

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, *Arabidopsis thaliana* 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, supposedly 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 downregulated, 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

hyperaccumulation, metal toxicity, metal transport, transcription factor, ZIP

1 | 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; Clemens, 2022; Hänsch & Mendel, 2009). 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 (Amini et al., 2022; Sinclair & Krämer, 2012; Stanton et al., 2022; Thiébaud 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 (Clemens, 2016; Hanikenne & Nouet, 2011; Krämer, 2010; 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 (Clemens, 2016; Hanikenne & Nouet, 2011; 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* (Hanikenne & Nouet, 2011; Krämer, 2010; Merlot et al., 2021; Talke et al., 2006). Among those, *AhNAS2* (*Nicotianamine Synthase 2*) contributes to enhanced zinc radial transport towards the root xylem and to root-to-shoot translocation (Cornu et al., 2014; Deinlein et al., 2012; Uraguchi et al., 2019) and *AhHMA4* (*Heavy Metal ATPase 4*) drives zinc and cadmium loading into the xylem (Courbot et al., 2007; Hanikenne et al., 2008; Hanikenne et al., 2013; Talke et al., 2006). *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; Fasani et al., 2017; Shahzad et al., 2013; Willems et al., 2007). 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 (Krämer et al., 2007; Lin et al., 2009; Talke et al., 2006). However, with the exception of *AhZIP6* (Spielmann et al., 2020; Spielmann, Detry, et al., 2022), 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 (Amini et al., 2022; Sinclair and Krämer, 2012; Stanton et al., 2022; Thiébaud and Hanikenne, 2022). This is most likely due to high functional redundancy among individual *ZIP* genes (Lee, Lee, et al., 2021). 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 (Barberon et al., 2011, 2014; Dubeaux et al., 2018; Spielmann, Cointy, et al., 2022; Vert et al., 2002). 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; Thiébaud 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 N-terminal 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 present in the promoter, and activate the transcription, of a small set of target genes, mostly *ZIPs* (e.g., *ZIP4*, *IRT3*, *ZIP9*) and two *NASs* (*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 (Hanikenne et al., 2008; Talke et al., 2006). 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 splicing 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.

2 | RESULTS

2.1 | *bZIP19* is alternatively spliced in *A. halleri*

Before 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 data set (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 (Figure 1a, Figure S1A and S1B). Because many genes involved in metal homeostasis are present in several copies in the *A. halleri* genome (Suryawanshi et al., 2016; Talke et al., 2006), 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 (Figure S2). These observations indicated that *AhbZIP19* undergoes an alternative splicing event, resulting in the presence of (i) a long transcript, hereafter

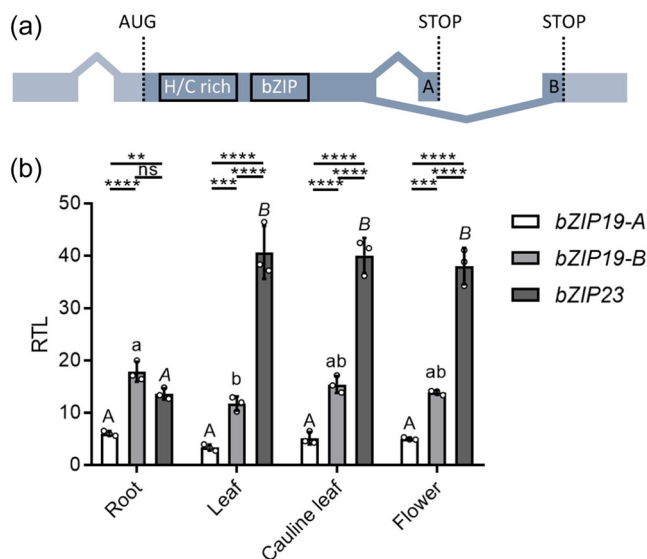


FIGURE 1 *AhbZIP19* and *AhbZIP23* transcript analysis. (a) Schematic representation of the two alternative *AhbZIP19* transcripts (*AhbZIP19-A* and *AhbZIP19-B*). The histidine and cysteine-rich motif and the bZIP domain are shown with black frames. Light and dark blue boxes represent untranslated transcribed 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 (5 μ M Zn). Values (mean \pm SEM; from three biological replicates each consisting of two plants) are relative to *AhEF1 α* 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) (** $p < 0.001$; *** $p < 0.0001$; **** $p < 0.0001$; ns, not significant) or different letters (between tissues) ($p < 0.05$).

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 (Figure 1a and Figure S1C).

2.2 | *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 ~ 3 x more expressed than *AhbZIP19-A* in all tested tissues (Figure 1b). In contrast, *AhbZIP23* was strongly expressed in aerial parts (leaves, cauline leaves and flowers) but only moderately expressed in roots (Figure 1b).

