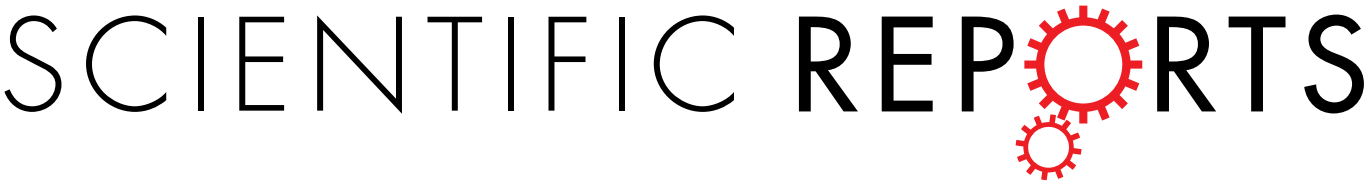

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# Hydrogen sulfide - cysteine cycle system enhances cadmium tolerance through alleviating cadmium-induced oxidative stress and ion toxicity in Arabidopsis roots

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**Cadmium ($Cd^{2+}$) is a common toxic heavy metal ion. We investigated the roles of hydrogen sulfide ($H_2S$) and cysteine (Cys) in plant responses to $Cd^{2+}$ stress. The expression of $H_2S$ synthetic genes *LCD* and *DES1* were induced by $Cd^{2+}$ within 3 h, and endogenous $H_2S$ was then rapidly released. $H_2S$ promoted the expression of Cys synthesis-related genes *SAT1* and *OASA1*, which led to endogenous Cys accumulation. The $H_2S$ and Cys cycle system was stimulated by $Cd^{2+}$ stress, and it maintained high levels in plant cells. $H_2S$ inhibited the ROS burst by inducing alternative respiration capacity (AP) and antioxidase activity. $H_2S$ weakened $Cd^{2+}$ toxicity by inducing the metallothionein (MTs) genes expression. Cys promoted GSH accumulation and inhibited the ROS burst, and GSH induced the expression of phytochelatin (PCs) genes, counteracting $Cd^{2+}$ toxicity. In summary, the $H_2S$ and Cys cycle system played a key role in plant responses to $Cd^{2+}$ stress. The $Cd^{2+}$ tolerance was weakened when the cycle system was blocked in *lcddes1-1* and *oasa1* mutants. This paper is the first to describe the role of the $H_2S$ and Cys cycle system in $Cd^{2+}$ stress and to explore the relevant and specificity mechanisms of $H_2S$ and Cys in mediating $Cd^{2+}$ stress.**

Cadmium ($Cd^{2+}$) is a common toxic heavy metal ion in the environment. It greatly affects the growth and development of plants and is harmful to human health through the food chain[1,2]. Because of its carcinogenic properties and its detrimental effects on the growth of organisms, $Cd^{2+}$ contamination of agricultural soil has become a critical concern. Preventing reduced growth and accumulation of $Cd^{2+}$ in harvested organs of plants growing on $Cd^{2+}$-contaminated soils has become an urgent task as it can contribute to food safety. Thus, it is important to explore plant stress defense mechanisms and to find ways to reduce the $Cd^{2+}$ accumulation in grains.

As a heavy metal not participating in redox reactions, $Cd^{2+}$ can easily dissolve in water and quickly be taken up by plant roots[3,4]. The physiological consequences of $Cd^{2+}$ toxicity in plants are chlorosis, stunted growth, and cell death, among others[5–7]. At the cellular level, $Cd^{2+}$ can alter protein structure and inhibit enzyme activity by binding to sulfhydryl and carbonyl groups and replacing essential co-factors of enzymes[7–9]. The overproduction of reactive oxygen species (ROS) is the primary response of plants to $Cd^{2+}$ with negative impact on cell function[10]. Further damage can be caused by ROS-independent, secondary mechanisms. Lipid peroxidation is the most deleterious effect caused by $Cd^{2+}$-induced ROS[4]. Malondialdehyde (MDA), one of the decomposition products of lipid peroxidation, can modify active substrates in plant cells, including nucleic acids, proteins and saccharides[11]. To become resistant to $Cd^{2+}$ toxicity, plants have developed several strategies, such as inducing the

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alternative respiratory pathway[11], activating antioxidants and glutathione (GSH)[12], and regulating the influx and efflux of heavy metals[13,14], as well as regulating the levels of heavy metal chelators, phytochelatins (PCs)[15] and metallothioneins (MTs)[16].

Hydrogen sulfide ($H_2S$) has been considered toxic gas for many years, which inhibits cytochrome oxidase activity in animal cell[17]. Recently, it has emerged as the third endogenous gasotransmitter, following the discovery of nitric oxide and carbon monoxide[17]. In plant systems, endogenous $H_2S$ is generated through enzymatic pathways. Cysteine (Cys) desulfhydrases (CDes) are key enzymes involved in $H_2S$ generation[18]. Cys synthesis occurs via two sequential phases catalyzed by serine acetyltransferase (SAT) and O-acetylserine(thiol)lyase (OAS-TL), both of which are encoded by multigene families[19]. L-Cys desulfhydrase (LCD) is the most understood CDes in Arabidopsis; it regulates L-Cys degradation into pyruvate, ammonia and $H_2S$[20]. OAS-TL regulates that $H_2S$ and O-acetylserine (OAS) synthetise L-Cys[20]. These physiological processes form $H_2S$ - Cys cycle system in cell. Recently, based on the sequence characteristics of DES1, it has been classified as an OAS-TL[21]. However, functional analysis of this enzyme revealed that DES1 has a higher affinity for L-Cys and degrades it to generate $H_2S$[21].

The alternative respiratory pathway is a unique pathway in the mitochondrial electron transport chain in higher plants that is regulated by alternative oxidase (AOX)[22]. A large body of evidence suggests that the enhanced alternative pathway could improve stress tolerance through limiting the ROS burst[22,23]. Recent research indicated that respiratory activity is regulated by endogenous $H_2S$ in *Escherichia coli*[24]. However, the relationship between $H_2S$ and the alternative respiratory pathway in plant responses to $Cd^{2+}$ stress is unclear.

Sulfur is an essential element that is taken up by plants in its oxidized state, reduced to $H_2S$, and first incorporated into Cys before it is used in metabolic processes. The products of sulfur metabolism, such as Cys, GSH, PCs, MTs and $H_2S$, have biological functions in plant responses to heavy metal stress and oxidative stress[25]. Recently, positive effects of $H_2S$ in response to several types of abiotic stress in plants have been found, such as osmotic stress, salt stress, heat shock stress and heavy metal stress[26–29]. It has been reported that a cross-talk between $H_2S$ and nitric oxide is responsible for the increased $Cd^{2+}$ tolerance in alfalfa and Bermuda grass plants[30,31]. In addition, $H_2S$ alleviates $Cd^{2+}$ toxicity by regulating cadmium transport in *Populus euphratica* cells[32]. $H_2S$ is also involved in the growth and development of plants through its effects on stomatal closure and seed germination and by increasing the growth rate[33–36]. Cys acts as a functional precursor for many important biologic activators, such as PCs and GSH, which can enhance the tolerance of plants to heavy metal stress[25,37]. In addition, $Cd^{2+}$ tolerance significantly decreases when Cys biosynthesis is blocked in *oasa1-1* and *oasa1-2* mutants[37].

Compelling evidence has suggested that $H_2S$ and Cys are involved in plant tolerance to heavy metal stress. The biosynthesis of $H_2S$ and Cys are interrelated. $H_2S$ is involved in the uptake of $SO_4^{2-}$ and in the biosynthesis of Cys. Cys is also involved in the generation of $H_2S$. Nevertheless, the relation and interaction between $H_2S$ and Cys are yet unknown under $Cd^{2+}$ stress. In this study, we aimed to clarify the common and independent functions of $H_2S$ and Cys in $Cd^{2+}$ stress. Additionally, we sought to demonstrate the working mechanisms of the $H_2S$ and Cys cycle system response to $Cd^{2+}$ stress in Arabidopsis.

