The two copies of the zinc and cadmium ZIP6 transporter of Arabidopsis halleri have distinct effects on cadmium tolerance

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Abstract
Plants have the ability to colonize highly diverse environments. The zinc and cadmium hyperaccumulator Arabidopsis halleri has adapted to establish populations on soils covering an extreme range of metal availabilities. The A. halleri ZIP6 gene presents several hallmarks of hyperaccumulation candidate genes: it is constitutively highly expressed in roots and shoots and is associated with a zinc accumulation quantitative trait locus. Here, we show that AhZIP6 is duplicated in the A. halleri genome. The two copies are expressed mainly in the vasculature in both A. halleri and Arabidopsis thaliana, indicative of conserved cis regulation, and acquired partial organ specialization. Yeast complementation assays determined that AhZIP6 is a zinc and cadmium transporter. AhZIP6 silencing in A. halleri or expression in A. thaliana alters cadmium tolerance, but has no impact on zinc and cadmium accumulation. AhZIP6-silenced plants display reduced cadmium uptake upon short-term exposure, adding AhZIP6 to the limited number of Cd transporters supported by in planta evidence. Altogether, our data suggest that AhZIP6 is key to fine-tune metal homeostasis in specific cell types. This study additionally highlights the distinct fates of duplicated genes in A. halleri.

KEYWORDS
Arabidopsis halleri, cadmium tolerance, gene duplication, hyperaccumulation, male sterility, sub-functionalization, zinc

Julien Spielmann and Hassan Ahmadi contributed equally to this study.

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1 | INTRODUCTION

All organisms have to maintain adequate metal ion homeostasis to ensure growth and development in varying environments. For sessile organisms such as plants, this represents an even more complicated challenge. Plants have developed a sophisticated and tightly regulated metal homeostasis network of transporters and chelating molecules to ensure sufficient metal supply while avoiding their toxicity (Palmer & Guerinot, 2009; Ricachevskiy, Menguer, Sperotto, & Fett, 2015; Sinclair & Krämer, 2012). This allows plants to develop in environments that substantially differ in metal availability. Hence, plants employ two main strategies to colonize natural or anthropogenic metal-polluted soils (Baker, 1981). The majority of plant species are excluders, limiting metal absorption, exporting metal ions in excess out of roots and limiting root-to-shoot translocation to protect the aerial photosynthetically active tissues. In contrast, about 720 species have developed a so-called hyperaccumulation strategy, where large amounts of metals are stored in shoot tissues without toxicity symptoms (Hanikenne & Nouet, 2011; Krämer, 2010; Merlot, de la Torre, & Hanikenne, 2018; Reeves et al., 2018; Verbruggen, Hermans, & Schat, 2009). Among those, only a few are zinc and cadmium hyperaccumulators, with commonly accepted hyperaccumulation thresholds of 3,000 μg g\(^{-1}\) of zinc and 100 μg g\(^{-1}\) of cadmium in leaf dry weight (Krämer, 2010; van der Ent, Baker, Reeves, Pollard, & Schat, 2013). Hyperaccumulator plants are regarded as models to unveil adaptation to extreme environments, but also to reveal homeostatic mechanisms controlling metal distribution in plants (Hanikenne & Nouet, 2011; Krämer, 2010).

With Noccaea caerulescens and two Sedum species, Arabidopsis halleri is a metal hyperaccumulator model (Merlot et al., 2018). It is a highly zinc and cadmium tolerant species and displays species-wide zinc hyperaccumulation, both in metallophilous (living on polluted soils) and in the more common non-metallophilous populations (Bert, Bonnin, Saumitou-Laprade, De Laguérie, & Petit, 2002; Stein et al., 2017). Cadmium hyperaccumulation is highly variable and restricted to a more limited number of A. halleri populations (Meyer et al., 2015; Stein et al., 2017). This suggests that zinc and cadmium hyperaccumulation rely on at least partially distinct pathways.

Physiologically, the hyperaccumulation process may be summarized to four main steps: (a) an active and efficient metal uptake from the soil, (b) an efficient radial transfer of metals to vascular tissues combined with low vacuolar storage in roots, (c) an enhanced xylem loading and root-to-shoot translocation and finally (d) an efficient detoxification process enabled by efficient shoot metal distribution and leaf vacuolar storage (Clemens, 2016; Hanikenne & Nouet, 2011; Merlot et al., 2018).

A. halleri genes with putative functions in these processes have been identified using quantitative trait locus (QTL) and/or cross-species transcriptomic analyses. Interspecific crosses between A. halleri and Arabidopsis lyrata ssp. petraea, a non-hyperaccumulator relative, were used to map QTLs for zinc tolerance (Willems et al., 2007) and accumulation (Frérot et al., 2010), as well as cadmium tolerance (Baliardini, Meyer, Salis, Saumitou-Laprade, & Verbruggen, 2015; Courbot et al., 2007) and accumulation (Willems et al., 2010), in back-cross 1 or F2 populations. Transcriptomics comparing A. halleri with Arabidopsis thaliana allowed the identification of about 50 metal homeostasis genes, encoding transporters or metal chelator synthesis enzymes, which are more highly expressed in A. halleri. High expression levels of a number of these genes, but not all (see Charlier et al., 2015) were linked to gene copy number expansion and/or cis-regulatory changes (Dräger et al., 2004; Fasani et al., 2017; Hanikenne et al., 2008; Shahzad et al., 2010; Suryawanshi et al., 2016). More recently, these approaches were also employed to examine the molecular basis of intraspecific variation in the zinc and cadmium hyperaccumulation and hypertolerance traits in A. halleri (Corso et al., 2018; Karam et al., 2019; Schwartzman et al., 2018).

