Chapter 12
Cadmium-Accumulating Plants

Hendrik Küpper and Barbara Leitenmaier

Contents

Abstract Plants are categorized in three groups concerning their uptake of heavy metals: indicator, excluder, and hyperaccumulator plants, which we explain in this chapter, the former two groups briefly and the hyperaccumulators in detail. The ecological role of hyperaccumulation, for example, the prevention of herbivore attacks and a possible substitution of Zn by Cd in an essential enzyme, is discussed. As the mechanisms of cadmium hyperaccumulation are a very interesting and challenging topic and many aspects are studied worldwide, we provide a broad overview over compartmentation strategies, expression and function of metal

H. Küpper (✉)
Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany
e-mail: hendrik.kuepper@uni-konstanz.de

B. Leitenmaier
Institut für Anorganische Chemie, Universität Zürich, Winterthurerstraße 190, CH-8057 Zürich, Switzerland

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transporting proteins and the role of ligands for uptake, transport, and storage of cadmium. Hyperaccumulators are not without reason a topic of great interest, they can be used biotechnologically for two main purposes which we discuss here for Cd: phytoremediation, dealing with the cleaning of anthropogenically contaminated soils as well as phytomining, i.e., the use of plants for commercial metal extraction. Finally, the outlook deals with topics for future research in the fields of biochemistry/biophysics, molecular biology, and biotechnology. We discuss which knowledge is still missing to fully understand Cd hyperaccumulation by plants and to use that phenomenon even more successfully for both environmental and economical purposes.

Keywords excluder plants • hyperaccumulator plants • indicator plants • natural overexpression of transport proteins • phytoremediation • phytomining • vacuolar metal sequestration

1 Introduction: Importance of Cadmium Accumulation in Plants

Certain heavy metals are well known to be essential microelements needed for plants to grow and complete their life cycle. Elevated concentrations of these metals, however, can be toxic and induce inhibition of various plant metabolic processes (reviewed, e.g., in [1–3]).

Cadmium can occur in very high concentrations in the soil that are detrimental or even lethal to most plants, as described in more detail in Chapters 2 and 13 of this book. Plants have developed a number of strategies to resist the toxicity of heavy metals (see [2–4] for recent reviews). Such strategies include the functioning of metal efflux pumps, sequestration in cells and intracellular compartments where metals can do the least harm, and binding of heavy metals inside the cells by strong ligands. According to the relation between the metal content in the soil or nutrient solution compared to the metal accumulated inside the plant, called "bioaccumulation coefficient" or "bioconcentration factor", plants are divided into three groups: excluder, indicator and hyperaccumulator plants (Figure 1).

1.1 Cadmium Accumulation in Indicator and Excluder Plants

Most heavy metal resistant plants belong to the so-called "excluders", they prevent the accumulation of heavy metals inside their tissues [5]. Metal exclusion can function in several different ways. The probably most simple way is a reduction of the unselective permeability of cells. This is typically reached by lignification of plant cell walls, and the enhancement of lignifying enzymes is a well-known response to cadmium toxicity [6]. In addition, exclusion can occur by precipitation

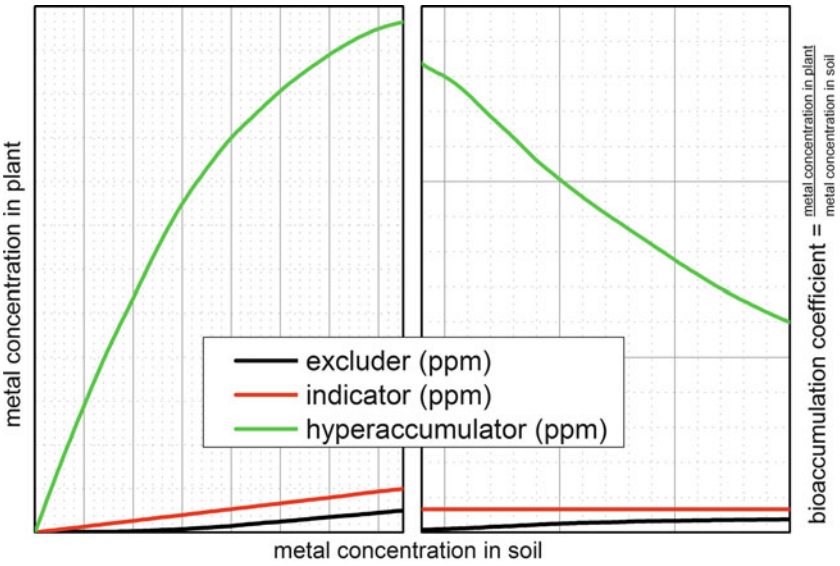

Figure 1 Three main types of heavy metal accumulation in plants: schematic correlations between metal in soil (or nutrient solution) and metal in the plants.

or binding of metals in the apoplast (cell walls) before they pass the plasma membrane. Third, plants can actively reduce concentrations of unwanted metals in their cells by pumping them out. Such ATP-dependent efflux pumps were first found in roots of *Silene vulgaris* [7], but later also in roots of other plants (e.g., [8]). Another efflux carrier is the Cd^{2+}-detoxifying *A. thaliana* Detoxification1 (AtDTX1 [9]).

