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Title

Zinc Homeostasis and Isotopic Fractionation in Plants: A review

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

38   DMA = Deoxymugineic acid

39   EXAFS = Extended X-Ray Absorption Fine Structure

40   FRO = Ferric Reductase Oxidase

41   HMA = Heavy Metal ATPase

42   IRT = Iron-Regulated Transporter

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

44   MTP = Metal Tolerance Protein

45   PS = Phytosiderophores

46   YS = Yellow Stripe

47   YSL = Yellow Stripe-Like

48   ZIF = Zinc-Induced Facilitators

49   ZIP = ZRT-IRT-like Protein

50

51   **Introduction**

52   Zinc is an essential micronutrient for living organisms with several crucial functions in the cell.

53   It is the only metal present in enzymes of all six major classes, playing catalytic, regulatory, and

54   structural roles (Vallee and Auld 1992; Coleman 1992). Zinc is furthermore involved in the

55   regulation of DNA transcription, and the transduction of intra- and intercellular signalling

56   (Broadley et al. 2007; Maret 2013). Unfortunately, Zn deficiency is widespread in arable soils

57   worldwide (Alloway 2009) due to various factors such as high pH (>7), low plant available Zn

58   content, prolonged flooding, low redox potential, and high contents of organic matter,

59   bicarbonate and phosphorus (P) (Neue and Lantin 1994; Ova et al. 2015). It is not surprising

60   that around 17% of the world population is at risk of insufficient Zn intake based on food

61   supply data, although actual deficiency rates are likely to be much higher (Wessells and Brown

62   2012; Kumssa et al. 2015). This makes Zn deficiency one of the most pressing causes of

63   malnutrition. The consequences for the public health and the economy of the affected areas

are severe. In young children, Zn deficiency leads to stunting and increased susceptibility to diarrhoea, pneumonia, and malaria, causing 800,000 early deaths yearly (Caulfield et al. 2006). Zinc deficiency in crops causes root apex necrosis, leaf disorders, reduction of biomass, delayed maturity, yield reduction, and high mortality (Van Breemen and Castro 1980; Wissuwa et al. 2006; Broadley et al. 2007; Singh and Singh 2011; Al-Fahdawi et al. 2014; Mattiello et al. 2015; Fu et al. 2015). Strategies like crop biofortification (increasing Zn content in edible parts during growth) or breeding for varieties tolerant to Zn-deficiency might help to overcome Zn deficiency in soils. To this end, it is crucial to increase our current level of understanding about the mechanisms involved in Zn uptake and metabolization by plants. The study of stable Zn isotope fractionation is a novel technique that is already helping us to understand better the mechanisms of Zn uptake, translocation, and tolerance in plants, and how these react to the environment. However, a comprehensive model linking Zn homeostasis and Zn isotopic fractionation in plants and considering the interactions with other micronutrients is still missing.

Here we review our present understanding of Zn isotopic fractionation in plants and compare it with other micronutrients and their isotopic systems, with the aim of advancing our current view of Zn homeostasis and interaction with other micronutrients.

Zinc isotopic fractionation in plants

The concentration of Zn in plant tissues must stay within a specific range to preserve the structural cohesion and metabolic functions of the cells. The lower end is typically around 15-20 µg Zn g^{-1} shoot dry matter, while the upper end is around 100-300 µg Zn g^{-1} (Marschner 1995; Broadley et al. 2012). In the cytoplasm of the plant cells, the concentration of free Zn^{2+} is kept very low (in the pM range) (Maret 2015), because it tends to bind to cellular components. Higher concentrations could eventually disrupt the cytosolic metabolism and restrict Zn transport to satisfy the demands of sink organs, tissues, cells, and organelles. Plants have

4

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).

116 Subsequent studies demonstrated that isotope discrimination by plants changes in response to

117 Zn availability in the environment. In rice, response to Zn deficiency resulted in changes in the

118 fractionation pattern, and the shoots of Zn-deficient plants accumulated more heavy isotopes

119 than the controls (Arnold et al. 2010). In reeds (*Phragmites australis* [Cav.] Trin. ex Steud.), Zn

120 excess also caused alteration of the isotopic fractionation, and the aerial parts of plants grown

121 in Zn-polluted solution were isotopically light as compared with the controls (Caldelas et al.

122 2011). Taken together, these findings show that Zn stable isotopes can be used to identify and

123 quantify metal uptake or transport mechanisms and to assess the influence of factors such as

124 environmental changes, physiological status, and species on these mechanisms.

125

126 **Determination of accurate and precise Zn stable isotope ratios in plants**

127 The MC-ICP-MS (multi-collector inductively coupled plasma – mass spectrometer) is a plasma

128 source mass spectrometer with an array of collectors used to measure the isotope ratios of

129 micronutrients. The instrument typically consists of three parts: an ion source, a mass analyser,

130 and a detector. The ion source is a high-temperature argon plasma that ionizes the element.

131 The mass analyser has two sectors (electrostatic and magnetic) that focus the ion beam and

132 separate the ions for their mass-to-charge ratio. The detector unit consists of an array of

133 Faraday cups that can measure different ion beams simultaneously. This multi-collector (MC)

134 array permits to measure all the isotopes of an element at the same time and increases

135 precision to 0.001% for the isotope ratios. An exhaustive description of the MC-ICP-MS

136 technique is found in Vanhaecke et al. 2009.

137 Mass spectrometers favour the transmission of the heavy isotopes inducing a mass-bias. The

138 sample preparation can also cause mass fractionation. There are different strategies to correct

139 for mass-bias shifts: direct sample-standard bracketing (SSB), doping with an external element,

140 and using a double-spike. In the direct SSB method, a standard is analysed before and after the

141 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]

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

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

156   [2]

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

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 μM Zn) or HEDTA- (2 μM Zn) solutions, and observed that ^{66}Zn was similarly enriched in the roots of all three species regardless of the nutrient solution ($\Delta^{66}Zn_{root-solution}$=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 $\Delta^{66}Zn_{root-solution}$ were obtained (-0.02 to 0.15‰) in EDTA solution with 1.6 or 0.62 μM 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

192   herbaceous and trees growing in a tropical watershed in Cameroon. All roots were isotopically

193   heavier than the soils (ranging between 0.09 and 0.64‰) (Fig. 2). In larch trees (*Larix gmelinii*

194   [Rupr.] Rupr.) from pristine Siberian forests, the fractionation between roots and soil was of

195   0.26‰ (Viers et al. 2015). Several other authors have reported the accumulation of heavy

196   isotopes in the roots (up to 0.8%) with respect to the source of Zn in a variety of species and

197   experimental set-ups (Aucour et al. 2011; Caldelas et al. 2011; Tang et al. 2012; Smolders et al.

