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Sulfur Metabolism and Cadmium Stress in Higher Plants

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ABSTRACT

Plant sulfur metabolism is deeply affected by cadmium (Cd) stress, mainly as a consequence of the activation of a wide range of adaptive responses involving glutathione (GSH) consuming activities. In fact, GSH not only acts as a direct or indirect antioxidant in mitigating Cd-induced oxidative stress, but also represents a key intermediate for the synthesis of phytochelatins (PCs), a class of cysteine-rich heavy metal-binding peptides involved in buffering cytosolic metal concentration. As a consequence, Cd exposure may result in a depletion of the cell GSH pools which in turn increases the plant demand for reduced sulfur compounds. In this condition the need for maintaining GSH homeostasis and an adequate PC biosynthesis rate is met by a general induction of enzyme activities directly or indirectly involved in sulfur metabolism. Transgenic plants overexpressing these enzymes exhibit a greater production of GSH and an increased Cd tolerance, confirming this pathway to be crucial for plant survival in metal polluted environments. In the present paper we analyze and discuss the biochemical and molecular mechanisms involved in the regulation of sulfate (the main sulfur source for plants) uptake and assimilation, and GSH synthesis during Cd detoxification.

Keywords: cysteine, detoxification, glutathione, heavy metals, phytochelatins, sulfate
Abbreviations: APS, 5′-adenylylsulfate; **Cd**, cadmium; **Cd·GS2**, bis(glutathionato)cadmium; **Cys**, cysteine; **γ-EC**, γ-glutamylcysteine; **Glu**, glutamate; **Gly**, glycine; **GSH**, glutathione; **GST**, glutathione *S*-transferase; **HMW**, high-molecular weight; **LMW**, low-molecular weight; **Met**, methionine; **OAS**, *O*-acetylserine; **PAPS**, 3′-phospho-5′-adenylylsulfate; **PC**, phytochelatin; **PCS**, phytochelatin synthase; **Ser**, serine; **STAS**, Sulfate Transporter/AntiSigma-factor antagonist

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PLANT SULFUR METABOLISM: AN OVERVIEW

Introduction

Sulfur is an ubiquitous and essential element for all living organisms. In plants it is found in two amino acids, cysteine (Cys) and methionine (Met), that are essential components of proteins, in iron-sulfur clusters, in polysaccharides and lipids, and in a broad variety of biomolecules such as vitamins (biotin and thiamine), cofactors (CoA and S-adenosyl-Met), peptides (glutathione and phytochelatins) and secondary products (allyl Cys sulfoxides and glucosinolates). Such molecules are involved in a multitude of essential biochemical and physiological processes, including enzyme activity regulation, redox cycles, heavy metal and xenobiotic detoxification, thought to be of crucial importance not only for growth and crop yield but also for adaptation to the adverse environmental conditions which plants may experience during their life. Excellent reviews regarding the cellular roles of sulfur containing compounds are available (Noctor *et al*. 1998; Leustek *et al*. 2000; Rausch and Wachter 2005).

Generally sulfur does not play specific structural roles in biological systems; in many cases the presence of sulfur atoms in biomolecules is responsible for their catalytic or electrochemical properties and thus for their involvement in specific biochemical mechanisms. For instance, the extreme nucleophilicity of the sulfhydryl group of Cys residues confers to many thiols the capacity to react with a broad spectrum of agents, ranging from free radicals, active oxygen species, and cytotoxic electrophilic organic xenobiotics, to heavy metals (Rabenstein 1989; Leustek *et al*. 2000), ac-

Received: 27 July, 2007. Accepted: 19 September, 2007.

Invited Review

counting for the unique roles that this amino acid plays in biological systems. Two Cys residues in a polypeptidic chain may interact in an oxidation reaction by forming a reversible disulfide bound which participates in maintaining protein structure and, sometimes, in the regulation of protein activity (Åslund and Beckwith 1999). The interconversion of two thiols in disulfide can also be involved in redox cycles where the electron transfer is thought to be essential in maintaining the cell redox status and in the physiological responses to the oxidative stress in all aerobic organisms. Such a simple cycle represents the chemical base making GSH a powerful cell redox buffer. Another interesting property of the sulfhydryl group in the GSH structure arises from its capability to readily react with a wide range of electrophylic compounds to form covalent bound glutathione *S*-conjugates (Leustek *et al*. 2000). This kind of reaction, catalyzed by glutathione *S*-transferases (GSTs), has been shown to be essential in the detoxification processes of toxins, xenobiotics and catabolic products (Marrs *et al*. 1995; Marrs 1996; Leustek *et al*. 2000). Finally, GSH is the direct precursor of phytochelatins (PCs), a class of enzymatically synthesized Cys-rich peptides in which the sulfhydryl groups of Cys residues are involved in buffering potentially toxic elements such as heavy metals (Zenk 1996).

Differently from animals which directly depend on organic sulfur compounds, plants have metabolic pathways that allow them to assimilate inorganic sulfur into organic sulfur compounds through a cascade of well characterized enzymatic steps. For this reason plant sulfur assimilatory pathways are thought to be the main sources of organic sulfur compounds for animal and human diets.

Although atmospheric sulfur dioxide can be taken up by leaves and assimilated into Cys, especially in polluted environments (de Kok 1990; de Kok *et al*. 1997), the main sulfur source for plants is the sulfate ion of the rhizosphere (Cram 1990; Clarkson *et al*. 1993; Marschner 1995), taken up by roots through specific high-affinity sulfate transporters localized on the plasma membrane of root cells. Once inside the plant, sulfate is allocated via the xylem and phloem routes to different sinks, and undergoes intracellular

Table 1 Inventory of the main characterized sulfate transporters in higher plants. For each transporter, the accession number of the relative cDNA as deposited in GenBank is reported.

Group	Name	Plant species	Accession number	Km	Notes	References
1	AtSultr1;1	*Arabidopsis thaliana*	AB018695	3.6 µM	Expressed in roots (tip, epidermis, root hairs, cortex) and hydathodes of cotyledons. Induced by limiting sulfur.	Takahashi *et al*. 2000
	ShST1	*Stylosanthes hamata*	X82255	10.0 µM	Expressed in roots. Induced by limiting sulfur.	Smith *et al*. 1995
	LeST1;1	*Lycopersicon esculentum*	AF347613	11.5 µM	Expressed in roots (epidermis, cortex and pericycle). Induced by limiting sulfur and in response to infection by *Verticillium dahliae*.	Howarth *et al*. 2003b
	HvST1	*Hordeum vulgare*	X96431	6.9 µM	Expressed in roots (endodermis, pericycle and xylem parenchyma). Induced by limiting sulfur and treatment with OAS.	Smith *et al*. 1997; Vidmar *et al*. 1999; Rae and Smith 2002
	ZmST1;1	*Zea mays*	AF355602	14.6 µM	Expressed in roots (epidermis and cell layer surrounding the central vascular bundle). Induced by limiting sulfur and Cd^{2+} exposure.	Bolchi *et al*. 1999; Nocito *et al*. 2002; Hopkins *et al*. 2004; Nocito *et al*. 2006
	AtSultr1;2	*Arabidopsis thaliana*	AB042322	6.9 µM	Expressed in roots (tip and cortex) and leaves (guard cells and hydathodes). Induced by limiting sulfur.	Shibagaki *et al*. 2002; Yoshimoto *et al*. 2002
	ShST2	*Stylosanthes hamata*	X82256	11.2 µM	Expressed in roots. Induced by limiting sulfur.	Smith *et al*. 1995
	LeST1;2	*Lycopersicon esculentum*	AF347614	9.8 µM	Expressed in roots (epidermis, cortex), stems and leaves. Induced by limiting sulfur.	Howarth *et al*. 2003b
	AtSultr1;3	*Arabidopsis thaliana*	AB049624	-	Expressed in the phloem of cotyledons, hypocotyls and roots. Induced in roots and leaves by limiting sulfur.	Yoshimoto *et al*. 2003
2	AtSultr2;1	*Arabidopsis thaliana*	AB003591	0.41 mM	Expressed in vascular tissues in both roots (xylem parenchyma and pericycle cells) and leaves (xylem parenchyma and phloem cells). Induced in the root tissues by limiting sulfur.	Takahashi *et al*. 1997, 2000; Kataoka *et al*. 2004a
	AtSultr2;2	*Arabidopsis thaliana*	D85416	≥1.2 mM	Expressed in roots (phloem cells) and leaves (vascular bundle sheath).	Takahashi *et al*. 2000
	ShST3	*Stylosanthes hamata*	X82454	99.2 µM	Expressed in roots and shoots. Induced in roots by limiting sulfur.	Smith *et al*. 1995
3	AtSultr3;1	*Arabidopsis thaliana*	D89631	-	Expressed in leaves.	Takahashi *et al*. 1999, 2000
	AtSultr3;2	*Arabidopsis thaliana*	AB004060	-	Expressed in leaves.	Takahashi *et al*. 1999, 2000
	AtSultr3;3	*Arabidopsis thaliana*	AB023423	-	Expressed in leaves.	Takahashi *et al*. 1999, 2000
	AtSultr3;4	*Arabidopsis thaliana*	AB054645	-	The expression pattern has not been determined.	Hawkesford 2003
	AtSultr3;5	*Arabidopsis thaliana*	AB061739	-	Constitutively expressed in roots (xylem parenchyma and pericycle cells) and shoots.	Kataoka *et al*. 2004a
4	AtSultr4;1	*Arabidopsis thaliana*	AB008782	-	Expressed in roots (pericycle and parenchyma cells of the vascular tissues), hypocotyls and leaves. Involved in the control of sulfate unloading from the vacuole. Induced by limiting sulfur.	Takahashi *et al*. 2000; Kataoka *et al*. 2004b
	AtSultr4;2	*Arabidopsis thaliana*	AB052775	-	Expressed in the mature part of roots (pericycle and parenchyma cells of the vascular tissues) and in the hypocotyls (vasculature, epidermis and cortical cells). Involved in the control of sulfate unloading from the vacuole. Induced by limiting sulfur.	Kataoka *et al*. 2004b
5	AtSultr5;1	*Arabidopsis thaliana*	NM_106680	-	The expression pattern has not been determined.	Hawkesford and de Kok 2006
	AtSultr5;2	*Arabidopsis thaliana*	NM_128127	-	The expression pattern has not been determined.	Hawkesford and de Kok 2006

channeling to chloroplast and vacuole, where it is assimilated into organic sulfur compounds or compartmentalized as sulfur store, respectively. Consequently, different sulfate transport systems are involved in mediating the sulfur fluxes through the whole plant in order to satisfy the sulfur requirement for growth, which varies during development and under different environmental conditions. The activity of these transporters is highly regulated and it is thought to be one of the main control points of sulfur metabolism (Hawkesford 2000).

