Heavy Metal Stress and Sulfate Uptake in Maize Roots

Fabio F. Nocito, Clarissa Lancilli, Barbara Crema, Pierre Fourcroy, Jean-Claude Davidian, and Gian Attilio Sacchi*

Dipartimento di Produzione Vegetale, Università degli Studi di Milano, 20133 Milan, Italy (F.F.N., C.L., B.C., G.A.S.); and Biochimie and Physiologie Moléculaire des Plantes, Institut National de la Recherche Agronomique (Unité 386), Centre National de la Recherche Scientifique (Unité Mixte de Recherche 5004), Ecole Nationale Supérieure Agronomique de Montpellier, Université Montpellier 2, 34060 Montpellier, cedex 1, France (P.F., J.-C.D.)

Agronomique de Montpellier, Université Montpellier 2, 34060 Montpellier, cedex 1, France (P.F., J.-C.D.)

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ZmST1;1, a putative high-affinity sulfate transporter gene expressed in maize (Zea mays) roots, was functionally characterized and its expression patterns were analyzed in roots of plants exposed to different heavy metals (Cd, Zn, and Cu) interfering with thiol metabolism. The ZmST1;1 cDNA was expressed in the yeast (Saccharomyces cerevisiae) sulfate transporter mutant CP154-7A. Kinetic analysis of sulfate uptake isotherm, determined on complemented yeast cells, revealed that ZmST1;1 has a high affinity for sulfate (Km value of 14.6 ± 0.4 µM). Cd, Zn, and Cu exposure increased both ZmST1;1 expression and root sulfate uptake capacity. The metal-induced sulfate uptakes were accompanied by deep alterations in both thiol metabolism and levels of compounds such as reduced glutathione (GSH), probably involved as signals in sulfate uptake modulation. Cd and Zn exposure strongly increased the level of nonprotein thiols of the roots, indicating the induction of additional sinks for reduced sulfur, but differently affected root GSH contents that decreased or increased following Cd or Zn stress, respectively. Moreover, during Cd stress a clear relation between the ZmST1;1 mRNA abundance increment and the entity of the GSH decrement was impossible to evince. Conversely, Cu stress did not affect nonprotein thiol levels, but resulted in a deep contraction of GSH pools. Our data suggest that during heavy metal stress sulfate uptake by roots may be controlled by both GSH-dependent or -independent signaling pathways. Finally, some evidence suggesting that root sulfate availability in Cd-stressed plants may limit GSH biosynthesis and thus Cd tolerance are discussed.

To minimize the detrimental effects of heavy metal accumulation, plants have evolved detoxification mechanisms, mainly based on chelation and subcellular compartmentalization (Clemens, 2001). The efficiency of these processes might result in the natural heavy metals tolerance and their basic understanding might be crucial for improving plant performances in phytoextraction of heavy metals from polluted soils (Salt et al., 1998; Pilon-Smits, 2005).

Chelation of heavy metals is a ubiquitous detoxification strategy described in a wide variety of plants (Zenk, 1996; Clemens, 2001). One of the principal classes of heavy metal chelators known in plants is phytochelatins (PCs), a family of Cys-rich peptides with the general structure $(\gamma$-Glu-Cys)$_n$-Gly $(n = 2–11)$. PCs are synthesized nontranslationally from reduced glutathione (GSH) in a transpeptidation reaction catalyzed by the enzyme PC synthase (PCS; Rea et al., 2004). Their synthesis is induced within minutes following exposure to different metals or metalloids; among these, Cd is the strongest inducer, whereas other metals such as Cu, Zn, Pb, and Ni are less effective and require higher external levels for induction (Grill et al., 1987; Maitani et al., 1996). Interestingly, some inducers, such as Zn, do not seem to serve as substrates for chelation (Clemens, 2001; Souza and Rauser, 2003).

The mechanism of heavy metal detoxification based on PC biosynthesis is well described in the case of Cd; the isolation of the Arabidopsis (Arabidopsis thaliana) cad1 mutant, which is defective in PCS activity and consequently Cd hypersensitive, has underlined the significance of this mechanism for plant survival in polluted soils (Howden et al., 1995).

It has been shown that Cd exposure and accumulation induce a transient depletion of GSH, which is used as substrate for a massive PC production (Tukendorf and Rauser, 1990). In this condition PCs rapidly become the most abundant class of nonprotein thiols (NPTs) in tissues, increasing the need for both Cys and GSH and thus the total sulfur request for plant growth, as indicated by the general alteration of the sulfur metabolic pathways induced by Cd (Tukendorf and Rauser, 1990; Schäfer et al., 1998; Heiss et al., 1999; Nocito et al., 2002). In fact, the genesis of additional sink for reduced sulfur, arising from PC biosynthesis, triggers a typical cell response, probably due to GSH depletion, which involves a demand-driven coordinate transcriptional regulation of genes for sulfate uptake and assimilation, and GSH biosynthesis (Schäfer et al., 1998; Heiss et al., 1999;...
RESULTS

Cloning and Functional Characterization of ZmST1;1

The ZmST1;1 (accession no. AF355602) coding sequence was amplified by reverse transcription (RT)-PCR from total RNA isolated from maize roots. Sequence analysis confirmed the identity of the cloned PCR product as the cDNA encoding the putative high-affinity sulfate transporter described by Hopkins et al. (2004) and previously named ZmST1-701, HAST, or ZmST1 (Bolchi et al., 1999; Nocito et al., 2002; Quaggiani et al., 2003).