AhbZIP19-A and *AhbZIP19-B* were upregulated in roots, upon high zinc exposure (300 μ M Zn) compared to control conditions (5 μ M Zn) (Figure 2a,b). In addition, *AhbZIP19-B* was downregulated

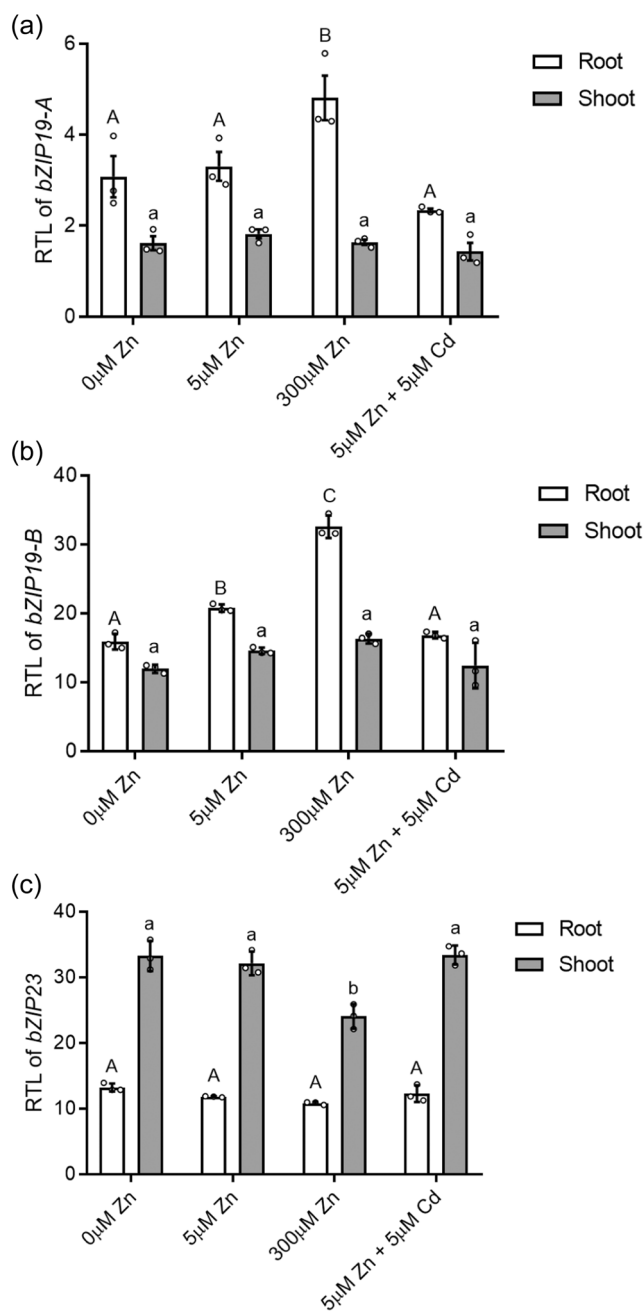


FIGURE 2 Regulation of *AhbZIP19* and *AhbZIP23* by zinc and cadmium availability. Relative transcript level (RTL) of (a) *AhbZIP19-A*, (b) *AhbZIP19-B* and (c) *AhbZIP23* in roots (white) and shoot (grey) of wild type *A. halleri* plants. The plants were grown in long days for 2 weeks in 1 μ M zinc Hoagland medium for rooting, then 2 weeks in control hydroponic medium (5 μ M Zn), then exposed for 3 weeks to zinc deficiency (0 μ M Zn), control (5 μ M Zn), high zinc (300 μ M Zn) or cadmium (5 μ M Zn + 5 μ M Cd) conditions. Values (mean \pm SEM; from three biological replicates each consisting of two plants) are relative to *AhEF1 α* 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 ($p < 0.05$) (root, capital letters; shoot, lower case letters).

in roots upon zinc deficiency (0 μM Zn) and in presence of cadmium (5 μM Cd) (Figure 2b). The *AhbZIP23* expression level was not affected by zinc or cadmium supply in roots and was downregulated in shoot by 300 μM Zn (Figure 2c). These results are in contrast to those previously reported in *A. thaliana* where *AtbZIP19* and *AtbZIP23* are upregulated by zinc deficiency and downregulated 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*.

2.3 | *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 (Figure S3) and grown on agar plates in control condition (1 μM Zn) or in zinc deficiency (0.05 μM 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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*.

2.4 | 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 (Hanikenne et al., 2008; Talke et al., 2006) with a single construct encoding two distinct artificial microRNAs (Ossowski et al., 2008;

