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| Abstract | As environmental issues become more and more stringent, the biotechnological approaches to maintain clean environments are receiving increasing attention. Heavy metal pollution is of great concern as it ultimately forces heavy metals into the food chain leading to serious ecological and health problems. |
Removal of excess heavy metals from contaminated sites could be achieved by means of organisms that bioaccumulate heavy metals without developing toxicity symptoms, features that are characteristic to hyperaccumulating plants. This review focuses on the applicability of hyperaccumulation phenomenon to heavy metal bioremediation as well as on the possibility to extend the hyperaccumulation concept to organisms other than plants.
Chapter 13
Hyperaccumulation: A Key to Heavy Metal Bioremediation

Ileana Cornelia Farcasanu, Mihaela Matache, Virgil Iordache, and Aurora Neagoe

13.1 Introduction

Industrialization, along with numerous benefits, brought up important issues such as environment awareness and environment protection. Strict regulations compel industries to find ways to limit the discharge of pollutants into the environment or to use eco-friendly approaches to clean up contaminated sites. Heavy metals are challenging pollutants, as they are natural components of the earth’s crust, they are persistent in the environment and are nondegradable. Regarding the interaction with the living organisms, they have a dualistic behavior. On the one hand, many of the heavy metals are essential in minute amounts for the normal metabolism, binding to and stabilizing biomolecules, or acting as cofactors for various enzymatic processes. On the other hand, heavy metals can be toxic in high concentrations, mainly by nonspecific binding to biomolecules or by interference to other metals’ metabolism. The sources of heavy metal pollution can be industrial effluents, automobile exhaustion, mining industry, leaching of metal ions from the soil into lakes, rivers and aquifers by acid rain, etc. Heavy metal contamination of soils and waters is alarming due to potential penetration through the food chain leading to serious health problems at all levels (Duruibe et al. 2007; Fraústo da Silva and Williams 2001; Sánchez 2008). The term “heavy metal” is ill defined and has raised controversy (Duffus 2002), but it is still commonly used when referring to any metallic chemical element that has a relatively high density and is toxic or poisonous at low concentrations. The term is usually applied not only to common transition metals, such as Cd, Co, Cu, Hg, Mn, Ni, Pb, Zn, but also to metalloids such as As or Se. Among these, Co, Cu, Mn, Ni, Zn, and Se are elements that are essential for life in...
very low amounts (essential trace elements). These are taken up by cells via intricate active transport systems and are maintained at relatively constant levels by means of strictly regulated homeostasis mechanisms. Other heavy elements such as Cd, Hg, Pb, As are not essential for life, but they can compete with the essential trace elements either for the transport systems or in binding to various biomolecules. Essential or not, when present in high concentrations, the heavy metals become toxic, causing serious damages to organisms.

Although avoidance of pollution should be a pre-requisite for any anthropomorphic activity, this is not always achieved. Cleaning polluted or contaminated sites is never an easy task, especially when classical physicochemical approaches are expensive or inefficient, produce secondary pollution, or simply fail. As pollution-related issues become more stringent, cost-effective, sustainable, and environmentally friendly methods for removal of hazardous substances are more and more needed. Bioremediation by microorganisms, algae or plants is often considered as inexpensive, safe and efficient way of cleaning up wastes, sediments, and soils. Heavy metal bioremediation may be regarded as the removal of the excess hazardous substance following the interaction between the pollutant and (1) one or more living species; (2) dead or inactivated biomass. Bioremediation can be achieved through a plethora of mechanisms, but the most actions against heavy metals involve biosorption and bioaccumulation, actions that can take place in situ or in bioreactors designed both for the hazardous substance and for the bioremediator. Biosorption implies binding of the heavy metal ions to the cell surface, usually by noncovalent, electrostatic forces and is considered a cost-effective biotechnology for the treatment of high-volume-and-low-concentration complex wastewater. Most times, biosorption refers to a property of certain types of inactive, dead, microbial biomass to bind and concentrate heavy metals from dilute aqueous solutions. The biomass exhibits this property, acting as an ion exchanger of biological origin, and it is particularly the cell wall structure of certain algae, fungi and bacteria, which was found responsible for this phenomenon. Bioaccumulation is another attractive possibility to remove heavy metals from the environment. In contrast to biosorption, bioaccumulation can be achieved only by living cells, requiring metabolically driven continuous uptake that needs to occur with a rate greater than the excretion rate. The retention of a chemical within the living cells is a key step for bioremediation and obtaining resistant strains is vital for such a process.

13.2 Hyperaccumulation as a Primary Tool for Heavy Metal Bioremediation

Bioaccumulation has bioremediation significance only when correlated with increased (gained) tolerance to the pollutant and easy separation of the bioremediator from the site to be decontaminated. Thus, an ideal heavy
metal bioremediator would have the following characteristics: tolerance to
nonphysiological metal concentrations, abundant growth on/in the contaminated
site, hyperaccumulating capacity, and facile separation from the bioremediated site.
Nevertheless, heavy metals inhibit very often the biological remediation processes
due to metal sensitivity of most organisms. Under such circumstances, strategies for
efficient operation have to be considered and heavy metal hyperaccumulating
plants seem to be the best models to follow when designing or developing a suitable
heavy metal bioremediator. Hyperaccumulation was a term first used by Brooks
et al. (1977) for plants that are endemic to metalliferous soils and are able to tolerate
and accumulate metals in their above-ground tissues. Metal hyperaccumulator
plants are naturally capable of accumulating trace elements, in their above-ground
tissues, without developing any toxicity symptoms (Baker 2002; Baker and Brooks
1989). The concentrations of these elements in dry leaf biomass are usually up to
100-fold higher than the concentrations in the soil (McGrath and Zhao 2003).
Natural hyperaccumulator species are often an indication of elevated soil heavy
metal concentrations, and hence they can function as bioindicators of contamina-
tion; their potential role in phytoremediation, phytoextraction, and phytomining has
been extensively studied (for review see Boyd 2010; Chaney et al. 2007; Cheng
2003; Krämer 2005; Lone et al. 2008). Although metal hyperaccumulator plants
seem very promising, most produce little biomass and are therefore used mainly as
model organisms for research purposes (Krämer 2005). In this paper, studies
concerning the use of hyperaccumulating plants for understanding the hyperaccu-
mulation and tolerance mechanisms, the use of transgenic approaches to obtain new
hyperaccumulators, as well as the possibility to extent the plant hyperaccumulation
concept to other organisms are reviewed.

