

# Potential preadaptation to anthropogenic pollution: evidence from a common quantitative trait locus for zinc and cadmium tolerance in metallicolous and nonmetallicolous accessions of *Arabidopsis halleri*

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## Summary

- As a drastic environmental change, metal pollution may promote the rapid evolution of genetic adaptations contributing to metal tolerance. In *Arabidopsis halleri*, genetic bases of zinc (Zn) and cadmium (Cd) tolerance have been uncovered only in a metallicolous accession, although tolerance is species-wide. The genetic determinants of Zn and Cd tolerance in a non-metallicolous accession were thus investigated for the first time.
- The genetic architecture of tolerance was investigated in a nonmetallicolous population (SK2) by using first backcross progeny obtained from crosses between SK2 and *Arabidopsis lyrata petraea*, a nonmetallophyte species.
- Only one significant and common quantitative trait locus (QTL) region was identified explaining 22.6% and 31.2% of the phenotypic variation for Zn and Cd tolerance, respectively. This QTL co-localized with *HEAVY METAL ATPASE 4* (*AhHMA4*), which was previously validated as a determinant of Zn and Cd tolerance in a metallicolous accession. Triplication and high expression of *HMA4* were confirmed in SK2. In contrast, gene duplication and high expression of *METAL TOLERANT PROTEIN 1A* (*MTP1A*), which was previously associated with Zn tolerance in a metallicolous accession, were not observed in SK2.
- Overall, the results support the role of *HMA4* in tolerance capacities of *A. halleri* that may have pre-existed in nonmetallicolous populations before colonization of metal-polluted habitats. Preadaptation to metal-contaminated sites is thus discussed.

## Introduction

Global change, namely the impact of human activities on the Earth, has become a major concern. In particular, the way in which habitat disturbances affect species diversity is now increasingly studied. Anthropogenic disturbances resulting in abrupt changes towards hostile environmental conditions (e.g. use of insecticides or herbicides, or soil, air and water pollution) are expected to locally reduce species diversity because they challenge the maintenance of species populations in local communities. Theoretically, population persistence may rely on the levels of phenotypic plasticity, dispersal or genetic adaptations. However, environmental changes are often so drastic that phenotypic plasticity and migration are not sufficient to avoid extinction. Accordingly, many examples suggest that population persistence, following human-induced environmental disturbances, implies

the rapid evolution of genetic adaptations (Reznick & Ghalambor, 2001). Thus, whereas strong selective pressures resulting from habitat modifications should cause population decline, adaptive evolutionary changes must occur quickly enough to restore population growth before extinction, a phenomenon known as evolutionary rescue (Gomulkiewicz & Holt, 1995; Gonzalez *et al.*, 2013; Carlson *et al.*, 2014). Whether populations can rapidly adapt – or undergo evolutionary rescue – actually depends on several extrinsic and intrinsic factors (Carlson *et al.*, 2014). Among them, intrinsic factors, such as population size, the initial levels of standing variation, and the initial degree of maladaptation, seem essential (Gomulkiewicz & Holt, 1995; Bell & Gonzalez, 2009; Carlson *et al.*, 2014).

Metal pollution is one of the major sources of anthropogenic disturbances inducing toxic environmental conditions for many plant populations, and is mainly caused by the use of agricultural fertilizers or the accumulation of residues from mining or smelting industries. Before the molecular biology era, several studies

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revealed that the maintenance of plant populations facing metal pollution and the associated selective pressures implied the rapid evolution of an adaptive trait called 'metal tolerance' (Wu *et al.*, 1975; Al-Hiyaly *et al.*, 1988). It was also shown that the evolution of metal tolerance in metal-tolerant (M) populations has occurred in pseudometallophyte species which displayed low frequencies of metal-tolerant individuals in nonmetal-tolerant (NM) populations growing in nonpolluted areas (Ingram, 1988, cited in Baker & Proctor, 1990). Therefore, the maintenance of M populations in those species suggested that they have undergone evolutionary rescue thanks to the rapid selection of favorable alleles from initial standing genetic variation (Bradshaw, 1991).

Such rapid evolution of metal tolerance supported the assumption that it may be controlled by a few major genes, although minor modifier genes could be secondarily selected (Macnair, 1983, 1997; Schat & Ten Bookum, 1992). Using quantitative trait locus (QTL) mapping, genetic studies have investigated zinc (Zn) and cadmium (Cd) tolerance in *Arabidopsis halleri*, a pseudometallophyte model species that develops on metal-polluted sites where pollution is only of anthropogenic origin. From an interspecific cross (BC1) between a Zn/Cd-tolerant M accession (Auby, France) and its nontolerant close relative *Arabidopsis lyrata petraea*, QTL analyses revealed that the genetic architecture of Zn and Cd tolerance traits involved a limited number of genomic regions accounting for a substantial part of phenotypic variance (Courbot *et al.*, 2007; Willems *et al.*, 2007). Two candidate genes co-localizing with these major QTLs were mainly detected. First, *HEAVY METAL ATPASE 4 (HMA4)*, encoding a plasma membrane metal pump involved in root-to-shoot metal translocation and cellular metal detoxification (Hanikenne *et al.*, 2008), co-localized with major QTLs for Zn and Cd tolerance. Second, paralogs of the *METAL TOLERANT PROTEIN 1* gene (*AbMTP1A1* and *AbMTP1A2* in tandem repeat and *AbMTP1B*), encoding Zn transporters involved in vacuolar sequestration (Dräger *et al.*, 2004; Shahzad *et al.*, 2010), co-localized with QTLs for Zn tolerance. More recently, *CATION EXCHANGER 1 (CAX1)*, a gene co-localizing with the second major QTL for Cd tolerance, was identified after fine-mapping. *CAX1* encodes a vacuolar  $\text{Ca}^{++}/\text{H}^{+}$  exchanger that was proposed to limit Cd-induced reactive oxygen species (ROS) accumulation under low-Ca conditions (Baliardini *et al.*, 2015). To date, only the contribution of *AbHMA4* has been validated by RNA interference (RNAi)-mediated silencing (Hanikenne *et al.*, 2008).

As QTLs were identified in an M accession, they might have been selected during the rapid adaptation of *A. halleri* to anthropogenic metal-polluted sites. However, it was demonstrated that NM populations of *A. halleri* exhibited (1) significant standing genetic variation (Meyer *et al.*, 2009) as well as (2) a high degree of Zn and Cd tolerance, so that metal tolerance in this pseudometallophyte species can be considered as species-wide or 'constitutive' (Pauwels *et al.*, 2006; Meyer *et al.*, 2010, 2015). Considering that M populations were probably founded from NM populations nearby (Pauwels *et al.*, 2005), it can be reasonably assumed that (1) initial (i.e. present in NM populations) levels of standing variation were sufficient and (2) the initial degree of maladaptation of

*A. halleri* to anthropogenic metal-polluted habitats was limited enough to promote the maintenance of M populations on metal-polluted soils. Nevertheless, significant differences in average tolerance levels between M and NM populations have been observed (Pauwels *et al.*, 2006; Meyer *et al.*, 2010, 2015). This means that the maintenance of M populations also has required specific adaptive changes, as expected in an evolutionary rescue scenario. However, it remains unclear whether *AbHMA4*, *AbMTP1A1/A2/B* and/or *AbCAX1* would participate in the local adaptation of M populations to the impacted environment or whether they would be involved in constitutive tolerance capacities shared by M and NM populations.

