Intraspecific Variation in Metal Tolerance of Plants

David W. M. Leung

Abstract Increased heavy metal pollution of soil and water threatens agricultural plant productivity and food safety as heavy metals could enter the food chain including the edible parts (the leaves or seeds, for example) of crop plants. Classical plant breeding is based on genetic variability in many traits, including disease resistance and yield. Few studies have focused on natural variation in metal tolerance of crop and non-food plants although knowledge from such studies could lead to identification of starter germplasm for plant breeding toward development of cultivars suitable for different practical applications. These include new non-food plant varieties with enhanced metal tolerance and increased heavy metal accumulating capacity for phytoremediation or those new food crops with reduced heavy metal bioaccumulation potential to limit the threat of food safety from heavy metal contamination of food crops. In addition, studies on natural variation in tolerance to selenium (Se, an essential trace metal for the benefit of human health) among lettuce and broccoli varieties showed that Se biofortification is a promising approach. Moreover, contrasting phenotypes identified in metal toxicity screening of different varieties of a plant could be applied to aid improved understanding of metal tolerance and accumulation in plants.

Keywords Biofortification · Genetic variability · Hyperaccumulating plants · Phytoremediation

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1 Introduction

Knowledge of genetic variability of plants in tolerance to and accumulation of heavy metals is important for studies to obtain a better understanding of the mechanisms in plant–metal interactions. About 400 metal hyperaccumulator plants are known (Rascio and Navari-Izzo 2011). Studies of the phytoremediation potential among the different natural populations of metal hyperaccumulating species such as *Thlaspi caerulescens* and Indian mustard (*Brassica junea*) have revealed that this is a variable character. For example, 15-day-old seedlings of 10 genotypes of Indian mustard were screened for variation in chromium (Cr) content following exposure to $K_2Cr_2O_7$ in a hydroponic culture system for 7 days (Diwan et al. 2008). Among the 10 genotypes tested, one accession stood out as the best accumulator which accumulated 1.68 mg Cr g^{-1} DW which was 15-fold higher than that in the lowest Cr accumulator genotype when grown in the presence of 100 μM Cr. This has implications in selecting suitable accessions for phytoremediation studies (Roosens et al. 2003; Assuncao et al. 2008; Diwan et al. 2008; Richau and Schat 2009). The metal hyperaccumulator plants generally producing little biomass are unlikely to be useful for direct application for phytoremediation of metal contaminants in soils. Instead, studying them would result in genes of interest that could be used in genetic engineering to enhance the potential of plant germplasm for soil phytoremediation (Seth 2012). The practical deployment of new transgenic plant varieties with enhanced phytoremediation potential could, however, be fought with issues and public concerns about growing transgenic plants in some countries, for example, in New Zealand.

Classical plant breeding of agricultural crops has a proven and long-running track record for generations of different types of agriculturally important plants including food crops and trees for cultivation. Intraspecific variation in many morphological characters and traits related to yield is known to occur. Diversity of plant germplasm in many traits including disease or pest resistance has been frequently studied. The ability of different genotypes of a crop plant to uptake, accumulate, and tolerate heavy metals is, however, in need of investigations

Table 1 Studies with focus on variation in metal toxicity of crop plants and model experimental plants that are not metal hyperaccumulators

Plant	Number of genotypes/accessions screened	Metal(s) studied	Reference
Pisum sativum L. (pea)	99	Cd	Belimov et al. 2003
Sorghum bicolor L. (sorghum)	10	Industrial sewage sludge (As, Cd, Pb, Zn)	Jamali et al. 2008
Populus clones and *Salix* clones (Poplar [P] and willow [S])	10P and 6S	Cd	Zacchini et al. 2009
Linum usitatissimum Flax/linseed	25	Cd and Zn	Smykalova et al. 2010
Lactuca sativa L. (lettuce)	30	Se	Ramos et al. 2011 a
Brassica oleracea var. *italic* (Broccoli)	38	Se	Ramos et al. 2011b
Arabidopsis thaliana L.	21	Ni	Agrawal et al. 2012
Arabidopsis thaliana L.	349	Cd	Chao et al. 2012

in-depth. Exploiting the diversity of the genotypes in metal tolerance is likely to be an effective method for metal biofortification or development of crop plant cultivars with reduced heavy metal bioaccumulation potential, thus addressing food safety concerns.

