

DEVELOPING TOOLS FOR PHYTOREMEDIATION: TOWARDS A MOLECULAR UNDERSTANDING OF PLANT METAL TOLERANCE AND ACCUMULATION

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Abstract. Certain plant species and genotypes are able to accumulate large quantities of heavy metals in their shoots. Based on this trait the concept of phytoremediation was developed, i.e. the use of metal hyperaccumulating plants for the cleansing of contaminated soils and water. In order to more efficiently use this capacity, an engineering of plants might be needed. However, very little is known about the underlying molecular mechanisms. Our work is focussing on the identification and characterization of plant genes involved in plant metal uptake, tolerance and accumulation.

Phytochelatinins are small glutathione-derived metal-binding peptides which are part of the plant metal detoxification system. Genes encoding phytochelatin synthases have been cloned and are now being studied with regard to their regulation, biochemistry and biotechnological potential. Another project aims at the dissection of metal responses in the metallophyte *Arabidopsis halleri*. This plant, a close relative to the model plant *Arabidopsis thaliana*, is Cd hypertolerant and Zn hyperaccumulating. It grows, for instance, on medieval mining sites in the Harz mountains in Germany and in many other metal-contaminated sites in Central Europe. We have isolated metal-regulated genes from *A. halleri* and molecularly analyzed interesting candidate genes with regard to their function and involvement in metal accumulation and tolerance.

Key words:

Plants, Metal tolerance, Metal accumulation, Phytoremediation

INTRODUCTION

Anthropogenic contamination with heavy metals began centuries ago. In Central Europe, for instance, mining activities during the Middle Ages left a number of metal-enriched sites behind. In such areas, and in soil that naturally contains high levels of metals, e.g. Zn-rich calamine soil or Ni-rich serpentine soil, a specialized vegetation evolved [1]. Plant species belonging to this flora display naturally selected higher levels of metal tolerance (= hypertolerance). Mostly, this hypertolerance is specific for certain metals.

Some plants not only tolerate higher levels of metals but hyperaccumulate them. About 400 different species belonging to a wide range of taxa have been described as hyperaccumulators [2]. Hyperaccumulators generally refer to plants able to accumulate >0.1% of dry weight of elements such as Ni, Co or Pb. For Zn the limit is >1%, and for Cd >0.01% of dry weight. Hyperaccumulation is mainly observed with Ni, Zn, Co and Se. Also, four hyperaccumulators of Pb and one hyperaccumulator each for Cd and As have been identified. It is interesting to note

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that approximately 75% of the hyperaccumulators characterized to date have been Ni hyperaccumulators [2].

The hyperaccumulation phenotype has recently attracted considerable attention and is the basis for the concept of phytoremediation, i.e. the use of metal hyperaccumulating plants for cleansing of metal-contaminated soil (and water) [3,4]. Phytoremediation is now considered a potential low-cost and environmentally friendly alternative to soil replacement, excavation and washing techniques. Initial field trials are under way now in different countries [e.g. 5]. In order to use more effectively phytoremediation approaches, an engineering of suitable plants will most likely be desirable [6].

Increasing the metal accumulation capacity of plants requires the molecular understanding of hyperaccumulation. However, very little is known to date about the five basic aspects of metal accumulation: mobilization, uptake, translocation within the plant, sequestration, and tolerance (Fig. 1). Mobilization of the more tightly bound fraction of metal ions from the soil represents one of the major limitations of phytoextraction of metals. Various projects are devoted to increasing mobilization by soil amendments such as EDTA or biosurfactants [7,8]. Uptake of solubilized metal ions is mediated by specialized transporters residing in the plasma membrane. Non-essential metal ions are likely to enter cells through cation transporters with a broad substrate specificity [9]. In recent years, a large number of plant metal transporters have been cloned based on the complementation of respective yeast mutants. Transporters of the ZIP family, for instance, which were first identified in plants and subsequently in other eukaryotes including humans [10], contribute to the uptake of Zn^{2+} and Fe^{2+} . Nramp transporters mediate uptake of Fe^{2+} , Mn^{2+} and other metal ions in yeast, plants and mammals [11,12]. Studies on the hyperaccumulator species *Thlaspi caerulescens* and its non-hyperaccumulating relative *T. arvense* indicate that increased expression of an uptake system may represent one of the factors determining metal accumulation rates [13].

