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Title

Zinc Homeostasis and Isotopic Fractionation in Plants: A review

Keywords

Copper, Iron, Nickel, Stable isotopes, Zinc deficiency, Zinc tolerance

Abstract

Aims: Recent advances in mass spectrometry have demonstrated that higher plants discriminate stable Zn isotopes during uptake and translocation depending on environmental conditions and physiological status of the plant. Stable Zn isotopes have emerged as a promising tool to characterize the plants response to inadequate Zn supply. The aim of this review is to build a comprehensive model linking Zn homeostasis and Zn isotopic fractionation in plants and advance our current view of Zn homeostasis and interaction with other micronutrients. *Methods:* The distribution of stable Zn isotopes in plants and the most likely causes of fractionation are reviewed, and the interactions with micronutrients Fe, Cu, and Ni are discussed. *Results:* The main sources of Zn fractionation in plants are i) adsorption, ii) low- and high-affinity transport phenomena, iii) speciation, iv) compartmentalization, and v) diffusion. We propose a model for Zn fractionation during uptake and radial transport in the roots, root-to-shoot transport, and remobilization. *Conclusions:* Future work should concentrate on better understanding the molecular mechanisms underlying the fractionations as this will be the key to future development of this novel isotope system. A combination of stable isotopes and speciation analyses might prove a powerful tool for plant nutrition and homeostasis studies.

Abbreviations

COPT = Copper Transporter

DMA = Deoxymugineic acid

EXAFS = Extended X-Ray Absorption Fine Structure

FRO = Ferric Reductase Oxidase

HMA = Heavy Metal ATPase

IRT = Iron-Regulated Transporter

MC-ICP-MS = Multi-Collector Induced Coupled Plasma Mass Spectrometry

MTP = Metal Tolerance Protein

PS = Phytosiderophores

YS = Yellow Stripe

YSL = Yellow Stripe-Like

ZIF = Zinc-Induced Facilitators

ZIP = ZRT-IRT-like Protein

Introduction

Zinc is an essential micronutrient for living organisms with several crucial functions in the cell. It is the only metal present in enzymes of all six major classes, playing catalytic, regulatory, and structural roles (Vallee and Auld 1992; Coleman 1992). Zinc is furthermore involved in the regulation of DNA transcription, and the transduction of intra- and intercellular signalling (Broadley et al. 2007; Maret 2013). Unfortunately, Zn deficiency is widespread in arable soils worldwide (Alloway 2009) due to various factors such as high pH (>7), low plant available Zn content, prolonged flooding, low redox potential, and high contents of organic matter, bicarbonate and phosphorus (P) (Neue and Lantin 1994; Ova et al. 2015). It is not surprising that around 17% of the world population is at risk of insufficient Zn intake based on food supply data, although actual deficiency rates are likely to be much higher (Wessells and Brown 2012; Kumssa et al. 2015). This makes Zn deficiency one of the most pressing causes of malnutrition. The consequences for the public health and the economy of the affected areas

64 are severe. In young children, Zn deficiency leads to stunting and increased susceptibility to
65 diarrhoea, pneumonia, and malaria, causing 800,000 early deaths yearly (Caulfield et al. 2006).
66 Zinc deficiency in crops causes root apex necrosis, leaf disorders, reduction of biomass,
67 delayed maturity, yield reduction, and high mortality (Van Breemen and Castro 1980; Wissuwa
68 et al. 2006; Broadley et al. 2007; Singh and Singh 2011; Al-Fahdawi et al. 2014; Mattiello et al.
69 2015; Fu et al. 2015). Strategies like crop biofortification (increasing Zn content in edible parts
70 during growth) or breeding for varieties tolerant to Zn-deficiency might help to overcome Zn
71 deficiency in soils. To this end, it is crucial to increase our current level of understanding about
72 the mechanisms involved in Zn uptake and metabolization by plants. The study of stable Zn
73 isotope fractionation is a novel technique that is already helping us to understand better the
74 mechanisms of Zn uptake, translocation, and tolerance in plants, and how these react to the
75 environment. However, a comprehensive model linking Zn homeostasis and Zn isotopic
76 fractionation in plants and considering the interactions with other micronutrients is still
77 missing.
78 Here we review our present understanding of Zn isotopic fractionation in plants and compare
79 it with other micronutrients and their isotopic systems, with the aim of advancing our current
80 view of Zn homeostasis and interaction with other micronutrients.
81
82 **Zinc isotopic fractionation in plants**
83 The concentration of Zn in plant tissues must stay within a specific range to preserve the
84 structural cohesion and metabolic functions of the cells. The lower end is typically around
85 15-20 µg Zn g^{-1} shoot dry matter, while the upper end is around 100-300 µg Zn g^{-1} (Marschner
86 1995; Broadley et al. 2012). In the cytoplasm of the plant cells, the concentration of free Zn^{2+} is
87 kept very low (in the p*M* range) (Maret 2015), because it tends to bind to cellular components.
88 Higher concentrations could eventually disrupt the cytosolic metabolism and restrict Zn
89 transport to satisfy the demands of sink organs, tissues, cells, and organelles. Plants have

developed several mechanisms to adapt to the fluctuations of the Zn available for them in the growth environment, and to maintain the intracellular levels of Zn stable within the optimal range. These are jointly known as Zn homeostasis. The amount of Zn taken up by the roots and transferred to the shoot is tightly controlled thanks to an intricate network of barriers, transporters, chelators, and compartments (Sinclair and Krämer 2012; Olsen and Palmgren 2014; Ricachenevsky et al. 2015). Zinc movement through the plants consequently leads to isotope discrimination.

Stable isotopes of light elements like C, N, O, and S have been long used to study plant physiology and its response to the environment (Mekhtiyeva and Pankina 1968; Deniro and Epstein 1979; Farquhar et al. 1982; Mariotti et al. 1982). A new generation of mass spectrometers (MC-ICP-MS) has enabled the use of stable isotopes to study Zn, copper (Cu), iron (Fe), nickel (Ni), calcium, and magnesium (Weiss et al. 2005; Wiegand 2005; Guelke and von Blanckenburg 2007; Black et al. 2008; Weinstein et al. 2011; Deng et al. 2014). Most progress has been made for Zn. Fractionation of Zn stable isotopes in plants was first reported during Zn uptake by the root and translocation to the shoots in hydroponically grown tomato (*Solanum lycopersicum* L.), lettuce (*Lactuca sativa* L.), and rice (*Oryza sativa* L.) (Weiss et al. 2005). Roots accumulated heavy isotopes relative to the solution, while the lighter isotopes were enriched in the shoots compared to the roots and differences in root-to-shoot fractionation were observed among species (Weiss et al. 2005). In soils, a survey of six species collected from a pristine water-shed in Cameroon showed that both roots and shoots were isotopically heavier than the top soil, with only one species showing root-to-shoot fractionation (*Megaphrynium macrostachyum*) (Viers et al. 2007). In the same survey, the leaves of trees were isotopically lighter than the rest of the plant, and a relationship between the height of the leaves and the magnitude of the fractionation was suggested. This hypothesis was later supported by a study using bamboo (*Phyllostachys aurea* Rivière & C. Rivière), where the light isotopes were progressively enriched in leaves with height (Moynier et al. 2009).

Subsequent studies demonstrated that isotope discrimination by plants changes in response to Zn availability in the environment. In rice, response to Zn deficiency resulted in changes in the fractionation pattern, and the shoots of Zn-deficient plants accumulated more heavy isotopes than the controls (Arnold et al. 2010). In reeds (*Phragmites australis* [Cav.] Trin. ex Steud.), Zn excess also caused alteration of the isotopic fractionation, and the aerial parts of plants grown in Zn-polluted solution were isotopically light as compared with the controls (Caldelas et al. 2011). Taken together, these findings show that Zn stable isotopes can be used to identify and quantify metal uptake or transport mechanisms and to assess the influence of factors such as environmental changes, physiological status, and species on these mechanisms.

Determination of accurate and precise Zn stable isotope ratios in plants

The MC-ICP-MS (multi-collector inductively coupled plasma – mass spectrometer) is a plasma source mass spectrometer with an array of collectors used to measure the isotope ratios of micronutrients. The instrument typically consists of three parts: an ion source, a mass analyser, and a detector. The ion source is a high-temperature argon plasma that ionizes the element. The mass analyser has two sectors (electrostatic and magnetic) that focus the ion beam and separate the ions for their mass-to-charge ratio. The detector unit consists of an array of Faraday cups that can measure different ion beams simultaneously. This multi-collector (MC) array permits to measure all the isotopes of an element at the same time and increases precision to 0.001% for the isotope ratios. An exhaustive description of the MC-ICP-MS technique is found in Vanhaecke et al. 2009.

Mass spectrometers favour the transmission of the heavy isotopes inducing a mass-bias. The sample preparation can also cause mass fractionation. There are different strategies to correct for mass-bias shifts: direct sample-standard bracketing (SSB), doping with an external element, and using a double-spike. In the direct SSB method, a standard is analysed before and after the sample and used to correct the shift. The mass-bias must be constant over time and the

142 sample must be very pure to minimize matrix effects. If mass fractionation changes over time,
143 a doping element with a mass similar to that of Zn (Cu is commonly used) may be mixed with
144 the sample to correct for mass bias. This external correction is based on the assumption that
145 the ratio of the mass fractionation of both elements stays constant during the analysis period
146 (Maréchal et al. 1999). In the double-spike correction, a spike containing two Zn isotopes is
147 added to the sample prior to sample preparation. The isotope ratio of the mixture is then
148 compared with that of the double-spike. The application of these various correction methods
149 to Zn stable isotope analysis has been described elsewhere(Albarède and Beard 2004).
150 Zinc isotope fractionation is commonly expressed using the delta notation, where the isotopic
151 ratio of the sample (e.g., $^{66}Zn/^{64}Zn$) is compared with that of a standard (e.g. the widely used
152 JMC 3-0729L Zn) and expressed in parts per thousand (‰) using Eqn. 1:
153 [1]

154 $$\delta^{66}Zn_{sample} = \left[\frac{\left({}^{66}Zn/{}^{64}Zn\right)_{sample}}{\left({}^{66}Zn/{}^{64}Zn\right)_{standard}} - 1\right] \cdot 10^3$$

155 The Zn isotopic fractionation between two samples (i and j) is calculated using Eqn. 2:
156 [2]

157 $$\Delta^{66}Zn_{i-j} = \delta^{66}Zn_i - \delta^{66}Zn_j$$

158
159 The IRMM 3702 standard has been proposed as the new first-choice reference material to
160 substitute the Johnson-Matthey Zn standard 3-0749L (JMC) (Moeller et al. 2012), of which
161 there is little left. However, JMC is still the standard most widely used in the literature and
162 most of authors did not analyse IRMM 3702. For this reason, all the $\delta^{66}Zn$ values in this review
163 are expressed relative to the JMC standard. Equation 3 was used to convert between
164 standards (Criss 1999):
165 [3]

$$\delta X_{JMC} = \delta X_{st} + \delta St_{JMC} + \frac{1}{10^3}(\delta X_{st}) \cdot (\delta St_{JMC})$$

, where δX_{JMC} is the $\delta^{66}Zn$ of the sample "X" relative to the standard JMC, $\delta^{66}X_{st}$ is the $\delta^{66}Zn$ of the same sample relative to the standard "St", and δSt_{JMC} is the $\delta^{66}Zn$ of the same standard relative to JMC. The data from each individual publication, was converted using the δSt_{JMC} provided by the authors. This was of 0.044±0.035‰ to 0.09±0.05‰ for the in-house standard Johnson-Matthey Purontronic™ Batch NH 27040 (Weiss et al. 2005; Arnold et al. 2010; Jouvin et al. 2012), 0.04±0.02‰ for the in-house standard London Zn (Smolders et al. 2013), and 0.27±0.08‰ to 0.28±0.05‰ for the reference material IRMM 3702 (Tang et al. 2012; Tang et al. 2016).

For Fe, Cu, and Ni the same δ notation is used, and δ values refer to the isotopic ratios $^{56}Fe/^{54}Fe$, $^{65}Cu/^{63}Cu$, and $^{60}Ni/^{58}Ni$, respectively, and are expressed relative to the standards IRMM-14 (Fe), NIST-SRM 976 (Cu), and NIST-SRM 986 (Ni).

