Zinc tolerance and hyperaccumulation are genetically independent characters

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The hyperaccumulation of metals by a rare class of plants is a fascinating and little understood phenomenon. No genetic analysis has been possible since no intraspecific variation is known for this character. Here, we report on crosses between the zinc-hyperaccumulating and -tolerant species *Arabidopsis halleri* and the non-hyperaccumulating, non-tolerant species *Arabidopsis petraea*. The F_2 segregates for both characters and it appears that the two characters are genetically independent. The data for tolerance are consistent with a single major gene for this character (although the number of genes for hyperaccumulation cannot be determined), and is probably not very large.

Keywords: hyperaccumulation; phytoremediation; *Arabidopsis halleri*

1. INTRODUCTION

The vast majority of plants, when grown in a medium containing elevated concentrations of heavy metals, will take up large amounts of metal into their roots, but translocate very little of this to their shoots, so that the root concentration is much higher than that in the shoot (Baker 1981). A rare class of plants (hyperaccumulators) do translocate significant quantities, such that the root and shoot concentrations are equivalent or the shoot concentrations are higher than the root concentrations (Kohl *et al*. 1997). These plants can accumulate some metals to per cent quantities in their aerial parts (particularly zinc, nickel and manganese) (Brooks 1994). The phenomenon has attracted considerable interest recently because of the potential of using such plants to phytoremediate metal-contaminated soils (Brooks 1998; Salt *et al*. 1998). However, in general, hyperaccumulators are of low biomass and not suitable candidates for phytoremediation. If there are only a small number of genes involved in hyperaccumulation, it may be possible to transform a high biomass species with genes for this character genetically and produce a plant that would be more effective for this technology. All known hyperaccumulators have been found on metalliferous soils (e.g. serpentine or old mine spoils) and also have the character of metal tolerance (Brooks 1998). Tolerance is a much better understood phenomenon and has proved a model system in which to study the process of genetic adaptation to a known environmental variable (Macnair 1993, 1997). Tolerance to a range of metals has evolved in many species exposed to elevated soil metal concentrations. For a number of metals, including zinc, copper and arsenic, genetic analysis has shown that this characteristic 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 & Macnair 1998). The relationship between tolerance and hyperaccumulation is unclear. In a recent review, Chaney *et al*. (1997) asserted that hypertolerance is the key property which makes hyperaccumulation possible, while Krämer *et al*. (1997) suggested that nickel hyperaccumulation is simply a manifestation of nickel tolerance in *Thlaspi goesingense*. To understand the relationship fully, we need to know whether the two characters are essentially governed by the same genes (as implied by Krämer *et al*. (1997)) or are genetically independent.

No genetic analysis of hyperaccumulation has been possible hitherto, because no intraspecific variation has been found. All populations of the species studied hitherto have either been all hyperaccumulating or all non-hyperaccumulating, though variation in the exact pattern of accumulation has been found (e.g. Lloyd-Thomas 1995) and this variation can be heritable (Pollard & Baker 1996). *Arabidopsis halleri* (=*Cardaminopsis halleri*) is a hyperaccumulator of zinc which is found exclusively on metal-contaminated sites in western Europe, though in eastern Europe it is found on both contaminated and uncontaminated sites (Fabiszewski 1986; Bert *et al*. 2000). It is closely related to and interfertile with *Arabidopsis petraea* (=*Cardaminopsis petraea* = *Arabis petraea*) which is both non-tolerant to zinc and a non-accumulator. This paper reports an investigation into the tolerance and accumulation patterns of F_2 plants derived from a cross between these two species.

2. MATERIAL AND METHODS

(a) *Provenance of the plants*

Arabidopsis halleri was collected as seed from plants growing on the banks of the River Innerste, 2 km north of the town of

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Langelsheim, Harz Mountains, Germany. The site is highly contaminated with zinc, lead and cadmium. *A. halleri* plants collected in the field had very high levels of zinc in their leaves ($\bar{x} = 1.7\%$ dry weight, $n = 18$ and range $= 0.8$–3.0%). Approximately 30 plants raised from the Innerste population were planted out in the garden at Exeter University and allowed to set seed. Seedlings grown from this seed were used in the uptake experiments, while a plant grown from wild-collected seed was used in the crosses. *A. petraea* was collected as both plants and seed from Unhošť, in the valley of Loděnice in Central Bohemia. This site is an uncontaminated woodland. Field-collected plants were used in the crossing programme, while plants raised from seed were used in the uptake experiments.

