1 **Natural variation in *Arabidopsis thaliana* reveals shoot ionome, biomass and gene**
2 **expression changes as biomarkers for zinc deficiency tolerance**
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4 Ana Carolina A. L. Campos[1,2#], Willem Kruijer[3], Ross Alexander[1,4], Robert C. Akkers[1],
5 John Danku[2#], David E. Salt[2#], Mark G. M. Aarts[1]*
6

7 [1]Laboratory of Genetics, Wageningen University, Droevendaalsesteeg 1, 6708 PB
8 Wageningen, The Netherlands; [2]Institute of Biological and Environmental Sciences,
9 University of Aberdeen, Cruickshank Building, Aberdeen, AB24 3UU United Kingdom;
10 [3]Biometris, Wageningen University and Research, P.O. Box 100, 6700AC Wageningen, The
11 Netherlands; [4]School of Life Sciences, John Muir Building, Heriot Watt University,
12 Edinburgh, EH14 4ASUnited Kingdom; [#]current address: School of Biosciences, University
13 of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD,
14 United Kingdom.
15

16 ana.atalalombelocampos@nottingham.ac.uk, willem.kruijer@wur.nl, r.alexander@hw.ac.uk,
17 robert.akkers@wur.nl, john.danku@nottingham.ac.uk, david.salt@nottingham.ac.uk
18

19 *corresponding author: Dr. Mark Aarts, mark.aarts@wur.nl, +31-317485413.
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23 **Highlight**: Arabidopsis genotypes with a better ability to grow and yield under Zn limiting
24 conditions can be distinguished based on the minimum Zn concentration required for growth
25 and the expression levels of Zn deficiency responsive genes.
26

31 **Abstract**

32 Zinc (Zn) is an essential nutrient for plants with a crucial role as a co-factor for many

33 enzymes. Approximately one third of the global arable land area is Zn deficient leading to

34 reduced crop yield and quality. To improve crop tolerance to Zn deficiency, it is important

35 to understand the mechanisms plants have adopted to tolerate suboptimal Zn supply. In this

36 study, physiological and molecular aspects of traits related to Zn deficiency tolerance were

37 examined in a panel of 19 *Arabidopsis thaliana* accessions. Accessions showed a larger

38 variation for shoot biomass than for Zn concentration, indicating that they have different

39 requirements for their minimal Zn concentration required for growth. Accessions with a

40 higher tolerance to Zn deficiency showed an increased expression of the Zn deficiency

41 responsive genes *ZIP4* and *IRT3* in comparison to Zn deficiency sensitive accessions.

42 Changes in the shoot ionome, as a result of the plants Zn treatment, were used to build

43 multinomial logistic regression model able to distinguish plants regarding their Zn

44 nutritional status. This set of biomarkers, reflecting the *A. thaliana* response to Zn

45 deficiency and Zn deficiency tolerance, can be useful for future studies aiming to improve

46 the performance and Zn-status of crop plants grown under suboptimal Zn concentrations.

47

48 **Key-words:** biofortification, biomarker, mineral concentration, plant ionome, shoot growth,

49 zinc usage index.

Introduction

Zinc (Zn) is an essential micronutrient required for plant growth and development. Many agricultural soils in the Middle East, India, and parts of Australia, America and Central Asia are Zn deficient, often due to poor Zn availability caused by high pH in calcareous soils. Zn deficient soils affect both crop yield and quality and can also result in human malnutrition through the intake of food containing low concentrations of Zn and other micronutrients (Alloway, 2009; Cakmak, 2007). The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations estimate that about one third of the world's population suffers from some form of Zn deficiency (Allen *et al.*, 2006). Since plants are often the main source of dietary Zn, improving plant Zn concentration and tolerance to Zn deficiency is an important goal in fighting this so called 'hidden hunger' (www.harvestplus.org).

Plants exposed to Zn deficiency show reduced growth. Severe deficiency results in extensive leaf chlorosis, wilting, stunting, leaf curling and reduced root elongation, while mild stress results in chlorosis in young leaves and early senescence of older leaves (Marschner, 1995). In *Arabidopsis thaliana*, all of these symptoms, as well as delayed flowering, are observed when plants are grown under Zn deficiency (Talukdar and Aarts, 2007). Zn deficiency also affects the function of enzymes such as copper/zinc superoxide dismutase (Cu/Zn SOD) and carbonic anhydrase (CA) leading to an accumulation of reactive oxygen species (ROS), which causes oxidative damage and a reduction in photosynthesis (Clemens, 2010; Ibarra-Laclette *et al.*, 2013).

The threshold Zn concentration below which plants are considered to be Zn deficient is around 15-20 μgg^{-1} dry biomass. This can vary from species to species and between plants of the same species (Marschner, 1995; White and Broadley, 2011). The ability of a plant to grow and yield under Zn limiting conditions compared to ideal growth conditions is defined as Zn Efficiency (ZnE). It is based on the difference in relative growth or yield between plants grown under control and Zn deficient conditions. Another parameter used is the Zn Usage Index (ZnUI), which quantifies the amount of dry matter produced per mg of Zn in the plant. The ZnUI is useful for the comparison of plant genotypes which do not show significant differences in Zn concentration, but differ in biomass production under Zn deficiency (Cakmak *et al.*, 1998; Genc *et al.*, 2006; Good *et al.*, 2004; Siddiqi and Glass, 1981).

84

85　To avoid problems associated with inappropriate Zn supply, plants have developed an

86　efficient homeostasis mechanism. Different genes act in the uptake of Zn from soil,

87　distribution over different organs, tissues, cells and organelles, and (re)mobilization through

88　the plant, to control Zn homeostasis (Sinclair and Kramer, 2012). While the actual Zn

89　deficiency sensor is not yet known, the Zn deficiency response in *A. thaliana* seems to start

90　with the activation of the transcription factors bZIP19 and bZIP23, the function of which is

91　essential for plants to survive Zn deficiency (Assunção *et al.*, 2013; van de Mortel *et al.*,

92　2006).

93

94　Zn is among the essential elements which compose the plant ionome (Salt *et al.*, 2008).

95　Previous studies have shown that the plant ionome profile reflects the physiological state of

96　plants under various genetic, developmental, and environmental backgrounds and can be

97　used as a biomarker for a particular physiological condition (Huang and Salt, 2016).

98　Ionome-based biomarker models have been used to determine differences in the plant

99　nutritional status among large sets of different genotypes and experimental batches (Baxter

100　*et al.*, 2008a). Natural variation for the concentration of elements composing the plant

101　ionome has been studied in *A. thaliana*, revealing important mineral homeostasis

102　mechanisms in plants (Baxter *et al.*, 2010; Baxter *et al.*, 2008a; Chao *et al.*, 2012; Kobayashi

103　*et al.*, 2008; Koprivova *et al.*, 2013; Loudet *et al.*, 2007; Morrissey *et al.*, 2009; Pineau *et*

104　*al.*, 2012; Rus *et al.*, 2006).

105

106　To efficiently improve the performance of crops grown under suboptimal Zn conditions and

107　increase the Zn content in their edible parts it is of paramount importance to understand the

108　physiological and molecular mechanisms underlying plants tolerance to Zn deficiency.

109　Aspects of natural variation for Zn deficiency tolerance have been described for several

110　plant species, including *A. thaliana* (Cakmak *et al.*, 1998; Genc *et al.*, 2006; Ghandilyan *et*

111　*al.*, 2012; Graham *et al.*, 1992; Hacisalihoglu *et al.*, 2004; Karim *et al.*, 2012; Rengel and

112　Graham, 1996). However, to date, a detailed study on natural variation of plants tolerance to

113　Zn deficiency involving both physiological and molecular mechanisms has not yet been

114　performed. In this study we evaluated natural variation among 19 diverse *A. thaliana*

115　accessions to identify physiological and molecular traits involved in the tolerance to Zn

116　deficiency. It shows that high-throughput screening of genetic variation for Zn deficiency

117　tolerance can be simplified by focusing on the combination of changes in the ionome

118   profile; the minimum Zn concentration required for growth; and the expression level of Zn

119   deficiency responsive genes.

