Chapter 12
Role of Sulfate and S-Rich Compounds in Heavy Metal Tolerance and Accumulation

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Abstract Plants can withstand the potentially toxic effects exerted by a number of heavy metals through exclusion, which consists in restricted metal transport into plant tissues, or accumulation of metals, accompanied by the development of concomitant internal tolerance mechanisms. Plant tolerance and accumulation to heavy metals are known to be related to sulfur assimilation. The presence of metals can differently modulate genes involved in sulfate uptake and induce the sulfate assimilatory pathway. The regulation of S assimilation may be necessary to ensure an adequate supply of sulfur compounds required for heavy metal detoxification. The individuation of limiting steps of the sulfate assimilatory pathway in heavy metal tolerance and accumulation allows the possibility of genetic engineering approaches in order to develop plants with augmented phytoremediation capacity.

1 Introduction

Plants can adapt to a wide range of environmental stress conditions using appropriate physiological responses. In the case of heavy metals stress, these mainly consist in modulation of the activity of plasma membrane and/or vacuolar transporters, secretion of metal chelating organic acids such as malate or citrate in the rhizosphere, and biosynthesis of intracellular metal chelators (Clemens 2006).

In this context, sulfur (S) plays a pivotal role because sulfate transporters can mediate the entry of sulfate-analogues into the cells (Maruyama-Nakashita et al. 2007), and S-containing compounds like glutathione (GSH), phytochelatins (PCs), and metallothioneins (MTs) can improve the tolerance of plants to several metals and metalloids through complexation and/or further sequestration of toxic forms inside cellular vacuoles (Xiang and Oliver 1998, Cobbett and Goldsbrough 2002, Hall 2002). Because of this, many studies have been focused on heavy metals

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uptake, accumulation, and tolerance in relation to sulfur availability to plants, and efforts have been made and others are underway to increase the phytoremediation potential of such species with traits that make them highly suitable for the clean up of heavy-metal-contaminated sites (Pilon-Smits and Pilon 2002, Cherian and Oliveira 2005, Pilon-Smits 2005, Pilon-Smits and Freeman 2006).

One promising approach to enhance plant metal accumulation and tolerance is genetic engineering, which allows the insertion of foreign genes of interest into plants. Such genes can be overexpressed by modulation of specific promoters, which leads to overproduction of desirable molecules involved in heavy metal tolerance and accumulation, like GSH, PCs, and MTs. Several transgenics developed in this way have been successful with respect to one or more metal(loids) in a number of trials.

2 Sulfate's Role in Heavy Metal Uptake

Heavy metal(loid)s like selenium (Se), chromium (Cr), and molybdenum (Mo) may cause sulfur deficiency in plants by inhibiting the uptake of sulfate from the environment when they are furnished to plants in the forms of selenate, chromate, and molybdate, respectively (Hopper and Parker 1999, Alhendawi et al. 2005, Maruyama-Nakashita et al. 2007, Schiavon et al. 2007, 2008). On the other hand, such elements as cadmium (Cd), zinc (Zn), and copper (Cu) have been reported to induce the absorption of sulfate for sustaining greater sulfur demand during the biosynthesis of the sulfur-containing compounds, glutathione and phytochelatins, notably involved in metal tolerance (Nocito et al. 2002, 2006).

On account of this, the interactions between sulfate and heavy metals must be considered when plants are going to be employed for the remediation of metal-contaminated sites, as the accumulation of contaminants in plant tissues might be altered by the sulfate concentration in the substrate where plants grow. Also, the use of sulfur-containing fertilizers during agronomic practices should be tightly managed to avoid excessive accumulation of nonessential elements and trace nutrients in crops.

2.1 Plant Availability of Chromate, Selenate, and Molybdate as Influenced by the Competing Ion Sulfate

The uptake of heavy metals and metalloids by plants is governed by many soil factors, which often include the presence of competitive ions (Hopper and Parker 1999). In particular, the group VI elements selenium and chromium are known to function as toxic analogues of sulfur (Mikkelsen and Wan 1990, Maruyama-Nakashita et al. 2007).