The molecular mechanisms governing the translocation of metal ions from root tissue to the above-ground parts are poorly understood. What we know is, that there is a huge

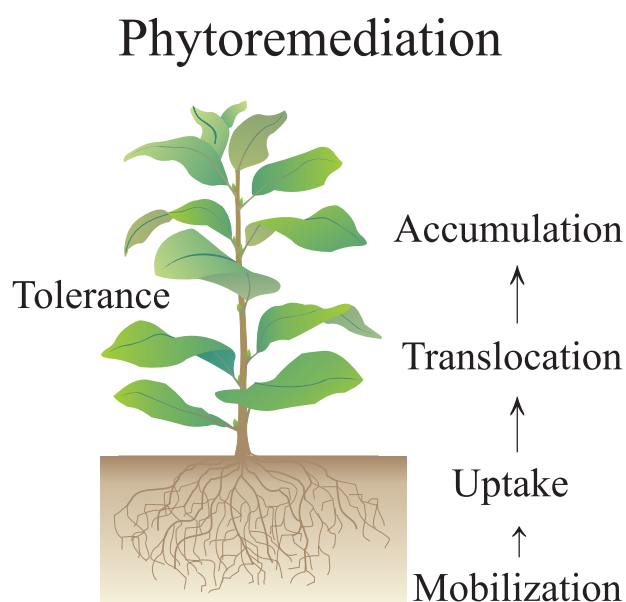


Fig. 1. The fundamental aspects of metal hyperaccumulation and its use for phytoremediation.

variation among plant species and even genotypes with respect to the root/shoot ratios of metal accumulation [14]. Tobacco leaves, for example, contain comparatively high amounts of Cd, which is the reason why tobacco smoke is a major route of Cd intake for humans [15]. A molecular understanding of metal translocation will potentially be beneficial not only for increasing metal accumulation rates in plants used for phytoremediation, but also for reducing the entry of toxic metals into crops. Metal sequestration and tolerance are related traits. Metal tolerance is a prerequisite for metal accumulation. It is mainly dependent on the chelation of metal ions in the cytosol and subsequent removal from the cytosol. In plant cells, large vacuoles – which are analogous to lysosomes – serve as storage sites for all kinds of toxic compounds. The best-studied response in plant cells to metal exposure is the formation of phytochelatins (PCs) [16,17]. PCs (named cadystins in *S. pombe*) (18) are small metal-binding peptides of the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2\text{--}11$) (Fig. 2), which are non-translationally synthesized by the enzyme phytochelatase [19] from glutathione as the precursor. Cd-PC complexes are transported into the vacuole, where high molecular weight complexes are formed under incorporation of sulfide [17]. The contribution of PC formation to Cd tolerance of

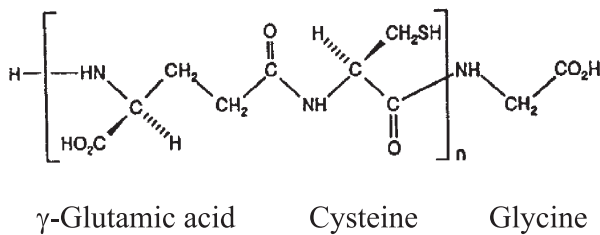


Fig. 2. Chemical structure of phytochelatins (PCs), glutathione-derived metal-binding peptides.

plants became apparent with the isolation of the *Arabidopsis* mutant *cad1*, which is PC-deficient and Cd-hypersensitive [20].

Phytochelatin formation is not the only tolerance mechanism. Like mammalian systems, plants express metallothioneins, which confer Cd or Cu tolerance when expressed heterologously. The actual function, however, has yet to be determined – again like for mammalian cells [21]. Other potential components include efflux pumps and vacuolar transporters. In eubacteria, those efflux pumps constitute the main metal tolerance mechanism. Most of the bacterial metal transporters belong to the family of CPx-type ATPases, a subclass of the P-type ATPases [22]. Well-studied examples are the Cd²⁺-specific pump CadA from *Staphylococcus aureus* and the Cu pumps CopA and CopB from *Enterococcus hirae*. Genes with similarity to those pumps have been found in the *Arabidopsis* genome. Vacuolar sequestration of metals plays a pivotal role for metal tolerance. Cd, Zn and Ni ions are transported into the vacuole. Candidates for the respective transport activities are members of the Cation Diffusion Facilitator family, present in prokaryotes and eukaryotes [23,24], or possibly metal/proton antiporters such as the CAX transporters [25,26].

