A long way ahead: understanding and engineering plant metal accumulation

Stephan Clemens, Michael G. Palmgren and Ute Krämer

Some plants can hyperaccumulate metal ions that are toxic to virtually all other organisms at low dosages. This trait could be used to clean up metal-contaminated soils. Moreover, the accumulation of heavy metals by plants determines both the micronutrient content and the toxic metal content of our food. Complex interactions of transport and chelating activities control the rates of metal uptake and storage. In recent years, several key steps have been identified at the molecular level, enabling us to initiate transgenic approaches to engineer the transition metal content of plants.

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The concept of phytoremediation has evoked considerable interest in plant metal accumulation [1]. Using hyperaccumulation as a means of cleaning up metal-contaminated soil and water has been proposed [2] based on well documented observations that several plant species not only tolerate otherwise toxic levels of certain metals in the soil but even hyperaccumulate them in their shoots [3]. Plants ideal for phytoremediation should possess multiple traits. They must be fast growing, have high biomass, deep roots, be easy to harvest and should tolerate and accumulate a range of heavy metals in their aerial and harvestable parts. To date, no plant has been described that fulfils all these criteria. However, a rapidly growing non-accumulator could be engineered so that it achieves some of the properties of hyperaccumulators. Recent progress in determining the molecular basis for metal accumulation and tolerance by hyperaccumulators has been significant, and provides us with a strong scientific basis to outline some strategies for achieving this goal.

Molecular understanding of plant metal accumulation determinants has numerous additional biotechnological implications. For instance, health-threatening human deficiencies in trace metals appear to be widespread in developing countries and possibly worldwide [4]. Engineering Zn accumulation in edible plant parts might help in enriching diets for Zn. Conversely, most of the toxic non-essential elements such as Cd enter the human body via plant-derived material (i.e. food or tobacco smoke) [5]. Because significant differences in Cd accumulation are known to occur between plant varieties, molecular identification of mechanisms governing this process could result in the development of useful markers, or of crops with reduced toxic metal content. Finally, because signal transduction cascades are hypothesised to have recruited metal homeostasis factors [6], a better understanding of plant metal handling might also lead to new insights into other fundamental aspects of plant physiology.

In this article, we are describing key aspects of our current understanding of plant metal accumulation determinants and selected engineering approaches to enhance metal accumulation capacity. The main focus is on Zn and Ni as the main hyperaccumulated metals, on Cu as a particularly toxic micronutrient, and on Cd, which is used as a ‘model’ non-essential metal of significant environmental relevance in many studies.

Metal homeostasis network

The existence of a complex metal homeostasis network has only recently come into focus [7,8]. It is a common characteristic of all life forms that some of the elements present in the environment are accumulated and others are rejected. The rates of accumulation are necessarily governed by physiological requirements rather than toxicity. Trace elements such as copper and zinc pose a specific dilemma to organisms. Their ions are essential for a vast number of metabolic processes yet are potentially dangerous. Both Cu(I) and Cu(II) ions show an extremely high affinity to various organic molecules. The redox-activity of Cu, which is exploited in many electron transfer reactions, for example, in photosynthesis, can lead to the generation of oxygen radicals. Among the divalent cations present in biological systems, Cd²⁺ ions, together with Ni²⁺ and Zn²⁺ ions, rank behind copper in their affinity for organic donors such as thiolates and amines [9]. Zn²⁺, in contrast to Ni²⁺, is widely used as a cofactor. Zinc ions are key structural or catalytic components in DNA binding proteins and hydrolytic enzymes, respectively. However, uncontrolled binding can result in the inactivation of proteins.

Thus, tight control and regulation of essential metal accumulation are vital at the organismal as well as at the cellular level. Metal requirements of different organelles vary tremendously because most Zn- or Cu-requiring proteins are located outside the cytoplasm. Coordinate function of uptake, buffering, translocation and storage processes is required to maintain essential metal concentrations in various tissues and compartments within the narrow physiological limits. Furthermore, because control of accumulation is imperfect, organisms have to cope with exposure to unwanted elements. A lack of specificity of uptake and distribution systems also leads to the accumulation of metals such as Cd, which is generally considered nonessential.
A fundamental aspect of transition metals is that during their passage through the plant only minute proportions, if any, are present as ‘free’ hydrated ions. Recent work has indicated that in yeast and bacterial cells, virtually all the cellular Cu and Zn, respectively, is bound to ligands and that any movement within the cell involves exchange reactions between ligands. This tight control over metal availability within the cell requires the existence of specific distribution systems for the trace elements. In the case of Cu, some of the distributors – the metallochaperones – have been identified in yeast, mammals and plants. For Zn, such molecules have yet to be identified.