The expression of ZmST1;1 in the yeast (Saccharomyces cerevisiae) double sulfate transporter mutant CP154-7A (Cherest et al., 1997) was able to revert the yeast mutant phenotype, allowing it to grow on a minimal medium containing 0.1 mM Na₂SO₄ as a sole sulfur source (Fig. 1). To further confirm the identity of the ZmST1;1 encoded polypeptide as sulfate transporter and to determine its affinity for sulfate, we performed sulfate uptake assays on complemented yeast cells. In Figure 2 the uptake of sulfate is reported as function of sulfate external concentration in the range 1 to 50 μM. Least squares fitting reveals that the resulting uptake isotherm can be properly described by a simple hyperbolic Michaelis-Menten function, allowing to calculate an apparent Kₘ for sulfate of 14.6 ± 0.4 μM.

Effect of Different Cd Concentrations on Thiol Compounds, Sulfate Uptake Capacity, and ZmST1;1 Transcript Levels in Maize Roots

To further investigate the role of GSH as putative signal involved in modulating sulfate uptake during Cd stress, we performed experiments on maize plants grown for 48 h in complete nutrient solutions in the absence (0 μM) or presence of CdCl₂ at different concentrations (1, 5, or 10 μM).

The analyses of thiol compounds showed that the NPT contents of the roots, expressed as GSH equivalents (Fig. 3A), progressively increased up to 5.5-fold with respect to the control, while at the same time a significant

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Figure 1. Phenotypic complementation of the yeast double sulfate transporter mutant CP154-7A by ZmST1;1. Yeast mutant cells expressing ZmST1;1 under the control of the inducible GAL10 promoter or harboring the empty pESC-TRP vector were grown at 28°C for 3 d on a minus-sulfur medium (−S) or on minimal media containing 0.1 mM sulfate (SO₄²⁻) or 0.1 mM homo-Cys (HCys) as sole sulfur sources.
contraction of total glutathione pools (reduced + oxidized form) of the root (Fig. 3B) was observed under all Cd-exposure conditions (about 50% as compared to the control). Moreover, Cd exposure significantly decreased the GSH pools but at the same time slightly increased the levels of glutathione disulfide (GSSG), affecting thus the GSH/GSSG ratio that in Cd-stressed roots had lower results than in the control (Fig. 3B). Northern-blot analysis, carried out on total RNA extracted from roots revealed that, upon Cd treatment, the ZmST1;1 transcript relative abundance significantly increased in relation to Cd concentration in the nutrient solution (Fig. 3C). Such a last response was positively correlated to an enhancement in the sulfate uptake capacity of the roots (Fig. 3D). The rate of 35S-sulfate uptake, measured at 0.2 mM SO4 \(^{2-}\) external concentration, increased up to 1.6-fold with respect to the control at the highest Cd external concentration (10 \(\mu\)M).

Cd Stress Versus Sulfate Deprivation

Since the above-reported data resulted from a long-term equilibrium between metabolite levels and gene expression, to better understand the relationship existing between Cd stress, sulfate transport, and thiol metabolism, we performed experiments aimed at comparing the short-term (\(\leq 12\) h) effects of Cd exposure with that of sulfate deprivation. In fact, like Cd exposure, sulfate starvation has been shown to modulate the levels of both ZmST1;1 transcript and thiol compounds (Bolchi et al., 1999; Nocito et al., 2002; Quaggiotti et al., 2003; Hopkins et al., 2004). To this purpose maize plants were grown for 3, 6, or 12 h in complete nutrient solutions, supplemented or not with 10 \(\mu\)M CdCl2, or in a minus-sulfate solution. As expected, both conditions were able to enhance the ZmST1;1 transcript abundance in the root, although with different time courses. In fact, following Cd exposure the ZmST1;1 mRNA levels started increasing over time already after 3 h of treatment, while the...
Effect of Zn and Cu on Thiol Compounds, Sulfate Uptake Capacity, and ZmST1;1 Transcript Levels in Maize Roots

The relationship between sulfur metabolism and ZmST1;1 expression pattern was also investigated in the roots of maize plants exposed to other heavy metals, Zn and Cu, which have been previously shown as affecting thiol metabolism and/or PC biosynthesis (Tukendorf, 1996; Schäfer et al., 1997; Xiang and Oliver, 1998; Souza and Rauser, 2003).