A number of candidate genes were commonly identified by the QTL and transcriptomics approaches (Hanikenne & Nouet, 2011; Krämer, 2010) but so far a direct contribution to hypertolerance and/or hyperaccumulation has been experimentally confirmed for only three of these genes by gene silencing in A. halleri. HMA4 (HEAVY METAL ATPase 4) has a major contribution to both zinc and cadmium hypertolerance and hyperaccumulation (Hanikenne et al., 2008), whereas NAS2 (Nicotianamine Synthase 2) contributes to zinc root-to-shoot translocation (Cornu et al., 2014; Deinlein et al., 2012; Uruguchi, Weber, & Clemens, 2019). Finally, CAX1 (CATION/HYDROGEN EXCHANGER 1) is required to minimize cadmium-triggered oxidative damages at low calcium supply (Ahmadi, Corso, Weber, Verbruggen, & Clemens, 2018; Baliardini et al., 2015).

Among uncharacterized candidate genes is ZIP6 (ZRT-IRT-like PROTEIN 6). It is 9- and 24-fold more highly expressed in roots and shoots of A. halleri compared with A. thaliana, respectively (Becher, Talke, Krall, & Krämer, 2004; Talke, Hanikenne, & Krämer, 2006) and is associated with a QTL for zinc accumulation in the presence of cadmium (Willems et al., 2010). It may be present in multiple copies in the genome (Suryawanshi et al., 2016; Talke et al., 2006). Moreover, it was found as highly expressed in roots and shoots of zinc hyperaccumulating F3 progenies of a A. halleri and A. lyrata ssp. petraea cross (Filatov et al., 2006). ZIP6 is also highly expressed in shoots of N. caerulescens, another zinc and cadmium hyperaccumulator (Hammond et al., 2006; Krämer, Talke, & Hanikenne, 2007) and, interestingly, it was part of a list of 12 genes whose expression correlated with cadmium accumulation among N. caerulescens populations (Halima et al., 2014; Wu et al., 2009).

ZIP6 belongs to the ZIP family of divergent metal cation transporters (Guerinot, 2000). ZIPs are described as cytoplasmic influx antiporters, which use the energy of the proton gradient to transport a wide variety of cations (zinc, iron, manganese, copper and cadmium) (Guerinot, 2000). The knowledge on metal specificity, subcellular localization and overall function of individual ZIP transporters in plant metal homeostasis remains very fragmentary (Ricachevskiy et al., 2015).

In contrast to most of its family members, ZIP6 expression is not induced upon zinc deficiency in A. thaliana (Talke et al., 2006) and zip6 mutant plants do not display obvious phenotypes (Wu et al., 2009). Overexpression of the N. caerulescens ZIP6 (ZNT6) in A. thaliana...
resulted in a weak increase of root cadmium sensitivity in one of three independent transgenic lines (Wu et al., 2009).

Here, we examined the contribution of the *A. halleri* ZIP6 (AhZIP6) gene to zinc and cadmium tolerance and hyperaccumulation. We showed a tandem duplication of ZIP6 in the genome of different *A. halleri* ecotypes and a partial tissue specialization of the two copies. We determined that AhZIP6 is a zinc and cadmium transporter, mainly expressed in vascular tissues. Using ZIP6 RNAi *A. halleri* lines and *A. thaliana* transgenic plants overexpressing AhZIP6, we demonstrated that AhZIP6 alters cadmium tolerance.

2 | RESULTS

2.1 | ZIP6 is duplicated in tandem in the *A. halleri* genome

Several highly expressed metal homeostasis genes are present in multiple copies in the *A. halleri* genome (e.g. MTP1 or HMA4) (Dräger et al., 2004; Hanikenne et al., 2008; Shahzad et al., 2010; Suryawanshi et al., 2016). To determine AhZIP6 gene copy number at a species-wide level, six ecotypes were selected from the two genetic units and

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**FIGURE 1** Analysis of the ZIP6 locus in different *Arabidopsis* species. (a) Determination of ZIP6 gene copy numbers in *A. thaliana* (Col-0), *A. lyrata* (Lyr) and different populations of *A. halleri* (I16, I30, Lan3.1, Lan5, Pl22 and Sk13) originating from the South-East (SE), North-West (NW) and Hydrid Zone (HZ) genetic units defined by Pauwels et al. (2012). Quantitative PCR was performed in triplicate on genomic DNA of pools of 10 individuals per population using two sets of primers (I and II). *A. thaliana* (Col-0) and two single copy genes (FRD3 and S13) were used to normalise gene copy numbers (means ± SEM; from one experiment representative of two independent experiments). (b) Schematic representation of the ZIP6 promoters. Based on multiple alignments, coloured boxes represent conserved DNA sequences with more than 75% of identity and a minimal length of 30 bp. Red, orange and yellow indicate the presence of conserved boxes in four, three and two promoters, respectively. Letters and numbers show boxes correspondence and position. Arrowheads represent the orientation of the boxes. All ZIP6 coding sequences are represented by black boxes and are not at the same scale. ZIP6 promoter sequences were collected from Phytozome (https://phytozome.jgi.doe.gov/) for *A. thaliana* and *A. lyrata* and were cloned from the Lan3.1 population for *A. halleri* in this study.
the hybrid zone identified within the geographic distribution of *A. halleri* in Europe to include both metallicolous (I16, Lan3.1, Lan5, P122) and non-metallicolous (I30, Sk13) populations (Pauwels et al., 2012). As expected, a single ZIP6 copy was detected in the *A. thaliana* and *A. lyrata* genomes (Figure 1a), whereas ZIP6 was duplicated in all *A. halleri* ecotypes, except in I16 where it was triplicated (Figure 1a).

To further examine the genomic architecture of the ZIP6 loci in the *A. halleri* genome, bacterial artificial chromosome (BAC) clones (Hanikenne et al., 2013) harbouring *AhZIP6* were isolated and analysed by quantitative PCR, which confirmed the gene duplication and indicated that both *AhZIP6* copies were on the same genome segment (Figure S1). PCR analyses determined a tandem and forward organization of the two copies separated by ~5 kb in the Lan3.1 genome (Figure 1b).

