Reduced expression of *bZIP19* and *bZIP23* increases zinc and cadmium accumulation in *Arabidopsis halleri*

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Abstract

Zinc is an essential micronutrient for all living organisms. When challenged by zinc-limiting conditions, plants use a strategy centered on two transcription factors, bZIP19 and bZIP23, to enhance the expression of several zinc transporters to improve their zinc uptake capacity. In the zinc and cadmium hyperaccumulator plant *Arabidopsis halleri*, highly efficient root-to-shoot zinc translocation results in constitutive local zinc deficiency in roots and in constitutive high expression of zinc deficiency-responsive *ZIP* genes, boosting zinc uptake and accumulation. Here, to disrupt this process and to analyze the functions of AhbZIP19, AhbZIP23 and their target genes in hyperaccumulation, the genes encoding both transcriptional factors were knocked down using artificial microRNAs (amiRNA). Although *AhbZIP19*, *AhbZIP23*, and their *ZIP* target genes were down-regulated, amiRNA lines surprisingly accumulated more zinc and cadmium compared to control lines in both roots and shoot driving to shoot toxicity symptoms. These observations suggested the existence of a substitute metal uptake machinery in *A. halleri* to maintain hyperaccumulation. We propose that the iron uptake transporter AhIRT1 participates in this alternative pathway in *A. halleri*.

Keywords: *Arabidopsis halleri*, bZIP19, bZIP23, Zinc, Cadmium, Hyperaccumulation, ZIP

Introduction

Zinc is an essential micronutrient with key functions in protein structure and catalytic activity. Indeed, zinc is the second most abundant trace metal element after iron in cells and it is estimated that >10% of the eukaryotic proteome uses or interacts with zinc, this represents for instance more than 2400 proteins in the model plant *Arabidopsis thaliana* (Andreini *et al.*, 2006, Broadley *et al.*, 2007, Hänsch and Mendel, 2009, Clemens, 2022). In soils, zinc concentrations largely vary, ranging from rare anthropogenically polluted areas with high zinc concentrations (Tóth *et al.*, 2012) to more widely distributed areas with poor zinc bioavailability (Alloway, 2008, Rengel *et al.*, 2023). To adapt to variable zinc availability and to limit toxicity or deficiency symptoms, respectively, plant evolved a complex and sophisticated network of transcription factors, chelators and transporters, enabling a tight control of zinc uptake, trafficking, storage and remobilization (Sinclair and Krämer, 2012, Amini *et al.*, 2022, Stanton *et al.*, 2022, Thiébaut and Hanikenne, 2022).

A small number of plant species have developed a so-called hyperaccumulation strategy to colonize and establish populations on soils with high metal concentrations. Hyperaccumulator plants are highly metal tolerant and actively take up and store extremely high metal amounts in aerial parts without toxicity symptoms (Krämer, 2010, Hanikenne and Nouet, 2011, Clemens, 2016, Merlot *et al.*, 2021). From a physiological point of view, metal hyperaccumulation requires four key steps: (i) an active metal absorption from the soil, (ii) an efficient radial transfer to vascular tissues with a low root vacuolar storage, (iii) an enhanced xylem loading leading to an efficient root-to-shoot translocation, and ultimately, (iv) sufficient detoxification processes including optimal shoot metal distribution and vacuolar storage (Hanikenne and Nouet, 2011, Clemens, 2016, Merlot *et al.*, 2021).

Arabidopsis halleri, a close relative of *A. thaliana*, is among the few zinc and cadmium hyperaccumulator plant species. Although these traits are highly variable within the species, it was shown to accumulate up to ~5.4% of zinc and/or ~0.36% cadmium of dry biomass in shoot tissues (Krämer, 2010, Stein *et al.*, 2017). Quantitative genetic and transcriptomic studies identified genes that are constitutively highly expressed and are (putative) actors of hyperaccumulation and hypertolerance in *A. halleri* (Talke *et al.*, 2006, Krämer, 2010, Hanikenne and Nouet, 2011, Merlot *et al.*, 2021). Among those, *AhNAS2* (*Nicotianamine Synthase 2*) contributes to enhanced zinc radial transport towards the root xylem and to root-to-shoot translocation (Deinlein *et al.*, 2012, Cornu *et al.*, 2014, Uraguchi *et al.*, 2019) and *AhHMA4* (*Heavy Metal ATPase 4*) drives zinc and cadmium loading into the xylem (Talke *et al.*, 2006, Courbot *et al.*, 2007, Hanikenne *et al.*, 2008, Hanikenne *et al.*, 2013). *AhMTP1* (*Metal Tolerance Protein 1*) is believed to play a key role in zinc vacuolar storage in the *A. halleri* shoot (Dräger *et al.*, 2004, Willems *et al.*, 2007, Shahzad *et al.*, 2013, Fasani *et al.*, 2017). Finally, several *ZIP* (*Zinc-regulated transporter, Iron-regulated transporter-like Proteins*) genes were suggested to contribute to enhanced zinc uptake by roots, zinc radial transport and zinc distribution in shoots (Talke *et al.*, 2006, Krämer *et al.*, 2007, Lin *et al.*, 2009). However, with the exception of *AhZIP6* (Spielmann *et al.*, 2020, Spielmann *et al.*, 2022b), experimental evidence for their exact roles in metal hyperaccumulation and tolerance is still lacking.

Since their identification in the mid 1990's, the characterization of the zinc-transporting ZIPs in *A. thaliana* is also lagging behind and their exact contribution to zinc homeostasis remains unclear (Sinclair and Krämer, 2012, Amini *et al.*, 2022, Stanton *et al.*, 2022, Thiébaut and Hanikenne, 2022). This is most likely due to high functional redundancy among individual *ZIP* genes (Lee *et al.*, 2021b). This is contrast to our very detailed knowledge of the functioning of another ZIP transporter, IRT1 (Iron Regulated Transporter 1), the main iron uptake systems by roots in dicots (Vert *et al.*, 2002, Barberon *et al.*, 2011, Barberon *et al.*, 2014, Dubeaux *et al.*, 2018, Spielmann *et al.*, 2022a). The transcriptional expression of zinc transporter encoding *ZIP* genes is strongly induced by zinc deficiency in many species, including *A. thaliana* (Amini *et al.*, 2022, Thiébaut and Hanikenne, 2022). The bZIP19 and bZIP23 (Basic Leucine Zipper) transcription factors were shown in *A. thaliana* to be key regulators of the zinc deficiency response (Assunção *et al.*, 2010, Inaba *et al.*, 2015). A *bzip19bzip23* double mutant is hypersensitive to zinc deficiency, which is lethal in the absence of zinc supplementation (Assunção *et al.*, 2010, Inaba *et al.*, 2015). Partially redundant, bZIP19 and bZIP23 act as bifunctional receptors and transcriptional factors, capable of, first, sensing the zinc status through direct zinc binding to a Nterminal histidine and cysteine-rich motif and then, binding to the promoters of zinc deficiency response genes (Assunção *et al.*, 2010, Lilay *et al.*, 2021). In presence of an optimal zinc concentration, zinc binding to bZIP19 and bZIP23 is believed to prevent their dimerization and represses their transcription factor activity (Assunção *et al.*, 2014, Lilay *et al.*, 2021). In contrast, upon zinc deficiency, bZIP19 and bZIP23 are free to dimerize and to bind Zinc Deficiency Response Elements (ZDRE) present in the promoter, and activate the transcription, of a small set of target genes, mostly *ZIP*s (e.g. *ZIP4*, *IRT3*, *ZIP9*) and two *NAS*s (*NAS2* and *4*) (Assunção *et al.*, 2010, Inaba *et al.*, 2015, Lilay *et al.*, 2021).

In *A. halleri*, highly efficient zinc loading into the xylem from pericycle cells, which is driven by high expression of *AhHMA4*, causes zinc depletion in the roots. It was hypothesized to trigger a local zinc deficiency response and constitutive high expression of the zinc deficiency-responsive *ZIP* genes, further increasing zinc uptake and accumulation (Talke *et al.*, 2006, Hanikenne *et al.*, 2008). The down-regulation of *ZIP* genes in *AhHMA4* RNAi lines with reduced *AhHMA4* expression and reduced zinc translocation to the shoot supports this hypothesis (Hanikenne *et al.*, 2008). Whether the *A. halleri* homologs of *bZIP19* and *bZIP23* control the local zinc deficiency response in roots and contribute to zinc hyperaccumulation has not been examined yet.

Here, we show that expression of two alternative spicing variants of *AhbZIP19* and of *AhbZIP23* partially complemented the growth defect of a *bzip19bzip23* double *A. thaliana* mutant upon zinc deficiency, indicating that bZIP19 and bZIP23 have conserved molecular functions in *A. thaliana* and *A. halleri*. Moreover, we aimed to unravel the function(s) of *AhbZIP19* and *AhbZIP23*, and their *ZIP* target genes, in *A. halleri*. We hypothesized that silencing both transcription factor genes using artificial microRNAs (amiRNAs) would indirectly silence *ZIP* genes and break high zinc uptake in *A. halleri*. In contrast, despite reduced expression of *ZIP* target genes, we showed that *AhbZIP19* and *AhbZIP23* double amiRNA lines accumulated higher zinc and cadmium concentrations in roots and shoot, leading to cadmium toxicity in shoot. Zinc, but not cadmium, radial transport in roots was also perturbed. Our results suggest that an alternative uptake pathway, in addition to ZIPs, exists in *A. halleri* to sustain strong metal uptake.