The analysis of thiol compounds of the root revealed that Zn and Cu had different effects on NPTs, GSH, and GSSG. In fact, compared to the control, the NPT levels dramatically increased following Zn exposure (+257% and +361% at 100 and 250 μM ZnCl₂, respectively) but were not affected by Cu (Fig. 5A). Moreover, a significant effect on total glutathione was evident only in the roots of plants exposed to 250 μM ZnCl₂ (+31% with respect to the control). Conversely, the GSH/GSSG ratio was significantly affected only by Cu exposure, which moved its value from 4.3 (control) to 0.2 (10 μM CuCl₂). Such a behavior was related to a dramatic decrease in the GSH level of the roots (Fig. 5B).

Northern-blot analysis showed that both heavy metals modulated the ZmST1;1 transcript levels. Following 48 h of ZnCl₂ (100 and 250 μM) or CuCl₂ (10 μM) exposure the ZmST1;1 mRNA relative abundances were significantly higher in the roots of Zn- and, although to a lesser extent, Cu-treated plants than in those of the controls (Fig. 5C). Also in these cases, the root sulfate uptake capacity was modulated by the heavy metal exposure. In the presence of Zn the rate of sulfate uptake by roots had significantly higher results (+42% and +71% at 100 and 250 μM ZnCl₂, respectively) than in the control (Fig. 5D); a similar effect was detected in Cu-treated roots, in which sulfate uptake was increased (+37%) by the presence of the heavy metal in the nutrient solution (Fig. 5D).

Sulfate Availability and Cd Tolerance

To establish whether Cd tolerance may be affected by the sulfate contents of the roots, we performed experiments on maize plants pregrown for 72 h at different sulfate concentrations (0, 0.2, and 2 mM) and then exposed for 48 h to 10 μM CdCl₂ in a complete nutrient solution containing 0.2 mM SO₄²⁻. The 72 h pregrowing period allowed us to modulate the total amount of sulfate stored in root tissues, which increased with sulfate concentration in the pregrowing solution (Table II). Table II also reports the effect of Cd on the relative growth of both shoots and roots, calculated referring to the growth of control plants not

Figure 4. Northern-blot analysis of ZmST1;1 expression in maize roots in response to sulfate deprivation or Cd exposure. Seedlings grown for 3 d in complete nutrient solutions were transferred and grown for additional 3, 6, and 12 h periods in complete nutrient solutions supplemented or not with 10 μM CdCl₂ or in a minus-sulfate solution. Total RNA was extracted from roots of control (C), sulfate deprived (−S), and Cd-exposed (Cd) plants. Thirty micrograms of total RNA were loaded onto each lane. Blots were hybridized with 32P-labeled ZmST1;1 probe. Ribosomal RNAs were stained on the gel with ethidium bromide (Et-Br) and used to check loading.
exposed to the heavy metal. Plant relative growth was closely dependent on the sulfate concentration in the pregrowing solution. In fact, the inhibitory effect exerted by Cd on plant growth diminished as sulfate external concentration increased; the growth of both shoots and roots of plants pregrown in the solution containing 2 mM SO$_4$$^{2-}$ was not affected by Cd exposure. The concentration of Cd in shoot and root tissues, measured at the end of the Cd-exposure period, was not influenced by the pregrowth at different sulfate concentrations (Table II). However, the amount of Cd removed by the plants from the nutrient solution, calculated considering the total biomass and the tissue Cd concentrations, increased as the sulfate concentration of the pregrowing solution increased (Table II). The plants pregrown in the solution containing 2 mM SO$_4$$^{2-}$ were more efficient in Cd extraction (0.77 ± 0.02 μmol plant$^{-1}$) than those pregrown in the solutions containing 0.2 mM or no sulfate (0.50 ± 0.02 and 0.38 ± 0.01 μmol plant$^{-1}$, respectively).

The pregrowth treatment influenced the total glutathione content of the roots, which increased with sulfate availability in the pregrowing solution (Fig. 6). Following Cd exposure, the total glutathione levels decreased in all conditions analyzed. However, at the end of the 48 h Cd-exposure period, a similar relation was still observed between the total glutathione levels of the roots and the sulfate concentration in the pregrowing solution (Fig. 6).

