

# Variability of zinc tolerance among and within populations of the pseudometallophyte species *Arabidopsis halleri* and possible role of directional selection

Claire-Lise Meyer<sup>1,\*</sup>, Alicja A. Kostecka<sup>2,\*</sup>, Pierre Saumitou-Laprade<sup>1</sup>, Anne Créach<sup>1</sup>, Vincent Castric<sup>1</sup>, Maxime Pauwels<sup>1</sup> and H  l  ne Fr  rot<sup>1</sup>

<sup>1</sup>Laboratoire de G  n  tique et   volution des Populations V  g  tales, UMR CNRS 8016, Universit   des Sciences et Technologies de Lille, B  timent SN2, F-59655 Villeneuve d'Ascq Cedex, France; <sup>2</sup>Department of Ecology, Institute of Botany Polish Academy of Sciences, Lubicz 46, PL-31 512 Cracow, Poland

## Summary

Author for correspondence:

Claire-Lise Meyer

Tel: +33 320434033

Email: [claire-lise.meyer@ed.univ-lille1.fr](mailto:claire-lise.meyer@ed.univ-lille1.fr)

Received: 5 June 2009

Accepted: 7 September 2009

*New Phytologist* (2010) **185**: 130–142

doi: 10.1111/j.1469-8137.2009.03062.x

**Key words:** *Arabidopsis halleri*, divergent selection,  $F_{ST}/Q_{ST}$ , local adaptation, quantitative trait, zinc tolerance.

- We estimated the level of quantitative polymorphism for zinc (Zn) tolerance in neighboring metallicolous and nonmetallicolous populations of *Arabidopsis halleri* and tested the hypothesis that divergent selection has shaped this polymorphism.
- A short-term hydroponic test was used to capture the quantitative polymorphism present between edaphic types, among and within populations. We measured six morphological and physiological traits on shoots and roots to estimate the response of *A. halleri* to Zn. In order to assess the adaptive value of Zn tolerance polymorphism, we compared differentiation of quantitative traits with that of molecular markers.
- Zinc tolerance of metallicolous populations was, on average, higher than that of nonmetallicolous populations according to the morphological and physiological traits measured. Phenotypic variability within edaphic types was very high and mainly explained by polymorphism among individuals within populations. Genetic differentiation for photosystem II yield of leaves (a measure of photosynthetic efficiency) was greater than the differentiation for microsatellite and thus, probably shaped by divergent selection.
- Overall, these results suggest that, in the sampled populations, Zn tolerance has been increased in metallicolous populations through selection on standing genetic variation within local nonmetallicolous ancestral populations.

## Introduction

Interaction between selection, gene flow and genetic drift is a key phenomenon because in the case of spatially heterogeneous selection it may result in local adaptation, a mechanism promoting speciation and maintaining adaptive variation. Therefore, a challenging goal of evolutionary biology is to evaluate the relative contribution of selection vs other forces in shaping phenotypic differentiation among populations. Among plants, attractive models to study phenotypic differentiation driven by natural selection are pseudometallophyte species, which have the ability to grow on soils contaminated by heavy metals as well as on

noncontaminated soils. In highly contaminated sites, extreme environmental conditions may promote rapid differentiation between metallicolous (M) and nonmetallicolous (NM) populations (Reznick & Ghalambor, 2001; Dechamps *et al.*, 2006; Jim  nez-Ambriz *et al.*, 2006). In this context, the trait typically chosen to investigate population differentiation is metal tolerance (i.e. the capacity to survive on soil with levels of metals toxic for most organisms; Antonovics *et al.*, 1971; Macnair & Baker, 1994). Previous investigations focused on this trait because it has been found to evolve rapidly following exposure to heavy metal stress (Wu *et al.*, 1975; Al-Hiyaly *et al.*, 1988) and because it showed intraspecific quantitative variation in several instances (Schat *et al.*, 1993; Meerts & Van Isaker, 1997; Smith & Macnair, 1998; Mengoni *et al.*,

\*These authors contributed equally to this work.

2001; Pauwels *et al.*, 2006). In addition, evolutionary studies have shown that metal tolerance could have evolved independently in geographically distant conspecific populations (Schat *et al.*, 1996; Vekemans & Lefebvre, 1997; Koch *et al.*, 1998; Mengoni *et al.*, 2001; Pauwels *et al.*, 2005).

Among the pseudometallophyte species, *Arabidopsis halleri* has recently emerged as a model species to study tolerance to heavy metals. This species, constitutively tolerant and hyperaccumulator of zinc (Zn) and cadmium (Cd) (Bert *et al.*, 2000, 2002; Küpper *et al.*, 2000; Pauwels *et al.*, 2006), is indeed the closest metal-tolerant relative of the model species *Arabidopsis thaliana* (Al-Shehbaz & O'Kane, 2002), with which it shows high nucleotide sequence identity and good synteny (Roosens *et al.*, 2008a). Differentiation for Zn tolerance was investigated at a broad scale by Pauwels *et al.* (2006) in 31 European populations using a hydroponic sequential test (Schat & Ten Bookum, 1992). They showed that despite its constitutive nature, Zn tolerance is a quantitative trait in this species, with significant differences in average tolerance among populations. In particular, a tendency towards higher tolerance and less polymorphism in M populations compared with NM ones was shown, suggesting that Zn tolerance could have evolved secondarily in M populations. Nevertheless, the possible role of directional selection in shaping the variability of Zn tolerance among *A. halleri* populations has not been established. These results and those of Pauwels *et al.* (2005), which showed that the M populations have probably been founded independently by the nearby NM populations, may imply distinct genetic bases for increased Zn tolerance in unrelated M populations (Pauwels *et al.*, 2006). Therefore, the role of gene flow, drift and selection in shaping variation of tolerance between edaphic types and among *A. halleri* populations should be investigated on a homogeneous genetic background (i.e. at a local scale in a network of populations with possible gene flow).

To investigate the variability of heavy metal tolerance among and within populations, most studies used tests of tolerance on short periods in hydroponic culture (Humphreys & Nicholls, 1984; Von Frenckell-Insam & Hutchinson, 1993; Schat & Vooijs, 1997; Assunção *et al.*, 2003; Pauwels *et al.*, 2006; Galardi *et al.*, 2007). These tests typically estimate only a part of the phenotypic variability responding to heavy metals (i.e. vegetative growth), but are still very useful because they show high repeatability in space and time (Schat & Ten Bookum, 1992) and allow to control the source of stress acting on the plants (one or a combination of different metals). In addition, when combined with genetic studies such as quantitative trait loci (QTL) or linkage disequilibrium (LD) mapping on large numbers of individuals, these tests provide the opportunity to identify the genetic bases of complex quantitative traits (Courbot *et al.*, 2007; Willems *et al.*, 2007).

Short-term hydroponic tests were initially based on either qualitative measures (ability of individuals to produce new roots) at a certain fixed concentration (Macnair, 1983) or quantitative measures (root growth in solutions with and without metal to estimate a tolerance index, Wilkins, 1978). Possible bias, owing to both innate root growth variation and choice of metal concentration, have led Schat & Ten Bookum (1992) to develop a sequential exposure test that uses the EC100 (lowest concentration for 100% growth inhibition) for root growth as an end point. This test has been successfully applied in several studies to estimate quantitative differences of tolerance between populations (Schat *et al.*, 1996; Schat & Vooijs, 1997; Bert *et al.*, 2000; Pauwels *et al.*, 2006). However, tolerance is then regarded as a binary trait (presence or absence of root growth at a sequence of fixed concentrations), when tolerance may actually show quantitative variation. In addition, the history of exposure during such a sequential test may actually lead to underestimation of the variability in the more tolerant populations (Schat & Ten Bookum, 1992).