Sulfate transport and sulfate transporter gene family

Sulfate transporters are classified as sulfate/proton cotransporters (Hawkesford 2003). Early studies report sulfate uptake as a process closely related to the transmembrane proton electrochemical gradient and thus dependent on the activity of plasma membrane H^+-ATPase (Lass and Ullrich-Eberius 1984; Hawkesford *et al.* 1993). More recently, several genes codifying sulfate transporters have been cloned and characterized in different plant species (Smith *et al.* 1995, 1997; Takahashi *et al.* 1997; Bolchi *et al.* 1999; Vidmar *et al.* 1999; Takahashi *et al.* 2000; Vidmar *et al.* 2000; Rae and Smith 2002; Shibagaki *et al.* 2002; Yoshimoto *et al.* 2002; Howarth *et al.* 2003b; Yoshimoto *et al.* 2003; Hopkins *et al.* 2004). An inventory of the most characterized sulfate transporters in higher plants is reported in **Table 1**. Most of these genes codify polypeptides of about 69-75 kDa, having a N-terminal region with 12 membrane spanning domains that constitutes the catalytic part of the protein, followed by a linking region that connects to a conserved C-terminal region, named STAS (Sulfate Transporter/AntiSigma-factor antagonist) domain because of its significant similarity to bacterial anti-sigma factor antagonists (Aravind and Koonin 2000; Hawkesford 2003). Several studies have suggested that the STAS domain is essential for the function and biogenesis of sulfate transporters, both facilitating their localization to plasma membrane and influencing the kinetic properties of the catalytic domain; an involvement of the STAS domain in mediating protein-protein interactions has also been suggested (Shibagaki and Grossman 2004; Rouached *et al.* 2005; Shibagaki and Grossman 2006).

The most extensively characterized plant is obviously *Arabidopsis thaliana*, where a multigenic family comprising 14 members has been described (for an extensive review see Hawkesford 2003). According to their amino acid sequences the members of the sulfate transporter family can be classified into five groups. The members of each group are suggested to have specialized functions for the uptake and distribution of sulfate in plants.

Transporters belonging to group 1 show high affinities for sulfate when expressed in yeast cells (Takahashi *et al.* 2000; Yoshimoto *et al.* 2002). AtSultr1;1 and AtSultr1;2 are predominantly expressed in root epidermis and cortex and are presumably involved in mediating primary sulfate uptake from the rhizosphere (Takahashi *et al.* 2000; Shibagaki *et al.* 2002; Yoshimoto *et al.* 2002). Both these transporters are regulated at transcriptional level in response to sulfate availability in the growing medium. Differently, AtSultr1;3 shows typical expression domains in sieve elements and companion cells of the phloem; according to this peculiar localization an involvement of this isoform in long distance sulfate translocation processes has been postulated (Yoshimoto *et al.* 2003).

The members of group 2, AtSultr2;1 and AtSultr2;2, have low-affinities for sulfate, are localized to vascular tissues of both roots and shoots and, thus, are presumably involved in controlling systemic sulfate translocation. Also the expression of these transporters has been shown to be regulated by sulfate availability (Takahashi *et al.* 2000).

Transporters falling into group 3 share significant sequence similarities and are expressed preferentially in leaves (Takahashi *et al.* 1999; Hawkesford 2003); however, their roles in plants are still unclear and await further investigations. Interestingly, it has been suggested that one of the five isoforms belonging to this group, AtSultr3;5, may form heterodimers with AtSultr2;1 facilitating the root-to-shoot transport of sulfate in the vasculature (Kataoka *et al.* 2004a). Thus, AtSultr3;5 could be considered as a component of the low-affinity sulfate uptake system controlling xylem sulfate translocation. This intriguing hypothesis mainly arises from these observations: i) AtSultr3;5 and AtSultr2;1 share the same expression domains in plant tissues; ii) the heterologous expression of AtSultr3;5 in a yeast mutant defective for sulfate uptake shows this protein as a non-functional transporter itself, whereas its co-expression with AtSultr2;1 enhances the sulfate uptake activity of AtSultr2;1.

Sulfate transporters of group 4 have been localized to the tonoplast. It has been proposed that these isoforms are involved in the control of sulfate unloading from the vacuole, playing a pivotal role in optimizing the distribution of sulfate within the cell (Kataoka *et al.* 2004b).

Finally, group 5 includes short amino acid sequences showing low similarity with all the other members of the sulfate transporter family (Hawkesford 2003; Hawkesford and de Kok 2006). To date no information are available about the contribution of these putative transporters in defining plant sulfate fluxes.

A similar number of genes is most likely present in other plant species (Buchner *et al.* 2004). The coordinated expression of this gene family is thought to facilitate optimum management of plant sulfate under varying conditions of supply and demand.

The sulfate assimilatory pathways

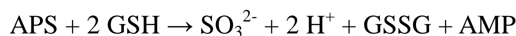
Once inside the cells sulfate can enter the metabolic pathways (**Fig. 1**) through an activation reaction catalyzed by ATP sulfurylase.

SO_4^{2-} + ATP → 5′-adenylylsulfate (APS) + PPi

This enzyme is prevalently localized in plastids, but also cytosolic isoforms have been described (Lunn *et al.* 1990; Renosto *et al.* 1993). The reaction product is an energy-rich mixed anhydride of phosphate and sulfate, the 5′-adenylylsulfate (APS), which represents a common intermediate for both reduction, in the reductive assimilatory pathway, and sulfation, in the non reductive assimilatory pathway.

The reductive assimilatory pathway consists of two phases: i) the reduction of APS to sulfide; ii) the assimilation of sulfide into Cys. All the enzymes involved in sulfide production are compartmentalized within the plastids (Brunold and Suter 1989).

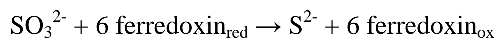
The first reaction of the reductive phase is catalyzed by APS reductase which transfers two electrons to APS, producing sulfite.



APS + 2 GSH → SO_3^{2-} + 2 H^+ + GSSG + AMP

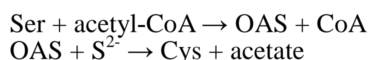
The electron donor in this reaction is the reduced glutathione (Bick *et al.* 1998; Kopriva and Koprivova 2004).

In a second step sulfite is reduced to sulfide following a six-electron transfer from reduced ferredoxin catalyzed by the enzyme sulfite reductase (Aketagawa and Tamura 1980; Krueger and Siegel 1982; Bork *et al.* 1996; Yonekura-Sakakibara *et al.* 1996).



SO_3^{2-} + 6 ferredoxin_{red} → S^{2-} + 6 ferredoxin_{ox}

Finally, in the assimilatory phase, sulfide is incorporated into *O*-acetylserine (OAS), a derivative of serine (Ser), leading to the formation of Cys, the end product of the reductive assimilatory pathway.