## Results

**Effect of $Cd^{2+}$ on root elongation, MDA and EL in Arabidopsis roots.** Arabidopsis seedlings (7-d-old) were transferred aseptically to 1/2 MS medium-containing $CdCl_2$, and the lengths of the primary roots were measured 5 d later. $Cd^{2+}$ stress led to toxicity symptoms and inhibited the elongation of Arabidopsis roots in a dose-dependent manner. As shown in Fig. 1a and b, root growth was slightly inhibited under 25 μM $Cd^{2+}$, but root elongation was significantly inhibited under 50 to 150 μM $Cd^{2+}$, exhibiting 53.5% to 34.9% inhibition, respectively. Malondialdehyde (MDA) and electrolyte leakage (EL) are considered as good indicators of stress-induced cell damage. $Cd^{2+}$ stress caused lipid peroxidation and MDA accumulation. When plants were treated with 50 to 150 μM $Cd^{2+}$, the MDA content of the roots increased by 117% to 200%, respectively (Fig. 1c). In the presence of 50 to 150 μM $Cd^{2+}$, EL increased by 131% to 217%, respectively (Fig. 1d). The 50 to 150 μM $Cd^{2+}$ treatment had significant effects on Arabidopsis roots. 100 and 150 μM $Cd^{2+}$ concentrations were too violent for plant growth and 150 μM $Cd^{2+}$ concentrations lead to the seedling etiolation in Fig. 1a. Thus, 50 μM $Cd^{2+}$ was chosen for further study of $Cd^{2+}$ stress.

**Effect of $Cd^{2+}$, NaHS and Cys on the $H_2S$ and Cys cycle system.** To explore the working mechanisms of the $H_2S$ and Cys cycle system's response to $Cd^{2+}$ stress in Arabidopsis roots, a time-course analysis of endogenous $H_2S$ and Cys contents was performed. Endogenous $H_2S$ and Cys contents undulated along with the time of $Cd^{2+}$ stress. $H_2S$ content was rapidly induced after treatment with $Cd^{2+}$ for 3 h, reached the highest level at 9 h, and then decreased at 12 h, but had another increase at 36 h (Fig. 2a). Treatment with Cys could enhance the $H_2S$ level and maintain $H_2S$ content at a high level in $Cd^{2+}$ stress (Fig. 2a). The Cys level slightly decreased in the initial stage under $Cd^{2+}$ treatment, but it increased after treatment with $Cd^{2+}$ for 12 h and maintained a high level from 24 to 36 h (Fig. 2b). Treatment with NaHS promoted Cys accumulation and a high level of Cys was maintained during $Cd^{2+}$ stress (Fig. 2b). When plants were treated with 50 or 100 μM $Cd^{2+}$ for a long time, both $H_2S$ and Cys levels were enhanced in Arabidopsis roots (Fig. 2c and d). As mentioned above, $H_2S$ and Cys contents were elevated by $Cd^{2+}$ stress, and $H_2S$ appeared to be an important mediator in the $Cd^{2+}$-induced increase of Cys, and the $H_2S$ and Cys cycle system was enhanced.

**The effects of $Cd^{2+}$, NaHS and Cys on synthetic genes of $H_2S$ and Cys.** To study the direct effects of $Cd^{2+}$, NaHS and Cys on genes regulating the synthesis of $H_2S$ and Cys, the Arabidopsis seedlings were exposed to various treatments for 3 h. The expressions of $H_2S$ synthetic genes *LCD* and *DES1* were markedly induced by $Cd^{2+}$ and Cys (Fig. 3a and b), but the expression of *D-CDES* was not significantly affected by $Cd^{2+}$ and Cys treatments (Fig. 3c). *SATs* and *OASs* are the important synthetic genes of Cys, but they had different responses to $Cd^{2+}$, NaHS and Cys. The expression levels of *SAT1* and *OASA1* were slightly increased in $Cd^{2+}$ stress, but they were

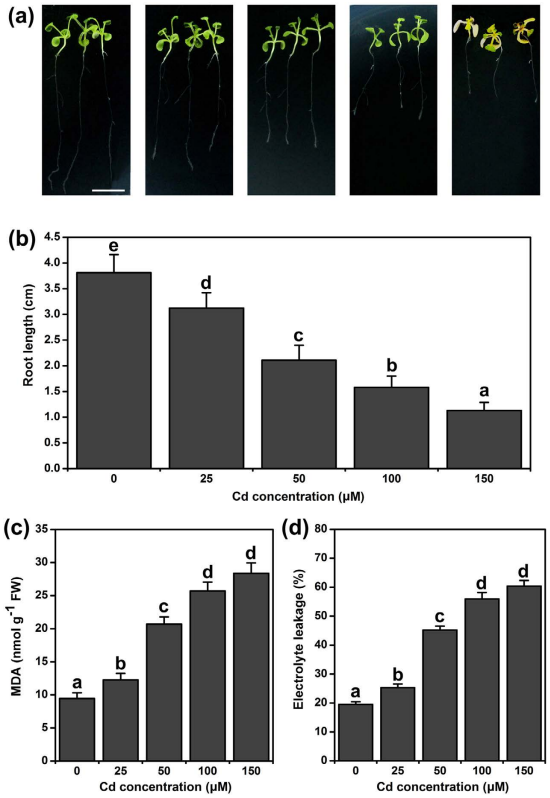


**Figure 1. The effect of $Cd^{2+}$ on root length, MDA and EL in Arabidopsis roots.** (**a**) Phenotype of root growth in WT seedlings. Bar = 1 cm. (**b**) The root lengths of WT seedlings (n > 25). (**c**) MDA contents in WT roots stressed by various concentrations of $Cd^{2+}$. (**d**) EL in WT roots stressed by various concentrations of $Cd^{2+}$. 7-d-old Arabidopsis seedlings were grown on 1/2 MS agar plates supplied with 0–150 μM $Cd^{2+}$ for 5 d, and the lengths of the primary roots, MDA contents and EL were recorded. Mean values and SE were calculated from three replicates. Within each set of experiments, bars with different letters are significantly different ($P < 0.05$, Duncan's multiple range tests).

markedly induced by treatment with NaHS (Fig. 3d and g). Additionally, the expression of *SAT5* was inhibited by Cys (Fig. 3f). *SAT3*, *OASB* and *OASC* did not respond to $Cd^{2+}$, NaHS or Cys (Fig. 3e,h and i).

**The effects of $Cd^{2+}$, NaHS and Cys on root elongation, MDA and EL in *lcddes1-1* and *oasa1* mutants.** Five-day-old Arabidopsis seedlings were transferred aseptically to $Cd^{2+}$-containing 1/2 MS medium, and the lengths of the primary roots were measured one week later. The root elongation of *lcddes1-1* mutant was shorter compared to WT root elongation under control conditions, but the root elongation of *oasa1* was the same as WT (Fig. 4a and b). The *lcddes1-1* and *oasa1* mutants were more sensitive to $Cd^{2+}$ stress. Application of NaHS or Cys recovered the $Cd^{2+}$-induced growth inhibition in WT. NaHS markedly recovered the effect of $Cd^{2+}$ in *lcddes1-1*, but it only partly recovered the effect of $Cd^{2+}$ in *oasa1* (Fig. 4a and b). On the contrary, treatment with Cys slightly recovered the effect of $Cd^{2+}$ in *lcddes1-1*, but it significantly recovered the effect of $Cd^{2+}$ in *oasa1* (Fig. 4a and b). Cys-mediated partial recovery the root length may be due to an independent physiological action of Cys in *lcddes1-1* because $H_2S$ production was blocked in the double mutant. NaHS or Cys treatment markedly decreased the EL level and the content of MDA under $Cd^{2+}$ stress in WT (Fig. 4c and d). NaHS strongly reduced the MDA content and the EL in *lcddes1-1* and *oasa1* (Fig. 4c and d). Cys prevented the effects of $Cd^{2+}$ on the MDA content and EL in *oasa1* but partly weakened the effects of $Cd^{2+}$ on the MDA content and EL in *lcddes1-1* (Fig. 4c and d).