13.3 Metals Commonly Hyperaccumulated by Plants

Most plants, when exposed to potentially toxic metals in their growing medium,
take up the metal into the root, but restrict its further translocation to the shoot. A
very rare class of plants, named hyperaccumulators, translocate substantial amounts
to their shoots, so that shoot:root ratios exceed unity (Baker 1981; Macnair 2003).
Most hyperaccumulators that have been identified so far hyperaccumulate nickel,
but hyperaccumulators of cadmium, arsenic and zinc are also well characterized
(Macnair 2003). To be considered a genuine hyperaccumulator, plants need to
accumulate high concentrations of metals in any part that grows above the ground
(Baker and Brooks 1989; Baker et al. 2000) in concentrations 10–500 times higher
than the same plant species from nonpolluted environments (Yanqun et al. 2005). In
addition, the shoot-to-root concentrations ratios must be higher than one (McGrath
and Zhao 2003; Yanqun et al. 2005), meaning higher concentrations in the plant
than in the soil. Most metal hyperaccumulators are endemic to soils with high
concentrations of metal (Baker and Brooks 1989; Pollard et al. 2002). Table 13.1
summarizes the most studied heavy metal plant hyperaccumulators. In particular,
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<tr>
<th>Plant name</th>
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<td>Ni(^{2+}/H^+) antiport V-ATPase at the tonoplast that can drive vacuolar accumulation of Ni through a secondary active transport mechanism</td>
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<td>Slow vacuolar (SV) channel activity</td>
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<td>Arabidopsis halleri</td>
<td>Cd, Ni, Zn</td>
<td>Metal chelation</td>
<td>One of the closest wild relatives of <strong>A. thaliana</strong></td>
<td>Becher et al. (2004), Farinati et al. (2009), Hanikenne et al. (2008), Küpper et al. (2000), Marquès et al. (2004), McGrath et al. (2006), Talke et al. (2006), Vera-Estrella et al. (2009), Weber et al. (2004), Zhao et al. (2006)</td>
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<td>Metal sequestration</td>
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<td><em>AhHMA4</em> increased activity</td>
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<td>Field and soil studies</td>
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<td><strong>AhHMA4</strong> increased activity</td>
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<td>Field and soil studies</td>
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<td>Athyrium yokoscense</td>
<td>As, Cd</td>
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<td>Avena strigosa</td>
<td>Cd</td>
<td>High activities of antioxidative enzymes</td>
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<td>High amounts of total soluble phenolics</td>
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<tr>
<td>Crotalaria juncea</td>
<td>Cd</td>
<td>High activities of antioxidative enzymes</td>
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<td>High amounts of total soluble phenolics</td>
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<td>Fagopyrum esculentum</td>
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<td>Organic acid chelation</td>
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<td>Gossia sp.</td>
<td>Mn/foliar</td>
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<td>Species</td>
<td>Metal(s)</td>
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<td><em>Maytenus founieri</em></td>
<td>Mn</td>
<td>Sequestration primarily in dermal tissues</td>
<td>Tree species, difficult to follow on short periods</td>
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<td><em>Phyllostachys asperata</em></td>
<td>Mn</td>
<td>Sequestration of Mn in leaf epidermis</td>
<td>Herbaceous Mn hyperaccumulator, with high biomass and fast growth</td>
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<td><em>Pteris vitata</em></td>
<td>As</td>
<td>Suppression of endogenous arsenate reduction in roots may serve to enhance root-to-shoot translocation of As</td>
<td>The plant transports 95% of the absorbed arsenic from roots to shoots</td>
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<td><em>Sedum alfredii</em></td>
<td>Cd, Pb, Zn</td>
<td>Enhanced root-to-shoot translocation</td>
<td>Transcriptomic analysis under Zn induction</td>
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<tr>
<td><em>Thlaspi caerulescens</em></td>
<td>Cd, Ni, Zn</td>
<td>Chelation by phytochelatins</td>
<td>One of the most studied model organisms for hyperaccumulation mechanisms</td>
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<td>Variations between populations and ecotypes</td>
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<td><em>Thlaspi goingense</em></td>
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<td>Increased expression of ZNT1, MTP1, and HMA4 genes</td>
<td>Field and soil studies</td>
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*Hyperaccumulation: A Key to Heavy Metal Bioremediation*
Thlaspi caerulescens and Arabidopsis halleri, have been studied extensively for their ability to hyperaccumulate several heavy metals, mainly Zn, Cd, and Ni, as well as the species from Alissum genus, known for their ability to hyperaccumulate Ni. To be considered a hyperaccumulator, the plants need to store more than 1,000 mg kg\(^{-1}\) (dry weight biomass) of metal for Ni, Cu, Co, Cr, or Pb, or 10,000 mg kg\(^{-1}\) for Zn or Mn (Baker and Brooks 1989). Some of the heavy metals more commonly stored by hyperaccumulator plants are presented below.