In addition to efflux pumps in roots, it has also been suggested that active efflux can be achieved by vesicle-mediated excretion of crystals in leaf hairs (trichomes) [10]. Because of the latter two mechanisms, excluder plants typically show only little increase of toxic metals inside their tissues at moderately toxic concentrations, but a steeper slope of the metal in soil/water *versus* metal in plant function at more toxic soil concentrations when the capacity of those efflux transporters is exceeded (Figure 1). Excluder plants cannot be used for phytoremediation in the strict sense of the word (dealt with in Section 4) as they would not remove much metal. But they are used for re-vegetation of toxic sites where extraction of the metal is not possible or not desired. In this case, the particularly low metal content in their above-ground parts is an advantage because grazing animals will not take up much of the toxic metal. In order to identify such plants for re-vegetation of contaminated sites, screening of plant species with such properties has been done since about 15 years with some success [11–13]. Further, exclusion of toxic metals is a desirable feature for crop plants, so that research identifying genes that determine toxic metal uptake, and modification of their expression by breeding or genetic engineering has been a

focus of recent research [12–16]. While screening for suitable low-metal cultivars already had considerable success [14,15], so far there are no genetically modified crop variants achieving this aim.

Indicator plants possess very little defence against uptake of toxic metals, so their internal metal content almost linearly reflects the metal concentration in their environment (Figure 1). Therefore, such plants have been suggested for monitoring environmental pollution already for a long time with research still continuing (e.g., [17–19]). More recent research in this direction deals with the quantitative aspects of the relation between environmental metal concentrations and accumulation in plants, and has shown linear relationships if species are well selected [20,21]. When rather metal-sensitive species are used, not only the element concentrations inside can be measured for quantitative analysis of environmental pollution, but in addition a screening for visual toxicity symptoms can be used as a quick and inexpensive test whether pollution already reached dangerous levels [21]. But even in indicator species, not all metals are taken up with the same efficiency, i.e., the slope of internal *versus* external metal concentration varies considerably, so that for a reliable assessment of environmental pollution with bioindicators an interspecies calibration is necessary [18,22]. Further, seasonal variations in growth of the indicator organisms, as well as abiotic environmental factors such as pH, which strongly influence metal uptake by organisms, lead to variations in this slope and have to be considered in the analysis of bioindicator data [23,24]. Also epiphytic bacteria can modify plant uptake, especially in water plants where they may cover a lot of the surface, compete for metal uptake and thus protect the plants from metal-induced damage [25].

1.2 Active Cadmium Hyperaccumulation

Some plants, called hyperaccumulators, actively take up large amounts of potentially toxic metals and store them in their above-ground tissues (first described by Risse in the article of Sachs in 1865 [26], the term "hyperaccumulator" was introduced by Brooks in 1977 [27]). These plants are the main topic of this chapter, so that important aspects of their ecological role, physiology, and biotechnological use will be described in detail in the following sections. There is no general agreement which level of Cd accumulation under which circumstances is enough for making a Cd-accumulating plant a true hyperaccumulator. But the highest naturally occurring Cd hyperaccumulation is clearly achieved by the southern French (from the Ganges region) ecotype of *Thlaspi caerulescens* = *Noccaea caerulescens* [28]. Shoots of this plant easily reach >2000 ppm Cd on natural soil [29] and >20000 ppm grown until mature state in nutrient solution [28]; the Cd bioaccumulation coefficient of this ecotype in its natural habitat is >70 [29]. This ecotype does not only accumulate most Cd, but is at the same time much more Cd-resistant than most other plants including other *T. caerulescens* ecotypes, maintaining an almost undiminished growth even at 30 μM Cd in nutrient solution [30].

To distinguish these very remarkable capabilities from the multitude of plant species that accumulate only a little Cd while being Cd-sensitive, but still are sometimes called "Cd hyperaccumulators" based on the old definition of Cd hyperaccumulation (">100 ppm in shoot dry weight in the natural habitat" [31]), it might be useful to re-define Cd hyperaccumulation as >500 ppm in shoots under environmentally relevant conditions with less than 20% growth reduction until the stage of maturity and a bioaccumulation coefficient >5 at a Cd concentration leading to >500 ppm in shoots.

