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Quantitative trait loci analysis of mineral element concentrations in an *Arabidopsis halleri* × *Arabidopsis lyrata petraea* F_2 progeny grown on cadmium-contaminated soil

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Summary

- This study describes the quantitative trait locus (QTL) analysis of cadmium (Cd), zinc (Zn), iron (Fe), potassium (K), magnesium (Mg) and calcium (Ca) accumulation in the pseudometallophyte *Arabidopsis halleri* under conditions of Cd excess using an interspecific *A. halleri* × *Arabidopsis lyrata* F_2 population.
- Our data provide evidence for the implication of one major QTL in Cd hyperaccumulation in *A. halleri*, and suggests that Cd tolerance and accumulation are not independent in *A. halleri*. Moreover, the major loci responsible for Zn hyperaccumulation in the absence of Cd appear to be the same when Cd is present at high concentrations.
- More than twofold higher Fe concentrations were measured in *A. halleri* shoots than in *A. lyrata*, suggesting a different regulation of Fe accumulation in the hyperaccumulator.
- With the exception of Ca, the accumulation of Cd was significantly correlated with the accumulation of all elements measured in the F_2 progeny, suggesting pleiotropic gene action. However, QTL analysis identified pleiotropic QTLs only for Cd, Zn and Fe. Mg accumulation was negatively correlated with Cd accumulation, as well as with dry shoot biomass, suggesting that it might indicate cellular damage.

Introduction

Cadmium (Cd) is a major environmental pollutant contaminating air, water and soil primarily in regions with high industrial activity. Except for diatoms that can use Cd for activation of carbonic anhydrase in depletion of zinc (Zn), no biological function has been attributed to Cd (Morel, 2008). The primary targets of Cd toxicity are not known. However, since it is chemically similar to essential elements, in particular Zn, but also calcium (Ca) and iron (Fe), Cd affects the homeostasis of the latter elements or causes their displacement from proteins, resulting in their inactivation (Verbruggen *et al.*, 2009a). Another important source of Cd toxicity is its high affinity for thiolates, causing depletion of reduced glutathione through the formation of Cd(II)-GS_2 complexes or Cd-induced phytochelatin synthesis. In yeast and human cells, Cd also acts as an important mutagen, as it inactivates DNA mismatch repair (Jin *et al.*, 2003), and the same mechanisms may act in plants.

Despite the toxicity of Cd, four plant species have been described that are able to accumulate very high Cd concentrations in their aerial biomass without displaying any symptom of toxicity (Verbruggen *et al.*, 2009b). This exceptional phenomenon, called hyperaccumulation, has been identified for essential as well as nonessential trace elements, in > 450 plant species. Growing on nonpolluted as well as polluted sites, *Arabidopsis halleri* ssp. *halleri* (*A. halleri*), one of the closest relatives of *Arabidopsis thaliana*,

shows constitutive Zn tolerance (Pauwels *et al.*, 2006) and, so far all populations that have been identified are able to hyperaccumulate Zn as well as tolerate Cd (Bert *et al.*, 2000, 2002, 2003). In some populations, Cd tolerance is accompanied by Cd hyperaccumulation (Bert *et al.*, 2000, 2002, 2003). The relationship between tolerance and hyperaccumulation traits has been investigated in this pseudometallophyte through interspecific crosses with its nontolerant nonaccumulator sister species *Arabidopsis lyrata* ssp. *petraea* (*A. lyrata*). Accumulation of, and tolerance to, Zn and Cd were reported to be, at least partially, independent based on the segregation of these traits in *A. halleri* × *A. lyrata* progenies (Macnair *et al.*, 1999; Bert *et al.*, 2003). A quantitative trait locus (QTL) analysis on an *A. halleri* × *A. lyrata* backcross progeny identified one common QTL region for Zn and Cd tolerance, colocalizing with the P-ATPase HMA4 (Courbot *et al.*, 2007; Willems *et al.*, 2007). The role of *AhHMA4* in Zn hyperaccumulation and Cd tolerance was demonstrated through RNAi-mediated silencing (Hanikenne *et al.*, 2008), where *A. halleri* plants, with a lower expression of *AhHMA4*, behaved similarly to wild-type *A. thaliana* plants with respect to their Zn and Cd tolerance and Zn accumulation abilities. On the other hand, *A. thaliana* overexpressing *AhHMA4* showed increased Zn translocation to the shoot, as well as increased transcript abundance of Zn deficiency genes in analogy with wild-type *A. halleri*. Shoot chlorosis, however, was observed in the latter transgenic plants, indicating hypersensitivity to both Cd and Zn excess as a result of the absence of sufficient mechanisms for the detoxification of metals in the shoots.

With the availability of high-throughput genotyping tools, ionomics gained considerable interest as it provides an efficient means to identify genes regulating element accumulation (Salt *et al.*, 2008; Baxter, 2009). In the current 'ionomics' era, hyperaccumulators deserve special interest, as these species naturally display severe homeostatic differences that probably cannot be obtained artificially. Therefore, analyzing the ionome of hyperaccumulators should significantly contribute to our understanding of the complex network of mechanisms regulating element accumulation. Ultimately, identifying the mechanisms underlying (hyper)accumulation can help in fighting mineral deficiency through biofortification of crops, that is, the process of increasing mineral content of edible parts of crop plants (Guerinot & Salt, 2001; Palmgren *et al.*, 2008).

In this study, the genetic architecture of Cd hyperaccumulation under Cd excess was investigated in *A. halleri* through a QTL analysis on an interspecific *A. halleri* × *A. lyrata* F_2 population. With the aim of identifying possible co-adaptive mechanisms of essential elements homeostasis, the accumulation of Zn, Fe, potassium (K), magnesium (Mg), and Ca under Cd excess was also investigated using QTL mapping.

Materials and Methods

Plant material

An interspecific F_2 mapping population was obtained starting from two different crosses between *A. halleri* ssp. *halleri* (henceforth called *A. halleri*) (pollen donor) and the closely related species *A. lyrata* ssp. *petraea* (henceforth called *A. lyrata*), as described in Frérot *et al.* (2010). In each of these crosses, two different *A. halleri* individuals, originating from an industrial metal-polluted site (Courcelles, France), as well as two different *A. lyrata* individuals, originating from an uncontaminated site in the Czech Republic (Unhost, Central Bohemia) (Macnair *et al.*, 1999), were used.

