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# Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*

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**Abstract**

The genetic basis of Cd tolerance and hyperaccumulation was investigated in *Arabidopsis halleri*. The study was conducted in hydroponic culture with a backcross progeny, derived from a cross between *A. halleri* and a non-tolerant and non-accumulating related species *Arabidopsis lyrata* ssp. *petraea,* as well as with the parents of the backcross. The backcross progeny segregates for both cadmium (Cd) tolerance and accumulation. The results support that (i) Cd tolerance may be governed by more than one major gene, (ii) Cd tolerance and Cd accumulation are independent characters, (iii) Cd and Zn tolerances co-segregate suggesting that they are under pleiotropic genetic control, at least to a certain degree, (iv) the same result was obtained for Cd and Zn accumulation.

## Introduction

Cadmium (Cd) is a widespread heavy metal, released into the environment by heating systems, metallurgic industries, waste incinerators, urban traffic, cement factories and as a contaminant of phosphate fertilizers (Sanita di Toppi and Gabbrielli, 1999). Cd is one of the four metals that have been mentioned to be a world-wide concern in terms of their importance in environmental quality and health (Sanita di Toppi and Gabbrielli, 1999). Its presence in the atmosphere, soil and water, can cause serious problems to all organisms, and heavy metal bioaccumulation in the food chain can be highly dangerous (Sanita di Toppi and Gabbrielli, 1999).

For a few years, remediation of metal-contaminated soils became a world preoccupation. Hyperaccumulator plants could represent a resource for phytoremediation of metal polluted soils, as they are able to extract metals from the soils and to concentrate them in their upper parts (Brooks, 1998). However, very little knowledge is available about this technology. Most of the hyperaccumulators are restricted to metal-enriched soils and also have the character of metal tolerance (Brooks, 1998). However, some hyperaccumulators, notably *Arabidopsis halleri* and *Thlaspi caerulescens*, have populations on normal soils, which are metal tolerant, too (e.g. Bert et al., 2000; Meerts and Van Isacker, 1997). For these species, several authors have shown that accumulation and tolerance are uncorrelated or inversely correlated characters (Bert et al., 2000; Escarré et al., 2000; Meerts and Van Isacker, 1997). Moreover, it is well established that a large variation exists within species for metal tolerance and accumulation in hyperaccumulators (Assunção et al., 2001). For a number of metals, including zinc, copper and arsenic, genetic analysis has shown that tolerance is controlled by a small number (one or two) of major genes, with additional modifiers determining the level of tolerance displayed (Schat et al., 1993; Smith and

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Macnair, 1998; van Hoof et al., 2001). Tolerance to one metal is generally controlled by gene(s) that are different from those which confer tolerance to other metals (Schat et al., 1996; Tilstone et al., 1997). In contrast, the genetics of hyperaccumulation and the precise genetic inter-relationships between tolerance and hyperaccumulation are poorly known. The limited references available refer to the hypothesis of the genetic independence of the two characters (Macnair et al., 1999). Metal tolerance and hyperaccumulation are still little understood at the molecular level. To evaluate the potential of hyperaccumulator-mediated remediation, genetics and physiology of tolerance and hyperaccumulation have to be first investigated.

*Arabidopsis halleri* (L.) O'Kane & Al Shehbaz, previously known as *Cardaminopsis halleri* (L.) Hayek, is one of the two species known to hyperaccumulate Cd (Brooks, 1998; Küpper et al., 2000). *A. halleri* is also a zinc (Zn) hyperaccumulator and usually occurs on Zn, Cd and Pb contaminated sites (Bert et al., 2000). Interestingly, it is closely related to and interfertile with *Arabidopsis lyrata* ssp. *petraea* (L.) O'Kane & Al Shehbaz that is both non-tolerant and a non-accumulator (Macnair et al., 1999). Using the F2 derived from the cross between *A. halleri* and *A. lyrata* ssp. *petraea,* Macnair et al. (1999) showed that Zn tolerance and Zn hyperaccumulation were genetically independent characters. In *Thlaspi caerulescens,* some populations combine high tolerance with high accumulation (Escarré et al., 2000; Lombi et al., 2000). Until now, the genetics of cadmium tolerance and hyperaccumulation in *Arabidopsis halleri* have not been investigated.

This work represents a first step in the analysis of the genetics of Cd tolerance and Cd hyperaccumulation in *A. halleri,* using a backcross progeny derived from the cross between *A. halleri* and *A. lyrata* ssp. *petraea*. More precisely, the following questions were addressed: What are the genetic bases of Cd tolerance and hyperaccumulation in *A. halleri*? Are Cd tolerance and Cd hyperaccumulation under pleiotropic genetic control? What is the genetic relationship between Cd tolerance and Zn tolerance, as well as between Cd accumulation and Zn accumulation?