To examine whether the genetic determinants of Zn and Cd tolerance that have been identified in the M accession of *A. halleri* were selected during adaptation to anthropogenic metal-polluted habitats or pre-existed in NM populations, the genetic architecture of Zn and Cd tolerance was explored for the first time using an NM *A. halleri* accession. For this purpose, QTL mapping of a BC1 progeny (called BC1 SK2) generated from a cross between one *A. halleri* individual from an NM Slovakian population (SK2) and *A. lyrata petraea* was performed. To allow comparison with the previous QTL mapping of the BC1 generated with the Auby accession (BC1 AU), the same phenotyping protocol as in Willems *et al.* (2007) and Courbot *et al.* (2007) was used. In addition, the genomic copy number and transcript levels of the candidate genes *HMA4* and *MTP1A* that were identified in BC1 AU were determined in BC1 SK2 and in the parental populations. By comparison with previous studies only involving M individuals, we discuss the possibility that the identified mechanisms are putative preadaptations to anthropogenic metalliferous soils.

## Materials and Methods

### Plant material

The NM SK2 population is a Slovakian population located in the Tatras Mountains that was initially referred to as SI2 (Bert *et al.*, 2002). It was chosen for this study because of its particularly low Zn and Cd tolerance levels compared with other studied *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz populations (Pauwels *et al.*, 2006; Meyer *et al.*, 2010, 2015). A single cross was performed between one individual from the SK2 population and one individual from the nontolerant species *Arabidopsis lyrata petraea* (L.) O'Kane & Al-Shehbaz (from Unhošť, Central Bohemia; Macnair *et al.*, 1999). Both species are self-incompatible and usually outcrossing. Therefore, to avoid any inbreeding depression effect, one randomly selected F1 individual was used as the pollen donor to fertilize a second *A. lyrata petraea* genotype, generating the interspecific backcross progeny (BC1). The BC1 population used for linkage map construction consisted of 335 individuals of which 129 and 70 were phenotyped for Zn and Cd tolerance, respectively. The different sample sizes resulted from difficulties in maintaining some genotypes by cutting.

## Evaluation of Zn and Cd tolerance

Zn and Cd tolerance were measured in two separate sequential growth tests as described in Willems *et al.* (2007) and Courbot *et al.* (2007). Genotypes were propagated by cutting and after 5 wk of growth on sand, three cuttings per BC1 genotype were transferred to vessels filled with nutrient solution. The Zn tolerance test was performed at the University of Lille (France) in a growth chamber with the following parameters: 13 h light  $d^{-1}$ , 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  irradiance, 20°C:18°C, day:night, and hygrometry of 65%. To minimize microenvironmental effects, vessels were randomly distributed in the chamber on a turntable (Rotoplan system; Strader, Saint Sylvain d'Anjou, France). Cd tolerance was measured under controlled glasshouse conditions (13 h light  $d^{-1}$ , 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  irradiance, and 20°C:17°C, day:night) at the Free University of Brussels. Vessels were randomly distributed in the chamber and moved around once a week during the change of nutrient solution. After 3 wk in nutrient solution, plants were sequentially transferred to increasing concentrations of Zn (10–3000  $\mu\text{M ZnSO}_4$ ) or Cd (10–250  $\mu\text{M CdSO}_4$ ). Individual tolerance levels were determined as the lowest concentration at which no increase in root length (for Zn tolerance) or fresh biomass (for Cd tolerance) was observed. This concentration is currently known as the effective concentration for 100% growth inhibition ( $EC_{100}$ ).

## Statistical analysis

For Zn tolerance and Cd tolerance, a one-way analysis of variance (ANOVA) was performed in SAS (GLM procedure; SAS Institute, Cary, NC) considering 'genotype' as a random effect. The normality of residuals was tested using the Kolmogorov–Smirnov  $D$  test and a normal quantile–quantile (Q–Q) plot. Departure from normality was low for both traits ( $D=0.15$  and  $D=0.14$ , respectively). For these reasons, and because normality was also difficult to improve, data sets were not transformed before parametric tests. The broad-sense heritability ( $H^2$ ) of Zn and Cd tolerance was then calculated by dividing the genetic variance (which includes additive genetic variance and other sources of genetic variance) by the total phenotypic variance. Variance components were calculated using the REML method of the VARCOMP procedure of SAS. The arithmetic mean of Zn and Cd tolerance for each genotype was then calculated for the three replicates. The correlation between Zn and Cd tolerances was estimated from genotype values using the Spearman nonparametric coefficient.

## Marker analysis

The genomic DNA of the four parental genotypes and 335 individuals of the BC1 progeny was extracted using a Kit NucleoSpin 96 Plant (Macherey–Nagel, Hoerd, France). Sixty single nucleotide polymorphism (SNP) or microsatellite markers were selected for genotyping (Supporting Information Table S1). Thirty-nine new microsatellite markers were developed as described in Frérot *et al.* (2010). Briefly, the markers were selected from a microsatellite-enriched genomic library developed

from an enrichment procedure with Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) (Glenn & Schable, 2005). Primer sequences were designed in flanking regions of *A. halleri* microsatellites using PRIMER3 software (<http://frodo.wi.mit.edu/>). In order to allow multiplexing of markers, primer combinations were selected according to a 60°C ( $\pm 5^\circ\text{C}$ ) melting temperature and compatibility of PCR product sizes (100–150, 150–250 and 250–350 bp). The microsatellite markers were combined in 12 multiplexes labeled with FAM, PET, NED or VIC fluorescent dyes. Multiplex PCR was carried out in 10- $\mu\text{l}$  reactions containing 1 $\times$  PCR buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM  $\text{MgCl}_2$ , 150 nM dNTP (Euromedex, Souffelweyersheim, France), 0.075  $\mu\text{M}$  of the forward-M13 primer, 0.375  $\mu\text{M}$  of the reverse-PIG primer, 1.5  $\mu\text{M}$  of fluorescent dye-labeled M13 (Applied Biosystems), 0.5 U of Qiagen Multiplex PCR kit, and 5  $\mu\text{l}$  of DNA (20–60 ng). The cycling conditions consisted of one initial denaturation step of 15 min at 95°C, followed by two touchdowns of five cycles each: denaturation for 45 s at 95°C, annealing at 68°C ( $-2^\circ\text{C}/\text{cycle}$ ) for 5 min for the first touchdown and at 58°C ( $-2^\circ\text{C}/\text{cycle}$ ) for 1 min for the second, and extension for 60 s at 72°C; then 27 cycles of 45 s at 95°C, 30 s at 47°C, and 60 s at 72°C; and a final extension of 10 min at 72°C. Amplification products were separated on an ABI Prism 3130 DNA sequencer (Applied Biosystems). Alleles at microsatellite loci were scored using GENEMAPPER software version 3.7 (Applied Biosystems).

In addition, re-sequencing data sets were used to design seven SNP markers. Illumina paired-end sequencing reads from six *A. halleri* populations (P. Saumitou-Laprade, unpublished data) were aligned to the Araly1 assembly of the *A. lyrata lyrata* genome (<http://genome.jgi.doe.gov/Araly1/Araly1.info.html>). Within regions of interest, the criteria used for the selection of SNPs were: interspecific polymorphism; fixed polymorphism within *A. halleri* or *A. lyrata*; 50-base flanking regions with low polymorphism within *A. halleri* and between *A. halleri* and *A. lyrata*; read depth >20. The SNPs were genotyped by KBioscience competitive allele-specific polymerase chain reaction (KASP) (LGC Genomics, Teddington, UK). KASP assays were performed in a final reaction volume of 8  $\mu\text{l}$  containing 4  $\mu\text{l}$  of KASP master mix V2 low ROX (LGC Genomics), 0.125  $\mu\text{l}$  of KASP mix assay (LGC Genomics) and *c.* 100 ng of genomic DNA. The PikoReal real-time PCR system (Thermo Scientific, Breda, the Netherlands) was used with the following cycling conditions: 15 min at 94°C; 10 touchdown cycles of 20 s at 94°C and 60 s at 61–55°C (the annealing temperature for each cycle being reduced by 0.6°C per cycle); and 26 cycles of 20 s at 94°C and 60 s at 55°C. Fluorescence detection was performed at the end of each cycle and the data sets were analyzed using the allelic discrimination RFU-based method of the PIKOREAL software 2.1.