Root growth was classically used to estimate heavy metal tolerance because, on one hand, early studies of metal tolerance showed that this trait was particularly sensitive to metals (Bradshaw, 1952). On the other hand, it was considered that the first organ in contact with metals (i.e. roots) is likely to be the one in which metal toxicity will first manifest. Nevertheless, shoot performance could be a more relevant trait according to the species under study. For example, for hyperaccumulator species in which metals are translocated very efficiently towards the leaves where they are stored, we might expect the most striking manifestation of metal toxicity to be found in the leaves. This was observed for *Thlaspi caerulescens*, in which the threshold metal exposure level for leaf chlorosis seems to be a good measure of metal tolerance (Assunção *et al.*, 2003) but not for the nickel hyperaccumulator *Alyssum bertolonii*. In this species the root was more sensitive to metal than the shoot (Galardi *et al.*, 2007), possibly because of a higher fraction of cytosolic metal in root cells. Overall, these results suggest that measuring tolerance is not necessarily a trivial task, such that using a number of different end points is needed to analyse the response of hyperaccumulator to toxic metal concentrations.

In the present study, we investigated the genetic variability of Zn tolerance in *A. halleri* at a local scale in a network of genetically and geographically close M and NM populations. We captured quantitative variation using tolerance indices based on shoot and root morphological and physiological traits. In order to assess the adaptive value of Zn tolerance, we compared population differentiation for each quantitative trait ( $Q_{ST}$  statistics) with that for neutral molecular markers ( $F_{ST}$  statistics). Departure from neutral expectations ( $F_{ST} \neq Q_{ST}$ ) can be used to distinguish between a history dominated by divergent selection

( $F_{ST} < Q_{ST}$ ) as opposed to stabilizing selection ( $F_{ST} > Q_{ST}$ ) (for review see Whitlock, 2008).

Using the tolerance test and the analysis of molecular markers we addressed three questions:

- On average, is Zn tolerance in metalicolous and nonmetalicolous populations different?
- What is the degree of polymorphism within and among populations?
- Have differences of Zn tolerance among *A. halleri* populations been shaped by natural selection, thus reflecting local adaptation?

## Materials and Methods

### Populations studied

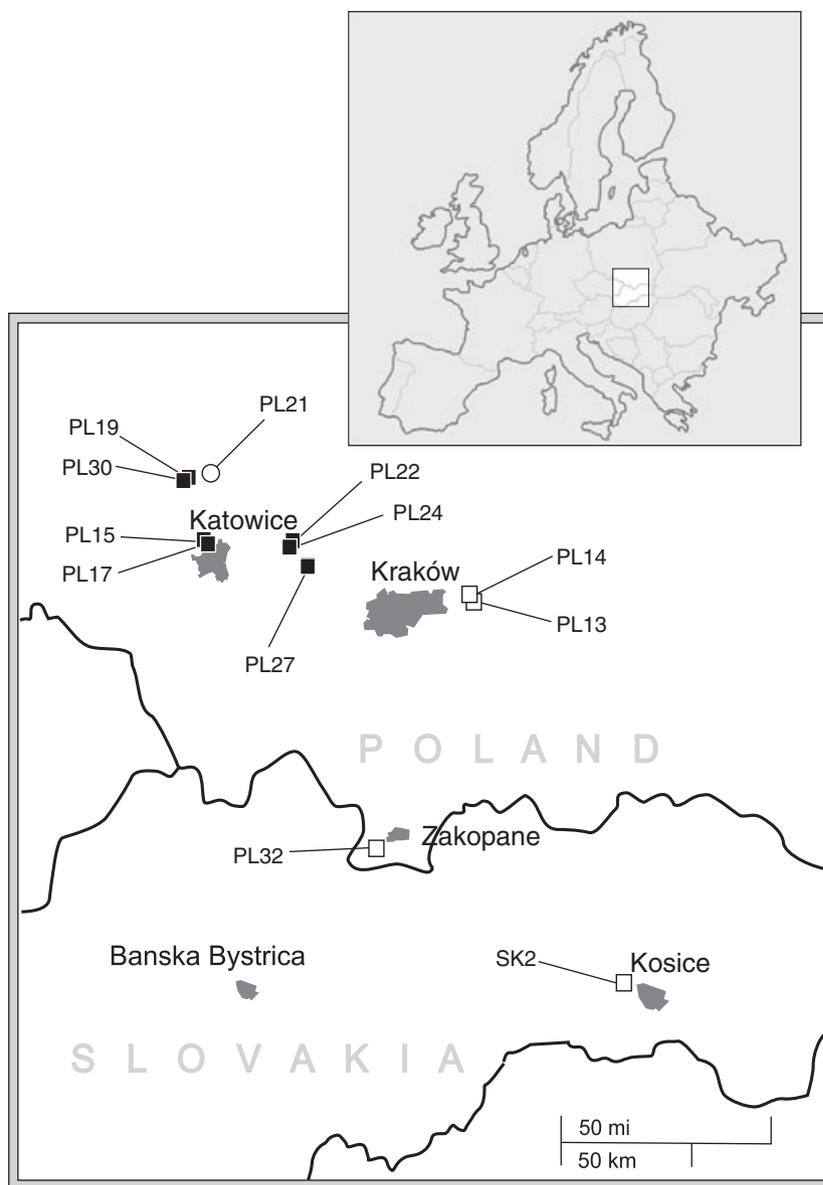
In June 2005, a total of 12 *A. halleri* (L.) (O'Kane & Al-Shehbaz, 2003) (syn. *Cardaminopsis halleri* (L.) Hayek) subsp. *halleri* populations were sampled in the south of Poland and in Slovakia (Fig. 1 and Table 1). A previous survey based on cpDNA variation has shown that populations in this region are genetically very similar (Pauwels *et al.*, 2005). Sampled populations were categorized as metalicolous (M) and nonmetalicolous (NM) according to the total concentration of Zn in soil (Bert *et al.*, 2002). The M populations were located in a small region of southern Poland on metallurgic sites (PL19, PL30, PL22, PL24) as well as in ancient spoil heaps of Zn smelters (PL15, PL17) or mines (PL27). In metalliferous sites, plants are rooted in natural and/or artificial substrates and other metallophytes are present in the vegetation. The distance among M populations ranged from 1 to 70 km. The NM populations were located either at low altitude in southern Poland (PL13, PL14) or at moderate altitude in the Tatra mountains (PL32), as well as in Slovakia (SK2). These populations were found in forest edges and meadows (Table 2). One NM population (PL21) was sampled near a polluted area, in close proximity (*c.* 7 km) to two sampled M populations (PL19 and PL30). This population will be referred to as 'nonmetalicolous in a polluted area' (NMP) according to Pauwels *et al.* (2006). Heavy metal contamination was extremely dissimilar between M and NM sites with total Zn concentrations in soil ranging from 1167 to 35 942  $\mu\text{g g}^{-1}$  for M populations, and concentrations  $< 169 \mu\text{g g}^{-1}$  for NM populations (Table 1). Mature individuals collected in each population (Table 1) were grown on nonpolluted compost in a glasshouse. In order to minimize the carry-over effects of the native population environment, each individual was propagated several times via cuttings.

### Zinc tolerance experiments

The experiment was performed in a controlled growth chamber under 13 h light  $\text{d}^{-1}$ , 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$

irradiance, 20°C d/18°C night and 80% humidity during the first 2 wk and 65% for the rest of the experiment. Light was generated by four metal halide lamps (Radium HRI-T 400W/N, wavelength from 400 to 700 nm, enriched in blue) and four high pressure sodium lamps (Osram Plantastar 400W, wavelength  $< 700$  nm, enriched in red). Six cuttings from each genotype were transferred directly into hydroponic conditions for rooting. Each cutting was grown in a 1 l polyethylene pot filled with standard nutrient solution containing: 20  $\mu\text{M}$  Fe-EDDHA (iron salt of ethylenediamine-di-o-hydroxyphenylacetic acid), 500  $\mu\text{M}$   $\text{MgSO}_4$ , 1 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.1  $\mu\text{M}$   $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ , 0.1  $\mu\text{M}$   $\text{CuSO}_4$ , 25  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 2  $\mu\text{M}$   $\text{MnSO}_4$ , 1  $\mu\text{M}$  KCl, 0.1  $\mu\text{M}$  NaCl, 2 mM  $\text{Ca}(\text{NO}_3)_2$ , 3 mM  $\text{KNO}_3$ , and 10  $\mu\text{M}$  Zn added as  $\text{ZnSO}_4$ . To ensure metal bioavailability, pH of the solution was buffered using 2 mM of MES (2-morpholinoethanesulphonic acid) adjusted to 5.5 with KOH. The nutrient solution was changed once per week. Pots were randomly distributed in the chamber and moved around during each change of nutrient solution. After a 2-wk period allowing plants to initiate roots and acclimatize to hydroponic conditions, the Zn tolerance experiment started. Three clones (i.e. ramets) per genotype were treated with the standard hydroponic solution added with 2000  $\mu\text{M}$  of Zn (contaminated condition, C), whereas three others were grown in the control solution with 10  $\mu\text{M}$  of Zn (noncontaminated condition, NC, according to Macnair *et al.*, 1999). The metal concentration in C condition was chosen on the basis of preliminary experiments (H. Schat, pers. comm.). The 2000  $\mu\text{M}$  Zn concentration in solution allowed all individuals to survive and revealed variability among individuals.

