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Expression of selected genes involved in cadmium detoxification in tobacco plants grown on a sulphur-amended metal-contaminated field

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ABSTRACT

We investigated the effect of a Cd solubilising soil treatment on the expression of genes regulating Cd uptake and detoxification in field-grown tobacco plants. Tobacco plants were grown on a heavy-metal contaminated soil to which elemental sulphur was applied to increase the phytoextraction of Cd. The expression of tobacco gene homologues for *A. thaliana MRP3*, *PDR8*, *ATM3*, *Sultr1*, *LAST*, *APR2*, *APR3*, *GSHI*, *GSHII*, and *NAS3* was assayed by qualitative RT-PCR.

Increased root and shoot Cd concentrations were associated with up-regulation of the putative Cd transporters and the genes involved in sulphur assimilation in root tissues. This is consistent with previous studies using hydroponics. However, unlike the previous studies, most of the genes tested in the leaves were unaffected by Cd concentration.

These differences may be due to the more complex stress situation that plants experienced here under field conditions. Moreover, unlike in hydroponic studies, our plants were sampled at maturity and not in the seedling stage. Our results indicate that hydroponic or agar experiments are useful predictors of effects that may be expected in the field. However, there is a need for studies investigating gene expression in response to multiple stresses representative for field conditions at later developmental stages.

1. Introduction

Cadmium (Cd) is a trace element that is non-essential for plants, animals and humans. It is toxic at elevated concentrations. Large areas of agricultural land are contaminated with Cd, which is readily taken up by plants and thus can easily enter food chains. Phytoextraction has been proposed to clean up such land (Chaney et al., 1997). Tobacco is a crop plant that produces a large biomass and accumulates relatively high concentrations of Cd (Lugon-Moulin et al., 2004). While the latter property exacerbates health problems for smokers, the combination of large biomass and high Cd accumulation also makes tobacco a candidate plant to be used for the remediation of agricultural soils with low to moderate Cd contamination. The application of elemental sulphur has shown to enhance phytoextraction of Cd on contaminated soil (Kayser et al., 2000; Wenger et al., 2002; Fässler et al., 2010b), as it increases solubility and plant availability of Cd. The Cd-mobilizing effect of sulphur addition results from the decrease in soil pH as elemental sulphur oxidized to sulphate by microbes (Tichý et al., 1997; Wang and Wu, 2006).

The expression of genes that are involved in the uptake, distribution and detoxification of Cd is affected by the exposure of plants to this element in the rhizosphere. Table 1 shows a list of genes that are suggested to be involved in Cd detoxification and/or transport in plants and their response to elevated concentrations of Cd in roots and shoots. Cadmium is taken up by plants due to (i) its chemical similarity with other cations that are essential for plants such as Ca and Zn and (ii) the lack of specificity of the uptake and distribution systems for these elements (Clemens et al., 2002). Genes that are involved in Cd-detoxification notably include ATP-binding

Abbreviations: ABC, ATP-binding cassette; APR, APS reductase; APS, adenosine 5′-phosphosulphate; ATM, ABC transporter of the mitochondria; BLAST, basic local alignment search tool; EDTA, ethylene diamine tetraacetic acid; EF, elongation factor; GSH, glutathione; LAST, low affinity sulphate transporter; MRP, multidrug resistance related proteine; NAS, nicotianamine synthase; PC, phytochelatine; PDR, pleiotropic drug resistance; RT-PCR, reverse transcription – polymerase chain reaction; Sultr, sulphate transporter.

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Table 1
Summary of studies in which Cd affects the expression of genes coding for enzymes that are involved in Cd uptake and detoxification.

Gene	Enzyme function[a]	Effects of trace elements or sulphur on gene expression						
		Study	System[b]	Plant species	Cd conc.	Effect		Exposure time/remarks
						Root	Shoot	
MRP3	Involved in Cd resistance (Tommasini et al., 1998)	Bovet et al. (2003)	H	*A. thaliana*	20 μM	↑	→	24 h
ATM3	Heavy-metal detoxification (Kim et al., 2006; Rea, 2007)	Kim et al. (2006)	A	*A. thaliana*	50 μM	↑	→	2 weeks
PDR8	Cd resistance by efflux pumping from root epidermal cells (Kim et al., 2007)	Kim et al. (2007)	A	*A. thaliana*	50 μM	↑	↑	12 h
						↑	n.d.	2, 10 and 24 h
Sultr1	Sulphur uptake by the roots (Smith et al., 1995; Yoshimoto et al., 2002)	Herbette et al. (2006)	H	*A. thaliana*	50 μM	→	n.d.	2, 6, and 30 h/Sultr1;2
		Herbette et al. (2006)	H	*A. thaliana*	50 μM	↑	n.d.	2, 6, and 30 h/Sultr1;1
LAST	Uptake, translocation, distribution and reallocation of sulphate in plants (Sun et al., 2005)	Sun et al. (2005)	H	*B. napus*	30 μM	↑	→	2 and 3 days
		Heiss et al. (1999)	H	*B. juncea*	25 μM	↓	→	48 h
		Sun et al. (2007)	H	*B. napus*	20 and 40 μM	↓	↑	72 h
					80 and 120 μM	↓	↓	
APR2	Reduction of APS to sulphite in sulphur assimilation, control function in sulphur pathway (Kopriva and Koprivova, 2004)	Herbette et al. (2006)	H	*A. thaliana*	5 μM	↑	n.d.	2 and 6 h, (→)
						↑	n.d.	30 h
					50 μM			2, 6 and 30 h
		Weber et al. (2006)	H	*A. thaliana*	10 and 50 μM	↑	n.d.	2 h
		Heiss et al. (1999)	H	*B. juncea*	25 μM	↑	↑	48 h
GSHI	Catalyses the first step in GSH synthesis (May et al., 1998b)	Xiang and Oliver (1998)	P	*A. thaliana*	100 μM[c]	n.d.	↑	12 h
		Xiang and Oliver (1998)	H	*A. thaliana*	25, 50, 100 μM	n.d.	↑	12 h
					400 μM	n.d.	↓	
		Schäfer et al. (1998)	H[d]	*B. juncea*	25 μM	↑	↑	10 days
		Kim et al. (2006)	A	*A. thaliana*	10 μM	(↑)	(↑)	2 weeks; ( ) = increased in the "seedlings"
		Sun et al. (2005)	H	*B. napus*	30 μM	↑	↑	2 and 3 days
		Herbette et al. (2006)	H	*A. thaliana*	5 and 50 μM	→	→	2, 6, and 30 h
GSHII	Catalyses the second step in GSH synthesis (May et al., 1998b)	Xiang and Oliver (1998)	P	*A. thaliana*	100 μM[c]	n.d.	↑	12 h
		Xiang and Oliver (1998)	H	*A. thaliana*	25, 50, 100 μM	n.d.	↑	12 h
					400 μM	n.d.	↓	
		Schäfer et al. (1998)	H[d]	*B. juncea*	25 μM	→	n.d.	10 days
NAS3	Transfer of excess metals from root to shoots (Sharma and Dietz, 2006)	Bovet et al. (2006)	H	*N. tabacum*	1 μM	↑	→	7 days
		Bovet et al. (2006)	H	*N. rustica*	1 μM	↑	→	7 days