## Linkage map construction

The *A. halleri* SK2  $\times$  *A. lyrata* *petraea* linkage map was constructed with the JOINMAP 3.0 program (Van Ooijen & Voorrips, 2001). The grouping of loci is based on a test for independence translated into a logarithm of odds (LOD) score. Linkage groups

were obtained at an LOD of 4. Markers along each linkage group were then ordered using the sequential method implemented in JOINMAP 3.0 in which loci are added one by one starting from the two most strongly linked loci. For each added locus, the best position is determined by comparing the goodness of fit of the resulting maps for each tested position. Kosambi's mapping function was used to translate recombination frequencies into map distances (Kosambi, 1944). When dealing with an interspecific cross, segregation distortion frequently occurs. Deviations from Mendelian ratios were assessed using a  $\chi^2$  test implemented in JOINMAP 3.0 at a locus-by-locus significance level of  $\alpha = 0.05$ .

### QTL mapping

Detection of QTLs was performed using the MAPQTL 4.0 software (Van Ooijen *et al.*, 2002). A Kruskal–Wallis rank test was first performed on each locus separately to find potential regions of QTLs. Interval mapping (IM) analysis then allowed finer detection by determining whether a QTL occurred and computing an LOD score for every centiMorgan (cM) along the linkage groups. The LOD score represents the 10-base logarithm of the quotient of two likelihoods: the likelihood of the presence of a segregating QTL (alternative hypothesis) divided by the likelihood of no segregating QTL (null hypothesis). To establish the occurrence of a QTL, the calculated LOD scores were compared with an LOD score threshold obtained by a permutation test (1000 permutations), which corresponds to a genome-wide empirical significance threshold at the 5% level (Churchill & Doerge, 1994). A multiple-QTL model (MQM) analysis was finally performed every cM, in which markers close to detected QTLs (by IM mapping) were selected as cofactors to take over the role of the nearby QTLs in the approximate multiple-QTL models used in the subsequent MQM analysis. This method reduces the residual variance and enhances the power of searching for other segregating QTLs. It also improves the precision of QTL positions. After manual selection of cofactors, an automatic selection of cofactors was executed to keep a restricted set of significant cofactors. The LOD score profiles showing QTLs with their one- and two-LOD support intervals were obtained using MAPCHART 2.1 (Voorrips, 2002).

Additionally, the power of analysis to detect the QTLs identified by Courbot *et al.* (2007) and Willems *et al.* (2007) was calculated using the R/QTLDESIGN software (Sen *et al.*, 2007).

### *HMA4* and *MTP1A* relative transcript levels and gene copy numbers

Eighteen BC1 progenies with different allelic combinations (*A. lyrata*/*A. lyrata* or *A. lyrata*/*A. halleri*) in the genomic region of *HMA4* (markers Chr2-06046 to Chr2-08800) and *MTP1A* (markers Chr2-17890 to Chr2-19598) were grown on soil in a controlled growth chamber (16 h light d<sup>-1</sup>, 100  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> irradiance, 20°C : 18°C, day : night and 70% humidity). Nine leaves of each genotype were harvested, immediately frozen in liquid nitrogen, homogenized and stored at -80°C. Total RNA was extracted with TRI Reagent (Sigma, St Louis, MO,

USA) and cDNA was synthesized from 1  $\mu$ g of DNaseI-treated (Promega, Fitchburg, WI, USA) total RNA using oligo-dT and the Goscript reverse transcription system (Promega). Real-time quantitative PCRs were conducted in 96-well plates with the PikoReal real-time PCR system using Takion no ROX SYBR Mastermix (Eurogentec, Cologne, Germany). Volumes of 2.5  $\mu$ l of cDNA were used for PCR in a 10- $\mu$ l mix containing 5  $\mu$ l of SYBR mastermix, 2  $\mu$ l of H<sub>2</sub>O and 0.5  $\mu$ M of each primer. A total of three technical repeats were run per cDNA and primer pair combination. Relative transcript levels of *HMA4* and *MTP1A* were calculated by normalization to *ELONGATION FACTOR 1 ALPHA (EF1 $\alpha$ )* as a constitutively expressed reference gene (Talke *et al.* 2006). *HMA4*, *MTP1A* and *EF1 $\alpha$*  primers were described previously (Talke *et al.*, 2006; Shazhad *et al.*, 2010; Hanikenne *et al.*, 2013). In the study by Shazhad *et al.* (2010), the *MTP1A* primers were designed for amplification on *A. halleri* DNA. By sequencing, we checked the specificity of these primers on *A. lyrata* DNA and then the PCR efficiency was evaluated from the analysis of 1 : 1, 1 : 3, 1 : 12 and 1 : 48 dilution series. Genomic copy numbers of *HMA4* and *MTP1A* were evaluated on genomic DNA of the BC1 SK2 parents. The single-copy gene *SHORT ROOT (SHR)* was used as a reference gene and a total of 15 technical repeats were run per DNA and primer pair combination according to the protocol previously described. All primers have been described previously (Baliardini *et al.*, 2015).

