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ORIGINAL ARTICLE

Glutathione homeostasis and Cd tolerance in the *Arabidopsis* *sultr1;1-sultr1;2* double mutant with limiting sulfate supply

Xiang Liu[1] · Fei-Hua Wu[1,2] · Jing-Xi Li[3] · Juan Chen[1] · Guang-Hui Wang[1] ·
Wen-Hua Wang[5] · Wen-Jun Hu[1,4] · Li-Jie Gao[3] · Zong-Ling Wang[3] ·
Jun-Hui Chen[3] · Martin Simon[1] · Hai-Lei Zheng[1]

Abstract

Key message Cadmium sensitivity in *sultr1;1-sultr1;2* double mutant with limiting sulfate supply is attributed to the decreased glutathione content that affected oxidative defense but not phytochelatins' synthesis.

Abstract In plants, glutathione (GSH) homeostasis plays pivotal role in cadmium (Cd) detoxification. GSH is synthesized by sulfur (S) assimilation pathway. Many studies have tried to investigate the role of GSH homeostasis on Cd tolerance using mutants; however, most of them have focused on the last few steps of S assimilation. Until now, mutant evidence that explored the relationship between GSH homeostasis on Cd tolerance and S absorption is rare. To further reveal the role of GSH homeostasis on Cd stress, the wild-type and a *sultr1;1-sultr1;2* double mutant which had a defect in two distinct high-affinity sulfate transporters were used in this study. Growth parameters, biochemical or zymological indexes and S assimilation-related genes' expression were compared between the mutant and wild-type *Arabidopsis* plants. It was found that the mutations of *SULTR1;1* and *SULTR1;2* did not affect Cd accumulation. Compared to the wild-type, the double mutant was more sensitive to Cd under limited sulfate supply and suffered from stronger oxidative damage. More importantly, under the same condition, lower capacity of S assimilation resulted in decreased GSH content in mutant. Faced to the limited GSH accumulation, mutant seedlings consumed a large majority of GSH in pool for the synthesis of phytochelatins rather than participating in the antioxidative defense. Therefore, homeostasis of GSH, imbalance between antioxidative defense and severe oxidative damage led to hypersensitivity of double mutant to Cd under limited sulfate supply.

Keywords Antioxidative defense · *Arabidopsis* ·
Cadmium toxicity · Glutathione homeostasis · Sulfate transporter · Sulfur assimilation-related genes

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✉ Hai-Lei Zheng
zhenghl@xmu.edu.cn

1   Key Laboratory for Subtropical Wetland Ecosystem Research of MOE, College of the Environment and Ecology, Xiamen University, Xiamen 361102, Fujian, People's Republic of China

2   Colleges of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, Zhejiang, People's Republic of China

3   Marine Ecology Research Center, First Institute of Oceanography, State Oceanic Administration, Qingdao 266061, Shandong, People's Republic of China

4   Sericultural Research Institute, Zhejiang Academy of Agricultural Science, Hangzhou 310021, Zhejiang, People's Republic of China

5   Fujian Key Laboratory of Subtropical Plant Physiology and Biochemistry, Fujian Institute of Subtropical Botany, Xiamen 361005, Fujian, People's Republic of China

Introduction

Cadmium (Cd) contamination is a widespread serious environmental problem, which has mainly originated from anthropogenic activities (Momodu and Anyakora 2010). In plants, Cd is easily taken up by roots in competition with other divalent ions, then translocated into other organs

(Baker et al. 2006). As a non-essential metal, Cd causes various phytotoxic symptoms including growth inhibition, leaf chlorosis, and nutrients deficiency (Ebbs and Uchil 2008).

At cellular level, Cd induces oxidative stress by disturbing the balance between pro-oxidants, for example, reactive oxygen species (ROS) and antioxidants (Xu et al. 2012). ROS cause oxidation of polyunsaturated fatty acids in membranes, depolymerization of nucleic acids, and breakage of peptide bonds. In addition, ROS participate in signaling cascades in defense responses, cell cycle, apoptosis or lignification of cell walls (Foyer and Noctor 2011). Therefore, it is necessary to maintain a certain level of ROS for cell metabolism. This is attained with a complex antioxidant system composed by low molecular weight metabolites like ascorbate (AsA) or glutathione (GSH), and antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidases (APX), catalases (CAT) or glutathione reductase (GR) (Hegedüs et al. 2001; Paradiso et al. 2008; Xu et al. 2009).

Sulfur (S) is an essential element and sulfate is the major form of inorganic S utilized by plants (Astolfi et al. 2004). Sulfate uptake depends on the help of sulfate transporters, among which SULTR1;1 and SULTR1;2 are two distinct high-affinity transporters (Takahashi et al. 2001; Vidmar et al. 2000). Absorbed sulfate undergoes S assimilation pathways which direct GSH synthesis (Saito 2000).

GSH homeostasis in plants is closely related to Cd tolerance (Nocito et al. 2006; Sun et al. 2007). Besides the antioxidant role of GSH, this tripeptide is also important in the Cd detoxification through the synthesis of phytochelatins (PCs) (Cobbett 2000). Numerous physiological, biochemical and genetic studies have confirmed that PCs are synthesized from GSH by PCs synthase (Rea 2012). Several studies have provided genetic evidence to elucidate the importance of GSH homeostasis on Cd detoxification. For instance, cad 1–1 exhibited Cd sensitivity due to the defect in PCs synthase (Howden et al. 1995); cad2–1 was another Cd-sensitive mutant with a lower level of PCs that was caused by deficiency in GSH (Cobbett et al. 1998). Recently, overexpression of Lycium chinense GSHS (GSH synthetase) in transgenic Arabidopsis resulted in improved tolerance to Cd stress compared to wild-type (Guan et al. 2015). However, these studies mainly concentrate on the GSH or PCs' synthesis, which belongs to the last few reactions of S assimilation. Until now, mutant evidence that explored the relationship between GSH homeostasis on Cd tolerance and S absorption process is rare.

In present study, an Arabidopsis double sultr1;1-sultr1;2 null mutant, which had defect in two distinct high-affinity sulfate transporters and displayed low sulfate uptake efficiency (Barberon et al. 2008) was used to investigate the possible role of GSH homeostasis on Cd tolerance under sulfate deficiency. Double mutant and wild-type Arabidopsis seedlings were first exposed to different sulfate supplies with or without Cd for 2 weeks. Then, we compared the root length, biomass, chlorophyll or soluble protein content, oxidative damage, antioxidative defense system, GSH homeostasis and several S assimilation-related genes expression between double mutant and wild-type Arabidopsis seedlings. So far, the potential role of GSH homeostasis on Cd tolerance in a sulfate uptake Arabidopsis has not been established. On one hand, our study reaffirms the importance of high-affinity sulfate transporters or sufficient sulfate supply for Cd tolerance. On the other hand, this research provides a genetic evidence to acknowledge the GSH homeostasis on Cd tolerance under limited sulfate supply. The use of genetic mutant allows us to establish the connections between Cd-induced growth reduction at a whole plant level and its negative effects on biochemical processes at cellular level.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana genotypes used for this study had "Columbia" background. The wild-type accession was obtained from the Nottingham Arabidopsis Stock Centre (N907), while the sultr1;1-sultr1;2 double mutant was obtained from Dr. Françoise Gosti (Institute National de la Recherché Agronomique, Universite Montpellier II, France). According to the methods by Barberon et al. (2008), double mutant was produced after crossing two single mutant (sultr1;1 and sultr1;2), three double mutant lines were selected by PCR, of which one line was chosen for further analysis. This line was then backcrossed three times in the original sel1–8, gl background and retained the homozygous gl mutation. For the sultr1;1 single mutant, it displays a T-DNA insertion in the 10th of the 13 exons of the SULTR1;1 gene (At4g08620) located in chromosome 4 (Barberon et al. 2008). While, the sultr1;2 single mutant (sel1–8 allele) harbors the I551T EMS-induced mutation in the SULTR1;2 gene (At1g78000) (Shibagaki et al. 2002).

Sterilized wild-type and double mutant Arabidopsis seeds were sown on half-strength (1/2) modified MS basal agar medium (Murashige and Skoog 1962) with various treatments in Petri dishes. Sulfate salts of Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} were substituted by equimolar amount of chloride salts (Astolfi et al. 2004). After breaking seed dormancy in dark at 4 °C for 3 days, seeds were geminated and cultivated in a controlled growth chamber with an 8-h light/16-h dark period cycle under a photon flux rate of 200 µmol m^{-2} s^{-1} at temperature of 22 °C and relative humidity of 80 %.

Treatments

Pre-experiments were conducted to select the proper limited or sufficient sulfate and Cd concentration. First, seeds of wild-type and double mutant were sown on 1/2 modified MS medium supplemented with 0, 25, 50, 100, 200, 400 or 1500 μM K_2SO_4, respectively. 1/2 standard MS medium (approximately 860 μM sulfate) was chosen as the control. Primary root length was considered as the basic selection parameter. Then, seeds of wild-type and double mutant were also sown on the medium containing 0, 100, 200, 400, 1/2 standard MS or 1500 μM K_2SO_4 together with 10, 20, 30, 40 or 50 μM $CdCl_2$. The phenotype and survival situations of both *Arabidopsis* lines were observed.

