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ACCUMULATION OF TRACE ELEMENTS AND SULFUR USE EFFICIENCY IN MODEL PLANTS

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“Alla mia famiglia, alla mia fidanzata e a tutti gli amici che mi sono stati vicini nei momenti più difficili di questo lungo percorso; mi avete spronato ed incoraggiato permettendomi di raggiungere questo traguardo: GRAZIE!!!”

ABSTRACT

Sulfur is an essential element for all living organisms. It is found in a broad variety of compounds [two amino acids (cysteine and methionine), glutathione (GSH), phytochelatins (PCs), vitamins, iron-sulfur clusters, cofactors and other molecules]. For plants, the main source of sulfur is sulfate ion that is taken up, by roots, from the soil solution. Once inside the cells, sulfate is reduced and assimilated into cysteine from which, GSH is synthetized enzymatically. This tripeptide (i.e., GSH) is involved in the maintaining of the redox homeostasis of the cells and in the detoxification of toxins. In plants not exposed to cadmium (Cd, a toxic not essential heavy metal), GSH represents the main thiol in the cells. However, under Cd exposure, plants, starting from GSH, immediately synthetize PCs, which, in turn, become the most abundant class of thiols. These Cys-rich peptides are able to chelate Cd, reducing the levels of free Cd ions in the cell and the damage induced by the metal. The large amount of PCs represents a sulfate additional sink that increases the request for Cys and GSH and, consequently, the total amount of sulfur necessary for both mitigation of stressing conditions and plant growth.

In this thesis, two experimental works are presented. The aim concerned the improvement of the knowledge on the molecular and physiological relationships existing among Cd accumulation, Cd tolerance, sulfur metabolism and sulfur use efficiency in two different model plants: barley and Arabidopsis.

In the first work, six barley cultivars widely differing for Cd tolerance, partitioning, and translocation were analyzed in relation to their thiol metabolism. The data analysis indicated that Cd tolerance was not clearly related to the total amount of Cd absorbed by plants, but it is closely dependent on the capacity of the cultivars to chelate and immobilize the metal at root level. Such behaviors suggested the existence of root mechanisms preserving shoots from Cd-induced oxidative damages, as indicated by the analysis of thiobarbituric acid-reactive substances (diagnostic indicators of oxidative stress), whose levels increased in the shoots and they were negatively related to Cd root retention and tolerance. Cd exposure differentially affected GSH and PC levels in the tissues of each barley cultivar. The capacity to produce PCs appeared as a specific characteristic of each barley cultivar, since it did not depend on Cd concentration in the roots and resulted negatively related to the concentration of the metal in the shoots, indicating the existence of a cultivar-specific interference of Cd on GSH biosynthesis, as confirmed by the presence of close positive linear relationships between the effect of Cd on GSH levels and PC accumulation in both roots and shoots. The six barley cultivars also differed for their capacity to load Cd ions into the xylem, which was negatively related to PC content in the roots. All these data indicated that the different capacity of

each cultivar to maintain GSH homeostasis under Cd stress may strongly affect PC accumulation and, thus, Cd tolerance and translocation.

Concerning the second work, plants of *Arabidopsis thaliana* were grown in complete hydroponic solutions containing different sulfate concentrations and exposed or not to different levels of Cd, for short or long period. Concerning shoot, long-term Cd exposure induced an increment of the external critical sulfate concentration ($[SO_4^{2-}]_{crit}$, i.e. the sulfate concentration in the growing medium that produced the 95% of the maximum amount of fresh weight). Moreover, in this experimental condition, shoot tolerance to relatively low Cd concentration increased as sulfate availability in the growing medium did, whilst at root level the strong inhibition induced by Cd was independent from external sulfate concentration. Conversely, under short-term Cd exposure, $[SO_4^{2-}]_{crit}$ did not change statistically in both shoot and roots and the inhibitory effect exerted by the metal on shoot and root growth was independent from external sulfate availability. Interestingly, the presence of Cd for both short and long period induced an increment of the relative expression levels of genes codifying for high-affinity sulfate transporters enhancing, consequently, the sulfate uptake. On the other hand, increases of the sulfate availability in the growing solution reduced the amount of sulfate taken up by roots. However, only under short-term Cd exposure the increments of sulfate uptake were coupled with increases of non-protein thiol levels indicating that long-term Cd exposure decreases the capacity of the Arabidopsis roots to efficiently use the available sulfate ions in the growing medium to promote the growth. Such a behavior is likely due to the effect exerted by Cd accumulation which, reducing the development of root apparatus, makes the adaptive response of the high-affinity sulfate transporters "*per se*" not enough to optimize the growth at sulfate external concentrations lower than $[SO_4^{2-}]_{crit}$.

Finally, reassuming, the main results show that the capacity of plant tissues to maintain GSH homeostasis under Cd stress may strongly affect PC accumulation and, thus, Cd tolerance and translocation. Moreover, such a capacity seems to be related to the total amount of sulfur available for plant nutrition in the growing medium, since adequate levels of sulfate modulate thiol metabolism and partitioning, reducing the negative effects produced by Cd at shoot level. This confirms that sulfur plays a pivotal role in the mechanisms involved in Cd detoxification suggesting that the manipulation of both sulfate transport and thiol metabolism may represent a useful strategy for the selection of low Cd-accumulating cultivars or more Cd-tolerant plants when grown in Cd-contaminated soils.

RIASSUNTO

Lo zolfo è un elemento essenziale per tutti gli organismi viventi ed è presente in un'ampia gamma di composti [in due amminoacidi (cisteina e metionina), glutatione (GSH), fitochelatine (PCs), vitamine, cluster ferro-zolfo, cofattori e altre molecole]. La principale fonte di zolfo per le piante è lo ione solfato che viene assorbito dalla soluzione circolante attraverso le radici. Una volta giunto all'interno delle cellule, lo ione solfato viene prima ridotto e poi assimilato sotto forma di cisteina. Da quest'ultima, viene sintetizzato enzimaticamente il GSH. Questo tripeptide (*i.e.*, GSH) è coinvolto sia nel mantenimento dell'omeostasi redox delle cellule che nei processi di detossificazione delle tossine. In piante non esposte a cadmio (Cd, un metallo pesante tossico e non essenziale), il GSH rappresenta il principale tiolo cellulare. Tuttavia, durante l'esposizione al Cd, le piante sintetizzano immediatamente, partendo dal GSH, le PCs che diventano la classe tiolica più abbondante. Questi peptidi, ricchi di residui cisteinici, sono in grado di chelare il Cd, riducendo sia il livello di ioni Cd liberi nelle cellule che il danno che questi possono produrre. È importante sottolineare che l'elevata quantità di PCs neosintetizzate rappresenta un sink addizionale di solfato che incrementa la richiesta di Cys e di GSH e, conseguentemente, la quantità di zolfo necessaria per mitigare le condizioni stressanti e garantire la crescita della pianta.

Questa tesi è composta da due lavori sperimentali il cui scopo generale è quello di incrementare la conoscenza riguardante le relazioni molecolari e fisiologiche esistenti tra l'accumulo di Cd, la tolleranza a questo metallo pesante, il metabolismo dello zolfo e l'efficienza d'uso di questo elemento essenziale in due differenti piante modello: orzo e Arabidopsis.

Nel primo lavoro, sei cultivar di orzo, che differiscono ampiamente per la tolleranza al Cd, la ripartizione e la traslocazione di questo metallo pesante, sono state analizzate in relazione al loro metabolismo tiolico. L'analisi dei dati ha rilevato che la tolleranza al Cd non dipendeva dal contenuto totale di questo metallo pesante assorbito dalla pianta, ma dalla capacità delle cultivar di immobilizzare il Cd nelle radici. Questi andamenti suggerivano l'esistenza di alcuni meccanismi, a livello radicale, capaci di preservare i germogli dai danni d'origine ossidativa indotti dal Cd, come indicato dall'analisi delle sostanze che reagivano con l'acido tiobarbiturico (che fungono da indicatori dello stress ossidativo), i cui livelli nei germogli aumentavano ed erano negativamente relazionati con la ritenzione radicale del Cd e con la tolleranza al metallo. Inoltre, l'esposizione al Cd influenzava il contenuto di GSH e PCs in modo differente in ogni cultivar di orzo. La capacità di produrre PCs sembrava essere una caratteristica specifica di ogni cultivar, poiché non dipendeva dalla concentrazione di Cd nelle radici. Altre osservazioni indicavano la presenza di un'interferenza esercitata dal Cd sulla biosintesi di GSH, la cui entità era cultivar-specifica. Infatti, esisteva una

correlazione lineare e negativa tra la diminuzione dei livelli di GSH dovuti alla presenza del Cd e l'accumulo di PCs nelle radici e nei germogli. Inoltre, le sei cultivar di orzo differivano per la quantità di Cd caricata nello xilema, che diminuiva all'aumentare del contenuto radicale di PCs. Tutti questi dati indicano che la differente capacità di ogni singola cultivar di mantenere l'omeostasi del GSH in presenza di Cd può influenzare pesantemente l'accumulo di PCs e, di conseguenza, la quantità traslocata di Cd verso i germogli incidendo anche sulla tolleranza verso questo metallo pesante tossico.

Per quanto riguarda il secondo lavoro sperimentale, piante di *Arabidopsis thaliana* sono state allevate in soluzioni idroponiche complete, contenenti differenti concentrazioni di solfato, ed esposte o meno a diversi livelli di Cd, sia per un breve che per uno lungo periodo di tempo. Durante gli esperimenti nei quali le piante subivano un'esposizione prolungata al Cd, la presenza del metallo pesante comportava, a livello dei germogli, un aumento della concentrazione critica di solfato presente nel mezzo esterno ($[SO_4^{2-}]_{crit}$, *i.e.*, la concentrazione di solfato nel mezzo di crescita che consentiva il raggiungimento del 95% del massimo peso fresco). Inoltre, in questa condizione sperimentale, per quanto riguarda i germogli, la tolleranza ad una concentrazione relativamente bassa di Cd incrementava all'aumentare della disponibilità di solfato nel mezzo esterno, mentre a livello radicale, il metallo pesante inibiva severamente la crescita indipendentemente dalla concentrazione di solfato esterno. Invece, negli esperimenti in cui le piante erano esposte per un breve lasso di tempo al Cd, la $[SO_4^{2-}]_{crit}$ rimaneva statisticamente invariata sia per quanto riguarda le radici che i germogli e l'effetto inibitorio dovuto al metallo pesante sulla crescita delle radici e dei germogli risultava indipendente dalla disponibilità esterna dell'anione. È interessante notare che durante le esposizioni al Cd, sia per un lungo che per un ridotto lasso di tempo, i livelli di espressione relativa dei geni codificanti i trasportatori ad alta affinità per il solfato incrementavano, portando al conseguente aumento dell'assorbimento di ioni solfato da parte delle radici. Invece, all'aumentare della disponibilità di solfato nel mezzo di crescita, la quantità di solfato assorbita dall'apparato radicale diminuiva. Tuttavia, solamente nelle piante esposte per un breve lasso di tempo al Cd, l'incremento dell'assorbimento di ioni solfato era abbinato ad un aumento dei livelli totali di tioli non proteici, portando alla conclusione che le esposizioni prolungate al Cd riducevano la capacità delle radici di Arabidopsis di utilizzare efficientemente il solfato disponibile nel mezzo esterno per sostenere la crescita della pianta. Questo potrebbe dipendere dall'effetto dell'accumulo del Cd che, riducendo lo sviluppo dell'apparato radicale, rende la risposta adattativa che coinvolge i trasportatori ad alta affinità per il solfato non sufficiente per ottimizzare la crescita a concentrazioni di solfato esterno inferiori alla $[SO_4^{2-}]_{crit}$.

Concludendo, i principali risultati mostrano che la capacità dei tessuti vegetali di mantenere l'omeostasi del GSH anche in presenza di Cd influenza fortemente l'accumulo di PCs e, di conseguenza, sia la traslocazione del metallo verso la parte aerea che la tolleranza al Cd. Quest'abilità pare essere relazionata alla quantità di solfato disponibile nel mezzo esterno, poiché adeguati livelli di questo anione modulano il metabolismo dei tioli e la loro ripartizione, riducendo gli effetti negativi esercitati dal Cd nella porzione aerea delle piante. Questo conferma che lo zolfo svolge un ruolo essenziale nei meccanismi coinvolti nella detossificazione del Cd suggerendo che manipolando il trasporto di solfato e il metabolismo tiolico si potrebbero selezionare cultivar accumulanti bassi livelli di Cd o maggiormente tolleranti quando coltivati in suoli contaminati da questo metallo pesante.

INDEX

1. INTRODUCTION

1.1. The essentiality of sulfur: an overview

Sulfur is a macronutrient and it is essential for all living organisms. It is found in a broad variety of metabolites:

a) two essential amino acids [cysteine (Cys) and methionine (Met)];
b) iron-sulfur clusters;
c) peptides [glutathione (GSH) and phytochelatins (PCs)] (Noctor et al. 1998; Leustek et al. 2000; Rausch and Wachter 2005);
d) membrane sulfolipids (Benning 1998);
e) cell wall components (Popper et al. 2011);
f) vitamins and cofactors (thiamine, biotin and coenzyme A);
g) glucosinolates and alliins, secondary metabolites characteristic of Brassicaceae and Alliaceae (Jones et al. 2004; Halkier and Gershenzon 2006).

Usually, sulfur in molecules does not have structural role, but it confers them specific catalytic and electrochemical properties. In fact, the sulfhydryl group has an extreme nucleophilicity and this feature allows to many thiols to react with a broad variety of electrophilic compounds (metals, reactive oxygen species, free radicals, xenobiotics; Rabenstein 1989; Leustek et al. 2000). Concerning metalloenzymes, the presence of Cys is essential to bind the metal, but when the concentration of metal ion is too much high or the type of the metal is not correct, the formation of a bind between the thiols and these chemicals can inactivate the enzyme.

Another specific feature of sulfhydryl groups present in the thiols is the capacity to form a covalent disulfide bound, which can readily reduce back to two sulfhydryl groups. This mechanism of reversible reduction plays a pivotal role in maintaining protein structure and in the regulation of protein activity (Åslund and Beckwith 1999). Moreover, reversible reduction of disulfide bound in two thiols is important to maintain the redox status in the cells: GSH represents the most important molecule involved in this mechanism of redox buffering. Concerning GSH, it also plays other important roles, for example:

a) it mitigates stresses, acting as a source of electrons for the enzyme glutathione peroxidase to deal with reactive oxygen species (Noctor et al. 2012; Sobrino-Plata et al. 2014);
b) it forms binds with toxins, by glutathione S-transferases (GSTs), inactivating them; these complexes (GS-toxin) can be bound in the extracellular matrix or compartmentalized in the vacuoles (Marrs 1996; Leustek et al. 2000);

c) it is the substrate for the PC synthesis. These peptides are rich in Cys residues and their sulfhydryl groups can bind different heavy metals (Zenk 1996; Pomponi et al. 2006, Brunetti et al. 2011).

Plants, differently from animals that need organic sulfur compounds, have well-characterized metabolic pathway that reduces sulfur, from sulfate to sulfide, and then assimilates sulfide into organic compounds. Consequently, plants are very important, as main source of organic sulfur, for animal and human diet.

Concerning plants, sulfate ions in the soil solution are the main source of sulfur and they are taken up by the roots (Clarkson et al. 1993; Marschner 1995). However, in the polluted environments, also sulfur dioxide in the atmosphere can be used as a source of sulfur and assimilated into Cys at leaf level (de Kok et al. 1997). At root level, sulfate ions are taken up against their electrochemical gradient through the activity of specific sulfate transporters (proton/sulfate cotransport systems, called SULTRs; Lass et al. 1984; Smith et al. 1995; Hawkesford 2010; Davidian and Kopriva 2010; Takahashi et al. 2011).

After the absorption into the cells, sulfate ion is transported, through xylem and phloem, to different sinks, where it is first reduced, in the chloroplast, and assimilated into Cys or compartmentalized in the vacuole as sulfur reserve. Consequently, it is logical to think about the existence of a plethora of different and specific sulfate transporters able to move sulfate ions throughout the plant and, in this way, satisfy the different requests for sulfur in every organ and tissue that can change during the flow of plant life and with the external conditions to which the plant is exposed. The activity of these transporters is finely regulated and represents one of the main control points of sulfur metabolism (Hawkesford 2000).

Concerning the conditions of sulfur deficiency and/or sulfur deprivation, they cause decrement in the osmotic potential (Kusaka et al. 2005), in the chlorophyll and Rubisco content, provoking chlorosis of young leaves (Gilbert et al. 1997; Lee et al. 2014; Muneer et al. 2014).

1.2. The sulfate transport along the plant

Plant sulfate transporters are classified as sulfate/proton cotransporters (Hawkesford 2003). Lass and Ullrich-Eberius (1984), studying *Lemna gibba*, found a probable $3H^+$/sulfate stoichiometry, enabling electrogenic transport across the inside negative plasma membrane. Other studies found that the H^+ gradient drove the transport of sulfate in yeasts expressing members of the *Stylosanthes hamata* sulfate transporter family (Hawkesford et al. 1993; Smith et al. 1995).

Sulfate transporters are encoded by multiple genes (Hawkesford, 2010). Most of these codify proteins of about 69-75 kDa, characterized by a N-terminal region with 12 membrane spanning domains, 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 the importance of the STAS domain for both function and biogenesis of sulfate transporters, since it probably facilitates the localization of the proteins to plasma membrane and influences the kinetic proprieties of the catalytic domains; moreover, an involvement of the STAS domain in mediating a possible protein-protein interaction that could control sulfate transport activity has also been suggested (Shibagaki and Grossman 2004; Rouached et al. 2005; Shibagaki and Grossman 2006).

In *Arabidopsis thaliana,* 14 genes have been described as members of the sulfate transporter gene family (Hawkesford 2003). According to their amino acid sequences, the members of the Arabidopsis sulfate transporter family can be broken down into five main groups. The members of each group are suggested to have specialized functions for the uptake and distribution of sulfate in the plant.

Group 1 refers to high-affinity sulfate transporters that are primarily responsible for the uptake of sulfate ions from the soil solution into the root cells; however, these transporters are also expressed in other tissues. The analysis of knockout mutants and heterologous expression in yeast indicated that AtSULTR1;1 and AtSULTR1;2 are high affinity sulfate transporters mainly expressed in root hairs, root epidermal and cortical cells and involved in the uptake of sulfate into roots especially under sulfur deficiency (Takahashi et al. 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002; Barberon et al. 2008; Hubberten et al. 2012; Liu et al. 2016). Although AtSULTR1;2 is similar to AtSULTR1;1, it is considered to mediate the major component of sulfate uptake into the roots. On the contrary, AtSULTR1;1 represents a more specialized component of the sulfate uptake system which may be involved in the acquisition of trace sulfate as it has a lower K_m value and is more strongly induced under sulfur limiting conditions (Takahashi et al. 2000; Yoshimoto et al.

2002). Differently, AtSULTR1;3, the third member belonging to the group 1, shows typical expression domains in sieve elements and companion cells of the phloem; according to its localization, Yoshimoto and coworkers (2003) hypothesized an implication of this isoform in long distance sulfate translocation processes.

Group 2 refers to low-affinity sulfate transporters (K_m > 100 μM) expressed only on the plasma membranes of cells of vascular tissues. The high K_m values support the hypothesis that these transporters contribute to translocation of sulfate ions within the plant vascular system, where the sulfate concentration would be high. Concerning AtSULTR2;1, it is expressed in the vascular tissues of the roots (xylem parenchyma and pericycle cells). Its induction under sulfur limitation suggests that AtSULTR2;1 plays a role in the control of the sulfate translocation from roots to shoots (Takahashi et al. 1997; Takahashi et al. 2000; Kataoka et al. 2004a). Moreover, this transporter is also suggested to control the flux of sulfate ions to developing seeds (Awazuhara et al. 2005). Concerning AtSULTR2;2, its role in the long distance transport of sulfate is not completely clear. In fact, it is expressed in the phloem cells of the roots and in the vascular bundle sheaths of the leaves (Takahashi et al. 2000).

G**roup 3** is poorly characterized and seems to have multiple functions (Takahashi et al. 1999; Hawkesford 2003). For AtSULTR3;5, one of the five isoforms belonging to this group, it has been suggested that this isoform can form heterodimers with AtSULTR2;1, facilitating the transport of sulfate ions from roots to shoots through xylem (Kataoka et al. 2004a). Therefore, it is possible to think that AtSULTR3;5 could be a component of the low-affinity sulfate uptake system involved in loading sulfate into xylem. This hypothesis is supported by the following observations:

a) AtSULTR3;5 and AtSULTR2;1 share the same expression domains in plant tissues;
b) AtSULTR3;5 is a non-functional transporter itself when it is expressed heterologously in a yeast mutant defective for sulfate uptake. Only the co-expression of AtSULTR3;5 and AtSULTR2;1 enhances the sulfate uptake activity of the latter.

Group 4 transporters are localized on the tonoplast and allow the efflux of sulfate from the vacuoles optimizing the distribution of the ion within the cell (Kataoka et al. 2004b).

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). The transporters belonging to this group are truncated sequences and possess little N or C-terminal regions beyond the transmembrane domains. Using green fluorescent protein technique, these transporters have been localized on internal membranes and it is thought that they may play a role in vacuolar loading, even if studies with knock out mutants do not show clear phenotypes. Since there are not papers that confirm sulfate transport through these transporters, either in plant or in

other expression systems, such as yeasts, there is the possibility that these proteins have substrates other than sulfate (Hawkesford 2008). For examples, AtSULTR5;2, renamed MOT1, functions as a molybdate transporter (Tomatsu et al. 2007; Baxter et al. 2008).

Taking into account the features of all these groups, the coordinated expression of these transporters enhances the optimal management of sulfate under different conditions of supply and request that can change during the flow of plant life and with the external conditions to which the plant is exposed.

1.3. Sulfate metabolism

1.3.1. From the sulfate to the sulfide

Once inside the cells, before reduction, sulfate has to be activated through an adenilation reaction catalyzed by ATP sulfurylase (ATPS). The resulting adenosine 5'-phosphosulfate (APS) forms a branching point where it can follow two different assimilative pathways:

a) the pathway of reductive sulfate assimilation, at the end of which Cys is synthesized;
b) the pathway of not reductive sulfate assimilation, at the end of which 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is synthesized. This molecule represents a donor of activated sulfate for many sulfation reactions.