DISCUSSION

In a previous work we hypothesized that sulfate transporter genes are involved in Cd-detoxification mechanisms based on PCs (Nocito et al., 2002) and closely dependent on the sulfate-assimilatory and GSH-biosynthesis pathways (Schäfer et al., 1998; Heiss et al., 1999; Lee and Leustek, 1999; Saito, 2004). In particular, we showed that Cd exposure, as well as sulfate deprivation, increases the sulfate uptake capacity of maize roots mainly by up-regulating the activity of HAST, a gene encoding a putative root-expressed high-affinity sulfate transporter, previously named ZmST1-701 and further indicated as ZmST1 (Bolchi et al., 1999; Nocito et al., 2002; Quaggiotti et al., 2003); the same gene was more recently studied by Hopkins et al. (2004), that properly proposed to call it ZmST1;1 (accession no. AF355602). The polypeptide encoded by ZmST1;1 has a significant similarity with the high-affinity H$^+$-dependant sulfate transporters belonging to the group 1 (Hopkins et al., 2004). It also shares with these members similar spatial distribution, being mainly expressed in the root epidermis and in the cell layer surrounding the central vascular bundle (Rae and Smith, 2002; Howarth et al., 2003; Hopkins et al., 2004). With this work we contribute to the integration of the picture painted by Hopkins et al. (2004) providing a kinetic characterization of this maize transporter by its expression in a heterologous system. The expression of the ZmST1;1 complete coding sequence in the yeast mutant strain CP154-7A, defective in its two sulfate transporters and thus unable to grow on media containing low concentrations of sulfate as sole sulfur source (Cherest et al., 1997), proved the capacity of ZmST1;1 to complement this mutant and to transport sulfate (Fig. 1). Kinetic analysis of the sulfate uptake isotherm obtained in a range of low sulfate external concentrations (1–50 μM; Fig. 2), revealed that ZmST1;1 has a high affinity for sulfate, with an apparent $K_m$ value (14.6 ± 0.4 μM) similar to those of other plant high-affinity sulfate transporters (Smith et al., 1995; Smith et al., 1997; Takahashi et al., 2000; Vidmar et al., 2000; Yoshimoto et al., 2002; Howarth et al., 2003). Taken as a whole, our findings suggest a role of ZmST1;1 in mediating the root high-affinity sulfate uptake from the soil solution.

The activity of sulfate transporters is transcriptionally regulated by signals reflecting the nutritional status of the plants (Hawkesford, 2000; Smith, 2001; Maruyama-Nakashita et al., 2004). In the regulation model proposed, some metabolites along the pathways of sulfate reductive assimilation and GSH biosynthesis, such as Cys and GSH, may act as signals...
in controlling sulfate transporter gene expression (Lappartient et al., 1999). An adequate level of reduced sulfur compounds would repress the expression of sulfate transporters through a negative feedback loop; vice versa, a contraction of the pools of these compounds would derepress sulfate transporter gene transcription (Hawkesford, 2000; Hawkesford and Wray, 2000). Such reversible down-regulation would result in a fine adaptation of sulfate fluxes suitable for plant survival in a wide range of environmental conditions (Maruyama-Nakashita et al., 2004).

Following Cd stress, a deep alteration of the sulfur nutritional status of maize plants occurs, mainly as a consequence of the Cd-induced PC biosynthesis. In fact, the levels of NPTs dramatically increase because of the massive biosynthesis of PCs, which rapidly become the most abundant class of thiols in the root cells with a consequent transient depletion of the GSH.

Figure 5. Effect of Zn and Cu on thiol compound content (A and B), ZmST1;1 transcript level (C), and sulfate uptake capacity (D) in maize roots. Seedlings grown for 3 d in complete nutrient solutions were transferred and grown for an additional 48 h period in complete nutrient solutions supplemented with ZnCl2 (100 or 250 μM) or CuCl2 (10 μM). NPT, total glutathione, GSH (white bars), and GSSG (black bars) levels are expressed as GSH equivalents. GSH/GSSG ratios are reported in parentheses above the histogram columns. Sulfate influxes were 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 0.2 mM SO$_4^{2-}$. Bars and error bars are means and SE of three experiments run in triplicate ($n=9$). Different letters indicate significant differences ($P<0.05$). For northern analysis, 30 μg of total RNA extracted from roots were loaded onto each lane. Blots were hybridized with $^{32}$P-labeled ZmST1;1 probe. Ribosomal RNAs were stained on the gel with ethidium bromide (Et-Br) and used to check loading.
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Table II. Effect of sulfate availability on Cd tolerance and accumulation in maize

Seedlings grown for 3 d in complete nutrient solutions were transferred and grown for an additional 72 h period in pregrowing solutions containing different sulfate concentrations (0, 0.2, and 2 mM). Plants were then exposed to 10 \( \mu M \) CdCl\(_2\) for 48 h in the complete nutrient solution. Root SO\(_4^{2-}\) contents were evaluated at the end of the pregrowing period. Relative growth was calculated referring to the growth of control plants not exposed to CdCl\(_2\). Values are means ± se of three experiments run in triplicate (n = 9). Different letters indicate significant differences (P < 0.05).