Selenium is an essential trace element in human and animal diets (Rayman 2000), as it is required for the production of selenoproteins involved in scavenging injurious free radicals (Zhang et al. 2003, Sors et al. 2005, Galeas et al. 2006). Although higher plants do not need Se for their metabolism (Novoselov et al. 2002, Sors et al. 2005),

they import Se from the environment mainly in the forms of selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), or organic Se compounds (Ellis and Salt 2003, Sors et al. 2005). The rate and form of Se uptake is strictly dependent on the concentration and chemical form of Se in the soil solution, as well as on the rhizosphere conditions (Sors et al. 2005). Generally, when selenate and selenite are present at equimolar concentrations, the uptake of selenate is greater than that of selenite (Mikkelsen et al. 1987, Zayed et al. 1998, Pilon-Smits et al. 1999a, Zhang et al. 2003).

The antagonistic interaction between sulfate and selenate for active transport into roots, as well as interference during their assimilation in plants, has long been investigated (Ferrari and Renosto 1972, Mikkelsen and Wan 1990, Bailey et al. 1995, Barak and Goldman 1997, Cherest et al. 1997, Shibagaki et al. 2002, White et al. 2004). Because of its close similarity in properties to sulfate, selenate is rapidly transported over membranes by the activity of sulfate transporters. Hence, it competitively inhibits the influx of sulfate and accesses the sulfur metabolic pathway for insertion into the Se nonprotein amino acid analogues selenocysteine (SeCys) and selenomethionine (SeMet) (Ferrari and Renosto 1972, Mikkelsen and Wan 1990, Hopper and Parker 1999, Ellis and Salt 2003). This competition is generally considered to cause the toxic reaction of plants to selenate, which can substitute sulfur in proteins and other sulfur compounds, leading to the reduced synthesis of important sulfur-molecules such as cysteine (Cys), methionine (Met), and glutathione (GSH), and to the disruption of several biochemical processes (Anderson and Scarf 1983, Bailey et al. 1995, Galeas et al. 2006, Kassis et al. 2007).

A number of mutants lacking sulfate transporter genes have been isolated from yeasts and plants by using the toxic effects of selenate as sulfate analogue (Cherest et al. 1997, Shibagaki et al. 2002). The finding that *A. thaliana sel* mutants were tolerant to selenate because they lacked root high-affinity sulfate transporter genes *SULTR1;1* or *SULTR1;2*, undoubtedly indicated a role for these transporters in taking up both sulfate and selenate from the soil solution into the root cells (Shibagaki et al. 2002, Yoshimoto et al. 2002, Kassis et al. 2007), and suggested that root growth and potentially root tip activity could represent a specific target of selenate toxicity in plants. Indeed, the selenate resistance phenotype of the *A. thaliana sel* mutants containing a lesion in the *SULTR1;2* gene was accompanied by a significant increase of root growth and root sulfate to selenate ratio under selenate treatment, while the sulfate uptake capacity of roots was clearly reduced, as well as the contents of sulfate and selenate in plant tissues (Kassis et al. 2007). The mechanism conferring the selenate resistance phenotype to the *A. thaliana sel* mutants is thought to be root-specific, as differences in sulfate to selenate ratio between mutants and wild-type were observed only in roots. The mechanism most likely consists of the combined effects of a restricted selenate uptake and a defensive role of sulfate against the toxicity of selenate induced on root growth.

In order to better exploit the use of plants in Se phytoremediation additional studies focused on Se uptake and accumulation versus sulfur availability in various plant species (Mikkelsen and Wan 1990, Bailey et al. 1995, Barak and Goldman 1997, Hopper and Parker 1999, White et al. 2004). In general, the provision of high sulfate concentrations to plants leads to a significant decline of selenate uptake and its

content in the aerial plant tissues (Bailey et al. 1995, White et al. 2004); in contrast, increasing selenate concentrations promoted the accumulation of both sulfur and selenium in leaves of *A. thaliana* (White et al. 2004), and of *Hordeum vulgare* and *Oryza sativa* at low sulfate concentration (Mikkelsen and Wan 1990).

The other analogue of sulfur, chromium, is regarded as one of the most noxious pollutants thought to pose a threat to animal and human health. In nature Cr is largely widespread and most frequently found in the trivalent (+3) and hexavalent (+6) oxidation states. Such forms, which can interchange depending upon several physical, biological, and chemical processes occurring in waters and soils (Kotas and Stasicka 2000), differ significantly in toxicity and environmental hazard. In particular, Cr^{6+} compounds (chromates and dichromates) are considered extremely toxic and harmful for living organisms due to their powerful oxidizing and carcinogenic properties. They can presumably react with cellular reducing cofactors to produce Cr^{3+} ions and valence intermediate-Cr ion species, which are most likely involved in chromium genotoxicity (Maruyama-Nakashita et al. 2007). Since a role for Cr in plant metabolism has not been evidenced so far, it seems reasonable to suppose that plants have not evolved specific mechanisms to take up this metal ion from external substrates (Zayed and Terry 2003).

