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 (Cd²⁺) is a common toxic heavy metal ion. We investigated the roles of hydrogen sulfide (H₂S) and cysteine (Cys) in plant responses to Cd²⁺ stress. The expression of H₂S synthetic genes LCD and DES1 were induced by Cd²⁺ within 3 h, and endogenous H₂S was then rapidly released. H₂S promoted the expression of Cys synthesis-related genes SAT1 and OASA1, which led to endogenous Cys accumulation. The H₂S and Cys cycle system was stimulated by Cd²⁺ stress, and it maintained high levels in plant cells. H₂S inhibited the ROS burst by inducing alternative respiration capacity (AP) and antioxidase activity. H₂S weakened Cd²⁺ 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 Cd²⁺ toxicity. In summary, the H₂S and Cys cycle system played a key role in plant responses to Cd²⁺ stress. The Cd²⁺ 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 H₂S and Cys cycle system in Cd²⁺ stress and to explore the relevant and specificity mechanisms of H₂S and Cys in mediating Cd²⁺ stress.
alternative respiratory pathway, activating antioxidants and glutathione (GSH), and regulating the influx and efflux of heavy metals, as well as regulating the levels of heavy metal chelators, phytochelatins (PCs) and metallothioneins (MTs).

Hydrogen sulfide (H₂S) has been considered toxic gas for many years, which inhibits cytochrome oxidase activity in animal cells. Recently, it has emerged as the third endogenous gasotransmitter, following the discovery of nitric oxide and carbon monoxide. In plant systems, endogenous H₂S is generated through enzymatic pathways. Cysteine (Cys) desulphhydrases (CDes) are key enzymes involved in H₂S generation. Cys synthesis occurs via two sequential phases catalyzed by serine acetyltransferase (SAT) and O-acetylseryltransferase (OAT), both of which are encoded by multigenic families. L-Cys desulphhydrase (L-CDes) is the most understood CDes in Arabidopsis; it regulates L-Cys degradation into pyruvate, ammonia and H₂S. AOX regulates H₂S and O-acetylseryl (OAT) synthesize L-Cys. These physiological processes form H₂S - Cys cycle system in cell. Recently, based on the sequence characteristics of DES1, it has been classified as an OAS-TL. However, functional analysis of this enzyme revealed that DES1 has a higher affinity for L-Cys and degrades it to generate H₂S.

The alternative respiratory pathway is a unique pathway in the mitochondrial electron transport chain in higher plants that is regulated by alternative oxidase (AOX). A large body of evidence suggests that the enhanced alternative pathway could improve stress tolerance through limiting the ROS burst. Recent research indicated that respiratory activity is regulated by endogenous H₂S in Escherichia coli. However, the relationship between H₂S and the alternative respiratory pathway in plant responses to Cd stress is unclear.

Sulfur is an essential element that is taken up by plants in its oxidized state, reduced to H₂S, and first incorporated into Cys before it is used in metabolic processes. The products of sulfur metabolism, such as Cys, GSH, PCs, MTs and H₂S, have biological functions in plant responses to heavy metal stress and oxidative stress. Recently, positive effects of H₂S in response to several types of abiotic stress have been found, such as osmotic stress, salt stress, heat shock stress and heavy metal stress. It has been reported that a cross-talk between H₂S and nitric oxide is responsible for the increased Cd toxicity in alfalfa and Bermuda grass plants. In addition, H₂S alleviates Cd toxicity by regulating cadmium transport in Populus euphratica cells. H₂S is also involved in the growth and development of plants through its effects on stomatal closure and seed germination.

Compelling evidence has suggested that H₂S and Cys are involved in plant tolerance to heavy metal stress. The biosynthesis of H₂S and Cys is interrelated. H₂S is involved in the uptake of SO₄ and in the biosynthesis of Cys. Cys is also involved in the generation of H₂S. Nevertheless, the relation and interaction between H₂S and Cys are yet unknown under Cd stress. In this study, we aimed to clarify the common and independent functions of H₂S and Cys in Cd stress. Additionally, we sought to demonstrate the working mechanisms of the H₂S and Cys cycle system response to Cd stress in Arabidopsis.

