Cadmium-Induced Hydrogen Sulfide Synthesis Is Involved in Cadmium Tolerance in *Medicago sativa* by Reestablishment of Reduced (Homo) glutathione and Reactive Oxygen Species Homeostases

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Abstract

Until now, physiological mechanisms and downstream targets responsible for the cadmium (Cd) tolerance mediated by endogenous hydrogen sulfide (H₂S) have been elusive. To address this gap, a combination of pharmacological, histochemical, biochemical and molecular approaches was applied. The perturbation of reduced (homo) glutathione homeostasis and increased H₂S production as well as the activation of two H₂S-synthetic enzymes activities, including L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD), in alfalfa seedling roots were early responses to the exposure of Cd. The application of H₂S donor sodium hydrosulfide (NaHS), not only mimicked intracellular H₂S production triggered by Cd, but also alleviated Cd toxicity in a H₂S-dependent fashion. By contrast, the inhibition of H₂S production caused by the application of its synthetic inhibitor blocked NaHS-induced Cd tolerance, and destroyed reduced (homo)glutathione and reactive oxygen species (ROS) homeostases. Above mentioned inhibitory responses were further rescued by exogenously applied glutathione (GSH). Meanwhile, NaHS responses were sensitive to a (homo)glutathione synthetic inhibitor, but reversed by the cotreatment with GSH. The possible involvement of cyclic AMP (cAMP) signaling in NaHS responses was also suggested. In summary, LCD/DCD-mediated H₂S might be an important signaling molecule in the enhancement of Cd toxicity in alfalfa seedlings mainly by governing reduced (homo) glutathione and ROS homeostases.

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Introduction

Cadmium (Cd) contamination is a non-reversible accumulation process, with the estimated half-life and high plant-soil mobility, thus resulting in a serious threat to human health through food chains. Normally, Cd exposure leads to the inhibition of plant growth, decrease of crop yield, and even plant cell death [1,2]. Indirectly stimulated generation of reactive oxygen species (ROS) that modify the antioxidant defence and bring out oxidative stress is ascribed to one of the Cd toxicities in plants, and therefore lipid peroxidation is considered as a hallmark of Cd exposure [3].

In plants, there are a lot of antioxidant defence mechanisms, which could keep the normally formed ROS at a low level and prevent them from exceeding toxic thresholds [3,4]. The glutathione (GSH) and ascorbate were subsequently recognized as the heart of the redox hub [5]. In plants, GSH is synthesized by two ATP-dependent steps: γ-glutamylcysteine (γ-EC) is synthesized from L-glutamate and L-cysteine by γ-glutamyl cysteine synthetase (γ-ECS, also called as γ-GCS); and the second step, glycine is conjunct to γ-EC by glutathione synthetase (GS) [6,7]. In soybean and alfalfa plants, GSH homolog homoglutathione (hGSH) synthesized by homoglutathione synthetase (hGS) from β-alanine and γ-EC, is more abundant than GSH [8]. The rate of glutathione reductase (GR) reaction was the same with either oxidized glutathione (GSSG) or oxidized homoglutathione (hGSSGh) as the substrate [7]. Upon Cd exposure, it was confirmed that the rapid accumulation of peroxides and depletion of GSH and hGSH causes redox imbalance in *Medicago sativa* [9]. Subsequent experiments with comparing ten pea genotypes showing that, activities of ascorbate peroxidase (APX) decreased, but concentrations of GSH increased in the less Cd-sensitive genotypes [10].

Another sulphur-containing compound, hydrogen sulfide (H₂S), previously known as a toxic gas, has been progressively recognized as a gaseous signaling molecule with multiple functions in animals [11,12]. For example, H₂S has been revealed as a cytoprotectant...
and a regulator in various biological processes, such as oxidative stress suppression, smooth muscle relaxation, proliferation inhibition and apoptosis triggering [13–16]. Meanwhile, although previous reports observed that many plants can emit H2S [17–19], there have been few studies on the physiological role of H2S in *plants* during the last century.