## Materials and methods

### *Plant material*

Seeds of *Arabidopsis halleri* were collected from plants growing on a site highly contaminated with Zn, Cd and Pb located in the North of France (Bois des Asturies, Département du Nord). Seeds of *Arabidopsis lyrata* ssp.*petraea* originated from an uncontaminated site in Czech Republic (Unhost, Central Bohemia; Macnair et al., 1999). One *A. halleri* plant was crossed, as a male, with one *A. lyrata* ssp. *petraea* plant (*A. petraea* 1) to produce the F1. Previous results on F1 have established the dominant character of Zn tolerance (Macnair et al., 1999). In this study, we have used a test-cross by back crossing the F1 with *A. lyrata* ssp. *petraea*. In order to prevent an inbreeding depression expected in these strongly outbreeding species, the backcross involved a new *A. lyrata* ssp. *petraea* (*A. petraea* 2) individual. Moreover, in order to avoid mixture of BC1 and F2 seeds in the progeny, we took advantage of the strong self-incompatibility observed in *A. lyrata* ssp. *petraea* and collected BC1 seeds only on *A. petraea* 2. From this second cross, a backcross progeny was obtained. The validity of the F1 and the backcross progeny was checked by analysis with ten polymorphic microsatellite markers (data not shown).

Using the property of vegetative propagation of both *A. halleri* and *A. lyrata* ssp. *petraea*, 6–9 cuttings per plant were generated. Cuttings were then dipped in rooting hormone and planted on sand moistened with deionised water, for five weeks. Rooted cuttings were transferred to 4-L vessels containing a nutrient solution based on the one used by Chaney and Bell (1987) and consisting of 2 m*M* $MmgSO_4$, 0.5 m*M* $Ca(NO_3)_2$, 0.5 m*M* $KNO_3$, 0.1 m*M* $K_2HPO_4$, 0.2 $\mu$M $CuSO_4$, 2 $\mu$M $MnCl_2$, 10 $\mu$M $H_3BO_3$, 0.1 $\mu$M $MoO_3$ and 10 $\mu$M FeEDDHA. The nutrient solution was changed every week. All vessels were continuously aerated. Twenty plants were grown in each vessel. Cadmium was added as $CdCl_2$ at concentrations ranging from 10 to 250 $\mu$M. pH of the nutrient solution was between 5.5 and 6. The experiment was performed in a greenhouse from March to April 2001 (24/22 °C day/night; 16 h/8 h day/night; not below 300 $\mu$mol $m^{-2}$ $s^{-1}$ at the plant level; 70% relative humidity).

### *Evaluation of Cd tolerance*

A first experiment was conducted on *A. halleri*, *A. petraea* 1, *A. petraea* 2 and the F1 in order to determine their level of Cd tolerance. Each genotype was cloned (*A. halleri*: *n*=20; *A. petraea* 1: *n*=22; *A.*

*petraea* 2: *n*=11; F1: *n*=22) and transferred to hydroponic solution. In order to assess Cd tolerance, plants were sequentially transferred to increasing concentrations of Cd. The range of Cd concentrations tested was 10, 25, 50, 75, 100, 150 and 250 $\mu$M. At the end of each week, roots of each plant were gently dried with tissue paper and the whole plant was weighed. In addition, root length was measured. Tolerance was determined as the lowest concentration at which no new root length was produced, and at which no increase in biomass was observed. In order to characterise the Cd tolerance of the backcross progeny, another experiment was conducted with 100 plants from the backcross progeny as well as *A. halleri*, *A. petraea* 1 and 2, and F1 plants. Six clonal replicates of each plant individual were obtained. Three clones were grown in presence of Cd whereas the three others were grown in the absence of Cd (control; data not shown). Replicates were randomly placed in the vessels.

*Evaluation of Zn tolerance*

The Zn tolerance of the backcross progeny was determined by the method of Schat and Ten Bookum (1992), which measures the tolerance of a plant by determining the lowest concentration at which no new root growth is produced (=$EC_{100}$). Three clones of each genotype were grown in 10 $\mu$M Zn for one week. Roots of all plants were blackened with activated charcoal and rinsed in deionised water to remove the excess powder. The plants were returned to 10 $\mu$M Zn for a further week. Roots of the plants with new root growth visible beyond the charcoal coated roots were reblackened and plants transferred in successive weeks to 25, 50, 75, 100, 150, 250, 500, 1000, 2000 and 3000 $\mu$M Zn.