Mineral analysis

Cadmium accumulation was assessed on four *A. halleri* individuals, three *A. lyrata* genotypes, as well as 158 individuals of the F_2 progeny. Since the *A. halleri* and *A. lyrata* individuals originally used for the generation of the F_2 progeny were not maintained, *A. halleri* and *A. lyrata* individuals originating from closely related (*A. halleri*) or the same (*A. lyrata*) populations were used. One to three clones of each F_2 genotype, and one to four clones of the *A. halleri* and *A. lyrata* genotypes were obtained through vegetative propagation, and grown in the glasshouse on general-purpose compost for 6 wk. Subsequently, the plants were transferred to 1 l plastic pots and grown on general-purpose compost amended with 10 mg Cd kg^{-1} fresh soil. Mineral elements were extracted with 1 N ammonium acetate-EDTA (pH 4.65) for 30 min (10 g dry soil in 50 ml). The supernatant was filtered and analyzed by inductively coupled plasma-optical emission spectrometers (ICP-OES) (Vista MPX; Varian Inc., Palo Alto, CA, USA) for all elements. Total element concentrations were 19, 22, 775, 2310, 9061 and 1062 mg kg^{-1} dry soil, respectively, for Zn, Cd, Fe, Mg, Ca and K. After 6 wk shoots were harvested, washed three times for 5 min with demineralized water to remove any soil particles, and dried at 65°C for 72 h. Dried shoot samples (250 mg or less, if total dry weight < 250 mg) were digested with HNO_3 (65%). In addition to Cd, the concentrations of Zn, Fe, K, Mg, and Ca were measured by ICP-OES.

Statistical analyses

Analysis of variance (ANOVA) of each element was performed in order to verify the genotype effect of each mineral concentration in the F_2 population using PROC GLM in SAS. Type III sums of squares were used as the dataset is unbalanced and the main factor genotype was considered a random factor. Genetic (V_G) and total phenotypic variance ($V_G + V_E$) were estimated using PROC

VARCOMP and method Type 1 in SAS 9.2 (SAS Institute, 2002). Broad-sense heritability was calculated by dividing the genetic variance by the total phenotypic variance. The average element concentration per genotype was obtained by calculating the arithmetic mean of the mineral concentrations over all clones per genotype. Correlations between the mean element concentrations, as well as between total dried shoot mass of the F_2 individuals used in this experiment and the mineral element concentrations, were verified using Spearman's rank correlation tests in R (R Development Core Team, 2009).

QTL mapping

The QTL intervals of the mineral concentrations were defined on the *A. halleri* × *A. lyrata* F_2 genetic linkage map (Frérot *et al.*, 2010) using MapQTL 6.0 software (Van Ooijen, 2009). Mean element concentrations were used in the QTL analysis. Before QTL mapping, a genome-wide logarithm of odds (LOD) score threshold for QTL detection at $\alpha = 0.05$ was calculated for each element based on three independent permutation tests (1000 permutations) implemented in MapQTL. LOD score significance thresholds for QTL detection were 3.7 for Zn, Fe, K, Ca and Mg concentrations, and 3.8 for Cd accumulation. Interval mapping was performed on all elements to identify those for which markers showing a LOD score exceeding the significance threshold were obtained. For those elements, automatic cofactor selection, implemented in MapQTL, was performed on each linkage group. The final set of markers obtained through automatic cofactor selection was then used in Multiple QTL Model (MQM) analysis. MQM analysis was performed several times as necessary to obtain the best possible set of QTLs, that is, showing maximal LOD scores. To test for significant interactions between the QTLs, ANOVA analysis was performed using the markers closest to, or at, the QTL positions as random factors in the model. LOD support intervals associated with the QTLs were obtained using Mapchart 2.1 (Voorrips, 2002). Additive and dominance effects, as well as the degree of dominance of the QTLs, were calculated as described in Frérot *et al.* (2010).

Results

Mineral element composition of the F_2 progeny

A highly significant difference in Cd ($P = 0.002$), Zn ($P < 0.001$), and Fe ($P < 0.001$) concentrations was observed between *A. halleri* and *A. lyrata* parental individuals grown on Cd-contaminated soil. For these elements, mean *A. halleri* shoot concentrations were 2.4-fold (Fe), 3.9-fold (Cd) and 22.4-fold (Zn) higher than mean *A. lyrata* concentrations. Calcium, Mg and K concentrations were higher in *A. lyrata* than in *A. halleri*. The difference was slightly significant for Ca ($P < 0.05$), while no significant difference between the parental lines was found in K and Mg concentrations between *A. halleri* and *A. lyrata*. The F_2 progeny showed a clear segregation for the six minerals quantified in this study; the largest variation was found for Zn concentration (Fig. 1). Moreover, transgressive segregation in the *A. halleri* × *A. lyrata* F_2 population was observed for all element concentrations with the exception of Zn. A highly significant genotype effect was detected for all elements analyzed in this study ($P < 0.0001$). The highest broad-sense heritability was obtained for Zn concentration (0.82), while Cd content showed a broad-sense heritability of 0.62. Broad-sense heritability of the other mineral concentrations was 0.33, 0.41, 0.49 and 0.45 for Fe, Ca, Mg and K, respectively. Spearman's correlation tests identified significant positive correlations between Cd and Zn, K and Fe, whereas a significant negative correlation was identified between Cd and Mg (Table 1). In addition to the positive correlation with Cd, Zn was positively correlated with Ca and Fe. Significant correlations were also identified between Fe and K, Mg and Ca. The elements K and Mg were negatively correlated, whereas the opposite was observed between Mg and Ca (Table 1). Spearman's rank correlation tests identified a positive correlation between total dry shoot weights of F_2 genotypes growing on Cd excess and Cd ($r^2 = 0.30$, $P < 0.0001$), Zn ($r^2 = 0.12$, $P < 0.05$) and K ($r^2 = 0.18$, $P < 0.001$) concentrations. Mg concentrations were, by contrast, negatively correlated with total dry shoot weight ($r^2 = -0.30$, $P < 0.0001$).

QTL mapping of mineral element concentrations

Since interval mapping did not reveal any markers above the LOD score threshold for Mg and Ca content, QTL detection through MQM analysis was only performed for the elements Cd, Zn, Fe and K. One QTL for Cd accumulation on linkage group 3 was identified explaining 21.4% of the phenotypic variance or 34.3% of the genetic variance (Figs 2–4, Table 2). In addition, regions with LOD scores close to the threshold, hereafter referred to as suggestive QTLs, were detected on the bottom of linkage groups 2 and 7. These suggestive QTLs account together for 12.9% of the phenotypic variance or 20.8% of the genetic variance observed for Cd accumulation in the F_2 population. Four QTLs were detected for Zn accumulation on linkage groups 2, 3, 4 and 7 (Figs 2–4, Table 2), explaining 54.7% of the genetic variance observed for this trait in the mapping population. Contributing to almost half of the total genetic variance, QTL Znconc-2 is clearly the major QTL for Zn hyperaccumulation in the *A. halleri* × *A. lyrata* F_2 progeny in this soil with normal Zn concentrations. One suggestive QTL was also detected on linkage group 6, explaining 6.0% of the genetic variance. The QTL analysis of Fe

