DOI: 10.1534/genetics.106.064485

The Genetic Basis of Zinc Tolerance in the Metallophyte *Arabidopsis halleri* ssp. *halleri* (Brassicaceae): An Analysis of Quantitative Trait Loci

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Manuscript received August 8, 2006
Accepted for publication February 22, 2007

ABSTRACT

The species *Arabidopsis halleri*, an emerging model for the study of heavy metal tolerance and accumulation in plants, has evolved a high level of constitutive zinc tolerance. Mapping of quantitative trait loci (QTL) was used to investigate the genetic architecture of zinc tolerance in this species. A first-generation backcross progeny of *A. halleri* ssp. *halleri* from a highly contaminated industrial site and its nontolerant relative *A. lyrata* ssp. *petraea* was produced and used for QTL mapping of zinc tolerance. A genetic map covering most of the *A. halleri* genome was constructed using 85 markers. Among these markers, 65 were anchored in *A. thaliana* and revealed high synteny with other Arabidopsis genomes. Three QTL of comparable magnitude on three different linkage groups were identified. At all QTL positions zinc tolerance was enhanced by *A. halleri* alleles, indicating directional selection for higher zinc tolerance in this species. The two-LOD support intervals associated with these QTL cover 24, 4, and 13 cM. The importance of each of these three regions is emphasized by their colocalization with *HMA4*, *MTP1-A*, and *MTP1-B*, respectively, three genes well known to be involved in metal homeostasis and tolerance in plants.

METAL tolerance in plants has been considered "an example of more powerful evolution in action than industrial melanism in moths" (Antonovics *et al.* 1971). Therefore it has been the focus of many evolutionary studies, in which it was argued that metal tolerance could evolve rapidly following exposure to heavy metal stress (Wu *et al.* 1975; Al-Hiyali *et al.* 1988). Some heavy metals, like zinc and copper, are oligo-nutrients and thus essential in small quantities for normal plant development. To avoid metal toxicity, all plants have evolved basic tolerance mechanisms. Binding by proteins or nonprotein thiol peptides in the cytoplasm and subsequent sequestration in the vacuole are the major component processes in the cellular heavy metal detoxification (Clemens 2001; Krämer 2005). However, at so-called metalliferous sites, heavy metals can occur at highly elevated concentrations in the soil, either through ancient natural processes, as in nickel-rich serpentine soils, or through recent human activities, as in zinc- and cadmium-rich calamine soils surrounding smelters. The total metal content of contaminated sites, depending on the metal, can be up to 10- to 1000-fold higher than that of uncontaminated sites (Bert *et al.* 2002). At these extreme concentrations, both essential and nonessential heavy metals become toxic (Clemens 2001; Hall 2002) and only a small number of plant species have evolved tolerance to such concentrations. These species have been classified as either absolute (strict or eu-) or facultative (pseudo-) metallophytes, according to their occurrence either on contaminated sites only or on both metalliferous and nonmetalliferous soils (Lambinon and Auquier 1964).

The genetic basis of adaptive quantitative traits is still the matter of strong debates among evolutionists. Current questions concern species differences in the genetic architecture of traits related to adaptation and focus mainly on the following points: How many genes are involved? How large are their phenotypic effects? Are they involved in a pleiotropic effect (*e.g.*, tolerance to different metals)? What are the dynamics of the alleles present at these genes? This emphasis on number of genes and sizes of effects reflects one of the oldest problems in evolutionary biology: the complexity of genetic changes underlying phenotypic evolution. Recent theoretical developments of the Fisher–Orr model

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suggest that fewer genes than expected according to the Fisher infinitesimal model could be involved in adaptation and that the dynamics of allele substitution at selected loci follow an approximate geometric sequence (Orr 1998a, 1999, 2002): large-effect mutations typically substitute early, whereas smaller-effect ones substitute later during adaptive walk. Heavy metal tolerance is a trait of particular interest for documenting genetic changes during adaptive walk, as high heavy metal concentration can easily be measured in soils and represents a strong directional selective pressure, resulting in the substitution of tolerance alleles at some loci.

The resolution of quantitative traits into discrete Mendelian loci analysis has made substantial progress since the development of genetic linkage maps. Quantitative trait loci (QTL) mapping has proved to be very powerful in examining complex adaptive traits (Doebley *et al.* 1997; Alonso-Blanco *et al.* 1998; Ungerer *et al.* 2002; Weinig *et al.* 2003); it provides an efficient means for determining the number of genes implicated in a trait as well as their effects and interactions, which are important for understanding the evolutionary history of a trait (Mackay 2001; Barton and Keightley 2002; Erickson *et al.* 2004). By identifying specific chromosomal regions where genetic variation can be associated with measurable phenotypic variation (Tanksley 1993; Doerge 2002), QTL mapping can help to detect or validate candidate genes underlying complex traits (Flint and Mott 2001; Yano 2001; Glazier *et al.* 2002).

In the case of heavy metal tolerance in plants, two pseudometallophyte species, *Arabidopsis halleri* (L.) (O'Kane & Al-Shehbaz) [syn. *Cardaminopsis halleri* (L.) Hayek] and *Thlaspi caerulescens* J. & C. Presl. recently emerged as model species (Assunção *et al.* 2003). These species belong to the Brassicaceae and are able to tolerate and hyperaccumulate zinc (Zn) and cadmium (Cd). A recent study performed on 33 metallicolous (M) and nonmetallicous (NM) *A. halleri* populations clearly established the occurrence of constitutive, or fixed, Zn tolerance in *A. halleri* and of quantitative variation of the degree of Zn tolerance among populations (Pauwels *et al.* 2006). Parsimony suggests that fixed Zn tolerance has occurred only once, probably early in the species history. Consequently, the genes underlying this tolerance are expected to be shared by all populations, irrespective of whether there is metal contamination at the site of population origin. Taking advantage of a wide range of resources that are available for its wild nontolerant close relative *A. thaliana* (Mitchell-Olds 2001), *A. halleri* can also be considered the most promising model for identifying the genetic basis of adaptation to metalliferous soil.

A global comparison of *A. halleri* and *A. thaliana* through transcription profiling was recently performed to identify genes differentially expressed and/or regulated in *A. halleri* compared to *A. thaliana* under various Zn concentrations. These studies identified *A. halleri* homologs of *A. thaliana* genes potentially involved in Zn tolerance (Becher *et al.* 2004; Weber *et al.* 2004). However, differential expression and/or regulation may just as well be the primary cause of heavy metal tolerance as its consequence or might simply be the result of the divergence time separating *A. halleri* and *A. thaliana*. Thus, definitive evidence for their implication in heavy metal tolerance is still missing.

In this study, we have applied a QTL approach to investigate the genetic basis underlying Zn tolerance in *A. halleri* and to define genomic regions containing the genes underlying the constitutive Zn tolerance in *A. halleri* as well as those involved in the recent adaptation to industrial polluted sites. The application of a QTL approach in *A. halleri* was highly promoted by the recent publication of the genetic linkage maps of its close relatives *A. l. petraea* and *A. l. lyrata* (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005). The extensive conservation of marker order reported between *A. lyrata* subspecies and the model *A. thaliana* (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005) made the prospect of transferring these resources to *A. halleri* even more attractive for gaining insights into adaptive evolution of heavy metal tolerance and hyperaccumulation (Clauss and Koch 2006). We performed an interspecific cross between *A. halleri* from a highly contaminated industrial site and *A. l. petraea* to generate a first-generation backcross (BC_1). These progeny, segregating for Zn tolerance, were used to construct a molecular linkage map (the first reported for a cross between these two species) and to identify QTL regions for Zn tolerance in *A. halleri*, making full use of the previous mapping experiments conducted on *A. lyrata* subspecies (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005) and of recent and current functional analyses of metal homeostasis genes in *A. halleri* (Becher *et al.* 2004; Weber *et al.* 2004; Filatov *et al.* 2006).

MATERIALS AND METHODS

Plant material: A single cross was performed between one individual from the Zn-tolerant species *A. halleri* ssp. *halleri* (henceforth called *A. halleri*) (pollen donor) and one from the nontolerant species *A. lyrata* ssp. *petraea* (*A. l. petraea* 1) (pollen recipient). The *A. halleri* individual ($2n = 16$) originated from a site highly contaminated with Zn, Cd, and lead (Pb) (Auby, France) (Van Rossum *et al.* 2004). The *A. l. petraea* 1 individual ($2n = 16$) originated from an uncontaminated site in the Czech Republic (Unhost, Central Bohemia) (Macnair *et al.* 1999). Both species are self-incompatible and usually outcrossing. Therefore, to avoid any inbreeding depression effect, one randomly selected F_1 individual was used as the male parent to fertilize a second *A. l. petraea* genotype (*A. l. petraea* 2), generating the interspecific backcross progeny (BC_1). The BC_1 population used for linkage map construction and QTL mapping consisted of 199 individuals. Both parental and progeny genotypes are easily maintained in time and multiplied for replication by cuttings.