Isotopic fractionation of Zn during uptake by plants

Zinc binding to the cell wall

The first evidence of Zn fractionation during Zn uptake by plants was provided by Weiss and colleagues (Weiss et al. 2005). They used rice, lettuce, and tomato grown hydroponically in EDTA- (1 µ*M* Zn) or HEDTA- (2 µ*M* Zn) solutions, and observed that ^{66}Zn was similarly enriched in the roots of all three species regardless of the nutrient solution ($\Delta^{66}Zn_{root\text{-}solution}$=0.08 to 0.16‰)(Fig. 1). This distribution of Zn isotopes was attributed to ^{66}Zn preferential adsorption onto the root surface or binding to cell walls, together with the preferential uptake of isotopically light Zn^{2+} into the root cells. Subsequently, in durum wheat (*Triticum durum* Desf.) and tomato, very similar $\Delta^{66}Zn_{root\text{-}solution}$ were obtained (-0.02 to 0.15‰) in EDTA solution with 1.6 or 0.62 µ*M* Zn (Jouvin et al. 2012). These results from hydroponic studies agree with data obtained from the natural environment. Viers et al. 2007 surveyed six plant species including

herbaceous and trees growing in a tropical watershed in Cameroon. All roots were isotopically heavier than the soils (ranging between 0.09 and 0.64‰) (Fig. 2). In larch trees (*Larix gmelinii* [Rupr.] Rupr.) from pristine Siberian forests, the fractionation between roots and soil was of 0.26‰ (Viers et al. 2015). Several other authors have reported the accumulation of heavy isotopes in the roots (up to 0.8%) with respect to the source of Zn in a variety of species and experimental set-ups (Aucour et al. 2011; Caldelas et al. 2011; Tang et al. 2012; Smolders et al. 2013; Houben et al. 2014; Couder et al. 2015; Aucour et al. 2015; Tang et al. 2016). Since these studies submitted plants to conditions of either Zn deficiency or Zn excess, they will be discussed in detail in sections 4.3 and 6.

Strong evidence of isotopic fractionation during Zn adsorption to cell walls has been obtained from laboratory experiments conducted with diatoms. Four species of marine and freshwater diatoms showed accumulation of ^{66}Zn in the unwashed cells with respect to the solution, with $\Delta^{66}Zn_{diatom\text{-}solution}$ ranging between 0.08 and 0.43‰ (Gélabert et al. 2006). The offset was attributed to adsorption of Zn onto the cell surface, and Zn adsorption modelling showed that Zn would mostly bind to the carboxylate groups of the cell walls. In the same line, the unwashed cells of the marine diatom *Thalassosiera oceanica* (Hasle) were isotopically heavier ($\delta^{66}Zn$ = -0.05 to 0.38‰) than EDTA-washed cells (-0.79 to -0.16‰) (John et al. 2007). The fraction of Zn adsorbed to *T. oceanica* was isotopically heavier than the solution, and the fractionation increased linearly with Zn concentration [Zn] ($\Delta^{66}Zn_{diatom\text{-}solution}$ from 0.09‰ at $10^{-11.5}$ *M* to 0.52‰ at $10^{-8.5}$ *M*)(John et al. 2007). The $\Delta^{66}Zn_{diatom\text{-}solution}$ from both studies is remarkably similar, and points to the preferential adsorption of heavy Zn isotopes onto the cell surface, probably to the carboxylate groups of the cell walls. Zinc binding to carboxyl and hydroxyl groups of pectin and to hydroxyl groups of cellulose in the cell walls of roots has been confirmed in tobacco plants (*Nicotiana tabacum* L.) using chemical extracts and Extended X-Ray Absorption Fine Structure (EXAFS) spectrometry (Straczek et al. 2008). It is thus very likely that the enrichment in heavy Zn isotopes observed in plants roots is mostly generated

during Zn binding to the hydroxyl and carboxyl groups of the cell walls, similarly to what happens in diatoms. The isotopic fractionation between plants roots and the solution where they grow ($\Delta^{66}Zn_{root\text{-}solution}$ = -0.15‰ to 0.8‰) is in a similar range to that of diatoms relative to the solution ($\Delta^{66}Zn_{diatom\text{-}solution}$ = 0.08‰ to 0.52‰). The wider spread of $\Delta^{66}Zn_{root\text{-}solution}$ could be explained by the larger [Zn] in the solutions (up to 10^{-5} *M*), and species-specific differences in the composition and adsorption capacity of the cell walls. To test this hypothesis, we need to constrain the isotopic fractionation during Zn binding to the cell walls of plants and their majoritarian components cellulose and pectine.

Low- and high-affinity transport phenomena

The shoots of hydroponically grown rice, tomato, and lettuce were depleted in ^{66}Zn relative to ^{64}Zn ranging from -0.25 to -0.56‰ and differing between species (Weiss et al. 2005)(Fig. 1). Analogous results were later obtained in tomato and durum wheat with $\Delta^{66}Zn_{shoot\text{-}root}$ ranging between -0.29 and -0.56‰ (Jouvin et al. 2012; Smolders et al. 2013). This shift was attributed to the preferential transport of free Zn^{2+} across cell membranes by transport proteins. The cell walls of root cells closely touch one another forming a single extracellular space termed root apoplast, in which water and solutes can circulate freely. This movement is restricted by the endodermis, a single layer of cells that surrounds the conductive tissue of the root. The Casparian strip, a ring of impermeable material found in the cell walls of the endodermis prevents Zn from reaching the shoots via the apoplastic pathway. Hence, Zn has to be transported across the cell membrane of the root cells. The cell membrane consists of a phospholipid bilayer, and its hydrophobic nature impedes the passive diffusion of dissolved ions into the cell. Ion uptake must be facilitated by transporter proteins, which allow plant cells to control their concentration in the cytoplasm. Zinc transport across the cell membranes is tightly controlled by plasma membrane associated proteins, mainly those from the ZIP family (ZRT-IRT-like Proteins). Transporters AtIRT1 and AtIRT3 (Iron Regulated Transporter) in

244 arabidopsis (Arabidopsis thaliana [L.] Heynh.), HvZIP7 in barley (*Hordeum vulgare* L.), and
245 OsIRT1, OsZIP1, OsZIP3, and OsZIP5 in rice are expressed in the root epidermis and involved in
246 Zn uptake from the rhizosphere into the cell (Korshunova et al. 1999; Vert et al. 2002; Ishimaru
247 et al. 2006; Lin et al. 2009; Lee et al. 2010; Tiong et al. 2014).
248 There are no isotope data on isolated plant cells (protoplasts) to help us understand how the
249 membrane transporter proteins could discriminate Zn isotopes. Experiments on diatoms
250 (single cell algae) might be a good approximation, since the functioning of Zn transporters is
251 similar. Early work in diatoms suggested that plasma membrane transporters discriminate
252 between Zn isotopes (Gélabert et al. 2006; John et al. 2007). Cells of marine diatoms washed in
253 EDTA to remove the extracellular Zn were isotopically light as compared with the solution,
254 which was attributed to fractionation during Zn uptake. Moreover, the $\Delta^{66}Zn_{solution\text{-}diatom}$
255 increased with [Zn], following a sigmoidal curve (John et al. 2007). The switch of this curve
256 took place around 10^{-10} *M* Zn, coinciding with the switch between suggested "high- and
257 low-affinity" Zn uptake reported for marine diatoms (Sunda and Huntsman 1992). Transport
258 characterized as "high-affinity" predominates at low [Zn], whereas low-affinity transport
259 prevails at high [Zn]. In the algae study conducted by John and co-workers, high-affinity Zn
260 uptake generated an isotopic fractionation of up to -0.2‰ at Zn levels below $10^{-10.5}$ *M* (John et
261 al. 2007). In contrast, low-affinity transport caused a much greater fractionation (up to -0.8‰)
262 at [Zn] above $10^{-9.5}$ *M*. It was argued that during higher efficiency transport most of the Zn
263 within reach is transported regardless of the isotope. This would explain the smaller isotopic
264 fractionation during Zn uptake when high-affinity transport predominates (John et al. 2007).
265 High- and low-affinity transport phenomena have been described in rice, wheat, and other
266 plants (Hacisalihoglu et al. 2001; Milner et al. 2012; Meng et al. 2014). The high-affinity
267 transport was reported to predominate at less than 10^{-8} *M* Zn in the growth medium for wheat
268 (Hacisalihoglu et al. 2001) and less than 10^{-7} *M* in rice (Meng et al. 2014). The [Zn] in the
269 hydroponic studies was around 10^{-7}-10^{-6} *M* (Weiss et al. 2005; Jouvin et al. 2012; Smolders et

al. 2013). At these Zn levels a higher contribution of low-affinity transport would be expected in wheat. In the above studies the root-to-shoot fractionation ($\Delta^{66}Zn_{shoot\text{-}root}$) was mainly attributed to Zn uptake into the plant by the root cells, so we can compare it to $\Delta^{66}Zn_{in\text{-}ex}$ in diatoms. The $\Delta^{66}Zn_{shoot\text{-}root}$ ranged from -0.25 to -0.56‰ for all plants, approximately between that of high- and low-affinity transport in marine diatoms (-0.2‰ and -0.8‰, respectively) (John et al. 2007). Moreover, wheat had lower $\Delta^{66}Zn_{shoot\text{-}root}$ (-0.29‰ to -0.51‰) than rice (-0.25‰ to -0.29‰) (Weiss et al. 2005; Jouvin et al. 2012)(Fig. 1). It is noteworthy that the extent of the fractionation between shoots and roots increased with increasing [Zn], as $\Delta^{66}Zn_{in\text{-}ex}$ did in diatoms (John et al. 2007). It is highly plausible that the ion selectivity of the membrane transport proteins like ZIP transporters is the predominant molecular mechanism responsible for the isotopic fractionation observed during Zn uptake at the root.

The use of $\Delta^{66}Zn_{shoot\text{-}root}$ as a proxy of isotopic fractionation during Zn uptake by plants serves well the purpose of elucidating the contribution of plants to isotope partitioning during Zn biogeochemical cycling. However, neither $\Delta^{66}Zn_{shoot\text{-}root}$ nor $\Delta^{66}Zn_{shoot\text{-}solution}$ separate the isotopic effect of Zn uptake from those caused by the mechanisms of Zn transfer from the root to the shoot (discussed in section 5.1). Therefore these parameters provide an insufficient level of detail for future physiological studies. To better quantify the discrimination of Zn isotopes occurring during Zn uptake into the root symplast we need to isolate this isotopic effect from any others. This could be achieved by reproducing the diatom studies using protoplasts, comparing the isotope ratios of wild type plants with mutant lines defective for known Zn transporters, measuring isotopic fractionation during Zn binding to relevant ligands, and analysing the isotope ratios of the cytoplasm, the vacuoles, and the xylem sap.