In mammals, the majority of endogenous H2S was produced by two enzymes, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1), from γ-cysteine [20]. Cysteine-degrading enzymes such as cysteine desulphydrases are hypothesized to be involved in H2S release in plants [21]. Previously, two specific desulphydrases, γ-cysteine desulphydrase (LCD, EC 4.4.1.1.; also called L-CDes or D-DES) and γ-cysteine desulphydrase (DCD, EC 4.4.1.15; also called D-CDes or D-DES), have been isolated and partially analyzed from *Arabidopsis thaliana* [22–24]. The LCD, which is considered as the most important enzyme with H2S production in plants, shares a 100% sequence homolog with CSE in mammals [25]. By using sodium hydrosulfide (NaHS) as a H2S donor, ample evidence further suggested that H2S can protect plants against various stress-induced damage, such as salinity stress [26], drought [27–29], heavy metal exposure [30,31], and heat shock [32]. Additionally, H2S can act as an inducer in several developmental processes, including adventitious root formation [33] and flower senescence [34]. However, exogenously applied H2S donor without checking the kinetics of H2S synthesis including corresponding metabolic functions of cAMP signaling in H2S-alleviated Cd stress in plants was not fully replicate the function of endogenous H2S in plants.

Cyclic AMP (adenosine 3’, 5’-cyclic monophosphate, cAMP) is a well-known second messenger playing important roles in many physiological processes. The cAMP is synthesized by adenylyl cyclase and broken down by cNMP phosphodiesterase. Dioxoyadenosine (DDA) and 1,3-diazanine-2,4,5,6-tetrone (alloxan) are well characterized as the inhibitors of adenylyl cyclase. Likewise, cNMP phosphodiesterase is sensitive to the inhibitor 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione (IBMX) [35,36]. In animals, there are ample evidences to show H2S-activated cAMP level or H2S-regulated cAMP homeostasis [37,38]. It was found that H2S acted via cAMP-mediated PI3K/Akt/p70S6K signal pathways to inhibit hippocampal neuronal apoptosis and protect neurons from OGD/R-induced injury [39]. However, the functions of cAMP signaling in H2S-attenuated Cd stress in plants are still poorly understood.

Thus, the aim of this study was to investigate the signaling role of endogenous H2S in the tolerance of *Medicago sativa* seedlings to Cd stress. For this purpose, we preliminarily investigated the synthesis of endogenous H2S under Cd stress, which has not been fully performed. Furthermore, the effects of H2S on GSH and hGSH metabolism, as well as ROS homeostasis were checked. Our results further indicated that Cd stress triggered endogenous H2S production catalyzed by LCD/DCD pathways, and the elevated H2S acts as a signal improving the homeostasis of GSH pool and keeping ROS under control, both of which finally contributed to Cd tolerance. Finally, the possible involvement of cAMP signaling in NaHS responses was also suggested.

**Materials and Methods**

**Plant material, growth condition**

Commercially available alfalfa (*Medicago sativa* L. *Victoria*) seeds were surface-sterilized with 5% NaClO for 10 min, and rinsed extensively in distilled water before being germinated for 1 d at 25°C in the darkness. Uniform seedlings were then selected and transferred to the plastic chambers and cultured with nutrient medium (quarter-strength Hoagland’s solution) in the illuminating incubator (14 h light with a light intensity of 200 μmol·m⁻²·s⁻¹, 25±1°C, and 10 h dark, 23±1°C). Five-day-old seedlings were then incubated in quarter-strength Hoagland’s solution with or without varying concentrations of NaHS (Sigma-Aldrich; St Louis, MO, USA) or the other indicated chemicals (2 mM p-propargylglycine (PAG), 1 mM GSH, 1 mM γ-buthionine-sulfoximine (BSO), 50 μM 8-Br-cAMP (8Br), 200 μM alloxan (All), 1 mM DDA, and 500 μM IBMX) alone, or the combination of treatments for 6 h followed by the indicated time points of incubation in 200 μM CdCl₂. Seedlings without chemicals were used as the control (Con). The pH for both nutrient medium and treatment solutions was adjusted to 6.0.