Quantitative variation of Zn tolerance was investigated measuring several morphological and physiological traits expected to reflect Zn toxicity (Broadley *et al.*, 2007): length and biomass of roots as a measure of inhibition of cell division and elongation, shoot biomass and width of leaves as a measure of inhibition of growth by metal, photosystem II yield ( $\Phi_{\text{PSII}}$ ) as a measure of the decrease of photosynthetic efficiency and the relative content of chlorophyll as a measure of chlorosis. Measurements were performed after 6 wk of Zn treatment for each plant in NC and C conditions. The width of leaves was measured as the maximum width of the blades of the three largest leaves. Physiological traits were measured on the three youngest leaves that were large enough to be measured. Chlorophyll fluorescence was measured under ambient light (of the growth chamber) with a PAM-2100 modulated fluorometer (Walz, Effeltrich, Germany). The fiber optic of the fluorometer was mounted in the Arabidopsis Leaf Clip 2060-B (Walz) applied to the young leaves in such a way that the distance between the fiber optic and the leaves was constant and standard. Moreover, the fiber optic formed a 60° angle with the sample, avoiding awkward shading or darkening. In order



**Fig. 1** Geographic locations of *Arabidopsis halleri* populations surveyed in the present study. Open squares, nonmetallicolous (NM) populations; closed squares, metallicolous (M) populations; circle, nonmetallicolous population in polluted area (NMP).

to estimate response of plants under normal growing and steady-state conditions, actinic light irradiance used to drive photosynthesis was equivalent to growth irradiance ( $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and measures were performed after a minimal exposition to the actinic light of 8 h. The effective quantum yield of photosystem II ( $\Phi_{\text{PSII}}$ ) was calculated according to Genty *et al.* (1989) as:  $(F_m' - F_t)/F_m'$  where  $F_t$  is the fluorescence steady-state level under ambient light and  $F_m'$  the maximum level of fluorescence measured during a saturating pulse ( $0.8 \text{ s}$ ,  $8500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Relative chlorophyll content was measured using the CL-01 Chlorophyll Content Meter (Hansatech Instruments, Kings Lynn, UK), which determines the relative content using dual wavelength optical absorbance ( $620 \text{ nm}$  and  $940 \text{ nm}$ ). This equipment allowed us to compare the responses of the

plants to Zn contamination and to highlight a potential evolution toward chlorosis. For traits measured on three leaves (leaves width, photosystem II yield and relative content of chlorophyll), the median was used to evaluate the value per ramet. To estimate dry biomass, shoots and roots were separated and dried at  $60^\circ\text{C}$  during 72 h.

To eliminate most of the variation in plant responses unrelated to metal treatment, we calculated a tolerance index (TI) for each trait. The TI could be calculated in two different ways: by dividing the value for a ramet in the C condition by either a randomly chosen value among one of the three ramets of the same genotype in the NC condition or by statistic (mean or median, the latter being more suitable for small samples) summarizing the set of value over the three ramets in the NC condition. Owing to the weak

**Table 1** Geographic location and edaphic type of the *Arabidopsis halleri* investigated natural populations

Type	Name	Location	Altitude (m)	Latitude	Longitude	Zinc total concentration in soil ( $\mu\text{g g}^{-1}$ )	Ni est.	<i>n</i>
M	PL22	Bukowno	339	50°16'58.08" N	19°28'43.38" E	3969	< 1000	6
M	PL24	Bolesław	334	50°17'00.18" N	19°29'05.64" E	14 964	< 1000	4
M	PL27	Galman	447	50°11'36.78" N	19°32'15.12" E	35 942	< 500	22
M	PL15	Wełnowiec	302	50°17'12.96" N	19°01'32.04" E	10 163	100–500	5
M	PL17	Wełnowiec	297	50°16'57.12" N	19°01'46.98" E	10 642	> 1000	8
M	PL19	Miasteczko Śląskie	308	50°30'12.84" N	18°56'08.34" E	1167	< 500	7
M	PL30	Miasteczko Śląskie	325	50°30'10.03" N	18°56'20.02" E	1481	< 1000	4
NMp	PL21	Bibiela	300	50°29'45.66" N	18°59'00.12" E	327	< 100	4
NM	PL13	Nieposłomice Forest	206	50°06'35.64" N	20°21'40.26" E	160	> 500	25
NM	PL14	Nieposłomice Forest	188	50°06'31.80" N	20°22'02.88" E	169	> 500	9
NM	PL32	Western Tatra Mts.	970	49°16'26.94" N	19°52'41.76" E	125	> 500	11
NM	SK2	Kosicka Bela	690	48°46'10.20" N	21° 7'48.60" E	51*	> 500	17

M, metallicolous; NM, nonmetallicolous; NMp, nonmetallicolous in polluted area; Ni est., approximate population size; *n*, number of phenotyped individuals; \* in Bert *et al.* (2002).

Populations are all located in Poland (PL) except one (SK2 is located in Slovakia)

**Table 2** Ecological background of the 12 sampled sites.

Type	Name	Habitat	Origin of contamination	Substrate		Vegetation*	Vegetative cover (%)
				Natural	Artificial	Dominant species	
M	PL22	Woody area	Mining activities and metallurgic	X	X	<i>Agrostis gigantea</i>	100
M	PL24	Meadow	activities since 13 <sup>th</sup> and 19 <sup>th</sup> century, respectively	X	X	<i>Achillea millefolium</i> , <i>Lotus corniculatus</i>	100
M	PL27	Forest	Mining activities during the 19 <sup>th</sup> century	X		<i>Silene vulgaris</i> , <i>Carex hirta</i>	100
M	PL15	Meager grassland	Waste heap from Zn smelter built		X	<i>Festuca ovina</i>	60
M	PL17	Woody area	during the 19 <sup>th</sup> and 20 <sup>th</sup> century		X	<i>Festuca ovina</i>	100
M	PL19	Degraded woody area	Metallurgic activities since 1966		X	<i>Arabidopsis arenosa</i>	60
M	PL30	Degraded woody area			X	<i>Arabidopsis halleri</i>	80
NMp	PL21	Ditch along a roadside	/	X		<i>Calamagrostis epigejos</i>	100
NM	PL13	Wet forest edge	/	X		<i>Aegopodium podagraria</i>	100
NM	PL14	Wet forest edge	/	X		<i>Aegopodium podagraria</i>	100
NM	PL32	Forest edge and meadow	/	X		<i>Picea abies</i> , <i>Sanicula europaea</i>	100
NM	SK2	Meadow	/	X		NA	100

\*Krystyna Grodzińska pers. comm.; M, metallicolous; NM, nonmetallicolous; X, presence of this substrate; NA, not available

between-ramet variance in the NC condition (*c.* 10% of the total variance), we found identical results using both procedures. As a consequence, only results using the median of the three ramets in the NC condition for TI estimates are presented in the paper.

### Analysis of phenotypic differentiation

Because of the unbalanced sample (from 4 to 25 individuals/population), we performed nonparametric exact tests (STATXACT v.8 Cytel Studio 2007, MA, USA) to analyse phenotypic differentiation among edaphic types, among populations within edaphic type and within populations.