The focus of this chapter is restricted to the genotypic variation in crop plants, perennial trees and the model experimental plant, *Arabidopsis thaliana* (Table 1). The implications of these studies for plant breeding and basic investigations into mechanisms of metal–plant interactions underpinning metal tolerance in crop plants will be discussed. The genetic basis of variation in metal tolerance has been discussed elsewhere (Grativol et al. 2012). Here, the focus is on studies that have already shown the potential of exploiting the natural variation in metal tolerance of plants. In particular, these studies provided: (1) an evaluation of the genetic variability of agricultural crops in tolerance to heavy metals; (2) an analysis of the relationships between genetic variability in tolerance to heavy metals and in heavy metal accumulation in agricultural crops and non-food plants including the model experimental plant, *Arabidopsis thaliana*; and (3) an analysis aiming to identify the genotypes with contrasting phenotypes which can be used to delineate biochemical and molecular basis of heavy metal tolerance and accumulation. Together, these studies will help the identification of starter material for breeding aiming to improve metal tolerance of heavy metals in agricultural crops, or for trace metal biofortification or with low heavy metal bioaccumulation potential. This also has implications for selecting the genotypes of non-food crops intended for practical phytoremediation.

2 Assessment of Variation in Metal Tolerance and Accumulation

Heavy metal toxicity or tolerance assays were conducted with seedlings raised from seeds and grown in a soil-less nutrient solution (in a hydroponic system) or in sand supplied with a nutrient solution with or without added heavy metal ions. In one approach, variation in the time, if any, taken for the particular genotypes/ varieties or accessions to die in a particular toxic concentration of exogenous heavy metal was noted (Belimov et al. 2003). The distribution in the mortality time of the different varieties was analyzed statistically to uncover if it varied from the expected normal distribution. Another more common approach was to investigate the relation between the exposure concentration of a heavy metal and the growth inhibition response it produced. Since seedlings were the favorite experimental materials, variation in the response to a concentration of an exogenously supplied heavy metal on postgerminative root growth, particularly root length, was determined. Then, tolerance index (TI), for example, in root length [= (root length in control—root length in metal-treatment)/root length in control] of different varieties against heavy metal toxicity could be calculated and compared.

2.1 In Vitro Screening Approach

Large-scale screening of the cultivars of a plant to uncover useful variation for metal phytoremediation purposes, or for reduced heavy metal bioaccumulation or for metal biofortification carried out in the hydroponic, sand or soil cultures requires a lot of growth room and glasshouse spaces and the associated expenses. Moreover, there are the potential complications from microbial communities in the growth medium on pH and utilization of nutrients and/or uptake of the added heavy metals. These microbial processes might directly or indirectly influence availability of heavy metals to plants in a hydroponic or soil culture. In vitro culture may be a cheaper alternative and more practical in regard to the requirement for growth spaces and could minimize the complications from microbial influences. For example, in vitro plant regeneration from hypocotyl explants from 25 flax and linseed varieties available in the Czech Republic was found to be useful for studying differences in Cd and Zn tolerance and accumulation among the different varieties investigated (Smykalova et al. Smykalova I, Vrbova M, Tejklova E, Vetrovcova M, Griga M 2010).

3 Evaluation of Variability of Different Genotypes in Response to Exogenous Application of Heavy Metals

Natural variation in pea varieties of different geographical origin in response to cadmium toxicity was investigated. Ninety-nine wild-growing or primitive pea varieties from many different areas around the world were included in a study to reveal natural variation of pea germplasm in cadmium tolerance (Belimov et al. 2003). Seedlings of an unspecified age were grown in pots of quartz sand and supplied with a nutrient solution with or without addition of cadmium in the form of $CdCl_2$. Fifteen varieties (15 %) were very sensitive to 13 mg Cd kg^{-1} of sand and died within 10 days of treatment while 10 varieties (10 %) were found to be most tolerant to this lethal concentration of Cd and died after 25–30 days. Statistical analysis showed that the observed distribution of pea varieties in relation to the time of mortality differed significantly from the expected normal distribution. In response to a lower concentration of Cd (7 mg kg^{-1} of sand) a few varieties exhibited a tolerance index of 30 % while some exhibited a tolerance index of 90 %.

