# Natural variation in *Arabidopsis thaliana* reveals shoot ionome, biomass and gene expression changes as biomarkers for zinc deficiency tolerance

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Running title: Natural variation for Arabidopsis Zn deficiency tolerance

**Highlight**: Arabidopsis genotypes with a better ability to grow and yield under Zn limiting conditions can be distinguished based on the minimum Zn concentration required for growth and the expression levels of Zn deficiency responsive genes.

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**Abstract**

Zinc (Zn) is an essential nutrient for plants with a crucial role as a co-factor for many enzymes. Approximately one third of the global arable land area is Zn deficient leading to reduced crop yield and quality. To improve crop tolerance to Zn deficiency, it is important to understand the mechanisms plants have adopted to tolerate suboptimal Zn supply. In this study, physiological and molecular aspects of traits related to Zn deficiency tolerance were examined in a panel of 19 *Arabidopsis thaliana* accessions. Accessions showed a larger variation for shoot biomass than for Zn concentration, indicating that they have different requirements for their minimal Zn concentration required for growth. Accessions with a higher tolerance to Zn deficiency showed an increased expression of the Zn deficiency responsive genes *ZIP4* and *IRT3* in comparison to Zn deficiency sensitive accessions. Changes in the shoot ionome, as a result of the plants Zn treatment, were used to build multinomial logistic regression model able to distinguish plants regarding their Zn nutritional status. This set of biomarkers, reflecting the *A. thaliana* response to Zn deficiency and Zn deficiency tolerance, can be useful for future studies aiming to improve the performance and Zn-status of crop plants grown under suboptimal Zn concentrations.

**Key-words:** biofortification, biomarker, mineral concentration, plant ionome, shoot growth, 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 $\mu g g^{-1}$ 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).

To avoid problems associated with inappropriate Zn supply, plants have developed an efficient homeostasis mechanism. Different genes act in the uptake of Zn from soil, distribution over different organs, tissues, cells and organelles, and (re)mobilization through the plant, to control Zn homeostasis (Sinclair and Kramer, 2012). While the actual Zn deficiency sensor is not yet known, the Zn deficiency response in *A. thaliana* seems to start with the activation of the transcription factors bZIP19 and bZIP23, the function of which is essential for plants to survive Zn deficiency (Assunção *et al.*, 2013; van de Mortel *et al.*, 2006).

Zn is among the essential elements which compose the plant ionome (Salt *et al.*, 2008). Previous studies have shown that the plant ionome profile reflects the physiological state of plants under various genetic, developmental, and environmental backgrounds and can be used as a biomarker for a particular physiological condition (Huang and Salt, 2016). Ionome-based biomarker models have been used to determine differences in the plant nutritional status among large sets of different genotypes and experimental batches (Baxter *et al.*, 2008a). Natural variation for the concentration of elements composing the plant ionome has been studied in *A. thaliana*, revealing important mineral homeostasis mechanisms in plants (Baxter *et al.*, 2010; Baxter *et al.*, 2008a; Chao *et al.*, 2012; Kobayashi *et al.*, 2008; Koprivova *et al.*, 2013; Loudet *et al.*, 2007; Morrissey *et al.*, 2009; Pineau *et al.*, 2012; Rus *et al.*, 2006).

To efficiently improve the performance of crops grown under suboptimal Zn conditions and increase the Zn content in their edible parts it is of paramount importance to understand the physiological and molecular mechanisms underlying plants tolerance to Zn deficiency. Aspects of natural variation for Zn deficiency tolerance have been described for several plant species, including *A. thaliana* (Cakmak *et al.*, 1998; Genc *et al.*, 2006; Ghandilyan *et al.*, 2012; Graham *et al.*, 1992; Hacisalihoglu *et al.*, 2004; Karim *et al.*, 2012; Rengel and Graham, 1996). However, to date, a detailed study on natural variation of plants tolerance to Zn deficiency involving both physiological and molecular mechanisms has not yet been performed. In this study we evaluated natural variation among 19 diverse *A. thaliana* accessions to identify physiological and molecular traits involved in the tolerance to Zn deficiency. It shows that high-throughput screening of genetic variation for Zn deficiency 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.

## Material and methods

### Plant material and hydroponic growth

A set of 19 *A. thaliana* accessions was selected based on their diverse site of origin (Supplementary Table S1). Seeds were surface-sterilized with chlorine vapour and sown in petri dishes on wet filter paper followed by a 4-day stratification treatment at 4 $^{o}$C in the dark, to promote uniform germination. Seeds were transplanted to 0.5% (w/v) agar-filled tubes, of which the bottom was cut off, and placed in a modified half-strength Hoagland nutrient solution for hydroponic growth (Assunção *et al.*, 2003): 3 mM $KNO_3$, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 µM KCl, 25 µM $H_3BO_3$, 2 µM $MnSO_4$, 0.1 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe(Na)EDTA. The solution pH was set at 5.5 using KOH and buffered with 2 mM MES (2-(N-morpholino) ethanesulfonic acid). Plants were grown hydroponically in two experiments performed separately. In experiment one, referred to as the mild Zn deficiency experiment, we compared plants grown for 41 days under control (2 µM $ZnSO_4$) and mild Zn deficiency (0.05 µM $ZnSO_4$). In experiment two, referred to as the severe Zn deficiency experiment, we compared plants grown for 31 days under control (2 µM $ZnSO_4$) and severe Zn deficiency (no Zn added). Plants were grown in a climate-controlled chamber set at 70 % relative humidity, with a 12-h day (120 µmol photons $m^{-2}s^{-1}$) and 20$^{o}$C/15$^{o}$C day/night temperatures. The hydroponic system consisted of plastic trays (46 x 31 x 8 cm) holding 9 L nutrient solution, covered with a non-translucent 5-mm-thick plastic lid with evenly spaced holes in a 7 x 10 format holding the agar-filled tubes with plantlets. The nutrient solution was refreshed once a week. Shoot fresh weight (SFW) was measured in all samples during harvesting. Some samples were immediately frozen in liquid nitrogen and stored at -80$^{o}$C for gene expression and element concentration analysis. The shoot dry weight (SDW) of these samples was calculated based on a fresh weight/dry weight correction factor obtained from additional plants which were dried for 72 h at 60$^{o}$C.For each trait, the treatment versus control relative values were determined as Rel_trait = (trait Zn deficiency/trait control)*100.The ZnUI was calculated based on the following formula:

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

### Mineral elemental analysis

For each treatment, the shoot ionome profile was determined for five biological replicates of each *A. thaliana* accession. Samples were first dried for 72 h at 60$^{o}$C, transferred to 96-well plates with tubes containing one 5-mm glass bead and homogenized for 5 minutes at 30 Hz with a Qiagen96-well plate mixer mill. 2 - 4 mg of leaf tissue was transferred to Pyrex test tubes (16 x 100 mm) and digested with 0.9 ml of concentrated nitric acid (Baker Instra-Analyzed; Avantor Performance Materials; http://www.avantormaterials.com) for 5 h at 115$^{o}$C. Samples were diluted to 10 mL with 18.2 MΩcmMilli-Q water. Elemental analyses were performed with an inductively coupled plasma mass spectrometry, ICP-MS (Elan DRC II; PerkinElmer, http://www.perkinelmer.com) for Li, B, Na, Mg, P, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo and Cd. A reference, composed of pooled samples of digested leaf material, was prepared and included every 9$^{th}$ sample in all sets of 70 samples to correct for variation between and within ICP-MS analysis runs. Seven samples from each sample set were weighed and used during the iterative weight normalization process to estimate the weight of the remaining 63 samples from the set (Danku *et al.*, 2013). The following elements were not added to the nutrient solution: Li, Co, Ni, As, Se, Rb, Sr and Cd and, except for Cd, their concentrations are not shown.