### 13.3.1 Cadmium

Cd is nonessential but a major pollutant that is extremely toxic to organisms. Soils can be contaminated with Cd as a result of fertilization with phosphates as well as from mining and smelting industries (McGrath et al. 2001; Sanità di Toppi and Gabrielli 1999). Daily consumption of Cd-contaminated foods poses a risk to human health (Watanabe et al. 2000). The principal plant used for Cd phytoetraction is Thlaspi caerulescens, a Zn/Cd hyperaccumulator (Baker et al. 2000) owing its potential to the exceeding bioconcentration factor of Cd and Zn in the shoots that enables a remarkable yield of both metals from contaminated soil (McGrath and Zhao 2003; McGrath et al. 2001). Usually, Cd overlaps the Zn tolerance and hyperaccumulation (Uraguchi et al. 2006; Yang et al. 2004). Apart from T. caerulescens, Arabidopsis halleri (Küpper et al. 2000), Sedum alfredii (Yang et al. 2004; Zhao et al. 2006), and Athyrium yokscense, (Morishita and Boratynski 1992) have been reported as evident Cd-hyperaccumulator plants. These are all wild metal-accumulators, growing slowly in the field. Uraguchi et al. (2006) studied the behavior of crop species and found two Cd hyperaccumulators, Avena strigosa and Crotalaria juncea. In this case, the Cd tolerance seems to be the result of enhanced activities of antioxidative enzymes and of augmentation in total soluble phenolics, both well-known antioxidante defense strategies. In plant cells, Cd tends to be stored in the apoplast and in vacuoles. In addition to the metal compartmentation mechanism, antioxidative ability might play an important role in the tolerance. Cd is a redox-inactive metal that is incapable of producing reactive oxygen species (ROS) directly, but can indirectly promote oxidative stress by disrupting physiological processes. Also, phytochelatins (PCs) and other thiol (SH)-containing compounds have been proposed to play an important role in the detoxification and tolerance of some heavy metals. For instance, glutathion may be responsible for Cd and tolerance in mine population of Sedum alfredii (Sun et al. 2007).

### 13.3.2 Copper

Cu is an essential element, and Cu poisoning is rarely an issue of environmental risk. Nevertheless, sources of Cu contamination are the electroplating industry, smelting
13 Hyperaccumulation: A Key to Heavy Metal Bioremediation

and refining, mining, biosolids (Liu et al. 2005). There are not many Cu hyperaccumulators described, and most of them are not Cu-specific, like some varieties of Thlaspi caerulescens, or Athyrum yokoscense (Cheng 2003; Honjo et al. 1984).

13.3.3 Manganese

Manganese, an essential trace element that is found in varying amounts in all tissues, is one of the most widely used metals in industry. Exposure to excess manganese results in manganese toxicity, including Parkinson-like symptoms (Chan et al. 2000; Erikson and Aschner 2003; Gerber et al. 2002), and abnormalities of the immune system (Vartanian et al. 1999). Manganese hyperaccumulation has been arbitrarily defined by a threshold foliar concentration of 10,000 mg kg\(^{-1}\) dry weight (Baker and Brooks 1989). Plants that hyperaccumulate Mn are predominantly woody and hence unsuited to short-term controlled study (Fernando et al. 2008). The number of species has varied with taxonomic changes, and currently nine are recognized worldwide (Bidwell et al. 2002; Reeves and Baker 2000; Xue et al. 2004). The heterogeneity of the Mn-hyperaccumulative trait was studied in Phytolacca acinosa a herbaceous species, under controlled conditions (Xue et al. 2005) and in natural populations of the tree Gossia bidwillii (Fernando et al. 2007). Recently, Fernando et al. (2009) demonstrated up to seven new Mn hyperaccumulators, mostly tropical rainforest species from the Gossia genus, one of them exhibiting also elevated foliar Ni concentrations. Among the Mn hyperaccumulating plants, P. acinosa is a good candidate for phytoremediation of Mn polluted soil for its high biomass and fast growth. Xu et al. (2006) determined Mn distribution within the hyperaccumulating plant and found that the highest Mn content was in the vascular tissues of root, stem, petiole and midrib and that, Mn content in leaf epidermis was higher than that in mesophyll, which suggested that the sequestration of Mn in leaf epidermis might be one of the detoxification mechanisms of P. acinosa.

13.3.4 Nickel

Approximately 450 species of metal-hyperaccumulating plants are currently known, of which more than 330 are Ni-hyperaccumulators (Reeves 2003; Reeves and Adiguzel 2004; Reeves and Baker 2000). Ni is ubiquitously distributed in nature and constitutes a trace element in most living cells. In high concentrations, it is toxic to most cells and is also listed as a possible human carcinogen (group 2B) and associated with reproductive problems and birth defects. Ni-hyperaccumulators are able to take up more than 1,000 mg kg\(^{-1}\) dry weight (Baker et al. 1994) without significant detrimental effect on plant survival or health. In terms of remediation using classical chemical approaches, Ni is one of the most recalcitrant pollutants; therefore, Ni hyperaccumulating plants have received special attention. Ni-hyperaccumulators belong to a group of taxonomically diverse plants that can
accumulate Ni to concentrations in excess of 0.1% shoot dry biomass (Baker and Brooks 1989; Reeves and Baker 2000). The genus Alyssum accounts for 48 of the known Ni hyperaccumulator species, and includes Alyssum lesbiacum which is capable of accumulating Ni to over 3% shoot dry biomass (Baker et al. 2000; Reeves and Baker 2000). Ni accumulates predominantly in the aerial tissues of hyperaccumulators, and the shoot epidermal cells are a major site of Ni deposition in hyperaccumulator plants, including several Alyssum species, Cleome heratensis, Hybanthus floribundus and Senecio coronatus (Asemaneh et al. 2006; Bidwell et al. 2004; de la Fuente et al. 2007; Küpper et al. 2001; Marquès et al. 2004; Mesjasz-Przybyłowicz et al. 1994).