Determinants of transition metal accumulation

Generally speaking, accumulation of a given metal is a function of uptake capacity and intracellular binding sites. In a multicellular organism, the situation is complicated by tissue- and cell-specific differences and also by intercellular transport. Figure 1 illustrates the processes that are assumed to be influencing metal accumulation rates in plants: mobilization and uptake from the soil, compartmentation and sequestration within the root, efficient of xylem loading and transport, distribution between metal sinks in the aerial parts, sequestration and storage in leaf cells. At every level, concentration and affinities of chelating molecules, as well as the presence and selectivity of transport activities, affect metal accumulation rates.

This tightly knit network of determinants of metal accumulation makes the development of feasible engineering approaches targeting the primary limiting steps a big challenge. Important insights have been obtained from the study of model metallophytes, often in comparison to closely related, non-metallophyte plants. The major model metallophytes being studied are various Zn-, Ni- or Cd-hyperaccumulating ecotypes of *Thlaspi caerulescens*, some Ni-hyperaccumulating taxa of the genus *Alyssum*, and Zn-hyperaccumulating populations of *Arabidopsis halleri*.

Mobilization

The elements essential for life are also among the most abundant on Earth. However, the actual bioavailability of some metals is limited because of low solubility in oxygenated water and strong binding to soil particles. Iron, the most extreme example, is mainly present as Fe(III), which forms insoluble hydroxides. Availability of Zn, which is required in similar quantities as Fe, is less restricted, whereas the bioavailability of some of the target metals in phytoremediation, particularly Pb, is limited.

With the notable exception of Fe, which is solubilized by either reduction to Fe(II) or extrusion of Fe(III)-chelating phytosiderophores, little is known about active mobilization of trace elements by plant roots. Possibly, mechanisms assisting in the acquisition of phosphorus contribute to increasing the bioavailability of certain micronutrients. Both the acidification of the rhizosphere and the exudation of carboxylates are considered potential targets for enhancing metal accumulation. Because organic acid secretion also protects plant roots from Al toxicity, transgenic plants were generated that express a bacterial citrate synthase. These plants were reported to show elevated citrate production and secretion, which resulted in enhanced Al tolerance. However, these results have now been questioned. Alternative approaches to increase carboxylate secretion include the engineering of phosphoenolpyruvate carboxylase, pyruvate dikinase or ATP-citrate lyase expression. Restricting the overproduction of organic acids to certain root zones by using specific promoters, as certain plants such as *Lupinus albus* are known to do, could significantly reduce the strain on plant resources. The same applies for the proposed modulation of plasma membrane H+-ATPase activity to support rhizosphere acidification. Regulated local increases in metal availability would also help to circumvent problems associated with applying chelators such as EDTA to the soil. The risk of this technique, which has also been applied in the phytoremediation of lead contaminations, is that metal-chelate complexes are leached into the groundwater.

Clearly, root-colonizing bacteria, as well as mycorrhiza, have a large impact on the availability of heavy metals for plant uptake. For instance, soil microorganisms significantly enhance Zn accumulation in the shoot of the hyperaccumulator *Thlaspi caerulescens*. Consequently, specific modifications of the rhizosphere could greatly enhance metal accumulation.

Uptake and sequestration

Following mobilization, a metal has to be captured by root cells. Metals are first bound by the cell wall, an ion exchanger of comparatively low affinity and low selectivity. Transport systems and intracellular high-affinity binding sites then mediate and drive uptake across the plasma membrane. Uptake of metal ions is likely to take place through secondary transporters such as channel proteins and/or H+-coupled carrier proteins. The membrane potential, which is negative on the inside of the plasma membrane and might exceed −200 mV in root epidermal cells, provides a strong driving force for the uptake of cations through secondary transporters.
Several cation transporters have been identified in recent years with the use of molecular techniques, largely owing to the complementation of *Saccharomyces cerevisiae* mutants. Most of the transporters thought to be involved in the uptake of micronutrients are in the ZIP (ZRT, IRT-like protein) and the Nramp (natural resistance-associated macrophage protein) family [21,22]. For some of them, expression in roots and up-regulation under deficiency conditions indicate a role in uptake from the soil. Direct evidence demonstrating the contribution of a specific transporter to transition metal acquisition is scarce.