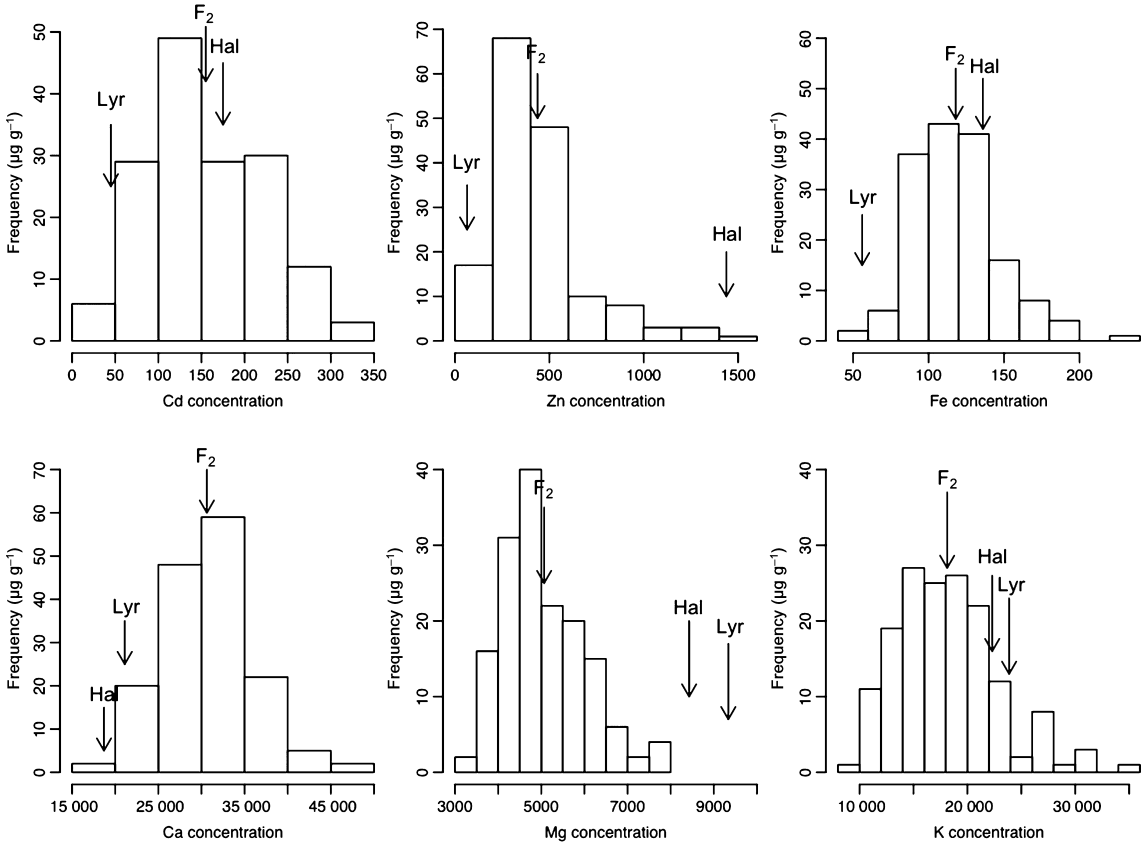

Fig. 1 Frequency distributions of mineral concentrations (µg g^{-1}) in the *Arabidopsis halleri* × *Arabidopsis lyrata* F_2 population. The mean concentrations obtained on the *A. halleri* and *A. lyrata* parental representatives, as well as the mean concentrations obtained on the interspecific *A. halleri* × *A. lyrata* F_2, are indicated by arrows. Hal, *Arabidopsis halleri* ssp. *halleri (A. halleri)*; Lyr, *Arabidopsis lyrata* ssp. *petraea (A. lyrata)*.

Table 1 Correlations between the different mineral element concentrations of *Arabidopsis halleri* × *Arabidopsis lyrata* F_2 individuals grown on Cd-contaminated soil

	Cd	Zn	Fe	K	Mg
Zn	0.5****				
Fe	0.424****	0.241**			
K	0.169*	−0.021	0.162*		
Mg	−0.364****	0.068	0.173*	−0.175*	
Ca	0.080	0.278**	0.173*	−0.047	0.573****

****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$.

revealed three QTLs on linkage groups 1, 2, and 3, explaining 35.3% of the phenotypic variance, which corresponds to the total genetic variance observed for this trait in the F_2 progeny. A suggestive QTL contributing an additional 5.5% to the phenotypic variance observed for this trait in the mapping population was identified on linkage group 8. Three QTLs located on linkage groups 1, 3 and 6 were detected for K content (Figs 2–4, Table 2). These QTLs explained together 37.1% of the phenotypic variance for K concentration, which corresponds to 82.13% of the genetic variance observed for this element in the F_2 progeny. One suggestive QTL was identified on linkage group 2, accounting for 5.4% of the phenotypic variance, or 12% of the genetic variance. For Cd accumulation, evidence of epistasis was found between the major QTL Cdconc-1 and the two suggestive QTLs ($P < 0.01$) (Table 3). For Zn, Fe and K concentrations, epistatic interactions between QTLs were not significant.

A. halleri provides trait-enhancing alleles at all QTLs for Cd and Zn accumulation, but not for Fe and K accumulation

As indicated by the negative additive effects at the QTLs for Cd and Zn accumulation, only *A. halleri* carries trait-enhancing alleles at those QTLs (Table 4). By contrast, at the QTLs for Fe and K concentrations, both parental species *A. halleri* and *A. lyrata* have complementary alleles (Table 4). At the QTL Feconc-1, for instance, the *A. lyrata* alleles tend to increase Fe concentration, whereas at the other QTLs Feconc-2 and Feconc-3 the inverse is observed. In the case of K accumulation also, a negative additive effect is reported for the QTL Kconc-2, whereas the QTLs Kconc-1 and Kconc-3 both show positive additive effects

Table 2 Quantitative trait loci (QTLs) for Cd, Zn, Fe and K concentrations

Trait	QTL[a]	LG/marker[b]	Position[c]	LOD score[d]	R^{2e}
Cd	Cdconc-1	LG3/2-08286	80.784	8.28	21.4
	Cdconc-2	*LG2/1-22940*	*4*	*3.08*	*6.7*
	Cdconc-3	*LG7/4-13275*	*10.25*	*2.83*	*6.2*
Zn	Znconc-1	LG2/1-26746	47.702	5.08	8.1
	Znconc-2	LG3/2-08286	80.784	4.91	7.9
	Znconc-3	LG4/2-12846	45.382	5.29	8.4
	Znconc-4	LG7/4-17540	1	11.61	20.5
	Znconc-5	*LG6/5-08352*	*57.709*	*3.43*	*4.9*
Fe	Feconc-1	LG1/1-04488	8.739	4.93	10.5
	Feconc-2	LG2/1-24380	20.247	5.74	12.2
	Feconc-3	LG3/2-08286	80.784	5.82	12.6
	Feconc-4	*LG8/5-21773*	*37.286*	*2.89*	*5.5*
K	Kconc-1	LG1/1-10858	35.922	4.56	9.8
	Kconc-2	LG3/3-08806	30.075	6.83	14.8
	Kconc-3	LG6/5-08352	57.769	5.73	12.5
	Kconc-4	*LG2/1-26746*	*43.642*	*2.82*	*5.4*

[a]QTLs are named by the trait, and ordered. Suggestive QTLs are indicated in italic.
[b]The linkage group on which the QTL is mapped, and the marker closest to or at the maximum logarithm of odds (LOD) score or QTL.
[c]The position (in centiMorgans, cM) of the QTL on the linkage group.
[d]The LOD score at the QTL.
[e]The percentage of explained variance at the QTL calculated by Multiple QTL Model (MQM) mapping.