According to the root length and phenotype results, 200 or 1500 μM K_2SO_4 and 20 μM $CdCl_2$ were finally chosen as the limited or sufficient sulfate and Cd treatment concentration. For the subsequent experiments, seedlings were divided into four groups: (1) 1/2 modified MS medium supplemented with 200 μM K_2SO_4 as the limited sulfate supply (LS); (2) 1/2 modified MS medium supplemented with 200 μM K_2SO_4 and 20 μM $CdCl_2$ as the combined stress of LS and Cd stress (LS + Cd); (3) 1/2 modified MS medium supplemented with 1500 μM K_2SO_4 as the control treatment of sufficient sulfate supply (SS); (4) 1/2 modified MS medium supplemented with 1500 μM K_2SO_4 and 20 μM $CdCl_2$ as the ameliorative effect of SS under Cd stress (SS + Cd). After treatment for 2 weeks, seedlings were harvested, frozen in liquid nitrogen and stored at −80 °C until used, with exception of some fresh seedling materials used for element or chlorophyll content measurement.

Primary root length measurements

After ten days growth, primary root length of seedlings was measured by digitalizing Petri dishes pictures via a scanner (HP ScanJet 7400C; Hewlett-Packard Company) and subsequently analyzing the images by Scion Image software (version 4.02). Forty plants were estimated for each treatment.

Total RNA extraction and gene expression analysis

Total RNA was isolated from wild-type and double mutant seedlings (0.2 g) under different treatments with 1 ml TRIZOL reagent (Invitrogen, Inc., CA, USA) according to manufacturer's procedure. The RNA integrity was confirmed by 1 % agarose gel electrophoresis and the concentration was determined by an ultraviolet spectrophotometer (Cary 50, Varian, USA). First-strand cDNA was synthesized with M-MLV reverse transcriptase (TaKaRa, Dalian, China) with an oligo $d(T)_{18}$ primer.

Quantitative real-time PCR (qRT-PCR) was used to analyze genes expression by StepOnePlus[TM] Real-Time PCR System (Applied Biosystems). A 10 μl real-time PCR system was adopted according to Chen et al. (2011). Primers listed in Supporting Information Table S1 were designed according to the known sequences in NCBI database. Relative quantification values for each target gene were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Gene expression levels were standardized using *actin2* as the internal control. The mRNA quantity from SS-treated sample was set as "1" for each gene and other treated samples were expressed relative to the corresponding control. The average expression folds of three biological replicates, including two technical replicates for each biological replicate were calculated. Then, hierarchical clustering of the expression profiles was performed on the log base 2 average expression fold values using Cluster software (version 3.0). Complete linkage algorithm was enabled and the results were plotted employing the Treeview software (version 1.1.3).

Determination of chlorophyll and soluble protein contents

Chlorophyll content was measured by the method of Lichtenthaler (1987) with some modifications. After extraction using 5 ml of 80 % (v/v) aqueous acetone, the total chlorophyll was calculated from the absorbance of leaf chlorophyll extracts at 470, 646, and 663 nm. Soluble protein content was measured according to Bradford (1976) using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard protein.

Measurement of total S and Cd contents

Fresh *Arabidopsis* seedlings were washed thoroughly with deionized water, then dried at 70 °C until constant weight. The dried seedlings were ground to a fine power with an agate mortar and pestle. About 10 mg of subsamples were weighted using an ultra-microbalance (Model 4504MP8, Sartorius Crop., Göttingen, Germany) and S elemental content was analyzed by an Carbon–Hydrogen–Nitrogen–Sulfur (CHNS) Elemental Analyzer (Model FlashEA 1112, Thermo Finnigan, San Josa, CA) (Chen et al. 2012b). For Cd elemental measurement, seedling roots and shoots were separated and washed for 10 min in 5 mM ice-cold $CaCl_2$ solution to displace extracellular Cd (Nocito et al. 2002). Then samples were washed twice with deionized water, followed by the digestion with 5 ml concentrated nitric acid in a microwave digestion system (CEM, Inc., Mars-V). The solution was finally diluted to 50 ml with deionized water (Zhang et al. 2010). Cd content was determined

by an inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700 series, Agilent Co. Inc., USA).

Measurement of lipid peroxidation and hydrogen peroxide content

Lipid peroxidation level was determined in terms of malondialdehyde (MDA) content by the thiobarbituric acid reaction (Yan et al. 2010). Hydrogen peroxide (H_2O_2) was measured spectrophotometrically according to Alexieva et al. (2001).

Analysis of antioxidant enzyme activities

Fresh seedling samples were homogenized on an ice bath with 1 ml of 50 mM phosphate buffer solution (pH 7.0) containing 1 mM ascorbic acid and 1 mM EDTA. The homogenate was centrifuged at 15,000 g for 15 min at 4 °C and the supernatant was used for further assays.

SOD (EC 1.15.1.1) activity was determined by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). The 3 ml of reaction mixture consisted of 20 μM riboflavin, 150 mM L-methionine, 600 μM NBT, 400 μl of H_2O and 200 μl of enzyme extracts. The reaction was started with the addition of riboflavin and illuminated for 20 min under irradiance of 180 μmol photons m^{-2} s^{-1} provided by a white fluorescent lamp. The system without enzyme extracts was conducted as the negative control. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT when measured at 560 nm.

CAT (EC 1.11.1.6) activity was detected at 240 nm in 1.9 ml of 50 mM phosphate buffer solution (pH 7.0) containing 1 ml of 0.1 % H_2O_2 and 50 μl enzyme extracts, respectively. The enzyme activity was calculated from the initial rate of the reaction using the extinction coefficient of H_2O_2 at 40 M^{-1} cm^{-1} (Dhindsa et al. 1981).

APX (EC 1.11.1.11) activity was conducted by the method of Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 1 mM sodium ascorbate and 150 μl of enzyme extracts. The reaction was started by the addition of 0.5 mM H_2O_2. The enzyme activity was calculated from the initial rate of the reaction using the extinction coefficient of ascorbate which is 2.8 mM^{-1} cm^{-1} at 290 nm.

Peroxidase (POD, EC 1.11.1.7) activity was assayed in 2 ml of 100 mM potassium phosphate buffer (pH 6.5) containing 40 mM guaiacol, 10 mM H_2O_2 and 100 μl of enzyme extract. Activity of enzyme was evaluated by the rate of tetraguaiacol production using an extinction coefficient of 25.5 mM^{-1} cm^{-1} at 436 nm (Chen et al. 2012a).

Analysis of antioxidant compounds

Total glutathione (Total GSH), reduced glutathione (GSH), and disulphide glutathione (GSSG) were estimated using a total GSH/GSSH reagent kit (Jian Cheng Bioengineering Institute, Nanjing, China) according to manufacturer's instructions. Plant sample (0.3 g) was ground with 1 ml ice-cold freshly made 5 % (w/v) m-phosphoric acid (Semane et al. 2007). GSH was estimated from difference between total GSH and GSSG. GSH/GSSG ratio was calculated by two compound values.

Total ascorbate (Total AsA), reduced ascorbate (AsA), and dehydroascorbate (DHA) were determined spectrophotometrically according to Hodges and Forney (2000). For each sample, DHA was estimated from the difference between total AsA and AsA. AsA/DHA ratio was calculated by two compound values.

Determination of thiols

Cysteine (Cys) in plants was measured according to Gaitonde (1967) with some modifications. Plant materials (0.3 g) were homogenized in 5 % chilled perchloric acid and centrifuged at 10,000 g for 15 min at 4 °C (Mishra et al. 2006). Cys content in supernatant was measured using acid/ninhydrin reagent at 560 nm. Total non-protein thiols (NPTs) content in plants was measured and GSH was used as the standard (Chen et al. 2011). Monobromobimane-labeled PCs content was analyzed by high-performance liquid chromatography (HPLC) (Li et al. 2014; Sneller et al. 2000). Detailed PCs measurement procedures were described in Supporting Information Methods: Determination of PCs. PCs content was expressed as GSH equivalent concentration.

Statistical analysis

For root length measurement, forty seedlings were used. For physiological and biochemical measurements, three replicates were used. Statistical treatment of the data was carried out by one-way analysis of variance (One-way ANOVA) with SPSS 19.0 package (SPSS, Chicago, Illionis USA). Duncan's post-test ($P < 0.05$) was used for multiple comparisons and Student's t-test (two tailed) was used for pairwise comparisons. Data are mean ± SE. In all of figures and tables, different capital letters indicate significant differences between different treatments, while the lowercase letters indicate significant differences between two kinds of plant materials under the same treatment.

Results

Effects of Cd on genes expression of sulfate transporter in wild-type *Arabidopsis*

To investigate the effect of Cd on sulfate uptake in wild-type *Arabidopsis* seedlings, gene expression levels of *SULTR1;1* and *SULTR1;2* were analyzed by qRT-PCR (Fig. 1). Limited sulfate treatment (LS) caused seven- or three-fold higher genes expression than control treatment (SS). Meanwhile, 20 μM Cd up-regulated the *SULTR1;1* and *SULTR1;2* genes expression. *SULTR1;1* expression level increased approximately 44-fold under LS + Cd treatment than SS treatment, and it was about four times higher under SS + Cd treatment (Fig. 1a). Similarly, the expression level of *SULTR1;2* showed 5- or 1.5-fold increase under LS + Cd or SS + Cd treatment compared to control (Fig. 1b), which is much lower than *SULTR1;1*. Genes expression results also showed that these two genes were suppressed completely in *sultr1;1-sultr1;2* double mutant in each condition analyzed (Fig. 1a, b).