Similar to selenate, the transport of chromate into the cells is active and appears to be mediated by sulfate carriers (Shewry and Peterson 1974, Skeffington et al. 1976, Cherest et al. 1997, Kleiman and Cogliatti 1997, Kim et al. 2005, Maruyama-Nakashita et al. 2007, Schiavon et al. 2007). The influx of chromate showed the Michaelis–Menten kinetics when the external concentration of Cr varied from 10 to 160 μM, and was competitively inhibited by 10 μM sulfate (Skeffington et al. 1976). The drastic inhibition (up to 75%) of chromate uptake by 250 μM sulfate or other Group VI anions (selenate, molybdate, and arsenate) was also observed by Shewry and Peterson (1974) in barley seedlings, except when chromate and sulfate were provided to plants at equimolar concentrations.

Further investigation on the uptake of chromate, in wheat reported the enhancement of chromate absorption in sulfate-deprived plants within 5 hours of exposure to either 1 or 5 μg ml^{-1} chromate, most due to likely due to the lack of sulfate-competition (Kleiman and Cogliatti 1997). The increase of chromate uptake was also dependent upon the duration of the sulfate deprivation pretreatment, as 5 days rather than 2 days of sulfur absence in the nutrient solution could cause a stronger de-repression of the sulfate transport system, which in turn induced the activity of the plasma membrane transporters implied in the uptake of sulfate and sulfate-chemical analogues like chromate, as already observed for selenate (Lee 1982).

A recent work suggests that chromate may affect the sulfate uptake capacity in *Zea mays* plants by competing for the active binding site of sulfate transporters and/or repressing the high-affinity sulfate transport system at transcriptional level (Schiavon et al. 2007). Indeed, short-term exposure to chromate at concentrations ranging within 0.05-1 mM was found to specifically inhibit the rate of sulfate influx in maize-pretreated S-deprived plants, and 2 days of 200 μM chromate treatment strongly repressed the gene expression of the maize root high-affinity sulfate

transporter *ZmST1;1*. Sustaining a role of chromate as competitor of sulfate for transport into the cells, the maximum chromium accumulation in maize plant tissues was recorded under S limitation as a result of the higher rate of the chromate uptake observed in S-starved plants following few hours of Cr exposure. Concomitantly, chromate lowered the concentration of sulfur and sulfate in S-provisioned plants to the basal level of S-starved, thus suggesting that maize plants may respond to chromate stress by reducing S accumulation (Schiavon et al. 2007). Similar results were also obtained in *B. juncea*, where the inhibition of the root low-affinity sulfate transporter and the reduction of the sulfate uptake capacity were observed concomitantly with the enhancement of sulfur assimilation as well as of GSH biosynthesis (Schiavon et al. 2008).

The involvement of sulfate transporters in the chromate uptake has been also corroborated by investigation on transgenics. The constitutive expression of the root high-affinity sulfate transporter encoded by the SHST1 gene from *Stylosanthes hamata*, resulted in enhanced capacity to take up chromate in *Brassica juncea* seedlings and mature plants (Lindblom et al. 2006). Transgenic tobacco plants overexpressing a putative yeast transcriptional activator MSN1 involved in chromium accumulation (Chang et al. 2003) showed greater accumulation of both Cr and S and higher tolerance to chromate. At the same time, the overexpression of MSN1 in transgenics increased the transcript level of the *NtST1* gene (*Nicotiana tabacum* high affinity sulfate transporter 1), therefore finally indicating that chromate and sulfate share a common transport system to enter into cells. In support of this, the expression of *NtST1* in *Saccharomyces cerevisiae* increased the yeast ability to accumulate Cr and S (Kim et al. 2005).