The promoter of the *AhZIP6* copy 1 (pAhZIP6-1, 3,038 bases upstream the ATG codon) of Lan3.1 was cloned by PCR thanks to sequence conservation with the *A. thaliana* (pAtZIP6) and *A. lyrata* (pAlZIP6) genomic sequences (Figure 1b). No sequence information was available for the *AhZIP6* copy 2 promoter. BAC clone Illumina sequencing and draft assemblies identified putative sequences that were then cloned by PCR from Lan3.1 genomic DNA (pAhZIP6-2, 3,116 bases upstream

**FIGURE 2** Localization of promoter activity and transcript level of *AtZIP6* or two *AhZIP6* copies in *A. thaliana* and *A. halleri*. (a) Histochemical detection of GUS activity (blue) directed by the pAtZIP6, pAhZIP6-1 or pAhZIP6-2 promoters in roots and leaves in *A. thaliana* (Top) and *A. halleri* (Bottom). Pictures are representative of six independent lines per construct. (b) Relative transcript levels (RTL) of *AhZIP6-1*, *AhZIP6-2* and both copies (*AhZIP6-Tot*) in wild-type (Lan3.1) *A. halleri* plants grown 7 weeks (long days) in Hoagland hydroponic medium in control condition (5 μM Zn). Values (mean ± SEM; from three biological replicates of two plants each) are relative to *EF1α* and *UBQ13*. (c) ZIP6 RTL in transgenic homozygous *A. thaliana* plants expressing pAhZIP6-1::*AhZIP6* (AhZIP6-1), pAhZIP6-2::*AhZIP6* (AhZIP6-2) or in Col-0 (AtZIP6) grown 6 weeks (short days) in Hoagland hydroponic medium control condition (1 μM Zn). Values (means ± SEM; from two biological replicates of two plants each for three independent lines per construct) are relative to *EF1α* and At1g58050. Data were analysed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means are indicated by asterisks (**p < .01, ***p < .001) or different letters (p < .05) [Colour figure can be viewed at wileyonlinelibrary.com]
the ATG codon). Sequence analysis revealed that pAhZIP6-1 was closely related to pAhZIP6 (seven blocks covering >2 kb and sharing an average of 89.3% of identity, excluding gaps) (Figure 1b and Figure S2, blocks a–g) and to a lesser extent to pAtZIP6 (five blocks covering ~1.1 kb with 80.4% average identity) (Figure 1b and Figure S2, blocks a, b, d, g and j). In contrast, the sequence of pAhZIP6-2 was highly divergent compared to the three others, with only two small conserved segments of 67 bp located in the 5'UTR and 34 bp including a putative TATA box, respectively (Figure 1b and Figure S2, red blocks). Using differences in promoter sequences and transcriptome data (from the PI22 ecotype) (Schwartzman et al., 2018), the coding sequences (CDS) of the 2 AhZIP6 copies were cloned from Lan3.1 genomic DNA and were highly similar with 99.4% of amino acid sequence identity and closely related to A. thaliana (~97%) and A. lyrata (~97%) ZIP6 sequences (Figure S3). The two sequences only diverged in a poly-glycine stretch (Figure S3C), outside conserved motifs found in transmembrane segments IV and V in ZIP proteins (Eng, Guerinot, Eide, & Saier, 1998; Guerinot, 2000), suggesting that polymorphisms among the two AhZIP6 proteins sequences are unlikely to result in functional differences. The version with the shortest Gly-stretch (8 Gly) was used in all downstream functional analysis.

2.2 | The AhZIP6 copies display partial tissue specialization

Next, the expression profile and expression level of the AhZIP6 copies were determined. Firstly, the pAhZIP6-1 and pAhZIP6-2 promoters, as well as pAtZIP6 for comparison, were fused to the GUS reporter gene for stable transformation in A. thaliana and A. halleri. A highly similar expression profile was observed for all three promoters in vegetative tissues of both species: ZIP6 was mainly expressed in the root and leaf vascular tissues, but was also expressed in the root cap, in leaf mesophyll cells and in hydathodes (Figure 2a and Figure S4). This profile was consistent with the AtZIP6 expression data available at Genevestigator (https://genevestigator.com/gsv/).

Secondly, copy-specific primers were designed and the expression level of each AhZIP6 copy was determined in wild-type A. halleri plants (ecotype Lan3.1). In roots, AhZIP6-2 was threefold more expressed than AhZIP6-1. In contrast, AhZIP6-1 was 1.7-fold more expressed than AhZIP6-2 in cauline leaves and both copies showed similar expression level in shoots (Figure 2b). As control, the AhZIP6 expression level detected with primers targeting simultaneously both copies was roughly similar to the addition of the AhZIP6-1 and AhZIP6-2 specific expression.

Finally, A. thaliana T3 homozygous transgenic lines expressing AhZIP6 under the control of either pAhZIP6-1 (AhZIP6-1 lines) or pAhZIP6-2 (AhZIP6-2 lines) were obtained. Both AhZIP6 copies were more highly expressed than AtZIP6 in roots and shoots of A. thaliana (Figure 2c). In roots, AhZIP6-2 was again predominant and was, respectively, 2- and 10-fold more expressed than AhZIP6-1 and AtZIP6. In contrast, in shoots and cauline leaves, AhZIP6-1 was dominant (1.5-fold higher than AhZIP6-2 and 20-33-fold higher than AtZIP6, respectively) (Figure 2c).

Altogether, these analyses indicated a higher expression of both AhZIP6 copies compared with AtZIP6 and suggested overall conserved cis regulatory mechanisms between A. halleri and A. thaliana, with a partial organ specialization of each copy: AhZIP6-1 was predominant in aerial parts and AhZIP6-2 in roots in both species.