Uptake of Zn complexes in Zn-deficient plants

295 Quantum mechanics predicts that the mass of atoms affects the strength of chemical bonds
296 (White 2015). The molecule with the heavy isotope has lower vibrational frequency and hence
297 lower dissociation energy. This generally leads to the accumulation of heavy isotopes in the
298 complexed form, as seen during the formation of Zn complexes with EDTA ($\Delta^{66}Zn_{Zn\text{-}L-}$
299 $_{Zn^{2+}}$=0.33%) and deoxymugineic acid (DMA) (0.30‰)(Markovic et al. 2016). In a field study
300 using soil with low Zn available, ^{66}Zn was enriched in the shoots of a rice variety tolerant to Zn
301 deficiency (RIL46) compared with the soil ($\Delta^{66}Zn_{shoot\text{-}soil}$=0.21‰) and the shoots of intolerant
302 plants ($\Delta^{66}Zn_{int\text{-}tol}$=0.13‰)(Arnold et al. 2010)(Fig. 2). This was tentatively attributed to Zn
303 uptake in form of complexes with DMA. The roots of RIL46 release more DMA in response to
304 Zn deficiency than a Zn-deficiency sensitive rice variety, IR74 (1.2 vs 0.4 μmol DMA g^{-1} root DW
305 4 h^{-1})(Widodo et al. 2010). Phytosiderophores (PS) like DMA are small molecular weight
306 compounds excreted by the roots of plants from the Poaceae family (Strategy II plants) in
307 response to Fe deficiency (Takagi 1976; Marschner et al. 1986; Takagi et al. 2008). The PS
308 solubilize Fe from soil and form PS-Fe complexes, which are then taken up by the root cells by
309 means of specific plasma membrane transporters of the OPT (Oligopeptide Transporter) family
310 (Lubkowitz 2011). Examples are ZmYS1 in maize (*Zea mays* L.), HvYS1 in barley, and OsYSL15 in
311 rice (Murata et al. 2006; Ueno et al. 2009; Inoue et al. 2009; Suzuki et al. 2012). To date no
312 Zn-PS specific transport activity has been described, but ZmYS1 can transport DMA complexes
313 with Zn, Cu, Mn, Ni, and Cd when expressed in yeast (*Saccharomyces cerevisiae* Meyen ex
314 E.C.Hansen) and frog oocytes (*Xenopus laevis*) (Schaaf et al. 2004). Besides, the mutant maize
315 ys1 (defective for ZmYS1, the Fe-PS transporter) cannot absorb Zn complexes with either DMA
316 or eHMA (epi-hydroxymugineic acid) (Von Wiren et al. 1996). This suggests that Zn might share
317 the same uptake pathway as Fe in Strategy II plants under Zn deficiency. In agreement, several
318 surveys report a significant increase in PS secretion in Zn-deficient wheat (*Triticum aestivum*
319 L.), barley, triticale (×*Triticosecale* Wittm. ex A.Camus), and rye (*Secale cereale* L.) (Zhang et al.
320 1989; Cakmak et al. 1994; Gries et al. 1995; Cakmak et al. 1996; Erenoglu et al. 1996; Cakmak

et al. 1998a; Cakmak et al. 1998b; Rengel 1999; Erenoglu et al. 2000; Suzuki et al. 2006), which reverts to control levels within 72 hours after Zn is resupplied (Zhang et al. 1989). Moreover, many Zn-efficient varieties of wheat, barley, and rice have shown higher PS release rate during Zn-deficiency than the inefficient ones (Cakmak et al. 1994; Cakmak et al. 1996; Cakmak et al. 1998a; Rengel et al. 1998; Rengel and Römheld 2000; Erenoglu et al. 2000; Tolay et al. 2001; Widodo et al. 2010; Neelam et al. 2010; Daneshbakhsh et al. 2013). In contrast, analogous experiments found no significant difference in PS release in Zn-deficient rice, wheat, and barley (Pedler et al. 2000; Suzuki et al. 2008; Widodo et al. 2010), or between Zn-efficient and inefficient wheat and barley (Erenoglu et al. 1996; Cakmak et al. 1998b; Pedler et al. 2000). These inconsistencies in the literature might indicate that Zn-efficiency in crops is determined by various factors, including PS release by the roots and possibly others. Interestingly, in some instances when Zn-efficiency and PS release did not correlate, Zn-efficient plants still took up more Zn and had higher root-to-shoot translocation rates (Von Wiren et al. 1996; Cakmak et al. 1998b). It was suggested that Zn-efficient varieties might have higher rates of Zn-PS uptake and Zn export to the shoot, while the release rates were not necessarily increased.

A central question is if the secretion rates observed during Zn deficiency and the Zn-PS complexes subsequently formed could account for the Zn budget of plants. The stability constant of Fe(III)-PS complexes ($10^{18.4}$) is significantly higher than that of Zn-PS complexes ($10^{12.9}$)(Murakami et al. 1989), which might make it difficult for the latter to form in presence of Fe(III). The amount of PS secreted during Fe-deficiency is much higher than during Zn-deficiency, up to 25 fold in rice (Suzuki et al. 2008), 23 fold in barley (Suzuki et al. 2006), and 16 fold in wheat (Tolay et al. 2001). In a recent study in wheat (cv Tamaro) grown in calcareous Fe-deficient soils, PS secretion from the roots ranged from 0.2 to 41 pmol DMA g^{-1} root DW s^{-1}, which is about 50 times less than solution-grown plants typically secrete (Oburger et al. 2014). It was argued that the PS release data from solution-grown plants in zero [Zn] or [Fe] might be grossly over-estimated, since these extreme conditions are not realistic in the

347 field. In soils, at least a small amount of those metals will be available for plants, reducing the
348 need for PS release. The PS release in soil-grown wheat was strongly increased by soil
349 characteristics like salinity and low trace element availability (Oburger et al. 2014). Salinity had
350 been previously seen to increase PS release rates in solution-grown wheat (Daneshbakhsh et
351 al. 2013). To the best of our knowledge, there are no data available of PS release in Zn-
352 deficient soils. This information is crucial to determine if the PS make a significant contribution
353 to Zn uptake, and build an accurate model of Zn uptake by plants in Zn-deficient soils.
354 Furthermore, since Fe and Zn might compete for the binding sites of PS and the uptake of the
355 resulting complexes, the interaction between Fe and Zn needs to be better understood for the
356 correct interpretation of Zn isotopic fractionation in Zn-deficient graminaceous plants. In the
357 particular case of rice, the effect of flooding and anoxia on the soil chemistry and plant
358 physiology needs to be considered, since this crop is usually grown in inundated fields. In
359 waterlogged soils, Fe^{2+} in soil solution is high while Zn availability is reduced by precipitation
360 (Becker and Asch 2005). Mathematical modelling has shown that the rate of DMA release
361 observed in Zn-deficient rice (≈10 pmol DMA g^{-1} root FW s^{-1}) can fully account for the Zn
362 uptake observed in a Zn-deficient submerged soil (Ptashnyk et al. 2011). However, the PS
363 release data used for the model were obtained from solution-grown rice in aerobic conditions
364 with low Fe^{2+} (Suzuki et al. 2006; Widodo et al. 2010). Unfortunately, no PS release data have
365 been obtained in conditions which simulate anaerobic soil high in Fe^{2+} and low Zn. Analysis of
366 gene expression has revealed that Fe^{2+} toxicity can down-regulate the expression of genes
367 involved in PS synthesis and Fe-PS uptake in rice roots after short exposure times (three days)
368 (Quinet et al. 2012). However, it is a short-lived effect. The expression levels are equal to the
369 control or even higher after longer exposure times (1 to 3 weeks) (Quinet et al. 2012; Müller et
370 al. 2015). Moreover, the stability constant of Zn-PS ($10^{12.9}$) is higher than that of Fe(II)-PS
371 ($10^{10.5}$) (Murakami et al. 1989). It is thus possible that PS have a role in Zn-uptake by Zn-
372 deficient rice in waterlogged soils rich in Fe^{2+}. An interesting feature of plants tolerant to

373 submersion is that they can have their roots covered by an iron plaque. This is a coat of
374 precipitated Fe(III) hydroxides formed thanks to the oxygen leaked from the roots. Up to 25 g
375 of iron plaque per Kg of root DW might favour Zn uptake in rice, whereas a higher amount
376 interferes with Zn uptake (Zhang et al. 1998). The effect of the Fe plaque is larger in plants
377 previously grown in Fe-deficient solution, and the Fe-deficient plants had a higher [Zn] in
378 shoots. It was concluded that the PS might promote Zn uptake in plants with iron plaque by
379 mobilizing Zn adsorbed to it.
380 Besides PS, plant roots exudate a mixture of organic acids and amino acids that enhance Zn
381 dissolution from the soil by lowering the pH around the roots and binding to Zn (Rasouli-
382 Sadaghiani et al. 2011). This strategy is also present in plant families that do not synthesize PS,
383 where Zn and other metals can be taken up in form of complexes (Degryse et al. 2007).
384 Furthermore, root exudates solubilize Zn from Zn-containing minerals like smithsonite ($ZnCO_3$)
385 (Houben and Sonnet 2012). For example, tomato seedlings were grown in resin-buffered
386 solution at two external [Zn] (10^{-6} or $1.5x10^{-8}$ *M*) and two pot sizes, to manipulate the
387 concentration of root exudates (Smolders et al. 2013). The highest [Zn] in the solution after
388 plant growth was recorded in the experimental conditions that most favoured the
389 accumulation of root exudates in the solution (low Zn and small pot). This suggested that root
390 exudates can also have an important role in mobilizing Zn in dicots. Besides, the shoots at 10^{-6}
391 *M* Zn were enriched in light isotopes (-0.52‰ to -0.56‰). By contrast, plants grown at $1.5x10^{-8}$
392 *M* had a similar isotopic composition as the roots ($\Delta^{66}Zn_{shoot-root}$=-0.06‰ to -0.09‰)(Smolders
393 et al. 2013). It was hypothesized that at high Zn supply uptake was dominated by the
394 facilitated diffusion of free Zn^{2+}, which favours ^{64}Zn, whereas at low Zn supply most of the Zn
395 was taken up as Zn complexes. The heavier isotopes accumulate in the complexes because
396 they form stronger bonds. The resulting complexes do not undergo isotopic fractionation
397 during transport because the relative difference of mass (isotope vs complex) is too small. Zinc
398 is preferentially taken up by plants as free Zn^{+2} (Marschner and Marschner 1995). However,

399 the uptake of entire Zn complexes has been reported in barley, potato (*Solanum tuberosum*
400 L.), *Brassica juncea* (L.) Czern., and *Lupinus albus* L. (Collins et al. 2002). Specific transporters
401 like AtHMA2 or members of the YSL (Yellow Stripe-like) family might facilitate the uptake of Zn
402 complexes with ligands in the root exudates, but direct evidence is still missing (Schaaf et al.
403 2004; Eren and Argüello 2004).
404 Plants adapted to low-phosphorus soils can emit large amounts of carboxylates in response to
405 phosphorus (P) deficiency (Gerke 2015). These carboxylates can increase the availability of Zn
406 and other metals from the rhizosphere (Duffner et al. 2012; Lambers et al. 2015). Zinc content
407 in the leaves of *Hakea prostrata* R.Br. increased with the development of cluster roots, which
408 was attributed to the high release of carboxylates typical of these type of roots (Shane and
409 Lambers 2005). In the same study, Zn concentration in leaves decreased with increasing P
410 availability. Similar antagonistic interaction between Zn and P has been repeatedly observed in
411 the literature (Imran et al. 2016; Zhang et al. 2016). This suggests that P availability could
412 affect the Zn isotopic composition of the plant by increasing the proportion of Zn taken up in
413 form of Zn complexes with OA. This interesting possibility has not been explored so far, and
414 would be useful for the study of Zn-P interaction.
415
416 **Isotope fractionation of Zn during transport to the aerial parts**
417 *Zinc transfer from the root to the stem*
418 The $\Delta^{66}Zn_{shoot\text{-}root}$ of Zn-sufficient hydroponically grown rice, tomato, and wheat is in the
419 range -0.56 to -0.25‰ (Weiss et al. 2005; Jouvin et al. 2012; Smolders et al. 2013). This shift in
420 favour of the light isotopes has been attributed to Zn uptake facilitated by transporters at the
421 membrane of the root cells, as discussed in section 4.2. However, in soil-grown plants the stem
422 was sometimes isotopically heavier than the roots ($\Delta^{66}Zn_{stem\text{-}root}$ from -0.33 to 0.25‰) (Viers et
423 al. 2007; Moynier et al. 2009; Viers et al. 2015). This could be explained by plants taking up a
424 greater proportion of Zn-complexes due to a lower availability of Zn in the natural

environment. However, we must also pay attention to the different sampling methods used. In the hydroponic studies (Weiss et al. 2005; Jouvin et al. 2012; Smolders et al. 2013) all the aerial parts were sampled together, whereas in the field studies (Viers et al. 2007; Moynier et al. 2009; Viers et al. 2015) stems and leaves were analyzed separately. The contribution of the leaves might then explain the shift between the solution-grown and the soil-grown plants. Besides, a number of processes take place during Zn transfer from the root symplast to the aerial parts that are likely to discriminate Zn isotopes. In the intra-cellular fluid (cytosol) the pH is close to neutral (7.2-7.5), and Zn^{2+} will be kept at a very low concentration (in the p*M* range)(Maret 2015) to avoid precipitation and misplaced binding. There are several low-weight molecules that have been proposed as important Zn ligands in the cytosol, such as nicotinamine (NA), histidine, organic acids, and small peptides (for a review, see Sinclair and Krämer 2012). Of these, the non proteinogenic amino acid NA is considered the main Zn ligand in the cytosol, and a key factor in enhancing Zn mobility in the symplast (Clemens et al. 2013). The fractionation of Zn isotopes during the formation of Zn complexes with NA has not been explored. However, *ab initio* calculations have shown that the partitioning of heavy isotopes between citrates, malates, phosphates, histidine, and other Zn species can account for part of the isotopic fractionation observed in plants, which will be further discussed in section 6.2 (Fujii and Albarède 2012; Fujii et al. 2014). The computational studies suggested that heavy isotopes tend to bind to oxygen donors, while light isotopes accumulate in Zn complexes with sulphur donors, and Zn complexes with nitrogen donors would be between the two or isotopically heavier than with oxygen donors (Fujii et al. 2014). Recent experimental evidence has confirmed that the heavy isotopes accumulate in Zn complexes with DMA, and structurally similar ligands (Markovic et al. 2016). Another potential source of isotope discrimination is Zn sequestration in the vacuoles. The activity of the vacuolar transporters regulates the amount of Zn in the cytosol available for translocation by either increasing or decreasing the amount of Zn sequestered in the vacuoles. Several studies conclude that MTP1 and MTP3 (Metal