198   2013; Houben et al. 2014; Couder et al. 2015; Aucour et al. 2015; Tang et al. 2016). Since these

199   studies submitted plants to conditions of either Zn deficiency or Zn excess, they will be

200   discussed in detail in sections 4.3 and 6.

201   Strong evidence of isotopic fractionation during Zn adsorption to cell walls has been obtained

202   from laboratory experiments conducted with diatoms. Four species of marine and freshwater

203   diatoms showed accumulation of ^{66}Zn in the unwashed cells with respect to the solution, with

204    $\Delta^{66}Zn_{diatom-solution}$ ranging between 0.08 and 0.43‰ (Gélabert et al. 2006). The offset was

205   attributed to adsorption of Zn onto the cell surface, and Zn adsorption modelling showed that

206   Zn would mostly bind to the carboxylate groups of the cell walls. In the same line, the

207   unwashed cells of the marine diatom *Thalassosiera oceanica* (Hasle) were isotopically heavier

208   ($\delta^{66}Zn$ = -0.05 to 0.38‰) than EDTA-washed cells (-0.79 to -0.16‰) (John et al. 2007). The

209   fraction of Zn adsorbed to *T. oceanica* was isotopically heavier than the solution, and the

210   fractionation increased linearly with Zn concentration [Zn] ($\Delta^{66}Zn_{diatom-solution}$ from 0.09‰ at

211    $10^{-11.5}$ *M* to 0.52‰ at $10^{-8.5}$ *M*)(John et al. 2007). The $\Delta^{66}Zn_{diatom-solution}$ from both studies is

212   remarkably similar, and points to the preferential adsorption of heavy Zn isotopes onto the cell

213   surface, probably to the carboxylate groups of the cell walls. Zinc binding to carboxyl and

214   hydroxyl groups of pectin and to hydroxyl groups of cellulose in the cell walls of roots has been

215   confirmed in tobacco plants (*Nicotiana tabacum* L.) using chemical extracts and Extended

216   X-Ray Absorption Fine Structure (EXAFS) spectrometry (Straczek et al. 2008). It is thus very

217   likely that the enrichment in heavy Zn isotopes observed in plants roots is mostly generated

218   during Zn binding to the hydroxyl and carboxyl groups of the cell walls, similarly to what

219   happens in diatoms. The isotopic fractionation between plants roots and the solution where

220   they grow ($\Delta^{66}Zn_{root\text{-}solution}$ = -0.15‰ to 0.8‰) is in a similar range to that of diatoms relative to

221   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

222   explained by the larger [Zn] in the solutions (up to 10^{-5} M), and species-specific differences in

223   the composition and adsorption capacity of the cell walls. To test this hypothesis, we need to

224   constrain the isotopic fractionation during Zn binding to the cell walls of plants and their

225   majoritarian components cellulose and pectine.

226

227   *Low- and high-affinity transport phenomena*

228   The shoots of hydroponically grown rice, tomato, and lettuce were depleted in ^{66}Zn relative to

229   ^{64}Zn ranging from -0.25 to -0.56‰ and differing between species (Weiss et al. 2005)(Fig. 1).

230   Analogous results were later obtained in tomato and durum wheat with $\Delta^{66}Zn_{shoot\text{-}root}$ ranging

231   between -0.29 and -0.56‰ (Jouvin et al. 2012; Smolders et al. 2013). This shift was attributed

232   to the preferential transport of free Zn^{2+} across cell membranes by transport proteins. The cell

233   walls of root cells closely touch one another forming a single extracellular space termed root

234   apoplast, in which water and solutes can circulate freely. This movement is restricted by the

235   endodermis, a single layer of cells that surrounds the conductive tissue of the root. The

236   Casparian strip, a ring of impermeable material found in the cell walls of the endodermis

237   prevents Zn from reaching the shoots via the apoplastic pathway. Hence, Zn has to be

238   transported across the cell membrane of the root cells. The cell membrane consists of a

239   phospholipid bilayer, and its hydrophobic nature impedes the passive diffusion of dissolved

240   ions into the cell. Ion uptake must be facilitated by transporter proteins, which allow plant

241   cells to control their concentration in the cytoplasm. Zinc transport across the cell membranes

242   is tightly controlled by plasma membrane associated proteins, mainly those from the ZIP family

243   (ZRT-IRT-like Proteins). Transporters AtIRT1 and AtIRT3 (Iron Regulated Transporter) in

arabidopsis (Arabidopsis thaliana [L.] Heynh.), HvZIP7 in barley (*Hordeum vulgare* L.), and OsIRT1, OsZIP1, OsZIP3, and OsZIP5 in rice are expressed in the root epidermis and involved in Zn uptake from the rhizosphere into the cell (Korshunova et al. 1999; Vert et al. 2002; Ishimaru et al. 2006; Lin et al. 2009; Lee et al. 2010; Tiong et al. 2014).

There are no isotope data on isolated plant cells (protoplasts) to help us understand how the membrane transporter proteins could discriminate Zn isotopes. Experiments on diatoms (single cell algae) might be a good approximation, since the functioning of Zn transporters is similar. Early work in diatoms suggested that plasma membrane transporters discriminate between Zn isotopes (Gélabert et al. 2006; John et al. 2007). Cells of marine diatoms washed in EDTA to remove the extracellular Zn were isotopically light as compared with the solution, which was attributed to fractionation during Zn uptake. Moreover, the $\Delta^{66}Zn_{solution-diatom}$ increased with [Zn], following a sigmoidal curve (John et al. 2007). The switch of this curve took place around 10^{-10} M Zn, coinciding with the switch between suggested "high- and low-affinity" Zn uptake reported for marine diatoms (Sunda and Huntsman 1992). Transport characterized as "high-affinity" predominates at low [Zn], whereas low-affinity transport prevails at high [Zn]. In the algae study conducted by John and co-workers, high-affinity Zn uptake generated an isotopic fractionation of up to -0.2‰ at Zn levels below $10^{-10.5}$ M (John et al. 2007). In contrast, low-affinity transport caused a much greater fractionation (up to -0.8‰) at [Zn] above $10^{-9.5}$ M. It was argued that during higher efficiency transport most of the Zn within reach is transported regardless of the isotope. This would explain the smaller isotopic fractionation during Zn uptake when high-affinity transport predominates (John et al. 2007).