<table>
<thead>
<tr>
<th>[SO(_4^{2-})] ( \text{mM} )</th>
<th>Root SO(_4^{2-}) Content</th>
<th>Relative Growth</th>
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<th>[Cd(^{2+})]</th>
<th>Total Cd(^{2+}) Removed</th>
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<td>0</td>
<td>0.57 ± 0.02(^a)</td>
<td>58.3 ± 1.6(^a)</td>
<td>48.0 ± 2.0(^a)</td>
<td>0.83 ± 0.03(^a)</td>
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<tr>
<td>0.2</td>
<td>2.56 ± 0.08(^b)</td>
<td>70.3 ± 2.0(^b)</td>
<td>69.4 ± 2.5(^b)</td>
<td>0.85 ± 0.05(^b)</td>
<td>0.33 ± 0.03(^b)</td>
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<tr>
<td>2</td>
<td>3.40 ± 0.11(^c)</td>
<td>98.3 ± 4.0(^c)</td>
<td>97.1 ± 3.6(^c)</td>
<td>0.93 ± 0.04(^c)</td>
<td>0.36 ± 0.02(^c)</td>
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poles (Tukendorf and Rauser, 1990; Rüegsegger and Brunold, 1992; Nocito et al., 2002). In this condition, the need for maintaining an adequate sulfur flux through the assimilatory pathway may be satisfied by increasing the root sulfate uptake capacity, via the derepression of a putative high-affinity sulfate transporter gene, named HAST, or more properly ZmST1;1 (Nocito et al., 2002). Nevertheless, more data are required to clarify the link existing between the transcriptional regulation of sulfate transporter genes and sulfur metabolism during Cd stress, since the relative abundance of ZmST1;1 mRNA seems to be more clearly related to the strength of the Cd-induced additional sinks for thiols compounds than to the total glutathione levels (Nocito et al., 2002).

With the aim to investigate the role of GSH in modulating sulfate transport activity during Cd stress we carried out a set of experiments with plants exposed to different Cd concentrations, to induce additional sinks for thiols compounds with different strength, and then GSH-consuming activities that could result in different GSH levels. Results suggest that the extent of the Cd-induced sulfate uptake is closely related to the strength of the Cd-induced additional sink for thiols compounds, which increases with the heavy metal concentration in the nutrient solution. In fact, a progressive enhancement in the NPT and ZmST1;1 mRNA levels and in the rate of sulfate uptake occurred as the external Cd concentration increased (Fig. 3). These responses were accompanied by a contraction of the root total glutathione contents, whose values do not seem clearly related to the strength of the additional sinks for thiols induced by Cd stress. In fact, as shown in Figure 3, moving from 1 to 10 \( \mu M \) Cd\(^{2+}\) external concentration, the NPT levels of the root increased from 78% to 454% with respect to the control, while the total glutathione levels fell down to about 0.5-fold the value of the control at the lower concentration analyzed and then remained constant as Cd concentration increases. A similar behavior was evident also considering the levels of GSH, a putative signal involved in sulfate transport modulation; in fact, the GSH/GSSG ratio in all Cd-exposure conditions, although lower than that of the control, remained constant.

Since this picture resulted from the establishment of a long-term equilibrium between metabolite levels, enzyme activities, and gene expression, speculating on the nature of GSH depletion and then on putative signals involved in ZmST1;1 gene expression appears difficult. In fact, the above-described behavior is consistent with the induction of GSH-consuming activities such as PC biosynthesis (Zenk, 1996), but does not exclude a direct effect of Cd on enzyme activities involved in GSH synthesis, i.e. GS (Rauser et al., 1991; Schneider and Bergmann, 1995). Moreover, under Cd stress the regulation of GSH biosynthesis undergoes deep changes, mainly at transcriptional level, as indicated by the increase of the \( \gamma \)-EC synthetase and GS mRNA relative abundances, probably caused by the transient GSH depletion itself (Schäfer et al., 1998; Xiang and Oliver, 1998).

To thoroughly analyze the putative role of GSH in controlling ZmST1;1 expression, we performed short-term experiments aimed at comparing Cd-treated and sulfate-starved roots; both conditions are indeed able to positively modulate ZmST1;1 transcription (Bolchi...

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et al., 1999; Nocito et al., 2002; Quaggiotti et al., 2003; Hopkins et al., 2004). Cd exposure was more efficient than sulfate withdrawal in promoting ZmST1;1 expression (Fig. 4), but in both conditions the increase in the ZmST1;1 transcript relative abundance began to be evident only when the root GSH contents dropped off (Fig. 4; Table I). Nevertheless, also in these cases we cannot evince a clear relation between the increment in the ZmST1;1 mRNA relative abundance and the entity of the GSH decrement.

Such a behavior may reflect any difficulties in maintaining GSH homeostasis during Cd stress and could be ascribed to a direct effect of Cd on GS activity, as previously reported by Schneider and Bergmann (1995). In fact, following the short-term exposures to Cd, the decline in the GSH levels was accompanied by a progressive accumulation of Cys and γ-EC in the root tissues (Table I).