2.3 | The localization of the AhZIP6 protein remains elusive

GUS staining and qRT-PCR provide relevant information on expression profile and expression level. However, subcellular localization is important to elucidate the protein function. Green fluorescent protein (GFP) was thus fused to the ZIP6 genomic DNA sequence (AhZIP6g). Genomic DNA was used instead of cDNA to resolve a problem of apparent bacterial toxicity during cloning. A. thaliana plants were stably transformed with two constructs (pAhZIP6-1::AhZIP6g::GFP and pAhZIP6-2::AhZIP6g::GFP) and five independent T2 lines per construct were observed by confocal microscopy. Intense fluorescence was only distinguished for pAhZIP6-1::AhZIP6g::GFP lines. In the epidermis of mature roots, fluorescent spots were observed on the cell edges and in some points co-localized with propidium iodide, a fluorescent dye staining the cell wall (Figure 3). However, observation in developing roots (root tip and elongation zone) where vacuoles are smaller and the cytoplasm wider, detected a dotted cytoplasmic fluorescence that did not co-localize with plasma membrane (Figure 3). AhZIP6g::GFP was probably expressed in small cytoplasmic vesicles, which were squeezed against the plasma membrane when vacuoles were fully developed. Additional efforts using A. thaliana protoplasts transformed with various tagged versions of AhZIP6-1 under control of the 35S promoter yielded equally ambiguous results. Protoplasts showed both cytosolic signals and in some cases, a staining pattern that could be interpreted as plasma membrane (Figure S5A–C). Note, however, that the functionality of the GFP fusion has to be questioned based on the expression in yeast (see next section).

2.4 | AhZIP6 is a zinc and cadmium transporter in yeast

As A. halleri is a zinc and cadmium hyperaccumulator plant, we next tested the transport ability of the AhZIP6 protein upon constitutive expression in yeast- (zrc1cot1) and cadmium- (ycf1) sensitive yeast mutants, defective for vacuolar metal transport, and the corresponding wild-type strains. Metal tolerance of the transformed yeast cells was assessed upon growth both on agar plates and in liquid medium contaminated with a range of zinc or cadmium concentrations, respectively (Figure 4). The expression of AhZIP6 had no effect on growth of either strain in control conditions (Figure 4). In contrast, AhZIP6 increased the zinc sensitivity of the zrc1cot1 strain, both in drop tests and in liquid cultures, with a complete inhibition of growth at 200 μM zinc in liquid culture for instance (Figure 4a,b).
However, zinc concentration did not differ between AhZIP6-expressing and empty vector-transformed yeast cells, respectively (Figure S6A). Similarly, AhZIP6 expression increased cadmium toxicity in the ycf1 mutant (Figure 4c,d). Additionally, the growth of the wild-type strain expressing AhZIP6 was also affected when exposed to the highest cadmium concentration (25 μM) in liquid cultures (Figure 4d). Again, no major difference in cadmium concentration in cells was found between the different yeast genotypes (Figure S6). As AhZIP6 seemed to localize to endomembranes in yeast cells when fused to GFP (Figure S5D), these data suggested that AhZIP6 was able to transport zinc and cadmium and was possibly involved in compartmentalization rather than cellular zinc and cadmium uptake. Note, however, that expression of the AhZIP6::GFP fusion in yeast did not cause the zinc and cadmium sensitivity phenotype observed with the native protein, questioning its functionality.

2.5 | Silencing ZIP6 expression in A. halleri does not affect zinc and cadmium accumulation

As expected from previous observations (Talke et al., 2006), AhZIP6 expression levels were only marginally regulated in A. halleri upon zinc deficiency (0 μM Zn), high zinc (300 μM) or cadmium (5 μM) exposure (Figure 5). Noticeably, the two AhZIP6 copies displayed a similar behaviour, with the exception of a repression of AhZIP6-2 at high zinc, amounting to a global down-regulation of AhZIP6 in shoots (Figure Sb).

To more directly address the physiological role of AhZIP6 in metal hyperaccumulation and hypertolerance, a vegetatively propagated individual from the Langelsheim population was transformed with an RNAi construct targeting the two AhZIP6 copies. Thirty-one independent transgenic lines were obtained and screened for AhZIP6 total transcript abundance in shoots using primers matching both AhZIP6
copies. Two lines with nearly 90% suppression of AhZIP6 transcript (lines 7 and 11) were selected for further analysis (Figure 6a). Analysis of the RNAi effect on the two AhZIP6 copies separately using specific primers confirmed a suppression of about 90% for both copies in the selected transgenic lines compared with the wild type (Figure S7). The RNAi effect was also confirmed in comparison to a transgenic line expressing GFP under control of the 35S promoter as an additional control besides wild-type plants (Figure 6 and Figure S7) since it had undergone the transformation procedure including prolonged phases of tissue culture.

Wild-type and transgenic lines were grown in hydroponic culture under control conditions (10 μM Zn). After 3 weeks, micronutrient contents were determined for roots and leaves. Typical strong accumulation of zinc in leaves was apparent but no differences between lines were observed for zinc, iron and manganese (Figure S8). Similarly, no genotype-dependent differences were found when plants were cultivated in excess zinc (300 μM Zn) (Figure S8).

Next, the two AhZIP6-suppressed lines and the two controls were grown in native A. halleri soil from the metalliferous site in Bestwig (for GPS and soil data, see Höreth et al., 2020) and in the phenotyping soil used for the characterization of field-collected individuals of European A. halleri populations (Stein et al., 2017). Growth as determined by shoot biomass was comparable between the metalliferous site soil and the phenotyping soil, indicating that the metal-contaminated soil did not exert toxic effects on the plants. No significant differences between genotypes were apparent (Figure S9). After cultivation in Bestwig soil, zinc accumulation in all lines exceeded by far the zinc hyperaccumulation threshold of 3,000 μg g⁻¹ of dry weight, whereas in phenotyping soil with very limited zinc phytoavailability, accumulation stayed below this threshold (Figure S10). In contrast, cadmium accumulation was higher in phenotyping soil exceeding the hyperaccumulation threshold of 100 μg g⁻¹ of dry weight, but not in Bestwig soil (Figure S10). Again, however, no significant differences were observed between the lines, arguing against a major direct impact of AhZIP6 on zinc or cadmium hyperaccumulation.