13.3.5 Lead

Pb is a nonessential heavy metal discharged in the environment from mining and smelting of metalliferous ores, burning of leaded gasoline, municipal sewage, industrial wastes enriched in Pb, paints (Gispert et al. 2003; Seward and Richardson 1990) There are certain cultivars of Indian mustard (Brassica juncea) (Kumar et al. 1995), the fern Athyrium yokoscense (Honjo et al. 1984), or the leguminous shrub Sesbania drummondii (Sahi et al. 2002) that have been reported as Pb hyperaccumulators. Tamura et al. (2005) found that common buckwheat (Fagopyrum esculentum Moench), known as an aluminum (Al) accumulator (Ma and Hiradate 2000; Ma et al. 1997; Shen et al. 2002), can accumulate a high concentration of lead (Pb) in the shoot and especially in the leaf. Since buckwheat can grow with relatively high biomass productivity this plant may prove to be a useful phytoremediator of Pb-contaminated soils around the world.

13.3.6 Zinc

Zn is another essential trace element that has deleterious effect on organisms when present in excess. Zn pollution sources can be electroplating industry, smelting and refining, mining, biosolids (Liu et al. 2005). Around 14 species have been described as Zn hyperaccumulators defined as containing more than 10 g kg$^{-1}$ shoot dry weight (Baker et al. 2000). Thlaspi caerulescens has been widely studied for its remarkable properties to tolerate toxic levels of Zn as well as Cd, and sometimes Ni (reviewed by Milner and Kochian 2008). Along with Arabidopsis halleri, T. caerulescens has been in the primelight for studies concerning the hyperaccumulation process. Because T. caerulescens is a slow-growing plant species that does not generate significant shoot biomass, it has been used primarily as a model system for the investigation and identification of the molecular and physiological mechanisms of hyperaccumulation, with the ultimate goal of transferring these mechanisms to higher biomass plant species. A. halleri has been described as constitutively zinc (Zn)-tolerant as well as Zn-hyperaccumulating
(Bert et al. 2000, 2002, 2003; Macnair 2002; Pauwels et al. 2006). The Zn-accumulating capacity of this plant is quite remarkable (Kashem et al. 2010). Another plant reported to accumulate Zn is Sedum alfredii Hance. When grown on an old lead/zinc mining site, Long et al. (2009) found this plant to significantly hyperaccumulate zinc and cadmium under field conditions.

13.3.7 Arsenic

Arsenic (As) has been categorized as a toxic and carcinogenic element and contribute to environmental and human health problems worldwide; the highest number of cases has been reported in South-East Asian countries (Mandal and Suzuki 2002). Drinking water from wells located in areas with As-rich underground sediments has been shown to be the source of arsenocosis (Patel et al. 2005; Yang et al. 2002). Ma et al. (2001) discovered the first known vascular plant, Pteris vittata L. commonly known as Chinese brake fern to hyperaccumulate arsenic. The brake fern takes up high concentrations of arsenic (as high as 2.3%) from soil and allocated most of it to the above-ground pars for final storage (Tu and Ma 2002). Moreover, the hyperaccumulation of arsenic is accompanied by an increased biomass of the above-ground plant parts, an important characteristic, which is indispensable for phytoremediation (Ma et al. 2001; Tu and Ma 2002). Other desirable characters permitting brake fern as an ideal plant for phytoremediation include its perennial growth habit, disease and pest resistance, fast vigorous growth, and its preference for soils with high pH where arsenic exists in high abundance (Bondada and Ma 2003; Bondada et al. 2004). Studies on arsenic hyperaccumulation by P. vittata were diverse (Caille et al. 2004; Wei et al. 2006, 2007) and other species of ferns, including Pteris cretica and Pityrogramma calomelanos, have also been determined to be As-hyperaccumulators and show great potential in phytoremediation (Francesconi et al. 2002; Visoottiviseth et al. 2002; Wei et al. 2006). The root systems of the As hyperaccumulating fern P. vittata possess a higher affinity for arsenate uptake than those of a related nonaccumulator fern species, and a suppression of endogenous arsenate reduction in roots may serve to enhance root-to-shoot translocation of As (Dhankher et al. 2002). Phytochelatins, metal-chelating molecules synthesized by the ubiquitous plant enzyme phytochelatin synthase are known to contribute to As detoxification in As hyperaccumulator plants (Zhao et al. 2003).

13.4 What is Responsible for the Heavy Metal Tolerance of the Hyperaccumulating Plants?

At least three processes appear to make a major contribution to the ability of certain species to hyperaccumulate metals: enhanced uptake, root-to-shoot translocation, and detoxification via chelation and sequestration (Clemens et al. 2002; Pollard...
et al. 2002). In addition, hyperaccumulator plants appear to have highly effective antioxidant systems to protect against the potentially damaging effects of metal-induced ROS (Freeman et al. 2004).