Concerning the pathway of reductive sulfate assimilation, the first reaction is catalyzed by APS reductase (APR) which transfers two electrons to APS, producing sulfite. The electron donor in this reaction is the reduced glutathione (Bick et al. 1998; Leustek et al. 2000; Kopriva and Koprivova 2004; Saito 2004). APR of flowering plants is a multidomain protein consisting of an N-terminal reductase domain and C-terminal thioredoxin/glutaredoxin-like part (Gutierrez-Marcos et al. 1996, Setya et al. 1996; Bick et al. 1998; Kopriva and Koprivova 2004). The reductase domain binds a Fe_4S_4 cluster as a cofactor (Kopriva et al. 2001; Kopriva et al. 2002; Kim et al. 2006). Even if the properties of the cluster are well-described, its exact function in the reaction mechanism of APR remains unknown (Kopriva et al. 2002; Kim et al. 2006).

In the 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; Leustek et al. 2000; Saito 2004). This enzyme is dependent on siroheme and FeS centers as prosthetic groups (Krueger and Siegel 1982).

It is also important to note that in this scheme of sulfate assimilation a sulfite oxidase was identified (Eilers et al. 2001). This enzyme is localized in the peroxisome, possesses a molybdenum cofactor and is able to oxidase the sulfite to sulfate, transferring the electrons to molecular oxygen forming hydrogen peroxide (Hänsch et al. 2006). However, until now, the importance of this enzyme to sulfur flux in the cell and its biological function is not clear, except for its capacity to enhance the resistance to high levels of sulfur dioxide deriving from the atmosphere (acid rain), or during the catabolism of sulfur-containing amino acids (Brychkova et al. 2007).

1.3.2. Cysteine biosynthesis

For the Cys biosynthesis, the sulfide is used as substrate (Saito 2004; Hell and Wirtz 2008). The first step is the activation of serine by the enzyme serine acetyltransferase (SAT), that, transferring acetyl coenzyme A to serine, forms *O*-acetylserine (OAS). In a second step, *O*-acetylserine (thiol) lyase (OAS-TL) catalyzes a β-replacement reaction between the acetyl moiety and sulfide (Leustek et al. 2000, Saito 2004). Both the enzymes (SAT and OAS-TL) are ubiquitously expressed in plant cells and are encoded by several nuclear genes (Hell and Wirtz 2008); thus, Cys can be synthesized in the cytosol, plastids, and mitochondria (Wirtz et al. 2004). However, from different studies on various SAT mutants, it has been discovered that OAS is mainly synthesized in the mitochondria and cytosol, but not in the plastids, which, instead of, are the sites where Cys synthesis is predominantly (Haas et al. 2008; Watanabe et al. 2008). Thus, taking into account these observations, it is possible to image that, at leaf level:

a) sulfide is produced in the chloroplasts as final product of the reductive process;
b) OAS is synthesized in the mitochondria;
c) the synthesis of major part of Cys occurs in the cytosol (Haas et al. 2008; Watanabe et al. 2008).

Cys represents the final metabolite of the pathway of reductive sulfate assimilation, but it also represents the substrate for production of other molecules containing reduced sulfur, such as: Met, GSH and a broad variety of other metabolites (Saito 2004).

1.3.3. Synthesis of glutathione and its functions

γ-glutamylcysteinylglycine (GSH) is a tripeptide that represents the main thiol in the cells of plants not exposed to heavy metals (Kunert and Foyer 1993). It plays a pivotal role in both defense and protection against oxidative damages produced by biotic and abiotic stresses (Noctor et al. 1998; Rausch and Wachter 2005; Foyer and Noctor 2009). In fact, in the cells, GSH acts as redox buffer:

a) by counteracting the detrimental effects provided by reactive oxygen species, that can be produced in response to stresses (Rausch et al. 2007; Astolfi and Zuchi 2013);
b) by maintaining a correct redox potential in the cells.

Moreover, GSH is involved in other important processes, such as the regulation of sulfur metabolism and inter-organ sulfur allocation (Lappartient and Touraine 1996), the control of development and cell cycle (May et al. 1998; Vernoux et al. 2000), calcium signaling (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 detoxification of xenobiotic and heavy

metals (Rauser 1995; Marrs 1996; Coleman et al. 1997). Changes in the intracellular concentration of GSH can produce important consequences for cells, through modification of redox status, gene transcription and metabolic functions.

The enzymatic steps need to synthesize GSH from Cys are well known and involve two ATP-dependent reactions (Lu et al. 2013). During the first step the enzyme γ-EC synthetase, through the formation of a peptide bond between the amine group of Cys and the γ-carboxyl group of the glutamate (Glu) side chain, synthesizes γ-glutamylcysteine (γ-EC; γ-Glu-Cys; Wachter et al. 2005). Both structure and activity of this enzyme are redox sensitive: when γ-EC synthetase is fully reduced, its activity is very low; on the contrary, the full oxidation leads to an higher activity (Hell and Bergmann 1990). From the study of the 3D structure of γ-EC synthetase, it was possible to observe that changes in the cell redox status modified the conformation of the enzyme by the formation/breaking of two intramolecular disulfide bridges (Jez et al. 2004; Hothorn et al. 2006; Hicks et al. 2007; Gromes et al. 2008). Successively, during the second step, a glycine (Gly) is added to the C-terminal of γ-EC to produce GSH. This reaction is catalyzed by GSH synthetase (Lu et al. 2013). These two reactions happen in both cytosol and chloroplasts. Anyway, both γ-EC and GSH can be transported by the membrane proteins CLTs, from chloroplast to cytosol (Maughan et al. 2010), while only GSH can enter the plastids (Pasternak et al. 2008). These fluxes allow to maintain, in every cellular compartment, the GSH homeostasis.

Concerning the GSH turnover, it is regulated by γ-glutamyltransferase (GGT) activities. GGT1 and GGT2 have high similarity and sequence identity and are localized on the outer surface of the plasma membrane. It seems that these two enzymes catalyze the GSH uptake and the long-distance transport, respectively (Ferretti et al. 2009), whilst GGT3 is considered a non-functional and truncated sequence. Finally, GGT4 is involved in the cleaving of glutathione-S-conjugates at the internal side of the tonoplast, only after that the glutathione-S-conjugates are transported into the vacuole through MRP-type ABC transporters (Grzam et al. 2007).

1.3.4. Regulation of sulfur metabolism is modulated by the request for S-containing molecules

Cys is the molecule from which many other sulfur-containing metabolites are synthesized (Met, GSH and PCs; Saito 2004), so its biosynthesis is highly regulated to meet the metabolic request for Cys. Consequently, also the sulfate flux along the pathway of reductive sulfate assimilation has to be finely modulated to guarantee the correct amount of required Cys, which can change under the different environmental conditions that plants experience during their growth. For example, plants exposed to biotic and/or abiotic stresses can need of higher levels of some compounds deriving from Cys, such as GSH and PCs, triggering an increment of the activity of

some enzymes involved in the pathway of reductive sulfate assimilation (Rausch and Wachter 2005), the typical response documented and studied also during sulfate starvation experiments (Lappartient and Touraine 1996; Lappartient et al. 1999). In these last conditions, the induction of some genes involved in the pathway is not triggered to meet the increasing request for Cys by plant metabolism, that contrariwise remains constant, but to satisfy the need of Cys and sulfur-containing compounds. This happens because, after the removal of sulfate from the growing medium, the levels of sulfate and all sulfur-containing compounds decrease producing an induction of sulfate transporters and some enzymes along the pathway of reductive sulfate assimilation (Lappartient and Touraine 1996; Lappartient et al. 1999). The increment of the activity of the sulfate transporters and key enzymes along the assimilatory pathway derives from their transcriptional induction. In fact, under sulfate limiting conditions, many papers report transcript accumulation of genes encoding sulfate transporters, ATPS and APR. Conversely, the addition of sulfate or sulfur-compounds to the growing medium, represses transcription (Lappartient and Touraine 1996; Smith et al. 1997; Takahashi et al. 1997; Bolchi et al. 1999; Lappartient et al. 1999). Taking into account all these observations, it is possible to hypothesize the existence of a mechanism able to regulate the Cys biosynthesis in order to satisfy the amount of sulfur-containing compounds needs to the plant. Furthermore, some papers report that the plants have the capacity to sense directly the own nutritional status rather than the composition of the growing medium (Lappartient and Touraine 1996; Lappartient et al. 1999) and this is possible because some terminal products of the pathway of reductive sulfate assimilation play the role of long distance repressor signals. Some researchers concluded that GSH could play this role as a phloem translocated signal (Herschbach and Rennenberg 1991; Lappartient and Touraine 1996; Lappartient et al. 1999), whilst Bolchi et al. (1999) found that, in the maize roots, Cys acted as a repressor.

From all these considerations, it is possible to build a model for the sulfate uptake and assimilation in plants, where some reduced sulfur-containing metabolites along the pathways of reductive sulfate assimilation and GSH biosynthesis increase or decrease the expression of some key genes and the activity of some enzymes. In this model, adequate levels of reduced sulfur-containing compounds (Cys and GSH) would be able to reduce gene expression through a negative feedback loop that would prevent the overcoming of the requests for sulfur-containing compounds and, consequently, energetic wastes. On the other hand, a reduction in the levels of reduced sulfur-containing compounds de-represses gene transcription increasing the flux of sulfate into the pathways of reductive sulfate assimilation. This regulation allows plants to adapt sulfur metabolism to the different environmental conditions.

Another molecule, involved in the regulation of S metabolism, is OAS. This metabolite plays a role as de-repressor of the transcription of sulfur responsive genes when nitrogen and carbon supply exceeds sulfur availability in the cells (Neuenschwander et al. 1991; Smith et al. 1997; Kim et al. 1999; Yamaguchi et al. 1999; Ohkama-Ohtsu et al. 2004). In these conditions, OAS accumulation, caused by the insufficient amount of sulfide not enable to inhibit the SAT activity, partially overrides the negative feedback provided by the reduced sulfur-containing compounds on gene transcription (Hawkesford 2000; Hawkesford and Wray 2000).

Concerning Cys biosynthesis, it is controlled also at post-translational level through the reversible formation of an enzymatic complex between SAT and OAS-TL (Kredich 1996; Saito 2004; Wirtz and Hell 2006; Hell and Wirtz 2008). Normally, in all cellular compartments, the concentration of OAS-TL is much higher than that of SAT (Lunn et al. 1990; Droux et al. 1992; Rolland et al. 1993), and only a small number of OAS-TL can form enzymatic complexes with SAT. These complexes are constituted by a homotetrameric SAT associated with two homodimeric OAS-TL. The formation of this bi-enzyme complex is promoted by sulfide, while OAS accumulation facilitates its dissociation (Saito 2004; Wirtz and Hell 2006; Hell and Wirtz 2008). When SAT is associated to OAS-TL, its kinetic properties are enhanced. On the other hand, OAS-TLs bound with SATs in the bi-enzyme complex lose in the catalytic efficiency. Thus, the Cys formation is mainly due to the free OAS-TLs (Droux et al. 1998). These observations provide evidences of the existence of a regulatory mechanism, where the bound form of OAS-TL acts as a positive regulatory subunit of SAT in the enzymatic complex. Moreover, Cys plays a role as controller of the synthesis of OAS through a negative feedback loop exerted on specific isoforms of SAT (Urano et al. 2000; Noji and Saito 2002; Wirtz and Hell 2003). This regulatory model is essential to both finely set the Cys biosynthesis and coordinate the OAS synthesis from Ser and sulfate reduction.

Even if all these complex strategies of regulation explain the maintaining of the homeostasis of the main sulfur-containing compounds, little is known about the modalities of signal perception and transduction. Some studies suggest different hormones (auxin, methyl jasmonate, abscisic acid, cytokinins, and salicylate) as molecules involved in the signal transduction pathways (Hirai et al. 2003; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003; Maruyama-Nakashita et al. 2004a; Rausch and Wachter 2005; Yakimova et al. 2006; Maksymiec 2011; Masood et al. 2012; Stroiński et al. 2013).

Concerning the transcriptional regulation of some sulfur responsive genes, potential sulfur responsive elements (SUREs) have been identified in their promoter regions (Awazuhara et al. 2002; Kutz et al. 2002), even if Maruyama-Nakashita and coworkers (2005) demonstrated that only

a 5 bp sequence in the promoter region of *AtSULTR1;1* was enough to promote the expression of this gene under sulfur starvation suggesting the involvement of SUREs in the transcriptional control of a group of genes required for adaptation to sulfur limiting conditions.

In 2006, Maruyama-Nakashita and coworkers discovered SLIM1, a transcriptional regulator, which controlled both the activation of sulfate acquisition and degradation of glucosinolates during sulfur starvation. Furthermore, SLIM1 induced miR395, a microRNA involved in the regulation of sulfur metabolism. During sulfur limiting conditions, in the cells, miR395 accumulates and targets three ATPS isoforms and AtSULTR2;1, causing the posttranscriptional degradation of transcripts of these genes (Jones-Rhoades and Bartel 2004; Kawashima et al. 2009).

1.3.5. Factors controlling glutathione synthesis

GSH accumulation in plants is significantly influenced by sulfur availability and assimilation (Maruyama-Nakashita et al. 2003; 2006; Yoshimoto et al. 2007; Jozefczak et al. 2012) and its biosynthesis is controlled by γ-EC synthetase activity and the amount of available Cys. In fact, in plants of Arabidopsis, reduction of the transcriptional levels of γ-EC synthetase using antisense strategy causes a decrement of GSH content at leaf level. On the other hand, the overexpression of this gene increases the GSH content at leaf level (Xiang et al. 2001). It is important to note that Arabidopsis plants overexpressing γ-EC synthetase do not show a reduction of the Cys amount due to enhanced GSH biosynthesis, suggesting the presence of a coordinate regulation of both Cys and GSH biosynthesis (Xiang et al. 2001). Furthermore, the activity of γ-EC synthetase is modulated at post-translational level through a negative feedback exerted by GSH, allowing to control GSH concentration and homeostasis (Hell and Bergmann 1990; Noctor et al. 1998, Noctor et al. 2002).

1.3.6 Sulfate Use Efficiency

The Sulfate Use Efficiency (SUE) is a measure of how a plant uses the available sulfur (Baraniecka and Kopriva 2014). It can be defined as yield (biomass) per unit of sulfur input. SUE is a complex trait, since it considers many factors, such as:

a) the ability to take up the sulfur source from the soil (or growing medium);
b) the sulfur transport;
c) the sulfur storage;
d) the sulfur mobilization;
e) the sulfur use within the plants.

SUE is of primary interest for crop improvement. In fact, the enhancing of this trait is a pre-requisite for:

a) reducing the sulfate fertilization;
b) increasing crop production of marginal lands, where the soil fertility is low.

1.4. The relationship between sulfur metabolism and cadmium

The capacity of a plant to finely regulate sulfur metabolism is essential for its survival in a wide range of environmental conditions, since some sulfur-containing compounds play a pivotal role in mitigation of both biotic and abiotic stresses (May et al. 1998; Rausch and Wachter 2005). Several studies report that, under different stressing conditions, many genes, expressed during sulfur starvation, are induced, and this behavior suggests the presence of a regulatory mechanism able to satisfy the increment of reduced sulfur-containing compounds requested by plants (Heiss et al. 1999; Vanacker et al. 2000; Noctor et al. 2002; Hirai et al. 2003; Howarth et al. 2003a; Howarth et al. 2003b; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003; Herbette et al. 2006; Nocito et al. 2006). This response is induced by stressing conditions which, in turn, produce additional sinks for reduced sulfur-containing compounds. These additional sinks increase the request for both Cys and GSH and, consequently, the total amount of sulfur necessary for both mitigation of the stressing condition and plant growth (Nocito et al. 2002; Rausch and Wachter 2005; Nocito et al. 2006).

1.4.1. The toxicity of cadmium

Heavy metals are a class of metals having a density higher than 5 g cm^{-3} (Elmsley 2001). They can be divided into two groups: essential and not essential for plants. For the essential ones (such as iron, copper and zinc), small quantities are required for the correct development and plant growth, since they are cofactors for many enzymes. On the other hand, cadmium (Cd), a not essential heavy metal as mercury and lead, is not necessary for plant growth, but rather it can produce deleterious effects. Once Cd is entered into the root cells, through transport systems specific for essential cations, Cd may alter the cellular functions interacting with sulfur and nitrogen atoms of amino acids, modifying protein structures and activities. These negative effects can be also exerted by essential heavy metals when, in the cells, their concentration is too much high. This is due to their chemical reactivity which can negatively affect metabolism and physiology of living organisms. For example, higher levels than those requested for free essential redox-active metals, such as iron and copper, are able to generate highly reactive hydroxyl radicals by a Fentom-catalyzed Haber-Weiss reaction (Halliwell and Gutteridge 1984, 1990).

When, in the cells, Cd accumulates, it may produce a broad variety of symptoms ranging from chlorosis, wilting, growth reduction, nutrients deficiency, until to cell death (Wójcik and Tukiendorf 2004; Mohanpuria et al. 2007; Ebbs and Uchil 2008). The negative effects exerted by Cd at cellular level depend on the capacity of this metal to interfere with: enzyme catalysis (van

Assche and Clijsters 1990), nitrate absorption and reduction (Hernandez et al. 1996), carbohydrate metabolism (Sanità di Toppi and Gabbrielli 1999), water balance (Costa and Morel 1994; Perfus-Barbeoch et al. 2002) and photosynthetic processes (Siedlecka and Krupa 1996; Pietrini et al. 2003). These Cd induced deleterious effects are mainly due to the ability of this metal to form bonds with sulfhydryl groups of proteins, causing the inactivation of the enzymes (Asgher et al. 2015). Furthermore, even if Cd is not a redox-active metal, its presence in the cells can displace from the proteins redox-active metals (Stohs and Bagchi 1995) which, in turn, can induce oxidative stress due to the formation of reactive oxygen species, such as superoxide anion and hydrogen peroxide (Romero-Puertas et al. 2004; Yadav 2010; Lin and Aarts 2012; Clemens et al. 2013, Choppala et al. 2014).

Among not essential heavy metals, the most studied one is Cd since it is highly mobile in both soil and plant; this makes it one of the major toxic pollutants very dangerous not only for plants and environment, but also for all living organisms (Clemens 2006; Nawrot et al. 2006; Järup and Akesson 2009; Gallego et al. 2012; Clemens et al. 2013; Asgher et al. 2014; Choppala et al. 2014). In the soil, it can be naturally present, even if it can be accidentally added by anthropogenic sources, such as atmospheric depositions from mining activities, phosphate fertilizers and manures, municipal sewage wastes, urban composts, and industrial sludges (Alloway and Steinnes 1999; McLaughlin et al. 1999; DalCorso et al. 2010; Momodu and Anyakora 2010; Gill et al. 2012; Nazar et al. 2012).

Cd is taken up by roots in competition with other divalent ions, through specific transporters for essential metals, such as members of ZIP and Nramp families or Ca^{2+} channels and transporters (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; Baker et al. 2006). To reduce the amount of Cd translocated to the shoots preventing Cd accumulation into the seeds, most plant species have a well conserved firewall system at the root level (Jarvis et al. 1976; Wagner 1993; Lozano-Rodríguez et al. 1997; Puig and Peñarrubia 2009; Verbruggen et al. 2009; Ueno et al. 2010; Nocito et al. 2011). When Cd ions enter into the cells, they are rapidly blocked at root level through binding sites with high affinity for the metal or through compartmentalization into the vacuoles (Clemens 2006; Ueno et al. 2010; Nocito et al. 2011). Only the Cd ions escaped by this firewall system may be loaded, through P_{1B}-ATPases, into the xylem and successively translocated to the shoot. In Arabidopsis, AtHMA2 and AtHMA4 are two transporters involved in the loading of free Cd ions into the xylem (Wong and Cobbett 2009). So, the Cd amount translocated to the shoot depends on a complex equilibrium between different biochemical and physiological processes, such

as: Cd chelation, compartmentalization, adsorption, and translocation (Nocito et al. 2011). Many actors play a role in this firewall system:

a) the processes of Cd chelation that involve GSH and PCs and the consecutive vacuolar compartmentalization of Cd-PC complexes (Cobbett 2000; Cobbett and Goldsbrough 2002; Clemens 2006; Seth et al. 2012; Choppala et al. 2014);
b) the adsorption of Cd ions to cellular matrices or apoplast components (Weigel and Jäger 1980; Khan et al. 1984);
c) the transport-mediated sequestration of Cd ions into the vacuole (Ueno et al. 2010; Satoh-Nagasawa et al. 2013);
d) the P_{1B}-type ATPase-mediated Cd loading into the xylem (Wong and Cobbett 2009; Nocito et al. 2011; Mills et al. 2012; Satoh-Nagasawa et al. 2012; Takahashi et al. 2012; Satoh-Nagasawa et al. 2013; Tan et al. 2013; Fontanili et al. 2016).

Through these ATPases (AtHMA2 and AtHMA4; Hussain et al. 2004; Verret et al. 2004; Wong and Cobbett 2009), Cd is loaded into the xylem and can reach the shoot, whilst, concerning Cd accumulation in the developing seeds, the phloem plays a pivotal role since the reproductive tissues have a reduced transpiration ratio limiting the xylematic contribute (Bauer and Hell 2006).

1.4.2. Phytochelatins: importance, structure and synthesis

Focusing the attention on PCs, the implication of these molecules in the chelation and subcellular compartmentalization of metal ions is a well conserved mechanism in the plants and its involvement in the buffering of cytosolic metal concentrations (Rauser 1999) and in the natural heavy metal tolerance contributing to the survival of the plants in wide range of soil conditions is known (Clemens 2001).

PCs are heavy metal chelators. They are Cys-rich peptides constituted by only three amino acids: Glu (E), Cys (C) and Gly (G), with Glu and Cys residues linked through a γ-carboxylamide bond. PCs are formed by at least two γ-EC units followed by a terminal Gly residue. The general formula of PCs is: $(\gamma\text{-Glu-Cys})_n$-Gly, where *n* ranges from 2 to 11 (most frequently 2-5).

When the plants are exposed to different metals or metalloids, the PC synthesis is rapidly induced (within minutes). Among the metals, Cd is the strongest inducer, consequently very low concentrations of this metal are required for the induction of PC biosynthesis (Grill et al. 1987; Grill et al 1989; Maitani et al. 1996). Finally, the studies of Howden and coworkers (1995a, 1995b) confirmed the pivotal role of PCs in the Cd detoxification, since mutants of Arabidopsis deficient in PC synthesis and in the formation of Cd-PC complexes were Cd hypersensitive.