**Gene expression**

Gene expression analysis was performed for eight accessions with different ZnUI values selected from the 19 accessions grown under mild Zn deficiency conditions. Frozen leaf material from plants grown under mild and severe Zn deficiency and their respective control treatments was used, in three biological replicates, each consisting of material from three plants. Total RNA was extracted using the method of Onate-Sanchez and Vicente-Carbajosa (2008). cDNA was synthesized from 1 µg of total RNA using the iScript cDNA synthesis kit from BioRad as per the manufacturer's instructions. Following synthesis, cDNA was diluted 10-fold. qRT-PCRs were performed in triplicate with iQ SYBR Green Supermix (BioRad) using an iQ Real Time PCR machine (BioRad). Relative transcript levels of selected genes were determined by qRT-PCR. The expression of *IRT3* (At1g60960), *ZIP3* (At2g32270), *ZIP4* (At1g10970), *bZIP19* (At4g35040), *CSD2* (At2g28190), and *CA2* (At5g14740) was measured. The oligonucleotides used for each gene are shown in Supplementary Table S2. Amplicon lengths were between 80 and 120 bp and all primer combinations had at least 95% efficiency. Reaction volumes were 10 µL (5 µL SYBR green qPCR mix, 300 nmol of each primer and 4 µL of cDNA template). Cycling parameters were 4 minutes at 95˚C, then 40 cycles of 15 seconds at 95˚C and 30 seconds at 55˚C. Gene expression values were

normalized to the reference genes *PEX4* (At5g25760), *SAND* (At2g28390) and *18S*. Gene expression levels relative to the average of the reference genes for each accession under mild and severe Zn deficiency and their respective control treatments were calculated based on ΔCT values. Gene expression levels of accessions exposed to mild and severe Zn deficiency relative to their respective control treatment, were calculated based on ΔΔCT values (Livak and Schmittgen, 2001).

**Statistical analysis**

For all shoot traits and gene expression levels relative to reference gene expression, a two-way ANOVA was performed to test for significant differences between treatments, accessions and the interaction between treatments and accessions. A one-way ANOVA was performed to test for significant differences between accessions for relative gene expression values, relative change in SDW, Zn concentration and Zn content. A one-way ANOVA was also performed to test for significant differences in element concentrations between the four treatments used (mild and severe Zn deficiency and their respective controls). Element concentration values were log10-transformed and a Benjamini-Hochberg correction of the p-values was performed. When significant differences were found, a Tukey's HSD post-hoc test with a significance level of 0.05 was performed. Broad-sense heritability was calculated as the ratio between estimated genetic variance and total phenotypic variance (Kruijer *et al.*, 2015).

**Multivariate analysis and classification**

To predict the Zn deficiency nutritional status of accessions based on their ionomic profile, various multinomial logistic regression (MLR) models were used, similar to the model described by Baxter *et al.* (2008b). In all cases, 11 elements (B, Mg, P, S, K, Ca, Mn, Fe, Cu, Zn and Mo) were considered of which the concentrations were reliably measured. At first, element concentrations were log10-transformed and the transformed element concentration values in the severe or mild Zn deficient plants are normalized to their respective control treatment by subtracting the means of the control group. Thereafter, plants from the control treatment of the two experiments are considered to have the same 'control' status. Hence, plants can either be in a control, mild or severe Zn deficiency state. These states have different probabilities, which were modelled as a linear function of the element concentrations. The prediction for the state of a new plant was defined as the state with the highest probability. Finally, the prediction performance of the following MLR models were

compared: (a) univariate MLR models, for each element; (b) a multivariate MLR model, including all elements; and (c) a multivariate MLR model with all elements except Zn. The multivariate models included a LASSO penalty, which is a multiple of the absolute values of the regression coefficients. The level of penalization was chosen by 10-fold cross-validation. The prediction performance of all models was assessed by drawing 100 times a training set of 199 plants from the total of 398 plants, while the remaining 199 plants were used as a validation set. Each training set was drawn in a stratified manner, respecting the number of plants in the Zn sufficiency (2x100), mild (99) and severe Zn deficiency treatment (99) categories. A penalized logistic regression model was fit for each training set using the R-package "glmnet" (Friedman *et al.*, 2010), and used to predict the status of the 199 plants in the validation set. Prediction performance was estimated by averaging the proportion of correctly classified plants over the 100 validation sets.

## Results

### Natural variation in Zn deficiency response for physiological and morphological traits

*A. thaliana* accessions were grown hydroponically under control conditions (2 μM $ZnSO_4$) and either mild (0.05 μM $ZnSO_4$) or severe Zn deficiency (no Zn added). After 31 days of exposure to severe Zn deficiency, plants showed clear deficiency symptoms compared to plants in the control treatment. This was primarily visible as reduced growth, leaves curling and the presence of chlorotic and necrotic spots (Fig. 1A and B). After 31 days in the mild Zn deficiency treatment, accessions did not show any sign of Zn deficiency, hence they were grown for an additional 10 days. Even then, only a few accessions had visual symptoms of Zn deficiency, mainly slight chlorosis in leaves and reduced growth (Fig. 1C and D), confirming that the treatment was indeed mild.

Accessions showed significant phenotypic variation for most traits analysed which varied according to the trait and Zn treatment (Supplementary Tables S3 and S5).Plants in the severe Zn deficiency treatment had shoot Zn concentrations close to the reported minimum required for growth, which is around 15-20 μg $g^{-1}$ dry biomass (Marschner, 1995). Shoot Zn concentrations under mild Zn deficiency were approximately two times higher than under severe Zn deficiency (Fig. 2). In addition, plants in the mild Zn deficiency experiment had a higher SDW than plants in the severe Zn deficiency experiment, as they were grown for 10 days longer. From all shoot traits only Zn concentration was significantly correlated between

the controls of the two Zn deficiency experiments, indicating that during the additional 10 days of growth between experiments other factors such as the growth rate of accessions affected their shoot biomass and Zn content in a different manner (Supplementary table S8). Accession Cvi-0 had to be excluded from further analysis as it had established poorly and too many plants were lost from especially the mild Zn deficiency experiment.