Evaluation of Zn tolerance: Twelve replicates of the four parental genotypes (*A. halleri*, *A. l. petraea* 1, *A. l. petraea* 2, and

the F_1 individual) and three replicates of each of the BC_1 individuals were obtained by vegetative propagation. They were grown in the greenhouse on sand, and 8 weeks after cloning were transferred to 10-liter polycarbonate trays containing a nutrient solution in a controlled growth chamber (temperature, 20° day:15° night; light, 14 hr day:10 hr night). The plants were randomly assigned in the trays (48 plants/vessel, including one copy of each parental genotype), which in turn were rotated in the growth chamber twice a week. The nutrient solution consisted of 0.5 mM $Ca(NO_3)$, 0.2 mM $MgSO_4$, 0.5 mM KNO_3, 0.1 mM K_2HPO_4, 0.2 μM $CuSO_4$, 2 μM $MnCl_2$, 10 μM H_3BO_3, 0.1 μM MoO_3, 10 μM FeEDDHA, and 10–3000 μM Zn added as $ZnSO_4$. The pH of the solution was set at 6.5.

Zn tolerance was measured by a sequential test established by Schat and Ten Bookum (1992). This test provides a measure for tolerance by sequentially transferring plants into increasing concentrations of Zn and determining for each individual the lowest concentration at which no new root growth is produced (the EC100). Roots of plants were blackened with activated charcoal to observe new root growth more easily. The plants were grown on 10 μM of Zn for the first 3 weeks. After verification of their root growth at 10 μM of Zn, 6–12 replicates of each parental genotype and 1–3 replicates of each BC_1 individual were transferred in successive weeks to 25, 50, 75, 100, 150, 250, 500, 1000, 2000, and 3000 μM of Zn. Root growth of the plants was evaluated at the end of each week. Plants were observed for at least 2 weeks after reaching their EC100, to ensure that no new root growth occurred.

Marker analysis: Genomic DNA of the four parental genotypes and of the 199 individuals of the BC_1 was extracted for marker analysis using a slightly modified Dellaporta method (Saumitou-Laprade *et al.* 1999). The BC_1 progeny were genotyped using 65 sequence-based markers anchored in *A. thaliana* and 18 AFLP markers (Table 1).

The anchored markers consisted of microsatellites, indels, and polymorphisms revealed by cleaved amplified polymorphisms (CAPS), RFLP, and SSCP analysis. Some markers were selected because of their potential implication in metal homeostasis (*FRD3*, *HMA4*, *MTP1*, *MTP3*, *NRAMP3*, *NRAMP4*, and *MT2B*). Others had been identified by transcription profiling analyses conducted on *A. halleri* (*At1g46768*, *HMA4*, *NRAMP3*, *At2g40140*, *At2g43010*, *MTP1*, *At3g28220*, *At4g33160*, *At4g38220*, and *At5g08160*) (Becher *et al.* 2004; Weber *et al.* 2004; Craciun *et al.* 2006; S. Mari, unpublished data). The remainders were selected using their position in the *A. thaliana* genome to improve coverage of the *A. halleri* genome. Of the 65 anchored markers, 40 were previously reported for *A. thaliana* and/or *A. l. petraea* (Bell and Ecker 1994; Clauss *et al.* 2002; Kuittinen *et al.* 2002, 2004); 31 of the 40 markers had already been mapped in *A. l. petraea* (Kuittinen *et al.* 2004). In addition, 25 newly defined markers were introduced in this study. For 11 of the 25 markers, primer design and/or genotyping of the BC_1 progeny were kindly performed by colleagues (Table 1). For the other 14 markers, we designed primers on the basis of the *A. thaliana* sequence (The Arabidopsis Information Resource database at http://www.arabidopsis.org/) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For 4 of these 14 markers (*At2-TCA1*, *At2-TA5*, *At4-GA2*, and *At4-TC1*), primer pairs were designed to amplify small fragments of 150–400 bp. For the other 10 markers (*At1g46768*, *At2g43010*, *At3g28220*, *At3g33530*, *At3g45810*, *At5g08160*, *At4g38220*, *At2g40140*, *At4g33160*, and *HMA4*), one primer was designed in an exonic region of the gene and the second primer was placed either in the following exon or in the 5′-upstream region of the start codon. In the latter case, the possible promoter region of the gene, supposed to be highly variable between species, was targeted. For amplification of *EMF2*, already mapped in *A. l. petraea*, new primer sequences were designed as described above and used instead of the ones defined by Kuittinen *et al.* (2004). To obtain labeled PCR products detectable on the automated genotyper Li-Cor 4200 (Li-Cor-ScienceTec), either the forward or the reverse primer contained a 5′-tail of 19 bp (forward primer) or 20 bp (reverse primer) homologous to the universal consensus M13 primer sequence, followed by the locus-specific sequence (Oetting *et al.* 1995).

The following polymerase chain reaction (PCR) conditions were applied for all 65 markers, except for those that were provided through collaborations (Table 1). PCR reactions were carried out in a total volume of 15 μl containing 20 ng of template DNA, 2 mM $MgCl_2$, 0.2 mg/ml BSA, 0.2 mM dNTP, 0.2 μM of each unlabeled primer and 0.15 μM of the M13 fluorescently labeled primer (either IRD-700 or IRD-800), 20 mM Tris–HCl (pH 8.3), 50 mM KCl, and 0.4 unit of AmpliTaq DNA Polymerase (Applied Biosytems, Foster City, CA). PCR was performed on a Perkin-Elmer (Norwalk, CT) Gene-Amp system 9700 under the following conditions: 94° for 5 min, followed by locus-specific amplification; 94° for 30 sec, annealing temperature for 45 sec, 72° for 40 sec, for eight cycles, followed by M13 labeling amplification; and 94° for 30 sec, 50° for 20 sec, 72° for 40 sec, for 30 cycles, and a final extension 72° for 7 min. The annealing temperature of the locus-specific cycles varied between 50° and 60°, depending on the locus. For CAPS markers, restriction was carried out in a total volume of 20 μl containing 10 μl of PCR product, 0.2 mM spermidine, 1× specific enzyme buffer provided by the supplier and 1 unit of restriction enzyme. Restriction was carried out by incubation for at least 4 hr at the appropriate temperature in a Perkin-Elmer Gene-Amp system 9700. Polymorphisms were revealed on agarose or polyacrylamide gels using a Li-Cor genotyper.

Amplified fragment length polymorphism (AFLP) marker analysis was performed as described by Vos *et al.* (1995), using *Eco*RI/*Mse*I restriction enzymes. For preamplification, one nucleotide was added to *Eco*RI and *Mse*I. For selective amplification, three and two nucleotides were added to *Eco*RI and *Mse*I, respectively. The *Eco*RI selective primer was fluorescently labeled with either IRD-700 or IRD-800 for visualization of the AFLP bands on a Li-Cor genotyper 4200 (Li-Cor-ScienceTec). Polymorphic and segregating bands were scored using the program RFLPSCAN 3.0 (Scanalytics). Their sizes were determined by comparison with an appropriately labeled molecular weight marker (50–700 bp, Li-Cor-ScienceTec). AFLP markers were named according to the selective nucleotides used in selective amplification and their size.

Linkage map construction: The *A. halleri* × *A. l. petraea* (*Ah* × *Alp*) linkage map was constructed with the Joinmap 3.0 program (Van Ooijen and Voorrips 2001). Individuals lacking information for >25% of all markers were excluded from the analysis. Linkage groups were obtained at a logarithm-of-odds (LOD) score threshold of 4. Markers along each linkage group were ordered using the sequential method implemented in Joinmap. In this method, the best order was determined by comparing the goodness of fit of the resulting map for each tested order using a threshold of 0.5 and 1.0 for the linkage groups and the loci, respectively. Kosambi's mapping function was used to translate recombination frequencies into map distances (Kosambi 1944).

Linkage map analysis: We performed a *t*-test for correlated samples (Minitab, State College, PA) to test for a significant difference in marker intervals between the *Ah* × *Alp* and the *A. l. petraea* maps using the markers common to both mapping experiments. A *t*-test for correlated samples (Minitab) was also performed to compare the linkage group lengths in the *Ah* × *Alp* and either the *A. l. petraea* or the *A. l. lyrata* maps.

Marker segregation: According to Mendelian inheritance, the *A. halleri* alleles are expected to segregate in a 1:1 ratio in the BC_1. When dealing with an interspecific cross, segregation distortion frequently occurs. Deviations from the Mendelian ratios were tested using a chi-square test implemented by Joinmap 3.0 at a locus-by-locus significance level of $\alpha = 0.05$ (Van Ooijen and Voorrips 2001).

Statistical analysis and QTL mapping: We performed a Kruskal–Wallis test, based on Wilcoxon rank scores of the data, to test for significant differences of Zn tolerance among the four parental genotypes using the NPAR1WAY procedure in SAS (1999). A one-way analysis of variance (ANOVA) using the GLM procedure in SAS (1999) was performed on the EC100 values obtained for the replicates of the 199 BC_1 individuals to determine the genotype effect. The main factor, *i.e.*, the BC_1 progeny, was considered a random effect, because the BC_1 individuals tested for Zn tolerance represent a random sampling of the total BC_1 population. The broad-sense heritability of Zn tolerance was calculated by dividing the genetic variance by the total phenotypic variance, using the mean square values (MS) from the ANOVA ($h^2 = MS_{genot}/(MS_{genot} + MS_{error})$. Type III sums of squares were used because the data set was unbalanced, due to an unequal number of clones of each BC_1 individual.