2 Ecological Role of Cadmium Hyperaccumulation

In nature, heavy metal hyperaccumulation can serve as a defence against pathogens and herbivores [32–38]. For the best Cd/Zn hyperaccumulator, *T. caerulescens*, unfortunately most studies in this direction were carried out with Zn, but given the even higher toxicity of Cd to animals, fungi, and bacteria, it is almost certain that the results apply at least as much to the ecological role of Cd hyperaccumulation in this species. Herbivores that were given the choice between *T. caerulescens* plants grown on different zinc levels [39] or belonging to ecotypes with different abilities of Zn hyperaccumulation [40] were shown to choose those plants which accumulate the lowest amount of metal. Pathogenic bacteria affected *T. caerulescens* less if it was grown with increasing Zn, and in this study also Cd was tested at least in cell cultures and had the expected effect [41]. A truly Cd-dedicated study on elemental defense in *T. caerulescens* was performed by Jiang et al. [42], who found that Cd accumulation deters thrips (*Frankliniella occidentalis*) from feeding on *T. caerulescens* leaves. These effects of accumulated metals are often additive if more than one metal is accumulated, and a particularly strong effect was found for the Cd+Zn combination where no larvae pupated while the same metal concentrations alone only reduced the likelyhood of pupation [43]. The same study showed that at least sometimes metal effects are further enhanced by organic defence chemicals produced by the plants. Further, already metal concentrations below the hyperaccumulation limit are active in this direction [44].

While it is clear from these studies that hyperaccumulation does protect against a broad range of herbivores and pathogens, like any other defence strategy it has limitations. Zinc accumulation did not protect the Cd/Zn hyperaccumulator *Arabidopsis halleri* from attack by snails [45], the same was found for snails feeding on *T. caerulescens* [46]. It looks like snails are less metal-sensitive than the various herbivores and pathogens that are efficiently deterred from hyperaccumulators, but it remains to be seen whether this applies also to defence by Cd as so far no studies investigating the feeding of snails on Cd-treated *T. caerulescens* have been performed.

Since the amount of heavy metal content accumulated in the plant can easily be controlled by the concentration of the metal in the growth medium [39],

hyperaccumulators may be an ideal model for a systematic study of plant-pathogen/herbivore interactions, as discussed by Pollard [47].

In addition to the protection against herbivores and pathogens, it was proposed that hyperaccumulation may serve as "elemental allelopathy" [48]. This study suggested that hyperaccumulators increase the metal concentration in the surface soil next to them and thereby inhibit the growth of non-accumulators competing for space and nutrients. At the same time, the elevated metal concentrations would encourage growth of hyperaccumulator seedlings [49–52]. Perronnet et al. [52] have shown that the hyperaccumulated metals in leaves of *T. caerulescens* easily become bioavailable again after incorporation of the leaves into the soil. These interesting ideas definitely deserve further investigations.

Another alternative hypothesis about the biological role of hyperaccumulation was the increase of osmotic pressure for increased tolerance to the drought stress that often characterizes the natural habitats of hyperaccumulators. This hypothesis, however, was falsified by a study with the Ni hyperaccumulator *Alyssum murale* and the Cd/Zn hyperaccumulator *T. caerulescens* [53].

Besides all the aforementioned benefits hyperaccumulated metals (may) have for the plants, there is also one recent study that strongly suggests a much more direct role in metabolism – it may be an alternative active center in carboanhydrase of *T. caerulescens*, which is normally a Zn-dependent enzyme [54]. Such a functional replacement of Zn by Cd, leading to growth increase upon addition of Cd, was previously found in the marine alga *Thalassiosira weissflogii* [55], from which Cd-carboanhydrase was even purified and its three-dimensional structure resolved [56]. This case is described in detail in Chapter 16 of this book.

Whatever the main ecological benefit of hyperaccumulation is, plants doing it treat the accumulated metal as something valuable, which becomes obvious during leaf senescence. As it is generally known for metals that are essential plant nutrients (e.g., [57]), also hyperaccumulators recycle beneficial metals, which seem to include the hyperaccumulated heavy metals; Cd concentrations were found to be lower in senescent compared to mature and young leaves of *T. caerulescens* [58,59]. Furthermore, roots of *T. caerulescens* have been found to grow towards rather than away from heavy metals [60].

3 Mechanisms of Cadmium Hyperaccumulation

The mechanisms by which plants hyperaccumulate heavy metals in their shoots and prevent phytotoxicity of these metals have been the subject of many studies. Nonetheless, many of these mechanisms are still under debate.

While the following sections deal with mechanisms of hyperaccumulation on the cellular and molecular level, it should also be noted that hyperaccumulation is modified by interactions between plants and arbuscular mycorrhizal fungi [61]. In this study, the colonisation of the plant roots with these fungi reduced Cd uptake and thus increased metal tolerance of the plants, but in studies on other plants

such a correlation was not observed (e.g., [62]). Another controversial point concerning interactions in metal hyperaccumulation is the interaction between different metals. According to several studies, Cd and Zn compete for uptake due to their well-known chemical similarity in the Zn-hyperaccumulating Prayon ecotype of *T. caerulescens*, but independence of the transport processes of these metals was found in the Cd/Zn-hyperaccumulating Ganges ecotype of *T. caerulescens* [28,63,64]. The Cd-Zn competition in the Prayon ecotype was questioned by a more recent study, where even an enhancement of Cd uptake during growth on high Zn was found [65]. Further, it was reported that Cd uptake in *T. caerulescens* Ganges increased under iron deficiency [66].