In both Zn deficiency treatments, most accessions showed reduced SDW relative to their respective control treatments, while few had a higher SDW and apparently were not affected by the reduced Zn supply (Fig. 3A and B). All accessions had a reduction in shoot Zn concentration of approximately 80% in both Zn deficiency treatments relative to their respective controls (Fig. 3C and D). Also, accessions with high shoot Zn concentrations were not always among the ones with a high shoot total Zn content, due to differences in SDW. Tsu-0, Col-0 and Mt-0 were the best performing accessions under mild Zn deficiency in terms of having similar Zn concentrations as the other accessions and higher SDW across the Zn deficiency and control treatments. Thus, these accessions seem to be able to maintain growth under Zn deficiency albeit with some reduction in shoot Zn concentration. Conversely, Pa-2, C24 and Li-5:2 performed poorly under mild Zn deficiency, with a strong reduction in growth in comparison to the other accessions though with a small reduction in shoot Zn concentrations in both Zn deficiency treatments. These accessions appear to have a poor ability to take up Zn both under control and Zn deficient conditions which results in a limited capacity to grow and to maintain cellular Zn levels. Only accession Bor-4 showed an increase in SDW under severe Zn deficiency relative to its control treatment even though not statistically different from most of the other accessions (Fig. 3A, Supplementary table S8). Bor-4 also showed an increase in SDW under mild Zn deficiency, as did Shah. However, it is important to note that these two accessions were among the ones with the lowest SDW in their respective control treatments, which could explain their lower sensitivity to Zn deficiency.

**Accessions with contrasting tolerance to Zn deficiency show differences in the expression of Zn deficiency responsive genes**

The Zn Usage Index (ZnUI) was used to determine the amount of biomass produced per unit of tissue Zn concentration (Fig. 4). In accordance with the results previously shown for SDW and Zn concentration the accessions Mt-0 and Tsu-0 had the highest ZnUI values for

both Zn deficiency treatments andC24 and Pa-2 had the lowest values. Even though only in the mild Zn deficiency treatment, these accessions had significantly higher or lower ZnUI values when compared to the other accessions (Supplementary Table S6). Eight accessions with different ZnUI values in the mild Zn deficiency treatment were then selected to examine if natural variation for Zn deficiency tolerance is reflected at the gene expression level. Mild Zn deficiency was favoured over the severe treatment as the variation between accessions for SDW was larger in the mild treatment. In addition, mild Zn deficiencies are more likely to be found in nature. The accessions Tsu-0 and Col-0 had high ZnIU values, accessions Ge-0, Bur-0 and Can-0 were intermediate and Pa-2, C24 and Per-1 had low ZnUI values. Accessions with higher ZnUI values were considered to be more tolerant to Zn deficiency (Fig. 4).

The expression of six genes involved in the plant Zn deficiency and oxidative stress response was determined in Zn deficiency tolerant and sensitive accessions (Fig. 5; Supplementary Fig. S1). *bZIP19* encodes one of the two redundant bZIP transcription factors which control the Zn deficiency response in *A. thaliana*. We also looked at the expression of the *IRT3*, *ZIP4* and *ZIP3* transcriptional targets genes of bZIP19, all encoding ZIP-like Zn transport proteins, strongly induced following Zn deficiency (Assunção *et al.*, 2010). The expression of the *CSD2* gene, encoding a Cu/Zn superoxide dismutase (SOD) which needs Zn as a structural component to function (Sharma *et al.*, 2004), and the *CA2* gene, encoding a carbonic anhydrase (CA) requiring Zn as co-factor, were also determined. *CSD2* is needed for detoxification of superoxide radicals, while *CA2* facilitates the diffusion of $CO_2$ through the liquid phase of the cell to the chloroplast, important for photosynthesis (Li *et al.*, 2013; Randall and Bouma, 1973). Both *CSD2* and *CA2* are expected to decrease in expression under Zn deficiency exposure due to the reduced concentration of Zn in the cells (Ibarra-Laclette *et al.*, 2013).

There was a significant effect of both the mild and severe Zn deficiency treatments on the expression level of most studied genes. The exceptions were *bZIP19* and *CA2* in the severe Zn deficiency treatment (Supplementary Table S3). The Zn deficiency responsive genes *IRT3*, *ZIP4* and *ZIP3* were up-regulated in all accessions under both Zn deficiency treatments, confirming that the plants sensed Zn deficiency (Fig. 5; Supplementary Fig. S1). Especially *ZIP4* and *IRT3* were in general higher expressed in the more Zn deficiency tolerant accessions than in the more Zn deficiency sensitive accessions, with especially Tsu-

0 showing strong induction of these genes under severe Zn deficiency. The expression of *ZIP3*, which is predominantly expressed in roots (van de Mortel *et al.*, 2006), is the least prominent of the three Zn transporter genes in shoots. The expression levels of *CSD2* and *CA2* were generally low and variable in both Zn deficiency treatments, but especially under mild Zn deficiency, these genes are down-regulated. The Zn deficiency tolerant accessions Ge-0 and Bur-0 had the highest induction of the *CA2* and *CSD2* genes under severe Zn deficiency (Fig. 5). Significant accession by treatment interaction was found for all genes tested, except for *bZIP19*, in at least one of the Zn deficiency experiments (Supplementary Table S3), indicating that gene expression differences between accessions response to Zn deficiency are pronounced.

To further understand the relation between the expression levels of Zn deficiency responsive genes and Zn deficiency tolerance traits a correlation analysis was performed. Under severe Zn deficiency we found a significant positive correlation between the expression levels of *IRT3* and *CSD2* with ZnUI and of *ZIP4* with shoot fresh weight (SFW) (Supplementary Table S9).

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

The shoot ionome of the 19 *A. thaliana* accessions was then determined. Box plots of the combined results per element showed a substantial variation between treatments for almost all the elements measured (Fig.6, Supplementary Table S7). Significant differences between treatments were observed for Zn, Mg, Mo, Cu and Cd concentrations in both the mild and severe Zn deficiency experiments. B, Na and Ca concentrations were significantly different between treatments only in the mild Zn deficiency experiment and Mn and Fe concentrations only in the severe Zn deficiency experiment. When comparing Zn concentrations across the four treatments, there was a significant difference between severe and mild deficiency but not between their respective control treatments.

Broad sense heritability ($H^2$) values were calculated to estimate the genetic contribution to the observed phenotypic variation (Table 1). $H^2$ values were generally higher in the mild compared to the severe Zn deficiency experiment and in plants exposed to Zn deficiency in comparison to their control treatments. The heritability for ZnUI was highest in the mild Zn deficiency treatment, suggesting that under those conditions a large part of the observed

variation is due to genetic differences between accessions. Fe concentration had the lowest heritability in both control treatments, whereas Mo concentration had the highest heritability across the treatments. Even though the Zn concentrations of plants grown under severe Zn deficiency were very low, there was substantial heritability for both Zn concentration and Zn content, with values of 0.49 and 0.41 respectively, indicating that the minimal Zn concentration/content levels are subject to genetic variation.

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

The univariate model (i.e. with a single element as the only predictor) performed poorly as a predictor of plant nutritional status, for most elements, and often mistakenly identified plants under Zn deficiency as being control (Table 2). As expected, only the Zn concentration was able to separate the three classes very well, with prediction accuracies ranging from 0.92 for the plants under severe Zn deficiency to 0.99 for the control plants. Cu also had a good prediction performance for severe Zn deficiency, while Ca was the only element (apart from Zn) that identified a substantial number of the plants under mild Zn deficiency (Table 2). Mg, Mn, Fe, and Mo performed only marginally well, having some ability to identify plants under severe and mild Zn deficiency. For the other elements (B, P, S, and K) the univariate model performed no better than a naïve classifier that would always predict control conditions.