We performed a Kolmogorov–Smirnov test (Minitab) on the $EC100_{mean}$ values (the arithmetic mean of the EC100 values of the clones) of the BC_1 genotypes as well as on the $EC100_{mean}$ values after logarithmic (log) transformation to test for deviation from a normal distribution. The QTL analysis for Zn tolerance was performed using the MapQTL 4.0 program (Van Ooijen *et al.* 2002). The LOD score threshold for QTL detection was set at 2.3 ($\alpha = 0.05$) and obtained by a permutation test on the quantitative data in MapQTL. A first QTL analysis was performed using interval mapping (IM) as implemented in MapQTL. The LOD score representing the likelihood of a QTL being present has been calculated every centimorgan within the intervals along the linkage groups. Markers for which the LOD score exceeded the significance threshold were identified in each linkage group. Automatic cofactor selection was performed by MapQTL on these markers for their use as cofactors in multiple QTL models (MQM) analysis. We performed MQM mapping twice while adjusting the selection of the cofactors to obtain the best possible set of QTL, *i.e.*, showing maximal LOD scores. One- and two-LOD support intervals were obtained using Mapchart 2.1 (Voorrips 2002). The estimated additive genetic effect (a) and the percentage of variance explained by each QTL (R^2) were calculated in IM. We tested for significant interactions between QTL using the GLM procedure in SAS (1999) on both raw and log-transformed $EC100_{mean}$ values. The model involved three factors corresponding to the genotypes of the BC_1 individuals at the markers (*HMA4*, *MTP1*-A, and *MTP1*-B) closest to or at the three QTL. These factors were considered either random or fixed. A box plot analysis was performed on the markers *HMA4*, *MTP1*-A, and *MTP1*-B. We performed a Kruskal–Wallis test, based on Wilcoxon rank scores of the data, to test for significant differences of Zn tolerance among the eight genotypic groups using the NPAR1WAY procedure in SAS (1999).

RESULTS

Linkage map construction: Of the 199 BC_1 individuals, 196 could be genotyped successfully at >75% of all markers and were used for the map construction. A total of 85 markers were assigned to eight linkage groups (LG1–LG8) by using a LOD score threshold of 4 (Table 1). The lengths of the linkage groups varied from 57 to 80 cM and summed to a total of 567 cM. The average distance between two adjacent markers was 6.6 cM, ranging from 1 to 27 cM (Figure 1).

Comparative analysis of the *A. halleri* × *A. l. petraea* map with the maps of *A. l.* petraea and *A. l. lyrata*: The transition from eight chromosomes in the *Ah* × *Alp* map to five chromosomes in the *A. thaliana* genome can be explained by five main chromosomal rearrangements as described for *A. l. petraea* (Kuittinen *et al.* 2004; Koch and Kiefer 2005) and *A. l. lyrata* (Yogeeswaran *et al.* 2005). These consist of three fusions between the linkage groups LG1/LG2, LG3/LG4, and LG7/LG8 and two reciprocal translocations between LG3/LG5 and LG6/LG7 (Figure 1). Marker order in the *Ah* × *Alp* and *A. thaliana* maps was generally similar. The order of the 31 marker loci shared by the *Ah* × *Alp* and *A. l. petraea* linkage maps was identical, with one exception on LG1. The positions in the interspecific map of the loci (*PhyA* and *AXR1*) did not agree with those reported for *A. l. petraea*, but rather with those expected from *A. thaliana*.

The marker distances obtained on the marker intervals between the markers common to the *Ah* × *Alp* and *A. l. petraea* maps were not significantly different in both mapping experiments ($P = 0.27$). The linkage group lengths differed significantly between the *Ah* × *Alp* map and either the *A. l. petraea* ($P = 0.03$) or the *A. l. lyrata* map ($P = 0.02$) (Table 2).

Two duplication events of the *MTP1* gene (a single-copy gene on *A. thaliana* chromosome 2 and *A. lyrata* LG4) were detected in *A. halleri*. We mapped the three copies identified in *A. halleri* (*MTP1-A*, *MTP1-B*, and *MTP1-C*) (Dräger *et al.* 2004) on three different linkage groups (LG4, LG6, and LG1, respectively). The *A. halleri MTP1-A* copy mapped to the lower arm of LG4 beyond the marker *ICE11* (the expected position from *A. thaliana*) and can therefore be considered as the ortholog of the *A. lyrata* and *A. thaliana MTP1* gene.

Markers in segregation distortion: At a locus-by-locus significance level of 0.05, 34 markers (40%) showed distorted segregation and were found on six of the eight linkage groups (Figure 1). The segregation ratio bias was highly directional. Of the 34 distorted markers, 31 showed an excess of the *A. l. petraea* 1/*A. l. petraea* 2 homospecific (*i.e.*, originating from the same species) allelic combination compared to the *A. halleri*/*A. l. petraea* 2 heterospecific (*i.e.*, originating from different species) genotype. Only three markers, all located on linkage group LG5, showed the opposite pattern, *i.e.*, an excess of the heterospecific combination. With a single exception (on LG5), distorted markers were always linked to markers distorted in the same direction, indicating that the segregation bias was due to meiotic events rather than genotyping errors.

Evaluation of Zn tolerance: A Kruskal–Wallis test showed a highly significant difference among the tolerance levels of the four parental lines of the BC_1 ($P <$

TABLE 1

Markers used in linkage map construction

Locus name	Identification/ BAC location in *A. thaliana*	Type of polymorphism	Annealing temperature (°)	Forward primer	Reverse primer
			Linkage group 1		
AthACS	F22L4	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
AAC/CG-88		AFLP			
AXR1	At1g05180	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
ACA/CG-105		AFLP			
PhyA	At1g09570	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
ICE10	F12F1	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
ICE13	At1g13220	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
GI	At1g22770	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
AGG/CT-198		AFLP			
ATTS0392	At1g30630	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
ACA/CG-228		AFLP			
VIP1	At1g43700	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
At1g46768	At1g46768	Indel	50	AGGTTGATCATTTTCTA AAAGTTCTTG	TTCCTCCTCCGTATCCCTCT
MTP1-C		RFLP/SSCP		Dräger *et al.* (2004)	Dräger *et al.* (2004)
			Linkage group 2		
F19K23	At1g62050	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
ACA/CG-87		AFLP			
ACA/CG-160		AFLP			
ACA/CG-320		AFLP			
AAC/CG-84		AFLP			
SLL2	At1g66680	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
lyr132[a]	T23K23	Microsat	50	GCCGTGAGATT AAAGAAGACG	GCAAGAGCTGA TCTCCATCC
ACA/CG-71		AFLP			
nga111	F28P22	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
At1g75830[b]	At1g75830		55	CATATCTATGCAAATT GTGTTTAATATA	ACATGGGAAGTAACA GATACACTTATGA
ADH1	At1g77120	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
			Linkage group 3		
At3g08040[c]	At3g08040	Indel	60	TACCAACCAGC CACAGCAACC	CGCTTTGTTTCCACT ATTTGACTTTG
MDC16	MDC16	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
nga162	MDC16	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
DMC1	At3g22880	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
At2-TCA1	T9F8	Microsat	50	CAACCACACCCCTTTAGCTT	GAGAGCCCATGGAGATGAAG
HMA4	At2g19110	Indel	62	TGACCTGAAAATGA AAGGTGGTC	TGCATAACTCCTGCAACAGCT
ICE14	F11A3	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
ACA/CG-310		AFLP			
			Linkage group 4		
con	At2g21320	*Mse*I		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
At2g22430[d]	At2g22430	Indel	55	AGTTCAGATTCAGTGGGTGG	GTAGATCTGTGAAACTCCGG
Ck2-alpha2	At2g23080	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
NRAMP3[e]	At2g23150	*Psi*I	55	TTGGATGTTTGGTCAAGCT AAGCCAAGTG	TGCCACGAGCAATGAGGT AGAGGATGAAT
At2-TA5	T19L18	Microsat	50	TCATCGGATCCAT ATTTGTTTG	CATTGTTGGTC GTGGCTATG
ELF3	At2g25930	Microsat		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
At2g28700[d]	At2g28700	Indel	55	TGGGAACATGGGAGA TTTTGTTATG	TGTCTTGCCCTTCAC TGAAAAAGAG

(*continued*)

TABLE 1

(Continued)