These tests make no assumptions about distributions and are suitable for small and/or unbalanced samples. Wilcoxon–Mann–Whitney exact tests for two independent groups were used to analyse phenotypic differences between NM and M groups of populations in NC and C conditions, and differences between NM and M groups of populations for tolerance indices. Differences within each edaphic type were investigated with Kruskal–Wallis exact test for *k* independent groups. Monte Carlo approximations for *P*-values were obtained using 10 000 permutation tests. In order to test for differences between pairs of population within edaphic type we used a nonparametric *post-hoc* test for multiple comparisons, according to Siegel &

Castellan (1988). Within edaphic type, we also estimated the partial variances explained by differences among populations and within population using the VARCOMP procedure (method TYPE 1 which does not assume normality of data) of the sas program v. 9.1 (SAS Institute Cary, NC USA). Correlations between tolerance indices were examined using the Spearman coefficient estimated with the CORR procedure (SAS Institute).

The variability observed for tolerance indices was summarized using a canonical discriminant analysis on tolerance indices and populations using the CANDISC procedure (SAS Institute). This linear combination maximizes differentiation between populations and allows one to test if populations group according to their edaphic type. This analysis was performed on genotypes to respect assumption of independent data of discriminant analysis (Mundry & Sommer, 2007). All data were square-rooted before the canonical analysis to fit a normal distribution.

We estimated the broad sense heritability or clonal repeatability of the tolerance indices as  $H^2 = V_G/V_P$  ( $V_G$  is total genetic variance, which includes additive genetic variance ( $V_A$ ) and other sources of genetic variance (dominance and epistasis);  $V_P$  is the total phenotypic variance). Both  $V_G$  and  $V_P$  were estimated using the TYPE 1 method of the VARCOMP procedure (SAS Institute, 2002). The model considered the edaphic type (NM or M) as fixed factor and the population within the edaphic type and the genotype within the population as random factors.

### Comparisons of genetic differentiation for quantitative traits and molecular markers

Owing to an imbalance in our data (see Table 1), measures of differentiation for quantitative traits ( $Q_{ST}$ ) were computed with a Bayesian analysis following the procedure described by Waldmann *et al.* (2005). The  $Q_{ST}$  statistics was estimated as  $V_b/(V_b + 2V_w)$  where  $V_b$  is the component of genetic variance between populations and  $V_w$  the component within populations. The model considers edaphic type as a fixed factor and population and genotype as random factors. We used a Gamma distribution (0.001, 0.001) as priors for the inverse of the variance ( $1/V_b$ ,  $1/V_w$  and  $1/V_{genotype}$ ). To calculate confidence intervals we ran two chains for 1 000 000 iterations, with a 500 000 burn-in. Four populations (PL15, PL24, PL30 and PL21) of our data set had sample sizes inferior to five individuals per population. Consequently, to avoid bias in the estimation of genetic differentiation we excluded these populations from the comparison of  $Q_{ST}/F_{ST}$ .

To estimate population differentiation at neutral molecular markers, 10 nuclear microsatellite loci were scored for a total of 203 individuals representing the eight populations studied. These samples partly overlapped with those used for phenotypic traits, *c.* 50% of the individuals genotyped

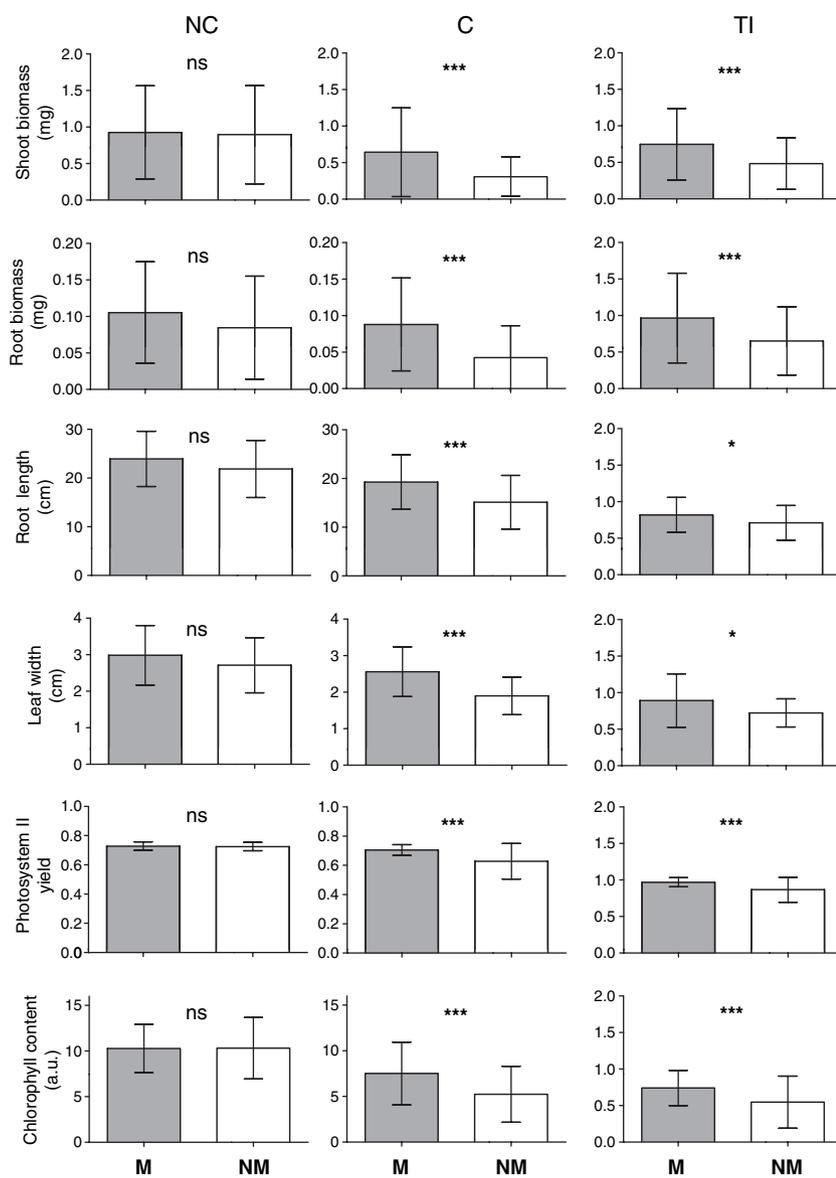
were also phenotyped. We used five microsatellite (*ATH*, *GC16*, *LYR132*, *LYR133*, *LYR104*) previously described in Van Rossum *et al.* (2004) and five others (*GC22*, *NGA112*, *ICE 13*, *MDC16*, *NGA361*) recently transferred from *A. thaliana* and combined in a multiplex (Llaurens *et al.*, 2008). For each microsatellite we estimated the level of polymorphism (number of alleles and total gene diversity  $H_T$ ) and the inbreeding coefficient ( $F_{IS}$ ) using FSTAT 2.9.3 (Goudet, 1995). Measures of differentiation for molecular markers were calculated based on  $F_{ST}$  (Weir & Cockerham, 1984) with the software FSTAT. The 95% confidence interval for the  $F_{ST}$  was estimated using 1000 bootstrap resampling of individuals.

## Results

### Morphological and physiological response to zinc of M and NM populations

Under NC conditions, morphological and physiological trait values of NM populations did not differ from those of M populations (Fig. 2). By contrast, in the C conditions, trait values in M populations were all significantly higher than those in NM populations. The NM plants had less dry biomass, shorter roots, narrower leaves, lower photosystem II yields and lower relative chlorophyll contents. Variances of traits were relatively similar between NC and C conditions except for the photosystem II yield that showed higher variance in C than in NC conditions.