Tolerance Proteins) facilitate the efflux of Zn^{2+} from the cytosol into the vacuole (reviewed by Ricachenevsky et al. 2013). The vacuole has an acidic pH around 5.2 (Shen et al. 2013) and Zn can stay soluble as Zn^{2+}. Similarly, AtZIF1 is a tonoplast transporter that carries Zn complexes with NA into the vacuoles (Haydon et al. 2012). Transporter AtNRAMP4 facilitates Zn influx from the vacuole back into the cytoplasm (Lanquar et al. 2010). Additionally, Zn^{2+} can diffuse from one cell to another across the plasmodesmata, small openings in the cell walls that allow the cytoplasm of adjacent cells to communicate. This is a kinetically controlled process that will favour the light isotopes (Criss 1999).

To reach the shoot, Zn needs to leave the root symplast and enter the xylem, a conductive tissue formed by the walls of dead cells containing no cytoplasm. Transporters HMA2 and HMA4 are plasma membrane transporters of the HMA family (Heavy Metal ATPases) expressed in the vasculature of the root, and are thought to export Zn from the adjacent cells to the xylem in arabidopsis (Eren and Argüello 2004). Of these, HMA2 transports Zn bound to a ligand while HMA4 transports Zn^{2+}. The xylem sap is considered an acidic environment with pH around 5.5 where Zn^{2+} could stay in solution, although it can be alkalinized in response to drought, flooding, bicarbonates, nutrients, light, change of season, daily rhythms, and disease (Wilkinson, Janet E. Corlett, Ludovic Oger et al. 1998). Little is known about how these fluctuations affect Zn xylem loading and forms in the xylem sap. In the xylem sap of strategy I plants Zn is predominantly transported as Zn^{2+} with the remaining fraction bound to organic acids (Salt et al. 1999; Monsant et al. 2011; Lu et al. 2014). The aminoacids histidine (Kozhevnikova et al. 2014) and NA (Cornu et al. 2015) have also been proposed a role in Zn-binding in the xylem. In strategy II plants, Zn-DMA complexes have been identified in root extracts of Fe-deficient wheat (Xuan et al. 2006), and in the shoots of japonica rice (Tsednee et al. 2016), which suggests that Zn could be transported up the xylem as Zn-PS complexes. All of these steps might contribute to the isotope ratios of roots and shoots.

Zinc translocation to the leaves

The few experiments that analysed leaves separately found generally isotopically lighter Zn compared to the stems ($\Delta^{66}Zn_{leaves\text{-}stem}$ -1.67 to 0.10‰)(Viers et al. 2007; Moynier et al. 2009; Caldelas et al. 2011; Viers et al. 2015). Furthermore, the leaves of various plant species were increasingly depleted in ^{66}Zn the further they were from the root (Fig. 3). First, tree leaves collected from a pristine tropical drainage basin were recorded as isotopically lighter ($\delta^{66}Zn_{leaves}$ -0.03‰ to -0.91‰) than the leaves of the herbaceous species in the same area (0.26 to 0.63‰) (Viers et al. 2007). The observations by Viers et al. 2007 were confirmed in bamboo leaves collected at various heights (20, 50, and 80 cm), which showed a progressive decrease of $\delta^{66}Zn$ with distance (-0.19‰, -0.32‰, and -0.55‰ respectively)(Moynier et al. 2009). Both studies proposed that the negative $\delta^{66}Zn$ of the leaves could be explained as the sum of two processes: i) Faster transport of ^{64}Zn across the plasma membranes, throughout Zn transfer from the roots to the leaves, and ii) preferential unload of ^{66}Zn from the xylem sap into the adjacent cells by low-affinity transporters, leaving the xylem solution progressively enriched in ^{64}Zn. Zinc binding to the cell walls lining the xylem vessels might also occur, favouring the depletion of heavy isotopes in the xylem sap via ion exchange. A similar trend was observed in reeds, where a fractionation of up to -0.44‰ was measured between leaves sampled at 5 and 100 cm (Caldelas et al. 2011). The extent of Zn fractionation with height was very consistent: -0.005‰ cm^{-1} in reed (Caldelas et al. 2011) and -0.006‰ cm^{-1} in bamboo (Moynier et al. 2009).

To leave the xylem and enter the leaf symplast Zn must first cross the membranes of the companion cells, a step mediated by transporters yet to be characterized. Membrane transporters AtIRT3 and OsZIP4 are expressed in the vasculature of the leaves and might facilitate both xylem unloading and phloem loading of Zn^{2+} (Ishimaru et al. 2005; Lin et al. 2009). The xylem unloading of Zn^{2+} could contribute to the enrichment of light isotopes observed in the leaves relative to the stem. The xylem loading, transport, and unloading of Zn

503 in form of Zn-NA or other Zn complexes is not expected to discriminate Zn isotopes.
504 Alternatively, Zn can be remobilized from older tissues and transported to the leaves via the
505 phloem. Excess Zn is stored in the vacuoles of the leaves in form of Zn complexes with citrate,
506 malate, or NA (Aucour et al. 2011; Tang et al. 2012). The formation of those Zn complexes
507 would likely favour the accumulation of heavy Zn isotopes in the complexes in the vacuole,
508 leaving an isotopically lighter Zn^{2+} pool available for transport in the cytosol. Tonoplast
509 transporters NRAMP3 and NRAMP4 in Arabidopsis facilitate Zn^{2+} efflux from the vacuole and
510 are expressed in leaves (Lanquar et al. 2010). Zinc facilitated diffusion across the tonoplast
511 should be faster for ^{64}Zn, which would contribute further to the accumulation of light isotopes
512 in the Zn^{2+} pool available for transport. However, the pH in the cytosol is close to neutral
513 (7.2-7.5), while the phloem sap is slightly alkaline (7.3-8.5) (Dinant et al. 2010). In these
514 conditions Zn must bind to an intracellular ligand to stay soluble, probably NA (von Wiren et al.
515 1999; Nishiyama et al. 2012). This would probably lead to the accumulation of ^{66}Zn in the
516 Zn-NA complexes, although this has not been tested. Metal loading from the cytosol of the
517 companion cells into the phloem is likely facilitated by plasma membrane transporters of the
518 YSL family, which transport metal complexes with NA. Transporters OsYSL2, AtYSL1, and
519 AtYSL3 transport NA complexes with Fe and other metals and are expressed in the vasculature
520 of the leaves (Koike et al. 2004; Chu et al. 2010). Analogous YSL transporters might be involved
521 in Zn loading from the phloem, but they have not been identified yet. In the phloem, Zn is
522 predominantly found as Zn-NA complexes (Nishiyama et al. 2012; Hazama et al. 2015). The
523 mechanisms of Zn unloading from the phloem at the leaves are poorly known. The phloem
524 loading, transport, and unloading of Zn in form of Zn-NA complexes is not expected to
525 discriminate Zn isotopes. Taking all the evidence together, it is yet not clear if Zn
526 remobilization from older leaves to young ones could account for the enrichment of light
527 isotopes in leaves with height. To advance our understanding of Zn isotopic fractionation from
528 the stem to the leaves, and from older to younger leaves, we need to first determine the Zn

529 forms in all the relevant Zn pools in the leaves, and to characterize the transporters that move
530 Zn between those pools.
531
532 *Zinc allocation to the seeds*
533 Evidence of isotopic fractionation during Zn transfer to the seeds is scarce. A recent study
534 found that in soil-grown rice the seeds were isotopically lighter than the shoots ($\Delta^{66}Zn_{seed-shoot}$ -0.8 to -0.7‰)(Arnold et al. 2015). This was attributed to Zn remobilization from the stem
536 during grain filling. However, the specific causes for this enrichment of the light isotopes are
537 not clear, because there is insufficient information of the molecular mechanisms and Zn
538 species involved in Zn transfer to the seeds in rice. Zinc in the rice grain is delivered via two
539 routes: i) phloem loading after remobilization from the leaves and stems (as discussed above),
540 and ii) direct xylem-to-phloem transfer in the stem and the nodes of the seed panicle, the
541 loosely-branched cluster of seeds (Yoneyama et al. 2010; Wu et al. 2010). The plasmatic
542 membrane transporter OsHMA2 is highly expressed at the nodes, and is believed to be
543 involved in the xylem-to-phloem transfer of Zn to the panicle (Yamaji et al. 2013). Tonoplast
544 transporters OsVIT1 and OsVIT2 (Vacuolar Iron Transporters) facilitate the influx of Zn into the
545 vacuole and are mainly expressed in the flag leaves, suggesting a primary role in the regulation
546 of Zn export to the seeds (Zhang et al. 2012). The activity of these transporters could
547 contribute to the observed enrichment of the light isotopes in seeds relative to shoots in rice,
548 but additional fractionation processes likely contribute. To allocate Zn to the developing seeds,
549 Zn must exit the phloem and cross a series of species-specific apoplastic barriers between the
550 maternal transfer cells, specialized in the transfer of solutes, and the endosperm of the seed,
551 the tissue that surrounds and nourishes the embryo (Olsen and Palmgren 2014). These
552 apoplastic barriers and the processes that control Zn transport across them are poorly known.
553 In barley, a detailed model of Zn trafficking to the seed has been proposed based on RNA
554 expression data from seed tissues (Tauris et al. 2009), which suggests that Zn would enter the

555 maternal transfer cells from the phloem in form of Zn-NA complexes. In the cytosol of the
556 transfer cells Zn appears to be in form of Zn-NA or Zn-PS complexes, as indicated by the high
557 expression of two NA synthases (NAS5-2 and NAS9), and one NA aminotransferase (NAATB,
558 that converts NA into DMA) (Tauris et al. 2009). The symplastic movement of Zn-NA complexes
559 from the phloem into the transfer cells would not cause any isotopic fractionation, whereas
560 the formation of Zn-complexes in the cytosol of the transfer cells would lead to the
561 accumulation of heavy isotopes in the complexes relative to the Zn^{2+} fraction. The vacuolar
562 transporters MTP1, MTPc4, VIT1-1, and CAX1a,b are highly expressed in the transfer cells, and
563 thought to promote Zn^{2+} flow into the vacuole (Tauris et al. 2009). Transporting Zn^{2+} into the
564 vacuole would likely result in the accumulation of light Zn isotopes in the vacuole relative to
565 the cytosol. During grain filling, Zn^{2+} stored in the vacuole of the transfer cells would be
566 remobilized by the vacuolar transporter NRAMP3 (Tauris et al. 2009). This transporter might
567 further enrich the light isotopes in the remobilized fraction relative to the vacuole. Thus Zn
568 storage and remobilization might cause the enrichment of light isotopes in the Zn^{2+} fraction
569 available for export to the seed relative to the Zn-NA fraction in the cytosol. Zinc efflux from
570 the cytosol of the transfer cells would be facilitated by plasma membrane transporters HMA2,
571 4, and 8, which are proposed to pump Zn^{2+} out to the endosperm cavity (Tauris et al. 2009) and
572 might cause further enrichment of the light isotopes in the Zn pool allocated to the seed.
573 Tauris and co-workers proposed that Zn would be in form of Zn^{2+} or Zn-complexes with NA or
574 DMA in the endosperm cavity, while Zn^{2+} would diffuse towards the seed, a
575 kinetically-controlled process that favours the light isotopes. In the aleurone (the outermost
576 layer of the endosperm) and the embryo, Zn^{2+} uptake would be facilitated by transporters of
577 the ZIP family, while YSL6, 9, and 12 would do the same with the Zn-complexes. Zinc efflux
578 from the seed back to the endosperm cavity was probably minimal, since the HMA
579 transporters involved in Zn efflux from the cytosol back into the apoplast showed much lower
580 transcription rates in the aleurone, endosperm, and embryo than in the maternal transfer cells

(Tauris et al. 2009). This suggests that Zn movement and compartmentalization within the seed after Zn uptake in the aleurone would not substantially add to the fractionation of Zn isotopes between the seed and the shoot.

