with observations in Arabidopsis (Rai *et al.*, 2014), but differentially accumulate Pi in shoots under Zn excess (Pongrac *et al.*, 2020b). The putative Zn transporter-encoding gene Bo4g045850, known to be induced in low Zn conditions (see above), as well as *Bo1g0221870* and *Bo2g036550*, encoding a Fe^{2+} transporter and an NAS, respectively, are differentially expressed at various ratios of Zn/Pi supplies (Pongrac *et al.*, 2020b). This study indicates that Pi soil concentration, together with the interplay between Zn, Fe, and Pi homeostasis, has to be considered in biofortification approaches (see Box 1).

Another important factor for nutrient absorption and crosstalk is the presence of other microorganisms. Rhizosphere organisms influence nutrient availability and can play an important role in Zn absorption (González-Guerrero *et al.*, 2016). These organisms also require Zn. For example, the rhizosphere microorganisms improve Zn uptake in some species, such as the Zn hyperaccumulator *A. halleri* (Muehe *et al.*, 2015), or the Zn uptake-efficient *Solanum nigrum* (Marques *et al.*, 2006). The bacterium *Burkholderia pyrrocinia* can even improve the tolerance of tomato to Zn excess (Min *et al.*, 2021).

In the context of Zn deficiency in dicotyledonous crops, microorganism–plant interactions are influenced by or influence Zn homeostasis in multiple ways. For instance, Zn deficiency increases the susceptibility of the plant to pathogens (e.g. in soybean, Helfenstein *et al.*, 2015). In other cases, Zn is necessary for pathogen virulence (López-Berges, 2020). A special case concerns legumes, such as soybean and bean, which among dicotyledonous species have the capacity to symbiotically interact with rhizobia bacteria via the formation of root nodules. This symbiotic interaction permits the plant to use the atmospheric nitrogen (N_2) fixed by the bacteria, in exchange for other nutrients (Garg and Geetanjali, 2007; Clarke *et al.*, 2014). This capacity is an important characteristic in agriculture, as it permits the reduction of the use of N fertilizers, and nodulation generally increases yield (Dupont *et al.*, 2012). The research on nodules has extensively used *Medicago truncatula* as a model. In this species, the Zn^{2+} transporters MtMTP2 and MtZIP6 are necessary for Zn distribution to nodules or Zn homeostasis in nodules. (Abreu *et al.*, 2017; León-Mediavilla *et al.*, 2018). Both transporters are necessary for nodule development, nitrogenase activity, and thus for plant growth in symbiotic conditions (Abreu *et al.*, 2017; León-Mediavilla *et al.*,

Box 2. Zn deficiency responses in monocotyledonous and dicotyledonous species

Mono- and dicotyledonous plants have distinct developmental pathways and anatomical features that have a direct impact on Zn homeostasis, with, for instance, the presence of an exodermis cell layer and its Casparian strip in roots or the existence of nodes acting as hubs for nutrient distribution to young vegetative and reproductive tissues in monocotyledonous plants. These anatomical differences impact symplastic and apoplastic Zn pathways and the numbers of barriers that have to be crossed by Zn to reach seeds from the soil.

At the molecular level, it is more and more clear that a similar set of metal transporter families (ZIPs, MTPs, HMAs, etc.) and metal chelators (e.g. NA) contribute to Zn homeostasis in mono- and dicotyledonous plants. In monocotyledonous plants, these players were mostly characterized in rice (Ricachenevsky *et al.*, 2015). However, phylogenetic studies indicating conservation of F-group bZIPs and ZDREs suggest that the Zn deficiency responsiveness of *ZIP* genes, and the transcriptional control of this responsiveness by F-group bZIP TFs are the most widely conserved mechanisms of Zn deficiency responses among mono- and dicotyledonous plants (see for instance Castro *et al.*, 2017; Lilay *et al.*, 2020). As in Arabidopsis, a Zn HMA pump has been suggested to play a key role in Zn loading into seed filial tissues from the mother plant in barley (Tauris *et al.*, 2009; Mills *et al.*, 2012).

Monocotyledonous species use distinct Fe uptake strategies and Fe chelators, when compared with dicotyledonous plants (Kobayashi and Nishizawa, 2012; Kobayashi *et al.*, 2019), which determines distinct interactions between Zn and Fe homeostasis (Hanikenne *et al.*, 2021; Kumar *et al.*, 2021). Briefly, in dicotyledonous plants, upon soil acidification and Fe^{3+} reduction, Fe is taken up in roots as Fe^{2+} by IRT1, a ZIP transporter, which has a broad specificity for divalent metal cations, including Zn^{2+}. In contrast, in graminaceous monocotyledonous plants, Fe^{3+} is chelated in the soil by so-called phytosiderophores (PSs), and PS–Fe complexes are taken up by YSL transporters (Marschner *et al.*, 1986; Kobayashi and Nishizawa, 2012). PS production is induced upon Zn deficiency, and PS–Zn transport into roots plays an important role in Zn uptake in monocotyledonous plants. PS, together with NA, also acts in radial root transport and long-distance transport of both Zn and Fe (Kobayashi and Nishizawa, 2012; Kobayashi *et al.*, 2019).

Altogether, tolerance to Zn deficiency is highly variable among monocotyledonous species, as it is in dicotyledonous plants (Table 1). Hence, if maize and rice are highly sensitive to Zn deficiency, barley shows intermediate sensitivity, whereas wheat is only mildly affected by Zn deficiency (Alloway, 2008*a*; Nielsen, 2012; Noulas *et al.*, 2018), indicative of variation of Zn homeostasis mechanisms in these species as well, and the need for detailed characterization of Zn homeostasis in individual major crops.

2018). Nodule function also requires Fe, which is transported by MtFPN2 and MtNRAMP1, responsible for Fe import into nodules and into rhizobia-infected cells, respectively (Tejada-Jiménez *et al.*, 2015; Escudero *et al.*, 2020). In soybean, the nodule number was shown to be affected by Zn deficiency, reducing yield (Shittu and Ogunwale, 2012). *GmZIP1*, the only fully characterized Zn transporter gene, is responsible for Zn import to nodules (Moreau *et al.*, 2002). The Zn requirement for nodulation has also observed in other dicotyledonous species, such as horse gram [*Dolichos biflorus* (Linn.)] (Edulamudi *et al.*, 2017). In soybean, Zn and Pi availability also influence arbuscular mycorrhizal fungi colonization, which in turn affects growth and yield (Yu *et al.*, 2020).

Conclusion

Although dicotyledonous crop species make up an important part of crop production and consumption worldwide, translation of the knowledge acquired on the Zn deficiency mechanisms in Arabidopsis has been slower towards dicotyledonous crops than monocotyledonous species. However, to address Zn deficiency as a global agronomic and health issue, both mono- and dicotyledonous crops should be considered as important for biofortification approaches (see, for instance, Castro *et al.*, 2017). Rapidly improving genomic resources, thanks to major progress in sequencing technologies, combined with the exploitation of ZUE natural variation among cultivars will allow uncovering the molecular mechanisms underlying tolerance to Zn deficiency at a much quicker pace in the future (Kumawat *et al.*, 2016; Sanjeeva Rao *et al.*, 2020).

Author contributions

MH and NT: conceptualization; NT: making the figures with input from MH; NT and MH: writing the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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