2.6 Silencing ZIP6 expression in A. halleri specifically increases cadmium tolerance

Shoot growth in Bestwig soil had not been inhibited by high metal content. To assess possible metal tolerance effects of AhZIP6 silencing...
directly on roots and in a more targeted, element-specific manner, plants were exposed to growth-inhibiting concentrations of different metals in hydroponic culture. Conditions tested were 600 μM zinc, 5 and 10 μM nickel and 10 and 20 μM cadmium. These concentrations were chosen in order to cause a comparable range of root growth inhibition effects on wild-type plants. Zinc concentrations could not be increased any further without precipitation of zinc salts. Importantly, AhZIP6-suppressed plants grew significantly better than control plants specifically in the presence of cadmium (Figure 6b–d). The gain in tolerance was restricted to root length and root fresh weight, and seemed specific to cadmium as all genotypes were equally sensitive to zinc and nickel (Figure 6b–d, Figures S11 and S12).

The higher cadmium tolerance of AhZIP6-suppressed lines could be due to a reduction in root cadmium uptake activity. Thus, we measured short-term cadmium uptake by roots of hydroponically grown plants. Experiments performed with non-toxic concentrations of 2 and 5 μM cadmium showed indeed that uptake by roots of AhZIP6-suppressed plants was strongly reduced compared with control plants (Figure 7). Thus, AhZIP6 appears to represent a cadmium uptake pathway in A. halleri.

When cadmium accumulation after long-term exposure in hydroponic culture was assayed, however, no differences between control and AhZIP6-RNAi plants were observed (Figure S13). This confirmed the findings of the soil experiments.

2.7 | In A. thaliana, AhZIP6 impacts the growth under cadmium exposure

To define the function of each AhZIP6 copy, wild-type (Col-0) and T3 homozygous A. thaliana plants expressing AhZIP6 under the control of either pAhZIP6-1 (AhZIP6-1 lines) or pAhZIP6-2 (AhZIP6-2 lines) were grown for 3 weeks in control hydroponic medium (1 μM Zn), then exposed for 3 weeks to the following conditions: control (1 μM Zn), zinc deficiency (0 μM Zn), zinc excess (20 μM Zn) or presence of cadmium (0.05 μM). Although the expression of both AhZIP6 copies was not altered by the zinc status or by cadmium concentration in the medium (Figure 8a,b), the fresh shoot biomass of all genotypes was impacted by the treatments (Figure 8c). Zinc deficiency and zinc excess decreased the shoot biomass with, for instance, 22 and 35% biomass reduction for Col-0, respectively, but no differences were observed between genotypes (Figure 8c). AhZIP6 expression in A. thaliana had overall a very limited impact on the plant ionome, across all culture conditions (Figure S14). Indeed, only a small but significant reduction (8%) of zinc concentration was found in shoots of AhZIP6 lines upon zinc excess (Figure S14A).

In contrast to zinc, cadmium exposure differentially impacted shoot biomass in the different genotypes: shoots of AhZIP6-2 plants had indeed 23% higher biomass than Col-0, which in turn had 30% higher biomass than AhZIP6-1 shoots (Figure 8c). Thus, high expression of AhZIP6 in roots triggered by the AhZIP6-2 construct contributed to increased shoot cadmium tolerance, whereas high expression of AhZIP6 in shoots triggered by the AhZIP6-1 construct increased shoot cadmium sensitivity. This occurred despite similar cadmium accumulation in roots and shoots among genotypes (Figure S14C). As for A. halleri RNAi lines, short-term cadmium uptake by roots of hydroponically grown plants was measured in Col-0 and in AhZIP6-2 lines. AhZIP6-2 lines were used as this copy is the most highly expressed in roots (Figure 2b,c). Cadmium uptake by roots was found to be similar between the two genotypes (Figure S15).

3 | DISCUSSION

In spite of their long suspected importance for zinc acquisition and other aspects of zinc homeostasis, very little is known about the physiological roles of ZIP transporters (Ricachenevsky et al., 2015). Plant
ZIP proteins were detected in different cellular membranes and various, sometimes contradictory, phenotypes were reported for yeast cells expressing ZIPs. With the exception of the IRT proteins, hardly any phenotype could be associated with a loss or gain of ZIP function in plants.

We analysed AhZIP6 because ZIP6 genes from *A. halleri* and *N. caerulescens* show all the known hallmarks of a metal hyperaccumulation candidate gene. As many other metal homeostasis genes, they are highly expressed in *A. halleri* and *N. caerulescens* compared with non-accumulator relatives and, more importantly, AhZIP6 was found to be associated with a QTL for zinc accumulation in *A. halleri* (Becher et al., 2004; Filatov et al., 2006; Hammond et al., 2006; Talke et al., 2006; Weber, Harada, Vess, Roepenack-Lahaye, & Clemens, 2004; Willems et al., 2010).