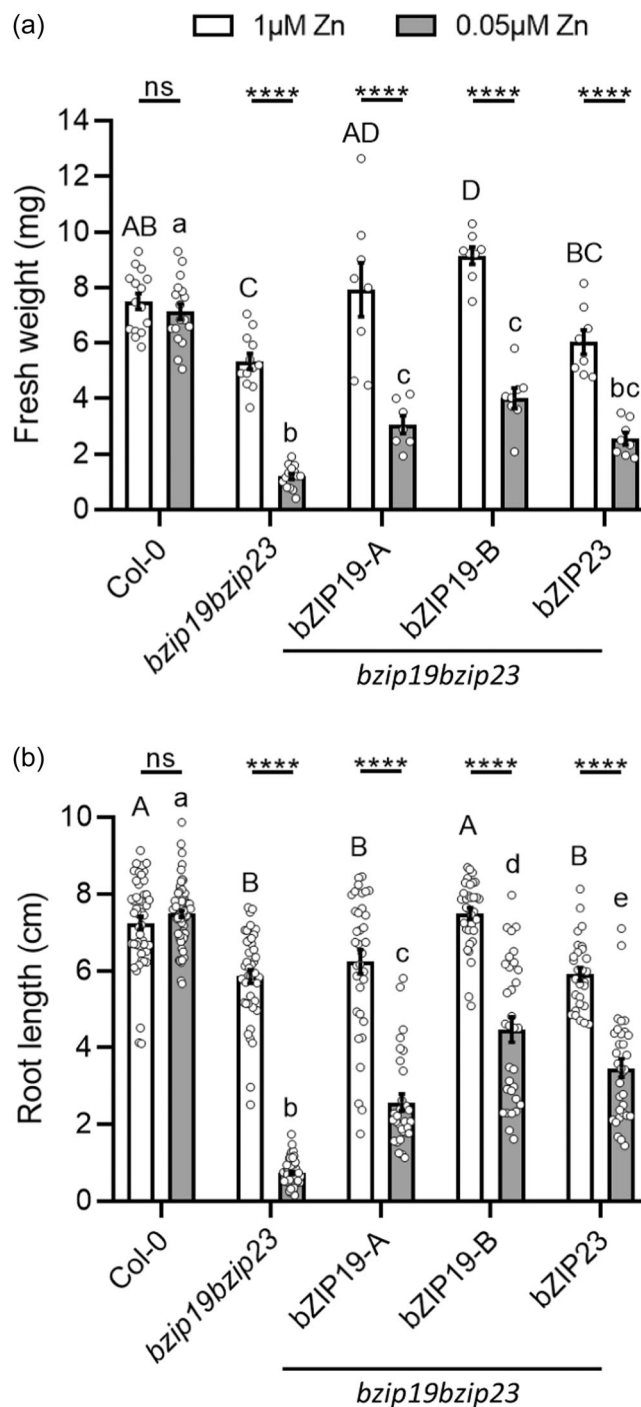


FIGURE 3 Complementation of the *Arabidopsis thaliana* double mutant *bzip19bzip23* with *AhbZIP19-A*, *AhbZIP19-B* and *AhbZIP23*. (a) Shoot fresh weight and (b) root length of Col-0, *bzip19bzip23* double mutant and complemented lines expressing 35S::*AhbZIP19-A* (*bZIP19-A*), 35S::*AhbZIP19-B* (*bZIP19-B*) or 35S::*AhbZIP23* (*bZIP23*). Plants were grown 2 weeks in short days on control (1 μM Zn) or zinc deficiency (0.05 μM Zn) Hoagland agar medium. Histogram values are means \pm SEM from one line for each construct with similar transgene expression (Figure S3). White dots represent individual values from 28 to 68 seedlings (roots) or 7 to 17 pools of four 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) (**** $p < 0.0001$; ns, not significant) or different letters (between genotypes) ($p < 0.05$).

Schwab et al., 2006), 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 (Figure 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 lines) were identified (Figure 4) and used for further analyses.

The two control and the two amiRNA lines were then grown in Hoagland hydroponic culture. After 2 weeks of rooting (1 μM Zn), and 3 weeks of growth in control condition (5 μM Zn), plants were exposed to four different conditions: (i) zinc deficiency (0 μM Zn), (ii) control condition (5 μM Zn), (iii) high zinc (300 μM Zn) and (iv) cadmium exposure (5 μM Zn and 5 μM Cd) for 3 weeks. Despite the transcriptional regulation of *AhbZIP19* and *AhbZIP23* by high zinc (Figure 2), this condition had very limited effect on growth and zinc accumulation in both control and amiRNA genotypes (Figure 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 (Figure 5a). Indeed, chlorophyll concentrations dropped by 72% and 33% in amiRNA and control lines, respectively, upon cadmium treatment (Figure 5b). Moreover, amiRNA lines accumulated more cadmium in both roots (75%) and shoot (50%) than control lines after cadmium exposure (Figure 6a,b). The amiRNA lines similarly accumulated more zinc in roots (380–700%) and shoot (60–135%) in control and cadmium conditions, respectively, compared to control lines (Figure 6a,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 (Figure 6a,b). Finally, roots of amiRNA lines accumulated 93%, 68% and 38% more manganese than control lines in control, zinc deficiency and cadmium conditions, respectively (Figure S6B,C). None of the other measured elements were affected in roots or shoot of amiRNA lines (Figure 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.