Locus name	Identification/ BAC location in *A. thaliana*	Type of polymorphism	Annealing temperature (°)	Forward primer	Reverse primer
nga361	T16B12	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
At2g33010[d]	At2g33010	Indel	55	TGGAGCATATCGAGAA GAAGCACTC	GGACTTCTTGTGAAG GCAAATCAGA
ACT3	At2g37620	*Msp*I		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
ICE12	At2g39010	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
At2g40140	At2g40140	Indel	50	TCAAAAACCCCAACCACTTC	TCCACCGATGGATTCTCTTC
At2g43010	At2g43010	Indel	50	CGGACTCAT GGACTTGCTTT	TTCTGGGTTTG GGTTTGTTC
ACA/CG-130		AFLP			
ICE11	F11C10/F13A10	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
MTP1-A	At2g46800	RFLP/SSCP		Dräger *et al.* (2004)	Dräger *et al.* (2004)
			Linkage group 5		
nga1145	T16F16	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
At3g28220	At3g28220	Indel	50	TGGGTCCATTTCTTGTTGTT	CCAAGCCAATT GCTCCATAG
At3g33530	At3g33530	*Mfe*I	50	TGGTGGGATGTAACAACAGG	TTTAGACTGGGG CACAAAGC
AAC/CG-179		AFLP			
At3g45810	At3g45810	*Dra*I	50	TTTGCTGGTTATTGCCTACG	ACCTCTCGCT CTTGTTTCCA
F3H	At3g51240	*Hin*dIII		Kuittinen *et al.* (2002)	Kuittinen *et al.* (2002)
MTP3[c]	At3g58060	Indel	55	CCATGGTCACG GTCATAGTCAT	CGCTCTGTATCGA ATCTCCCAGCA
nga112	At3g62650	Microsat		Bell and Ecker (1994)	Bell and Ecker (1994)
			Linkage group 6		
At4-GA2	T18A10	Microsat	50	CCTCGGGTA AAGACAGAGCA	TGGTAACACC GGAAGTTTCA
LD	At4g02560	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
MTP1-B		RFLP/SSCP		Dräger *et al.* (2004)	Dräger *et al.* (2004)
AthDET1	At4g10180	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
ICE2	K18P6	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
AthCDPK9	MQM1	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
AAC/CG-336		AFLP			
ACA/CG-359		AFLP			
At5g08160	At5g08160	Indel	50	TTGTTGGTACG ACTTTTTCTCG	GCCGGATCCT TGACTTTCTT
MT2b		RFLP		Zhou and Goldsbourgh (1995)	Zhou and Goldsbourgh (1995)
			Linkage group 7		
At4g38220	At4g38220	Indel	50	GTGGTGGTGGGAGAAGAGAG	ACCGTGTTGATTCGGAGGTA
At4g33160	At4g33160	Indel	50	TTGGCCTAAG TTTTTCTTTTTG	GTCATCCATGGGGAATTCAG
cha15[f]	F11C18	Microsat	50	AATTCAAACAGGCGAAACCA	CTGCGAATCTCACGACTTCA
TSB2	At4g27070	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
At4-TC1	T19F6	Microsat	50	CAAGGTCGAATGTTGGAGACT	GCGCACTACAAAAATGAGAGG
SRK[g]	At4g21366	SNP		TCAAGATTGAAGCTGAGTGA	TACACAACCCGTCCCGCCAA
FCA	At4g16280	*Hin*fI		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
ACA/CG-266		AFLP			
ACA/CG-312		AFLP			
PhyC	At5g35840	Indel		Kuittinen *et al.* (2002)	Kuittinen *et al.* (2004)
ATTSO191	At5g37780	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
ICE9	At5g40340	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)

(*continued*)

TABLE 1

(Continued)

Locus name	Identification/ BAC location in *A. thaliana*	Type of polymorphism	Annealing temperature (°)	Forward primer	Reverse primer
			Linkage group 8		
ATCLH2	At5g43860	Indel		Kuittinen *et al.* (2004)	Kuittinen *et al.* (2004)
ACA/CT-86		AFLP			
EMF2	At5g51230	*Xho*I	54	GTTGCAGTTTGCAAAAACGA	CATGGAATGTGACCATCTGC
ACA/CG-387		AFLP			
MHJ24	MHJ24	Microsat		Clauss *et al.* (2002)	Clauss *et al.* (2002)
NRAMP4[e]	At5g67330	Indel	55	TTGGATGTTTGGTCAG ACGAAACCCAGTG	ATAAACTGTCCGGCGTA CGTACCTGTGAT

The names and identification of the loci in *A. thaliana*, the type of polymorphism scored, restriction enzyme if a CAPS marker, annealing temperature, and primer sequences if newly defined markers are shown.

[a] Primers provided by T. Mitchell-Olds (personal communication).

[b] Primers and genotyping results provided by M. Mirouze (personal communication).

[c] Primers and genotyping results provided by M. Hanikenne (personal communication).

[d] Primers and genotyping results provided by F. Varoquaux (personal communication).

[e] Primers and genotyping results provided by R. Oomen (personal communication).

[f] Primers provided by C. Schlötterer (personal communication).

[g] *A. halleri* allele-specific primers are given, and genotypes have been controlled by amplification with *A. l. petraea* 1 allele-specific primers (V. Castric, personal communication).

0.0001) (Figure 2, a and b). Pairwise comparisons revealed a significant difference in Zn tolerance between the *A. halleri* parental clones ($n = 12$; $EC100_{mean} = 2917$ μM Zn) and the *A. l. petraea* 1 parental clones ($n = 6$; $EC100_{mean} = 38$ μM Zn) ($P < 0.0001$). The Zn tolerance of the *A. halleri* parental clones was probably underestimated because, even at the highest concentration applied in the test (3000 μM Zn), all clones except one still showed new root growth. The Zn tolerance of the F_1 parental clones ($n = 12$; $EC100_{mean} = 1708$ μM Zn) differed significantly from the tolerance of the *A. l. petraea* 2 ($n = 8$; $EC100_{mean} = 44$ μM Zn) ($P < 0.0001$) and *A. halleri* parental clones ($P < 0.0001$). This indicates partial dominance of Zn tolerance in *A. halleri*, even though the underestimation of Zn tolerance for the *A. halleri* parental clones precludes any estimation of the dominance coefficient. No significant difference was identified between Zn tolerance of *A. l. petraea* 1 and *A. l. petraea* 2 parental clones ($P = 0.3519$). The genotype effect of Zn tolerance of the BC_1 individuals was highly significant ($F = 2.22$; $P < 0.0001$). Broad-sense heritability of Zn tolerance in the BC_1 was high ($h^2 = 0.69$). No transgressive segregation of Zn tolerance was observed (Figure 2c).

QTL mapping of Zn tolerance: Three QTL located on linkage groups LG3, LG4, and LG6 were identified by IM and subsequent MQM mapping (Figure 3). The QTL were named Zntol-1, -2, and -3, respectively, for linkage group LG3, LG4, and LG6. LOD scores of Zntol-1, -2, and -3, calculated by the MQM module of MapQTL, were 6.46, 7.28, and 4.52, respectively (Table 3). In the five other linkage groups, the LOD scores did not exceed the significance threshold value of 2.4 (Figure 3). The individual contribution of each QTL to the phenotypic variance was 12.2, 11.2, and 5.6%, respectively, for the QTL Zntol-1, -2, and -3 (Table 3). They accounted together for 29% of the total phenotypic variance, which represents 42% of the genetic variance. For each of the QTL, the *A. halleri* allele increased Zn tolerance (Table 3). Pairwise interactions between the QTL were significant at $\alpha = 0.05$ using the raw $EC100_{mean}$ data. However, loss of significance was observed using the log-transformed $EC100_{mean}$ values, indicating a statistical artifact (Table 4). Identical results were obtained independently of considering the main factors *HMA4*, *MTP1*-A, and *MTP1*-B fixed or random. As showed by box plots, eight genotypic groups were obtained by considering the markers *HMA4*, *MTP1*-A, and *MTP1*-B, closest to or at the three QTL positions, characterized by the presence or absence of the *A. halleri* allele at one, two, or three markers (Figure 4). A Kruskal–Wallis test revealed significant differences of Zn tolerance among the eight groups ($P < 0.0001$). For Zntol-1, -2, and -3, one-LOD support intervals of 19, 4, and 8 cM were reported, respectively. Two-LOD support intervals were 24, 4, and 13 cM for the three QTL, respectively (Figure 5). Six markers colocalized with the LOD support intervals. Markers *ICE14* and *HMA4* mapped in the LOD support interval of Zntol-1, markers *MTP1-A* and *ICE11* colocalized with Zntol-2, and markers *AthDET1* and *MTP1-B* colocalized with Zntol-3.

DISCUSSION

Previous combinations of classical functional and transcriptional analyses have identified several genes