120 **Material and methods**

121 **Plant material and hydroponic growth**

122 A set of 19 *A. thaliana* accessions was selected based on their diverse site of origin
123 (Supplementary Table S1). Seeds were surface-sterilized with chlorine vapour and sown in
124 petri dishes on wet filter paper followed by a 4-day stratification treatment at 4 ^{o}C in the
125 dark, to promote uniform germination. Seeds were transplanted to 0.5% (w/v) agar-filled
126 tubes, of which the bottom was cut off, and placed in a modified half-strength Hoagland
127 nutrient solution for hydroponic growth (Assunção *et al.*, 2003): 3 mM KNO$_3$, 2 mM
128 Ca(NO$_3$)$_2$, 1 mM NH$_4$H$_2$PO$_4$, 0.5 mM MgSO$_4$, 1 µM KCl, 25 µM H$_3$BO$_3$, 2 µM MnSO$_4$, 0.1
129 µM CuSO$_4$, 0.1 µM (NH$_4$)$_6$Mo$_7$O$_{24}$, 20 µM Fe(Na)EDTA. The solution pH was set at 5.5
130 using KOH and buffered with 2 mM MES (2-(N-morpholino) ethanesulfonic acid). Plants
131 were grown hydroponically in two experiments performed separately. In experiment one,
132 referred to as the mild Zn deficiency experiment, we compared plants grown for 41 days
133 under control (2 µM ZnSO$_4$) and mild Zn deficiency (0.05 µM ZnSO$_4$). In experiment two,
134 referred to as the severe Zn deficiency experiment, we compared plants grown for 31 days
135 under control (2 µM ZnSO$_4$) and severe Zn deficiency (no Zn added). Plants were grown in
136 a climate-controlled chamber set at 70 % relative humidity, with a 12-h day (120 µmol
137 photons m^{-2}s^{-1}) and 20°C/15°C day/night temperatures. The hydroponic system consisted of
138 plastic trays (46 x 31 x 8 cm) holding 9 L nutrient solution, covered with a non-translucent
139 5-mm-thick plastic lid with evenly spaced holes in a 7 x 10 format holding the agar-filled
140 tubes with plantlets. The nutrient solution was refreshed once a week. Shoot fresh weight
141 (SFW) was measured in all samples during harvesting. Some samples were immediately
142 frozen in liquid nitrogen and stored at -80°C for gene expression and element concentration
143 analysis. The shoot dry weight (SDW) of these samples was calculated based on a fresh
144 weight/dry weight correction factor obtained from additional plants which were dried for 72
145 h at 60°C.For each trait, the treatment versus control relative values were determined as
146 Rel_trait = (trait Zn deficiency/trait control)*100.The ZnUI was calculated based on the
147 following formula:

148

$$ZnUI = \left(\frac{shoot\ biomass\ (mg)}{shoot\ Zn\ concentration\ (ppm)} \right)$$

149

150 **Mineral elemental analysis**

151 For each treatment, the shoot ionome profile was determined for five biological replicates of

152 each *A. thaliana* accession. Samples were first dried for 72 h at 60°C, transferred to 96-well

153 plates with tubes containing one 5-mm glass bead and homogenized for 5 minutes at 30 Hz

154 with a Qiagen96-well plate mixer mill. 2 - 4 mg of leaf tissue was transferred to Pyrex test

155 tubes (16 x 100 mm) and digested with 0.9 ml of concentrated nitric acid (Baker Instra-

156 Analyzed; Avantor Performance Materials; http://www.avantormaterials.com) for 5 h at

157 115°C. Samples were diluted to 10 mL with 18.2 MΩcmMilli-Q water. Elemental analyses

158 were performed with an inductively coupled plasma mass spectrometry, ICP-MS (Elan DRC

159 II; PerkinElmer, http://www.perkinelmer.com) for Li, B, Na, Mg, P, S, K, Ca, Mn, Fe, Co,

160 Ni, Cu, Zn, As, Se, Rb, Sr, Mo and Cd. A reference, composed of pooled samples of

161 digested leaf material, was prepared and included every 9th sample in all sets of 70 samples

162 to correct for variation between and within ICP-MS analysis runs. Seven samples from each

163 sample set were weighed and used during the iterative weight normalization process to

164 estimate the weight of the remaining 63 samples from the set (Danku *et al.*, 2013). The

165 following elements were not added to the nutrient solution: Li, Co, Ni, As, Se, Rb, Sr and

166 Cd and, except for Cd, their concentrations are not shown.

167

168 **Gene expression**

169 Gene expression analysis was performed for eight accessions with different ZnUI values

170 selected from the 19 accessions grown under mild Zn deficiency conditions. Frozen leaf

171 material from plants grown under mild and severe Zn deficiency and their respective control

172 treatments was used, in three biological replicates, each consisting of material from three

173 plants. Total RNA was extracted using the method of Onate-Sanchez and Vicente-Carbajosa

174 (2008). cDNA was synthesized from 1 µg of total RNA using the iScript cDNA synthesis kit

175 from BioRad as per the manufacturer's instructions. Following synthesis, cDNA was diluted

176 10-fold. qRT-PCRs were performed in triplicate with iQ SYBR Green Supermix (BioRad)

177 using an iQ Real Time PCR machine (BioRad). Relative transcript levels of selected genes

178 were determined by qRT-PCR. The expression of *IRT3* (At1g60960), *ZIP3* (At2g32270),

179 *ZIP4* (At1g10970), *bZIP19* (At4g35040), *CSD2* (At2g28190), and *CA2* (At5g14740) was

180 measured. The oligonucleotides used for each gene are shown in Supplementary Table S2.

181 Amplicon lengths were between 80 and 120 bp and all primer combinations had at least 95%

182 efficiency. Reaction volumes were 10 µL (5 µL SYBR green qPCR mix, 300 nmol of each

183 primer and 4 µL of cDNA template). Cycling parameters were 4 minutes at 95˚C, then 40

184 cycles of 15 seconds at 95˚C and 30 seconds at 55˚C. Gene expression values were

185 normalized to the reference genes *PEX4* (At5g25760), *SAND* (At2g28390) and *18S*. Gene

186 expression levels relative to the average of the reference genes for each accession under

187 mild and severe Zn deficiency and their respective control treatments were calculated based

188 on ΔCT values. Gene expression levels of accessions exposed to mild and severe Zn

189 deficiency relative to their respective control treatment, were calculated based on ΔΔCT

190 values (Livak and Schmittgen, 2001).

191

192 **Statistical analysis**

193 For all shoot traits and gene expression levels relative to reference gene expression, a two-

194 way ANOVA was performed to test for significant differences between treatments,

195 accessions and the interaction between treatments and accessions. A one-way ANOVA was

196 performed to test for significant differences between accessions for relative gene expression

197 values, relative change in SDW, Zn concentration and Zn content. A one-way ANOVA was

198 also performed to test for significant differences in element concentrations between the four

199 treatments used (mild and severe Zn deficiency and their respective controls). Element

200 concentration values were log10-transformed and a Benjamini-Hochberg correction of the p-

201 values was performed. When significant differences were found, a Tukey's HSD post-hoc

202 test with a significance level of 0.05 was performed. Broad-sense heritability was calculated

203 as the ratio between estimated genetic variance and total phenotypic variance (Kruijer *et al.*,

204 2015).

205

206 **Multivariate analysis and classification**

207 To predict the Zn deficiency nutritional status of accessions based on their ionomic profile,

208 various multinomial logistic regression (MLR) models were used, similar to the model

209 described by Baxter *et al.* (2008b). In all cases, 11 elements (B, Mg, P, S, K, Ca, Mn, Fe,

210 Cu, Zn and Mo) were considered of which the concentrations were reliably measured. At

211 first, element concentrations were log10-transformed and the transformed element

212 concentration values in the severe or mild Zn deficient plants are normalized to their

213 respective control treatment by subtracting the means of the control group. Thereafter, plants

214 from the control treatment of the two experiments are considered to have the same 'control'

215 status. Hence, plants can either be in a control, mild or severe Zn deficiency state. These

216 states have different probabilities, which were modelled as a linear function of the element

217 concentrations. The prediction for the state of a new plant was defined as the state with the

218 highest probability. Finally, the prediction performance of the following MLR models were

219   compared: (a) univariate MLR models, for each element; (b) a multivariate MLR model,

220   including all elements; and (c) a multivariate MLR model with all elements except Zn. The

221   multivariate models included a LASSO penalty, which is a multiple of the absolute values of

222   the regression coefficients. The level of penalization was chosen by 10-fold cross-validation.

223   The prediction performance of all models was assessed by drawing 100 times a training set

224   of 199 plants from the total of 398 plants, while the remaining 199 plants were used as a

225   validation set. Each training set was drawn in a stratified manner, respecting the number of

226   plants in the Zn sufficiency (2x100), mild (99) and severe Zn deficiency treatment (99)

227   categories. A penalized logistic regression model was fit for each training set using the R-

228   package "glmnet" (Friedman *et al.*, 2010), and used to predict the status of the 199 plants in

229   the validation set. Prediction performance was estimated by averaging the proportion of

230   correctly classified plants over the 100 validation sets.