**The effects of $Cd^{2+}$, NaHS and Cys on the alternative respiratory pathway.** The alternative respiratory pathway plays an important role in plant stress resistance by limiting the ROS burst[38]. In this study, we sought to elucidate the roles of NaHS and Cys in the alternative respiratory pathway under $Cd^{2+}$ stress. In general, the alternative pathway operates at a low level under normal conditions, but it can be significantly induced when plants are stimulated by various environmental stresses[23]. We first checked the expression of AOX genes after $Cd^{2+}$, NaHS and Cys treatments for 3 h. The expression of *AOX1A*, *AOX1C* and *AOX2* were increased in $Cd^{2+}$ stress (Fig. 5a,b and c). Interestingly, NaHS and Cys treatments also markedly enhanced the expression levels of *AOX1A, AOX1C* and *AOX2* in both control and $Cd^{2+}$ stress conditions (Fig. 5a,b and c). Furthermore, the total respiration capacity (TP), cytochrome respiration capacity (CP) and alternative respiration capacity (AP) were analyzed in WT and mutant plants. TP was slightly enhanced by 25 or 50 μM $Cd^{2+}$, but it was inhibited by 100 or 150 μM $Cd^{2+}$ in WT (Fig. 5d). Under $Cd^{2+}$ stress, CP was inhibited in a dose-dependent manner; however,

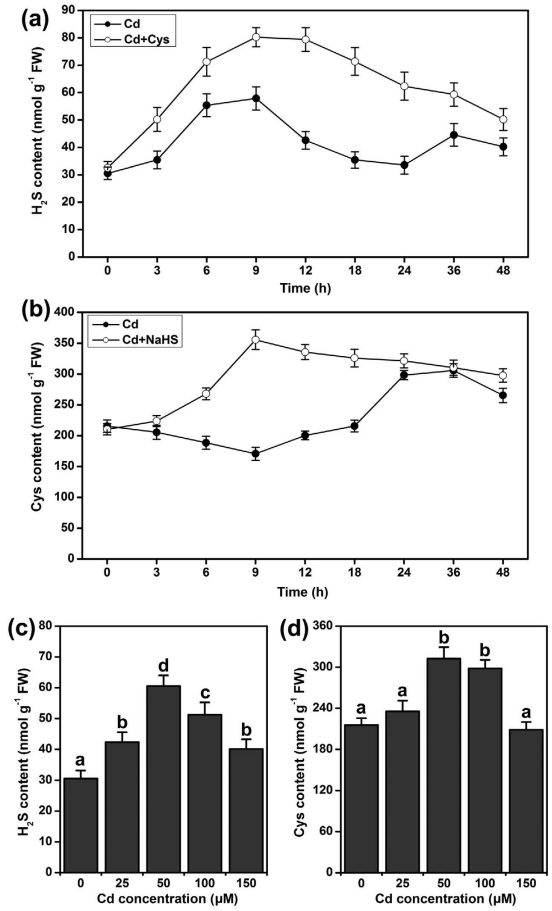


**Figure 2. Analysis of endogenous $H_2S$ and Cys contents in WT roots of Arabidopsis.** (**a**) Time-course of $H_2S$ content. (**b**) Time-course of Cys content. 7-d-old WT seedlings were treated with 50 μM $Cd^{2+}$, 50 μM $Cd^{2+}$ + 1 mM Cys and 50 μM $Cd^{2+}$ + 50 μM NaHS for 0 to 48 h. (**c**) Changes of $H_2S$ content in various $Cd^{2+}$ concentrations. (**d**) Changes of Cys content in various $Cd^{2+}$ concentrations. 7-d-old WT seedlings were supplied with 0–150 μM $Cd^{2+}$ for 5 d. Mean values and SE are calculated from three replicates. Within each set of experiments, bars with different letters are significant different ($P < 0.05$, Duncan's multiple range tests).

AP was increased under $Cd^{2+}$ stress, and AP achieved its maximum induction with the 50 μM $Cd^{2+}$ treatment (Fig. 5e). Similar to the pattern of expression of the AOX genes in response to NaHS or Cys treatments under $Cd^{2+}$ stress, AP was increased by NaHS or Cys under $Cd^{2+}$ stress (Fig. 5f). However, it was different in the mutant plants. Under $Cd^{2+}$ stress, the effects of Cys on AP were not observed in *lcd* and *des1-1*, and they were especially decreased in *lcddes1-1* (Fig. 5g,h and i). In *oasa1*, the effects of NaHS and Cys were the same as in WT under $Cd^{2+}$ stress (Fig. 5g).

**The effects of $Cd^{2+}$, NaHS and Cys on antioxidant enzyme activity and GSH level, and the relationship among AP, antioxidant enzyme activity, and GSH level in $Cd^{2+}$ stress.** Antioxidant enzymes depress the level of ROS. A previous study showed that $H_2S$ could enhance antioxidase activity in rice[39]. In addition, many studies suggested that AOX was important in maintaining the homeostasis of the redox state[22,38]. Therefore, the effects of $Cd^{2+}$, NaHS, Cys and AP on antioxidant enzyme activity were analyzed. As shown in Fig. 6a and c, after 12 h of 50 μM $Cd^{2+}$ treatment, the activities of SOD and CAT in plants were significantly higher than in the control plants in WT. NaHS or Cys treatments could enhance the antioxidase activity under unstressed conditions (Fig. 6b and d), and this enhancement was further strengthened under $Cd^{2+}$ stress in WT (Fig. 6a and c). However, treatment with n-propyl gallate (nPG) had no significant effect on the antioxidase activity of the plants either under $Cd^{2+}$ stress or under unstressed conditions. Furthermore, nPG did not affect the elevated antioxidase activity of the NaHS- and Cys-treated plants under $Cd^{2+}$ stress (Fig. 6a and c). The effects of $Cd^{2+}$ and Cys were altered in *lcddes1-1*; treatment with $Cd^{2+}$ or Cys did not enhance the antioxidase activity in *lcddes1-1* (Fig. 6b and d). Additionally, the effects of $Cd^{2+}$ on the SOD activity were also weakened, and CAT activity was negligible in *oasa1* (Fig. 6d). Contrarily, treatment with NaHS still enhanced the antioxidase activity in mutant plants (Fig. 6b and d).

GSH is the product of sulfur metabolism, and it has positive biological functions in plant responses to heavy metal stress and oxidative stress[25]. As shown in Fig. 6e, the GSH content was increased in $Cd^{2+}$ stress. NaHS and Cys also enhanced the GSH level in WT (Fig. 6e and f). Specially, Cys had a significant promoting effect on GSH content. The *oasa1* mutant did not respond to $Cd^{2+}$ and NaHS, and even had a reduced GSH level, but Cys still