A rather comprehensive study of interactions between uptake of different metals in *T. caerulescens* was done by Assunção et al. [67]. Via binary metal combinations they tested interactions between Cd and Zn, Cd and Ni, and Ni and Zn, which confirmed that the Ganges population expresses a highly Cd-specific high-affinity uptake system, which was not found in the other *T. caerulescens* populations. The other populations only had a high-affinity Zn-uptake system (present also in the Ganges population) and a low-affinity Cd/Zn/Ni-uptake system.

3.1 Compartmentation of Cadmium in Tissues, Cells, and Organelles

An enhanced uptake of metals into the root symplasm was found in *T. caerulescens* compared to the related non-accumulator, *T. arvense* [68,69], and a reduced sequestration into the root vacuoles was associated with the higher root to shoot translocation efficiency of *T. caerulescens* [49,69,70]. Xylem loading and xylem transport are key steps in Cd hyperaccumulation, as it will be discussed in detail in the section about transport proteins below, and as it was commented by White et al. already 10 years ago [71]. Also in non-hyperaccumulators, the degree of Cd accumulation in above-ground tissues mainly depends on xylem transport [72].

While metal uptake through the root is the first important step in hyperaccumulation, most of the metal is stored in the above-ground parts. Studies of cellular metal compartmentation have shown that in most hyperaccumulators the metal is sequestered preferentially into compartments where they can not damage metabolic processes, e.g., photosynthesis as a very cadmium-sensitive vital function of plants (see Chapter 13). Therefore, it is important for hyperaccumulators to keep the metal concentration in the cytoplasm of mesophyll cells as low as possible.

Many plants detoxify heavy metals by sequestering them, either as phytochelatin complexes or without specific ligands, in the vacuoles (for reviews see e.g., [73,74]). It makes sense for hyperaccumulating plants to store metal in the vacuoles as well because this organelle only contains enzymes like phosphatases, lipases, and proteinases [75] that were never identified as targets of heavy metal toxicity. Vacuole sequestration is driven to an extreme form in hyperaccumulators, where

the primary metal storage compartment of most species is clearly the leaf vacuoles [50,51,76–79]. This plant-specific (animal and bacterial cells do not possess this organelle) metal detoxification strategy provides an efficient form of protection because the vacuole does not contain any sensitive enzymes.

In most heavy metal-tolerant plants, the vacuolar sequestration mainly occurs in non-photosynthetic cells of the epidermis, reducing toxicity to the heavy metal sensitive photosynthetic apparatus [50,51,76,80–82]. For a general review on mechanisms of such differential ion accumulations in leaves, see Karley et al. [83]. Additionally it has been shown that high amounts of metals are stored specifically in the vacuoles of particularly large epidermal cells [50,51,76]. The approximate volume of this storage site multiplied by the metal concentration in it (data, e.g., for Zn from Küpper et al. [50]) indicates that about 70% of the total accumulated metal in mature leaves is stored in the epidermis of *T. caerulescens*. In the vacuoles of the epidermal metal storage cells, heavy metal concentrations of several hundred mmol.L^{-1} can be reached [50,51], showing that hyperaccumulation must involve active pumping of the metals into specific storage sites. The preferential heavy metal accumulation in epidermal storage cells, previously observed for several metals in intact leaves of various hyperaccumulator species, is due to differences in active metal transport and not differences in passive mechanisms like transpiration stream transport or cell wall adhesion [79]. Combining this with previous studies, it seems likely that the transport steps over the plasma and tonoplast membranes of leaf epidermal storage cells are driving forces behind the hyperaccumulation phenotype.

Like in many other cases, also in this one there is a famous exception from the rule. In the Zn hyperaccumulator *A. halleri*, which also has a limited accumulation capability for Cd, except for a few trichomes epidermal cells are rather small. Therefore, despite their high concentrations of Cd and Zn they contribute only a minor proportion to total storage of Cd and Zn in this species [84]. This may be the reason, however, why in this species Cd accumulation is limited by Cd toxicity as shown in the same study, because Cd accumulation in the mesophyll represents a danger in terms of Cd-induced inhibition of photosynthesis. A seemingly similar situation was recently reported for the Cd/Zn hyperaccumulator *Sedum alfredii* [85], where Cd is accumulated in the mesophyll of leaves beside the pith and cortex of stems [86]. Comparing this to other hyperaccumulators, however, it has to be kept in mind that *S. alfredii* has rather thick, succulent leaves, which means that it has exceptionally large vacuoles in the mesophyll, making Cd storage there safer than it would be in regular sized mesophyll cells.