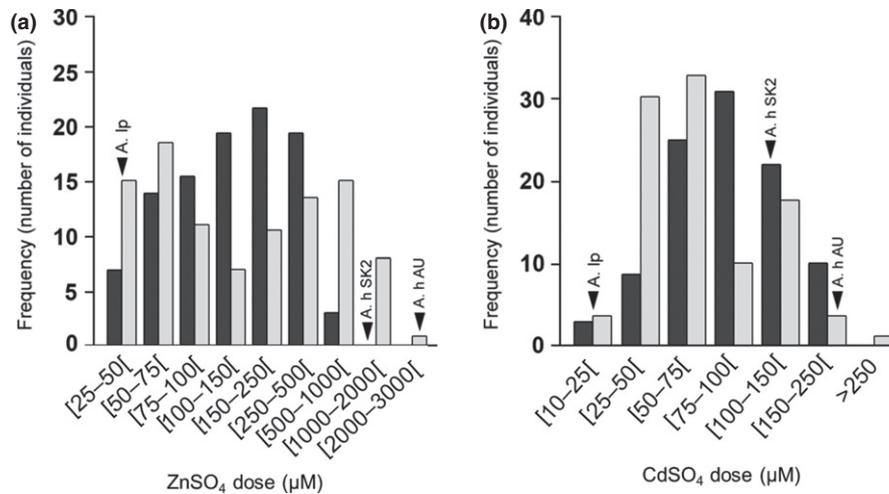
## Results

### Zn and Cd tolerance in BC1 SK2

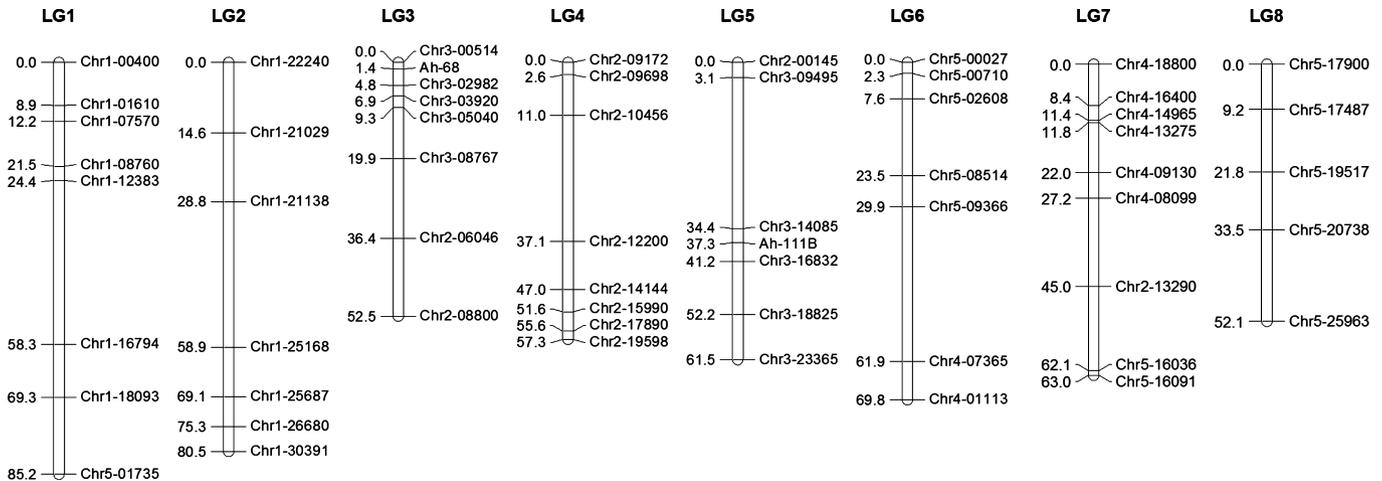
For both Zn and Cd tolerance, the broad-sense heritability ( $H^2$ ) was moderate: 0.54 and 0.50, respectively. The phenotypic distribution of these traits was examined in the BC1 population (Fig. 1). A slight departure from normality was observed for both traits ( $D=0.15$  and  $D=0.14$ , respectively;  $P$ -value < 0.01), with skewness (-0.13 and -0.15, respectively) and kurtosis (3.07 and 3.07, respectively) values barely different from those of a normal distribution (0 and 3, respectively). Seven individuals showed higher Cd tolerance values (EC<sub>100</sub> from 150 to 225  $\mu$ M CdSO<sub>4</sub>) than the average tolerance in the SK2 population (EC<sub>100</sub> = 133  $\mu$ M CdSO<sub>4</sub>; Meyer *et al.*, 2015). This result has to be treated with caution because parent representatives are not the original parents and some individuals from this population were able to survive up to 250  $\mu$ M CdSO<sub>4</sub> in the study of Meyer *et al.* (2015). For both Zn and Cd tolerance, the phenotypic distributions were rather different between the BC1 SK2 and the BC1 AU progenies, suggesting different genetic architectures (Fig. 1). The correlation between Zn and Cd was positive and significant ( $N=50$ ;  $r=0.29$ ;  $P=0.039$ ).

### Linkage map

The 60 markers were assigned to the eight linkage groups of the *A. halleri*  $\times$  *A. lyrata* *petraea* genetic map (Willems *et al.*, 2007; Frérot *et al.*, 2010). The length of each linkage group varied from 52.1 to 85.2 cM, while the marker number varied from five to



**Fig. 1** Segregation profile for Zn (a) and Cd (b) tolerance in backcross progenies generated either using one SK2 individual (dark gray bars) or one Auby individual (light gray bars; from Courbot *et al.* (2007) and Willems *et al.* (2007)). Parents' representatives are noted above the arrows. A.l.p, *Arabidopsis lyrata* ssp *petraea*; A.h SK2, *A. halleri* of population SK2; A.h AU, *A. halleri* from population AU (Auby).



**Fig. 2** Linkage map of the *Arabidopsis halleri* SK2 × *A. lyrata petraea* BC1 progeny. Markers are labeled to the right of linkage groups by their position in the *A. thaliana* genome (chromosome number – position in kb) except for some markers with unknown positions. Distances on the genetic map are expressed in cM. Markers in segregation distortion are underlined. LG, linkage group.

nine by linkage group (Fig. 2). The total length of the map (522 cM) was very similar to the previous estimations (567 and 526 cM; Willems *et al.*, 2007; Frérot *et al.*, 2010). The order of the markers in the present genetic map was very similar to that in *A. thaliana* and *A. lyrata* physical maps (Fig. 2; Table S1). Discrepancies among these three maps were only observed for one marker in linkage group 7 (Chr2-13290) and three markers at the beginning of linkage group 2 (markers Chr1-22240, Chr1-21029 and Chr1-21138). The order in this group of three markers was similar between the *A. lyrata* physical map and the present genetic map but the localization was different (end of scaffold 1 for *A. lyrata*; data not shown). At a significance threshold of 5%, 32 markers (i.e. 53% of markers) showed significant departure from the expected Mendelian ratio (Fig. 2). These markers were located mainly in LG1, LG2 and LG4 and showed distortion in the same direction (excess of the homospecific *A. l. petraea*/

*A. l. petraea* allelic combination) as was observed in the BC1 AU progeny (Willems *et al.*, 2007).

### QTLs of Zn and Cd tolerance

Only one significant QTL was detected for Zn tolerance and one for Cd tolerance (called SK2\_ZnTol1 and SK2\_CdTol1, respectively) with LOD scores higher than the threshold obtained by permutation (i.e. 2.3). These two QTLs overlapped and were located at the end of linkage group 3 between markers Chr2-08800 and Chr2-06046. They explained 22.6% and 31.2% of the total phenotypic variance for Zn and Cd tolerance, respectively (Table 1; Fig. 3). Negative additive effects were measured for both traits, meaning that the *A. halleri* alleles increased tolerance compared with the *A. lyrata petraea* alleles (Table 1). Interestingly, confidence intervals of SK2\_ZnTol1 and SK2\_CdTol1

**Table 1** Summary characteristics of quantitative trait loci (QTLs) detected for zinc (Zn) and cadmium (Cd) tolerance in the *Arabidopsis halleri* SK2 × *A. lyrata petraea* BC1 progeny

Tolerance to	QTL <sup>a</sup>	LG <sup>b</sup>	Marker <sup>c</sup>	LOD score <sup>d</sup>	R <sup>2</sup> e	a <sup>f</sup>
Zn	SK2_ZnTol1	3	Chr2-08800/ Chr2-06046	6.85	22.6	-178.50
Zn	<i>SK2_ZnTol2</i>	6	Chr5-08514	1.65	4.6	-79.61
Zn	<i>SK2_ZnTol3</i>	8	Chr5-17487	1.29	3.9	-75.00
Cd	SK2_CdTol1	3	Chr2-08800/ Chr2-06046	4.33	31.2	-48.74
Cd	<i>SK2_CdTol2</i>	1	Chr5-01735	1.55	10.5	-27.71
Cd	<i>SK2_CdTol3</i>	5	Chr3-14085	1.39	7.3	-23.30

<sup>a</sup>QTLs are named according to the trait and ordered according to their significance level. Suggestive QTLs are indicated in italics.  
<sup>b</sup>Linkage groups (LGs) where the QTLs were detected.  
<sup>c</sup>Marker closest to the higher logarithm of odds (LOD) score.  
<sup>d</sup>Maximum LOD score for the linkage group obtained by the multiple-QTL model (MQM) mapping method.  
<sup>e</sup>Percentage of variance explained by the QTL.  
<sup>f</sup>Additive effect of the QTL.