231

232   **Results**

233   **Natural variation in Zn deficiency response for physiological and morphological traits**

234   *A. thaliana* accessions were grown hydroponically under control conditions (2 μM $ZnSO_4$)

235   and either mild (0.05 μM $ZnSO_4$) or severe Zn deficiency (no Zn added). After 31 days of

236   exposure to severe Zn deficiency, plants showed clear deficiency symptoms compared to

237   plants in the control treatment. This was primarily visible as reduced growth, leaves curling

238   and the presence of chlorotic and necrotic spots (Fig. 1A and B). After 31 days in the mild

239   Zn deficiency treatment, accessions did not show any sign of Zn deficiency, hence they were

240   grown for an additional 10 days. Even then, only a few accessions had visual symptoms of

241   Zn deficiency, mainly slight chlorosis in leaves and reduced growth (Fig. 1C and D),

242   confirming that the treatment was indeed mild.

243

244   Accessions showed significant phenotypic variation for most traits analysed which varied

245   according to the trait and Zn treatment (Supplementary Tables S3 and S5).Plants in the

246   severe Zn deficiency treatment had shoot Zn concentrations close to the reported minimum

247   required for growth, which is around 15-20 µg g^{-1} dry biomass (Marschner, 1995). Shoot Zn

248   concentrations under mild Zn deficiency were approximately two times higher than under

249   severe Zn deficiency (Fig. 2). In addition, plants in the mild Zn deficiency experiment had a

250   higher SDW than plants in the severe Zn deficiency experiment, as they were grown for 10

251   days longer. From all shoot traits only Zn concentration was significantly correlated between

252 the controls of the two Zn deficiency experiments, indicating that during the additional 10

253 days of growth between experiments other factors such as the growth rate of accessions

254 affected their shoot biomass and Zn content in a different manner (Supplementary table S8).

255 Accession Cvi-0 had to be excluded from further analysis as it had established poorly and

256 too many plants were lost from especially the mild Zn deficiency experiment.

257

258 In both Zn deficiency treatments, most accessions showed reduced SDW relative to their

259 respective control treatments, while few had a higher SDW and apparently were not affected

260 by the reduced Zn supply (Fig. 3A and B). All accessions had a reduction in shoot Zn

261 concentration of approximately 80% in both Zn deficiency treatments relative to their

262 respective controls (Fig. 3C and D). Also, accessions with high shoot Zn concentrations

263 were not always among the ones with a high shoot total Zn content, due to differences in

264 SDW. Tsu-0, Col-0 and Mt-0 were the best performing accessions under mild Zn deficiency

265 in terms of having similar Zn concentrations as the other accessions and higher SDW across

266 the Zn deficiency and control treatments. Thus, these accessions seem to be able to maintain

267 growth under Zn deficiency albeit with some reduction in shoot Zn concentration.

268 Conversely, Pa-2, C24 and Li-5:2 performed poorly under mild Zn deficiency, with a strong

269 reduction in growth in comparison to the other accessions though with a small reduction in

270 shoot Zn concentrations in both Zn deficiency treatments. These accessions appear to have a

271 poor ability to take up Zn both under control and Zn deficient conditions which results in a

272 limited capacity to grow and to maintain cellular Zn levels. Only accession Bor-4 showed an

273 increase in SDW under severe Zn deficiency relative to its control treatment even though not

274 statistically different from most of the other accessions (Fig. 3A, Supplementary table S8).

275 Bor-4 also showed an increase in SDW under mild Zn deficiency, as did Shah. However, it

276 is important to note that these two accessions were among the ones with the lowest SDW in

277 their respective control treatments, which could explain their lower sensitivity to Zn

278 deficiency.

279

280 **Accessions with contrasting tolerance to Zn deficiency show differences in the**

281 **expression of Zn deficiency responsive genes**

282 The Zn Usage Index (ZnUI) was used to determine the amount of biomass produced per unit

283 of tissue Zn concentration (Fig. 4). In accordance with the results previously shown for

284 SDW and Zn concentration the accessions Mt-0 and Tsu-0 had the highest ZnUI values for

285   both Zn deficiency treatments andC24 and Pa-2 had the lowest values. Even though only in

286   the mild Zn deficiency treatment, these accessions had significantly higher or lower ZnUI

287   values when compared to the other accessions (Supplementary Table S6). Eight accessions

288   with different ZnUI values in the mild Zn deficiency treatment were then selected to

289   examine if natural variation for Zn deficiency tolerance is reflected at the gene expression

290   level. Mild Zn deficiency was favoured over the severe treatment as the variation between

291   accessions for SDW was larger in the mild treatment. In addition, mild Zn deficiencies are

292   more likely to be found in nature. The accessions Tsu-0 and Col-0 had high ZnIU values,

293   accessions Ge-0, Bur-0 and Can-0 were intermediate and Pa-2, C24 and Per-1 had low ZnUI

294   values. Accessions with higher ZnUI values were considered to be more tolerant to Zn

295   deficiency (Fig. 4).

296

297   The expression of six genes involved in the plant Zn deficiency and oxidative stress

298   response was determined in Zn deficiency tolerant and sensitive accessions (Fig. 5;

299   Supplementary Fig. S1). *bZIP19* encodes one of the two redundant bZIP transcription

300   factors which control the Zn deficiency response in *A. thaliana*. We also looked at the

301   expression of the *IRT3*, *ZIP4* and *ZIP3* transcriptional targets genes of bZIP19, all encoding

302   ZIP-like Zn transport proteins, strongly induced following Zn deficiency (Assunção *et al.*,

303   2010). The expression of the *CSD2* gene, encoding a Cu/Zn superoxide dismutase (SOD)

304   which needs Zn as a structural component to function (Sharma *et al.*, 2004), and the *CA2*

305   gene, encoding a carbonic anhydrase (CA) requiring Zn as co-factor, were also determined.

306   *CSD2* is needed for detoxification of superoxide radicals, while *CA2* facilitates the diffusion

307   of CO_2 through the liquid phase of the cell to the chloroplast, important for photosynthesis

308   (Li *et al.*, 2013; Randall and Bouma, 1973). Both *CSD2* and *CA2* are expected to decrease in

309   expression under Zn deficiency exposure due to the reduced concentration of Zn in the cells

310   (Ibarra-Laclette *et al.*, 2013).

311

312   There was a significant effect of both the mild and severe Zn deficiency treatments on the

313   expression level of most studied genes. The exceptions were *bZIP19* and *CA2* in the severe

314   Zn deficiency treatment (Supplementary Table S3). The Zn deficiency responsive genes

315   *IRT3*, *ZIP4* and *ZIP3* were up-regulated in all accessions under both Zn deficiency

316   treatments, confirming that the plants sensed Zn deficiency (Fig. 5; Supplementary Fig. S1).

317   Especially *ZIP4* and *IRT3* were in general higher expressed in the more Zn deficiency

318   tolerant accessions than in the more Zn deficiency sensitive accessions, with especially Tsu-

319   0 showing strong induction of these genes under severe Zn deficiency. The expression of

320   *ZIP3*, which is predominantly expressed in roots (van de Mortel *et al.*, 2006), is the least

321   prominent of the three Zn transporter genes in shoots. The expression levels of *CSD2* and

322   *CA2* were generally low and variable in both Zn deficiency treatments, but especially under

323   mild Zn deficiency, these genes are down-regulated. The Zn deficiency tolerant accessions

324   Ge-0 and Bur-0 had the highest induction of the *CA2* and *CSD2* genes under severe Zn

325   deficiency (Fig. 5). Significant accession by treatment interaction was found for all genes

326   tested, except for *bZIP19*, in at least one of the Zn deficiency experiments (Supplementary

327   Table S3), indicating that gene expression differences between accessions response to Zn

328   deficiency are pronounced.