In summary, it appears that the enrichment of light isotopes in the seeds relative to the shoots could originate from Zn^{2+} storage in the vacuoles of the transfer cells, efflux from transfer cells, diffusion in the endosperm cavity, and uptake by aleurone cells. Additionally, mechanisms that lead to the enrichment of light isotopes in the phloem sap relative to the bulk shoot, like Zn remobilization from the older tissues or direct xylem-to-phloem Zn transfer could contribute to make seeds isotopically lighter than the shoots. The $\delta^{66}Zn$ of the grain might prove an interesting tool for the development of Zn biofortification in crops, which could help us identify the key mechanisms involved in Zn allocation into the seed. However, there is a pressing need for more isotope data from seeds and a better knowledge of the molecular mechanisms behind Zn allocation to the seed and the Zn forms in the different reservoirs.

Fractionation of Zn isotopes associated with plants response to Zn excess

Accumulation in the roots of Zn-tolerant plants

Tolerant plants are those that can grow and develop when [Zn] in the environment would usually be deleterious. In tolerant plants exposed to an excessive amount of Zn in the environment, roots play an important role in Zn detoxification and sequestration to protect sensitive photosynthetic tissue. The response of tolerant plants to high Zn levels can cause significant changes in the distribution of Zn isotopes across the plant organs in some species. In reed (*Phragmites australis* [Cav.] Trin. ex Steud) grown hydroponically with sufficient Zn supply ($3x10^{-6}$ *M*) the roots, rhizomes, and shoots were similarly enriched in heavy isotopes relative to the growth solution (~0.2‰)(Caldelas et al. 2011)(Fig. 4a). By contrast, the addition of Zn in an excessive amount ($2x10^{-3}$ *M*) caused further enrichment of the heavy isotopes in roots relative to the growth solution ($\Delta^{66}Zn_{root\text{-}solution}$=0.47 ‰) and relative to the stems ($\Delta^{66}Zn_{stem\text{-}root}$=-0.84

607 to -0.94‰). The magnitude of both isotopic effects was larger than reported for
608 hydroponically grown crops ($\Delta^{66}Zn_{root\text{-}solution}$ -0.02 to 0.16‰, and $\Delta^{66}Zn_{shoot\text{-}root}$ -0.25
609 to -0.56‰)(Weiss et al. 2005; Jouvin et al. 2012; Smolders et al. 2013). Excess Zn can
610 precipitate with insoluble phosphates or silicates at the root epidermis, the intercellular
611 spaces, and the cell walls of the roots, a process termed biomineralization that might reduce
612 Zn influx into the symplast (Neumann and zur Nieden 2001; Straczek et al. 2008; Medas et al.
613 2015; De Giudici et al. 2015). In tobacco, Zn binds to the carboxyl and hydroxyl groups of
614 pectin and to the hydroxyl groups of cellulose in the cell walls of roots (Straczek et al. 2008).
615 The composition of the cell walls changes in response to excess Zn, modifying its permeability,
616 binding capacity, and affinity to enhance Zn tolerance (Lin and Aarts 2012). Inside the cell, Zn
617 binds to various ligands to limit its interaction with sensitive cellular components.
618 Nicotinamine forms complexes with Zn that are then sequestered in the vacuoles of the root
619 cells (Trampczynska et al. 2010; Haydon et al. 2012). Phytochelatins (PC) are small peptides
620 synthesized from gluthathione that bind to Zn and have a role in Zn tolerance and
621 accumulation in the roots (Tennstedt et al. 2009). Free Zn^{2+} and Zn-ligand complexes are
622 compartmentalized in the vacuole so they cannot interfere with the cell metabolism. This is
623 achieved by means of specific vacuolar transporters. Several tonoplast Zn transporters
624 involved in Zn sequestration in the vacuoles of the root cells have been described, belonging to
625 the protein families MTP, HMA, and ZIF (Zinc-Induced Facilitators)(reviewed by Peng and Gong
626 2014). Arabidopsis AtMTP1 and AtMTP3, OsMTP1 in rice, and HvMTP1 in barley are expressed
627 in the roots, facilitate Zn efflux to the vacuole, and have a role in Zn tolerance (Kobae et al.
628 2004; Arrivault et al. 2006; Podar et al. 2012; Menguer et al. 2013). A similar function has been
629 attributed to AtHMA3 and AtZIF2 in arabidopsis, which are mainly expressed in the roots and
630 are involved in Zn tolerance (Morel et al. 2009; Remy et al. 2014). Finally, AtZIF1 is a tonoplast
631 transporter in arabidopsis that carries Zn-NA complexes into the vacuoles and is mostly
632 expressed in the roots (Haydon et al. 2012).

633 The individual contribution of each of the mechanisms of Zn sequestration in the root
634 discussed above to the partitioning of Zn isotopes in plants is not yet clear. In *Phalaris*
635 *arundinacea* L. grown in soil receiving Zn-polluted stormwater, around 30-40% of the Zn in
636 roots was present in tetrahedral coordination, which may correspond to apoplasmic Zn binding
637 to the cell walls (Aucour et al. 2015)(Fig. 4a). The rest of the Zn fraction was coordinated in an
638 octahedral structure, probably binding to ligands like organic acids and sequestered in the
639 vacuoles. The $\Delta^{66}Zn_{shoot\text{-}root}$ in this study was -0.83‰, very similar to previous results in *P.*
640 *australis* ($\Delta^{66}Zn_{shoot\text{-}root}$=-0.84 to -0.94‰)(Caldelas et al. 2011), and to recent data in *Noccaea*
641 *caerulescens* (J.Presl & C.Presl) and *Thlaspi arvense* L. ($\Delta^{66}Zn_{shoot\text{-}root}$ =-0.79‰ in both species)
642 (Tang et al. 2016). In the later study Zn in roots was separated into symplastic and apoplastic
643 fractions by successive extractions. Most of Zn in roots was in the symplastic fraction (69 to
644 93%), and in both species the proportion of apoplastic Zn increased around 20% at high [Zn]
645 (50 µM for *N. caerulescens* and 5 µM for *T. arvense*, in accord with their different tolerance).
646 This evidence suggests a common pattern of Zn exclusion during Zn excess for *P. australis*, *P.*
647 *arundinacea*, *N. caerulescens* and *T. arvense*, where isotopically heavy Zn would be
648 sequestered in the apoplast. However, not all Zn-tolerant species show enhanced ^{66}Zn
649 accumulation in the roots when exposed to high Zn levels. For instance, *Silene vulgaris*
650 ([Moench.] Garcke.) growing in a contaminated soil showed no significant shift between soil,
651 roots, and shoot (Tang et al. 2012)(Fig. 4b). Furthermore,^{66}Zn was enriched in the roots of the
652 tolerant grass *Agrostis capillaris* (L.) grown in two different technosols compared to soils and
653 to shoots ($\Delta^{66}Zn_{root\text{-}solution}$=0.07-0.19‰, $\Delta^{66}Zn_{shoot\text{-}root}$=-0.24 to -0.40‰)(Houben et al. 2014)(Fig.
654 4d), but the magnitude of the fractionation was comparable to that of non-tolerant plants. The
655 same seems to hold for rapeseed (*Brassica napus* L.) and rye grass (*Lolium multiflorum* L.)
656 grown in pots containing three multi-polluted soils ($\Delta^{66}Zn_{root\text{-}solution}$=0.05-0.20‰,
657 $\Delta^{66}Zn_{shoot\text{-}root}$=-0.04 to -0.39‰)(Couder et al. 2015).

Besides the sequestration of Zn in the roots, plants display other responses to Zn excess that possibly discriminate Zn isotopes. Both *Arabidopsis thaliana* (L) and *Arabidopsis halleri* (L.) secrete NA to the rhizosphere, where it forms Zn-NA complexes, and *A. halleri* increases NA secretion when exposed to high Zn levels in the environment (Tsednee et al. 2014). In the same study, [Zn] in the roots of *A. thaliana* decreased by up to 60% when 50 µ*M* NA was added to the soil, indicating that the Zn-NA complexes were not taken up by the root cells. It was concluded that plants secrete NA to the rhizosphere to reduce Zn bioavailability in the soil. Moreover, the secretion of NA increased in *A. halleri* in response to excess Zn To illustrate the impact of root exudates on the isotopic composition of the soil solution, *Agrostis capillaris* was grown in columns filled with two technosols and compared with controls without plant cover (Houben et al. 2014). The columns were irrigated with nutrient solution in excess and allowed to drain, and the resulting leachates were collected and analyzed. Without plants, the leachates were isotopically lighter than the soil ($\Delta^{66}Zn_{leachates\text{-}soil}$ =-0.12 to -0.21‰), meaning that the leaching of free Zn^{2+} from the soil removed the light isotopes. By contrast, in columns planted with *A. capillaris* the leachates were isotopically heavier than the soil ($\Delta^{66}Zn_{leaches\text{-}soil}$ =0.14 to 0.04‰), with up to 4 times more Zn. This was attributed to the mobilization of Zn from an isotopically heavier pool in the soil facilitated by the root exudates. Further work is needed to determine whether the mobilized Zn is then taken up by the *A. capillaris* or remains in the soil. On top of stimulating the secretion of root exudates, an excessive Zn supply modifies the activity of some Zn transporters in the membrane of the root cells, which could have an impact in Zn isotope partitioning. High Zn levels inhibit the activity of plasma membrane transporters like AtIRT3 in arabidopsis, involved in Zn uptake by the roots (Lin et al. 2009). The Zn efflux transporter AtPCR2 in arabidopsis exports excess Zn from the cytoplasm to outside the cell, and its expression in yeast is increased at high Zn levels (Song et al. 2010). Another process contributing to the accumulation of ^{64}Zn in the aerial parts of Zn-tolerant plants under Zn stress has been suggested in *B. napus* and *L. multiflorum* grown in three