PC biosynthesis depends on GSH through nontranslational synthesis, catalyzed by PC synthase (PCS) in a stepwise reaction. The first step consists in the transpeptidation of the γ-Glu-Cys unit of a GSH molecule onto another GSH molecule to form PC_2; in the next steps, other γ-Glu-Cys units arising from GSH are transferred to PC_2 to form PC_3 and so on (Rea et al. 2004; Rea 2012).

Concerning the types of complexes deriving from the binding between PCs and Cd, a lot of studies have been conducted. Gel filtration analysis of alkaline extracts from plants grown under a broad range of Cd concentrations and for different times reveal that the most part of buffer-soluble Cd is present as Cd-PC complexes, resolvable as low- and high-molecular weight (LMW and HMW) complexes (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; Howden et al. 1995b; Rauser 2000; Rauser 2003). The composition of these Cd-PC complexes is not constant; in fact, many times the presence of acid-labile sulfide in different molar ratios with Cd^{2+} has been reported (Reese and Winge 1988; Speiser et al. 1992; Rauser and Meuwly 1995). Experiments of Cd exposure on plants of tomato, *Brassica juncea* and cells of *Schizosaccharomyces pombe* report that acid-labile sulfide predominates more in HMW than in LMW complexes (Reese et al. 1992; Speiser et al. 1992), showing the same conservative nature of Cd complexation mechanisms in plants and fungi.

LMW and HMW complexes appear within a few hours after Cd exposure and they are in a dynamic state depending on both exposure time and Cd concentration in the growing medium (Murasugi et al. 1981; Leopold et al. 1998; Leopold et al. 1999; Rauser 2003). LMW complexes are predominant in the early exposure phases; conversely, the HMW complexes are more abundant during the following phases. Furthermore, experiments on *S. pombe* confirm the essential role played by HMW complexes in the maximization of Cd detoxification, since the mere presence of LMW complexes is not sufficient (Mutoh and Hayashi 1988).

Taking into account all these previously reported observations, a general model of Cd detoxification is proposed. During the first phase of Cd chelation, cytosolic LMW complexes are compartmentalized into the vacuole, through the ABC (ATP-binding cassette)-type transporters, localized on the tonoplast (Vögeli-Lange and Wagner 1990; Ortiz et al. 1995; Park et al. 2012; Song et al. 2014). Once inside the vacuole, the LMW complexes incorporate S^{2-} and other Cd^{2+} ions, evolving into more stable HMW complexes. Thus, LMW and HMW Cd-PC complexes are part of a dynamic process, where the first ones are involved as cytosolic carriers whilst latter ones represent the major Cd storage forms into the cells (Ortiz et al. 1995). However, it seems that other transporters localized on the tonoplast could be involved in the PC-based Cd detoxification mechanism (Ortiz et al. 1995). It has been hypothesized that a part of Cd^{2+} present in the vacuole is

transported through Cd^{2+}/H^{+} antiporter (Salt and Wagner 1993) and it seems that the Arabidopsis antiporter CAX2 (calcium exchanger 2) could be involved in this function, since it has a broad substrate range and its expression increases the Cd amount translocated into the vacuoles (Hirshi et al. 2000). Moreover, also AtHMA3 and OsHMA3, two P_{1B}-ATPase transporters present in Arabidopsis and rice plants, respectively, are involved in the movement of Cd ions into the vacuole (Morel et al. 2009; Miyadate et al. 2011).

In 2008, Mendoza-Cózatl and coworkers found that in the phloem of several plant species Cd was mainly joined with GSH and PCs, but this discovery was unexpected since, for a long time, PCs were considered the molecules mediating only the Cd compartmentalization. In addition, X-ray analysis detected significant Cd levels associated in sulfur-containing complexes in the cytoplasm of companion cells (Van Belleghem et al. 2007), suggesting the role of these thiols as mediators for long-distance transport of metals along the phloem, even if the involved plasma membrane transporters remain unknown. Studies about transcriptome of Arabidopsis show that *phytochelatin synthase* (*PCS*) is highly expressed in companion cells (Mustroph et al. 2009). Since companion cells and sieve elements (phloem) are inter-connected through permeable plasmodesmata (Turgeon and Wolf 2009), the compounds synthesized in the companion cells, or transported into them, such as GSH or PCs, can enter easily the phloem and be transported to sink tissues, such as seeds and roots (Li et al. 2004; Chen et al. 2006; Li et al. 2006; Turgeon and Wolf 2009). In 2011, Mendoza-Cózatl and coworkers found in Arabidopsis seeds significant levels of GSH but no PCs suggesting that detected Cd in the seeds is conjugated with GSH, whilst the Cd-PC complexes loaded into phloem are sequestered at root level in the vacuoles by ABC-type transporters. Moreover, in sustain of this thesis, transcripts of ABC-type transporters are expressed 3-fold higher in the roots than in the shoots (Mustroph et al. 2009). Taking into account these observations, it seems that PCs contribute to the Cd transport out of the shoots in order to preserve photosynthetic apparatus from the detrimental effects caused by the heavy metal (Mendoza-Cózatl et al. 2008; Van Belleghem et al. 2007).

1.4.3. Regulation of phytochelatin biosynthesis

PC biosynthesis is regulated:

a) directly, through the level and activity of PCS;
b) indirectly, through the amount of available GSH.

Arabidopsis PCS is a constitutively expressed enzyme, since its expression level does not change under Cd exposure (Ha et al. 1999; Vatamaniuk et al. 1999; Cobbett 2000), whilst in *Triticum aestivum*, *TaPCS1* expression in the roots increased under Cd exposure (Clemens et al.

1999). As previously reported, PC biosynthesis starts some minutes after Cd exposure (Grill et al. 1987). PCS synthesizes PCs, from GSH, in the presence of metal ions and, among these, Cd is the strongest activator of this enzyme (Grill et al. 1989; Howden et al. 1995a; Howden et al. 1995b; Klapheck et al. 1995; Chen et al. 1997).

Rea and coworkers (2004) reported that the activation of PCS depends on the presence of GSH-like peptides containing blocked sulfhydryl groups (deriving, for example, but no necessary, from the formation of a heavy metal thiolate), and not on metal ions directly bound to the C-terminal region of the enzyme.

1.4.4. Regulation of sulfur metabolism during cadmium exposure

Given that PC biosynthesis depends on GSH availability, it is possible to speculate the existence of a relationship between sulfate assimilation, GSH biosynthesis and Cd detoxification. During Cd exposure and its following accumulation in plant tissues, the immediate PC synthesis causes a transient reduction of cellular GSH levels (Grill et al. 1987; Tukendorf and Rauser 1990), and, under protract Cd exposure, PCs become the most abundant class of non-protein thiols in plant tissues. In these stressing conditions, the PC concentration can reach values higher than those of GSH, which, in turn, represents the most abundant non-protein thiol in not Cd exposed plants (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). Consequently, since synthesized PCs can be considered as an additional sink for reduced sulfur, it appears clear that the Cd detoxification increases the need of the plants for total amount of reduced sulfur producing an increment in the sulfate assimilation rate. This relationship between Cd detoxification processes and sulfate assimilation was documented for the first time in 1988 (Nussbaum et al. 1988) on maize (*Zea mays*) seedlings, where ATPS and APR activity was induced by 50 μM Cd^{2+} exposure after only 24 hours. Moreover, other papers (Rüegsegger et al. 1990; Rüegsegger and Brunold 1992) report that, always in maize seedlings, the activity of two other enzymes, involved in the GSH biosynthesis (γ-EC synthetase and GSH synthetase), increased under Cd exposure and accumulation. The induction of activity of these two enzymes was triggered by the temporary decrement in the GSH levels caused by PC biosynthesis.

The request for reduced sulfur-containing compounds deriving from PC synthesis seems to be a common response in Cd exposed plants. For example, in Arabidopsis exposed to the metal, several genes involved in sulfate assimilation pathway, such as *SAT* (Howarth et al. 2003a), cytosolic *OAS-TL* (Dominguez-Solis et al. 2001), *ATPS* and *APR* (Harada et al. 2002), and in pathway of GSH synthesis, such as *γ-EC synthetase* and *GSH synthetase* (Xiang and Oliver 1998) were induced. Similarly, also in *B. juncea* plants exposed to Cd, transcriptional up-regulation of

ATPS, *APR* and *γ-EC synthetase* genes has also been reported (Schäfer et al. 1998; Heiss et al. 1999; Lee and Leustek 1999).

Until now, all these researches suggest that, during Cd exposure, the presence of an additional sink for reduced sulfur deriving from PC biosynthesis, increases the metabolic demand for Cys and GSH, generating a typical demand-driven coordinate transcriptional regulation of genes involved in sulfate assimilation and GSH biosynthesis. This adaptation is essential for plant survival when exposed to Cd. In fact, the regulation of genes involved in sulfur assimilation allows to maintain in the cells GSH homeostasis and to detoxify Cd through GSH consuming activities.

Moreover, Nocito and coworkers (2002 and 2006) found that, in the roots of maize seedlings exposed to Cd, the over-expression of *ZmST1;1*, a gene encoding a root-expressed high-affinity sulfate transporter, allowed an increment of the sulfate uptake root capacity. This demonstrated that, under Cd exposure, also the regulation of the sulfate influx into the roots contributed to the fully reaching of amount of sulfur requested by plant for PC biosynthesis and for maintaining of cellular GSH homeostasis. Furthermore, the entity of the modulation of this gene was related with the nutritional request for reduced sulfur, which, in turn, depended on the strength of Cd-induced additional sink for thiol compounds (Nocito et al. 2006). Thus, the regulation of the sulfate transporters, responsible for the sulfate uptake, can be considered the first point of control of an adaptive and essential response that ensures the correct sulfur supply for Cd detoxification. Also other papers report the transcription induction of two root sulfate transporters, *AtSULTR1;1* and *AtSULTR1;2* during Cd exposure (Herbette et al. 2006; Rouached et al. 2008; Villiers et al. 2012; Jobe et al. 2012).

Interestingly, also other sulfate transporters, not directly involved in the sulfate uptake from the growing medium, were transcriptionally regulated during Cd exposure (Herbette et al. 2006). For example, the overexpression of *AtSULTR4;1*, a sulfate transporter localized on the tonoplast and responsible for the flux of the anion from the vacuole to the cytosol, could be involved in mobilizing the vacuolar sulfate stores providing additional substrate for the pathway of assimilation. Moreover, in Cd treated plants, the sulfate concentration in the xylem sap increased through the upregulation of genes codifying sulfate transporters involved in sulfate loading into the xylem, suggesting that, under this experimental condition, demand-driven regulatory networks enhanced the translocation of sulfate (Yamaguchi et al. 2016).

Concerning the specific mechanism of perception of presence of Cd in the cells, it remains unclear. It could recognize the GSH depletion or the increment of the amount of reduced sulfur requested. In this way, the effect of Cd exposure and its accumulation inside the cells would simulate a sort of a sulfur limiting condition.

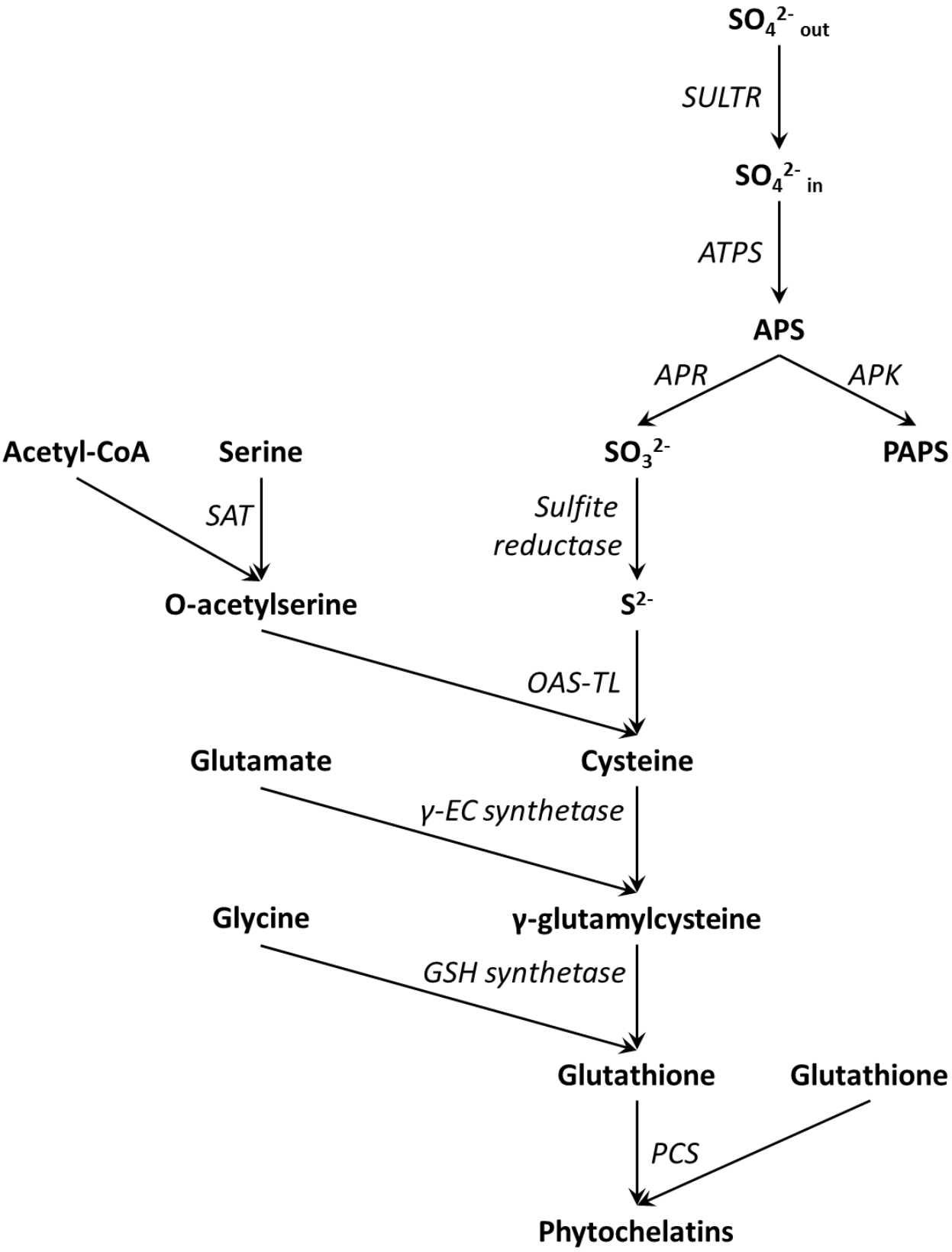
Since up-regulation of main sulfur responsive genes is induced by both sulfur limitation in the growing solution and Cd exposure, it appears evident the need to have multiple signaling pathways modulating sulfur nutrition in response to both sulfur demand and soil sulfate level. However, the nature of these signals needs further investigations, since only indirect evidences have been obtained. Xiang and Oliver (1998) suggested that, in Cd stressed Arabidopsis, jasmonate would control transcriptionally the genes involved in the GSH synthesis. Finally, it has been hypothesized that also the induction of sulfate uptake under heavy metal stress may be controlled through both GSH-dependent or GSH-independent signaling pathways (Nocito et al. 2006). This hypothesis derived from these observations:

a) in the roots of Cd stressed plants, variations in *ZmST1;1* transcript levels and sulfate uptake capacity are not in correlation with changes in GSH levels in the cells.
b) other metals, such as Zn, even promoting the genesis of additional sinks for thiol without negatively affecting GSH pools, can induce both *ZmST1;1* transcription and sulfate uptake by roots.

1.4.5. Possible strategies to enhance cadmium tolerance in higher plants

Since it seems that several bottlenecks along the Cd detoxification pathway could limit the tolerance to the metal, experiments on γ-EC synthetase and GSH synthetase activities confirm that these two enzymes are limiting factors of GSH biosynthesis in heavy metal stressed plants (Schäfer et al. 1998; Xiang and Oliver 1998). In plants not exposed to Cd, the limiting factor for GSH biosynthesis is assumed to be γ-EC synthetase, whose activity is negatively regulated by GSH levels (Noctor et al. 1998). Conversely, in Cd exposed plants, the transient reduction in the GSH levels induced transcriptional regulation of GSH biosynthesis, since Cd exerts an inhibitory effect on GSH synthetase activity (Schneider and Bergmann 1995; Schäfer et al. 1998; Xiang and Oliver 1998). From the experiments on *B. juncea* and sugar beet, the over-expression of *γ-EC synthetase* or *GSH synthetase* induced an higher synthesis of GSH resulting in an increment in the Cd tolerance (Zhu et al. 1999a, 1999b; Liu et al. 2015). These plants, when Cd stressed, had higher levels in PCs and GSH enhancing tolerance to the metal. Also the overexpression of *Lycium chinense GSH synthetase* in transgenic Arabidopsis plants resulted in improved tolerance to Cd stress compared to wild-type (Guan et al. 2015). All these findings confirm the central role of GSH and PCs in both stress tolerance and Cd accumulation. However, plants of Arabidopsis overexpressing the *PCS* gene are hypersensitive to Cd (Lee et al. 2003; Li et al. 2004). This could be caused by an excessive GSH utilization during PC synthesis induced by the metal, bringing to severe decrement in GSH levels. Finally, it is documented that in maize seedlings, sulfate availability in the growing medium

affected the toxic effects exerted by Cd accumulation (Astolfi et al. 2004; Nocito et al. 2006). In fact, at high sulfate concentrations in the growing medium, the root sulfate stores effect GSH biosynthesis, increasing the level of this metabolite (Nocito et al. 2006).



Scheme of sulfate assimilation pathway and PC biosynthesis.

2. GENERAL AIM OF PhD THESIS IN A NUTSHELL

In this PhD thesis, two experimental works are presented and discussed, with the aim of contributing to the improvement our knowledge on the molecular and physiological relationships existing among cadmium accumulation, cadmium tolerance, sulfur metabolism and sulfur use efficiency in two different model plants: barley and Arabidopsis. The specific aims of the experimental works are reported in the introduction of the two subsequent chapters.

3. ANALYSIS OF CADMIUM TRANSLOCATION, PARTITIONING AND TOLERANCE IN SIX BARLEY (*Hordeum vulgare* L.) CULTIVARS AS A FUNCTION OF THIOL METABOLISM

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3.1. Introduction

Cadmium (Cd) is one of the most toxic heavy metals present in soils from natural and anthropogenic sources, including atmospheric depositions from mining activities, phosphate fertilizers and manures, municipal sewage wastes, urban composts and industrial sludges (Alloway and Steinnes 1999; McLaughlin et al. 1999).

The presence of Cd in soils is an increasing concern with respect to human food chain accumulation, since it can be easily taken up by roots and accumulated in vegetative and reproductive plant organs: in this way, Cd-rich soils potentially result in Cd-rich foods.

Despite several efforts aimed at both reducing Cd input into agricultural soils and developing agronomic practices having the potential to reduce Cd bioavailability, breeding of low Cd-accumulating crops seems to be the most promising approach to minimize the dietary intake of Cd (Grant et al. 2008). Selection of novel cultivars with different Cd accumulation profiles should reduce not only the total amount of the heavy metal in the edible parts of the plants, but also the requirement for other management techniques. In such a context it appears evident the need to characterize and exploit the natural variation occurring in main crop species for their capacity to accumulate/exclude Cd from the edible parts, as well as to understand potential processes and molecular components that underlie these traits (Grant et al. 2008; Clemens et al. 2013).

Considerable natural variation in plant Cd accumulation occurs both between and within species (Guo et al. 1995; Grant et al. 1998; Cakmak et al. 2000; Clarke et al. 2002; Dunbar et al. 2003; Grant et al. 2008; Uraguchi et al. 2009). Most plant species retain much of the Cd taken up within roots by a conserved "firewall system" limiting the spread of Cd through the whole plant and preventing excessive Cd accumulation into seeds (Jarvis et al. 1976; Wagner 1993; Lozano-Rodríguez et al. 1997; Puig and Peñarrubia 2009; Verbruggen et al. 2009; Ueno et al. 2010; Nocito et al. 2011). The efficiency of this system is thought to be pivotal in determining the "Cd accumulation profiles" observed in crop species.

Once inside root cells Cd ions are trapped into roots through selective binding sites with high affinity for the metal, or through transfer across a membrane into an intracellular compartment (Clemens 2006; Ueno et al. 2010; Nocito et al. 2011). Only Cd ions escaping these trapping pathways may be potentially available to be loaded, by specific transport systems, into the xylem and translocated in a root-to-shoot direction. Thus, the ability of the root system to retain Cd should result from a complex equilibrium between different biochemical and physiological processes involved in Cd chelation, compartmentalization, adsorption and translocation (Nocito et al. 2011). Several actors have been described as active members of this firewall system, including: i) the

processes of Cd chelation and vacuolar compartmentalization based on the biosynthesis of phytochelatins (PCs) and related peptides (Cobbet 2000; Clemens 2006); ii) the adsorption of Cd ions to cellular matrices or apoplast components (Weigel and Jäger 1980; Khan et al. 1984); iii) the transport-mediated sequestration of Cd ions into the vacuole (Ueno et al. 2010; Satoh-Nagasawa et al. 2013); iv) the P_{1B}-type ATPase-mediated Cd loading into the xylem (Nocito et al. 2011; Satoh-Nagasawa et al. 2012, 2013; Mills et al. 2012; Takahashi et al. 2012; Tan et al. 2013).

Recent progress in understanding the molecular mechanisms controlling Cd allocation in rice makes realistic the development of low Cd-accumulating cultivars in an immediate future (Uraguchi and Fujiwara 2012; Clemens et al. 2013). Unfortunately, not nearly as much information is available for other major cereals, including barley, for which a significant increase in grain and flour consumption is expected in some critical arid and semiarid regions of North Africa (Bei et al. 2012). Although some report about genotypic diversity in barley grain Cd accumulation exists (Wu et al. 2003, 2007; Chen et al. 2008), scarce information about the physiological basis governing Cd distribution in the plant is available. Recently, it has been shown that the preferential retention of Cd in roots of barley is mainly due to immobilization processes mediated by S-ligands and reflects the accumulation of Cd-PC and Cd-S molecules in the vacuoles (Akhter et al. 2013).

In this paper we describe and compare six barley cultivars differing for their capacity to accumulate Cd in the shoot, with the specific aim to describe the role of thiol biosynthesis and metabolism in determining Cd partitioning and tolerance.