329

330   To further understand the relation between the expression levels of Zn deficiency responsive

331   genes and Zn deficiency tolerance traits a correlation analysis was performed. Under severe

332   Zn deficiency we found a significant positive correlation between the expression levels of

333   *IRT3* and *CSD2* with ZnUI and of *ZIP4* with shoot fresh weight (SFW) (Supplementary

334   Table S9).

335

336   **Zn deficiency affects the shoot ionomic profile of *A. thaliana* accessions**

337   The shoot ionome of the 19 *A. thaliana* accessions was then determined. Box plots of the

338   combined results per element showed a substantial variation between treatments for almost

339   all the elements measured (Fig.6, Supplementary Table S7). Significant differences between

340   treatments were observed for Zn, Mg, Mo, Cu and Cd concentrations in both the mild and

341   severe Zn deficiency experiments. B, Na and Ca concentrations were significantly different

342   between treatments only in the mild Zn deficiency experiment and Mn and Fe

343   concentrations only in the severe Zn deficiency experiment. When comparing Zn

344   concentrations across the four treatments, there was a significant difference between severe

345   and mild deficiency but not between their respective control treatments.

346

347   Broad sense heritability (H^2) values were calculated to estimate the genetic contribution to

348   the observed phenotypic variation (Table 1). H^2 values were generally higher in the mild

349   compared to the severe Zn deficiency experiment and in plants exposed to Zn deficiency in

350   comparison to their control treatments. The heritability for ZnUI was highest in the mild Zn

351   deficiency treatment, suggesting that under those conditions a large part of the observed

352 variation is due to genetic differences between accessions. Fe concentration had the lowest

353 heritability in both control treatments, whereas Mo concentration had the highest heritability

354 across the treatments. Even though the Zn concentrations of plants grown under severe Zn

355 deficiency were very low, there was substantial heritability for both Zn concentration and Zn

356 content, with values of 0.49 and 0.41 respectively, indicating that the minimal Zn

357 concentration/content levels are subject to genetic variation.

358

359 **Classification of the plant Zn deficiency state using multinomial logistic regression**

360 The univariate model (i.e. with a single element as the only predictor) performed poorly as a

361 predictor of plant nutritional status, for most elements, and often mistakenly identified plants

362 under Zn deficiency as being control (Table 2). As expected, only the Zn concentration was

363 able to separate the three classes very well, with prediction accuracies ranging from 0.92 for

364 the plants under severe Zn deficiency to 0.99 for the control plants. Cu also had a good

365 prediction performance for severe Zn deficiency, while Ca was the only element (apart from

366 Zn) that identified a substantial number of the plants under mild Zn deficiency (Table 2).

367 Mg, Mn, Fe, and Mo performed only marginally well, having some ability to identify plants

368 under severe and mild Zn deficiency. For the other elements (B, P, S, and K) the univariate

369 model performed no better than a naïve classifier that would always predict control

370 conditions.

371

372 The penalized multivariate model, fitted on all elements except Zn, performed much better

373 than the univariate model: the predicted accuracy for mild (0.6596) and severe Zn deficiency

374 (0.7750) was far higher than with any element alone (except Zn), and the accuracy for the

375 control treatments (0.8738) was still very good. When this model was fitted on all elements

376 (including Zn), it performed similarly to the univariate model fitted with Zn alone, the latter

377 having a higher accuracy for the controls and mild Zn deficiency treatments and less for the

378 severe Zn deficient plants (Table 2).

379

380 **Discussion**

381 The natural variation in the response of *A. thaliana* to two levels of Zn deficiency was

382 examined, with a focus on physiological and molecular traits. Analysis of genetic variation

383 indicated that: (1) accessions vary for the minimum Zn requirement for growth; (2) tolerance

384 to Zn deficiency seems to be related to an increased expression of genes encoding Zn

385   transmembrane transporter proteins (*ZIP4* and *IRT3*); (3) Zn deficiency results in changes in

386   the plant ionome which can be used as biomarker to predict the plant's physiological

387   condition.

388

389   **Natural variation of growth and Zn concentration in response to severe and mild Zn**
390   **deficiencies**

391   The tested *A. thaliana* accessions showed substantial diversity for all traits studied in both

392   Zn deficiency experiments (Figs. 1 – 3). Extreme accessions were identified for all traits,

393   confirming the existing large natural variation in *A. thaliana* response to Zn deficiency

394   conditions and endorsing this panel of representative accessions as a valuable resource to

395   study the plant response and tolerance to Zn deficiency. The response of *A. thaliana* to Zn

396   deficiency has previously been examined in the Ler x Cvi RIL population, in which large

397   variation in SDW and Zn concentration was observed (Ghandilyan *et al.*, 2012).

398

399   The mild Zn deficiency treatment is more suitable to reveal genetic variation underlying

400   plants response to Zn nutrition with higher heritability for most traits in comparison to the

401   other treatments (Table 1).The disadvantage of using this mild treatment was that plants

402   were 10 days older than in the severe Zn deficiency treatment, resulting in the initiation of

403   flowering in some accessions. Such change in development could include remobilization of

404   minerals from older to younger organs (e.g. from rosette leaves to developing fruits),

405   however, Waters and Grusak (2008) previously showed that the contribution of

406   remobilization is probably less than 10% of the seed mineral content, so we considered this

407   not much of a disturbing factor. In addition, this treatment seems better in representing Zn

408   deficient conditions likely to be encountered by *A. thaliana* in nature, with an average Zn

409   concentration in leaves of 26 ppm in comparison to 18 ppm in the severe Zn deficiency

410   treatment. To support this, Ghandilyan et al. (2012) observed leaf average Zn concentration

411   of 40 ppm when using a Zn deficient and nutrient-poor soil originating from Eskisehir,

412   Central Anatolia in Turkey to grow the *A. thaliana* Ler x Cvi RIL population. Furthermore,

413   the harshness of the severe Zn deficiency treatment seems to be beyond the genetic capacity

414   of most accessions to tolerate based on the extensive chlorosis displayed by nearly all

415   accessions in this treatment and their very low average leaf Zn concentration, which was

416   within or below the minimum Zn concentration range of 15-20 ppm required for growth as

417   suggested by Marschner (1995).

418

419 Heritabilities of most traits were higher in the severe and mild Zn deficiency treatments than

420 in their respective controls, further supporting the observed large genetic variation for all

421 traits in response to the Zn deficiency treatments. Contrary to these observations,

422 Ghandilyan et al. (2012) reported lower heritability values for shoot biomass and most

423 element concentrations in *A. thaliana* plants grown in Zn deficient soil compared to control

424 conditions. Yet, other studies show that heritabilities for the same trait can change according

425 to the growth conditions (Baxter *et al.*, 2012; Ghandilyan *et al.*, 2009; Richard *et al.*, 2011),

426 hence the importance of taking heritability into account when to select growth conditions

427 most amenable to detect genetic variation for a specific trait.

428

429 The control treatments of the two Zn deficiency experiments were significantly correlated

430 with respect to the Zn concentration, but not for SDW and Zn content (Supplementary Table

431 S8). This is probably due to differences in growth rate between the *A. thaliana* accessions

432 during the ten additional days of growth in the mild Zn deficiency experiment. Previous

433 studies have shown that growth rate is highly variable among plants; being affected by both

434 internal and external factors such as developmental processes and environmental conditions

435 (El-Lithy *et al.*, 2004; Zhang *et al.*, 2012). Differences in growth rate between accessions in

436 the mild and severe Zn deficiency experiments are likely caused by differences in the

437 initiation of flowering. Most accessions in the control treatment of the mild Zn deficiency

438 experiment were flowering or bolting at the harvesting day; while only three accessions, of

439 the control treatment, were flowering in the sever Zn deficiency experiment at the harvesting

440 day, which was 10 days earlier than in the mild Zn deficiency experiment (Fig. 1A and C).

441

442 **Physiological and molecular mechanisms of Zn deficiency tolerance in *A. thaliana***

443 *A. thaliana* accessions showed a larger variation for relative change in SDW than in Zn

444 concentration under both Zn deficiency treatments (Fig. 3). This indicates the presence of

445 genetic variation for their minimum Zn requirement and for the ability to tolerate low Zn

446 concentrations. This is not unique for *A. thaliana* though. Also for barley, bread and durum

447 wheat, common bean and rice, different genotypes are reported to have similar shoot Zn

448 concentrations with different levels of Zn deficiency tolerance (Cakmak *et al.*, 1998; Genc *et*

449 *al.*, 2002; Hacisalihoglu *et al.*, 2003; Rengel, 2001; Sadeghzadeh *et al.*, 2009; Wissuwa *et*

450 *al.*, 2006). Further indications that *A. thaliana* accessions vary for the minimum Zn

451 requirement is shown by a few accessions with slightly higher SDW in the Zn deficient

452 treatment relative to its control.