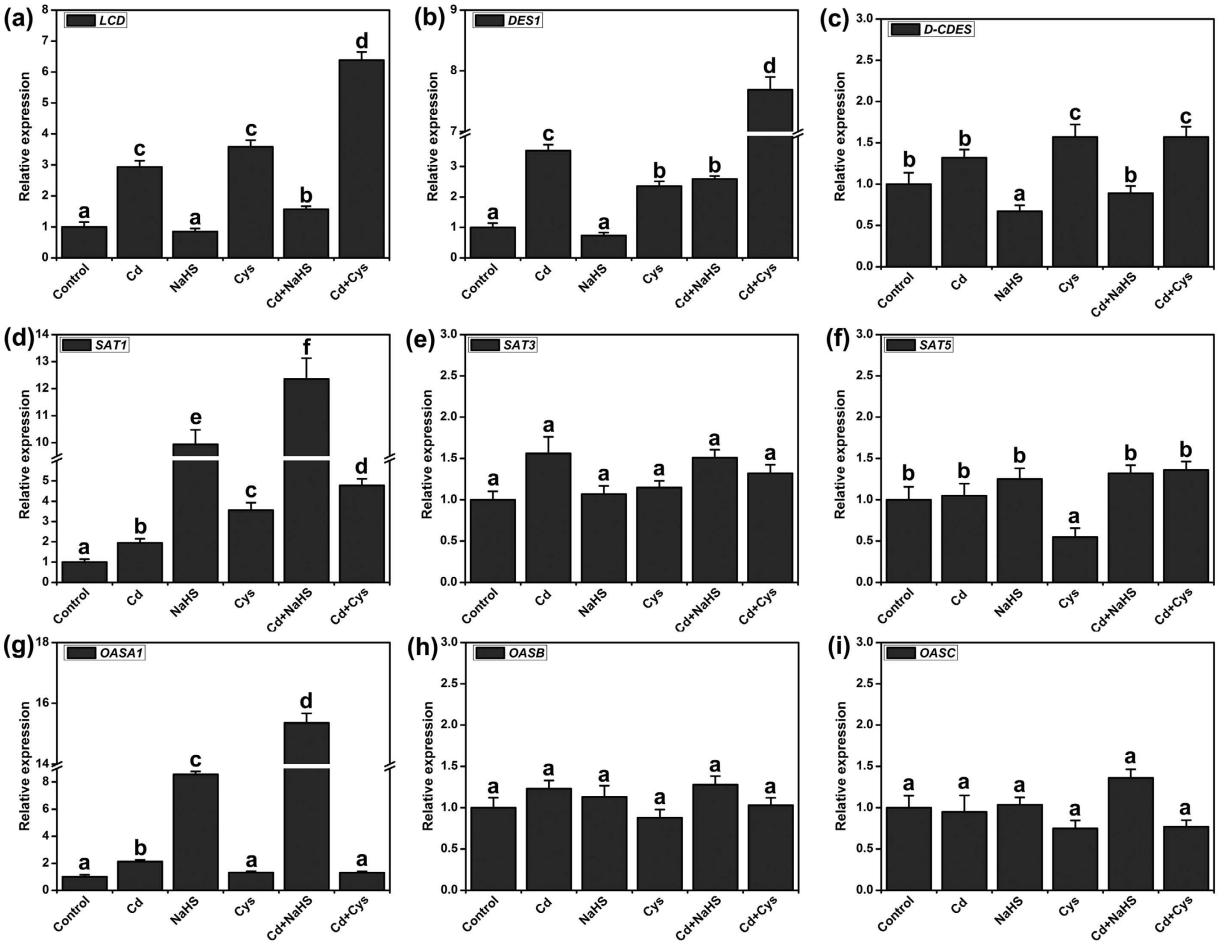


**Figure 3. qRT-PCR analysis the synthesis genes of $H_2S$ and Cys in Arabidopsis WT roots.** Relative expression levels were normalized with the internal standard *EF1a*. 7-d-old WT seedlings were grown on agar plates supplemented with 50 μM $Cd^{2+}$, 50 μM NaHS, 1 mM Cys, 50 μM $Cd^{2+}$ plus 50 μM NaHS, and 50 μM $Cd^{2+}$ plus 1 mM Cys for 3 h, respectively. Mean values and SE were calculated from three replicates. Within each set of experiments, bars with different letters are significant different ($P < 0.05$, Duncan's multiple range tests).

increased the GSH content in *oasa1* (Fig. 6f). Additionally, the effect of $Cd^{2+}$, NaHS and Cys on the GSH content in *lcddes1-1* was the same as WT plants (Fig. 6f).

**Effect of NaHS and Cys on ROS, and the relationship between AP and ROS in $Cd^{2+}$ stress.** To estimate the potential role of the $H_2S$ and Cys cycle in ROS homeostasis, we visualized the production of $H_2O_2$ in the roots under $Cd^{2+}$ stress. Over-accumulation of $H_2O_2$ was visualized by fluorescence labeling in the roots subjected to $Cd^{2+}$ stress (Fig. 7a). Conversely, NaHS or Cys treatment considerably diminished the accumulation of $H_2O_2$ in $Cd^{2+}$ stress (Fig. 7a and b). Additionally, inhibiting the alternative respiratory pathway with nPG caused an over-accumulation of $H_2O_2$ under $Cd^{2+}$ stress. The effects of NaHS and Cys were partly averted and slightly weakened by nPG in $Cd^{2+}$ stress, respectively (Fig. 7a and b). As shown in the time-course of $H_2O_2$, the ROS burst occurred during the early phase of $Cd^{2+}$ stress. Then, high levels of ROS were maintained from 4 to 8 h and declined after 12 h. $H_2S$ supplementation could maintain $H_2O_2$ at a low level during $Cd^{2+}$ stress. Treatment with Cys did not alter the burst of $H_2O_2$ in the early phase, but it prevented the over-accumulation of $H_2O_2$ after 6 h (Fig. 7c).

**Effect of NaHS and Cys on $Cd^{2+}$ accumulation.** The role of $H_2S$ and Cys in $Cd^{2+}$ homeostasis was investigated by measuring the percentage of $Cd^{2+}$ in the root. The results in Fig. 8a show that $Cd^{2+}$ accumulation increased in roots under $Cd^{2+}$ stress in WT and in the mutants, but the mutants accumulated more $Cd^{2+}$ than the WT. NaHS or Cys supplementation had inhibitory effects on $Cd^{2+}$ uptake and accumulation in WT and *oasa1*. Nevertheless, *lcddes1-1* did not respond to the effect of NaHS under $Cd^{2+}$ stress, but the effect of Cys on $Cd^{2+}$ uptake and accumulation was only partially reduced in *lcddes1-1*.

**Effect of NaHS and Cys on the expression of heavy metal chelator genes.** When plants were treated with $Cd^{2+}$ for 3 h, the expression of the heavy metal chelator genes *PCS1*, *PCS2*, *MT1A*, *MT1B* and *MT2B* was significantly up-regulated in WT. Cys supplementation promoted the expression of *PCS1* and *PCS2*, and

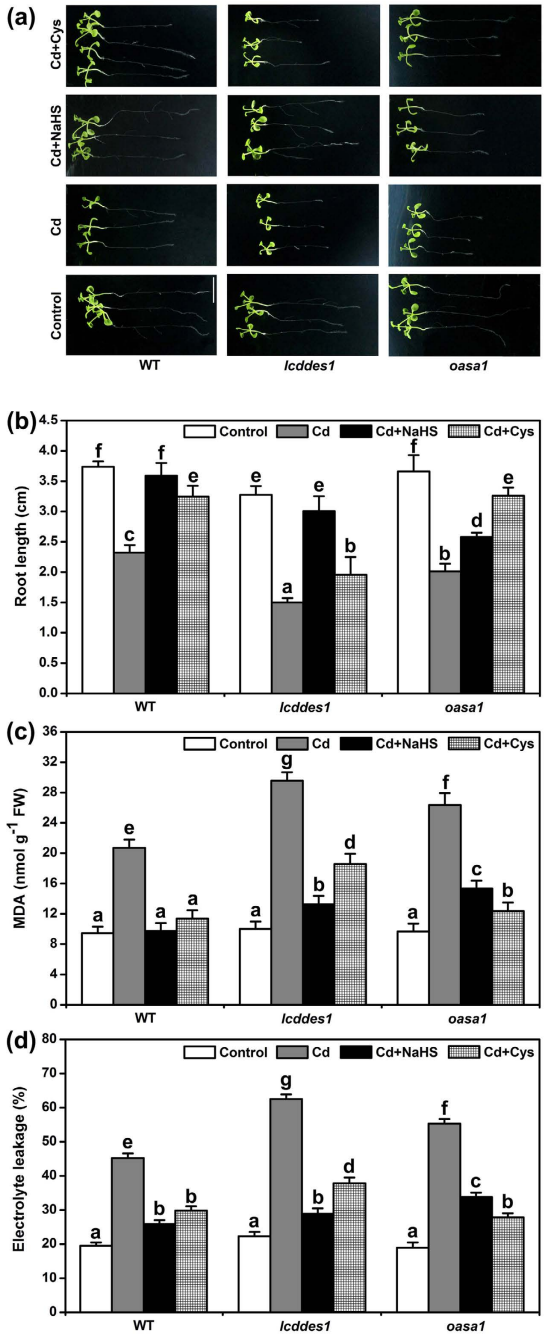


**Figure 4. The effect of $H_2S$ and Cys on root length, MDA and EL in *lcddes1-1* and *oasa1* mutant plants under $Cd^{2+}$ stress.** (**a**) Phenotype of Arabidopsis root growth. Bar = 1 cm. (**b**) The root lengths of Arabidopsis seedlings (n > 25). (**c**) MDA contents in Arabidopsis roots. (**d**) EL in Arabidopsis roots. 7-d-old Arabidopsis seedlings were grown on 1/2 MS agar plates supplied with 50 μM $Cd^{2+}$, 50 μM $Cd^{2+}$ plus 50 μM NaHS and 50 μM $Cd^{2+}$ plus 1 mM Cys for 5 d respectively, and the lengths of the primary roots, MDA contents and EL were recorded. Mean values and SE were calculated from three replicates. Within each set of experiments, bars with different letters are significantly different ($P < 0.05$, Duncan's multiple range tests).

NaHS promoted the expression of *MT1A*, *MT1B* and *MT2B* (Fig. 8b). To further study the effect of the $H_2S$ and Cys cycle system on the heavy metal chelator genes, the time-course of *PCS1* and *MT1A* gene expression was investigated. $Cd^{2+}$ was found to up-regulate the expression of *PCS1* and *MT1A* genes at 0.5 h, which then remained at a high expression level. Cys enhanced the expression of the *PCS1* gene at 0.5 h, which reached a maximum by 1 h, but NaHS enhanced the expression of the *PCS1* gene at 6 to 12 h. The expression of *MT1A* was different from *PCS1*. After 3 h of Cys supplementation, the expression of *MT1A* started to increase, reaching a maximum at 6 h, and NaHS enhanced the expression of *MT1A* gene at 0.5 h (Fig. 8c and d).