Results

bZIP19 is alternatively spliced in *A. halleri*

Prior to their characterization, the coding sequences (CDSs) of the *A. halleri bZIP19* and *bZIP23* (*AhbZIP19* and *AhbZIP23*) genes needed to be properly delineated. Blasting the *A. thaliana bZIP19* and *bZIP23* (*AtbZIP19* and *AtbZIP23*) CDSs against the *A. halleri* genome available on Phytozome (https://phytozome-next.jgi.doe.gov/) and against a publicly-available RNA-Seq dataset (Schvartzman *et al.*, 2018) identified two *AhbZIP19* and a single *AhbZIP23* CDSs, respectively. The two *AhbZIP19* CDSs were 100% identical until the end of the second exon, after which the sequences strongly diverged (Fig. 1A, S1A and S1B). Because many genes involved in metal homeostasis are present in several copies in the *A. halleri* genome (Talke *et al.*, 2006, Suryawanshi *et al.*, 2016), the *AhbZIP19* gene copy number was determined by quantitative PCR in *A. halleri* and in the two closely related species, *A. thaliana* and *Arabidopsis lyrata*. A single copy was detected in the three species (Fig. S2). These observations indicated that *AhbZIP19* undergoes an alternative splicing event, resulting in the presence of (i) a long transcript, hereafter named *AhbZIP19-A* and (ii) a short transcript, hereafter named *AhbZIP19-B* and sharing the highest sequence similarity to *AtbZIP19* and its ortholog in *Arabidopsis lyrata*, *AlbZIP19*, and an identical gene organization (Fig. 1A and S1C).

bZIP19 and *bZIP23* have distinct expression patterns in *A. halleri*

Quantitative RT-PCR using transcript specific primers for *AhbZIP19* revealed that the expression of both transcript variants was rather stable across different tissues. However, *AhbZIP19-B* was ~3x more expressed than *AhbZIP19-A* in all tested tissues (Fig. 1B). In contrast, *AhbZIP23* was strongly expressed in aerial parts (leaves, cauline leaves and flowers) but only moderately expressed in roots (Fig. 1B).

AhbZIP19-A and *AhbZIP19-B* were up-regulated in roots, upon high zinc exposure (300 µM Zn) compared to control conditions (5µM Zn) (Fig. 2A and B). In addition, *AhbZIP19-B* was down-regulated in roots upon zinc deficiency (0 µM Zn) and in presence of cadmium (5 µM Cd) (Fig. 2B). The *AhbZIP23* expression level was not affected by zinc or cadmium supply in roots and was down-regulated in shoot by 300 µM Zn (Fig. 2C). These results are in contrast to those previously reported in *A. thaliana* where *AtbZIP19* and *AtbZIP23* are up-regulated by zinc deficiency and down-regulated by zinc excess (Assunção *et al.*, 2010, Inaba *et al.*, 2015). Taken together, these observations confirmed that *AhbZIP19* and *AhbZIP23* have different expression patterns. Moreover, their transcriptional response to zinc status appeared to be different in *A. halleri* compared to *A. thaliana* which suggested that these transcription factors may have other functions in *A. halleri*.

***AhbZIP19* and *AhbZIP23* partially complement the *bzip19bzip23* double mutant**

To individually analyze the function of each *AhbZIP19* and *AhbZIP23* transcripts, the *A. thaliana* *bzip19bzip23* double mutant was stably transformed with either *AhbZIP19-A*, *AhbZIP19-B* or *AhbZIP23* for ectopic overexpression under the control of the constitutive *CaMV 35S* promoter (Assunção *et al.*, 2010, Lilay *et al.*, 2019). For each construct, a line with similar expression level of the transgene was selected among 3 independent homozygous T3 lines (Fig. S3) and grown on agar plates in control condition (1µM Zn) or in zinc deficiency (0.05µM Zn), together with *bzip19bzip23* double mutant and Col-0 plants used as controls. Similar growth (shoot weight and root length) were observed in both conditions for Col-0 (Fig. 3). In contrast, the *bzip19bzip23* mutant already showed reduced growth (lighter shoot and shorter roots than Col-0) in control condition and was strongly impacted by zinc deficiency (Fig. 3), as reported previously (Assunção *et al.*, 2010). In control condition, the *bzip19bzip23* mutant was fully complemented by *AhbZIP19-B* whereas root and shoot growth defects were only partially complemented upon zinc deficiency (Fig. 3). *AhbZIP19-A* fully complemented the shoot phenotype in control condition whereas partial complementation was observed in roots in control condition and in both roots and shoot upon zinc deficiency. For *AhbZIP23*, a partial complementation was obtained in both control and zinc deficiency conditions (Fig. 3). Overall, these observations indicated that the function of the *AhbZIP19* variants and of *AhbZIP23* in regulating the zinc deficiency response was, at least, partially conserved between *A. thaliana* and *A. halleri*.

Reduced expression of *AhbZIP19* and *AhbZIP23* in *A. halleri* results in cadmium toxicity, and increases zinc and cadmium accumulation

Double knock-down transgenic lines for both *AhbZIP19* and *AhbZIP23* were generated by transforming *A. halleri* plants (Talke *et al.*, 2006, Hanikenne *et al.*, 2008) with a single construct encoding two distinct artificial microRNAs (Schwab *et al.*, 2006, Ossowski *et al.*, 2008), targeting *AhbZIP19* and *AhbZIP23*, respectively. Thirteen independent transgenic lines were regenerated from transformed calli (Hanikenne *et al.*, 2008) and were screened for reduced *AhbZIP19* and *AhbZIP23* transcript abundance in leaves (Fig. S4). Two lines (Lines 13 and 10, hereafter called amiRNA I and amiRNA II, respectively) with a moderate, but significant, reduction of *AhbZIP19* (~50% in shoot and ~21% in roots) and *AhbZIP23* (~34% in shoot and ~24% in roots) expression compared to plants regenerated from untransformed calli (hereafter called control line) were identified (Fig. 4) and used for further analyses.

The two control and the two amiRNA lines were then grown in Hoagland hydroponic culture. After two weeks of rooting (1µM Zn), and three weeks of growth in control condition (5µM Zn), plants were exposed to four different conditions: (i) zinc deficiency (0µM Zn), (ii) control condition (5µM Zn), (iii) high zinc (300µM Zn) and (iv) cadmium exposure (5µM Zn and 5µM Cd) for three weeks. Despite the transcriptional regulation of *AhbZIP19* and *AhbZIP23* by high zinc (Fig. 2), this condition had very limited effect on growth and zinc accumulation in both control and amiRNA genotypes (Fig. S5) and was therefore abandoned after the first experimental replicate. The three remaining conditions were used to further characterize the physiological role of *AhbZIP19* and *AhbZIP23* in *A. halleri*. First, visual observation revealed that cadmium exposure led to a stronger chlorosis in amiRNA lines than in control lines (Fig. 5A). Indeed, chlorophyll concentrations dropped by 72% and 33% in amiRNA and control lines, respectively, upon cadmium treatment (Fig. 5B). Moreover, amiRNA lines accumulated more cadmium in both roots (75%) and shoot (50%) than control lines after cadmium exposure (Fig. 6A and B). The amiRNA lines similarly accumulated more zinc in roots (380 to 700%) and shoot (60 to 135%) in control and cadmium conditions, respectively, compared to control lines (Fig. 6A and B). In contrast, upon zinc deficiency, a slight increase (not significant) in zinc accumulation in roots (~16%) and a more substantial decrease (not significant) in shoot (64%) were observed (Fig. 6A and B). Finally, roots of amiRNA lines accumulated 93%, 68% and 38% more manganese than control lines in control, zinc deficiency and cadmium conditions, respectively (Fig. S6A-C). None of the other measured elements were affected in roots or shoot of amiRNA lines (Fig. S6A-B).

Thus, reduced expression of *AhbZIP19* and *AhbZIP23* perturbed zinc and manganese homeostasis as well as cadmium accumulation, leading to chlorosis likely resulting from increased cadmium (and/or zinc or manganese) accumulation or mislocalization.

Reduced expression of *AhbZIP19* and *AhbZIP23* in *A. halleri* reduces zinc root-to-shoot translocation

A. halleri, as most metal hyperaccumulator plants, is characterized by an efficient metal root-to-shoot translocation, often resulting in a shoot-to-root metal concentration ratio above 1 at least for zinc (Talke *et al.*, 2006, Hanikenne *et al.*, 2008). In all three tested conditions (zinc deficiency, control and cadmium exposure), amiRNA and control lines displayed zinc shoot-to-root ratios above 1. However, these ratios was lower (by 45, 66 and 73%, respectively) in amiRNA lines (Fig. 7A), suggesting that, in these lines overaccumulating zinc (Fig. 6), (i) a larger part of Zn was retained in roots and/or (ii) zinc translocation to the shoot may have been a limiting step. In contrast, the cadmium shoot-root ratio was non-significantly reduced (-20%) in amiRNA lines (Fig. 7B). Finally, calcium and magnesium (as well as manganese, but non-significantly) shoot-root ratios were reduced in amiRNA lines (Fig. S7).

Reduced expression of *AhbZIP19* and *AhbZIP23* in *A. halleri* impacts a myriad of genes involved in zinc homeostasis

Quantitative RT-PCR analysis showed that, in control conditions, genes putatively directly under the control of *AhbZIP19* and *AhbZIP23* (*AhIRT3*, *AhZIP4* and *AhZIP9*, Assunção *et al.,* 2010) were less expressed in both roots (31-55% reduction) and shoot (52-65% reduction) of amiRNA lines compared to control lines (Fig. 8A-C and 8H-J). Two genes involved in metal translocation to the shoot, *AhHMA4* and *AhFRD3*, displayed divergent regulation in amiRNA lines. *AhHMA4*, encoding a zinc and cadmium transporter which loads metals into the xylem (Hussain *et al.*, 2004, Hanikenne *et al.*, 2008), was not differently expressed in amiRNA lines compared to control lines (Fig. 8G-H). In contrast, the expression of *AhFRD3*, encoding a citrate transporter implicated in iron and zinc homeostasis and root-to-shoot translocation (Durrett *et al.*, 2007, Pineau *et al.*, 2012, Charlier *et al.*, 2015), was reduced by 35% in roots (Fig. 8L). Among genes involved in vacuolar metal storage, *AhMTP1*, encoding a zinc tonoplastic transporter (Dräger *et al.*, 2004, Desbrosses-Fonrouge *et al.*, 2005, Shahzad *et al.*, 2010, Fasani *et al.*, 2017), and *AhZIF1*, encoding a tonoplastic nicotianamine transporter involved in iron homeostasis and zinc tolerance (Haydon *et al.*, 2012), were more expressed in roots (+32% and +100% respectively) but not significantly affected in shoot of amiRNA lines (Fig. 8F, G, M, N). Finally, *AhIRT1*, encoding a broad spectrum metal transporter involved in metal uptake (including zinc and cadmium) (Vert *et al.*, 2002, Barberon *et al.*, 2011, Spielmann *et al.*, 2022a), was the most strongly (~300%) up-regulated gene among all tested genes in roots of amiRNA lines (Fig. 8O).