13.4.1 Metal Chelation

Regarding the deleterious effects of heavy metals on the living organisms, it is considered that mainly the osmotically free forms of the metal ions are genuinely toxic, as they are prone to bind nonspecifically to biomolecules or to interfere with the essential metals’ metabolism. To avoid the growth impairments caused by the potentially toxic heavy metals, hyperaccumulating plants must possess biochemical defense mechanisms. Plants developed a number of strategies to resist this toxicity, including active efflux, sequestration, and binding of heavy metals inside the cells by strong ligands. The primary antidote against the osmotically free ions may be the presence of chelating agents that form easily nontoxic complexes. Thus, an essential component of tolerance is the buffering of free metal ions in the cytoplasm via chelation with high-affinity ligands. The response of plants is complex with considerable variation between species. Several detoxification strategies are known to occur and different effects are observed with different metals and metal concentrations. Still and Williams (1980) first proposed that Ni hyperaccumulation might involve a ligand containing two nitrogen donor centers and one oxygen donor center because this would exhibit a sufficiently high affinity for Ni and could account for the observed preference for Ni over Co in these plants. This was demonstrated by Krämer et al. (1996), who observed a linear relationship between the concentrations of Ni and free histidine appearing in the xylem of hyperaccumulating species of Alyssum exposed to a range of Ni concentrations. Thus, it was found that the concentration of free histidine in the roots of the hyperaccumulator species A. lesbiacum could be several fold higher than in the nonaccumulator Brassica juncea, even in the absence of Ni (Kerkeb and Krämer 2003). Histidine forms soluble complex compounds with Ni, which are completely nontoxic. To determine the molecular basis of the histidine response and its contribution to Ni tolerance, Ingle et al. (2005a, b) analyzed the transcripts of the enzymes involved in histidine biosynthesis and found that the transcript levels were constitutively higher in the hyperaccumulating A. lesbiacum compared to the congeneric nonaccumulator A. montanum. This was noted especially for the first enzyme in the biosynthetic pathway, ATP-phosphoribosyltransferase (ATP-PRT); comparison with the weak hyperaccumulator A. serpyllifolium revealed a close correlation between Ni tolerance, root histidine concentration, and ATP-PRT transcript abundance. Thus, it seems that ATP-PRT expression plays a major role in regulating the pool of free histidine and contributes to the exceptional Ni tolerance of hyperaccumulator Alyssum species. Free histidine seems to be involved in Ni tolerance in other hyperaccumulators as well. Thus, histidine concentration in the roots is 17-fold higher in the Ni hyperaccumulator Thlaspi goingense than in
the nonaccumulator *T. arvense* (Persans et al. 1999). Nevertheless, it seems that the Ni hyperaccumulating phenotype in *T. goesingense* is not determined by the overproduction of His in response to Ni (Persans et al. 1999). Coordination with histidine is not a universal determinant for heavy metal hyperaccumulation, not even for Ni. Phytochelatins are other plant chelators that might be worth considering when metal ion buffering is desired. Complexation with phytochelatin peptides synthesized from glutathione has been identified as an important mechanism for detoxifying metals such as Cd, Pb, and Zn in nonhyperaccumulator plants and plant cell cultures (Cobbett 2000; Zenk 1996), but phytochelatins do not appear to be responsible for hyperaccumulation or metal tolerance in hyperaccumulator species (Ebbs et al. 2002; Krämer et al. 1996, 2000; Shen et al. 2000). Sun et al. (2005) investigated whether phytochelatins were differentially produced in mine populations of *Sedum alfredii* compared with a nonmine control of the same species and found that phytochelatins and cysteine were not responsible for Zn and Pb tolerance in the mine population; instead, Zn and Pb treatments resulted in the increase of glutathione, suggesting that glutathione, rather than phytochelatins, may be involved in Zn and Pb transport, hyperaccumulation/accumulation and tolerance in mine population of *S. alfredii*. There are many indications that organic acids are involved in heavy metal tolerance, transport and storage in plants, including for Cd, Ni, and Zn (Godbold et al. 1984; Krotz et al. 1989; Nigam et al. 2001; Yang et al. 1997). In hyperaccumulators, the levels of citric, malic, malonic, and oxalic acids have been correlated with elevated concentrations of Ni or Zn in the biomass Li et al. (2010; Tolrá et al. 1996). Determining the distribution of Cd and Ni in hairy roots of the Cd hyperaccumulator, *Thlaspi caerulescens* and the Ni hyperaccumulator, *Alyssum bertolonii* that contained high constitutive levels of citric, malic and malonic acids, Boominathan and Doran (2003) found that about 13% of the total Cd in *T. caerulescens* roots and 28% of the total Ni in *A. bertolonii* were associated with organic acids, while the hairy roots remained healthy and grew well. Nicotianamine (NA), a nonproteinogenous amino acid synthesized in all plants through the activity of the enzyme nicotianamine synthase (NAS) is another chelator that can be involved in heavy metal tolerance (Higuchi et al. 1994). Based on yeast screens, Mari et al. (2006) demonstrated root-to-shoot long-distance circulation of nicotianamine and nicotianamine–nickel chelates in the metal hyperaccumulator *Thlaspi caerulescens*, while NA–Ni chelates are absent in the nontolerant nonhyperaccumulator-related species *T. arvense*. Furthermore, *Arabidopsis thaliana* lines overexpressing *Tc* NAS cDNA produce a large amount of NA, correlated with a better resistance to the toxicity of this metal (Pianelli et al. 2005). An intriguing fact is that the Cd/Zn hyperaccumulator *Thlaspi caerulescens* is sensitive toward Cu, which may be a problem for phytoremediation of soils with mixed contamination, demonstrating that hyperaccumulation and metal resistance are highly metal specific. A few individuals of *T. caerulescens* that were more Cu resistant revealed that a large proportion of Cu in *T. caerulescens* leaves is bound by sulfur ligands, in contrast to the known binding environment of cadmium and zinc in the same species, which is dominated by oxygen ligands (Mijovilovich et al. 2009). This observation suggests that hyperaccumulators detoxify hyperaccumulated metals.
differently compared with nonaccumulated metals. Thus, it seems that hyperaccumulators have different strategies of detoxification for metals that are hyperaccumulated compared with nonhyperaccumulated metals. For the hyperaccumulated metals, detoxification is mainly based on active sequestration into the vacuoles of the epidermis, where they are stored only loosely associated with organic acids that are abundant in this organelle (Küpper et al. 2004). Strong ligands such as the phytochelatins and metallothioneins that detoxify heavy metals in nonaccumulator plants do not play a major role in the detoxification of hyperaccumulated metals in hyperaccumulator plants (Küpper et al. 2009; Mijovilovich et al. 2009). At the same time, Ni and Zn are possibly transported by similar ligands and thus competition for binding sites is likely to be a factor in the relationship between uptake of Zn and Ni when levels of both are high in the soil (Assunção et al. 2003).