3.2 Expression and Function of Metal Transport Proteins

In recent years, much progress has been made in identifying genes involved in metal transport. Thus it has been found that metal hyperaccumulation is mediated, at least in part, by an up to 200 times higher expression of metal transporter genes in

hyperaccumulators compared to related non-accumulator plants ([87–95], reviewed by Verbruggen et al. [96]). To achieve a bioaccumulation coefficient greater than one, the metals have to be pumped into their storage sites, i.e., vacuoles, against the concentration gradient. And already before, most likely many steps of the metal transport from root uptake until passage over the plasma membrane of the storage cells are against the concentration gradient or at least there wouldn't be a concentration gradient facilitating passive diffusion. Therefore, all these transport steps require an active, i.e., energy-consuming transport system [97]. Furthermore, specificity of the transport has to be tightly controlled in order to allow for the vast differences in concentrations between hyperaccumulated and non-accumulated metals in the same cell.

The early finding that root uptake and root to shoot translocation are strongly elevated in hyperaccumulators compared to non-accumulators (see Section 3.2. above) obtained a genetic basis in recent years, as it was found that the heavy metal ATPase HMA4 is strongly overexpressed in roots of the Cd/Zn hyperaccumulator plants *A. halleri* and *T. caerulescens* [90,91,98], and that this is linked to HMA4 gene multiplication [93,95,99]. Investigations of the biochemical and biophysical properties of the *T. caerulescens* version of HMA4, TcHMA4, have shown that the ATPase function of this transporter is activated most strongly by Cd and Zn. Gels and western blots (using an antibody specific for TcHMA4) of crude root extract and of the purified protein revealed a size of TcHMA4 of about 50 to 60 kDa, while the mRNA for the TcHMA4 gene predicts a single protein with a size of 128 kDa. This indicates the occurrence of post-translational processing [100]. In recent work by Leitenmaier et al. [101], TcHMA4 showed activity with Cu^{2+}, Zn^{2+}, and Cd^{2+} under various concentrations (0.03–10 μM tested), and all three metal ions activated the ATPase at a concentration of 0.3 μM. Notably, the enzyme worked best at rather high temperatures (optimum at 42°C). Arrhenius plots showed constant activation energy ($E_A = 38$ kJ.mol^{-1}) over the whole concentration range of Zn while it increased from 17 to 42 kJ.mol^{-1} with rising Cu concentration and decreased from 39 to 23 kJ.mol^{-1} with rising Cd concentration. According to EXAFS, TcHMA4 appeared to bind Cd mainly by thiolate sulfur from cysteine, and not by imidazole nitrogen from histidine.

Protein families involved in vacuolar sequestration may be the NRAMP's, CDF's, and CAX's (reviewed by Hall and Williams [102]) as well as CPx-type ATPases. Until now, already several transporters for vacuolar sequestration of zinc (and possibly cadmium) and nickel have been investigated and could be partially characterized [103–107]. Several CDF transporters for vacuolar sequestration of Zn (and possibly Cd and Co) have been characterized, all are homologous, almost identical in sequence. These are MTP1, ZAT, and ZTP (e.g., [88,89,103,108]). The strongly elevated expression of the CPx-type metal ATPase HMA3 was shown to play a decisive role in Cd accumulation not only in *T. caerulescens* [109], but also in rice [110,111], reconfirming also the importance of the sequestration into vacuoles for the hyperaccumulation phenotype. The natural over-expression of NRAMPs was identified both in rice and in *T. caerulescens* to play an important role in Cd tolerance and possibly Cd accumulation [112–114]. When vacuolar

sequestration is coupled to complexation by phytochelatins (i.e., not in hyperaccumulators, but in non-accumulator plants), transport of the Cd-phytochelatin complexes to the vacuoles is mediated by transporters of the ABC family, as recently shown by Mendoza-Cózatl et al. [115].

In contrast to the progress that has been made in finding genes that are expressed at higher levels in hyperaccumulators, the cell-specificity of their expression and regulation in hyperaccumulators has remained largely unknown. Therefore, it remains impossible to judge which of these genes are directly involved in hyperaccumulation by encoding transporters that pump the metal into storage sites, and which may be secondarily up-regulated to prevent Zn deficiency in other compartments. Two recent studies have indicated such secondary up-regulation for members of the IRT micronutrient transporter family: Küpper et al. [116] for ZNT1 in *T. caerulescens* by QISH analysis, and Hanikenne et al. [95] for ZIP4 and IRT3 by expressing HMA4 from the Zn hyperaccumulator *A. halleri* (AhHMA4) under its own promoter in the non-accumulator *A. thaliana*.