↑, up regulated; ↓, down regulated; →, expression not affected, ( ), see remarks; n.e., not expressed; n.d., not determined.
[a] Linked to Cd.
[b] A: agar; H: hydroponics; P: pot.
[c] Plants sprayed with Cd solution.
[d] In pots with sand, watered with nutrient solution.

cassette (ABC) proteins (Hall and Williams, 2003; Plaza and Bovet, 2008). Members of the ABC transporter family that are known to confer Cd tolerance to plants include MRP3 (multidrug-resistance-related protein) (Kolukisaoglu et al., 2002), ATM3 (ABC transporter of the mitochondria) (Kim et al., 2006), and PDR8 (pleiotropic drug resistance) (Kim et al., 2007).

An important role in Cd detoxification is played by phytochelatins (PC) (Howden et al., 1995), which are heavy metal-chelating polypeptides that are synthesized in the cells through a specific non-translational pathway from glutathione. The synthesis of GSH occurs in two ATP-dependent steps, which are catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (May et al., 1998b). These enzymes are encoded by the genes *GSHI* and *GSHII*, respectively (Zhu et al., 1999). An important component of GSH is sulphur (Leustek and Saito, 1999). Sulphur is taken up by plant roots primarily in the form of sulphate (SO_4^{2-}) through high-affinity sulphate transporters, such as the Sultr1 (SULphate TRansporter) subfamily in *A. thaliana* (Saito, 2004). Low-affinity sulphate transporters (LAST) are involved in the translocation of sulphate from roots to shoots (Sun et al., 2005). In the sulphur assimilatory pathway, sulphate is reduced in several steps to sulphite. The key step in sulphur assimilation is the reduction of adenosine 5′-phosphosulphate (APS), which is catalyzed by the enzyme APS reductase (APR) (Kopriva and Koprivova, 2004). Due to their role in Cd detoxification, it is no surprise that the expression of the genes coding for the respective enzymes, i.e. *Sultr1*, *LAST*, *APR*, *GSHI*, and *GSHII*, is also responsive to Cd exposure (Xiang and Oliver, 1998; Kopriva and Koprivova, 2004; Sun et al., 2005) (see also Table 1). Furthermore, although not fully understood, nicotianamines (NA) are involved in Cd chelation, transport and detoxification in plants (Sharma and Dietz, 2006). Nicotianamines are ubiquitously present in plants and synthesized from three molecules of methionine by nicotianamine synthase (NAS) (Sharma and Dietz, 2006).

Most studies investigating the expression of genes involved in Cd uptake and detoxification were carried out using *Arabidopsis thaliana*, *Brassica napus* and *Brassica juncea* in hydroponic or agar systems (Table 1). In contrast, there are only few studies on Cd-induced gene expression using *N. tabacum*, which is surprising given the health problems for smokers associated with the accumulation of Cd by this plant. The times over which seedlings were exposed to Cd in the experiments investigating Cd effects on gene expression generally ranged between 2 h and 2 weeks. Thus, in such experiments plants were generally harvested at an early vegeta-

tive state. Bovet et al. (2003) showed that it is not always possible to extrapolate data on gene expression from seedlings to mature plants. Furthermore, previous experiments were often performed with excessively high Cd concentrations (Ernst et al., 2008). No studies on Cd-related gene expression have so far been performed under field conditions, where plants experience many other stress factors and are grown to maturity.

The aim of this study was to investigate the effects of increased Cd phytoavailability on the expression of genes that are involved in Cd uptake, distribution and detoxification in tobacco plants grown in a phytoextraction experiment on a heavy-metal contaminated soil in comparison to published results from hydroponic or agar experiments. In this field experiment some plots were treated with elemental sulphur in order to increase the Cd solubility in the soil and thereby the phytoextraction of this soil contaminant.

2. Materials and methods

2.1. Experimental design

The plants were taken from a field experiment (Fässler et al., 2010b) at Witzwil in the Bernese Seeland, Switzerland, on a former wetland soil that had been converted to agricultural use after drainage at the end of the 19th century. The land had been contaminated in the first half of the 20th century through the application of organic but also other wastes from the city of Bern. In the field experiment, which was started in the year 2000, maize (*Zea mays*, cv. Magister), sunflower (*Helianthus annuus*, cv. Sanluca), and tobacco (*Nicotiana tabacum*, cv. Burley 92) were grown on three parallel plots in a 3-year scheme of annual rotation between these crops. Each plot was subdivided into 16 sub-plots (3 m × 12 m) on which four treatments affecting Cd solubility were applied to the soil in four replicates. All fields were fertilized according to the nutritional requirements of the plants. For the study of gene expression reported here, we focus on tobacco plants, which were sampled in 2006 from plots treated with elemental sulphur and from control plots that had not received any chemical treatment apart from fertilization. Over a period of 7 years (2000–2006), a total of 1.71 kg sulphur per m^2 had been added to the top soil (plough layer). This is more than usually needed by crop plants (4–6 g kg^{-1} (Bergmann, 1993)), but the S content remained in a range that is normal for soils of humid climatic zones, which is 0.02–2% (Scheffer et al., 2002).

2.2. Soil properties

Relevant soil properties, i.e. pH, $CaCO_3$ content, organic carbon content, soluble and total metal and nutrient concentrations are listed in Table 2. Soluble metals were extracted by 0.1 M $NaNO_3$. This extract is often used to determine the easily plant available fraction. Total metal concentrations were analysed in extracts obtained with boiling 2 M HNO_3. Further details about soil sampling and analysis are given in Fässler et al. (2010a), from where the soil data were taken.