Unlike Cr and Se, molybdenum (Mo) is an essential micronutrient for plant metabolism (Marschner 1995, Kaiser et al. 2005) functioning as constituent of the mononuclear Mo enzymes involved in detoxifying excess sulfite (sulfite oxidase), purine catabolism (xanthine dehydrogenase), nitrate assimilation (nitrate reductase), and phytohormones biosynthesis (aldehyde oxidase[s]) (Hale et al. 2001, Mendel and Hänsch 2002, Mendel and Florian 2006). Inside cells the metal itself is biologically inactive if not complexed by a special cofactor (Mendel and Schwarz 1999). With the exception of the bacterial nitrogenase, where Mo is included in the FeMo-cofactor, Mo is bound to a pterin, thereby forming the molybdenum cofactor (Moco), which is the active compound at the catalytic site of all other Mo-enzymes (Mendel and Florian 2006).

To date, Mo transport systems and mechanisms of homeostasis have been identified and characterized in bacteria and some lower-order eukaryotes (Mendel and Häensch 2002), while in plants they are still weakly understood (Zimmer and Mendel 1999, Kaiser et al. 2005). Solely in the model plant *Arabidopsis thaliana* has a Mo-specific metal transporter, AMA1, been cloned and characterized (Palmgren and Harper 1999).

However, similarities in physiological responses to Mo between prokaryotes and eukaryotes exist (Kaiser et al. 2005), and evidences from bacteria studies indicate that Mo is actively transported across the plasma membranes of cells in the form of molybdate (Kannan and Ramani 1978), most likely competing with the similar-sized anion sulfate for the binding transport sites in uptake at root surface (Stout

and Meagher 1948, Stout et al. 1951, Self et al. 2001). Sustaining this, the provision of sulfate to sulfur-starved plants altered the transport of Mo in the xylem sap of tomato (Alhendawi et al. 2005), lowering the Mo concentration of the sap and increasing sulfur levels. The analyses of Mo transport as measured by root pressure exudates also revealed that the absence of sulfate in the nutrient-growing medium could enhance Mo uptake by plants.

The effect of sulfate on molybdate uptake is not only detectable at the root/soil interface. Soybean plants showed decreased molybdenum levels in aerial plant tissues as the sulfate supply increased, even though molybdenum was applied as a foliar spray (Kannan and Ramani 1978). Following the first studies by Stout and Meagher (1948), sulfate has been proved to act as an effective regulator of molybdenum uptake in many plant species under a wide range of growing and environmental conditions (Macleod et al. 1997).

Recently, efforts to conceive plant-based tests for quantifying environmental concentration of heavy metals have been made due to their low cost and lack of requirement for technical means for analysis. From this standpoint, the employment of sulfur-responsive promoters of sulfate transporter genes may represent a promising tool for monitoring sulfate analogues. Maruyama-Nakashita et al. (2007) used a fusion gene construct consisting of a sulfur-responsive promoter region of the *A. thaliana* root high-affinity sulfate/selenate transporter SULTR1;2 and green fluorescent protein (GFP; $P_{SULTR1;2}$-GFP) to quantify the external levels of selenate and chromate via GFP accumulation. The $P_{SULTR1;2}$-GFP transgenics significantly increased in GFP following selenate or chromate treatment. The increase in GFP was linearly dependent upon the amount of Se and Cr in the growing medium, suggesting the potential use of $P_{SULTR1;2}$-GFP plants as indicators in detecting environmental selenate and chromate concentrations. However, for practical applications the sensitivity of $P_{SULTR1;2}$-GFP plants for selenate and chromate is still too low within the environmental limitations of Se and Cr, but more sensitive indicators could be designed through arranging the promoter regions and reporter proteins.

2.2 Enhanced Sulfate Uptake in Response to Cadmium, Zinc, or Copper Excess

Genes encoding sulfate transporters are involved in Cd-detoxification mechanisms, which imply the synthesis of phytochelatins (Nocito et al. 2002, 2006); they are strictly regulated by the accumulation of intermediates generated along the sulfate reductive assimilatory and GSH biosynthesis pathways, which may function as signals for the modulation of sulfate uptake (Hawkesford and De Kok 2006).

The induction of the sulfate uptake capacity in *Zea mays* plants exposed to toxic concentrations of either Cd, Zn, or Cu is closely dependent on the upregulation of the high-affinity sulfate transport system (HATS), as well as on the external metal concentration (Nocito et al. 2002, 2006). In particular, the enhancement in transcript accumulation of the maize root high-affinity sulfate transport gene, ZmST1;1,

was observed following all Cd, Zn, and Cu treatments. It occurred earlier and was more pronounced under 10 μM Cd exposure than that occurring upon sulfur starvation, which was compared during the same time courses (Nocito et al. 2002, 2006). Concomitantly with the induced sulfate uptake activity and sulfate transporters transcription, the content of nonprotein thiols (NPTs) significantly increased following Cd or Zn treatments, and was accompanied by a transient depletion or no variation of the GSH pools in plus Cd and Zn, respectively. Neither NPTs nor total GSH were affected by excess Cu; however, the ratio GSH to glutathione oxidized strongly varied, thus suggesting that Cu could induce sulfate uptake in response to a reduction in the root GSH pool.