Tolerance indices further showed that Zn had a negative effect on most of the morphological traits measured and on all physiological traits, regardless of the edaphic type of the populations (Fig. 2). However, toxicity of Zn was, on average, significantly more severe for NM individuals than for M individuals. The NM plants showed smaller tolerance indices for dry biomass ( $0.48 \pm 0.34$  vs  $0.78 \pm 0.48$  for shoot and  $0.65 \pm 0.46$  vs  $0.99 \pm 0.59$  for root), root length ( $0.71 \pm 0.23$  vs  $0.82 \pm 0.24$ ), leaf width ( $0.73 \pm 0.20$  vs  $0.89 \pm 0.35$ ), photosystem II yield ( $0.87 \pm 0.16$  vs  $0.97 \pm 0.06$ ) and relative content of chlorophyll ( $0.54 \pm 0.35$  vs  $0.74 \pm 0.23$ ). Broad sense heritabilities of the tolerance indices were high for all the traits ( $> 0.50$ ; see Table 3). The highest heritabilities were found for the relative content of chlorophyll (0.78) and the width of leaves (0.69). All the morphological indices were significantly correlated among each other, with  $r$  values ranging from 0.28 to 0.68 (Table 3). The highest correlation was observed for shoot and root dry biomass. Photosystem II yield was correlated with each index, except the one for leaf width. Relative chlorophyll content was correlated only with photosystem II yield and shoot biomass. Metal contamination is variable between metalliferous sites (Table 1), but we found no correlation between



**Fig. 2** Mean  $\pm$  SD from measures of morphological and physiological traits in metallophilous (M, tinted bars) and nonmetallophilous (NM, open bars) populations on zinc contaminated (C) and noncontaminated conditions (NC). Tolerance indices (TI) are shown on the right side of the figure. Results from exact permutation tests are indicated as follows: \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, nonsignificant.

tolerance indices and Zn soil concentration ( $P$ -value from 0.071 for leaf width to 0.759 for root length).

Canonical discriminant analysis showed that populations were differentiated for all tolerance indices ( $P < 0.0001$ ). Variations among populations were significantly explained by the first three axes, representing 47%, 21% and 15% of the variance. The first axis was mainly explained by the photosystem II yield and the root length, the second by the width of leaves and the third by the relative chlorophyll content (Table 4). The first axis, upon which populations are continuously distributed, separated populations according to their edaphic type (Fig. 3). The NM populations had smaller values on this axis than M populations with extreme values for populations SK2 and PL17. The second axis identified two M populations with a different behavior, PL30 and PL19; these showed high values for the width of

leaves. Interestingly, the NMp population (PL21) was less associated with the NM group than with the major M group.

#### Variability within edaphic type and populations in response to Zn

Apart from photosystem II yield, tolerance indices for all traits showed high variance within edaphic types (see Fig. 2). Because of these high variances, we observed a large overlap of values between the two edaphic groups. Indeed, some M and NM individuals showed comparable values for all tolerance indices (Fig. 2). The high variances within edaphic type were mainly explained by variation within populations. For all tolerance indices, the part of variance explained by difference among populations was weak compared with that

**Table 3** Broad-sense heritability ( $H^2$ ) of six zinc tolerance indices (TI) measured on 12 *Arabidopsis halleri* populations and correlation between these indices

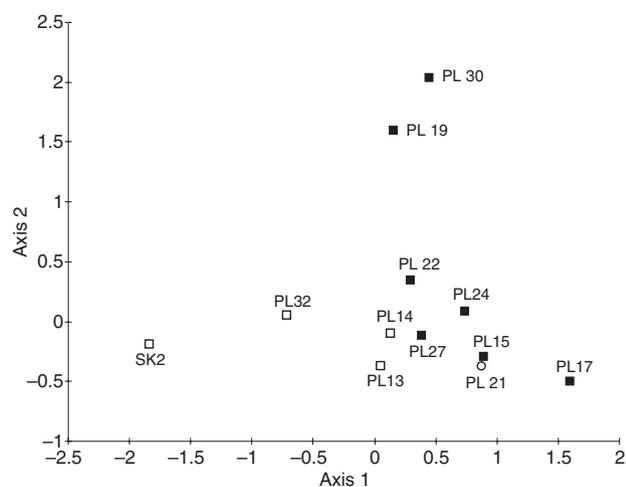
TI	$H^2$	Correlation				
		Leaf width	Root biomass	Root length	Photosystem II yield	Relative chlorophyll content
Shoot biomass	0.56	0.50***	0.68***	0.30***	0.27**	0.21*
Leaf width	0.69		0.38***	0.28**	0.09 ns	0.06 ns
Root biomass	0.57			0.40***	0.18*	0.02 ns
Root length	0.50				0.26*	0.06 ns
Photosystem II yield	0.63					0.33***
Relative Chlorophyll content	0.78					

*P*-values are represented as follows: \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ , ns, nonsignificant.

**Table 4** Canonical loadings and proportion of variance from discriminate analysis of population differentiation for zinc tolerance indices (TI)

TI	Axis 1	Axis 2	Axis 3
Shoot biomass	0.401	0.185	-0.493
Leaf width	0.129	0.896	-0.475
Root biomass	0.273	0.056	-0.053
Root length	0.538	-0.086	-0.166
Photosystem II yield	0.934	0.060	0.005
Relative chlorophyll content	0.309	0.055	0.802
Proportion of variance explained (%)	47	21	15

populations was significantly smaller than within the NM populations for two traits: the relative chlorophyll content and the photosystem II yield ( $P = 0.001$  and  $P = 0.003$ , respectively). We also found significant differences among populations within edaphic types. Namely, M populations showed different tolerance indices for photosystem II yield and relative chlorophyll content ( $P = 0.03$  and  $P = 0.012$ , respectively; Fig. 4), whereas NM populations differed significantly from each other only for photosystem II yield ( $P < 10^{-4}$ ; Fig. 4). The difference in photosystem II yield between NM and M populations was mainly explained by the populations PL32 and SK2.



**Fig. 3** Canonical discriminant analysis on six zinc tolerance indices in metalliculous (M, closed squares) and nonmetalliculous (NM, open squares) populations of *Arabidopsis halleri*. Axes 1 and 2 represent 47% and 21% of the variation between populations, respectively. The open circle corresponds to the nonmetalliculous population in polluted area (NMP).

explained by polymorphism within populations (Table 5). This demonstrates that a high variability is present within populations, irrespective of edaphic type. Variance within M