2.3. Cadmium and nutrient concentrations in tobacco

After 20 weeks of growth we took four randomly selected plants from a sulphur treated sub-plot and four plants from a control sub-plot. The leaves of each plant were grouped into bottom, middle and top leaves, taking the same amount of leaves for each category. Roots were excavated using a garden fork and washed carefully with water to remove soil particles. For gene expression analyses, some fresh material was taken from the root and leaf samples and conserved at −80 °C. The rest was cut into small pieces, oven-dried for three days at 60 °C and ground to a particle size of approximately 0.75 mm using a heavy-duty cutting mill (Retsch, SM1). The root samples were first coarsely ground using a knife mill (Fuchs, 180S), before they were passed through the cutting mill. A subsample of 0.5 g was taken from each sample and microwave-digested in a mixture of 5 ml HNO_3 (65%), 3 ml H_2O_2 (30%), and 2 ml H_2O. The digested samples were diluted by adding Millipore water to a total volume of 25 ml and then filtered (filter paper grade MN 640 d, average retention capacity 2–4 μm). Cadmium was analyzed by means of graphite furnace atomic absorption spectroscopy (GF-AAS, Varian GTA120/AA240Z). Concentrations of the micro- and macronutrient elements Cu, Fe, Mg, Mn, S and Zn were determined by inductively coupled plasma optical emission spectroscopy (ICP

Table 2
Total and soluble concentrations of selected metals, total S concentration, pH and $CaCO_3$ content of treated and control top soil samples (0–20 cm).

Property	Method[a]	Unit	Control	Sulphur treatment
Cd conc.	HNO_3	mg kg^{-1}	1.444 ± 0.095	1.366 ± 0.121
	$NaNO_3$	μg kg^{-1}	1.673 ± 0.439	3.493 ± 0.516*
Cu conc.	HNO3	mg kg^{-1}	588 ± 27	593 ± 30
	EDTA	mg kg^{-1}	284 ± 13	280 ± 14
Fe conc.	HNO_3	%	2.042 ± 0.112	2.019 ± 0.154
	EDTA	%	0.074 ± 0.002	0.100 ± 0.003*
Mg conc.	HNO_3	mg kg^{-1}	2954 ± 148	2678 ± 195
	EDTA	mg kg^{-1}	278 ± 10	291 ± 10
Mn conc.	HNO_3	mg kg^{-1}	999 ± 57	953 ± 70
	EDTA	mg kg^{-1}	131 ± 5	195 ± 5*
S conc.	XRF	mg kg^{-1}	3128 ± 81	7130 ± 336*
Zn conc.	HNO_3	mg kg^{-1}	683 ± 41	645 ± 55
	$NaNO_3$	μg kg^{-1}	81 ± 14	614 ± 88*
pH	H_2O		7.71 ± 0.03	6.48 ± 0.09*
$CaCO_3$ content[b]		%	2.72 ± 0.32	1.38 ± 0.14*
C_{org} content[c]		%	11.1 ± 0.3	11.0 ± 1.1

[a] HNO_3: extraction with boiling 2 M HNO_3; $NaNO_3$: extraction with 0.1 M $NaNO_3$; EDTA: extraction with 0.5 M ammonium acetate and 0.03 M EDTA, metal analysis of the extractions: Cd by means of GF-AAS (graphite furnace atomic absorption spectroscopy), analysis of all other elements by means of ICP-OES (inductively coupled plasma optical emission spectrometry); XRF: determination by means of X-ray fluorescence; H_2O: measurement of pH in H_2O at a soil:solution ratio of 1:2.5.
[b] Determination by means of calcimeter.
[c] Determination with potassium dichromate method.
* Asterisks indicate significant differences between the treatments.

Table 3
List of primers used for the qualitative RT-PCRs.

Gene	Forward primer	Reverse primer	*Arabidopsis thaliana* homolog
NtMRP_a	GAGGATGTTCCTCAGCTTCA	AGGTACGGGCCAACAAAAG	AtMRP3
NtATM_a	AATCAGAGATGCAAACGATGC	TTTCCAGAATGGGTGTCAAAG	AtATM3
NtPDR_a	GGGATCAATGTATGCTGCTG	TTCAAATCCAATCATAGCATAGACA	AtPDR8
NtSultr_a	CCCCAAAACAGGACATATTCA	CAATGGTGAGACCAGCAATAA	AtSultr1;2
NtLAST	TATTCTGGCTACCGGCTATTG	GATAATGACCCCACAATGTTCA	AtLAST
NtAPR_a	AAGGAGTGTGGATTGCACAAG	TTAACACCTGAACCAGCCAAC	AtAPR2
NtAPR_b	GCTAATTGGTGGTGATTTGGA	TGTGTAACCTCCCAGAGGATG	AtAPR3
NtGSHI[a]	TGCAGCCTATTGCTACAGCTC	ATGCACACAACCTTCTCCAAG	AtGSHI
NtGSHII[a]	GAACTGTTCCTGGAGTTGGA	AATTACCAACCTAGACAGTGATTGC	AtGSHII
NtNAS	GGCATCTGACCCTGACTTGTA	CGTCTGTTGGATGGTGGATAG	AtNAS3
NtTubulin	ATTTGTTGACTGGTGCCCAAC	TCTTCATCGTCAACTTCAGCA	AtTubulin
NtEF_a	TTGGAAATGGATATGCTCCAG	CACCAACAGCAACAGTTTGAC	AtEF1

[a] In the tobacco genome only 1 copy for each gene is apparently present.

OES, Varian Vista-MPX). For quality control certified material of Virginia tobacco leaves (CTA-VTL-2 (Dybczynski et al., 1998)) was digested and analyzed together with the samples. The maximal relative standard deviation of repeated measurements of the reference samples was 7% and the respective maximal relative bias was 5% for all elements.

