

Hydrogen Sulfide Alleviates Cadmium Toxicity by Restricting Cd²⁺ Influx in *Arabidopsis*

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Abstract As an emerging gasotransmitter, hydrogen sulfide (H₂S) is mainly produced by cysteine desulphydrases (CDes) responsible for the degradation of cysteine in plants. It has been reported that H₂S signal interacted with plant hormones in response to heavy metal cadmium (Cd) stress. However, the mechanism based on systematic study is still limited. In the present study, we evaluated the protective effect of H₂S against Cd-induced oxidative stress from different levels in *Arabidopsis* seedlings. The expression levels of CDes and H₂S production were gradually increased dependent on the concentration of CdCl₂. Cd stress caused a decrease of the dry weight of seedlings by about 33%, and an increase of hydrogen peroxide markedly and malondialdehyde content by about 110%. The activity of superoxide dismutase (SOD) increased by two fold, and catalase and glutathione reductase activities decreased by 27% and 21%, respectively, which caused the reduced glutathione content significantly. Prior to metal exposure, seedlings pretreated with NaHS as a H₂S donor significantly recovered all the above changes to control levels. H₂S treatment downregulated the expression of Cd transporter in plasma membrane (*HMA4* and *IRT1*), while upregulated those in vacuolar membrane (*MRP3* and *CAX2*). Non-invasive micro-test technique (NMT) data showed that NaHS inhibited Cd²⁺ influx, therefore the Cd content in leaves and roots was significantly decreased by 15% and 38.4%, respectively. Together, these results suggested that H₂S upregulated antioxidant enzymatic and non-enzymatic systems under Cd²⁺ stress and then scavenged the Cd²⁺-elicited H₂O₂ within cells. The regulation in Cd²⁺ transport and vacuolar sequestration by H₂S reduced the concentration of Cd²⁺, subsequently. In final, Cd²⁺ toxicity was restricted in *Arabidopsis*. It provides a new method for understanding the mechanism of crop response to heavy metal stress.

Key words hydrogen sulfide (H₂S); gasotransmitter; non-invasive micro-test technique; Cd²⁺ influx; *Arabidopsis*

硫化氢通过抑制镉离子内流降低拟南芥体内的镉毒性

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摘要 硫化氢(H₂S)作为一种新兴的气体信号分子,在植物体内主要由半胱氨酸脱巯基酶(CDes)降解半胱氨酸产生。已有报道表明,H₂S信号与植物激素共同作用增强植物的镉(Cd)耐受。然而,H₂S信号响应重金属Cd胁迫的作用机制尚缺乏系统研究。本文以拟南芥为实验材料,从不同水平探究H₂S分子对Cd胁迫诱导氧化应激的保护作用。结果表明,CDes基因表达量和H₂S的产

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率随 CdCl_2 浓度升高而逐渐增加。重金属 Cd 胁迫导致幼苗干重降低约 33%、体内过氧化氢显著增加、丙二醛含量升高约 110%、超氧化物歧化酶活性增加约 100%、谷胱甘肽还原酶活性和过氧化氢酶活性分别下降 27% 和 21%，还原性谷胱甘肽含量随之显著降低。生理浓度 NaHS (H_2S 供体) 预处理显著缓解以上 Cd 胁迫产生的影响, 使恢复到对照水平。同时, H_2S 处理可显著下调质膜中 Cd 转运蛋白 (HMA4 和 IRT1) 的表达, 同时上调液泡膜中 MRP3 和 CAX2 的表达。利用非损伤微测技术测定植物根系 Cd^{2+} 的流动速度和流动方向。结果显示, 生理浓度的 H_2S 显著抑制 Cd^{2+} 内流, 最终表现为植物叶片和根中的 Cd 含量显著降低, 分别下降了 15% 和 38.4%。总之, 在 Cd 胁迫条件下, H_2S 信号可激活植物体内的抗氧化酶促和非酶促系统, 以清除细胞内 H_2O_2 。 H_2S 对 Cd^{2+} 转运和液泡区式化的调节, 降低了体内 Cd^{2+} 的浓度, 减小 Cd 毒性对植物生长的影响。为理解农作物应对重金属胁迫的机制提供了新的思路。

