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Possible Molecular Mechanisms Involved in Nickel, Zinc and Selenium Hyperaccumulation in Plants

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Introduction

Most hyperaccumulator species are able to accumulate between 1–5% of their biomass as metal. However, these plants are often small, slow growing, and do not produce a high biomass. Phytoextraction, a cost-effective, *in situ*, plant-based approach to soil remediation takes advantage of the remarkable ability of hyperaccumulating plants to concentrate metals from the soil and accumulate them in their harvestable, above-ground tissues (Salt *et al.*, 1998). However, to make use of the valuable genetic resources identified in metal hyperaccumulating species, it will be necessary to transfer this material to high biomass, rapidly growing crop plants (Salt *et al.*, 1998). These plants would then be ideally suited to the phytoremediation process, having the ability to produce a large amount of metal-rich plant biomass for rapid harvest and soil cleanup.

It is becoming clear that the hyperaccumulator plant's genetic material could also be very valuable in enhancing the nutritional value of human foodstuffs. Malnutrition remains one of the most serious problems facing mankind and, although remarkable improvements in crop productivity have been made over the last twenty years, it is now clear that this has been made at the expense of the nutritional value of the foodstuff produced. Deficiencies in such micronutrients as iron, zinc, selenium, iodine and vitamin A are often referred to as the 'hidden hunger'. Substantial efforts

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Abbreviations: NAS, nicotianamine synthase; NAAT, nicotianamine aminotransferase; ZRT, zinc-regulated transporter; IRT, iron-regulated transporter; ZIP, ZRT, IRT like protein; ZAP, zinc-responsive activator protein; ZRE, zinc responsive element; SMF, suppressor of mif1; BSD, bypass SOD deficiency; Nramp, natural resistance associated macrophage protein; THG, *Thlaspi goesingense* hisG gene; THD, *Thlaspi goesingense* hisD gene; THB, *Thlaspi goesingense* hisB gene; ZnT, zinc transporter; ZAT, zinc transporter *Arabidopsis thaliana*; MTP, metal transporter protein; atMTP, *Arabidopsis thaliana* metal transport protein; XAS, X-ray absorption spectroscopy; PC, phytochelatin; SAT, serine acetyltransferase; SMT, selenocysteine methyl transferase; ATP, adenosine triphosphate.

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are now being made to rectify this situation, with the development of nutrient-fortified food crops. Hyperaccumulator plants are known that accumulate zinc and selenium, elements known to play very important roles in human health. Clearly, the genetic material governing the hyperaccumulation of these metals will provide a mechanism for the manipulation of both the concentration and chemical form of these elements in human food crops. These developments should lead to real gains in human health in the future.

Definition and physiology of hyperaccumulator plants

DEFINITION OF METAL HYPERACCUMULATION

Brooks *et al.* (1977) first defined a nickel hyperaccumulator as a plant that can accumulate, in its natural environment, a shoot nickel concentration of at least 1,000 μg nickel/g dry biomass (0.1%). This value is also applicable to cobalt and copper accumulators, and a base value for zinc has been set at 10,000 μg/g dry biomass (Baker and Brooks, 1989). These values are at least one order of magnitude higher than the concentrations of these metals found in non-accumulator species. Although there is some debate about the magnitude of these values, they are generally agreed upon as being indicative of a hyperaccumulating species. Characteristically, hyperaccumulators have a higher concentration of metal present in the shoot compared to the root. Based on this physiological property, it has been proposed that hyperaccumulating species be defined as plants that contain a shoot to root metal concentration ratio greater than 1, whereas the shoot to root metal ratio of a non-accumulator species would be less than 1 (Baker, 1981). Currently, nickel, zinc, cobalt, lead, copper, manganese, and selenium hyperaccumulators are known in nature (Baker and Brooks, 1989; Brown and Shrift, 1982). However, the validity of the lead and copper hyperaccumulators has been questioned and needs further study (Huang and Cunningham, 1996; Andrew J.C. Smith, personal communication).

The metal hyperaccumulation phenotype can be quite dramatic. This is well illustrated by the nickel hyperaccumulating tree, *Serbertia acuminata*. This tree, a native of New Caledonia, can accumulate up to 37 kg of nickel in a mature 15 metre tall tree (Sagner *et al.*, 1998). About 400 taxa of plants have been identified as having this extraordinary hyperaccumulation ability (Brooks, 1998). Approximately 75% of all known hyperaccumulators accumulate nickel (Baker and Brooks, 1989). In contrast, only about 4% of hyperaccumulator plant species have been identified as zinc hyperaccumulators (Baker and Brooks, 1989). The genus *Alyssum* contains the most nickel hyperaccumulating species, with over 48 currently known (Reeves, 1992). Certain field collected *Alyssum* species have been recorded with shoot nickel concentrations as high as 29,400 μg/g (Baker and Brooks, 1989), and this nickel accumulation capacity has been confirmed in laboratory studies (Krämer *et al.*, 1996).

The genus *Thlaspi* is also of interest because it contains species that accumulate nickel and others that accumulate zinc. For example, field collected specimens of *Thlaspi goesingense, Thlaspi montanum* var. *montanum*, and *Thlaspi montanum* var. *siskiyouense* have been recorded with shoot nickel concentrations as high as 12,000, 5,530, and 24,600 μg nickel/g dry biomass (1.2%, 0.55%, 2.46%), respectively (Baker and Brooks, 1989; Reeves, 1988). Recently, the hyperaccumulator status of

these species has been confirmed under laboratory conditions (Krämer *et al.*, 1997b; Boyd and Martens, 1998; Heath *et al.*, 1997). A direct comparison of the nickel accumulation capacity of *T. goesingense* and the common weedy species *Thlaspi arvense* revealed that the hyperaccumulator *T. goesingense* can accumulate 4-fold more nickel than the non-accumulator *T. arvense* over the same time period (Krämer *et al.*, 1997b).

Field specimens of the closely related zinc hyperaccumulator *Thlaspi caerulescens* have been recorded with shoot zinc concentrations as high as 27,300 μg/g (Baker and Brooks, 1989). Laboratory studies with this plant have shown that it is capable of accumulating up to 25,000–30,000 μg zinc/g dry biomass (2.5–3.0%) in its shoots without showing any symptoms of zinc toxicity (Brown *et al.*, 1995a,b; Lasat *et al.*, 1996; Shen *et al.*, 1997). When compared to non-accumulator species such as *T. arvense* (Lasat *et al.*, 1996) and *Thlaspi ochroleucum* (Shen *et al.*, 1997), under laboratory conditions, *T. caerulescens* accumulates 4-fold more zinc in the shoots than these other closely related non-accumulator species (Shen *et al.*, 1997; Lasat *et al.*, 1996).

Selenium accumulating plants are also known. Of particular note are several species of the genus *Astragalus*, specimens of which have been recorded to contain up to 6,000 μg/g selenium in their dry shoot biomass (Byers, 1936). Interestingly, the selenium in these species is predominantly as methylselenocysteine [CH_3 - Se - CH_2 - $CHNH_2$ - COOH] (Trelease *et al.*, 1960; Virupaksha and Shrift, 1965; Shrift and Virupaksha, 1965; Neuhierl and Böck, 1996).

This review will focus on plant species known to hyperaccumulate zinc, nickel and selenium. In particular, the zinc hyperaccumulator *T. caerulescens*, the nickel hyperaccumulator *T. goesingense*, and the selenium hyperaccumulator *Astragalus bisulcatus*. Because similar physiological and biochemical processes are known to be common to large groups of plants and other eukaryotic and prokaryotic organisms, we will also review mechanisms of metal uptake and transport in non-accumulator plants and other organisms.