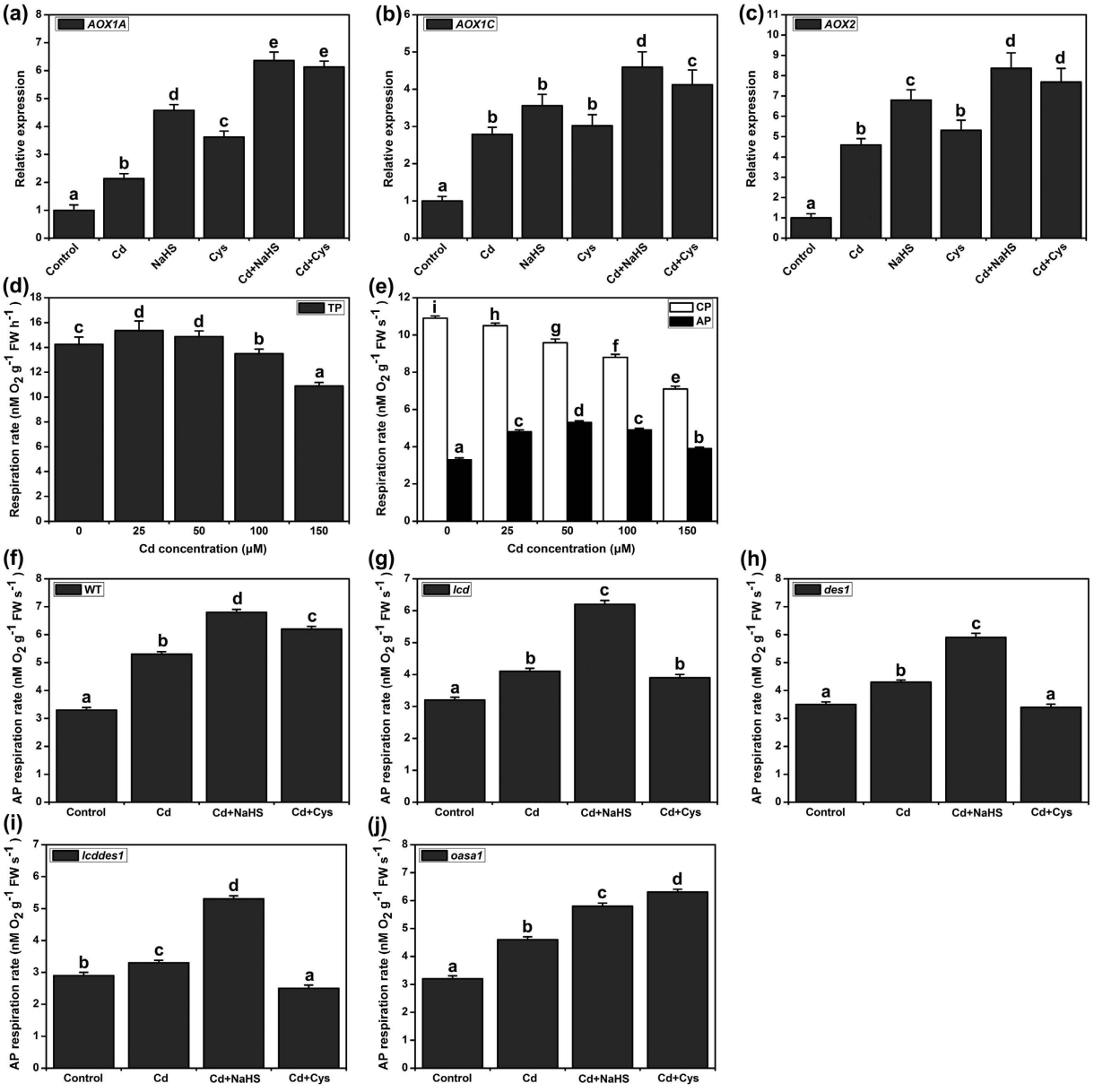


**Figure 5. Effect of $H_2S$ and Cys on the expression of AOX genes and the activity of TP, CP and AP in $Cd^{2+}$ stress.** (**a**–**c**) The expression of *AOX1A*, *AOX1C* and *AOX2*. 7-d-old WT seedlings were grown on 1/2 MS agar plates supplied with 50 μM $Cd^{2+}$, 50 μM NaHS, 1 mM Cys, 50 μM $Cd^{2+}$ plus 50 μM NaHS, and 50 μM $Cd^{2+}$ plus 1 mM Cys for 3 h, respectively. (**d**,**e**) Changes in TP, CP and AP activity in various $Cd^{2+}$ concentrations for 5 d in WT. (**f**–**l**) AP activity in WT and mutants. 7-d-old Arabidopsis seedlings were grown on 1/2 MS agar plates treated with 50 μM $Cd^{2+}$, 50 μM $Cd^{2+}$ plus 50 μM NaHS and 50 μM $Cd^{2+}$ plus 1 mM Cys for 5 d. Mean values and SE were calculated from three replicates. Within each set of experiments, bars with different letters are significantly different ($P < 0.05$, Duncan's multiple range tests).

## Discussion

The root is the primary organ that plants deploy to accumulate most of the heavy metals to which they are exposed[40,41]. Sulfur metabolism is required for the growth and development of plants, and the production of sulfur metabolites also plays a critical role in plant responses to heavy metal-induced stress[25]. $H_2S$ and Cys are important sulfur metabolism products that participate in suppressing heavy metal stress in plants[40]. In previous reports, $H_2S$ and Cys were always studied separately in plant responses to abiotic stress[39,42]. Recently, $H_2S$ and $H_2S$-induced Cys accumulation were reported to be critical in imparting $Cr^{6+}$ tolerance in Arabidopsis[43]. Therefore, the $H_2S$ and Cys cycle is an important system for regulating $H_2S$ and Cys functions in heavy metal stress. In this study, we used the *lcddes1-1* and *oasa1* Arabidopsis mutants to block the $H_2S$ and Cys cycle system. Then, we intensively researched the relevant and specificity roles of $H_2S$ and Cys in $Cd^{2+}$ stress. Our results indicated that $Cd^{2+}$ can rapidly accumulate in Arabidopsis roots and inhibit the primary root growth in a $Cd^{2+}$ concentration-dependent manner (Figs 1a and 7a), suggesting that $Cd^{2+}$ is easily absorbed and highly toxic.