2.5 | 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 (Hanikenne et al., 2008; Talke et al., 2006). In all three tested conditions (zinc deficiency, control & cadmium exposure), amiRNA and control lines displayed zinc shoot-to-root ratios above 1. However, these ratios were lower (by 45, 66 and 73%, respectively) in amiRNA lines (Figure 7a), suggesting that, in these lines over-accumulating zinc (Figure 6), (i) a larger part of zinc 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 (Figure 7b). Finally, calcium and magnesium (as well as manganese, but non-significantly) shoot-root ratios were reduced in amiRNA lines (Figure S7).

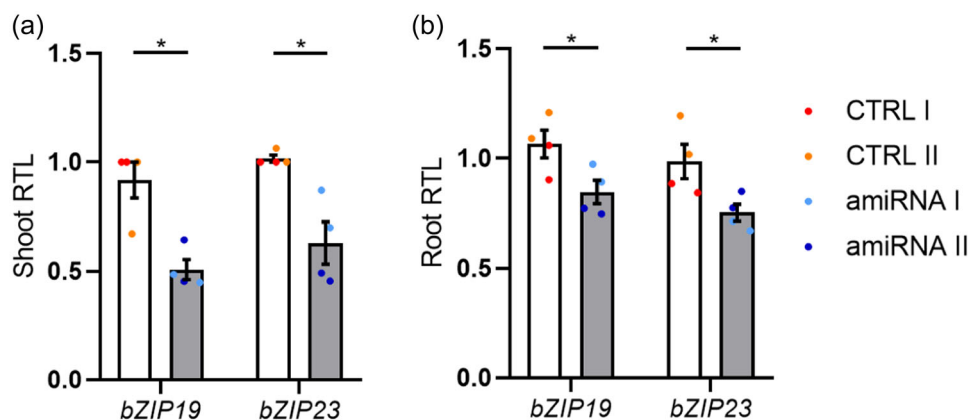


FIGURE 4 *AhbZIP19* and *AhbZIP23* expression in selected *Arabidopsis halleri* amiRNA lines. Relative transcript levels (RTL) of *AhbZIP19* and *AhbZIP23* were analyzed in shoots (a) and in roots (b) of two independent control lines (white columns: means \pm SEM; red dots: individual values for the CTRL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means \pm SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line) grown on hydroponic medium (5 μM Zn). Values (mean \pm SEM; from three biological replicates with two independent lines per genotype) are relative to *AhEF1a* 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) ($*p < 0.05$).

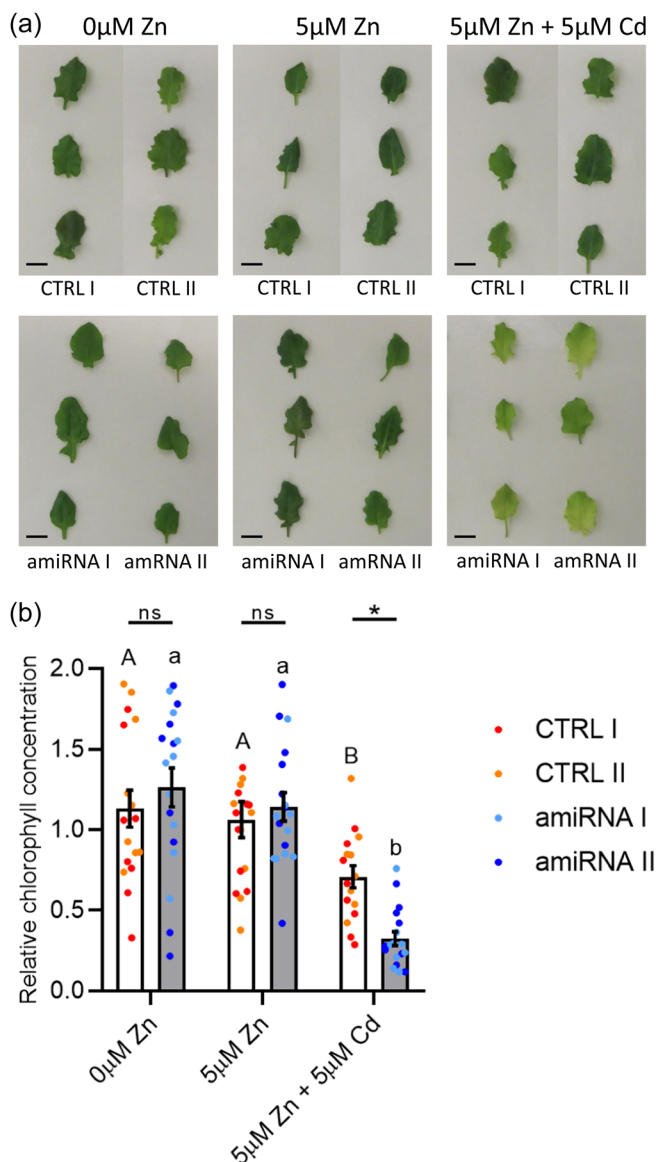


FIGURE 5 Cadmium tolerance of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *Arabidopsis halleri*. (a) Pictures of representative leaves (three leaves of each two independent lines) of control lines (CTRL I and CTRL II) and *AhbZIP19* and *AhbZIP23* amiRNA lines (amiRNA I and amiRNA II). (b) Relative chlorophyll concentration in control (white columns: means \pm SEM; red dots: individual values for the CTRL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means \pm 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 1 μ M Zn for 2 weeks then grown with 5 μ M Zn for 3 weeks and finally exposed for 3 weeks to control (5 μ M Zn) or cadmium (5 μ M Zn + 5 μ M Cd) conditions. Values are relative to control one line and are means \pm SEM from three independent experiments each including three 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) ($*p < 0.05$; ns, not significant) or different letters (between conditions) ($p < 0.05$).