(Table 4). Dominance effects at the QTLs take either a negative or positive value, suggesting dominance of either one of the parental alleles. At the QTLs Cdconc-1, Znconc-2, and Kconc-2, the dominance effects are positive, indicating that *A. halleri* alleles tend to be dominant at these QTLs (Table 4). As shown in Fig. 5, both heterospecific classes at these QTLs have a mean element concentration that exceeds the average concentration obtained on F_2 individuals that have two alleles from either *A. halleri* or *A. lyrata* at this QTL. *A. lyrata* alleles tend to be dominant at the QTLs Znconc-1, Feconc-2 and Kconc-1, as dominance effects at these QTLs are negative, and F_2 individuals carrying an allele from each of the parental species at these QTLs show similar accumulation capacities as those that have two *A. lyrata* alleles at the QTLs (Fig. 5, Table 4). At the QTLs Znconc-3, Znconc-4, Feconc-1, Feconc-3 and Kconc-3, the heterospecific F_2 individuals do not display the same behavior depending on the alleles they inherited (Fig. 5, Table 4). Whereas one group will behave as the homospecific *lyrata/lyrata* individuals, the other group behaves as the *halleri/halleri* F_2 genotypes (Fig. 5, Table 4). Over all QTLs, dominance degrees ranged from 0.052 to 1.241 (Table 4). The QTL Cdconc-1 was classified as either overdominant or dominant depending on the heterospecific class (Table 4). The QTL for Zn accumulation displayed dominance degrees ranging from 0.098 to 1.363, and were consequently classified as additive, partially dominant, dominant or overdominant (Table 4). Depending on the heterospecific group, QTLs Znconc-2 and Znconc-3 did not belong to the same category. Both QTLs were qualified as dominant if the heterospecific F_2 individuals carried *halleri*-2/*lyrata*-1 alleles at these QTLs. However, when *halleri*-1/*lyrata*-2 alleles were present at these QTLs, the QTLs Znconc-2 and Znconc-3 were classified as overdominant and additive, respectively (Table 4). As the QTL for Fe concentration displayed dominance degrees between 0.256 and 0.792, they were considered partially dominant (Table 4). Dominance degrees at the QTL for K concentration ranged from 0.052 to 0.808. The QTL Kconc-3 showed a totally additive gene action, whereas the QTL Kconc-2 was classified as partially dominant. The QTL Kconc-1 was considered dominant or partially dominant depending on the parental alleles that the heterospecific F_2 individuals carry at this QTL (Table 4).

Discussion

This study is the first to report a QTL analysis for Cd hyperaccumulation in the metallophyte *A. halleri* grown under conditions of Cd excess. In this aim, an interspecific *A. halleri* × *A. lyrata* F_2 population was created showing extensive segregation for Cd accumulation. In addition to Cd, the accumulation of the elements Zn, K, Fe, Mg and Ca was also investigated and analyzed through QTL mapping.

The detection of one major QTL for Cd accumulation as well as two regions with a LOD score nearby the significance threshold suggests a rather simple genetic architecture of shoot Cd accumulation in *A. halleri*, at least in conditions of Cd excess. A single major QTL for shoot Cd accumulation, explaining a similar portion of the variance, was also detected in an intraspecific *Thlaspi caerulescens* mapping population in conditions of Cd excess (Deniau *et al.*, 2006). Because the *T. caerulescens* genetic map was mainly composed of amplified fragment length polymorphism (AFLP)-based markers, the colocalization of both QTLs could not be verified.

At the QTL Cdconc-1 and the suggestive QTLs Cdconc-2 and Cdconc-3, the *A. halleri* alleles increased Cd concentrations, as was reported for Cd tolerance QTLs in an interspecific *A. halleri* × *A. lyrata* backcross progeny (Courbot *et al.*, 2007; Willems *et al.*, 2007). Additive effects of the same sign at QTLs are expected for traits that have evolved under continuous directional selection (Orr, 1998). Soils with high metal concentrations are believed to have driven the evolution of metal tolerance in *A. halleri*. However, that hyperaccumulation is an adaptation to excess metals by providing increased metal tolerance is not yet verified, and other selective pressures such as herbivory or drought have been hypothesized to be at the origin of this trait (Boyd & Martens, 1992). Despite the fact that the genes involved in