High- and low-affinity transport phenomena have been described in rice, wheat, and other plants (Hacisalihoglu et al. 2001; Milner et al. 2012; Meng et al. 2014). The high-affinity transport was reported to predominate at less than 10^{-8} M Zn in the growth medium for wheat (Hacisalihoglu et al. 2001) and less than 10^{-7} M in rice (Meng et al. 2014). The [Zn] in the hydroponic studies was around 10^{-7}-10^{-6} M (Weiss et al. 2005; Jouvin et al. 2012; Smolders et

270 al. 2013). At these Zn levels a higher contribution of low-affinity transport would be expected

271 in wheat. In the above studies the root-to-shoot fractionation ($\Delta^{66}Zn_{shoot-root}$) was mainly

272 attributed to Zn uptake into the plant by the root cells, so we can compare it to $\Delta^{66}Zn_{in-ex}$ in

273 diatoms. The $\Delta^{66}Zn_{shoot-root}$ ranged from -0.25 to -0.56‰ for all plants, approximately between

274 that of high- and low-affinity transport in marine diatoms (-0.2‰ and -0.8‰, respectively)

275 (John et al. 2007). Moreover, wheat had lower $\Delta^{66}Zn_{shoot-root}$ (-0.29‰ to -0.51‰) than rice

276 (-0.25‰ to -0.29‰) (Weiss et al. 2005; Jouvin et al. 2012)(Fig. 1). It is noteworthy that the

277 extent of the fractionation between shoots and roots increased with increasing [Zn], as

278 $\Delta^{66}Zn_{in-ex}$ did in diatoms (John et al. 2007). It is highly plausible that the ion selectivity of the

279 membrane transport proteins like ZIP transporters is the predominant molecular mechanism

280 responsible for the isotopic fractionation observed during Zn uptake at the root.

281

282 The use of $\Delta^{66}Zn_{shoot-root}$ as a proxy of isotopic fractionation during Zn uptake by plants serves

283 well the purpose of elucidating the contribution of plants to isotope partitioning during Zn

284 biogeochemical cycling. However, neither $\Delta^{66}Zn_{shoot-root}$ nor $\Delta^{66}Zn_{shoot-solution}$ separate the

285 isotopic effect of Zn uptake from those caused by the mechanisms of Zn transfer from the root

286 to the shoot (discussed in section 5.1). Therefore these parameters provide an insufficient

287 level of detail for future physiological studies. To better quantify the discrimination of Zn

288 isotopes occurring during Zn uptake into the root symplast we need to isolate this isotopic

289 effect from any others. This could be achieved by reproducing the diatom studies using

290 protoplasts, comparing the isotope ratios of wild type plants with mutant lines defective for

291 known Zn transporters, measuring isotopic fractionation during Zn binding to relevant ligands,

292 and analysing the isotope ratios of the cytoplasm, the vacuoles, and the xylem sap.

293

294 *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 ($\Delta^{66}Zn_{Zn-L-}$

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 ($\Delta^{66}Zn_{shoot-soil}$=0.21‰) and the shoots of intolerant

302 plants ($\Delta^{66}Zn_{int-tol}$=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

321 et al. 1998a; Cakmak et al. 1998b; Rengel 1999; Erenoglu et al. 2000; Suzuki et al. 2006), which

322 reverts to control levels within 72 hours after Zn is resupplied (Zhang et al. 1989). Moreover,

323 many Zn-efficient varieties of wheat, barley, and rice have shown higher PS release rate during

324 Zn-deficiency than the inefficient ones (Cakmak et al. 1994; Cakmak et al. 1996; Cakmak et al.

325 1998a; Rengel et al. 1998; Rengel and Römheld 2000; Erenoglu et al. 2000; Tolay et al. 2001;

326 Widodo et al. 2010; Neelam et al. 2010; Daneshbakhsh et al. 2013). In contrast, analogous

327 experiments found no significant difference in PS release in Zn-deficient rice, wheat, and

328 barley (Pedler et al. 2000; Suzuki et al. 2008; Widodo et al. 2010), or between Zn-efficient and

329 inefficient wheat and barley (Erenoglu et al. 1996; Cakmak et al. 1998b; Pedler et al. 2000).

330 These inconsistencies in the literature might indicate that Zn-efficiency in crops is determined

331 by various factors, including PS release by the roots and possibly others. Interestingly, in some

332 instances when Zn-efficiency and PS release did not correlate, Zn-efficient plants still took up

333 more Zn and had higher root-to-shoot translocation rates (Von Wiren et al. 1996; Cakmak et al.

334 1998b). It was suggested that Zn-efficient varieties might have higher rates of Zn-PS uptake

335 and Zn export to the shoot, while the release rates were not necessarily increased.

336 A central question is if the secretion rates observed during Zn deficiency and the Zn-PS

337 complexes subsequently formed could account for the Zn budget of plants. The stability

338 constant of Fe(III)-PS complexes ($10^{18.4}$) is significantly higher than that of Zn-PS complexes

339 ($10^{12.9}$)(Murakami et al. 1989), which might make it difficult for the latter to form in presence

340 of Fe(III). The amount of PS secreted during Fe-deficiency is much higher than during

341 Zn-deficiency, up to 25 fold in rice (Suzuki et al. 2008), 23 fold in barley (Suzuki et al. 2006),

342 and 16 fold in wheat (Tolay et al. 2001). In a recent study in wheat (cv Tamaro) grown in

343 calcareous Fe-deficient soils, PS secretion from the roots ranged from 0.2 to 41 pmol DMA g^{-1}

344 root DW s^{-1}, which is about 50 times less than solution-grown plants typically secrete (Oburger

345 et al. 2014). It was argued that the PS release data from solution-grown plants in zero [Zn] or

346 [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 ($\approx$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

submersion is that they can have their roots covered by an iron plaque. This is a coat of

precipitated Fe(III) hydroxides formed thanks to the oxygen leaked from the roots. Up to 25 g

of iron plaque per Kg of root DW might favour Zn uptake in rice, whereas a higher amount

interferes with Zn uptake (Zhang et al. 1998). The effect of the Fe plaque is larger in plants

previously grown in Fe-deficient solution, and the Fe-deficient plants had a higher [Zn] in

shoots. It was concluded that the PS might promote Zn uptake in plants with iron plaque by

mobilizing Zn adsorbed to it.

Besides PS, plant roots exudate a mixture of organic acids and amino acids that enhance Zn

dissolution from the soil by lowering the pH around the roots and binding to Zn (Rasouli-

Sadaghiani et al. 2011). This strategy is also present in plant families that do not synthesize PS,

where Zn and other metals can be taken up in form of complexes (Degryse et al. 2007).