Results

Effect of Cd on root elongation, MDA and EL in Arabidopsis roots. Arabidopsis seedlings (7-d-old) were transferred aseptically to 1/2 MS medium containing CdCl₂, and the lengths of the primary roots were measured 5 days later. Cd 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 stress, but root elongation was significantly inhibited under 50 to 150 µM Cd stress, 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 stress caused lipid peroxidation and MDA accumulation. When plants were treated with 50 to 150 µM Cd, the MDA content of the roots increased by 117% to 200%, respectively (Fig. 1c). In the presence of 50 to 150 µM Cd, EL increased by 131% to 217%, respectively (Fig. 1d). The 50 to 150 µM Cd treatment had significant effects on Arabidopsis roots. 100 and 150 µM Cd concentrations were too violent for plant growth and 150 µM Cd concentrations lead to the seedling etiolation in Fig. 1a. Thus, 50 µM Cd was chosen for further study of Cd stress.

Effect of Cd, NaHS and Cys on the H₂S and Cys cycle system. To explore the working mechanisms of the H₂S and Cys cycle system's response to Cd stress in Arabidopsis roots, a time-course analysis of endogenous H₂S and Cys contents was performed. Endogenous H₂S and Cys contents undulated along with the time of Cd stress. H₂S content was rapidly induced after treatment with Cd 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 H₂S level and maintain H₂S content at a high level in Cd stress (Fig. 2a). The Cys level slightly decreased in the initial stage under Cd treatment, but it increased after treatment with Cd 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 stress (Fig. 2b). When plants were treated with 50 or 100 µM Cd for a long time, both H₂S and Cys levels were enhanced in Arabidopsis roots (Fig. 2c and d). As mentioned above, H₂S and Cys contents were elevated by Cd stress, and H₂S appeared to be an important mediator in the Cd-induced increase of Cys, and the H₂S and Cys cycle system was enhanced.

The effects of Cd, NaHS and Cys on synthetic genes of H₂S and Cys. To study the direct effects of Cd stress, NaHS and Cys on genes regulating the synthesis of H₂S and Cys, Arabidopsis seedlings were exposed to various treatments for 3 h. The expressions of H₂S synthetic genes LCD and DES1 were markedly induced by Cd stress and Cys (Fig. 3a and b), but the expression of D-CDES was not significantly affected by Cd stress and Cys treatments (Fig. 3c). SATs and OASs are the important synthetic genes of Cys, but they had different responses to Cd stress, NaHS and Cys. The expression levels of SAT1 and OASA1 were slightly increased in Cd stress, but they were
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 Cd2⁺, NaHS or Cys (Fig. 3e,h and i).

The effects of Cd2⁺, NaHS and Cys on root elongation, MDA and EL in lcddes1-1 and oasa1 mutants. Five-day-old Arabidopsis seedlings were transferred aseptically to Cd2⁺-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 Cd2⁺ stress. Application of NaHS or Cys recovered the Cd2⁺-induced growth inhibition in WT. NaHS markedly recovered the effect of Cd2⁺ in lcddes1-1, but it only partly recovered the effect of Cd2⁺ in oasa1 (Fig. 4a and b). On the contrary, treatment with Cys slightly recovered the effect of Cd2⁺ in lcddes1-1, but it significantly recovered the effect of Cd2⁺ 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 H₂S production was blocked in the double mutant. NaHS or Cys treatment markedly decreased the EL level and the content of MDA under Cd2⁺ 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 Cd2⁺ on the MDA content and EL in oasa1 but partly weakened the effects of Cd2⁺ on the MDA content and EL in lcddes1-1 (Fig. 4c and d).