Ser + acetyl-CoA → OAS + CoA
OAS + S^{2-} → Cys + acetate

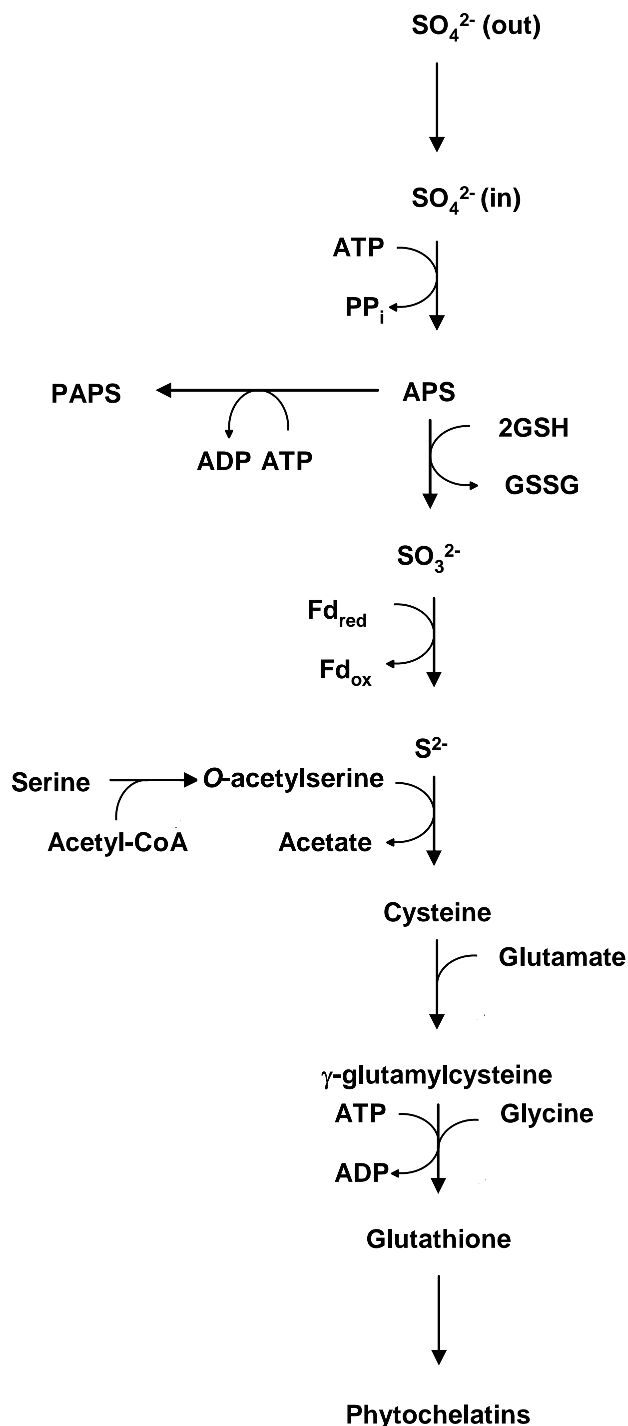


Fig. 1 Sulfate assimilation and GSH biosynthesis in higher plants. Sulfur is taken up as sulfate by means of specific sulfate transporters. Once inside the cell sulfate is activated to 5′-adenylylsulfate (APS) which enters the reductive assimilatory pathway where it is reduced to sulfite and then to sulfide. The overall reduction from sulfate to sulfide requires one ATP and eight electrons. Sulfide is then incorporated into *O*-acetylserine, a derivative of serine, yielding cysteine, the central intermediate from which most sulfur-containing compounds, such as glutathione and phytochelatins, are synthesized.

Two different enzymes are involved in sulfide assimilation: the Ser acetyltranferase, which produces OAS from Ser and acetyl-CoA, and OAS(thiol)-lyase, which transfers sulfide into the β-position of OAS. Both the enzymes are localized in cytosol, chloroplast and mitochondrion, differently from those of the reductive phase (Noji *et al.* 1998; Saito 2000; Hell *et al.* 2002; Saito 2004; Kawashima *et al.* 2005).

Cys is the terminal metabolite of the reductive pathway, but also represents the starting point for production of Met, GSH, and a broad variety of other sulfur metabolites (Saito 2004).

The non reductive pathway consists in the direct incorporation of sulfate (sulfation) into organic compounds. The sulfation reactions are promoted by cytosolic sulfotransferases which use 3′-phospho-5′-adenylylsulfate (PAPS) as sulfate donor to catalyze sulfation of a variety of compounds such as glucosinolates, flavonoids, jasmonates, brassinosteroids, peptides and extracellular polysaccharides (Varin *et al.* 1997). The production of PAPS from APS and ATP is catalyzed by the enzyme APS kinase (Lee and Leustek 1998).

The glutathione biosynthetic pathway

GSH, a tripeptide with the formula γ-Glu-Cys-Gly, represents the major form of nonprotein thiol compounds in plant cells (Kunert and Foyer 1993), where it plays pivotal functions in both defence and protection (Noctor *et al.* 1998; Rausch and Wachter 2005). Such a molecule indeed mainly acts as a redox buffer in protecting cells from oxygen reactive species which may accumulate in response to abiotic and biotic stresses, and in homeostatic adjustment of the cellular redox potential too. Furthermore, GSH has been described as involved in several other processes, including regulation of sulfur metabolism and inter-organ sulfur allocation (Lappartient and Touraine 1996), control of development and cell cycle (May *et al.* 1998; Vernoux *et al.* 2000), calcium signalling (Gomez *et al.* 2004), gene expression (Dron *et al.* 1988; Wingate *et al.* 1988; Herouart *et al.* 1993; Wingsle and Karpinski 1996; Baier and Dietz 1997; Ball *et al.* 2004), and xenobiotic and heavy metal detoxification (Rauser 1995; Marrs 1996). Changes in intracellular GSH concentration may thus be expected to have important consequences for cells, through modification of redox status, gene transcription and metabolic functions.

The pathway for GSH biosynthesis from Cys is well known and involves two sequential ATP-dependant reactions in plastids and cytosol (**Fig. 1**). In the first step of the pathway, γ-glutamylcysteine (γ-EC; γ-Glu-Cys) is synthesized by the enzyme γ-EC synthetase through the formation of an unusual peptide bond between the amine group of Cys and the γ-carboxyl group of the glutamate (Glu) side chain.

Glu + Cys + ATP → γ-Glu-Cys (γ-EC) + ADP + Pi

In the second step GSH synthetase catalyzes the addition of a glycine (Gly) to the C-terminal of γ-EC to produce GSH.

γ-Glu-Cys (γ-EC) + Gly + ATP → γ-Glu-Cys-Gly (GSH) + ADP + Pi

γ-EC synthetase activity and Cys availability are thought to be the main factors controlling GSH synthesis. In fact, it has been shown that the antisense suppression of γ-EC synthetase in *Arabidopsis* causes decrease in leaf GSH content; conversely, its overexpression increases the amount of leaf GSH (Xiang *et al.* 2001). Substantial increases in leaf GSH levels have also been observed in plants of poplar and tobacco overexpressing an *Escherichia coli* γ-EC synthetase (Noctor *et al.* 1996; Creissen *et al.* 1999). Interestingly, in *Arabidopsis* plants overexpressing γ-EC synthetase, the Cys pool is not depleted by the enhanced GSH biosynthesis, suggesting the existence of a coordinate regulation of Cys and GSH synthesis (Xiang *et al.* 2001). Moreover, like in animals, γ-EC synthetase is controlled through a negative feedback exerted by the end-product GSH on its activity (**Fig. 2**). Such post-translational control is thought to be crucial in controlling GSH concentration and homeostasis (Hell and Bergman 1990; Noctor *et al.* 1998, 2002).

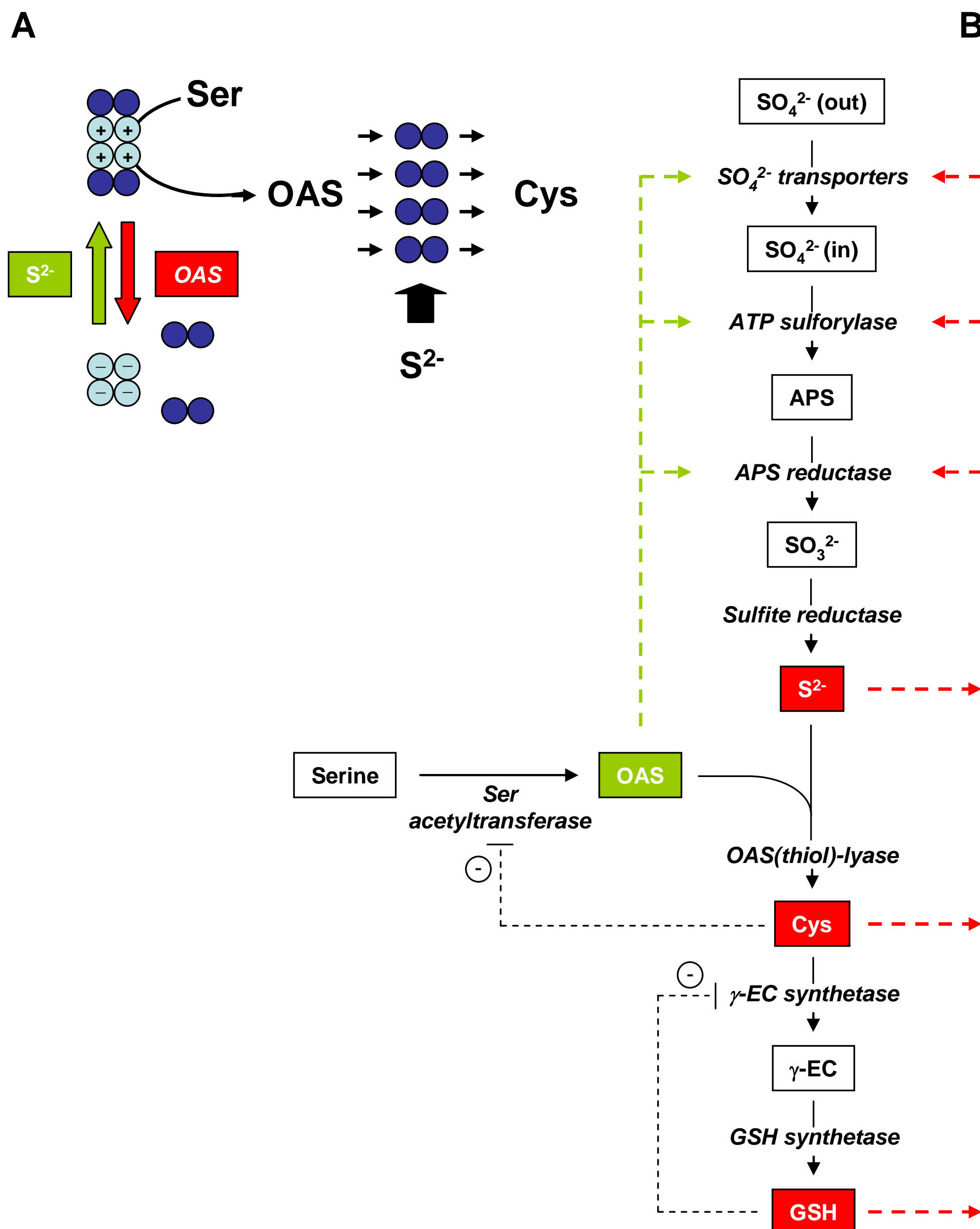


Fig. 2 Control of Cys and GSH biosynthesis. In the integrated regulatory model some intermediates along the pathways act as negative (red) or positive (green) signals in controlling the expression of key genes and the activity of some enzymes. (**A**) Regulation of Cys biosynthesis through the formation of a bi-enzyme complex between homotetrameric Ser acetyltransferase (light blue circles) and homodimeric OAS(thiol)-lyase (dark blue circles), in which OAS(thiol)-lyase acts as a regulatory subunit of Ser acetyltransferase. Sulfide availability promotes the formation of the complex, allowing OAS production and thus Cys biosynthesis; OAS accumulation disrupts the bi-enzyme complex. (**B**) Regulatory circuit controlling sulfur fluxes along the pathway of sulfate assimilation and GSH biosynthesis. Positive and negative regulatory loops controlling the transcription of key genes are shown in green and red, respectively. Negative allosteric regulations of Ser acetyltransferase and γ-EC synthetase are also shown.