3.2. Material and methods

3.2.1. Plant material, growth conditions and sampling

All the experiments were carried out on 6 varieties of barley (*Hordeum vulgare* L.) with six (Manel, Rihane, Martin, Souihli, Lemsi) or two rows (Roho) – selected among the most cultivated in Tunisia for their capacity to accumulate Cd in the shoot – provided by the National Research Agronomic Institute of Tunisia.

Surface sterilized caryopses were placed on a filter paper saturated with distilled water and incubated in the dark at 26 °C. Seven days later, seedlings were transplanted into 5 L plastic tanks (8 seedlings per tank) containing the following complete aerated nutrient solution: 1.5 mM $MgSO_4$, 1.6 mM KH_2PO_4, 0.4 mM K_2HPO_4, 3.0 mM KNO_3, 2.0 mM NH_4NO_3, 3.5 mM $Ca(NO_3)_2$, 62 µM Fe-tartrate, 9 µM $MnCl_2$, 0.3 µM $CuSO_4$, 0.8 µM $ZnSO_4$, 46 µM H_3BO_3, 0.1 µM $(NH_4)_6Mo_7O_{24}$ (pH 6.5). Seedlings were kept for 10 d in a growth chamber at 26 °C and 80% relative humidity during the 16-h light period and at 22 °C and 70% relative humidity during the 8-h dark period. Photosynthetic photon flux density was 400 µmol m^{-2} s^{-1}. At the end of this period, plants were treated or not (control) with Cd by supplementing the nutrient solution with $CdCl_2$ to reach the final concentration of 25 µM. The treatment period was 30 d long. All hydroponic solutions were renewed 3 times per week to minimize nutrient depletion.

Plants were harvested and roots were washed for 10 min in ice-cold 5 mM $CaCl_2$ solution to displace extracellular Cd (Rauser 1987), rinsed in distilled water and gently blotted with paper towels. Shoots were separated from roots and the tissues were frozen in liquid N_2 and stored at -80 °C, or analyzed immediately.

3.2.2. Determination of Cd

Dried samples of about 150 mg were digested in 10 mL of 65% (v:v) HNO_3 using a microwave digestion system (Anton Paar MULTIVAWE 3000). The mineralized material was diluted 1:40 (v:v) in Milli-Q water (to a final volume of 10 mL) and filtered on a 0.45 µm PVDF membrane. Cd content was measured by inductively coupled plasma mass spectrometry (ICP-MS; Bruker Aurora M90 ICP-MS).

3.2.3. Determination of thiols and thiobarbituric acid-reactive-substances

Samples (roots and shoots) were pulverized using mortar and pestle in liquid N_2 and stored frozen in a cryogenic tank. For total non-protein thiol (NPT) content, 400 mg of powders were extracted in 600 µL of 1 M NaOH and 1 mg mL^{-1} $NaBH_4$, and the homogenate was centrifuged for

15 min at 13000 *g* and 4 °C. Four hundred microliters of supernatant were collected, 66 µL of 37% HCl were added and then centrifuged again for 10 min at 13000 *g* and 4 °C. For the quantification, volumes of 200 µl of the supernatant were collected and mixed with 800 µL of 1 M K-Pi buffer (pH 7.5) containing or not 0.6 mM Ellman's reagent {[5,5′-dithiobis(2-nitrobenzoic acid); DTNB]}. The samples' absorbances at 412 nm were then spectrophotometrically measured. The level of total GSH was determined according to Griffith (1980). Phytochelatins and related peptides were evaluated as difference between NPT and GSH levels in both root and shoot of Cd exposed plants (Schäfer et al. 1997). All results were expressed as micromoles of GSH equivalents.

The thiobarbituric acid-reactive-substances (TBARS) assay was performed according to Hodges et al. (1999).

3.2.4. Analysis of root-to-shoot Cd translocation

At the end of the exposure period, shoots were cut at 2 cm above the roots with a microtome blade. Xylem sap exuded from the lower cut surface was collected by trapping into a 1.5 mL plastic vial filled with a small piece of cotton for 2 h. The amount of collected sap was determined by weighing and the Cd concentration was measured by ICP-MS.

3.2.5. Statistical analysis

Statistical analysis was carried out using SigmaPlot for Windows version 11.0 (Systat Software, Inc.). Quantitative values are presented as mean ± standard deviation of the mean (SD). Significance values were adjusted for multiple comparisons using the Bonferroni correction. Statistical significance was at $P < 0.05$. Student's *t*-test was used to assess the significance of the observed differences between control and Cd-exposed plants. The *P* value < 0.05 was considered to be significant.

3.3. Results and discussion

3.3.1. Cd tolerance and partitioning in six barley cultivars

Six Tunisian improved barley cultivars – Lemsi, Manel, Martin, Rihane, Roho and Souihli – derived from local (Tunisia, Algeria) landraces (Chaabane et al. 2009), were exposed to 25 µM Cd^{2+} for 30 days and then analyzed for Cd partitioning and tolerance.

At the end of the incubation period no visible symptoms of toxicity (necrosis or chlorosis) were detectable in the shoots of any of the six barley cultivars. Such observations were confirmed by chlorophyll analysis showing that the concentration of chlorophyll a/b in the shoots was unaffected by Cd exposure (data not shown). Conversely, the growth of the six cultivars was significantly ($P < 0.001$) influenced by Cd (**Figure 1**). Considering the shoots: i) Lemsi appeared to be the most sensitive cultivar, with a Tolerance Index (TI) – defined as the average weight of shoots in treated group × 100 / the average weight of shoots in control group – of 37%; ii) Roho, Martin and Souihli showed an intermediate sensitivity, with TIs of 63, 67 and 73%, respectively; iii) Manel and Rihane were the most tolerant cultivars, with TIs of 86 and 85%, respectively (**Figure 1A**). Root growth was generally less affected by Cd exposure: the percentage of growth inhibition ranged from 0 in Souihli to 37% in Lemsi (**Figure 1B**). Similar behaviors were evinced by referring to plant fresh weight, since Cd exposure did not affect tissue water contents (data not shown).

Wide differences were observed considering the concentration of Cd in the shoot: i) Lemsi and Manel showed the highest and the lowest values, respectively; ii) in Rihane the concentration was significantly ($p < 0.05$) higher than in Manel; iii) in Martin, Souihli and Roho the values of Cd concentration were intermediate with respect to Manel and Lemsi and significantly ($p < 0.05$) higher than in Rihane (**Figure 2A**). By contrast a moderate variability was observed with regard to root Cd concentration (**Figure 2B**). From these data set we calculated that: i) the total amount of Cd accumulated in the whole plant was significantly ($P < 0.05$) higher in Lemsi, Rihane, Manel, and Martin than in Roho and Souihli (Supplementary Table S1); ii) the Cd root retention (i.e. the percentage of the total Cd retained in the root) widely differed among the six cultivars (Supplementary Table S1). The lowest value of retention was observed in Lemsi (70.8%), whilst the highest one in Manel (85.9%); all the other cultivars had intermediate values.

It has been largely reported that plant responses to Cd exposure involve a plethora of constitutive and adaptive processes, which interactions at molecular, physiological and morphological level result in complex phenomena allowing the cells to protect themselves against the injury due to Cd accumulation, or allowing the plants to exclude Cd stress (Turner 1994; Gwozdz et al. 1997; Sanità di Toppi and Gabbrielli 1999; Nocito et al. 2007). Cd tolerance and Cd

root-to-shoot translocation are often negatively related (Verkleij et al. 1990; Wong and Cobbett 2009). However, although tolerance is often associated with a high capability to retain the metal into roots, it does not necessarily mean that increased root retention itself is the cause of tolerance, since intraspecific differences in Cd uptake might occur (Lombi et al. 2000; Assunção et al. 2003).

Considering our data, it is important to note that the fraction of the absorbed metal translocated to the shoot was 2.2-fold higher in Lemsi than in Manel, although they did not significantly ($P < 0.05$) differed for the total amount of Cd accumulated in the whole plant. Data analysis also revealed the lack of any clear relationship between the total amount of Cd absorbed by plant and the calculated TIs (**Figure 3A**), which instead increased as Cd root retention did (**Figure 3B**). Thus, at least in our conditions, the reduced capacity to absorb Cd showed by some barley cultivars - even if conceivable as a possible mechanism of stress avoidance – was not involved in Cd tolerance.

Taken as a whole this group of data suggests the existence of root mechanisms limiting Cd translocation from root to shoot and thus preserving the photosynthetic tissues from the detrimental effects that Cd may induce. In fact, although Cd is not a redox-reactive metal, its accumulation in plant tissues generally results in oxidative stress (Nocito et al. 2008; Sharma and Dietz 2009; Del Buono et al. 2014).

For this reason, to better understand the relationship between Cd root retention and Cd tolerance, we measured, at the end of the Cd exposure period, the levels of thiobarbituric acid-reactive-substances (TBARS) in the shoots, assuming these values as diagnostic indicators of the occurrence/severity of Cd-induced oxidative stress (Hodges et al. 1999). As reported in **Figure 4A**, Cd exposure increased the levels of TBARS in the shoots. However, such an increase strongly differed among the six barley cultivars – ranging from 171% (Manel) to 544% (Lemsi) – and resulted negatively related to Cd tolerance (**Figure 4B**), suggesting Cd root retention as a possible mechanism of stress avoidance which preserves shoot tissues from Cd-induced oxidative damages. Finally, the importance of such a mechanism in determining Cd tolerance is further supported by the following observations: i) TI values increased as Cd concentration in the shoot decreased (**Figure 2A** and **Figure 3**); ii) Cd-induced oxidative damages increased as Cd concentration in the shoot did (**Figure 2A** and **Figure 4**). In this way, the selection of novel genotypes with enhanced Cd root retention or/and lower Cd concentration in the shoot may represent a valuable strategy, not only to reduce Cd exposure through plant-derived food, but also to increase Cd tolerance.

3.3.2. Analyses of Cd partitioning and tolerance as a function of thiol metabolism

Plant sulfur metabolism and thiol biosynthesis are deeply affected by Cd stress, mainly

because of the activation of a wide range of adaptive responses involving glutathione (GSH) consuming activities (Nocito et al. 2006, 2007; Lancilli et al. 2014). 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, a class of cysteine-rich peptides able to form thiolate bonds with Cd ions in complexes that accumulate in the vacuoles (Cobbett 2000; Clemens 2006). Studies on maize, rice and barley showed that most of the total Cd retained by roots is bound in complexes containing PCs and related thiol compounds, revealing these peptides as crucial for Cd root retention in cereals (Rauser and Meuwly 1995; Rauser 2003; Nocito et al. 2011; Akhter et al. 2013). Since the activity of homeostatic mechanisms based on thiol biosynthesis has been shown to be involved in Cd tolerance and may potentially allow a different proportion of Cd to be retained in roots, we analyzed the effects of Cd exposure on GSH and non-protein thiol (NPT) levels in both roots and shoots of the six barley cultivars.

Cadmium exposure significantly ($P < 0.001$) reduced the levels of total GSH in both roots and shoots of all the cultivars (**Figure 5A,D**). Such an effect was likely due to a general alteration of thiol homeostasis as indicated by the analysis of the NPTs, which levels in both roots and shoots significantly ($P < 0.001$) increased following Cd stress and overcame those of GSH – the main non-protein thiol in non-stressed plant tissues – measured in the same conditions (**Figure 5B,E**).

Data analysis revealed that the entity of the GSH decrement induced by Cd was negatively related to the general tolerance of the six barley cultivars to Cd stress. In fact, the effect of Cd on GSH content was minimum (or absent) in Manel and maximum in Lemsi, considering both roots and shoots (Supplementary Figure S1A,B). Conversely, the increments in the NPT content induced by Cd were directly related to the Cd tolerance: the highest increase was observed in Manel (+359%), whilst the lowest one was measured in Lemsi (+10%; Supplementary Figure S1C,D). PC and related peptide contents (**Figure 5C,F**) were evaluated as difference between NPT and GSH levels in both roots and shoots of Cd-exposed plants (Schäfer et al. 1997). Results indicated that the six barley cultivars widely differed for their capacity to synthetize PCs and related peptides (**Figure 5C,F**). Also in this case the level of these compounds in both roots and shoots was closely related to the Cd tolerance of each cultivar (Supplementary Figure S1E,F).

Cd exposure rapidly induces PC biosynthesis in plant tissues as result of GSH polymerization through the constitutive enzyme phytochelatin synthase (Rea et al. 2004). Short-term exposures to Cd generally result in both PC accumulations and GSH depletions closely related to the total amount of the metal accumulated in the tissues. In such a context the decreases in GSH levels due to the induction of PC biosynthesis should be directly related to the amount of PCs accumulated in the tissues or, in other words, to the strength of the additional sinks for reduced

sulfur induced by Cd (Grill et al. 1987; Tukendorf and Rauser 1990; Mendoza-Cózatl and Moreno-Sánchez 2006). However, under long-term Cd exposures PCs rapidly become the most abundant class of non-protein thiols and the relative increase in the metabolic demand for both cysteine and GSH generates a typical demand driven coordinated transcriptional regulation of genes involved in sulfate uptake, sulfate assimilation and GSH biosynthesis (Nocito et al. 2007). Such a response is thought to be pivotal in a metabolic scenario in which the rate of GSH biosynthesis has to maintain not only GSH homeostasis but also PC-based Cd detoxification processes (Nocito et al. 2007).

The analysis of thiols revealed the existence of a general relationship between the capacity of the barley cultivars to synthetize PCs and their Cd tolerance (Supplementary Figure S1E,F), which however did not seem related to the total amount of Cd accumulated (**Figure 3A**), as previously reported by Persson et al. (2006). The capacity to produce and accumulate PCs appeared as a specific characteristic of each barley cultivar since it was not significantly related to Cd concentration in the roots and resulted negatively related to the quantity of Cd accumulated in the shoot (Supplementary Figure S1G,H). Moreover, considering GSH concentrations in both root and shoot of untreated plants (control) it appears evident the lack of any clear relationship between the total amount of reduced sulfur assimilated into GSH and the tolerance of each cultivar to Cd stress. These behaviors may reflect any difficulties in maintaining GSH homeostasis during Cd stress and could be ascribed to a direct and cultivar-specific interference of Cd on some activity along the pathways involved in sulfate uptake, sulfate assimilation and GSH biosynthesis.

Such a hypothesis seemed to be confirmed by the analyses of the changes in the GSH levels induced by Cd accumulation which showed the existence of close positive linear relationships between the effect of Cd on GSH levels and PC accumulation in both root and shoot (**Figure 6A,B**). In other words the ability of each barley cultivars to maintain GSH homeostasis during PC biosynthesis was crucial for Cd tolerance, as previously demonstrated by the analysis of transgenic *Brassica juncea* plants in which the over-expression of γ-glutamylcysteine synthetase or GSH synthetase – the two enzymes along the GSH biosynthetic pathway – enhanced Cd tolerance as a consequence of a greater production of GSH during Cd stress (Zhu et al. 1999a, 1999b). On the other hand, transgenic Arabidopsis plants expressing the cDNA for γ-glutamylcysteine synthetase in antisense orientation resulted hypersensitive to Cd as a consequence of a reduced capacity to synthetize both GSH and PCs under the exposure to the metal (Xiang et al. 2001).

3.3.3. Analysis of root-to-shoot Cd translocation as a function of thiol metabolism

To better understand the relationship existing between Cd root retention, thiol biosynthesis and root-to-shoot Cd translocation we measured the concentration of Cd in the xylem sap of the six

barley cultivars at the end of the exposure period. In these experiments Cd translocation was estimated as the amount of Cd ions loaded and transported in the xylem sap for 2 h, according to Nocito et al. (2011).

Results indicated that the six barley cultivars strongly differed for their capacity to load Cd ions into the xylem (**Figure 7A**). The amount of Cd transported in the xylem sap of the six barley cultivars during the observation period ranged from 55.3 (Manel) to 187.5 ng 2 h^{-1} (Lemsi), and was linearly related (r^2 = 0.817) to the total amount of Cd accumulated in the shoots over a 30 d period (**Figure 7B**).

Since the capacity of barley roots to retain Cd ions has been recently associated to immobilization processes mediated by S-ligands (Akhter et al. 2013), we analyzed Cd translocation as a function of GSH homeostasis and PC accumulation in the roots, with the aim to evince a general relationship describing how the "Cd translocation" trait depends on root thiol metabolism in different barley genotypes. Results revealed that Cd translocation was closely related to thiols since the amount of Cd ions loaded in the xylem sap linearly decreased as PC content in the roots increased (**Figure 7C**). Moreover, since the capacity of the roots to synthetize PCs was related to the capacity of each cultivar to maintain GSH homeostasis, it was also possible to evince a negative relation between Cd translocation and the negative effect exerted by Cd on GSH biosynthesis (**Figure 7D**). Such an analysis allows us to speculate that the genotypic differences observed in Cd translocation in the six barley cultivars could be partially due to a different sensitivity of GSH metabolism to Cd accumulation. In this view the different capacity of each barley cultivar to maintain GSH homeostasis during Cd stress should affect PC production and, thus, Cd translocation capacity, since, in the absence of any other significant differences in the main components of the firewall trapping Cd into the roots, the amount of Cd ions escaping thiol chelation may be considered as potentially available to be loaded into the xylem and translocated in a root-to-shoot direction.

Taken as a whole our analysis confirms the central role of both GSH and PCs in determining Cd tolerance and partitioning, and suggests that the effect of Cd on GSH biosynthesis may be potentially taken into account to develop indexes useful for the selection of low Cd-accumulating cultivars in barley. However, the molecular bases of such an effect need to be further investigated in order to individuate the main factor(s) – along the sulfur metabolic pathways – influencing the capacity of barley to maintain GSH homeostasis during Cd-induced PC biosynthesis. Interestingly, Schneider and Bergmann (1995) indicated the activity GSH synthetase as a possible limiting factor. Finally, our conclusions need to be validated in open field or glasshouse experiments, in where the activity of root exudation (Cesco et al. 2012) and the presence of rhizobacteria (Palacios et al. 2014)

may also influence plant Cd uptake and tolerance.

3.4. Figures

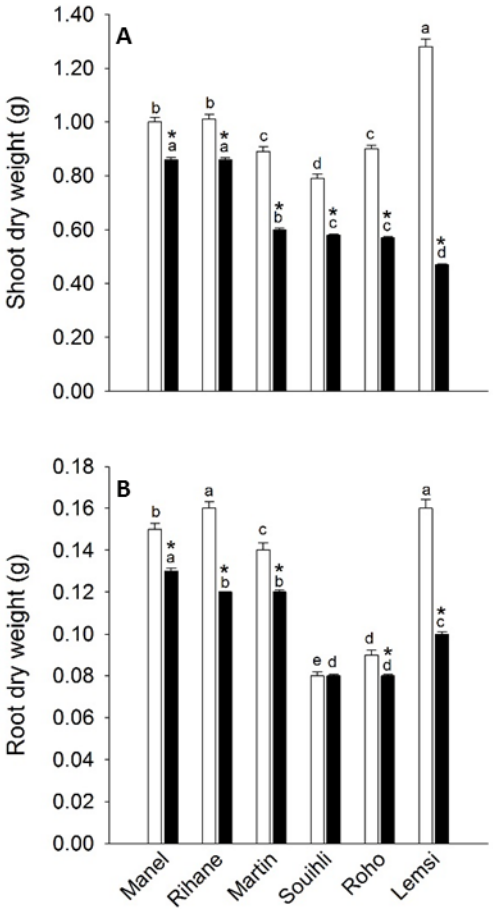

FIGURE 1. Effect of Cd exposure on growth of shoots (A) and roots (B) of six barley cultivars. Plants were grown for 30 days in a complete nutrient solution supplemented (black bars) or not (white bars) with 25 µM $CdCl_2$. Bars and error bars are means and SD of three experiments each performed with 4 plants (n = 3). Asterisks indicate significant differences between control and Cd-exposed plants (P < 0.001). Different letters indicate significant differences between the cultivars (P < 0.05).

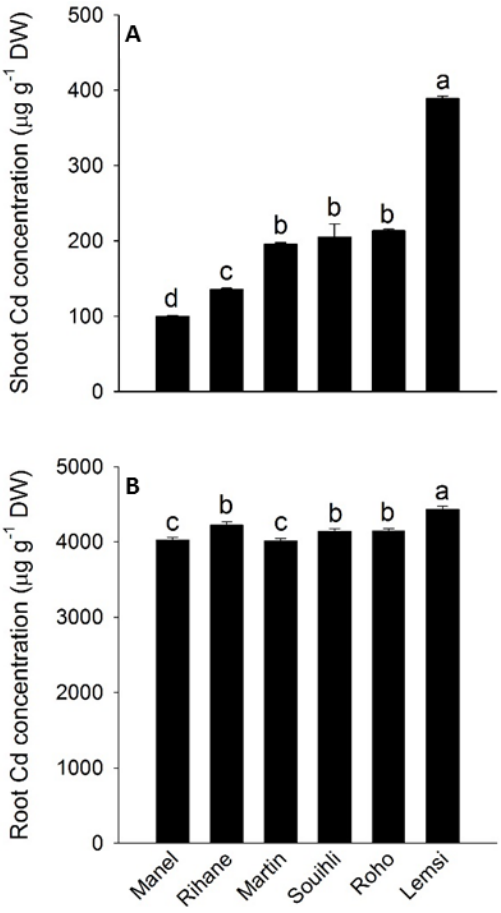

FIGURE 2. Cadmium accumulation in shoots (A) and roots (B) of six barley cultivars. Plants were grown for 30 days in a complete nutrient solution supplemented with 25 µM $CdCl_2$. Bars and error bars are means and SD of three experiments each performed with 4 plants (n = 3). Different letters indicate significant differences between the cultivars ($P < 0.05$).