*Evaluation of Cd accumulation*

A first experiment was conducted on *A. halleri*, *A. petraea* 1, *A. petraea* 2 and the F1 in order to determine Cd accumulation of each plant. Each genotype was cloned and transferred to hydroponic culture. After five weeks, roots of each plant were gently dried with tissue paper and the whole plant was weighed. Replicates of each genotype were then treated, for one week ,with 10 $\mu$M Cd (*A. halleri* (*n*=3), F1 (*n*=3), *A. petraea* 1 (*n*=3) and *A. petraea* 2 (*n*=3)) or without Cd (*A. halleri* (*n*=2), F1 (*n*=3), *A. petraea* 1 (*n*=3) and *A. petraea* 2 (*n*=3)). In order to determine Cd accumulation of the backcross progeny, a separate experiment was conducted upon non-lethal Cd treatment, with 29 plants from the backcross progeny, as well as *A. halleri*, *A. petraea* and F1 plants. After five weeks in hydroponics, roots of each plant were gently dried with tissue paper and the whole plant was weighed. Replicates of each genotype were then assigned to two treatments as follows: one replicate in 10 $\mu$M Cd for one week and one replicate in a vessel without Cd (control) for one week.

After one week, roots of each plant were dried with tissue paper and the whole plant was weighed. Significant biomass increase indicated that the plants were not suffering from phytotoxicity at this concentration. Shoots and roots were separated, washed carefully with deionised water and dried at 60°C until constant weight. No desorption of Cd, i.e. with $CaCl_2$, was performed on the roots of the plants. Dried plant materials (0.5–1 g) were digested with 10 mL $HNO_3$/$HClO_4$ (2/1 v/v). Concentrations of Cd and Zn were determined using flame atomic absorption spectrophotometry (FAAS).

*Data analysis*

One way analysis of variance (ANOVA), Student *t*-test and correlation analysis were performed using Statistica 5 package.

## Results

*Genetic basis of Cd tolerance in* Arabidopsis halleri

Before analysing the backcross progeny, Cd tolerance was first investigated in *A. halleri, A. petraea* 1 and 2 and the F1 (Figure 1). The level of Cd tolerance of *A. halleri* was $>$ 150 $\mu$M Cd. The two genotypes of *A. petraea* had low level of Cd tolerance. Indeed, *A. petraea* 1 was non-tolerant at 14 $\mu$M $\pm$ 1 and *A. petraea* 2 was non-tolerant at 19 $\mu$M $\pm$ 2 ($t_{obs}$=$-$2.3; df=31; $p<0.05$). Because of the large difference between *A. halleri* and *A. petraea* 1, a wide segregation is expected in the backcross progeny. Interestingly, the level of tolerance of the F1 was significantly lower than that of *A. halleri* ($t_{obs}$=5.6; df=40; $p<0.001$).

We further conducted an experiment to measure Cd tolerance in the backcross progeny. Similar levels of tolerance were again obtained for *A. halleri*, *A. petraea* 1 and 2 and the F1 (data not shown). Several genotypes of the backcross progeny died off whereas others were unable to grow more than two or three clones. In addition, for some genotypes, Cd tolerance thresholds of their respective clones appeared to be

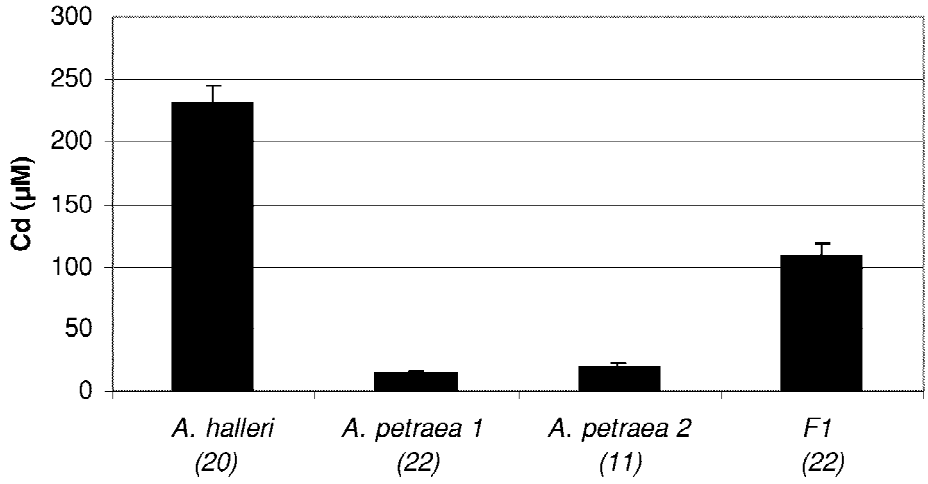


*Figure 1.* Cd tolerance thresholds (mean ± SE) of *A. halleri*, *A. petraea* 1, *A. petraea* 2 and the F1. Tolerance was assessed by exposing cuttings to increasing concentrations of Cd and determining the concentration at which no increase in fresh weight is observed. The number of clones tested is indicated below each plant (*n*).