Effects of Cd on biomass, root length, total chlorophyll, soluble protein, total S content and Cd accumulation in double mutant and wild-type *Arabidopsis* under LS and SS treatments

Compared to LS treatment, the seedling biomass of both lines was higher under SS treatment and there was no significant difference between wild-type and double mutant. Meanwhile, the biomass was obviously reduced by Cd in both *Arabidopsis* lines. Under LS treatment, Cd caused a 41 or 50 % decrease in wild-type and mutant seedlings (Table 1).

In both *Arabidopsis* lines, LS treatment inhibited the root elongation compared to SS treatment. Similarly, Cd

exposure also inhibited the root elongation significantly. Under LS treatment, decrease percentage induced by Cd reached 14.6 % in wild-type seedlings and 22 % in double mutant seedlings (Table 1).

In both *Arabidopsis* lines, the total leaf chlorophyll content reached the maximum under SS treatment and decreased under both LS and Cd treatments. Interestingly, under LS condition, the total chlorophyll content in double mutant seemed to be more affected by Cd compared to wild-type (Table 1).

In wild-type *Arabidopsis*, we found that the soluble protein content was almost not affected by different sulfate supplies. Furthermore, the protein content for LS + Cd-treated seedlings decreased by 24 % in comparison to LS-treated seedlings, while no difference was observed between SS and SS + Cd treatment. However, in double mutant, an obvious decrease of soluble protein content was found under LS treatment. The decrease percentage of soluble protein content induced by Cd reached 69 % under LS treatment (Table 1).

To examine the effects of Cd on S content in two lines of *Arabidopsis* seedlings under different sulfate supplies, we measured the total S content (Fig. 2a). Double mutant accumulated less S than wild-type. In wild-type *Arabidopsis*, it was more than two-fold higher under SS than LS treatment. In addition, Cd exposure enhanced S accumulation regardless of sulfate supply. Changes in double mutant were consistent with those in wild-type.

Cd accumulation was also measured as shown in Fig. 2b, c. In both *Arabidopsis* lines, SS treatment enhanced Cd accumulation in roots and shoots. Moreover, there was no noticeable difference between double mutant and wild-type seedlings grown under LS + Cd or SS + Cd treatment.
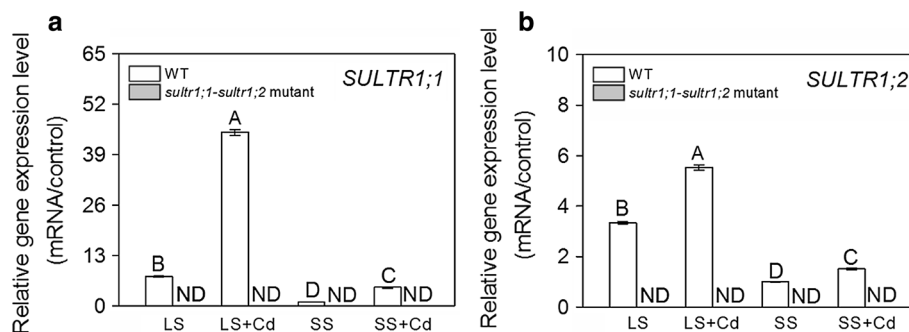

Fig. 1 Effects of Cd on *SULTR1;1* (**a**) and *SULTR1;2* (**b**) transcript level in 2-week-old wild-type and double *sultr1;1-sultr1;2* null mutant *Arabidopsis* seedlings under different treatment conditions. Data are mean ± SE of three independent replicates. The *SULTR1;1* and *SULTR1;2* mRNA transcriptional levels were suppressed completely in mutant. *ND* represents not detectable. *LS* limited sulfate supply treatment: 200 μM K_2SO_4; LS + Cd, limited sulfate supply with Cd treatment: 200 μM K_2SO_4 + 20 μM $CdCl_2$; *SS* sufficient sulfate supply treatment: 1500 μM K_2SO_4; SS + Cd, sufficient sulfate supply with Cd treatment: 1500 μM K_2SO_4 + 20 μM $CdCl_2$

Table 1 Effects of Cd on biomass, root length, total chlorophyll content, soluble protein content and Cd-caused decrease percentage of every parameters of wild-type and double *sultr1;1-sultr1;2* null mutant of *Arabidopsis* seedlings under limited or sufficient sulfate supply for 2 weeks

| | Wild-type | | | | | | Double mutant | | | | | |
| | Treatments | | | | Decrease percentage by Cd (%) | | Treatments | | | | Decrease percentage by Cd (%) | |
	LS	LS + Cd	SS	SS + Cd	LS	SS	LS	LS + Cd	SS	SS + Cd	LS	SS
Biomass (mg/seedling)	2.14 ± 0.03 Ba	1.26 ± 0.13 Da	2.54 ± 0.04 Ab	1.66 ± 0.05 Ca	41.12	34.65	1.56 ± 0.08 Bb	0.78 ± 0.11 Db	2.31 ± 0.18 Ab	1.29 ± 0.08 Cb	50.00	44.16
Root length (mm)	41.17 ± 0.95Ca	35.13 ± 0.07 Da	49.13 ± 0.37 Aa	46.93 ± 0.38Bb	14.60	4.48	15.71 ± 0.02Cb	12.25 ± 0.08Db	48.45 ± 0.01Ab	47.33 ± 0.07Bb	22.02	2.31
Total chlorophyll (mg/g FW)	0.62 ± 0.06 Ba	0.51 ± 0.09 Ba	0.92 ± 0.08 Aa	0.55 ± 0.08 Ba	17.74	40.22	0.57 ± 0.04 Ba	0.25 ± 0.04 Cb	0.89 ± 0.15 Aa	0.58 ± 0.02 Ba	56.14	34.83
Soluble protein (mg/g FW)	4.25 ± 0.03 Aa	3.23 ± 0.35 Ba	4.05 ± 0.23 Aa	3.86 ± 0.37 ABa	24.00	4.69	2.66 ± 0.22 Bb	0.81 ± 0.20 Cb	3.51 ± 0.09 Aa	3.36 ± 0.09 Aa	69.55	4.27

Data are mean ± SE of three independent replicates. *Different capital letters* indicate significant differences between different treatments, while the *lowercase letters* indicate significant differences between two kinds of plant material under the same treatment ($P < 0.05$, one-way ANOVA with Duncan's post-test for multiple comparisons and Student's *t*-test (two tailed) for pairwise comparisons
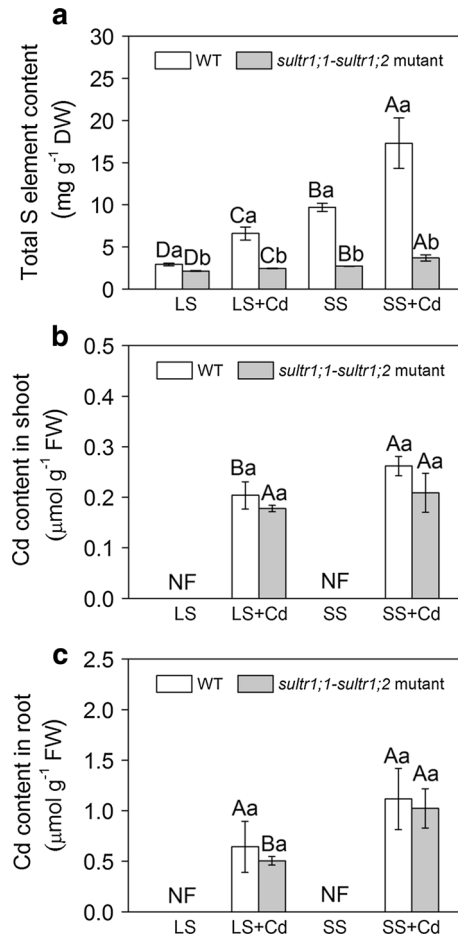

Fig. 2 Total sulfur (S) content (**a**) and Cd accumulation in shoots (**b**) or roots (**c**) of wild-type and double *sultr1;1-sultr1;2* null mutant of *Arabidopsis* seedlings under limited or sufficient sulfate supply for 2 weeks. *NF* represents not found. Data are mean ± SE of three independent replicates

Oxidative damage and quantification of enzymatic antioxidants in double mutant and wild-type *Arabidopsis* under LS and SS treatments with or without Cd

To investigate the oxidative damage induced by Cd, lipid peroxidation was estimated by measuring MDA content (Fig. 3a). In wild-type *Arabidopsis,* Cd exposure had no effect on MDA content under LS or SS treatment. However, for double mutant seedlings, MDA content was 76 % higher under LS + Cd combined stress compared to LS treatment, while only 23 % increase of MDA was measured at SS + Cd stress compared to SS treatment.