453

454 The ability to enhance the root Zn uptake and the root to shoot Zn transport are among the

455 proposed mechanisms underlying tolerance to Zn deficiency (Broadley *et al.*, 2007; Rengel,

456 2001), but the Zn deficiency signal may come from shoots. Indeed, accessions considered

457 tolerant to Zn deficiency had a higher expression of Zn deficiency responsive genes *ZIP4*

458 and *IRT3* in shoots (Fig. 5). These genes, encoding Zn transmembrane transporters (Grotz *et*

459 *al.*, 1998) are transcriptionally responsive to Zn deficiency and mainly expressed in roots,

460 but are also expressed in shoot tissue in response to low Zn, suggesting a role in both Zn

461 uptake and distribution (Jain *et al.*, 2013; Lin *et al.*, 2009). Our findings indicate that higher

462 tolerance to Zn deficiency may be the result of an increased, or more efficient, shoot Zn re-

463 allocation capacity, and that natural variation for it may reflect variation in the expression of

464 these and other Zn transport genes in *A. thaliana.*

465

466 Previous studies have shown that tolerance to Zn deficiency can also be affected by the plant

467 capacity to deal with the high levels of ROS produced under low Zn conditions (Rengel,

468 2001; Sinclair and Kramer, 2012). In this study a relationship was found between the

469 expression of *CA2* and ZnUI (Supplementary Table S9). Further studies examining the

470 ability of plants to tolerate ROS under Zn deficiency and other mechanisms not included in

471 this study, but thought to contribute to tolerance to Zn stress, will be useful for a more

472 complete understanding of the mechanisms involved in plant tolerance to Zn deficiency

473 (Cakmak *et al.*, 1996; Chen *et al.*, 2009; Gao *et al.*, 2005; Genc *et al.*, 2006; Hoffland *et al.*,

474 2006; Impa *et al.*, 2013a; Impa *et al.*, 2013b; Rengel, 2001; Wissuwa *et al.*, 2006). This

475 should include examining the ability of plants to increase the bioavailability of Zn^{2+} ions in

476 the soil; to improve the root system architecture to scavenge larger soil volumes; and a more

477 efficient utilization, compartmentalization and remobilization of Zn.

478

479 **Model to predict Zn deficiency status based on other elements concentration**

480 Exposing *A. thaliana* plants to different levels of Zn deficiency also affects the homeostasis

481 of other elements, which made it possible to develop a MLR model able to predict the Zn

482 deficiency status of a plant based on changes in other elements (Table 2). This approach is

483 analogous to the model used by Baxter et al. (2008b) to predict the physiological status of *A.*

484   *thaliana* plants exposed to Fe or P deficiency. Contrary to the MLR model developed for Zn

485   deficiency, Baxter et al. (2008b) found that changes in Fe concentration alone had no power

486   to detect Fe-deficiency and detection was totally dependent on analysis of other elements.

487   This difference could be caused by the two different Zn deficiency treatments used in this

488   study which incorporated more data points to the model, while only one deficiency

489   treatment was used in the Fe deficiency study (Baxter *et al.*, 2008b), but it could also be

490   because in that study the Fe concentrations in leaves of plants grown under low and normal

491   Fe did not differ, while in our study, the shoot Zn concentrations of plants grown under

492   severe and mild Zn deficiency were significantly different, next to having extremely low Zn

493   concentrations in comparison to control conditions. The Zn concentration thus appears to be

494   much less tightly controlled in *A. thaliana* than for Fe. In that respect, Zn corresponds more

495   with P, for which their model did incorporate P concentration (Baxter *et al.*, 2008b). This

496   analysis provides strong evidence that elements do not behave independently upon Zn

497   deficiency and it shows the power of using a combination of elements as a phenotype of

498   interest to detect a plant's nutritional status. The use of these traits to evaluate crops

499   tolerance to Zn deficiency has the potential to simplify and shorten the process of

500   identification of Zn deficiency tolerant varieties. However, further studies confirming the

501   application of comparable biomarkers as found for *A. thaliana* in the evaluation of Zn

502   deficiency tolerance in crops will be needed.

503

504   **Conclusion**

505   This study demonstrates that several physiological and molecular mechanisms underlie

506   differences in Zn deficiency tolerance in *A. thaliana*. These include the minimum Zn

507   concentration required for growth and the ability to take up and translocate Zn by inducing

508   the expression of Zn deficiency responsive genes. ZnUI, the reduction in SDW and the

509   expression level of Zn deficiency responsive genes such as *ZIP4* and *IRT3* are useful proxies

510   to evaluate plant tolerance to Zn deficiency in future studies. A mild Zn deficiency condition

511   is more amenable for genetic studies than a severe stress, with higher heritability values for

512   most studied traits and providing a more natural condition, at least for *A. thaliana*. Finally,

513   the shoot ionome profile is a useful predictor of the plant Zn deficiency status. Changes in

514   Zn concentration alone or in combination with other elements have an excellent capacity to

515   detect physiological plant Zn deficiency in the absence of other visible symptoms. While we

516   have shown this now for *A. thaliana*, a model plant species, the application of our findings

517 will be in crops. Although it will be more difficult to establish this, we expect our research

518 to inspire others to test the applicability of the described biomarkers in crops, under

519 experimental and field conditions.

520

525

Supplementary data file

Table S1: Detailed information of the *A. thaliana* accessions used in this study.

Table S2: Oligonucleotide PCR primer sequences.

Table S3: Two-way ANOVA of shoot dry weight, Zn concentration, total Zn content and gene expression of *A. thaliana* accessions exposed to control and Zn deficiency.

Table S4: Tukey pairwise multiple comparison between accessions for shoot dry weight, shoot Zn concentration, shoot Zn content and gene expression levels (relative to reference genes) in the severe and mild Zn deficiency experiments. Similar letters represent non-significant differences ($p > 0.05$) between accessions.

Table S5: One-way ANOVA of differences between *A. thaliana* accessions exposed to control and Zn deficiency for relative changes in shoot dry weight, Zn concentration, total Zn content, Zn usage index and gene expression.

Table S6: Tukey pairwise multiple comparison between accessions for relative change in shoot dry weight, shoot Zn concentration, shoot Zn content and gene expression values in the severe and mild Zn deficiency treatments relative to their respective control treatments. Similar letters represent non-significant differences ($p > 0.05$) between accessions.

Table S7: One-way ANOVA of the log10-transformed shoot element concentrations to determine significant differences between treatments.

Table S8: Pearson correlation coefficients for the comparison of shoot traits measured in the nineteen *A. thaliana* accessions grown under severe, mild Zn deficiency and their respective control treatments.

Table S9: Pearson correlation coefficients for the comparison of gene expression with shoot traits measured in eight *A. thaliana* accessions grown under severe or mild Zn deficiency and their respective control treatments.

Figure S1: Relative gene expression in leaves of eight *A. thaliana* accessions grown under severe and mild Zn deficiency, compared to their respective control treatments.

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Tables

Table 1: *Broad sense heritability (H^2) values for the traits measured in A. thaliana accessions grown under severe and mild Zn deficiency and their respective Zn sufficiency conditions.*

traits	mild		severe	
	control	Zn deficiency	control	Zn deficiency
SFW	0.44	0.62	0.41	0.66
SDW	0.68	0.78	0.40	0.48
ZnUI	0.65	0.81	0.40	0.57
SZnC	0.60	0.62	0.50	0.41
[Zn]	0.63	0.65	0.60	0.49
[Mn]	0.68	0.69	0.60	0.64
[Fe]	0.36	0.53	0.32	0.83
[Cu]	0.50	0.75	0.59	0.38
[Mo]	0.91	0.97	0.86	0.75
[Cd]	0.59	0.73	0.49	0.76
[B]	0.67	0.51	0.63	0.78
[Na]	0.48	0.37	0.55	0.60
[Mg]	0.59	0.71	0.55	0.46
[P]	0.62	0.71	0.44	0.72
[S]	0.45	0.59	0.53	0.58
[K]	0.51	0.65	0.46	0.48
[Ca]	0.72	0.69	0.42	0.52

SFW – shoot fresh weight (g); SDW - shoot dry weight (mg); ZnUI - Zn Usage Index; SZnC - shoot total Zn content (µg); and [X] - mineral element concentrations ($µg.g^{-1}$ dry weight).