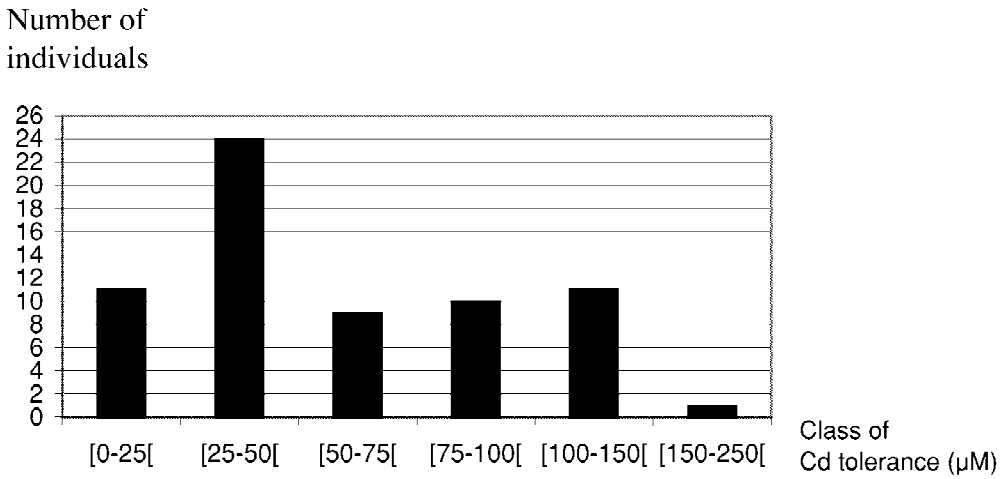


*Figure 2.* Distribution of Cd tolerance in the backcross progeny. Tolerance was assessed as in figure 1. The number of individuals in each class is indicated, each class representing an interval of Cd concentrations ($\mu$M).

different with more than one concentration. Results for these genotypes were discarded. Therefore, the Cd tolerance analysis was conducted on a total of 66 genotypes. As expected, the backcross progeny exhibited a large variation range in tolerance, including individuals with similar tolerance level as the parental species (Figure 2). Since *A. lyrata* ssp. *petraea* is not a tolerant plant, we conclude that the backcross progeny segregates into a 11:55 ratio of non-tolerant to tolerant. This ratio is consistent with both the 1:3 ratio and the 1:7 ratio expected for, respectively, a character controlled by two or three major genes with additive effect (1:3: $\chi^2$=2.4, ns and 1:7: $\chi^2$=1.05, ns, df=1). In contrast, the observed 11:55 ratio of non-tolerant to tolerant is statistically different from the 1:1 ratio expected for a character controlled by one major gene ($\chi^2$=29.2, $p<0.05$; df=1).

### *Cd accumulation*

After one week growth in 10 $\mu$M Cd, Cd concentration in shoot and root was determined for *A. halleri, A. petraea* 1 and 2 and the F1 (Figure 3). Cd concentrations were higher in roots compared to shoots in *A. halleri, A. petraea* 1 and 2 and the F1 plant.

*A. petraea* 1 and 2 did not differ in their Cd content in shoots ($t_{obs}$=0.7; df=4; $p>0.05$) and roots ($t_{obs}$=0.8;

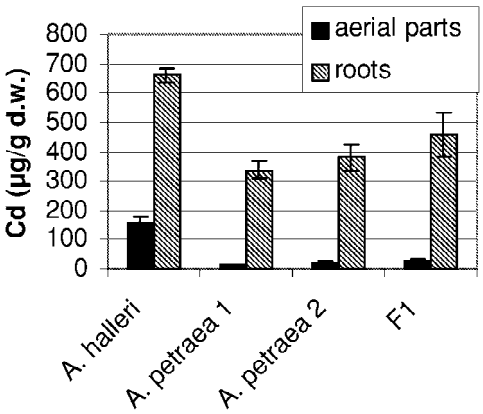


*Figure 3.* Cadmium concentration (mean ± SE) in aerial parts and roots of *A. halleri*, *A. petraea* 1, *A. petraea* 2 and the F1.

df=4; $p>0.05$). *A. halleri* had significantly higher Cd concentrations in shoots and roots compared to *A. petraea*. *A. halleri* and *A. petraea* differed significantly in their mean Cd concentration (shoot: *A. halleri*: 157 $\mu$g g$^{-1}$, *A. petraea*: 15 $\mu$g g$^{-1}$, $F_{2,6}$=32, $p<0.001$; root: *A. halleri*: 660 $\mu$g g$^{-1}$, *A. petraea*: 336 $\mu$g g$^{-1}$, $F_{2,6}$=27, $p<0.01$).