Furthermore, root exudates solubilize Zn from Zn-containing minerals like smithsonite ($ZnCO_3$)

(Houben and Sonnet 2012). For example, tomato seedlings were grown in resin-buffered

solution at two external [Zn] (10^{-6} or 1.5×10^{-8} M) and two pot sizes, to manipulate the

concentration of root exudates (Smolders et al. 2013). The highest [Zn] in the solution after

plant growth was recorded in the experimental conditions that most favoured the

accumulation of root exudates in the solution (low Zn and small pot). This suggested that root

exudates can also have an important role in mobilizing Zn in dicots. Besides, the shoots at 10^{-6}

M Zn were enriched in light isotopes (-0.52‰ to -0.56‰). By contrast, plants grown at 1.5×10^{-8}

M had a similar isotopic composition as the roots ($\Delta^{66}Zn_{shoot-root}$=-0.06‰ to -0.09‰)(Smolders

et al. 2013). It was hypothesized that at high Zn supply uptake was dominated by the

facilitated diffusion of free Zn^{2+}, which favours ^{64}Zn, whereas at low Zn supply most of the Zn

was taken up as Zn complexes. The heavier isotopes accumulate in the complexes because

they form stronger bonds. The resulting complexes do not undergo isotopic fractionation

during transport because the relative difference of mass (isotope vs complex) is too small. Zinc

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 $\Delta^{66}Zn_{shoot-root}$ 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 ($\Delta^{66}Zn_{stem-root}$ 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

425 environment. However, we must also pay attention to the different sampling methods used. In

426 the hydroponic studies (Weiss et al. 2005; Jouvin et al. 2012; Smolders et al. 2013) all the aerial

427 parts were sampled together, whereas in the field studies (Viers et al. 2007; Moynier et al.

428 2009; Viers et al. 2015) stems and leaves were analyzed separately. The contribution of the

429 leaves might then explain the shift between the solution-grown and the soil-grown plants.

430 Besides, a number of processes take place during Zn transfer from the root symplast to the

431 aerial parts that are likely to discriminate Zn isotopes. In the intra-cellular fluid (cytosol) the pH

432 is close to neutral (7.2-7.5), and Zn^{2+} will be kept at a very low concentration (in the pM

433 range)(Maret 2015) to avoid precipitation and misplaced binding. There are several low-weight

434 molecules that have been proposed as important Zn ligands in the cytosol, such as

435 nicotinamine (NA), histidine, organic acids, and small peptides (for a review, see Sinclair and

436 Krämer 2012). Of these, the non proteinogenic amino acid NA is considered the main Zn ligand

437 in the cytosol, and a key factor in enhancing Zn mobility in the symplast (Clemens et al. 2013).

438 The fractionation of Zn isotopes during the formation of Zn complexes with NA has not been

439 explored. However, *ab initio* calculations have shown that the partitioning of heavy isotopes

440 between citrates, malates, phosphates, histidine, and other Zn species can account for part of

441 the isotopic fractionation observed in plants, which will be further discussed in section 6.2

442 (Fujii and Albarède 2012; Fujii et al. 2014). The computational studies suggested that heavy

443 isotopes tend to bind to oxygen donors, while light isotopes accumulate in Zn complexes with

444 sulphur donors, and Zn complexes with nitrogen donors would be between the two or

445 isotopically heavier than with oxygen donors (Fujii et al. 2014). Recent experimental evidence

446 has confirmed that the heavy isotopes accumulate in Zn complexes with DMA, and structurally

447 similar ligands (Markovic et al. 2016). Another potential source of isotope discrimination is Zn

448 sequestration in the vacuoles. The activity of the vacuolar transporters regulates the amount

449 of Zn in the cytosol available for translocation by either increasing or decreasing the amount of

450 Zn sequestered in the vacuoles. Several studies conclude that MTP1 and MTP3 (Metal

451   Tolerance Proteins) facilitate the efflux of Zn^{2+} from the cytosol into the vacuole (reviewed by

452   Ricachenevsky et al. 2013). The vacuole has an acidic pH around 5.2 (Shen et al. 2013) and Zn

453   can stay soluble as Zn^{2+}. Similarly, AtZIF1 is a tonoplast transporter that carries Zn complexes

454   with NA into the vacuoles (Haydon et al. 2012). Transporter AtNRAMP4 facilitates Zn influx

455   from the vacuole back into the cytoplasm (Lanquar et al. 2010). Additionally, Zn^{2+} can diffuse

456   from one cell to another across the plasmodesmata, small openings in the cell walls that allow

457   the cytoplasm of adjacent cells to communicate. This is a kinetically controlled process that will

458   favour the light isotopes (Criss 1999).

459   To reach the shoot, Zn needs to leave the root symplast and enter the xylem, a conductive

460   tissue formed by the walls of dead cells containing no cytoplasm. Transporters HMA2 and

461   HMA4 are plasma membrane transporters of the HMA family (Heavy Metal ATPases)

462   expressed in the vasculature of the root, and are thought to export Zn from the adjacent cells

463   to the xylem in arabidopsis (Eren and Argüello 2004). Of these, HMA2 transports Zn bound to a

464   ligand while HMA4 transports Zn^{2+}. The xylem sap is considered an acidic environment with pH

465   around 5.5 where Zn^{2+} could stay in solution, although it can be alkalinized in response to

466   drought, flooding, bicarbonates, nutrients, light, change of season, daily rhythms, and disease

467   (Wilkinson, Janet E. Corlett, Ludovic Oger et al. 1998). Little is known about how these

468   fluctuations affect Zn xylem loading and forms in the xylem sap. In the xylem sap of strategy I

469   plants Zn is predominantly transported as Zn^{2+} with the remaining fraction bound to organic

470   acids (Salt et al. 1999; Monsant et al. 2011; Lu et al. 2014). The aminoacids histidine

471   (Kozhevnikova et al. 2014) and NA (Cornu et al. 2015) have also been proposed a role in

472   Zn-binding in the xylem. In strategy II plants, Zn-DMA complexes have been identified in root

473   extracts of Fe-deficient wheat (Xuan et al. 2006), and in the shoots of japonica rice (Tsednee et

474   al. 2016), which suggests that Zn could be transported up the xylem as Zn-PS complexes. All of

475   these steps might contribute to the isotope ratios of roots and shoots.

476

Zinc translocation to the leaves

478   The few experiments that analysed leaves separately found generally isotopically lighter Zn

479   compared to the stems ($\Delta^{66}Zn_{leaves-stem}$ -1.67 to 0.10‰)(Viers et al. 2007; Moynier et al. 2009;

480   Caldelas et al. 2011; Viers et al. 2015). Furthermore, the leaves of various plant species were

481   increasingly depleted in ^{66}Zn the further they were from the root (Fig. 3). First, tree leaves

482   collected from a pristine tropical drainage basin were recorded as isotopically lighter

483   ($\delta^{66}Zn_{leaves}$ -0.03‰ to -0.91‰) than the leaves of the herbaceous species in the same area

484   (0.26 to 0.63‰) (Viers et al. 2007). The observations by Viers et al. 2007 were confirmed in

485   bamboo leaves collected at various heights (20, 50, and 80 cm), which showed a progressive

486   decrease of $\delta^{66}Zn$ with distance (-0.19‰, -0.32‰, and -0.55‰ respectively)(Moynier et al.