Cd exposure induced the accumulation of H_2O_2 (Fig. 3b). In wild-type *Arabidopsis*, H_2O_2 content in seedlings grown under LS + Cd treatment increased by 11 % compared to LS treatment, while it was 32 % in double mutant. Interestingly, double mutant seedlings accumulated more H_2O_2 content than wild-type in each treatment group.

Fig. 3 Effects of Cd on MDA content (**a**), H_2O_2 content (**b**) and enzymatic antioxidants activities of superoxide dismutase (SOD) (**c**), catalase (CAT) (**d**), ascorbate peroxidase (APX) (**e**) and peroxidase (POD) (**f**) in wild-type and double *sultr1;1-sultr1;2* null mutant *Arabidopsis* seedlings under limited or sufficient sulfate conditions for 2 weeks. Data are mean ± SE of three independent replicates
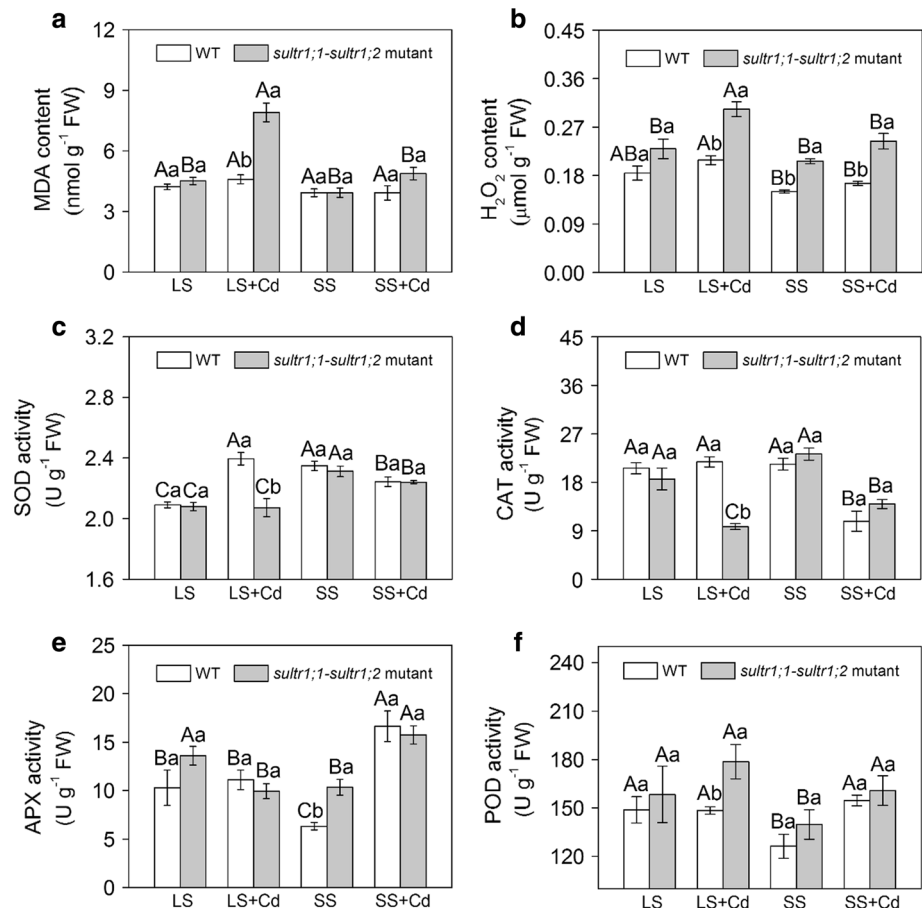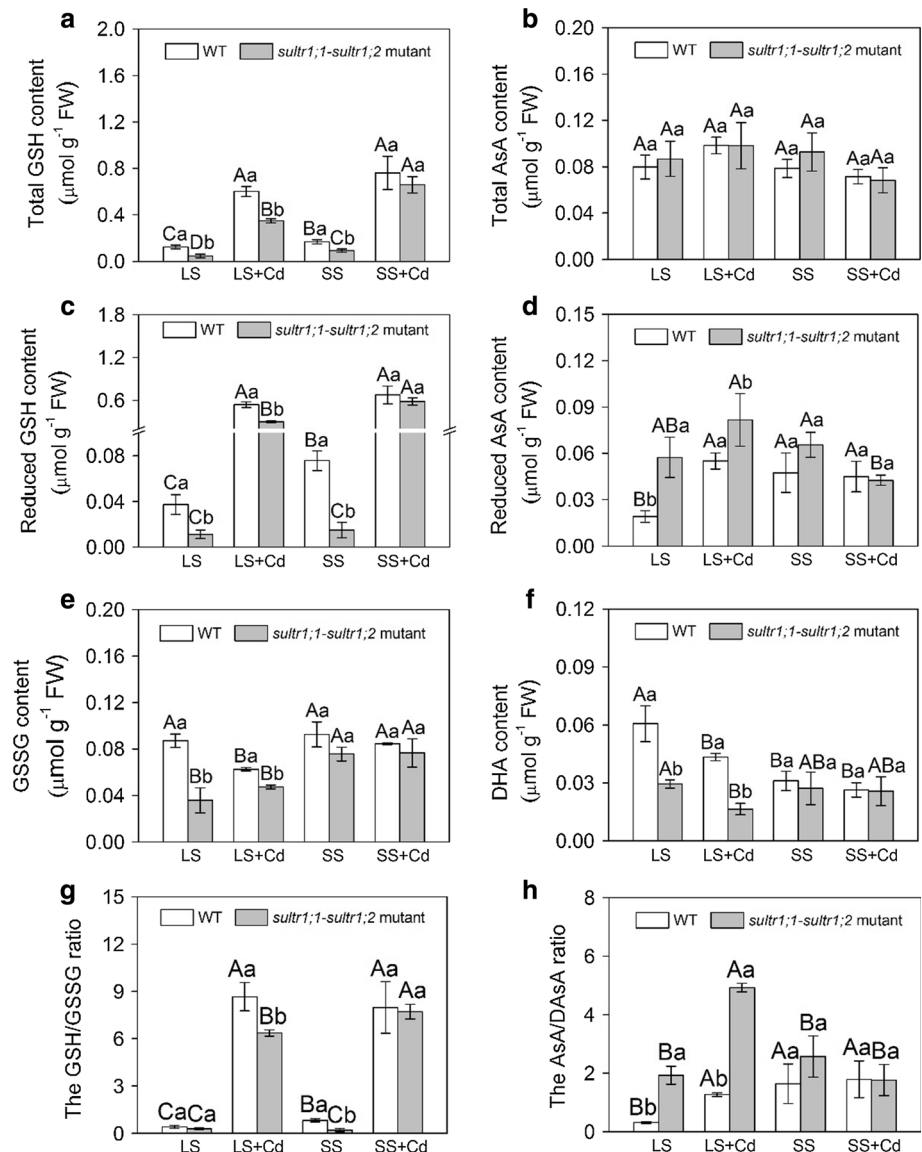

Compared to LS treatment, LS + Cd treatment increased the activity of SOD by 14 % in wild-type seedlings. However, in double mutant seedlings, SOD activity showed no marked change between LS and LS + Cd treatment (Fig. 3c). Unlike the changes pattern of SOD under LS and LS + Cd treatments, Cd caused 49 and 34 % decrease of SOD activity under SS treatment for wild-type and double mutant, respectively. The activity of CAT decreased by 58 % under LS + Cd combined stress compared to LS treatment in double mutant, while no significant difference was observed in wild-type (Fig. 3d). Furthermore, we also found that the CAT activity in double mutant was lower than those in wild-type under LS + Cd combined stress. No significant change in APX activity was measured in wild-type between LS and LS + Cd treatment, while Cd exposure caused an obvious increase at SS treatment (Fig. 3e). However, in double mutant, Cd exposure resulted in 27 % decrease or 52 % increase of APX activity under LS or SS treatment, respectively. In addition, we also examined the activity of POD as shown in Fig. 3f. Results suggested that Cd did not alter POD activity under LS treatment in both *Arabidopsis* lines, while Cd increased POD activity under SS treatment.

Non-enzymatic antioxidant defense in double mutant and wild-type *Arabidopsis* under LS and SS treatments with or without Cd

We further measured the changes of GSH and AsA which play an important role in ROS detoxification. For total GSH content, Cd exposure caused marked increase under LS or SS treatment in both *Arabidopsis* lines. Furthermore, double mutant kept lower total GSH than wild-type among all treatments except for SS + Cd (Fig. 4a). Similarly, double mutant also kept lower level of reduced GSH than wild-type under all treatments except for SS + Cd (Fig. 4c). Besides, Cd caused approximately 30- or 58-fold elevation in double mutant seedlings under LS or SS treatment, respectively, while the increase was only 18- or 10-fold higher in wild-type. Compared to LS treatment, Cd decreased GSSG content, the oxidized form of GSH, under LS + Cd combined stress in wild-type (Fig. 4e). However, in double mutant, there was no significant difference between LS + Cd and LS treatment (Fig. 4e). Finally, in wild-type seedlings, GSH/GSSG ratio was 0.83 in control, but it turned out to 0.42, 8.66 and 7.99 under LS, LS + Cd and SS + Cd treatment, respectively. Consistent with the

Fig. 4 Influence of Cd on glutathione–ascorbic acid metabolism in wild-type and *sultr1;1-sultr1;2* double mutant *Arabidopsis* seedlings under limited or sufficient sulfate conditions for 2 weeks. **a** total glutathione (total GSH) content, **c** reduced glutathione (GSH) content, **e** oxidized glutathione (GSSG) content, **g** the GSH/GSSG ratio, **b** total ascorbic acid (total AsA) content, **d** reduced ascorbic acid (AsA) content and **f** dehydroascorbic acid content (DHA), **h** the AsA/DHA ratio. Data are mean ± SE of three independent replicates

results from wild-type, Cd exposure also increased the ratio under LS or SS treatment in double mutant. Moreover, it was noteworthy that double mutant seedlings kept 26 % lower GSH/GSSG ratio under LS + Cd treatment compared to wild-type (Fig. 4g).