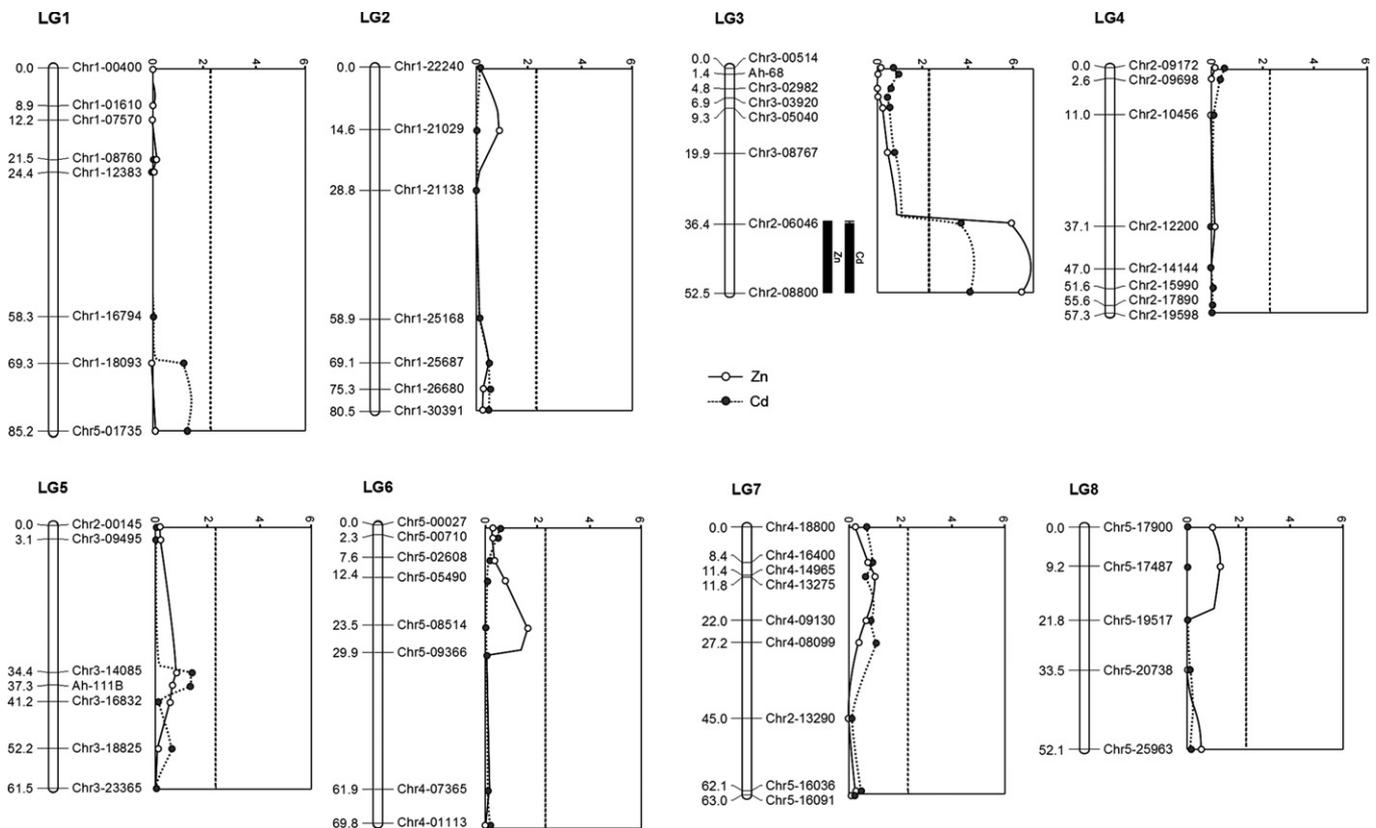
overlapped with the position of the *HMA4* gene (Chr2-08280 in the *Arabidopsis thaliana* genome). Additionally, two peaks near the LOD threshold were observed in linkage groups 6 and 8, and

1 and 5 for Zn and Cd tolerance, respectively (Fig. 3; Table 1) which may indicate minor-effect QTLs. These putative QTLs did not co-localize with the QTLs previously identified in BC1 AU. Together, they explained 8.5% and 17.8% of the Zn and Cd phenotypic variance, respectively.

Using 129 BC1 individuals, markers spaced *c.* 7 cM apart, broad-sense heritability of 0.89 and an LOD threshold of 2.3 for declaring significance, 99% power was found to detect QTLs with additive effects similar to those of ZnTol2 and ZnTol3 (Willems *et al.*, 2007). With the same parameters, 70 BC1 individuals and a broad-sense heritability of 0.79, 75% power to detect a QTL similar to CdTol2 was obtained and 50% power for a QTL similar to CdTol3 (Courbot *et al.*, 2007). These results indicated that the absence of QTLs similar to those of BC1 AU in the present study is not attributable to a lack of statistical power.

### Transcript levels and genomic copy numbers of *HMA4* and *MTP1A*

In order to confirm *HMA4* as a candidate gene for the overlapping QTLs SK2\_ZnTol1 and SK2\_CdTol1, relative expression and genomic copy numbers were examined in some BC1 SK2



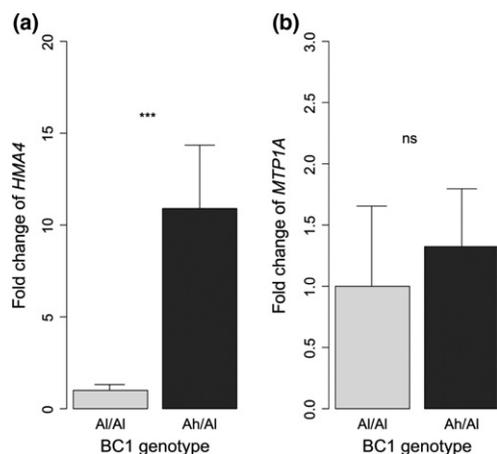
**Fig. 3** Quantitative trait locus (QTL) mapping for zinc (Zn) and cadmium (Cd) tolerance in the *Arabidopsis halleri* SK2 × *A. lyrata petraea* progeny obtained by the multiple QTL model (MQM) mapping method. Marker names are designated by the position on *A. thaliana* chromosomes except for some markers with unknown positions. Logarithm of odds (LOD) scores are indicated on the horizontal axes. The vertical dashed lines represent the LOD score threshold (2.3) at a 5% error level for QTL detection. The positions of QTLs are indicated by bars representing the one-LOD support intervals (one LOD score unit on either side of the QTL peak) and whiskers representing the two-LOD support intervals (two LOD score unit on either side of the QTL peak). Black line, LOD scores for Zn tolerance; dotted line, LOD scores for Cd tolerance.

individuals and in the parents of the cross. The same analysis was performed on *MTP1A* to further confirm that *MTP1* was not a candidate gene for Zn tolerance in SK2. Relative expression of *HMA4* and *MTP1A* was studied in the shoots of 18 BC1 individuals representing the two genotypic classes (*A. l. petraea*/*A. l. petraea* and *A. l. petraea*/*A. halleri*) in each genomic region. On average, 10-fold higher *HMA4* transcript levels were observed in the BC1 genotypes harboring the *A. halleri* allele at the QTLs SK2\_ZnTol1 and SK2\_CdTol1 than in the individuals with two *A. lyrata petraea* alleles in this interval (Fig. 4a). By contrast, *MTP1A* was similarly expressed in the different BC1 individuals independently of the presence of the *A. halleri* allele in the QTL region (Fig. 4b). Using quantitative PCR on genomic DNA, the copy number in the parents of the BC1 SK2 was estimated. As expected, three *HMA4* gene copies were found for *A. halleri* and two for *A. lyrata petraea* (Fig. 5a). It should be noted that one of the two copies detected in *A. lyrata* is a truncated pseudogene (Hanikenne *et al.*, 2013). Only one copy of *MTP1A* was detected in the parent *A. halleri* SK2 and in *A. lyrata petraea* (Fig. 5b).