The effects of Cd2⁺, NaHS and Cys on the alternative respiratory pathway. The alternative respiratory pathway plays an important role in plant stress resistance by limiting the ROS burst38. In this study, we sought to elucidate the roles of NaHS and Cys in the alternative respiratory pathway under Cd2⁺ 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 stresses25. We first checked the expression of AOX genes after Cd2⁺, NaHS and Cys treatments for 3 h. The expression of AOX1A, AOX1C and AOX2 were increased in Cd2⁺ 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 Cd2⁺ 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 Cd2⁺, but it was inhibited by 100 or 150 μM Cd2⁺ in WT (Fig. 5d). Under Cd2⁺ stress, CP was inhibited in a dose-dependent manner; however,
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 \(\text{lcd}^{-}\) and \(\text{des}1-1\), and they were especially decreased in \(\text{lcd}^{-}\text{des}1-1\) (Fig. 5g, h and i). In \(\text{oasa}1\), 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\(_2\)S 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 \(\text{lcd}^{-}\text{des}1-1\); treatment with Cd\(^{2+}\) or Cys did not enhance the antioxidase activity in \(\text{lcd}^{-}\text{des}1-1\) (Fig. 6b and d). Additionally, the effects of Cd\(^{2+}\) on the SOD activity were also weakened, and CAT activity was negligible in \(\text{oasa}1\) (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\(^{35}\). 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 \(\text{oasa}1\) mutant did not respond to Cd\(^{2+}\) and NaHS, and even had a reduced GSH level, but Cys still
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\(_2\)S and Cys cycle in ROS homeostasis, we visualized the production of H\(_2\)O\(_2\) in the roots under Cd\(^{2+}\) stress. Over-accumulation of H\(_2\)O\(_2\) 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\(_2\)O\(_2\) in Cd\(^{2+}\) stress (Fig. 7a and b). Additionally, inhibiting the alternative respiratory pathway with nPG caused an over-accumulation of H\(_2\)O\(_2\) under Cd\(^{2+}\) stress. The effects of NaHS and Cys were partly averted and slightly weakened by nPG in Cd\(^{3+}\) stress, respectively (Fig. 7a and b). As shown in the time-course of H\(_2\)O\(_2\), 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\(_2\)S supplementation could maintain H\(_2\)O\(_2\) at a low level during Cd\(^{2+}\) stress. Treatment with Cys did not alter the burst of H\(_2\)O\(_2\) in the early phase, but it prevented the over-accumulation of H\(_2\)O\(_2\) after 6 h (Fig. 7c).

**Effect of NaHS and Cys on Cd\(^{2+}\) accumulation.** The role of H\(_2\)S 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
NaHS promoted the expression of MT1A, MT1B and MT2B (Fig. 8b). To further study the effect of the H₂S and Cys cycle system on the heavy metal chelator genes, the time-course of PCS1 and MT1A gene expression was investigated. Cd²⁺ 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 at 6 h, and NaHS enhanced the expression of MT1A gene at 0.5 h (Fig. 8c and d).
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\(_2\)S and Cys are important sulfur metabolism products that participate in suppressing heavy metal stress\(^{40}\). In previous reports, H\(_2\)S and Cys were always studied separately in plant responses to abiotic stress\(^{39,42}\). Recently, H\(_2\)S and H\(_2\)S-induced Cys accumulation were reported to be critical in imparting Cr\(^{6+}\) tolerance in Arabidopsis\(^{43}\). Therefore, the H\(_2\)S and Cys cycle is an important system for regulating H\(_2\)S and Cys functions in heavy metal stress. In this study, we used the \textit{lcddes1-1} and \textit{oasa1} Arabidopsis mutants to block the H\(_2\)S and Cys cycle system. Then, we intensively researched the relevant and specificity roles of H\(_2\)S 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.
We observed that endogenous H$_2$S 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$_2$S and Cys levels. Endogenous H$_2$S was first induced by Cd$_{2+}$ stress, and then Cys levels increased. On this account, we suppose that H$_2$S 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$_2$S release but that Cys accumulation is a secondary effect of Cd$_{2+}$ stress. Data for the expression of H$_2$S and Cys synthetic genes supports this hypothesis. The expression of H$_2$S synthetic genes was directly induced by Cd$_{2+}$, and then, exogenous H$_2$S supplementation induced the upregulation of Cys synthetic-related genes (Fig. 3). Additionally, exogenous H$_2$S 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$_2$S - Cys cycle system was triggered by Cd$_{2+}$ and that H$_2$S and Cys could promote the production of each other.