As other non-enzymatic antioxidants, total AsA content showed no significant difference among all treatments in this study (Fig. 4b). In both *Arabidopsis* lines, Cd exposure increased the content of reduced AsA under LS treatment. Furthermore, AsA content in double mutant was much higher than that in wild-type under LS + Cd combined treatment (Fig. 4d). For the oxidized form, DHA content decreased under LS + Cd combined stress compared to LS treatment in both *Arabidopsis* lines; however, it was much lower in double mutant than that in wild-type under LS + Cd combined stress (Fig. 4f). Besides, the AsA/

DHA ratio increased after Cd exposure under LS treatment in both plants, especially in double mutant (Fig. 4h). No evident changes were observed between SS and SS + Cd treatment.

Thiol compound changes in double mutant and wild-type *Arabidopsis* under LS and SS treatments with or without Cd

In wild-type, LS treatment increased Cys content compared to control, while Cd addition further promoted Cys synthesis, with increment of 1.3- or 1-fold, respectively, when seedlings were treated by LS or SS. The changes in mutant were similar as described above in wild-type. However, under LS + Cd combined stress, double mutant seedlings accumulated 53 % more Cys than wild-type (Fig. 5a).

Then we evaluated total content of NPTs. In both plants, different sulfate concentrations did not affect the NPTs content (Fig. 5b). However, after Cd exposure, NPTs content in wild-type or double mutant seedling was 3.45- or 2.78-fold higher than that under LS treatment, while which was approximately 7.25- or 2.57-fold higher than that under SS treatment.

Finally, the content of phytochelatin 2 (PC2), phytochelatin 3 (PC3) or phytochelatin 4 (PC4) was analyzed by HPLC, but only PC2 and PC3 were detected in our study (Fig. 5c, d). In both *Arabidopsis* lines, Cd stress induced production of PC2 and PC3, especially under SS treatment. Interestingly, under LS + Cd combined stress, double mutant seedlings could accumulate equivalent PCs compared to wild-type.

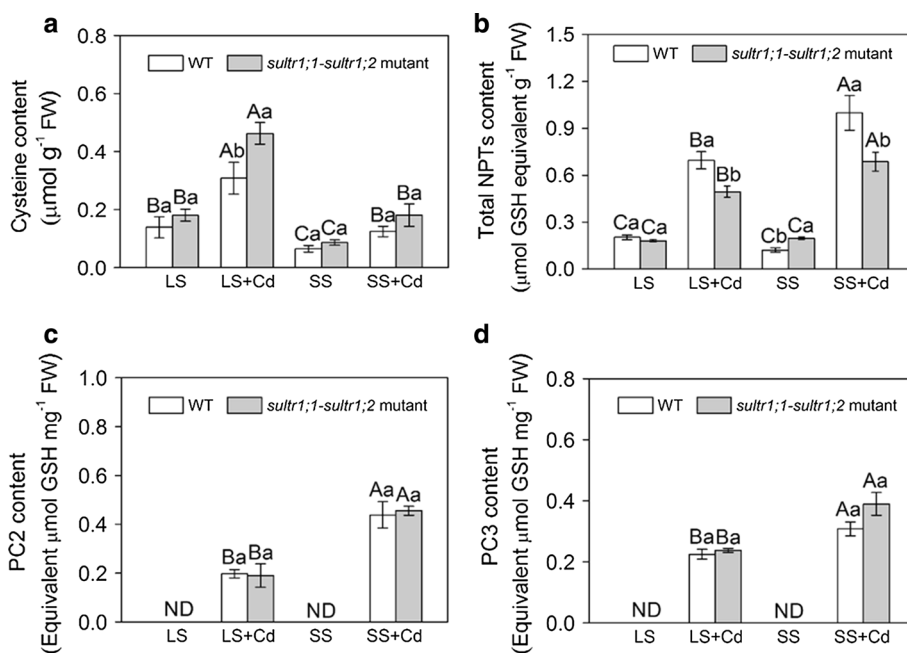## S assimilation-related genes expression

Cluster analysis of transcriptional fold changes for S assimilation-related genes is shown in Fig. 6. Original genes transcriptional changes are listed in Supporting Information Table S2. Genes expression of five important sulfate transporters was analyzed first. In both *Arabidopsis* lines, LS treatment down-regulated *SULTR2;1*, *SULTR 1;3*, *SULTR 3;4* and *SULTR 3;5* expression levels. Compared to LS treatment, Cd did not affect the transcript levels of these four genes in wild-type but caused up-regulation in double mutant. Interestingly, *SULTR 4;2* expression level was up-regulated in both *Arabidopsis* lines under LS and LS + Cd treatments.

Cd led to significant increase of ATP sulfurylase (*ATPS*), adenosine 5′-phosphosulfate reductase (*APR2*), sulfide reductase (*SIR*) and O-acetylserine(thiol)lyase (*OASTL*) mRNA levels under LS treatment in both *Arabidopsis* lines (Fig. 6). Generally, the transcriptional levels of these four genes were much lower in double mutant than those in wild-type (Fig. 6).

For the GSH biosynthesis-related genes, Cd did not affect the genes expression level of γ-glutamylcysteine synthase (*ECS1*) and glutathione synthetase 2 (*GSH2*) in wild-type. On the contrary, Cd led to increment of *GSH2* gene expression level in double mutant seedlings under LS or SS treatment. Meanwhile, no significant difference was observed for expression of *ECS1* between LS and LS + Cd treatment in double mutant.

For the GSH metabolism-related genes expression, we detected that Cd up-regulated the expression levels of encoding glutathione reductase (*GR1*), encoding monodehydroascorbate reductase (*DHAR1*) and encoding glutathione-S-transferase (*GST1*) in wild-type. Differentially, *GST1* was down-regulated by Cd in double mutant under LS + Cd combined stress. Overall, the transcriptional levels of these three genes were much lower in double mutant than those in wild-type. We also analyzed the genes encoding PCs synthase (*PCS1*). In both *Arabidopsis* lines, *PCS1* was slightly up-regulated after Cd exposure. Interestingly, no significant difference was observed between the wild-type and double mutant seedlings under LS + Cd stress.

Fig. 5 Effects of Cd on three major thiol compounds content in wild-type and double *sultr1;1-sultr1;2* null mutant *Arabidopsis* seedlings under limited or sufficient sulfate conditions for 2 weeks. **a** cysteine (Cys) content, **b** total non-protein thiols (NPTs) content, **c** phytochelatin 2 (PC2) content and **d** phytochelatin 3 (PC3) content. Data are mean ± SE of three independent replicates. *ND* represents not detectable
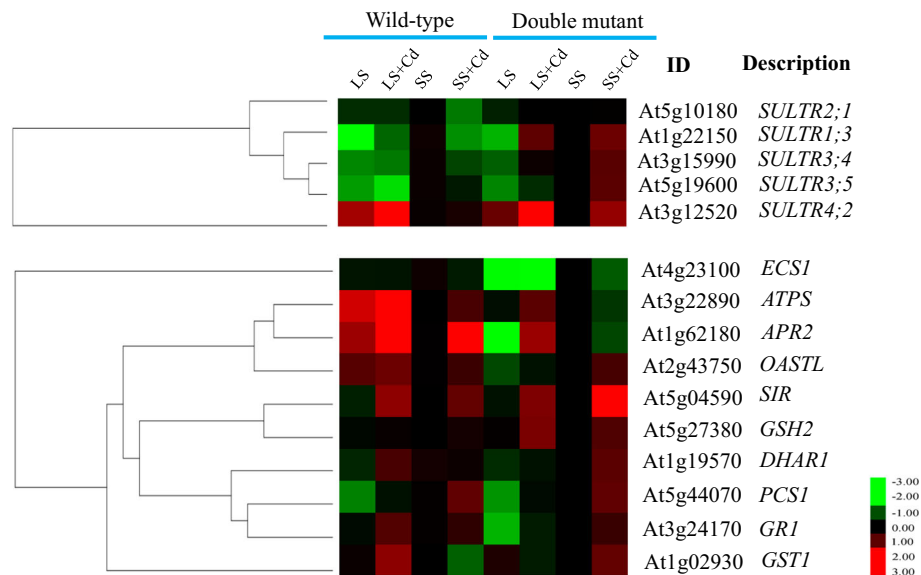
Fig. 6 Cluster analyses of S metabolism-related genes expression in wild-type and double *sultr;1-sultr;2* null mutant *Arabidopsis* seedlings under limited or sufficient sulfate conditions for 2 weeks. Each *horizontal line* displays the abundance of one gene. *Red* and *green* indicate up- and down-regulation in treated plants compared to control, respectively. Intensity of the colors is proportional to the absolute value of the fold difference. The SS-treated sample mRNA quantity was set at "1" for each gene and other treated samples were expressed relative to the corresponding control. Sulfate transporters (SULTR 2;1, At5g10180; SULRE1;3, At1g22150; SULTR3;4 At3g15990; SULTR3;5, At5g19600; SULTR4;2, At3g12520), ATP sulfurylase (ATPS, At3g22890), adenosine 5′-phosphosulfate reductase (APR2, At1g62180), sulfide reductase (SIR, At5g04590) and *O*-acetylserine(thiol)lyase (OASTL), γ-glutamylcysteine synthase (ECS1, At5g23100), GSH synthase (GSH2, At5g27380) phytochelatin synthase (PCS, At5g44070), GSH reductase (GR, At3g24170), GSH S-transferase (GST, class phi, At1g02930) (color figure online)