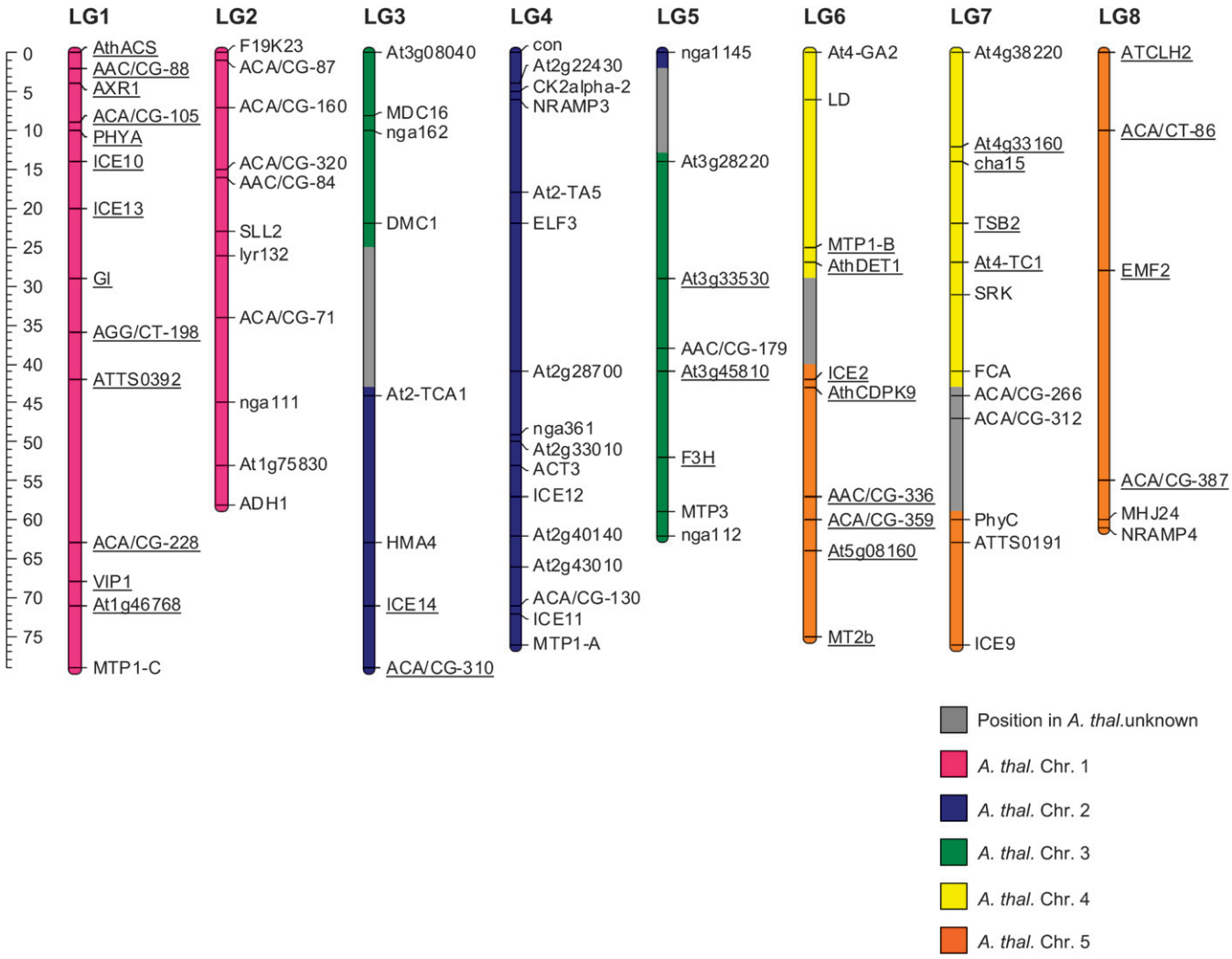

FIGURE 1.—Linkage map of the *A. halleri* × *A. l. petraea* BC_1 progeny constructed with Joinmap 3.0. The homology with the *A. thaliana* chromosomes is indicated by the colors. The regions where translocation occurred are in gray. The position in *A. thaliana* of the anonymous markers was inferred by integration of the *A. l. petraea* and *A. l. lyrata* mapping experiments. Markers in segregation distortion are underlined.

potentially involved in the adaptation of *A. halleri* to high heavy metal concentrations. However, definitive evidence for their implication in heavy metal tolerance has not yet been provided, and the genetic mechanisms underlying this trait are still largely unknown. This article contributes to a better understanding of the genetic architecture of Zn tolerance by applying a QTL mapping approach to the Zn-tolerant species *A. halleri*. The linkage map, constructed on *A. halleri* × *A. l. petraea* BC_1 progeny, revealed three QTL regions determining Zn tolerance, in which we would expect genes involved in the adaptation of *A. halleri* to high heavy metal concentrations to be located.

Genetic architecture of Zn tolerance in *A. halleri*: Until recently, the constitutive nature of Zn tolerance in *A. halleri* (PAUWELS *et al.* 2006) rendered its genetic analysis inaccessible. MACNAIR *et al.* (1999) was the first to circumvent this major handicap by analyzing the segregation of Zn tolerance in interspecific crosses performed between *A. halleri* and its closest nontolerant relative *A. l. petraea*. On the basis of segregation analysis of one F_2 progeny, the authors hypothesized a single major gene determining Zn tolerance in *A. halleri*, as already described for other metals and species (SCHAT *et al.* 1993; SMITH and MACNAIR 1998). Considering the two modes observed in the frequency distribution of the $EC100_{mean}$ values of the BC_1 individuals, one might expect this distribution to indicate also the presence of a single major gene in the determinism of Zn tolerance. Our results based on a QTL analysis firmly establish that three additive QTL located on three different linkage groups are involved in the evolution of Zn tolerance in *A. halleri*. We believe that the results of the QTL analysis and the distribution of the phenotypic data are not contradictory for two main reasons. First, a significant deviation from the distribution expected under the hypothesis of a single major gene governing Zn tolerance was observed ($P < 0.001$). Second, the QTL

TABLE 2

Comparison of mean linkage group length in the *A. halleri* × *A. l. petraea*, the *A. l. petraea*, and the *A. l. lyrata* maps

Linkage group	*A. halleri* × *A. l. petraea*[a]	*A. l. petraea*[a]	*A. l. lyrata*[a]
LG1	78	74	39
LG2	57	58	6
LG3	80	64	69
LG4	76	67	47
LG5	63	54	60
LG6	75	61	76
LG7	76	78	49
LG8	62	59	61

[a] Lengths of the linkage groups are given in centimorgans.

analysis performed on log-transformed EC100 values, which is expected to improve the normal distribution of the phenotypic data, did not modify the results (data not shown). With the exception of a minor QTL on LG8 slightly exceeding the LOD score threshold, no other QTL and no major variation of the explained variance for the identified QTL were detected.

Different arguments can also be proposed to explain the discrepancy between our results and Macnair's conclusions. First, the methodologies to assess tolerance were different. In Macnair's study, Zn tolerance was evaluated at a single fixed concentration (250 μM) (Macnair *et al.* 1999), whereas in our study a multiple concentration test was applied to measure tolerance of the BC_1 individuals; the latter is assumed more appropriate for assessing quantitative variations in tolerance levels (Schat and Ten Bookum 1992). Moreover, Macnair *et al.* (1999) used the lack of chlorosis as a subjective measure of tolerance rather than root growth. Second, the *A. halleri* genotypes used in both studies did not originate from the same metalliferous site. The *A. halleri* individual used in the QTL analysis was collected from Auby (France), a site with a very high metal contamination of relatively recent date (the beginning of the 20th century) resulting from the proximity to a Zn smelter factory (Van Rossum *et al.* 2004). In contrast,

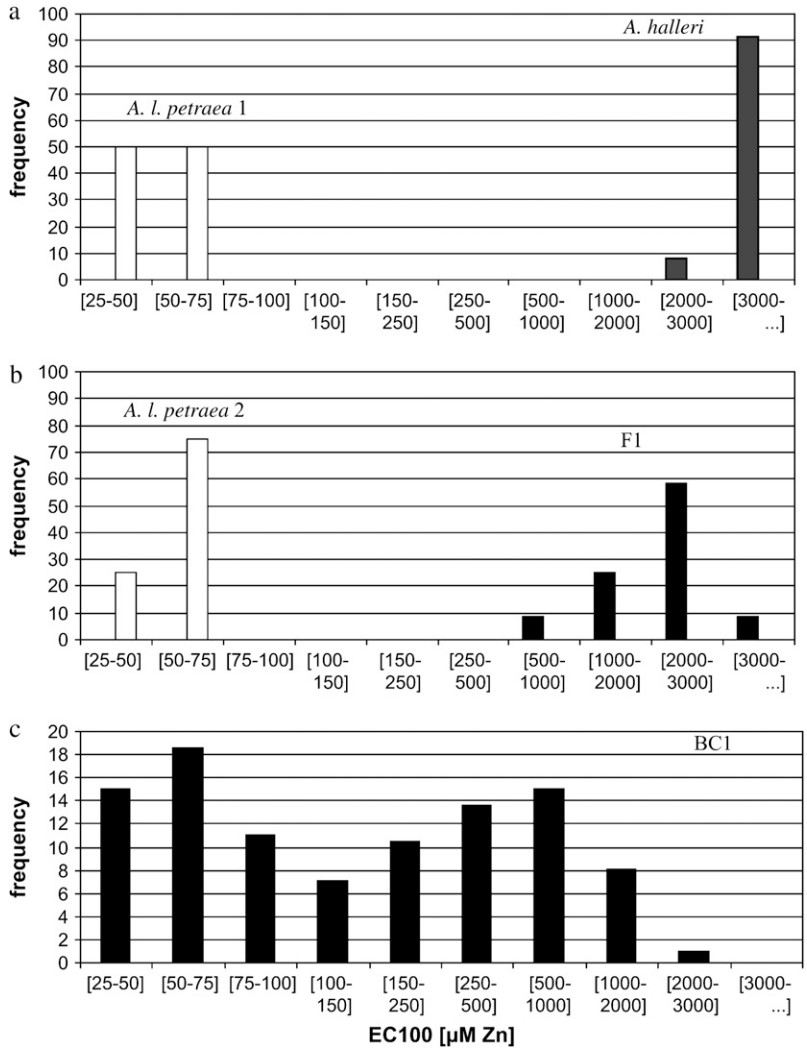

Figure 2.—Frequency distribution of Zn tolerance of the parental clones *A. halleri*, *A. l. petraea* 1, *A. l. petraea* 2, and F_1, and the BC_1 derived from *A. halleri* × *A. l. petraea*. Zn tolerance was measured by a sequential Zn exposure test in hydroponic solution. (a) Frequency distribution of Zn tolerance of the *A. halleri* and *A. l. petraea* 1 parental genotypes. (b) Frequency distribution of Zn tolerance of the *A. l. petraea* 2 and F_1 parental genotypes. (c) Frequency distribution of Zn tolerance of the BC_1 progeny; $EC100_{mean}$ values were used.