## Discussion

### Genetic architecture of Zn and Cd tolerance in an NM *A. halleri* accession

The genetic architecture of Zn and Cd tolerance was investigated for the first time in progeny from an interspecific cross between an NM accession of *A. halleri* (SK2) and *A. lyrata petraea*, a non-metallophyte close relative species. Previous QTL analyses suggested a genetic architecture involving six major genetic determinants for Zn and Cd tolerance, called Zntol-1, Zntol-2, Zntol-3, Cdtol-1, Cdtol-2 and Cdtol-3 (Courbot *et al.*, 2007; Willems *et al.*, 2007). Associated percentages of explained variance were moderate for Zn tolerance (12.2%, 11.2% and 5.6%, respectively) and higher for Cd tolerance (42.9%, 23.7% and 15.9%, respectively). By contrast, the genetic architecture



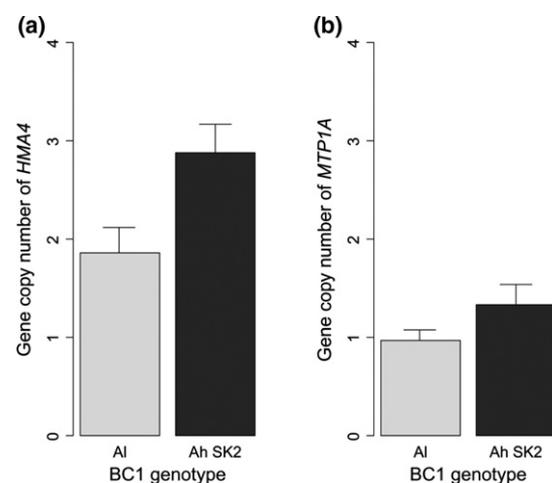
**Fig. 4** Leaf transcript levels of *HEAVY METAL ATPASE 4* (*HMA4*) and *METAL TOLERANT PROTEIN 1A* (*MTP1A*) in BC1 SK2 individuals displaying the *Arabidopsis lyrata petraea* or *A. halleri* alleles (AI and Ah, respectively). Values (mean ± SE;  $n = 9$ ) are given relative to transcript levels of the allelic class AI/AI. Asterisks indicate significant differences: \*\*\*,  $P < 0.001$ ; ns, nonsignificant.

detected here was apparently simpler, as only one significant QTL region was detected for each trait (SK2\_ZnTol1 and SK2\_CdTol1, respectively). The associated explained variances were reasonably high (22.6% and 31.2%, respectively), even though they may be overestimated as a result of sample size effects (Beavis, 1994). Interestingly, however, SK2\_ZnTol1 co-localized with Zntol-1 in Willems *et al.* (2007) and SK2\_CdTol1 co-localized with Cdtol-1 in Courbot *et al.* (2007). Moreover, SK2\_ZnTol1 and SK2\_CdTol1 co-localized with each other and represented the only QTL region that was shared between Zn and Cd tolerance in previous studies (Fig. 3). Additionally, two peaks near the LOD threshold which may indicate minor-effect QTLs explained 8.5% and 17.8% of the Zn and Cd phenotypic variance, respectively. These putative QTLs did not co-localize with the QTLs previously identified with BC1 AU.

The phenotypic distributions in BC1 SK2 were unimodal with an intermediate mode between the values of the parents' representatives (Fig. 1). By contrast, bimodal distributions of phenotypic values were observed for Zn and Cd tolerance of the BC1 AU progeny (Courbot *et al.*, 2007; Willems *et al.*, 2007). A bimodal distribution is commonly interpreted as evidence for one major effect locus in association with a few minor effect loci. Nevertheless, a unimodal – even normal – distribution is not evidence of genetic control by more than a single major gene (Lynch & Walsh, 1998). The phenotypic distributions in BC1 SK2 thus remained in accordance with the detection of one major-effect QTL, even though a contribution of additional weak-effect genes for Zn and Cd tolerance in *A. halleri* is very likely.

### Putative role of *AhHMA4* in constitutive tolerance in *A. halleri*

SK2 showed among the lowest levels of Zn and Cd tolerance compared with other NM and M *A. halleri* accessions (Pauwels



**Fig. 5** Gene copy number in the parents of the *Arabidopsis halleri* SK2 × *A. lyrata petraea* BC1 progeny for (a) *HEAVY METAL ATPASE 4* (*HMA4*) and (b) *METAL TOLERANT PROTEIN 1A* (*MTP1A*). Values (mean ± SE;  $n = 15$  technical replicates) are given relative to the single-copy gene *SHORT ROOT* (*SHR*). *Arabidopsis lyrata petraea* (AI) genomic DNA served as a calibrator. Note that, among the AI*HMA4* copies, one is a truncated pseudogene (Hanikenne *et al.*, 2013).

*et al.*, 2006; Meyer *et al.*, 2010, 2015). This suggests that the tolerance capacities of SK2 are representative of the constitutive capacities that are shared by the whole species and must have evolved very early after the species emergence (Bert *et al.*, 2000; Pauwels *et al.*, 2006). Accordingly, we assume that genetic bases of constitutive background tolerance are species-wide and, *a fortiori*, shared among M and NM populations. As SK2\_ZnTol1 and SK2\_CdTol1 (the overlapping QTLs) corresponded to the common QTL region identified for BC1 AU and BC1 SK2, this region may be involved in constitutive tolerance in *A. halleri*.

Thus, considering the co-localization of SK2\_ZnTol1 and SK2\_CdTol1 with Zntol-1 (Willems *et al.*, 2007) and Cdtol-1 (Courbot *et al.*, 2007), *AbHMA4* can be considered as a relevant candidate gene for constitutive tolerance. Although the wide confidence intervals of the QTL regions mean that we cannot rule out the possibility that one or more genes in linkage disequilibrium may also be valid candidates, strong evidence supports *AbHMA4* as a candidate gene for constitutive tolerance. First, *AbHMA4* was already identified as a candidate gene underlying Zntol-1 and Cdtol-1 in the BC1 AU studied by Willems *et al.* (2007) and Courbot *et al.* (2007). A contribution of *HMA4* to Zn and Cd tolerance traits was further demonstrated by RNAi-mediated silencing in the German M accession of Langelsheim (Hanikenne *et al.*, 2008). In addition, high *HMA4* expression was associated with tandem triplication of *AbHMA4*, whereas the gene is present as a single copy in nontolerant relatives (Hanikenne *et al.*, 2008). A combination of demographic and molecular evolution approaches suggested that *AbHMA4* triplication occurred well before the establishment and colonization of anthropogenic metal-polluted habitats (Roux *et al.*, 2011). Actually, the first duplication event of *AbHMA4* could have occurred very close to the time of the split between *A. lyrata* and *A. halleri*, and was subsequently followed by the second *AbHMA4* duplication (Roux *et al.*, 2011). More recently, a population survey suggested that triplication was shared among M and NM accessions from Germany (Hanikenne *et al.*, 2013). Our results confirmed that *AbHMA4* was also triplicated in SK2, a population that can be considered to be genetically isolated from M accessions than have been characterized so far (Pauwels *et al.*, 2012). Taken together, previous findings and the results of the present study strongly support the assumption that the tandem triplication and the consecutive overexpression of *AbHMA4* may be a major determinant of constitutive Zn and Cd tolerance in *A. halleri*.