The penalized multivariate model, fitted on all elements except Zn, performed much better than the univariate model: the predicted accuracy for mild (0.6596) and severe Zn deficiency (0.7750) was far higher than with any element alone (except Zn), and the accuracy for the control treatments (0.8738) was still very good. When this model was fitted on all elements (including Zn), it performed similarly to the univariate model fitted with Zn alone, the latter having a higher accuracy for the controls and mild Zn deficiency treatments and less for the severe Zn deficient plants (Table 2).

**Discussion**

The natural variation in the response of *A. thaliana* to two levels of Zn deficiency was examined, with a focus on physiological and molecular traits. Analysis of genetic variation indicated that: (1) accessions vary for the minimum Zn requirement for growth; (2) tolerance to Zn deficiency seems to be related to an increased expression of genes encoding Zn

transmembrane transporter proteins (*ZIP4* and *IRT3*); (3) Zn deficiency results in changes in the plant ionome which can be used as biomarker to predict the plant's physiological condition.

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

The tested *A. thaliana* accessions showed substantial diversity for all traits studied in both Zn deficiency experiments (Figs. 1 – 3). Extreme accessions were identified for all traits, confirming the existing large natural variation in *A. thaliana* response to Zn deficiency conditions and endorsing this panel of representative accessions as a valuable resource to study the plant response and tolerance to Zn deficiency. The response of *A. thaliana* to Zn deficiency has previously been examined in the Ler x Cvi RIL population, in which large variation in SDW and Zn concentration was observed (Ghandilyan *et al.*, 2012).

The mild Zn deficiency treatment is more suitable to reveal genetic variation underlying plants response to Zn nutrition with higher heritability for most traits in comparison to the other treatments (Table 1).The disadvantage of using this mild treatment was that plants were 10 days older than in the severe Zn deficiency treatment, resulting in the initiation of flowering in some accessions. Such change in development could include remobilization of minerals from older to younger organs (e.g. from rosette leaves to developing fruits), however, Waters and Grusak (2008) previously showed that the contribution of remobilization is probably less than 10% of the seed mineral content, so we considered this not much of a disturbing factor. In addition, this treatment seems better in representing Zn deficient conditions likely to be encountered by *A. thaliana* in nature, with an average Zn concentration in leaves of 26 ppm in comparison to 18 ppm in the severe Zn deficiency treatment. To support this, Ghandilyan et al. (2012) observed leaf average Zn concentration of 40 ppm when using a Zn deficient and nutrient-poor soil originating from Eskisehir, Central Anatolia in Turkey to grow the *A. thaliana* Ler x Cvi RIL population. Furthermore, the harshness of the severe Zn deficiency treatment seems to be beyond the genetic capacity of most accessions to tolerate based on the extensive chlorosis displayed by nearly all accessions in this treatment and their very low average leaf Zn concentration, which was within or below the minimum Zn concentration range of 15-20 ppm required for growth as suggested by Marschner (1995).

Heritabilities of most traits were higher in the severe and mild Zn deficiency treatments than in their respective controls, further supporting the observed large genetic variation for all traits in response to the Zn deficiency treatments. Contrary to these observations, Ghandilyan et al. (2012) reported lower heritability values for shoot biomass and most element concentrations in *A. thaliana* plants grown in Zn deficient soil compared to control conditions. Yet, other studies show that heritabilities for the same trait can change according to the growth conditions (Baxter *et al.*, 2012; Ghandilyan *et al.*, 2009; Richard *et al.*, 2011), hence the importance of taking heritability into account when to select growth conditions most amenable to detect genetic variation for a specific trait.

The control treatments of the two Zn deficiency experiments were significantly correlated with respect to the Zn concentration, but not for SDW and Zn content (Supplementary Table S8). This is probably due to differences in growth rate between the *A. thaliana* accessions during the ten additional days of growth in the mild Zn deficiency experiment. Previous studies have shown that growth rate is highly variable among plants; being affected by both internal and external factors such as developmental processes and environmental conditions (El-Lithy *et al.*, 2004; Zhang *et al.*, 2012). Differences in growth rate between accessions in the mild and severe Zn deficiency experiments are likely caused by differences in the initiation of flowering. Most accessions in the control treatment of the mild Zn deficiency experiment were flowering or bolting at the harvesting day; while only three accessions, of the control treatment, were flowering in the sever Zn deficiency experiment at the harvesting day, which was 10 days earlier than in the mild Zn deficiency experiment (Fig. 1A and C).

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

*A. thaliana* accessions showed a larger variation for relative change in SDW than in Zn concentration under both Zn deficiency treatments (Fig. 3). This indicates the presence of genetic variation for their minimum Zn requirement and for the ability to tolerate low Zn concentrations. This is not unique for *A. thaliana* though. Also for barley, bread and durum wheat, common bean and rice, different genotypes are reported to have similar shoot Zn concentrations with different levels of Zn deficiency tolerance (Cakmak *et al.*, 1998; Genc *et al.*, 2002; Hacisalihoglu *et al.*, 2003; Rengel, 2001; Sadeghzadeh *et al.*, 2009; Wissuwa *et al.*, 2006). Further indications that *A. thaliana* accessions vary for the minimum Zn

requirement is shown by a few accessions with slightly higher SDW in the Zn deficient treatment relative to its control.

The ability to enhance the root Zn uptake and the root to shoot Zn transport are among the proposed mechanisms underlying tolerance to Zn deficiency (Broadley *et al.*, 2007; Rengel, 2001), but the Zn deficiency signal may come from shoots. Indeed, accessions considered tolerant to Zn deficiency had a higher expression of Zn deficiency responsive genes *ZIP4* and *IRT3* in shoots (Fig. 5). These genes, encoding Zn transmembrane transporters (Grotz *et al.*, 1998) are transcriptionally responsive to Zn deficiency and mainly expressed in roots, but are also expressed in shoot tissue in response to low Zn, suggesting a role in both Zn uptake and distribution (Jain *et al.*, 2013; Lin *et al.*, 2009). Our findings indicate that higher tolerance to Zn deficiency may be the result of an increased, or more efficient, shoot Zn re-allocation capacity, and that natural variation for it may reflect variation in the expression of these and other Zn transport genes in *A. thaliana*.

Previous studies have shown that tolerance to Zn deficiency can also be affected by the plant capacity to deal with the high levels of ROS produced under low Zn conditions (Rengel, 2001; Sinclair and Kramer, 2012). In this study a relationship was found between the expression of *CA2* and ZnUI (Supplementary Table S9). Further studies examining the ability of plants to tolerate ROS under Zn deficiency and other mechanisms not included in this study, but thought to contribute to tolerance to Zn stress, will be useful for a more complete understanding of the mechanisms involved in plant tolerance to Zn deficiency (Cakmak *et al.*, 1996; Chen *et al.*, 2009; Gao *et al.*, 2005; Genc *et al.*, 2006; Hoffland *et al.*, 2006; Impa *et al.*, 2013a; Impa *et al.*, 2013b; Rengel, 2001; Wissuwa *et al.*, 2006). This should include examining the ability of plants to increase the bioavailability of $Zn^{2+}$ ions in the soil; to improve the root system architecture to scavenge larger soil volumes; and a more efficient utilization, compartmentalization and remobilization of Zn.