Average Cd concentration in shoot was significantly lower in the F1 compared to *A. halleri* ($t_{obs}=-5$; $p<0.01$; df=4). In contrast, no significant difference was found in average Cd concentration in shoot and root of the F1 compared to *A. petraea* 1 and 2 (one way ANOVA: shoot: $F_{2,6}$=1.9, $p>0.05$; root: $F_{2,6}$=1.4, $p>0.05$).

Extensive segregation was observed in the backcross progeny, including genotypes with Cd concentration similar to the one of *A. halleri*, as well as genotypes that accumulated very little Cd (Figure 4). In addition, there was no significant correlation between Cd concentration in shoot and root in the progeny ($r=-0.13$; $n$=20, $p>0.05$).

### *Relationship between Cd tolerance and Cd accumulation*

There was no significant difference between the mean Cd concentrations of the shoots of the plants with varying levels of Cd tolerance ($F_{3,23}$=1.8; $p>0.05$), indicating that the most tolerant plants were not accumulating more Cd than the less tolerant plants (Figure 5). Similar results were obtained for roots ($F_{3,15}$=0.2; $p>0.05$).

### *Correlation between Cd and Zn tolerance*

Plants from the backcross progeny were characterised for Zn tolerance and Cd tolerance (Figure 6). Interestingly, the result of the correlation analysis showed a positive and significant relationship between the two characters ($r$=0.55; $p<0.001$; $n$=66), indicating that the most tolerant plants for Cd are also the most tolerant plants for Zn. However, a large variation in Zn tolerance among the more Cd-tolerant progeny can be observed (Figure 6).

### *Correlation between Cd and Zn accumulation*

Zn was also measured in shoot and root of plants grown in the presence of Zn (1 $\mu$M) in the nutrient solution (Figure 7). In contrast to Cd, shoot:root ratio for Zn is $> 1$ in *A. halleri*, indicating that Zn is highly translocated from roots to shoots. The same pattern was obtained for the F1. In contrast, restricted translocation of Zn from roots to shoots was observed in *A. petraea*.

In the backcross progeny, a significant positive correlation was found between Zn and Cd shoot concentration ($r$=0.5; $n$=29; $p<0.01$; Figure 8). The correlation was not significant in roots ($r$=0.25; $n$=20; $p<0.05$).

## Discussion

### *Genetic basis of Cd tolerance in* Arabidopsis halleri

Cadmium tolerance was investigated both in the parents of the backcross and in the backcross progeny. The level of tolerance of the F1 was significantly lower than the one of *A. halleri*. This result will have to be verified on a larger sample of F1. At this stage, it indicates that Cd tolerance may be a partially dominant character.

The study of the backcross progeny suggests that Cd tolerance is a more complex character than Zn tolerance in *A. halleri* and might be governed by more than one single major gene. In a previous study, Macnair et al. (1999), working on F2 plants from a cross between *A. halleri* and *A. lyrata* ssp. *petraea*, have proposed that Zn tolerance is controlled by a single major gene. For other metals and species, genetic analysis has shown that tolerance is controlled by one or two major genes, with additional modifiers determining the level of tolerance (Schat et al., 1993; Smith and Macnair, 1998, van Hoof et al., 2001). In