### Role of standing genetic variation in adaptive evolution to anthropogenic metal-polluted habitats

The subtractive comparison of QTL regions detected for BC1 AU and BC1 SK2 should be informative about the genetic mechanisms involved in the local adaptation to anthropogenic metal-polluted habitats. It is remarkable that Zntol-2 and Zntol-3, which are commonly associated with *AbMTP1A* and *AbMTP1B*, respectively (Willems *et al.*, 2007), Cdtol-2, which is associated with *AbCAX1* (Baliardini *et al.*, 2015), and Cdtol-3 were not detected in BC1 SK2. It is also remarkable that *AbMTP1A* was not overexpressed in SK2 compared with control sensitive species

(Fig. 4b). This might indicate that those candidate genes may rather contribute to enhanced metal tolerance observed in M populations instead of constitutive tolerance. We suggest that the maintenance of M populations in anthropogenic polluted sites (i.e. their evolutionary rescue) might have involved selection of particular variants of those genes, either in the coding or in the promoting region. Considering *AbMTP1*, this would suggest that an improvement of Zn detoxification mechanisms, through enhanced sequestration in leaf cell vacuoles, would have been advantageous for plants exposed to higher concentrations of Zn in soils. In contrast, effective Zn detoxification is not necessary on nonmetalliferous soils, and basic activity of *AbMTP1* should be sufficient to ensure Zn homeostasis.

More generally, most adaptive genetic changes are expected to result from standing variation, that is, the selection of neutral or mildly deleterious alleles present at variable frequency in populations (Matuszewski *et al.*, 2015). Based on investigations of within-population genetic variation, it has already been suggested that M populations of *A. halleri* may have evolved from large standing variation existing in NM populations (including SK2) rather than from new mutations (Pauwels *et al.*, 2006; Meyer *et al.*, 2010). Several authors came to the same conclusion regarding tolerance polymorphism within NM populations of *Noccaea caerulea* (Meerts and Van Isacker, 1997; Escarré *et al.*, 2000).

### Preadaptation of *A. halleri* to anthropogenic metal-polluted habitats

Apart from selection of standing variation, local adaptation may also benefit from the existence of advantageous alleles that are already at high frequency or even fixed in populations. In this case, individuals can be considered as 'preadapted'. In some pseudometallophytes, it has already been suggested that adaptation to metal-polluted soils may have benefited from preadapting processes. For example, preadaptation to high soil Zn concentrations was reported for Canadian populations of *Deschampsia cespitosa* as all NM individuals tested revealed quite high Zn tolerance levels (Cox & Hutchinson, 1981). Similarly, the widespread tolerance to high magnesium : calcium ratios and nickel concentrations covered by a granite outcrop population of *Phacelia dubia* suggested that this species showed some degree of preadaptation to serpentine habitats (Taylor & Levy, 2002). In *A. halleri*, the pre-existence of constitutive tolerance and of *AbHMA4* copy number expansion (Roux *et al.*, 2011; Hanikenne *et al.*, 2013) suggests that the species was somehow preadapted to anthropogenic metal-polluted habitats. It also interrogates the evolutionary 'raison d'être' of constitutive metal tolerance in a pseudometallophyte. Evidence of positive selection in the *AbHMA4* genomic region in M as well as NM populations implies that *AbHMA4* paralogs may have played a role in a non-metalliferous environment (Hanikenne *et al.*, 2013). However, the selective pressure that may have favored the selection of *AbHMA4* overexpression remains unclear.

A preadaptation may occur when a trait that is currently associated with a particular adaptation was previously selected for another function in response to a different selective pressure

(Gould & Vbra, 1982). The current trait is thus considered as an 'exaptation', in comparison with an 'adaptation' which is an original trait that evolved for its present function. A preadaptation is thus a potential exaptation if it becomes selected for a different function. Considering *AbHMA4* paralogs, available data indicate that they may not only be involved in tolerance of toxic metals in soils. In particular, *AbHMA4* gene copies have been shown to participate to Zn and Cd hyperaccumulation (Hanikenne *et al.*, 2008; Frérot *et al.*, 2010; Willems *et al.*, 2010), that is, the abnormal concentrations of these trace elements in aerial plant parts (for recent reviews, see Krämer, 2010; van der Ent *et al.*, 2013; Verbruggen *et al.*, 2009). Indeed, *HMA4* is a plasma membrane pump that plays a role in Zn and Cd xylem loading and cellular detoxification of Zn and Cd excess (Hanikenne *et al.*, 2008). The selective pressures that promote the evolution of hyperaccumulation have been, and are still, a subject of debate (Boyd & Martens, 1992; Pollard *et al.*, 2014). Interestingly, some of these putative selective pressures could act in a nonpolluted environment. For example, elemental defense against herbivores and pathogens or elemental allelopathy have been suggested as potential roles for metal hyperaccumulation (Boyd & Martens, 1992, 1998).

Finally, our study provides consistent arguments in favor of some degree of preadaptation to Zn and Cd tolerance in *A. halleri*. These arguments will be strengthened when the occurrence of *AbHMA4* copies, associated with significant Zn and Cd tolerance capacities, is demonstrated in several NM populations. Undoubtedly, the more integrated NM accessions are in future molecular studies, the greater will be the potential to elucidate the evolutionary origin of tolerance capacities in *A. halleri*. Other important implications of our work relate to investigations into mechanisms of local adaptation to metal-polluted soils. In this regard, it should be enlightening (1) to pursue the identification of candidate genes associated with the QTL regions specific to BC1 AU, (2) to explore the association between their expression and quantitative variations for Zn and Cd tolerance in natural populations of *A. halleri*, and (3) to examine the consequence of local adaptation to metal-polluted sites for the population structure of genetic diversity at those genes.

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## Author contributions

C-L.M., H.F. and N.V. planned and designed the research, analyzed data and wrote the manuscript; C-L.M., L.B. and C.G. performed experiments; M.P. helped with the *MTP1* genotyping and revised the manuscript; A.B. and P.S. maintained plant collections; D.S. performed preliminary experiments.

## References

- Al-Hiyaly SA, McNeilly T, Bradshaw AD. 1988. The effect of zinc contamination from electricity pylons – evolution in a replicated situation. *New Phytologist* 110: 571–580.
- Baker AJM, Proctor J. 1990. The influence of cadmium, copper, lead, and zinc on the distribution and evolution of metallophytes in the British Isles. *Plant Systematics and Evolution* 173: 91–108.
- Baliardini C, Meyer C-L, Salis P, Saumitou-Laprade P, Verbruggen N. 2015. CAX1 co-segregates with Cd tolerance in the metal hyperaccumulator *Arabidopsis halleri* and plays a role in limiting oxidative stress in *Arabidopsis*. *Plant Physiology* 169: 549–559.
- Beavis WD. 1994. The power and deceit of QTL experiments: lessons from comparative QTL studies. *Proceedings of the 49th Annual Corn & Sorghum Industry Res. Conf. Am Seed Trade Assoc.*, Chicago, IL, USA, 250–266.
- Bell G, Gonzalez A. 2009. Evolutionary rescue can prevent extinction following environmental change. *Ecology Letters* 12: 942–948.
- Bert V, Bonnin I, Saumitou-Laprade P, de Laguérie P, Petit D. 2002. Do *Arabidopsis halleri* from nonmetalliferous populations accumulate zinc and cadmium more effectively than those from metalliferous populations? *New Phytologist* 155: 47–57.
- Bert V, Macnair MR, De Laguérie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metalliferous and nonmetalliferous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* 146: 225–233.
- Boyd RS, Martens SN. 1992. The *raison d'être* for metal hyperaccumulation by plants. In: Baker AJM, Proctor J, Reeves RD, eds. *The ecology of ultramafic (Serpentine) soils*. Andover, UK: Intercept, 279–289.
- Boyd RS, Martens SN. 1998. Nickel hyperaccumulation by *Thlaspi montanum* var. *montanum* (Brassicaceae): a constitutive trait. *American Journal of Botany* 85: 259–265.
- Bradshaw AD. 1991. Genostasis and the limit to evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* 333: 289–305.
- Carlson SM, Cunningham CJ, Westley PAH. 2014. Evolutionary rescue in a changing world. *Trends in Ecology & Evolution* 29: 521–530.
- Churchill GA, Doerge RW. 1994. Empirical threshold values for quantitative trait mapping. *Genetics* 138: 963–971.
- Courbot M, Willems G, Motte P, Arvidsson S, Roosens N, Saumitou-Laprade P, Verbruggen N. 2007. A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase. *Plant Physiology* 144: 1052–1065.
- Cox RM, Hutchinson TC. 1981. Multiple and co-tolerance to metal in the grass *Deschampsia cespitosa*: adaptation, preadaptation and the cost. *Journal of Plant Nutrition* 3: 731–741.
- Dräger DB, Desbrosses-Fonrouge A-G, Krach C, Chardonnes AN, Meyer RC, Saumitou-Laprade P, Krämer U. 2004. Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *Plant Journal* 39: 425–439.
- van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H. 2013. Hyperaccumulators of metal and metalloid trace elements: facts and fiction. *Plant and Soil* 362: 319–334.
- Frérot H, Faucon M-P, Willems G, Godé C, Courseaux A, Darracq A, Verbruggen N, Saumitou-Laprade P. 2010. Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL × environment interactions. *New Phytologist* 187: 355–367.
- Glenn TC, Schable NA. 2005. Isolating microsatellite DNA loci. *Methods in Enzymology* 395: 202–222.