BIOLOGICAL ROLE OF METAL HYPERACCUMULATION

There are several ideas that have been put forward on the biological role of metal hyperaccumulation. The most plausible of these is the suggestion that plants accumulate toxic metals or non-metals as a protective mechanism against herbivory (Reeves *et al.*, 1981). The most obvious example of this is the occurrence of 'alkali disease' in cattle that graze on the selenium enriched shoots of various selenium hyperaccumulator species (Davis, 1972). However, perhaps of more evolutionary significance is the protective effect nickel and zinc hyperaccumulation affords against bacterial and fungal pathogens (Boyd *et al.*, 1994) and insect herbivory (Boyd and Martens, 1994; Martens and Boyd, 1994; Pollard and Baker, 1997).

PHYSIOLOGY OF METAL HYPERACCUMULATION

Several steps are required to acquire, transport, and sequester hyperaccumulated metals or non-metals in the plant (Salt and Krämer, 1999) (*Figure 14.1*). Firstly, metals and non-metals must cross from the soil solution across root cell plasma

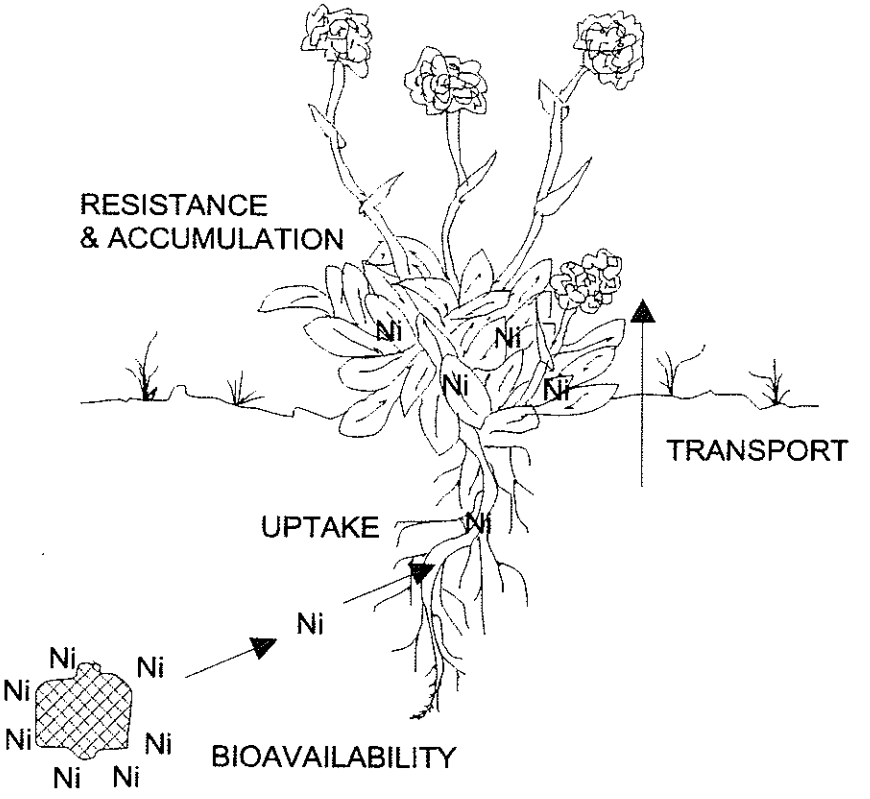

Figure 14.1. Pathway of metal assimilation in hyperaccumulator plants. Uptake and assimilation of nickel by the hyperaccumulator *Thlaspi goesingense*.

membrane into the roots. Secondly, the metals and non-metals must be loaded into the xylem for translocation to the shoots, in the transpiration stream. Low molecular weight metal-chelate molecules such as an amino acid or organic acid may mediate xylem loading. Thirdly, metals and non-metals arriving in the shoot must cross the leaf cell plasma membrane and enter the leaf cell. Finally, inside the leaf cell the metals and non-metals are detoxified, most probably by compartmentalization in the vacuole or transformation into less toxic forms. Transport of metals to the vacuole may also be facilitated by low molecular weight metal-chelates.

This general outline of the physiological processes involved in the hyperaccumulation of metals and non-metals will act as a guide to the topics that will be discussed in the following sections. Each step of the hyperaccumulation process, as delineated above (*Figure 14.1*) will be addressed at the molecular level. It is not the focus of this review to provide an in-depth analysis of the physiological mechanisms of metal and non-metal hyperaccumulation. For a complete description of these processes the reader is referred to Salt and Krämer (1999).

Molecular aspects of hyperaccumulation

PLANT MEDIATED SOIL BIOAVAILABILITY OF METALS

The transition of metals and non-metals from the abiotic to the biotic world takes place at the root/soil interface. For root uptake to occur, metals and non-metals must be released from the solid phase of the soil. One possible explanation for the enhanced metal and non-metal accumulation observed in hyperaccumulator plants could be the existence of an efficient system for the mobilization of metals or non-metals in the rhizosphere of the hyperaccumulator plant. However, at present there is no direct evidence to support this (Bernal and McGrath, 1994; Bernal *et al.*, 1994; McGrath *et al.*, 1997). Non-accumulator plants have evolved several strategies to enhance the solubility of metals and non-metals within the soil, and it is possible that hyperaccumulator species may have enhanced these mechanisms to allow for hyperaccumulation of these elements.

Plants increase the soil solubility of cationic micronutrients by either secreting specialized metal-chelating molecules or acidifying the rhizosphere (Römheld, 1987; Cakmak and Marschner, 1990). Rhizosphere acidification is achieved through the activity of the plasma membrane H^+-pumping ATPase. This enzyme acts to pump H^+ from the cytoplasm into the rhizosphere at the expense of ATP hydrolysis. To increase the availability of anionic non-metal nutrients, plants secrete various hydrolytic enzymes to release bound nutrients from organic molecules. For example, plants secrete phosphatases into the rhizosphere under phosphorous deficiency. These enzymes mobilize phosphate bound to organic molecules, making phosphate more available to the plant (Tadano and Sakai, 1991). A study of these mechanisms should shed some light on the possible genetic modification that may have occurred in hyperaccumulator plants.

Phytosiderophores are a class of metal-chelating compounds produced by roots of graminaceaous species to enhance the solubility and uptake of certain cationic micronutrients in the rhizosphere. These compounds have a high binding affinity for iron(III), and the intact phytosiderophore-iron(III) complex is directly transported across the root plasma membrane by specialized membrane transport proteins (Von Wiren *et al.*, 1994, 1995, 1996). There is also evidence that phytosiderophores are capable of enhancing the uptake of copper(II), zinc and manganese(II) (Römheld, 1991).

Nicotianamine is the main precursor compound for the biosynthesis of the phytosiderophores 2'-deoxymugineic acid and avenic acid. All three of these compounds have the ability to chelate iron(III) and are involved in the mobilization of iron for uptake into the plants (Von Wiren *et al.*, 1999). Several related genes, including NAS1, have been cloned from barley that encode nicotianamine synthase, an enzyme which catalyzes the synthesis of nicotianamine from *S*-adenosylmethionine (Higuchi *et al.*, 1999). Expression of the NAS genes is induced in iron-deficient roots, and repressed under iron-sufficient conditions. However, the NAS genes are not expressed in shoots under either iron-deficient or -sufficient conditions (Higuchi *et al.*, 1999). Also, expression of NAS1 in *E. coli* was found to be sufficient to allow *E. coli* to synthesize nicotianamine from exogenously supplied *S*-adenosylmethionine. The next gene in the phytosiderophore synthesis pathway, nicotianamine aminotransferase (NAAT), has also been cloned from barley and is induced by iron-deficiency (Higuchi *et al.*, 1999).