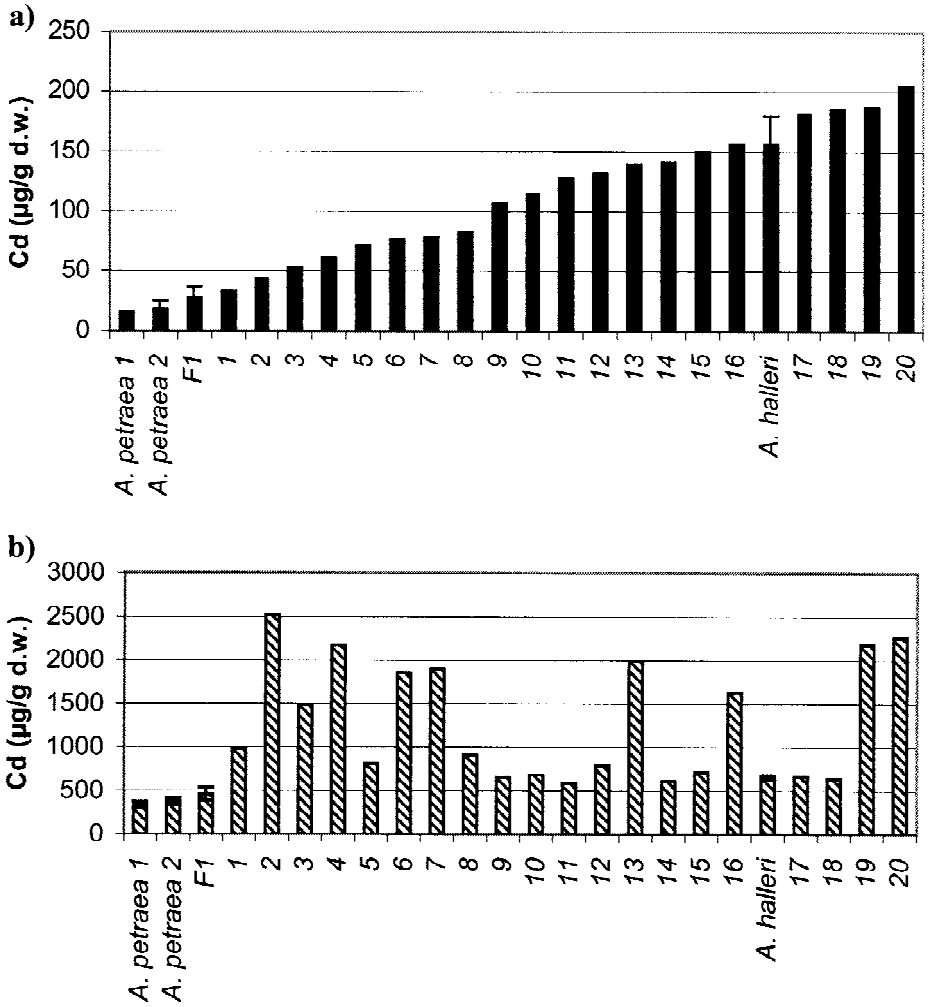


*Figure 4.* Cadmium concentration in shoots (a) and roots (b) of *A. halleri*, *A. petraea* 1, *A. petraea* 2, the F1 and the backcross progeny, after one week in 10 $\mu$M Cd. Means $\pm$ SE are indicated for *A. halleri*, *A. petraea* 1, *A. petraea* 2 and the F1. The backcross progeny is numbered from 1 to 20. In (a), genotypes are ranked by increasing Cd concentration in the shoot.

the case of Cd tolerance in *A. halleri*, a much more extensive breeding programme will be required to enable a reliable estimation of the number of genes involved.

*Cd accumulation: A complex character?*

The study of Cd accumulation in the backcross progeny was not finished when this work was presented at ICOBTE (6th, Guelph, Canada, August 2001). In addition, another experiment, conducted with 66 genotypes from the backcross progeny and nine cuttings per genotype, is in progress in order to increase and replicate the data sets.

In the backcross progeny, a significant positive correlation was found between Zn and Cd shoot concentration ($r$=0.5; $n$=29; $p<0.01$; Figure 8). The correlation was not significant in roots ($r$=0.25; $n$=20; $p>0.05$).

The Cd concentration of the F1 was significantly lower than the one of *A. halleri* and similar to the one of *A. petraea,* suggesting that Cd accumulation in *A. halleri* might be a recessive character in *A. halleri.* This should be verified on a larger sample of F1. However, this result suggests that Cd accumulation in *A. halleri* has a complex genetic basis.

In *A. halleri*, the Cd concentration is higher in roots than in shoots, whereas Zn is much higher in shoots than in roots. These results have also been obtained by Küpper et al. (2000). The same pattern was found in

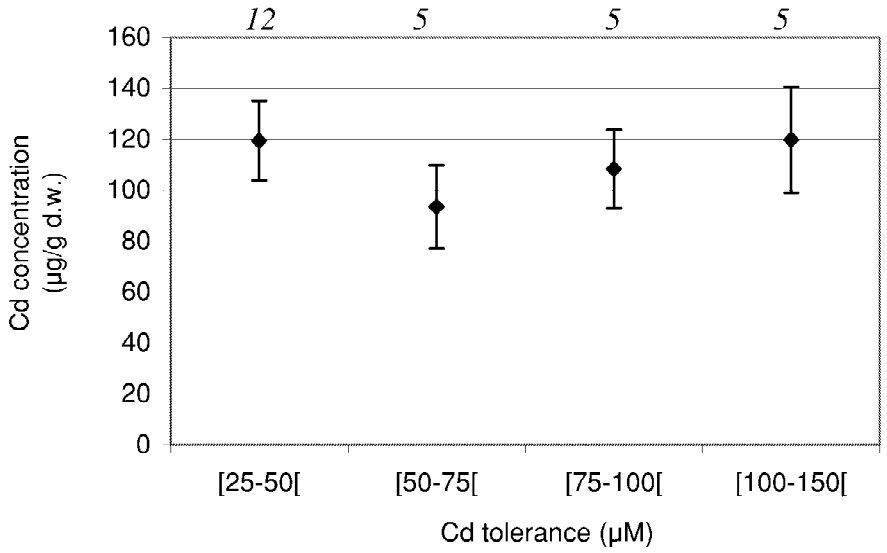


*Figure 5.* Cadmium concentrations (means ± SE) in the aerial parts of plants from the backcross progeny of varying Cd tolerance, measured as the concentration at which no increase in fresh weight is observed. Plants were analysed after one week in 10 $\mu$M Cd. Sample sizes are indicated above each bar.