684 different soils with high Zn (Couder et al. 2015). A significant negative correlation (R^2=0.83,
685 p=0.01) was found between the $\Delta^{66}Zn_{shoot\text{-}root}$ and the transpiration per total dry biomass. The
686 authors proposed that bulk mass flow driven by transpiration controlled Zn flux from the soil
687 into the plant under high Zn supply. However, the precise mechanism by which the
688 fractionation could be generated during the convective transport of Zn up the shoot remains
689 obscure. The preferential binding of ^{66}Zn to the cell walls of the xylem vessels might cause this
690 effect, but experimental evidence is missing. Besides, it has been suggested that Zn might
691 enter the root xylem via the apoplastic pathway (White et al. 2002). Using literature data for *N.*
692 *caerulescens* during high Zn supply (Lasat et al. 1996; Pence et al. 2000; Lombi et al. 2001),
693 White and coworkers argue that Zn influx to the cells is smaller than Zn flux to the xylem, and
694 insufficient to account for the Zn content of the shoots (White et al. 2002). A better
695 understanding of plants response to Zn excess is crucial to discuss the distribution of Zn
696 isotopes associated with it and to identify the predominant mechanisms.
697
698 *Increased uptake and sequestration in the aerial parts of hyperaccumulators*
699 Some soils are naturally high in Zn due to the composition of the parent rock (e.g. calamine or
700 serpentine soils) (Kazakou et al. 2010; Escarré et al. 2010), and plants growing on them have
701 adapted to these conditions. These metallicolous plants tolerate Zn levels that would cause
702 death in other plants, and sometimes display unique hyperaccumulation traits: an increased
703 rate of Zn uptake, a more efficient root-to-shoot translocation, and a higher sequestration
704 capacity in the leaves (>3,000 µg Zn g^{-1} shoot dry matter) without showing any toxicity
705 symptoms (Lasat et al. 2000; Broadley et al. 2007). In these plants, Zn is compartmentalized in
706 the leaf cell walls and vacuoles (Küpper et al. 1999). The Zn-hyperaccumulator *Arabidopsis*
707 *halleri* and the non-accumulator *Arabidopsis petraea (A. lyrata subsp. petraea* [L.] O'Kane &
708 Al-Shehbaz) were grown hydroponically to compare the partitioning of Zn isotopes under Zn
709 excess (10^{-5} *M*)(Aucour et al. 2011)(Fig. 4c). The heavier Zn isotopes were enriched in the roots

of *A. halleri* compared with the solution (10 or 250 µ*M* Zn, $\Delta^{66}Zn_{root\text{-}solution}$=0.4-0.8‰) and the shoots ($\Delta^{66}Zn_{shoot\text{-}root}$=-0.7‰), in line with results obtained for *P. australis* (Caldelas et al. 2011). By contrast, the roots of the related non-accumulator *A. petraea* had a similar $\delta^{66}Zn$ as the solution (Aucour et al. 2011). The enrichment of heavier Zn isotopes in the roots of *A. halleri* relative to the roots of *A. petraea* was explained as the result of Zn storage in the vacuoles of the root cells in form of Zn-phosphates (Aucour et al. 2011). Previous spectroscopic work had revealed that in *A. halleri* Zn binds mostly to phosphates in the roots, and to citrate or malate in the shoots (Sarret et al. 2002). Using *ab initio* calculations, Fujii and Albarède compared the $\delta^{66}Zn$ of those three Zn species at total concentrations of 0.05 *M* (Zn), 0.01 *M* (citrate and malate), and 1 *M* (phosphate). At pH around neutral, Zn-phosphates were expected to enrich isotopically heavier Zn with respect to the solution, whilst Zn-malates and Zn-citrates would both concentrate light isotopes relative to the solution (Fujii and Albarède 2012). The $\Delta^{66}Zn$ between Zn-citrates (or Zn-malates) and Zn-phosphates would be around -0.8 to -0.9‰, consistent with the $\Delta^{66}Zn_{shoot\text{-}root}$ of *A. halleri* (-0.7‰) and *P. australis* (-0.8 to -0.9‰)(Aucour et al. 2011; Caldelas et al. 2011). By contrast, at pH below 5 all three Zn complexes would have a similar isotopic composition as the solution (Fujii and Albarède 2012), in agreement with the roots of *A. petraea* having a similar $\delta^{66}Zn$ as the source (Aucour et al. 2011; Tang et al. 2012). Fujii and Albarède suggested that *A. halleri* might maintain the pH of the root cells around neutral to promote Zn complexation with phosphates as a tolerance mechanism, which would cause most of the heavy enrichment of the roots relative to the soil in this species (Fujii and Albarède 2012). The predominant Zn species in *P. australis* roots and shoots remain elusive, but the distribution of Zn isotopes is similar to that of *A. halleri*. This suggests that Zn complexation with phosphate in the roots could be a key tolerance mechanism in *P. australis*, which is not a hyperaccumulator species.

Compared with the plant-available Zn of the soil, ^{66}Zn was enriched in the roots of the hyperaccumulator *N. caerulescens* collected from a Zn-contaminated soil, a serpentine soil,

736 and a non-metalliferous soil (0.40-0.72‰)(Tang et al. 2012)(Fig. 4b). Analysis of the Zn
737 speciation in *N. caerulescens* has shown that in the roots Zn is accumulated as Zn-phytate (a
738 polyphosphate) or Zn-histidine (Monsant et al. 2011). In the leaves, a mixture of Zn-citrate and
739 Zn-malate predominates in the epidermis while Zn-NA is the main form in the mesophyll
740 (Schneider et al. 2013). According to *ab initio* calculations, Zn complexes with histidine are
741 isotopically lighter than with citrates, malates, and phosphates by roughly -0.4, -0.3, and -1‰,
742 respectively (Fujii et al. 2014). Besides, Zn-phosphates are isotopically heavier than Zn-citrates
743 and Zn-malates by around 0.8‰ (Fujii and Albarède 2012). The isotopic signature of Zn-NA
744 complexes found in this hyperaccumulator is not yet known. With the information available,
745 the isotope partitioning observed between the shoot and the root in *N. caerulescens* could be
746 explained by the distribution of phosphates, citrates, and malates. A different interpretation
747 was proposed by Tang and co-workers for the accumulation of ^{66}Zn in the roots of *N.*
748 *caerulescens*: the preferential transfer of ^{64}Zn to the xylem by means of the NcHMA4
749 transporter (Tang et al. 2012). This plasma membrane transporter orthologous to arabidopsis
750 AtHMA4 is highly expressed in the vascular tissue of *N. caerulescens* due to multiple gene
751 copies (Ó Lochlainn et al. 2011), and is likely to be responsible for the increased export of Zn to
752 the xylem in this species that is part of the hyperaccumulation response (Papoyan and Kochian
753 2004; Craciun et al. 2012). Two other transporters, NcZNT1 and NcMTP1, are up-regulated in
754 *N. caerulescens*. The plasma membrane protein NcZNT1 is expressed in the root epidermis and
755 vasculature, and is involved both in Zn uptake in the root and Zn transport to the shoot. The
756 tonoplast transporter NcMTP1 (=NcZTP1, TcZTP1) is expressed mainly in leaves and
757 contributes to Zn sequestration in the vacuoles (Küpper and Kochian 2010; Milner et al. 2012).
758 Similar transporters have been identified in other Zn hyperaccumulators, like AhMTP1 and
759 AhHMA3 in *A. halleri*, or NgMTP1 in *Noccaea goesingensis* ([Halácsy] F.K.Mey] (Becher et al.
760 2004; Shahzad et al. 2010). The increased activity of the above transporters could explain the
761 accumulation of ^{64}Zn in the shoots of hyperaccumulators.

762

763 **Isotopic fractionation of iron by plants**

764 *Iron uptake in strategy I and II plants*

765 Iron can change its oxidation state during uptake or translocation within the plant, and those
766 redox conversions induce large isotopic fractionation. Strategy I plants (non-graminaceous
767 species) use the proton ATPase AHA2 localized to the plasma membrane to release protons to
768 the rhizosphere during Fe deficiency, increasing the solubility of Fe(III) (Santi and Schmidt
769 2009). The ferric reductase oxidase FRO2 then reduces the Fe(III) complexes at the plasma
770 membrane to aqueous Fe(II) (Robinson et al. 1999). Finally, Fe(II) is taken up by the roots via
771 the plasma membrane protein IRT1, the main high-affinity Fe transporter of the plant root
772 (Vert et al. 2002). By contrast, strategy II plants secrete PS to the rhizosphere to solubilise
773 Fe(III) during Fe deficiency (Takagi et al. 2008). The Fe(III)-PS complexes are transported into
774 the root symplast by plasma membrane transporters of the OPT family like ZmYS1 in maize,
775 HvYS1 in barley, and OsYSL15 in rice (Murata et al. 2006; Ueno et al. 2009; Inoue et al. 2009;
776 Suzuki et al. 2012). In a survey involving ten species grown in agricultural soil, the stems,
777 leaves, and grains of strategy I plants accumulated ^{54}Fe compared to the soil ($\Delta^{56}Fe_{x\text{-}soil}$ up
778 to -1.6‰, relative to the IRMM-014 standard), while those of strategy II plants (grasses) were
779 isotopically heavier ($\Delta^{56}Fe_{x\text{-}soil}$ up to 0.2‰) (Guelke and von Blanckenburg 2007). In aqueous
780 solutions, there is a strong fractionation of the isotopes between Fe(III) and Fe(II)
781 (Δ^{56}Fe(III)-Fe(II) = 2.8‰), with the light isotopes accumulating in the Fe(II) (Johnson et al.
782 2002). The enrichment of ^{54}Fe in the aerial parts of strategy I plants was attributed to the
783 reduction of Fe(III) in the soil by FRO2 previous to high-affinity uptake. The enrichment of ^{56}Fe
784 in the aerial parts of strategy I plants was best explained by isotopically heavy Fe(III) binding to
785 PS in the soil.

786 However, Strategy II plants produce little PS in absence of Fe-deficiency (Cakmak et al. 1994;
787 Suzuki et al. 2006; Suzuki et al. 2008). The alpine species *Oxyria digyna* ([L.] Hill) and *Rumex*

788 *scutatus* (L.), both strategy I, showed an enrichment of the light isotopes in the entire plants
789 relative to the soil ($\Delta^{56}Fe_{plant\text{-}soil}$ -0.60 for *O. digyna* and -1.03‰ for *R. scutatus*), while in the
790 graminaceous *Agrostis gigantea* (Roth) there was very little fractionation (-0.07‰)(Kiczka et al.
791 2010b). The partitioning of Fe isotopes in the two dicots was attributed to H^+ and
792 ligand-promoted Fe dissolution from the soil, which favours light isotopes (Wiederhold et al.
793 2006; Chapman et al. 2009; Kiczka et al. 2010a). The smaller fractionation in *A. gigantea* was
794 ascribed to different strategy II phenomena not involving PS, like Fe binding to other root
795 exudates. The authors argued that the amount of available Fe in the soil (1500 µg g^{-1}) was very
796 high, and the Fe content of plant samples was not indicative of Fe deficiency, making PS
797 contribution to Fe uptake unlikely. In beans (*Phaseolus vulgaris* L., strategy I) and oats (*Avena*
798 *sativa* L., strategy II) grown in a nutritive solution containing 20 µ*M* Fe(III)-EDTA (Fe-sufficient),
799 the light isotopes were enriched in both species, but the magnitude of $\Delta^{56}Fe_{plant\text{-}solution}$ was
800 larger in bean (-1.2‰) than in oats (-0.5‰) (Guelke-Stelling and von Blanckenburg 2011). The
801 accumulation of light isotopes in both plants was explained by Fe(III)-EDTA reduction followed
802 by the uptake of Fe^{2+} on the surface of the roots. Reduction of Fe(III)-chelates is a mechanism
803 present both in strategy I and II plants in absence of Fe-deficiency (Bienfait et al. 1983;
804 Bruggemann and Moog 1989). To account for the smaller magnitude of $\Delta^{56}Fe_{plant\text{-}solution}$ in oats,
805 Guelke-Stelling and von Blankenburg proposed that two mechanisms could compete with the
806 reduction of Fe(III)-complexes: i) direct uptake of Fe(III)-EDTA complexes, or ii) Fe(III) binding
807 to PS or phosphates in the apoplast. The uptake of either Fe(III)-EDTA or Fe(III)-PS would not
808 fractionate Fe isotopes, but the ligand exchange (from EDTA to PS) likely would. The Fe(III)-PS
809 complexes have a lower stability constant (10^{18})(Murakami et al. 1989) than the Fe(III)-EDTA
810 complexes (10^{25})(Smith and Martell 1989), which means that the lighter isotopes would
811 accumulate in the PS complexes, the species with the weaker bonds (Criss 1999). The direct
812 uptake of Fe(III)-chelates has been reported in strategy I and II species (Römheld and
813 Marschner 1981; Orera et al. 2010). Strategy II plants secrete PS in absence of Fe-deficiency,