2.6 | 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 (Figure 8a–c,h–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 (Hanikenne et al., 2008; Hussain et al., 2004), was not differently expressed in amiRNA lines compared to control lines (Figure 8d,k). In contrast, the expression of *AhFRD3*, encoding a citrate transporter implicated in iron and zinc homeostasis and root-to-shoot translocation (Charlier et al., 2015; Durrett et al., 2007; Pineau et al., 2012), was reduced by 35% in roots (Figure 8l). Among genes involved in vacuolar metal storage, *AhMTP1*, encoding a zinc tonoplasmic transporter (Desbrosses-Fonrouge et al., 2005; Dräger et al., 2004; Fasani et al., 2017; Shahzad et al., 2010), and *AhZIF1*, encoding a tonoplasmic 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 (Figure 8f,g,m,n). Finally, *AhIRT1*, encoding a broad spectrum metal transporter involved in metal uptake (including zinc and cadmium) (Barberon et al., 2011; Spielmann, Cointy et al., 2022; Vert et al., 2002), was the most strongly (~300%) upregulated gene among all tested genes in roots of amiRNA lines (Figure 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 nontarget genes such as *AhFRD3*, *AhIRT1*, *AhMTP1*, or *AhZIF1* were indirectly impacted, possibly to compensate the misregulation of *AhbZIP19* and *AhbZIP23* target genes.

3 | 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 ZIP target genes, and is sufficient to result in major phenotypic outcomes.

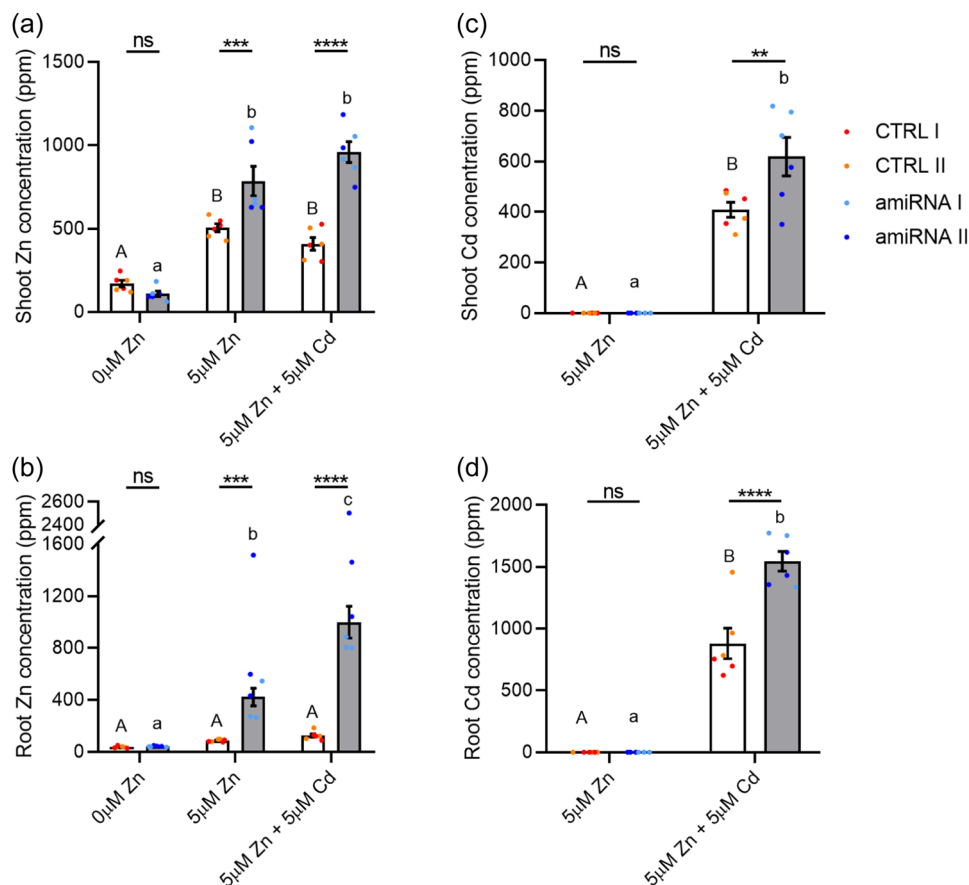


FIGURE 6 Zinc and cadmium accumulation in *AhbZIP19* and *AhbZIP23* double amiRNA lines in *Arabidopsis halleri*. (a) Shoot and (b) root zinc concentrations, (c) shoot and (d) root cadmium concentrations in control (white columns: means \pm SEM; red dots: individual values for the CTRL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means \pm SEM; turquoise dots: individual values for the amiRNA I line, blue dots: individual values for the amiRNA II line). Plants were rooted in hydroponic medium supplemented with 1 μ M Zn for 2 weeks then grown with 5 μ M Zn for 3 weeks and finally exposed for 3 weeks to zinc deficiency (0 μ M Zn), control (5 μ M Zn), or cadmium (5 μ M Zn + 5 μ M Cd) conditions. Values are means \pm SEM from three independent experiments each including one 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 < 0.01; *** p < 0.001; **** p < 0.0001; ns, not significant) or different letters (between conditions) (p < 0.05).