2.4. Gene expression analysis

Gene expression was analysed by qualitative RT-PCR using frozen plant material. All RT-PCRs were repeated twice on each plant sample. The design of primers was based on tobacco sequences available from the National Center for Biotechnology Information (NCBI) and the University of Fribourg access to the tobacco genome initiative TGI (NC State University, 2008) (Table 3 gives a complete list of the primers). The selection of tobacco genes was based on the literature listed in Table 1 and on a search using the sequence similarity search program BLAST (Basic Local Alignment Search Tool) (Ye et al., 2006). For a few accessions only partial sequences were available. Using deduced amino acid sequence homologies from putative coding sequence of more than 200 bp (size of the fragments tested by qualitative RT-PCR), we were able to find significant degrees of homology (>85%) with Arabidopsis protein, thereby attesting that the sequences selected were close to the sequences of Arabidopsis genes. The BLAST analysis gave a sequence of the tobacco genome that belongs to the *Sultr* family and is close to the sequences of the *AtSultr1;1*, *AtSultr1;2* and *AtSultr1;3* genes. A specific *Sultr* homologue for tobacco could not be assigned. Therefore, the designation *AtSultr1* was chosen. Specific tobacco *APR2* homologues could not be ascertained either. Sequences similar to *AtAPR2* and *AtAPR3* were chosen therefore. Total RNA was purified from the root and leaf samples using the RNeasy Plant Mini Kit (Qiagen, Basel, Switzerland) and stored at −80 °C after quantification by spectrophotometry. After DNAse treatment (RQ1, RNase free, Promega Catalys, Wallisellen, Switzerland), cDNAs were prepared using M-MLV reverse transcriptase (RNase H minus, point mutant, Promega Catalys) as prescribed by the manufacturer and stored at −20 °C. An aliquot of the cDNAs was diluted 1:10 in water and used for PCR. After 2 min denaturation at 95 °C, 35 PCR cycles (95 °C for 30 s, 50–58 °C (depending primers Tm) for 30 s and 72 °C for 30 s) were run. PCR was performed in a final volume of 15 μL containing the following mixture: PCR buffer 1×, 5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of both forward and reverse primers, 1 U Go Taq DNA polymerase (Promega Catalys). Thirty-five PCR cycles were performed in order to ensure detection of all transcripts. By using the same number of cycles of RT-PCR for all genes, a qualitative comparison of the level of gene expression was possible. For PCR adjustment, we used the housekeeping gene "elongation factor A" (*NtEFA*), the tobacco homolog of the *AtEF1* gene, for the root samples and the *NtTubulin* gene homolog to *At4G14960* for the shoot samples.

2.5. Statistical analysis

Treatment effects and variations between plant parts were analysed by means of one-way analysis of variance (ANOVA) and tested for significance between group means by the Bonferroni post hoc test using SPSS version 15. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Treatment effects on soil properties

The pertinent changes in soil parameters that were found in response to the sulphur treatment are shown in Table 2. The acidity generated by the oxidation of the applied sulphur markedly decreased the soil pH from 7.7 to 6.5. Buffering of the acidity by dissolved carbonate led to a reduction of the calcium carbonate content from 27 to 14 g kg^{-1}. The acidification of the soil caused soluble Zn to increase by an order of magnitude from 81 to 614 μg kg^{-1}, while soluble Cd was doubled from 1.67 to 3.49 μg kg^{-1}. In addition, soluble Fe and Mn slightly increased, whereas there were no significant effects on soluble Cu and Mg. The total S concentration was approximately doubled in the sulphur treated plots.

3.2. Treatment effects on element uptake by the plants

The sulphur treatment had no visible effect on plant phenotypes and did not affect the biomass production (data not shown). The plants showed no signs of toxicity and grew well in both treatments. The mean leaf Cd concentration increased from 1.42 ± 0.09 mg kg^{-1} in the controls to 2.30 ± 0.13 mg kg^{-1} in the sulphur treatments. These values are in the concentration range of tobacco plants grown on uncontaminated land and used for cigarette production (Lugon-Moulin et al., 2004). The sulphur treatment significantly increased leaf Cd concentrations along the entire length of the stem (Fig. 1). The concentration was the highest in the bottom leaves but the differences were not significant between the positions. In contrast, Lugon-Moulin et al. (2004) reported clearly higher concentrations in bottom than in top leaves. Root Cd concentrations on the other hand, did not respond to the sulphur treatment in our field experiment. As a result, the leaf/root Cd concentration ratio increased from 5.5 in the control to 7.2 in the sulphur treatment. This suggests that the sulphur treatment stimulated the xylem loading of Cd. Cellular detoxification in the roots generally includes both compartmentalization of Cd complexes and xylem loading (Clemens et al., 2002).

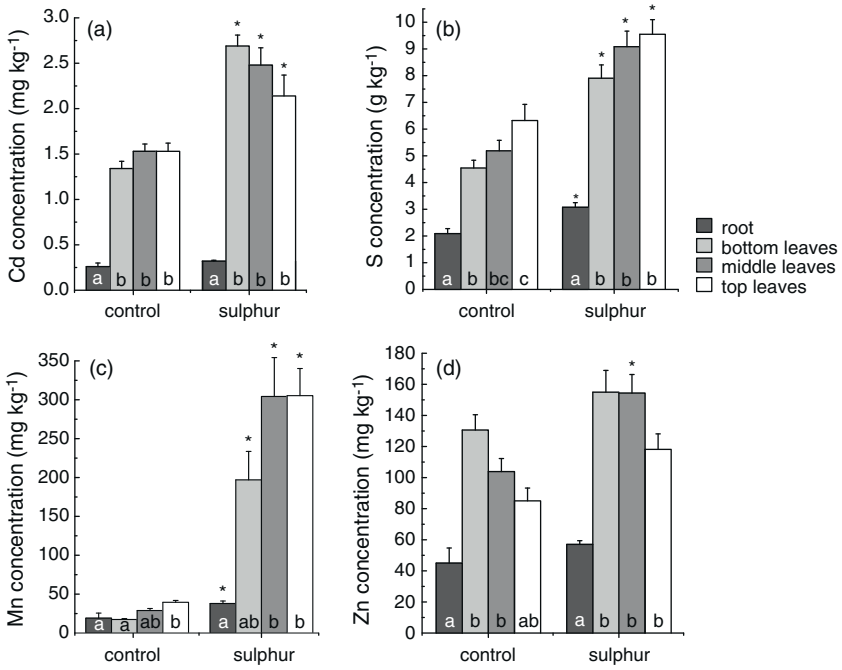

Fig. 1. Concentrations of Cd (a), S (b), Mn (c) and Zn (d) in field-grown tobacco leaves and roots in control and sulphur treatments. Letters indicate significant differences between plant parts within a treatment; asterisks indicate significant differences between treatments for the respective plant parts.

