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ORIGINAL ARTICLE

Sulfate influx transporters in *Arabidopsis thaliana* are not involved in arsenate uptake but critical for tissue nutrient status and arsenate tolerance

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Abstract

Main conclusion **Arsenic, a non-nutrient metalloid is toxic to plants but many details on the physiology of plant adaptation to arsenic stress are not well understood. This work provides new insights about the role of sulfur assimilation in arsenate uptake, growth and arsenic tolerance. Research reported here indicates that two high affinity sulfate transporters in *Arabidopsis thaliana* are not involved in root uptake of arsenate. Further this study revealed that sulfate status influenced thiol levels, elemental nutrients, growth and arsenate tolerance.**

The hypothesis that arsenate may be transported via sulfate transporters, SULTR1;1 and SULTR1;2 in *Arabidopsis,* was tested. The double mutant of s*ultr1;1* s*ultr1;2* exhibited significantly less growth than the wild-type or the single mutants. The double mutant's sulfur content was significantly lower than the wild-type but the single mutants were similar to the wild-type confirming the redundant functions of *SULTR1;1* and *SULTR1;2*. Gene expression analyses indicated that the double mutant's sulfate uptake could be explained by the expressions of *SULTR1;3*, *SULTR2;1*, and *SULTR2;2* in its roots. Following arsenate supply to the roots, the double mutant accumulated significantly less arsenic in the roots and the shoots than did the single mutants and the wild-type. The double mutant accumulated significantly less potassium and phosphorus also. ^{35}S sulfate supplied to wild-type or double mutant roots showed that sulfate uptake was not inhibited by arsenate. Taken together, these results indicate that root uptake of arsenate is probably not via sulfate transporters, but the poor growth of the double mutant of s*ultr1;1* and s*ultr1;2* was due to its poor sulfate status and decreased levels of thiols, which had pleiotropic effects on the root uptake and translocation of potassium and phosphorus and arsenic tolerance.

Keywords *Arabidopsis* · Arsenic · Gene expression · Mutants · Phosphorus · Potassium · Sulfur nutrition

Abbreviations

As(V)	Arsenate
As(III)	Arsenite
DTNB	5,5' Dithiobis (2-nitrobenzoic acid)
ICP-AES	Inductively coupled plasma atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
RT-PCR	Reverse transcription-polymerase chain reaction
SULTR	sulfate transporter

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Introduction

Arsenic (As) is a nonessential and toxic metalloid to living organisms. It is found in the environment commonly in two inorganic forms: arsenate [As (V)] and arsenite [As (III)]. Natural processes like weathering of rocks and volcanic emissions, as well as anthropogenic sources such as fossil fuel combustion, mining and smelting of ores, and application of arsenical pesticides, are the main sources of As contamination in the environment (Ali et al. 2009).

Long-term exposure to As causes skin diseases and cancers in humans. In most As-contaminated areas, As intake by humans is derived from groundwater that is naturally contaminated with As (Chowdhury 2000). This is especially serious in West Bengal, India, and Bangladesh. The major ingestion pathway of As is drinking As-contaminated water, followed by eating foods grown with contaminated water. Crops cultivated with As-containing groundwater accumulate As in their edible parts (Meharg 2004). Rice is the major crop in these areas and has great propensity to accumulate high levels of As in its grains.

The inorganic form of arsenic is more toxic than the organic forms (Kamiya et al. 2009). These inorganic forms As(V) and As(III) are interconvertible, depending on the redox condition of the medium. Plants take up As mainly as arsenate, the dominant form of phytoavailable As in aerobic soils. Upon absorption, most arsenate is rapidly reduced to arsenite, due to an arsenate reductase activity. Hence, the arsenate cytoplasmic concentration is generally not high enough to exert toxicity (Dho et al. 2010).

Both As species interfere with various metabolic pathways. Arsenate as an analogous chemical to phosphate, may replace phosphate in the ATP and in various phosphorylation reactions, leading to the disruption of the energy flow in cells. The toxicity of arsenite results from its reaction with sulfhydryl groups in many enzymes and tissue proteins interfering with their functions (Meharg and Hartley-Whitaker 2002).

Excessive uptake of As by crop plants may present a food safety problem. This is exemplified by findings that *Oryza sativa* is particularly efficient in As uptake from paddy soil, leading to accumulation in rice grain at concentrations that may pose a health risk (Zhu et al. 2008). Understanding how plants take up and metabolize As is important for developing mitigation measures to counter the problem of food chain contamination by As (Zhao et al. 2009).

In plants, a number of studies have shown the physiological properties and molecular mechanisms of As uptake. In rice, uptake kinetics of arsenate and arsenite follow the Michaelis–Menten equation, suggesting the presence of a transporter (Kamiya et al. 2009). In *Arabidopsis thaliana*, phosphate transporters, *PHT1;1* and *PHT1;2,* have been implicated for As(V) uptake (Quaghebeur and Rengel 2004; Shin et al. 2004; Catarecha et al. 2007). Although it has been shown that phosphate transporters also take up As(V) in plant roots, phosphate transporter mutations did not completely prevent As uptake (Catarecha et al. 2007), suggesting that other transporters could be involved in As uptake in plants as well. It is logical to argue that sulfate transporters may also be involved in As uptake considering the positions occupied by As, Se, P, and S on the periodic table. The molecular characteristics of high-affinity sulfate transporters (SULTRs) have been studied extensively in *Arabidopsis*. The *Arabidopsis* SULTR1;1 and SULTR1;2 sulfate transporter proteins show 70–84 % identity at the amino acid sequence level. They are localized in the epidermis and cortex of roots, suggesting physiological roles in the initial uptake of sulfate (Takahashi et al. 2000; Yoshimoto et al. 2002; Maruyama-Nakashita et al. 2004a, b). In addition, *Arabidopsis* SULTR1;2 was suggested to play a regulatory role in response to sulfur-nutrient status (Zhang et al. 2014).