A study of a smaller sample size comprising 10 different sorghum varieties (*Sorghum bicolor* L.) grown in the same experimental plot supplied with untreated industrial sewage sludge which was effectively a form of heavy metal-contaminated fertilizer containing both essential trace elements (Ca, Fe, K, Mg, etc.) and toxic heavy metals (As, Cd, Pb, Zn, etc.). The contents of trace elements and toxic heavy metals in matured grains produced by the plants of these different sorghum varieties were determined. The data obtained were subjected to basic statistical correlation analysis, and other multivariate analysis including pattern recognition statistical tools such as principal component and cluster analysis (Jamali et al. 2008). Significant variability among the 10 sorghum varieties in accumulation of the different trace elements and heavy metals was evident. One variety appeared to be the most promising as it accumulated the least levels of toxic heavy metals. However, there was no accompanying data about the effects of the contaminants on plant growth and grain yield. The sorghum variety identified in this small-scale study might not be the best genotype to start plant breeding or genetic engineering to develop sorghum varieties with reduced concentrations of toxic heavy metals without affecting the uptake of other essential trace elements. Nevertheless, based on this finding a larger study beyond this type of "proof-of-concept" study seems justified.

Perennial tree species are more favorable candidates for phytoremediation than the known herbaceous metal hyperaccumulating plants because these trees including poplars can produce a large biomass, deep root systems, and have a great adaptability to grow in marginal soils. In a comparative study of 10 different poplar clones and 6 willow clones, variation in response to growing in a hydroponic solution with 50 μM cadmium sulfate for 3 weeks was noted. Four clones of poplars were particularly sensitive with more drastic reduction in leaf area than others, while two poplar clones showed less inhibition than others (Zachinni et al. 2009). Other root growth measurements were also determined. Unfortunately, the sample size in this study was relatively small and no correlation analysis between response of each growth measurement and the Cd concentration in plant tissues was made. Therefore, it was not possible to identify promising starter clones for breeding or genetic engineering. It was also not possible to make recommendations for phytoremediation trials or design more relevant basic studies to gain a better understanding of the mechanism of Cd tolerance, accumulation, and translocation in poplars and willows.

3.1 Toward Selenium (Se) Biofortification

For the first time, 30 diverse lettuce cultivars (*Lactuca sativa* L.) from the lettuce database maintained by the United States Department of Agriculture (USDA) were used in a study to investigate the diversity of growth responses to a nutrient solution with, or without selenium in the form of 15 µM Na_2SeO_4 (selenate) or Na_2SeO_3 (selenite) under hydroponic conditions (Ramos et al. 2011a). Seven cultivars were not affected by either form of selenium. In another group comprising 18 cultivars, their shoot fresh weight was only inhibited by selenite but not by selenate when compared to the untreated control. In the third group of five cultivars, an average 13 % increase in their shoot fresh weight was found following the selenate treatment. In the plants grown in the nutrient solution without either form of the selenium (the untreated control), Se was not detectable. At least over a 2-fold difference in Se contents between high and low Se accumulating lettuce cultivars in response to exogenous Se application were found showing that Se biofortification efficacy could be related to cultivar differences.

Among the lettuce cultivars that showed no growth inhibition in response to exogenous selenate application, some also accumulated relatively high levels of Se. Thus, it is possible to select and develop better lettuce cultivars with relatively high capacity for Se accumulation in the edible parts (leafy shoots) without negative effects on plant growth. There was no negative correlation between relatively high Se accumulation and amino acid contents, thus suggesting that it could be possible to select lettuce cultivars responsive to Se biofortification without adverse effects on other desirable nutritional attributes. Following identification of relatively high or low Se accumulating lettuce cultivars, these would be useful for studies to gain a better understanding of Se metabolism.