3.1 | 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 (Charlier et al., 2015; Dong et al., 2018; Remy et al., 2014). 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 (Dong et al., 2018; Fanara et al., 2022; Zhang et al., 2014).

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 (Corso et al., 2021; Schwartzman et al., 2018) 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

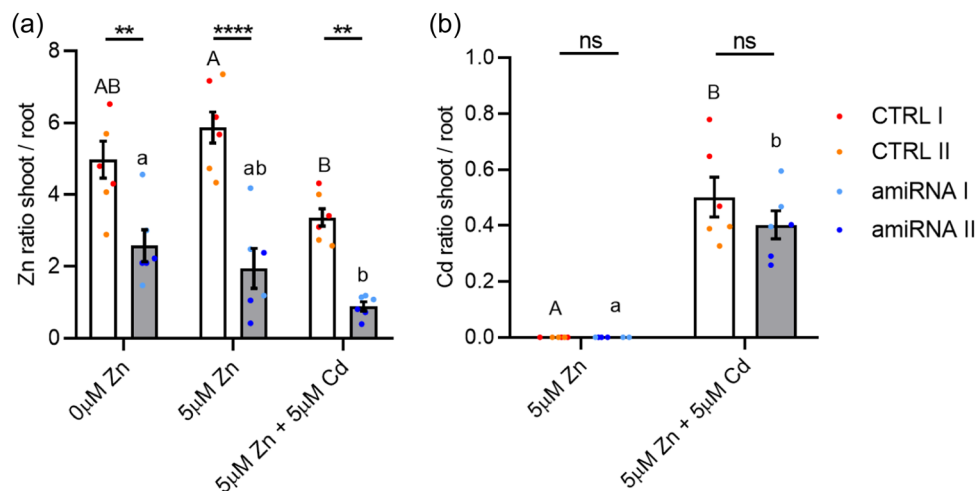


FIGURE 7 Zinc and cadmium shoot-to-root concentration ratios in *AhbZIP19* and *AhbZIP23* double amiRNA lines in *Arabidopsis halleri*. (a) Zinc and (b) cadmium shoot-to-root ratios in control (white columns: means \pm SEM; red dots: individual values for the CTRL I line; orange dots: individual values for the CTRL II line) and *AhbZIP19* and *AhbZIP23* amiRNA lines (grey columns: means \pm 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 Figure 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) (** $p < 0.01$; **** $p < 0.0001$; ns, not significant) or different letters (between conditions) ($p < 0.05$).

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; Evens et al., 2017; Inaba et al., 2015; Lilay et al., 2020, 2021; Nazri et al., 2017), using a 35S promoter to drive the ectopic over-expression 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.

3.2 | 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 turn induces a zinc deficiency response and the high expression of zinc uptake *ZIP* genes, further supporting hyperaccumulation (Hanikenne & Nouet, 2011; Hanikenne et al., 2008; Krämer, 2010; 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 caerulea* 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* (Barberon et al., 2011; Spielmann, Cointy, et al., 2022; Vert et al., 2002), whose transcriptional expression is increased in *AhbZIP19/AhbZIP23* double amiRNA lines.