Table 2: *Estimated prediction performance values for elements used in the logistic regression model to predict plant nutritional Zn status.*

univariate models	control	Zn deficiency		average
		severe	mild	
B	0.914	0.002	0.0837	0.4804
Mg	0.901	0.364	0	0.5442
P	1	0	0	0.5025
S	0.993	0	0.0061	0.5005
K	0.991	0	0.0102	0.5005
Ca	0.949	0	0.2673	0.5427
Mn	0.882	0.412	0	0.5467
Fe	0.977	0.278	0	0.5608
Cu	0.877	0.716	0.0286	0.6276
Zn	0.996	0.92	0.9857	0.9744
Mo	0.911	0.204	0	0.509
multivariate models				
All elements except Zn	0.8738	0.7750	0.6596	0.7962
All elements	0.9921	0.9332	0.9549	0.9681

Figure legends

Figure 1: *Comparison of A. thaliana accessions grown under control and severe or mild Zn deficient conditions. Representative examples of A. thaliana accessions grown in hydroponic medium under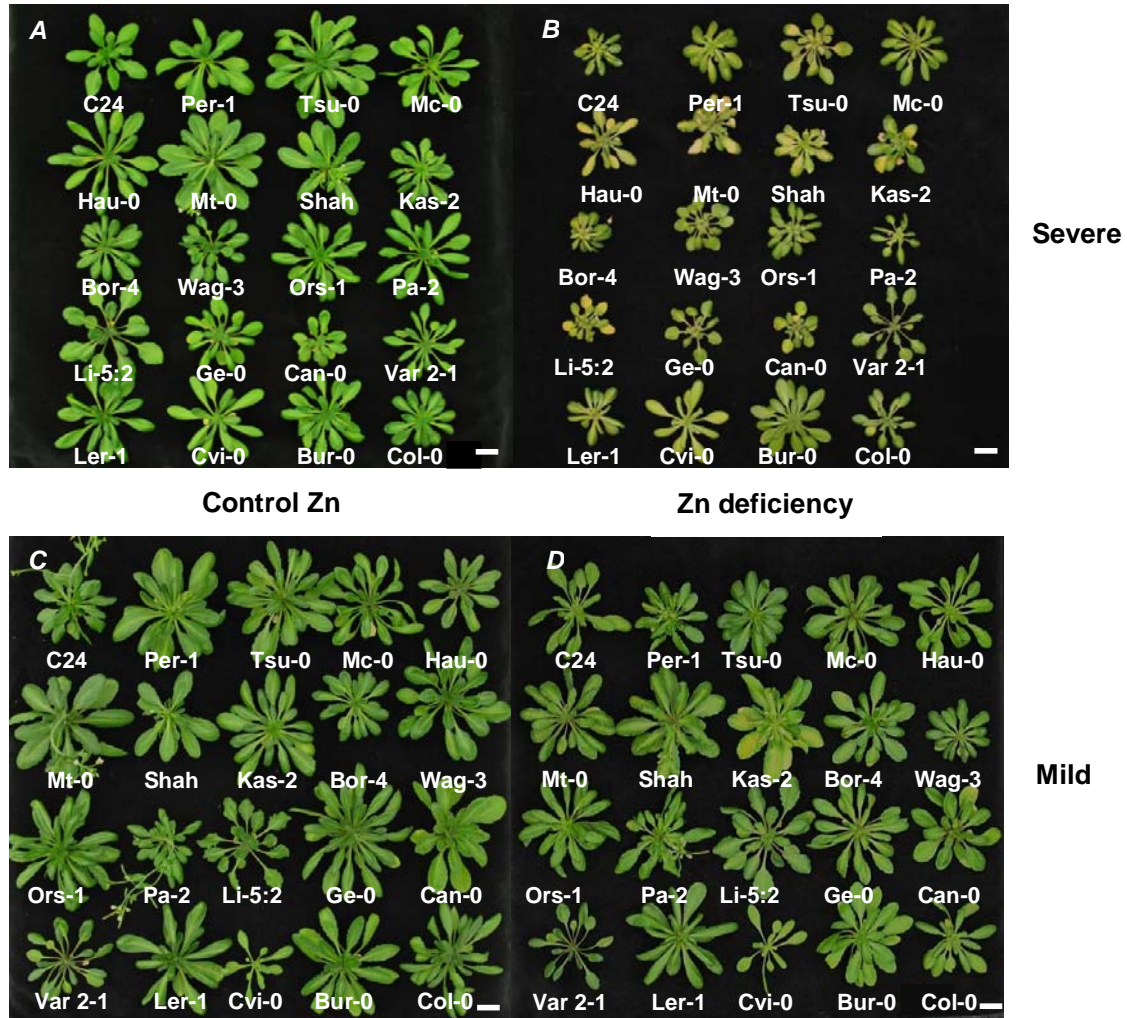 Zn sufficient control conditions (2 µM ZnSO₄)(A and C) or severe (no Zn added) (B) and mild Zn deficiency (0.05 µM ZnSO₄)(D). Plants in A and B are grown for 31 days, plants in C and D are grown for 41 days. Accessions from left to right in rows from top to bottom: C24, Per-1, Tsu-0, Mc-0, Hau-0, Mt-0, Shah, Kas-2, Bor-4, Wag-3, Ors-1, Pa-2, Li-5:2, Ge-0, Can-0, Var 2-1, Ler-1, Cvi-0, Bur-0 and Col-0. Bars indicate 2 cm.*
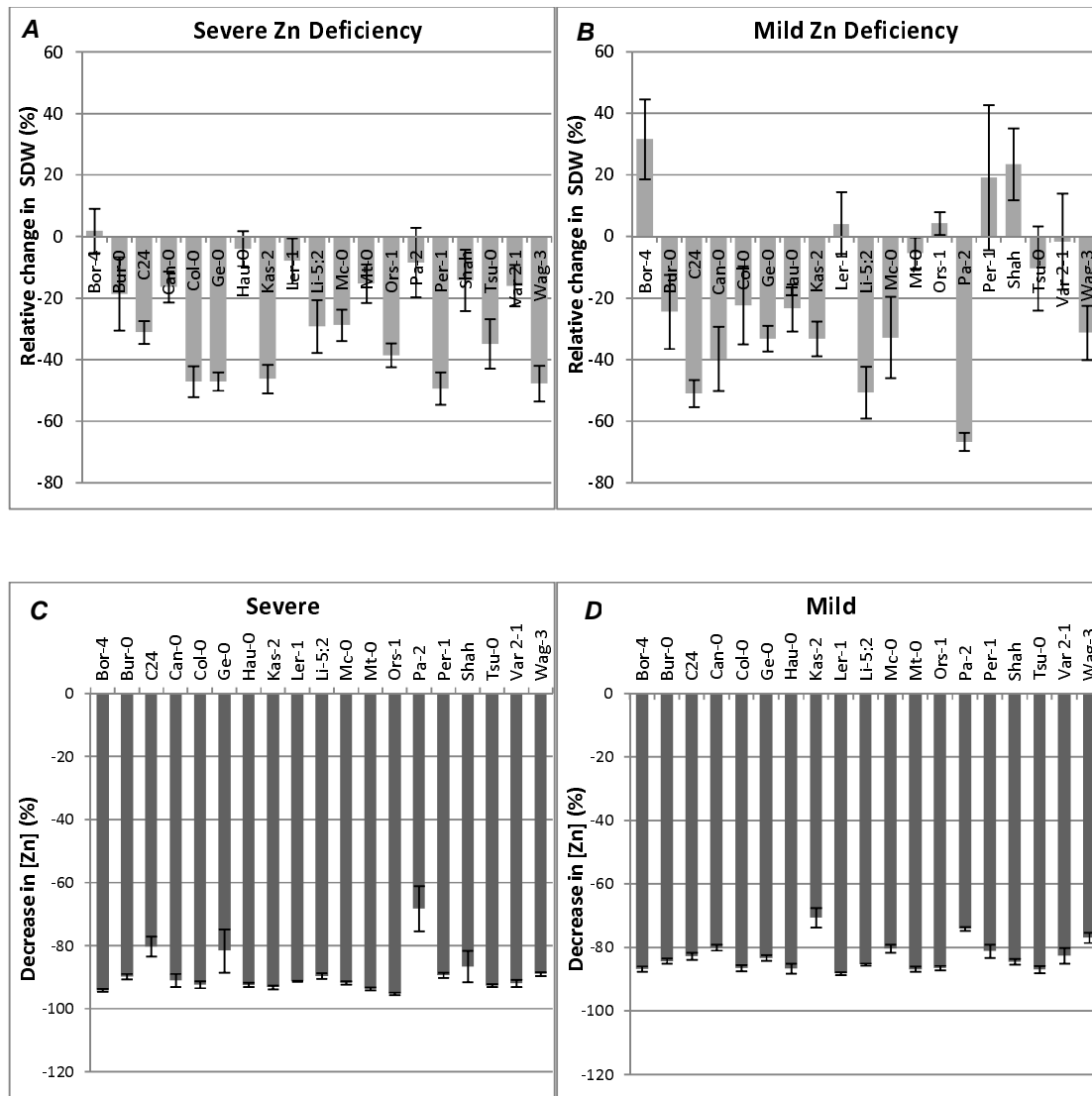
Figure 2: *Relations between shoot dry weight and Zn concentration of 19 A. thaliana accessions grown under Zn deficiency.*
Shoot dry weight (SDW) is expressed in mg and Zn concentration in $µg.g^{-1}$ dry weight. See Supplementary Table S1 for the list of accessions. Data for plants grown under severe Zn deficiency (no Zn added; A) or mild Zn deficiency (0.05 µM ZnSO₄; B) are indicated with grey dots and plants grown under their respective control conditions (2 µM ZnSO₄) with black dots. Plants used for A grew for 31 days, plants used for B grew for 41 days.