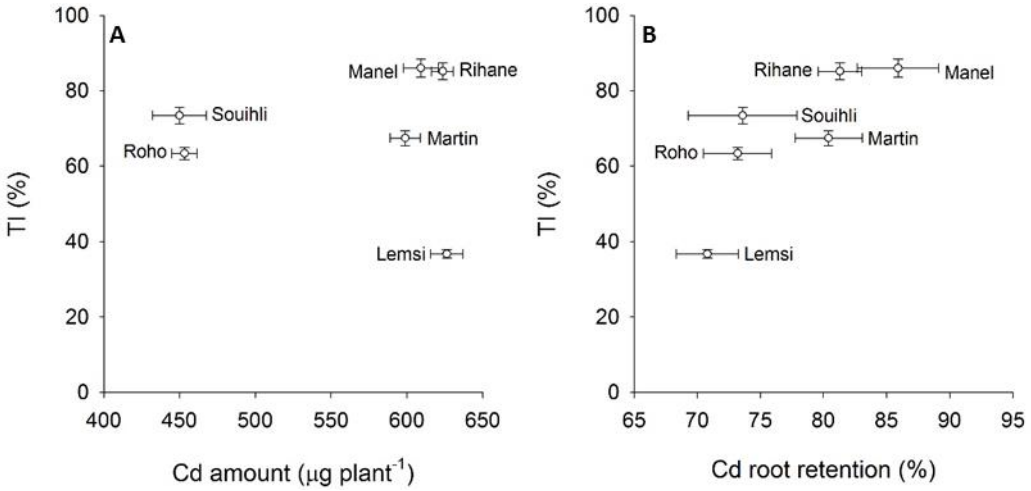

FIGURE 3. Analysis of Cd tolerance as a function of the total amount of Cd absorbed by plants (A) or Cd root retention (B) in six barley cultivars. Plants were grown for 30 days in a complete nutrient solution supplemented or not with 25 µM $CdCl_2$. Data are means and SD of three experiments each performed with 4 plants (n = 3). TI, tolerance index.

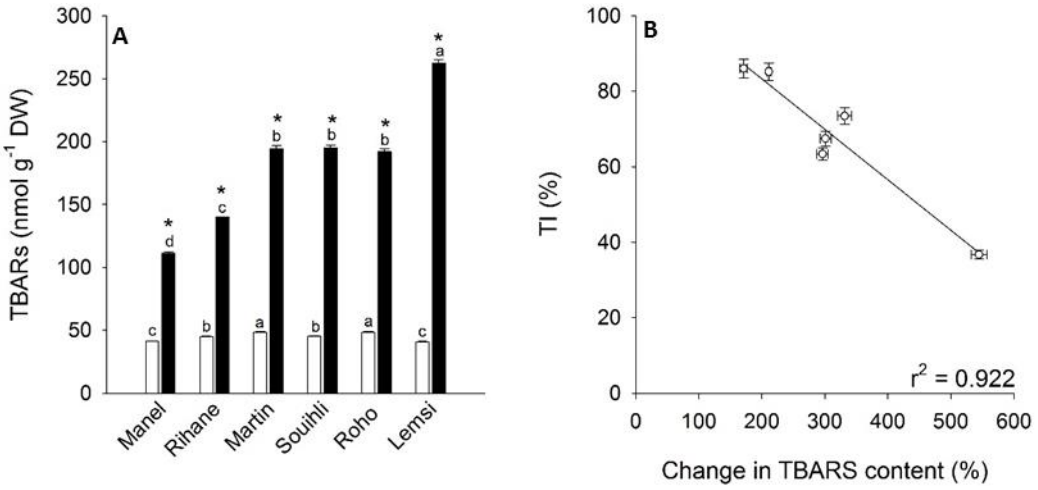

FIGURE 4. Effect of Cd exposure on the levels of TBARS in the shoots of six barley cultivars (A) and analysis of Cd tolerance as a function of changes in TBARS content (B). Plants were grown for 30 days in a complete nutrient solution supplemented (black bars) or not (white bars) with 25 µM $CdCl_2$. Data are means and SD of three experiments each performed with 4 plants (n = 3). TI, tolerance index. Asterisks indicate significant differences between control and Cd-exposed plants ($P < 0.001$). Different letters indicate significant differences between the cultivars ($P < 0.05$).

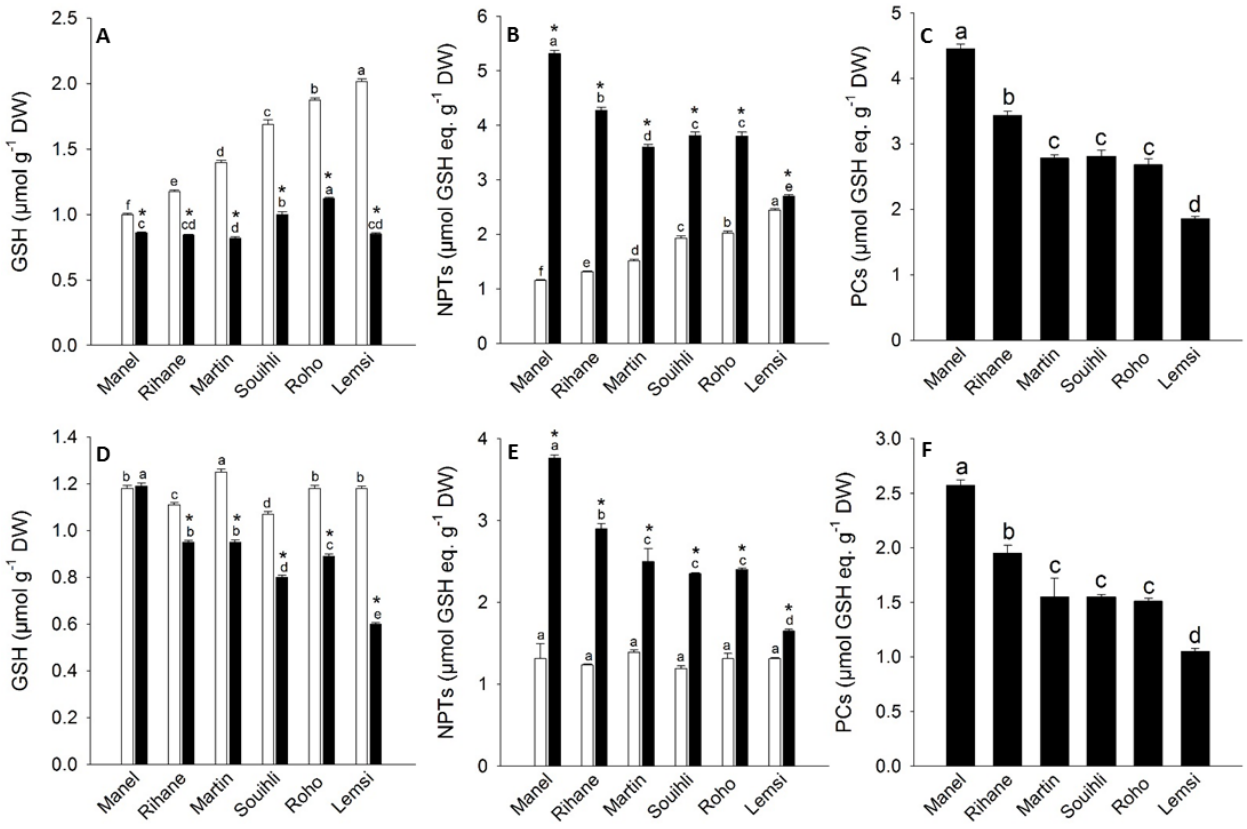

FIGURE 5. Effect of Cd exposure on the level of thiols in roots (A, B, C) and shoot (D, E, F) of six barley cultivars. Plants were grown for 30 days in a complete nutrient solution supplemented (black bars) or not (white bars) with 25 µM $CdCl_2$. NPT contents are expressed as GSH equivalents. PCs were evaluated as difference between NPT and GSH levels in both roots and shoots of Cd-exposed plants. Bars and error bars are means and SD of three experiments each performed with 4 plants (n = 3). Asterisks indicate significant differences between control and Cd-exposed plants ($P < 0.001$). Different letters indicate significant differences between the cultivars ($P < 0.05$).

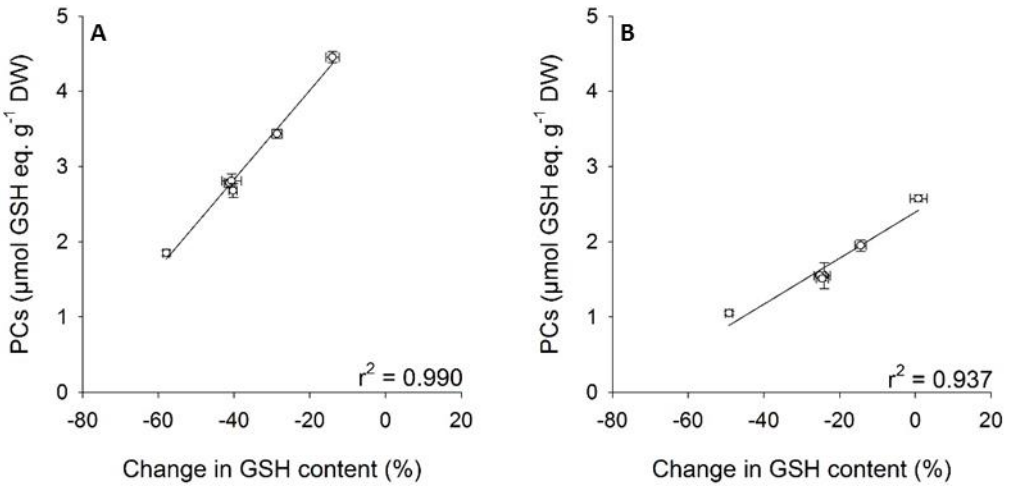

FIGURE 6. Analysis of PC content as a function of the effect of Cd on GSH levels in roots (A) and shoots (B) of six barley cultivars. Plants were grown for 30 days in a complete nutrient solution supplemented or not with 25 µM $CdCl_2$. Changes in GSH content were calculated comparing the GSH contents both roots and shoots of control and Cd-exposed plants. PCs were evaluated as difference between NPT and GSH levels in both roots and shoots of Cd-exposed plants. Data are means and SD of three experiments each performed with 4 plants (n = 3).

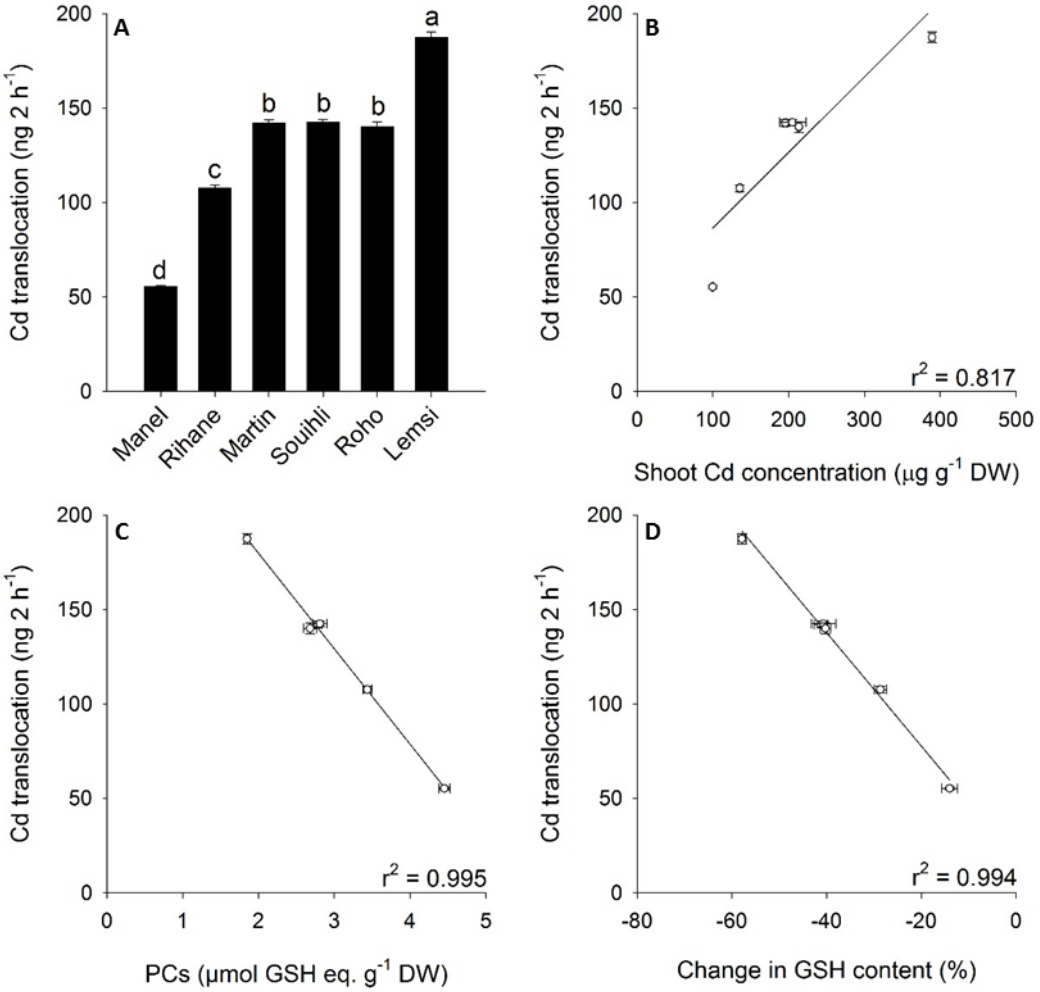

FIGURE 7. Analysis of Cd translocation in six barley cultivars. Plants were grown for 30 days in a complete nutrient solution supplemented or not with 25 µM $CdCl_2$. At the end of the exposure period, shoots were separated from roots and the xylem sap exuded from the cut (root side) surface was collected. **(A)** Cd ions loaded and transported in the xylem sap during 2 h. Data are means and SD of three experiments each performed with 4 plants (n = 3). Different letters indicate significant differences between the cultivars ($P < 0.05$). **(B, C, D)** Relationships between Cd ions loaded in the xylem sap, Cd concentration in shoots, and changes in root thiol content after a 30 d period of Cd exposure. Data are means and SD three experiments each performed with 4 plants (n = 3).

3.5. Supplementary materials

Cultivar	Cd amount			Cd root retention (%)
	Shoot (µg plant^{-1})	Root (µg plant^{-1})	Plant (µg plant^{-1})	
Manel	85.6 ± 1.7 (c)	523.6 ± 9.8 (a)	609.2 ± 11.5 (a)	85.9 ± 3.2 (a)
Rihane	116.6 ± 2.3 (b)	507.0 ± 4.9 (a)	623.6 ± 7.2 (a)	81.3 ± 1.7 (ab)
Martin	117.3 ± 2.2 (b)	481.6 ± 7.9 (b)	598.9 ± 10.0 (a)	80.4 ± 2.7 (ab)
Souihli	118.9 ± 11.3 (b)	331.1 ± 6.4 (d)	450.0 ± 17.7 (b)	73.6 ± 4.3 (bc)
Roho	121.7 ± 2.2 (b)	331.6 ± 6.2 (d)	453.3 ± 8.4 (b)	73.2 ± 2.7 (bc)
Lemsi	182.9 ± 3.1 (a)	443.4 ± 7.8 (c)	626.3 ± 10.9 (a)	70.8 ± 2.5 (c)

TABLE S1. Cadmium amount and Cd root retention in six barley cultivars. Plants were grown for 30 days in a complete nutrient solution supplemented with 25 µM $CdCl_2$. Cd root retention was calculated as the percentage of the total Cd retained by roots. Data are means and SE of three experiments each performed with 4 plants (n = 3). Different letters indicate significant differences between the cultivars ($P < 0.05$).

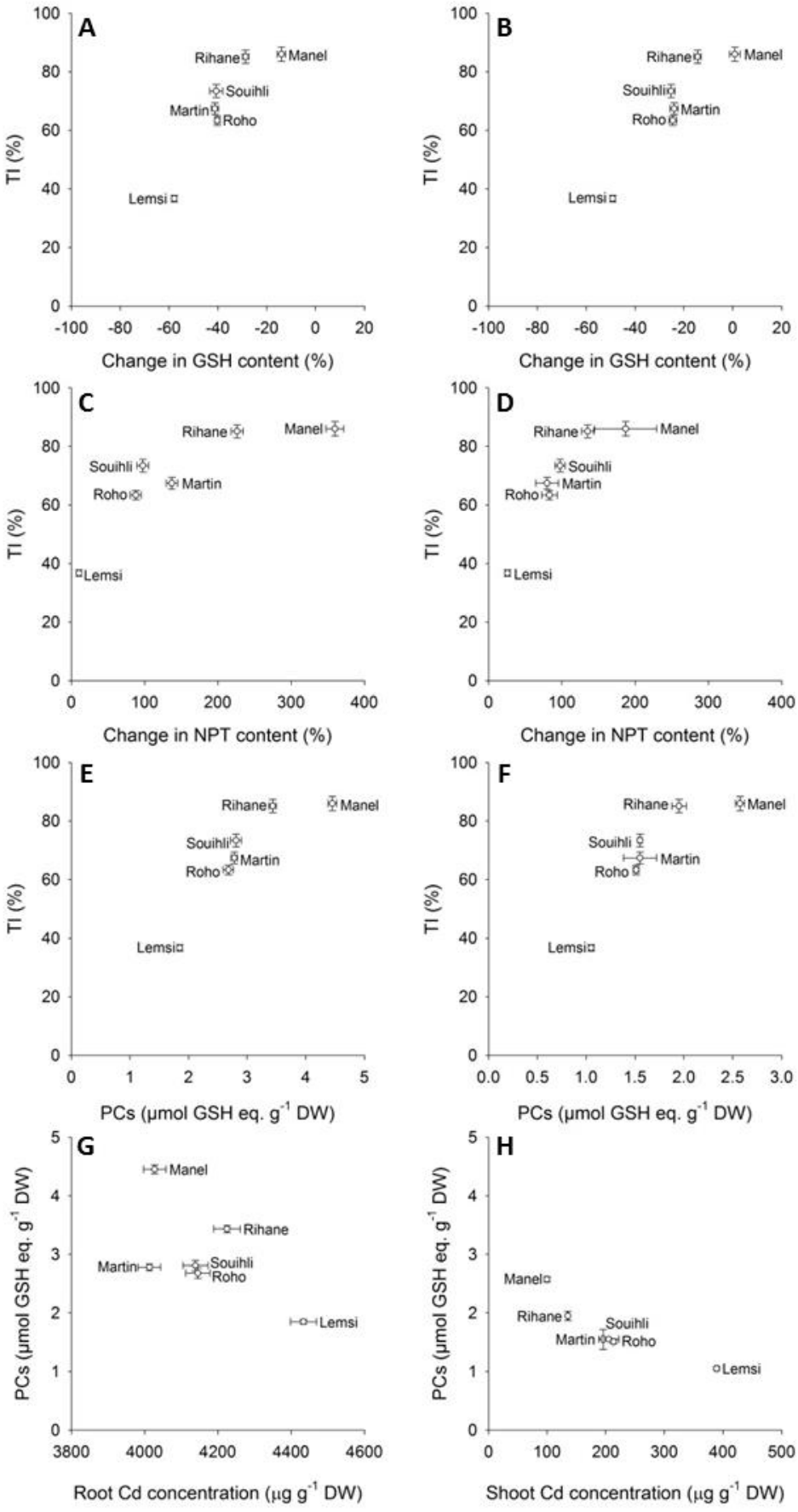
A
B
C
D
E
F
G
H
TI (%)
Change in GSH content (%)
Change in NPT content (%)
PCs (µmol GSH eq. g-1 DW)
PCs (µmol GSH eq. g-1 DW)
Root Cd concentration (µg g-1 DW)
Shoot Cd concentration (µg g-1 DW)
Rihane
Manel
Souihli
Martin
Roho
Lemsi

FIGURE S1. Analysis of Cd tolerance as a function of thiol metabolism. Plants were grown for 30 days in a complete nutrient solution supplemented or not with 25 µM $CdCl_2$. **(A, C, E)** Relationships between Cd tolerance and changes in root thiol content after a 30 d period of Cd exposure. **(B, D, F)** Relationships between Cd tolerance and changes in shoot thiol content after a 30 d period of Cd exposure. **(G, H)** Relationships between PC content and Cd concentration in roots and shoots. Data are means and SE of three experiments each performed with 4 plants (n = 3).

4. LONG-TERM EXPOSURE TO CADMIUM NEGATIVELY AFFECTS THE SULFATE USE EFFICIENCY IN *Arabidopsis thaliana*

4.1. Introduction

Plants have evolved a complex network of adaptation mechanisms that allow them to minimize the damages from exposure to nonessential and potentially toxic metal ions (Clemens 2001; Clemens 2006). Such mechanisms involve the main transport, chelation and sequestration processes controlling metal homeostasis into the cells and along the whole plant.

The synthesis of cysteine (Cys)- rich metal binding peptides – such as phytochelatins (PCs) – appears as the most conserved and ubiquitous process used by plants for cadmium (Cd) detoxification. PCs are a class of small peptides consisting of repeating units of γ-glutamylcysteine (γ-Glu-Cys) followed by a C-terminal glycine (Gly): the general structure of these peptides is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n = 2 to 11 (Grill 1987; Zenk 1996; Cobbett 2000; Cobbett and Goldsbrough 2002). The presence of γ-glutamyl linkages in these peptides implies that they are non-translationally synthesized using reduced glutathione (GSH) as direct precursor in a transpeptidation reaction catalyzed by the enzyme PC synthase (Rea et al. 2004; Rea 2006). Once synthetized in the cytoplasm PCs form thiolate bound with Cd^{2+} ions, and resulting Cd-PC complexes are subsequently sequestered to the vacuole (Cobbett 2000). In this way cells may control the concentration of the free Cd^{2+} ions in the cytosol, limiting the potential damages due to the their overaccumulation. The relevance of this mechanism for the natural Cd tolerance of the plants has been underlined by the analysis of the Arabidopsis PC-deficient mutants *cad1* and *cad2-1*, which resulted more sensitive to Cd than wild-type plants (Howden 1995a,b).

The interactions between Cd accumulation and sulfur (S) metabolism in higher plants have been exhaustively described and reviewed in several papers (Nocito et al. 2002; Mendoza-Cózatl et al. 2005; Nocito et al. 2007; Ernst et al. 2008; Lancilli et al. 2014; Jozefczak et al. 2014; Khan et al. 2016). In particular, it has been shown that the increases in the metabolic request for both Cys and GSH – generated by Cd-induced PC biosynthesis – produce a demand-driven coordinated transcriptional regulation of genes involved in sulfate uptake, sulfate assimilation and GSH biosynthesis. Such an activation is thought to be pivotal for ensuring – at least in the early phases of Cd accumulation – both GSH homeostasis and adequate fluxes of reduced S to help Cd detoxification processes. In fact, the large amount of PCs produced following Cd exposure may generate additional sinks for thiols which, in turn, increase the total nutritional demand for S by plant. However, some aspects of this model need to be further elucidated, since it is not clear whether the early adaptive responses to Cd – involving the S assimilation pathway – are enough to maintain adequate S levels to promote plant growth under prolonged exposures to the metal, i.e., whether plants growing in the presence of Cd need a higher amount of S in the soil to maximize

their growth. Considering these aspects, here we present and discuss two sets of growing experiments with Arabidopsis aimed at studying the effect of both short- and long-term exposure to Cd on the plant capability to optimize its growth under a wide range of sulfate concentrations in the growing medium, showing that long-term exposure to Cd negatively affects this trait.