Altogether, these observations indicated that reduced expression of *AhbZIP19* and *AhbZIP23* had a wider impact, beyond their direct target genes (*AhZIP4*, *AhZIP9*, *AhIRT3*). Hence non-target genes such as *AhFRD3*, *AhIRT1, AhMTP1* or *AhZIF1* were indirectly impacted, possibly to compensate the misregulation of *AhbZIP19* and *AhbZIP23* target genes.

Discussion

Here, we examined the role of bZIP19 and bZIP23, and indirectly of their *ZIP* target genes, in the zinc and cadmium hyperaccumulation process in *A. halleri*. Although the expression of *AhbZIP19* and *AhbZIP23* was only partially reduced, especially in roots, amiRNA lines displayed striking phenotypes with increased zinc and cadmium accumulation, accompanied by cadmium toxicity symptoms. Transcription factors are generally lowly expressed (Czechowski *et al.*, 2004), and our results suggest that moderate alteration of the *AhbZIP19* and *AhbZIP23* gene dosage has a stronger impact on the expression of their target genes, and is sufficient to result in major phenotypic outcomes.

The *AhbZIP19* splicing variants and *AhbZIP23* only partially complement the *bzip19bzip23 A. thaliana* double mutant

A strategy used by organisms to respond to a stress is to synthesize from a single gene, thanks to alternative splicing, different transcript variants, which may display distinct mRNA stability or translation efficiency and encode proteins with distinct sequences and functions (Remy *et al.*, 2014, Charlier *et al.*, 2015, Dong *et al.*, 2018). For instance, retention of an intron in the 5' untranslated region (UTR) of the mRNA encoding the ZIF2 transporter in response to zinc excess results in enhanced ZIF2 translation rate and increased zinc tolerance (Remy *et al.*, 2014). Moreover, mutations affecting splicing factors were shown to alter nutrient homeostasis as well as cadmium tolerance in plants (Zhang *et al.*, 2014, Dong *et al.*, 2018, Fanara *et al.*, 2022).

In the Phytozome database, the *AtbZIP19* gene is described as producing four alternative transcripts, initiated from two alternative sites and differing in 3'UTRs. To date, these alternative transcripts have not been experimentally confirmed and it is unknown whether they have distinct functions. Here, the *AhbZIP19* gene was shown to undergo alternative splicing in a german population (LAN) of *A. halleri*, producing transcripts that diverge at the 3' end of their coding sequences and in 3'UTRs. The analysis of publicly available RNA-Seq data (Schvartzman et al., 2018, Corso et al., 2021) identified sequencing reads supporting the presence of the two *AhbZIP19* splicing variants in polish (PL22) and italian (I16 and I30) populations. In contrast, no sequence equivalent to AhbZIP19-A is found in the genome sequence of *A. lyrata* available on Phytozome. The alternative splicing event of AhbZIP19 appears thus to be specific to *A. halleri* and to be conserved in populations originating from all 3 genetic units of the species (as defined by Pauwels et al. 2012) and from metalliferous and non-metalliferous sites (Table S1). These two variants, *AhbZIP19-A* and *AhbZIP19-B*, displayed some ability to complement a *bzip19bzip23 A. thaliana* double mutant (Assunção *et al.*, 2010). However, *AhbZIP19-A,* the *A. halleri*-specific variant, afforded weaker complementation, which suggested that the alternative 3' end affected, at least partially, the function of the transcription factor in regulating the zinc deficiency response. Moreover, although this strategy has been used in all studies characterizing plant homologs of AtbZIP19 and AtbZIP23 in the *bzip19bzip23 A. thaliana* background (Assunção *et al.*, 2010, Inaba *et al.*, 2015, Evens *et al.*, 2017, Nazri *et al.*, 2017, Lilay *et al.*, 2020, Lilay *et al.*, 2021), using a 35S promoter to drive the ectopic overexpression of both transcripts may have hidden additional differences due to mRNA stability or translation efficiency. Fully elucidating the biological role of the *AhbZIP19-A* and *AhbZIP19-B* variants will require more investigation, such as *bzip19bzip23* complementation using a *bZIP19* native promoter/terminator and/or knocking-out/-down each *AhbZIP19* variant directly in *A. halleri*.

In addition to *AhbZIP19-A* and *AhbZIP19-B*, *AhbZIP23* also had the ability to complement the growth defect of the *bzip19bzip23* double mutant upon zinc deficiency, indicating that the *AhbZIP19* and *AhbZIP23* function in regulating the zinc deficiency response was, at least partially, conserved between *A. thaliana* and *A. halleri*. However, in our experimental conditions, expression of either of the *AhbZIP19* or *AhbZIP23* genes was not sufficient to fully complement the growth defect of the *bzip19bzip23* double mutant. This indicated that, as in *A. thaliana* (Inaba *et al.*, 2015, Lilay *et al.*, 2019), both transcription factors are not fully redundant in *A. halleri*, which together with distinct expression patterns and regulation by zinc and cadmium suggests that they could have distinct regulatory activities.

Metal uptake is enhanced in amiRNA lines

The current model of zinc hyperaccumulation in *A. halleri* proposes that the high rate of zinc translocation to the shoot by AhHMA4 causes zinc depletion in the roots, which in turns induces a zinc deficiency response and the high expression of zinc uptake *ZIP* genes, further supporting hyperaccumulation (Hanikenne *et al.*, 2008, Krämer, 2010, Hanikenne and Nouet, 2011, Merlot *et al.*, 2021). Beyond an important role of *AhHMA4* as well (Courbot *et al.*, 2007, Hanikenne *et al.*, 2008), how this pathway controls cadmium uptake and shoot translocation is far more unclear. The observations reported here somehow challenge this model. Indeed, if *AhbZIP19/AhbZIP23* double amiRNA lines displayed reduced expression of several AhbZIP19 and AhbZIP23 putative *ZIP* target genes (*AhIRT3*, *AhZIP4* and *AhZIP9*), they concomitantly accumulated more zinc and cadmium in roots and shoot, as well as more manganese in roots only, and displayed increased cadmium sensitivity, when compared to wild-type plants. Manganese accumulation was shown to be increased in roots of an *A. thaliana* mutant for *ZIP1*, a bZIP19 and bZIP23 target (Assunção *et al.*, 2010, Milner *et al.*, 2013). A non-synonymous mutation in *bZIP19* in *Noccaea caerulescens* was recently shown to result in increased shoot Mn accumulation (Wang *et al.*, 2022). This indicated that reducing the expression of *AhbZIP19/AhbZIP23* unexpectedly resulted in increased zinc, manganese and cadmium uptake from the medium, and suggested that in addition to zinc deficiency-responsive *ZIPs*, an additional uptake pathway exists for these metals in *A. halleri*.

Zinc, manganese and cadmium are substrates of the broad spectrum metal transporter IRT1 (Vert *et al.*, 2002, Barberon *et al.*, 2011, Spielmann *et al.*, 2022a), whose transcriptional expression is increased in *AhbZIP19/AhbZIP23* double amiRNA lines. Variations in gene expression, protein level and/or functionality of IRT1 were linked to differential zinc and/or cadmium shoot accumulation among populations of two zinc and cadmium hyperaccumulator plants, *A. halleri* (Corso *et al.*, 2018, Schvartzman *et al.*, 2018) and *N. caerulescens* (Halimaa *et al.*, 2014). It is therefore tempting to speculate that IRT1 may constitute this alternative uptake pathway, compensating the loss of *ZIP* gene expression, and may contribute to increased metal accumulation in *AhbZIP19/AhbZIP23* double amiRNA lines.

AtIRT1 is subjected to post-transcriptional regulation to limit the excess uptake of non-iron metals (including zinc, manganese and cadmium) and avoid toxicity (Barberon *et al.*, 2011, Barberon *et al.*, 2014, Dubeaux *et al.*, 2018, Spielmann *et al.*, 2022a, Spielmann et al., 2023). Upon excess, AtIRT1 directly binds these non-iron metals thanks to a histidine-rich motif localized in a so-called cytosolic regulatory loop (Dubeaux *et al.*, 2018, Spielmann *et al.*, 2022a). AtIRT1 loop-metal interactions leads to phosphorylation of several key IRT1 residues, initiating a process of IRT1 endocytosis and vacuolar degradation (Dubeaux *et al.*, 2018). Comparing the sequences of the AtIRT1 and AhIRT1 loops was a first step to assess whether a similar post-transcriptional regulation may occur in *A. halleri*. This revealed two differences between the two orthologous proteins (Fig. S8): (i) a serine, which is predicted to be phosphorylated in presence of non-iron metals and plays a role in AtIRT1 post-translational regulation (Dubeaux *et al.*, 2018), was substituted by a proline in AhIRT1; and (ii) the histidine-rich motif was truncated in AhIRT1, with the presence of only three histidine residues instead of four in AtIRT1. It was recently demonstrated that mutation of this histidine-rich motif inhibits AtIRT1 non-iron metal-mediated endocytosis and degradation leading to root non-iron metal accumulation and non-iron metal-related toxicity symptoms (Dubeaux *et al.*, 2018, Spielmann *et al.*, 2022a). These observations suggest that the post-translational regulation of AhIRT1 by non-iron metals may be altered or at least less efficient in the Langelsheim *A. halleri* population, possibly enabling sustained transport activity even in the presence of high zinc or cadmium concentrations in the medium. Fully characterizing how the mutations found in the cytoplasmic loop of AhIRT1 impact metal binding and/or its post-transcriptional regulation will require more investigations.