High expression of these metal homeostasis genes was often found to be associated with gene copy number amplification (Dräger et al., 2004; Fasani et al., 2017; Hanikenne et al., 2008; Shahzad et al., 2010; Suryawanshi et al., 2016). For instance, both HMA4, critical for zinc root-to-shoot translocation (Courbot et al., 2007; Hanikenne et al., 2008; Hanikenne et al., 2013; Nouet et al., 2015), and MTP1, involved in zinc vacuolar storage in shoots (Dräger et al., 2004; Fasani et al., 2017; Krämer, 2005; Shahzad et al., 2010; Willems}

**FIGURE 6** Identification of AhZIP6-RNAi *A. halleri* lines and cadmium tolerance test. (a) AhZIP6 transcript levels were analysed in shoots of wild-type, a control GFP-transformed line and two AhZIP6-suppressed (7–2 and 11–1) *A. halleri* lines grown on normal soil. Tissues were harvested after 6 weeks of cultivation and analysed by quantitative RT-PCR. Transcript abundance was expressed relative to EF1α. Values are means ± SD from two independent experiments including a pool of six replicate clones per genotype for each data point. Cadmium tolerance was determined by measuring root length (b), and root weight (c) relative to control conditions of plants grown hydroponically for 3 weeks in 1/10 Hoagland medium supplemented with 0 (control), 10 or 20 μM cadmium. Pictures of the representative plants at the end of the experiment are shown (d). Values (means ± SD, N = 12 replicates from three independent experiments) are relative to the control condition and were analysed by ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means are indicated by asterisks (*p < .05, **p < .01, ***p < .001) or different letters (p < .05) [Colour figure can be viewed at wileyonlinelibrary.com]
et al., 2007), are present in multiple copies in the A. halleri genome. Here, we showed that ZIP6 is a multicyclic gene (Figure 1a) in both metallicolous and non-metallicolous A. halleri populations representing the geographic distribution of the species in Europe (Pauwels et al., 2012). For most genotypes, our analysis revealed that the number of ZIP6 copies in the A. halleri genome is not fixed. Three ZIP6 copies were indeed detected in the I16 Italian metallicolous population (Figure 1a) that colonizes a highly zinc-, cadmium- and lead-contaminated environment and displays a cadmium exclusion strategy (Corso et al., 2018; Fasani et al., 2017; Meyer et al., 2015; Schrartzman et al., 2018). Unfixed gene copy number among populations was also found for MTP1 (Fasani et al., 2017; Meyer et al., 2016; Shahzad et al., 2010). Gene copy number variation among and within populations may reflect ongoing local adaptation processes. For instance, the third copy might differentially contribute to cadmium tolerance or accumulation in the I16 population (Corso et al., 2018).

As for HMA4 (Hanikenne et al., 2008), the two ZIP6 copies are found in tandem in the A. halleri genome (Figure 1b), at an even closer distance, and remain highly conserved (Figure S3). This suggests, as for HMA4 (Hanikenne et al., 2013), that the ZIP6 copies may undergo ectopic gene conversion enabling concerted evolution of the paralogous ZIP6 coding sequences. In contrast, the promoter sequences of the ZIP6 copies have considerably diverged, again reminiscent of HMA4 and MTP1 (Fasani et al., 2017; Hanikenne et al., 2008; Shahzad et al., 2010), with pAhZIP6-1 retaining the ancestral sequence most similar to the A. lyrata and A. thaliana ZIP6 promoters (Figure 1b). However, unlike MTP1 (Shahzad et al., 2010), both ZIP6 copies are highly expressed in A. halleri, compared with A. thaliana (Figure 2). In contrast to HMA4 (Hanikenne et al., 2008), a (partial) sub-functionalization of the two copies occurred after the duplication. While the two copies retained a highly similar tissue-specificity of expression, at least in vegetative tissues (Figure 2a), AhZIP6-1 and AhZIP6-2 are differentially expressed in root and shoot tissues (Figure 2b).}

**FIGURE 7** Short-term cadmium uptake in wild-type and AhZIP6-RNAi A. halleri lines. Cadmium concentration in roots of wild-type, control GFP-transformed line and two independent AhZIP6-suppressed lines (7–2 and 11–1) grown hydroponically for 2 weeks in 1/10 Hoagland medium and then exposed for a short term (15 or 30 min) to media containing 2 μM (a) and 5 μM (b) cadmium at 4°C or 25°C. Values (means ± SD) are from three independent experiments, each including 4–5 replicate clones per genotype. Data were analysed by two-way ANOVA followed by Bonferroni multiple comparison post-test. Statistically significant differences between means are indicated by different letters (p < .05)

Taken together, the genomic situation and expression analyses further substantiated AhZIP6 as an important candidate gene possibly contributing to zinc and/or cadmium hyperaccumulation and hyper-tolerance in A. halleri. We therefore transformed an A. halleri individual from the metallicolous Langelsheim population with an RNAi construct and obtained lines with strong suppression of both AhZIP6 transcripts (Figure 6a, Figure S7). However, no significant changes in root or leaf ionomes were detected in these lines after hydroponic or soil cultivation in a wide range of conditions (Figures S7–S10), arguing against a direct role of AhZIP6 in metal hyperaccumulation and against AhZIP6 being the causal gene detected in hyperaccumulation QTLs (Filatov et al., 2006; Willems et al., 2010).

Surprisingly, tolerance assays with AhZIP6-suppressed plants and respective controls revealed a gain in cadmium tolerance when the two AhZIP6 copies are knocked down (Figure 6). This effect appeared to be specific for cadmium, over zinc or nickel (Figure S12). A gain in cadmium tolerance upon the suppression of a gene could be indicative of cadmium uptake activity. ZIP transporter-mediated cadmium transport has been described for ZIP proteins from other kingdoms before. For example, loss-of-function mutations in mouse ZIP8 (SLC39A8) explain the resistance of particular inbred strains to cadmium-induced testicular necrosis (Dalton et al., 2005) because ZIP8 represents the main cadmium uptake pathway. Mouse ZIP14 was shown to transport cadmium into mouse kidney cells (Fujishiro, Yano, Takada, Tanihara, & Himeno, 2012).