487   2009). Both studies proposed that the negative $\delta^{66}Zn$ of the leaves could be explained as the

488   sum of two processes: i) Faster transport of ^{64}Zn across the plasma membranes, throughout Zn

489   transfer from the roots to the leaves, and ii) preferential unload of ^{66}Zn from the xylem sap

490   into the adjacent cells by low-affinity transporters, leaving the xylem solution progressively

491   enriched in ^{64}Zn. Zinc binding to the cell walls lining the xylem vessels might also occur,

492   favouring the depletion of heavy isotopes in the xylem sap via ion exchange. A similar trend

493   was observed in reeds, where a fractionation of up to -0.44‰ was measured between leaves

494   sampled at 5 and 100 cm (Caldelas et al. 2011). The extent of Zn fractionation with height was

495   very consistent: -0.005‰ cm^{-1} in reed (Caldelas et al. 2011) and -0.006‰ cm^{-1} in bamboo

496   (Moynier et al. 2009).

497   To leave the xylem and enter the leaf symplast Zn must first cross the membranes of the

498   companion cells, a step mediated by transporters yet to be characterized. Membrane

499   transporters AtIRT3 and OsZIP4 are expressed in the vasculature of the leaves and might

500   facilitate both xylem unloading and phloem loading of Zn^{2+} (Ishimaru et al. 2005; Lin et al.

501   2009). The xylem unloading of Zn^{2+} could contribute to the enrichment of light isotopes

502   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 ($\Delta^{66}Zn_{seed-}$

535 $_{shoot}$ -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

581 (Tauris et al. 2009). This suggests that Zn movement and compartmentalization within the

582 seed after Zn uptake in the aleurone would not substantially add to the fractionation of Zn

583 isotopes between the seed and the shoot.

584 In summary, it appears that the enrichment of light isotopes in the seeds relative to the shoots

585 could originate from Zn^{2+} storage in the vacuoles of the transfer cells, efflux from transfer cells,

586 diffusion in the endosperm cavity, and uptake by aleurone cells. Additionally, mechanisms that

587 lead to the enrichment of light isotopes in the phloem sap relative to the bulk shoot, like Zn

588 remobilization from the older tissues or direct xylem-to-phloem Zn transfer could contribute to

589 make seeds isotopically lighter than the shoots. The $\delta^{66}Zn$ of the grain might prove an

590 interesting tool for the development of Zn biofortification in crops, which could help us

591 identify the key mechanisms involved in Zn allocation into the seed. However, there is a

592 pressing need for more isotope data from seeds and a better knowledge of the molecular

593 mechanisms behind Zn allocation to the seed and the Zn forms in the different reservoirs.

594

595 **Fractionation of Zn isotopes associated with plants response to Zn excess**

596 *Accumulation in the roots of Zn-tolerant plants*

597 Tolerant plants are those that can grow and develop when [Zn] in the environment would

598 usually be deleterious. In tolerant plants exposed to an excessive amount of Zn in the

599 environment, roots play an important role in Zn detoxification and sequestration to protect

600 sensitive photosynthetic tissue. The response of tolerant plants to high Zn levels can cause

601 significant changes in the distribution of Zn isotopes across the plant organs in some species. In

602 reed (*Phragmites australis* [Cav.] Trin. ex Steud) grown hydroponically with sufficient Zn supply

603 ($3x10^{-6}$ *M*) the roots, rhizomes, and shoots were similarly enriched in heavy isotopes relative to

604 the growth solution (~0.2‰)(Caldelas et al. 2011)(Fig. 4a). By contrast, the addition of Zn in an

605 excessive amount ($2x10^{-3}$ *M*) caused further enrichment of the heavy isotopes in roots relative

606 to the growth solution ($\Delta^{66}Zn_{root-solution}=0.47$ ‰) and relative to the stems ($\Delta^{66}Zn_{stem-root}=-0.84$

607   to -0.94‰). The magnitude of both isotopic effects was larger than reported for

608   hydroponically grown crops ($\Delta^{66}Zn_{root-solution}$ -0.02 to 0.16‰, and $\Delta^{66}Zn_{shoot-root}$ -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 $\Delta^{66}Zn_{shoot-root}$ in this study was -0.83‰, very similar to previous results in *P.*

640 *australis* ($\Delta^{66}Zn_{shoot-root}$=-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. ($\Delta^{66}Zn_{shoot-root}$ =-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 ($\Delta^{66}Zn_{root-solution}$=0.07-0.19‰, $\Delta^{66}Zn_{shoot-root}$=-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 ($\Delta^{66}Zn_{root-solution}$=0.05-0.20‰,

657 $\Delta^{66}Zn_{shoot-root}$=-0.04 to -0.39‰)(Couder et al. 2015).

658   Besides the sequestration of Zn in the roots, plants display other responses to Zn excess that

659   possibly discriminate Zn isotopes. Both *Arabidopsis thaliana* (L) and *Arabidopsis halleri* (L.)

660   secrete NA to the rhizosphere, where it forms Zn-NA complexes, and *A. halleri* increases NA

661   secretion when exposed to high Zn levels in the environment (Tsednee et al. 2014). In the

662   same study, [Zn] in the roots of *A. thaliana* decreased by up to 60% when 50 µ*M* NA was

663   added to the soil, indicating that the Zn-NA complexes were not taken up by the root cells. It

664   was concluded that plants secrete NA to the rhizosphere to reduce Zn bioavailability in the soil.

665   Moreover, the secretion of NA increased in *A. halleri* in response to excess Zn To illustrate the

666   impact of root exudates on the isotopic composition of the soil solution, *Agrostis capillaris* was

667   grown in columns filled with two technosols and compared with controls without plant cover

668   (Houben et al. 2014). The columns were irrigated with nutrient solution in excess and allowed

669   to drain, and the resulting leachates were collected and analyzed. Without plants, the

670   leachates were isotopically lighter than the soil ($\Delta^{66}Zn_{leachates-soil}$ =-0.12 to -0.21‰), meaning

671   that the leaching of free Zn^{2+} from the soil removed the light isotopes. By contrast, in columns

672   planted with *A. capillaris* the leachates were isotopically heavier than the soil ($\Delta^{66}Zn_{leaches-soil}$

673   =0.14 to 0.04‰), with up to 4 times more Zn. This was attributed to the mobilization of Zn

674   from an isotopically heavier pool in the soil facilitated by the root exudates. Further work is

675   needed to determine whether the mobilized Zn is then taken up by the *A. capillaris* or remains

676   in the soil. On top of stimulating the secretion of root exudates, an excessive Zn supply

677   modifies the activity of some Zn transporters in the membrane of the root cells, which could

678   have an impact in Zn isotope partitioning. High Zn levels inhibit the activity of plasma

679   membrane transporters like AtIRT3 in arabidopsis, involved in Zn uptake by the roots (Lin et al.