As a consequence of increased uptake by roots, the combined increases in cadmium and zinc accumulation in shoots of the *AhbZIP19/AhbZIP23* double amiRNA lines likely exceeded the tolerance capacities of the plants, resulting in chlorosis. Extensive variation in zinc and cadmium accumulation and tolerance has been reported among *A. halleri* populations (Bert *et al.*, 2000, Bert *et al.*, 2002, Bert *et al.*, 2003, Meyer *et al.*, 2015, Stein *et al.*, 2017, Corso *et al.*, 2018, Schvartzman *et al.*, 2018). The Langelsheim population originates from a metalliferous site, with moderate soil cadmium concentration (Meyer *et al.*, 2015), but displays relatively high tolerance to cadmium, compared for instance to non-metallicolous populations (Meyer *et al.*, 2015).

Zinc is retained in roots of *AhbZIP19/AhbZIP23* amiRNA lines

Although reduced expression of AhbZIP19 and AhbZIP23 increased zinc accumulation in both roots and shoot, shoot-to-root zinc concentration ratios indicated that a larger part of the taken-up zinc remained in roots, whereas only a smaller part was translocated to the shoot. This can result from either of two hypotheses, that are not necessarily exclusive: (i) limited zinc translocation from the roots to the shoot and/or (ii) increased zinc retention in roots. Based on the observations reported here, the first hypothesis does not appear to be viable. Indeed, zinc accumulation in shoots of *AhbZIP19/AhbZIP23* double amiRNA lines exposed to high zinc (300 µM Zn) was ~8-fold higher than in control conditions (5 µM Zn). Moreover, the expression of *AhHMA4*, which is a key actor controlling zinc loading into the xylem and shoot accumulation in *A. halleri* (Hanikenne *et al.*, 2008, Frérot *et al.*, 2010, Hanikenne *et al.*, 2013, Nouet *et al.*, 2015) remained high and unaffected in *AhbZIP19/AhbZIP23* double amiRNA lines. These observations indicated that zinc translocation capacities were very high and not limiting in these lines.

Increased metal retention in the roots, on the other hand, can result from increased vacuolar metal storage and/or lower radial transport to the vascular tissues. In roots of the amiRNA lines, higher expression of *ZIF1* and *MTP1*, encoding nicotianamine and a zinc vacuolar transporters, respectively (Dräger *et al.*, 2004, Desbrosses-Fonrouge *et al.*, 2005, Shahzad *et al.*, 2010, Fasani *et al.*, 2017;Haydon *et al.*, 2012), both likely contributed to increased zinc vacuolar storage. The ~50% reduction *IRT3*, *ZIP4* and *ZIP9* expression may further contribute to zinc retention in roots of amiRNA *A. halleri* lines. Indeed, *irt3zip4zip9* triple and *irt3zip4zip6zip9* quadruple *A. thaliana* mutants recently shown to display increased zinc accumulation in roots, altered zinc radial transport and reduced zinc shoot-to-root ratios (Lee *et al.*, 2021b), as the *AhbZIP19/AhbZIP23* amiRNA lines in *A. halleri*. However, in contrast to the *A. halleri* amiRNA lines, the *A. thaliana* mutants accumulated less zinc in shoots and did not display increased zinc uptake by roots (Lee *et al.*, 2021b). Thus, with a combination of increased zinc uptake and perturbed radial transport in roots of *AhbZIP19/AhbZIP23* amiRNA lines, zinc still reached the root vascular tissues in sufficient amount to allow xylem loading by AhHMA4 and accumulation in the shoot.

Zinc and cadmium use different pathways for radial movement in roots in *A. halleri*

Cadmium is a non-essential and toxic element for organisms, and to be taken up and transported in plant tissues, it highjacks transporters of other divalent metal cations, zinc transporter in particular (Verbruggen *et al.*, 2009). If reduced *AhbZIP19* and *AhbZIP23* expression increased root and shoot cadmium accumulation, similar to zinc, it did not alter the cadmium shoot-to-root ratio, suggesting that in contrast to zinc, cadmium radial movement in roots was unaffected. It further suggests that (i) ZIP transporters, such as AhIRT3, AhZIP4 or AhZIP9 do not contribute to such cadmium transport, as shown for zinc by Lee *et al.* (2021b) and (ii) cadmium root radial transport is independent of AhbZIP19 and AhbZIP23 target genes. Zinc and cadmium would therefore employ distinct pathways to reach the root vascular tissues. This confirms earlier genetic findings showing that zinc and cadmium accumulation do not fully segregate in *A. halleri* and, with the exception of AhHMA4, are associated to distinct quantitative trait loci (Courbot *et al.*, 2007, Willems *et al.*, 2007, Frérot *et al.*, 2010, Willems *et al.*, 2010, Baliardini *et al.*, 2015). Identifying the mechanism controlling cadmium root radial transport in *A. halleri* may allow disconnecting zinc and cadmium accumulation and specifically reducing cadmium translocation to the shoot. This knowledge may prove useful to design zinc biofortification strategies in crops grown on land with high background levels of cadmium (Tóth *et al.*, 2012).

Conclusions and perspectives

Taken together, the observations reported here suggest that the (partial) loss of the AhbZIP19 and AhbZIP23 function, and the consequent (partial) loss of the expression of their putative *ZIP* target genes, is compensated in *A. halleri*. This compensation mechanism is efficient enough to increase up to 135% and 50% zinc and cadmium shoot accumulation, respectively (Fig. 9). This somehow suggests, that in contrast to their translocation to the shoot which is essentially driven by AhHMA4 (Hanikenne *et al.*, 2008), a backup system evolved in *A. halleri* to ensure sustained zinc (and cadmium) uptake. We hypothesize that the upregulation of *AhIRT1* contributes at least in part to this increased uptake and accumulation. If correct, this hypothesis implies that sensing and regulation mechanisms underlying the induction of *AhIRT1* exist and will need to be identified. Several actors involved in the iron deficiency response in *A. thaliana* (such as BRUTUS and related proteins) were recently shown to impact zinc tolerance and to some extent accumulation under zinc excess (Chen *et al.*, 2018, Zhu *et al.*, 2022, Hanikenne and Bouché, 2023, Stanton *et al.*, 2023).

It is interesting to note that, in addition to *AhIRT1*, other genes previously involved in interactions between the homeostasis of zinc and iron [i.e. *ZIF1*, *FRD3*, (Hanikenne *et al.*, 2021)] are also deregulated in *AhbZIP19/AhbZIP23* amiRNA lines (Fig. 9). This is reminiscent of the observations made when exploring the mechanisms underlying the variation of the zinc and cadmium hyperaccumulation and tolerance traits in *A. halleri* (Corso *et al.*, 2018, Schvartzman *et al.*, 2018, Corso *et al.*, 2021, Lee *et al.*, 2021a). Emerges thus the picture that two types of metal homeostasis genes are highly expressed in *A. halleri*: core genes (e.g. *AhHMA4*, *AhMTP1*) associated with the early evolution of hypertolerance and hyperaccumulation in the species (Krämer, 2010, Hanikenne and Nouet, 2011) and whose overexpression is (almost) strictly constitutive, and additional genes (e.g. *AhIRT1*, *AhZIF1*, *AhFRD3*, …), whose expression is much more flexible and who are involved in fine-tuning these processes (Merlot *et al.*, 2021).

Methods

Plant material and growth conditions

A. halleri ssp. *halleri* plants, from the Langelsheim population (Talke *et al.*, 2006, Hanikenne *et al.*, 2008) were used for all experiments. Plants were grown on soil (wild-type) or *in vitro* (transgenic lines and untransformed regenerated plants) on solid agar (0.8% w/v Select Agar, Sigma Aldrich) ½ MS medium (Duchefa Biochimie) supplemented with sucrose (1% w/v, Duchefa Biochimie) and were propagated by cuttings. For experiments, cuttings were transferred into hydroponic trays (Araponics) and were grown for two weeks in 1 µM zinc Hoagland medium for rooting, then two weeks in 5 µM zinc Hoagland medium, then submitted to experimental conditions for three weeks. In all hydropony experiments, the nutrient solution was exchanged with fresh medium once a week and last 3 days before harvest. The control Hoagland medium was modified as in (Talke *et al.*, 2006, Hanikenne *et al.*, 2008) and included 10 µM FeIII-HBED [N, N' – di (2-hydroxybenzyl) ethylenediamine N, N' - diacetic acid monohydrochloride]. Zinc was omitted from the medium for deficiency experiments and zinc ($ZnSO_4.H_2O$) and cadmium ($CdSO_4.8/3H_2O$) were added as indicated for excess experiments. Plants were cultivated in climate-controlled growth chambers at 20°C (night and day), with a photoperiod of 16h (long days) light (100 µmol photon $m^{-2}s^{-1}$).

Wild-type, *bzip19bzip23* double mutant (Assunção *et al.*, 2010) and complemented lines were from the Columbia-0 (Col-0) background for all *A. thaliana* experiments. In a small hermetic chamber, seeds were surface sterilized for 2 hours using chloral vapor produced by a mix of 50 ml of 14% sodium hypochlorite (VWR) with 3 ml of 37% HCL (VWR) in an Erlenmeyer. Then, seeds were sown on solid agar (0.8% w/v; Select Agar; Sigma Aldrich) Hoagland medium (modified as above) with either 1 µM zinc (control) or 0.05 µM zinc (zinc deficiency) and incubated at 4°C for two days. Plants were then cultivated in climate-controlled growth chambers at 20°C (night and day), with a photoperiod of 8h (short days) light (100 µmol photon $m^{-2}s^{-1}$) for 14 days. Root lengths were determined using the tool "segmented lines" of the ImageJ software. Shoot fresh weights were measured on a precision balance by pooling the shoots of 4 seedlings.

Details on experimental replication are provided in figure legends.

Plant transformation

*Agrobacterium tumefaciens (*GV3101, pMP90) was used to perform stable transformation of *A. thaliana* and *A. halleri* using the floral dip method (Clough and Bent, 1998) or a tissue-culture based procedure (Hanikenne *et al.*, 2008, Ahmadi *et al.*, 2018), respectively. In both cases, the pMDC32 vector (see cloning section below) was used (Curtis and Grossniklaus, 2003) and transgenic plants were selected using hygromycin B (Duchefa).