Both transporters were upregulated under sulfate deficiency (-S), and studies have shown that a compensatory role exists between them when either of these sulfate transporters is knocked out (Maruyama-Nakashita et al. 2003). Further research showed that *sultr1;1* and *sultr 1;2* plants displayed unequal redundancy for selenate tolerance and selenate accumulation (Shibagaki et al. 2002; Barberon et al. 2008), indicating that *SULTR1;1* and *SULTR1*;*2* are involved in both sulfate and selenate uptakes.

It is therefore the aim of this research to evaluate the involvement of sulfate transporters, *SULTR1;1* and *SULTR1;2,* in arsenate uptake using *A. thaliana* as a model species.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana genotypes used for this study had 'Columbia' background. The wild-type (WT) accession and the single mutants [*sultr1;1* (SALK_093256) and *sultr1;2* (SALK_133651)] were from the *Arabidopsis* Information Resource (TAIR), while the double mutant *sultr1;1-sultr1;2* was obtained from the Institute National de la Recherche Agronomique, Universite Montpellier II, France.

For the hydroponic experiments, two nutrient solutions based on *Arabidopsis*-standardized, Hoagland's nutrient solution (Hoagland and Arnon 1941) modified from Heeg et al. (2008) were used as a growth medium. The first is sulfate-containing solution (+S): 2 mM $Ca(NO_3)_2{\cdot}4\ H_2O$, 2 mM KNO_3, 0.5 mM NH_4NO_3, 0.5 mM $MgSO_4{\cdot}7\ H_2O$,

0.25 mM KH_2PO_4, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnCl_2{\cdot}4H_2O$, 2 μM $ZnSO_4{\cdot}7\ H_2O$, 0.5 μM $CuSO_4{\cdot}5H_2O$, 0.15 μM $CoCl_2{\cdot}6H_2O$, 0.075 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 40 μM Fe-EDTA. The second medium was sulfate free (-S) and contained: 2 mM $Ca(NO_3)_2{\cdot}4\ H_2O$, 2 mM KNO_3, 0.5 mM NH_4NO_3, 0.5 mM $MgCl_2$, 0.25 mM KH_2PO_4, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnCl_2$, $4H_2O$, 2 μM $ZnCl_2$, 0.5 μM $CuCl_2$, 0.15 μM $CoCl{\cdot}6H_2O$, 0.075 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 40 μM Fe-EDTA. The pH of the nutrient solutions was 5.9 (initially adjusted with 5 N KOH or 5 N HCl). Solutions were changed once a week to avoid the excessive depletion of any particular ion. The plants were grown under 16-h-light photoperiod at 150 μmol s^{-1} m^{-2} supplied by cool white fluorescent lights at 24/20 °C day/night temperatures.

Seeds of the four genotypes were germinated on plugs of rockwool partially submerged in aerated half-strength Hoagland's solution containing sulfate, and seedlings were allowed to grow in this medium for 3 weeks. After this period, a set of uniform seedlings was starved of sulfate for 10 days by replacing the nutrient solution with a modified Hoagland's solution devoid of sulfate. Then seedlings, from sulfate-containing and sulfate-depleted media, were exposed to 0.1 mM arsenate (supplied as Na_2HAsO_4) for 24 h. At the end the experimental period, to remove arsenic adsorbed on the roots, the plants were transferred into distilled water and rinsed before harvesting with ice cold phosphate buffer (1 mM Na_2HPO_4, 10 mM MES, and 0.5 mM $Ca(NO_3)_2$, pH 5.7). Then all were washed once again with distilled water and blotted dry with tissue paper. Then, the harvested plants were separated into roots and shoots in three groups (three replicates each). For biomass determination, one group was placed in paper bags and weighed after oven drying at 65 °C for 3 days. The other two groups were frozen directly in liquid N_2 and stored at −80 °C for total RNA extraction and the analysis of total thiols.

For agar media experiment, seeds were surface sterilized using 20 % (v/v) commercial bleach and 0.01 % (v/v) Silvet™ for 10 min, rinsed five times with sterile water, and chilled for 2 days at 4 °C. The seeds were then transferred using a pipette onto the growth media solidified in Petri plates. The medium was made of 0.5 × Murashige and Skoog (MS) salts (Murashige and Skoog 1962) with 2 % (w/v) sucrose, pH was adjusted to 5.7, and solidified using 0.8 % (w/v) agar. Seedlings were grown on vertically placed plates at 23–24 °C with light intensity of 80 μmol m^{-2} s^{-1} and 16:8 h light/dark photoperiods.

Semiquantitative reverse transcription (RT)-PCR analysis

To verify *A. thaliana* double mutant *sultr1;1, -sultr1;2* and to examine the expression of SULTRs in roots, total RNA was extracted from frozen root samples of WT and double mutant plants grown under hydroponic conditions. RNA was extracted using RNeasy Mini Kit with on-column DNase digestion (Qiagen, Cat #74904). Approximately 2 μg of total RNA was used for RT-PCR reactions. RNA RT and DNA amplification by PCR were done using one-step RT-PCR kit (Takara Bio Inc., Cat #RR055A) based on a 30-cycle program. Each cycle consisted of a denaturation step at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 2 min. Five genes coding for sulfate transporters were tested in this study, including *SULTR1;1*, *SULTR1;2*, *SULTR1;3*, *SULTR2;1*, and *SULTR2;2*. *ACTIN2* was used as a control. Information about the primers is included in Table 1.