关键词 硫化氢; 气体信号分子; 非损伤微测技术; 镉离子内流; 拟南芥

中图分类号 Q942.5

Cadmium (Cd^{2+}) is a toxic element that normally exists at low concentrations in soils. The metal is taken up quickly by plant roots^[1] and generally causes growth inhibition and even plant death by reducing enzyme activities^[2], photosynthesis^[3], and water and nutrient uptake^[4]. It has been suggested that root growth inhibition by Cd^{2+} is due to a direct effect on the nucleus or to interaction with hormones^[5] and growth retardation of aerial parts is due to inhibition of photosynthesis^[4]. The export of Cd^{2+} from the cytoplasm can occur either into internal (vacuoles) or apoplastic compartments via specific transporters. In plants, the ATP-binding cassette (ABC) transporter (s) involved in cadmium transport has not been identified. However, the transcript level of one multidrug-resistance-related protein (MRP) gene *AtMRP3* is increased after Cd^{2+} treatment^[6]. There are data to support the idea that other classes of transporters, such as iron-regulated metal transporter 1 (IRT1), are involved in Cd distribution within plants^[7]. Xylem loading of Cd has been shown to be mediated by the P-type ATPase transporter *AtHMA4* in *A. thaliana*^[8].

A great variety of abiotic stressors including drought, salinity, UV light, air pollutants and heavy metals cause molecular damage to plants, either directly or indirectly through the formation of reactive oxygen species (ROS). Although the mechanism by which metals caused damage is not clearly understood in plant, increasing evidence suggests that metal toxicity is partly due to oxidative damage. Redox metals, such as copper or iron, may produce free radicals directly^[9]. Cd^{2+} is a non-redox metal and unable to participate in Fenton-type reactions, but can induce oxidative stress in cells^[10]. In plants, several reports have described metal-induced alterations in both the activities of antioxidative enzymes and the levels of soluble antioxidants^[11-13], accompanied by an enhancement of lipid peroxidation^[14,15]. Depending on its concentration, Cd^{2+} can either inhibit or stimulate the activity of several antioxidative enzymes before any

visible symptoms of toxicity appear^[16]. The production of ROS will cause oxidative damage if not counteracted by increased antioxidative capacity^[17]. Plant cells are normally protected against oxidative damage by a broad spectrum of radical scavenger systems, including antioxidative enzymes such as ascorbate peroxidase (APX), GR and SOD, as well as non-enzymatic compounds like GSH, carotenoids, ascorbate and α -tocopherol^[18].

It has been shown that H_2S is the third gasotransmitter identified in animals, in addition to NO and CO^[19]. Two pyridoxal-5'-phosphate (PLP)-dependent enzymes in the trans-sulfuration pathway, cystathionine γ -lyase (CSE, EC4.4.1.1) and cystathionine β -synthase (CBS, EC4.2.1.22), have consistently been demonstrated to produce H_2S in mammalian tissues with L-cysteine or homocysteine as the main substrate. In the plant kingdom, NO and CO have already been identified as signaling molecules involved in antioxidative defense. However, the role of H_2S in antioxidative protection of plants remains unclear, although H_2S emission has already been observed in many species. L-cysteine desulphydrase (LCD, EC4.4.1.1) and D-cysteine desulphydrase (DCD, EC4.4.1.15) in *Arabidopsis* have been found to catalyze the production of H_2S in plants^[20]. As a novel gasotransmitter, H_2S has potential regulatory functions on the development of plants and alleviation various biotic and abiotic stresses^[19]. Under Cd^{2+} stress, H_2S decreases reactive oxygen species levels and enhances the antioxidant capacity in alfalfa^[21], *Populus euphratica* cells^[22] and bermudagrass^[23], respectively. More recently, our research group reported that H_2S might be a downstream signal molecule in SA-induced Cd^{2+} tolerance^[24] and CDPKs enhanced the tolerance to Cd through H_2S signal in *Arabidopsis*^[25]. Additionally, H_2S and cooperated with proline and methyl jasmonate to alleviate Cd-damage in foxtail millet^[26,27]. However, it was still insufficient how H_2S responded to Cd^{2+} stress in model plants

systematically and directly.