Broccoli is considered to be an Se-accumulating crop as it accumulates many times more Se than the non-accumulating crops (Ramos et al. 2011b). Genotypic variation in different broccoli accessions that affect exogenous selenate treatment on Se accumulation, plant growth, and the levels of other beneficial nutrients was first studied by Ramos et al. (2011b). Thirty eight broccoli accessions from the Plant Genetic Resources Unit at Geneva, NY were grown in a hydroponic solution with 20 µM sodium selenate for 2 weeks. At this dosage of selenate treatment, no negative effect on plant growth was observed. However, differences in Se contents ranging from 801 $\mu g\ g^{-1}$ to 1798 $\mu g\ g^{-1}$ dry weight among the broccoli accessions were found. This was expected to have a significant dietary Se impact.

Further to this sort of genotypic variation study, it seems possible to breed a cultivar simultaneously with enhanced Se accumulation (without simultaneously harming plant health) and without a negative effect on other beneficial nutrients such as sulfur-containing glucosinolates (GLS) and essential micronutrients such as Fe, Cu, Zn, and Mn by taking advantage of Se fertilizer application (the biofortification approach).

4 Physiological, Biochemical, and Molecular Differences Related to Metal Tolerance

There is no naturally occurring metal hyperaccumulating plant with genetic background as well characterized as that of *A. thaliana*. The utility of *A. thaliana* as a model experimental plant has contributed to the rapid development of and contributions to a better understanding of many areas of plant biology. *Arabidopsis* "ecotypes" or "accessions" are basically selfing and therefore a collection of these represent lines of separate genotypes. This collection is a significant genetic resource for investigations of complex genetic interactions in most areas of plant biology (Chao et al. 2012). *A. thaliana* is not a metal hyperaccumulating plant but unlike the well-known metal hyperaccumulating plants it is a plant with its genome completely sequenced. Relevant experiments using its accessions as a genetic resource can be of value in understanding metal–plant interactions. For example, the effects of excess exogenous nickel (Ni) concentrations on *Arabidopsis* accessions were used for the first time to identify Arabidopsis accessions of contrasting abilities regarding Ni tolerance and accumulation (Agrawal et al. 2012). Seedlings of 21 different accessions were grown aseptically in a medium containing 75 μM $Ni(NO_3)_2$ for 7 to 10 days. Like some of the agricultural crop plants, Ni tolerance index based on root length inhibition was also found to be variable ranging from 0.23 to 0.62.

From the screening of 21 accessions, four *Arabidopsis* ecotypes which fell into two groups of plants displaying contrasting phenotypes when exposed to 75 μM $Ni(NO_3)_2$ were selected for basic investigations. The first group comprised two ecotypes considered to be relatively resistant to this concentration of Ni were Ler-0 and S.C., which had similar TI values for Ni (0.62 and 0.48 respectively). Another group comprised WT Col-0 and Di-0/G which were considered to be the most Ni sensitive ecotypes with much lower TI values (0.35 and 0.23 respectively). In terms of Ni contents, there was no significant difference in the roots among these four ecotypes, suggesting that the tolerant ecotypes have mechanisms to tolerate Ni toxicity. In the tolerant ecotype S.C., the Ni content in the leaves was higher than the Ni levels in the sensitive ecotypes. This lends support to the suggestion that this tolerant ecotype has a different mechanism to handle higher levels of Ni in the leaves to reduce the severity of Ni toxicity as evident in the more sensitive ecotypes.

The two resistant *Arabidopsis* ecotypes had different Ni contents in their leaves, suggesting that Ler-0 with a lower level of Ni than S.C. exhibited an Ni excluder-type mechanism, whereas S.C. had activated an Ni tolerance mechanism. These findings have implications for selecting materials for designing relevant experiments for phytoremediation (aiming for higher metal accumulation and therefore higher tolerance capability as well). There are also implications for designing experiments in relation to food safety perspective (aiming for reduced heavy metal bioaccumulation and therefore the excluder-type mechanism).