(b) *Crossing programme*

One *A. halleri* plant (derived from wild seed) was crossed (as a male) with two separate *A. petraea* plants to produce the F_1. From each family, one F_1 plant was chosen and they were inter-crossed to give the F_2. The validity of the F_1s was checked by analysis of microsatellite markers (data not shown). All crosses were done by hand.

(c) *Plant cultivation*

The seeds were germinated on sand and transferred to a hydroponic culture in a constant environment chamber (10 h day, 25 °C in the day and 15 °C at night). Plants were grown in 12 l plastic trays with 35 plants per tray. All trays were continuously aerated. The nutrient solution was based on the one given in Chaney & Bell (1987) and consisted of 2 mM $MgSO_4$, 0.5 mM $Ca(NO_3)_2$, 0.5 mM KNO_3, 0.1 mM K_2HPO_4, 0.2 mM $CuSO_4$, 2 mM $MnCl_2$, 10 mM H_3BO_3, 0.1 mM MoO_3 and 10 mM FeEDDHA. Zinc was added (as zinc sulphate) at various concentrations between 10 and 1000 mM; 10 mM is a non-toxic concentration that permits all plants to grow healthily. The pH of all solutions was between 6.5 and 7.0.

(d) *Determination of the zinc content of plants*

The zinc content of leaves was determined using the colorimetric reagent zincon (Sigma Chemicals, Gillingham, UK) with the methods of Macnair & Smirnoff (1999). Small leaf samples (10–40 mg) were frozen in liquid nitrogen, extracted with 2% sulphosalicylic acid and the zinc concentration measured at 606 nm on a Shimadzu UV-2401PC spectrophotometer at pH 9.5. Each datum is the mean of two independent samples per plant. Zinc concentrations are expressed as micromoles per gram of fresh weight. The use of fresh weights leads to inaccuracy if plants differ substantially in their water status. However, all plants were grown under identical conditions in hydroponics and there was no evidence that the the zinc treatments caused systematic variation in plant water status, since all plants maintained full turgor throughout the experiment.

(e) *Experiment 1*

A sample of the F_2 ($n = 59$), together with samples of the parent species (*A. petraea*, $n = 11$ and *A. halleri*, $n = 14$), were grown for 26 days in a low concentration of zinc (10 μM) and the zinc concentration of the leaves determined. The plants were transferred to a toxic (to *A. petraea*) concentration of zinc (250 μM). Within four days, 19 of the F_2 plants were suffering severe toxicity symptoms (chlorosis of the base of the leaves) and were removed from the experiment and potted in normal soil. These plants were deemed non-tolerant. The other plants were able to survive for four weeks at this concentration without

showing toxicity symptoms and were classified as tolerant. The zinc concentration of the leaves of all tolerant plants was determined weekly. The rate of accumulation of zinc was defined as the regression of zinc concentration on time. After four weeks at 250 μM the plants were removed from the experiment and potted in normal soil.

(f) *Experiment 2*

Most of the F_2s from experiment 1 were grown for four months in soil and a sample ($n = 25$, including 18 tolerant and seven non-tolerant plants) cloned. The plants were chosen on the basis of biomass (sufficient sideshoots to clone from) and success in rooting. The plants chosen were representative of the tolerant and non-tolerant classes. The plants were cloned by removing sideshoots, dipping in rooting hormone (Seradix; May & Baker Ltd, Dagenham, UK) and placing in sand under a mist unit. Rooted cuttings were removed after three weeks, washed carefully to remove the sand and established in a hydroponic system as in experiment 1. Between two and six ramets of each plant were grown for ten days at 10 μM and the zinc concentration of the leaves determined. Two ramets of each of the 25 plants were then transferred to 250 μM zinc and grown for six weeks at this concentration. The zinc content of the leaves was measured weekly and the rate of accumulation calculated as the regression of zinc concentration on time.