In this study, we try to provide the basic theory to explore the production of H_2S in plants under Cd^{2+} stress. The effects of H_2S signal on ROS elimination, expression of Cd^{2+} transporters, Cd^{2+} flux in roots and enrichment in plants under Cd^{2+} stress were also explored.

1 Materials and Methods

1.1 Plant materials and growth condition

Seeds of *Arabidopsis* (Col-0) were surface sterilized with 70 % ethanol for 30 s followed by 5 % (W/V) sodium hypochlorite for 8 minutes. After three washes with distilled water, seeds (50 ~ 100 per bottle) were cultivated in 1/2 Murashige-Skoog (1/2MS) medium supplemented with 0.75 % (W/V) agar, and 1 % (W/V) sucrose, pH 5.8. A growth chamber with the condition of $160\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of light, 60 % relative humidity, $23\pm1\ ^\circ\text{C}$, 16/8 hours light/dark was used for plant growth. According to the previous studies, the seedlings after 7 days germination were treated with or without 50 $\mu\text{mol/L}$ NaHS for 6 hours, respectively^[24, 28]. Subsequently, treated seedlings were transferred to 1/2 MS medium containing 200 $\mu\text{mol/L}$ CdCl_2 for 72 hours. 50 seedlings per group were sampled and dried at 105 $^\circ\text{C}$

Table 1 List of primers for RT-PCR

Gene	Accession number	Primer pairs
LCD	At3g62130	5'-CAATGGAGGCGGGAGAGCGGCGCAATG-3' 5'-CTACAATGCAGGAAGGTTTGTACAAG-3'
DCD1	At1g48420	5'-CAATGAGGGACGAAGCTTGACACTCTC-3' 5'-CTAGAACCTTTTCCCAACACATCT-3'
DCD2	At3g26115	5'-TCCCCTGCAGGATGAAGGTCCAACGGTCA-3' 5'-TCCCCTGCAGGCTAATCTTTCAAGTTAGTG-3'
GSH1	At4g23100	5'-GGTCAGTTCGAGCTTAGTGG-3' 5'-GTGCGGTCTTGTGAGTGTCT-3'
GSH2	At5g27380	5'-TTCCCTCACATCATCTCCATA-3' 5'-AAGCAGTCGCAGTGGTTTATT-3'
HMA4	At2g19110	5'-TACAATCTTCCCGACACTATCTC-3' 5'-ATCACCGAATGAAAGTATCTCCA-3'
IRT1	At4g19690	5'-CAGCTAGAAGATCCACGAGTGCC-3' 5'-CCCGCAAATGATGTTACCTTACC-3'
MRP3	At3g13080	5'-TACAATCTTCCCGACACTATCTC-3' 5'-ATCACCGAATGAAAGTATCTCCA-3'
CAX2	At3g13320	5'-TGACATTACCAACTTACTTCTGC-3' 5'-CTCCTCCAACCTCTGATAGCTTCTC-3'
ACTIN	At3g46520	5'-CTCAGCACCTTCCAACAGATGTGA-3' 5'-CCAAAAAATGAACCAAGGACCAAA-3'

1.4 Determination of H_2S production rate

The total protein extracts were collected from 4-week-old leaves, then activities of cysteine desulphydrases were measured by the rate of H_2S production from L-cysteine and D-cysteine as described previously^[26].

1.5 Measurement of H_2O_2 and MDA content

to a constant weight.

1.2 Stress and chemical treatment

Seeds were surface sterilized and sown in plastic trays filled with vermiculite. The growth condition was kept the same as described above, watered with Hoagland solution for 4 weeks. Ten identical replicate trays were prepared and arranged in a completely randomized block design, dividing into two groups. One group was watered with Hoagland (Control), and another group with Hoagland plus 50 $\mu\text{mol/L}$ NaHS (NaHS) for 7 days, then watered half of each group with Hoagland plus 200 $\mu\text{mol/L}$ CdCl_2 , for 10 days. Therefore, there are treatments with various chemicals (Control, 50 $\mu\text{mol/L}$ NaHS, 200 $\mu\text{mol/L}$ CdCl_2 , 200 $\mu\text{mol/L}$ CdCl_2 + 50 $\mu\text{mol/L}$ NaHS). All determinations were made using rosette leaves.