The plant S concentrations were significantly increased by the increased sulphur supply (Fig. 1). However, the ratio between leaf and root S concentrations was not affected. In each treatment, the root S concentration was less than half that of the leaf concentration, indicating that the S taken up by the roots was effectively translocated into the shoots. As in the case of Cd, the increased concentration of EDTA-soluble Mn and $NaNO_3$-soluble Zn in the S-treated soil were associated with enhanced accumulation of these elements in the shoots (Fig. 1). The increase in Zn accumulation was marginal and significant only in younger leaves. However, leaf Mn concentrations increased by an order of magnitude. In the roots the concentration of this element was doubled by the sulphur treatment, whereas the concentration of Zn was not significantly increased. The sulphur treatment also increased the concentration of Mg from a mean leaf concentration of $4.34 \pm 0.16\,g\,kg^{-1}$ to $5.18 \pm 0.08\,g\,kg^{-1}$, although no change was observed in the EDTA-extractable Mg concentration of the soil (Table 2). The root Mg concentration was $1.25\,g\,kg^{-1}$, with and without sulphur treatment. On the other hand, there was no change in Fe accumulation, although EDTA extractable soil Fe concentrations were significantly increased by the S-treatment. For Fe we measured 140 ± 34 mg in the roots and 81 ± 6 mg in the shoots per kg dry weight, pooled over both treatments (i.e. with and without sulphur application). In addition, no treatment effects on Cu accumulation were observed. The concentration in roots and shoots were $20.1 \pm 2.6\,mg\,kg^{-1}$ and $26.0 \pm 1.3\,mg\,kg^{-1}$, respectively, in both treatments.

3.3. Gene expression

In the control plants none of the genes involved in Cd transport and detoxification was expressed in the roots (Fig. 2a). However, most of them were constitutively expressed in the shoots (Fig. 2b). The sulphur treatment induced the expression of *NtMRP_a*, *NtATM_a*, and *NtPDR_a* also in the roots. The activation of the ABC transporters in the roots was likely linked to heavy metal transport and may explain the increased root-shoot translocation of Cd that was indicated by the increased shoot/root Cd concentration ratio.

In the leaves, *NtMRP_a* and *NtATM_a* were constitutively expressed in all positions with and without sulphur treatment. This is in agreement with studies of other authors who found no changes in shoot *AtMRP3* and *AtATM3* expression upon addition of Cd to the nutrient solution (Bovet et al., 2003; Kim et al., 2006). *NtPDR_a* expression in leaves showed considerable variation between replicates, independently of treatment and leaf age (or position along the stem), suggesting that *NtPDR_a* responded to local stress conditions in the field, e.g. pathogens, and not to S treatment or Cd uptake. Stein et al. (2006) reported that AtPDR8 transporter in the leaves of *A. thaliana* also play a role in pathogen infection.

In the roots of the control plants we detected no expression of the genes involved in sulphur uptake, transport or assimilation. Only the house-keeping gene *NtEF_a* was clearly expressed. Whereas *NtLAST* was not expressed at all (data not shown), *NtSultr_a*, *NtAPR_a*, and *NtAPR_b* were strongly expressed in the shoots of the control plants. In the sulphur treatment all three genes were up-regulated in the roots, while *NtAPR_a* and *NtAPR_b* tended to be down-regulated in the bottom leaves (in 1–2 out of 3 replicates in each case). It may be noteworthy that this tendency increased with the leaf S content (data not shown). The observed increases in root gene expression caused by the sulphur treatment agree with findings reported by Herbette et al. (2006) and Weber et al. (2006). As *NtSultr1* was up-regulated in the roots of our tobacco plants, and Herbette et al. (2006) found a strong up-regulation of *Sultr1;1* expression in *Arabidopsis* exposed to elevated Cd concentrations, while *Sultr1;2* remained unaffected by Cd, *NtSultr_a* seems to be more closely related to *AtSultr1;1* than to *AtSultr1;2*.

The sulphur treatment only marginally induced *NtGSHI* expression in the roots, while there was no expression of root *NtGSHII*, neither in the control, nor in the sulphur treatment. It is possible that the base level of glutathione was either sufficient for Cd detoxification, or glutathione production was stimulated otherwise. May et al. (1998b) and Herbette et al. (2006) related increased GSHI activity in response to Cd exposure to post-transcriptional up-regulation of *GSHI*. Both genes were more strongly expressed in younger (top) than in the older (bottom) leaves independent of the

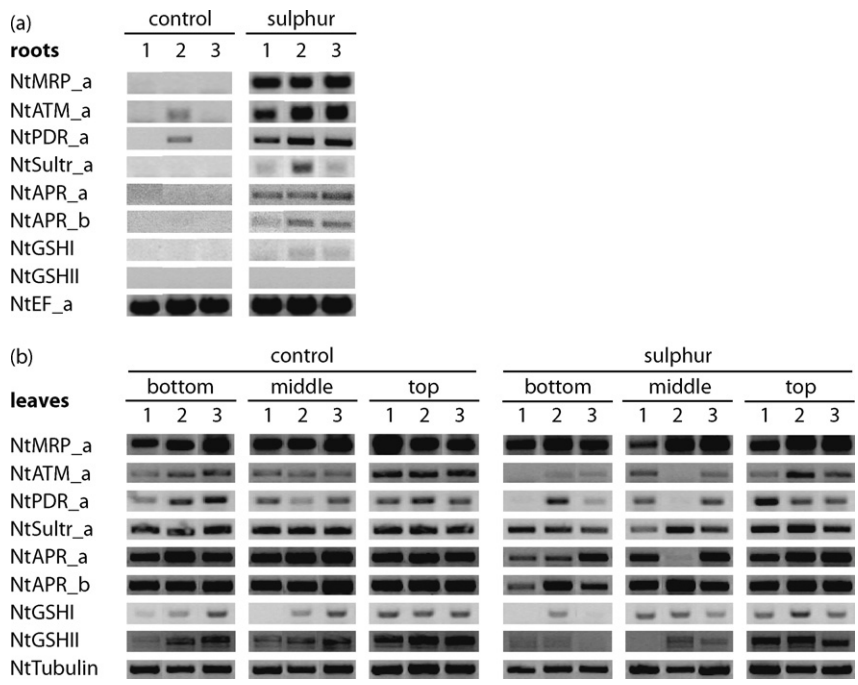

Fig. 2. Gene expression (qualitative RT-PCR) in roots (a) and shoots (b) of tobacco plants grown in a Cd-contaminated field after 6 years of annually repeated sulphur amendments (sulphur) and under control conditions (control). Genes possibly coding for cadmium (Cd) transport (*NtMRP_a*, *NtATM_a* and *NtPDR_a*), transporters and enzymes involved in sulphate assimilation (*NtSultr_a*, *NtAPR_a*, *NtAPR_b*) and in glutathione synthesis (*NtGSHI*) were tested. These genes as well as the house-keeping elongation factor (*NtEF_a*) and tubulin (*NtTubulin*) were amplified by PCR. Results for three replicate plants (1–3 in each treatment) are shown. Each plant was divided into roots and bottom, middle and top leaves.

treatment. The *NtNAS_a* gene was not expressed in both root and shoot (data not shown).