Gene expression analyses

Total RNA was extracted from 100 mg of plant tissues using the NucleoSpin® RNA Plant Kit (Macherey-Nagel). cDNAs were synthesized with the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific) using Oligo(dT) and 1 µg of total RNAs. cDNAs were 50-fold diluted and quantitative PCR, quality control and primer efficiency correction were performed as described (Spielmann *et al.*, 2020). Relative transcript level normalization was performed using *EF1a* (Talke *et al.*, 2006) and *UBQ13* (Spielmann *et al.*, 2020). Primer sequences are provided in Table S2.

Determination of gene copy number

Gene copy number were determined by quantitative PCR on *A. thaliana*, *A. halleri* and *A. lyrata* genomic DNA as previously described (Spielmann *et al.*, 2020). Primer sequences are provided in Table S2.

Cloning and DNA manipulation

Full-length coding sequences of *AhbZIP19-A*, *AhbZIP19-B* and *AhbZIP23* were amplified by PCR using *A. halleri* [Lan3.1 individual, (Talke *et al.*, 2006, Hanikenne *et al.*, 2008)] shoot cDNA as template, the *Fusion* DNA polymerase (Thermo Scientific) and primers containing appropriate restriction sites (Table S3). For *AhbZIP19-A*, *AhbZIP19-B* and *AhbZIP23*, PCR fragments were gel-purified and cloned into the *Asc*I/*Pac*I sites of the pMDC32 vector under the control of a 2x35S promoter (Curtis and Grossniklaus, 2003).

The DNA fragment encoding the cytoplasmic loop of AhIRT1 was amplified by PCR using *A. halleri* [Lan3.1 individual, (Talke *et al.*, 2006, Hanikenne *et al.*, 2008)] genomic DNA as template, the *Fusion*

DNA polymerase (Thermo Scientific) and appropriate primers (Table S3). After a column purification, the PCR fragment was sequenced.

Two amiRNAs, targeting either *AhbZIP19* or *AhbZIP23*, were designed using the "Designer" tool at http://wmd3.weigelworld.org/. amiRNAs synthesis were performed by PCR as describe on http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Downloads;project=stdwmd, using the pRS300 vector as template, the *Pfu* polymerase (Promega) and appropriate primers (Table S3). Once synthesized, amiRNAs were cloned into the pJET1.2/blunt vector (Thermos Scientific) before adding *Kpn*I/*Sac*II sites by PCR with the appropriate primers (Table S3). PCR fragments were gel-purified and cloned into the *Kpn*I/SacII sites of the pMDC32 vector under the control of a 2x35S promoter (Curtis and Grossniklaus, 2003).

Mineral analyses

Upon harvest, root samples were desorbed and shoot samples were washed as described (Nouet *et al.*, 2015). All samples were dried at 60°C for four days. 20-30 mg of dried tissues were digested with 3 ml of ≥65% HNO_3 (Sigma-Aldrich) using a DigiPrep Graphite Block Digestion System (SCP Science) as follows: 10 min at 45˚C, 10 min at 65˚C and 90 min at 105 ˚C. Once cooled, sample volumes were adjusted to 10 ml with distilled water and 200 µl of ≥65% HNO_3 (Sigma-Aldrich) were added. Metal concentrations were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a Vista-AX instrument (Varian, Melbourne, Australia).

Chlorophyll concentration measurements

To determine chlorophyll concentrations, 30-100 mg of *A. halleri* leaves were placed in a 1.5 ml Eppendorf and dark-incubated with 1 ml of Ethanol 96% (VWR) for 40h at room temperature. Samples were diluted 10 times in 96% Ethanol and absorbances were measured at 649 and 665nm. Total chlorophyll concentrations were calculated as followed $Chl_{Tot} = (((6.1 \times A665) + (20.4 \times A649)) \times 10)/$ fresh weight (mg) (Wintermans and De Mots, 1965), then presented as values relative to a control line.

Statistical analysis

All Statistical analyses were performed using the GraphPad Prism 8.01 software. Normal distribution of the values were analyzed with the Shapiro-Wilk's test. To analyze variance homogeneity, the tests recommended by the GraphPad Prism 8.01 software were used (Spearman's test for two-way ANOVA; Brown-Forsythe test for one-way ANOVA; F-test for *Student's* t-tests). Parametric and non-parametric tests used to assess statistical significance are indicted in figure legends.

Supplemental materials

Table S1. Alternative splicing of the *bZIP19* gene in Arabidopsis relatives.

Table S2. List of primers used for quantitative (RT)-PCR.

Table S3. List of primers used for cloning.

Figure S1. bZIP19 sequence similarity analyses.

Figure S2. Determination of *bZIP19* gene copy numbers in *A. thaliana* (Col-0), *A. lyrata* (Lyr) and *A. halleri* (Lan3.1).

Figure S3. *AhbZIP19* and *AhbZIP23* expression in transgenic *A. thaliana* lines.

Figure S4. *AhbZIP19* and *AhbZIP23* expression in *A. halleri* amiRNA lines.

Figure S5. Zinc accumulation in *AhbZIP19* and *AhbZIP23* double amiRNA lines exposed to 300µM Zn.

Figure S6. Metal concentrations in shoot and roots of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*.

Figure S7. Metal shoot-to-root concentration ratios in *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*.

Figure S8. Amino acid sequence alignment of the cytosolic regulatory loops of the *A. thaliana* and *A. halleri* IRT1 proteins.

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Author contributions

MH conceived and directed the research. MH and JS designed the experiments. JS and MS performed experiments. JS and MH analysed the data. JS made the figures. JS and MH wrote the manuscript, with comments of MS.

References

Ahmadi, H., Corso, M., Weber, M., Verbruggen, N. and Clemens, S. (2018) CAX1 suppresses Cd-induced generation of reactive oxygen species in *Arabidopsis halleri*. *Plant, Cell and Environment*, **41**, 2435-2448.

Alloway, B. (2008) Zinc in soils and crop nutrition. *International Fertilizer Industry Association, and International Zinc Association*, Second edition, 1-139.

Amini, S., Arsova, B. and Hanikenne, M. (2022) The molecular basis of zinc homeostasis in cereals. *Plant, Cell and Environment*, **45**, 1339-1361.

Andreini, C., Banci, L., Bertini, I. and Rosato, A. (2006) Zinc through the Three Domains of Life. *Journal of Proteome Research*, **5**, 3173-3178.

Assunção, A.G., Herreroa, E., Lina, Y.-F., Huettelb, B., Talukdara, S., Smaczniakc, C., Immink, R.G.H., van Eldike, M., Fierse, M., Schatf, H. and Aarts, M.G. (2010) *Arabidopsis thaliana* transcription factors bZIP19 and bZIP23 regulate the adaptation to zinc deficiency. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 10296–10301.

Assunção, A.G., Schat, H. and Aarts, M.G. (2014) Regulation of the adaptation to zinc deficiency in plants. *Plant Signaling and Behavior*, **5**, 1553-1555.

Baliardini, C., Meyer, C.L., Salis, P., Saumitou-Laprade, P. and Verbruggen, N. (2015) *CATION EXCHANGER1* cosegregates with cadmium tolerance in the metal hyperaccumulator

Arabidopsis halleri and plays a role in limiting oxidative stress in *Arabidopsis* Spp. *Plant Physiology*, **169**, 549-559.

Barberon, M., Dubeaux, G., Kolb, C., Isono, E., Zelazny, E. and Vert, G. (2014) Polarization of IRON-REGULATED TRANSPORTER 1 (IRT1) to the plant-soil interface plays crucial role in metal homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 8293-8298.

Barberon, M., Zelazny, E., Robert, S., Conéjéro, G., Curie, C., Friml, J. and Vert, G. (2011) Monoubiquitin-dependent endocytosis of the IRON-REGULATED TRANSPORTER 1 (IRT1) transporter controls iron uptake in plants. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 450-458.

Bert, V., Bonnin, I., Saumitou-Laprade, P., De Laguérie, P. and Petit, D. (2002) Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist*, **155**, 47-57.

Bert, V., Macnair, M.R., De Laguerie, P., Saumitou-Laprade, P. and Petit, D. (2000) Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist*, **146**, 225-233.

Bert, V., Meerts, P., Saumitou-Laprade, P., Salis, P., Gruber, W. and Verbruggen, N. (2003) Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil*, **249**, 9-18.

Broadley, M.R., White, P.J., Hammond, J.P., Zelko, I. and Lux, A. (2007) Zinc in plants. *New Phytologist*, **173**, 677-702.

Charlier, J.B., Polese, C., Nouet, C., Carnol, M., Bosman, B., Krämer, U., Motte, P. and Hanikenne, M. (2015) Zinc triggers a complex transcriptional and post-transcriptional regulation of the metal homeostasis gene *FRD3* in *Arabidopsis* relatives. *Journal of Experimental Botany*, **66**, 3865-3878.

Chen, C.L., Cui, Y., Cui, M., Zhou, W.J., Wu, H.L. and Ling, H.Q. (2018) A FIT-binding protein is involved in modulating iron and zinc homeostasis in *Arabidopsis*. *Plant, Cell and Environment*, **41**, 1698-1714.

Clemens, S. (2016) How metal hyperaccumulating plants can advance Zn biofortification. *Plant and Soil*, **411**, 111-120.

Clemens, S. (2022) The cell biology of zinc. *Journal of Experimental Botany*, **73**, 1688-1698.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal*, **16**, 735-743.

Cornu, J.Y., Deinlein, U., Horeth, S., Braun, M., Schmidt, H., Weber, M., Persson, D.P., Husted, S., Schjoerring, J.K. and Clemens, S. (2014) Contrasting effects of nicotianamine synthase knockdown on zinc and nickel tolerance and accumulation in the zinc/cadmium hyperaccumulator *Arabidopsis halleri*. *New Phytologist*, **206**, 738-750.

Corso, M., An, X., Jones, C.Y., Gonzalez-Doblas, V., Schvartzman, M.S., Malkowski, E., Willats, W.G.T., Hanikenne, M. and Verbruggen, N. (2021) Adaptation of *Arabidopsis halleri* to extreme metal pollution through limited metal accumulation involves changes in cell wall composition and metal homeostasis. *New Phytologist*, **230**, 669-682.