Concerning the mechanisms of sulfate transport regulation some physiological considerations need to be taken into account. Our knowledge of sulfate transport regulation mainly arises from extensive studies conducted on sulfur-starved plants. As expected, in this condition the absence or the reduced availability of sulfate in the external medium limits the sulfur flux through the assimilatory pathway with a consequent GSH biosynthesis impairment, which in turn may act as a signal in promoting the up-regulation of high-affinity sulfate transporters. Several works have shown the strength of these responses to be related to the entity of the decrement in the levels of GSH and Cys, which, in these conditions, represent the principal NPTs in plant tissues (Smith et al., 1997; Bolchi et al., 1999). On the other hand, the exposure to Cd induces sulfate uptake, although in the presence of adequate sulfate supply, probably as a consequence of an increase in the sulfur request by plant, as indicated by the dramatic variation of the NPT levels that result several fold higher than those of Cys and GSH (Table I; Nocito et al., 2002). Taking into account the wide variety of soils and environmental conditions experienced by plants, it appears clear the need for plants to have multiple signaling pathways modulating sulfate uptake by roots in response to both sulfur requirement in different phases of their life and soil sulfate level. Our data confirm the pivotal role of GSH levels in triggering Cd-induced ZmST1;1 expression and sulfate uptake and suggest the existence of other factors able to modulate the ZmST1;1 responses. Results obtained in the experiment performed with Zn-treated plants also support this last conclusion. In fact, Zn exposure promoted ZmST1;1 derepression and sulfate uptake by roots and generated additional sink for thiol compounds, without negatively affecting the GSH pools (Fig. 5). The increases in the NPT levels observed during Zn exposure seem to be related to the Zn concentrations in the nutrient solution and may be ascribed to the synthesis of PCs. It has been shown that maize, differently from other species, synthesizes PCs in response to Zn accumulation, although this metal does not seem to be chelated by PCs (Tukendorf, 1996; Souza and Rauser, 2003). Moreover, our data revealed that also Cu exposure promoted ZmST1;1 derepression and sulfate uptake by roots (Fig. 5). However, the treatment with Cu did not influence neither NPT nor total glutathione levels of the roots but deeply affected the cell redox potential as indicated by the dramatic variation of GSH/GSSG ratio (Fig. 5). Such a behavior suggests that a redox-reactive heavy metal, like sulfate starvation, may induce sulfate uptake in response to a contraction in the GSH pool of the roots.

Taken as a whole, our data suggest that during heavy metal-induced stress, sulfate uptake by roots may be controlled through GSH-dependent or -independent signaling pathways. However, the nature of these last signals needs to be further investigated. Several works have shown that other metabolic intermediates along the pathways of sulfate reductive assimilation and GSH biosynthesis, such as O-acetyl-serine (OAS), the carbon skeleton used for $\text{S}_2^-$ assimilation, may play a role as signals in controlling both sulfate transporter and sulfur-responsive gene expression (Hawkesford, 2000; Koprivova et al., 2000; Leustek et al., 2000; Hirai et al., 2003, 2004; Saito, 2004). Such a molecule, synthesized from Ser and acetyl-CoA in a reaction catalyzed by Ser acetyltransferase, accumulates under sulfur-limiting conditions or, in other words, when there is not enough $\text{S}_2^-$ available for Cys biosynthesis (Hawkesford, 2000; Saito, 2004; Hopkins et al., 2005).

Experimental evidence suggests that OAS acts as a positive transcription regulator of sulfate transporter genes and that its presence in the growing medium partially overrides the negative feedback provided by the reduced sulfur compounds on gene transcription (Smith et al., 1997). From our data we can exclude that under Cd or Zn exposure the reduction of sulfate to sulfide may limit Cys biosynthesis, since in these conditions the NPT levels of the roots were several fold higher than those of the control. Moreover, following Cd exposure the level of Cys into the root progressively increased (Table I); since such a molecule negatively acts on Ser acetyltransferase activity through a feedback mechanism (Saito, 2004) it seems unlikely that OAS accumulates under Cd stress.

Considering the effect of Cd on sulfate uptake and thiol metabolism, a natural question arises: May sulfate acquisition limit GSH biosynthesis and thus Cd tolerance and accumulation? The experiments conducted with plants pregrown at different sulfate concentrations suggested that the toxic effects exerted by Cd accumulation can be alleviated maintaining high sulfate concentrations in the root tissues. In fact, following 48 h of Cd exposure, the total biomass produced by plants increased with sulfate concentration in the pregrowing solution (Table II). Such a behavior was likely due to an effect of root sulfate stores on the synthesis of GSH, since the levels of this metabolite were positively related to those of sulfate in the pregrowing solutions (Fig. 6). Similar results have been obtained in plants of Brassica juncea overexpressing GS,
which maintain, in the absence or presence of Cd, GSH levels higher than those of the wild type (Zhu et al., 1999a). The increase in Cd tolerance did not influence the plant capacity to accumulate Cd, since Cd concentration in both shoot and root was not affected by the sulfate concentration in the pregrowing solution (Table II). However, since Cd content in the total biomass produced is the most important parameter for phyto-
remediation, it appears interesting to calculate this value for each plant. As reported in Table II, the increased tolerance shown by the high-sulfate-grown plants resulted in a greater capacity to extract Cd from the growing medium.