After various treatments, above-ground parts and root tissues of seedlings were sampled immediately or flash-frozen in liquid nitrogen, and stored at −80°C for further analysis. Among these, above-ground parts and root tissues of 240 seedlings were respectively used for the determination of Cd contents. Seedling root tissues were also used for fresh weight determination (10 seedlings), thiobarbituric acid reactive substances (TBARS) content determination (120 seedlings), and other indicated tests (30 seedlings).

**Determination of H2S content, LCD and DCD activity**

Hydrogen sulfide content was determined according to the method previously reported [19,34]. 100 mg of alfalfa seedling roots from 30 seedlings were ground under liquid nitrogen and extracted by 1 ml phosphate buffered saline (50 mM, pH 6.8) containing 0.1 M EDTA and 0.2 M ascorbic acid. After centrifugation at 13000 g for 15 min at 4°C, 400 μl of the supernatant was injected to 200 μl 1% zinc acetate and 200 μl 1 N HCl. After 30 min reaction, 100 μl 5 mM dimethyl-p-phenylenediamine dissolved in 7 mM HCl was added to the trap followed by the injection of 100 μl 50 mM ferric ammonium sulfate in 200 mM HCl. After 15 min incubation at room temperature, the amount of H2S was determined at 567 nm. Solutions with different concentrations of Na₂S were used in a calibration curve.

100 mg of alfalfa seedling roots from 30 seedlings were used for activity determination. The activities of LCD and DCD were determined as described by the methods previously reported [23,40]. γ-cysteine desulphydrase (LCD) activity was measured by the release of H2S from γ-cysteine in the presence of dithiothreitol (DTT). The formation of methylene blue was determined at 670 nm. To removal of the background, content of H2S in the extracted protein solution was measured by same way with 50% trichloroacetic acid (TCA) instead of γ-cysteine. The final LCD activity was calculated from the difference between the measured LCD activity and the background. γ-cysteine desulphydrase (DCD) activity was measured by the same method with following modifications: γ-cysteine instead of γ-cysteine, the pH of Tris-HCl was 8.0 rather than 9.0. Solutions with different concentrations of Na₂S were prepared, treated in the same way as the assay samples and were used for the quantification of enzymatically formed H2S.

**Determination of thiobarbituric acid reactive substances (TBARS), (h)GSH and (h)GSSG(h) contents**

Lipid peroxidation was estimated by measuring the amount of TBARS, (h)GSH and (h)GSSG(h) contents

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concentration of lipid peroxides together with oxidatively modified proteins of plants were thus quantified in terms of TBARS amount using an extinction coefficient of 155 mM·cm⁻¹·μmol⁻¹ and expressed as nmol g⁻¹ fresh weight (FW).

(h)GSH (GSH + hGSH) and [h]GSSG[h] (GSSG + hGSSGh) contents were measured by the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)-glutathione reductase (GR) recycling assay [31,42]. Frozen root tissues from 30 seedlings were homogenized in cold 5% 3-sulfosalicylic acid. The homogenate was centrifuged at 12,000×g for 20 min at 4°C and the supernatant was collected. Total glutathione (h)GSH plus [h]GSSG[h]) was determined in the homogenates spectrophotometrically at 412 nm, using GR, DTNB, and NADPH. [h]GSSG[h] contents were determined by the same method in the presence of 2-vinylpyridine and [h]GSH contents were calculated from the difference between total glutathione and [h]GSSG[h].

Thiol analysis by reversed-phase HPLC
Low-molecular-weight thiols and their corresponding disulfides contents in root tissues from 30 seedlings were measured according to the methods previously reported [43–45], through derivatization with monobromobimane (mBBr) after reduction with DTT with or without previously blocked with N-ethylmaleimide (NEM), and separation by reversed-phase HPLC (Agilent Technologies, 1200 series Quaternary, Foster city, USA).

Histochemical analyses
Histochemical detection of lipid peroxidation and loss of plasma membrane integrity was performed with Schiff’s reagent and with Evans blue described by previous reports [41,45].

Real-time quantitative RT-PCR analysis
Total RNA from root tissues of 30 seedlings was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. DNA-free total RNA (2 μg) from different treatments was used for first-strand cDNA synthesis in a 20-μL reaction volume containing 2.5 units of avian myeloblastosis virus reverse transcriptase XI (TakaraRa) and oligo dT primer.