From yeast studies, it is apparent that most of the cation transporters show a rather broad substrate range, enabling even non-essential metals such as Cd to enter cells. IRT1 expression, for instance, rescues Fe, Mn and Zn uptake-deficient *S. cerevisiae* strains and causes Cd hypersensitivity [23,24]. The wheat Ca transporter LCT1 mediates uptake of Na and Cd in yeast [25,26]. However, for phytoremediation, specificity and affinity of transport systems have to be considered in relation to relative abundance of different substrates. Non-target elements such as Ca might out-compete target elements. Also, little is known about the actual physiological substrates for uptake. For example, the metals might be taken up as hydrated ions and in metal-chelate complexes. The recent molecular identification of the first Fe-phytosiderophore transporter ysl1 from maize, and the finding that the *Arabidopsis* genome contains several ysl-like sequences [27], should help in addressing this question.

At least three different engineering approaches to enhance metal uptake can be envisioned. The number of uptake sites could be increased, the specificity of uptake systems could be altered so that competition by unwanted cations is reduced, and the sequestration capacity could be enhanced by increasing the number of intracellular high-affinity binding sites or the rates of transport into organelles. A correlation between uptake capacity and hyperaccumulation was found for ZIP family members in *Thlaspi caerulescens*. Under Zn-replete conditions, ZNT1 and ZNT2 are expressed at significantly higher levels in roots of different *T. caerulescens* accessions than in roots of the non-hyperaccumulating relative *Thlaspi arvense* [28,29]. This observation fits well with the four-to-fivefold higher Zn uptake measured for *T. caerulescens* roots [30]. Thus, overexpression of uptake systems might result in enhanced accumulation of transition metals. Substrate specificity can be engineered. By substituting various conserved residues in ZIP family transporters with alanine produces mutant versions of IRT1 that apparently no longer transport Fe, Mn and Zn yet retain Zn transport activity [24].

Intracellular binding and sequestration drive the passage of transition metal ions across the plasma membrane. Several processes known to contribute to metal tolerance are associated with metal accumulation at the same time. *S. cerevisiae* cells synthesizing phytochelatins, glutathione-derived metal-binding peptides, show significantly higher Cd tolerance and increased Cd accumulation even at subtoxic concentrations [31]. Phytochelatin synthesis is the principal response of plants and many fungi to toxic metal exposure, and apparently also of certain animals [32–34]. Phytochelatin deficiency is correlated with Cd, Cu and As hypersensitivity. Therefore, numerous attempts have been made to boost phytochelatin formation by overexpressing enzymes involved in the synthesis of the phytochelatin precursor glutathione. In some cases, this led to a slight enhancement of Cd tolerance and accumulation [35], whereas in others it did not [36]. In spite of the recent cloning of phytochelatin synthase genes (PCS) [31,37,38], the effects of PCS overexpression have not been reported in plants. It remains to be determined which step limits the rate of phytochelatin formation and whether engineering phytochelatin synthesis can indeed be exploited to enhance tolerance and accumulation. The best target might be the transport of phytochelatin-metal complexes into the vacuole, which in fission yeast is mediated by an ABC-type transporter, Hmt1 [39]. However, in plants the corresponding proteins have yet to be identified.

Other potential mediators of metal sequestration and accumulation include members of the Cation Diffusion Facilitator family (CDF). In eukaryotic systems, they have been implicated in moving Zn, Cd and Co from the cytosol into cellular compartments [40]. Studies on various hyperaccumulators and their respective close relatives show a possible correlation between differences in either expression levels or substrate specificities of CDFs and the hyperaccumulation phenotype [29,41]. *Arabidopsis* plants overexpressing the transporter ZAT become more Zn tolerant and accumulate more Zn [42]. Thus, engineering metal-tolerance mechanisms, such as the formation of metal-chelating molecules or transporter-mediated vacuolar sequestration, might result in enhanced accumulation.