680   2009). The Zn efflux transporter AtPCR2 in arabidopsis exports excess Zn from the cytoplasm

681   to outside the cell, and its expression in yeast is increased at high Zn levels (Song et al. 2010).

682   Another process contributing to the accumulation of ^{64}Zn in the aerial parts of Zn-tolerant

683   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 $\Delta^{66}Zn_{shoot-root}$ 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

710   of *A. halleri* compared with the solution (10 or 250 µM Zn, Δ^{66}Zn$_{root-solution}$=0.4-0.8‰) and the

711   shoots (Δ^{66}Zn$_{shoot-root}$=-0.7‰), in line with results obtained for *P. australis* (Caldelas et al. 2011).

712   By contrast, the roots of the related non-accumulator *A. petraea* had a similar δ^{66}Zn as the

713   solution (Aucour et al. 2011). The enrichment of heavier Zn isotopes in the roots of *A. halleri*

714   relative to the roots of *A. petraea* was explained as the result of Zn storage in the vacuoles of

715   the root cells in form of Zn-phosphates (Aucour et al. 2011). Previous spectroscopic work had

716   revealed that in *A. halleri* Zn binds mostly to phosphates in the roots, and to citrate or malate

717   in the shoots (Sarret et al. 2002). Using *ab initio* calculations, Fujii and Albarède compared the

718   δ^{66}Zn of those three Zn species at total concentrations of 0.05 M (Zn), 0.01 M (citrate and

719   malate), and 1 M (phosphate). At pH around neutral, Zn-phosphates were expected to enrich

720   isotopically heavier Zn with respect to the solution, whilst Zn-malates and Zn-citrates would

721   both concentrate light isotopes relative to the solution (Fujii and Albarède 2012). The Δ^{66}Zn

722   between Zn-citrates (or Zn-malates) and Zn-phosphates would be around -0.8 to -0.9‰,

723   consistent with the Δ^{66}Zn$_{shoot-root}$ of *A. halleri* (-0.7‰) and *P. australis* (-0.8 to -0.9‰)(Aucour et

724   al. 2011; Caldelas et al. 2011). By contrast, at pH below 5 all three Zn complexes would have a

725   similar isotopic composition as the solution (Fujii and Albarède 2012), in agreement with the

726   roots of *A. petraea* having a similar δ^{66}Zn as the source (Aucour et al. 2011; Tang et al. 2012).

727   Fujii and Albarède suggested that *A. halleri* might maintain the pH of the root cells around

728   neutral to promote Zn complexation with phosphates as a tolerance mechanism, which would

729   cause most of the heavy enrichment of the roots relative to the soil in this species (Fujii and

730   Albarède 2012). The predominant Zn species in *P. australis* roots and shoots remain elusive,

731   but the distribution of Zn isotopes is similar to that of *A. halleri*. This suggests that Zn

732   complexation with phosphate in the roots could be a key tolerance mechanism in *P. australis*,

733   which is not a hyperaccumulator species.

734   Compared with the plant-available Zn of the soil, ^{66}Zn was enriched in the roots of the

735   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

Isotopic fractionation of iron by plants

Iron uptake in strategy I and II plants

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

However, Strategy II plants produce little PS in absence of Fe-deficiency (Cakmak et al. 1994; 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 ($\Delta^{56}Fe_{plant-soil}$ -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 $\Delta^{56}Fe_{plant-solution}$ 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 $\Delta^{56}Fe_{plant-solution}$ 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 ($\Delta^{56}Fe_{shoot-soil}$

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 ($\Delta^{56}Fe_{stem-stele}$ 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

840 of Fe in the symplast is Fe(II)-NA, while Fe in the xylem is Fe(III)-citrate (Rellán-Alvarez et al.

841 2008; Rellán-Alvarez et al. 2010). This is in agreement with *ab initio* calculations of isotopic

842 fractionation between Fe species, which predict that Fe(II)-NA would be isotopically lighter

843 (≈2‰) than Fe(III)-citrate (Moynier et al. 2013). Moynier and co-workers noted that while

844 redox changes accounted for the largest isotopic fractionations (≈3‰), speciation only could

845 explain isotopic fractionations up to 1.5‰.

846 In beans (strategy I) grown in 20 μM Fe(III)-EDTA solution δ^{56}Fe decreased progressively from

847 the stem to the leaves and the grains (δ^{56}Fe -0.31, -0.69, and -1.90‰ respectively) (Guelke-

848 Stelling and von Blanckenburg 2011). By contrast, no discrimination was observed between the

849 aerial parts and the roots in oats (strategy II) in the same study. The authors attributed the

850 fractionation pattern of beans to the reduction of Fe(III) in the xylem to Fe(II) during transfer

851 to the symplast of leaves and grains. The absence of fractionation in the above-ground organs

852 in oats is compatible with the translocation of Fe(III)-PS complexes from the root to the shoot,

853 and with the transfer of Fe(III) from Fe(III)-PS or Fe(III)-EDTA to NA without changing the redox

854 form. In strategy II plants Fe(III) can be transported up the shoot as Fe(III)-NA complexes

855 without a previous reduction step (von Wiren et al. 1999).

856

857 *Fe remobilization from older leaves to developing organs*

858 In two Fe-deficient soils, the leaves of beans became depleted of ^{56}Fe between the first harvest

859 and the fourth by up to -0.7‰ (Guelke and von Blanckenburg 2007). The remobilization of Fe

860 from older leaves to developing organs was proposed as the origin of this pattern. Excess Fe is

861 oxidized to Fe(III) and stored as phytoferritin in the plastids of plant cells, from where it can be

862 mobilised upon demand (van der Mark et al. 1982). It was argued that a non-quantitative

863 sequestration of Fe in the phytoferritin complexes would favour the accumulation of

864 isotopically heavy Fe(III) in the phytoferritin, leaving a pool of isotopically light Fe(II) in the

865 cytoplasm available for export to the developing tissues, and isotopically heavier older leaves.