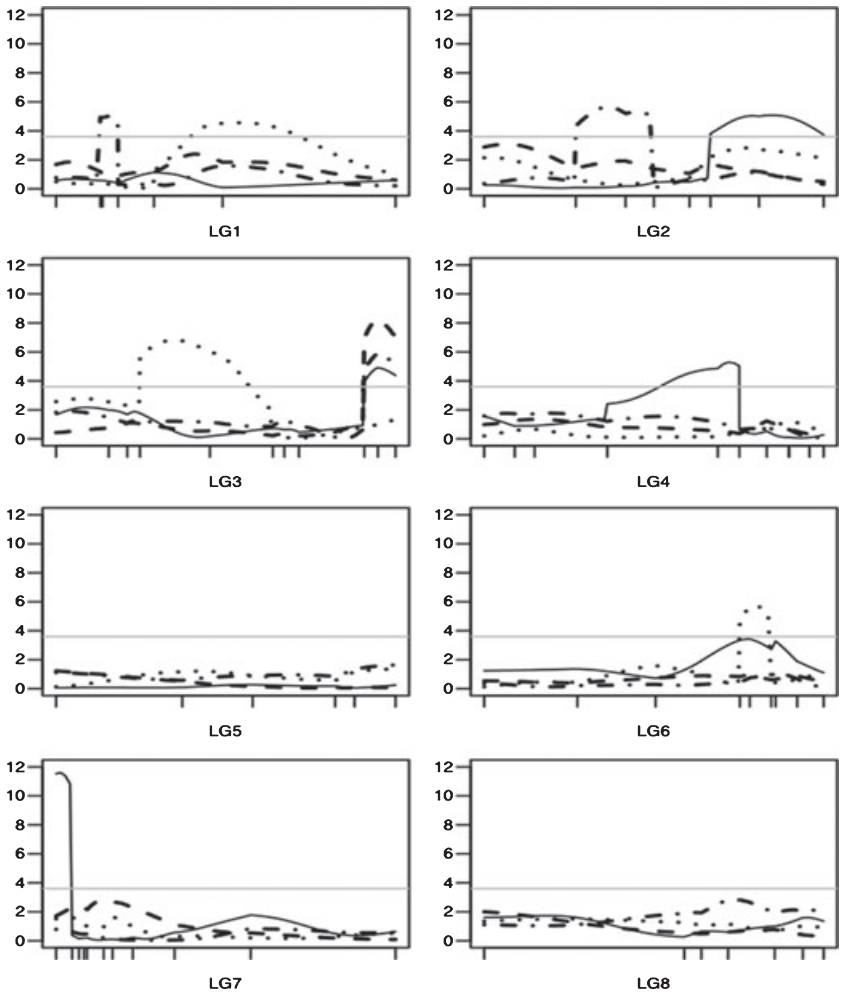

Fig. 2 Logarithm of odds (LOD) score profiles for Cd, Zn, Fe and K concentrations obtained by Multiple Quantitative Trait Loci Model (MQM) analysis. Marker positions are indicated along the *x*-axis, LOD scores along the *y*-axis. LOD score profiles obtained for Cd are indicated by the dashed lines, for Zn by the solid lines, for Fe by the dot-dash lines, and for K by the dotted lines. As different LOD score significance thresholds were applied for the QTL analysis of each of the mineral concentrations, only the lowest LOD score threshold corresponding to 3.6 was reported by the horizontal gray line. LG1–LG8, linkage groups 1–8.

Cd hyperaccumulation are probably under selection in *A. halleri*, it cannot be excluded that several alleles are segregating at these genes in *A. halleri*, as inferred from the different dominance effects and dominance degrees that were detected, depending on the parental alleles inherited at the suggestive QTLs in the heterospecific F_2 individuals. Because of the absence of selective pressures in *A. lyrata* for metal hyperaccumulation, it is nevertheless more likely that the *A. lyrata* parent carries different alleles at the QTLs than *A. halleri*.

The relationship between metal tolerance and hyperaccumulation in metallophytes has been the subject of numerous studies (Macnair *et al.*, 1999; Bert *et al.*, 2003; Frérot *et al.*, 2005). In *A. halleri*, independence of Cd tolerance and accumulation was suggested based on the segregation of these characters in an *A. halleri* × *A. lyrata* backcross progeny (Bert *et al.*, 2003). The results obtained in the present QTL analysis do not corroborate this hypothesis, since the major QTL for Cd hyperaccumulation, Cdconc-1, was previously identified as the major QTL involved in Cd tolerance in an *A. halleri* × *A. lyrata* backcross progeny (Courbot *et al.*, 2007). However, evidence against a complete dependence of Cd accumulation and tolerance has been provided since two QTLs specific to Cd tolerance were detected in addition to the major QTL common to Cd hyperaccumulation (Courbot *et al.*, 2007).

Although only fine-mapping and functional validation can provide conclusive evidence of the gene underlying the QTL, we checked for the presence of potential candidate genes in the QTL intervals through comparative mapping as described in Roosens *et al.* (2008). The transfer of the QTL Cdconc-1 and its associated interval from the *A. halleri* × *A. lyrata* map to the *A. thaliana* genome revealed that the genetic marker with which the peak of the QTL Cdconc-1 colocalized was defined within the coding sequence of the heavy metal ATPase HMA4. This protein was shown to be involved in Zn and Cd efflux from the cytoplasm in *A. thaliana*, and more precisely in root-to-shoot translocation of these metals (Hussain *et al.*, 2004; Mills *et al.*, 2005; Wong & Cobbett, 2009). In *A. halleri*, the analysis of RNAi lines with reduced expression of *HMA4* supports the role of HMA4 in Zn hyperaccumulation

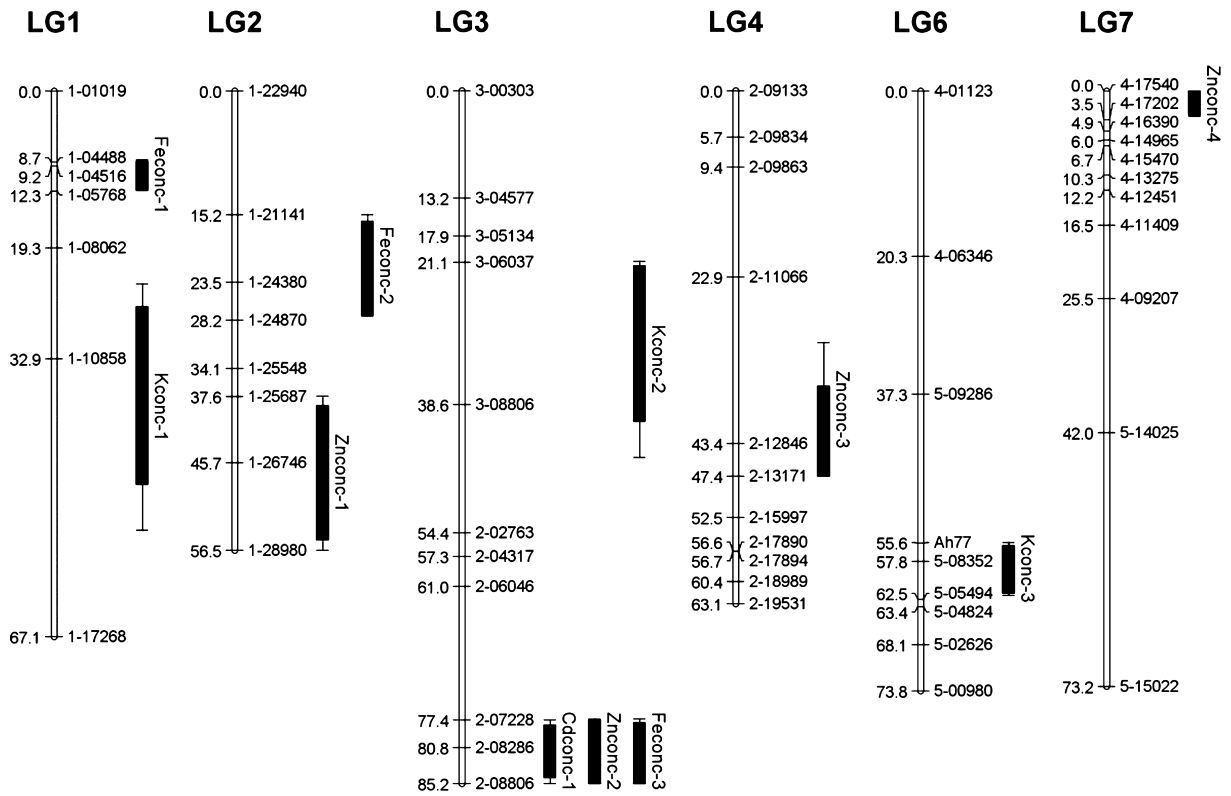

Fig. 3 Confidence intervals associated with the quantitative trait loci (QTLs) for Cd, Zn, Fe and K accumulation. The linkage groups (LG1–LG7) of the *Arabidopsis halleri* × *Arabidopsis lyrata* map on which QTLs for mineral concentrations were detected are displayed. Markers along the linkage groups are indicated with the positions in centiMorgans (cM). One- and two-LOD support intervals around QTLs, calculated in Mapchart, are displayed on the left of each linkage group.