Corso, M., Schvartzman, M.S., Guzzo, F., Souard, F., Malkowski, E., Hanikenne, M. and Verbruggen, N. (2018) Contrasting cadmium resistance strategies in two metallicolous populations of *Arabidopsis halleri*. *New Phytologist*, **218**, 283-297.

Courbot, M., Willems, G., Motte, P., Arvidsson, S., Roosens, N., Saumitou-Laprade, P. and Verbruggen, N. (2007) A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with *HMA4*, a gene encoding a heavy metal ATPase. *Plant Physiology*, **144**, 1052-1065.

Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology*, **133**, 462-469.

Czechowski, T., Bari, R.P., Stitt, M., Scheible, W-R. and Udvardi, M.K. (2004) Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant Journal*, **38**, 366-379

Deinlein, U., Weber, M., Schmidt, H., Rensch, S., Trampczynska, A., Hansen, T.H., Husted, S., Schjoerring, J.K., Talke, I.N., Krämer, U. and Clemens, S. (2012) Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation. *The Plant cell*, **24**, 708-723.

Desbrosses-Fonrouge, A.G., Voigt, K., Schroder, A., Arrivault, S., Thomine, S. and Kämer, U. (2005) *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Letters*, **579**, 4165-4174.

Dong, C., He, F., Berkowitz, O., Liu, J., Cao, P., Tang, M., Shi, H., Wang, W., Li, Q., Shen, Z., Whelan, J. and Zheng, L. (2018) Alternative Splicing Plays a Critical Role in Maintaining Mineral Nutrient Homeostasis in Rice (*Oryza sativa*). *The Plant Cell*, **30**, 2267-2285.

Dräger, D.B., Desbrosses-Fonrouge, A.G., Krach, C., Chardonnens, A.N., Meyer, R.C., Saumitou-Laprade, P. and Krämer, U. (2004) Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal*, **39**, 425-439.

Dubeaux, G., Neveu, J., Zelazny, E. and Vert, G. (2018) Metal sensing by the IRT1 transporter-receptor orchestrates its own degradation and plant metal nutrition. *Molecular Cell*, **69**, 953-964 e955.

Durrett, T.P., Gassmann, W. and Rogers, E.E. (2007) The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiology*, **144**, 197-205.

Evens, N.P., Buchner, P., Williams, L.E. and Hawkesford, M.J. (2017) The role of ZIP transporters and group F bZIP transcription factors in the Zn-deficiency response of wheat (*Triticum aestivum*). *Plant Journal*, **92**, 291-304.

Fanara, S., Schloesser, M., Hanikenne, M. and Motte, P. (2022) Altered metal distribution in the *sr45-1* Arabidopsis mutant causes developmental defects. *Plant Journal*, **110**, 1332-1352.

Fasani, E., DalCorso, G., Varotto, C., Li, M., Visioli, G., Mattarozzi, M. and Furini, A. (2017) The *MTP1* promoters from *Arabidopsis halleri* reveal *cis*-regulating elements for the evolution of metal tolerance. *New Phytologist*, **214**, 1614-1630.

Frérot, H., Faucon, M.P., Willems, G., Gode, C., Courseaux, A., Darracq, A., Verbruggen, N. and Saumitou-Laprade, P. (2010) Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL x environment interactions. *New Phytologist*, **187**, 355-367.

Gruber, B.D., Giehl, R.F., Friedel, S. and von Wiren, N. (2013) Plasticity of the Arabidopsis root system under nutrient deficiencies. *Plant Physiology*, **163**, 161-179.

Halimaa, P., Lin, Y.F., Ahonen, V.H., Blande, D., Clemens, S., Gyenesei, A., Häikiö, E., Kärenlampi, S.O., Laiho, A., Aarts, M.G., Pursiheimo, J.P., Schat, H., Schmidt, H., Tuomainen, M.H. and Tervahauta, A.I. (2014) Gene expression differences between *Noccaea caerulescens* ecotypes help to identify candidate genes for metal phytoremediation. *Environmental Science and Technology*, **48**, 3344-3353.

Hanikenne, M. and Bouché, F. (2023) Iron and zinc homeostasis in plants: a matter of trade-offs. *Journal of Experimental Botany*, **74**, 5426-5430.

Hanikenne, M., Esteves, S.M., Fanara, S. and Rouached, H. (2021) Coordinated homeostasis of essential mineral nutrients: a focus on iron. *Journal of Experimental Botany,* **72**, 2136-2153.

Hanikenne, M., Kroymann, J., Trampczynska, A., Bernal, M., Motte, P., Clemens, S. and Krämer, U. (2013) Hard selective sweep and ectopic gene conversion in a gene cluster affording environmental adaptation. *PLoS Genetics*, **9**, e1003707.

Hanikenne, M. and Nouet, C. (2011) Metal hyperaccumulation and hypertolerance: a model for plant evolutionary genomics. *Current Opinion in Plant Biology*, **14**, 252-259.

Hanikenne, M., Talke, I.N., Haydon, M.J., Lanz, C., Nolte, A., Motte, P., Kroymann, J., Weigel, D. and Krämer, U. (2008) Evolution of metal hyperaccumulation required *cis*-regulatory changes and triplication of *HMA4*. *Nature*, **453**, 391-395.

Hänsch, R. and Mendel, R.R. (2009) Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Current Opinion in Plant Biology*, **12**, 259-266.

Haydon, M.J., Kawachi, M., Wirtz, M., Hillmer, S., Hell, R. and Krämer, U. (2012) Vacuolar nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in *Arabidopsis*. *The Plant Cell*, **24**, 724-737.

Hussain, D., Haydon, M.J., Wang, Y., Wong, E., Sherson, S.M., Young, J., Camakaris, J., Harper, J.F. and Cobbett, C.S. (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. *The Plant Cell*, **16**, 1327-1339.

Inaba, S., Kurata, R., Kobayashi, M., Yamagishi, Y., Mori, I., Ogata, Y. and Fukao, Y. (2015) Identification of putative target genes of *bZIP19*, a transcription factor essential for *Arabidopsis* adaptation to Zn deficiency in roots. *Plant Journal*, **84**, 323-334.

Krämer, U. (2010) Metal hyperaccumulation in plants. *Annual review of plant biology*, **61**, 517-534.

Krämer, U., Talke, I.N. and Hanikenne, M. (2007) Transition metal transport. *FEBS Letters*, **581**, 2263-2272.

Lee, G., Ahmadi, H., Quintana, J., Syllwasschy, L., Janina, N., Preite, V., Anderson, J.E., Pietzenuk, B. and Krämer, U. (2021a) Constitutively enhanced genome integrity maintenance anddirect stress mitigation characterize transcriptome ofextreme stress-adapted *Arabidopsis halleri*. *Plant Journal*, **108**, 896-911.

Lee, S., Lee, J., Ricachenevsky, F.K., Punshon, T., Tappero, R., Salt, D.E. and Guerinot, M.L. (2021b) Redundant roles of four ZIP family members in zinc homeostasis and seed development in Arabidopsis thaliana. *Plant Journal*, **108**, 1162-1173.

Lilay, G.H., Castro, P.H., Campilho, A. and Assunção, A.G. (2019) The Arabidopsis bZIP19 and bZIP23 activity requires zinc deficiency - insight on regulation from complementation lines. *Frontiers in Plant Science*, **9**, 1955.

Lilay, G.H., Castro, P.H., Guedes, J.G., Almeida, D.M., Campilho, A., Azevedo, H., Aarts, M.G.M., Saibo, N.J.M. and Assuncao, A.G.L. (2020) Rice F-bZIP transcription factors regulate the zinc deficiency response. *Journal of Experimental Botany*, **71**, 3664-3677.

Lilay, G.H., Persson, D., Humberto Castro, P., Liao, F., Alexander, R., Aarts, M.G. and Assunção, A.G. (2021) Arabidopsis bZIP19 and bZIP23 act as zinc sensors to control plant zinc status. *Nature Plants*, **7**, 137–143.

Lin, Y.F., Liang, H.M., Yang, S.Y., Boch, A., Clemens, S., Chen, C.C., Wu, J.F., Huang, J.L. and Yeh, K.C. (2009) Arabidopsis IRT3 is a zinc-regulated and plasma membrane localized zinc/iron transporter. *New Phytologist*, **182**, 392-404.

Merlot, S., Garcia de la Torre, V.S. and Hanikenne, M. (2021) Physiology and Molecular Biology of Trace Element Hyperaccumulation. In *Agromining: Farming for Metals, Mineral Resource Reviews*, pp. 93-116.

Meyer, C.L., Juraniec, M., Huguet, S., Chaves-Rodriguez, E., Salis, P., Isaure, M.P., Goormaghtigh, E. and Verbruggen, N. (2015) Intraspecific variability of cadmium tolerance and accumulation, and cadmium-induced cell wall modifications in the metal hyperaccumulator *Arabidopsis halleri*. *Journal of Experimental Botany*, **66**, 3215-3227.

Milner, M.J., Seamon, J., Craft, E. and Kochian, L.V. (2013) Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis. *Journal of Experimental Botany*, **64**, 369-381.

Nazri, A.Z., Griffin, J.H.C., Peaston, K.A., Alexander-Webber, D.G.A. and Williams, L.E. (2017) F-group bZIPs in barley-a role in Zn deficiency. *Plant, Cell and Environment*, **40**, 2754-2770.

Nouet, C., Charlier, J.-B., Carnol, M., Bosman, B., Farnir, F., Motte, P. and Hanikenne, M. (2015) Functional Analysis of the three *HMA4* copies of the metal hyperaccumulator *Arabidopsis halleri*. *Journal of Experimental Botany*, **66**, 5783-5795.

Ossowski, S., Schwab, R. and Weigel, D. (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant Journal*, **53**, 674-690.

Pineau, C., Loubet, S., Lefoulon, C., Chalies, C., Fizames, C., Lacombe, B., Ferrand, M., Loudet, O., Berthomieu, P. and Richard, O. (2012) Natural Variation at the *FRD3* MATE Transporter Locus

Reveals Cross-Talk between Fe Homeostasis and Zn Tolerance in A*rabidopsis thaliana*. *PLoS Genetics*, **8**, e1003120.