In *Brassica juncea* the downregulation of a low-affinity sulfate transporter most likely involved in the translocation of sulfate, *BjST1*, was observed in response to Cd exposure (Heiss et al. 1999). The authors interpreted this effect with the need of plants to retain high sulfate amounts in roots, which may be required for filling the greater demand of GSH and S-chelating compounds to cope Cd stress.

3 Cellular Signaling of Heavy Metals: Activation of Internal S-Related Chelating Mechanisms

Several transition metal ions are essential for plants and algae but, together with nonessential heavy metal ions, they can be toxic to living cells when present in excess. Excess metal ions activate redox mechanisms, leading to the formation of hydroxyl radicals through Haber-Weiss and Fenton reactions (Halliwell and Gutteridge 1990).

In plants, the response to the uptake and accumulation of heavy metals involves a variety of potential mechanisms, including the biosynthesis of two classes of metal-chelating compounds, phytochelatins (PCs) and metallothioneins (MTs), which bind metals in thiolate complexes (Cobbett and Goldsbrough 2002).

As the accumulation of metals rises, plants must cope with the enhanced requirement for amino acids, in particular cysteine, needed for the formation of MTs and PCs. Their synthesis is energy expensive and requires increasing amounts of sulfur and nitrogen. Therefore, the increasing demand for the synthesis of S-containing metal ligands might affect plant growth and consequently limit the use of certain plant species as possible phytoremediators (Tong et al. 2004). On metal-rich soil certain plant species and varieties have adapted a very pronounced hypertolerance to toxic metal concentration. More than 400 plant species are known to hyperaccumulate metals, including Cu, Ni, Zn, Cd, As, and Se (Baker and Brooks 1989)

3.1 Gluthatione and Phytochelatins

The tripeptide (γ-GluCysGly) glutathione (GSH) plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction,

conjugation of metabolites, detoxification of xenobiotics (Xiang et al. 2001), and the expression of stress-responsive genes (Mullineaux and Rausch 2005). Indeed, GSH acts as an antioxidant, quenching the reactive oxygen species (ROS) generated in response to stress before they cause damages to cells (Xiang et al. 2001). When present in excess, several heavy metals may induce variation in plant GSH content (Rauser 2001). In plants exposed to Cd the cellular GSH concentration transiently declined (Steffens 1990), inducing an increased activity of the two enzymes of GSH synthesis, γ -glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS) (Rüegsegger et al. 1990, Rüegsegger and Brunold 1992).

The availability of cysteine may restrict the rate of GSH synthesis (Noctor et al. 1996), and therefore an increased assimilatory flux for sulfur should be required. Upstream of GSH synthesis is the assimilation of sulfate as well as the biosynthesis of cysteine. The transcriptional upregulation of several steps in these pathways has been thoroughly described. These include the uptake of sulfate (Nocito et al 2002, 2006), the sulfate activation catalyzed by ATP sulfurylase, and the following reduction to sulfite by adenosine 5-phosphosulfate reductase (Heiss et al 1999), the synthesis of cysteine promoted by O-acetylserine(thiol)lyase (Dominguez-Solis et al 2001), and the biosynthesis of GSH involving two enzymes, γ-glumatyl synthetase and GSH synthetase (Xiang and Oliver 1998). However, the mechanisms at the basis of the upregulation are still poorly understood. In one study, under metal excess jasmonate was found to be involved in the transcriptional control of GSH biosynthesis genes (Xiang and Oliver 1998), with the upregulation of the genes encoding γ-ECS, GS and GSH reductase (GR). It remains unclear whether the upregulation is directly triggered by metal sensing or by the increased requirement for sulfur caused by GSH enhanced synthesis.