4.2. Materials and methods

4.2.1. Plant materials, growth conditions and experimental design

Arabidopsis thaliana (Ler-0) seeds were washed under continuous shaking in 0.5 mL 0.01% (v/v) Tween 20 for 20 min, surfaced-sterilized by adding an equal volume of commercial bleach (4% active chlorine) for 5 min, and then rinsed four-times with sterile distilled water. Seeds were sown on a sterile 3M™ paper sheet – imbibed with sterile distilled water and lay down into a Petri dish – and then incubated for 4 days, in the dark, at 4 °C to remove dormancy.

Vernalized seeds were sown, with a toothpick, on small pieces of rockwool (Grodan®) placed into appropriate seed holders, obtained by cutting 1 mL pipette tips at 2 and 12 mm from the tip. The seed holders were transferred into pipette tip boxes, filled with distilled water – to maintain imbibed the rockwool – and finally incubated at 22 °C under continuous light to allow seed germination. Seven days after sowing, seedlings – selected for uniform growth – were transferred into 3 L plastic tanks (41 seedlings per tank) containing non-sterile aerated complete nutrient solutions and kept for 22 days in a growth chamber maintained at 22 °C and 80% relative humidity, with a 12-h light period.

For the short-term Cd exposure experiments plants were grown for 19 days in hydroponic solutions [250 µM $NH_4H_2PO_4$, 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 25 µM Fe-tartrate, 46 µM H_3BO_3, 9 µM $MnCl_2$, 0.8 µM $ZnCl_2$, 0.3 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, pH 6.5] containing different sulfate concentrations (5, 25, 50 and 150 µM $MgSO_4$). At the end of this pre-growing period plants were maintained in the same solutions and exposed, or not, to three concentrations of Cd^{2+} (0.1, 1 and 10 µM $CdCl_2$) for 72 h.

For the long-term Cd exposure experiments plants were grown for 22 days in hydroponic solutions [250 µM $NH_4H_2PO_4$, 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 25 µM Fe-tartrate, 46 µM H_3BO_3, 9 µM $MnCl_2$, 0.8 µM $ZnCl_2$, 0.3 µM $CuCl_2$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, pH 6.5] under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM $MgSO_4$), in the presence or absence of 0.1 µM $CdCl_2$.

In all the experiments, $MgCl_2$ was added to maintain the same concentration (500 µM) of the Mg^{2+} ions in each solution. All hydroponic solutions were renewed daily to minimize sulfate depletion. At the end of the growing periods, plants were used for the *in vivo* experiments or harvested to be further analyzed. In this case, roots were washed for 10 min in an ice-cold 5 mM $CaCl_2$ solution to displace extracellular Cd (Rauser 1987), rinsed in distilled water and gently blotted with paper towels; shoots were separated from roots and the tissues were weighted before to be frozen in liquid N_2 and stored at -80 °C.

4.2.2. Plant growth analysis

The curves describing the growth of both shoot and roots as a function of the sulfate concentration in the growing medium were drown by fitting the equation $y = y_0 + a(1 - e^{-bx})$ to the data obtained by weighting the shoot and the roots of the Arabidopsis plants at the end of each experiment. The sulfate concentration in the growing medium that produced the 95% of the maximum amount of fresh weight for shoot or roots (sulfate critical concentration; $[SO_4^{2-}]_{crit}$) in each experiment was calculated as follows:

$$[SO_4^{2-}]_{crit} = \frac{ln\left[\frac{0.05(y_0 + a)}{a}\right]}{-b}$$

4.2.3. Determination of thiols and cadmium content

Shoot and roots were pulverized using mortar and pestle in liquid N_2. Total non-protein thiols (NPTs) and Cd contents were determined as described by Fontanili et al. (2016). Total GSH, reduced GSH and oxidized GSH (GSSG) were measured according to Griffith (1980). Phytochelatins and related peptides were evaluated as difference between NPT and total GSH levels in both shoot and roots of Cd-exposed plants (Sghayar et al. 2015). All results were expressed as nanomoles of GSH equivalents.

4.2.4. Sulfate influx assay

Sulfate influx into the roots was measured by determining the rates of ^{35}S uptake, over a 15 min pulse in a complete nutrient solution labeled with the radiotracer. Briefly, a single plant was placed onto 10 mL of a fresh acclimation nutrient solution with the same ionic composition of those used for plant growth, containing 150 µM $MgSO_4$, supplemented or not with different concentrations of $CdCl_2$ (0.1, 1 and 10 µM Cd^{2+} for the short-term exposure experiments; 0.1 µM Cd^{2+} for the long-term exposure experiments); each solution was maintained aerated and thermoregulated at 22 °C. Radioactive pulses were started by adding ^{35}S-labeled Na_2SO_4 to the uptake solutions. Specific activity was 4.7 kBq µmol^{-1}. At the end of the pulse period, roots were rinsed twice for 1 min in 10 mL of a 4 mM $CaSO_4$ nonradioactive solution at 4 °C, blotted with paper towels, weighed, and then heated for 20 min at 80 °C in 5 mL 0.1 N HNO_3. Radioactivity was measured on aliquots of the extracting solution by liquid scintillation counting in a β counter (LS 6000SC, Beckman).

4.2.5. RNA extraction and qRT-PCR analysis

Total RNA was extracted from roots using TRIzol® Reagent (LifeTechnologies) and then purified using PureLink® RNA Mini Kit (LifeTechnologies), according to the manufacturer's instructions. Contaminant DNA was removed on-column using PureLink® DNase (LifeTechnologies). First-strand cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen), according to the manufacturer's instructions.

qRT-PCR analysis of *SULTR1;1* (At4g08620) and *SULTR1;2* (At1g78000) was performed on first-strand cDNA in a 20 µL reaction mixture containing GoTaq® qPCR Master Mix (Promega) and the specific primers, using an ABI 7300 Real-Time PCR system (Applied Biosystems). The relative transcript level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method using the expression of the *S16* (At4g34620) gene as reference. Primers for qRT-PCR are listed in (Supplementary Table S1).

4.2.6. Statistical analysis

Statistical analysis was carried out using SigmaPlot for Windows version 11.0 (Systat Software, Inc.). Quantitative values are presented as mean ± standard error of the mean (SE). Significance values were adjusted for multiple comparisons using the Bonferroni correction. Statistical significance was at $P < 0.05$. Student's *t*-test was used to assess the significance of the observed differences between control and Cd-exposed plants. The *P* value < 0.05 was considered to be significant.

4.3. Results

4.3.1. Effect of short-term exposure to Cd under different sulfate concentrations on growth, thiol content, Cd accumulation, and sulfate uptake of Arabidopsis plants

For short-term experiments, Arabidopsis plants were pre-grown for 19 days under four sulfate concentrations (5, 25, 50 and 150 µM) and then exposed for 72 h to three concentrations of Cd^{2+} (0.1, 1 and 10 µM). For each Cd concentration used in the experiments, we produced a set of curves showing the dependence of shoot (**Figure 1A**) or root (**Figure 1B**) fresh weight on the sulfate availability in the growing medium. The curves – properly described by exponential rise to maximum functions – approached saturation (95% of the maximum amount of fresh weight) at external sulfate concentrations ($[SO_4^{2-}]_{crit}$) of about 26 or 21 µM, for shoot or roots, respectively. The inhibitory effect of Cd on plant growth was concentration-dependent, as indicated by the comparison of the plants grown under the same sulfate concentration. On the other hand, the effect of each Cd concentration on shoot or root fresh weight was independent of the sulfate concentration, as indicated by the analyses of the growth normalized with respect to the control (i.e., relative growth; Supplementary Figure S1).

Short-term exposure to Cd significantly changed the NPT levels of both shoot and roots, which increased as Cd concentration did under all the sulfate concentrations analyzed (**Figure 2A,B**). Such trends were mainly related to the accumulation of PCs, which became the most abundant class of thiols in the tissues of Cd-exposed plants (**Figure 2E,F**). Opposite behaviors were observed as regards the effects of Cd on the total GSH levels of shoot and roots. In fact, for each sulfate concentration, the levels of total GSH significantly increased or decreased, considering shoot or roots, respectively, as the Cd concentration in the growing medium increased (**Figure 2C,D**). Moreover, for each Cd concentration, the dependence of the NPT, total GSH and PC levels on the external sulfate was described by typical exponential rise to maximum curves, approaching the saturation at sulfate concentrations very close to the critical ones. Finally, Cd concentration was higher in the roots than in the shoot; its concentration in the shoot or root tissues was dependent on the level of the metal in the growing medium, but resulted unaffected by the sulfate concentration (**Figure 2G,H**).

The capacity of the Arabidopsis roots to take-up sulfate was deeply affected by the sulfate availability in the growing medium, as well as by the presence of Cd, as indicated by the values of ^{35}S-sulfate uptake measured at 150 µM SO_4^{2-} external concentration (**Figure 3A**). In the control plants, the rate of sulfate uptake increased up to 1.2 fold, moving the external sulfate concentration from 150 to 5 µM. A Cd-dependent increase in the rate of sulfate uptake was also observed in the

Arabidopsis plants grown under the same sulfate concentration in the media. Sulfate uptake increases of 1.7, 2.0, 2.2, and 1.6 fold were measured in plants grown under 5, 25, 50, and 150 µM external sulfate, respectively, moving the Cd^{2+} concentration from 0 to 10 µM. These behaviors were closely associated to changes in the relative transcript levels of *SULTR1;1* (**Figure 3B**) and *SULTR1;2* (**Figure 3C**), the two Arabidopsis genes involved in sulfate uptake by roots (Maruyama-Nakashita et al., 2004b).

4.3.2. Effect of long-term exposure to Cd under different sulfate concentrations on growth, thiol content, Cd accumulation, and sulfate uptake of Arabidopsis plants

For long-term exposure experiments, we grew Arabidopsis plants for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM $MgSO_4$), in the presence or absence of 0.1 µM $CdCl_2$. As previously shown, the data set distribution of shoot and root fresh weights, obtained in each condition, as a function of the external sulfate concentration was properly described by an exponential rise to maximum function, which allowed to calculate the relative $[SO_4^{2-}]_{crit}$ maximizing the growth in each experimental condition. Such a value, calculated for the shoot (**Figure 4A**), was significantly higher in Cd-treated (40.9 ± 1.2 µM) than in control plants (28.8 ± 0.6 µM), whilst, for the roots (**Figure 4B**), resulted independent of the presence of Cd (19.4 ± 0.3 µM and 18.3 ± 0.5 µM, for control and Cd-treated plants, respectively). Interestingly, the analysis of the growth curves also revealed that Cd exposure exerted inhibitory effects on shoot growth only at sulfate concentrations lower than $[SO_4^{2-}]_{crit}$. At sulfate external concentrations higher than $[SO_4^{2-}]_{crit}$ the presence of Cd did not affect shoot growth (**Figure 4A**). Moreover, the effects produced by Cd on shoot growth were closely dependent on the sulfate concentration, since they reduced as the sulfate concentration in the medium increased up to the value of $[SO_4^{2-}]_{crit}$ (Supplementary Figure S2A). Conversely, the effects of Cd on root growth were independent of the sulfate concentration in the growing medium (Supplementary Figure S2B).

The relationship between the NPT level and the sulfate concentration in the medium exhibited a saturation behavior for both shoot and roots (**Figure 5A,B**). In particular, the NPT levels measured in the shoot were significantly higher in Cd-exposed than in control plants at sulfate external concentrations higher than $[SO_4^{2-}]_{crit}$; no significant effects were observed at sulfate concentrations lower than $[SO_4^{2-}]_{crit}$ (**Figure 5A**). On the other hand, the NPT levels in the Cd-exposed roots were significantly higher than in the control under all the sulfate concentrations analyzed (**Figure 5B**). Such behaviors were associated to deep changes in the balance among the different classes of thiols, whose relative abundance seemed to be dependent on: i) the presence/absence of Cd in the growing medium; ii) the sulfate external concentration; iii) the plant

tissues we considered. The analyses of the curves describing the dependence of the total GSH levels of the shoot on the sulfate external concentration revealed that the inhibitory effects exerted by Cd on the GSH accumulation gradually decreased moving the external sulfate concentration up to the critical value calculated for this condition. At sulfate external concentrations higher than $[SO_4^{2-}]_{crit}$ the presence of Cd did not affect the total GSH levels, whose values resulted similar to those measured in the control (**Figure 5C**). Such behavior was closely related to the synthesis of PCs, whose levels in the shoot tissues progressively decreased as the sulfate concentration increased, up to reach a constant value at sulfate concentrations higher than $[SO_4^{2-}]_{crit}$ (**Figure 5E**). A different picture was evinced by analyzing the dependence of total GSH and PC levels of the roots on the external sulfate concentration. Interestingly, in Cd-exposed roots the total GSH levels resulted significantly higher than in the control and did not show any apparent dependence on the sulfate external concentration (**Figure 5D**), differently from the PC levels, whose dependence on the sulfate external concentration was properly described by a saturation curve (**Figure 5F**). Finally, it is worthy to note that the concentration of Cd in both shoot and roots was not constant under all the sulfate concentration analyzed, showing a dependence on sulfate external concentration similar to that described for the concentrations of PCs in each apparatus (**Figure 5G,H**).

Since reduced GSH not only represents the key intermediate for the synthesis of PCs, but also plays a pivotal role as an antioxidant in controlling the cellular redox status, we measured the GSH/GSSG ratio in all the experimental conditions, assuming this value as a marker for oxidative stress. Results (**Figure 6**; Supplementary Figure S3) revealed that also in this case the relationship between the GSH/GSSG ratio measured in both shoot and roots and the sulfate concentration in the medium was described, in each condition, by a saturation curve, indicating that the optimal cellular redox status was reached at sulfate concentrations higher than the respective critical value. In particular, the GSH/GSSG ratio measured in the shoot was significantly lower in Cd-exposed than in control plants at sulfate external concentrations lower than $[SO_4^{2-}]_{crit}$; no significant effects were observed at sulfate concentrations higher than $[SO_4^{2-}]_{crit}$ (**Figure 6A**). Conversely, the GSH/GSSG ratio in the Cd-exposed roots were significantly lower than in the control under all the sulfate concentrations analyzed (**Figure 6B**).

Finally, the rate of sulfate uptake – measured at 150 μM SO_4^{2-} external concentration at the end of the exposure period – increased as the sulfate concentration in the growing medium decreased, but resulted significantly higher in Cd-exposed than in control plants under all the sulfate concentrations analyzed (**Figure 7A**). Close relationships between the rate of sulfate uptake and the relative transcript levels of *SULTR1;1* (**Figure 7B**) and *SULTR1;2* (**Figure 7C**) were observed also in this set of experiments.

4.4. Discussion

Several papers report that early Cd stress triggers a wide range of adaptive mechanisms – involving GSH consuming activities – which may increase the metabolic demand for sulfate, sulfur metabolites and carbon skeletons (Lee and Leustek 1999; Nocito et al. 2006, 2008). In fact GSH not only is polymerized to form PCs in response to Cd accumulation (Xiang et al. 2001; Rea et al. 2004), but also acts as an antioxidant in mitigating the oxidative stress produced by free Cd^{2+} ions into the cells (Cuypers et al. 2011; Noctor et al. 2012). In such a context, the need to maintain GSH homeostasis and continuous Cd chelation induces responses allowing plants to increase sulfate uptake by roots and sulfate entry in the reductive assimilation pathway, as well as to modulate sulfate allocation among the different tissues and organs. Such responses are mainly controlled at transcriptional levels and involve transcript accumulation of genes that encode sulfate transporters and activities involved in sulfate assimilation and GSH biosynthesis (Lee and Leustek 1999; Nocito et al. 2002, 2006; Lancilli et al. 2014; Yamaguchi et al. 2016). The pivotal importance of sulfate uptake in the plant adaptation to Cd stress has been recently underlined by the analysis of the Arabidopsis *sultr1;1-sultr1;2* double mutant – defective in two distinct high-affinity sulfate transporters (SULTR1;1 and SULTR1;2) involved in root sulfate uptake from the rhizosphere – which resulted, under limited sulfate supply, more sensitive to Cd-induced oxidative stress than the wild type (Liu et al. 2016). Moreover, analyses of Arabidopsis mutants defective in thiol metabolism and accumulation revealed that both oxidative stress and thiol depletion are necessary to induce the transcription of *SULTR1;2* during early Cd stress (Jobe et al. 2012). Thus, while the contribute of Cd-induced sulfate uptake to the early phases of Cd-detoxification appears evident, the role of sulfate uptake and S nutrition in maintaining plant growth under prolonged Cd exposure still need to be elucidated, since long-term exposure to Cd may permanently affect thiol homeostasis and allocation to different sinks, ultimately affecting the plant capacity to optimize its growth at a given sulfate concentration in the external medium and then the S use efficiency of the plants.

The analyses of the dependence of both shoot and root fresh weight on the amount of external sulfate available for the growth, may provide some educated guesses on how plants optimize the growth at a given sulfate concentration, then on the possible effects of Cd stress on S use efficiency. For this reason we calculate for each condition the $[SO_4^{2-}]_{crit}$ (i.e., the sulfate concentration in the growing medium that produced the 95% of the maximum amount of fresh weight for shoot or roots), assuming that changes in this value necessarily reflect changes in the plant ability to use the external S sources to promote the growth.

Data analysis reveals that short-term exposure to Cd negatively affects plant growth but does not produce any significant effects on the growth pattern of shoot or roots in relation to the external sulfate, as indicated by the invariance of the $[SO_4^{2-}]_{crit}$ determined for shoot or root growth under each experimental condition (**Figure 1**; Supplementary Figure S1). On the other hand, long-term exposure to Cd significantly changes the pattern of fresh weight accumulation of the shoot in relation to the external sulfate, and significantly enhances the $[SO_4^{2-}]_{crit}$ maximizing the growth (**Figure 4A**). It is also worthy to note that in this condition increasing in the sulfate external concentration up to reach the $[SO_4^{2-}]_{crit}$ progressively reduces the inhibitory effects exerted by the same concentration of Cd on shoot growth (Supplementary Figure S2A), indicating that – at least in our conditions – plant tolerance to relatively low Cd concentrations is dependent to the S nutritional status, as previously observed in maize seedlings grown under different sulfate availabilities (Nocito et al. 2006). Such a behavior seems to be related to multiple and complex effects induced by the increase of the sulfate concentration in a range of sub-optimal availability for plant growth, which affecting thiol biosynthesis produces deep effects on PC accumulation, Cd partitioning between shoot and roots, and cellular redox state (**Figure 5** and **Figure 6**). In such a scenario, the increase in the root PC levels induced by enhancing sulfate (**Figure 5F**) progressively results in a greater capacity to retain Cd within the roots (**Figure 5H**), and thus reduces the amount of free Cd^{2+} ions that – escaping chelation – is potentially available to be translocated via the xylem in a root-to-shoot direction (Wong and Cobbett 2009; Nocito et al. 2011). The analysis of changes in the shoot Cd concentration in relation to the external sulfate (**Figure 5G**) further supports this conclusion, underlining that the sulfate-induced enhancement in Cd root retention contributes to reduce Cd accumulation and injury in the shoot tissues (**Figure 4A** and **Figure 5G**; Supplementary Figure S2A). Moreover, the increase in the sulfate external concentration progressively reduces the negative effect of Cd on the level of reduced GSH in the shoot (Supplementary Figure S3A), enhancing the cellular capacity to cope with Cd-induced oxidative stress. Such an effect allows the shoot tissues to progressively contrast the oxidative damage exerted by Cd, until to reach the complete recovery at sulfate concentrations higher than $[SO_4^{2-}]_{crit}$, i.e., where the cells of the shoot tissues reached the optimal redox status, as indicated by the values of GSH/GSSG ratio that we assume as indicators of oxidative stress (**Figure 6A**). Conversely, the lack in the roots of a "sulfate-induced recovery" from Cd damages (**Figure 4B**; Supplementary Figure S2B) indicates that the increase in the total GSH levels of the roots induced by long-term exposure to Cd could be not enough to fully sustain Cd detoxification processes and thus to efficiently contrast the redox imbalance produced by Cd in the root tissues (**Figure 5D** and **Figure 6B**).

Taken as a whole the so far discussed behaviors clearly indicate the existence of sulfate-dependent adaptive responses to long-term exposure to Cd which prevent excessive Cd accumulation in the shoot tissues. The efficiency of these mechanisms seems to be related to the concentration of the sulfate ions in the growing medium, since the shoot recovery from Cd stress requires a higher sulfate concentration than that required to maximize shoot growth in the absence of Cd.