Our results suggest that root sulfate availability could represent a limiting factor for GSH biosynthesis and for plant Cd tolerance. Moreover, since other genes encoding high- and low-affinity sulfate transporters have been shown to be modulated in their expression following Cd exposure and accumulation (Heiss et al., 1999; Nocito et al., 2005), it is becoming clear that under Cd stress the sulfate fluxes along the whole plant undergo deep changes to ensure detoxi-
fication and survival. Considering these aspects, it seems particularly important to improve root sulfate uptake and translocation capacities as well as sulfur metabolism to improve plant tolerance to Cd and to envisage the production of Cd-tolerant plants explo-
itable in phytoremediation programs.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Experimental Design

Maize (Zea mays L. cv Dekalb DK 300) caryopses were sown on filter paper saturated with distilled water and incubated at 26°C in the dark. Three days later, seedlings selected for uniform growth were transplanted into 5 L plastic tanks (18 seedlings per tank) containing an aerated complete nutrient solution (200 mM KNO₃, 200 mM Ca(NO₃)₂, 40 mM KH₂PO₄, 200 mM MgSO₄, 25 mM Fe-
tartrate, 30 mM H₂BO₃, 5 mM MnCl₂, 1 mM CuCl₂, 1 mM ZnCl₂, and 0.1 mM (NH₄)₂MoO₄, pH 6.5) and kept for 3 d in a growth chamber maintained 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.

Two experimental systems were used to study the involvement of GSH as metabolic signal in promoting ZmST1;1 derepression or the role of sulfate acquisition in Cd tolerance and accumulation. In the first, 3 d after seedling transplanting, the complete nutrient solution was supplemented with CdCl₂, ZnCl₂, or CuCl₂ at different concentrations, or substituted with a minus-sulfate solution, where MgSO₄ was replaced by an equimolar amount of MgCl₂. The treatment period varied from 3 to 48 h. Plants were then sampled and frozen in liquid nitrogen or immediately used for further experiments (see sulfate influxes assay).

In the second experimental system, 3 d after transplanting, seedlings were pregrown for an additional 72-h period in a nutrient solution (see above) containing different sulfate concentrations (0, 0.2, and 2 mM); in the 0 mM SO₄²⁻ pregrowing solution MgSO₄ was replaced by an equimolar amount of MgCl₂. Plants were then exposed to 10 μM CaCl₂ for 48 h in the complete nutrient solution containing 0.2 mM MgSO₄. Relative growths of both shoots and roots were calculated referring to the growth of control plants not exposed to CaCl₂.

All hydroponic solutions were renewed daily to minimize nutrient depletion. Total hydroponic solution was extracted from roots of 5-d sulfur-starved plants using Trizol Reagent (Invitrogen) and first-strand cDNA synthesis was carried out using SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. PCR was carried out on the first-strand cDNA using 18S rDNA polymerase (Promega) and the amplified EcoRI-ended fragment was cloned into the EcoRI site of pBluescript KS vector (Stratagene). The identity of the PCR product was verified by sequencing both the strands.

Heterologous Expression of ZmST1;1 in Yeast and Sulfate Uptake Assay

The EcoRI-ended fragment of ZmST1;1 cDNA was subcloned in the EcoRI site of the yeast (Saccharomyces cerevisiae) expression vector pESC-TRP (Strata-
tagene) under the control of GAL10 promoter. The chimeric vector was used to transform the yeast double sulfate transporter mutant CP154-7A (MATa his3 leu2 ura3 ade2 trpl sul11 leu2 ura3 his4; Cherest et al., 1997) using the standard lithium acetate method (Gietz et al., 1992). CP154-7A transformed with the empty pESC vector was used as negative control. Trp⁺ recombinant yeast cells were selected on minimal media containing 2% (w/v) Gal, 40 mg L⁻¹ adenine sulfate, and required amino acids (Sherman, 1991).

For the complementation test, Trp⁺ cells were grown to approximately 1 A₅₉₀ unit in a Trp-free rich medium containing yeast nitrogen base and required amino acids. Yeast cells were then washed twice with sterile distilled water and resuspended to a final absorbance of 1 A₅₉₀ unit in a selective synthetic minimal medium, named B medium, containing 15 mM NH₄Cl, 6.6 mM KH₂PO₄, 0.5 mM K₂HPO₄, 2 mM MgCl₂, 0.7 mM NaCl, 0.68 mM CaCl₂, 80 mM H₂BO₃, 6 μM KI, 4 μM ZnCl₂, 2 μM CuCl₂, 1.8 μM FeCl₃, 2% (w/v) Gal, 200 mg L⁻¹ His, 40 mg L⁻¹ adenine, 20 mg L⁻¹ inositol, 2 mg L⁻¹ calcium pantothenate, 2 mg L⁻¹ thiamine HCl, 2 mg L⁻¹ pyridoxine HCl, and 0.02 mg L⁻¹ biotine. Ten microliters of drops of the final cell suspension were dropped on B medium, containing either 0.1 mM Na₂SO₄ or 0.1 mM d-t-homo-Cys as the sole sulfur sources, solidified with 1% (w/v) low-sulfate-containing agarose (Invitrogen). Yeast cells were incubated at 28°C for 3 d and then analyzed for the growth.