Although heavy metal hyperaccumulators have been widely studied in the last years with the aim of better understanding the biochemical and molecular basis of metal tolerance, the molecular signaling pathways that control these mechanisms are not fully understood. In plants of *Thlaspi* Ni-hyperaccumulators, the elevated levels GSH found to be involved in Ni-tolerance were due to the constitutively increased activity of Ser acetyltransferase (SAT) (Freeman et al. 2004).

GSH serves an additional function in plant responses to heavy metal stress as a precursor of metal-chelating oligopeptides, the phytochelatins (PCs), which are synthesized by a reaction of transpeptidation of γ-glutamyl-cysteinyl units catalyzed by phytochelatin synthase (PCS) (Grill et al. 1989). Plants produce phytochelatins (PCs) in response to excess of several metal ions, including Ni, As, Cd, Zn, Ag, Sb, Cu, Hg, Pb, and Te (Cobbett 2003). PCs are small peptides of the general structure (-Glu-Cys)nGly, where n = 2–11, although (γ-Glu-Cys)2 -Gly (PC2) and (γ-Glu-Cys)3-Gly (PC3) are the most common (Cobbett and Goldsbrough 2002). No other environmental factors are known that would induce PC accumulation.

The vacuole is generally the main storage site for metals in yeast and plant cells, and there is evidence that phytochelatin-metal complexes are pumped into the plant vacuole (Salt and Rauser 1995).

PCS is activated through metal ions and/or metal-GS complexes (Vatamaniuk et al. 2000), and no signaling cascade seems to be involved in the activation of the

enzyme since only the substrate is needed for the functioning of the enzyme in vitro (Clemens 2006).

However, the precise mechanism of enzyme activation by either free heavy metal ions or metal-thiolate complexes is still a matter of debate (Ducruix et al. 2006).

The most convincing evidence of the role of PCs in heavy metal detoxification was obtained by Ha et al. (1999) by comparing the relative sensitivity of PC synthase-deficient mutants of *Arabidopsis* and *Schizosaccharomyces pombe* to different heavy metals. PC-deficient mutants of both organisms were sensitive to Cd and arsenate, while for many other metals (Zn, Cu, Hg, Ni, Ag) and selenite, little or no sensitivity was observed. Vatamaniuk et al. (2000) showed that purified tagged AtPCS1 catalyzed PC synthesis in the presence of Cu, Zn, Mg, Ni, or Co, while Oven et al. (2002) found that purified untagged AtPCS1 did not show any activity with Mg, Ni, or Co. The regulation of PCS has been extensively studied. In *Thlaspi* Ni-hyperaccumulators Freeman et al. (2005) showed that salicylic acid activated Ser acetyltransferase (SAT), enhancing GSH accumulation and GR activity, while inhibiting PCS activity. This finding was explained as a need for hyperaccumulators to maintain high levels of GSH acting as antioxidant. The paradoxical observation that overexpression of *Arabidopsis* phytochelatin synthase provides Cd sensitivity is a confirmation that depletion of GSH pools for phytochelatin synthesis can reduce metal tolerance (Lee et al. 2003). In the same plant species, the cellular pool of available glycine constituted a limiting factor for the synthesis of PCs and iso-PCs under Cd stress (Ducruix et al. 2006).

3.2 Metallothioneins

Metallothioneins (MTs) are a superfamily of low molecular mass cysteine-rich, metal-binding proteins. Their ability to bind such metal ions as Cd, Zn, Cu, Co, Ag, and Hg is attributable to the arrangement of the Cys residues. According to Cobbett and Goldsbrough (2002) MTs can be classified into three classes, based on the arrangement of Cys residues: Class-I MTs are monomers with two Cys-rich clusters separated by a spacing region; Class-II MTs are translational monomers in which Cys residues are scattered throughout the entire sequence, and are further subdivided into four types (MT1, MT2, MT3, MT4) based on amino acid sequence; and Class-III MTs consist of peptide chains of variable length.