**Xylem transport**

The apoplastic continuum of the root epidermis and cortex is readily permeable for solutes. The cell walls of the endodermal cell layer act as a barrier for apoplastic diffusion into the vascular system. In general, solutes have to be taken up into the root symplasm before they can enter the xylem [43]. Subsequent to metal uptake into the root symplasm, three processes govern the movement of metals from the root into the xylem: sequestration of metals inside root cells, symplastic transport into the stele and release into the xylem. The transport of ions into the xylem is generally a tightly controlled process mediated by membrane transport proteins [44]. The metal transporters involved in xylem loading have yet to be identified.
Inside the root, the presence of elevated amounts of exogenously supplied histidine, which is able to chelate Ni²⁺, results in a 50-fold increase in the rate of transport of Ni into the xylem of *Alyssum montanum* [45]. In agreement with this, several Ni hyperaccumulators respond to Ni²⁺ exposure by a large dose-dependent increase in histidine concentrations in the xylem. Thus, chelation with certain ligands, for example histidine, nicotianamine [46] and citrate [47], appears to route metals primarily to the xylem. By contrast, chelation with other ligands, such as phytochelatins or metallothioneins, might route metals predominantly to root sequestration [48]. Inside the xylem, a pH-dependent equilibrium exists between low-molecular weight chelators, free hydrated metal cations and metal chelates in the mobile transpiration stream, and stationary metal-binding sites in the cell wall material surrounding the xylem vessels [49]. Xylem-unloading processes are the first step in controlled distribution and detoxification of metals in the shoot, as well as in a possible re-distribution of metals via the phloem [50].

A promising engineering approach aimed at enhancing xylem transport rates of metals is to increase the steady-state concentrations of a chelator that promotes root-to-shoot mobility of a metal. With this aim, transgenic plants have been generated that contain elevated levels of wild-type or mutant ATP phosphoribosyltransferases, catalyzing the first rate-limiting step in the histidine biosynthetic pathway (U. Krämer *et al.*, unpublished). Because an elevated histidine content, whether of exogenous or endogenous origin, is known to increase Ni tolerance [45], this approach targets at least two of the determinants of plant metal accumulation. The identification of novel proteins involved in root-to-shoot transport of metals or its regulation should provide researchers with novel targets for engineering metal accumulation.

**Unloading, trafficking and storage**

Transition metals reach the apoplast of leaves in the xylem sap, from where they have to be scavenged by leaf cells [12]. Transporters mediate uptake into the symplast, and distribution within the leaf occurs via the apoplast or the symplast [51]. Trafficking of metals occurs inside every plant cell, maintaining the concentrations within the specific physiological ranges in each organelle and ensuring delivery of metals to metal-requiring proteins. Metallochaperones, such as CCH1, [52] and pumps, such as P-type ATPases, are probably involved in these processes. For instance, it has been suggested that RAN1 pumps Cu into post-Golgi vesicles and is required for Cu delivery to the ethylene receptor and other proteins [53, 54].

In the *Arabidopsis* genome, there are several additional P-type ATPase genes encoding pumps of unknown specificity and unknown subcellular localization. Some of these resemble bacterial pumps transporting heavy metals such as Ag, Zn, Co, Cd and Pb [55].

Excess essential metals, as well as non-essential metals, are sequestered in leaf cell vacuoles [56, 57]. Intriguingly, different leaf cell types show pronounced differential accumulation. The distribution pattern varies with plant species and element. Zn accumulation in *T. caerulescens* leaves is 5.0–6.5-fold higher in epidermis cells than in mesophyll cells [58], whereas in metal-treated *A. halleri*, the mesophyll cells are thought to contain more Zn and Cd than the epidermal cells [59]. Furthermore, trichomes apparently play a major role in storage and detoxification of metals. For the phytoremediation candidate species *Brassica juncea*, Cd accumulation was reported to be >40-fold higher in trichomes compared with the leaf total [60]. A significant proportion of Ni and Zn are found in trichomes of the hyperaccumulators *Alyssum lesbiacum* [61] and *Arabidopsis halleri* [58], respectively.
Molecularly, the factors governing differential metal accumulation and storage are unknown. It is likely that vacuolar uptake is important. Therefore, engineering vacuolar transporters, preferably in specific cell types, might result in significantly increased accumulation rates. Alternatively, creating artificial metal sinks in the shoot by enhancing the number of metal-binding sites, via expression of cell wall proteins with high-affinity metal-binding sites for instance, might suggest to the plant metal homeostatic network an increased metal demand in the shoot, resulting in enhanced root-to-shoot translocation. Also, there might be a need to concomitantly repress metal sinks in the roots, for example, by decreasing transport into root vacuoles.