Real-time quantitative RT-PCR reactions were performed with Mastercycler realexplex² real-time PCR system (Eppendorf, Hamburg, Germany) using the SYBR Premix Ex Taq (TakaraRa) according to the user manual. The cDNA was amplified using primers (Table S1). The expression levels of the genes are presented as values relative to the corresponding control samples under the indicated conditions, with normalization of data to the geometric average of two internal control genes MSC27 and Actin2 [46].

Visualization of endogenous ROS by LSCM
Endogenous ROS was imaged using the fluorescent probe H2DCFDA, and then scanned described by [45,47].

Statistical analysis
Values are means ± SD of three different experiments with three replicated measurements. Differences among treatments were analysed by one-way ANOVA, taking P<0.05 as significant according to Duncan’s multiple range test.

Results
(h)GSH depletion and increased endogenous H₂S synthesis triggered by Cd stress
Considering alfalfa plants contain a thiol tripeptide homolog, hGSH, instead of or in addition to GSH [8,9], we detected the concentrations of GSH and hGSH. As shown in Table 1, the content of hGSH in alfalfa seedling roots under the control conditions, was about 8-fold higher than that of GSH. Similarly, hGSSGh is the main component of [h]GSSG[h] (total of hGSSGh and GSSG), because the GSSG content was almost negligible.

To further elucidate the correlation among GSH pool, H₂S and Cd tolerance, the time course of (homo)glutathione ([h]GSH; total of hGSH and GSH, and [h]GSSG[h]) contents, and H₂S synthesis were investigated in alfalfa seedling roots upon Cd stress. As expected, a decrease of [h]GSH content (especially hGSH) and an increase of [h]GSSG[h] (especially hGSSGh) level were progressively triggered by Cd stress within 12 h, thus leading to a decreased [h]GSH/[h]GSSG[h] ratio (12 h, Figure 1A-C), an important parameter for the intracellular redox status in plants [3,45]. The ratio of hGSH/hGSSGh exhibited the similar tendency (Table 1). These results were consistent with the observed Cd toxicity, confirmed by the histochemical staining detecting the aggravated loss of plasma membrane integrity and lipid peroxidation with Evans blue and Schiff’s reagent, increased TBARS content and growth stunt of seedling roots (Figure S1).

Because H₂S synthesis could be induced by oxidative stress and depletion of GSH both in animals and plants [48–50], we simultaneously investigated the production of H₂S in seedling roots after the exposure to Cd. Similar to the recent report [51], the production of H₂S was continuously increased after the exposure to Cd alone for 12 h (Figure 1D). The changes in activities of two H₂S synthetic enzymes LCD and DCDD displayed similar tendencies (Figure 1E and F). Apparently, the reduced (homo)glutathione depletion and increased endogenous H₂S synthesis preceded Cd toxicity in alfalfa seedlings.

NaHS not only mimics intracellular H₂S content, but also alleviates Cd toxicity
Previous results revealed that the exogenously applied NaHS, a H₂S donor, alleviates Cd toxicity in bermudagrass seedlings [51]. Therefore, a preliminary work was carried out to compare the oxidative damage and growth performance of alfalfa seedlings upon Cd exposure with or without the indicated concentrations of NaHS pretreatment. Firstly, the results of histochemical staining and TBARS contents revealed that NaHS at 100 (in particular) and 500 μM was able to significantly decreased Cd-induced lipid peroxidation (Figure S1A and B). These beneficial roles were also supported by the changes of fresh weight of ten alfalfa seedling roots, showing that NaHS at 100 and 500 μM had the greatest effects on the alleviation of the inhibition of root growth caused by Cd stress (Figure S1C). The beneficial roles of 100 μM NaHS alone were also observed. Subsequent work confirmed that H₂S rather than other sulphur-containing derivatives and sodium exhibited the cytoprotective role in the improvement of Cd toxicity by using a series of sulphur- and sodium-containing chemicals including Na₂S, Na₂SO₄, Na₂SO₃, NaHSO₄, NaHSO₃, and NaAc, in comparison with the positive roles of NaHS (Figure S2).