4. Discussion

In Table 4 we compared the expression of Cd-induced genes between tobacco cultivated in sulphur amended soil with observations on gene expression in other studies as listed in Table 1. The expression of the genes in the roots of our plants mostly agreed with the reported results. Exceptions were *NtLAST*, *NtGSHII* and *NtNAS_a*, which were not expressed at all.

The strong up-regulation effects in the roots on genes that are putatively involved in Cd detoxification indicate that the ATPase and the ABC transporters might have been activated by both the increased Cd concentration and the increased availability of sulphur. The influence of sulphur supply on the expression of these genes in plants has received scant attention. Kolukisaoglu et al. (2002) and Glombitza et al. (2004) found that leaf *AtMRP3* was not only inducible by Cd, but also by other stressors such as xenobiotics and oxidative stress. Considering that an increased S supply would enhance glutathione production and that glutathione is involved in the reduction of oxidative stress in plants (May et al., 1998a), the Cd effect on *NtMRP_a* may have been partially compensated by the increase in soil S concentration in the sulphur treatments.

In the leaves, however, most of the genes studied here were either not affected or down-regulated, as in the case of the bottom leaves, while up-regulation or no effects were expected according to the literature. The down-regulation of the genes in the bottom leaves in our plants may have been due to a senescence effect. In other studies the experimental plants were harvested well before maturity and not divided into bottom, middle and top leaves. The bottom leaves of our plants showed first signs of senescence at harvest. Down-regulation has been reported for many genes in senescent leaves (Quirino et al., 2000). Down-regulation of genes involved in sulphur uptake and assimilation might also result from changes in sulphur supply. Many genes involved in the acquisition and assimilation of sulphate from soil are known to respond to the S status of a plant (Leustek and Saito, 1999; Hawkesford, 2000; Saito, 2000). Sulphur starvation has been found to activate sulphur acquisition and assimilation mechanisms in the same way as the exposure of plants to Cd (Yoshimoto et al., 2002; Herbette et al., 2006). The expression of *AtAPR* was found to be up-regulated in the roots and shoots of *A. thaliana* under sulphur deficiency (Takahashi et al., 1997; Hirai et al., 2003). Little is known about the effects of excessive sulphur supply, a condition that was more likely the case in our field experiment than sulphur deficiency, on gene expression. An excess of SO_4^{2-} in nutrient solution was found to decrease the activity of APS sulphurylase (Brunold et al., 1987). This enzyme was recently found to be identical with APR (Suter et al., 2000). Thus, it is plausible that the expression of *NtAPR_a* and *NtAPR_b* became down-regulated in older leaves of the field-grown tobacco plants in our experiment in response to an over-supply of sulphate. This is in

Table 4
Summary of the effects of sulphur application on gene expression in roots and shoots of field-grown tobacco plants in comparison to expectation based on the literature discussed in the text.

Gene	Observed effects		Expected Cd effect	
	Root	Shoot	Root	Shoot
NtMRP_a (*AtMRP3*)	↑	→	↑	→
NtATM_a (*AtATM3*)	↑	→	↑	→
NtPDR_a (*AtPDR8*)	↑	→	↑	↑
NtSultr_a (*AtSultr1*)	↑	→	○	○
NtLAST (*BnLAST*)	■	■	○	○
NtAPR_a (*AtAPR2*)	↑	↓[a]	↑	↑
NtAPR_b (*AtAPR3*)	↑	↓[a]	↑	↑
NtGSHI (*AtGSHI*)	↑	→	↑	○
NtGSHII (*AtGSHII*)	■	→	→	○
NtNAS_a (*AtNAS3*)	■	■	↑	→

↑: up-regulated; ○: ambiguous; ↓: down-regulated; ■: not expressed; →: unchanged.

[a] In the older leaves.

line with the observation that down-regulation tended to increase with leaf S content.

Gene expression may also have been affected by the increase in the solubility of Fe, Mg, Mn, and Zn. There are few studies investigating Zn, Fe and Mg effects on the expression of the genes that were under study here. While, e.g. Xiang and Oliver (1998) and Kim et al. (2006) *NtMRP_a* and *NtATM_a* found no effects of Zn on the expression of *AtGSHI*, *AtGSHII* and *AtATM3* in *A. thaliana*, Bovet et al. (2003) found a slight up-regulation effect of Zn on *AtMPR3* expression. Similar to Cd, Zn is known to up-regulate the expression of *Sultr1;1* in roots (Ernst et al., 2008). Zinc may thus also have been involved in the up-regulation of these genes in the S-treatment of our field experiment. Magnesium effects on the expression of the investigated genes have not been reported. The detoxification of cells through ABC transporters is mediated by MgATP-energized GS-conjugate transport (Rea, 1999, 2007) but this does not necessarily indicate that there are changes in the gene expression when the soluble Mg concentration in the soil changes. However, the increase in plant Fe was not significant, and the increases in plant Zn and Mg were not significant in all leave fractions, either. It is thus unlikely that these metals had a substantial effect on gene expression. There are no reports of the effects of Mn on the expression of the studied genes. Manganese was the element of which the accumulation was increased most by the sulphur treatment.

The decrease in soil pH itself might have an effect. Changes in rhizosphere pH can affect a wide range of proteins such as ABC-transporters (Reeve et al., 2004) or enzymes of bacteria and plants involved in sulphur assimilation (APR) (Setya et al., 1996). Sulphate transporters are pH-dependent (Saito, 2004), and glutathione production in *Escherichia coli* bacteria was found to decrease with external pH (Smirnova et al., 2003). The impacts of changes in soil pH on gene expression have not been tested for the genes that were studied here.