13.4.2 Metal Transport, Distribution, Compartmentalization

Plants developed different strategies to grow on soils rich in heavy metals. Many of them, the excluders, are able to restrict root uptake, and in particular, root-to-shoot translocation of heavy metals (Baker 1981; Küpper et al. 2000, 2001). On the opposite side, hyperaccumulators store high amounts of heavy metals in the above-ground parts. Understanding how metal transport and accumulation processes differ between normal and hyperaccumulator plants is important. “Normal” nonhyperaccumulator plants tend to store the absorbed heavy metals in the roots, whereas hyperaccumulator plants are capable of transporting most of the accumulated heavy metals to the shoots (Lasat et al. 1998, 2000). Hyperaccumulator plants exhibit stronger influx of heavy metals into the roots than the nonaccumulator species do (Lasat et al. 1996). Studies examining the competitive effects between Zn and Cd transport in *Thlaspi caerulescens* suggested that, at least in the leaf, the heavy metal Cd is transported via cellular Zn transporters (Cosio et al. 2004). While Zn and Cd are transported via the same transporter in the case of the Prayon ecotype, Cd transport in the Ganges ecotype (which hyperaccumulates Cd to a higher degree) takes place through a separate transporter (Lombi et al. 2001). In contrast, in nonaccumulator plants, Zn and Cd uptake and accumulation are negatively correlated (Hart et al. 2002; Wu et al. 2003). Papoyan et al. (2007) suggested that xylem loading may be one of the key sites responsible for the hyperaccumulation of Zn and Cd accumulation in *Thlaspi caerulescens*. Interestingly, the expression of one of the metal transporter involved, (HMA4) in *Arabidopsis thaliana* is downregulated upon exposure to heavy metals (Mills et al. 2003), while its expression in *T. caerulescens* is upregulated upon exposure to high concentrations of Cd and Zn (Papoyan and Kochian 2004). In a quantitative study of cell compartmentation, Küpper et al. (2000) showed that the major storage site for Zn and Cd hyperaccumulation in *Arabidopsis halleri* are the leaf mesophyll cells. In *Sedum alfredii* Hance, altered Zn transport across the tonoplast in the root stimulate Zn
uptake in the leaf cells, being the major mechanisms involved in the strong Zn hyperaccumulation observed (Yang et al. 2006). At the cellular level, a major role has been ascribed to vacuolar compartmentalization of excess cytosolic metals. Likewise, hyperaccumulation is connected to the ability to transport large amounts of metals into leaf vacuoles (Krämer et al. 2000; Persans et al. 2001). Shira et al. (2009) investigated the transport properties of the Slow Vacuolar (SV) channel identified in leaf vacuoles of Alyssum bertolonii Desv. Accumulation of Ni in epidermal cells seemed to be a common feature in the leaves of Ni-hyperaccumulators, such as Thlaspi goesingense, Alyssum species and Berkheya codii (Broadhurst et al. 2004; Kerkeb and Krämer 2003; Krämer et al. 1996; Küpper et al. 2000, 2001; McNear et al. 2005). The sequestration of toxic metals in leaf epidermis is not a universal detoxification mechanism in all hyperaccumulators. Cu in Elsholtzia splendens (Shi et al. 2004) and Mn in Gossia bidwillii (Fernando et al. 2006) seem to be more abundant in the mesophyll than in the epidermis of leaves. The cellular distribution of Pb in leaves is less studied. The Pb-As co-hyperaccumulator Viola principis H. de Boiss has similar Pb and As compartmentalization patterns in the leaves, as both elements accumulate in the bundle sheath and the palisade mesophyll, while the vacuolar bundle and the epidermis contained lower levels of Pb and As. The palisade enrichment of Pb and As indicates that V. principis H. de Boiss. may have a special mechanism on detoxification of toxic metals within the mesophyll cells (Lei et al. 2008). Apart from complexation with organic ligands, cellular compartmentation is likely to be involved in heavy metal tolerance of the hyperaccumulators. Krämer et al. (2000) estimated that approximately 70% of Ni in Thlaspi goesingense was associated with the apoplast, while Küpper et al. (2001) reported that the majority of Ni was localized in the vacuoles in leaf tissue of Alissum lesbiacum and Thlaspi goesingense. This apparent discrepancy may be due in part to the different methods used to study Ni localization (Smart et al. 2007). The vacuole is the largest subcellular compartment, occupying 70–80% of the total volume of mature parenchymatous plant cells, and plays an important role in the storage of inorganic ions (Martinoia et al. 2007). It is rich in carboxylic acids, such as citric and malic acid, which may serve to chelate Ni with moderately high affinity (Saito et al. 2005; Smart et al. 2007). If the vacuole is a site of Ni sequestration in hyperaccumulators, some form of active transport will be required at the tonoplast membrane to move Ni into the vacuole against its electrochemical gradient. Ingle et al. (2008) highlighted a Ni\(^{2+/H^+}\) antiport system at the tonoplast of Alissum lesbiacum that can drive vacuolar accumulation of Ni via a secondary active transport mechanism.