Accordingly, we observed that the treatment with 100 μM NaHS for 3 h resulted in the enhancement of endogenous H₂S level in alfalfa seedling roots, which also mimicked a physiological response elicited by Cd alone for 12 h (Figure 2A). The addition of Cd to the NaHS-pretreated plants further strengthened the increased H₂S content. Therefore, 100 μM NaHS was used to mimic the physiological role of intracellular H₂S in the subsequent experiments.
Table 1. Concentrations of low molecular weight thiols and their disulfides, and hGSH/hGSSGh ratio in root tissues.

| Treatment            | cysteine (nmol g⁻¹ FW) | cysteine disulfide (nmol g⁻¹ FW) | γ-EC (nmol g⁻¹ FW) | γ-EC disulfide (nmol g⁻¹ FW) | GSH (nmol g⁻¹ FW) | GSSG (nmol g⁻¹ FW) | hGSH (nmol g⁻¹ FW) | hGSSGh (nmol g⁻¹ FW) | hGSH/hGSSGh |
|----------------------|------------------------|----------------------------------|-------------------|-----------------------------|-----------------|-----------------|------------------|------------------|---------------|
| Con→Con              | 30±1 d                 | 3.8±0.8 c                        | 10±1 d            | 15±0.1                      | 27±2 b          | 0.2±1.9         | 252±16 b         | 28±2 c           | 8.86          |
| Con→Cd               | 33±1 cd                | 5.7±0.8 b                        | 14±2 d            | 17±0.1                      | 21±2 c          | 0.2±1.4         | 112±13 f         | 33±1 bc          | 3.41          |
| NaHS→Cd              | 40±2 c                 | 4.0±0.6 c                        | 18±2 bc           | 14±0.5                      | 26±4 c          | 0.1±1.4         | 163±14 de        | 33±4 bc          | 4.89          |
| NaHS→Con             | 34±2 cd                | 3.4±0.7 c                        | 8±0 e             | 12±0.5                      | 36±1 b          | 0.2±1.1         | 39±14 a          | 30±2 bc          | 10.23         |
| NaHS + PAG→Cd        | 54±7 b                 | 4.3±0.4 bc                       | 21±3 d            | 12±0.5                      | 29±5 b          | 0.3±0.7         | 144±8 e          | 41±6 a           | 3.55          |
| NaHS + PAG + GSH→Cd  | 65±8 a                 | 4.7±1.6 bc                       | 27±1 a            | 14±0.5                      | 46±11 a         | 0.7±0.6         | 179±7 d          | 36±5 ab          | 4.91          |
| PAG→Cd               | 52±6 b                 | 7.4±0.7 a                        | 21±1 b            | 16±0.2                      | 29±1 bc         | 0.9±0.9         | 82±12 g          | 33±3 bc          | 2.41          |
| PAG→Con              | 56±4 b                 | 4.0±0.3 c                        | 17±3 cd           | 12±0.5                      | 29±2 bc         | 0.7±0.7         | 206±28 c         | 36±2 ab          | 5.67          |

Seedlings were pretreated with or without 100 μM NaHS, 2 mM PAG, 1 mM GSH, individual or combination for 6 h, and then exposed to 200 μM CdCl₂ for another 12 h. Values are means ± SD of three independent experiments with three replicates for each. Different letters within columns indicate significant differences (P<0.05) according to Duncan’s multiple range test.

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Transcripts of representative antioxidant defense genes were sensitive to PAG, but rescued by GSH

Since ROS homeostasis was reestablished by NaHS in stressed conditions, the real-time RT-PCR test of corresponding genes involved in their metabolism, i.e. Cu, Zn-SOD, APX1, and GPX [3,5], were analysed. The results of Figure 6 revealed that in comparison with Cd alone samples, NaHS pretreatment followed by Cd exposure resulted in the enhancement in the transcript levels of Cu, Zn-SOD, APX1, and GPX in alfalfa seedling roots. The addition of PAG, however, significantly blocked the increases in the transcripts levels of these representative antioxidant enzymes induced by NaHS, all of which were reversed when GSH was added together with PAG.