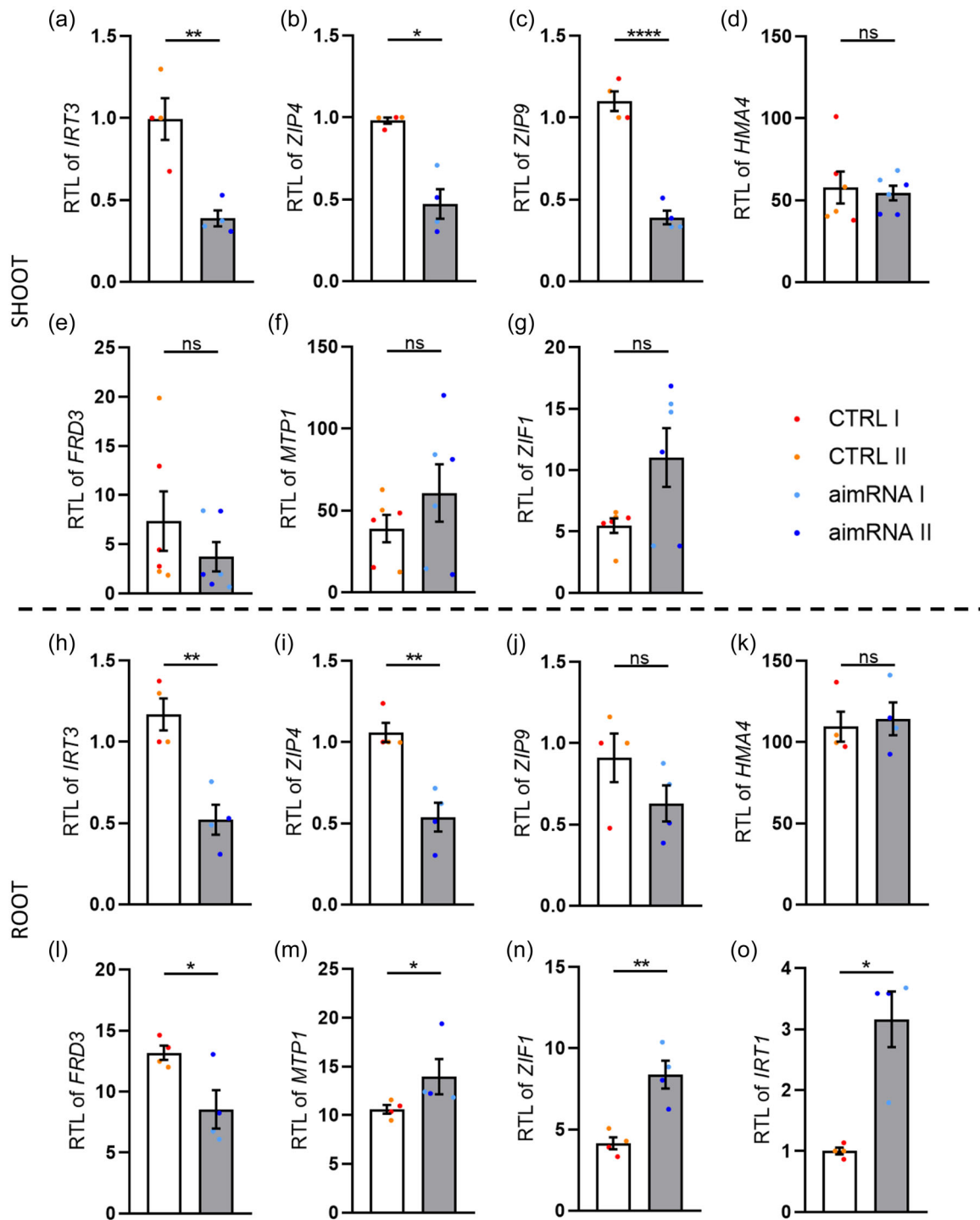


FIGURE 8 Expression profiling zinc homeostasis genes in shoot and roots of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *Arabidopsis halleri*. Relative transcript levels (RTL) of *AhIRT3* (a, h), *AhZIP4* (b, i), *AhZIP9* (c, j), *AhHMA4* (d, k), *AhFRD3* (e, l), *AhMTP1* (f, m), *AhZIF1* (g, n), and *AhIRT1* (o) in shoots (a–g) and roots (h–o) of control (white columns: means ± SEM; red dots: individual values for the CTRL 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*. Plants were rooted in hydroponic medium supplemented with 1 μM Zn for 2 weeks then grown with 5 μM Zn for 8 weeks. Values are means ± SEM from two (a–c, h–o) to three (d–g) independent experiments each including one 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* < 0.05; ***p* < 0.01; *****p* < 0.0001; ns, not significant).

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 hyper-accumulator plant species, *A. halleri* (Corso et al., 2018; Schwartzman 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, Cointry, et al., 2022; 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, Cointry, et al., 2022). 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 (Figure 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, Cointry, et al., 2022). 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; Corso et al., 2018; Meyer et al., 2015; Schwartzman et al., 2018; Stein et al., 2017). 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).

3.3 | 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 ~eightfold 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* (Frérot et al., 2010; Hanikenne et al., 2008; 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 (Desbrosses-Fonrouge et al., 2005; Dräger et al., 2004; Fasani et al., 2017; Haydon et al., 2012; Shahzad et al., 2010), 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 were recently shown to display increased zinc accumulation in roots, altered zinc radial transport and reduced zinc shoot-to-root ratios (Lee, Lee, et al., 2021), 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, Lee, et al., 2021). 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.

3.4 | 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 hijacks transporters of other divalent metal cations, zinc transporters 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, Lee, et al. (2021) 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 (Baliardini et al., 2015; Courbot et al., 2007; Frérot et al., 2010; Willems et al., 2007; Willems et al., 2010). 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).

4 | 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 (Figure 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; Hanikenne & Bouché, 2023; Stanton et al., 2023; Zhu et al., 2022).

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 (Figure 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, 2021; Lee, Ahmadi, et al., 2021; Schwartzman et al., 2018). 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 (Hanikenne & Nouet, 2011; Krämer, 2010) and whose overexpression is (almost) strictly constitutive, and additional genes (e.g., *AhIRT1*, *AhZIF1*, *AhFRD3*, ...), whose expression is much more flexible and which are involved in fine-tuning these processes (Merlot et al., 2021).