Discussion

Cd up-regulates genes' expression of sulfate transporter in wild-type *Arabidopsis* seedlings

The increased expression of sulfate transporter genes in response to sulfate deficiency has been considered as one of the most important mechanisms to improve sulfate utilization by plants (Hawkesford 2000). SULTR1;1 and SULTR1;2 are two important high-affinity transporters and their transcriptions are responsive to sulfate deprivation in *Arabidopsis* (Takahashi et al. 2001). Our results showed that LS treatment enhanced *SULTR1;1* and *SULTR1;2* transcript abundance (Fig. 1). Similar results have been certified by Hubberten et al. (2012). Based on their results, the expression of *SULTR1;1* and *SULTR1;2* was up-regulated ten- and three-fold in LS-treated *Arabidopsis* roots. Besides, in our study, Cd exposure also up-regulated expression of these two genes regardless of sulfate concentration (Fig. 1). In accordance with the results by Nocito et al. (2006), Cd exposure increased *ZmST1;1* expression in maize roots, which was confirmed as a high-affinity sulfate transporter. It was noteworthy that Cd-induced genes' expression increased significantly in seedlings under LS treatment (Fig. 1). These findings were consistent with recent studies in *Brassica juncea* using

semi-quantitative RT-PCR (Lancilli et al. 2014). Their results indicated that *BjSultr1;1* and *BjSultr1;2* genes' expression could be elevated by Cd exposure and sulfate limitation. Moreover, the transcript level of *SULTR1;1* was higher than *SULTR1;2* for seedlings treated by LS or Cd toxicity, suggesting that *SULTR1;1* was easily induced by sulfate deficiency or Cd toxicity. Reports by Yoshimoto et al. (2002, 2007) have proved that these two sulfate transporters exhibited slight differences in mRNA inducibility. Under LS condition, *SULTR1;1* mRNA was predominantly detected, whereas *SULTR1;2* was found abundantly even when plants were supplied with adequate sulfate and was less responsive to the fluctuation of S status compared to *SULTR1;1*.

The double *sultr1;1-sultr1;2* null mutant shows Cd hypersensitivity under LS treatment

Since *SULTR1;1* and *SULTR1;2* are the main genes involved in sulfate uptake in *Arabidopsis*, double mutation results in an even much lower sulfate uptake. Poor sulfate status in *sultr1;1-sultr1;2* double mutant led to reduced growth, but mutant did flower and produced viable seeds. It is noteworthy that the mutant did not always show reduced growth, as revealed by the progressive recovery observed after increasing the sulfate concentration (Barberon et al.

2008). In recent years, this mutant has been utilized to investigate the relationship between sulfate transporters and metal tolerance. For example, Barberon et al. (2008) reports that double mutant plants possess selenate tolerance; El-Zohri et al. (2015) also reports that poor sulfate status could influence arsenate tolerance in mutant. Nevertheless, our study focused on the correlation between sulfate transporter and Cd tolerance using the double *sultr1;1-sultr1;2* null mutant.

Aiming to select a proper limited or sufficient sulfate concentration, the primary root length under a set of K_2SO_4 concentration was measured in two *Arabidopsis* lines. In mutant, root length was shorter than that in wild-type except when 1500 µM sulfate was added into medium (Supporting Information Fig. S1). Based on this result, 1500 µM K_2SO_4 was chosen as the SS treatment. Then, different Cd concentrations were added in the medium containing a gradient of K_2SO_4 concentration, and we mainly observed the survival situations (data were not shown). We found mutant seedlings could not grow normally in presence of Cd when sulfate added in medium was equal or lower to 100 µM. Similarly, mutant seedlings also could not survive in 30, 40 and 50 µM Cd when supplied with 200, 400 and 1/2 standard MS medium sulfate. To avoid the early death of double mutant seedlings under LS + Cd combined stress and ensure the samples needed for subsequent experiments, 200 µM K_2SO_4 was used as the LS treatment in this study, while 20 µM $CdCl_2$ was selected as the Cd stress.

Sanita di Toppi and Gabbrielli (1999) report that the main visualized symptoms induced by Cd are root growth inhibition, biomass reduction and leaf chlorosis. Soluble protein content in organisms, an important indicator of reversible and irreversible changes in metabolism, is known to respond to a wide variety of stressors such as natural and xenobiotic (Singh and Tewari 2003). Our data showed that 20 µM $CdCl_2$ decreased the biomass, root length, chlorophyll and soluble protein content in both wild-type and double mutant plants under LS treatment (Table 1). Similarly, in maize seedlings, these growth parameters were inhibited by 100 µM Cd (Astolfi et al. 2004). Besides, Cd also resulted in a significant decrease of protein level in duckweed (Hou et al. 2007).

More importantly, under LS + Cd treatment, Cd-induced growth inhibition was much greater in mutant than that in wild-type (Table 1 and Supporting Information Fig. S2b, f), suggesting the mutant is more sensitive to Cd stress under LS condition. However, all reductions caused by Cd could be alleviated under SS treatment (Table 1 and Supporting Information Fig. S2f, h). Based on these results, we conclude that double mutant is significantly more impaired in presence of Cd under LS. Moreover, the sensitivity could be recovered by SS treatment.

Cd in the growth medium is easily taken up by plant tissues (Gill et al. 2012). Compared to LS treatment, SS promoted roots and shoots Cd accumulation in both *Arabidopsis* lines, suggesting that SS could help plant to take up more Cd from cultural medium. In addition, it was worth noticing that Cd content had no significant difference between double mutant and wild-type seedlings under LS + Cd or SS + Cd treatment (Fig. 2c, d), indicating the mutations of *SULTR1;1* and *SULTR1;2* did not affect the capacity of Cd accumulation.

Functions of antioxidative enzymes in Cd sensitivity for *sultr1;1-sultr1;2* double mutant under LS + Cd combined stress

Being a non-transition metal, Cd leads to oxidative stress characterized by increased H_2O_2 level and lipid peroxidation (Hsu and Kao 2007; Paradiso et al. 2008). In *Arabidopsis*, oxidative stress after exposure to Cd is due to H_2O_2 accumulation (Cho and Seo 2005). In present study, compared with wild-type, higher MDA and H_2O_2 accumulation was found in double mutant under LS + Cd combined stress (Fig. 3a, b), implying that Cd hypersensitivity in double mutant can be attributed to severe oxidative damage under LS + Cd combined stress. Cd-induced increase of thiobarbituric acid and H_2O_2 content was also detected in leaves of *Lepidium sativum* (Gill et al. 2012).

Enzymatic antioxidant system, such as SOD, CAT, APX and POD, is capable to scavenge excess ROS and prevent plants from lipid peroxidation (Hegedüs et al. 2001; Salin 2007). SOD is a key enzyme against oxidative stress as it catalyzes the dismutation of $O_2^{.-}$ to H_2O_2 and O_2, which is essential for quenching ROS (Chu et al. 2005). In our study, Cd elevated SOD activity under LS condition in wild-type seedlings but did not alter SOD activity in mutant at the same treatment. Compared to wild-type, mutant even held lower SOD activity under LS + Cd treatment (Fig. 3c). These results indicate that double mutant fails to increase SOD activity under LS + Cd. The decrease of SOD activity might be caused by the inactivation of enzyme by overproduced H_2O_2, low availability of Cys residue and failure or limitation in iron (Fe)–S cluster synthesis (Sandalio et al. 2001; Ryan et al. 2010). CAT, APX and POD, other three antioxidative enzymes, play an important role in removal of H_2O_2 (Gratão et al. 2005). In wild-type seedlings, Cd did not alter CAT activity under LS treatment, while in double mutant, CAT activity was decreased. Under LS + Cd combined stress, CAT activity was much lower in mutant than wild-type (Fig. 3d). Consistent with the results of Bashir et al. (2013), Cd failed to stimulate SOD and CAT activities in *Arabidopsis* under S deficiency. The decline of CAT activity

reflects reduced H_2O_2 scavenging capacity (Azpilicueta et al. 2007), which results in H_2O_2 accumulation. Besides, in wild-type, the activity of APX was not influenced by Cd under LS condition, but it showed a slight decrease in double mutant (Fig. 3e). Decreased APX activity induced by Cd also led to H_2O_2 accumulation in double mutant under LS treatment. Surprisingly, the POD activity in wild-type seedlings was much lower than that in double mutant under LS + Cd (Fig. 3f), suggesting mutant still needs to retain enough POD to encounter H_2O_2. The contradictory responses of POD and APX to Cd stress indicate that different mechanisms may be involved in their operations against oxidative stress (Ali et al. 2002).