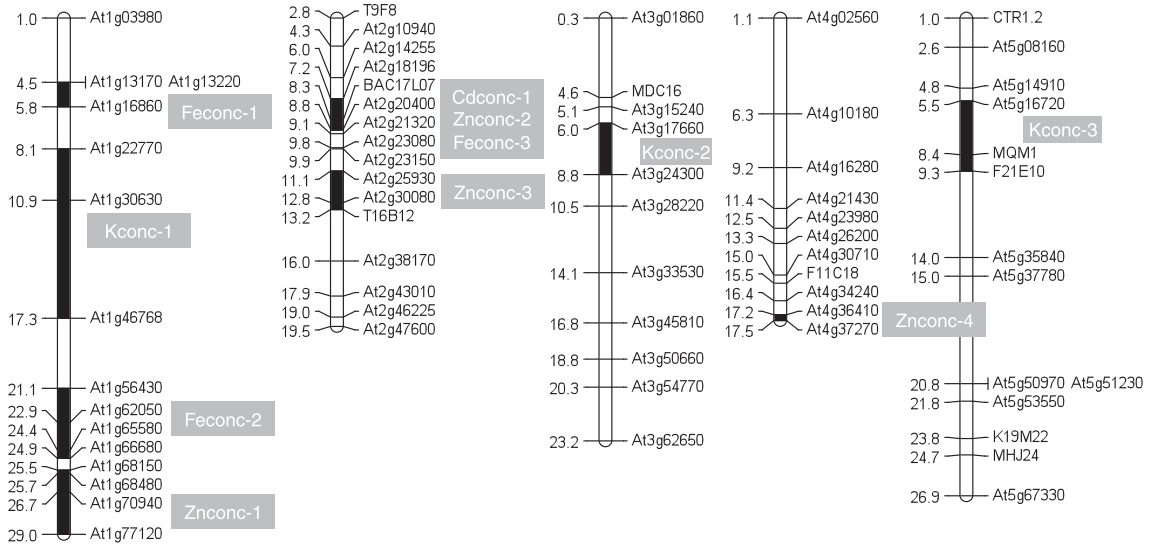

Fig. 4 Quantitative trait loci (QTLs) intervals transferred onto the *Arabidopsis thaliana* genome. The *A. thaliana* chromosomes are displayed, on which the QTL intervals of the mineral concentrations detected in the *Arabidopsis halleri* × *Arabidopsis lyrata* F_2 mapping population were transferred by taking the markers closest to the lower and upper bounds. Markers that were mapped on the *A. halleri* × *A. lyrata* linkage map are indicated along the five *A. thaliana* chromosomes, with the position in kilobases, and named according to the gene or Bacterial Artificial Chromosome (BAC) to which the marker mapped.

Table 3 Epistasis between quantitative trait loci (QTLs) for Cd accumulation

Source	df	Sum of squares	Mean square	*F*-value
Cdconc-1	3	134 061.5506	44 687.1835	15.46****
Cdconc-2	3	46 916.6795	15 638.8932	5.41**
Cdconc-3	3	34 451.2285	11 483.7428	3.97*
Cdconc-1 **Cdconc-2*	9	27 613.8763	3068.2085	1.06
Cdconc-1 **Cdconc-3*	9	12 771.2745	1419.0305	0.49
*Cdconc-2 *Cdconc-3*	9	17 315.5634	1923.9515	0.67
Cdconc-1 **Cdconc-2 *Cdconc-3*	22	131 107.5504	5959.4341	2.06**
Residuals	88	254 314	2890	

****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$.
Suggestive QTLs are indicated in italic.

through the root-to-shoot translocation of Zn (Hanikenne *et al.*, 2008). The *A. halleri* individual used to engineer RNAi lines was not a Cd hyperaccumulator. Nonetheless, the *AtHMA4* silenced lines were more sensitive to Cd and translocated less Cd to the shoot (Hanikenne *et al.*, 2008). These data therefore support the colocalization of the QTL Cdconc-1 with *AhHMA4*. However, the possibility that another gene is causal to the QTL Cdconc-1 cannot, of course, be excluded.

In contrast to the relatively simple genetic determinism of Cd accumulation, a more complex genetic architecture was obtained for Zn accumulation, as four QTLs and one suggestive QTL were detected in the *A. halleri* × *A. lyrata* F_2 progeny for this trait. At all QTLs detected for Zn accumulation, the *A. halleri* alleles increased Zn concentrations, which might reflect strong directional selection that acts on Zn accumulation in *A. halleri*, as was previously reported for Zn tolerance in this metallophyte (Willems *et al.*, 2007). In addition, this study suggests that the alleles at the QTL for Zn hyperaccumulation are not fixed in the species *A. halleri* and/or *A. lyrata*, because dominance effects and dominance degrees vary depending on the parental alleles inherited at the QTLs in the heterospecific F_2 individuals.

As reported in an *A. halleri* × *A. lyrata* BC1 progeny (Bert *et al.*, 2003), a significant positive correlation was observed in the F_2 progeny between Cd and Zn accumulation, suggesting that both characters are under pleiotropic control. This finding was further supported by the QTL analysis of those traits since the QTL Znconc-2 colocalized exactly with the major QTL for Cd accumulation Cdconc-1. As for Cd accumulation, the metal ATPase HMA4 is likely to be the candidate gene underlying the QTL Znconc-2. Indeed, RNAi-mediated silencing of *HMA4* in *A. halleri* resulted in a lower Zn translocation from the root to the shoot, whereas *A. thaliana AhHMA4* transformants showed increased Zn shoot concentrations (Hanikenne *et al.*, 2008). As three QTLs were identified in addition to the one colocalizing with *HMA4*, Zn translocation from the roots to the shoots is certainly not the only factor determining Zn hyperaccumulation in *A. halleri*. The peaks of the QTLs Znconc-3 and Znconc-4 colocalized exactly with genetic markers defined within the coding sequence of the metal homeostasis genes *ZIP6* and *HMA1*, respectively. In *A. halleri*, expression of *ZIP6* is predominant in the shoots, where it is believed to be involved in xylem unloading and cellular uptake of the metals Zn and Fe (Becher *et al.*, 2004; Talke *et al.*, 2006; Kramer *et al.*, 2007). Similarly to *ZIP6*, *HMA1* is primarily expressed in *A. halleri* shoots, and *AtHMA1* is suggested to have a role in Zn detoxification by reducing the Zn content of plastids (Kim *et al.*, 2009). Both *ZIP6* and *HMA1* have higher expression levels in *A. halleri* than in *A. thaliana* under low and high Zn conditions, and these were related, at least for *ZIP6*, to the presence of multiple copies of this gene in the *A. halleri*