Plants also secrete chelating compounds into the rhizosphere to reduce the uptake of potentially toxic metals. Chelation of metals has the effect of reducing the effective concentration of the toxic free ionic form of the metal in the soil solution, reducing the overall toxicity of the metal. For example, aluminium resistance is achieved by the secretion of citrate or malate into the rhizosphere. Both organic acids are capable of chelating ionic aluminium, thereby reducing the effective soil solution concentration of the toxic free ionic form (Kochian, 1995). The release of these organic acids is induced by aluminium (Miyasaka *et al.*, 1991; Delhaize *et al.*, 1993). Interestingly, overexpression of citrate synthase in tobacco was found to be sufficient to impart aluminium resistance in the transgenic plants (Manuel de la Fuente *et al.*, 1997). A *Pseudomonas* citrate synthase gene was fused to a cauliflower mosaic virus 35S promoter and constituitively over-expressed in tobacco and papaya. The level of citrate synthase activity in the transgenic plants was found to be between 3- and 10-fold higher than wild type plants. Over-production of citrate caused an increase in citrate efflux from the roots of between 2- and 4-fold. Roots of transgenic plants over-producing citrate accumulated less aluminum than wild-type plants. This decreased accumulation was thought to be due to a decrease in aluminium availability in the rhizosphere induced by an increased concentration of extracellular citrate.

The production of metal-chelating compounds may be a general strategy non-accumulator plants use to prevent the uptake of potentially toxic metals. In support of this, the non-accumulator *T. arvense* was found to increase the amount of histidine and citrate produced in root exudates in response to treatment with nickel (Salt *et al.*, 1999a). Histidine concentrations in the root exudate increased by at least 10-fold after exposure to nickel. In contrast to the non-accumulator, the nickel hyperaccumulator *T. goesingense* showed no increase in histidine or citrate in root exudate when exposed to nickel. An intriguing possibility is that in hyperaccumulators this metal exclusion system has been suppressed, thereby allowing for an increased uptake of metals from the soil.

MOLECULAR ASPECTS OF METAL AND NON-METAL TRANSPORT ACROSS THE PLASMA MEMBRANE

Regardless of their soil solubility, metals and non-metals must cross the root cell plasma membrane to gain entry into the plant. There have been several physiological studies on bacteria, yeast, and plants which have revealed the existence of specialized systems for the transport of metals and non-metals across the plasma membrane (Watt and Ludden, 1999; Ross, 1995; Lasat *et al.*, 1996; Smith *et al.*, 1995b).

The *Saccharomyces cerevisiae* genes ZRT1 and ZRT2 encode proteins necessary for high affinity (K_M = 1 μM) and low affinity (K_M = 10 μM) plasma membrane zinc transport, respectively (Zhao and Eide, 1996a,b). The ZRT1 and ZRT2 genes have eight putative transmembrane domains, as well as a putative metal-binding domain that is rich in histidine residues thought be located on the cytoplasmic surface of the plasma membrane (Zhao and Eide, 1996a). When expressed in a zrt1 mutant, the ZRT1 gene is able to restore high affinity zinc transport activity (Zhao and Eide, 1996a). Similarly, when ZRT2 is expressed in a zrt2 mutant, low affinity zinc transport activity is restored (Zhao and Eide, 1996b).

Both transporters are highly specific for zinc. The zinc transport activity of ZRT1

and ZRT2 was not inhibited by cobalt, magnesium, manganese(II), iron(III) or nickel. However, zinc transport activity was inhibited by both copper(II) and iron(II), implying that ZRT1 and ZRT2 are capable of also transporting copper(II) and iron(II) (Zhao and Eide, 1996b). ZRT1 expression is induced in zinc starved cells and is repressed in cells that have sufficient zinc. In contrast to ZRT1, ZRT2 expression is not as tightly regulated by zinc. At zinc concentrations 4 times higher than that required to repress ZRT1 activity, ZRT2 zinc transport activity is unaffected. Interestingly, the yeast zrt1/zrt2 double mutant is viable when supplemented with 1,000-fold more zinc than required by wild-type yeast, suggesting that yeast also have a third, lower affinity, zinc uptake system.

Homologues of these plasma membrane transporters have been found in plants. A ZRT-like gene has been recently cloned from *Arabidopsis thaliana* by functionally complementing a yeast fet3/fet4 mutant on iron(II) deficient media. This gene has been designated IRT1 for iron regulated transporter (Eide *et al.*, 1996). Like the ZRT proteins, IRT1 appears to have eight membrane-spanning domains and a putative histidine-rich, metal-binding domain (Eide *et al.*, 1996). When assayed for function in a yeast fet3/fet4 mutant background, the IRT1 protein was found to be specific for iron(II) uptake and had almost no affinity for iron(III). A 10-fold molar excess of strontium, nickel, copper(II), cobalt, zinc, manganese(II), and cadmium were used in a competitive inhibition assay for iron transport. It was found that cadmium was an effective inhibitor of iron(II) transport, reducing transport activity by over 60% (Eide *et al.*, 1996). At a 100-fold molar excess, the cobalt, manganese(II) and zinc were also found to inhibit iron(II) transport activity. These results indicate that, in addition to iron(II), IRT1 is also capable of transporting cadmium, cobalt, manganese(II) and zinc, with a lower affinity. Further characterization of the metal specificity of the IRT1 transporter was achieved by studying the ability of IRT1 to complement various metal transport mutants of yeast (Korshunova *et al.*, 1999). IRT1 was found to complement both the smf1 high affinity manganese(II) transport mutant and the zrt1/zrt2 double mutant of yeast. This confirms the observation that IRT1 is capable of transporting both manganese(II) and zinc.

IRT1 is highly expressed in roots of iron-deficient plants (Eide *et al.*, 1996). However, its expression is repressed in roots when plants are supplied with sufficient iron. IRT1 is not expressed in shoots of either iron-sufficient or iron-deficient plants. Interestingly, *A. thaliana* plants that express IRT1 under iron-deficient conditions were found to accumulate increased amounts of manganese(II), zinc and cobalt in roots, although IRT1 expression is not regulated by these metals (Korshunova *et al.*, 1999). Iron-deficient *Pisum sativum* have also been observed to accumulate 7-fold higher concentrations of cadmium in their roots compared to iron-sufficient plants (Cohen *et al.*, 1998). This increase in cadmium uptake correlated with increased expression of the IRT1 homologue RIT1. The increased accumulation of manganese(II), zinc, cobalt and cadmium is thought to be due to the ability of IRT1, and its homologues, to transport these other metals into the plant root.

A related family of IRT1-like genes has recently been cloned from *A. thaliana* (Grotz *et al.*, 1998). This family includes ZIP1, ZIP2, ZIP3 and ZIP4. These genes, along with IRT1, ZRT-1, and ZRT-2, have been placed in the metal transporter gene superfamily designated ZIP for ZRT, IRT-like proteins (Eng *et al.*, 1998). Subfamily I contains genes isolated from plants (currently 13) and two genes from yeast.

Subfamily II contains genes cloned from animals (currently 11). It should be noted that these genes form a group that is distinct from other known membrane metal transporters such as the Nramp family, ATP-dependent metal transporters, membrane ion channels (Fox and Guerinot, 1998), and ABC-type transporters (Rea *et al.*, 1998).