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

Exposing *A. thaliana* plants to different levels of Zn deficiency also affects the homeostasis of other elements, which made it possible to develop a MLR model able to predict the Zn deficiency status of a plant based on changes in other elements (Table 2). This approach is analogous to the model used by Baxter et al. (2008b) to predict the physiological status of *A.*

*thaliana* plants exposed to Fe or P deficiency. Contrary to the MLR model developed for Zn deficiency, Baxter et al. (2008b) found that changes in Fe concentration alone had no power to detect Fe-deficiency and detection was totally dependent on analysis of other elements. This difference could be caused by the two different Zn deficiency treatments used in this study which incorporated more data points to the model, while only one deficiency treatment was used in the Fe deficiency study (Baxter *et al.*, 2008b), but it could also be because in that study the Fe concentrations in leaves of plants grown under low and normal Fe did not differ, while in our study, the shoot Zn concentrations of plants grown under severe and mild Zn deficiency were significantly different, next to having extremely low Zn concentrations in comparison to control conditions. The Zn concentration thus appears to be much less tightly controlled in *A. thaliana* than for Fe. In that respect, Zn corresponds more with P, for which their model did incorporate P concentration (Baxter *et al.*, 2008b). This analysis provides strong evidence that elements do not behave independently upon Zn deficiency and it shows the power of using a combination of elements as a phenotype of interest to detect a plant's nutritional status. The use of these traits to evaluate crops tolerance to Zn deficiency has the potential to simplify and shorten the process of identification of Zn deficiency tolerant varieties. However, further studies confirming the application of comparable biomarkers as found for *A. thaliana* in the evaluation of Zn deficiency tolerance in crops will be needed.

## Conclusion

This study demonstrates that several physiological and molecular mechanisms underlie differences in Zn deficiency tolerance in *A. thaliana*. These include the minimum Zn concentration required for growth and the ability to take up and translocate Zn by inducing the expression of Zn deficiency responsive genes. ZnUI, the reduction in SDW and the expression level of Zn deficiency responsive genes such as *ZIP4* and *IRT3* are useful proxies to evaluate plant tolerance to Zn deficiency in future studies. A mild Zn deficiency condition is more amenable for genetic studies than a severe stress, with higher heritability values for most studied traits and providing a more natural condition, at least for *A. thaliana*. Finally, the shoot ionome profile is a useful predictor of the plant Zn deficiency status. Changes in Zn concentration alone or in combination with other elements have an excellent capacity to detect physiological plant Zn deficiency in the absence of other visible symptoms. While we 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
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**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 | | Zn deficiency | | average |
|---|---|---|---|---|
| | control | 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 $ZnSO_4$)(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.*

***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 $ZnSO_4$; 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 S6. See Supplementary Table S1 for the list of accessions. 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 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 $ZnSO_4$) 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 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 $P \leq 0.05$. The results of this ANOVA are shown in Supplementary Table S7.*