### Comparison of phenotypic and molecular differentiation

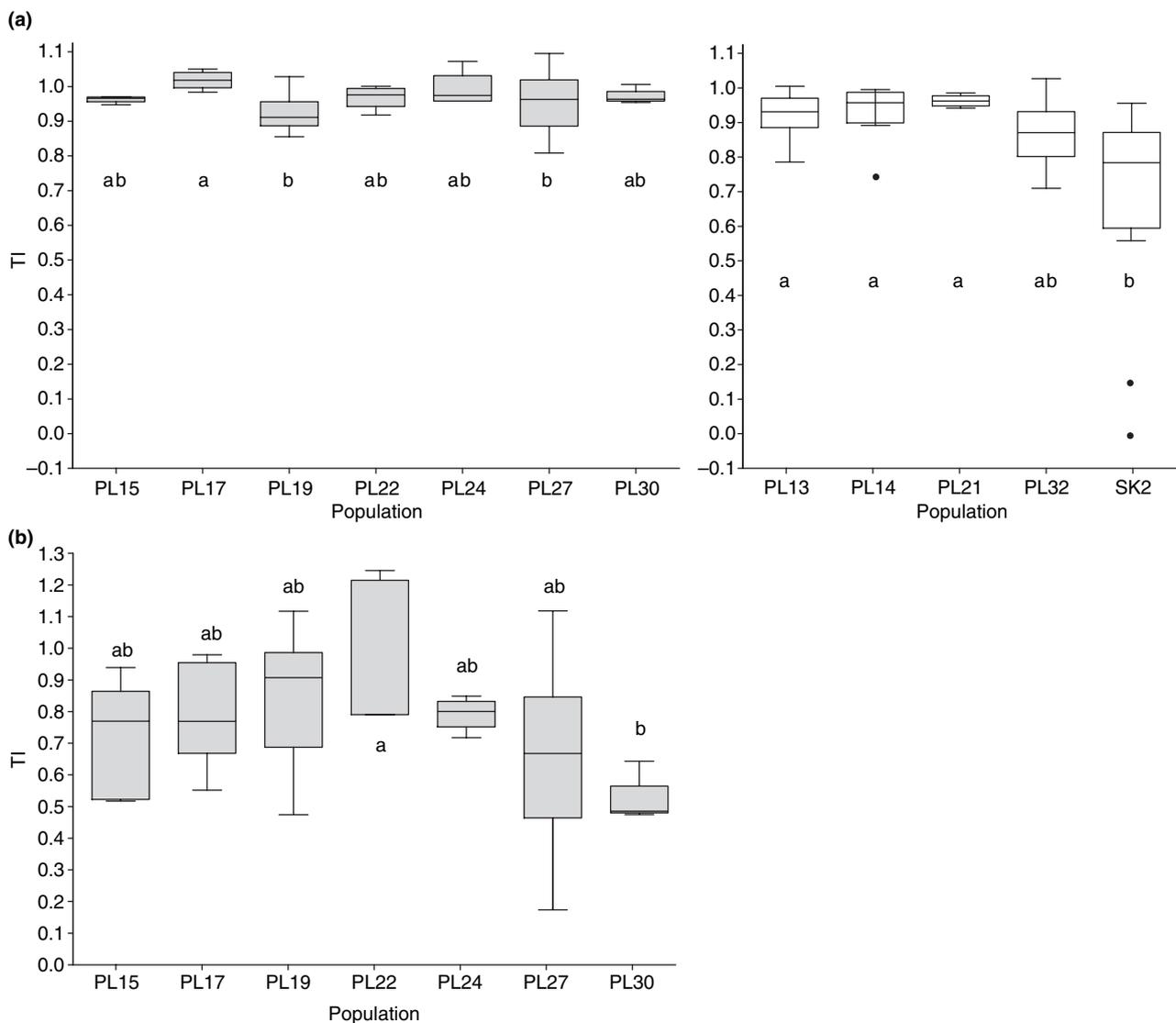
The 10 microsatellites showed 5–15 alleles (average = 8.5), and were polymorphic in all the populations. Statistics of population genetic diversity ( $H_T$  and  $F_{IS}$ ) are presented in Table S1 for each of these loci. Total gene diversity was relatively high for all loci (mean  $H_T = 0.599$ ) and inbreeding coefficient ranged from -0.122 to 0.269. Across all eight populations, the differentiation at microsatellite loci ( $F_{ST}$ ) was, on average, 0.146 with a 95% confidence interval (CI) from 0.116 to 0.171. This estimate of genetic differentiation at molecular markers was higher than those obtained in the same area in a previous survey using AFLP (average  $F_{ST}$  of 0.066 in Meyer *et al.*, 2009).

The highest  $Q_{ST}$  was observed for photosystem II yield (average = 0.33) with a 95% CI from 0.13 to 0.67 (Fig. 5). Overlap between this CI and that of molecular markers was very weak. Hence, these results suggest that photosystem II yield has probably experienced divergent selection. Shoot biomass, root length and relative chlorophyll content also showed  $Q_{ST}$  higher than  $F_{ST}$ , although their wide CI overlapped largely with the CI of the  $F_{ST}$ . Consequently, we could not make a conclusion about the role of selection in shaping variability at these traits. On average, the differentiation levels for root biomass and leaf width were

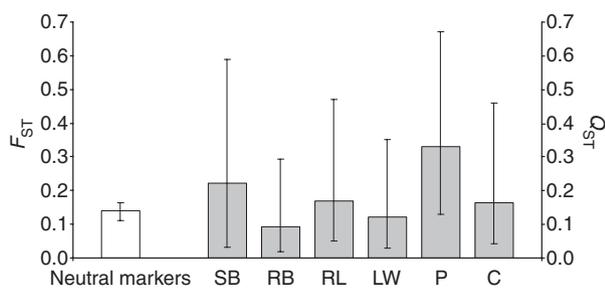
**Table 5** Part of variance explained by variability among and within populations for zinc (Zn) tolerance indices (TI)

TI	Variance components					
	Within M type			Within NM type		
	Among populations	Within populations	Total	Among populations	Within populations	Total
Shoot biomass	0.0087	0.1376	0.3660	0.0001	0.0362	0.0831
Leaf width	0.0044	0.0582	0.1171	0.0068	0.1582	0.2482
Root biomass	0.0273	0.2572	0.9251	0.0037	0.0371	0.0683
Root length	0.0081	0.0436	0.0713	0.0009	0.0229	0.0619
Photosystem II yield	0.0005	0.0021	0.0045	0.0100	0.0229	0.0328
Relative chlorophyll content	0.0169	0.0444	0.0920	0.0042	0.0526	0.1174

M, metallicolous; NM, nonmetallicolous.



**Fig. 4** Zinc (Zn) tolerance indices showing significant differences among metallicolous (M, tinted bars) or nonmetallicolous (NM, open bars) populations. (a) Index based on photosystem II yield; (b) index based on relative chlorophyll content. The box represents the 25th and 75th percentiles of the data and the mean is indicated by the horizontal line. Vertical lines and bullets show the highest and lowest data and the outlier values, respectively. Different letters indicate significant differences at the 5% level.



**Fig. 5** Comparison of genetic differentiation for molecular markers ( $F_{ST}$ ) and quantitative traits ( $Q_{ST}$ ). Tinted bars correspond to estimates for different characters: SB, shoot biomass; RB, root biomass; RL, root length; LW, leaf width; P, photosystem II yield; C, relative content of chlorophyll. The 95% confidence interval for genetic differentiation is represented by the vertical lines.

all smaller than the molecular differentiation, suggesting neutral evolution or stabilizing selection.

## Discussion

### Evaluating variability of Zn tolerance with root and shoot measurements

Overall, our results demonstrated and quantified variability of Zn tolerance at a local scale in the pseudometallophyte species *A. halleri*. In the 12 populations sampled, the morphological (shoot and root dry biomass, root length and leaf width) and physiological (leaf photosystem II yield and relative chlorophyll content) traits used to estimate Zn tolerance showed congruent trends towards higher mean values for M populations. Nevertheless, it is the response of shoot, mainly through photosystem II yield, that seemed to capture most variability of tolerance, in particular among populations. These results are congruent with those reported by Assunção *et al.* (2003) on *Thlaspi caerulescens* and support the hypothesis that the shoot is more sensitive than the root in hyperaccumulator species because of a preferential accumulation of metal in the leaves (Assunção *et al.*, 2001, 2003). Among all the measures we performed, the most informative trait to evaluate tolerance in our study species seems to be the photosystem II yield of leaves. This trait showed high heritability, weaker variability within M than NM populations and clear differences among populations. The photosystem II yield, an indicator of functional photosynthesis and vitality of the photosynthetic tissue, has been used to investigate the mechanisms of heavy metal toxicity in previous studies (Barylá *et al.*, 2001; Küpper *et al.*, 2007), and to estimate the difference of metal tolerance between hyperaccumulator and nonhyperaccumulator species using incubation of leaf slices (Cho *et al.*, 2003). We showed that this physiological measure was also reliable to estimate polymorphism of Zn tolerance in a pseudometallophyte hyperaccumulator species. Macnair (1983) and Schat & Ten

Bookum (1992) suggested that integration of possible innate variation may bias tolerance indices. However, in our study on quantitative polymorphism of Zn tolerance, such bias seems to be limited because M and NM plants showed very similar trait values under NC control conditions. This similar response of M and NM plants is interesting because it may suggest that enhanced tolerance does not imply a cost in terms of elevated Zn requirements. These results contrast with the classical hypothesis that the most tolerant and hyperaccumulator plants requires more metal for normal growth (van de Mortel *et al.*, 2006). Nevertheless, this possible absence of cost concerns only the Zn concentration tested in our survey (i.e. 10  $\mu\text{M}$ ) and cannot be inferred to apply to lower concentrations. Overall, in order to rigorously demonstrate this hypothesis it would be essential to measure survival and reproductive parameters as was achieved for *T. caerulescens* (Dechamps *et al.*, 2006).