866 In the same study, the leaves of oats and wheat did not show substantial fractionation

867 between the first and the second harvest (Guelke and von Blanckenburg 2007). The authors

868 proposed that in strategy II plants Fe could move in the phloem as Fe(III) binding to heavy

869 mass ligands such as NA. Iron (III) can form complexes with NA (von Wiren et al. 1999), but a

870 study of the chemical forms of Fe in the phloem sap of rice has detected Fe(III)-PS complexes

871 instead of Fe(III)-NA complexes (Nishiyama et al. 2012). In the Fe-deficient soils where oats and

872 wheat were grown in the Guelke and von Blankenburg study a strong contribution of PS to Fe

873 uptake can be expected, so Fe(III) could move in the phloem as Fe(III)-PS. In agreement, the

874 grains of rice grown both in aerobic and anaerobic Fe-deficient soils had a similar composition

875 as the shoot (Arnold et al. 2015).

876 Similar results were obtained in beans and oats grown in Fe-sufficient nutritive solution where

877 Fe was given as Fe(III)-EDTA. The youngest leaves and the seeds of beans became isotopically

878 lighter as plants grew (up to -1.75‰), but those of oats did not (Guelke-Stelling and von

879 Blanckenburg 2011). By contrast, the leaves of both strategy I and II alpine species grown in an

880 Fe-rich soil (where very little contribution from PS to Fe uptake could be expected)

881 accumulated ^{56}Fe with age (up to 1.5‰) (Kiczka et al. 2010b). Therefore it appears that in

882 strategy II species the source of Fe determines the isotopic fractionation during remobilization.

883 The uptake of Fe(III) binding to strong ligands like EDTA or PS causes no fractionation during Fe

884 movement in the phloem, while the uptake of non-complexed Fe leads to a fractionation

885 pattern analogous to that of strategy I species, indicative of a series of redox conversions.

886 The partitioning of Fe isotopes between older and younger leaves is similar to that previously

887 discussed in section 5.2 for Zn. The light Zn isotopes become enriched in the leaves with

888 height. This effect could be an evidence of Zn isotope partitioning during Zn remobilization

889 from older leaves to developing ones, analogous to that observed in Fe. While excess Fe is

890 stored in the plastids in form of phytoferritin, excess Zn is stored in the vacuoles of the leaves

891 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. $\Delta^{65}Cu_{shoot-seed}$ of -0.34‰ (Weinstein et al. 2011). This effect was identical to that

903 reported for Zn in the same experiment, i.e. $\Delta^{66}Zn_{shoot-seed}$ = -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 ($\Delta^{65}Cu_{shoot-solution}$ -1.06 to -0.34‰), and the roots generally had the same isotopic composition

916 as the shoots ($\Delta^{65}Cu_{root-solution}$ -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 ($\Delta^{56}Fe_{root-solution}$ = -1.0 to -0.5‰),

918   which was attributed to Fe(III) reduction during Fe uptake (Guelke-Stelling and von

919   Blanckenburg 2011). By contrast, Zn in the roots is typically enriched in heavy isotopes

920   ($\Delta^{66}Zn_{root-solution}$ up to 0.8‰)(Weiss et al. 2005; Viers et al. 2007; Aucour et al. 2011). It was

921   concluded that the enrichment of ^{63}Cu in the roots was due to the reduction of Cu(II) to Cu(I)

922   during Cu uptake by the root cells. Leaching of Cu(I) minerals has shown that ^{65}Cu accumulates

923   in the aqueous Cu(II) ($\Delta^{65}Cu_{mineral-solution}$ -1.18 to -0.94‰)(Kimball et al. 2009). The fractionation

924   during Cu(II) reduction to Cu(I) is similar to the fractionation observed during Cu uptake by the

925   root by Jouvin and co-workers ($\Delta^{65}Cu_{root-solution}$ -0.84 to -0.11‰). The smaller fractionation in

926   plants indicates a combination of reduction and another uptake mechanism that does not

927   fractionate isotopes or takes a shift towards the heavy. In arabidopsis, Cu(II) reduction to Cu(I)

928   is a pre-requisite for the high-affinity uptake of Cu by means of the transporter COPT1, and is

929   mediated by the ferric reductase oxidases FRO5 and FRO4 (Bernal et al. 2012). The reductase

930   FRO2 is responsible for Fe(III) reduction to Fe(II) prior to uptake mediated by the IRT1

931   transporter, as discussed in the previous section. The reductase activity of the FRO family thus

932   constitutes an important link between Cu and Fe homeostasis. Still, transporter COPT1 is

933   responsible for only 40-60% of Cu uptake in arabidopsis under adequate Cu nutrition

934   (Sancenón et al. 2004). Plants take up Cu(II) in form of Cu^{2+} or PS-Cu(II) complexes (Roberts et

935   al. 2004; Hötzer et al. 2012). The transport of Cu^{2+} across the cell membrane during uptake in

936   the roots is probably facilitated by cation transporters like ZIP2 in arabidopsis, and IRT1 and

937   IRT2 in tomato (Wintz et al. 2003; George et al. 2012). The uptake of PS complexes with Cu(II)

938   is mediated by plasma membrane transporters in the root epidermis like YS1 in maize (Roberts

939   et al. 2004). In agreement, the roots of tomato and oats grown in Fe-sufficient nutrient

940   solution were enriched in light isotopes relative to the solution, and the shift was much larger

941   in tomato ($\Delta^{65}Cu_{root-solution}$ -1.43‰) than in oats (-0.20‰)(Ryan et al. 2013). In both species the

942   fractionation decreased during Fe-deficiency (-1.05‰ and -0.12‰ respectively) indicating a

943   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 (Δ^{65}Cu$_{shoot-root}$ 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 (Δ^{65}Cu$_{leaves-stem}$ 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.

Nickel

Very little is known about Ni isotopic fractionation in plants, but it is still worth discussing it here because many metalicollous plants are Zn and Ni hyperaccumulators (Prasad and De Oliveira Freitas 2003). A survey of Ni isotopic fractionation in plants including a non-hyperaccumulator (*Thlaspi arvense* L.), a Ni hyperaccumulator *(Alyssum murale* Waldst. & Kit.), and a Ni and Zn hyperaccumulator *(N. caerulescens)* revealed that plants generally accumulate light isotopes during uptake ($\Delta^{60}Ni_{plant-solution}$ -0.90 to -0.21‰, relative to NIST-SRM 986)(Deng et al. 2014). The hyperaccumulators showed a larger fractionation (-0.90 to -0.63‰), which points to a greater permeability of the low-affinity transporters in these species (Deng et al. 2014). Interestingly, a high supply of Zn (50×10^{-6} *M*) suppressed the observed fractionation in all the three species (-0.11 to -0.07‰). This indicates that Zn and Ni compete for the same uptake mechanisms. The root to shoot transfer of Ni resulted in further enrichment of ^{58}Ni in the hyperaccumulators (-0.47 to -0.14‰), while the shoots of the non-hyperaccumulator accumulated ^{60}Ni relative to the roots (0.25‰). This suggests the existence of different Ni translocation mechanisms in hyperaccumulators, but these remain poorly understood.