Data analysis also reveals that the induction of sulfate uptake is a common adaptive response to both short- and long-term exposure to Cd (**Figure 3A** and **Figure 7A**). In fact, under all the sulfate concentrations analyzed the presence of Cd in the growing medium modulates the well-known effects of the sulfate external concentration on the capacity of the Arabidopsis roots to take-up sulfate (Takahashi et al. 1997; Shibagaki et al. 2002; Rouached et al. 2008; Yoshimoto et al. 2002, 2007). Such a modulation seems to be related to a differential regulation of the transcription of *SULTR1;1* and *SULTR1;2*, the two Arabidopsis genes involved in sulfate uptake by roots (**Figure 3B,C** and **Figure 7B,C**), probably as a consequence of the Cd-induced changes in thiol metabolism and partitioning. However, a careful comparison of the amounts of the NPTs accumulated in each plant, in the presence or absence of Cd, clearly revels the existence of a differential and time-dependent effect of Cd exposure on thiol accumulation, and thus on the nutritional need for S generated by Cd (Supplementary Figure S4). In our experiments, short-term exposure to Cd induces additional sinks for thiols whose strengths are closely dependent on the concentration of the metal in the growing medium (Supplementary Figure S4A), as previously reported by Lancilli et al. (2014). In such a condition, the increase in the relative expression of *SULT1;1* and *SULTR1;2* seems to be due to homeostatic mechanisms driven by the Cd-induced increase in the total NPT levels per plant, since under all the sulfate concentrations analyzed the presence of 0.1, 1 or 10 μM Cd^{2+} positively affected both sulfate transporter gene expression and total NPT levels (**Figure 8A,B**). Conversely, long term-exposure to Cd does not produce additional sink for thiols. In this condition the total amount of NPTs per plant, calculated for each sulfate concentration, was not significantly affected by the presence of 0.1 μM Cd^{2+} (Supplementary Figure S4B), and the relationship between changes in the relative expression of *SULTR1;1* or *SULTR1;2* transcript and the total amount of NPTs per plant appears to be more complex than those described under short-term exposure to Cd (**Figure 8C,D**). Considering the plots in **Figure 8C** and **Figure 8D** we can easily evince that, under the same sulfate concentration, the Cd-induced increase in transcript level of *SULTR1;1* or *SULTR1;2* does not produce any significant changes in the total amount of NPTs per plant. Such a finding indicates that long-term exposure to Cd, even if results in a greater rate of sulfate uptake (**Figure 7A**), negatively affects the capacity of the entire root

apparatus to efficiently absorb the external sulfate at concentration lower than $[SO_4^{2-}]_{crit}$, probably because of the dramatic effect produced by Cd on the pattern of fresh weight accumulation of the roots (**Figure 4B**). In fact, under long term-exposure to Cd the relative growth of the roots was about 52.5% with respect to the control, under all the sulfate concentrations analyzed, whilst the fold change for the potential capacity of the roots to take-up sulfate (measured at saturation) ranged from 0.2 to 1, moving the sulfate external concentration from 1 to 150 µM. From these data we can calculate that Cd-exposed plants became able to absorb the same amount of sulfate than control plants at 56.9 µM external SO_4^{-2}, i.e., where the fold change for the potential capacity of the roots to take-up sulfate reached the value of 0.9 (**Figure 8E**). In these conditions ($[SO_4^{-2}]_{out} \geq 56.9$ µM), the induction of sulfate uptake is potentially able to balance the negative effects of Cd on root growth and then to assure an adequate sulfate amount for optimizing shoot growth and thiol metabolism.

In conclusion our results indicate that long term-exposure to Cd, although induces sulfate uptake, decrees the capacity of the Arabidopsis roots to efficiently use the sulfate ions available in the growing medium to promote the growth. Such a behavior is likely due to an effect exerted by Cd accumulation which – reducing the development of the root apparatus – makes the adaptive response of the high-affinity sulfate transporters "*per se*" not enough to optimize the growth at sulfate external concentrations lower than $[SO_4^{2-}]_{crit}$.

4.5. Figures

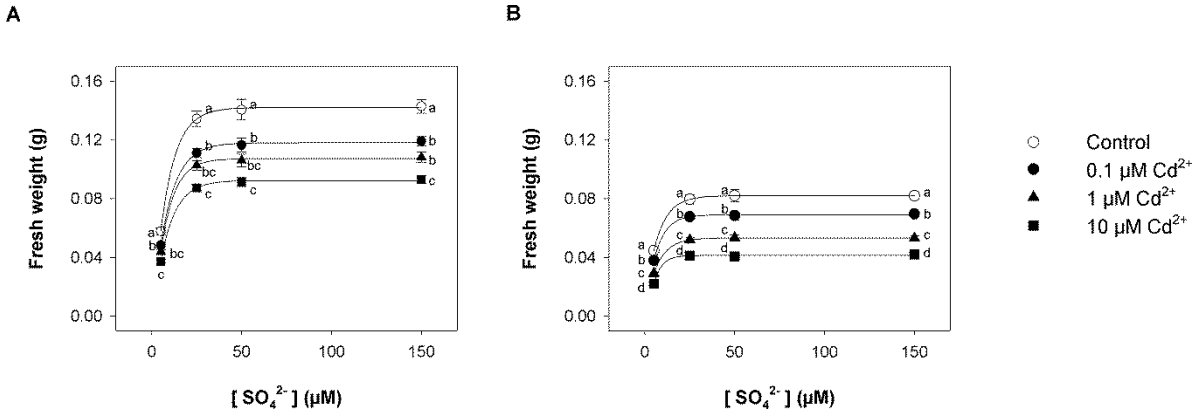

FIGURE 1. Effect of short-term exposure to Cd on shoot and root growth as a function of the sulfate concentration in the external medium. Arabidopsis plants were pre-grown for 19 days under four sulfate concentrations (5, 25, 50 and 150 µM) and then exposed for 72 h to three concentrations of Cd^{2+} (0.1, 1 and 10 µM). **(A)** Characteristic curves describing shoot fresh weight accumulation in relation to the external sulfate. **(B)** Characteristic curves describing root fresh weight accumulation in relation to the external sulfate. Data are means and SE of two experiments run in triplicate ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

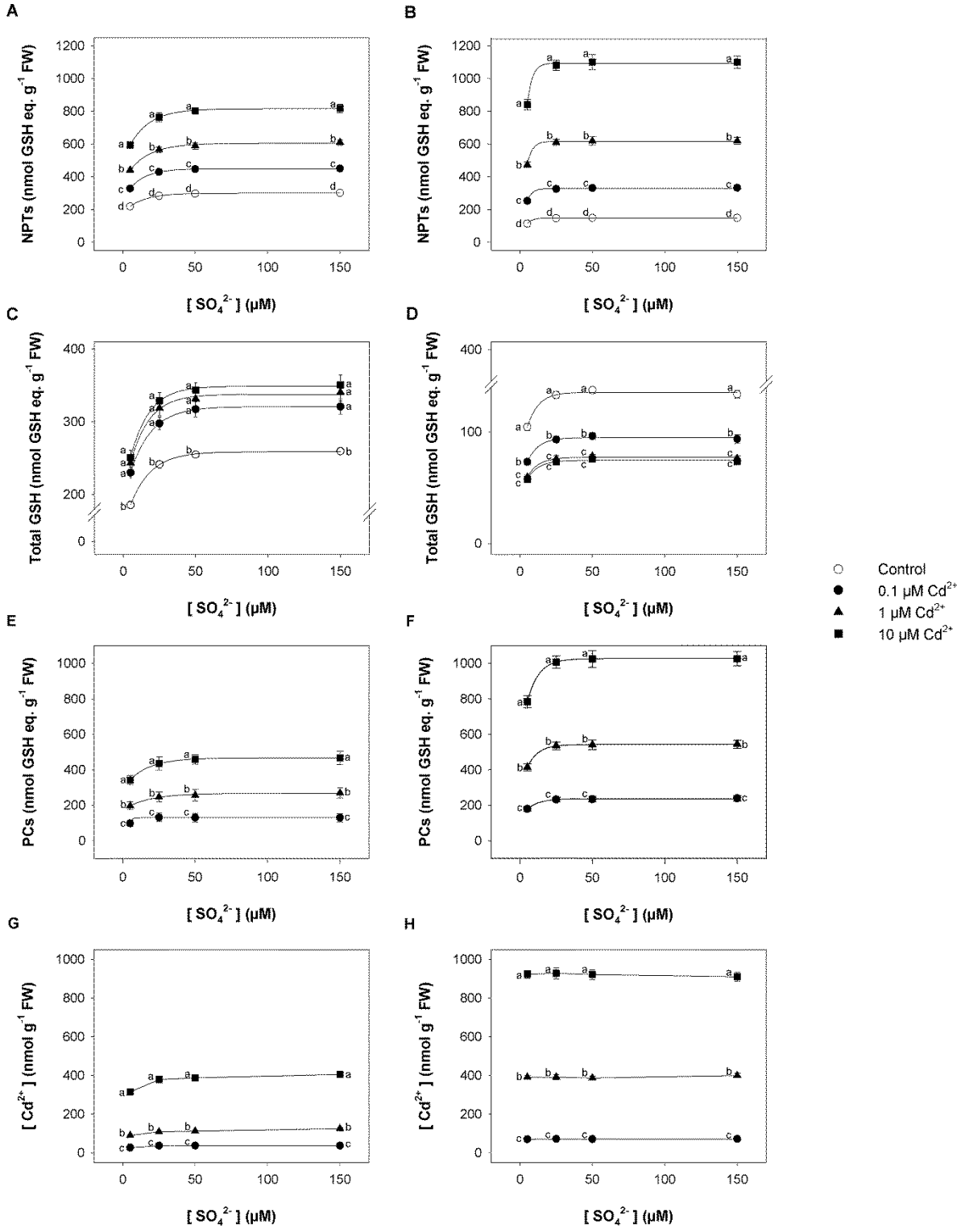

FIGURE 2. Effect of short-term exposure to Cd on thiol and Cd levels in shoot and roots. Arabidopsis plants were pre-grown for 19 days under four sulfate concentrations (5, 25, 50 and 150 µM) and then exposed for 72 h to three concentrations of Cd^{2+} (0.1, 1 and 10 µM). NPT levels in shoot **(A)** and roots **(B)**; total GSH levels in shoot **(C)** and roots **(D)**; PC levels in shoot **(E)** and roots **(F)**; Cd contents in shoot **(G)** and roots **(H)**. Data are means and SE of two experiments run in triplicate ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

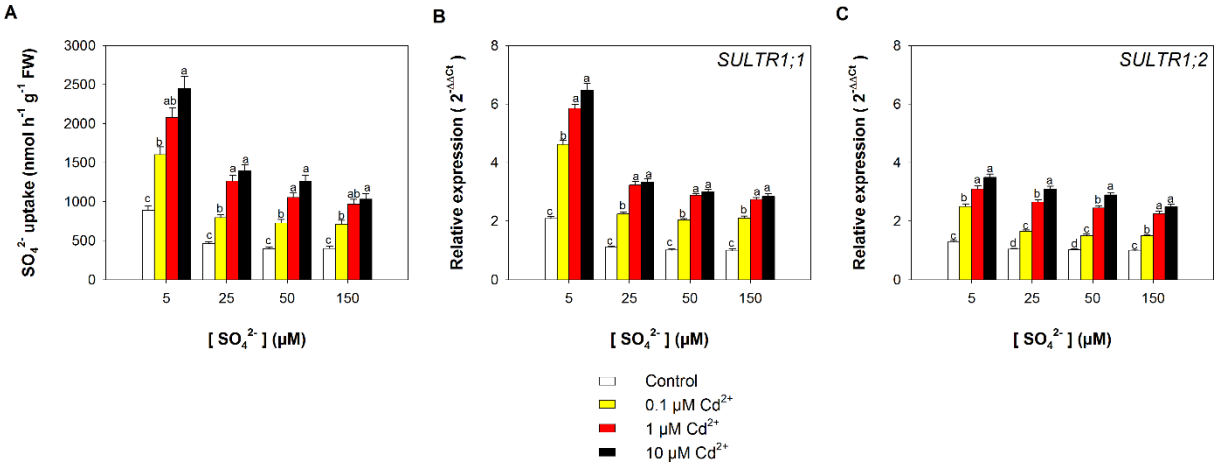

FIGURE 3. Effect of short-term exposure to Cd on the sulfate uptake capacity of the roots. Arabidopsis plants were pre-grown for 19 days under four sulfate concentrations (5, 25, 50 and 150 µM) and then exposed for 72 h to three concentrations of Cd^{2+} (0.1, 1 and 10 µM). **(A)** Sulfate uptake capacity was evaluated by measuring the rate of $^{35}SO_4^{2-}$ absorption into roots of intact plants over a 15 min pulse. The incubation solutions contained 150 µM SO_4^{2-}. **(B,C)** Changes in the relative transcript levels of *SULTR1;1* and *SULTR1;2* in the roots. Bars and error bars are means and SE of two experiments run in triplicate ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

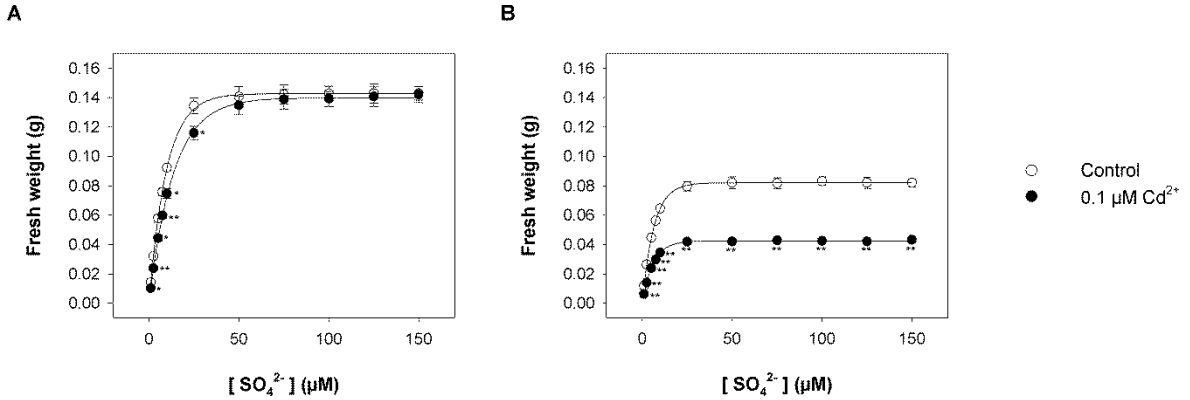

FIGURE 4. Effect of long-term exposure to Cd on shoot and root growth as a function of the sulfate concentration in the external medium. Arabidopsis plants were grown for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM) in the presence or absence of 0.1 µM Cd^{2+}. **(A)** Characteristic curves describing shoot fresh weight accumulation in relation to the external sulfate. **(B)** Characteristic curves describing root fresh weight accumulation in relation to the external sulfate. Data are means and SE of two experiments run in triplicate ($n = 6$). Asterisks indicate significant differences (Student's *t*-test; * $0.001 \leq P < 0.05$; ** $P < 0.001$) between control and Cd-exposed plants grown under the same sulfate external concentration.

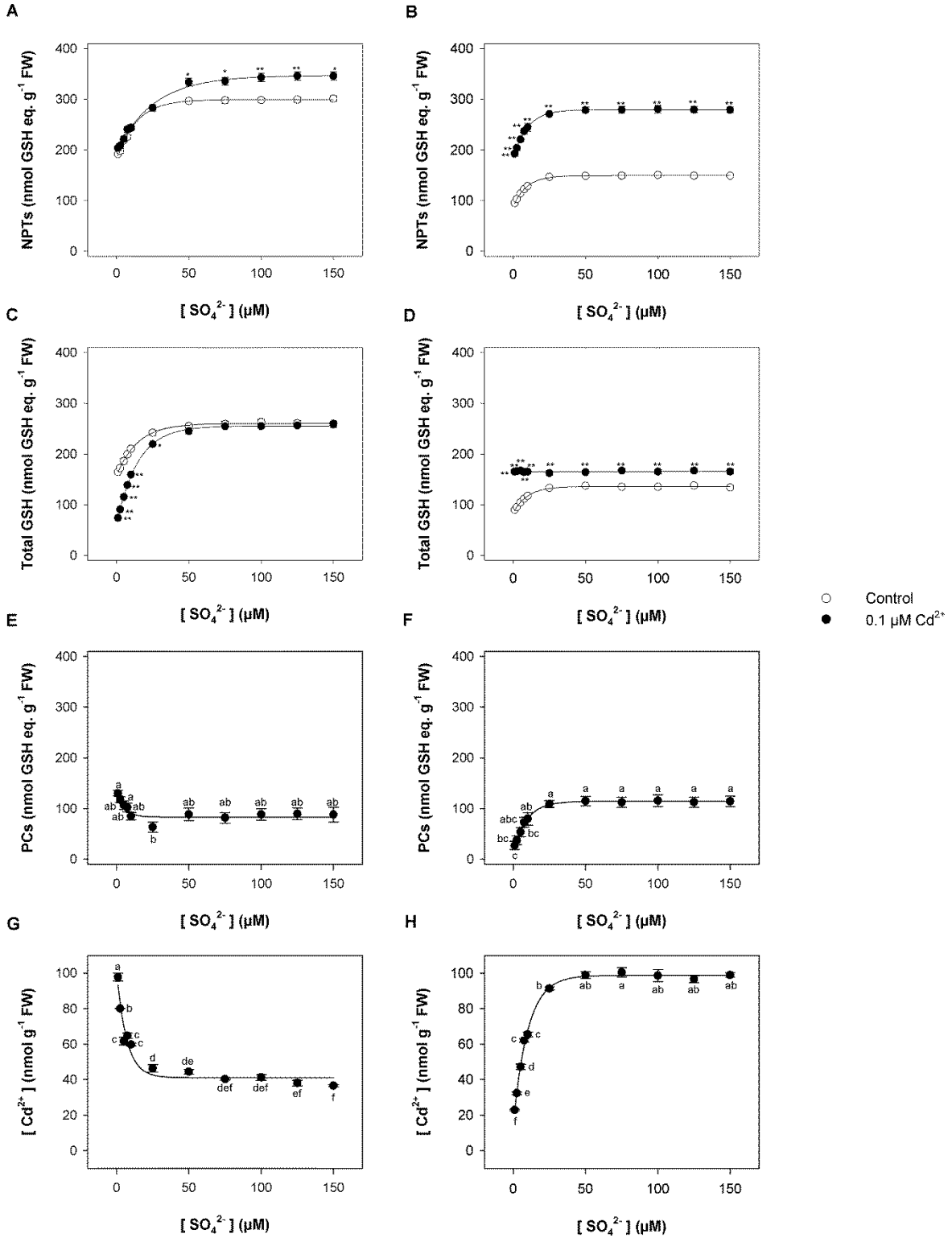

FIGURE 5. Effect of long-term exposure to Cd on thiol and Cd levels in shoot and roots. Arabidopsis plants were grown for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM) in the presence or absence of 0.1 µM Cd^{2+}. NPT levels in shoot **(A)** and roots **(B)**; total GSH levels in shoot **(C)** and roots **(D)**; PC levels in shoot **(E)** and roots **(F)**; Cd contents in shoot **(G)** and roots **(H)**. Data are means and SE of two experiments run in triplicate ($n = 6$). Different letters indicate significant differences ($P < 0.05$). Asterisks indicate significant differences (Student's *t*-test; * $0.001 \leq P < 0.05$; ** $P < 0.001$) between control and Cd-exposed plants grown under the same sulfate external concentration.

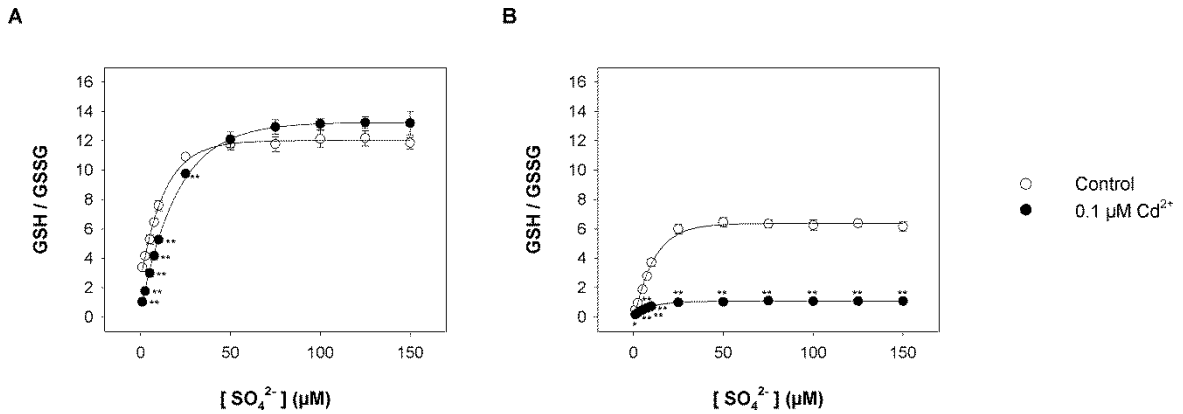

FIGURE 6. Effect of long-term exposure to Cd on the GSH/GSSG ratio in shoot (A) and roots (B). Arabidopsis plants were grown for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM) in the presence or absence of 0.1 µM Cd^{2+}. The GSH/GSSG ratios were calculated using data about reduced GSH and GSSG reported in Supplementary Figure S3. Data are means and SE of two experiments run in triplicate ($n = 6$). Asterisks indicate significant differences (Student's *t*-test; * $0.001 \leq P < 0.05$; ** $P < 0.001$) between control and Cd-exposed plants grown under the same sulfate external concentration.

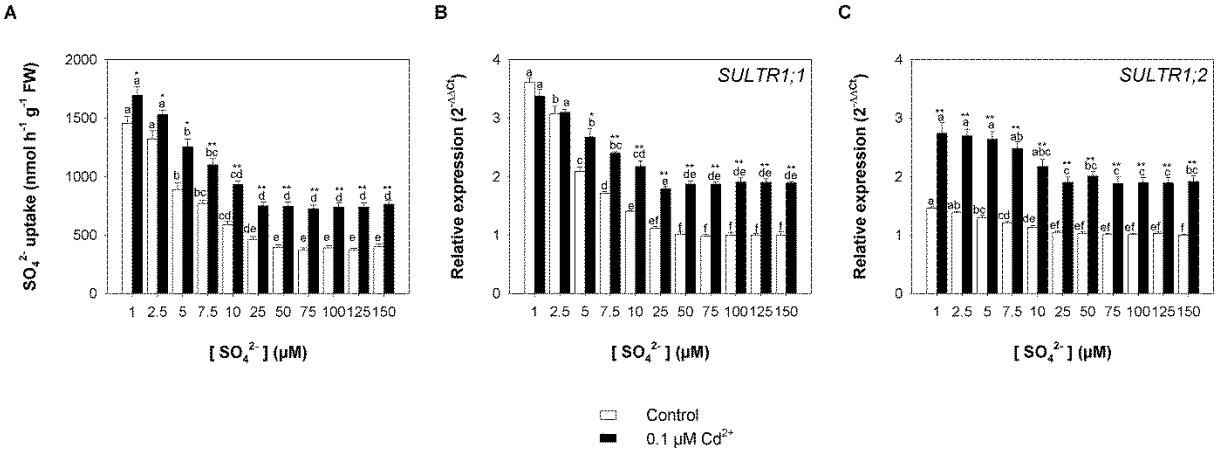

FIGURE 7. Effect of long-term exposure to Cd on the sulfate uptake capacity of the roots. Arabidopsis plants were grown for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM) in the presence or absence of 0.1 µM Cd^{2+}. **(A)** Sulfate uptake capacity was evaluated by measuring the rate of $^{35}SO_4^{2-}$ absorption into roots of intact plants over a 15 min pulse. The incubation solutions contained 150 µM SO_4^{2-}. **(B,C)** Changes in the relative transcript levels of *SULTR1;1* and *SULTR1;2* in the roots. Bars and error bars are means and SE of two experiments run in triplicate ($n = 6$). Different letters indicate significant differences ($P < 0.05$). Asterisks indicate significant differences (Student's *t*-test; * $0.001 \leq P < 0.05$; ** $P < 0.001$) between control and Cd-exposed plants grown under the same sulfate external concentration.