ZIP1, ZIP2 and ZIP3 genes were isolated by functional complementation of a zrt1/zrt2 yeast mutant. Searching the *A. thaliana* genome database for genes similar to ZIP1-3 identified ZIP4, which was cloned by PCR amplification. These ZIP proteins have a similar structure to both the ZRT proteins of yeast and the IRT1 protein of *A. thaliana* in that they appear to have eight membrane-spanning domains and a putative histidine-rich, metal-binding domain (Grotz *et al.*, 1998). The ZIP1-3 proteins appear to have predicted plasma membrane targeting sequences, while ZIP4 has a potential chloroplast targeting sequence (Grotz *et al.*, 1998).

The ZIP1-3 genes have been shown to functionally complement the zrt1/zrt2 yeast double mutant. Therefore, it was concluded that they are able to transport zinc (Grotz *et al.*, 1998). This conclusion was confirmed by direct zinc transport assays in the zrt1/zrt2 yeast double mutant expressing the ZIP1-3 genes. Interestingly, the ZIP4 gene was unable to complement the zrt1/zrt2 double mutant, and it was suggested that this might be due to the ZIP4 protein being targeted to an internal yeast membrane system rather than the plasma membrane (Grotz *et al.*, 1998). The ZIP1-4 genes were found not to functionally complement a fet3/fet4 yeast double mutant unable to acquire iron(II) from the media, suggesting that proteins of the ZIP family are unable to transport iron(II) into cells. Ten-fold excess concentrations of manganese(II), nickel, iron(II), and cobalt were found to have no inhibitory effect on zinc transport by ZIP1. Only cadmium and copper were found to be able to partially inhibit zinc transport. ZIP1 is therefore a highly specific zinc transporter (Grotz *et al.*, 1998). ZIP2 zinc transport activity was strongly inhibited by cadmium and copper(II), and these metals were almost as effective as nonradioactive zinc at inhibiting radio-labelled zinc transport. ZIP3 has the broadest range of metal transport specificities of the ZIP protein family members. Its transport activity can be inhibited by manganese(II), iron, cobalt, cadmium and copper(II), but inhibition was found to be not greater than 50% for any of these metals. The results of the metal specificity assays for the ZIP transporters are similar to the IRT1 transporter in that the proteins have a high affinity for one particular metal (ie zinc or iron) but may also, with some lower affinity, transport other metals.

Expression of ZIP1 and ZIP3 is strongly induced in the roots of zinc-deficient plants. However, these genes are not expressed in zinc-sufficient roots. Neither ZIP1 nor ZIP3 are expressed in shoots (Grotz *et al.*, 1998). The ZIP2 mRNA was undetectable in all plant tissues investigated and zinc conditions tested. The ZIP4 mRNA is induced in both the roots and shoots of zinc-deficient plants, but absent in the roots and shoots of zinc-sufficient plants (Grotz *et al.*, 1998).

At the transcriptional level, the ZIP family member ZRT1 is regulated by the transcription factor ZAP1 (Zhao and Eide, 1997). This gene was identified by searching for yeast mutants that lacked the ability to regulate the expression of ZRT1 in response to zinc. A zap1 yeast mutant was found to be unable to induce expression of ZRT1 in response to zinc starvation, and it required 1,000 times more zinc than wild-type yeast to grow optimally. ZAP1 also regulates the expression of ZRT2. However, its inductive effect on ZRT2 expression is much lower than for ZRT1.

Expression of ZAP1 is itself regulated by zinc. In zinc-starved cells, its transcription is activated and in zinc-replete cells its transcription is repressed. Another allele of ZAP1 was found and designated ZAP1-1^{up}. This allele is unregulated by zinc, and was always transcribed regardless of zinc nutritional status. A single nucleotide change in ZAP1-1^{up}, mutating a cysteine to a serine in the protein coding sequence was found to affect the protein's ability to respond to zinc. This mutation caused the transcription of ZAP1, ZRT1 and ZRT2 to be unregulated by the zinc nutritional status.

The ZAP1 protein has features that classify it as a member of the transcriptional activator protein family. The carboxyl terminal portion of the protein contains 5 zinc finger domains and a non-canonical 6th zinc finger domain. The N-terminus contains two acidic domains rich in glutamate and aspartate that could be transcriptional activation domains. There are also regions that are rich in asparagine, serine, and threonine, motifs that have been found in other transcription factors. The protein also contains a consensus nuclear localization motif (KNRR).

The ZAP1 protein was found to bind to zinc responsive elements (ZRE) contained within the ZAP1, ZRT1, and ZRT2 gene promoters (Zhao *et al.*, 1998). These ZRE elements contain the consensus sequence ACCYYNAAGGT. This sequence is both necessary and sufficient to activate the transcription of ZAP1, ZRT1, and ZRT2 in response to zinc. The promoters of the ZRT1, ZRT2, and ZAP1 genes were found to contain three, two, and one ZRE elements, respectively. The expression levels of these three genes in zinc-starved yeast paralleled the number of ZRE's present in the promoters. ZRT1 was expressed at the highest level, ZRT2 at a somewhat lower level and ZAP1 at the lowest level. This suggests that multiple ZRE elements have an additive effect on the level of gene expression.

Interestingly, there are members of the ZIP family that are also post-translationally regulated by metals. In yeast, the ZRT1 protein is present in the yeast cell plasma membrane in zinc-starved cells, but in zinc-replete cells it is targeted to the vacuole for degradation by proteases (Gitan *et al.*, 1998). Cadmium and cobalt also initiate removal of the ZRT1 protein from the plasma membrane and its targeting to the vacuole for degradation, but they are less effective than zinc.

Another family of plasma membrane metal transporters that warrants comment is the Nramp (Natural resistance associated macrophage protein) family. Two genes, SMF1 and SMF2 were originally isolated from yeast mutants that had a lethal mutation in the PEP4 processing enhancing protein of mitochondria (West *et al.*, 1992). Further studies indicated that the SMF1 and SMF2 genes were plasma membrane localized manganese(II) transporters (Supek *et al.*, 1996). The SMF1 gene encodes the high affinity manganese(II) transporter, while SMF2 encodes the low affinity manganese(II) transporter. SMF1 has been shown to be capable of transporting both copper(II) and cadmium in addition to manganese(II) (Liu *et al.*, 1997). SMF2 is capable of transporting cobalt in addition to manganese(II), but it cannot transport copper(II) or cadmium (Liu *et al.*, 1997). Proteins of the Nramp family contain ten membrane-spanning domains (Fox and Guerinot, 1998) in contrast to the eight membrane-spanning domains of proteins in the ZIP family.

The transcriptional and post-translational regulation of the SMF1 and SMF2 genes in yeast is very similar to the regulatory processes that govern ZRT1 and ZRT2 transcription and protein stability. SMF1 expression is activated in manganese(II)-deficient cells and down-regulated in manganese(II)-replete cells (Liu and Culotta,

1999a). However, it is not known if this regulatory effect is mediated by a transcription factor similar to ZAP1. In manganese(II)-replete cells, expression of the SMF1 and SMF2 proteins is post-translationally regulated by targeting of the proteins to the vacuole for degradation (Liu *et al.*, 1997). This vacuolar targeting is mediated in part by the BSD2 gene (Liu and Culotta, 1999a). Manganese(II) replete yeast cells that are mutant for BSD2 are unable to efficiently target SMF1 and SMF2 to the vacuole for degradation. Bsd2 mutant yeast can accumulate toxic levels of cadmium, copper(II) and cobalt, and this accumulation is mediated by increased, unregulated, expression of the SMF1 and SMF2 proteins (Liu and Culotta, 1999a). Additionally, mutations within the SMF1 gene can abolish post-translational regulation of the protein by manganese(II) and BSD2. However, these mutations also destroyed SMF1 metal transport activity (Liu and Culotta, 1999b). This suggests that metal transport activity is essential for the regulation of SMF1 protein levels by manganese(II) and BSD2.