THE CLONING OF PLANT METAL TOLERANCE GENES

The lack of knowledge about molecular components of plant metal tolerance led us to initiate a search for respective genes by expression cloning in *S. cerevisiae*. From a wheat root cDNA library we isolated a cDNA that conferred a 20-fold increase in Cd²⁺ tolerance when

expressed in baker's yeast [27]. Homologs of this cDNA, now called *TaPCS1*, were found in *Arabidopsis thaliana* and *Schizosaccharomyces pombe* (fission yeast) thanks to the genome sequencing programs. After cloning the *Arabidopsis* and the fission yeast homolog they were also expressed in *S. cerevisiae* and found to generate a similar Cd²⁺ tolerance phenotype. These PCS genes constitute a new family of genes with no significant homology to other known gene families.

TaPCS1 was characterized further in *S. cerevisiae* cells. Cd²⁺ accumulation experiments showed that TaPCS1-expressing cells accumulate about 40–50% more Cd²⁺ than control cells in the presence of non-toxic Cd²⁺ concentrations. This indicated, that TaPCS1-mediated Cd²⁺ tolerance is probably not attributable to efflux activity. It also demonstrated that the PCS genes potentially represent valuable tools for phytoremediation. Heterologous expression results in increased tolerance and accumulation. A series of experiments involving various yeast vacuolar mutants demonstrated that TaPCS1-expression results in elevated Cd²⁺ tolerance even in the absence of a pH gradient across the vacuolar membrane or the absence of functional vacuoles altogether.

In order to examine the physiological relevance of PCS genes for metal tolerance, we took advantage of the fact that there is only one PCS homolog in the *S. pombe* genome. A knock-out strain was constructed by disruption of the *SpPCS* gene via homologous recombination. This strain displayed enhanced growth inhibition in the presence of Cd²⁺, thereby clearly demonstrating the contribution of PCS genes to Cd²⁺ tolerance not only when overexpressed in a heterologous system. Also, the $\Delta SpPCS$ cells provided a valuable tool for additional functional studies. The indications for a PCS-dependent catalytic process in the cytosol pointed towards the synthesis of phytochelatins, even more so as the gene encoding phytochelatin synthase had not been isolated to that date.

When challenged with Cd²⁺ or other metal ions, *S. pombe* cells start to form phytochelatins. These can be detected by a fluorescence detector following derivatization with monobromobimane and separation by HPLC. Predominantly formed are PC2 and PC3. Both were found

to be completely absent from extracts of Cd²⁺-treated $\Delta SpPCS$ *S. pombe* cells. In the reverse experiment, Cd²⁺-treated *S. cerevisiae* cells, which under the experimental conditions used do not produce detectable amounts of PCs (and lack a *PCS* homologous gene), do form phytochelatin when they express *PCS* genes. To prove that the *PCS* associated PC formation is directly attributable to *PCS* enzyme activity, SpPCS was expressed in the $\Delta SpPCS$ strain with a hemagglutinin (HA) tag. The recombinant protein was purified by affinity chromatography on a column with a monoclonal anti-HA antibody coupled to sepharose. It was shown to possess *PCS* activity.

With that, this new family of metal tolerance genes was assigned a function. Two groups independently arrived at the same conclusion and at about the same time. Ha et al. [28] positionally cloned the *cad1* gene from *Arabidopsis*. It is identical to the *AtPCS1* gene isolated by Vatamaniuk et al. [29] from a yeast expression library following a strategy similar to the one outlined above. It is interesting to note, that *PCS* homologs are present in the *C. elegans* genome [27]. This might indicate, that PCs could play a role for metal tolerance also in animals.

MOLECULAR DISSECTION OF METAL HYPERACCUMULATION

PCS genes are ubiquitous in the plant kingdom. However, as mentioned above, metallophytes growing on metal-contaminated soil display a degree of metal tolerance by far exceeding that of normal plants. In order to identify molecular components of plant hypertolerance and hyperaccumulation we are establishing *Arabidopsis halleri* (syn. *Cardaminopsis halleri*) as a model system to study plant metal responses. *A. halleri* is a close relative of the model species of plant molecular genetics, *A. thaliana*. It grows on metal-contaminated sites in Central and Eastern Europe. For instance, it can be found on medieval mining sites in the Harz mountains. *A. halleri* is a known Zn hyperaccumulator [30] and also significantly more Cd²⁺ tolerant than *A. thaliana*. One of the approaches we have been following is the identification of metal-regulated

genes in *A. halleri* by cDNA-AFLP [31]. Followed by *in silico* analysis and confirmation of expression patterns by RT-PCR, this has led to identification of a number of putative signal transduction components such as MAP kinases, protein phosphatases and transcription factors (Vess and Clemens, unpublished data). These findings will hopefully enable us to contribute to the understanding of plant metal signal transduction, as well as plant metal tolerance and accumulation.

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