Metal storage cells were furthermore found to display a strongly elevated expression of the metal transporters MTP1 and ZNT5 [117]. But for most of these genes the cellular expression pattern and its metal-dependent regulation remains unknown. Quantitative mRNA *in situ* hybridization (QISH) revealed that transporter gene expression changes not only dependent on metal nutrition/toxicity, but even more so during plant and leaf development [117]. Main mRNA abundances found: ZNT1: mature leaves of young plants; ZNT5: young leaves of young plants: MTP1 (= ZTP1 ≈ ZAT): young leaves of both young and mature plants. Surprisingly different cellular expression patterns were found for ZNT1 and ZNT5, both belonging to the ZIP family of transition metal transporters. ZNT1: photosynthetic mesophyll and bundle sheath cells; ZNT5: non-photosynthetic epidermal metal storage cells and bundle sheath cells. Thus, ZNT1 may function in micronutrient nutrition while ZNT5 may be involved in metal storage associated with hyperaccumulation. The latter is in agreement with experiments of knock-outs and heterologous expression of TcZNT5 in *A. thaliana* [118].

3.3 Role of Metal Ligands in Cadmium Uptake, Transport, and Storage

Cadmium is highly toxic to plants (see Chapter 13), so that the extreme Cd accumulation in Cd hyperaccumulators immediately raises the question in which chemical form it is present in these plants, i.e., whether its toxicity is diminished by strong ligands that reduce the likelyhood of binding to proteins. Most reliable information about Cd ligands in plants was gained by X-ray absorption spectroscopy (XAS), especially X-ray absorption near edge structures (XANES) and extended X-ray absorption fine structure (EXAFS), as reviewed, e.g., by Gardea-Torresday et al. [119] and Saraswat and Rai [120]. The big advantage of XAS

compared to chromatographic methods is the possibility to exclude artefactual exchange of Cd ligands during sample preparation and analysis. Crushing cells for tissue/cell fractionation and extraction inevitably destroys membranes that separate the metals accumulated in the vacuole from various proteins in the cytoplasm and from the cell wall in the apoplast. This membrane destruction will immediately result in a change of Cd speciation because very strong Cd ligands are found in the cytoplasm (e.g., active sites of proteins) and cell wall, while vacuoles are well-known to contain weak ligands like organic acids.

For using XAS it is not necessary to extract metal-ligand complexes from their natural compartment (e.g., the vacuole), and using rapid-freeze techniques in combination with measurement of frozen-hydrated samples artefacts of re-distribution can be efficiently prevented. This has been done for Cd by Küpper et al. [59], showing that in mature leaves as the main storage sites of Cd in hyperaccumulators this metal is bound predominantly by weak oxygen ligands such as organic acids. This result was later confirmed via ^{113}Cd NMR [121] and further XAS studies [122,123]. Also by other methods, sulfur-containing metal-binding ligands such as phytochelatins were shown not to be relevant for cadmium or zinc storage or detoxification in *T. caerulescens*. For example, phytochelatin levels were shown to be lower in this plant than in the related non-accumulator *T. arvense* [124], and inhibition of phytochelatin synthesis in hyperaccumulators did not affect their Cd resistance [125]. Nevertheless, metallothionein genes have been found to be highly expressed in *T. caerulescens* [90,126], and differences between *T. caerulescens* and *A. thaliana* metallothioneins have been examined [126,127], but their function remains unclear.

For long-term storage in the vacuoles, hyperaccumulated metals are bound only to weak ligands like organic acids [59,128,129]. So the main detoxification strategy in hyperaccumulators is clearly not binding to strong ligands, but sequestration of the hyperaccumulated heavy metals. Also other genes, e.g., those related to stress responses, are much more highly expressed in hyperaccumulators than in related non-accumulators, but their relevance for hyperaccumulation is not clear.

Only in young, not fully expanded leaves, in stems and petioles as Cd transport organs, as well as in seeds a higher percentage of sulfur ligands was found around Cd [59,123]. In this way, less Cd-accumulating tissues of hyperaccumulators somewhat resemble the situation known from non-accumulator plants, in which most of the Cd is bound to strong ligands, especially phytochelatins (reviewed by Cobbett and Goldsbrough [4]).

4 Biotechnological Use of Cadmium Hyperaccumulators

In all cases of anthropogenic contamination of soils with heavy metals, the highest heavy metal concentrations are found rather close to the surface, although not directly in the uppermost few mm to cm as these are leached by rain like in natural heavy metal sites [130,131]. For this reason, a decontamination of such areas is, in principle,

possible in several ways. The classic way would be the removal of the topsoil and leaching of it in a chemical or microbial way in special facilities. Due to extremely high costs, however, this is only a realistic option for very small (and at the same time economically or socially very important) spots. For larger areas, decontamination by plants seems to be the most attractive option, as on fertile ground (which would be a most attractive kind of site for decontamination as it could be agriculturally valuable) plants should grow well without too much human effort. But it is hotly debated what kind of plants should be used for this task. In principle, three main strategies exist: (i) The use of naturally occurring metal hyperaccumulator plants, probably combined with classical breeding, (ii) the use of high biomass non-accumulator plants, and (iii) the transfer of genes from hyperaccumulator plants to turn originally non-accumulating high biomass plants into high biomass metal hyperaccumulators. We would like to summarize work on these strategies from the perspective of our own work on metal metabolism in plants.