Nramp genes were originally found in mammals and yeast, but several representatives have now been cloned from plants. Three Nramp genes have been cloned from rice, OsNramp1, OsNramp2 and OsNramp3 (Belouchi *et al.*, 1997). The predicted protein sequence of these three genes were found to have high sequence identity (65–70%) with Nramp proteins from mammals, and they also share the predicted transmembrane regions observed in the mammalian and yeast Nramp proteins. Three Nramp genes have also recently been cloned from *A. thaliana*, AtNramp1, AtNramp2 and AtNramp3. Individual expression of each of these genes in yeast was able to functionally complement a smf1 mutation (Thomine *et al.*, 1999).

Expression of OsNramp1 was found to be primarily in roots with only slight expression in the shoots. In contrast, the expression pattern of OsNramp2 was reversed, with more expression in the shoots than in the roots. OsNramp3 is equally expressed in both roots and shoots. The OsNramp1 and OsNramp2 proteins may, therefore, be unique to their respective tissues, whereas the OsNramp3 protein may be present in both roots and shoots. In *A. thaliana*, all 3 AtNramp genes were found to be equally expressed in roots and shoots, and their expression was regulated in response to metal availability (Thomine *et al.*, 1999).

An analysis of the function and regulation of the transport genes described above, including the ZIP and Nramp family members, should help direct research into possible ion transport processes occurring in hyperaccumulator plants. It is possible that a mutation(s) in one or several of the genes described above would allow for altered transport specificity or the gain of function by a transport protein. This might result in an increased flux of metal ions into the plant. Mutations that alter the regulation of metal transporter genes might also lead to an increased metal flux into the plant. For example, defects in control by a transcription factor such as ZAP1 may result in the constitutive expression of metal transporter genes, regardless of the metal status of the plant. Transporter proteins would then accumulate to higher levels in the plasma membrane, increasing the flux of metal ions into cells. Alternatively, mutations in genes that control the degradation of transporter proteins in response to metal status would increase the cellular lifetime of the proteins, allowing for increased metal transport. An example of this process is evident in the bsd2 yeast mutant. The SMF1 and SMF2 proteins in cells of this mutant have increased cellular stability, allowing these cells to accumulate toxic concentrations of cadmium, copper(II) and cobalt.

Interestingly, similar changes in the regulation of zinc transport have been postulated to occur in the zinc hyperaccumulator *T. caerulescens*. Roots of *T. caerulescens* have a zinc transport rate 4½ times faster than the non-accumulator *T. arvense* (Lasat *et al.*, 1996). However, the K_M for zinc of this transport is similar in both species. This implies that *T. caerulescens* might have more zinc transporters in the plasma membrane of its root cells than *T. arvense*. Recently, a high affinity zinc transporter gene (ZNT1) has been cloned from the zinc hyperaccumulator *T. caerulescens* by functional complementation of a yeast zinc uptake mutant (Letham *et al.*, 1999). This gene is highly expressed in roots under zinc-sufficient conditions, and expression is increased further in response to zinc starvation. In contrast, a much lower level of expression of a ZNT1 homologue was found in the roots of the non-accumulator *T. arvense*. The *T. caerulescens* ZNT1 gene was also found to require a much higher zinc concentration to repress its expression than the ZNT1 homologue from *T. arvense* (Letham and Kochian, 1999). Altered regulation of the ZNT1 gene in *T. caerulescens* could account for the increased zinc influx observed in *T. caerulescens*, and ultimately contribute to its zinc hyperaccumulation phenotype.

In contrast to metals, the non-metal selenium is accumulated from the soil solution via the root plasma membrane sulphate transport system (Lauchli, 1993). In physiological studies of plant root plasma membrane sulphate transporters, selenate was found to compete with sulphate for root uptake (Ferrari and Renosto, 1972). Additionally, yeast in which the high affinity sulphate transporter gene (SUL1) has been deleted were found to be resistant to selenate (Smith *et al.*, 1995a). There have been several plant sulphate transporter genes cloned, and the proteins encoded by these genes may provide an avenue for selenate entry into plants (Smith *et al.*, 1995b; Smith *et al.*, 1997; Takahashi *et al.*, 1996, 1997).

XYLEM LOADING AND TRANSLOCATION TO THE SHOOTS

The next step that an assimilated metal or non-metal undergoes in a hyperaccumulator plant is loading into the xylem and translocation to the shoot. In general, the shoot to root metal concentration ratio is greater than one in hyperaccumulator plants. For non-accumulator species, the converse is true. This observation implies that hyperaccumulator plants have a more efficient root to shoot translocation system for metals than non-accumulators (Baker *et al.*, 1994; Lloyd-Thomas, 1995; Krämer *et al.*, 1996; Shen *et al.*, 1997; Lasat *et al.*, 1996). However, this may not always be the case. It has been demonstrated that the nickel hyperaccumulator *T. goesingense* translocates nickel at the same rate as the non-accumulator *T. arvense*, at concentrations of nickel that are non-toxic to either plant species (Krämer *et al.*, 1997b).

Xylem sap concentrations of histidine and nickel have been found to be linearly correlated in several nickel hyperaccumulating *Alyssum* species (Krämer *et al.*, 1996). Based on this, and other evidence, it has been suggested that histidine might complex nickel and facilitate its loading into the xylem in *Alyssum* nickel hyperaccumulators. In contrast, increased histidine concentrations in response to nickel do not appear to play a significant role in nickel hyperaccumulation in *T. goesingense* (Persans *et al.*, 1999a). Analysis of the expression levels of three key histidine biosynthetic genes, THG1, THB1 and THD1, in *T. goesingense* revealed that they are not regulated in either the roots or shoots by nickel treatment (Persans *et*

al., 1999a). Biochemical analysis of xylem sap and root and shoot tissues also revealed no increases in histidine concentrations after nickel exposure. X-ray absorption spectroscopy (XAS) of xylem exudate from both *T. caerulescens* and *T. goesingense* showed that the majority of the zinc and nickel in these hyperaccumulator species is translocated in the xylem as the hydrated cation (Salt *et al.*, 1999b; Salt and Pickering, unpublished), and not coordinated with histidine. However, the majority of nickel in roots of *T. goesingense* and the non-accumulator *T. arvense*, and zinc in *T. caerulescens*, was found to be coordinated by a histidine-like molecule (Krämer *et al.*, 1999; Salt *et al.*, 1999b). This suggests that histidine may play a more general role in xylem loading of divalent cations such as zinc and nickel in both non-accumulator and hyperaccumulator plant species.

SHOOT SEQUESTRATION OF METALS AND NON-METALS

It has been found that metals appear to be concentrated in the sub-epidermal and epidermal cells of hyperaccumulators (Heath *et al*, 1997; Küpper *et al.*, 1999). Energy-dispersive X-ray microanalysis of *Thlaspi montanum* var. *siskiyouense* leaves showed that the nickel accumulated by this plant was localized to epidermal subsidiary cells located next to guard cells in the leaf epidermis (Heath *et al.*, 1997). In *T. caerulescens*, the vacuolar sap of epidermal cells contained 5 to 6.5 times more zinc than the vacuolar sap of the mesophyll cells (Küpper *et al.*, 1999). Scanning proton microscopy has also revealed leaf nickel to be localized in epidermal and subepidermal tissues in shoots of the nickel hyperaccumulators *Senecio coronatus* (Mesjasz-Przybyłowicz *et al.*, 1994) and *A. lesbiacum* (Krämer *et al.*, 1997a). The highest nickel concentrations were found in the unicellular stellate trichomes covering the leaf surface in *A. lesbiacum*. Interestingly, several non-accumulator plant species have also been observed to accumulate cadmium (Salt *et al.*, 1995), copper, nickel and zinc (Neumann *et al.*, 1995), lead (Martell, 1974), and manganese (Blamey *et al.*, 1986) in leaf trichomes.