DEMAND-DRIVEN REGULATION OF SULFUR METABOLISM

Since Cys is the key intermediate for the synthesis of a variety of sulfur metabolites, it is not surprising that the rate of its biosynthesis is finely modulated to meet the demand for Cys consuming activities, which largely contribute to define the total sulfur request by plants. Such a demand may consistently vary under the different environmental conditions that plants experience during their growth. For instance, biotic and abiotic stresses may increase the demand for some Cys derivative compounds, causing an increase in the activity of sulfate assimilatory pathway (Rausch and Wachter 2005). A similar metabolic activation has been largely described under sulfate limitation (Lappartient and Touraine 1996; Lappartient *et al.* 1999). In this condition plant sulfur needs to sustain the growth do not vary and then the induction of sulfate assimilatory pathway reflects some difficulties in maintaining both adequate rate of Cys biosynthesis and sulfur containing compound homeostasis. In fact, following sulfate withdrawal from the growing medium, the levels of sulfate, Cys, and GSH in plant tissues dramatically decrease leading to the induction of sulfate transporter systems and key enzymes along the assimilatory pathway (Lappartient and Touraine 1996; Lappartient *et al.* 1999). The most part of these responses are regulated at transcriptional level, as indicated by transcript accumulation of genes encoding sulfate transporters, ATP sulfurylase and APS reductase, and may be rapidly reverted by restoring optimal sulfate level in the medium or feeding plants with reduced sulfur containing compounds (Lappartient and Touraine 1996; Takahashi *et al.* 1996; Smith *et al.* 1997; Takahashi *et al.* 1997; Bolchi *et al.* 1999; Lappartient *et al.* 1999). From these observations it appears clear that a demand-driven homeostatic mechanism operates in order to match the rate of Cys biosynthesis with the total sulfur needs of the whole plant. Moreover, split root experiments on *Brassica napus* provide evidences supporting a regulatory mechanism for sulfate uptake and assimilation which results from direct sensing of the plant nutritional status rather than of the composition of the external solution supplied to the roots (Lappartient and Touraine 1996; Lappartient *et al.* 1999). This control necessary involves an inter-organ signalling mechanism in which a terminal product of the assimilatory pathway may act as long distance repressor signal. Experimental evidences suggest GSH as a phloem-translocated signal responsible for this regulation (Herschbach and Rennenberg 1991; Lappartient and Touraine 1996, 1997; Lappartient *et al.* 1999). However, in maize roots also Cys has been shown to act as a repressor (Bolchi *et al.* 1999).

An integrated and wide accepted model for regulation of sulfate uptake and assimilation in plants is reported in

Fig. 2. In this model some metabolites along the pathways of sulfate assimilation and GSH biosynthesis may act as negative or positive signals in controlling both the expression of key genes and the activities of some enzymes. Adequate levels of reduced sulfur containing compounds, such as Cys and GSH, would repress gene expression through a negative feedback loop which prevents excessive sulfate uptake and reduction, and useless energetic wasteful; *vice versa* a contraction of the pools of these compounds would de-repress gene transcription allowing sulfate to enter the pathway. Such a reversible regulation would result in a fine adaptation of sulfate fluxes suitable for plant survival in a wide range of environments.

A second regulatory loop, involving OAS as a key intermediate, may act in promoting gene de-repression when nitrogen and carbon supply exceeds sulfur availability within the cells. In this condition, since sulfide availability is not enough for Cys biosynthesis or for the allosteric inhibition of Ser acetyltranferase activity, OAS accumulates and partially overrides the negative feedback provided by the reduced sulfur containing compounds on gene transcription (Hawkesford 2000; Hawkesford and Wray 2000). Different experimental evidences support this OAS regulatory role: i) nitrogen limitation blocks transcript accumulation for ATP sulfurylase and APS reductase normally induced by limiting sulfur (Yamaguchi *et al.* 1999) and OAS accumulation (Kim *et al.* 1999); ii) exogenous supply of OAS increases both APS reductase activity and thiol content in *Lemna minor* and induces both sulfate uptake and coordinate transcript accumulation for an high-affinity sulfate transporter in the presence of high sulfate levels in the growing media (Neuenschwander *et al.* 1991; Smith *et al.* 1997); iii) an *Arabidopsis* mutant with increased levels of OAS shows higher transcript levels of sulfur responsive genes with respect to the wild-type (Ohkama-Ohtsu *et al.* 2004).

Moreover, Cys biosynthesis is also controlled at post-translational level through an unique regulatory mechanism involving the reversible formation of an enzyme complex between Ser acetyltransferase and OAS(thiol)-lyase. Since OAS(thiol)-lyase exceeds over Ser acetyltransferase molecules in all cell compartments where protein synthesis occurs (Lunn *et al.* 1990; Droux *et al.* 1992; Rolland *et al.* 1993), only a fraction of OAS(thiol)-lyase may form complexes with Ser acetyltransferase. *In vitro* studies, with recombinant proteins, have shown that homotetrameric Ser acetyltransferase associates with two molecules of homodimeric OAS(thiol)-lyase to form a bi-enzyme complex. Sulfide promotes the complex formation, whereas OAS counteracts the action of sulfide triggering the dissociation of the bi-enzyme complex. It has also been shown that the complex formation strongly affects the kinetic properties of Ser acetyltranferase, which move from a typical Michaelis-Menten model, in the free form of the enzyme, to one displaying positive kinetic cooperativity with respect to Ser and acetyl-CoA. Finally, the formation of the bi-enzyme complex results in a dramatic decrease in the catalytic efficiency of bound OAS(thiol)-lyase, suggesting that a large amount of free OAS(thiol)-lyase is responsible for the actual Cys formation (Droux *et al.* 1998). Such data provide evidences for a regulatory mechanism in which the bound form of OAS(thiol)-lyase acts as a positive regulatory subunit of Ser acetyltransferase in the bi-enzyme complex. In this way, when the sulfide production by the assimilatory pathway limits Cys biosynthesis, OAS accumulates and thus prevents its further production by promoting bi-enzyme complex disruption. *Vice versa*, when sulfur availability is not limiting, the sulfide-promoted formation of the complex results in a stimulation of OAS synthesis which allows to sustain Cys production. Furthermore, OAS synthesis may be controlled through a negative feedback loop exerted by Cys on specific isoforms of Ser acetyltransferase (Urano *et al.* 2000; Noji and Saito 2002; Wirtz and Hell 2003). Such a regulatory model accounts not only for the fine tuning of Cys biosynthesis and homeostasis, but also for the coordination of OAS synthesis from Ser and sulfate reduction.

Despite this elegant model explaining the general mechanisms involved in both sensing sulfur nutritional status and maintaining homeostasis of the main sulfur containing compounds (i.e. Cys and GSH), the most part of the aspects related to signal perception and transduction remains largely unknown. Several studies indicate different hormones, such as auxin and methyl jasmonate, as possible components of the signal transduction pathways (Hirai *et al.* 2003; Maruyama-Nakashita *et al.* 2003; Nikiforova *et al.* 2003). Expression profiling analyses in *Arabidopsis* reveal that several sulfur related genes are induced by treatment with methyl jasmonate (Jost *et al.* 2005). Cytokinins also appear to be involved in the regulation of gene expression, as suggested by their negative effect on transcript accumulation of an *Arabidopsis* high affinity sulfate transporter (Maruyama-Nakashita *et al.* 2004).

Recently, potential sulfur responsive elements (SUREs) have been identified in the promoter regions of some sulfur responsive genes (Awazuhara *et al.* 2002; Kutz *et al.* 2002; Maruyama-Nakashita *et al.* 2005), although no consensus sequences have been shown yet. However, an interesting study on *Arabidopsis AtSultr1;1* promoter demonstrates that a 5 bp sequence is essential to promote sulfur response of *AtSultr1;1* (Maruyama-Nakashita *et al.* 2005). Interestingly, such a sequence also appears in the promoter regions of many sulfur responsive genes, suggesting its involvement in the transcriptional control of a gene set required for adaptation to sulfur limiting conditions.

SULFUR METABOLISM UNDER STRESS: THE EXAMPLE OF CADMIUM

Introduction

Adaptation of sulfate uptake and assimilation is assumed to be a crucial determinant for plant survival in a wide range of adverse environmental conditions since different sulfur containing compounds are involved in plant responses to both biotic and abiotic stresses (May *et al.* 1998; Rausch and Wachter 2005). Interestingly, comparative analyses of data produced from different experiments reveal that most of the main sulfur-responsive genes involved in sulfate transport and assimilation or in related metabolisms are also induced under different stress conditions, suggesting the existence of a general adaptive responses to an increase in the cell demand for reduced sulfur (Heiss *et al.* 1999; Vanacker *et al.* 2000; Noctor *et al.* 2002; Hirai *et al.* 2003; Howarth *et al.* 2003a, 2003b; Maruyama-Nakashita *et al.* 2003; Nikiforova *et al.* 2003; Herbette *et al.* 2006; Nocito *et al.* 2006). In fact, it has been largely described that several stresses can induce additional sinks for sulfur-compounds which in turn increase cell metabolic demand for both Cys and GSH and thus the total sulfur request for plant growth and adaptation (Nocito *et al.* 2002; Rausch and Wachter 2005; Nocito *et al.* 2006). Some aspects of these responses, with particular emphasis on Cd stress, are focused in the second part of the present paper, where the general contribute of plant sulfur metabolism in heavy metal stress tolerance is reviewed.