Table 4 Additive effects, dominance effects, and dominance degrees at the quantitative trait loci (QTLs) for Cd, Zn, Fe and K accumulation

QTL	*a*[a]	*d halleri*-1/*lyrata*-2[b]	d *halleri*-2/*lyrata*-1[b]	ld/al *halleri*-1/*lyrata*-2[c]	ld/al *halleri*-2/*lyrata*-1[c]
Cdconc-1	−33.82	41.959	30.905	1.241	0.914
Znconc-1	−66.05	−66.05	−66.05	1	1
Znconc-2	−40.96	56.06	39.13	1.369	0.955
Znconc-3	−75.12	−7.38	63.06	0.098	0.839
Znconc-4	−95.95	−95.95	78.94	1	0.823
Feconc-1	15.42	−6.878	4.363	0.446	0.283
Feconc-2	−13.31	−10.541	−7.665	0.792	0.576
Feconc-3	−14.66	3.750	−7.448	0.256	0.508
Kconc-1	2021.15	−1632.950	−1443.650	0.808	0.714
Kconc-2	−2990.15	1778.750	1163.850	0.595	0.389
Kconc-3	2570.30	375.400	−132.900	0.146	0.052

[a]The additive effect at the QTL.
[b]The dominance effect at the QTL. As there are two heterospecific classes at each QTL, the dominance effect was calculated for each of the heterospecific classes.
[c]Dominance degree at the QTL. Dominance degrees were calculated for each heterospecific class.

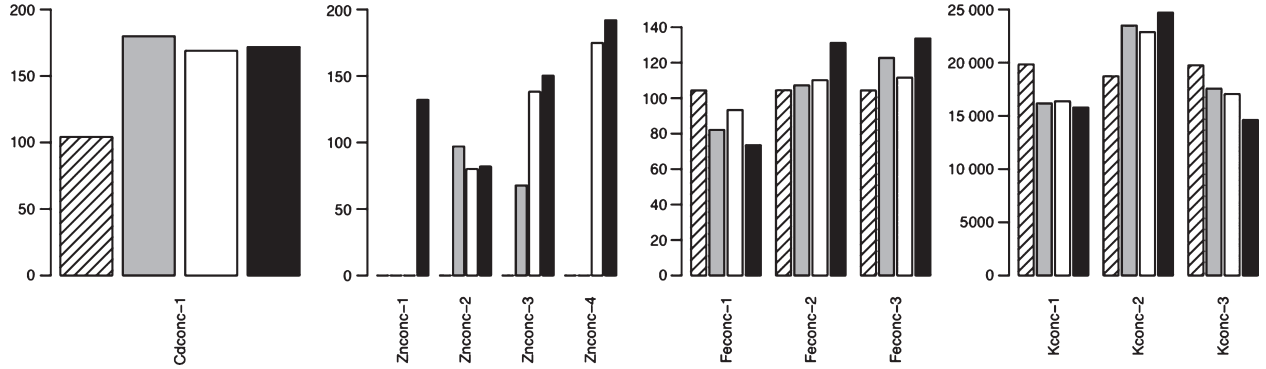

Fig. 5 Bar plots of mean concentrations (μg g^{-1} shoot dry weight), estimated by Multiple Quantitative Trait Loci Model (MQM) in MapQTL, of the four genotype groups at the quantitative trait loci (QTLs) for Cd, Zn, Fe and K accumulation. If for any of the elements MapQTL estimated negative concentrations, they were interpreted as zero. Homospecific *Arabidopsis lyrata*/*A. lyrata* and *Arabidopsis halleri*/*A. halleri* groups are represented by the dashed and black bars, respectively, and the two heterospecific *A. halleri*/*A. lyrata* genotype classes are indicated by the gray and white bars.

genome (Becher *et al.*, 2004; Talke *et al.*, 2006). No known metal homeostasis genes were detected in the *A. thaliana* genome in the region corresponding to the confidence interval associated with the QTL Znconc-1.

The comparison of the low-polluted Zn treatment without Cd addition performed by Frérot *et al.* (2010) and this work allows us to study the impact of Cd excess on the genetic determinants of Zn hyperaccumulation. Whereas the QTLs Znconc-2 and Znconc-4 colocalized exactly with the QTLs ZnAcLP-1 and ZnAcLP-3, partial overlap of the confidence intervals of the QTLs Znconc-3 and ZnAcLP-5 was observed. Also close to the QTL Znconc-1 a suggestive QTL was detected for Zn accumulation in low-polluted soil, while the suggestive QTL Znconc-5 was identified nearby the QTL ZnAcLP-2 on linkage group 6. These results consequently suggest that the major players in Zn hyperaccumulation in the absence of Cd are still the same when Cd is present at high concentrations.

This work suggests that *A. halleri* is distinct from the nonhyperaccumulator species *A. lyrata* not only in its extraordinary capacity to accumulate Zn and Cd to high foliar concentrations, but also in the accumulation of other minerals, such as Fe, since more than twofold higher Fe concentrations were observed in *A. halleri* shoots compared with *A. lyrata*. Modification in homeostasis of metals other than the ones that are hyperaccumulated has previously been reported for hyperaccumulator species (Becher *et al.*, 2004; Roosens *et al.*, 2004; Weber *et al.*, 2004; Talke *et al.*, 2006; Van de Mortel *et al.*, 2006). In *A. halleri*, more specifically, genes involved in Fe homeostasis were found to be more highly expressed when compared with the nonhyperaccumulator species *A. thaliana* in control conditions or, present in multiple copies in the *A. halleri* genome (Becher *et al.*, 2004; Roosens *et al.*, 2004; Weber *et al.*, 2004; Talke *et al.*, 2006; Verbruggen *et al.*, 2009b). Despite the 2.4-fold higher Fe content in Cd-treated shoots of *A. halleri* compared with *A. lyrata*, additive effects at the QTLs indicate that *A. halleri* is not the only parental species contributing trait-enhancing alleles.