Several studies in hyperaccumulating plants have localized intracellularly accumulated nickel and zinc to the vacuole (Krämer *et al.*, 1999; Vazquez *et al.*, 1992, 1994; Lasat *et al.*, 1998; Küpper *et al.*, 1999). In the hyperaccumulator *T. goesingense*, 75% of the intracellular leaf nickel was found localized to the vacuole (Krämer *et al.*, 1999). A direct comparison of the nickel contents of vacuoles from the non-accumulator *T. arvense* and the hyperaccumulator *T. goesingense* revealed that vacuoles from the hyperaccumulator contained approximately double the nickel of the non-accumulator vacuoles.

The vacuole has also been shown to be important for metal tolerance in yeast. Nickel resistant yeast appears to have an enhanced ability to sequester nickel in the vacuole (Joho *et al.*, 1995), with 70% of the intracellular nickel being found in the vacuole of this nickel resistant strain. The pH gradient across the vacuolar membrane is important in vacuolar sequestration of nickel in yeast (Nishimura *et al.*, 1998). Yeast mutants either lacking vacuoles or lacking a functional vacuolar H^+-ATPase are more sensitive to a wide range of metals (Ramsay and Gadd, 1997), and yeast mutants defective in the vacuolar H^+-ATPase accumulated only 50% of the wild-type levels of nickel in vacuoles; yeast cells appear to require a vacuole for sequestration and a pH gradient to energetically drive metal ions into the vacuole.

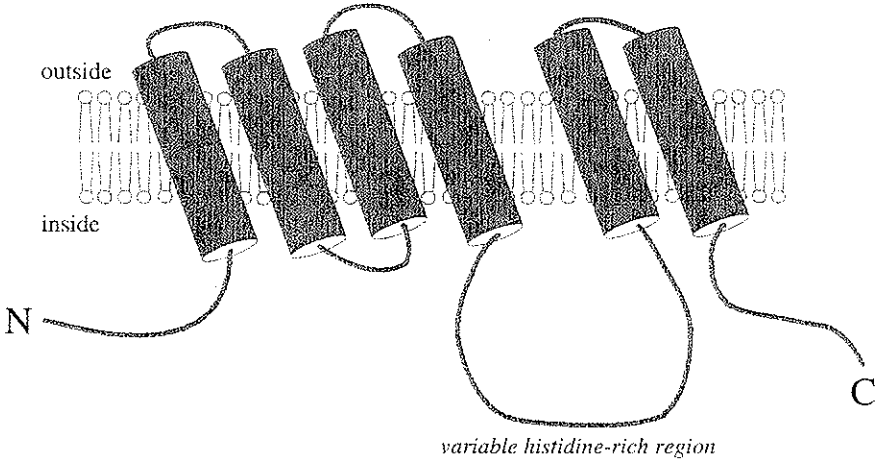

Figure 14.2. Predicted membrane topography of the *Arabidopsis thaliana* ZAT protein. Demonstrating the predicted orientation of the protein, the six transmembrane helixes, an intracellular variable histidine-rich region and an extensive intracellular C-terminus. Constructed using the Tmpred program (Hofmann and Stoffel, 1993) at http://www.ch.embnet.org/software.TMPRED_form.html.

Little is known about the molecular biology of intracellular metal sequestration in plants. However, a Cd/H antiport activity has been characterized across the vacuole membrane in plants (Salt and Wagner, 1993), and progress has recently been made in the purification of the protein involved in this activity (Koreníkov *et al.*, 1999). However, there have been no genes cloned to date that have been confirmed to be involved in the tonoplast transport of hyperaccumulated metals such as nickel and zinc.

At present, only thirteen genes have been identified as being homologues of, or potential candidate genes for, transport proteins that may be involved in the intracellular transport of hyperaccumulated metals. Four are from mammals (ZnT1-4), two are from yeast (COT1 and ZRC1), four are from *A. thaliana* (ZAT and atMTP2-4), and three are from *T. goesingense* (MTP1, MTP2 and MTP3). All of these proteins have six putative transmembrane domains, a histidine-rich region between transmembrane domains four and five, and a long C-terminal tail (*Figure 14.2*). All are distinctly different from previously identified metal transporter proteins. The interrelatedness of these genes is shown in the dendrogram in *Figure 14.3*. The genes that cluster together are from similar organisms. The rat and mouse genes cluster together at the top, the genes from yeast near the middle, and genes from plants cluster near the bottom of the dendrogram. All of the *Thlaspi* genes are closely related to one another and these genes are most closely related to the ZAT gene from *A. thaliana* (Van der Zaal *et al.*, 1999).

ZnT1 has been characterized as a plasma membrane protein that effluxes zinc from rat cells (Palmiter and Findley, 1995). Cells lacking this gene are more sensitive to zinc because the zinc cannot be effluxed from the cells efficiently. Overexpression of the ZnT1 gene imparted some level of increased zinc resistance to cells. Even though ZnT1 had been localized to the plasma membrane, there are homologues of ZnT1 that are localized to intracellular membrane systems. ZnT2 is very similar to ZnT1 in

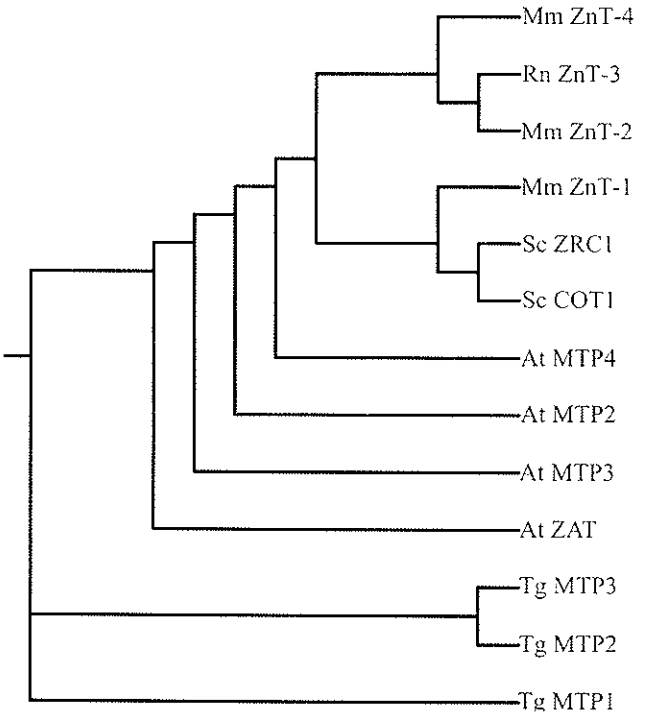

Figure 14.3. Dendrogram of the interrelatedness of the ZnT, COT1, ZRC1, ZAT, and MTP genes. Rn = *Rattus norvegicus*; Mm = *Mus musculus*; Sc = *Saccharomyces cerevisiae*; At = *Arabidopsis thaliana*; Tg = *Thlaspi goesingense*. Constructed using the CLUSTAL W (Thompson *et al.*, 1994) and the PHYLIP (Felsenstein, 1993) programs of the Biology Workbench suite of programs at http://biology.ncsa.uiuc.edu.

that it has six membrane-spanning domains, an intracellular histidine-rich loop, and a long C-terminal tail (Palmiter *et al.*, 1996). However, unlike ZnT1, which is localized to the plasma membrane, ZnT2 is localized in intracellular vesicular membranes. Overexpression of ZnT2 in zinc-sensitive cells increases their zinc resistance. The mechanism of this resistance was found to be the enhanced sequestration of zinc into intracellular vesicles. Another ZnT homologue, ZnT3, was shown to be localized to synaptic vesicles, and it is proposed that ZnT3 pumps zinc into synaptic vesicles as a storage pool of zinc to be released upon excitation of the neuron (Wenzel *et al.*, 1997). A fourth ZnT homologue (ZnT4) has been isolated from mice that are defective in zinc transport into milk (Huang and Gitschier, 1997). In mammals, the ZnT4 transporter is responsible for effluxing zinc from the mammary cells into the secreted milk, and it is expressed at high levels in the mammary tissue. Interestingly, ZnT4 is also expressed at high levels in brain cells, and it might perform a similar function to ZnT3 in these tissues (Huang and Gitschier, 1997).