Cadmium toxicity in higher plants

Heavy metals, a class of metals whose densities exceed 5 g cm^{-3} (Elmsley 2001), can be classified into essential or non essential for plants and many other organisms. Small quantities of essential heavy metals, such as iron, copper and zinc, are nutritionally required for plant growth since they are cofactors in many enzyme activities and ligand interacttions. Conversely, non essential heavy metals, such as cadmium, mercury and lead are not required for plant growth and in many cases may exert toxic effects on plants; in fact, they can enter the cells, through the same transport systems used by essential heavy metals, and alter then cellular functions mainly interacting with sulfur and nitrogen atoms in

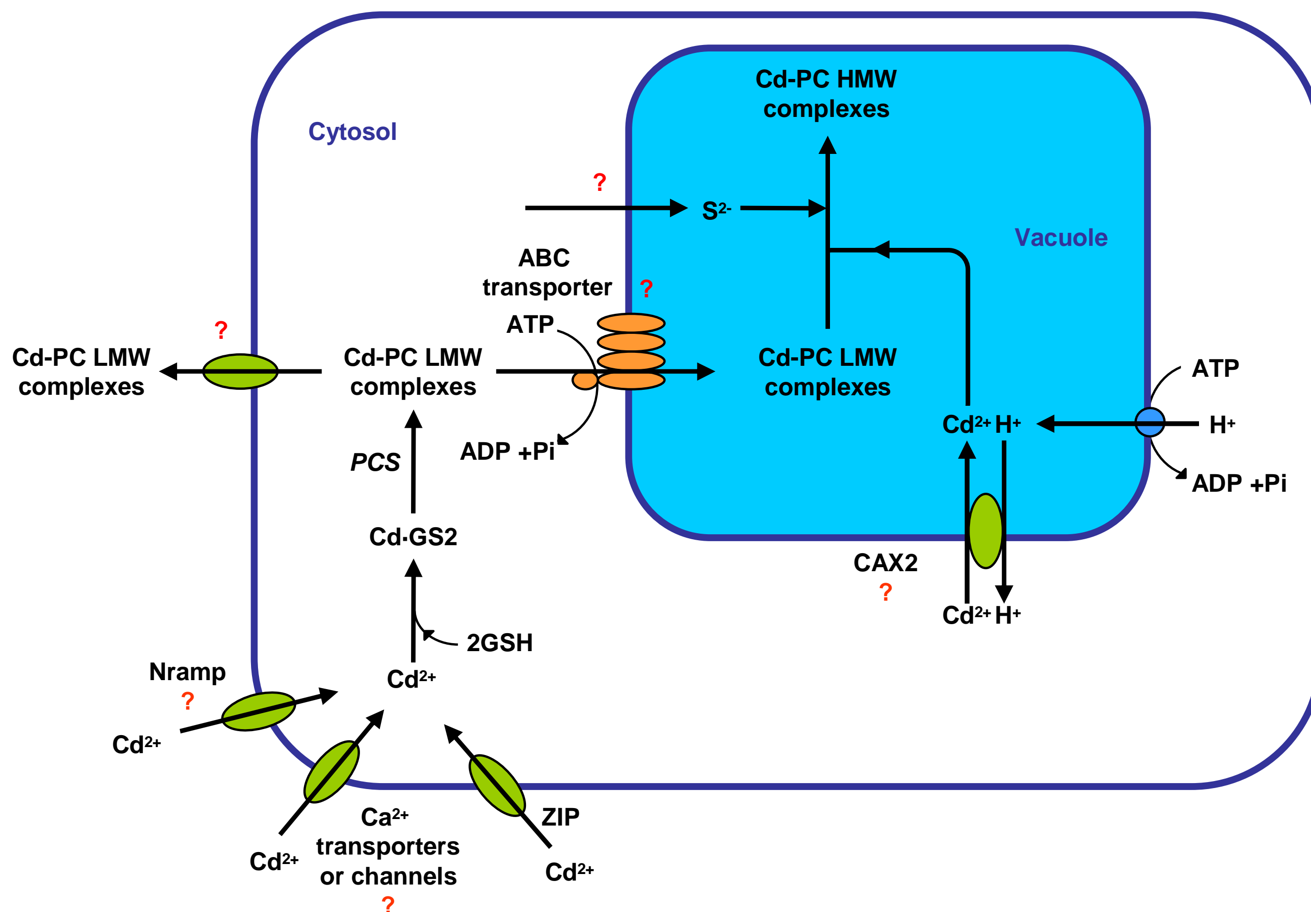


Fig. 3 Scheme of the processes involved in Cd uptake and detoxification. Cd^{2+} is taken up into plant cells by transport systems involved in the uptake of essential nutrients (members of ZIP and Nramp families or Ca^{2+} channels and transporters). Once inside the cytosol, Cd^{2+} ions interact with GSH to form bis(glutathionato)cadmium (Cd·GS2), which in turn activates the synthesis of PCs responsible for Cd chelation. The products of cytosolic Cd chelation are Cd-PC low-molecular-weight (LMW) complexes, which are compartmentalized into the vacuole, by means of transporters localized in the tonoplast (probably ABC-type transporters). Once inside the vacuole, the LMW complexes incorporate S^{2-} and other Cd^{2+} ions evolving into Cd-PC high-molecular-weight (HMW) complexes. Probably, part of cytosolic Cd^{2+} ions could be directly sequestered into the vacuole through a Cd^{2+}/H^+ antiporter (CAX2).

amino acid side chains. However, under physiological conditions, also supraoptimal levels of essential metals, by virtue of their chemical reactivity, may negatively affect metabolism and physiology of living organisms. For instance, excesses of free essential redox-active metals, such as iron and copper, are thought to elicit the generation of highly reactive hydroxyl (OH·) radicals by a Fenton-catalyzed Haber-Weiss reaction (Halliwell and Gutteridge 1984, 1990).

Among the class of non essential metals, Cd is one of the major concern with respect to both plant exposure and human food-chain accumulation (McLaughlin *et al.* 1999). Its relevance as environmental pollutant and its relatively high mobility in the soil-plant system have made this toxic metal the most widely studied in plants (Clemens 2006).

Cd, as well as other nonessential metal ions, is thought to be assumed by plants via cation transport systems normally involved in the uptake of essential elements, such as members of ZIP and Nramp families or Ca^{2+} channels and transporters (**Fig. 3**; Clemens *et al.* 1998; Grotz *et al.* 1998; Korshunova *et al.* 1999; Pence *et al.* 2000; Thomine *et al.* 2000; Lombi *et al.* 2001; Perfus-Barbeoch *et al.* 2002).

Accumulation of Cd in plant tissues may cause a variety of toxicity symptoms ranging from chlorosis, wilting, and growth reduction, to cell death. Cd cellular toxicity may result from interferences with many processes, such as carbohydrate metabolism (Sanità di Toppi and Gabrielli 1999), nitrate absorption and reduction (Hernandez *et al.* 1996), enzyme catalysis (van Assche and Clijsters 1990), water balance (Costa and Morel 1994; Perfus-Barbeoch *et al.* 2002) and photosynthesis (Siedlecka and Krupa 1996; Pietrini *et al.* 2003). For the most part these effects are thought to be linked to Cd extreme capability to bind sulfhydryl groups of proteins, leading to enzyme inactivation. Moreover, Cd accumulation induces oxidative stress as evidenced by the formation of reactive oxygen species, such as superoxide anion and hydrogen peroxide (Romero-Puertas *et al.* 2004). However, since Cd is not a redox-active metal, oxidative stress could result from both interferences with GSH metabolism and redox-active metal displacement from proteins (Stohs and Bagchi 1995).

Phytochelatins: Structure, significance and synthesis

In order to minimize the detrimental effects of cell heavy metals accumulation, plant have evolved detoxification mechanisms, mainly based on chelation and subcellular compartmentalization. The efficiency of these processes is thought to result in the natural heavy metal tolerance and may contribute to plant survival in adverse soil conditions (Clemens 2001).

An important and recurrent general mechanism for heavy metal detoxification in plant and other organisms is based on the synthesis of chelators buffering cytosolic metal concentration. To date, a broad range of metal-binding molecules has been identified and reviewed elsewhere for their functional roles in plants (Rauser 1999).

One of the principle classes of heavy metal chelators known in plants and fungi is phytochelatins, a family of Cys-rich small peptides consisting of only three amino acids: Glu, Cys and Gly, with the Glu and Cys residues

linked through a γ-carboxylamide bond. In PCs two or more repeating γ-EC units are followed by a terminal Gly residue giving the general structure (γ-Glu-Cys)$_n$-Gly, where n ranges from 2 to 11 (most commonly 2-5). Variants of this general rule with β-Ala (homo-PCs), Ser (hydroxymethyl-PCs) or Glu (iso-PCs) instead of the terminal Gly residue have also been identified in some plant species; even PC variants without the C-terminal Gly (desGly-PCs) have been found in plants and some yeasts (Rauser 1995; Zenk 1996; Kubota *et al.* 2000; Oven *et al.* 2002).