### Variability of Zn tolerance at a local scale and evolutionary inference

*Arabidopsis halleri* is a species constitutively tolerant to Zn (Bert *et al.*, 2000) that shows at a broad-scale continuous variation from NM to M populations (Pauwels *et al.*, 2006). Our results revealed that this pattern of variation is also present at a local scale, among populations that are likely to exchange genes. Hence, these results suggest that gene flow, in the network of populations considered in our survey, is probably not intense enough to prevent increasing tolerance in response to the high concentrations of metals at metalliferous sites. We also found important variability of tolerance within edaphic types, which was mostly explained by variability among individuals within populations. Such a high level of variability could be interpreted in two different ways. First, it is consistent with gene flow between NM and M populations, whereby NM individuals with tolerance close to that of M individuals would have acquired enhanced tolerance from M populations through gene flow of metal tolerance genes. This observation and the fact that the less tolerant populations (PL32 and SK2) were the most distant from the M populations may indicate that gene flow plays an important role in the distribution of this quantitative trait. Second, the large variation of Zn tolerance observed within NM populations could suggest that these *A. halleri* populations have the genetic potential to evolve towards a higher tolerance. Thus, the enhanced tolerance in recently founded M populations may result from selection on standing variation existing in NM populations rather than on newly arisen mutations. Indeed, recurrent mutations, genetic drift and, particularly in this case, gene flow could preserve a relatively high amount of neutral or deleterious variation upon which selection may proceed (Barrett & Schluter, 2007). The fact that polluted sites, which are

dramatically altered environments, become rapidly colonized is suggestive of pre-existent suitable genetic variants from standing variation in the surrounding NM populations. In addition, alleles with small effects, proposed to be involved in the variability of heavy metal tolerance among populations (Macnair *et al.*, 2000), are expected to largely contribute to adaptation from standing variation.

It is interesting to note that populations PL19 and PL30, which are geographically very close to one another (< 1 km) and are both at less Zn-contaminated metalliferous sites, clearly differed from other M populations and from NM populations in the canonical analysis of the TI. These results suggest possible different mechanisms of adaptation among M populations in our system study. Convergent evolution has often been assumed to concern distantly related species rather than closely related species or populations. Nevertheless, empirical studies of the genetics of adaptation have shown that different populations within a species may use different genetic solutions to solve similar ecological problems (for review see Arendt & Reznick, 2007). This was observed by Hoekstra *et al.* (2006) in Atlantic and Gulf coast populations of mice, for an extreme pigmentation phenotype. They showed that the mutation *Mc1r* implicated in the light coloration of the Gulf coast mice was not present in the pale Atlantic coast mice. In the context of heavy metal tolerance, Smith & Macnair (1998), using crosses between two lines that differed in copper tolerance and a single nontolerant plant, showed that several modifier genes explain different levels of tolerance in the metallophyte plant *Mimulus guttatus*. The genetic architecture of heavy metal tolerance in *A. halleri* was investigated in a cross between one M individual from *A. halleri* and one from the nontolerant species *A. lyrata* (Courbot *et al.*, 2007; Willems *et al.*, 2007). They identified several QTLs for Zn and Cd tolerance, probably implicated in different pathways (Roosens *et al.*, 2008b), which means that genetic change at either of them can, in itself, enhance metal tolerance. Evolution of Zn tolerance by population specific mechanisms is thus a possibility in *A. halleri*. Moreover, based on a genome scan approach, Meyer *et al.* (2009) showed that different loci may be involved in adaptation in two M populations present in our survey (PL22 and PL27), consistent with a scenario of convergent evolution towards increased tolerance in these two populations.

Our results for the NMp population are congruent with those obtain by Pauwels *et al.* (2006). Indeed, they showed that at the European scale, Zn tolerance in these populations was intermediate between M and NM populations. They proposed two hypotheses to explain the features of the NMp populations. These populations could be founded from neighboring M populations, followed by selection for lower tolerance owing to a possible cost for excessive tolerance. Conversely, the phenotype of NMp populations could also be explained by intense gene flow of metal tolerance

genes. In these two hypotheses the NMp populations could be in a situation of maladaptation. Therefore, to gain a better knowledge of the role of gene flow, selection and drift in shaping Zn tolerance in *A. halleri*, it would be interesting to investigate the genetic structure and the variability of tolerance on larger samples of NMp populations.

### Adaptive value of Zn tolerance variability

To assess the role of natural selection in shaping the variability of Zn tolerance among *A. halleri* populations, we compared differentiation of this quantitative trait to differentiation of neutral molecular markers. The higher value of  $Q_{ST}$  for tolerance indices compared with  $F_{ST}$ , particularly for leaf photosystem II yield, lends support to the hypothesis that divergent selection at metalliferous and nonmetalliferous sites has played a role in phenotypic differentiation. Another observation supporting this hypothesis, and congruent with the results of Pauwels *et al.* (2006), is the higher variance observed within NM populations compared with M populations. Indeed, reduction of genetic diversity is typically considered as one of the most striking signatures of directional selection (Nielsen, 2005). Hence, our survey supports the view that tolerance to Zn, the usual trait used to explore adaptation to metal-polluted sites and the genetic basis underlying such adaptation, has probably been shaped by divergent selection within the species. However, the conclusion of our  $Q_{ST}$ – $F_{ST}$  approach has to be confirmed because of first, the large confidence interval of  $Q_{ST}$ , and second, the possible genetic  $\times$  environment effect. In *Thlaspi caerulescens*, a pseudometallophyte that tolerates and hyperaccumulates Zn and Cd, Jiménez-Ambríz *et al.* (2006) and Dechamps *et al.* (2006) have shown that several life history traits differed between edaphic types probably through divergent selection. Similarly, in *A. halleri*, life-history traits, particularly reproductive ones, could be involved in adaptation to metalliferous sites and thus will have to be investigated in parallel with Zn tolerance.

### Acknowledgments

We are grateful to Angélique Bourceaux and Anna Szczerbinska for technical support. We are also grateful to Krystyna Grodzińska for collecting ecological data. We thank Henk Schat for advice on the experimental design and helpful comments on the manuscript and Patrick Waldmann for providing the script for Bayesian estimates and further assistance on its application. This work was supported by funding from the Contrat de Plan Etat/Région Nord-Pas de Calais (PRC), from the Fondation pour la Recherche en Biodiversité (FRB) (contract BRG No. 92), from the INSU-CNRS program ACI ECCO (contract no. 04 2 9 FNS) and from the POLONIUM program (contract no. 6209.I/2005). C.L.M. was funded by the French

Ministry of Research and Technology. A.A.K. was founded by the French Ministry of Foreign and European Affairs by 'Eiffel excellence Doctorate Scholarship' (contract no. 494706A/P490826L) and by Scholarship for PhD students founded by the President of the Polish Academy of Sciences (contract no 13/S/2004).