These ZnT proteins are distinct from transporters in the ZIP family in that they are involved in effluxing metal out of cells or into intracellular compartments. Although intracellular vesicles in a mammalian cell are somewhat different than the plant vacuole, both compartments function to sequester metals, and one might infer that

proteins similar to ZnT2 and ZnT3 might be performing such a function in the plant vacuole.

Two related genes, COT1 (Conklin *et al.*, 1992) and ZRC1 (Conklin *et al.*, 1994), have been isolated from yeast. These genes share the six putative membrane-spanning domains of the ZnT genes and also the histidine-rich region. COT1 is involved in cobalt resistance, while ZRC1 is involved in zinc and cadmium resistance. Preliminary evidence suggests that COT1 may be localized to the mitochondria. However, the subcellular location of both COT1 and ZRC1 remains uncertain. Yeast deletion mutants of either gene show increased sensitivity to cobalt (COT1 deletion), or zinc and cadmium (ZRC1 deletion). Overexpression in yeast of COT1 and ZRC1 leads to an increase in resistance to cobalt (COT1) and zinc (ZRC1). Overexpression of ZRC1 also causes cross-resistance to cobalt, and overexpression of COT1 causes cross-resistance to zinc. Although there is strong genetic evidence that COT1 and ZRC1 are involved in metal homeostasis in yeast, the exact mechanism of action remains elusive. Further studies on the transport activity and localization of these genes needs to be performed to verify their role in metal transport and sequestration. There are now a growing number of plant homologues of COT1 and ZRC1, suggesting that they are good candidate genes for study of metal resistance mechanisms in plants.

A plant homologue of the ZnT, COT1 and ZRC1 genes has recently been cloned from *A. thaliana* (Van der Zaal *et al.*, 1999) and designated ZAT for zinc transporter of *Arabidopsis thaliana*. Like the other genes described previously, ZAT has six putative transmembrane domains, a histidine-rich region, and a long C-terminal tail (*Figure 14.3*); and probably belongs to the same gene family. ZAT was not found to be regulated by zinc, nor was expression in yeast able to impart increased resistance to zinc, cadmium or cobalt (Van der Zaal *et al.*, 1999). Therefore, transgenic plants were made overexpressing the ZAT gene to determine if elevated levels of the ZAT protein could result in increased zinc resistance. A slight increase in zinc resistance was qualitatively seen in examining the phenotype of wild-type versus transgenic plants, but a root growth assay indicated that the transgenic plants were about two-fold more zinc resistant than wild-type plants. These data are encouraging, and point to a role for ZAT in zinc metabolism in plants, but more extensive study is needed to further elucidate the significance of ZAT in metal transport and hyperaccumulation.

A search of the *A. thaliana* genomic sequence database revealed another three homologues of ZAT which we have designated atMTP (2-4) for *A. thaliana* metal transport protein. Also, a partial EST cDNA from *Brassica campestris* ssp. *pekinensis* was found that also shows homology to COT1, ZRC1 and ZAT (Lim *et al.*, 1996). A homologue of the gene represented by this EST was also found to be up-regulated in young seedlings of *Brassica juncea* after treatment with either nickel, zinc or cadmium (Persans *et al.*, 1999b). This suggests that homologues of the ZAT, COT1, ZRC1, ZnT gene family might be involved in metal resistance in this commonly used phytoremediation plant.

Recently, we have cloned three ZAT homologues from the metal hyperaccumulator species *T. goesingense* (Persans *et al.*, 1999b). They are designated MTP1, MTP2, and MTP3 (for metal transporter protein) and, like ZAT, they have six predicted membrane-spanning domains. These three genes are highly similar to each other and the ZAT gene, except that the histidine-rich region between transmembrane domains four and five is variable in size. There appears to be two histidine-rich regions located

in this variable region. MTP1 only has the first region, MTP2 and MTP3 have both regions, although the second histidine region in MTP3 is slightly larger than in MTP2. Perhaps these changes reflect different metal specificity and/or metal regulation of these proteins. Expression of the MTP genes was not induced by either high level, short-term nickel exposure (500 μM nickel for 24–48hrs) or long-term, low level nickel exposure (100 μm nickel for 1–4 weeks). However, these genes were expressed at a high level in shoots, even though their expression was not influenced by nickel (Persans *et al.*, 1999b).

The ZnT, COT1, ZRC1, ZAT and the MTP gene families are promising candidates for vacuolar metal transporters. However, much more extensive work is required before any of the genes in this family can be shown to have a role in the sequestration of metals in the vacuole in hyperaccumulating plants.

Several genes have now been characterized which encode proteins involved in vacuolar membrane transport of cadmium chelated by either phytochelatins or glutathione (Ortiz *et al.*, 1995; Li *et al.*, 1996, 1997). These transporters are members of the ABC-type transporter super-family, and they have a very different structure from the proteins described above. Given that phytochelatins and glutathione are not involved in zinc or nickel hyperaccumulation in plants (Krämer *et al.*, 1996; Salt *et al.*, 1999b; Krämer *et al.*, 1999), these types of proteins are probably not involved in plant hyperaccumulation of these metals.

It is now fairly well established that hyperaccumulator plants show increased sequestration of metals in the vacuole. To achieve this increased sequestration, it is possible that regulation of the expression of genes encoding vacuolar metal transport proteins may be modified in hyperaccumulator plants. This could occur in much the same way as suggested for plasma membrane transporters. Mutations at the transcriptional or post-translational levels could affect the level of transporter mRNAs and stability of the vacuolar membrane transport proteins. Both types of changes could result in increased fluxes of metal ions into the vacuole.

Any discussion of the vacuolar localization of metals in hyperaccumulating plants would not be complete without considering how metal ions cross the cytoplasm and are safely delivered to the vacuolar membrane. It has already been suggested that histidine is involved in shuttling nickel into the xylem, and it is possible that histidine may also be involved in transporting nickel and zinc across the cytoplasm and into the vacuole. X-ray absorption spectroscopy (XAS) has identified a 'histidine-like' molecule as being involved in chelating both zinc (Salt *et al.*, 1999b) and nickel (Krämer *et al.*, 1999) in shoots of *T. caerulescens* and *T. goesingense*, respectively. Based on the binding affinities of histidine for nickel and zinc, we would predict that histidine would make a very effective chelate molecule at the neutral pH of the cytoplasm. However, to date there is no direct evidence to implicate histidine in this process.