Many natural hyperaccumulators, i.e., plants that actively accumulate several percent of heavy metals in the dry mass of their above-ground parts, have a good potential to be used for phytoremediation, i.e., to extract and remove heavy metals from anthropogenically contaminated soils, which was first proposed by Chaney [132]. Some of them even allow for commercially profitable phytomining, i.e., the extraction of metals from naturally heavy metal rich soils (that are not directly usable as metal ores) with subsequent burning of the plants, the ash of which can be used as a metal ore (first proposed by Baker, Brooks, and Reeves [133]). These applications of metal phytoextraction have been a subject to extensive research (for reviews see [3,31,132,134–141].

For cadmium, the Cd/Zn hyperaccumulator *T. caerulescens* seems to be the best known candidate for phytoremediation. Although it has a rather small biomass of 2–5 $t.ha^{-1}$ [136,142], the extreme bioaccumulation coefficient of its southern French ecotypes [28,29,143] allows it to significantly lower metal concentrations in soil solution [144]. When grown in field conditions, this yields Cd extraction rates high enough for cleaning up moderately Cd-contaminated soils within a few years as tested in the field by Robinson et al. [142], Hammer and Keller [145], and McGrath et al. [146]. Phytoremediation by *T. caerulescens* was furthermore shown to enhance microbial life in soil [147]. The high copper sensitivity of *T. caerulescens*, however, may limit its use; concentrations that occur in multi-contaminated soils were found to strongly inhibit its growth [148]. This might be alleviated by selection of copper-resistant individuals that occur in natural populations of this species [149]. For zinc, the Chinese Cd/Zn hyperaccumulator *Sedum alfredii* may be the most promising candidate for phytoremediation and possibly even commercial phytomining because of its correlation of high zinc accumulation with relatively high biomass [150,151]. In comparison, *T. caerulescens* has a rather low biomass and at high soil zinc concentrations also a low bioaccumulation coefficient [142,143], so that its use in zinc phytoremediation is generally limited to moderate levels of contamination. Indeed, while field trials on moderately contaminated soil were successful [152], those on more heavily Zn-contaminated

soil failed [145]. Metal accumulation by hyperaccumulators may be further enhanced by root-colonising bacteria, as shown for Cd + Zn in *S. alfredii* [153].

In addition to true hyperaccumulator plants, various other plants have been proposed for use in soil phytoremediation. One idea is to use high-biomass plants for absorbing the metals; it is argued that the much higher biomass will yield higher metal extraction per area of land compared to hyperaccumulators, despite the much lower metal content of non-accumulator plants (e.g., [137,138,154]. Those who argue for such an approach, however, mostly ignore that such a strategy would dilute the extracted metal in a much larger amount of toxic biomass compared to hyperaccumulator plants; this biomass would be too toxic for use as compost and would not contain enough metal to make a recycling of the phytoextracted metal feasible (discussed, e.g., by Chaney et al. [155] and Williams [156]). In addition, the bioaccumulation coefficient of metals in non-accumulator plants is usually so low that hundreds of crops would be required for phytoremediation of even a moderately contaminated soil [136,151,155]. Those who argue for this approach because of the low biomass of many hyperaccumulators should also keep in mind the following facts.

(i) The biomass yield of nonaccumulator plants on contaminated soils is reduced by phytotoxicity of the contaminating metal [155,157]; this applies also to the slightly Cd accumulating poplar (*Populus sp.*) that is a popular suggestion for phytoremediation because of its high biomass [158].
(ii) The biomass of hyperaccumulators can be rather easily improved by selecting suitable ecotypes and individuals within the natural population [159,160], breeding [161], and fertilization (2–3 times increase: [159,160,162–164]).
(iii) The metal accumulation of hyperaccumulators can further be optimized by selection. Many recent studies pointed out the more than twentyfold variation of bioaccumulation coefficient for the same metal between ecotypes/populations [28,30,143,165–169]. Furthermore, the accumulation efficiency is not directly correlated to the metal content of the habitat [167], and strong variation of metal bioaccumulation coefficients as well as metal resistance exists even within one population [149,169]. Finally, accumulation is higher on the average moist agricultural land compared to their dry natural habitats [160,170].