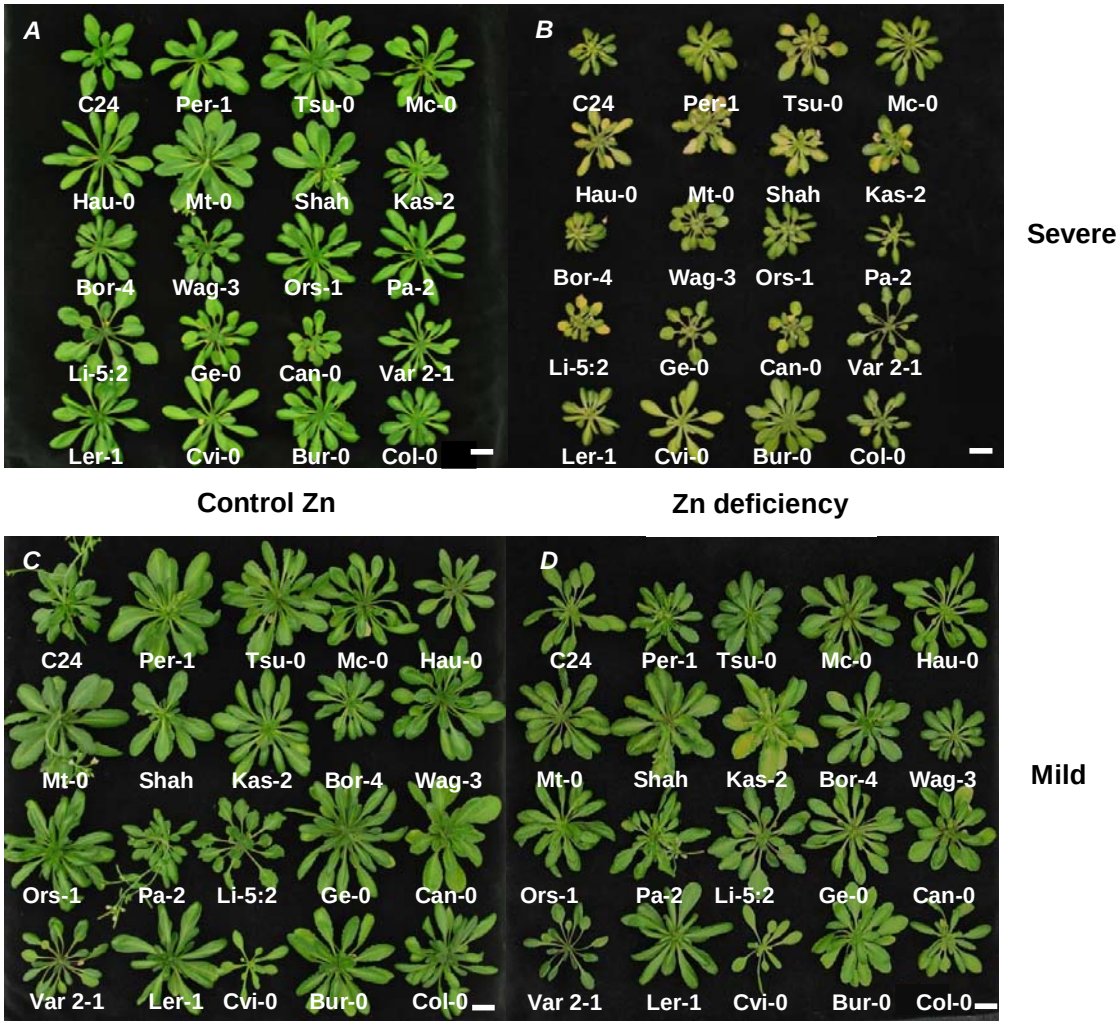



***Figure 1:*** *Comparison of A. thaliana accessions grown under control and severe or mild Zn deficient conditions.*

*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.*

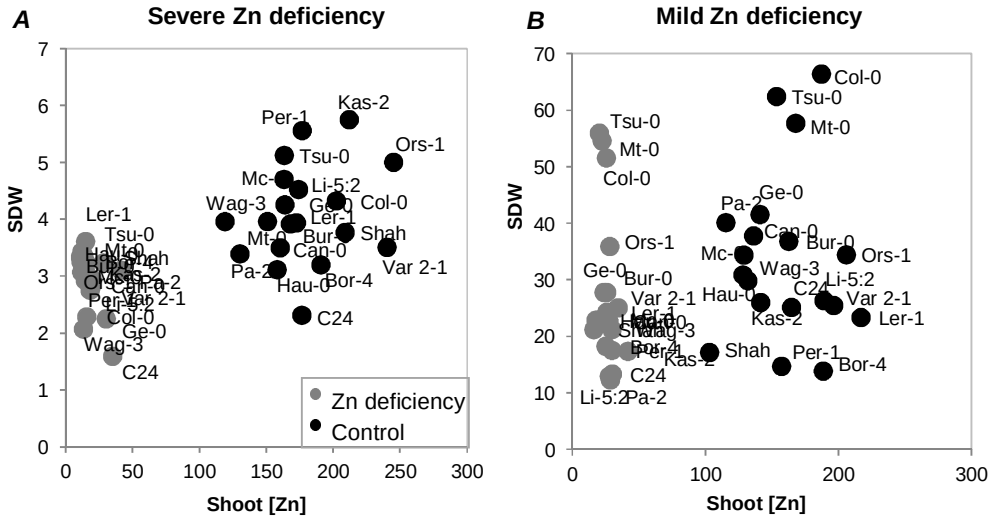



***Figure 2:*** *Relations between shoot dry weight and Zn concentration of 19 A. thaliana accessions grown under Zn deficiency.*

*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 $ZnSO_4$; 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.*

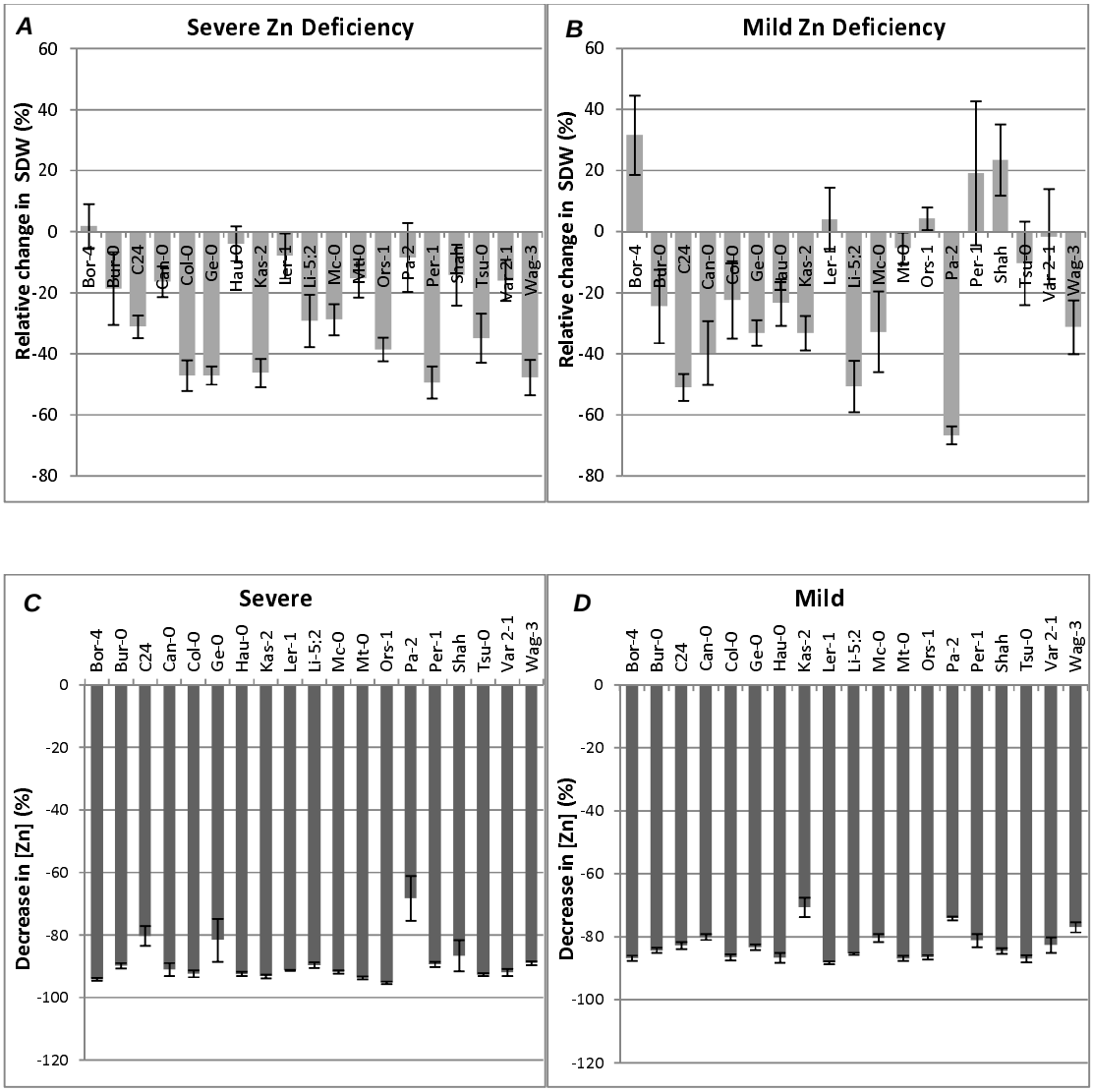



***Figure 3:*** *Relative changes in shoot dry weight and Zn concentration of 19 A. thaliana accessions grown under severe (A and C) and mild (B and D) Zn deficiency, compared to their respective control treatments.*

*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 $ZnSO_4$) and severe (no Zn added) or mild Zn deficiency (0.05 µM $ZnSO_4$). Plants in A and C are grown for 31 days, plants in B and D are grown for 41 days.*