Determination of total metal contents

About 50 mg of oven-dried root and shoot samples, grown under hydroponic conditions, were added into glass vials containing 3 mL of concentrated HNO_3 and heated at 105 °C in the digester block for 15 h. After cooling, 2 mL of 30 % (v/v) H_2O_2 was added. Then, the samples were filtered through 0.2 μm membrane and diluted with 1 % HNO_3. Total As, K, and P in the digests were determined using inductively coupled plasma-mass spectrometry (ICP-MS, PerkinElmer 5300DV, Waltham, MA, USA) using EPA method 2007 (Chen and Ma 1998). The total sulfur content of the extract was analyzed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Zhao et al. 1994). Blanks and positive controls were included for quality assurance.

Table 1 Oligonucleotide sequence of primers used in RT-PCR reactions

Primer name	PCR product size (bp)	Forward primer (5′–3′)	Reverse primer (5′–3′)
SULTR1;1	468	CACCAGTCGTTCGTCAGAGA	TGGCAGTGAAGACAAGTCGG
SULTR1;2	536	AGTCTCCTGTTCTGTTTGCAGAG	GTGAAGGCAAGGCGGAGATA
SULTR1;3	402	CTCCGCTTAGCCTTCACCTCGCT	CTTGTCGGCTCGGGTTATGT
SULTR2;1	528	TTGCCAATGCCAGTTCA	ACAAACACACCCATATCGATCCT
SULTR2;2	606	TTCCACTCGCTTCACCATCC	AAGCGCCGAGAGGATTATGG
ACTIN2	239	TGGGCAAGTCATCACGATTGGT	TGCTTGGTGCAAGTGCTGTGAT

Sulfate uptake by *A. thaliana* wild type and the double mutant

WT and double mutant for *sultr1;1* and *sultr1;2* of *A. thaliana* were grown in hydroponics under sulfate-sufficient condition. After one month of growth under 16 h light cycles at room temperature, the plants were moved to sulfate-free medium for 7 days. Roots from these plants were sampled for uptake experiments using ^{35}S. Sodium sulfate (^{35}S, 3.7×10^{10} Bq mg^{-1} (43 Ci/mg)-specific activity from American Radiolabeled Chemicals Inc., MO, USA) was used at the rate of 3700 Bq (1 μCurie) per excised root sample (~50–150 mg). Labeled tracer sodium sulfate was incubated with the root sample in a solution containing 0.5 mM potassium sulfate and 0, 0.5, or 1.0 mM sodium arsenate for 5 h at 28 °C on a shaker incubator. The roots were washed in 1 mM potassium sulfate solution, blotted dry, and counted using a liquid scintillation counter (Beckman). Each treatment had three replicates, and two independent experiments were done with similar results.

Evaluation of arsenic tolerance using agar media

To evaluate the tolerance levels of the four genotypes to arsenic toxicity with reference to root growth, a bioassay was used. Seeds were surface sterilized and grown on solid 0.5 × MS medium for 3 days. Then seedlings were transferred to plates containing sterile 0.5 × MS medium spiked with 0.1 mM arsenate. Plates were incubated vertically for three more days and root length of the plants was measured. Half-strength MS medium without arsenate served as control.

Determination of total thiols

Ellmańs spectrophotometric method was used for the determination of sulfhydryl (-SH) groups (Ellman 1959). The seedlings of WT, single mutants, and the double mutant were grown under hydroponic conditions. Frozen plant tissues (roots/shoots, approximately 0.1 g of FW) were ground to powder in liquid N_2. Then 1.5 mL of 0.1 M K-phosphate buffer (pH 7.4) containing 0.02 M EDTA was added, and the sample was homogenized for 5 min. After mixing using a vortex, the homogenate was centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatant (0.5 mL) was transferred to a polyethylene test tube and mixed with 1.5 mL of 0.2 M Tris buffer (pH 8.2) and 100 μL of 0.01 M 5,5́ dithiobis (2 -nitrobenzoic acid) (DTNB) prepared in 0.05 M Na-acetate. Reaction was allowed to develop for 20 min at room temperature before absorbance was measured. A sample blank (without adding DTNB) and reagent blank (without sample) were prepared and measured in the same manner. Absorbance was measured at 405 nm. Thiol contents were calculated using an extinction coefficient of 13,100 M^{-1} cm^{-1} (Nagalakshmi and Prasad 2001).

Statistical analyses

Data are presented as the means of all replicates, and error bars represent one-standard error either side of the mean. All statistical analyses were carried out using Duncan's multiple range mean comparisons at 5 % alpha using SAS statistical package.

Results

We used well-characterized mutants of high-affinity sulfate influx transporters to investigate whether sulfate transporters, SULTR1;1 and SULTR1;2, were involved in arsenate transport in *A. thaliana*. RT-PCR experiments using root total RNA confirmed the absence of expressions of *sultr1;1* and *sultr1;2* genes in the double mutant and their expression in the WT roots of plants exposed to sulfate-deplete medium (Fig. 1a). *SULTR1;1* and *SULTR1;2* primers amplified additional weak products of unknown origin in addition to the expected products of 468 and 536 bp, respectively (Fig. 1a).