814 although the release rate is smaller (Erenoglu et al. 2000). However, PS release in oats greatly
815 depends on the cultivar, with Fe-inefficient varieties apparently not capable of producing
816 much PS even in Fe-deficient conditions (Jolley and Brown 1989). The amount of PS released to
817 the solution, the forms of Fe predominant in the shoots, and the oats cultivar used were not
818 indicated in the Guelke-Stelling and von Blankenburg study. This makes it difficult to establish
819 which of the two proposed uptake routes (reduction combined with direct uptake of
820 Fe-chelates, or uptake of Fe-PS complexes) predominates in Fe-sufficient oats.
821 Another mechanism for Fe uptake has been suggested for rice. The shoots of rice grown in
822 aerobic and anaerobic conditions had the same fractionation relative to the soil ($\Delta^{56}Fe_{shoot\text{-}soil}$
823 up to -0.5‰) in spite of the likely difference in Fe redox forms and concentration of Fe(III)-PS
824 complexes (Arnold et al. 2015), and the magnitude of the fractionation was smaller than in
825 previous work (Guelke and von Blanckenburg 2007; Kiczka et al. 2010b). It was suggested that
826 a substantial amount of Fe was absorbed directly as Fe^{2+} and translocated up the shoot with no
827 redox conversions (Arnold et al. 2015), since rice can take Fe^{2+} up from the soil in aqueous
828 form directly and without previous solubilization by PS (Ishimaru et al. 2006). Future work
829 must be addressed to test this hypothesis by complementing the isotope data with additional
830 data, including Fe speciation in plants and soils, and PS release.
831
832 *Xylem loading and unloading of Fe*
833 Further discrimination of the isotopes occurs during Fe translocation within the plant. In
834 strategy I and II plants growing in Fe-sufficient soil, where a smaller contribution from PS could
835 be expected, the conductive tissue of the root (stele) was isotopically lighter than aerial parts
836 of the plant ($\Delta^{56}Fe_{stem\text{-}stele}$ up to 3.0‰) (Kiczka et al. 2010b). This was attributed to the
837 reduction of apoplastic Fe(III) and transfer of Fe(II) across the cell membrane, and to the
838 oxidation of Fe(II) to Fe(III) during the formation of Fe(III)-citrate complexes, which would then
839 be exported to the xylem leaving the symplast of the stele isotopically lighter. The main form

of Fe in the symplast is Fe(II)-NA, while Fe in the xylem is Fe(III)-citrate (Rellán-Alvarez et al. 2008; Rellán-Alvarez et al. 2010). This is in agreement with *ab initio* calculations of isotopic fractionation between Fe species, which predict that Fe(II)-NA would be isotopically lighter (≈2‰) than Fe(III)-citrate (Moynier et al. 2013). Moynier and co-workers noted that while redox changes accounted for the largest isotopic fractionations (≈3‰), speciation only could explain isotopic fractionations up to 1.5‰.

In beans (strategy I) grown in 20 µ*M* Fe(III)-EDTA solution δ^{56}Fe decreased progressively from the stem to the leaves and the grains (δ^{56}Fe -0.31, -0.69, and -1.90‰ respectively) (Guelke-Stelling and von Blanckenburg 2011). By contrast, no discrimination was observed between the aerial parts and the roots in oats (strategy II) in the same study. The authors attributed the fractionation pattern of beans to the reduction of Fe(III) in the xylem to Fe(II) during transfer to the symplast of leaves and grains. The absence of fractionation in the above-ground organs in oats is compatible with the translocation of Fe(III)-PS complexes from the root to the shoot, and with the transfer of Fe(III) from Fe(III)-PS or Fe(III)-EDTA to NA without changing the redox form. In strategy II plants Fe(III) can be transported up the shoot as Fe(III)-NA complexes without a previous reduction step (von Wiren et al. 1999).

Fe remobilization from older leaves to developing organs

In two Fe-deficient soils, the leaves of beans became depleted of ^{56}Fe between the first harvest and the fourth by up to -0.7‰ (Guelke and von Blanckenburg 2007). The remobilization of Fe from older leaves to developing organs was proposed as the origin of this pattern. Excess Fe is oxidized to Fe(III) and stored as phytoferritin in the plastids of plant cells, from where it can be mobilised upon demand (van der Mark et al. 1982). It was argued that a non-quantitative sequestration of Fe in the phytoferritin complexes would favour the accumulation of isotopically heavy Fe(III) in the phytoferritin, leaving a pool of isotopically light Fe(II) in the cytoplasm available for export to the developing tissues, and isotopically heavier older leaves.

In the same study, the leaves of oats and wheat did not show substantial fractionation between the first and the second harvest (Guelke and von Blanckenburg 2007). The authors proposed that in strategy II plants Fe could move in the phloem as Fe(III) binding to heavy mass ligands such as NA. Iron (III) can form complexes with NA (von Wiren et al. 1999), but a study of the chemical forms of Fe in the phloem sap of rice has detected Fe(III)-PS complexes instead of Fe(III)-NA complexes (Nishiyama et al. 2012). In the Fe-deficient soils where oats and wheat were grown in the Guelke and von Blankenburg study a strong contribution of PS to Fe uptake can be expected, so Fe(III) could move in the phloem as Fe(III)-PS. In agreement, the grains of rice grown both in aerobic and anaerobic Fe-deficient soils had a similar composition as the shoot (Arnold et al. 2015).

Similar results were obtained in beans and oats grown in Fe-sufficient nutritive solution where Fe was given as Fe(III)-EDTA. The youngest leaves and the seeds of beans became isotopically lighter as plants grew (up to -1.75‰), but those of oats did not (Guelke-Stelling and von Blanckenburg 2011). By contrast, the leaves of both strategy I and II alpine species grown in an Fe-rich soil (where very little contribution from PS to Fe uptake could be expected) accumulated ^{56}Fe with age (up to 1.5‰) (Kiczka et al. 2010b). Therefore it appears that in strategy II species the source of Fe determines the isotopic fractionation during remobilization. The uptake of Fe(III) binding to strong ligands like EDTA or PS causes no fractionation during Fe movement in the phloem, while the uptake of non-complexed Fe leads to a fractionation pattern analogous to that of strategy I species, indicative of a series of redox conversions.

The partitioning of Fe isotopes between older and younger leaves is similar to that previously discussed in section 5.2 for Zn. The light Zn isotopes become enriched in the leaves with height. This effect could be an evidence of Zn isotope partitioning during Zn remobilization from older leaves to developing ones, analogous to that observed in Fe. While excess Fe is stored in the plastids in form of phytoferritin, excess Zn is stored in the vacuoles of the leaves in form of Zn complexes with citrate, malate, or NA (Aucour et al. 2011; Tang et al. 2012).

892 Moreover, Zn can bind to more than 1,000 proteins in the cytoplasm of plant cells (Broadley et
893 al. 2007). If the heavy isotopes were enriched in the Zn bound to organic acids, amino acids,
894 and proteins, this would leave an isotopically lighter Zn^{2+} pool available for transport. The
895 constant mobilization of micronutrients from older to younger leaves as new leaves appear
896 could thus explain the gradation in the isotope composition of the leaves with age for both Fe
897 and Zn.
898
899 **Copper**
900 Fewer studies have focused on the isotopic fractionation of Cu in plants. Lentils (*Lens culinaris*
901 Med.) germinated in distilled water showed a marked accumulation of ^{63}Cu in shoots relative
902 to seeds, i.e. $\Delta^{65}Cu_{shoot\text{-}seed}$ of -0.34‰ (Weinstein et al. 2011). This effect was identical to that
903 reported for Zn in the same experiment, i.e. $\Delta^{66}Zn_{shoot\text{-}seed}$ = -0.34‰ (Moynier et al. 2009). In
904 the study conducted by Weinstein and co-workers, aerial parts of *Elymus virginicus* (L.) were
905 isotopically lighter than soil (-0.94 to -0.33‰), and leaves lighter than stems (-0.39 to -0.13‰).
906 Furthermore, the $\delta^{65}Cu_{NIST976}$ of the leaves of *Carex hirsutella* (Mackenzie) decreased with
907 height (from 7 to 43.5 cm), and the linear relationship between them ($\delta^{65}Cu$=-0.01*H-0.07,
908 where H is the height in cm) was remarkably similar to that previously noted for Zn in bamboo
909 ($\delta^{66}Zn$=-0.01*H-0.06)(Moynier et al. 2009; Weinstein et al. 2011). The authors concluded that
910 both Cu and Zn were submitted to the same fractionation mechanisms during uptake and
911 translocation, probably during transport across the cell membranes. However, a distinct
912 pattern of fractionation during Cu uptake was later described in rice, lettuce, tomato, and
913 durum wheat grown hydroponically (Jouvin et al. 2012). In these species, shoots accumulated
914 ^{63}Cu relative to the solution in a range very similar to that of the Weinstein study
915 ($\Delta^{65}Cu_{shoot\text{-}solution}$ -1.06 to -0.34‰), and the roots generally had the same isotopic composition
916 as the shoots ($\Delta^{65}Cu_{root\text{-}solution}$ -0.84 to -0.11‰). The accumulation of ^{63}Cu in the roots was
917 similar to that previously reported for ^{54}Fe in beans and oats ($\Delta^{56}Fe_{root\text{-}solution}$ = -1.0 to -0.5‰),

which was attributed to Fe(III) reduction during Fe uptake (Guelke-Stelling and von Blanckenburg 2011). By contrast, Zn in the roots is typically enriched in heavy isotopes ($\Delta^{66}Zn_{root\text{-}solution}$ up to 0.8‰)(Weiss et al. 2005; Viers et al. 2007; Aucour et al. 2011). It was concluded that the enrichment of ^{63}Cu in the roots was due to the reduction of Cu(II) to Cu(I) during Cu uptake by the root cells. Leaching of Cu(I) minerals has shown that ^{65}Cu accumulates in the aqueous Cu(II) ($\Delta^{65}Cu_{mineral\text{-}solution}$ -1.18 to -0.94‰)(Kimball et al. 2009). The fractionation during Cu(II) reduction to Cu(I) is similar to the fractionation observed during Cu uptake by the root by Jouvin and co-workers ($\Delta^{65}Cu_{root\text{-}solution}$ -0.84 to -0.11‰). The smaller fractionation in plants indicates a combination of reduction and another uptake mechanism that does not fractionate isotopes or takes a shift towards the heavy. In arabidopsis, Cu(II) reduction to Cu(I) is a pre-requisite for the high-affinity uptake of Cu by means of the transporter COPT1, and is mediated by the ferric reductase oxidases FRO5 and FRO4 (Bernal et al. 2012). The reductase FRO2 is responsible for Fe(III) reduction to Fe(II) prior to uptake mediated by the IRT1 transporter, as discussed in the previous section. The reductase activity of the FRO family thus constitutes an important link between Cu and Fe homeostasis. Still, transporter COPT1 is responsible for only 40-60% of Cu uptake in arabidopsis under adequate Cu nutrition (Sancenón et al. 2004). Plants take up Cu(II) in form of Cu^{2+} or PS-Cu(II) complexes (Roberts et al. 2004; Hötzer et al. 2012). The transport of Cu^{2+} across the cell membrane during uptake in the roots is probably facilitated by cation transporters like ZIP2 in arabidopsis, and IRT1 and IRT2 in tomato (Wintz et al. 2003; George et al. 2012). The uptake of PS complexes with Cu(II) is mediated by plasma membrane transporters in the root epidermis like YS1 in maize (Roberts et al. 2004). In agreement, the roots of tomato and oats grown in Fe-sufficient nutrient solution were enriched in light isotopes relative to the solution, and the shift was much larger in tomato ($\Delta^{65}Cu_{root\text{-}solution}$ -1.43‰) than in oats (-0.20‰)(Ryan et al. 2013). In both species the fractionation decreased during Fe-deficiency (-1.05‰ and -0.12‰ respectively) indicating a greater uptake of Cu(II), probably as Cu(II) complexes with root exudates. The smaller