5. Conclusion

The up-regulation observed in the expression of most of the investigated genes in the roots agreed well with findings reported from hydroponic experiments in the literature. However, there were also differences, in particular the lack of effects on gene expression in the shoots. These findings indicate that factors other than increased exposure to Cd played an important role, in particular the higher concentrations of S and soluble Mn, Fe and Zn in the soil, and the lower soil pH. The leaves investigated here were obtained from mature plants, while hydroponically grown plants are usually harvested at an early stage. Nevertheless, our results indicate that hydroponic or agar experiments are useful predictors of gene expression effects that may be expected in field conditions. In order to become more comparable to field situations, future laboratory studies on the expression of genes expected to respond to environmental stresses should also look into the effects of combinations of different stress factors and investigate such effects also at later developmental stages.

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References

Bergmann, W., 1993. Ernährungsstörungen bei Kulturpflanzen, 3rd ed. Gustav Fischer Verlag, Jena/Stuttgart.

Bovet, L., Eggmann, T., Meylan-Bettex, M., Polier, J., Kammer, P., Marin, E., Feller, U., Martinoia, E., 2003. Transcript levels of *AtMRPs* after cadmium treatment: induction of *AtMRP3*. Plant Cell Environ. 26, 371–381.

Bovet, L., Rossi, L., Lugon-Moulin, N., 2006. Cadmium partitioning and gene expression studies in *Nicotiana tabacum* and *Nicotiana rustica*. Physiol. Plant. 128, 466–475.

Brunold, C., Suter, M., Lavanchy, P., 1987. Effect of high and low sulfate concentrations on adenosine 5′-phosphosulfate sulfotransferase activity from *Lemna minor*. Physiol. Plant. 70, 168–174.

Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., Baker, A.J.M., 1997. Phytoremediation of soil metals. Curr. Opin. Biotechnol. 8, 279–284.

Clemens, S., Palmgren, M.G., Krämer, U., 2002. A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci. 7, 309–315.

Dybczynski, R., Polkowska-Motrenko, H., Samczynski, Z., Szopa, Z., 1998. Virginia Tobacco Leaves (CTA-VTL-2)—new polish CRM for inorganic trace analysis including microanalysis. Anal. Bioanal. Chem. 360, 384–387.

Ernst, W.H.O., Krauss, G.J., Verkleij, J.A.C., Wesenberg, D., 2008. Interaction of heavy metals with the sulphur metabolism in angiosperms from an ecological point of view. Plant Cell Environ. 31, 123–143.

Fässler, E., Robinson, B.H., Gupta, S.K., Schulin, R., 2010a. Uptake and allocation of plant nutrients and Cd in maize, sunflower and tobacco growing on contaminated soil and the effect of soil conditioners under field conditions. Nutr. Cycl. Agroecosyst..

Fässler, E., Robinson, B.H., Stauffer, W., Gupta, S.K., Papritz, A., Schulin, R., 2010b. Phytomanagement of metal-contaminated agricultural land using sunflower, maize and tobacco. Agric. Ecosyst. Environ. 136, 49–58.

Glombitza, S., Dubuis, P.H., Thulke, O., Welzl, G., Bovet, L., Götz, M., Affenzeller, M., Geist, B., Hehn, A., Asnaghi, C., Ernst, D., Seidlitz, H.K., Gundlach, H., Mayer, K.F., Martinoia, E., Werck-Reichhart, D., Mauch, F., Schäffner, A.R., 2004. Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. Plant Mol. Biol. 54, 817–835.

Hall, J.L., Williams, L.E., 2003. Transition metal transporters in plants. J. Exp. Bot. 54, 2601–2613.

Hawkesford, M.J., 2000. Plant responses to sulphur deficiency and the genetic manipulation of sulphate transporters to improve S-utilization efficiency. J. Exp. Bot. 51, 131–138.

Heiss, S., Schäfer, H.J., Haag-Kerwer, A., Rausch, T., 1999. Cloning sulfur assimilation genes of *Brassica juncea* L.: cadmium differentially affects the expression of a putative low-affinity sulfate transporter and isoforms of ATP sulfurylase and APS reductase. Plant Mol. Biol. 39, 847–857.

Herbette, S., Taconnat, L., Hugouvieux, V., Piette, L., Magniette, M.L.M., Cuine, S., Auroy, P., Richaud, P., Forestier, C., Bourguignon, J., Renou, J.P., Vavasseur, A., Leonhardt, N., 2006. Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. Biochimie 88, 1751–1765.

Hirai, M.Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noji, M., Saito, K., 2003. Global expression profiling of sulfur-starved *Arabidopsis* by DNA macroarray reveals the role of *O*-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J. 33, 651–663.

Howden, R., Goldsbrough, P.B., Andersen, C.R., Cobbett, C.S., 1995. Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol. 107, 1059–1066.

Kayser, A., Wenger, K., Keller, A., Attinger, W., Felix, H.R., Gupta, S.K., Schulin, R., 2000. Enhancement of phytoextraction of Zn, Cd, and Cu from calcareous soil: the use of NTA and sulfur amendments. Environ. Sci. Technol. 34, 1778–1783.

Kim, D.Y., Bovet, L., Kushnir, S., Noh, E.W., Martinoia, E., Lee, Y., 2006. AtATM3 is involved in heavy metal resistance in *Arabidopsis*. Plant Physiol. 140, 922–932.

Kim, D.Y., Bovet, L., Maeshima, M., Martinoia, E., Lee, Y., 2007. The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Plant J. 50, 207–218.

Kolukisaoglu, H.Ü., Bovet, L., Klein, M., Eggmann, T., Geisler, M., Wanke, D., Martinoia, E., Schulz, B., 2002. Family business: the multidrug-resistance related protein (MRP) ABC transporter genes in *Arabidopsis thaliana*. Planta 216, 107–119.

Kopriva, S., Koprivova, A., 2004. Plant adenosine 5′-phosphosulphate reductase: the past, the present, and the future. J. Exp. Bot. 55, 1775–1783.

Leustek, T., Saito, K., 1999. Sulfate transport and assimilation in plants. Plant Physiol. 120, 637–643.

Lugon-Moulin, N., Zhang, M., Gadani, F., Rossi, L., Koller, D., Krauss, M., Wagner, G.J., 2004. Critical review of the science and options for reducing cadmium in tobacco (*Nicotiana tabacum* L.) and other plants. Adv. Agron. 83, 111–180.