Remy, E., Cabrito, T.R., Batista, R.A., Hussein, M.A., Teixeira, M.C., Athanasiadis, A., Sa-Correia, I. and Duque, P. (2014) Intron retention in the 5'UTR of the novel ZIF2 transporter enhances translation to promote zinc tolerance in arabidopsis. *PLoS Genetics*, **10**, e1004375.

Rengel, Z., Cakmak, I. and White, P.J. (2023) Marschner's mineral nutrition of plants.*4th edn. London: Academic Press.*

Schvartzman, M.S., Corso, M., Fataftah, N., Scheepers, M., Nouet, C., Bosman, B., Carnol, M., Motte, P., Verbruggen, N. and Hanikenne, M. (2018) Adaptation to high zinc depends on distinct mechanisms in metallicolous populations of *Arabidopsis halleri*. *New Phytologist*, **218**, 269-282.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N. and Weigel, D. (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. *The Plant Cell*, **18**, 1121-1133.

Shahzad, Z., Gosti, F., Frérot, H., Lacombe, E., Roosens, N., Saumitou-Laprade, P. and Berthomieu, P. (2010) The five *AhMTP1* zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. *PLoS Genetics*, **6**, e1000911.

Shahzad, Z., Ranwez, V., Fizames, C., Marques, L., Le Martret, B., Alassimone, J., Gode, C., Lacombe, E., Castillo, T., Saumitou-Laprade, P., Berthomieu, P. and Gosti, F. (2013) Plant Defensin type 1 (PDF1): protein promiscuity and expression variation within the Arabidopsis genus shed light on zinc tolerance acquisition in Arabidopsis halleri. *New Phytologist*, **200**, 820-833.

Sinclair, S.A. and Krämer, U. (2012) The zinc homeostasis network of land plants. *Biochimica et Biophysica Acta*, **1823**, 1553-1567.

Sinclair, S.A. and Krämer, U. (2020) Generation of effective zinc-deficient agar-solidified media allows identification of root morphology changes in response to zinc limitation. *Plant Signaling and Behavior*, **15**, 1687175.

Spielmann, J., Ahmadi, H., Scheepers, M., Weber, M., Nitsche, S., Carnol, M., Bosman, B., Kroymann, J., Motte, P., Clemens, S. and Hanikenne, M. (2020) The two copies of the zinc and cadmium ZIP6 transporter of *Arabidopsis halleri* have distinct effects on cadmium tolerance. *Plant, Cell and Environment*, **43**, 2143-2157.

Spielmann, J., Cointry, V., Devime, F., Ravanel, S., Neveu, J. and Vert, G. (2022a) Differential metal sensing and metal-dependent degradation of the broad spectrum root metal transporter IRT1. *Plant Journal,* **112**, 1252-1265.

Spielmann, J., Detry, N., Thiebaut, N., Jadoul, A., Schloesser, M., Motte, P., Perilleux, C. and Hanikenne, M. (2022b) *ZRT-IRT-Like PROTEIN 6* expression perturbs local ion homeostasis in flowers and leads to anther indehiscence and male sterility. *Plant, Cell and Environment*, **45**, 206-219.

Spielmann, J., Fanara, S., Cotelle, V., and Vert, G. (2023) Multilayered regulation of iron homeostasis in Arabidopsis. *Frontiers in Plant Science*. **14**, 1250588.

Stanton, C., Rodriguez-Celma, J., Kraemer, U., Sanders, D. and Balk, J. (2023) BRUTUS-LIKE (BTSL) E3 ligase-mediated fine-tuning of Fe regulation negatively affects Zn tolerance of Arabidopsis. *Journal of Experimental Botany*. **74**, 5767-5782.

Stanton, C., Sanders, D., Kramer, U. and Podar, D. (2022) Zinc in plants: Integrating homeostasis and biofortification. *Molecular Plant*, **15**, 65-85.

Stein, R.J., Horeth, S., de Melo, J.R., Syllwasschy, L., Lee, G., Garbin, M.L., Clemens, S. and Krämer, U. (2017) Relationships between soil and leaf mineral composition are element-specific, environment-dependent and geographically structured in the emerging model *Arabidopsis halleri*. *New Phytologist*, **213**, 1274-1286.

Suryawanshi, V., Talke, I.N., Weber, M., Eils, R., Brors, B., Clemens, S. and Krämer, U. (2016) Between-species differences in gene copy number are enriched among functions critical for adaptive evolution in *Arabidopsis halleri*. *BMC Genomics*, **17**, 1034.

Talke, I.N., Hanikenne, M. and Krämer, U. (2006) Zinc-Dependent Global Transcriptional Control, Transcriptional Deregulation, and Higher Gene Copy Number for Genes in Metal Homeostasis of the Hyperaccumulator *Arabidopsis halleri*. *Plant Physiology*, **142**, 148-167.

Thiébaut, N. and Hanikenne, M. (2022) Zinc deficiency responses: bridging the gap between Arabidopsis and dicotyledonous crops. *Journal of Experimental Botany*, **73**, 1699-1716.

Tóth, B., Veres, S., Bakonyi, N., Gajdos, E., Marozsan, M. and Levai, L. (2012) Industrial side-products as possible soil-amendments. *Journal Environmental Biology*, **33**, 425-429.

Uraguchi, S., Weber, M. and Clemens, S. (2019) Elevated root nicotianamine concentrations are critical for Zn hyperaccumulation across diverse edaphic environments. *Plant, Cell and Environment*, **42**, 2003-2014.

Verbruggen, N., Hermans, C. and Schat, H. (2009) Mechanisms to cope with arsenic or cadmium excess in plants. *Current Opinion in Plant Biology*, **12**, 364-372.

Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M.L., Briat, J.-F. and Curie, C. (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *The Plant Cell*, **14**, 1223-1233.

Wang, Y., Salt, D.E., Koornneef, M. and Aarts, M.G.M. (2022) Construction and analysis of a *Noccaea caerulescens* TILLING population. *BMC Plant Biology*, **22**, 360.

Willems, G., Dräger, D.B., Courbot, M., Gode, C., Verbruggen, N. and Saumitou-Laprade, P. (2007) The genetic basis of zinc tolerance in the metallophyte *Arabidopsis halleri ssp. halleri* (Brassicaceae): an analysis of quantitative trait loci. *Genetics*, **176**, 659-674.

Willems, G., Frérot, H., Gennen, J., Salis, P., Saumitou-Laprade, P. and Verbruggen, N. (2010) Quantitative trait loci analysis of mineral element concentrations in an *Arabidopsis halleri* x *Arabidopsis lyrata petraea* F_2 progeny grown on cadmium-contaminated soil. *New Phytologist*, **187**, 368-379.

Wintermans, J.F. and De Mots, A.S. (1965) Spectrophotometric Characteristics of Chlorophylls a and b and Their Phaeophytins in Ethanol. *Biochimica et Biophysica Acta*, **109**, 448-453.

Zhang, W., Du, B., Liu, D. and Qi, X. (2014) Splicing factor SR34b mutation reduces cadmium tolerance in Arabidopsis by regulating iron-regulated transporter 1 gene. *Biochemical and Biophysical Research Communications*, **455**, 312-317.

Zhu, Y., Dai, Y., Jing, X., Liu, X. and Jin, C. (2022) Inhibition of BRUTUS Enhances Plant Tolerance to Zn Toxicity by Upregulating Pathways Related to Iron Nutrition. *Life* **12**, 216.

Figure legends

Figure 1. *AhbZIP19* and *AhbZIP23* transcript analysis. (A) Schematic representation of the two alternative *AhbZIP19* transcripts (*AhbZIP19-A* and *AhbZIP19-B*). The histidine and cysteine-rich motif and the bZIP domain are shown with black frames. Light and dark blue boxes represent untranslated transcribed and coding sequences, respectively. AUG and STOP correspond to the translation initiation and termination codons within the coding sequences, respectively. (B) Relative transcript levels (RTL) of *AhbZIP19-A* (white), *AhbZIP19-B* (light grey) and *AhbZIP23* (dark grey) in different plant tissues in wild type *A. halleri* plants. The plants were grown for 7 weeks in long days in control hydroponic medium (5µM Zn). Values (mean ± SEM; from three biological replicates each consisting of 2 plants)

are relative to *AhEF1α* and *AhUBQ13*. Individual values are represented by white dots. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between gene) (**p<.001; ***p<.0001, p**** < .0001; ns = not significant) or different letters (between tissues) (p < .05).

Figure 2. Regulation of *AhbZIP19* and *AhbZIP23* by zinc and cadmium availability. Relative transcript level (RTL) of (A) *AhbZIP19-A*, (B) *AhbZIP19-B* and (C) *AhbZIP23* in roots (white) and shoot (grey) of wild type *A. halleri* plants. The plants were grown in long days for two weeks in 1 µM zinc Hoagland medium for rooting, then two weeks in control hydroponic medium (5µM Zn), then exposed for 3 weeks to zinc deficiency (0µM Zn), control (5µM Zn), high zinc (300µM Zn) or cadmium (5µM Zn + 5µM Cd) conditions. Values (mean ± SEM; from three biological replicates each consisting of 2 plants) are relative to *AhEF1α* and *AhUBQ13*. Individual values are represented by white dots. Data were analyzed between conditions by one-way ANOVAs followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by different letters (p < .05) (root, capital letters; shoot, lower case letters).

Figure 3. Complementation of the *A. thaliana* double mutant *bzip19bzip23* with *AhbZIP19-A*, *AhbZIP19-B* and *AhbZIP23*. (A) Shoot fresh weight and (B) root length of Col-0, *bzip19bzip23* double mutant and complemented lines expressing 35S::*AhbZIP19-A* (bZIP19-A), 35S::*AhbZIP19-B* (bZIP19-B) or 35S::*AhbZIP23* (bZIP23). Plants were grown 2 weeks in short days on control (1µM Zn) or zinc deficiency (0.05µM Zn) Hoagland agar medium. Histogram values are means ± SEM from one line for each construct with similar transgene expression (Fig. S3). White dots represent individual values from 28-68 seedlings (roots) or 7-17 pools of 4 seedlings (shoot). Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between conditions) (****p< .0001; ns = not significant) or different letters (between genotypes) (p < .05).