Figure 3: *Relative changes in shoot dry weight and Zn concentration of 19 A. thaliana accessions grown under severe (A and C) and mild (B and D) Zn deficiency, compared to their respective control treatments.*
Relative changes are expressed as percentages of the control (%). One-way ANOVA of these data and pairwise comparisons between accessions are provided in Supplementary Tables S5 and S6. See Supplementary Table S1 for the list of accessions. Plants were grown in hydroponic medium under Zn sufficient control conditions (2 µM ZnSO₄) and severe (no Zn added) or mild Zn deficiency (0.05 µM ZnSO₄). Plants in A and C are grown for 31 days, plants in B and D are grown for 41 days.

Figure 4: *Shoot Zn Usage Index (ZnUI) of A. thaliana accessions grown in severe (A) and mild (C) Zn deficiency and their respective control treatments (B and D). The letters above each bar indicates if the accession was already bolting (B) or flowering (F) when harvested. The ZnUI is defined as shoot biomass (in mg)/shoot Zn concentration (in ppm). Plants were grown in hydroponic medium under Zn sufficient control conditions (2 µM ZnSO₄) and severe*

(no Zn added) or mild Zn deficiency (0.05 μM ZnSO$_4$). Plants in A and B are grown for 31 days, plants in C and D are grown for 41 days. One-way ANOVA of these data and pairwise comparisons between accessions are provided in Supplementary Tables S3 and S4.

Figure 5: Normalized gene expression levels of bZIP19, IRT3, ZIP3, ZIP4, CSD2 and CA2 in rosette leaves of eight A. thaliana accessions under Zn deficiency (Zn-) and control treatments (Zn+ control) in the severe (left) and mild Zn deficiency experiments (right). Accessions are ranked from left to right according to decreasing Zn Usage Index values under mild Zn deficiency (see Fig. 4). Plants were grown in hydroponic medium under Zn sufficient control conditions (2 μM ZnSO4) and severe (no Zn added) or mild Zn deficiency (0.05 μM ZnSO4), for respectively 31 or 41 days. Error bars represent standard errors of the mean, one-way ANOVA and pairwise comparisons between accessions are provided in Supplementary Tables S3 and 4.

Figure 6: Box plots comparing mineral element concentrations in shoots of 19 A. thaliana accessions grown under severe and mild Zn deficiency and their respective control treatments.

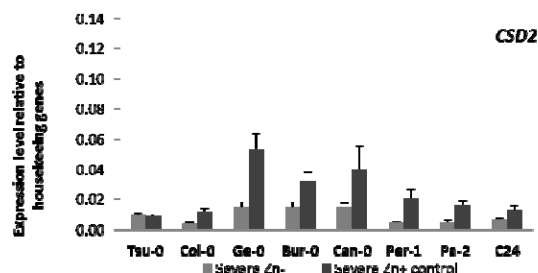*Plants were grown in hydroponic medium under Zn sufficient control conditions (2 μM ZnSO4) and severe (no Zn added) or mild Zn deficiency (0.05 μM ZnSO4). Plants in the severe Zn deficiency condition were grown for 31 days, plants in the mild Zn deficiency condition were grown for 41 days. For each concentration the box represents the interquartile range (IQR), the bisecting line represents the median, the whiskers indicate 1.5 times the IQR and the open circles indicate outlier points. Lower case letters denote statistically different groups when comparing the four treatments using a one-way ANOVA with groupings by Tukey's HSD test with a significance level of P≤0.05. The results of this ANOVA are shown in Supplementary Table S7.*

Figure 1: *Comparison of A. thaliana accessions grown under control and severe or mild Zn deficient conditions.*

Representative examples of A. thaliana accessions grown in hydroponic medium under Zn sufficient control conditions (2 µM ZnSO$_4$)(A and C) or severe (no Zn added) (B) and mild Zn deficiency (0.05 µM ZnSO$_4$)(D). Plants in A and B are grown for 31 days, plants in C and D are grown for 41 days. Accessions from left to right in rows from top to bottom: C24, Per-1, Tsu-0, Mc-0, Hau-0, Mt-0, Shah, Kas-2, Bor-4, Wag-3, Ors-1, Pa-2, Li-5:2, Ge-0, Can-0, Var 2-1, Ler-1, Cvi-0, Bur-0 and Col-0. Bars indicate 2 cm.

A Severe Zn deficiency

B Mild Zn deficiency

Figure 2: *Relations between shoot dry weight and Zn concentration of 19 A. thaliana accessions grown under Zn deficiency.*

Shoot dry weight (SDW) is expressed in mg and Zn concentration in $\mu g.g^{-1}$ dry weight. See Supplementary Table S1 for the list of accessions. Data for plants grown under severe Zn deficiency (no Zn added; A) or mild Zn deficiency (0.05 μM ZnSO$_4$; B) are indicated with grey dots and plants grown under their respective control conditions (2 μM ZnSO$_4$) with black dots. Plants used for A grew for 31 days, plants used for B grew for 41 days.

Figure 3: *Relative changes in shoot dry weight and Zn concentration of 19 A. thaliana accessions grown under severe (A and C) and mild (B and D) Zn deficiency, compared to their respective control treatments.*
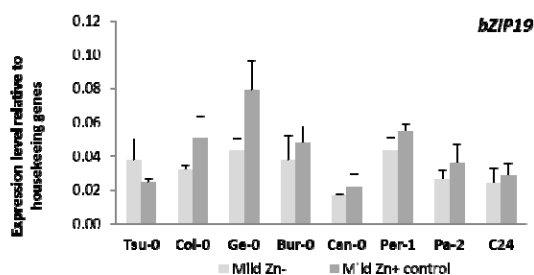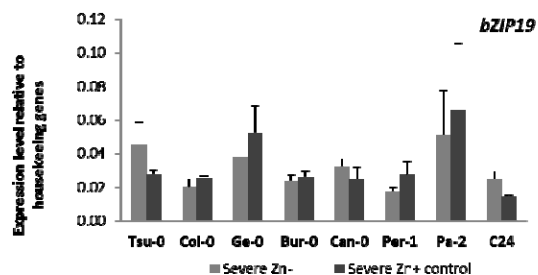
Relative changes are expressed as percentages of the control (%). One-way ANOVA of these data and pairwise comparisons between accessions are provided in Supplementary Tables S5 and 6. See Supplementary Table S1 for the list of accessions. Plants were grown in hydroponic medium under Zn sufficient control conditions (2 μM ZnSO₄) and severe (no Zn added) or mild Zn deficiency (0.05 μM ZnSO₄). Plants in A and C are grown for 31 days, plants in B and D are grown for 41 days.

Figure 4: Shoot Zn Usage Index (ZnUI) of A. thaliana accessions grown in severe(A) and mild (C) Zn deficiency and their respective control treatments (B and D). The letters above each bar indicate if the accession was already bolting (B) or flowering (F) when harvested. The ZnUI is defined as shoot biomass (in mg)/shoot Zn concentration (in ppm). Plants were grown in hydroponic medium under Zn sufficient control conditions (2 μM ZnSO$_4$) and severe (no Zn added) or mild Zn deficiency (0.05 μM ZnSO$_4$). Plants in A and B are grown for 31 days, plants in C and D are grown for 41 days. One-way ANOVA of these data and pairwise comparisons between accessions are provided in Supplementary Tables S3 and 4.