The observation that *A. halleri* alleles lead to increased Fe content at the QTLs Feconc-2 and Feconc-3 supports an at least partial positive correlation between the capacity to maintain high Cd and Fe concentrations in *A. halleri*. This suggests the presence of proteins in *A. halleri* able to transport both elements, and/or that these transporters do not compete much for both elements. The hypothesis of pleiotropic gene action for Cd and Fe accumulation is reinforced by the colocalization of the QTLs Feconc-3 and Cdconc-1. However, in contrast to Cd and Zn, a role in Fe transport has not been reported thus far for the metal ATPase HMA4. Furthermore, RNAi-induced silencing of *HMA4* did not affect the partitioning and accumulation of metals other than Zn and Cd in *A. halleri* (Hanikenne *et al.*, 2008). Except for a metal ion binding protein that has not been functionally characterized, no other metal homeostasis gene was found in the *A. thaliana* genomic region corresponding to the QTL interval Feconc-3. Comparison of the position of Feconc-1 and its interval with the syntenic region in *A. thaliana* did not reveal any potential candidate gene for Fe homeostasis. In the QTL interval associated with Feconc-2, on the other hand, three metal homeostasis genes, *NICOTIANAMINE SYNTHASE 4* (*NAS4*), *IRON-REGULATED TRANSPORTER 3* (*IRT3*) and *Yellow Stripe-Like7* transporter (*YSL7*), were found. *IRT3*, which was located closest to the QTL peak of Feconc-2, was reported in *A. halleri* roots at higher transcript abundances than in *A. thaliana* roots in control conditions (Becher *et al.*, 2004; Talke *et al.*, 2006). Given its localization to the plasma membrane, and its ability to complement Zn and Fe-uptake double mutants in yeast, *AhIRT3* is believed to be involved in Fe and Zn uptake in the roots (Becher *et al.*, 2004; Talke *et al.*, 2006; Lin *et al.*, 2009). Nicotianamine (NA) is a metal chelator of both Zn and Fe, and seems to be involved in hyperaccumulation in *A. halleri*

as several NAS genes were more highly expressed in this metallophyte and higher NA concentrations were measured (Weber *et al.*, 2004). *A. thaliana* mutant analysis suggested that *NAS4* is required for correct supply of seeds with Fe (Klatte *et al.*, 2009). The YSL proteins belong to the superfamily of oligopeptide transporters and are believed to be involved in metal hyperaccumulation in *A. halleri* and *T. caerulescens*, and more precisely in the long-distance transport of metals through and between vascular tissues (Gendre *et al.*, 2007). In *A. halleri*, high expression levels were reported for *YSL6* (Talke *et al.*, 2006), but no additional proof for their contribution to metal hyperaccumulation has been provided as yet. In *T. caerulescens*, no metal transport activity was measured for *TcYSL7* upon expression in yeast (Gendre *et al.*, 2007). Comparable concentrations were measured for the elements Mg, Ca and K in *A. halleri* and *A. lyrata*, suggesting that homeostasis of these elements is not affected in the hyperaccumulator parent. Despite a comparable, though relatively low, broad-sense heritability for all three elements, QTLs were only detected for K concentrations. Nonetheless the presence of multiple small effect QTLs in Mg and Ca accumulation should not be excluded, as those would probably remain undetected in the present study because of limited power. QTL analysis is generally also more successful for traits that are under selection, which is probably not the case for Mg and Ca accumulation in *A. halleri* and *A. lyrata* (Falconer & Mackay, 1996). In the *A. thaliana* regions corresponding to the QTLs and their confidence intervals, potential candidate genes for K accumulation were identified near the peaks of the QTLs Kconc-2 (*K^+ uptake permease 10* (*KUP10*); *CBL-interacting protein kinase 23* (*CIPK23*)), and Kconc-3 (*putative pyruvate kinase* (AT3G25960)).

A significant correlation was reported between Cd and Mg content, as well as with K content, though pleiotropic gene action in Cd and K accumulation was not supported by the QTL analysis since none of the detected QTLs colocalized. In contrast to Cd accumulation, Zn accumulation showed a significant correlation with Ca, but not with the other two mineral contents. Interestingly, Mg was the only element in this experiment for which the accumulation was negatively correlated with the accumulation of Cd. In the Cd hyperaccumulator *T. caerulescens* enhanced Mg uptake in leaves upon Cd excess was reported (Dechamps *et al.*, 2005; Molitor *et al.*, 2005; Kupper & Kochian, 2009), and believed to be a defense reaction preventing Cd binding to the chlorophyll (Kupper & Kochian, 2009). The studies in *T. caerulescens,* however, were performed on Cd-tolerant populations or on intraspecific crosses involving Cd-tolerant *T. caerulescens* plants. This experiment, by contrast, used an interspecific progeny originating from a cross between a Cd-sensitive and Cd-tolerant species. Combining two different genomes that have diverged from each other *c.* 5 million yr ago may cause severe changes on the epigenetic level (Ha *et al.*, 2009). Because metal homeostasis genes seem to be differently regulated in hyperaccumulator and in nonaccumulator species (Becher *et al.*, 2004; Weber *et al.*, 2004; Talke *et al.*, 2006; Hanikenne *et al.*, 2008), profound changes in the homeostasis of Mg in F_2 individuals because of the reshuffling of *A. lyrata* and *A. halleri* alleles might explain why this study reported a negative correlation between Cd and Mg, rather than a positive correlation as was observed in the other experiments. However, adding Cd to the soil might also affect the uptake of the other elements, such as Mg, as was reported in winter wheat (Kikuchi *et al.*, 2009). The authors obtained a suppressive effect on Cd uptake by MgO, which they attributed to its high soil-neutralizing capacity (Kikuchi *et al.*, 2009). It is also interesting to note that correlations between element concentrations and shoot biomass in this experiment were all positive with the exception of Mg content. This suggests that Mg accumulation may be associated with cellular damage upon Cd excess. Among all elements, the strongest correlation was detected between Ca and Mg accumulation. In *Brassica olereacea*, pleiotropic QTLs for Ca and Mg accumulation were reported (Broadley *et al.*, 2008), and in *A. thaliana* slow vacuolar channels encoded by *TPC1* are Ca^{2+}-permeable as well as Mg^{2+}-permeable (Pottosin & Schonknecht, 2007), which could explain pleiotropic gene action for Ca and Mg accumulation.

To conclude, the extraordinary capacity of *A. halleri* to accumulate Cd in its leaves can be partially explained by xylem loading affected by *HMA4*, which could encode the major QTL Cdconc-1. In contrast to the previous report of the independent segregation of Cd tolerance and accumulation in an *A. halleri* × *A. lyrata* BC_1 progeny, the colocalization of the major QTL for Cd hyperaccumulation and tolerance provides evidence for a partial dependence of both traits. The QTL common to Cd tolerance and accumulation also colocalized with the QTLs identified for Zn tolerance and accumulation, suggesting that these traits are all partially dependent in *A. halleri*. Zn hyperaccumulation is not severely affected by the presence of excess Cd in the growth medium, since all QTLs identified under Cd excess showed partial to complete colocalization with the QTLs identified by Frérot *et al.* (2010). In addition to Cd and Zn, homeostasis of Fe is distinct in *A. halleri* and the nonaccumulator species *A. lyrata*, and characterized by enhanced Fe accumulation in *A. halleri* shoots.

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