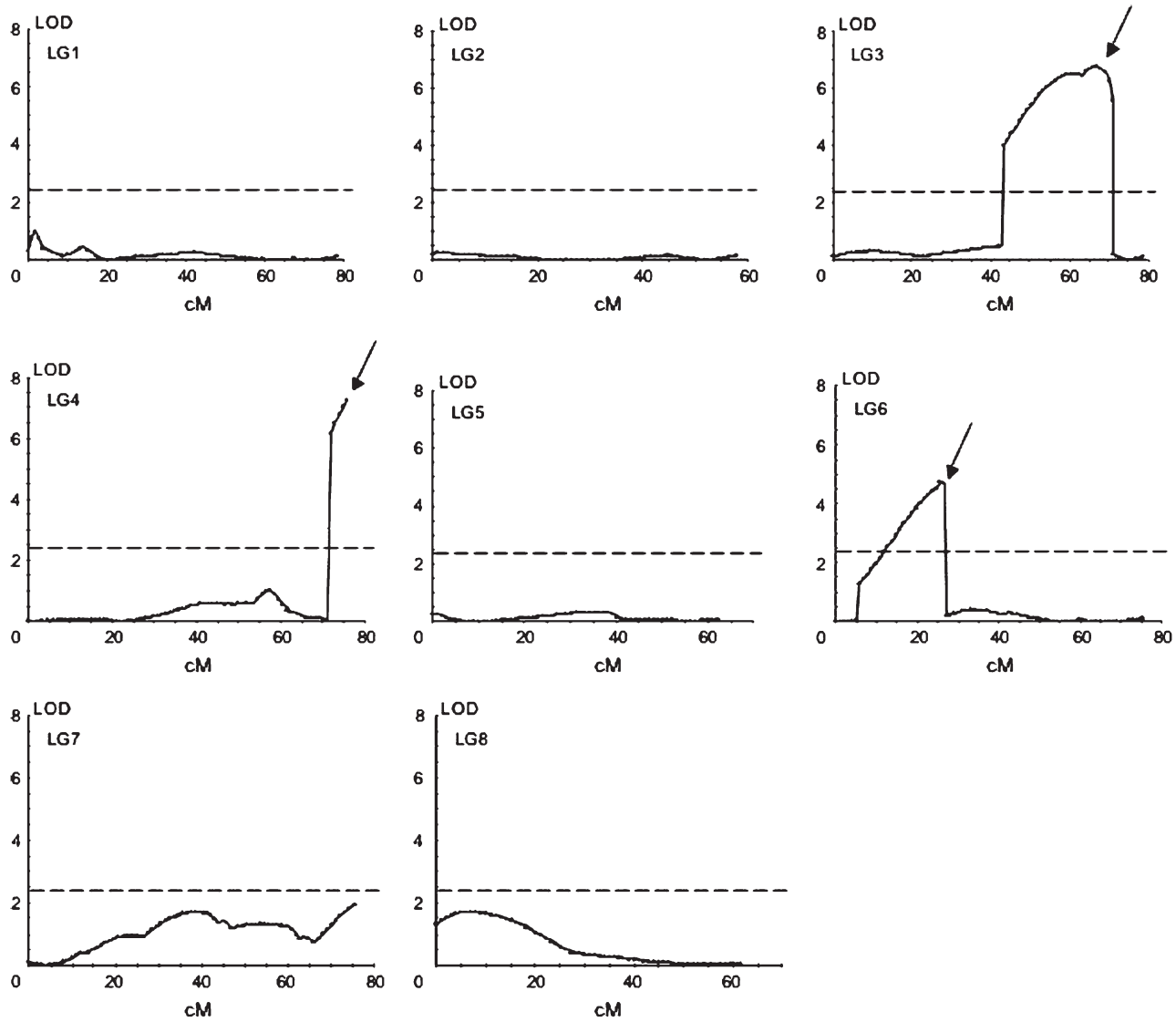

Figure 3.—LOD score profiles for Zn tolerance. Names of linkage groups are given at the top left corner of each graph. Map positions are plotted along the abscissa. LOD scores are plotted along the *y*-axis. Dashed lines correspond to the LOD score threshold (2.3) for QTL detection at an error level of $\alpha = 0.05$. QTL are indicated by arrows above the LOD score profile.

the *A. halleri* genotype used in Macnair's study (Macnair *et al.* 1999) originated from a suburb of Langelsheim (Germany); this site was reported to have become contaminated because of medieval metal-mining activities (Weber *et al.* 2004) and is characterized today by lower metal contamination levels. However, historical and genealogical data show high genetic relationships between German and French populations and suggest that *A. halleri* has been introduced in France from M sites such as Langelsheim located in Germany (Pauwels *et al.* 2005). Consequently, the different origin of *A. halleri* in both studies is not expected to contribute significantly to the discrepancies observed, even though the existence of specific local adaptations to metal contamination in both *A. halleri* populations might not be excluded.

The three QTL identified in this study explain 42% of the genetic variance of Zn tolerance, which means that we could have missed some QTL. The so-called "Beavis effect" predicts that in experiments using progeny sizes of ~100 individuals, fewer QTL are identified than with larger progeny sizes of ~400 (Beavis 1994). Moreover, estimates of genetic effects were reported to be inflated in experiments using progeny sizes of 100 compared to the ones using progenies of 400 individuals (Beavis 1994; Kearsey and Farquhar 1998; Xu 2003). According to the "Beavis effect," a progeny of intermediate size (~200 individuals), such as the one used in this QTL analysis of Zn tolerance, still suffers from a reduction in QTL detection power and an inflation of the estimates of the QTL effects (Beavis 1994; Kearsey and Farquhar 1998; Xu 2003). Such a reduction in power leading to a failure to detect a number of QTL in this experiment could explain the difference observed between broad-sense heritability of Zn tolerance in the

TABLE 3

QTL for Zn tolerance

QTL[a]	LG/Marker[b]	Position[c]	LOD Score[d]	R^{2e}	a^f
Zntol-1	LG3/HMA4	64.6	6.46	12.2	−280.861
Zntol-2	LG4/MTP1-A	75.5	7.28	11.2	−266.055
Zntol-3	LG6/MTP1-B	24.7	4.52	5.6	−197.275

[a] QTL are named by the trait and ordered from 1 to 3.
[b] Linkage group on which QTL were mapped and marker at QTL position or closest to QTL position.
[c] Position of marker at or closest to QTL (in centimorgans).
[d] LOD score of marker at or closest to QTL.
[e] Percentage of explained variance of the marker at or closest to QTL.
[f] Additive effects are given as the difference between the means of the two genotypic groups of BC_1 individuals (negative value implies that the *A. halleri* allele increases Zn tolerance compared to the *A. l. petraea* allele).

BC_1 progeny and the variance explained by the QTL. Segregation distortion is also believed to reduce the power of QTL detection and to affect the estimates of QTL effects, because it reduces the effective size of the progeny by reducing the size of one genotypic class (Bradshaw *et al.* 1998). Segregation distortion was reported for 40% of all markers in the BC_1 population. It is therefore possible that some QTL for Zn tolerance, probably of minor effect, have not been detected. Among the QTL that have been detected, only the QTL Zntol-3 is located in a distorted region and showed a deficit in heterospecific allelic combinations. It is highly probable that this affected the estimation of the QTL effect since the mean Zn tolerance value was calculated on less heterospecific genotypes than the one calculated on the homospecific genotypes.

Evolutionary dynamics of metal tolerance in *A. halleri*: At all three QTL positions, the tolerance-enhancing allele originated from the *A. halleri* parent, as expected under continuous directional selection (Orr 1998b). Because we used an *A. halleri* individual of metallicolous origin in our study, the identified QTL might reflect the constitutive tolerance observed in M and NM populations and the enhanced tolerance occurring in M populations on recently colonized industrial sites (Pauwels *et al.* 2006). In the present state of our knowledge, it remains unclear whether specific alleles at all three QTL are involved in enhanced tolerance or whether one or more QTL are specific for the constitutive tolerance. Crosses involving NM accessions might provide conclusive results to distinguish between these two hypotheses.