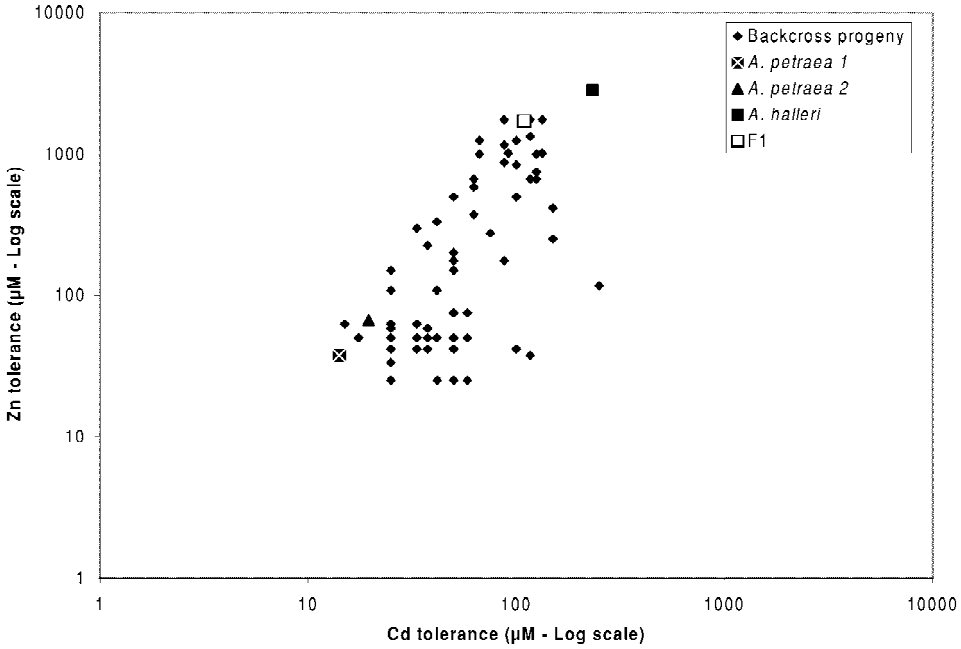


*Figure 6.* Relationship between Zn and Cd tolerance in the backcross progeny ($n$=66).

the Zn/Cd hyperaccumulator *T. caerulescens* (Lombi et al., 2000). This finding does not change the Cd hyperaccumulor status of *A. halleri* since Cd hyperaccumulators are defined as plants able to accumulate $> 0.01\%$ of dry weight of Cd in their aerial parts (Brooks, 1998). In our study, *A. halleri* was found to accumulate $>100$ $\mu$g g$^{-1}$ Cd in its shoots. Küpper et al. (2000) reported an accumulatation of up to 2700 mg Cd kg$^{-1}$ in shoots without phytotoxicity. Also, in its natural habitat, *A. halleri* can accumulate more than 100 mg kg$^{-1}$ Cd in shoots (Bert et al., 2002; Dahmani-Muller et al., 1999).

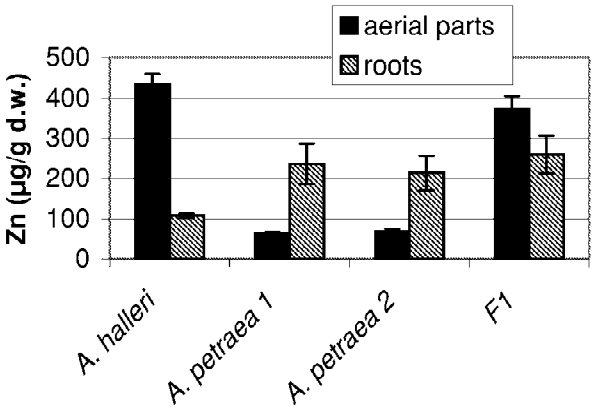


*Figure 7.* Zinc concentrations (means ± SE) in aerial parts and roots of *A. halleri*, *A. petraea* 1, *A. petraea* 2 and the F1. Zn was measured in plants that grew in the nutrient solution containing 10 $\mu$M Cd and 1 $\mu$M Zn.