1.3 Extraction of total RNA and RT-PCR

After treatment with NaHS (50 $\mu\text{mol/L}$) and CdCl_2 (0, 100, 200, 300 $\mu\text{mol/L}$) for 6 hours^[24, 28], the 4-week-old leaves were collected. Total RNA samples were extracted and semi-quantitative RT-PCR was carried out as described previously^[25, 28]. All RT-PCR expression assays were performed and analyzed at least three times in independent experiments, using *ACTIN* as an internal control. The primers (Table 1) were shown as follows:

The leaves above (described in 2.2) were collected and placed in 1 mg/mL of 3,3'-diaminobenzidine (DAB, Sigma, MO, USA) solution, then incubated in dark for 1 hour and in light for 12 hours in a growth chamber. After washing with distilled water three times, the leaves were boiled in 95% ethanol for 10 minutes. H_2O_2 was

visualized as a reddish-brown coloration. Determination of Malondialdehyde (MDA) and GSH were carried out as previously described^[25].

1.6 Enzyme preparation and assay

Leaves in the four groups described in 2.2 were harvested. The activities of SOD (EC 1.15.1.1), GR (EC 1.6.4.2) and CAT (EC 1.11.1.6) were assayed as described previously^[26]. The data presented are the means \pm SE from three different experiments which included at least three replicate measurements.

1.7 Determination of net Cd²⁺ fluxes in the roots

Net Cd²⁺ fluxes in the roots were measured in the YoungerUSA (Xuyue Beijing) NMT Service Center using Non-invasive Micro-test Technology and iFluxes/imFluxes 1.0 Software (NMT100 Series, YoungerUSA LLC, Amherst, MA, USA; Xuyue (Beijing) Sci. & Tech. Co., Ltd., Beijing, China) as described previously^[29]. Cd²⁺-selective electrodes were prepared as the protocol; the micropipettes were filled with a backfilling solution (10 mmol/L Cd(NO₃)₂ and 0.1 mmol/L KCl) and then front filled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909, Sigma-Aldrich, St. Louis, MO, USA). The 4-week-old seedlings were pretreated with various chemicals (Control, 50 μ mol/L NaHS, 200 μ mol/L CdCl₂, 200 μ mol/L CdCl₂ + 50 μ mol/L NaHS) for 6 hours, and then the fine roots were cut and transferred immediately to a Petri dish containing 10 mL of the measuring solution (0.075 mmol/L CdCl₂, 0.1 mmol/L KCl, 0.5 mmol/L NaCl, 0.3 mmol/L MES, 0.2 mmol/L Na₂SO₄, pH 5.5) for 10 minutes. Then the net Cd²⁺ fluxes were recorded 100 μ m from the apex and conducted along the root axis until 2300 μ m, at intervals of 200 μ m, and then calculated using Fick's law of diffusion: $J = -D (dc / dx)$, where J represents the ion flux in the x direction, dc/dx is the ion concentration gradient, and D is the ion diffusion constant in a particular medium.

1.8 Analysis of Cd accumulation

Determination of the Cd²⁺ concentration was performed according to the method described previously^[27]. Briefly, the 4-week-old leaf and root tissue were deactivated for 30 minutes at 100 °C and then dried for 48 hours at 80 °C, weighed, ground into a fine powder, digested with HNO₃ and HClO₄ (3:1, V/V), and dissolved in deionized water. The Cd²⁺ concentrations were determined using an atomic absorption spectrophotometer (AA240 VARIAN, Palo Alto, CA, USA).

1.9 Statistical analysis

All data presented are the mean values of five independent sets of experiments. Each value is presented as $\bar{x} \pm s$ with a minimum of three replicates. Statistical assays were carried out using one-way

ANOVA using the Tukey test to evaluate whether the means were significantly different. $P < 0.05$ was considered as statistically significant.

2 Results

2.1 NaHS pretreatment alleviated the growth inhibition under Cd²⁺ stress

In the preliminary experiment, Cd²⁺ stress inhibited the elongation of *Arabidopsis* roots in a dose-dependent manner (from 0 to 300 μ mol/L). The lengths of the primary roots showed the significant decrease in the presence of 200 μ mol/L CdCl₂ compared with control (Fig. 1A). According to the previous results, less than 100 μ mol/L was the physiological concentration of NaHS as the H₂S donor. Therefore, 200 μ mol/L of CdCl₂ and 50 μ mol/L of NaHS were selected for the treatment of *Arabidopsis* seedlings in the subsequent experiments.