May, M.J., Vernoux, T., Leaver, C., Van Montagu, M., Inzé, D., 1998a. Glutathione homeostasis in plants: implications for environmental sensing and plant development. J. Exp. Bot. 49, 649–667.

May, M.J., Vernoux, T., Sánchez-Fernández, R., Van Montagu, M., Inzé, D., 1998b. Evidence for posttranscriptional activation of γ-glutamylcysteine synthetase during plant stress responses. Proc. Natl. Acad. Sci. U.S.A. 95, 12049–12054.

NC State University, 2008. Tobacco genome initiative. http://tgi.ncsu.edu.

Plaza, S., Bovet, L., 2008. Cadmium detoxification in plants: involvement of ABC transporters. In: Prasad, M.N.V. (Ed.), Trace Elements as Contaminants and Nutrients: Consequences in Ecosystems and Human Health. Wiley, Weinheim, pp. 449–469.

Quirino, B.F., Noh, Y.S., Himelblau, E., Amasino, R.M., 2000. Molecular aspects of leaf senescence. Trends Plant Sci. 5, 278–282.

Rea, P.A., 1999. MRP subfamily ABC transporters from plants and yeast. J. Exp. Bot. 50, 895–913.

Rea, P.A., 2007. Plant ATP-binding cassette transporters. Annu. Rev. Plant Biol. 58, 347–375.

Reeve, W.G., Tiwari, R.P., Guerreiro, N., Stubbs, J., Dilworth, M.J., Glenn, A.R., Rolfe, B.G., Djordjevic, M.A., Howieson, J.G., 2004. Probing for pH-regulated proteins in

Sinorhizobium medicae using proteomic analysis. J. Mol. Microbiol. Biotechnol. 7, 140–147.
Saito, K., 2000. Regulation of sulfate transport and synthesis of sulfur-containing amino acids. Curr. Opin. Plant Biol. 3, 188–195.
Saito, K., 2004. Sulfur assimilatory metabolism. The long and smelling road. Plant Physiol. 136, 2443–2450.
Schäfer, H.J., Haag-Kerwer, A., Rausch, T., 1998. cDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy-metal accumulator *Brassica juncea* L.: evidence for Cd-induction of a putative mitochondrial γ-glutamylcysteine synthetase isoform. Plant Mol. Biol. 37, 87–97.
Scheffer, F., Schachtschabel, P., Blume, H.-P., 2002. Lehrbuch der Bodenkunde, 15th ed. Spektrum Akademischer Verlag, Heidelberg.
Setya, A., Murillo, M., Leustek, T., 1996. Sulfate reduction in higher plants: molecular evidence for a novel 5′-adenylylsulfate reductase. Proc. Natl. Acad. Sci. U.S.A. 93, 13383–13388.
Sharma, S.S., Dietz, K.J., 2006. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J. Exp. Bot. 57, 711–726.
Smirnova, G.V., Torkhova, O.A., Oktyabr'skii, O.N., 2003. The status and the role of glutathione under disturbed ionic balance and pH homeostasis in *Escherichia coli*. Mikrobiology 72, 542–547.
Smith, F.W., Ealing, P.M., Hawkesford, M.J., Clarkson, D.T., 1995. Plant members of a family of sulfate transporters reveal functional subtypes. Proc. Natl. Acad. Sci. U.S.A. 92, 9373–9377.
Stein, M., Dittgen, J., Sánchez-Rodríguez, C., Hou, B.H., Molina, A., Schulze-Lefert, P., Lipka, V., Somerville, S., 2006. *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18, 731–746.
Sun, X., Sun, X.M., Yang, Z.M., Li, S.Q., Wang, J., Wang, S.H., 2005. Expression of *Brassica napus* L. γ-Glutamylcysteine synthetase and low- and high-affinity sulfate transporters in response to excess cadmium. J. Integr. Plant Biol. 47, 243–250.
Sun, X.M., Lu, B., Huang, S.Q., Mehta, S.K., Xu, L.L., Yang, Z.M., 2007. Coordinated expression of sulfate transporters and its relation with sulfur metabolites in *Brassica napus* exposed to cadmium. Bot. Stud. 48, 43–54.
Suter, M., von Ballmoos, P., Kopriva, S., den Camp, R.O., Schaller, J., Kuhlemeier, C., Schürmann, P., Brunold, C., 2000. Adenosine 5′-phosphosulfate sulfotransferase and adenosine 5′-phosphosulfate reductase are identical enzymes. J. Biol. Chem. 275, 930–936.
Takahashi, H., Yamazaki, M., Sasakura, N., Watanabe, A., Leustek, T., De Almeida Engler, J., Engler, G., Van Montagu, M., Saito, K., 1997. Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U.S.A. 94, 11102–11107.
Tichý, R., Fajtl, J., Kužel, S., Kolář, L., 1997. Use of elemental sulphur to enhance a cadmium solubilization and its vegetative removal from contaminated soil. Nutr. Cycl. Agroecosyst. 46, 249–255.
Tommasini, R., Vogt, E., Fromenteau, M., Hörtensteiner, S., Matile, P., Amrhein, N., Martinoia, E., 1998. An ABC-transporter of *Arabidopsis thaliana* has both glutathione-conjugate and chlorophyll catabolite transport activity. Plant J. 13, 773–780.
Wang, T.L., Wu, M., 2006. An ATP-binding cassette transporter related to yeast vacuolar ScYCF1 is important for Cd sequestration in *Chlamydomonas reinhardtii*. Plant Cell Environ. 29, 1901–1912.
Weber, M., Trampczynska, A., Clemens, S., 2006. Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd^{2+}-hypertolerant facultative metallophyte *Arabidopsis halleri*. Plant Cell Environ. 29, 950–963.
Wenger, K., Kayser, A., Gupta, S.K., Furrer, G., Schulin, R., 2002. Comparison of NTA and elemental sulfur as potential soil amendments in phytoremediation. Soil Sediment Contam. 11, 655–672.
Xiang, C.B., Oliver, D.J., 1998. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10, 1539–1550.
Ye, J., McGinnis, S., Madden, T.L., 2006. BLAST: improvements for better sequence analysis. Nucleic Acids Res. 34, W6–W9.
Yoshimoto, N., Takahashi, H., Smith, F.W., Yamaya, T., Saito, K., 2002. Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. Plant J. 29, 465–473.
Zhu, Y.L., Pilon-Smits, E.A.H., Jouanin, L., Terry, N., 1999. Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. Plant Physiol. 119, 73–79.