No significant differences were observed in plant dry biomasses between the WT and single mutant *sultr1;1*, both under sulfate-replete or sulfate-deplete conditions (Fig. 1b). A significant reduction was recorded in terms of dry biomass accumulation in the double mutants compared to WT and single mutants, both under sulfate-replete or sulfate-deplete conditions (Fig. 1b). Double mutant plants had a mean root dry weight (DW) of 0.011 g per plant compared with the single mutants and the WT, which had mean roots of 0.026 and 0.029 g per plant, respectively when grown in hydroponics without sulfate (Fig. 1b). Similar trend was observed for plant shoots, where double mutant shoot DW reduced by about 68.5 % less than single mutants and WT (Fig. 1c). Accordingly, when sulfur content in the studied genotypes was analyzed under sulfate-depleted conditions, it was observed that the double mutant's root and shoot had 0.08 and 0.2 mg per g DW, respectively, compared to 3 and 4.8 mg per g DW in WT and 2.5 and 4.3 mg per g DW in single mutants, respectively (Fig. 2a, b).

To explain the limited amount of sulfur accumulation in the double mutant, the expression of other SULTRs was explored under sulfate deficiency in roots of the double mutant and the WT. There are 14 putative sulfate transporter genes in Arabidopsis genome, and Buchner et al. (2004) subdivided the *Arabidopsis* sulfate transporter

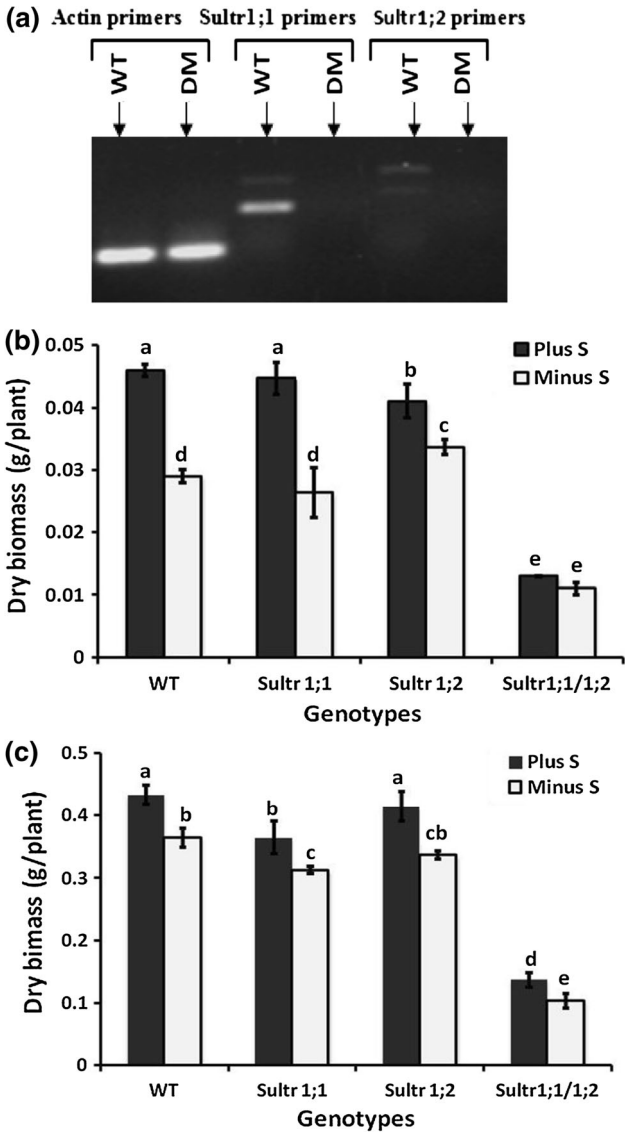

Fig. 1 Effects of sulfate deficiency on the expression of sulfate transporters and plant growth in *Arabidopsis thaliana*. **a** Verification of expression of sulfate transporters in wild-type (WT) and double mutant (*sultr1;1, sultr1;2*) using semiquantitative RT-PCR. Total RNA from the roots of WT, double mutant (DM) were used as template using primers for *ACTIN*2 (control), *SULTR1;1* and *SULTR1;2* genes. Expected PCR products were of the following sizes: *ACTIN2* 239 bp, *SULTR1;1* 468 bp, and *SULTR1;2* 536 bp. **b**, **c** Dry biomass of roots and shoots, respectively, of 3-week-old WT and sulfate transporter mutants of *A. thaliana* (*sultr1;1, sultr1;2 and sultr1;1, sultr1;2*) grown on sulfate-replete and sulfate-deplete hydroponic medium for 10 days. Values are mean ± SE ($n = 3$). *Different lowercase letters* above *bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