PC synthesis is induced within minutes following exposure to different metals or metalloids; among these, Cd is the strongest inducer, whereas other metals such as Cu, Zn, Pb, and Ni are less effective and require higher external levels for induction (Grill *et al.* 1987, 1989; Maitani *et al.* 1996).

The significance of PCs for Cd detoxification in plants has been underlined by the isolation of the *Arabidopsis cad* mutants, deficient in the formation of Cd-PC complexes and in PC biosynthesis, and consequently Cd hypersensitive (Howden *et al.* 1995a, 1995b).

PCs are nontranslationaly synthesized from GSH in a stepwise reaction, catalyzed by the enzyme PC synthase (PCS; γ-Glu-Cys dipeptididyl transpeptidase). In a first step the reaction involves the transpeptidation of the γ-Glu-Cys unit of a GSH molecule onto another GSH molecule to form PC_2; in the later stages, after the accumulation of sufficient level of PCs, other γ-Glu-Cys units arising from GSH are transferred to PC_n to form PC_{n+1} (Rea *et al.* 2004).

The molecular identity of PCS has been identified by cloning and characterizing the corresponding genes in *Arabidopsis thaliana*, *Triticum aestivum* and *Schizosaccharomyces pombe* (Clemens *et al.* 1999; Ha *et al.* 1999; Vatamaniuk *et al.* 1999) using different approaches. The three identified genes (designed as *AtPCS1*, *TaPCS* and *SpPCS*, respectively) codify 50- to 55-kD polypeptides sharing 40% to 50% of sequence similarity to each other (Rea *et al.* 2004). Functional characterization experiments have shown AtPCS1 and TaPCS polypeptides able to suppress the Cd-hypersensitive phenotypes of *Arabidopsis* or yeast mutants (Ha *et al.* 1999; Vatamaniuk *et al.* 1999), or to confer Cd tolerance when heterologous expressed in yeast (Clemens *et al.* 1999). Moreover, *SpPCS* disrupted mutant of *S. pombe* has shown to be hypersensitive to heavy metals and deficient in cellular PCs (Ha *et al.* 1999). Take as a whole these experiments definitively proved the role of cloned PCS in both PC biosynthesis and cell Cd detoxification mechanisms.

A comparison of the predicted amino acid sequences of cloned PCS revealed a highly conserved N-terminal region, which is presumed to be the catalytic domain, and a C-terminal region containing multiple Cys residues which shows little apparent conservation of amino acid sequence (Cobbett 2000; Rea *et al.* 2004).

Insight into the mechanism of cadmium detoxification

The mechanism by which PCs bind heavy metals reducing their free amounts into cells has been largely investigated in fungi and plants (**Fig. 3**). Since *in vitro* and *in vivo* studies showed Cd as the principal inducer, it is not surprising that the most part of the available information arises from works on Cd-PC complexes (Rauser 1995).

Gel filtration analyses of alkaline extracts from plants exposed to a variety of Cd concentrations for different times have shown that the most part of buffer-soluble Cd occurs as Cd-PC complexes, resolvable as low- and high-molecular weight (LMW and HMW) complexes on the bases of their migration during the chromatography (Murasugi *et al.* 1981; Jackson *et al.* 1984; Grill *et al.* 1987; Kneer and Zenk 1992; Reese *et al.* 1992; Speiser *et al.* 1992; Howden *et al.* 1995a, 1995b; Rauser 2000, 2003). The exact molecular weights of these complexes remain unclear because they are susceptible to change with the ionic strength of the elution buffer (Grill *et al.* 1987).

Composition analyses of Cd-PC complexes found in different organisms have shown they are aggregates of a variety of PCs and Cd^{2+}; many times the presence of acid-labile sulfide in different molar ratios with Cd^{2+} has also been reported (Reese and Winge 1988; Speiser 1992; Rauser and Meuwly 1995). In Cd-PC complexes isolated from tomato plants grown under 100 µM Cd^{2+}, acid-labile sulfide predominate more in HMW (S^{2-}:Cd^{2+} molar ratio 0.15-0.41) than in LMW (S^{2-}:Cd^{2+} molar ratio 0.04-0.13) complexes (Reese *et al.* 1992). Similar results have been obtained in a comparative study on *Brassica juncea* and the fission yeast *S. pombe* (Speiser *et al.* 1992), showing the conservative nature of Cd complexation mechanisms in plant and fungi.

Analyses of the X-ray diffraction patterns of HMW Cd-PC complexes from yeasts indicated the presence of CdS crystallite in which about 80 CdS units are coated by about 30 molecules of different PCs (Dameron *et al.* 1989). Such complexes show a S^{2-}:Cd^{2+} molar ratio greater than 0.4 and appear as dense aggregates of 20 Å diameter. Crystallite formation in HMW complexes represents an important example of biomineralisation, a process in which biopolymers provide an ordered structure to other components facilitating, in this way, the formation of a specific crystal. Incorporation of acid-labile sulfide into the Cd-PC complexes has been interpreted as a mechanism increasing both thermodynamic stability and Cd binding capacity of the aggregates (Reese and Winge 1988).

Studies on dynamic of Cd chelation have shown that Cd-PC complexes appear within a few hours following the exposure to the metal and that LMW and HMW complexes are in a dynamic state which depends on both time of exposure and Cd concentration in the external medium (Murasugi *et al.* 1981; Leopold *et al.* 1998, 1999; Rauser 2003). The LMW complexes predominate in the early stages of the exposure, whereas the HMW ones become prevalent in more advanced stages. Moreover, HMW complexes have also been shown as essential for maximal Cd detoxification and tolerance in *S. pombe*, while the LMW alone seems to be not sufficient (Mutoh and Hayashi 1988).

Ortiz and co-workers (1995) proposed a general model for the cell mechanism of Cd detoxification which accounts for all previously reported observations (**Fig. 3**). Briefly, in this model the first products of Cd chelation are cytosolic LMW complexes which are compartmentalized into the vacuole, by means of specific transporters localized in the tonoplast. Once inside the vacuole the LMW complexes incorporate S^{2-} and other Cd^{2+} ions, evolving into the more stable HMW complexes. Thus, in a population of dividing cells or in a growing organ exposed to Cd, the two complexes are part of a dynamic process in which LMW Cd-PC complexes function as cytosolic carriers and the vacuolar HMW complexes represent the major Cd storage forms into the cells. Such a model is mainly supported by studies on a Cd-sensitive mutant of *S. pombe*, *hmt1* (*heavy metal tolerance 1*), unable to produce HMW complexes since defective in an ABC (ATP-binding cassette)-type transporter involved in the vacuolar compartmentalization of LMW Cd-PC complexes (Ortiz *et al.* 1992, 1995). *In vitro* experiments on vacuolar membrane vesicle preparations from *hmt1* and wild-type yeasts demonstrate this protein able to transport PCs and both LMW and HMW Cd-PC complexes in an ATP-dependent manner into vacuolar vesicles. However, sulfide-poor LMW are transported more efficiently than sulfide-rich HMW complexes (Ortiz *et al.* 1995). Moreover, overexpression of HMT1 polypeptides in yeast results in HMW complex formation and enhances Cd tolerance and accumulation (Ortiz *et al.* 1992).

Several features of the Ortiz and co-workers' model have also been found in plants (**Fig. 3**). Studies on isolated mesophyll protoplasts from tobacco have shown that most of Cd and PCs are found into the vacuoles (Vögeli-Lange and Wagner 1990). Sequestration of Cd-PC complexes has also been observed in preparations of tonoplast-enriched vesicles from oat roots in which an ATP-dependent transporter,

similar to HMT1, has been described. Such a transporter mediates the MgATP-dependent movement of PCs and Cd-PC complexes into the vacuole (Salt and Rauser 1995); however, the molecular nature of this transporter remains unclear.

Other tonoplast transporters are thought to be involved in the PC-based Cd detoxification mechanism (Ortiz *et al.* 1995). For instance, it has been hypothesized that part of Cd^{2+} incorporated into the evolving vacuolar HMW complexes reaches the vacuole through a Cd^{2+}/H^{+} antiporter (Salt and Wagner 1993). The activity of this transporter could be involved in removing cytosolic Cd excess when PC levels are low, or when the metal concentration exhausts the short-term capacity of the cells to synthesize stoichiometric amounts of PCs. Molecular evidences suggest that the *Arabidopsis* antiporter CAX2 (calcium exchanger 2), which has a broad substrate range, may be a key mediator of this process (**Fig. 3**). In fact, transgenic tobacco plants expressing CAX2 accumulate almost three times the total Cd in root tissues as the control plants. Moreover, the expression of CAX2 in tobacco increases Cd tonoplast transport activity, as indicated by *in vitro* experiments where tonoplast vesicles isolated from transgenic plants showed a Cd accumulating capability from 1.6- to 2.1-fold higher than those prepared from the control (Hirschi *et al.* 2000).