Figure 6. Effect of H$_2$S 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 50μM Cd$_{2+}$, 50μM NaHS, 1 mM Cys, and 200μM 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).
forming a cycle of activation. Finally, treatment with Cd2⁺ for 5 d, H₂S 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 H₂S treatment, and the H₂S 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 H₂S synthesis; thus, lcd, des1-1, lcedes1-1 and oasa1 were used to study the H₂S and Cys cycle system in Cd²⁺ stress. The Cd²⁺-induced root shortening and increases in MAD and EL were markedly enhanced in mutant plants, suggesting that the Cd²⁺ resistance was weakened when the H₂S and Cys cycle was blocked. Exogenous H₂S or Cys supplementation only partly restored the root growth, MAD and EL levels, suggesting that H₂S or Cys alone could not replace the function of the H₂S and Cys cycle in plant cells. Additionally, the H₂S and Cys system is also important for stress caused by other heavy metals, such as Cr⁶⁺; it was reported that NaHS treatment increases the expression levels of the Cys synthesis-related genes. However, different heavy metal stress condition lead to the difference MTs genes expression. The details regarding the mechanism of H₂S in heavy metal resistance requires further study.

Excessive Cd²⁺ can induce the production of ROS, which is highly toxic to biomembranes, nucleic acids and proteins. The alternative respiratory pathway plays an important role in stress conditions by repressing the production of ROS. Our study also found that the CP and AP activities were altered by Cd²⁺ stress (Fig. 5e). Plant signaling molecules, such as nitric oxide, can regulate the alternative respiratory pathway in stress conditions. Whether an H₂S signal or Cys could affect AP activity was not previously known; our analysis found that exogenous H₂S or Cys supplementation could further induce the activity of AP in Cd²⁺ stress. However, in H₂S synthesis mutants, the effect of Cys was negligible, and in Cys synthesis mutants, the effect of H₂S was not altered.
These data imply that H2S is a direct trigger of AP activity and that Cys might play an indirect role in Cd2+ stress. The expression of AOX genes was also induced by H2S within 3 h, but not by Cys. Antioxidases are also one of the central elements in maintaining ROS levels in plant cells. 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 Cd2+ stress. The activities of SOD and CAT were induced by Cd2+ and increased Cd2+ resistance (Fig. 6). H2S or Cys biosynthesis was necessary for the increase in SOD and CAT activities in response to Cd2+ stress because Cd2+-induced activities of SOD and CAT were weakened in H2S and Cys synthesis mutants. We further studied the relationship of H2S and Cys in this physiological process. H2S supplementation could remedy the deficiency.
was induced by H2S (Fig. 8b). However, only long-term supplementation of Cys or H2S induced the expression of PCs, leading to raised PC activity, which counteracts Cd2+ toxicity.

Figure 9. A diagram representing Cd2+-induced toxicity and protective mechanism of the H2S 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 H2S and that Cys indirectly affects the activities of SOD and CAT by promoting the generation of H2S.

GSH performs numerous physiological functions in the plant response to heavy metal stress. Cys is a precursor of GSH, which stores and transports GSH via the γ-glutamyl cycle. In this study, supplementation with exogenous H2S or Cys strengthened Cd2+-mediated GSH elevation in WT plants (Fig. 6f). It is interesting that the effects of Cd2+ and H2S were reversed in oasa1, but the effects of Cd2+ and H2S were not altered in lcddes1-1 (Fig. 6f). These results suggest that Cys is a direct regulatory factor of GSH, and H2S 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 Cd2+ stress.