Sulfate uptake kinetic was determined on complemented yeast cells by measuring the rate of sulfate uptake in a range of low sulfate external concentrations. To this purpose the complemented cells were grown in B medium containing 0.1 mM d-t-homo-Cys as sole sulfur source up to reach an absorbance of 0.5 A₅₉₀ units. At the end of the growing period cells were washed twice with sterile distilled water and then resuspended to a final absorbance of 0.7 A₅₉₀ units in a sulfurless B medium. The uptake assay was performed by adding 0.5 mL of cell suspension to 0.5 mL of B medium containing different Na₂SO₄ concentrations and labeled with 37 kBq Na₂¹⁵SO₄. The mixture was incubated at 28°C for 30 and 210 s; for each time the incubation was stopped by the addition of 10 mL of ice-cold 0.1 mM Na₂SO₄, cells were filtered on a GF/C glass filter (Whatman), and then washed three times with 10 mL of ice-cold 0.1 mM Na₂SO₄. Radioactivity retained on each filter was measured by liquid scintillation counting in a β counter (Beckman LS 6000SC). Kinetic analysis of sulfate uptake was performed by fitting the Michaelis-Menten equation to the experimental data in the concentration range 1 to 30 mM.

Northern-Blot Analysis

Roots of heavy metal-exposed, sulfur-starved, and control plants were prepared, using mortar and pestle in liquid nitrogen and total RNA was extracted using Trizol Reagent (Invitrogen). Thirty micrograms of total RNA per lane were separated by electrophoresis at 5 V cm⁻¹ in a 1.3% (w/v) agarose gel containing 6% (w/v) formaldehyde, transferred to Hybond-N+ nylon membrane (Amersham Bioscience) by capillary blotting in 20 × SSC and then fixed by UV cross-linking. The blot was hybridized with 32P-labeled cDNA probe for the entire coding sequence of the high-affinity sulfate transporter ZmST1;1. Prehybridization and hybridization were carried out in buffer containing the nylon membrane manufacturer’s instructions. Membrane was washed for 10 min with 2 × SSC in 0.1% (w/v) SDS at room temperature, with 1 × SSC in 0.1% (w/v) SDS at 65°C for 20 min and then for 10 min with 0.1 × SSC in 0.1% (w/v) SDS at 65°C.
Sulfate Influxes Assay

Sulfate influxes into the roots were measured by determining the rates of 35S uptake, over a 15 min pulse in incubation solutions labeled with the radiotracer. Three plants were placed onto 400 mL of a fresh complete nutrient solution, containing 200 μM MgSO4, supplemented or not with CdCl2, ZnCl2 or CuCl2 at different concentrations, aerated and thermostated at 25°C. Radioactive pulses were started by adding 35S-labeled Na2SO4 to the uptake solutions. Specific activity was 4.7 kBq mmol−1. At the end of the pulse period, roots were excised from shoots, rinsed twice for 1 min in 400 mL of a 4 mm CaSO4 nonradioactive solution at 4°C, blotted with paper towels, and then heated for 20 min at 80°C in 0.1 N HNO3 (10 mL g−1 fresh weight). Radioactivity was measured on aliquots of the extracting solution by liquid scintillation counting in a β counter (Beckman LS 6000SC).

Determination of Thiols

Roots were pulverized using mortar and pestle in liquid nitrogen and stored frozen in a cryogenic tank. For Cys, γ-EC, and total glutathione determination, frozen powdered samples were extracted in 1:2 (w/v) ice-cold 0.1 mM HCl, 1 mM Na2EDTA, and the homogenates were centrifuged for 15 min at 15,000g and 4°C. The supernatants were collected and immediately subjected to thiol analysis. Cys, γ-EC, and total glutathione contents were measured, after reduction with diithiothreitol and derivatization with monomobromobimane, by reverse-phase HPLC and fluorescence detection as described by Schupp and Rennenberg (1988). GSH and GSSG were measured according to Griffith (1980). NPTs were determined according to Nagalakshmi and Prasad (2001). All results were expressed as nmol of GSH equivalent g−1 fresh weight.

Determination of Sulfate

Roots were rinsed three times in distilled water and blotted with paper towels. Sulfate was extracted by homogenizing the samples in 1:10 (w/v) ice-cold 0.1 N HNO3. After heating at 80°C for 20 min, the extracts were filtered and the sulfate contents were then determined according to the turbidimetric method described by Tabatabai and Bremmer (1970).

Determination of Cd

Plants were harvested and roots were washed for 10 min in ice-cold 5 mM CaCl2 solution to displace extracellular Cd (Rauser, 1987). Roots were excised from shoots and gently blotted with paper towels. Cd content was measured, after complete mineralization of root and shoot tissues in a mixture of nitric, sulfuric, and perchloric acid (5:1:1, v/v/v) by atomic absorption spectrophotometry (SpectrAA-20, Varian).

Statistical Analysis

ANOVA, Bonferroni’s, and Student’s t tests were calculated using SigmaStat for Windows (Version 3.11).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF355602.

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LITERATURE CITED


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