Model of Zn isotope discrimination by plants

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

relative to the soil solution, because the Zn-complexes themselves are isotopically heavier than the unbound Zn^{2+}. Efflux transporters would probably carry isotopically light Zn^{2+} to the rhizosphere, but their contribution is likely to be small. In Zn-sufficient environments, the main sources of isotope separation at the soil-root interface identified were Zn speciation in the soil solution, binding to the cell walls, and low-affinity transport phenomena. In Zn-deficient conditions, these were substituted by high-affinity transport phenomena, Zn binding to root exudates, and the uptake of the resulting complexes. The two latter are especially relevant in Strategy II plants. Finally, Zn isotopic fractionation at the soil-root interface with high Zn supply originated mostly from Zn binding to the cell wall.

Jouvin and co-workers addressed only briefly some of the mechanisms that could lead to isotopic fractionation during Zn transfer from the root to the shoot: i) complexation with various ligands in the xylem and phloem, especially NA, ii) membrane crossings, iii) diffusion, and iv) ion exchange (Jouvin et al. 2012). Here we would like to build on their preliminary model and provide a detailed account of all the likely sources of fractionation at work during Zn export to the shoots and movement between above-ground organs. Following Zn uptake by the root, Zn^{2+} in the cytosol binds to small weight compounds, most likely NA (Fig. 6). Heavy isotopes are thought to accumulate in the resulting Zn-complexes relative to the Zn^{2+} fraction. Unbound Zn^{2+} can diffuse radially over small distances in the apoplast and the symplast, passing from the cytoplasm of one cell to another across the plasmodesmata. Lighter isotopes will diffuse faster due to their smaller mass. In our model, we propose that the vacuoles might have a key role in determining the isotopic composition of the Zn pool of the cytosol. Excess Zn^{2+} can be removed from the cytosol and stored in the vacuole by means of vacuolar transporters, wherefrom it can be remobilized. In this manner, vacuolar transporters control the amount of Zn available for export to the aerial parts. The isotopic composition of the vacuolar Zn pool is unknown and probably very dynamic, determined by the joint activity of the vacuolar transporters and Zn speciation in the vacuole.

1048   Zinc will be loaded onto the xylem sap from the cytosol of the adjacent cells, a step mediated

1049   by membrane transporters that seem to favour the lighter isotopes. Zinc in the xylem sap

1050   mostly consists of Zn^{2+}, but a substantial amount of Zn-complexes with OA, histidine, NA, and

1051   PS might be present, especially under low Zn supply (Fig. 7). The xylem sap moves up the stem

1052   thanks to the mass flow of water driven by transpiration and maintained by the cohesion of

1053   the water molecules and their adhesion to the cellulose walls. This movement might result in

1054   the enrichment of light isotopes in the leaves with height, although it is not yet clear how the

1055   isotopes could be discriminated. Adsorption of Zn^{2+} onto the cellulose walls of the xylem

1056   should result in a progressive depletion of the heavier isotopes in the xylem sap relative to the

1057   root symplast. From the xylem, Zn will enter the leaf symplast via Zn transporters at the

1058   plasma membrane of the bundle sheath cells, specialized in xylem unloading. Xylem unloading

1059   might favour the accumulation of lighter isotopes in the leaves relative to the xylem sap.

1060   In the leaf symplast Zn^{2+} will bind to various ligands, mainly NA, accumulating heavy isotopes in

1061   the complexes relative to free Zn^{2+}. Ligand exchange might also occur during Zn unloading

1062   from the xylem to the symplast, but the isotopic effects derived from it are not known. In

1063   addition, preferential diffusion of isotopically light Zn^{2+} in the symplast could lead to further

1064   discrimination of Zn isotopes during Zn movement in the leaves. The activity of the vacuolar

1065   transporters and Zn complexation in the cytosol and the vacuole regulate $[Zn^{2+}]$ in the leaf

1066   symplast. These processes might leave an isotopically lighter pool of Zn^{2+} available for

1067   remobilization to developing leaves and grains. Zinc remobilization from source to sink tissues

1068   involves phloem loading, xylem-to-phloem direct transfer, and phloem unloading of Zn. All

1069   three processes are thought to be mediated by transporters at the plasma membrane, and

1070   likely favour the lighter isotopes.

1071

1072   **Conclusions**

1073 The purpose of this review is to provide an overview of Zn isotopic fractionation in plants, in

1074 relation with our current knowledge of Zn homeostasis. The research debated here clearly

1075 indicates that Zn uptake, transport to the aerial parts, and transfer to the leaves can induce

1076 isotopic fractionation relative to the Zn source, and between the plant organs. The isotopic

1077 effects observed are species-specific and concentration-dependent, and are attributed mainly

1078 to Zn speciation, compartmentalization, and transporters. In future, isotope ratios may serve

1079 to elucidate the predominant Zn species taken up by the roots, and found in the various Zn

1080 pools in the plant. Furthermore, isotope ratios could be used to identify the major processes

1081 controlling Zn transfer to the aerial parts and to quantify the extent of remobilization from

1082 source to sink organs, for instance during grain filling. Moreover, analysing the isotope ratios

1083 of Zn and other metals with similar biochemistry could reveal their competitive interactions.

1084 Additionally, changes in the patterns of Zn isotopic partitioning in response to environmental

1085 conditions might be used to study plant physiology and to identify desired traits like Zn

1086 efficiency or hypertolerance.

1087 Future efforts must be directed towards the identification of the individual mechanisms

1088 responsible for the observed isotopic effects. To achieve this objective, research needs to solve

1089 several knowledge gaps. Probably the most urgent task is to constrain the isotopic effect

1090 associated with Zn binding to key ligands, like phytosiderophores, cellulose, pectine, OA, NA,

1091 and phosphates. Besides, the δ^{66}Zn of subcellular compartments, plant tissues, and some

1092 organs (fruits, seeds, and rhizomes) has not been properly investigated yet, which is

1093 hampering our understanding of Zn flows in plants. Finally, the influence of many factors

1094 important for plant nutrition like soil biochemistry, Zn interactions with other nutrients,

1095 mycorrhizae, root iron plaque, and environmental stressors on the patterns of Zn isotope

1096 discrimination is still unexplored.

1097

1098 **Aknowledgements**

1103

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