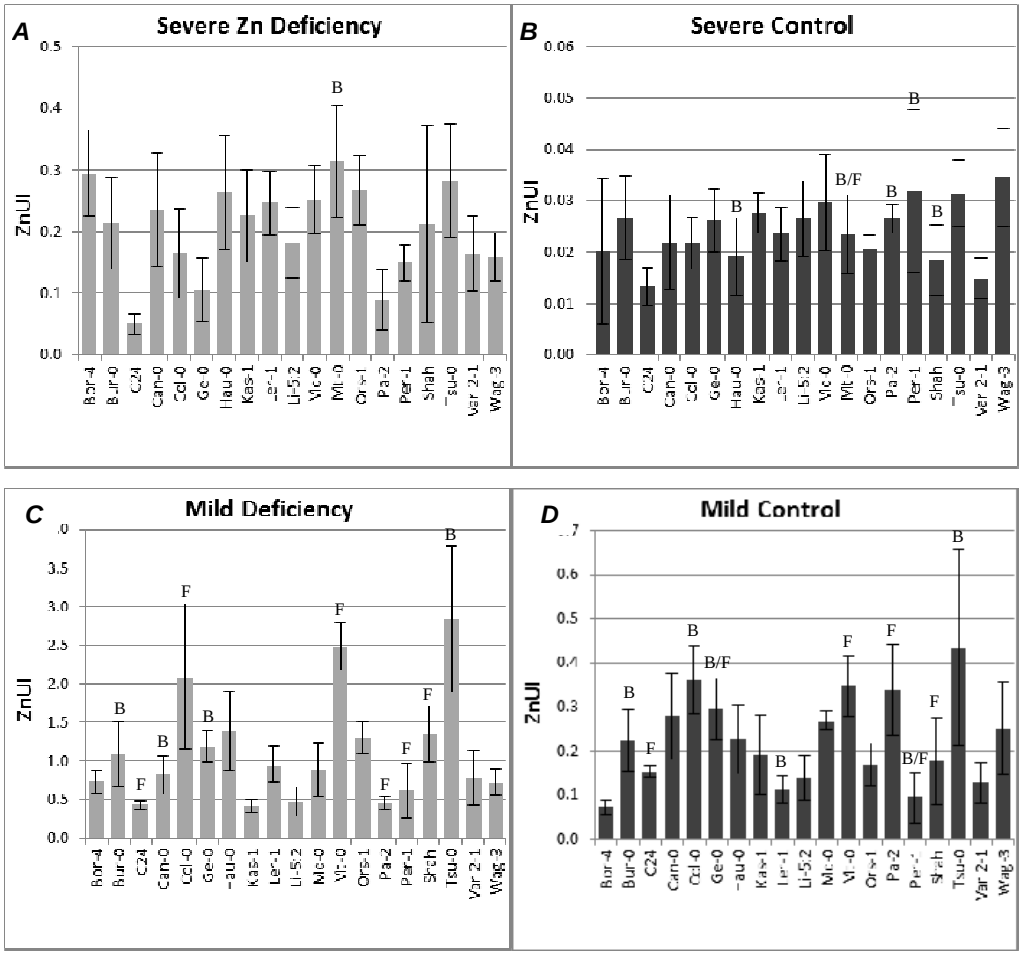



***Figure 4:*** *Shoot Zn Usage Index (ZnUI) of A. thaliana accessions grown in severe(A) and mild (C) Zn deficiency and their respective control treatments (B and D). The letters above each bar indicate if the accession was already bolting (B) or flowering (F) when harvested. The ZnUI is defined as 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.*

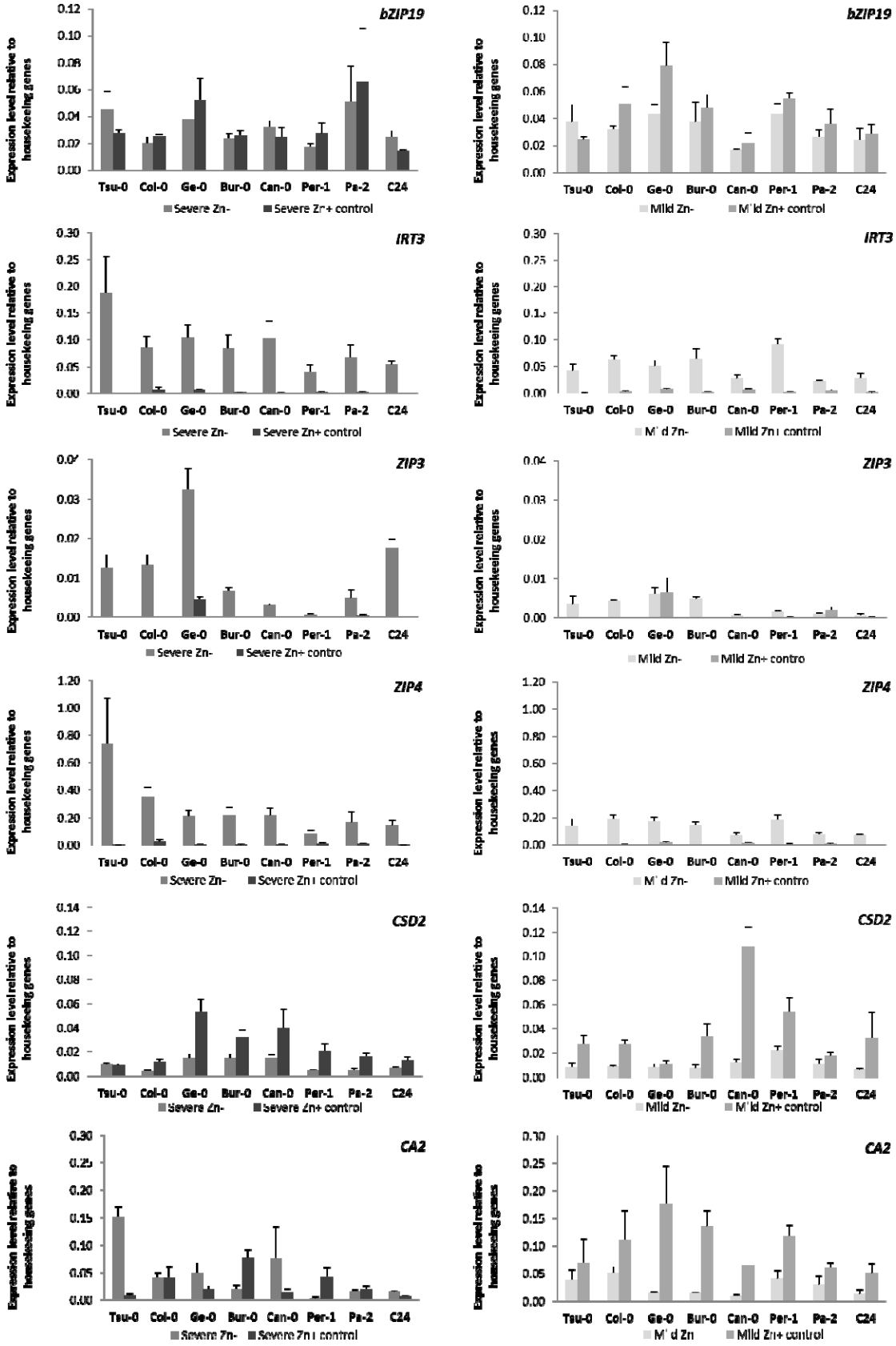
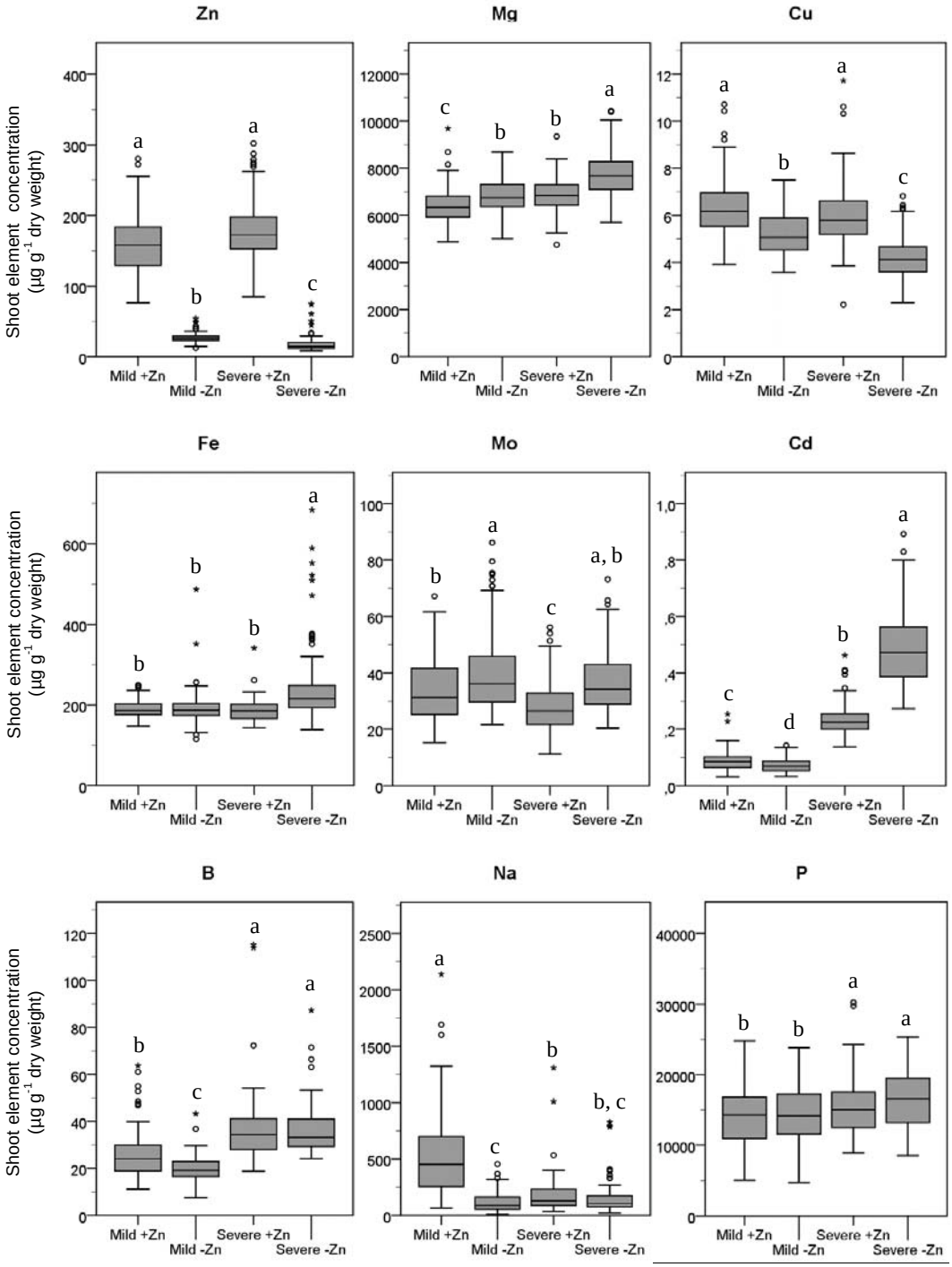