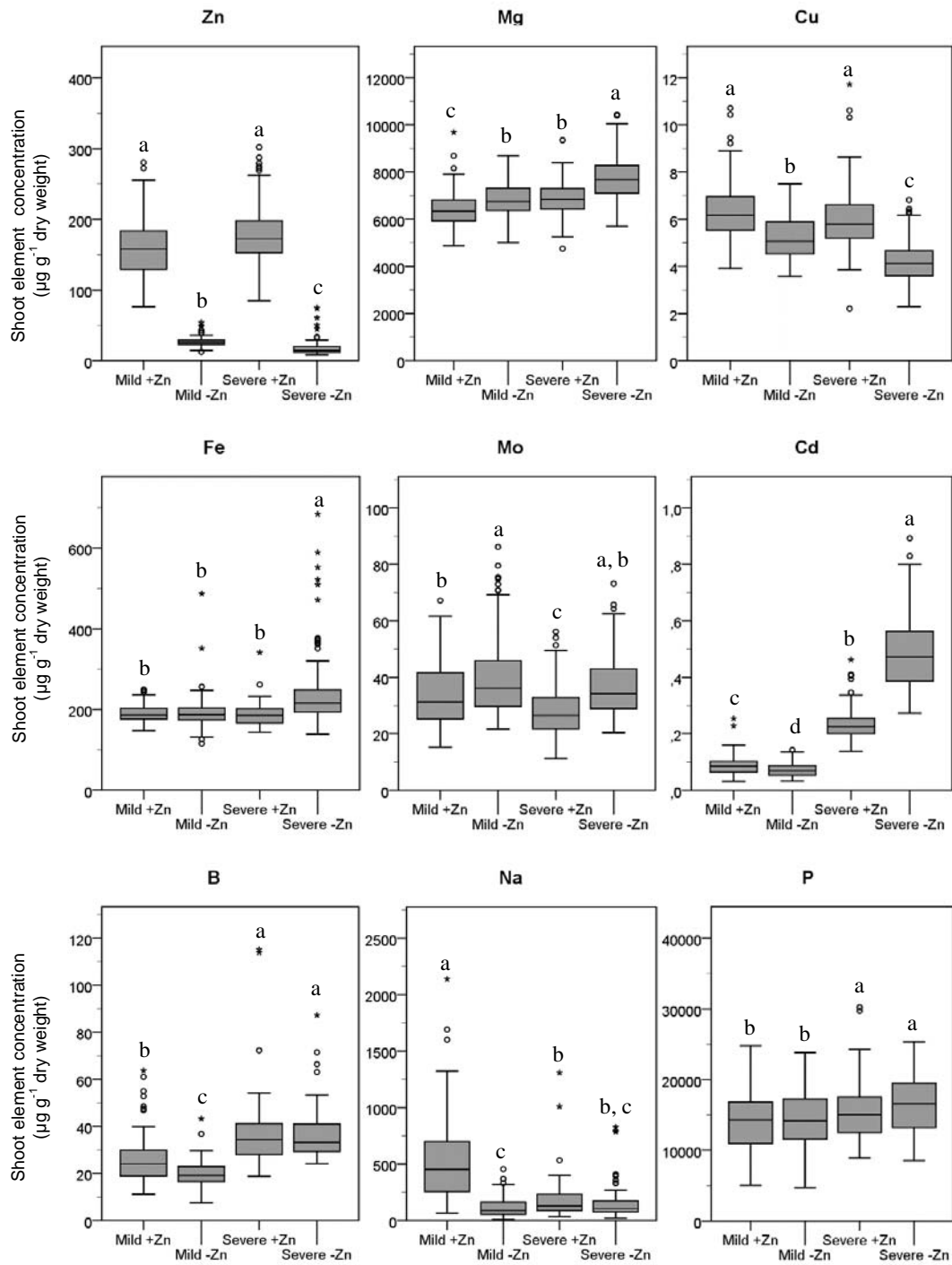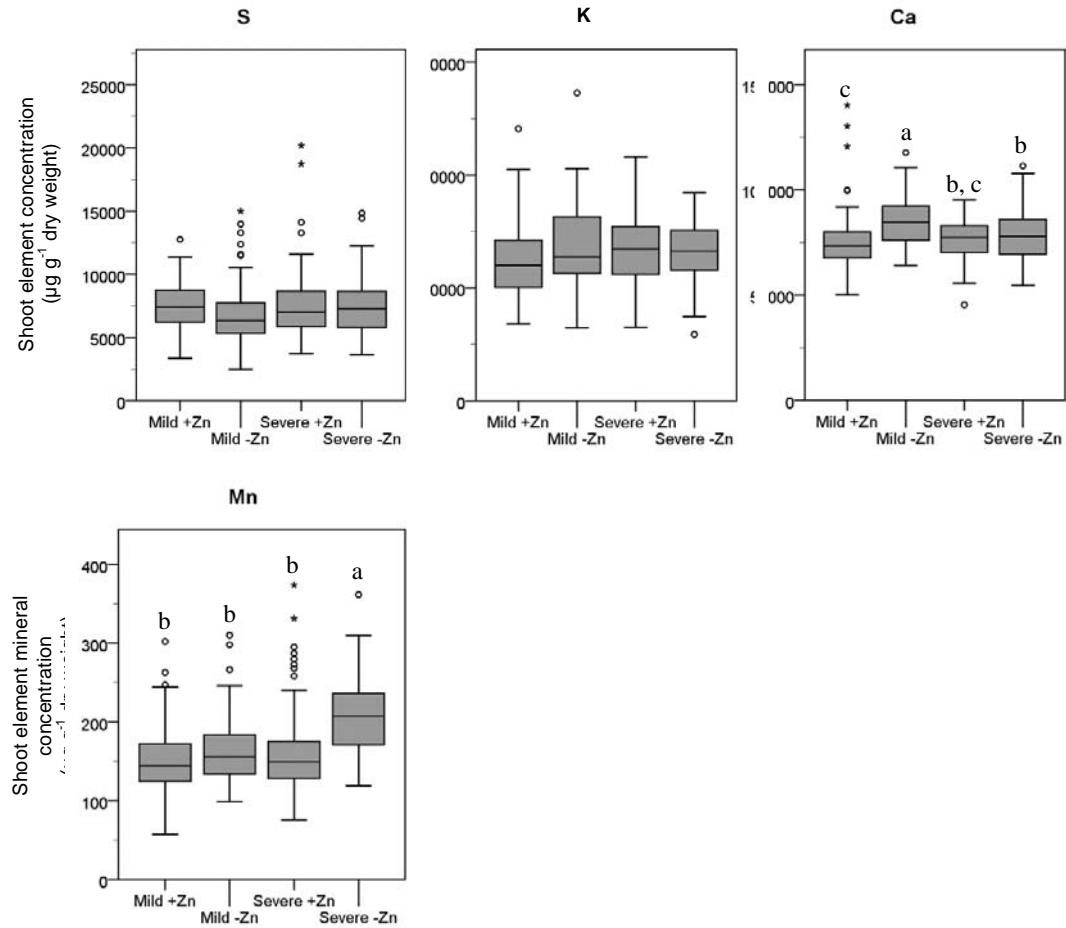# Natural variation for Arabidopsis Zn deficiency tolerance

Figure 5: Normalized gene expression levels of bZIP19, IRT3, ZIP3, ZIP4, CSD2 and CA2 in rosette leaves of eight A. thaliana accessions under Zn deficiency (Zn-) and control treatments (Zn+ control) in the severe (left) and mild Zn deficiency experiments (right). Accessions are ranked from left to right according to decreasing Zn Usage Index values under mild Zn deficiency (see Fig. 4). Plants were grown in hydroponic medium under Zn sufficient control conditions (2 μM ZnSO4) and severe (no Zn added) or mild Zn deficiency (0.05 μM ZnSO4), for respectively 31 or 41 days. Error bars represent standard errors of the mean, one-way ANOVA and pairwise comparisons between accessions are provided in Supplementary Tables S3 and 4.

Figure 6: *Box plots comparing mineral element concentrations in shoots of 19 A. thaliana accessions grown under severe and mild Zn deficiency and their respective control treatments.*

Plants were grown in hydroponic medium under Zn sufficient control conditions (2 µM ZnSO4) and severe (no Zn added) or mild Zn deficiency (0.05 µM ZnSO4). Plants in the severe Zn deficiency condition were grown for 31 days, plants in the mild Zn deficiency condition were grown for 41 days. For each concentration the box represents the interquartile range (IQR), the bisecting line represents the median, the whiskers indicate 1.5 times the IQR and the open circles indicate outlier points. Lower case letters denote statistically different groups when comparing the four treatments using a one-way ANOVA with groupings by Tukey's HSD test with a significance level of P≤0.05. The results of this ANOVA are shown in Supplementary Table S7.