Short-term cadmium uptake assays indeed demonstrated a significant reduction in roots of AhZIP6-suppressed plants (Figure 6). Thus, AhZIP6 can be added to the small list of plant transporters for which
indicated by asterisks (*p < .05) post-test. Statistically significant differences between means are by two-way ANOVA followed by Bonferroni multiple comparison for each three independent line per construct. Data were analysed representative of two independent experiments, with 14 of shoot fresh weight. Values (means ± SEM; from one experiment letters (p < .05, ***p < .001) or different letters (p < .05) cadmium transport activity has been directly shown. These include IRT1 in A. thaliana (Vert et al., 2002) and OsNramp5 in rice (Ishimaru et al., 2012; Sasaki, Yamaji, Yokosho, & Ma, 2012). Indirect support for a cadmium, and zinc, transport activity of AhZIP6 was also derived from yeast expression (Figure 4). Furthermore, AhZIP6-dependent cadmium uptake is consistent with the reported higher cadmium sensitivity of A. thaliana plants overexpressing the ZIP6 ortholog from N. caerulescens (Wu et al., 2009).

Unlike OsNramp5 loss-of-function mutants, however, which accumulate much less cadmium in above-ground tissues than respective wild-type plants (Ishimaru et al., 2012; Sasaki et al., 2012), AhZIP6-knock-down plants did not show any alterations in cadmium accumulation behaviour (Figures S10 and S13). We therefore hypothesize that (a) several pathways for cadmium uptake into A. halleri roots exist that mask the effects of AhZIP6 knock-down when integrating uptake over a long period of time in accumulation experiments, and that (b) AhZIP6 may mediate transport of cadmium into particularly sensitive root cells, thus explaining the tolerance phenotype.

Cadmium hypertolerance is a species-wide trait in A. halleri (Meyer et al., 2015; Stein et al., 2017). Thus, it is difficult to explain why AhZIP6 should be more highly expressed in A. halleri and possibly still subject to local adaptation processes (see above) when the activity of AhZIP6 renders plants more cadmium sensitive. Arguably, the simplest explanation would be that AhZIP6 is involved in other processes important for metal hyperaccumulation, for example, by fine-tuning metal distribution within tissues. Cadmium transport by AhZIP6 would in such a scenario be a tolerable side effect, consistent with the lack of any biomass reduction of AhZIP6 RNAi plants after cultivation in native metalliferous A. halleri soil (Figure S9).

Most plant ZIP transporters have so far been indirectly implicated in zinc transport, making zinc the most likely candidate metal to guide the search for other suspected ZIP6 functions. As was the case for practically all studies on plant ZIP transporters to date, however, our A. halleri experiments did not uncover zinc ionome phenotypes upon suppression of ZIP gene function. Most available evidence for zinc transport by plant ZIPs has been obtained in overexpression studies, for example, OsZIP7 (Ricachenevsky et al., 2018) and HvZIP7 (Tiong et al., 2014). We therefore generated A. thaliana lines expressing the AhZIP6 gene copies under their native promoters (Figure 8), allowing copy-specific analyses not possible in A. halleri.

Overall, subtle metal-related phenotypes were observed for the transgenic lines. AhZIP6 expression affected cadmium tolerance, albeit in opposite directions for the two copies. AhZIP6-2 is responsible for higher ZIP6 expression in roots and conferred increased cadmium tolerance to the entire plant without modifying the ionomics profile in tissues (Figures 2b and 8, Figure S14) or contributing measurably to root cadmium uptake (Figure S15). AhZIP6-1 expression, in contrast, rendered plants slightly more cadmium sensitive (Figure 8). Taken together, these observations provided further indirect support for a role of AhZIP6 in cadmium transport. It thus seems that high expression of AhZIP6 in an organ conferred cadmium sensitivity locally (e.g. in roots of A. halleri, Figure 6, or in shoots for AhZIP6-1 in A. thaliana, Figure 8c), but could also
act on cadmium tolerance of another organ (i.e. AhZIP6-2 in A. thaliana, Figure 8c). This distinction could only be revealed by analysing the two copies separately in A. thaliana, whereas the phenotypic output in A. halleri resulted from the combined actions of the two copies.

At the end of this study, the cellular localization of AhZIP6 remains unclear. A protein mediating short-term cadmium uptake would be expected to reside in the plasma membrane (Figure 7). On the contrary, the absence of ionome phenotype in yeast (Figure S6), A. thaliana (Figures S14 and S15) or A. halleri (Figures S10 and S13) over or underexpressing AhZIP6 may equally rule out plasma membrane localization and rather support a role for AhZIP6 in intracellular cadmium distribution. In this case, altered cadmium distribution in cells may indirectly trigger the increased cadmium uptake observed at short term in A. halleri. Neither of these hypotheses could be confirmed or falsified by our localization experiments. AhZIP6 fused to a fluorescent reporter protein in C-terminal seems to localize in vesicular endomembranes in yeast cells (Figure SSD) and in Arabidopsis plants or protoplasts (Figure 3, Figure S5A–C). The localization of AhZIP6 is somewhat reminiscent of those of IRT1 (Dubéaux, Neveu, Zelazny, & Vert, 2018) and NRAMP1 (Agorio et al., 2017), which were described to undergo intracellular trafficking and whose localization on endomembranes or the plasma membrane is regulated by iron and other metal availability (Agorio et al., 2017; Dubéaux et al., 2018). Whether the localization of AhZIP6 is submitted to such dynamic processes depending on cadmium and/or zinc availability will need to be examined in the future. The observations reported here have, however, to be interpreted with caution as the AhZIP6 fusion protein appeared no longer functional in yeast. These ambiguous observations may result from a possible toxicity of the protein—molecular cloning of tagged or untagged versions of AhZIP6 has been strongly hampered by toxicity in E. coli in the course of this study.