### 13.5 Molecular Studies

Most hyperaccumulators are slow-growing plant species that do not generate significant shoot biomass. This is why they are used mainly as model systems for the investigation and identification of the underlying molecular and physiological
mechanisms of hyperaccumulation, with the ultimate goal of transferring these mechanisms to higher biomass plant species. A number of genes involved in the hyperaccumulating phenotype were identified. Among them, a major role for \textit{AhHMA4} in naturally selected Zn hyperaccumulation and associated Cd and Zn hypertolerance in \textit{Arabidopsis halleri} was demonstrated (Hanikenne et al. 2008). The function of identified genes was also checked by complementation analysis in model organisms such as \textit{Arabidopsis thaliana} or \textit{Saccharomyces cervisiae} (Table 13.2). The molecular basis of the widely used hyperaccumulator \textit{Thlaspi caerulescens} was thoroughly reviewed by Milner and Kochian (2008). More widely, systemic studies such as transcriptomic and proteomic studies are expected to shed light on the intricate mechanisms that lead to hyperaccumulation.

### 13.5.1 Transcriptomic Studies

A comparative transcriptome analysis between the Zn/Cd hyperaccumulator \textit{Arabidopsis halleri}, and the nonaccumulator \textit{Arabidopsis thaliana} using the gene chip arrays revealed that hyperexpression may be a general property of metal hyperaccumulators, as a large number of genes were found to be more highly expressed in \textit{A. halleri} compared with \textit{A. thaliana} (Becher et al. 2004; Weber et al. 2004). In a separate study, a set of candidate genes for Zn hyperaccumulation, Zn and Cd hypertolerance, and the adjustment of micronutrient homeostasis in \textit{A. halleri} was identified using a combination of genome-wide cross species microarray analysis and real-time PCR (Talke et al. 2006). In this study, 18 putative metal homeostasis genes were newly identified to be more highly expressed in \textit{A. halleri} than in \textit{A. thaliana}, and 11 previously identified candidate genes were confirmed (Talke et al. 2006). Interestingly, the study showed that in the steady state, \textit{A. halleri} roots, but not the shoots, act as physiologically Zn-deficient under conditions of moderate Zn supply. An analysis of the first EST collection from \textit{Thlaspi caerulescens} obtained from Zn-exposed roots and shoots revealed that \textit{T. caerulescens} expresses a relatively large number of genes, which are expressed at a very low level in \textit{A. thaliana}, and 8% of the total set of expressed uni-genes did not have an \textit{Arabidopsis} ortholog (Rigola et al. 2006). Interestingly, some of the genes shown to be more highly expressed in \textit{A. halleri} also exhibited elevated expression in \textit{T. caerulescens}, suggesting this set of genes might be important for hyperaccumulation in both plant species. Also, cDNA-AFLP analysis of inducible gene expression in the Zn hyperaccumulator \textit{Sedum alfredii} Hance under zinc induction was reported (Chao et al. 2010).
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<td>Arabidopsis thaliana</td>
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<td>Ni tolerance</td>
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<td>Expression in Ar resulted in a significant increase in both plant Ni tolerance and Ni accumulation in the shoot</td>
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<td>ATP-PRT cDNA/Alissum lesbiacum</td>
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<td>CPx-ATPase</td>
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<td>TcHMA4/Thlaspi caerulescens</td>
<td>Cd tolerance</td>
<td>CPx-ATPase</td>
<td>Expression in Tc is induced by both Zn-deficiency and high-Zn treatments, as well as in response to high Cd</td>
<td>Bernard et al. (2004), Papoyan and Kochian (2004)</td>
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<td>(metallothionein)/Thlaspi caerulescens</td>
<td>Cd and Cu tolerance</td>
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<td>Maintains normal Cu homeostasis under high cytoplasmic Cd and Zn</td>
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<td><em><em>TgMTP1</em>/Thlaspi goesingense</em>*</td>
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<td>Tolerance to Cd, Co, and Zn</td>
<td>Vacuolar metal ion transporter</td>
<td><em>TgMTP1</em> transcripts are highly expressed in <em>T. goesingense</em> compared with orthologs in the nonaccumulators <em>Arabidopsis thaliana</em>, <em>Thlaspi arvense</em>, and <em>Brassica juncea</em></td>
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<td>Tolerance to Ni</td>
<td>Vacuolar metal ion transporter</td>
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13.5.2 Proteomic Studies