5 | METHODS

5.1 | Plant material and growth conditions

A. halleri ssp. *halleri* plants, from the Langelsheim population (Hanikenne et al., 2008; Talke et al., 2006) 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 2 weeks in 1 µM zinc Hoagland medium for rooting, then 2 weeks in 5 µM zinc Hoagland medium, then submitted to experimental conditions for 3 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 Hanikenne et al., 2008; Talke et al., 2006) 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 (ZnSO₄·H₂O) and cadmium (CdSO₄·8/3H₂O) 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 16 h (long days) light (100 µmol photon m⁻²s⁻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 h 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 2 days. Plants were then cultivated in climate-controlled growth chambers at 20°C (night and day), with a photoperiod of 8 h (short days) light (100 µmol photon m⁻²s⁻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.

5.2 | Plant transformation

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

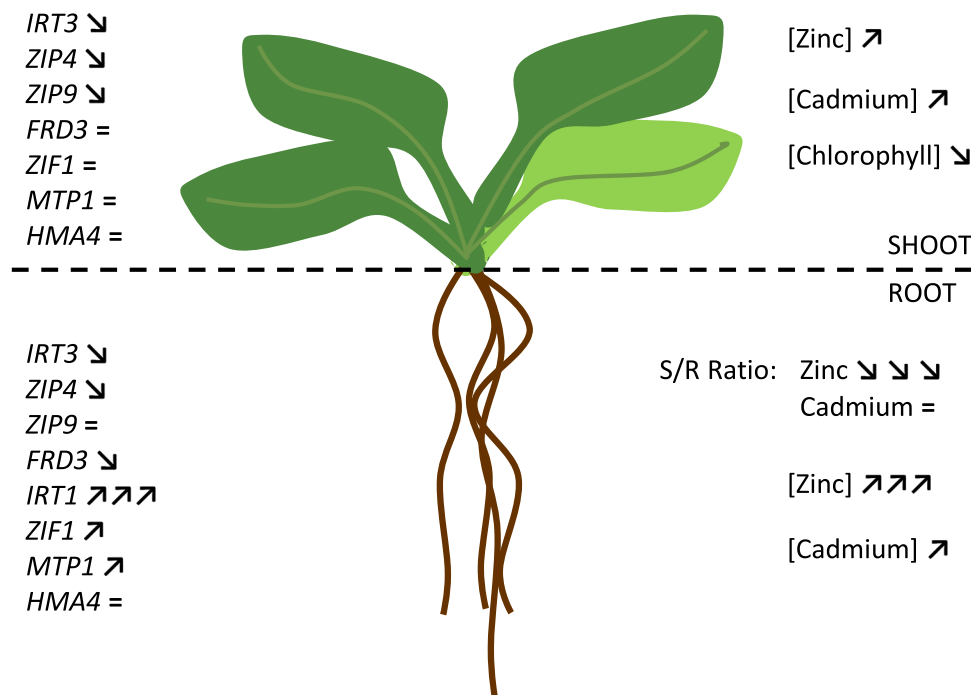


FIGURE 9 Schematic representation of major phenotypes of *AhbZIP19* and *AhbZIP23* double amiRNA lines in *Arabidopsis halleri*. Are represented the effects of *AhbZIP19* and *AhbZIP23* amiRNAs on the expression of metal homeostasis genes (left) and on zinc, cadmium and chlorophyll concentrations, as 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. [Color figure can be viewed at wileyonlinelibrary.com]

5.3 | 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.

5.4 | 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.

5.5 | 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, [Hanikenne et al., 2008; Talke et al., 2006]) 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 *Ascl/PacI* sites of the pMDC32 vector under the control of a 2x35S promoter (Curtis & Grossniklaus, 2003).

The DNA fragment encoding the cytoplasmic loop of AhIRT1 was amplified by PCR using *A. halleri* [Lan3.1 individual, (Hanikenne et al., 2008; Talke et al., 2006)] 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 *KpnI/SacII* sites by PCR with the appropriate primers (Table S3). PCR fragments were gel-purified and cloned into the *KpnI/SacII* sites of the pMDC32 vector under the control of a 2x35S promoter (Curtis & Grossniklaus, 2003).

5.6 | 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 4 days. 20–30 mg of dried tissues were digested with 3 ml

of $\geq 65\%$ 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 $200\ \mu\text{L}$ of $\geq 65\%$ 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).

5.7 | 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 40 h at room temperature. Samples were diluted 10 times in 96% Ethanol and absorbances were measured at 649 and 665 nm. Total chlorophyll concentrations were calculated as followed $\text{Chl}_{\text{Tot}} = ((6.1 \times A_{665}) + (20.4 \times A_{649})) \times 10 / \text{fresh weight (mg)}$ (Wintermans and De Mots, 1965), then presented as values relative to a control line.

5.8 | 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 indicated in figure legends.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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