Figure 4. *AhbZIP19* and *AhbZIP23* expression in selected *A. halleri* amiRNA lines. Relative transcript levels (RTL) of *AhbZIP19* and *AhbZIP23* were analyzed in shoots (A) and in roots (B) of two independent control lines (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line) grown on hydroponic medium (5µM Zn). Values (mean ± SEM; from three biological replicates with two independent lines per genotype) are relative to *AhEF1α* and *AhUBQ13* and to one of the control line. Colored dots represent individual datapoints. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between conditions) (p* < .05).

Figure 5. Cadmium tolerance of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*. (A) Pictures of representative leaves (3 leaves of each 2 independent lines) of control lines (CTRL I and CTRL II) and *AhbZIP19* and *AhbZIP23* amiRNA lines (amiRNA I and amiRNA II). (B) Relative chlorophyll concentration in control (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line). Plant were rooted in hydroponic medium supplemented with 1µM Zn for 2 weeks then grown with 5µM Zn for 3 weeks and finally exposed for 3 weeks to control (5µM Zn) or cadmium (5µM Zn + 5µM Cd) conditions. Values are relative to control one line and are means ± SEM from three independent experiments each including 3 plants of two independent lines per genotype. Colored dots represent individual datapoints. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between genotypes) (*p< .05; ns = not significant) or different letters (between conditions) (p < .05).

Figure 6. Zinc and cadmium accumulation in *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*. (A) Shoot and (B) root zinc concentrations, (C) shoot and (D) root cadmium concentrations in control (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line). Plant were rooted in hydroponic medium supplemented with 1µM Zn for 2 weeks then grown with 5µM Zn for 3 weeks and finally exposed for 3 weeks to zinc deficiency (0µM Zn), control (5µM Zn), or cadmium (5µM Zn + 5µM Cd) conditions. Values are means ± SEM from three independent experiments each including 1 plant of two independent lines per genotype. Colored dots represent individual datapoints. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between genotypes) (**p< .01; ***p< .001; ****p< .0001; ns = not significant) or different letters (between conditions) (p < .05).

Figure 7. Zinc and cadmium shoot-to-root concentration ratios in *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*. (A) Zinc and (B) cadmium shoot-to-root ratios in control (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line). The data used in this figure are described in Fig. 6. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between genotypes) (**p< .01; ****p< .0001; ns = not significant) or different letters (between conditions) (p < .05).

Figure 8. Expression profiling zinc homeostasis genes in shoot and roots of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*. Relative transcript levels (RTL) of *AhIRT3* (A,H), *AhZIP4* (B,I), *AhZIP9* (C,J), *AhHMA4* (D,K), *AhFRD3* (E,L), *AhMTP1* (F,M), *AhZIF1* (G,N), and *AhIRT1* (0) in shoots (A-G) and roots (H-0) of control (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line) in *A. halleri*. Plant were rooted in hydroponic medium supplemented with 1µM Zn for 2 weeks then grown with 5µM Zn for 8 weeks. Values are means ± SEM from two (A-C, H-O) to three (D-G) independent experiments each including 1 plant of two independent lines per genotype. Colored dots represent individual datapoints. Data were analyzed by pairwise comparisons using *Student's* t-tests (A,C,D,F,H,I,J,L,N,O) or Mann-Whitney tests (B,E,G,K,M). Statistically significant differences between means are indicated by asterisks (between genotypes) (*p<.05; **p< .01; ****p< .0001; ns = not significant).

Figure 9. Schematic representation of major phenotypes of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*. Are represented the effects of *AhbZIP19* and *AhbZIP23* amiRNAs on the expression of metal homeostasis genes (left) and on zinc, cadmium and chlorophyll concentrations, as well as the shoot-to-root zinc concentration ratio (S/R ratio) (right). =: no change, ↗: increase and ↘: decrease relative to wild-type control plants, respectively. The light green leaf symbolizes chlorosis occurring upon cadmium exposure.

Supplemental Materials

Figure S1. bZIP19 sequence similarity analyses. Alignment of *AtbZIP19, AlbZIP19*, *AhbZIP19-A* and *AhbZIP19-B* coding sequences at nucleotide (A) and amino acid (B) levels. Red and blue letters correspond to conserved nucleotides/amino acid in four and three sequences, respectively. In (A), green and violet boxes correspond to start (ATG) and stop (TGA) codons, respectively. The sequences were aligned using the multalin tool (http://multalin.toulouse.inra.fr/multalin/). (C) Pair-wise comparisons of *AtbZIP19*, *AlbZIP19, AhbZIP19-A* and *AhbZIP19-B* coding sequences. Numbers correspond to percentages of identity at amino acid or nucleotide levels.

Figure S2. Determination of *bZIP19* gene copy numbers in *A. thaliana* (Col-0), *A. lyrata* (Lyr) and *A. halleri* (Lan3.1). Quantitative PCRs were performed in four replicates on genomic DNA. *A. Lyrata* (Lyr) and two single copy genes (*FRD3* and *S13*) were used to normalize gene copy numbers (mean ± SEM). Individual values are represented by white dots. Data were analyzed by one-way ANOVA followed by Bonferroni multiple comparison post-tests. No statistically significant differences have been identified between means (ns = not significant, $p < .05$).

Figure S3. *AhbZIP19* and *AhbZIP23* expression in transgenic *A. thaliana* lines. Relative transcript levels (RTL) of *AhbZIP19* (white) and *AhbZIP23* (grey) were analyzed in *bzip19bzip23* double mutant and complemented lines expressing 35S::*AhbZIP19-A*, 35S::*AhbZIP19-B* or 35S::*AhbZIP23*. Values (mean ± SEM; from three biological replicates for one line for each construct with similar transgene expression) are relative to *AhEF1α* and AT1G58050. White dots represent individual values from 3 seedlings. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by different letters ($p < .05$).

Figure S4. *AhbZIP19* and *AhbZIP23* expression in *A. halleri* amiRNA lines. Relative transcript levels (RTL) of *AhbZIP19* (grey) and *AhbZIP23* (white) were analyzed in shoots of 13 independent *AhbZIP19* and *AhbZIP23* amiRNA lines and one control line (CTRL I) grown on hydroponic medium (5µM Zn). Values (mean ± SEM; from one biological replicates and three technical replicates) are relative to

AhEF1α and *AhUBQ13* and to the control line. Lines 13 (amiRNA I) and 10 (amiRNA II) were selected for further analyses due to the lowest relative transcript levels of *AhbZIP19* and *AhbZIP23*, respectively.

Figure S5. Zinc accumulation in *AhbZIP19* and *AhbZIP23* double amiRNA lines exposed to 300µM Zn. Plant were rooted in hydroponic medium supplemented with 1µM Zn for 2 weeks then grown with 5µM Zn for 3 weeks and finally exposed for 3 weeks to high zinc (300µM Zn) conditions. Values are means ± SEM from one biological replicate including 2 plants of two independent lines per genotype. (A) Pictures of representative leaves (3 leaves of each 2 independent lines) of control lines (CTRL) and *AhbZIP19* and *AhbZIP23* amiRNA lines. (B) Relative chlorophyll concentrations in control (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line). Values are means ± SEM from one experiments including 3 plants of two independent lines per genotype. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. No statistically significant differences were observed between genotypes (ns = not significant) or different between conditions (same letters: $p > .05$). (C) Shoot, (D) root zinc concentrations and (E) shoot-to-root concentration ratios in control (white column; red dots: CRTL I; orange dots: CTRL II) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns; turquoise dots: amiRNA, blue dots: amiRNA II). Values are means ± SEM from one experiments including 1 plants of two independent lines per genotype. Data were analyzed by Mann-Whitney test. No statistically significant differences have been identified between means (ns = not significant, $p < .05$). (B-E) Colored dots represent individual datapoints. For comparison, leaf pictures at 5µM Zn are duplicated from Figure 5.

Figure S6. Metal concentrations in shoot and roots of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*. Metal concentrations in shoot (A) and roots (B) of *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots:

individual values for the amiRNA II line) relative to control lines (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line). Plant were rooted in hydroponic medium supplemented with 1µM Zn for 2 weeks then grown with 5µM Zn for 6 weeks. (C) Manganese concentrations in roots of in control (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line). Plant were rooted in hydroponic medium supplemented with 1µM Zn for 2 weeks then grown with 5µM Zn for 3 weeks and finally exposed for 3 weeks to zinc deficiency (0µM Zn), control (5µM Zn), or cadmium (5µM Zn + 5µM Cd) conditions. (A-C) Values are means ± SEM from three independent experiments each including 2 plants of two independent lines per genotype. Colored dots represent individual datapoints. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between genotypes) (***p< .001; ****p< .0001; ns = not significant) or different letters (between conditions) (p < .05).

Figure S7. Metal shoot-to-root concentration ratios in *AhbZIP19* and *AhbZIP23* double amiRNA lines in *A. halleri*. Shoot-to-root concentration ratios of aluminum (Al), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn) and molybdenum (Mo) in control (white columns: means ± SEM; red dots: individual values for the CRTL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means ± SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line). The data used in this figure are described in Fig. S6. Data were analyzed by two-way ANOVA followed by Bonferroni multiple comparison post-tests. Statistically significant differences between means are indicated by asterisks (between genotypes) (**p< .01; ****p< .0001; ns = not significant) or different letters (between conditions) (p < .05).

Figure S8. Amino acid sequence alignment of the cytosolic regulatory loops of the *A. thaliana* and *A. halleri* IRT1 proteins. Red letters correspond to amino acids conserved in the two sequences. Black and blue letters correspond to non-conserved amino acids. Green and violet horizontal bars correspond to the histidine-rich motif (essential for metal binding) and the serine/threonine amino acids believed to be phosphorylated upon excess of non-iron metals in *A. thaliana*, respectively. The sequences of AtIRT1 and AhIRT1 cytoplasmic loops were aligned using the multalin tool (http://multalin.toulouse.inra.fr/multalin/).