TABLE 4

Epistatic interactions between QTL for Zn tolerance

	On raw $EC100_{mean}$ data		On log-transformed $EC100_{mean}$ data	
Source	F^a	P^b	F^a	P^b
Zntol-1	31.53	<0.0001	16.83	<0.0001
Zntol-2	41.85	<0.0001	30.43	<0.0001
Zntol-3	30.23	<0.0001	25.56	<0.0001
Zntol-1 × Zntol-2	4.75	0.0306	0.03	0.8664
Zntol-1 × Zntol-3	5.51	0.0200	0.38	0.5592
Zntol-2 × Zntol-3	3.96	0.0482	0.11	0.7428

[a] Values of *F*-statistic, considering the markers fixed or random factors.
[b] Significance levels, considering the markers fixed or random factors.

a

group	markers HMA4	MTP1-A	MTP1-B	n
1	Alp/Alp	Alp/Alp	Alp/Alp	30
2	Ah/Alp	Alp/Alp	Alp/Alp	21
3	Alp/Alp	Ah/Alp	Alp/Alp	38
4	Alp/Alp	Alp/Alp	Ah/Alp	25
5	Ah/Alp	Ah/Alp	Alp/Alp	27
6	Ah/Alp	Alp/Alp	Ah/Alp	15
7	Alp/Alp	Ah/Alp	Ah/Alp	13
8	Ah/Alp	Ah/Alp	Ah/Alp	13

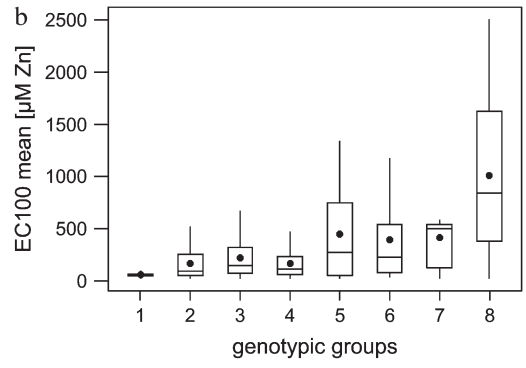

Figure 4.—Box plot of Zn tolerance. (a) Genotypic groups of BC_1 individuals at the markers HMA4, MTP1-A, and MTP1-B are numbered from 1 to 8. The number of BC_1 individuals in each group is given by "n." (b) Box plot of Zn tolerance by genotypic groups. The boxes represent the interquartile range, with arithmetic means indicated by solid circles and medians indicated by horizontal lines. Whiskers connect the nearest observations within 1.5 times the interquartile ranges of the lower and upper quartiles.

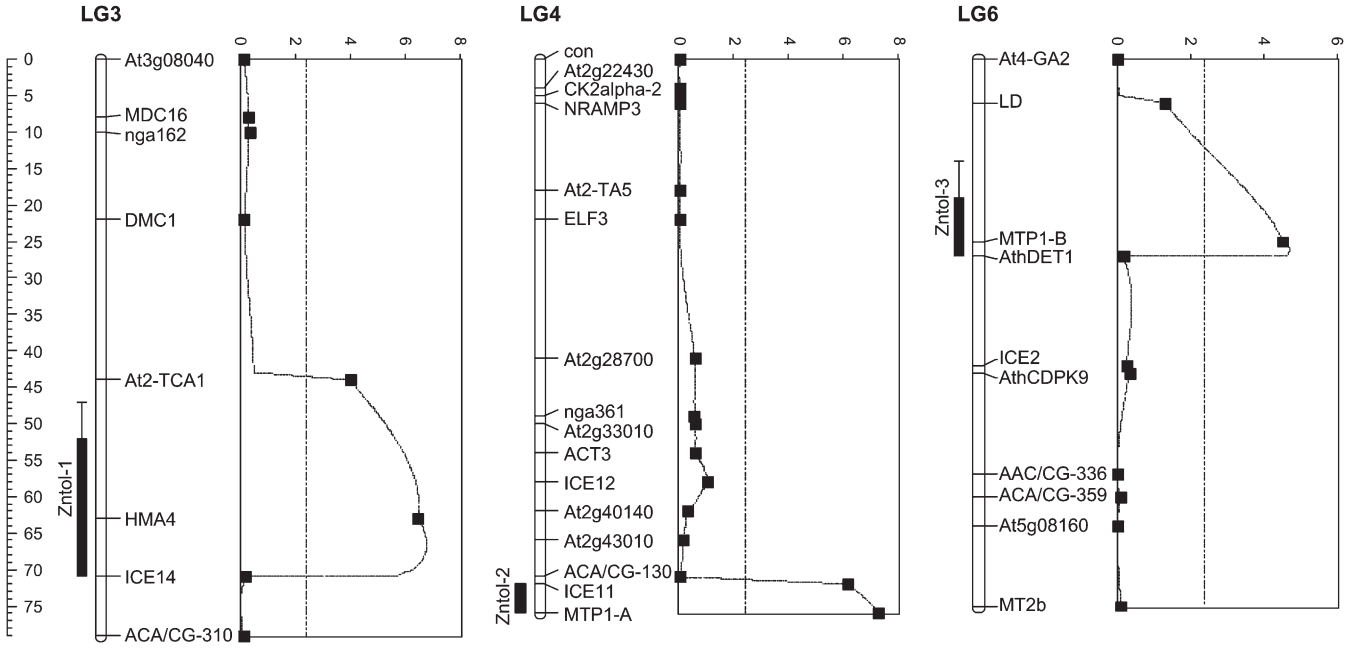

FIGURE 5.—LOD score profiles and LOD support intervals for the QTL for Zn tolerance in the BC_1 progeny. The linkage groups LG3, LG4, and LG6 on which a QTL for Zn tolerance was detected are represented. The LOD score profiles along the linkage groups are given at the right of each linkage group. Map positions are plotted along the vertical axis. LOD scores are plotted along the horizontal axis. Dashed lines correspond to the LOD score threshold (2.3) for QTL detection at an error level of $\alpha = 0.05$. LOD support intervals are given at the left of each linkage group. The position of a QTL is shown as the interval over which the LOD score is within 1 or 2 log units of its maximum value, *i.e.*, at the most likely position of the QTL. Bars indicate 1-LOD (10-fold) support intervals and whiskers (lines extending beyond bars) indicate 2-LOD (100-fold) support intervals.

The QTL analysis of Cd tolerance was recently addressed in a subset of the interspecific BC_1 population used in this study (M. COURBOT, G. WILLEMS, P. MOTTE, S. ARVIDSSON, P. SAUMITOU-LAPRADE and N. VERBRUGGEN, unpublished results). Among the QTL identified for Zn and Cd tolerance, one was involved in both adaptive traits as inferred from the colocalization of the QTL Zntol-1 and the major QTL (>45% of genetic variance explained) for Cd tolerance. Because Zn and Cd are very often associated in soils naturally enriched in Zn (ERNST 1974), it is most parsimonious to hypothesize that Zn constitutive tolerance initially evolved in *A. halleri* refuge through the fixation of a QTL conferring tolerance to both metals. Increased tolerance to Zn and Cd might have been achieved more recently on industrial sites surrounding Zn smelters. In these sites, the concentrations of Zn and Cd are much higher than ever observed in naturally metal-enriched sites and this could have driven the selection of mechanisms specific to either Zn or Cd tolerance. The results presented here are in fair agreement with the predictions of the Fisher–Orr model of adaptation that describes the entire adaptive walk taken by a population to move toward a new fitness optimum and suggests that the size of the first factor fixed is fairly large (ORR 2005). However, the validity of this hypothesis should be verified using QTL mapping of heavy metal tolerance in recombinant populations from interspecific crosses between *A. l. petraea* and *A. halleri* from NM populations, which should reveal mainly the QTL associated with first adaptation (constitutive tolerance), and from intraspecific crosses between independently founded M and NM populations, which should detect QTL associated with the more recent adaptation to industrial polluted sites (PAUWELS *et al.* 2005).

The interspecific map covers most of the *A. halleri* genome and synteny with other Arabidopsis genomes is high: The extensive conservation of marker order between the interspecific *Ah* × *Alp* map and *A. thaliana* was in line with previous results reported for the *A. l. petraea* (KUITTINEN *et al.* 2004; KOCH and KIEFER 2005) and *A. l. lyrata* (YOGEESWARAN *et al.* 2005) maps. The only discrepancy in marker order between the *A. l. petraea* and the interspecific map was observed for the loci *PhyA* and *AXR1* (LG1/AL1), but, as suggested by KUITTINEN *et al.* (2004), their order in *A. l. petraea* "could well be consistent with the order expected from *A. thaliana*." The large inversion observed on AL6 of the *A. l. petraea* linkage map (KUITTINEN *et al.* 2004) and confirmed in *A. l. lyrata* (YOGEESWARAN *et al.* 2005) as well as in another close relative, *Capsella rubella* (BOIVIN *et al.* 2004), was not detected on the corresponding linkage group LG6 on the *Ah* × *Alp* map. Nevertheless, the low marker density in this region probably precluded the detection of this inversion.

Less efficient recombination has been reported in interspecific crosses due to the genetic divergence between the parental lines belonging to different species (Williams *et al.* 1995; Bernacchi and Tanksley 1997). However, the nonsignificant difference of the marker interval sizes between the *Ah* × *Alp* and the *A. l. petraea* map suggests that the recombination between the *A. halleri* and *A. l. petraea* genomes was as efficient in the interspecific hybrid as in the intraspecific cross. Significant differences of linkage group lengths were observed between the *Ah* × *Alp* map and either the *A. l. petraea* map or the *A. l. lyrata* map: the total length was increased in our mapping experiment compared to the *A. lyrata* maps. Since the inverse would be rather expected in such interspecific crosses in which absence of homology could reduce recombination and subsequent observed genome size, we assume that this just indicates the less complete saturation of the *A. lyrata* genetic linkage maps. We believe that our interspecific map covers most of the *A. halleri* genome, since markers situated near the extremities of *A. thaliana* chromosomes were used in the interspecific cross for map construction. Moreover, the markers located on the extremity of the LG4 (AL4, AlyLG3), LG6 (AL6, AlyLG7), and LG8 (AL8, AlyLG8) lower arms were nearer to the *A. thaliana* chromosome extremities in the interspecific map than in the *A. l. petraea* and the *A. l. lyrata* maps (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005).