A working hypothesis is that AhZIP6 is key to fine-tune metal distribution at local scale, controlling metal homeostasis in specific cell types. Considering two scenarios for the protein localization, high expression of AhZIP6 would act on metal distribution between tissues (plasma-membrane localization) or between compartments within cells (endomembrane localization), producing the tolerance phenotype reported in this study. Moreover, AhZIP6 contributes to root cadmium uptake, although it is mostly expressed in vascular tissues (Figure 2). It has been shown for other transporters that expression in the vascular system can contribute to metal uptake. For instance, high expression of HMA4 in A. halleri root vascular tissues activates zinc uptake by ZIPs (Hanikenne et al., 2008). Another example is NRAMP1, which although mostly expressed in the endodermis, contributes together with IRT1 to iron uptake in A. thaliana roots (Cailliatte, Schlöker, Briat, Mari, & Curie, 2010; Castaing, Caquot, Loubet, & Curie, 2016).

In conclusion, ZIP6 is a duplicated gene in A. halleri, expressed mainly in the vasculature and encoding a zinc and cadmium transporter. Each copy acquired some extent of organ specialization, in aerial parts (AhZIP6-1) and in roots (AhZIP6-2). The expression pattern of the AhZIP6 copies, and the activity of their promoters, was overall conserved in both A. halleri and A. thaliana, suggesting a conservation of cis regulatory mechanisms, as for HMA4 (Hanikenne et al., 2008). Overexpression of the AhZIP6 copies in A. thaliana or their extinction in A. halleri altered cadmium tolerance but had no impact on zinc tolerance or the ionome of the plants, suggesting a function of AhZIP6 in controlling tissue or cellular metal distribution. Altogether, this study highlights the diversity and complexity of mechanisms underlying the evolution of hyperaccumulation and the distinct fates of duplicated genes in A. halleri. Obvious hyperaccumulation candidate genes do not necessarily show a directly measurable loss-of-function impact on metal concentrations when the still tedious generation of respective knock-down lines is achieved. Our study also confirms that functional characterization of ZIPs in plants remains a challenge.

## 4 METHODS

### 4.1 Plant material, growth conditions and transformation

A. thaliana (accession Columbia-0, Col-0) and A. halleri ssp. halleri (accession Langelsheim) were used for all experiments. Growth conditions in hydroponic Hoagland medium and on soil are detailed in Text S1. A. thaliana transformation was performed by floral dip (Clough & Bent, 1998) and Agrobacterium tumefaciens (GV3101, pMP90) mediated stable transformation of A. halleri was performed using a tissue-culture based procedure (Ahmadi et al., 2018; Hanikenne et al., 2008).

### 4.2 Determination of gene copy number and gene expression analysis

Gene copy numbers were determined by quantitative PCR as described (Hanikenne et al., 2013) using primers listed in Table S1. Gene expression analyses were performed by quantitative RT-PCR (Table S1). Data analysis and normalization were conducted as described (Talke et al., 2006; Nouet et al., 2015; Lekeux et al., 2018). Procedures are detailed in Text S1.

### 4.3 Cloning, DNA manipulation and sequence analysis

The identification and characterization of the two AhZIP6 copies from A. halleri BACs (Hanikenne et al., 2008) using quantitative PCR, BAC sequencing and phylogenetic analysis (Kumar, Stecher, Li, Knyaz, & Tamura, 2018; Nei & Kumar, 2000) are detailed in Text S1. Vector constructs for expression of (un)tagged versions of AhZIP6 in yeast or plants and reporter constructs obtained using standard molecular cloning procedures and primers listed in Table S2 are described in Text S1.
4.4 Yeast tolerance and complementation assays

Zinc and cadmium yeast assays were conducted using the zrc1cot1 and ycf1 mutants and their respective parental strains BY4741 and BY4742 (Euroscarf), the pFL38 vectors (Text S1) and LSP-Leu medium added with a range of zinc or cadmium concentrations (Talke et al., 2006).

4.5 Protoplast isolation and transformation

Protoplasts were isolated from A. thaliana wild-type (Col-0) leaves and were transformed using a PEG solution as described in Text S1.

4.6 Short-term uptake experiment

To examine the effect of AhZIP6 suppression or over-expression on transport activity for cadmium in A. halleri or A. thaliana, respectively, a short-term (15 and 30 min) cadmium uptake experiment, as described in (Sasaki et al., 2012), was performed (Text S1).

4.7 Analysis of metal contents

After desorption, plant and yeast samples were digested with HNO3 (Sigma-Aldrich) as described (Scheepers et al., 2020) and analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a Vista-AX instrument (Varian, Melbourne, Australia) or an iCAP 6,500 Series instrument (Thermo-Fisher, Waltham, MA) (yeast, roots and shoots) (Text S1).

4.8 Confocal microscopy analysis

Confocal images were recorded using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) or a Leica TCS SP5 (Leica Microsystems) (Text S1).

4.9 Histochemical staining

GUS staining was performed as described (Jefferson, Kavanagh, & Bevan, 1987) and tissues were then fixed and discoloured (Hanikenne et al. (2008)).

4.10 Accession numbers

The genomic sequences of AhZIP6-1 and pAhZIP6-2, including the promoter sequences, were deposited in the NCBI Genbank database with the accession numbers MT155929 and MT155930, respectively.

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AUTHOR CONTRIBUTIONS

Marc Hanikenne, Julien Spielmann and Stephan Clemens designed the research. Julien Spielmann, Hassan Ahmadi, Maxime Scheepers, Michael Weber, Sarah Nitsche, Monique Carnol, Bernard Bosman, Juergen Kroymann performed the experiments. Julien Spielmann, Hassan Ahmadi, Marc Hanikenne, Michael Weber, Stephan Clemens, Patrick Motte analysed the data. Julien Spielmann and Hassan Ahmadi made the Figures. Julien Spielmann, Marc Hanikenne, Stephan Clemens wrote the manuscript. All authors read and approved the manuscript.

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**SUPPORTING INFORMATION**

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

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