Identification of ecotypes with contrasting tolerance phenotypes is also useful to test specific hypothesis or previously established correlations (between two parameters) related to the possible mechanism involved in metal tolerance and accumulation. For example, the four *Arabidopsis* ecotypes were used to test the previously held notion that malic acid was important for Ni hyperaccumulation in *Alyssum murale* (McNear et al. 2010). Whether the four *Arabidopsis* ecotypes were susceptible or resistant to 75 μM $Ni(NO_3)_2$ or not, they accumulated similar levels of Ni in the roots. A possible correlation of this with the amounts of malic acid in the root exudates of the four ecotypes was investigated. No significant difference in the malic acid in the root exudates among the two contrasting groups of Ni resistant ecotypes was found, suggesting that malic acid secretion to the root zone played an equal role in facilitating Ni uptake by all four ecotypes. However, only the more tolerant ecotypes were found to have significantly more Ni in the roots, presumably by having more malic acid to bind with Ni and thereby minimizing Ni toxicity in the root cells of the tolerant ecotypes. Therefore, the availability of these ecotypes with contrasting phenotypes is useful in showing that malic acid is more important in differential Ni tolerance than uptake at least in Arabidopsis and possibly in other plants as well. Testing this in other species would therefore probably benefit from screening genotypic variation and use of the identified genotypes in similar ways as shown in the *Arabidopsis* study.

In a more comprehensive study on 349 *Arabidopsis* accessions collected from the wild around the world, a 4-fold variation in leaf Cd content among the accessions when grown under the same experimental conditions was uncovered (Chao et al. 2012). This was correlated with the natural variation in the DNA sequences at the heavy metal ATPase3 (*HMA3*) locus which codes for the HMA3 protein, a transporter involved in sequestration of heavy metals such as Cd into vacuoles (Park et al. 2012). The DNA changes of this gene (leading to changes in HMA3 protein variants) in some accessions was found to be causally related to the natural variation in leaf Cd accumulation (Chao et al. 2012). The broad implication of this finding is that there might be a similar primary genetic basis for natural variation in Cd accumulation in other plants.

The expression of many genes has been linked to metal uptake, translocation, accumulation, and resistance and detoxification mainly from studies on *A. thaliana* (Seth 2012). Arabidopsis mutants in relation to metal tolerance have been used to gain a better understanding of the relationship between expression patterns of many metal-resistance genes and response to metal stress (Wang et al. 2011; Lv et al. 2012). Besides, studies beyond this model plant system, particularly those on the variability in response to metal stress among cultivars of a plant species have also provided relevant materials with contrasting phenotypes in response to metal stress for further studies. For example, poplars (*Populus nigra* L.) has already been shown to have a promising potential for use in phytoremediation. Two genotypes identified from a study on natural variation of poplar germplasm with differing response to Cd treatment were used to investigate the relationship in the expression of some candidate genes and their differing response to Cd stress (Gaudet et al. 2011).

Rooted stem cuttings of two *P. nigra* genotypes (Poli and 58–861 with contrasting TI values, 0.91 and 0.42, respectively) from two contrasting natural growth environments in Italy were grown in a controlled climate chamber for 3 weeks with or without 50 μM $CdSO_4$ added to a hydroponic nutrient solution. Poli was more tolerant than 58–861 to the Cd treatment (Gaudet et al. 2011). There was no significant difference in the Cd levels in the leaves of both genotypes. Cd treatment did not affect the leaf biomass of Poli but reduced more than half the leaf biomass of 58–861. Net leaf photosynthesis was reduced by 30 % in Poli but by 70 % in 58–861 under Cd stress. These growth and photosynthesis effects under Cd stress were correlated with a significantly elevated level of the glutathione S-transferase gene transcript in Poli but not in 58–861 in response to Cd treatment. While the transcript levels of other candidate genes involved in the ascorbate–glutathione cycle (ascorbate peroxidase and glutathione reductase) did not differ between the two genotypes. Thus the study of genotypes with contrasting phenotypes under Cd stress is an effective way to help understand the relative contributions of key metal-resistance genes or alleles.

5 Conclusion

There have been relatively few studies investigating intraspecific variation in metal tolerance of crop plants. The crop plant studies discussed in this review often screened far too few genotypes but already genotypes differing in metal tolerance and accumulation were identified. These could be directly applied to start plant breeding toward cultivar development for metal biofortification or for reduction in heavy metal bioaccumulation. Also, they could be used to improve our understanding of metal–crop plant interactions and not purely based on studies on unrelated model experimental plants or other metal hyperaccumulating plants.

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