NaHS responses were sensitive to a (h)GSH synthetic inhibitor, but reversed by the added GSH

The involvement of (h)GSH homeostasis in NaHS-induced cytoprotective against Cd stress were further investigated using a (h)GSH synthetic inhibitor and GSH applied exogenously. Pretreatment with NaHS, and L-buthionine-sulfoximine (BSO) at 1 mM, a concentration expected to be effective [52], exhibited an aggravated Cd toxicity, which was confirmed by the severe growth stunt and TBARS overproduction, in comparison with Cd plus NaHS (Figure 7A and B). Similarly, NaHS-mediated reestablishment of (h)GSH homeostasis in Cd stressed alfalfa seedling roots was also perturbed by BSO (Figure 7C and D), which was confirmed by the significant decreased (h)GSH content and the ratio of (h)GSH/(h)GSSG(h), respect to Cd alone. By contrast, above BSO responses were sensitive to the addition of GSH when
applied together. Above results clearly indicated a requirement for GSH homeostasis in NaHS-mediated alleviation of Cd toxicity.

cAMP signaling might be involved in NaHS responses

To test the hypothesis that H2S response is associated with cAMP signaling pathway, a pharmacological approach was used to manipulate endogenous cAMP. Results presented in Figure 8A and B indicated that the pretreatment with 8-Br-cAMP, a membrane-permeable analogue of cAMP, alleviated Cd-induced decrease of fresh weight and increase of TBARS content in alfalfa seedling roots. Both of two adenyl cyclase inhibitors, alloxan and DDA, blocked NaHS-alleviated Cd stress. Moreover, similar to the beneficial actions of 8-Br-cAMP (when was cotreated with PAG followed by Cd stress), a cNMP phosphodiesterase inhibitor IBMX also reversed the PAG responses in the aggravation of fresh weight loss and lipid peroxidation caused by Cd stress. Results from the real-time RT-PCR showed that 8-Br-cAMP and IBMX pretreatments followed by Cd stress, mimicked the effect of NaHS on GR1 up-regulation, regardless of whether PAG was added or not.

Figure 2. NaHS increased endogenous H2S and (h)GSH contents, and the ratio of (h)GSH/(h)GSSG(h) upon Cd stress. Endogenous H2S concentration in root tissues (A) was detected at 3 h after the beginning of 100 μM NaHS pretreatment (~3 h), and 200 μM CdCl2 or chemical-free control treatments for 12 h (~12 h). Meanwhile, contents of (h)GSH (B) and the ratio of (h)GSH/(h)GSSG(h) (C) in root tissues were detected at the indicated time points of treatments. Values are means ± SD of three independent experiments with three replicates for each. Bars denoted by the same letter did not differ significantly at P<0.05 according to Duncan’s multiple range test. doi:10.1371/journal.pone.0109669.g002

Figure 3. Time course of transcripts responsible for (h)GSH metabolism regulated by NaHS and Cd. Seedlings were pretreated with or without 100 μM NaHS for 6 h and then exposed to 200 μM CdCl2 for another 24 h. The expression levels of ECS (A), GS (B) and GR1 (C) in root tissues analyzed by real-time RT-PCR are presented as values relative to the control at the beginning of pretreatment, normalized against expression of two internal reference genes in each sample. Values are means ± SD of three independent experiments with three replicates for each. doi:10.1371/journal.pone.0109669.g003
not (Figure 8C). Two inhibitors alloxan and DDA partially blocked NaHS plus Cd-induced GR1 transcripts. A similar tendency was found in the changes in GPX transcripts (Figure 8F). Results presented in Figure 8D and E further revealed the negative effects of adenylyl cyclase inhibitors on the transcripts of Cu, Zn-SOD and APX1 in NaHS-pretreated seedling roots upon Cd, in comparison with the positive responses of 8-Br-cAMP and IBMX in the presence or absence of PAG.