## 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.
- Al-Shehbaz IA, O’Kane SLJ. 2002. Taxonomy and phylogeny of *Arabidopsis* (Brassicaceae). In: Somerville CR, Meyerowitz EM, eds. *The Arabidopsis book*. Rockville, MD, USA: American Society of Plant Biologists. doi: 10.1199/tab.0001, <http://www.aspb.org/publications/arabidopsis/>
- Antonovics J, Bradshaw AD, Turner RG. 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* 7: 1–85.
- Arendt J, Reznick D. 2007. Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? *Science* 23: 26–32.
- Assunção AGL, Da Costa Martins P, De Folter S, Vooijs R, Schat H, Aarts MGM. 2001. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* 24: 217–226.
- Assunção AGL, Ten Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO. 2003. Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* 159: 411–419.
- Barrett RDH, Schluter D. 2007. Adaptation from standing variation. *Trends in Ecology and Evolution* 23: 38–44.
- Baryla A, Carrier P, Franck F, Coulomb C, Sahun C, Havaux M. 2001. Leaf chlorosis in oilseed rape plants (*Brassica napus*) grown on cadmium-polluted soil: causes and consequences for photosynthesis growth. *Planta* 212: 696–709.
- Bert V, Macnair MR, De Laguerie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri*. *New Phytologist* 146: 225–233.
- Bert V, Bonin I, Saumitou-Laprade P, de Laguerie P, Petit D. 2002. Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* 155: 47–57.
- Bradshaw AD. 1952. Populations of *Agrostis tenuis* resistant to lead and zinc poisoning. *Nature* 169: 1098.
- Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007. Zinc in plants. *New Phytologist* 173: 677–702.
- Cho M, Chardonnens AN, Dietz K-J. 2003. Differential heavy metal tolerance of *Arabidopsis halleri* and *Arabidopsis thaliana*: a leaf slice test. *New Phytologist* 158: 287–293.
- 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.
- Dechamps C, Lefebvre C, Noret N, Meerts P. 2006. Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. *New Phytologist* 173: 191–198.
- Galardi F, Corrales I, Mengoni A, Pucci S, Barletti L, Barzanti R, Arnetoli M, Gabbriellini R, Gonnelli C. 2007. Intra-specific differences in nickel tolerance and accumulation in the Ni-hyperaccumulator *Alysum bertolonii*. *Environmental and Experimental Botany* 60: 377–384.
- Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990: 87–92.
- Goudet J. 1995. Fstat version 1.2: a computer program to calculate F statistics. *Journal of Heredity* 86: 485–486.
- Hoekstra HE, Hirschmann RJ, Bunday RA, Insel PA, Crossland JP. 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science* 313: 101–104.
- Humphreys MO, Nicholls MK. 1984. Relationships between tolerance to heavy metals in *Agrostis capillaris* L. (A. tenuis Sibth.). *New Phytologist* 98: 177–190.
- Jiménez-Ambríz G, Petit C, Bourrié I, Dubois S, Olivieri S, Ronce O. 2006. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* 173: 199–215.
- Koch M, Mummenhoff K, Hurka H. 1998. Systematics and evolution history of heavy metal tolerant *Thlaspi caerulescens* in Western Europe: evidence from genetic studies based on isozyme analysis. *Biochemical Systematics and Ecology* 26: 823–838.
- Küpper H, Lombi E, Zhao F-J, McGrath SP. 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* 212: 75–84.
- Küpper H, Parameswaran A, Leitenmaier B, Trtílek M, Šetlík I. 2007. Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* 175: 655–674.
- Llaurens V, Castric V, Austerlitz F, Vekemans X. 2008. High paternal diversity in the self-incompatible herb *Arabidopsis halleri* despite clonal reproduction and spatially restricted pollen dispersal. *Molecular Ecology* 17: 1577–1588.
- Macnair MR. 1983. The genetic control of copper tolerance in the yellow monkey flower, *Mimulus guttatus*. *Heredity* 50: 283–293.
- Macnair MR, Baker JM. 1994. Metal-tolerant plant: an evolutionary perspective. In: Farago ME ed. *Plants and the chemical elements, biochemistry, uptake, tolerance and toxicity*. New York, NY, USA: VCH, 68–83.
- 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* 266: 2175–2179.
- Macnair MR, Tilstone GH, Smith SE. 2000. The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Banuelos G, Vangronsveld J, eds. *Phytoremediation of contaminated soil and water*. Boca Raton, FL, USA: CRC Press, 235–250.
- Meerts P, Van Isaker I. 1997. Heavy metal tolerance and hyperaccumulation in metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* 133: 221–231.
- Mengoni A, Barabesi C, Gonnelli C, Galardi F, Gabbriellini R, Bazzicalupo M. 2001. Genetic diversity of heavy metal-tolerant populations in *Silene paradoxa* L. (Caryophyllaceae): a chloroplast microsatellite analysis. *Molecular Ecology* 10: 1909–1916.
- Meyer CL, 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.
- van de Mortel JE, Villanueva LA, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM. 2006. Large expression differences in genes for iron and Zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* 142: 1127–1147.
- Mundry R, Sommer C. 2007. Discriminant function analysis with nonindependent data: consequence and an alternative. *Animal Behaviour* 74: 965–976.
- Nielsen R. 2005. Molecular signatures of natural selection. *Annual Review of Genetics* 39: 197–218.

- O'Kane SL, Al-Shehbaz IA. 2003. Phylogenetic position and generic limits of *Arabidopsis* (Brassicaceae) based on sequences of nuclear ribosomal DNA. *Annals of the Missouri Botanical Garden* **90**: 603–612.
- Pauwels M, Saumitou-Laprade P, Holl C, Petit D, Bonin I. 2005. Multiple origins of metalcolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14**: 4403–4414.
- Pauwels M, Frérot H, Bonin I, Saumitou-Laprade P. 2006. A broad-scale analysis of population differentiation for Zn tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae). *Journal of Evolutionary Biology* **19**: 1838–1850.
- 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.
- Roosens NCJ, Willems G, Saumitou-Laprade P. 2008a. Using *Arabidopsis* to explore zinc tolerance and hyperaccumulation. *Trends in Plant Science* **13**: 208–215.
- Roosens NCJ, Willems G, Godé C, Courseaux A, Saumitou-Laprade P. 2008b. The use of comparative genome analysis and syntenic relationships allows extrapolating the position of Zn tolerance QTL regions from *Arabidopsis halleri* into *Arabidopsis thaliana*. *Plant and Soil* **306**: 105–116.
- Schat H, Ten Bookum WM. 1992. Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68**: 219–229.
- Schat H, Vooijs R. 1997. Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. *New Phytologist* **136**: 489–496.
- Schat H, Kuiper E, Ten Bookum WM, Vooijs R. 1993. A general model for the genetic control of copper tolerance in *Silene vulgaris*: evidence from crosses between plants from different tolerant populations. *Heredity* **70**: 142–147.
- Schat H, Vooijs R, Kuiper E. 1996. Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. *Evolution* **50**: 1888–1895.
- Siegel S, Castellan NJ. 1988. *Non parametric statistics for the behavioural sciences*. New York, NY, USA: MacGraw-Hill.
- Smith SE, Macnair MR. 1998. Hypostatic modifiers cause variation in degree of copper tolerance in *Mimulus guttatus*. *Heredity* **80**: 760–768.
- Van Rossum F, Bonnini I, Fénot S, Pauwels M, Petit D, Saumitou-Laprade P. 2004. Spatial genetic structure within a metalcolous population of *Arabidopsis halleri*, a clonal, self-incompatible, and heavy metal tolerant species. *Molecular Ecology* **13**: 2959–2967.
- Vekemans X, Lefèvre C. 1997. On the evolution of heavy-metal tolerant populations in *Armeria maritima*: evidence from allozyme variation and reproductive barriers. *Journal of Evolutionary Biology* **10**: 175–191.
- Von Frenckell-Insam BAK, Hutchinson TC. 1993. Occurrence of heavy metal tolerance and co-tolerance in *Deschampsia cespitosa* (L.) Beauv. From European and Canadian populations. *New Phytologist* **125**: 555–564.
- Waldmann P, Garcia-Gil MR, Sillanpää MJ. 2005. Comparing Bayesian estimates of genetic differentiation of molecular markers and quantitative traits: an application to *Pinus sylvestris*. *Heredity* **94**: 623–629.
- Weir BS, Cockerham CC. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.
- Whitlock MC. 2008. Evolutionary inference from  $Q_{ST}$ . *Molecular Ecology* **17**: 1885–1896.
- Wilkins DA. 1978. The measurement of tolerance to edaphic factors by means of root growth. *New Phytologist* **80**: 623–633.
- Willems G, Dräger DB, 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.
- 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 in the online version of this article.

### Table S1 Summary statistics of the 10 microsatellites

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.



## About New Phytologist

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at [www.newphytologist.org](http://www.newphytologist.org).
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *Early View* – our average submission to decision time is just 29 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £151 in Europe/\$279 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office ([newphytol@lancaster.ac.uk](mailto:newphytol@lancaster.ac.uk); tel +44 1524 594691) or, for a local contact in North America, the US Office ([newphytol@ornl.gov](mailto:newphytol@ornl.gov); tel +1 865 576 5261).