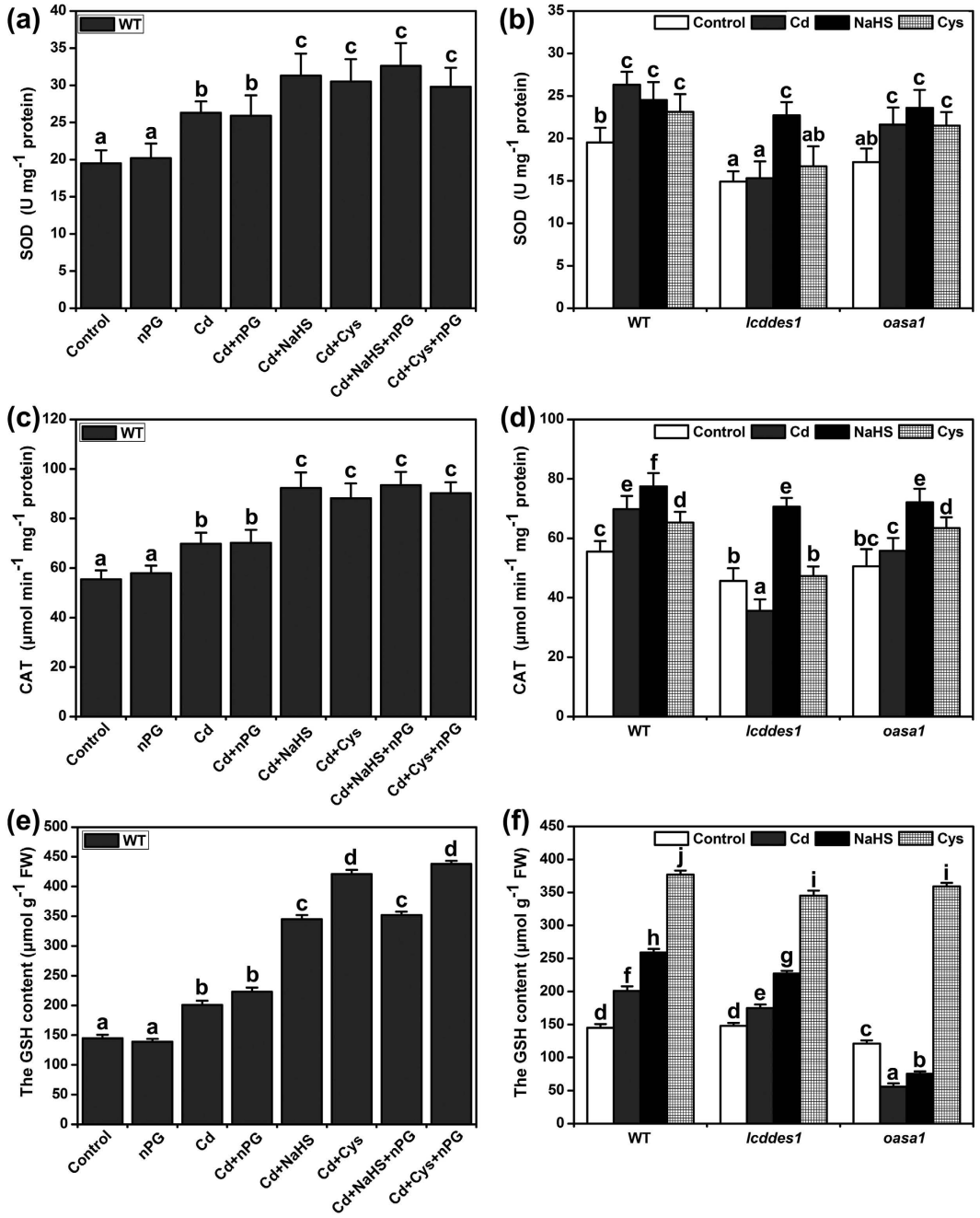


**Figure 6. Effect of $H_2S$ and Cys on antioxidant enzymes activity and GSH level in $Cd^{2+}$ stress.** 7-d-old Arabidopsis seedlings were grown on 1/2 MS agar plates supplemented with 50 μM $Cd^{2+}$, 50 μM NaHS, 1 mM Cys, and 200 μM nPG for 6 h, and SOD activity (**a**,**b**), CAT activity (**c**,**d**) and GSH content (**e**,**f**) were recorded. Mean values and SE were calculated from three replicates. Within each set of experiments, bars with different letters are significantly different ($P < 0.05$, Duncan's multiple range tests).

We observed that endogenous $H_2S$ and Cys levels undulate from 3 to 48 h under $Cd^{2+}$ stress (Fig. 2). However, the endogenous patterns of change were different for $H_2S$ and Cys levels. Endogenous $H_2S$ was first induced by $Cd^{2+}$ stress, and then Cys levels increased. On this account, we suppose that $H_2S$ is produced rapidly under $Cd^{2+}$ stress and that it acts as second messenger to activate the synthesis of Cys, implying that $Cd^{2+}$ stress could be the direct cause of endogenous $H_2S$ release but that Cys accumulation is a secondary effect of $Cd^{2+}$ stress. Data for the expression of $H_2S$ and Cys synthetic genes supports this hypothesis. The expression of $H_2S$ synthetic genes was directly induced by $Cd^{2+}$, and then, exogenous $H_2S$ supplementation induced the upregulation of Cys synthetic-related genes (Fig. 3). Additionally, exogenous $H_2S$ or Cys supplementation during $Cd^{2+}$ stress could rapidly induce mutual endogenous levels of the other contents in $Cd^{2+}$ stress. These results suggested that the $H_2S$ - Cys cycle system was triggered by $Cd^{2+}$ and that $H_2S$ and Cys could promote the production of each other,

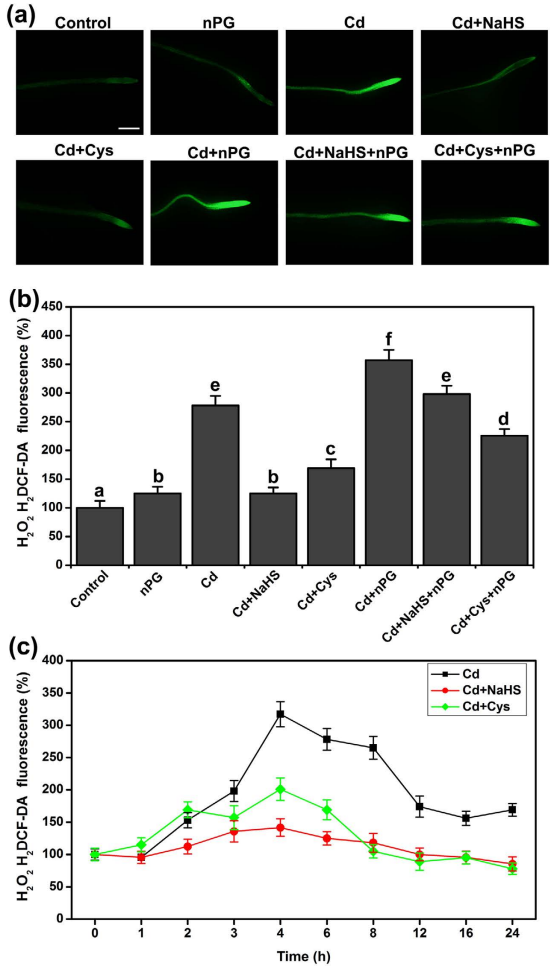


**Figure 7. The changes of the endogenous $H_2O_2$ level in Arabidopsis root.** (**a**) $H_2O_2$ $H_2$DCF-DA fluorescence in WT roots (bar = 100 μm). (**b**) Quantification of $H_2O_2$ $H_2$DCF-DA fluorescence intensity. (**c**) Time-course of $H_2O_2$ level. 7-d-old Arabidopsis seedlings were grown on 1/2 MS agar plates supplemented with 50 μM $Cd^{2+}$, 50 μM NaHS, 1 mM Cys, and 200 μM nPG for 6 h (**a**,**b**), 0–24 h (**c**). $H_2$DCF-DA fluorescence intensity data represents mean grey values. SE was calculated from measurements of at least five roots for each treatment, and the experiments were repeated three times. Within each set of experiments, bars with different letters are significantly different ($P < 0.05$, Duncan's multiple range tests).

forming a cycle of activation. Finally, treatment with $Cd^{2+}$ for 5 d, $H_2S$ and Cys contents increased significantly in Arabidopsis roots.

The expression levels of the Cys synthesis-related genes *OASA1* and *SAT1* were up-regulated significantly by $H_2S$ treatment, and the $H_2S$ synthesis genes *LCD* and *DES1* were up-regulated significantly by Cys treatment (Fig. 3). *OASA1* directly regulated Cys synthesis, and *LCD* and *DES1* directly regulated $H_2S$ synthesis; thus, *lcd*, *des1-1*, *lcddes1-1* and *oasa1* were used to study the $H_2S$ and Cys cycle system in $Cd^{2+}$ stress. The $Cd^{2+}$-induced root shortening and increases in MAD and EL were markedly enhanced in mutant plants, suggesting that the $Cd^{2+}$ resistance was weakened when the $H_2S$ and Cys cycle was blocked. Exogenous $H_2S$ or Cys supplementation only partly restored the root growth, MAD and EL levels, suggesting that $H_2S$ or Cys alone could not replace the function of the $H_2S$ and Cys cycle in plant cells. Additionally, the $H_2S$ and Cys system is also important for stress caused by other heavy metals, such as $Cr^{6+}$; it was reported that NaHS treatment increases the expression levels of the Cys synthesis-related genes[43]. However, different heavy metal stress condition lead to the difference genes expression include MTs genes[43], thus $Cd^{2+}$ and $Cr^{6+}$ condition also could lead to the difference MTs genes expression. The details regarding the mechanism of $H_2S$ in heavy metal resistance requires further study.

Excessive $Cd^{2+}$ can induce the production of ROS, which is highly toxic to biomembranes, nucleic acids and proteins[11]. The alternative respiratory pathway plays an important role in stress conditions by repressing the production of ROS[22,23,42]. Our study also found that the CP and AP activities were altered by $Cd^{2+}$ stress (Fig. 5e). Plant signaling molecules, such as nitric oxide, can regulate the alternative respiratory pathway in stress conditions[44]. Whether an $H_2S$ signal or Cys could affect AP activity was not previously known; our analysis found that exogenous $H_2S$ or Cys supplementation could further induce the activity of AP in $Cd^{2+}$ stress. However, in $H_2S$ synthesis mutants, the effect of Cys was negligible, and in Cys synthesis mutants, the effect of $H_2S$ was not altered.