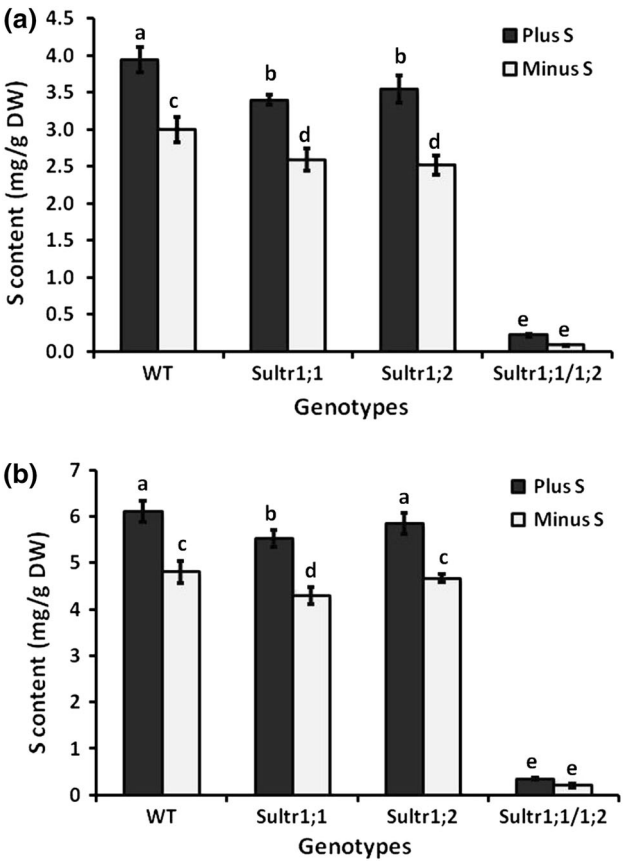

Fig. 2 Sulfur content in the roots (**a**) and shoots (**b**) of wild-type (WT) and sulfate transporter mutants of *A. thaliana* (*sultr1;1, sultr1;2 and sultr1;1, sultr1;2*). Three-week-old seedlings were grown hydroponically on sulfate-replete and sulfate-deplete media for 10 days. Then plant seedlings were transferred to hydroponic medium containing 0.1 mM arsenate for 1 day both under sulfate-replete and sulfate-deplete conditions. *Different lowercase letters* above *bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

family into four closely related groups. Sulfate transport activity has been demonstrated only for group 1 and 2 transporters which include *SULTR1;1*, *SULTR1;2*, *SULTR1;3*, *SULTR2,1,* and *SULTR2;2* (Buchner et al. 2004). Therefore, in the present study, we further investigated the expression patterns of *SULTR1;3*, *SULTR2;1,* and *SULTR2;2* in the double mutant and the WT using RT-PCR with gene-specific primers (Table 1). The gene expression analysis indicated that *SULTR1;3* and *SULTR2;1* were expressed in the double mutant and the WT. However, in the WT, *SULTR2;1* primers amplified an additional product origin of which is unknown. Interestingly, *SULTR2;2* transcript was detected in the roots of the double mutant, but not in the WT (Fig. 3). While *SULTR2;1* and *SULTR1;3*'s expression levels were comparable to *ACTIN2* expression level, *SULTR2;2*'s expression was greatly reduced (Fig. 3).

To test whether arsenate uptake was altered in the sulfate transporter mutants, WT, single, and double mutants were first grown hydroponically under sulfate-sufficient conditions, then exposed to sulfate-free nutrient medium for 10 days, and finally exposed to arsenate for 1 day (Fig. 4). In plant roots, no significant difference was

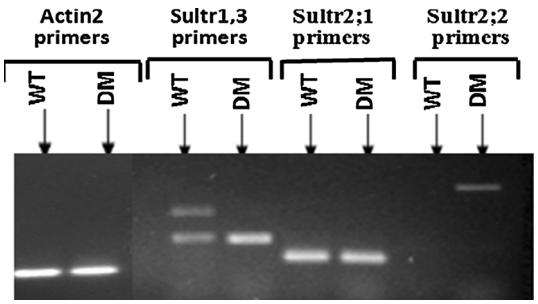

Fig. 3 Semiquantitative RT-PCR analysis for the expression of *SULTR1;3, SULTR2;1* and *SULTR2;2* in the roots of wild-type (WT) and double mutant (DM) grown under sulfate deficiency for 10 days. *ACTIN2* primers were used as a control

observed in arsenic concentration between the WT and single mutants either under sulfate-repleted or sulfate-depleted media (Fig. 4a). The arsenic concentration in plant roots revealed that the double mutant took up 0.8 µmol per g DW, which was significantly lower than the values observed for the WT and the single mutants, which had 5.7 and 5.9 µmol per g DW, respectively, under sulfate-depleted conditions (Fig. 4a). Similarly, the double mutant accumulated significantly less arsenic in its shoot compared with WT and single mutants (Fig. 4b). Arsenic concentration in the double mutant's shoot was 0.6 µmol per g DW, while in WT and single mutant's shoots, they were 1.15 and 1.13 µmol per g DW, respectively (Fig. 4b). Arsenic translocation capacity (from root to shoot), of all of the studied genotypes under sulfate-deplete conditions, was much higher than that under sulfur-replete condition (Fig. 4b). Arsenic accumulation in the shoots of WT and single mutants under sulfur deficiency was around threefold higher than that under sulfur-replete conditions, while in the double mutant, this induction was 4.6-fold (Fig. 4b).

In addition to sulfur, to estimate whether the arsenic treatment differentially altered the accumulation of other essential elements in the studied genotypes, the concentrations of K and P were also measured (Fig. 5). No significant differences were recorded between the single mutants and WT in K and P contents both under adequate S-nutrition and sulfate-limitation conditions. However, K and P contents of the roots and shoots of the double mutant were significantly lower than those in WT and single mutants (Fig. 5a–d). In plant roots, this reduction was more noticeable for K content than that for P content. K and P contents in the double mutant root reduced by about 73 and 28 %, respectively, less than those in WT root (Fig. 5a, c). However, in plants shoots, the reductions in K and P contents in the double mutant were more or less similar in values, around 35 % less than WT under sulfate-deplete media (Fig. 5b, d).