- Gomulkiewicz R, Holt RD. 1995. When does evolution by natural selection prevent extinction? *Evolution* 49: 201–207.
- Gonzalez A, Ronce O, Ferriere R, Hochberg ME. 2013. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* 368: 20120404.
- Gould SJ, Vbra ES. 1982. Exaptation – a missing term in the science of form. *Paleobiology* 8: 4–15.
- Hanikenne M, Kroymann J, Trampczynska A, Bernal MP, Motte P, Clemens S, Krämer U. 2013. Hard selective sweep and ectopic gene conversion in a gene cluster affording environmental adaptation. *PLoS Genetics* 9: e1003707.
- Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U. 2008. Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature* 453: 391–396.
- Kosambi D. 1944. The estimation of map distances from the recombination values. *Annals of Eugenics* 12: 172–175.
- Krämer U. 2010. Metal hyperaccumulation in plants. *Annual Review of Plant Biology* 61: 517–534.
- Lynch M, Walsh B. 1998. *Genetics and analysis of quantitative traits*. Sunderland, MA, USA: Sinauer Associates.
- Macnair MR. 1983. The genetic control of copper tolerance in the yellow monkey flower, *Mimulus guttatus*. *Heredity* 50: 283–293.
- Macnair MR. 1997. The evolution of plants in metal-contaminated environments. In: Bijlsma R, Loeschcke V, eds. *Environmental stress, adaptation and evolution*. Basel, Switzerland: Birkhäuser-Verlag, 3–24.
- Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D. 1999. Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London B: Biological Sciences* 266: 2175–2179.
- Matuszewski S, Hermisson J, Kopp M. 2015. Catch me if you can: adaptation from standing genetic variation to a moving phenotypic optimum. *Genetics* 200: 1255–1274.
- Meyer C-L, Juraniec M, Huguet SP, Chaves-Rodriguez E, Salis P, Isaure M-P, Goormaghtigh E, Verbruggen N. 2015. Intraspecific variability of cadmium tolerance and accumulation, and cadmium-induced cell wall modifications in the metal hyperaccumulator *Arabidopsis halleri*. *Journal of Experimental Botany* 66: 3215–3227.
- Meyer C-L, Kostecka AA, Saumitou-Laprade P, Créach A, Castric V, Pauwels M, Frérot H. 2010. Variability of zinc tolerance among and within populations of the pseudometallophyte *Arabidopsis halleri* and possible role of directional selection. *New Phytologist* 185: 130–142.
- Meyer C-L, Vitalis R, Saumitou-Laprade P, Castric V. 2009. Genomic pattern of adaptive divergence in *Arabidopsis halleri*, a model species for tolerance to heavy metal. *Molecular Ecology* 18: 2050–2062.
- Pauwels M, Frérot H, Bonnin I, Saumitou-Laprade P. 2006. A broad-scale study of population differentiation for Zn-tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae). *Journal of Evolutionary Biology* 19: 1838–1850.
- Pauwels M, Saumitou-Laprade P, Holl AC, Petit D, Bonnin I. 2005. Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* 14: 4403–4414.
- Pauwels M, Vekemans X, Godé C, Frérot H, Castric V, Saumitou-Laprade P. 2012. Nuclear and chloroplast DNA phylogeography reveals vicariance among European populations of the model species for the study of metal tolerance, *Arabidopsis halleri* (Brassicaceae). *New Phytologist* 193: 916–928.
- Pollard AJ, Reeves RD, Baker AJM. 2014. Facultative hyperaccumulation of heavy metals and metalloids. *Plant Science* 217–218: 8–17.
- Reznick DN, Ghalambor CK. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. *Genetica* 112–113: 183–198.
- Roux C, Castric V, Pauwels M, Wright SI, Saumitou-Laprade P, Vekemans X. 2011. Does speciation between *Arabidopsis halleri* and *Arabidopsis lyrata* coincide with major changes in a molecular target of adaptation? *PLoS ONE* 6: e26872.
- Schat H, Ten Bookum WM. 1992. Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* 68: 219–229.
- Sen S, Satagopan JM, Broman KW, Churchill GA. 2007. R/qtlDesign: inbred line cross experimental design. *Mammalian Genome* 18: 87–93.
- Shahzad Z, Gosti F, Frérot H, Lacombe E, Roosens N, Saumitou-Laprade P, Berthomieu P. 2010. The five AhMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. *PLoS Genetics* 6: e1000911.
- Taylor SI, Levy F. 2002. Responses to soils and a test for preadaptation to serpentine in *Phacelia dubia* (Hydrophyllaceae). *New Phytologist* 155: 437–447.
- Talke IN, Hanikenne M, Krämer U. 2006. Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* 142: 148–167.
- Van Ooijen JW, Boer MP, Jansen RC, Maliepaard C. 2002. *MapQTL 4.0: software for the calculation of QTL positions on genetic maps*. Wageningen, the Netherlands: Plant Research International.
- Van Ooijen JW, Voorrips RE. 2001. *Joinmap 3.0: software for the calculation of genetic linkage maps*. Wageningen, the Netherlands: Plant Research International.
- Verbruggen N, Hermans C, Schat H. 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* 181: 759–776.
- Voorrips RE. 2002. Mapchart: software for the graphical presentation of linkage maps and QTLs. *Heredity* 93: 77–78.
- Willems G, Dräger D, Courbot M, Godé C, Verbruggen N, Saumitou-Laprade P. 2007. The genetic basis of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri* (Brassicaceae): an analysis of quantitative trait loci. *Genetics* 176: 659–674.
- Willems G, Frérot H, Gennen J, Salis P, Saumitou-Laprade P, Verbruggen N. 2010. Quantitative trait loci analysis of mineral element concentrations in an *Arabidopsis halleri* × *Arabidopsis lyrata petraea* F<sub>2</sub> progeny grown on cadmium-contaminated soil. *New Phytologist* 187: 368–379.
- Wu L, Bradshaw AD, Thurman DA. 1975. The potential for evolution of heavy metal tolerance in plants. III. The rapid evolution of copper tolerance in *Agrostis stolonifera*. *Heredity* 34: 165–187.

## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

### Table S1 List of markers used in linkage map construction

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