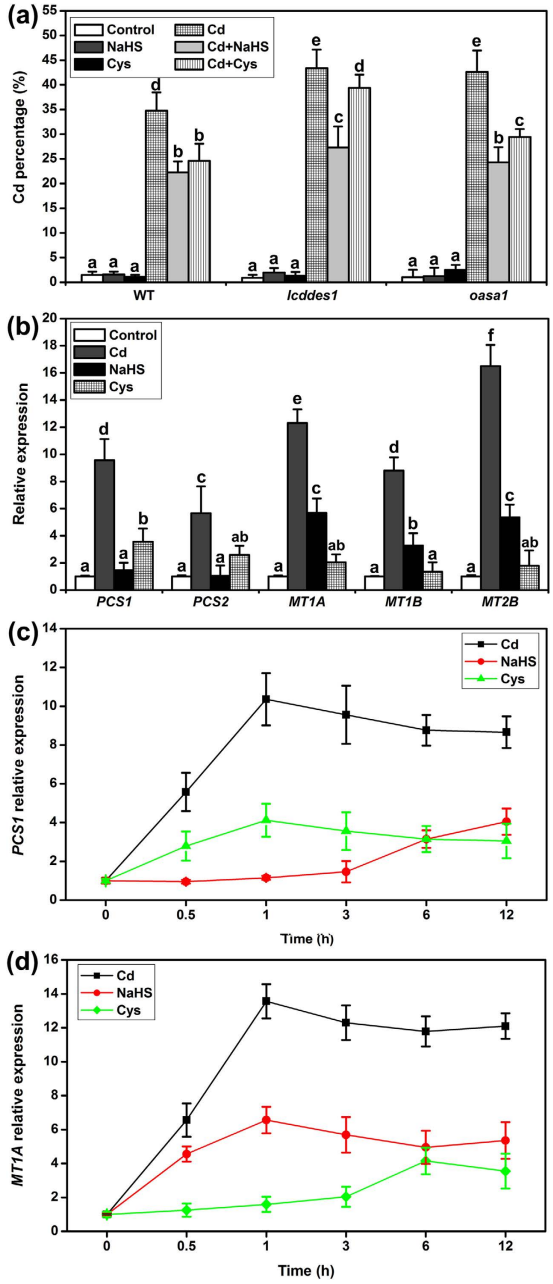


**Figure 8. Effect of NaHS and Cys on $Cd^{2+}$ percentage and qRT-PCR analysis the expression of heavy metal chelators genes in Arabidopsis roots.** (**a**) $Cd^{2+}$ accumulation in Arabidopsis roots. (**b**) The expression of *PCS1*, *PCS2*, *MT1A*, *MT1B* and *MT2B* in WT. (**c**) Time-course of *PCS1* gene expression. (**d**) Time-course of *MT1A* gene expression. 7-d-old Arabidopsis seedlings were grown on 1/2 MS agar plates supplied with 50 μM $Cd^{2+}$, 50 μM NaHS and 1 mM Cys for 5 d (**a**), 3 h (**b**), 0–12 (**c**,**d**). Mean values and SE were calculated from three replicates. Within each set of experiments, bars with different letters are significantly different ($P < 0.05$, Duncan's multiple range tests).

These data imply that $H_2S$ is a direct trigger of AP activity and that Cys might play an indirect role in $Cd^{2+}$ stress. The expression of AOX genes was also induced by $H_2S$ within 3 h, but not by Cys.

Antioxidases are also one of the central elements in maintaining ROS levels in plant cells[45]. We investigated the connection between the alternative respiratory pathway and antioxidases, but we found that the activities of SOD and CAT were not altered when the alternative respiratory pathway was inhibited by nPG (Fig. 6a and c), suggesting that the alternative respiratory pathway and antioxidases have independent functions in response to $Cd^{2+}$ stress. The activities of SOD and CAT were induced by $Cd^{2+}$ and increased $Cd^{2+}$ resistance (Fig. 6). $H_2S$ or Cys biosynthesis was necessary for the increase in SOD and CAT activities in response to $Cd^{2+}$ stress because $Cd^{2+}$-induced activities of SOD and CAT were weakened in $H_2S$ and Cys synthesis mutants. We further studied the relationship of $H_2S$ and Cys in this physiological process. $H_2S$ supplementation could remedy the deficiency

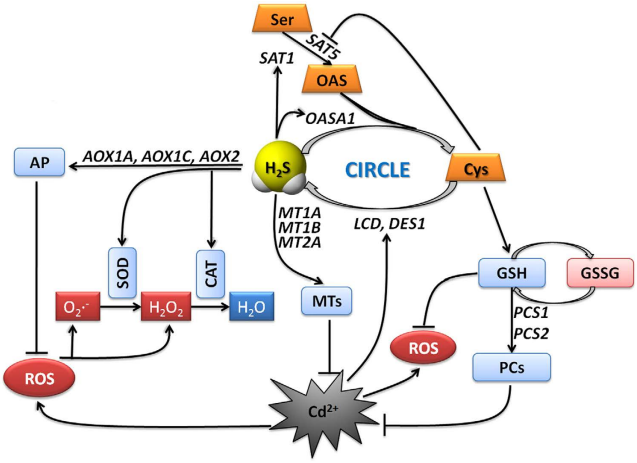


**Figure 9. A diagram representing $Cd^{2+}$-induced toxicity and protective mechanism of the $H_2S$ and Cys cycle system in Arabidopsis roots.** Arrows indicate enhanced effects and hyphens indicate suppressed effects.

of Cys biosynthesis and increase the activities of SOD and CAT in *oasa1* mutants, but Cys supplementation could not. These data suggest that the activities of SOD and CAT are directly regulated by $H_2S$ and that Cys indirectly affects the activities of SOD and CAT by promoting the generation of $H_2S$.

GSH performs numerous physiological functions in the plant response to heavy metal stress[46]. Cys is a precursor of GSH, which stores and transports GSH via the γ-glutamyl cycle[47]. In this study, supplementation with exogenous $H_2S$ or Cys strengthened $Cd^{2+}$-mediated GSH elevation in WT plants (Fig. 6f). It is interesting that the effects of $Cd^{2+}$ and $H_2S$ were reversed in *oasa1*, but the effects of $Cd^{2+}$ and $H_2S$ were not altered in *lcddes1-1* (Fig. 6f). These results suggest that Cys is a direct regulatory factor of GSH, and $H_2S$ affects GSH levels indirectly. Additionally, the GSH content was not altered by nPG (Fig. 6e), suggesting that the alternative respiratory pathway and GSH are not related in their responses to $Cd^{2+}$ stress.

$Cd^{2+}$ enrichment was also observed in this study (Fig. 8a). Inhibiting $Cd^{2+}$ uptake and enhancing $Cd^{2+}$ efflux are the main defense strategies that plant cells use to prevent $Cd^{2+}$ toxicity. Exogenous $H_2S$ or Cys supplementation effectively inhibited the accumulation of $Cd^{2+}$ (Fig. 8a). When endogenous $H_2S$ or Cys synthesis was blocked, $Cd^{2+}$ over-accumulation occurred (Fig. 8a), suggesting that the $H_2S$ and Cys cycle system is important for inhibiting $Cd^{2+}$ uptake or enhancing $Cd^{2+}$ efflux. Additionally, the effect of Cys was partly inhibited in the *lcddes1-1* mutant, implying that the role of $H_2S$ in the $H_2S$ and Cys cycle might be to directly regulate $Cd^{2+}$ uptake or efflux.

The generation of chelators is also an effective pathway in plant cells for avoiding $Cd^{2+}$ toxicity. *PCS1*, *PCS2*, *MT1A*, *MT1B* and *MT2B* are mainly expressed in roots and regulate PCs and MTs synthesis; the expression of these chelators is generally induced by numerous heavy metal ions[42,43,48]. Interestingly, the expression of *PCS1* and *PCS2* was found to be induced by Cys in a very short time, and the expression of *MT1A*, *MT1B* and *MT2B* was induced by $H_2S$ (Fig. 8b). However, only long-term supplementation of Cys or $H_2S$ induced the expression of *PCS1* and *MT1A* (Fig. 8c,d). These data suggest that the generation of chelators can be regulated differently in plant cells. Cys and $H_2S$ played different roles in the physiological process, but when combined Cys and $H_2S$ mutually promoted the expression of chelator synthesis genes to a level higher than when they were used as separate supplements.