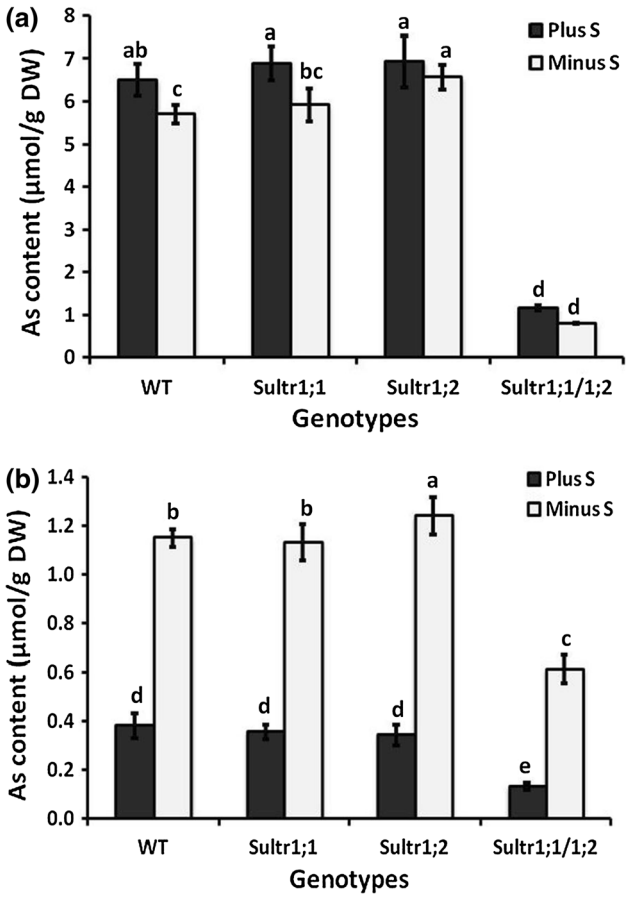

Fig. 4 Arsenic content in the roots (**a**) and shoots (**b**) of wild-type (WT) and sulfate transporter mutants of *A. thaliana* (*sultr1;1, sultr1;2 and sultr1;1, sultr1;2*). Three-week-old seedlings initially grown on sulfate-replete media were grown hydroponically on sulfate-replete and sulfate-deplete media for 10 days. Then seedlings were transferred to hydroponic medium containing 0.1 mM arsenate for 1 day both under sulfate-replete and sulfate-deplete conditions. *Different lowercase letters* above *bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

To test potential competition between arsenate and sulfate, we measured the root uptake of radio-labeled sulfate and its potential competition with arsenate. While sulfate uptake was 14,800 Bq per g fresh weight (FW)/4 h in the double mutant, the WT roots had 74,000 Bq per g FW/4 h. It was observed that exposure to different concentrations of arsenate at 0.5 and 1 mM had no significant effect on S uptake (Fig. 6), indicating that SULTR1;1 and SULTR1;2 proteins had a dominant role in root uptake of sulfate, and arsenate did not interfere with sulfate uptake.

In support of this idea, when we assessed root elongation with or without arsenate in the growth medium, the double mutant was not more resistant to arsenate than the WT, but was significantly more sensitive to arsenate than the WT (Fig. 7). The double mutant root length was reduced by about 73 % less than WT under arsenate treatment (Fig. 7).