944 fractionation in oats, a strategy II plant, points to a substantial uptake of PS-Cu(II) complexes in
945 this species.
946 Further isotope effects have been reported during Cu transport to stem and leaves. In tomato,
947 the translocation of Cu up the shoots favoured ^{65}Cu ($\Delta^{65}Cu_{shoot\text{-}root}$ up to 1.0‰), suggesting that
948 Cu(I) was oxidised to Cu(II) before its export to the xylem (Ryan et al. 2013). In agreement, Cu
949 is mostly present as Cu(II)-NA complexes in the xylem (Curie et al. 2009). By contrast, the
950 transfer of Cu from the stem to the leaves favoured the light isotopes ($\Delta^{65}Cu_{leaves\text{-}stem}$ up
951 to -0.37‰), which points to reduction of Cu(II) to Cu(I) during Cu unloading from the xylem
952 (Ryan et al. 2013). Transporter COPT6 in arabidopsis is expressed in the vasculature of the
953 shoot and might be involved in Cu(I) import into the leaf symplast (Jung et al. 2012). This
954 transporter has a high specificity for Cu(I) and requires the previous reduction of Cu(II) to Cu(I).
955 This reduction is probably catalysed by the reductase FRO3, which is expressed in the
956 vasculature of the shoot in response to both Fe and Cu deficiency (Mukherjee et al. 2006). In
957 oats, roots, stems, and leaves had the same isotopic composition (Ryan et al. 2013). This
958 suggests that strategy II plants might have different translocation mechanisms for Cu not
959 involving redox conversions, as discussed for Fe in the previous section. The Cu(II) in PS
960 complexes might be transferred to NA in the xylem with no further redox conversion. In
961 strategy II plants, Fe could be transported up the shoot as Fe(III)-NA complexes without a
962 previous reduction step (von Wiren et al. 1999). In rice, the transporter YSL16 can transport
963 Cu(II)-NA complexes from the xylem directly into the phloem to nourish the developing leaves
964 (Zheng et al. 2012). Finally, the green leaves of tomato were enriched in light isotopes relative
965 to the chlorotic ones by -0.30‰, suggesting that the remobilization of Cu to the developing
966 organs favours the light isotopes, as previously discussed for Fe and Zn (Ryan et al. 2013).
967 However, in oats the green leaves were enriched in heavy isotopes as compared with the
968 chlorotic ones by 0.27‰, which points to Cu reduction during remobilization of nutrients in
969 oats.

970

971 **Nickel**

972 Very little is known about Ni isotopic fractionation in plants, but it is still worth discussing it
973 here because many metalicollous plants are Zn and Ni hyperaccumulators (Prasad and De
974 Oliveira Freitas 2003). A survey of Ni isotopic fractionation in plants including a
975 non-hyperaccumulator (*Thlaspi arvense* L.), a Ni hyperaccumulator *(Alyssum murale* Waldst. &
976 Kit.), and a Ni and Zn hyperaccumulator *(N. caerulescens)* revealed that plants generally
977 accumulate light isotopes during uptake ($\Delta^{60}Ni_{plant\text{-}solution}$ -0.90 to -0.21‰, relative to NIST-SRM
978 986)(Deng et al. 2014). The hyperaccumulators showed a larger fractionation (-0.90
979 to -0.63‰), which points to a greater permeability of the low-affinity transporters in these
980 species (Deng et al. 2014). Interestingly, a high supply of Zn ($50x10^{-6}$ *M*) suppressed the
981 observed fractionation in all the three species (-0.11 to -0.07‰). This indicates that Zn and Ni
982 compete for the same uptake mechanisms. The root to shoot transfer of Ni resulted in further
983 enrichment of ^{58}Ni in the hyperaccumulators (-0.47 to -0.14‰), while the shoots of the
984 non-hyperaccumulator accumulated ^{60}Ni relative to the roots (0.25‰). This suggests the
985 existence of different Ni translocation mechanisms in hyperaccumulators, but these remain
986 poorly understood.

987

988 **Model of Zn isotope discrimination by plants**

989 The isotope data recently published permit to confirm and improve upon the model developed
990 by Jouvin and co-workers (Jouvin et al. 2012) (Fig. 5), which identified the most likely sources
991 of isotope discrimination during Zn uptake by plants roots. Zinc in the soil solution will bind
992 with suitable ligands, such as the low-molecular weight compounds in the root exudates (PS,
993 OA, etc.) and the cellulose and pectin of the cell walls. Alternatively, Zn might bind covalently
994 to phosphates in the apoplast as proposed by Fujii and Albarède 2012, or to silicates (Medas et
995 al. 2015). In the light of the experimental evidence discussed above, these reactions seem to

996 favour the accumulation of heavy isotopes in the resulting Zn-complexes relative to free Zn^{2+}.
997 Unbound Zn^{2+} can move by diffusion over small distances in the un-stirred layer around the
998 roots, a kinetically controlled reaction that would favour the lighter isotopes (Criss 1999). The
999 fractionation of Zn isotopes caused by the joint activity of the plasma membrane transporters
1000 would depend on the concentration and predominant Zn species in the rhizosphere. Jouvin
1001 and co-workers proposed in their model that low-affinity uptake of Zn^{2+} mediated by ion
1002 channels would work towards the accumulation of light isotopes in the cytosol of the root cells
1003 relative to the apoplast, due to the faster diffusion of the light isotopes, whereas high-affinity
1004 uptake would favour the heavy isotopes due to the covalent binding of Zn with these
1005 transporters (Jouvin et al. 2012). This latter notion has been recently challenged by Tang and
1006 co-workers, who proposed that high-affinity uptake would not fractionate Zn isotopes (Tang et
1007 al. 2016). They observed a small enrichment of the light isotopes in plants relative to the
1008 solution which was larger in the non-accumulator *T. arvense* (-0.16 to -0.26‰) than in the
1009 hyperaccumulator *N. caerulescens* (-0.06 to -0.12‰). They attributed their results to i)
1010 diffusional fractionation caused by a depletion zone around the roots in hyperaccumulators
1011 and ii) a mixture of high- and low-affinity transport in non-accumulators. Experiments in
1012 diatoms have shown that the light isotopes are enriched in the cell relative to the growth
1013 solution during high-affinity uptake (John et al. 2007). Besides, it is not yet clear if the ZIP
1014 transporters mainly responsible for Zn^{2+} uptake at the root function as ion channels or as
1015 carrier proteins, how Zn binds to these proteins, or how Zn is transported across the
1016 membrane. In our view, both low- and high-affinity Zn^{2+} transporters discriminate in favour of
1017 the light isotopes, but the magnitude of this effect would be smaller during high-affinity
1018 uptake because the higher efficiency of these transporters would make them less selective of
1019 the isotopes (as proposed by John et al. 2007). Finally, the uptake of Zn-complexes would not
1020 further discriminate Zn isotopes because of the large mass of the complexes. However, non-
1021 quantitative uptake of Zn-complex would result in the enrichment of heavy isotopes in plants

1022 relative to the soil solution, because the Zn-complexes themselves are isotopically heavier
1023 than the unbound Zn^{2+}. Efflux transporters would probably carry isotopically light Zn^{2+} to the
1024 rhizosphere, but their contribution is likely to be small. In Zn-sufficient environments, the main
1025 sources of isotope separation at the soil-root interface identified were Zn speciation in the soil
1026 solution, binding to the cell walls, and low-affinity transport phenomena. In Zn-deficient
1027 conditions, these were substituted by high-affinity transport phenomena, Zn binding to root
1028 exudates, and the uptake of the resulting complexes. The two latter are especially relevant in
1029 Strategy II plants. Finally, Zn isotopic fractionation at the soil-root interface with high Zn supply
1030 originated mostly from Zn binding to the cell wall.
1031 Jouvin and co-workers addressed only briefly some of the mechanisms that could lead to
1032 isotopic fractionation during Zn transfer from the root to the shoot: i) complexation with
1033 various ligands in the xylem and phloem, especially NA, ii) membrane crossings, iii) diffusion,
1034 and iv) ion exchange (Jouvin et al. 2012). Here we would like to build on their preliminary
1035 model and provide a detailed account of all the likely sources of fractionation at work during
1036 Zn export to the shoots and movement between above-ground organs. Following Zn uptake by
1037 the root, Zn^{2+} in the cytosol binds to small weight compounds, most likely NA (Fig. 6). Heavy
1038 isotopes are thought to accumulate in the resulting Zn-complexes relative to the Zn^{2+} fraction.
1039 Unbound Zn^{2+} can diffuse radially over small distances in the apoplast and the symplast,
1040 passing from the cytoplasm of one cell to another across the plasmodesmata. Lighter isotopes
1041 will diffuse faster due to their smaller mass. In our model, we propose that the vacuoles might
1042 have a key role in determining the isotopic composition of the Zn pool of the cytosol. Excess
1043 Zn^{2+} can be removed from the cytosol and stored in the vacuole by means of vacuolar
1044 transporters, wherefrom it can be remobilized. In this manner, vacuolar transporters control
1045 the amount of Zn available for export to the aerial parts. The isotopic composition of the
1046 vacuolar Zn pool is unknown and probably very dynamic, determined by the joint activity of
1047 the vacuolar transporters and Zn speciation in the vacuole.

Zinc will be loaded onto the xylem sap from the cytosol of the adjacent cells, a step mediated by membrane transporters that seem to favour the lighter isotopes. Zinc in the xylem sap mostly consists of Zn^{2+}, but a substantial amount of Zn-complexes with OA, histidine, NA, and PS might be present, especially under low Zn supply (Fig. 7). The xylem sap moves up the stem thanks to the mass flow of water driven by transpiration and maintained by the cohesion of the water molecules and their adhesion to the cellulose walls. This movement might result in the enrichment of light isotopes in the leaves with height, although it is not yet clear how the isotopes could be discriminated. Adsorption of Zn^{2+} onto the cellulose walls of the xylem should result in a progressive depletion of the heavier isotopes in the xylem sap relative to the root symplast. From the xylem, Zn will enter the leaf symplast via Zn transporters at the plasma membrane of the bundle sheath cells, specialized in xylem unloading. Xylem unloading might favour the accumulation of lighter isotopes in the leaves relative to the xylem sap.

In the leaf symplast Zn^{2+} will bind to various ligands, mainly NA, accumulating heavy isotopes in the complexes relative to free Zn^{2+}. Ligand exchange might also occur during Zn unloading from the xylem to the symplast, but the isotopic effects derived from it are not known. In addition, preferential diffusion of isotopically light Zn^{2+} in the symplast could lead to further discrimination of Zn isotopes during Zn movement in the leaves. The activity of the vacuolar transporters and Zn complexation in the cytosol and the vacuole regulate [Zn^{2+}] in the leaf symplast. These processes might leave an isotopically lighter pool of Zn^{2+} available for remobilization to developing leaves and grains. Zinc remobilization from source to sink tissues involves phloem loading, xylem-to-phloem direct transfer, and phloem unloading of Zn. All three processes are thought to be mediated by transporters at the plasma membrane, and likely favour the lighter isotopes.

Conclusions

The purpose of this review is to provide an overview of Zn isotopic fractionation in plants, in relation with our current knowledge of Zn homeostasis. The research debated here clearly indicates that Zn uptake, transport to the aerial parts, and transfer to the leaves can induce isotopic fractionation relative to the Zn source, and between the plant organs. The isotopic effects observed are species-specific and concentration-dependent, and are attributed mainly to Zn speciation, compartmentalization, and transporters. In future, isotope ratios may serve to elucidate the predominant Zn species taken up by the roots, and found in the various Zn pools in the plant. Furthermore, isotope ratios could be used to identify the major processes controlling Zn transfer to the aerial parts and to quantify the extent of remobilization from source to sink organs, for instance during grain filling. Moreover, analysing the isotope ratios of Zn and other metals with similar biochemistry could reveal their competitive interactions. Additionally, changes in the patterns of Zn isotopic partitioning in response to environmental conditions might be used to study plant physiology and to identify desired traits like Zn efficiency or hypertolerance.

Future efforts must be directed towards the identification of the individual mechanisms responsible for the observed isotopic effects. To achieve this objective, research needs to solve several knowledge gaps. Probably the most urgent task is to constrain the isotopic effect associated with Zn binding to key ligands, like phytosiderophores, cellulose, pectine, OA, NA, and phosphates. Besides, the δ^{66}Zn of subcellular compartments, plant tissues, and some organs (fruits, seeds, and rhizomes) has not been properly investigated yet, which is hampering our understanding of Zn flows in plants. Finally, the influence of many factors important for plant nutrition like soil biochemistry, Zn interactions with other nutrients, mycorrhizae, root iron plaque, and environmental stressors on the patterns of Zn isotope discrimination is still unexplored.

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