Many genes and cDNAs encoding MTs have been isolated in plants (Cobbett and Goldsbrough 2002), and functional complementation has been useful to show a relation between plant MT genes and plant metal tolerance (Giritch et al. 1998, Ma et al. 2003, Kohler et al. 2004, Zhang et al. 2004, Castiglione el al. 2006). Although the possible involvement of MTs in plant metal detoxification and homeostasis has been thoroughly studied and a number of metal responsive elements (MRE) have been identified in the promoter regions of the MTs genes and characterized in yeasts and mammalian (Cobbett and Goldsbrough 2002), little is known about the transcriptional regulation of plant MTs genes in response to heavy metals. To date the promoter

PvSR2 isolated in tobacco (Qi et al. 2007) represents the only example of plant MRE sequences-containing promoters that is heavy-metal-specific-responsive. No evidences of other MRE or MRE-like sequences conferring heavy-metal-specific-responsiveness to MT genes in plants have yet been provided. Therefore, alternative mechanisms of sensing, such as signal transduction pathways that involve mitogen-activated protein kinases (MAPKs), may be hypothesized to induce the transcription of defense genes, including MT genes. Indeed, several distinct MAPK pathways have been recently corroborated to be activated in response to copper and cadmium stress (Jonak et al. 2004).

4 Enhancement of Heavy Metal Accumulation Through Overexpression of Enzymes Involved in S-Assimilation and GSH/PCs Biosynthesis

Naturally occurring metal hyperaccumulators are promising candidates for the phytoextraction of heavy metals from contaminated sites. However, factors such as slow growth, shallow root system, and small biomass production often limit their use (Chaney et al. 1997, Pilon-Smits 2005).

Genetic engineering approaches represent a powerful tool to increase the ability of plants to remediate environmental pollutants (Pilon-Smits and Pilon 2002, Tong et al. 2004, Pilon-Smits 2005). On account of this, two possible strategies can be used: enhancing the biomass productivity of hyperaccumulators or improving the tolerance and/or the accumulation of heavy metals in high biomass producing and fast-growing plants (Pilon-Smits and Pilon 2002, Pilon-Smits and Freeman 2006). The latter is more easily achievable, as plant productivity is under the control of several genes and is fairly hard to obtain through a single gene insertion (Pilon-Smits and Pilon 2002).

The enhancement of plant metal tolerance and/or accumulation can be carried out by the acceleration of an existing plant process that is limiting for metal remediation or by the transfer and overexpression of a new pathway from other organisms, ranging from bacteria to mammalian, into plants (Clemens et al 2002, Pilon-Smits and Pilon 2002, Cherian and Oliveira 2005). The foreign genes introduced are usually integrated in the nuclear genome or targeted to the chloroplasts, and appropriate promoters are used to modulate their expression (Pilon-Smits and Freeman 2006).

The understanding of the mechanisms involved in metal tolerance and accumulation is required to develop transgenics that may be successfully employed in phytoremediation. To date, a number of metal detoxification systems have been genetically and functionally characterized at the molecular level in plants, yeasts, and bacteria, and several works have utilized the overexpression approach to dissect the involvement of enzymes in alleviating heavy metal stress in plants. In particular, some transgenics with enhanced metal tolerance and accumulation have been

developed by overexpression of sulfate transporters (Lindblom et al. 2006), enzymes involved in the sulfate assimilation (Pilon-Smits et al. 1999b, Dominiguez-Solis et al. 2001, Dominiguez-Solis et al. 2004, Kawashima et al. 2004, Van Huysen et al. 2004, Wangeline et al. 2004), and biosynthesis of GSH, PCs, and MTs (Hasegawa et al. 1997, Zhu et al. 1999ab, Lee et al. 2003, Li et al. 2004).

The overexpression of the *Escherichia coli gshI* and *gshII* genes encoding γ-ECS and GS respectively, conferred to the metal accumulator *B. juncea* increased Cd tolerance and accumulation (Zhu et al. 1999ab). Specifically, overexpression of *gshII* in the cytosol increased Cd concentrations in the shoot up to 25% and total Cd accumulation per shoot up to threefold compared with the wild-type, and promoted the synthesis of GSH and PCs. The overexpression of *E. coli gshI* targeted to the plastids resulted in transgenic plants that well tolerated and accumulated shoot Cd concentration 40%–90% higher than in the wild-type. This was most likely because of greater production of GSH (1.5- to 2.5-fold) and PCs. Overexpression of γ-ECS also led to greater accumulation of total sulfur in the shoot of *B. juncea* as already found in poplar, thus indicating an added benefit of enhanced sulfur metabolism (Arisi et al. 1997, Zhu et al. 1999a).

The induced synthesis of thiols in response to Cd was also observed in *A. thaliana* plants overexpressing OASTL and, together with the increased rate of cysteine biosynthesis, is thought to be responsible for the augmented Cd tolerance and accumulation (Dominiguez-Solis et al. 2001, Dominguez-Solis et al. 2004).