A class of compounds that has been shown to be involved in transporting cadmium across the tonoplast membrane into vacuoles are the phytochelatins (Salt and Rauser, 1995; Ortiz *et al.*, 1995). These small molecular weight compounds are synthesized by plants in response to a number of metals, including cadmium, lead, zinc, nickel, and mercury (Grill *et al.*, 1985, 1987). The coordinating atom for the metal in these complexes is sulphur (Strasdeit *et al.*, 1991; Pickering *et al.*, 1999). Phytochelatins are enzymatically synthesized from the precursors γ-glutamylcysteine and glutathione by

PC synthase. Recently, genes for all the biosynthestic steps required to convert cysteine, glutamate and glycine into phytochelatins have been cloned (May and Leaver, 1994; Rawlins *et al.*, 1995; Schäfer *et al.*, 1998; Clemens *et al.*, 1999; Ha *et al.*, 1999; Vatamaniuk *et al.*, 1999). Expression of the genes encoding γ-glutamylcysteine synthetase and glutathione synthetase appear to be regulated by cadmium and copper(II) (Schäfer *et al.*, 1997, 1998; Xiang and Oliver, 1998). However, expression of the gene encoding the enzyme which catalyses the final step in phytochelatin synthesis, phytochelatin synthase, appears to be unregulated by cadmium (Clemens *et al.*, 1999; Ha *et al.*, 1999; Vatamaniuk *et al.*, 1999).

Interestingly, overexpression of GSH synthetase in *B. juncea* increased the amount of cadmium accumulated by the transgenic plants (Zhu *et al.*, 1999) by increasing the concentration of both glutathione and phytochelatins. Also, overexpression of the gene encoding phytochelatin synthase in yeast resulted in the overproduction of phytochelatins, leading to increased cadmium tolerance (Clemens *et al.*, 1999; Ha *et al.*, 1999; Vatamaniuk *et al.*, 1999). Although phytochelatins are not involved in either zinc (Salt *et al.*, 1999b) or nickel (Krämer *et al.*, 1996; Krämer *et al.*, 1999) hyperaccumulation, manipulation of the genes involved in phytochelatin synthesis should allow the engineering of cadmium, and possibly other metal tolerances, in plants.

Could hyperaccumulator species contain unique metal chelating compounds that have not yet been characterized? To address this question a *T. goesingense* cDNA library was expressed in *E. coli*, and transformed cells selected for nickel resistance. Of the 200,000 colonies screened, approximately 2,000 were found to be nickel resistant (Persans *et al.*, 1999b). One hundred of these nickel tolerant colonies were randomly sampled and all were found to contain the cDNA encoding serine acetyltransferase (SAT). Three isoforms were represented and all had an equal ability to confer nickel resistance in *E. coli*. Cells expressing SAT were about 3-fold more resistant than wild-type *E. coli* containing the expression vector alone. Is it possible that overexpression of SAT in *E. coli* causes increased production of a chelate molecule that is capable of binding nickel and protecting the cells from nickel toxicity? The product of SAT, *O*-acetyl serine, has been shown to regulate sulphur assimilation and metabolism in *E. coli* (Kredich, 1996). It is possible that increased assimilation of sulphur causes the production of some, as yet unknown, chelate molecule capable of binding nickel. The obvious candidate for this compound would be some type of sulphur containing ligands. However, X-ray absorption spectroscopy (XAS) of nickel exposed *E. coli* cells overexpressing SAT has revealed that the majority of the nickel in these transgenic cells is not coordinated by sulphur (Salt, Prince and Pickering, unpublished). Further studies are ongoing to determine the mechanism of this SAT-induced nickel tolerance, and to establish its significance *in planta*.

In contrast to metal ions, the non-metal selenium is capable of being transformed into less toxic forms. In non-accumulator species, selenium is incorporated into selenocysteine and selenomethionine, which are both toxic to the plant. However, in selenium hyperaccumulator plants, selenium is funnelled into the non-toxic, non-protein amino acids, methylselenocysteine and selenocystathionine (Lauchli, 1993; Neuhierl and Böck, 1996).

Until recently, little was known about how *Astragalus* selenium hyper-

accumulator species were able to produce the non-protein amino acid methylselenocysteine. However, a gene from *Astragalus bisulcatus* (SMT for selenocysteine methyl transferase) was recently cloned and found to encode an enzyme capable of methylating selenocysteine *in vitro* or when expressed in *E. coli* (Neuhierl *et al.*, 1999). This enzyme is unusual in that it utilizes *S*-methyl methionine as its preferred methyl donor instead of the more common *S*-adenosyl methionine. The enzyme was found to be very specific for selenium compounds and preferentially methylated these over sulphur containing compounds. Using antibodies, the SMT protein was found to be expressed in the roots, hypocotyl, cotyledons and leaves of *A. bisulcatus*. This suggests that the SMT enzyme activity is present in all tissues, and that selenocysteine is detoxified in all parts of the plant. Analysis of SMT expression at the mRNA level also showed that SMT gene expression is not regulated by selenium (Persans, Bubner and Salt, unpublished). Interestingly, even though SMT gene expression appears to be equal in both old, medium and young leaf tissue (Persans, Bubner and Salt, unpublished), the capacity of these tissues to synthesis methylselenocysteine is very different. Using X-ray absorption spectroscopy, we have shown that selenate is the predominant form of selenium in old leaf tissue of *A. bisulcatus*, whereas, in the youngest leaf tissue, all the selenium is found to be assimilated into organic forms (Pickering, Prince and Salt, unpublished). These results suggest that SMT is not the rate-limiting step in selenium assimilation into methylselenocysteine in *A. bisulcatus*. Selenium assimilation studies in the non-accumulator *B. juncea* have shown that, if the sulphate-activating enzyme, ATP sulphurylase, is overexpressed in these plants, selenium is more efficiently assimilated into organic forms (Pilton-Smits *et al.*, 1999). This suggests that ATP sulphurylase might be a rate-limiting step in the assimilation of selenate in plants.

POSSIBLE MECHANISM FOR THE EVOLUTION OF HYPERACCUMULATION

In comparison to a non-accumulator species, it would appear that hyperaccumulators have undergone several genetic changes that allow them to resist and accumulate toxic metals. These changes presumably arose because they imparted a selective advantage to the plant. A simple evolutionary scenario for the development of metal or non-metal hyperaccumulation in plants might be as follows. A pioneer plant, from an uncontaminated pasture, without any special adaptations for the exclusion of metals, arrived on a metal enriched site by random seed dispersal. By chance, this individual had the genetic capacity to resist a limited accumulation of metals in its shoots. The selective advantage this slight accumulation conferred allowed the plant to prosper in this new environment. Further mutations, leading to a parallel reduction in metal exclusion and increased cellular metal resistance, allowed for increased metal accumulation and a concomitant increase in fitness. At a certain point, the cost of metal accumulation and the selective advantage gained would be balanced, and no further increase in metal accumulation would be expected to occur. This type of scenario, though hypothetical, highlights the limited number of genetic changes we can envisage are required for the evolution of metal hyperaccumulation. These changes could involve mutations in the regulation or structure of particular metal transporters and biosynthetic enzymes, as outlined in this review.

Conclusion

Clearly, metal hyperaccumulation is an extraordinary property of a very limited number of specialized plant species and, as such, it deserves our attention as plant scientists. However, the exploitation of the genetic material of these specialized plants also offers us the opportunity to enhance both the nutritional properties of crop plants and engineer plants ideally suited to phytoremediation. Although progress is being made in understanding the genetic basis of metal hyperaccumulation, a more complete understanding will be necessary before we can take full advantage of the genetic potential of these plants.

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PART 5

Antibody Biotechnology