Based on the data described above, a signal pathway model was developed and is depicted in Fig. 9. It shows the specific roles of $H_2S$ and Cys in regulating plant responses to $Cd^{2+}$ stress and their interaction. $H_2S$ is activated much earlier than Cys in plant responses to $Cd^{2+}$ stress, acting as a secondary messenger to increase Cys accumulation by regulating the transcription levels of *SAT1* and *OASA1*. In addition, the production of $H_2S$ might deplete the endogenous Cys pool, which might subsequently increase the expression of *SAT1* or *OASA1*. Furthermore, once the $H_2S$ and Cys cycle is initiated, it works to maintain elevated $H_2S$ and Cys levels. $H_2S$ inhibits the ROS burst by promoting CP and antioxidase activities, and it weakens $Cd^{2+}$ ion toxicity by inducing the gene expression of MTs. Cys acts as a precursor of GSH to promote GSH accumulation, which then contributes to inhibiting the ROS burst. GSH also induces genes expression of PCs, leading to raised PC activity, which counteracts $Cd^{2+}$ ion toxicity. In sum, the $H_2S$ and Cys cycle system is a key regulator of the response to $Cd^{2+}$ stress in plants that acts to induce and maintain levels of bioactive molecules ($H_2S$, Cys, GSH, PCs, and MTs) that improve plant resistance to $Cd^{2+}$ stress.

## Materials and Methods

**Plant material and chemical treatments.** This study was carried out on *Arabidopsis thaliana,* including wild ecotypes Columbia (Col-0) and the *lcd* (SALK_082099), *des1-1* (SALK_103855), *lcddes1-1* and *oasa1* (SALK_074242c) mutants. Seeds were surface sterilized with 70% ethanol for 30 s and 15% sodium hypochlorite for 15 min and were washed at least five times with sterilized water before sowing on solid 1/2 Murashige and Skoog (MS) medium (pH 5.7), which contained 1% (w/v) sucrose, and 0.8% (w/v) agar. After that, the seeds were vernalized for 48 h at 4 °C. Then, the seedlings were grown in a growth room, which had the temperature set at

22 °C and a 14/10 h light/dark photoperiod under a photon flux density of 120 mmol $m^{-2}s^{-1}$. The Arabidopsis plants used throughout this work were grown routinely in a growth chamber under 50–60% humidity.

Following 7 d growth, Arabidopsis seedlings were transferred to the following mediums: (1) 1/2 MS agar medium, (2) 1/2 MS agar medium containing 25–150 μM $CdCl_2$, 50 μM sodium hydrosulfide (NaHS), 1 mM Cys, or 200 μM n-propyl gallate (nPG), respectively. The $H_2S$ donors NaHS, Cys and nPG were purchased from Sigma (USA).

**Root elongation assays.** Seven-day-old Arabidopsis seedlings grown on the vertical 1/2 MS agar plates were transferred to the 1/2 MS agar medium containing various chemicals for the different treatments. Root elongation was measured after 5 d of various treatments. All experiments were repeated at least three times, with photographs collected at 7 d from one representative experiment being shown. The root length was measured with ImageJ.

**Electrolyte leakage assay.** Measurement of ion leakage was determined according to Sairam and Srivastava (2002) with some modifications[43]. The 7-d-old Arabidopsis seedlings were treated for 5 d on the 1/2 MS agar medium containing different chemicals. Following the treatments, the roots were collected and washed in deionized water three times to remove surface-adhered electrolytes. Then, they were immersed in 10 ml deionized water for 3 h at 25 °C in test tubes. After the incubation, the conductivity in the bathing solution was determined ($C_1$), and the conductivity of deionized water was also determined ($C_0$). The samples were heated in boiling water for 1 h before the total conductivity was measured in the bathing solution ($C_2$). Relative ion leakage was expressed as a percentage of the total conductivity after heating in boiling water [relative ion leakage $= (C_1 - C_0)/(C_2 - C_0) \times 100$].

**MDA and GSH content assays.** The chemical treatments were the same as the measurements of ion leakage. Following the treatments[49], Arabidopsis roots were collected. Lipid peroxidation of the roots was measured by estimating the MDA content according to the method of Heath and Packer. The GSH content was measured based on a previously described method[49].

**Measurement of $H_2S$ content.** $H_2S$ quantification was performed as described by Nashef *et al.*[50]. The chemical treatments were the same as the methods of ion leakage. Following the treatments, the seedling roots were collected with liquid nitrogen and ground into fine powder with mortar and pestle; 0.3 g of frozen tissue was homogenized in 1 ml 100 mM potassium phosphate buffer (pH = 7), which contained 10 mM ethylenediaminetetra-acetic acid (EDTA). The homogenates were centrifuged at 15,000 × *g* for 20 min at 4 °C, and 100 μl of supernatant was used for the quantification of $H_2S$ in an assay mixture containing 1,880 μl extraction buffer and 20 μl of 20 mM 5,5′-dithiobis (2-nitrobenzoic acid), for a total volume of 2 ml. The assay mixture was incubated at room temperature for 2 min, and the absorbance was read at 412 nm. $H_2S$ was quantified based on a standard curve of known concentrations of NaHS.

**Measurement of the Cys content.** The chemical treatments were the same as the measurements of ion leakage. Following the treatments, Arabidopsis roots were collected. Cys can react specifically with acid ninhydrin, and the product was extracted by methylbenzene, which has a maximum absorbance at 560 nm. The reaction is highly sensitive for Cys determination. Thus, the Cys content could be determined as described previously[51].

**RNA isolation and qRT-PCR.** Seven-day-old Arabidopsis seedlings were transferred to the 1/2 MS agar medium containing different chemicals and treated for 0–12 h. Following the treatments, roots of Arabidopsis were harvested to extract total RNA for real-time polymerase chain reaction (RT-PCR). Total RNA was extracted using an RNAprep pure plant kit (Tiangen, Beijing) and was treated with RNase-free DNase reagent (RNase-free DNase kit, Tiangen). The total RNA was reverse-transcribed into first-strand cDNA using PrimeScript™ Reverse Transcriptase (Takara, Japan) and Oligo $(dT)_{15}$ primer (Takara) following the manufacturer's instructions. The samples were amplified using SYBR Green I (SYBR® Premix Ex Taq™ Kit, Takara). The housekeeping gene EF1A was used as an internal control. The thermal cycle used was as follows: 95 °C for 10 s, and 40 cycles of 95 °C for 5 s and 59 °C for 25 s. This was followed by 80 cycles of 10 s during the time elapsed at 55–95 °C. The PCR amplifications for each gene were performed in triplicate. The results were analyzed by Rotor-Gene Real-Time Analysis Software 6.1 (Build 81).

**Extraction and assay of antioxidant enzymes.** Seven-day-old Arabidopsis seedlings were transferred to the 1/2 MS agar medium containing different chemicals and treated for 6 h. Following the treatments, Arabidopsis roots were collected and enzymes extracted according to the method of Mostofa *et al.*[51]. Activities of antioxidase and glyoxalase were determined by the standard methods reported in Mostofa and Fujita[52] for SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6). The protein standard was bovine serum albumin (BSA), which was employed to determine the protein content.

**Determination of $H_2O_2$ contents.** $H_2O_2$ was visualized using the specific $H_2O_2$ fluorescent probe dichlorofluorescein diacetate ($H_2$DCF-DA) according to the method described by Maffei *et al.*[53]. Seven-day-old Arabidopsis seedlings were transferred to the 1/2 MS agar medium containing different chemicals and treated for 0–24 h. Following the treatments, Arabidopsis seedlings were incubated in the reaction buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-NaOH (pH 7.5) and 10 μM H2DCF-DA for 15 min at 25 °C. Thereafter, the roots were washed three times with the HEPES-NaOH buffer (pH 7.4) prior to visualization using a laser confocal scanning microscope (Leica SM IRBE Multisync FE 1250). Excitation was at 480 nm and emission was at 520 nm. Images were processed and analyzed using the Leica Tcs SP2 software.

**Statistical analysis.** Each experiment was repeated at least three times and with three replications each time. Values were expressed as the mean ± SE. Experiments that required an analysis of variance were analyzed using SPSS 17.0 for one-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparisons. The confidence coefficient was set at 0.05.

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## Author Contributions

J.S.L. and H.L.J. designed research; J.S.L. and H.L.J. performed research; X.F.W., Y.H.D. and D.L. screened mutant plants; H.L.J., W.T.S., H.F., C.Z., S.L.C. and J.J.X. analyzed data; and J.S.L. and H.L.J. wrote the paper.

## Additional Information

**Supplementary information** accompanies this paper at http://www.nature.com/srep

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