Overall, H_2O_2 scavenging ability was challenged by LS + Cd combined stress in double mutant, which directly caused excess H_2O_2 accumulation.

Role of glutathione homeostasis in Cd hypersensitivity for *sultr1;1-sultr1;2* double mutant under LS + Cd combined stress

The functions of GSH due to its unique structural properties, abundance, broad redox potential, and wide distribution in most living organisms have been drawn (May et al. 1998). Being a non-protein S-containing tripeptide, GSH is related to the sequestration of heavy metals and is also an essential component of the cellular antioxidative defense system which keeps ROS under control (Sobrino-Plata et al. 2014). In past few years, the power of mutant analysis in elucidation role of GSH homeostasis on heavy metal tolerance has been demonstrated in various species. In *Arabidopsis*, *cad2-1* (*Cd sensitive 2–1*) with a reduced capacity to produce GSH was found to be hypersensitive to both Cd and copper (Cu) (Cobbett et al. 1998); The phytoalexin-deficient 2–1 (*pad2–1*) and ascorbate-deficient 2–1 (*vtc2–1*) mutants showed enhanced susceptibility to Cd, which was also related to lower subcellular GSH content (Koffler et al. 2014); In roots of mercury (Hg)-treated *regulator of APX2 1–1* (*rax1–1*) *Arabidopsis* mutant, there was a strong accumulation of GSH (Sobrino-Plata et al. 2014). Similar studies have been addressed in cultivated plants of economic importance. For example, a GSH-deficient mutant of grass pea, *gshL-1*, also exhibited Cd sensitivity (Talukdar 2012); Liu et al. (2015) indicated that overexpression of *StGCS-GS* (encoding γ-glutamylcysteine synthetase-glutathione synthetase in *Streptococcus thermophilus*) was an efficient mean of enhancing heavy metal tolerance in transgenic sugar beet. Until now, mutant evidence that explored the relationship between GSH homeostasis on Cd tolerance and S absorption process is rare. In present study, we assessed the possible role of GSH homeostasis on Cd tolerance in double *sultr1;1-sultr1;2* null mutant, especially under LS treatment.

Regulation of GSH biosynthesis

We measured the content of total S, which is necessary for the biosynthesis of thiol compounds through S assimilation pathway (Saito 2000). In both *Arabidopsis* lines, Cd increased sulfate uptake independently (Fig. 2a). This result is consistent with previous work reported in *Brassica napus* (Sun et al. 2007). Although the expression of *SULTR1;1* and *SULTR1;2* was suppressed, Cd stress could still enhance sulfate absorption in double mutant at low efficiency. Possible explanations are that plants might accumulate sulfate via a nonspecific anion uptake mechanism (Frachisse et al. 1999) or by activation of other sulfate transporters.

The first step of S assimilation pathway is sulfate uptake by roots and transport to other different organs (Kopriva et al. 2009). To study the roles of sulfate transporters, transcriptional levels of five genes were analyzed (Fig. 6). In wild-type seedlings, Cd down-regulated the expression levels of *SULTR2;1*, *SULTR1;3*, *SULTR3;4* and *SULTR3;*5 under LS treatment. Conversely, Cd increased the expression levels of these four genes in mutant seedlings under LS treatment, indicating the mutations of *SULTR1;1* and *SULTR1;2* did not restrict sulfate transport completely. Cd-induced increase expression of other sulfate transporters was highly important for double mutant. This viewpoint has already been proved by El-Zohri et al. (2015) recently. In their research, *sultr1;1-sultr1;2* double mutant also had drastically very low S contents in its root and shoot tissues. The low amounts of S in the double mutant could be explained by the expression of other sulfate transporters, such as *SULTR 1;3*, *SULTR2;1*, and *SULTR 2;2*. In double mutant, compensation process is responsible for adequate S supply for Cd tolerance.

The second step in the S assimilation pathway is the sulfate activation followed by its reduction to sulfide, catalyzed by ATPS and APR; while, the rate-limiting step for GSH biosynthesis is considered to be the availability of reduced S for needed Cys synthesis that occurs at the last step of the S reduction pathway (Cobbett 2000). Regardless of sulfate supply, Cd increased the expression levels of *ATPS*, *APR, SIR* and *OASTL* in wild-type seedlings (Fig. 6). Similar results were observed in both *Arabidopsis* (Harada et al. 2001) and other plants such as *Brassica juncea* (Heiss et al. 1999; Lee and Leustek 1999). Even though up-regulation induced by Cd was also observed in double mutant under LS treatment, the transcriptional levels of these four genes were much lower in double mutant than wild-type (Figs. 6, 8). Lower capacity of S assimilation in mutant after loss of functions of *SULTR1;1* and *SULTR1;2* could not meet enough GSH needs to tolerate Cd. Increment of GSH biosynthesis was closely related with Cd response (Semane et al. 2007).

Biosynthesis of GSH occurs in two steps: first, synthesis of γ-glutamylcysteine (γ-EC) catalyzed by γ-EC synthetase (ECS); then, synthesis of GSH catalyzed by GSH synthetase. In our study, mutations of *SULTR1;1* and *SULTR1;2* did not affect gene expression of *ECS1* under LS + Cd stress, but it was worth noticing that the *GSH2* transcriptional level was increased (Fig. 6). This result suggests that *GSH2* might be a potential gene responsible for maintaining GSH content when mutant plants were faced to lower S assimilation capacity.

Regulatory role of AsA–GSH pathway

Thiol groups, such as in GSH, possess the particularity of high affinity to Cd. In presence of Cd, an imbalance of the available GSH pool can lead to the disturbance of the AsA–GSH cycle (Di Cagno et al. 2001; Noctor and Foyer 1998; Jozefczak et al. 2012).

In the present study, enhanced GSH content was related to Cd tolerance in *Arabidopsis* (Fig. 4a), and the change in total GSH mainly resulted from the increase of the reduced form (GSH) rather than the oxidized form (GSSG) (Fig. 4a, c, e). In cells, GSH is supplemented by regeneration from GSSG reduction catalyzed by GR (Semane et al. 2007). Since Cd induced *GR1* expression (Fig. 6), we suppose up-regulated expression of *GR1* may promote this reaction. Although the GSH content was increased obviously in presence of Cd, double mutant seedlings accumulated less GSH than wild-type (Fig. 4c). Considering GSH functions directly as a free radical scavenger by reacting with ROS, GSH deficiency also contributes to the severe oxidative damage to double mutant. GSH/GSSG ratio is often used to indicate the redox state in organisms. Lower GSH/GSSG ratio implies more severe oxidative stress (Smeets et al. 2005). In present study, double mutant maintained lower GSH/GSSG ratio in comparison with wild-type under LS + Cd stress (Fig. 4g). This result was relevant to the excess H_2O_2 accumulation observed in Fig. 3b. Decreased GSH generation in mutant under LS + Cd was also related to the down-regulated *GR1* transcriptional abundance (Fig. 6). Lower expression of *GR1* transcript suggested that the function of GR was challenged by Cd in double mutant under S-deficiency condition. In other words, normal conversion between GSH and GSSG is impaired by combined stress. Additionally, GST, a group of dimeric, multifunctional enzymes, catalyzes conjugation of GSH with xenobiotic compounds for detoxification (Marrs 1996). As an important cellular detoxifier of metabolites, it is also involved in alleviating Cd-induced oxidative stress (Aravind and Prasad 2005). In our study, the expression of *GST1* was down-regulated by Cd in double mutant under LS + Cd stress (Fig. 6), which might result in severe oxidative damage in double mutant.

AsA takes part in growth process, electron transport and removal of H_2O_2 through APX (Semane et al. 2007). In plants, GSH can be used as electron donor by dehydroascorbate reductase (DHAR) to recycle DHA to AsA (Semane et al. 2007). In general, reduced conversion procedure from GSSG to GSH was accompanied with low DHAR activity, which directly resulted in low AsA and high DHA accumulation. In our study, under LS + Cd, *DHAR1* mRNA transcript level was lower in mutant than wild-type (Fig. 6). *DHAR1* transcript level has been correlated to plants' resistance to stress (Chen and Gallie 2004; Ushimaru et al. 2006). Reduced *DHAR1* reflects the poor resistance of double mutant to Cd under LS. Surprisingly, compared to wild-type, AsA content was slightly higher in double mutant, while its oxidized form, DHA content exhibited lower content when seedlings were all exposed to LS + Cd (Fig. 4d, f). We suppose DHAR may undergo post-transcriptional regulation (Chen and Gallie 2004). Increase of AsA and decrease of DHA resulted in the highest AsA/DHA ratio in double mutant under LS + Cd stress (Fig. 4h). One hypothesis to explain the highest ratio is the increased accumulation of H_2O_2. H_2O_2 is reduced to H_2O by APX using AsA as the specific electron donor (Semane et al. 2007). The above-mentioned Cd-reduced APX activity in double mutant under LS treatment led to AsA accumulation. Moreover, it is possible that regeneration of DHA from AsA by DHAR using GSH as an electron donor could not be maintained at a sufficient level due to decreased GSH content.