Research on plant PCs has mainly focused on the role of these peptides in metal sequestration and compartmentalization, since they are supposed to act in a local detoxification mechanism within the cells in which they are produced. However, PCs have recently been shown to undergo long-distance transport in both root-to-shoot and shoot-to-root directions (Gong *et al.* 2003; Chen *et al.* 2006). Such an intriguing finding has been obtained in two sets of experiments in which a *T. aestivum* PCS, *TaPCS1*, was specifically targeted to the roots or the shoots of the PCS defective *Arabidopsis cad1-3* mutant, under the control of the promoters of *Adh* (alcohol dehydrogenase; for targeting to the roots) or *CAB2* (chlorophyll A/B-bindig protein 2; for targeting to the shoots). The main results obtained in these studies demonstrate that: i) the heterologous expression of *TaPCS1* in both roots or shoots suppresses the Cd sensitivity of *cad1-3* mutant; ii) PCs specifically produced in roots or shoots can be transported to shoots or roots, respectively; iii) the expression of *TaPCS1* in roots or shoots of *cad1-3* mutant reduces Cd accumulation in roots or shoots, respectively, and enhances long distance root-to-shoot or shoot-to-root Cd transport, compared with the wild-type. Even if still unclear, the significance of this unexpected PC-mediated Cd systemic movement has been interpreted as an overflow protection mechanism involved in maintaining low metal concentrations in Cd-challenged plant tissues and organs (Gong *et al.* 2003). In fact, the expression of PCS does not result in an increase of Cd content in targeted organs, as predictable on the basis of the Ortiz's model, but conversely causes a reduction of Cd accumulation. Such evidences also underline that the PC-based Cd detoxification model derived from unicellular organisms is not completely applicable to multicellular organisms where PCs may assume different roles in the heavy metal processing pathways.

Regulation of phytochelatin biosynthesis

PC biosynthesis may be regulated in several ways, through a direct control of PCS level and activity or, indirectly, by a fine tuning of biosynthetic pathways leading to GSH production (see next paragraph).

Studies on *Arabidopsis AtPCS1* indicate PCS as a constitutively expressed enzyme. This is supported by Northern or RT-PCR analysis of the expression of PCS genes which show their steady-state mRNA levels as not influenced by Cd exposure (Ha *et al.* 1999; Vatamaniuk *et al.* 1999; Cobbett 2000). However, RT-PCR analysis of *TaPCS1* expression in roots of plants of *T. aestivum* indicated increased levels of mRNA as a response to Cd exposure (Clemens *et al.* 1999). More recently Heiss and co-workers (2003) showed that prolonged Cd exposures may affect BjPCS1 (a PCS from *B. juncea*) expression, as indi-cated by a progressive accumulation of the protein in leaves which nevertheless does not correspond to increases in the *BjPCS1* transcript levels. Taken as a whole these findings suggest the existence of some mechanisms controlling the expression of PCS at transcriptional and post-transcriptional level; however, since they seem to be species-dependent mechanisms, direct control of PCS activity is likely expected to be the main point at which PC synthesis is regulated.

First kinetic studies on plant cell suspensions indicated that PC biosynthesis occurs within minutes following Cd and other heavy metal exposure (Grill *et al.* 1987). In their pioneering paper Grill and co-workers (1989) described the capability of a partially purified preparation of PCS from *Silene cucubalus* cell suspension to synthesize PCs, starting from GSH, only in the presence of a variety of metal ions. Among these, Cd was by far the best metal activator of the enzyme followed by Ag, Bi, Pb, Zn, Cu, Hg, and Au. Interestingly, these metal ions were the same known to induce PC biosynthesis in plant cells (Grill *et al.* 1987). Similar PCS activities have been described in tomato cell culture (Chen *et al.* 1997) and in intact plants such as pea (Klapheck *et al.* 1995) and *Arabidopsis* (Howden *et al.* 1995a, 1995b).

The mechanism by which metals activate PC biosynthesis has been recently reviewed in two interesting and exhaustive papers (Rea *et al.* 2004; Rea 2006). The speculative initial model proposed assumed PCS activation as a consequence of direct binding of metal ions to the Cys residues in the C-terminal region of the enzyme (Cobbett 2000). Such a model was consistent with the presence of conserved Cys residues in the C-terminal region potentially able to coordinate metal ions such as Cd^{2+}, Cu^{2+}, and/or Hg^{2+}, and most of all with the observation that three *Arabidopsis cad1* alleles have amino acid substitutions in this region (Ha *et al.* 1999). However, evidences supporting this model are little or indirect and, above all, the model *per se* fails in explaining how potentially enzyme-inactivating agents like heavy metals may control the activity of a key enzyme in detoxification mechanisms.

More recently Rea and co-workers proposed an experiment-based model derived from studies on *Arabidopsis* recombinant PCS1. The main results obtained in these studies demonstrate that the presence of free metal ions is not essential for catalysis, although PCS is able to bind them directly (Vatamaniuk *et al.* 2000). In particular it has been estimated that under the conditions in which AtPCS1 catalyzes high rates of PC synthesis from GSH, the concentration of free Cd in the reaction medium is approximately six orders of magnitude lower than that required for direct binding to the enzyme. This because the 98% of total Cd added to the reaction medium is associated with GSH as its corresponding bidentate thiolate, bis(glutathionato)cadmium (Cd·GS2). Kinetic analyses showed that AtPCS1 activity approximates a bisubstrate-substituted enzyme mechanism where micromolar Cd·GS2 and free GSH act as high-affinity and low-affinity substrates, respectively. Finally, recombinant AtPCS1 has shown to be able to catalyze the net synthesis of *S*-alkyl-PCs starting from a variety of *S*-alkyl-glutathione derivatives (such as *S*-methylglutathione, *S*-ethylglutathione, *S*-propylglutathione, *S*-butylglutathione and *S*-hexylglutathione) even in metal ion devoided media. From these data the authors concluded that the dependence of PCS activity on the presence of metal ions in media containing GSH arises from the peculiar enzyme's requirement for GSH-like peptides containing blocked sulfhydryl groups, and not from a direct binding of metal ions to the C-terminal region of the enzyme. Such an intriguing model both accounts for the kinetic properties of plant PCS and obviates any problems due to direct interaction of PCS with potentially enzyme-inactivating metal ions.

Regulation of sulfur metabolism during cadmium stress

Since PC biosynthesis has shown to be closely dependent on GSH, it is likely to suppose the existence of a general relationship between sulfate assimilation, GSH biosynthesis and Cd detoxification mechanisms. Early studies on *Rauvolfia serpentina* cell suspension cultures and maize roots indicate that Cd exposure and accumulation is accompanied by a transient depletion of cellular GSH, directly used as substrate for PC production (Grill *et al.* 1987; Tukendorf and Rauser 1990). Moreover, a prolonged exposure results in a massive synthesis of PCs, which rapidly become the most abundant class of nonprotein thiols in plant tissues. Comparative analyses of thiol compound levels in plants exposed to Cd stress reveal that PCs concentration (expressed as GSH equivalent) may reach values several fold higher than those of their direct precursor GSH, which instead represents the main nonprotein thiol in non stressed plants (**Table 2**; Heiss *et al.* 1999; Zhu *et al.* 1999b; Nocito *et al.* 2002; Drążkiewicz *et al.* 2003; Ranieri *et al.* 2005; Sun *et al.* 2005; Nocito *et al.* 2006). Thus, the need to sustain Cd detoxification processes strongly increases the total amount of reduced sulfur request by plants.

The large amount of PCs produced by Cd stressed plants may represent an additional sink for reduced sulfur able to increase the rate of sulfate assimilation. Nussbaum and co-workers (1988) first demonstrated the existence of a close relationship between Cd accumulation and sulfate assimilation by describing a positive effect of the metal on the extractable activity of both ATP sulfurylase and APS reductase of maize roots. Such an effect was significantly detectable after 24 hours of treatment with 50 μM Cd^{2+}. In this condition the activities of ATP sulfurylase and APS reductase reached values 2.4- and 4-fold respectively higher than those of the control. Other physiological works demonstrated the existence of a generalized response of sulfur metabolism to Cd exposure which also involves enzyme activities along the pathway of GSH biosynthesis. In particular, γ-EC synthetase and GSH synthetase have been described as the major responsive enzymes to Cd exposure and accumulation, indicating a cellular response to the transient GSH depletion during PC biosynthesis (Rüegsegger *et al.* 1990; Rüegsegger and Brunold 1992). A significant and progressive positive effect of the metal on the extractable activity of γ-EC synthetase of maize roots was measured after 4 days of incubation at 5 micromolar or higher Cd^{2+} concentrations in the nutrient solution. Such a behaviour was accompanied by a progressive accumulation of γ-EC in the roots, whose concentrations reached values from about 2.5- to 36-times higher than those of the control (Rüegsegger and Brunold 1992). Similarly, appreciable increases in the extractable activity of GSH synthetase from pea roots were described in plants exposed for 48 hours to 5 to 50 μM Cd^{2+} external concentrations (Rüegsegger *et al.* 1990).

The increased demand for reduced sulfur-containing compounds arising from the activation of the PC-based Cd detoxification system seems to be a common response in plant exposed to Cd stress, as indicated by different studies on the transcriptional responses of the main metabolic sulfur responsive genes. In *Arabidopsis*, Cd exposure has been described to induce several genes along the pathway of sulfate assimilation, such as different isoforms of Ser acetyltransferase (Howarth *et al.* 2003a), cytosolic OAS(thiol)-lyase (Dominguez-Solis *et al.* 2001), ATP sulfurylase, APS reductase (Harada *et al.* 2002), as well as γ-EC synthetase and GSH synthetase (Xiang and Oliver 1998). Similarly, transcriptional up-regulation of ATP sulfurylase, APS reductase and γ-EC synthetase genes has also been reported in studies on *B. juncea* plants exposed to different Cd concentrations (Shäfer *et al.* 1998; Heiss *et al.* 1999; Lee and Leustek 1999).

Taken as a whole these studies indicate that the genesis of additional sinks for reduced sulfur, arising from PC biosynthesis, increases the metabolic request for both Cys and GSH, generating a typical demand-driven coordinate transcriptional regulation of genes involved in sulfate assimilation and GSH biosynthesis. Such a response is crucial for plant survival in heavy metal polluted environments, since a fine modulation of sulfur fluxes along the metabolic pathways is thought to be essential to satisfy two contrasting needs: i) maintaining cell GSH homeostasis; ii) detoxifying heavy metals by means of GSH consuming activities.