(g) *Experiment 3*

A further sample of seed from the same F_2 family was sown and 114 seedlings were established in hydroponics (10 μM zinc) for four weeks. Their tolerance was determined by the methods of Schat & Ten Bookum (1992). This technique measures the tolerance of a plant by sequentially transferring plants into increasing concentrations of metals and determining the concentration at which no new root growth is produced (the EC_{100}). The technique is not directly comparable to single concentration tests of tolerance (such as that used in experiment 1) since there is the opportunity for plants to acclimate to the increasing concentrations of metal. However, if acclimation occurs, the effect is much smaller than genetic differences in innate tolerance and, using this technique, it has proved possible to study the genetics of tolerance in species which do not clone easily (Schat & Ten Bookum 1992). The roots of all F_2 plants were blackened with activated charcoal and rinsed under deionized water to remove the excess powder. The plants were returned to 10 mM zinc for a further week after which new root growth was visible beyond the charcoal-coated roots. The roots were then reblackened and the plants transferred in successive weeks to 25, 50, 75, 100, 150, 200, 250, 500 and 1000 μM zinc. The zinc concentration of the leaves of each plant was determined before each transfer as before except for the 500 and 1000 μM treatments. At each transfer, those plants which had reached their EC_{100} were removed from the experiment and deemed to be non-tolerant at this concentration. However, eight plants which reached their EC_{100} at 50 mM zinc were kept in the experiment for a further week (i.e. at 75 μM) with the 106 other plants so that the zinc content of all plants could be determined at this particular concentration, which is the maximum EC_{100} of *A. petraea*.

3. RESULTS

(a) *Experiments 1 and 2*

Using lack of chlorosis as a subjective measure of tolerance in experiment 1, the F_2 segregated into a 40:19 ratio

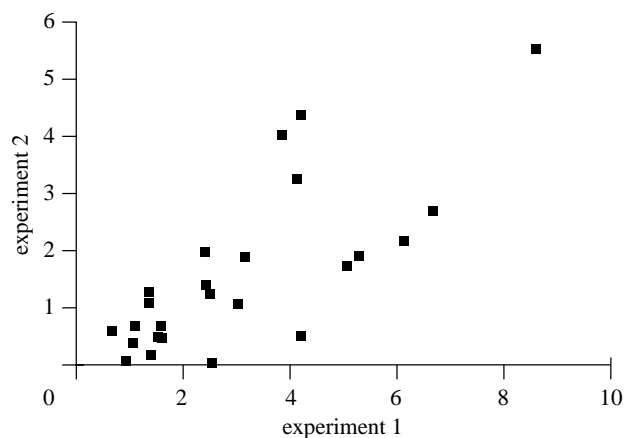Figure 1. The zinc content of 25 F_2 plants ($\mu mol\,g^{-1}$ fresh weight) when grown in $10\,\mu M$ zinc in two separate experiments. In experiment 1, plants were tested after 26 days' growth in hydroponics, while in experiment 2 they were tested after ten days.

of tolerant to non-tolerant. This ratio is consistent with the 3:1 ratio expected from a single major gene for this characteristic ($\chi^2 = 1.63$, n.s.) as has been found for other metals and species (Macnair 1993; Schat *et al.* 1996).

When tested after growth at $10\,\mu M$ zinc, the two parent species differed substantially in their mean zinc concentrations in the leaves (*A. petraea* $n = 11$, $\bar{x} = 0.88 \pm 0.04$ and range 0.6–$1.1\,\mu mol\,g^{-1}$ fresh weight and *A. halleri* $n = 14$, $\bar{x} = 3.81 \pm 0.52$ and range 1.9–$7.0\,\mu mol\,g^{-1}$ fresh weight) and the F_2 was highly variable (range 0.85–$9.24\,\mu mol\,g^{-1}$ fresh weight), though there was no difference between the zinc concentrations in the leaves of the F_2 plants which were subsequently determined to be tolerant or non-tolerant (tolerant $\bar{x} = 3.39 \pm 0.37\,\mu mol\,g^{-1}$ fresh weight and non-tolerant $\bar{x} = 2.85 \pm 0.32\,\mu mol\,g^{-1}$ fresh weight; one-way ANOVA $F_{1,57} = 0.92$, n.s.). After four weeks in $250\,\mu M$ zinc, the variation in the tolerant plants had increased (final leaf concentration range of 3.1–$46.9\,\mu mol\,g^{-1}$ fresh weight), with rates of accumulation in the range of 0.01–$11.6\,\mu mol\,g^{-1}$ fresh weight per week. Note that the final mean zinc concentration of the *A. halleri* plants was $46.5\,\mu mol\,g^{-1}$ fresh weight and their rate of accumulation was $14.9\,\mu mol\,g^{-1}$ fresh weight per week. Thus, the tolerant F_2 produced very wide segregation, including individuals that had similar phenotypes to the accumulating parent, as well as individuals which accumulated very little (note that it is not possible to establish the accumulation phenotype of *A. petraea* at $250\,\mu M$ since this concentration is toxic to this species).