Discussion

Although H2S is a hazardous gaseous molecule with a strong odor of rotten eggs, it has been described as an important regulator with a variety of biological roles in animals and recently in plants [11–16,25–34,53–56]. Moreover, recent works on Populus euphratica cells [57] and bermudagrass seedlings [51], demonstrated that exogenously applied NaHS, a H2S donor, resulted in an enhanced Cd tolerance in these species. However, possible physiological mechanisms and downstream targets responsible for the observed Cd tolerance triggered by intracellular H2S remain elusive. In this report, we discovered endogenous H2S production in response to Cd stress, and further provided evidence demonstrating a requirement of (h)GSH and ROS homeostases, at least partially, in the intracellular H2S-mediated plant adaptation.
against Cd toxicity. Therefore, our results presented in this work are vital for both fundamental and applied plant biology.

Endogenous H$_2$S production in response to Cd stress: the possible involvement of LCD/DCD

In animals, it was previously reported that diverse stress-inducing stimuli could result in the production of H$_2$S, including oxidative stress [49], depletion of cysteine (or its derivatives) [58] and glutathione [50]. Recent work in Arabidopsis [25] and bermudagrass seedlings [51] reported drought- and Cd-induced H$_2$S production. Because the signal compound H$_2$S is very reactive [53], the rapid regulation of the activity of H$_2$S biosynthetic enzymes seems essential to fulfill H$_2$S-dependent functions. In this work, we further showed that Cd-triggered endogenous H$_2$S production might be related to LCD/DCD pathways (Figure 1D-F), since the similar increasing changes in the levels of intracellular...
H2S as well as LCD/DCD activities were observed in the seedling roots of alfalfa challenged with Cd for 12 h. Meanwhile, similar to previous reports in wheat [30], bermudagrass [51], Spinacia oleracea seedlings [59], and strawberry plants [60], NaHS-induced H2S production in alfalfa plants was also observed (Figure 2A).

In plants, both LCD and DCD are hypothesized to be involved in intracellular H2S synthesis [21,27]. Several LCD/DCD candidates have been cloned and partially analyzed from the model plant Arabidopsis to Brassica napus [24,61]. Our above findings are consistent with those reported by Bloem et al. [40], in which they found that Brassica napus was able to react to Pyrenopeziza brassicae infection with a greater potential to release H2S, which was reflected by an increasing LCD activity with fungal infection. More recently, auxin-induced DES-mediated H2S generation was also found to be involved in lateral root formation in tomato seedlings [62]. In view of the fact that all H2S synthetic enzymes are not fully elucidated, our results suggested that LCD/DCD pathways might be, at least partially, related to Cd-induced H2S production in alfalfa seedlings. In a future study, the role of other enzymatic and non-enzymatic pathways-mediated induction of H2S synthesis in alfalfa seedlings upon Cd stress need be further elucidated.

The mechanism underlying the role of intracellular H2S in the alleviation of Cd toxicity: reestablishment of reduced (homo)glutathione and ROS homeostases