Studies on Cd exposed maize plants demonstrate that the need for maintaining a high rate of PC biosynthesis and adequate GSH levels under Cd stress may be also met by modulating sulfate influx into the root, and thus the total amount of sulfur taken up from the environment. This finding arises from the observation that both short and prolonged exposures to micromolar Cd concentrations increase sulfate uptake capacity of the roots by the up-regulation of *ZmST1;1*, a gene encoding a root-expressed high-affinity sulfate transporter (Nocito *et al.* 2002, 2006). Moreover, the modulation of sulfate uptake seems to be dependent on the nutritional request for reduced sulfur, as suggested by the observation that both changes in *ZmST1;1* transcript levels and sulfate uptake capacity of the roots are closely related to the strength of Cd-induced additional sink for thiol compounds (Nocito *et al.* 2006). The regulation of sulfate uptake may thus represent the first step of the adaptive process required to ensure an adequate sulfur supply for Cd detoxification.

Preliminary data resulting from transcriptomic analyses in *Arabidopsis* reveal that other sulfate transporters, not functionally involved in sulfate uptake from the external medium, can be transcriptionally regulated following Cd exposure (Herbette *et al.* 2006). Although significance of the most part of these transcriptional regulations remains unclear, it is interesting to underline the authors' suggestion that the Cd-induced up-regulation of the tonoplast transporter Sultr4;1 could be involved in mobilizing the vacuolar sulfate stores in order to provide additional substrate for the assimilatory pathway. Finally, these data also suggest that modulation of sulfate fluxes along the whole plant could play an important role in Cd detoxification and tolerance.

How plants sense the presence of Cd inside the cell and activate sulfur metabolism is still unclear. To date the most characterized system able to perceive Cd is PCS. However,

Table 2 Comparative analysis of the main thiol compound levels in different plant species grown in the absence or in the presence of Cd. The values signed with an asterisk have been directly deduced from the original figures published in each paper; n.d., not detectable.

Plant species	Notes	Control		Cadmium		References
		GSH	PCs	GSH	PCs	
		(*nmol GSH equivalent g^{-1}*)				
Brassica juncea	Roots of plants exposed to 25 μM Cd^{2+} for 48 h.	87	n.d.	53	587	Heiss *et al.* 1999
Brassica juncea	Leaves of plants exposed to 25 μM Cd^{2+} for 48 h.	98	n.d.	51	862	Heiss *et al.* 1999
Brassica juncea	Shoots of seedlings exposed to 200 μM Cd^{2+} for 11 d.	117*	n.d.	152*	1742*	Zhu *et al.* 1999b
Rauvolfia serpentina	Cell suspensions exposed to 200 μM Cd^{2+} for 9 h.	$7.5 \cdot 10^3$*	n.d.	$2.7 \cdot 10^3$*	$5.3 \cdot 10^3$*	Grill *et al.* 1987
Zea mays	Roots of plants exposed to 10 μM Cd^{2+} for 48 h.	188	n.d.	105	1009	Nocito *et al.* 2002
Triticum aestivum	Roots of plants exposed to 54 μM Cd^{2+} for 14 d.	120*	-	970*	4800*	Sun *et al.* 2005
Triticum aestivum	Roots of plants exposed to 1 mM Cd^{2+} for 10 d.	$1.4 \cdot 10^3$*	n.d.	$0.7 \cdot 10^3$*	$5.0 \cdot 10^3$*	Ranieri *et al.* 2005
Zea mays	Leaves of plants exposed to 200 μM Cd^{2+} for 14 d.	449	n.d.	509	1366	Drążkiewicz *et al.* 2003

activation of PC biosynthesis is a post-translational response which in turn may induce increases of the cell metabolic request for GSH. Several experimental results discussed in this review have often been interpreted by postulating the existence of a coordinate transcriptional regulation mechanisms triggered by GSH depletion or by an increased demand for reduced sulfur. In this way the presence of Cd inside the cells would simulate metabolic and nutritional conditions similar to those induced by sulfur starvation.

Concerning these aspects, some physiological considerations need to be taken into account: i) the knowledge on the transcriptional regulation of the main sulfur responsive genes arise from studies conducted on sulfur-starved plants where the reduced availability of sulfate in the growing medium limits sulfur fluxes through the assimilatory pathway and thus GSH pools of the cells; ii) Cd exposure induces sulfur-responsive gene transcription in the presence of adequate sulfate sources, probably as a consequence of an increase in the sulfur request by plant. Taking also into account the wide variety of soils and environmental conditions experienced by plants, it appears clear the need to have multiple signalling pathways modulating sulfur nutrition in response to both sulfur requirement and soil sulfate level. However, the nature of these signals needs to be further investigated, since only indirect evidences have been obtained in a few studies. For instance, jasmonate has been described as involved in the transcriptional control of GSH biosynthesis genes in *Arabidopsis* metal stressed plants (Xiang and Oliver 1998). Finally, it has been hypothesised that also the induction of sulfate uptake under heavy metal stress may be controlled through both GSH-dependent or GSH-independent signalling pathways (Nocito *et al.* 2006). Such a suggestion mainly arises from the following observations: i) changes in both *ZmST1;1* transcript levels and sulfate uptake capacity in heavy metal stressed roots do not correlate with changes in cell GSH content; ii) other metals, such as Zn, promoting the genesis of additional sinks for thiol without negatively affecting GSH pools, are also able to induce *ZmST1;1* up-regulation and sulfate uptake by roots.

Enhancing cadmium tolerance in higher plants

Considering the effect of Cd on sulfate uptake and assimilation as well as on GSH biosynthesis, a natural question arises: may sulfate metabolism limit GSH biosynthesis and thus Cd tolerance and accumulation? Such a question is of crucial importance to improve those plant species thought to be useful for the environmental clean-up of heavy metal polluted soils. In fact, performances in phytoextraction are not only based on plant ability to take up, translocate, and accumulate heavy metals, but also on mechanisms able to alleviate their toxic effects (Salt *et al.* 1998).

The coordinate transcriptional regulation of metabolic sulfur genes observed under Cd stress is thought to reflect the existence of several bottlenecks along the Cd detoxification pathway, which in turn may limit metal tolerance.

Since γ-EC synthetase and GSH synthetase activities have been shown as limiting factors for GSH biosynthesis in heavy metal stressed plants (Schäfer *et al.* 1998; Xiang and Oliver 1998), they are expected to be good candidates for manipulation. In the absence of heavy metals, the rate limiting step for GSH synthesis is thought to be the reaction catalyzed by γ-EC synthetase, because the activity of this enzyme is feedback regulated by GSH (**Fig. 2**; Noctor *et al.* 1998). However, under Cd stress the regulation of GSH biosynthesis undergoes deep changes, mainly at transcriptional level, probably caused by the transient GSH depletion itself and the inhibitory effect exerted by Cd on GSH synthetase activity (Schneider and Bergmann 1995; Schäfer *et al.* 1998; Xiang and Oliver 1998). Experiments carried out on transgenic plants of *B. juncea* overexpressing γ-EC synthetase or GSH synthetase from *E. coli*, clearly demonstrate that a greater production of GSH may result in plants with an enhanced tolerance to Cd stress (Zhu *et al.* 1999a, 1999b). In non treated γ-EC synthetase transgenic seedlings the levels of GSH in the shoots were from 1.5- to 2.5-fold higher than those of the wild-type. Such a feature was maintained in Cd stressed seedlings (200 μM Cd^{2+} for 7 days), resulting in a greater capability to both synthesize PCs (about + 30%) and tolerate Cd accumulation; this last feature was evaluated by measuring root length, which resulted in 2-fold longer transgenic seedlings than in the wild-type at the end of the exposure period. Furthermore, when tested in a hydroponic system, γ-EC synthetase transgenic plants resulted significantly more efficient in accumulating Cd in the shoots; the observed increase in Cd accumulation ranged from 40 to 90% (Zhu *et al.* 1999b). Similarly, GSH synthetase transgenic plants exposed for 10 days to 100 μM Cd^{2+} showed higher concentrations of GSH and PCs, which were about 5- and 2-fold higher than those of the wild-type plants, and resulted more tolerant to Cd (Zhu *et al.* 1999a). Moreover, experiments conduced by Bennett and co-workers (2003) in open field like conditions on a mine tailing podded soil polluted with a mixture of heavy metals (Cd, Cr, Cu, Mn, Pb and Zn) demonstrate the better capability of these transgenic lines to absorb and accumulate in the shoot different heavy metals as compared to the wild-type. These findings confirm the central role of GSH and PCs in both stress tolerance and Cd sequestration and also suggest the implication of thiol compounds in determining plant phytoextraction capacity even in aged polluted soils, not only for Cd, but also for other metals which do not form complexes with PCs. Interestingly, two different papers report that ectopic overexpression of a PCS gene (*AtPCS1*) in *Arabidopsis* paradoxically results in a Cd-hypersensitive phenotype (Lee *et al.* 2003; Li *et al.* 2004). Such an unexpected behaviour might result from an excessive GSH depletion during Cd-induced PC biosynthesis, which in turn makes the plants more sensitive to other metal-related stresses. Finally, indirect evidences obtained on maize plants fed in different sulfate availability suggest that the toxic effect exerted by Cd accumulation can be alleviated maintaining high sulfate concentrations in the root tissues. Such a behaviour is likely due to an effect of root sulfate stores on GSH biosynthesis, since the levels of this metabolite result positively related to those of sulfate in the feeding solutions (Nocito *et al.* 2006).

ACKNOWLEDGEMENTS

This work was supported by grants from the Italian Ministry of Education, University, and Research (Ministero dell'Istruzione, dell'Università e della Ricerca, Progetti di Ricerca di Interesse Nazionale 2006).

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