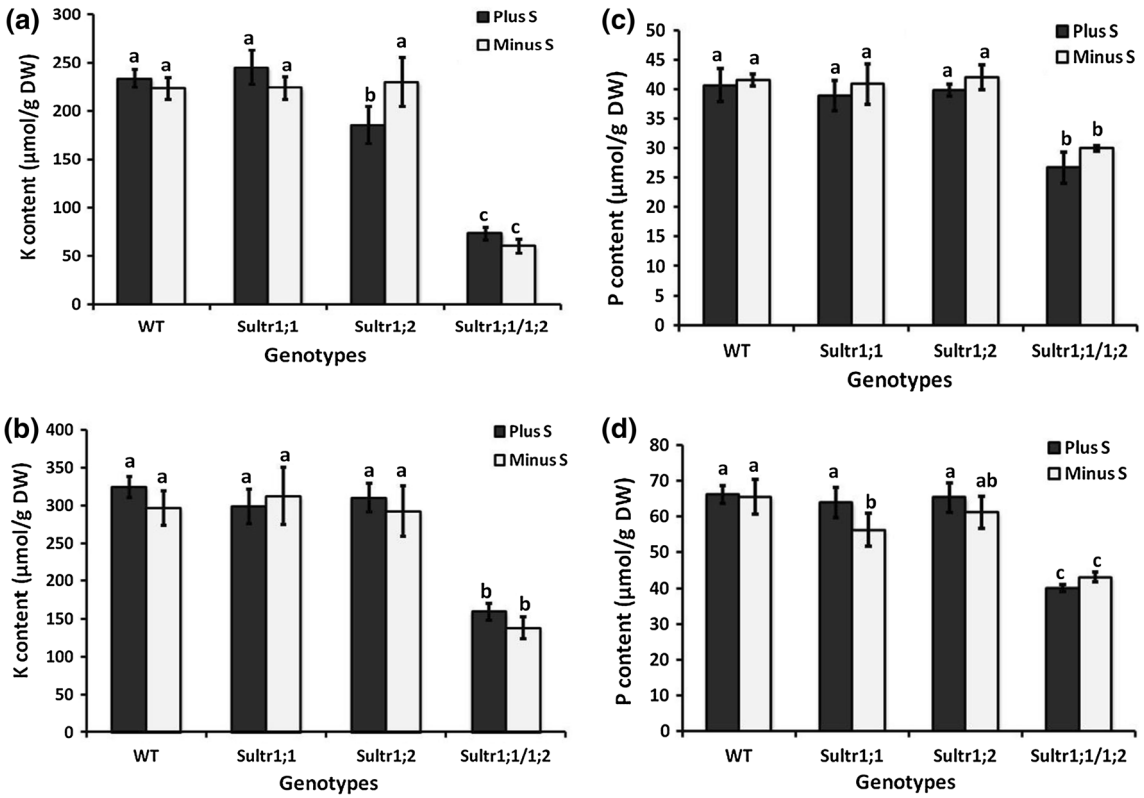

Fig. 5 Potassium content in the roots (**a**) and shoots (**b**) and phosphorus content in the roots (**c**) and shoots (**d**) of wild-type (WT) and sulfate transporter mutants of *A. thaliana (sultr1;1, sultr1;2 and sultr1;1, sultr1;2)*. Three-week-old seedlings initially grown on sulfate-replete media were grown hydroponically on sulfate-replete and sulfate-deplete media for 10 days. Then seedlings were transferred to hydroponic medium containing 0.1 mM arsenate for 1 day both under sulfate-replete and sulfate-deplete conditions. *Different lowercase letters* above *bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

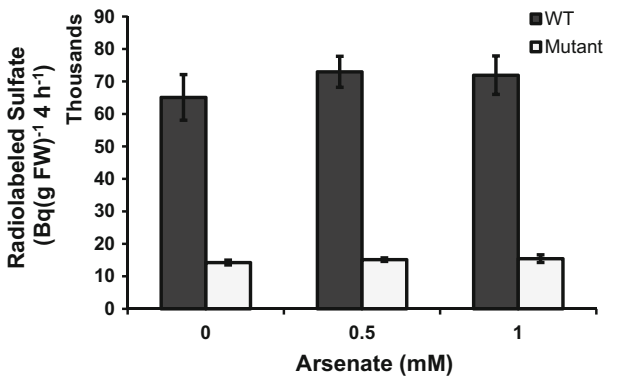

Fig. 6 Arsenate did not affect sulfate uptake in *A. thaliana*. Uptake of ^{35}S sodium sulfate following incubation of excised roots in the absence or the presence of arsenates (0.5 and 1.0 mM). Values are mean ± SE ($n = 4$). *Different lowercase letters* above *bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

To assess the relevance of sulfur deficiency in the studied genotypes on sulfur-related metabolites, we estimated total thiols in the double mutant, single mutants, and WT grown

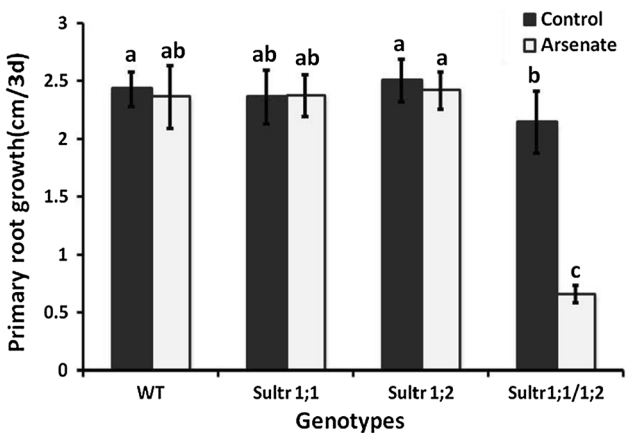

Fig. 7 Primary root growth of wild-type (WT) and sulfate transporter mutants of *A. thaliana* (*sultr1;1, sultr1;2 and sultr1;1, sultr1;2*). Three-day-old seedlings were transferred to vertical plates containing 0.5 strength MS medium (control) or on the same medium supplied with arsenate. Primary root length was measured after 3 days of treatment. Values are mean ± SE ($n = 7$). *Different lowercase letters* above *bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