Cd2+ enrichment was also observed in this study (Fig. 8a). Inhibiting Cd2+ uptake and enhancing Cd2+ efflux are the main defense strategies that plant cells use to prevent Cd2+ toxicity. Exogenous H2S or Cys supplementation effectively inhibited the accumulation of Cd2+ (Fig. 8a). When endogenous H2S or Cys synthesis was blocked, Cd2+ over-accumulation occurred (Fig. 8a), suggesting that the H2S and Cys cycle system is important for inhibiting Cd2+ uptake or enhancing Cd2+ efflux. Additionally, the effect of Cys was partly inhibited in the lcddes1-1 mutant, implying that the role of H2S in the H2S and Cys cycle might be to directly regulate Cd2+ uptake or efflux.

The generation of chelators is also an effective pathway in plant cells for avoiding Cd2+ 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. 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 H2S (Fig. 8b). However, only long-term supplementation of Cys or H2S 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 H2S played different roles in the physiological process, but when combined Cys and H2S 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 H2S and Cys in regulating plant responses to Cd2+ stress and their interaction. H2S is activated much earlier than Cys in plant responses to Cd2+ stress, acting as a secondary messenger to increase Cys accumulation by regulating the transcription levels of SAT1 and OASA1. In addition, the production of H2S might deplete the endogenous Cys pool, which might subsequently increase the expression of SAT1 or OASA1. Furthermore, once the H2S and Cys cycle is initiated, it works to maintain elevated H2S and Cys levels. H2S inhibits the ROS burst by promoting CP and antioxidase activities, and it weakens Cd2+ 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 Cd2+ ion toxicity. In sum, the H2S and Cys cycle system is a key regulator of the response to Cd2+ stress in plants that acts to induce and maintain levels of bioactive molecules (H2S, Cys, GSH, PCs, and MTs) that improve plant resistance to Cd2+ 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 CdCl₂, 50 μM sodium hydrosulfide (NaHS), 1 mM Cys, or 200 μM n-propyl gallate (nPG), respectively. The H₂S 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. 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 (C₁), and the conductivity of deionized water was also determined (C₀). The samples were heated in boiling water for 1 h before the total conductivity was measured in the bathing solution (C₂). Relative ion leakage was expressed as a percentage of the total conductivity after heating in boiling water [relative ion leakage = (C₁ − C₀) / (C₂ − C₀) × 100].

MDA and GSH content assays. The chemical treatments were the same as the measurements of ion leakage. Following the treatments, 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. 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 material was homogenized in 1 ml 100 mM potassium phosphate buffer (pH = 7), which contained 10 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was removed for the quantification of MDA as described previously. After the treatment, the seedling roots were collected with liquid nitrogen and ground into fine powder with mortar and pestle; 0.3 g of frozen material was homogenized in 1 ml 100 mM potassium phosphate buffer (pH = 7), which contained 10 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was removed for the quantification of MDA in the 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. H₂S 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. Following the treatment, the seedling roots were collected and washed in deionized water before the total conductivity was measured in the bathing solution (C₀). Relative ion leakage was expressed as a percentage of the total conductivity after heating in boiling water [relative ion leakage = (C₁ − C₀) / (C₂ − C₀) × 100].

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. Activities of antioxidase and glyoxalase were determined by the standard methods reported in Mostofa and Fujita for SOD (EC 1.15.1.1) and CAT (EC 1.1.1.6). The protein standard was bovine serum albumin (BSA), which was employed to determine the protein content.

Determination of H₂O₂ contents. H₂O₂ was visualized using the specific H₂O₂ fluorescent probe dichlorofluorescein diacetate (H₂DCF-DA) according to the method described by Maffei et al. 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

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Additional Information

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