The genome sizes reported for *A. thaliana* (0.16 pg) and its close relatives (~ 0.26 pg) (Johnstone *et al.* 2005) indicate that one or more deletion events might have accompanied the transition from eight to five chromosomes, characterizing the genome of *A. thaliana*. Ideally, the comparative analyses of the *Ah* × *Alp* and the *A. lyrata* maps with the *A. thaliana* genome should take these events into account. In this regard, the sequencing project on *A. l. lyrata* (http://www.jgi.doe.gov/sequencing/why/CSP2006/AlyrataCrubella.html) will be very valuable, since this will provide us with an exhaustive knowledge of the genome of the Arabidopsis relatives.

Segregation distortion: At a significance threshold of 0.05, we might expect 5% of all markers to show distorted segregation by chance; in the BC_1 population, we greatly exceeded this proportion. A failure to show the expected Mendelian ratios is rather common in interspecific crosses for different reasons (Zamir and Tadmor 1986; Bernacchi and Tanksley 1997; Jenczewski *et al.* 1997). In the BC_1 progeny, segregation bias could be explained, at least partially, by the divergence time between the parental lines, which has been estimated to be about half (X. Vekemans, personal communication) the divergence time between *A. thaliana* and *A. l. petraea* (5.8 MYA) (Koch *et al.* 2001). Outbreeding depression, which in our case is supported by the large majority of the distorted markers (92%) showing an excess of homospecific *vs.* heterospecific allelic combinations, could also be at the origin of the high segregation distortion observed in the BC_1 progeny. Finally, because the F_1 and all the BC_1 individuals belong to maternal progenies collected on *A. l. petraea*, the highly directional segregation bias observed in the BC_1 could also indicate a negative interaction between the *A. halleri* alleles at the nuclear loci (or at closely linked loci) and the maternally inherited cytoplasmic genotype corresponding to *A. l. petraea* (Fishman *et al.* 2001).

Linkage map construction and, more precisely, estimation of recombination frequencies, can be affected by segregation distortion (Fishman *et al.* 2001; Kuittinen *et al.* 2004). However, because we applied stringent goodness-of-fit thresholds to minimize the effects of segregation distortion on the linkage map construction and observed macrosynteny between the *Ah* × *Alp* map and the *A. l. petraea* map, as well as between the *Ah* × *Alp* map and *A. thaliana*, we believe that the current map is quite robust.

Colocalization of known heavy metal homeostasis genes with the QTL for Zn tolerance: The length of the LOD support intervals associated with the QTL for Zn tolerance reported in this experiment precludes the direct identification of the underlying genes. In *A. thaliana*, for instance, 1 cM has been reported to correspond to an average of 250 kb or ~40 genes (Mauricio 2001). However, the correspondence between genetic and physical distances in the close relatives of *A. thaliana* is not known. In this context, the sequencing of the *A. l. lyrata* genome (http://www.jgi.doe.gov/sequencing/why/CSP2006/AlyrataCrubella.html) also will be very useful. On the current map, we reported the colocalization of three genes, *HMA4*, *MTP1-A*, and *MTP1-B*, with the three QTL regions for Zn tolerance.

HMA4 is a member of the family of P-type ATPases and colocalized with the QTL Zntol-1. In *A. thaliana*, *HMA4* is expressed mainly in the roots and was shown to be involved in the root-to-shoot transport of Zn and Cd (Mills *et al.* 2003; Hussain *et al.* 2004). Compared to the *A. thaliana* orthologous gene, *HMA4* was shown to be highly overexpressed in the roots of the Zn/Cd-tolerant and hyperaccumulator species *T. caerulescens*, indicating a possible role in translocation, as well as in the shoots where *HMA4* may be involved in Zn/Cd detoxification (Bernard *et al.* 2004; Papoyan and Kochian 2004).

The metal homeostasis genes *MTP1-A* and *MTP1-B* mapped to the QTL Zntol-2 and Zntol-3, respectively. These genes are homologous to *MTP1* from *A. thaliana*, formerly known as *ZAT* (van der Zaal *et al.* 1999), and to *ZTP1* from *T. caerulescens* (Assunção *et al.* 2001), a cation diffusion facilitator, and were clearly shown by functional analysis to interact with zinc homeostasis in *A. halleri* (Dräger *et al.* 2004; Krämer 2005). In microarrays hybridized with labeled shoot cRNA, normalized signal intensities for *ZAT/AtMTP1* were between 14- and 23-fold higher in *A. halleri* compared to *A. thaliana* (Becher *et al.* 2004). Under control conditions (1 μM

Zn), the root steady-state *MTP1* transcript levels in *A. halleri* were approximately equivalent to those in *A. thaliana*. Nevertheless, after exposure to 100–300 μM Zn, root *MTP1* transcript abundance increased incrementally in *A. halleri*, but not in *A. thaliana* or in *A. l. petraea* (Dräger *et al.* 2004). Using the same BC_1 population as the one investigated in this study, to separately analyze the expression of the MTP1 loci, Dräger *et al.* (2004) performed semiquantitative RT–PCR on RNA extracted from the shoots of selected BC_1 individuals containing the different copies. In three BC_1 individuals harboring the copies *AhMTP1-B* and *AhMTP1-C*, the transcript level of *AhMTP1-B* on average was 5.7-fold higher than the one of *AhMTP1-C*. The expression of *AhMTP1-C* was more or less equivalent to the expression of the *MTP1* gene in *A. l. petraea*. In BC_1 individuals carrying the copies *AhMTP1-A* and *AhMTP1-B*, the transcript levels of both loci together on average were 11.1-fold higher than the one of *A. l. petraea MTP1* (Dräger *et al.* 2004).

The colocalization with the QTL for Zn tolerance (this study) and the differential expression and/or regulation demonstrated for *AhMTP1-A* and *AhMTP1-B* in response to Zn (Dräger *et al.* 2004) provide strong arguments in favor of adaptive modifications of these specific metal homeostasis genes (or their regulatory regions) in relation with Zn tolerance in *A. halleri*. This may also be the case for *HMA4*, which was described previously in *T. caerulescens* (Bernard *et al.* 2004; Papoyan and Kochian 2004). These genes can consequently be considered as good candidates for Zn tolerance and are currently being submitted to deeper investigations.

Zn tolerance and Zn accumulation remain unlinked in *A. halleri*: Recently, an interspecific crossing scheme between *A. halleri* and *A. l. petraea* was used for identifying QTL involved in Zn accumulation in *A. halleri* (Filatov *et al.* 2006). By comparing gene expression of Zn-accumulating F_3 families to non-Zn-accumulating F_3 families, the authors identified 237 genes that were more expressed in the accumulating progenies. Deducing the chromosomal position of these genes from the *A. l. petraea* linkage map reported by Kuittinen *et al.* (2004), the authors identified 20 and 18 adjacent genes, respectively, belonging to two regions located on chromosomes 3 and 7 (Filatov *et al.* 2006), corresponding to LG3 and LG7 of the *Ah* × *Alp* map. None of these regions were identified in the QTL analysis of Zn tolerance reported here. These results confirm previous studies in which Zn hyperaccumulation and tolerance were shown to segregate independently in an *A. halleri* × *A. l. petraea* F_2 progeny (Macnair *et al.* 1999) and suggest that in *A. halleri* both traits are expected to be governed, at least partially, by different genes.

In conclusion, our search for QTL controlling Zn tolerance in *A. halleri* revealed three genomic regions in which three metal homeostasis genes colocalized. To minimize the LOD support intervals associated with the QTL, we are currently increasing the marker density of the *Ah* × *Alp* map and producing second-generation backcross progenies. Finally, the *Ah* × *Alp* map constitutes a powerful tool available to the scientific community working on metal homeostasis genes: any gene of interest can be mapped on our material and characterized for its relationships with the QTL of Zn tolerance in *A. halleri*.

We thank Robert Dron, Claire Feutrie, and Eric Schmitt for technical advice and support in taking care of the plants and Maxime Pauwels and Stéphane Fenart for help in phenotyping. We are very grateful to Mark Macnair for providing *A. lyrata* seeds and to Vincent Castric, Adrian Radu Craciun, Marc Hanikenne, Charles Langley, Michel Lebrun, Stéphanie Loubet, Marie Mirouze, Tom Mitchell-Olds, Ronald Oomen, Outi Savolainen, Christian Schlötterer, Sébastien Thomine, and Fabrice Varoquaux for providing primer pairs and/or help in genotyping. We are also very grateful to Steve Barnes, Joel Cuguen, Ute Krämer, Patrick de Laguérie, Henk Schat, Pascal Touzet, and Xavier Vekemans for scientific discussions and helpful comments on the manuscript. This research was supported by the Contrat de Plan Etat/Région Nord-Pas de Calais (Programme de Recherché Concerté), the European Fonds Européens de Développement Régional (contract no. 79/1769), the Programme National/Action Concertée Incitative du Fond National de la Science ECCO (contract no. 04 2 9 FNS), the Belgian Science Policy (Interuniversity Attraction Pole Programme V/13), and by a grant from the Fonds National de la Recherche Scientifique (FRFC 2.4565.02), in addition to a doctoral fellowship to G.W. from the European Research Training Network Metalhome (HPRN-CT-2002-00243).

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Communicating editor: O. Savolainen