There are numerous checkpoints along the way (Fig. 2) as a transition metal makes its way from the soil to the storage sites in the leaf. We now have some molecular understanding of the key processes in metal homeostasis in general, and of the phenotype of metallophytes in particular. We are beginning to understand the trafficking of metals at the cellular level and the roles of increased uptake rates and chelator synthesis in hyperaccumulation. However, many steps of the way remain enigmatic. The transport and storage forms of transition metals are largely unknown. Many transport systems have yet to be analyzed with respect to mechanism, localization, function and structure. Key components, such as proteins involved in xylem loading and the differential distribution between leaf cells, have not been identified.

References
15 Delhaize, E. et al. (2001) Expression of a Pseudomonas aeruginosa citrate synthase gene in tobacco is not associated with either enhanced citrate accumulation or efflux. Plant Physiol. 125, 2059–2067

20 Hirsch, R.E. et al. (1998) A role for the AKT1 potassium channel in plant nutrition. Science 280, 918–921
23 Korshunova, Y.O. et al. (1999) The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. Plant Mol. Biol. 40, 37–44
31 Clemens, S. et al. (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J. 18, 3325–3333
34 van der Zaal, B.J. et al. (1999) Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiol. 119, 1047–1056

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Impact of phyto-oxylipins in plant defense

Elizabeth Blée

Phyto-oxylipins are metabolites produced in plants by the oxidative transformation of unsaturated fatty acids via a series of diverging metabolic pathways. Biochemical dissection and genetic approaches have provided compelling evidence that these oxygenated derivatives actively participate in plant defense mechanisms. During the past decade, interest in this field was focused on the biosynthesis of jasmonic acid (one branch of C18 polyunsaturated fatty acid metabolism) and on its relationship to the other plant defense-signaling pathways. However, recently, antisense strategies have revealed that oxylipins other than jasmonates are probably also essential for the resistance of plants to pathogens.

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Intriguing analogies can be found between the biosynthesis and the functions of oxylipins in vertebrates and plants. Oxylipins are produced during the metabolism of unsaturated fatty acids via their oxidation by one, two or four atoms of oxygen catalyzed by cytochrome P450, lipoxygenase (LOX) and cyclo-oxygenase-like activities, respectively (Fig. 1). In mammals, oxylipins are formed mainly via the arachidonic acid cascade (a C20 fatty acid); they play a major role in inflammatory processes and, in general, in stress responses to infection, allergy and exposure to food, drugs and environmental xenobiotics.

Plants are devoid of the intricate protective mechanisms provided by the immune system in animals. Nevertheless, they have evolved an efficient armory of defense systems against attacks by pathogens or other life-threatening events. These means of defense can be constitutive and/or inducible, and include physical barriers and production of toxic compounds. Phyto-oxylipins are believed to play a pivotal role in these defenses because they act as signal molecules and/or protective compounds such as antibacterial and wound-healing agents, or as constituents of cutin (the framework of the cuticle that protects all the aerial parts of a plant from its environment). Plants can respond in different ways to the various stimuli that result in tissue damage caused by abiotic factors or pathogens. These stress responses usually include the production of specific oxylipins, which have many different biological functions. Therefore, knowledge of the processes that regulate the biosynthesis and localization of such bioactive lipid derivatives in plant cells is crucial to the understanding of the biology of this still-growing family of metabolites. Recent studies, including the characterization of new catalytic activities and the cloning of some important biosynthetic enzymes, have provided new insights into these processes. Here, these recent findings are reviewed and the possible regulation of the phyto-oxylipins biosynthetic pathways during stress conditions is discussed.