### *Cd tolerance and Cd accumulation: Independent characters?*

The low sample size of the backcross progeny analysed in this study does not allow us to establish the precise relationship between Cd tolerance and Cd accumulation. Nevertheless, our results suggest independent segregation of these two characters. In *A. halleri,* Zn tolerance and Zn hyperaccumulation are also independent characters (Macnair et al., 1999). In *Thlaspi caerulescens*, non-metallicolous populations were significantly less tolerant and accumulated Zn to significantly higher concentration compared to metallicolous populations, evoking the independence of Zn tolerance and Zn hyperaccumation in this species (Assunção et al., 2001; Escarré et al., 2000; Meerts and Van Isacker, 1997). In contrast, for Cd, the best Cd hyperaccumulator populations were also the most tolerant populations (Escarré et al., 2000). Lombi et al. (2000), studying metallicolous populations of *T. caerulescens*, showed that Cd tolerance and Cd accumulation are combined. Thus, they proposed that Cd hypertolerance and Cd hyperaccumulation were somehow linked although conclusive genetic evidence is lacking in their article.

### *Cd and Zn tolerance are genetically related characters*

The significant co-segregation of Cd and Zn tolerance indicates that these characters are controlled by common major genes or by linked genes. However, the large variation observed in Zn tolerance among the more Cd tolerant plants suggests that nonpleiotropic genes or modifiers are likely to be involved too. In *Silene vulgaris*, Schat et al. (1996) and Schat and Vooijs (1997) have shown that Zn tolerance and Cd tolerance were under the control of different genes.

### *Cd and Zn accumulation*

As specified above, Cd and Zn accumulation experiments are being repeated with more progenies to increase the data sets. In this study, we have found that Cd and Zn were co-accumulated in aerial parts of the plants. This shows that Cd and Zn uptake are genetically correlated, suggesting that the metals are taken up (partly, at least) by the same transporter(s) or that their transporters, when different, are controlled by common regulators. Recently, several plant transporters have been identified that show affinity for both Zn and Cd. By complementation of a yeast Zn-transport-defective mutant with a *T. caerulescens* cDNA library, Lasat et al. (2000) cloned the *ZNT1* cDNA, which encodes a high affinity Zn transporter. However, ZNT1 can also mediate low affinity Cd transport (Lasat et al., 2000; Pence et al., 2000). Based on the study of two *T. caerulescens* ecotypes, Lombi et al. (2001) suggested that Cd may be transported in the low Cd accumulation ecotype via ZNT1 but, conversely, that Cd may be mediated in the high accumulation ecotype via a high affinity Cd transporter. Additional studies in yeast showed that IRT1, an iron transporter belonging to the ZIP family, has a broad substrate range and also transports $Zn^{2+}$ and possibly $Cd^{2+}$ (Clemens, 2001; Korshunova et al., 1999). Furthermore, AtNramp3, an *Arabidopsis* metal transporter involved in iron metal uptake, showed $Cd^{2+}$ transport activity (Thomine et al., 2000). In the CDF (cation diffusion facilitator) family, the *Arabidopsis* ZAT transporter involved in Zn sequestration, may be able to transport other metals including Cd (Williams et al., 2000).

In conclusion, our genetic analysis suggests that Cd accumulation and Cd tolerance are under independent genetic control in *A. halleri*. On the other hand, the tolerance to Cd and Zn, seem to be pleiotropic, at least to a certain degree. The analysis of the backcross progeny has allowed the selection of extreme genotypes, that is to say, the most and the less Cd tolerant plants and the most and the less Cd accumulating plants. The comparative study of these plants will facilitate the identification of genes involved in Cd tolerance or hyperaccumulation in *A. halleri*.

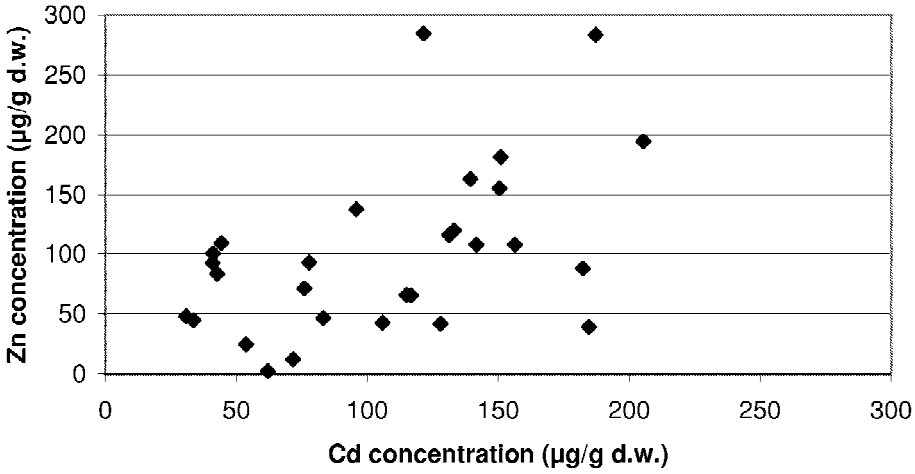


*Figure 8.* Relationship between Cd and Zn accumulation in the shoots of the backcross progeny ($n$=29).

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