The overexpression of adenosine triphosphate sulfurylase (APS) led to increased selenate uptake, reduction and tolerance in *B. juncea* with rates of Se accumulation in shoots 2- to 3-fold and 1.5-fold higher in shoots and roots, respectively, compared to wild-type (Pilon-Smits et al. 1999b). Similarly, transgenic *B. juncea* plants overexpressing the enzymes cystathionine-γ-synthase and selenocysteine methyltransferase promoting selenium (Se) organication and volatilization showed increased tolerance to Se and higher rates of Se volatilization (LeDuc et al. 2004, Van Huysen et al. 2004). Also, glutathione reductase (GR), an enzyme that regenerates GSH, was overexpressed in *B. juncea* and was found to be most effective in providing chloroplastic tolerance. Indeed, GR transgenic plants had increased Cd tolerance of the plastids when the gene was targeted there, while the whole plant Cd tolerance was not affected (Pilon-Smits et al. 2000).

In another study, transgenic *B. juncea* plants overexpressing γ-ECS, GS, and APS showed improved phytoremediation ability when tested on soil contaminated with mixtures of metals (Bennett et al. 2003). The ECS and GS transgenics accumulated more Cd (+50%) and Zn (+45%for GS and +93%for ECS) in shoot than wild-type. Additionally, the ECS transgenics contained greater amounts of Cr (+170%), Cu (+140%), and Pb (+200%) relative to wild-type plants.

Other efforts to increase heavy metal tolerance and accumulation in plants have focused on the enhancement of the plant ability to sequester metals in nontoxic forms inside cells through the overexpression of genes directly encoding metal-chelating compounds like PCs or MTs. Paradoxically, the overexpression of the phytochelatins synthase *AtPCS1* in *A. thaliana* did not enable plants to be more

tolerant and/or to accumulate more Cd and Zn; rather it induced hypersensitivity to these metals, which the authors explain as a result of the toxicity of supraoptimal levels of PCs generated when compared with GSH levels (Lee et al. 2003). Conversely, *AtPCS1* overexpressing *A. thaliana* plants showed more tolerance to arsenate and high expression of many unknown thiol products (Li et al. 2004). However, no significant accumulation of As occurred in the above-ground plant tissues. Gisbert et al. (2003) reported that the overexpression in *Nicotiana glauca* of a phytochelatins synthase encoding gene from wheat, TaPCS1, greatly improved its tolerance to such metals as lead (Pb) and Cd. The significant increase of Pb concentrations in up to 50% in the shoot and 85% in roots indicates that *Nicotiana glauca* represents a promising candidate for Pb and Cd phytoextraction from soil.

The overexpression of MTs often led to enhanced plant tolerance to the metals tested. Transgenic tobacco and rapeseed overexpressing the *MT2* human gene resulted in greater tolerance to Cd (Misra and Gedamu 1989), as well as the overexpression of the *CUP1* yeast gene in cauliflower (Hasegawa et al. 1997). In a related study, the overexpression of *CUP1* enabled tobacco plants to taken up Cu at higher rates, up to threefold relative to wild-type (Thomas et al. 2003). Greater Cu accumulation was also observed in *A. thaliana* transformants overexpressing the pea MT gene *PsMTA* (Evans et al. 1992).

While most of the above-mentioned investigations using transgenics have been realized in the laboratory and greenhouse, only one trial has been done in the field (Banuelos et al. 2005). This study successfully confirmed the results obtained in laboratory experiments with transgenics overexpressing the enzyme involved in sulfate/selenate reduction, which showed fivefold higher accumulation of Se in the field when grown on soil polluted with Se, boron (B), and other salts. Other field experiments with transgenics are presently underway (Pilon-Smits and Freeman 2006).

5 Concluding Remarks

Much progress in understanding the role of sulfur in heavy metals uptake and accumulation in plants has been made in the last years. However, a comprehensive picture of the signaling network necessary for a coordinated cellular response to heavy metals is still lacking, mainly because of the complex cross talk existing between heavy metals and other stress-signaling pathways, which involve redox mechanisms and antioxidant molecules.

Future research should be aimed at identifying new metal-responsive genes and elucidating the regulatory mechanisms of plant responses to heavy metal stress. The comprehension of these mechanisms will be helpful for engineering plants with enhanced ability for metal tolerance and accumulation, considering the success obtained in the field trials by using transgenics in metal remediation.

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