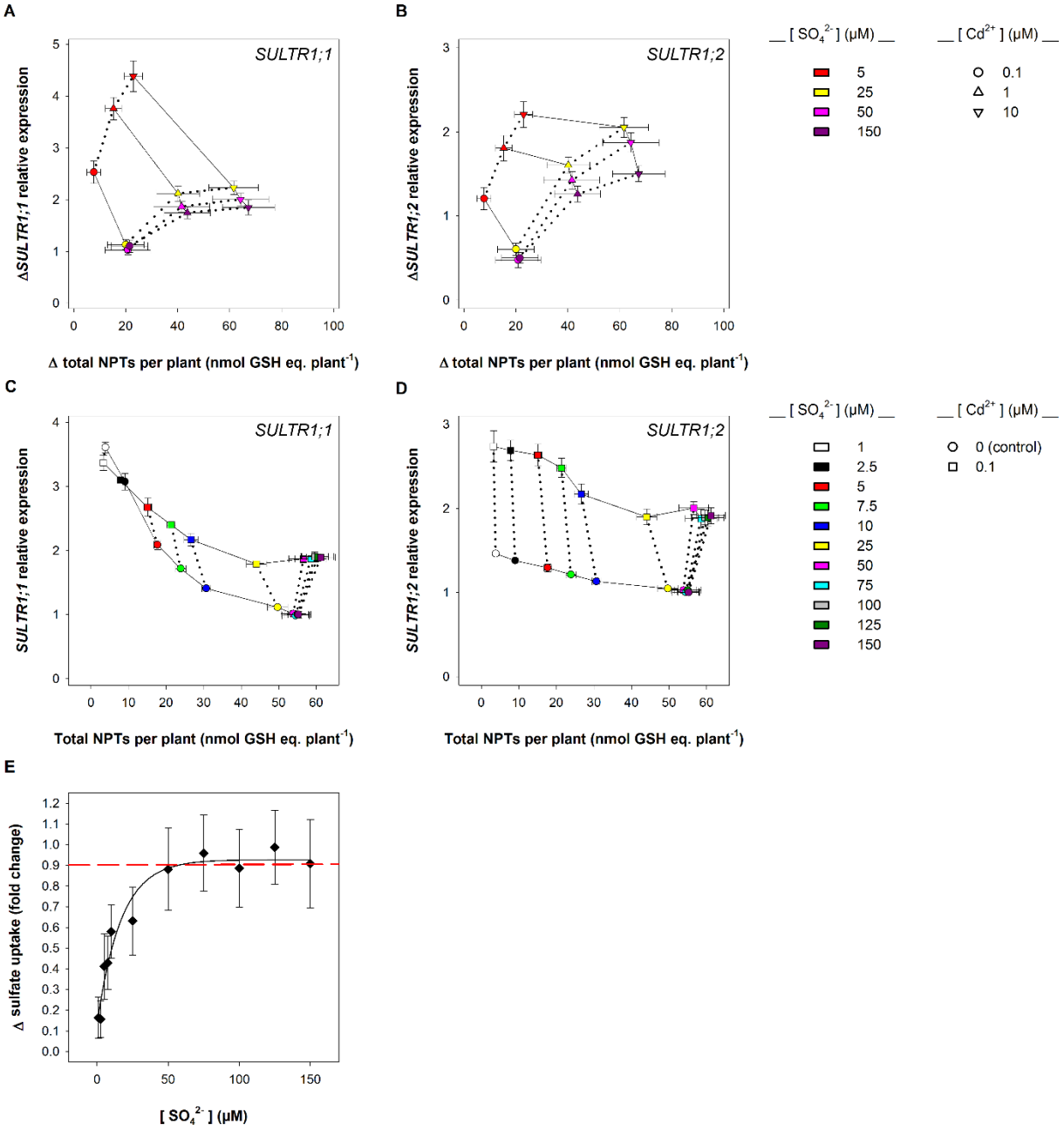

FIGURE 8. Analysis of the relationships between the expression of the sulfate transporter genes (*SULTR1;1* and *SULTR1;2*) and the total NPT levels per plant. The relationships between the relative expression of *SULTR1;1* or *SULTR1;2* transcript and the total amount of NPTs per plant were evinced using data reported in Figures 3, 7, S4. **(A,B)** Changes in the relative expression of *SULTR1;1* or *SULTR1;2* transcript vs changes in the total amount of NPTs per plant under short-term exposure to Cd. Solid lines link data about plants exposed to the same Cd concentration (circles, 0.1 μM Cd^{2+}; triangles up, 1 μM Cd^{2+}; triangles down, 10 μM Cd^{2+}). Dotted lines link data about plants grown under the same sulfate concentration. **(C,D)** Relative expression of *SULTR1;1* or *SULTR1;2* transcript vs total amount of NPTs per plant under long-term exposure to Cd. Solid lines link data about plants grown under different sulfate concentrations in the absence (circles) or presence of 0.1 μM Cd^{2+} (squares). Dotted lines link data about plants grown under the same sulfate concentration. **(E)** Cd-induced changes in the potential capacity of the roots to take up sulfate as a function of the sulfate external concentration. The analyses was performed using data reported in Figure 7A. The red line indicates the threshold over which sulfate uptake is potentially able to balance the negative effects of Cd on root growth and then to assure an adequate sulfate amount for optimizing shoot growth and thiol metabolism. Data reported in each plot are means and SE of two experiments run in triplicate ($n = 6$).

4.6. Supplementary materials

Gene	Primer name	Sequence
SULTR1;1 (At4g08620)	Sultr1;1for	GCCATCACAATCGCTCTCCAA
	Sultr1;1rev	TTGCCAATTCCACCCATGC
SULTR1;2 (At1g78000)	Sultr1;2for	GGATCCAGAGATGGCTACATGA
	Sultr1;2rev	TCGATGTCCGTAACAGGTGAC
S16 (At4g34620)	S16for	CGCCGATCGAGCTTTATCAG
	S16rev	CACCAGGACCACCAAACTTCTT

TABLE S1. Primers used for qRT-PCR analysis.

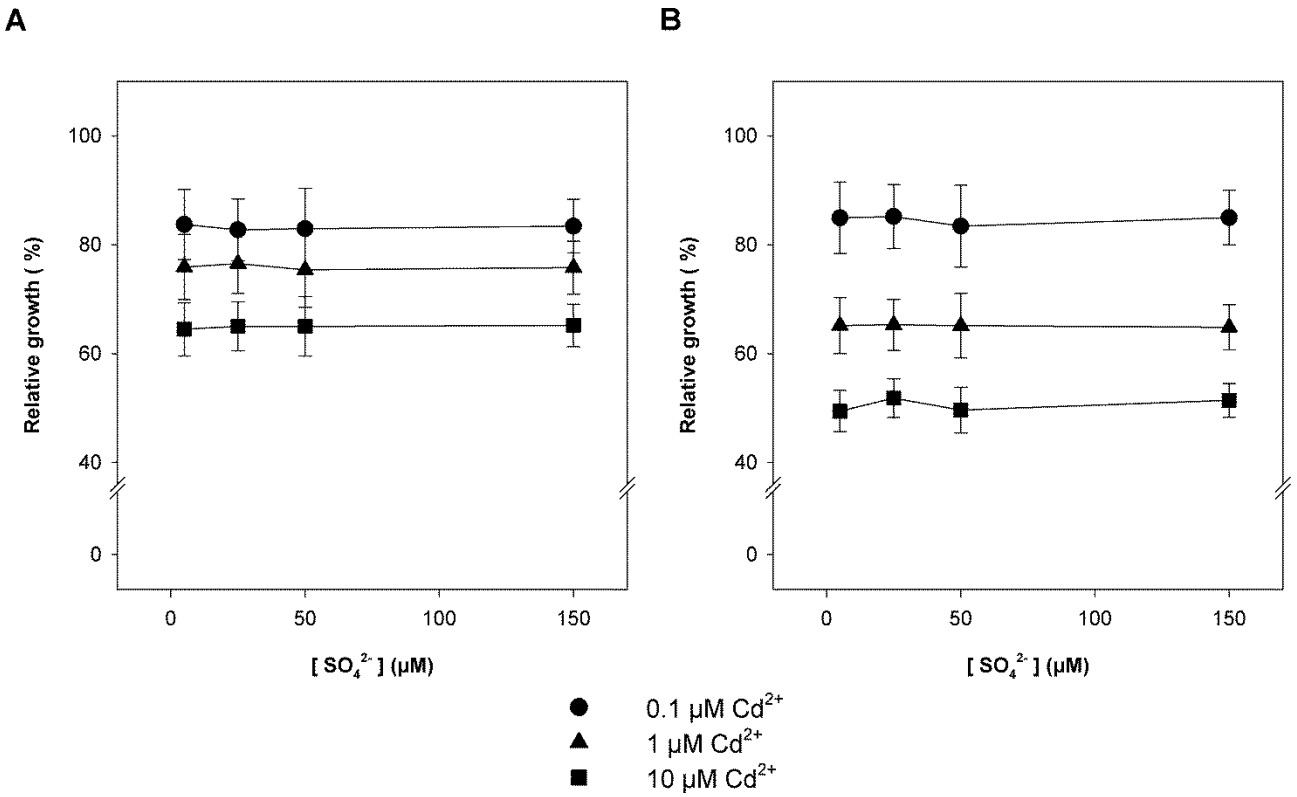

FIGURE S1. Shoot (A) and root (B) relative growth under short-term exposure to Cd. Arabidopsis plants were pre-grown for 19 days under four sulfate concentrations (5, 25, 50 and 150 µM) and then exposed for 72 h to three concentrations of Cd^{2+} (0.1, 1 and 10 µM). Relative growths for shoot and roots were calculated using data reported in Figure 1, by normalizing the growth of Cd-exposed plants with respect to the control. Data are means and SE of two experiments run in triplicate ($n = 6$).

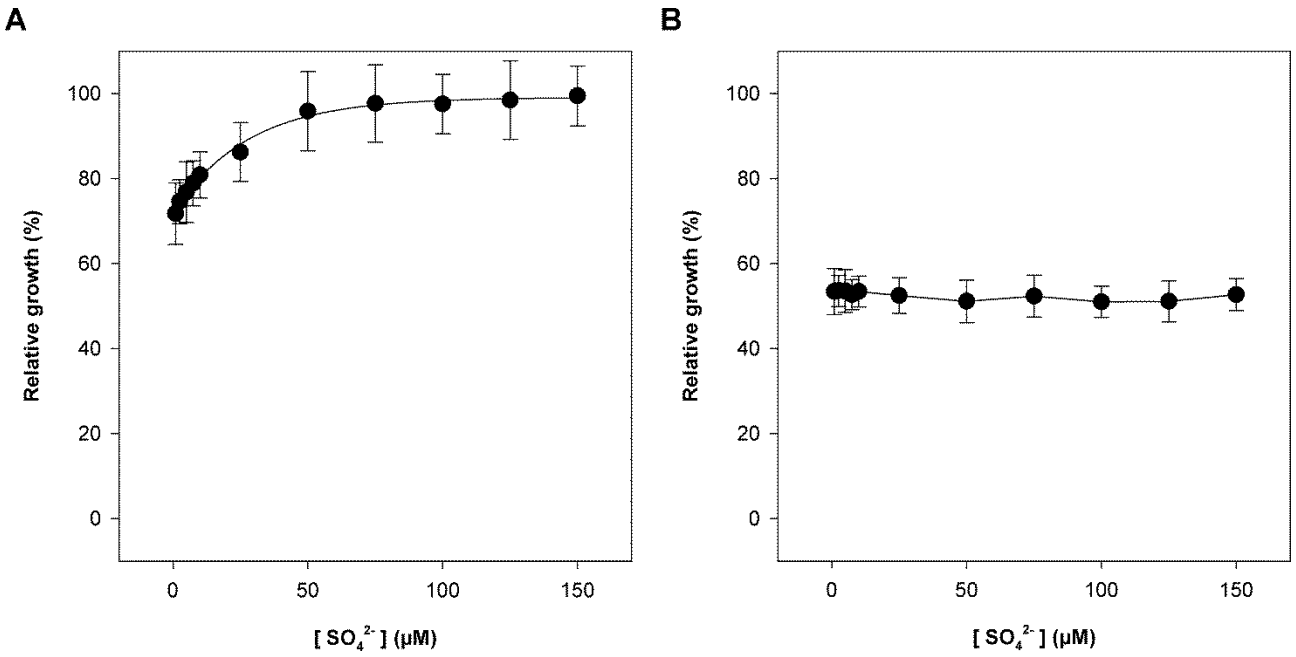

FIGURE S2. Shoot (A) and root (B) relative growth under long-term exposure to Cd. Arabidopsis plants were grown for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM) in the presence or absence of 0.1 µM Cd^{2+}. Relative growths for shoot and roots were calculated using data reported in Figure 4, by normalizing the growth of Cd-exposed plants with respect to the control. Data are means and SE of two experiments run in triplicate ($n = 6$).

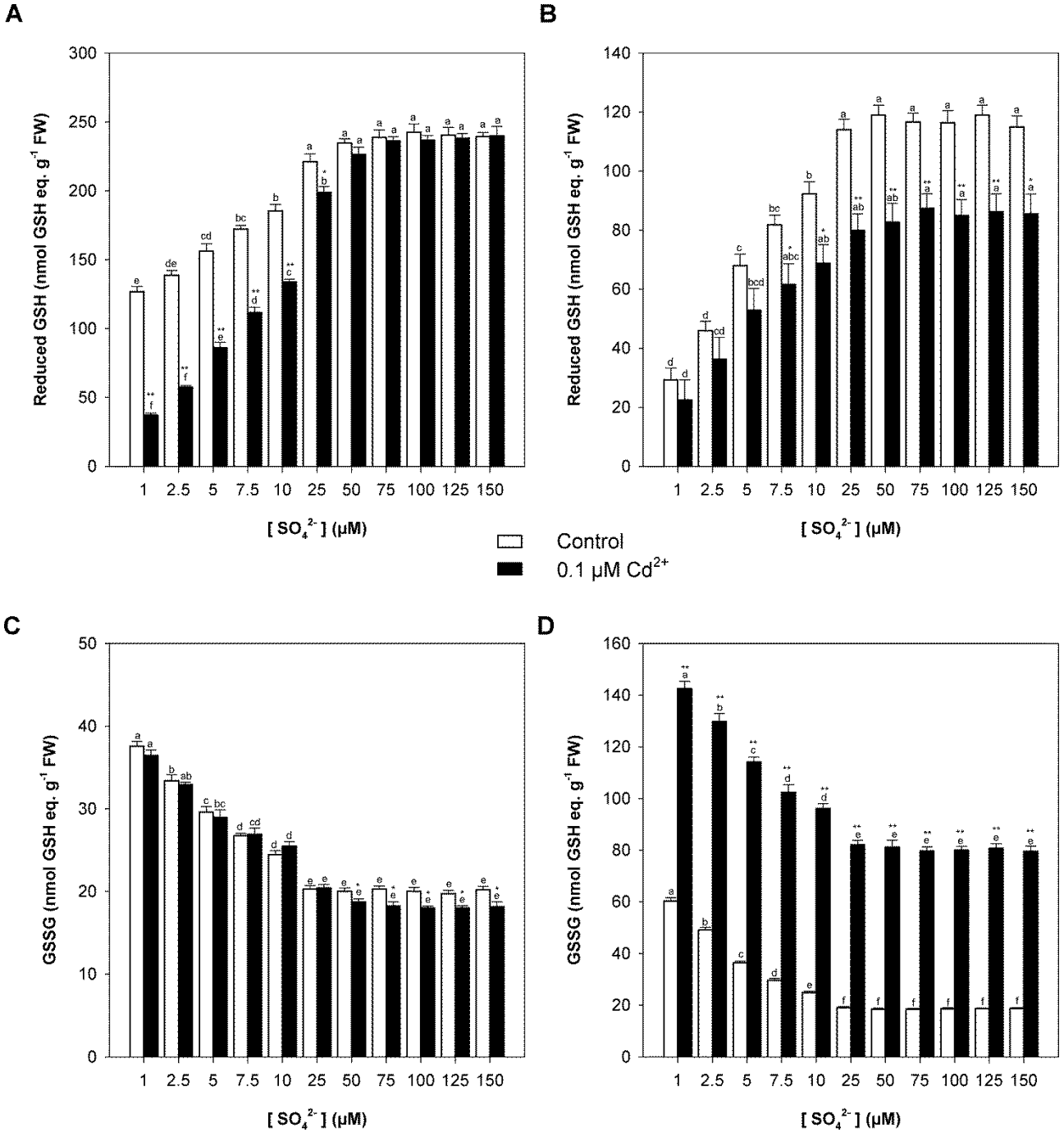

FIGURE S3. Effect of long-term exposure to Cd on reduced GSH and GSSG levels in shoot and roots. Arabidopsis plants were grown for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM) in the presence or absence of 0.1 µM Cd^{2+}. Reduced GSH levels in shoot **(A)** and roots **(B)**; GSSG levels in shoot **(C)** and roots **(D)**. Bars and error bars are means and SE of two experiments run in triplicate ($n = 6$). Different letters indicate significant differences ($P < 0.05$). Asterisks indicate significant differences (Student's *t*-test; * $0.001 \leq P < 0.05$; ** $P < 0.001$) between control and Cd-exposed plants grown under the same sulfate external concentration.

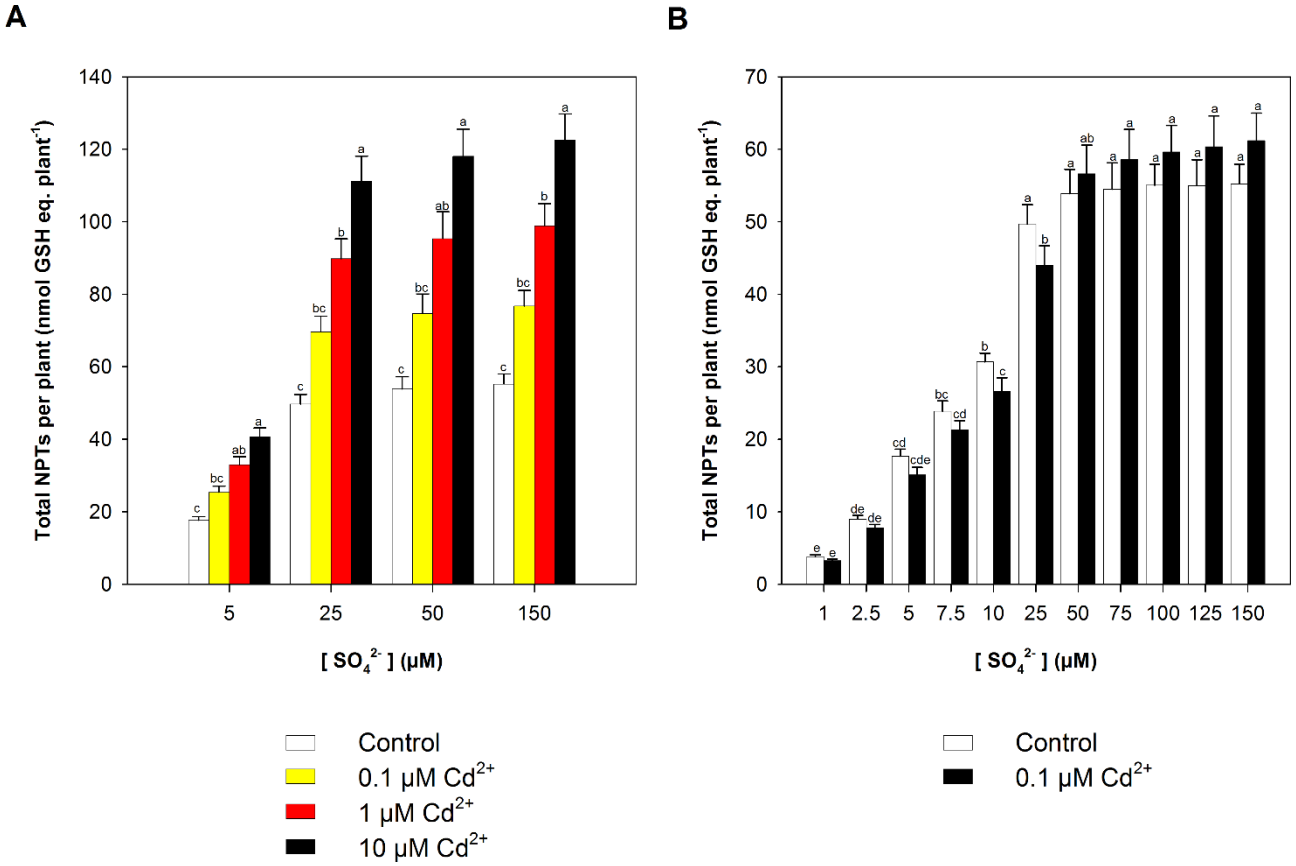

FIGURE S4. Effect of short- (A) and long-term (B) exposure to Cd on the total NPT levels per plant. For the short-term exposure to Cd, Arabidopsis plants were pre-grown for 19 days under four sulfate concentrations (5, 25, 50 and 150 µM) and then exposed for 72 h to three concentrations of Cd^{2+} (0.1, 1 and 10 µM). For the long-term exposure to Cd, Arabidopsis plants were grown for 22 days under a wide range of sulfate concentrations (1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 125 and 150 µM) in the presence or absence of 0.1 µM Cd^{2+}. The total NPT levels per plant were calculated using data reported in Figures 1, 2(A,B), 4, 5(A,B). Bars and error bars are means and SE of two experiments run in triplicate ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

5. CONCLUSIONS AND REMARKS IN A NUTSHELL

The main results obtained during my PhD thesis clearly indicate that the capacity of plant tissues to maintain glutathione homeostasis under cadmium stress may strongly affect phytochelatin accumulation and, thus, cadmium tolerance and translocation. Moreover, such a capacity seems to be related to the total amount of sulfur available for plant nutrition in the growing medium, since adequate levels of sulfate modulate thiol metabolism and partitioning, reducing the negative effects produced by cadmium accumulation in the shoot. Finally, these results confirm the central role of sulfur metabolism in the mechanisms involved in cadmium detoxification and suggest that the manipulation of both sulfate transport and thiol metabolism may represent a useful strategy for the selection of low cadmium-accumulating cultivars or to enhance plant performances in cadmium-contaminated soils.

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