Broader molecular characterization of hyperaccumulators has been performed mainly by transcriptomics. In proteomics, even though large and hydrophobic transporters and low abundant or small polypeptides may remain undetected in 2-DE-based proteomics, many other proteins, such as regulatory proteins and those contributing to stress protection that appear to have importance in the hyperaccumulation phenotype might be detected (Farinati et al. 2009; van de Mortel et al. 2006; Verbruggen et al. 2009). In addition, posttranscriptional regulation could be very important, and there is often no proportionality between the transcript and protein abundance. Different profiling techniques are thus clearly complementary, and the proteomics approach is of increasing interest in exploring the hyperaccumulation phenomenon. A proteomic analysis of the Ni hyperaccumulator plant *Alyssum lesbiacum* was carried out to identify proteins that may play a role in the Ni tolerance and accumulation characteristic. As very few polypeptides were found to change in abundance in root or shoot tissue after plants were exposed to conditions representing the optimum for growth and hyperaccumulation of Ni in the shoot, it was concluded that constitutively expressed genes may be sufficient to allow for effective chelation and sequestration of Ni without the need for additional protein synthesis (Ingle et al. 2005b). Tuomainen et al. (2006) identified differences in protein intensities among three *Thlaspi caerulescens* accessions with pronounced differences in tolerance, uptake and root-to-shoot translocation of Zn and Cd, noticing clearest differences mainly among the *Thlaspi* accessions, while the effects of metal exposures were less pronounced. When looking at protein profiles of *Thlaspi caerulescens*, two accessions, and lines derived from the two accession proteins that showed co-segregation with high or low Zn accumulation were manganese superoxide dismutase, glutathione S-transferase, S-formyl glutathione hydrolase (Tuomainen et al. 2010).

13.6 The Hyperaccumulation Concept Applied to Organisms Other than Plants

Searching through literature uncovers the fact that the heavy metal hyperaccumulation seems to be restricted to plants, as seen from the number of studies or from the unequivocal definition of a hyperaccumulator. Hyperaccumulation is a phenomenon that generated the idea of phytoremediation and phytoextraction, immediately related to eco-friendly bioremediation processes. Even if the large-scale application of hyperaccumulating plants in bioremediation is in its infancy, it is undeniable that this is somehow restricted to soils. Phycoremediation, or the use of algae in bioremediation is another increasing possibility that would enlarge the applicability to contaminated waters (Olguín 2003). Marine organisms are of increasing interest and exceptionally high levels of trace metals have been reported in specific tissues.
of certain *Polychaetes* (Gibbs et al. 1981; Ishii et al. 1994; Fattorini et al. 2005, 2010; Sandrini et al. 2006). In a remarkable review, Gifford et al. 2007 introduced the concept of zooremediation and defined an animal heavy metal hyperaccumulator by similitude with plants as animal species known to accumulate $>100$ mg kg$^{-1}$ Cd, Cr, Co or Pb; or $>1,000$ mg kg$^{-1}$ Ni, Cu, Se, As or Al; or $>10,000$ mg kg$^{-1}$ Zn or Mn. Even though this field would probably be limited to invertebrates for ethical reasons, emerging data are tempting and self-financing zoo remediation models such as pearl oysters, sponges are proposed as models (Gifford et al. 2007). Work concerning the use of zooremediation is less intense than in plants, one system is already in use for the recovery of Cd in waste scallop tissue: in scallops (Seki and Suzuki 1997; Shiraishi et al. 2003, http://www.unirex-jp.com/engcadmium/engcadmium.htm). Although microorganisms are the most common group of organisms used for bioremediation, biosorption is the main governing process used to remove heavy metals from contaminated sites. Manipulating heavy metal resistance of bacteria by overexpressing genes from hyperaccumulating plants has been reported (Freeman et al. 2005), but the term hyperaccumulation may be awkward to apply in this case. Molecular mechanisms concerning heavy metal metabolism are widely studied in bacteria, and expressing various bacterial genes into plants to obtain hyperaccumulating transgenics is a promising approach (Dhankher et al. 2002, 2003; Rugh et al. 1996). The genus *Saccharomyces* looks like the group to which the term is worth extrapolating, mainly because of the general tendency to extend plant molecular processes to model organisms such as *Saccharomyces cerevisiae* (Table 13.2). The ease of growth and the exceptional elegance of genetic manipulation make the yeast cells a versatile tool for biotechnology. *S. cerevisiae* is not a heavy metal accumulator, and attempts to obtain tolerant hyperaccumulating in *S. cerevisiae* yeast strains failed, noticing a biunivocal relationship tolerance-exclusion, hyperaccumulation-sensitivity. Nevertheless, there are studies indicating that heavy metal sensitive but accumulating *kamikaze* strains could be used for heavy metal bioremediation through bioaccumulation (Ruta et al. 2010). Moreover, the cell surface of yeast cells can be engineered for heavy metal increases absorption capacity using the molecular display (arming) technology in which heterologous proteins that are expected to offer novel functionality to cell wall can be expressed on the surface of the cell (Georgiou et al. 1997; Murai et al. 1997; Shibasaki et al. 2009). Using such technology, cells with improved heavy metal biosorption abilities were obtained (Kambe-Honjoh et al. 2000; Nakajima et al. 2001; Kotrba and Rumi 2010). Combining improved biosorbents with bioaccumulative induction may be the basis of obtaining hyperaccumulating yeast strains.

### 13.7 Conclusions

As heavy metal pollution poses serious problems and needs to be overcome in a friendly, noninvasive way, the extraordinary phenotype of hyperaccumulating plants seems to be the appropriate tool to design bioremediation systems capable...
of removing the contaminating heavy metals from various sites. In recent years, major scientific progress has been made in understanding the physiological and molecular mechanisms of metal uptake and transport in these plants. General metal hyperaccumulators, however, may be inadequate for bioremediation technology because of their small size and slow growth rates. At present, therefore, the focus is on searching for new metal hyperaccumulators or on trying to improve metal uptake in plants and other organisms using metal chelators and on producing transgenic organisms that show metal hyperaccumulation.

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