Relationship between PCs and GSH in Cd hypersensitivity

Binding mechanism of thiol compounds is crucial to heavy metal detoxification. One of the most important compounds for binding metal ions is non-protein thiol (NPTs). Many studies have indicated that Cd enhances NPTs formation (Cho and Seo 2006; Vögeli-Lange and Wagner 1996). Similarly, in our study, Cd also enhanced NPTs formation and SS treatment could promote this biosynthesis process in both *Arabidopsis* lines (Fig. 5b). Double mutant seedlings accumulated less NPTs than wild-type. These results suggest that S content and/or loss function of two high-affinity sulfate transporters are closely related to NPTs accumulation.

Cys, as predominant NPTs and the precursor molecule for GSH synthesis, plays an important role in plant stress responses (Sobrino-Plata et al. 2014). Since Cys is a potent chelator of heavy metals ions, Cd exposure increases the Cys demands (Heiss et al. 1999). In our study, LS and LS + Cd treatments triggered Cys accumulation, while double mutant accumulated much more Cys under LS + Cd combined stress (Fig. 5a). It has been reported that unstressed cells contain low Cys concentrations and
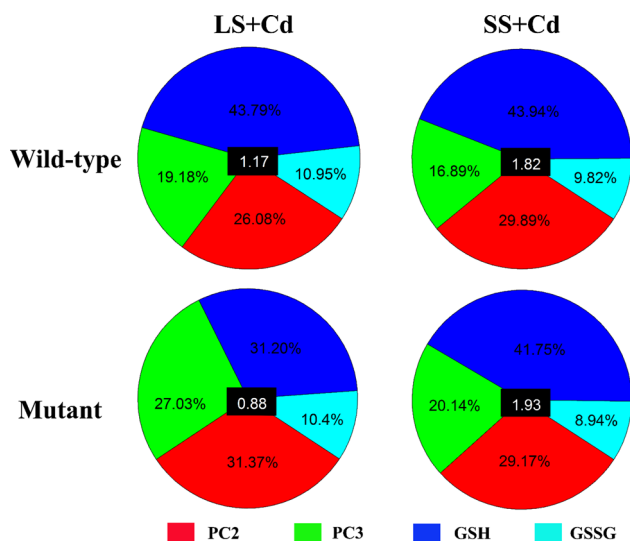
Fig. 7 Total GSH pool concentration (*black boxes*) and proportion (%) of reduced GSH (*Blue part*), GSSG (*light blue part*), PC2 (*red part*) and PC3 (*green part*) in wild-type and *sultr1;1-sultr1;2* double mutant *Arabidopsis* seedlings under limited or sufficient sulfate with Cd conditions for 2 weeks (color figure online)

free Cys levels would be increased in response to various abiotic stresses (Ruiz and Blumwald 2002). However, accumulation of free Cys is not always a benefit for plant.

Cys–metal ion complex can trigger the Fenton reaction, thereby producing the highly toxic •OH radical (Zagorchev et al. 2013). From these viewpoints, overaccumulation of Cys could be another important reason why mutant seedlings suffered from severe oxidative stress under LS + Cd.

Chelation by PCs is considered as another efficient mechanism against Cd toxicity. PCs bind Cd by thiol groups of Cys. After chelation, Cd–PCs complexes are transported into vacuole through some ATP binding cassette transporters for sequestration of Cd, thereby reducing Cd toxicity (Park et al. 2012). We detected the *PCS1* was up-regulated after Cd exposure. In previous studies, it has been reported that *PCS1* expression was constitutively expressed and post-transcriptionally regulated by activation of the enzyme in presence of metal (Cobbett 2000). Interestingly, we observed that double mutant seedlings suffered from LS + Cd combined stress maintained comparable PC2 and PC3 content as wild-type seedlings (Fig. 5c). Both Cd accumulation and PCs chelation were not influenced by lower GSH content induced by mutations of *SULTR1;1* and *SULTR1;2*. Correspondingly, regarding the expression level of *PCS1*, there was no difference between double mutant and wild-type under LS + Cd stress (Fig. 6). These results suggest that the chelation of
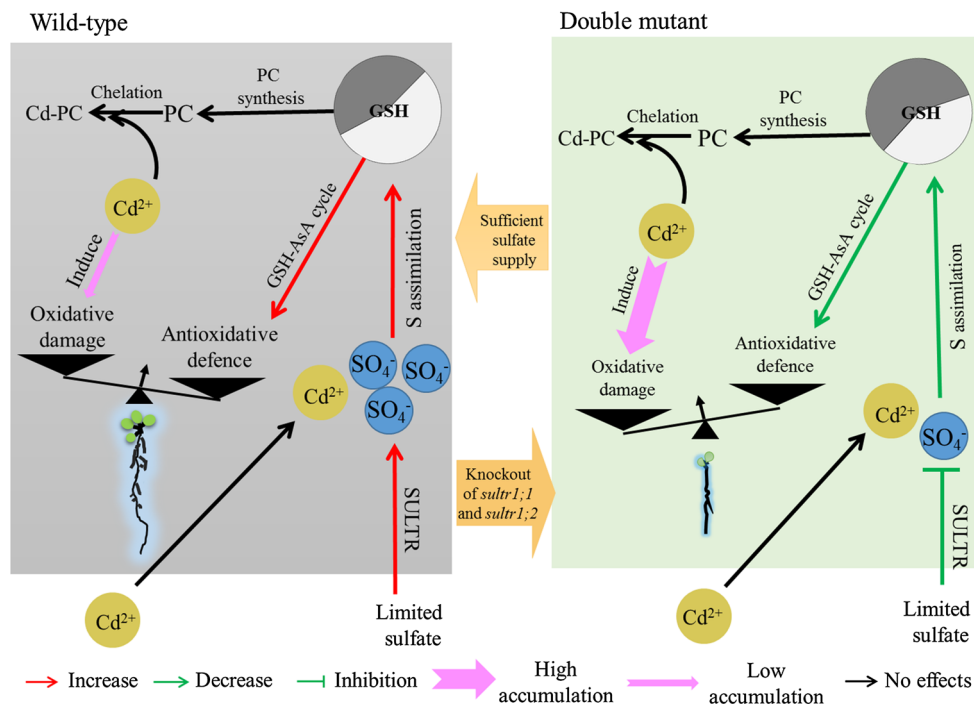

Fig. 8 A model of differential processes involved in Cd tolerance in mutant and wild-type *Arabidopsis* under LS + Cd combined stress. Mutations of *SULTR1;1* and *SULTR1;2* affected sulfate uptake, then sulfur (S) deficiency reduced S assimilation genes expression level, which ultimately resulted in decrease of GSH content. Having faced the deficiency of GSH, a large proportion of GSH was used for the PCs' synthesis to chelate Cd rather than participating in antioxidative

defense. Finally, imbalance between antioxidative defense and severe oxidative damage led to Cd hypersensitivity in mutant. Combined stress-induced Cd hypersensitivity can be turned over by additional sufficient sulfate in medium. In small GSH pie chart, *white* section means percentage of GSH involved in antioxidative reaction, the *dark gray* section means the percentage involved in PCs' synthesis (color figure online)

PCs to Cd is not affected in double mutant, even under LS + Cd combined stress.

To know the amount of GSH involved in PCs' synthesis, we referred to the data processing method according to Sobrino-Plata et al. (2014) and designed an additional pie chart (Fig. 7). New figure exhibited the total GSH pool (black boxes) and proportion (%) of GSH, GSSG, PC2, PC3 in wild-type and double mutant. We calculated the total GSH pool by adding up the content of reduced GSH, two-fold GSSG, PC2 and PC3. It was obvious that compared to wild-type under LS + Cd, total GSH pool in mutant was lower. More importantly, having faced the GSH pool deficiency condition, 31 and 27 % of GSH was used to synthesize PC2 and PC3, respectively, while the percentage was 26 and 19 % in wild-type. Thus, it can be seen that a majority of GSH in pool is consumed for the synthesis of PCs.

In summary, to further acknowledge the role of GSH homeostasis on Cd stress, a double *sultr1;1-sultr1;2* mutant was used in our study. Compared to wild-type, double mutant was more sensitive to Cd under limited sulfate supply. More importantly, the sensitive phenotype was mainly attributed to the decreased GSH content that affected oxidative defense but not PCs' synthesis (Fig. 8).

Compliance with ethical standards

Author contribution statement X.L., F.H.W. and H.L.Z. designed the experiments and wrote the manuscript. X.L., F.H.W., J.X.L., J.C., G.H.W., W.H.W., W.J.H., L.J.G., Z.L.W. and J.H.C. conducted experiments. X.L. and M.S. analyzed data. F.H.W. and W.H.W. provided technical assistance. All authors read and approved the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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