In summary, presently it is not the phytoremediation by hyperaccumulators that is a ‘hype’, but the use of non-accumulating plants for this task. The only way that non-hyperaccumulating plant species may become a better alternative would be creating (by genetic engineering or better traditional breeding) metal-accumulating cultivars, or to search for new naturally Cd-accumulating plants with improved properties. Search for high biomass Cd accumulators led to the finding of rather good Cd accumulation by the tropical tree *Averrhoa carambola* and some ecotypes of willow (*Salix alba*) with Cd bioaccumulation coefficients above 10 [158,171], although this is still little compared to the Ganges population of *T. caerulescens* (see Section 3.2).

It is often argued that instead of using natural hyperaccumulators for phytoremediation and phytomining, genetically engineered plants should be used. Looking at the results of classical selection breeding of hyperaccumulators *versus* attempts to create transgenic hyperaccumulators, the former approach appears much more promising, for the following reasons. Research on the mechanisms of hyperaccumulation has revealed that this process involves many different steps in diverse parts of the plant, starting from enhanced uptake into the roots [68] and continuing via enhanced xylem loading [90], translocation to the shoots possibly by transport ligands (e.g., [172]), unloading from the veins and finally sequestration into vacuoles of usually epidermal storage cells [50,51,76,79] as reviewed, e.g., by Küpper and Kroneck [3,141]. Furthermore, individual members of metal transport protein families display vastly different tissue-, age-, and metal nutrition-dependent regulation in the same plant [117]. Therefore, to re-create a hyperaccumulator by genetic engineering, one would have to modify the expression of many genes, in a tissue-specific way and probably at particular stages of plant and leaf ontogenesis. This has not been achieved, not even in an approximation, in any study so far (reviewed, e.g., by Chaney et al. [140]). Therefore, it is not surprising that in all attempts of creating hyperaccumulators by genetic engineering at best a few times enhancement of metal accumulation compared to the original non-accumulator wild-type was achieved, while true (natural) hyperaccumulators usually have hundreds of times higher metal bioaccumulation coefficients than those non-accumulators (see Section 3.2. and for reviews, e.g., [3,140,141]). And such transgenics are not useful to apply, for the same reasons as explained for wild-type non-accumulators. Unless someone finds a general "switch gene" that leads to the changed expression pattern of all the other genes involved in hyperaccumulation, transgenic plants that really extract as much metal per hectare as hyperaccumulators will remain science fiction.

In contrast, field trials have shown that the biomass of natural hyperaccumulators can be dramatically increased by addition of fertilizer, natural selection, and classical breeding to reach levels that are economically attractive (reviewed by Chaney et al. [139]). As a source for selecting species that are suitable for a specific phytoextraction tasks, conservation of metallophyte biodiversity is of prime importance as outlined by Whiting et al. [173].

5 Outlook

This chapter summarized recent advances and earlier important works dealing with the accumulation of cadmium in plants, with a special focus on Cd hyperaccumulators. As described in detail above, a lot of progress has been made in

(i) the analysis of the uptake of Cd from soils into plants, including uptake from contaminated soils into crops and hyperaccumulator plants in terms of phytoremediation.

(ii) the investigation of mechanisms involved in the hyperaccumulation phenotype, including the analysis of tissue- and cell-level metal sequestration patterns and screening of genes that are involved in species- and ecotype-specific difference in Cd accumulation and resistance.

Based on these advances, several attempts have been made to improve plants for phytoremediation, and to modify the metal uptake into crops. The at best partial success of the latter efforts, however, have shown that many questions concerning Cd (and other metals as well) accumulation still remain open and have to be answered before the aims of making better crop plants, improving phytoremediation technology, and understanding general principles of metal metabolism, can be fully reached. In our opinion, based on what we described in this chapter, future efforts should put a strong focus on

(i) the biochemical/biophysical mechanism of function of metal transport proteins, as these proteins are clearly the decisive factor in differential metalaccumulation, but knowledge about almost all of them is still restricted to knowing their genes and knowing effects of their knockout and/or over-expression.
(ii) the regulation of metal transport genes dependent on the nature and concentration of the accumulated metal, on the age of the plant, the age and type of the tissue. Like the previous point, this will greatly help in understanding general principles of metal metabolism, and at the same time generate a basis for the next point concerning phytoremediation.
(iii) the breeding of improved variants of species that already have been demonstrated to be practically useful for phytoremediation, i.e., mainly *T. caerulescens* for Cd and *S. alfredii* for zinc.

Abbreviations

ABC transporters	ATB binding casette transporters
ATP	adenosine 5'-triphosphate
CDF	cation diffusion facilitator
CAX	cation exchanger
E_A	energy of activation in kJ.mol^{-1}
EXAFS	extended X-ray absorption fine structure
HMA	heavy metal ATPase
IRT transporters	iron regulated transporters
mRNA	messenger RNA
NMR	nuclear magnetic resonance
NRAMP	natural resistance-associated macrophage proteins
QISH	quantitative *in situ* hybridization
XANES	X-ray absorption near edge structure
XAS	X-ray absorption spectroscopy

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