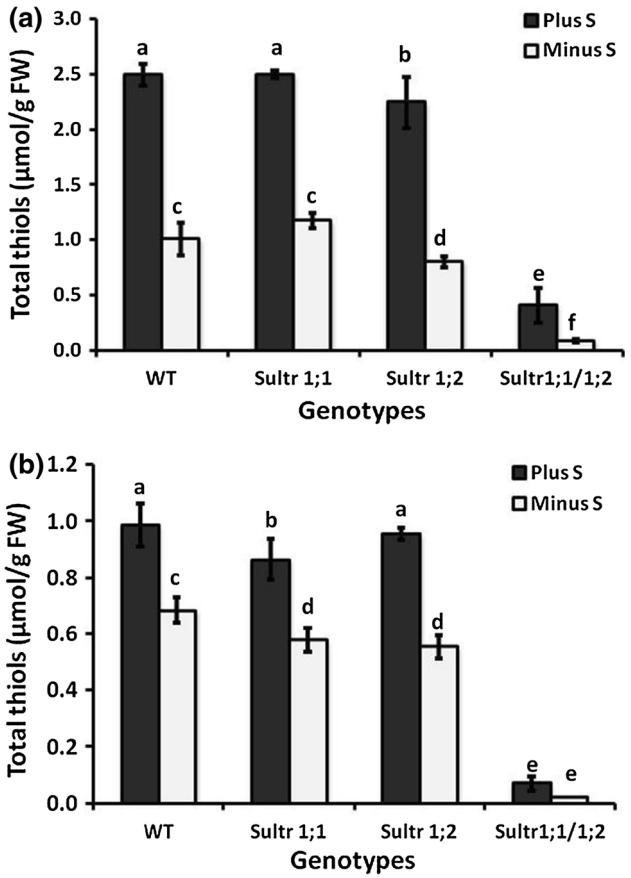

Fig. 8 Total thiol content in the roots (**a**) and shoots (**b**) of wild-type (WT) and sulfate transporter mutants of *A. thaliana* (*sultr1;1, sultr1;2 and sultr1;1, sultr1;2*). Three-week-old seedlings were grown hydroponically on sulfate-replete and sulfate-deplete media for 10 days. Then seedlings were transferred to hydroponic medium containing 0.1 mM arsenate for 1 day both under sulfate-replete and sulfate-deplete conditions. *Different lowercase letters* above *bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

under sulfur-replete and sulfur-deplete conditions using Ellman's spectrophotometric procedure. Total thiol content in the double mutant was significantly lower than those in single mutants and WT (Fig. 8). In the double mutant, total thiol concentrations were 0.09 and 0.02 µmol per g FW in roots and shoots, respectively, while these values were 1 and 0.7 µmol per g FW in WT and 1.1 and 0.6 µmol per g FW in single mutants, in roots and shoots, respectively (Fig. 8a, b).

Discussion

Previous research showed that SULTR1;1 and SULTR1;2 are predominantly expressed in plant roots, and had an overlapping, but critical role in high-affinity root uptake of sulfate (Barberon et al. 2008). Prior to the use of these mutants in this study, we have verified the lack of gene expression of the mutated genes in the double mutant (Fig. 1a). Because sulfur is an essential element, the growth of the double mutant was severely reduced (Fig. 1b, c), but the mutant did flower and produced viable seeds, indicating that uptake from other sources of sulfur must have been responsible for the low amount of sulfur in the tissue for growth and development. In accordance with the mutant phenotype, the double mutant had drastically very low sulfur contents in its root and shoot tissues (Fig. 2a, b). The low amounts of sulfur in the double mutant could be explained by the expression of other sulfate transporters SULTR1;3, SULTR2;1, and SULTR2;2 (Fig. 3). In this regard, expression of SULTR2;2 in the double mutant but not in the WT roots suggests that this transcript for a transporter previously characterized to be a low-affinity sulfate transporter that was phloem specific (Takahashi et al. 2000) was inducible by severe sulfur deficiency as experienced by the double mutant.

When the roots were exposed to sodium arsenate in the hydroponic medium for 24 h, arsenic concentrations in the root and shoot tissues on a DW basis were not significantly different from those of WT plants and the single mutants (Fig. 4a, b). However, the root and shoot tissues of the double mutant had significantly lower levels of arsenic compared to those of the WT and single mutants under both sulfate-sufficient and sulfate-deficient conditions (Fig. 4a, b). This could be interpreted as the simultaneous involvement of *sultr1;1* and *sultr1;2* in root uptake of arsenate. However, several lines of evidence suggest that this is not likely. First, when potassium and phosphate levels were measured in the root and shoot tissue samples from the experiment shown in Fig. 4, the double mutant had significantly lower amounts of these elements as well (Fig. 5a–d), indicating a negative effect on elemental uptake in the double mutant. Second, radiotracer experiments on sulfate uptake indicated that, the double mutant was significantly impaired in sulfate uptake, but high arsenate concentrations had no effect on sulfate uptake by WT roots (Fig. 6). The fact that the double mutant was more sensitive to arsenate than the WT instead of being tolerant to it (Fig. 7) was consistent with this observation. On the other hand, other studies have shown that the mutations in sulfate transporters resulted in selenate resistance and there was a direct relationship between seedling selenate content and selenate resistance (Barberon et al. 2008). As we have observed the sulfate limitation's pleiotropic effects on the levels of phosphorus and potassium (Fig. 5), others found that sulfate uptake and assimilation were affected by other ions such as iron and vice versa (Ciaffi et al. 2013; Paolacci et al. 2014).

Unlike the results for selenate and sulfate competition, results presented here indicate that arsenate was not

directly competing for uptake via SULTR1;1 and SULTR1;2. The high levels of sensitivity to arsenate and lower levels of tissue arsenic were most probably due to pleiotropic effects of poor sulfur status in the mutant, as sulfur compounds including glutathione, phytochelatins, and cysteine have important roles in plant defense against arsenic-induced oxidative stress (Zhao et al. 2009). This conclusion was supported by our estimation of total thiols which indicated that the double mutant's shoot and root tissues had very low thiol contents (Fig. 8).

Although our study was designed with the objective to test the role of two sulfate transporters in arsenate uptake, we also observed that under sulfate-deplete conditions, the arsenic concentrations in shoots with reference to corresponding arsenic levels in roots have significantly increased in all the genotypes tested (Fig. 4). The above observation suggests that sulfate deficiency likely influenced root-to-shoot translocation of arsenate or other aspects of arsenic metabolism hitherto unknown. Future studies should focus on revealing the gene(s) and protein(s) in these processes regulated by sulfur deficiency, as arsenic translocation in plants remains a poorly understood physiology (Wang et al. 2010; Srivastava et al. 2014) that has important implications for arsenic food safety.

Author contribution BR and LM designed the study; ME and VO executed the experimental work; and ME, VO, LM, and BR wrote the manuscript.

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