Experiment 2 was performed to check the repeatability of the results in experiment 1. Figure 1 shows the relationship between the zinc concentrations of the leaves found at the permissive concentration in experiments 1 and 2. There was a correlation of 0.756 (d.f. $= 23$ and $p < 0.001$) between the concentrations found in experiments 1 and 2 (see figure 1), which indicates a high repeatability for this measure of accumulation. There was also a significant correlation between the final concentrations attained by the tolerant F_2s in the two experiments ($r = 0.47$ and $p < 0.05$). There was no difference in the rates of accumulation between tolerant and non-tolerant plants in experiment 2 (non-tolerant $x = 2.25 \pm 0.414\,\mu mol\,g^{-1}$ fresh weight per week
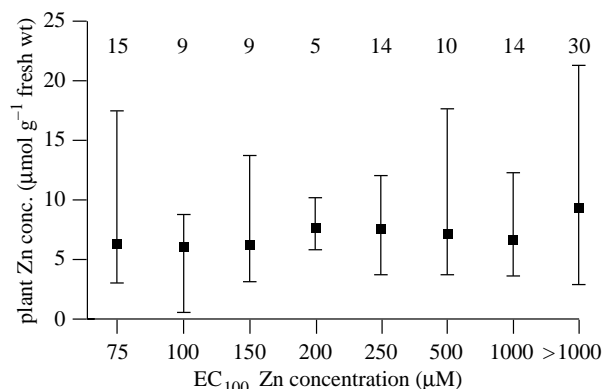

Figure 2. Means and ranges of the zinc concentrations in the leaves ($\mu mol\,g^{-1}$ fresh weight) of F_2 plants of varying zinc tolerance as shown by their EC_{100} values. Plants were tested when all were grown at $75\,\mu M$ zinc in the growth medium. The sample sizes are included above each sample.

and tolerant $x = 2.65 \pm 0.324\,mmol\,g^{-1}$ fresh weight per week; one-way ANOVA $F_{1,23} = 0.47$, n.s.). Finally, there was a significant correlation ($r = 0.55$, d.f. $= 23$ and $p < 0.01$) between the rate of accumulation (at $250\,\mu M$) of all clones calculated in experiment 2 and the zinc concentration of the leaves when grown at $10\,\mu M$ in experiment 1. This shows that the accumulation of plants in the permissive zinc concentration is a reflection of the ability of plants to accumulate zinc in more toxic conditions and that there does not appear to be a separate accumulation process that is only operative in a high zinc environment.

(b) *Experiment 3*

The preliminary experiments showed that the two parent species differed substantially in their tolerance as defined by their EC_{100}s. All *A. petraea* plants ($n = 19$) ceased rooting at 50 or $75\,\mu M$ while all *A. halleri* plants ($n = 19$) were able to grow roots in concentrations of greater than $1\,mM$. The F_2s had very variable EC_{100}s, 23 plants showing EC_{100}s of $75\,\mu M$ or less and 30 plants having EC_{100}s of greater than $1\,mM$ (figure 2). The zinc content of all plants was measured at $75\,\mu M$, the maximum EC_{100} value for *A. petraea*. Figure 2 shows the means and ranges of the zinc concentrations of plants as classified by their EC_{100}s. There was no significant difference between the mean zinc concentrations of the plants with EC_{100}s of $75\,\mu M$ or greater ($F_{7,98} = 1.93$ and $p > 0.05$), indicating that the most tolerant plants were not accumulating more zinc at this concentration. However, when the eight plants with EC_{100}s of $50\,mM$ were included, there was a difference between the samples ($F_{8,105} = 3.46$ and $p < 0.001$), suggesting that plants whose roots had stopped growing accumulated less zinc than those whose roots were still growing (and, thus, presumably functioning more normally).