bZIP19
IRT3
ZIP3
ZIP4
CSD2
CA2
Expression level relative to housekeeping genes
Tsu-0 Col-0 Ge-0 Bur-0 Can-0 Per-1 Pa-2 C24
Severe Zn-
Severe Zn+ control
Mild Zn-
Mild Zn+ control

***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.*


Zn
Mg
Cu
Fe
Mo
Cd
B
Na
P
Shoot element concentration (µg g-1 dry weight)
Mild +Zn
Mild -Zn
Severe +Zn
Severe -Zn

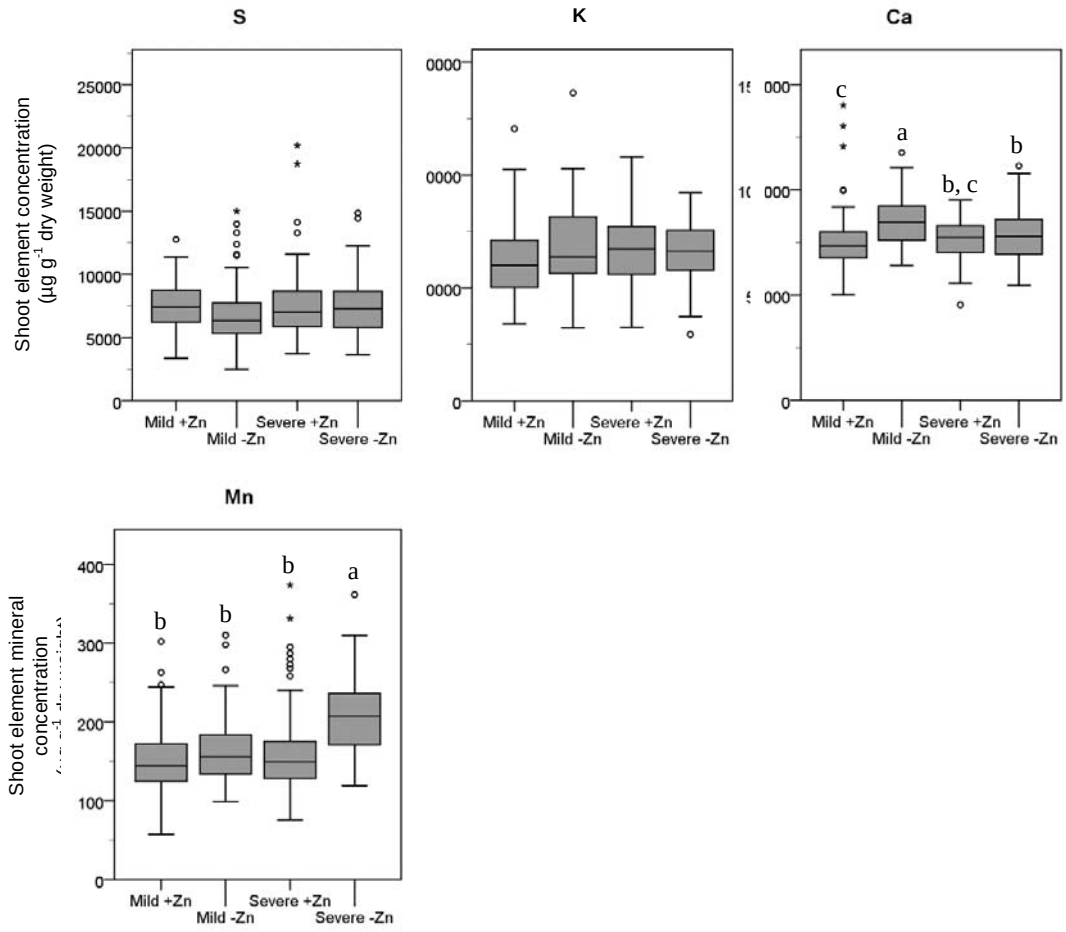



***Figure 6:*** *Box plots comparing mineral element concentrations in shoots of 19 A. thaliana accessions grown under severe and mild Zn deficiency and their respective control treatments.*

*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.*