Ample evidence revealed a clear relationship between metal stress and redox homeostasis and antioxidant capacity.
be associated with the cAMP pathway. This conclusion is based on reestablishment of (h)GSH and ROS homeostases, which might (Figure 4A, Figure S1C). (ii) Application of a H$_2$S-releasing compound NaHS showed the crucial role of PCs, especially their precursor GSH in responding to Cd challenge. (Figure S1A, S3 and S4; Figures 4A and 5). Our further experiments provide strong evidence to support the existence of a causal relationship between the endogenous H$_2$S signal and the alleviation of Cd toxicity in alfalfa seedlings partly by reestablishment of (h)GSH and ROS homeostases, which might be associated with the cAMP pathway. This conclusion is based on several pieces of evidence: (i) increased H$_2$S metabolism as well as the perturbation of (h)GSH homeostasis in alfalfa seedling roots are two early responses to the exposure of Cd (Figure 1A, Table 1). These changes were consistent with the phenotypes of Cd toxicity (Figure 4A, Figure S1C). (ii) Application of a H$_2$S-releasing compound NaHS (also called as H$_2$S donor), not only mimics intracellular H$_2$S content triggered by Cd, but also alleviates Cd toxicity (Figures 2 and 4). Consistently, we also detected reestablishment of (h)GSH homeostasis, which was reflected by a higher (h)GSH content and ratio of (h)GSH/(h)GSSG(h) upon Cd stress. The observed Cd tolerance might be due to the available (h)GSH by the up-regulation of (h)GSH synthesis related genes, EGS and GS (Figure 3A and B), as well as GR1 (Figure 3C), because besides the synthesis of PCs, availability of GSH and concerted activity of GR seem to play a important role for plants to combat oxidative stress and Cd toxicity [7,72,73]. While, the inhibition of H$_2$S production caused by its synthetic inhibitor PAG blocked NaHS-induced Cd tolerance and reestablishment of (h)GSH and ROS homeostases, the latter of which was confirmed by the histochemical staining detecting the alleviation of plasma membrane integrity and lipid peroxidation, decreased ROS content and up-regulation of Cu,Zn-SOD, APX1 and GPX transcripts, as well as declined TBARS level (Table 1, Figures 2–6, and Figures S1, S3 and S4). (iii) Above mentioned PAG responses were further rescued by exogenously applied GSH (Table 1, Figures 4–6). (v) NaHS responses were sensitive to a (h)GSH synthetic inhibitor, but reversed by the added GSH (Figure 7), both of which suggesting a requirement of (h)GSH homeostasis for NaHS cytoprotective roles; and (v) Previous reports in animals showed H$_2$S-activated cAMP level or H$_2$S-regulated cAMP homeostasis [37,38]. Here, we found that two adenylyl cyclase inhibitors, alloxan and DDA, blocked the beneficial responses conferred by NaHS in alfalfa seedlings subjected to Cd stress (Figure 8). On the contrary, an analogue of cAMP 8-Br-cAMP and a cNMP phosphodiesterase inhibitor IBMX mimicked the effects of NaHS on the alleviation of Cd toxicity as well as the regulation of (h)GSH homeostasis and ROS metabolism (GR1, Cu,Zn-SOD, APX1, and GPX, etc.). Above pharmacological evidence indicated the involvement of cAMP signaling in NaHS responses. Additionally, NaHS-triggered cytoprotective roles were confirmed to act as a H$_2$S-dependent fashion (Figure S2). Above results clearly established a casual link between intracellular H$_2$S in the alleviation of Cd toxicity and reestablishment of (h)GSH and ROS homeostases.

Conclusions

In summary, our pharmacological, histochemical, biochemical and molecular evidence suggested that the intracellular H$_2$S was able to ameliorate Cd toxicity in alfalfa seedlings at least partly by reestablishment of (h)GSH and ROS homeostases. Figure 9 illustrates a simplified scheme of mechanisms involved in Cd tolerance by LCD/DCD-produced H$_2$S-modulated (h)GSH and ROS homeostases. Abbreviations: NaHS, sodium hydrosulfide; PAG, o-propargylglycine; LCD, L-cysteine desulphydrase; DCD, D-cysteine desulphydrase; H$_2$S, hydrogen sulfide; ROS, reactive oxygen species; (h)GSH, reduced (homol)glutathione; BSO, L-buthionine-sulfoximine; cAMP, cyclic AMP. The dashed line denotes possible signaling cascade. 

Figure 9. Simplified scheme of mechanisms involved in Cd tolerance by LCD/DCD-produced H$_2$S-modulated (h)GSH and ROS homeostases. 

 Supporting Information

Figure S1 NaHS pretreatment alleviates Cd toxicity. (DOC)

Figure S2 H$_2$S or HS$^-$, but not other compounds derived from NaHS contribute to NaHS responses. (DOC)

Figure S3 Effects of NaHS, PAG and GSH pretreatments on the fresh weight (A) and TBARS concentrations (B) in alfalfa seedling roots upon Cd stress. (DOC)

Figure S4 Effects of NaHS, PAG and GSH pretreatments on Cd concentrations in alfalfa seedlings upon Cd stress. (DOC)
Table S1 The sequences of primers for real-time RT-PCR.

Author Contributions
Conceived and designed the experiments: WS. Performed the experiments: WC KJ QJ. Analyzed the data: WC Y. Xia JW. Wrote the paper: WC HG WS.
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