This effect is explored more fully in figure 3. As the plants were placed in a higher zinc concentration each week to determine their EC_{100}s, the zinc content of their leaves was determined. In all cases, the plants which had reached their EC_{100} and whose roots had therefore stopped growing had a lower zinc concentration than the plants whose roots were still functioning. However, at each concentration, there was no difference between the
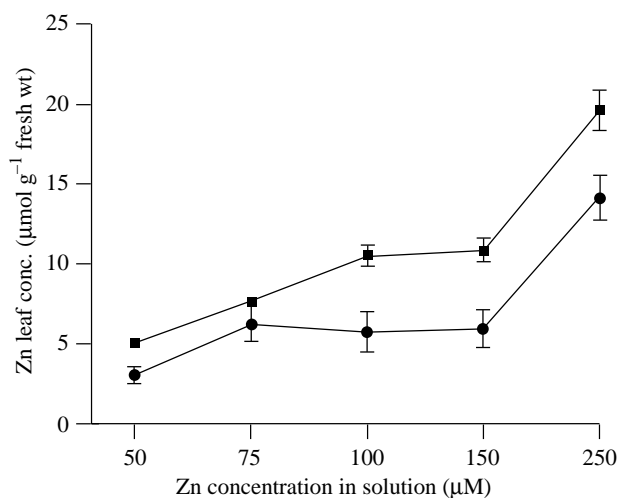
Figure 3. The mean zinc concentrations in the leaves ($\mu mol\,g^{-1}$ fresh weight) ($\pm$ s.e.) of the F_2 plants grown in experiment 3 at the different concentrations used for determining the EC_{100} values of the plants. At each external concentration, the mean zinc content of plants that have not reached their EC_{100} (squares) is compared with the content of plants that have stopped rooting at this concentration (circles). These latter plants were then removed from the experiment and are not included in the data for higher concentrations.

zinc contents of the plants belonging to the different classes which had EC_{100}s higher than the current test concentration (note that these statistical tests are not formally independent of the test performed in figure 2, since the same plants are involved and so are not presented here).

4. DISCUSSION

These results show that zinc tolerance and hyperaccumulation are genetically independent characters. Tolerance is a phenomenon involving the response of plants to external zinc concentrations and does not appear to be influenced by the internal zinc concentrations of aerial parts. Toxicity symptoms occurred very swiftly after the plants were exposed to $250\,\mu M$ zinc and were unrelated to the zinc concentration of the leaves. Hyperaccumulation involves the translocation of zinc from roots to shoots and there is no association between the tolerance of a plant and its ability to translocate this metal. However, plants whose roots are not functioning properly appear to translocate less zinc than those which are still growing, but this only happens after their roots are affected. There is no evidence that separate hyperaccumulation genes to those causing elevated concentrations in aerial parts at low external concentrations are operative at high external levels of zinc.

The data are consistent with there being a single major gene for zinc tolerance in this species, as has been found in *Silene vulgaris* (Schat *et al.* 1996) and for other metals (Macnair 1993). However, only a much more extensive breeding programme will enable this hypothesis to be confirmed. The number of genes for hyperaccumulation cannot be determined from these data, but the wide segregation in the F_2 might suggest that there are not very many.

These results have consequences for consideration of the mechanisms, applications and evolution of hyperaccumulation. If the characteristics are genetically independent, then it follows that different genes and mechanisms are responsible for the two characteristics. This would cast doubt on the findings of Krämer *et al.* (1997) who suggested that nickel hyperaccumulation is simply a manifestation of nickel tolerance. However, it remains possible that nickel hyperaccumulation is controlled differently to zinc hyperaccumulation. Van der Zaal *et al.* (1999) recently isolated a putative zinc transporter (ZAT1) from *Arabidopsis thaliana*, which is similar in sequence to animal zinc transporters. They found that, when this gene was overexpressed in *A. thaliana*, it resulted in increased tolerance and accumulation in the roots. Our results suggest that it is unlikely that a single gene such as ZAT1 is responsible for both the tolerance and hyperaccumulation patterns in natural hyperaccumulators.

Since there are two separate characteristics involved in hyperaccumulation, accumulation and tolerance, it is unwise to discuss hyperaccumulation as a single characteristic. It is known that species and populations occur which are tolerant only; it may also be possible to find species or populations that accumulate only but, since they do not occur on highly polluted soils, they do not reach the very high concentrations which have been arbitrarily chosen to define this phenomenon. Thus, for the purposes of phytoremediation, many workers (e.g. Chaney *et al.* 1997; Salt *et al.* 1998) have discussed the merits of hyperaccumulators in extracting metals from soils. However, it may not be necessary to possess both characteristics, except on very highly polluted soils which cannot be phytoremediated anyway: accumulation only is the key to extracting metals from soils. Likewise, when discussing the evolution of hyperaccumulation, most reviews (e.g. Boyd & Martens 1992; Martens & Boyd 1994) have reified the phenomenon and sought to find an adaptive explanation for it. However, if accumulation evolved first and tolerance evolved later, then hyperaccumulation may in fact be an epiphenomenon which does not have an adaptive explanation as such. Only a detailed phylogenetic analysis of the evolution of both characteristics will enable this hypothesis to be tested.

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