With or without pretreatment of NaHS, 7-day-old *Arabidopsis* seedlings were transferred to 1/2 MS medium containing CdCl₂, respectively. Then the dry weight of the seedlings was measured 10 days later. As shown in Fig. 1A, Cd²⁺ stress led seedlings to toxicity symptoms; chlorosis and growth inhibition. Plants pretreated with NaHS and subsequently exposed to CdCl₂ (NaHS + Cd) showed a slight reduction in growth, as compared with control plants. The dry weight of 50 seedlings each group was compared in Fig. 1B, which reduced by 33% in presence of CdCl₂ as compared with control. However, plants pretreated with NaHS before CdCl₂ exposure (NaHS + Cd) reversed to the control level, with a 27% increase compared with CdCl₂ treatment alone.

2.2 Upregulation of CDes expression and H₂S production rate was dependent on CdCl₂ concentration

In order to investigate the response of H₂S on Cd²⁺ stress, the expression of CDes and the production rate of H₂S in 4-week-old *Arabidopsis* plants were analyzed. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed, and *ACTIN* gene was used as an internal control. As shown in Fig. 2A and 2B, after treatment with different concentrations of CdCl₂ for 1 week, the expression patterns of CDes were similar, and the expression levels gradually increased with Cd²⁺ concentration from 0 to 300 μ mol/L. As expected, the rate of H₂S production was significantly higher in the presence of Cd²⁺ compared with control, in a dose-dependent manner not only based on L-cysteine but also D-cysteine (Fig. 2C).

2.3 Effect of H₂S on H₂O₂ production and antioxidant enzyme activities

Over accumulation of H₂O₂ is a critical effect of Cd toxicity in plant cells. To determine the role of physiological concentrations of H₂S on Cd stress, the

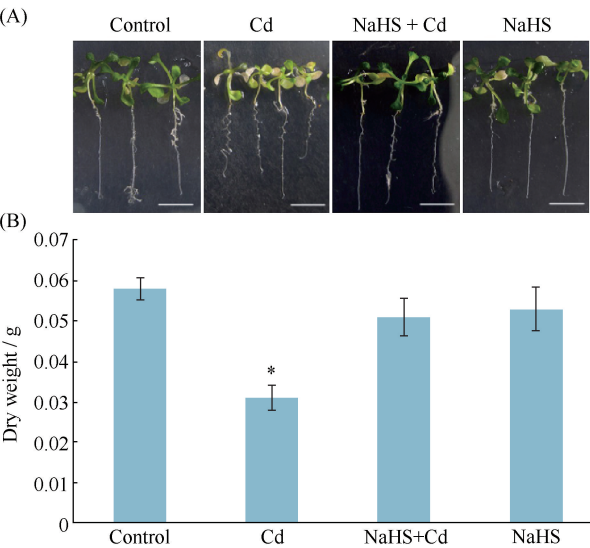


Fig.1 Effect of Cd and NaHS pretreatment on plant growth (A) and dry weight (B) C: Control, Cd: 200 μmol/L CdCl₂, NaHS+Cd: 50 μmol/L NaHS plus 200 μmol/L CdCl₂, NaHS: 50 μmol/L NaHS. (A) Plants were either pretreated with or without 50 μmol/L of NaHS for 7 days on 1/2 MS medium, whole wild-type plants were transferred to 1/2 MS medium and treated with 200 μmol/L of CdCl₂ or not for 10 days. Bars=1 cm. (B) The leaves of 20 plants described in section 2.1 were used for the dry weight measurements. Values are the means of three different experiments with five replicate measurements, and bars indicate S.E. * Significant differences ($P < 0.05$), according to Tukey’s multiple range test.

leaves of 4-week-old plants were treated with various chemicals for 24 hours. As shown in Fig.3 A, treatment with Cd²⁺ alone induced a dramatic increase in H₂O₂ production in the leaves compared with control. Pretreatment with NaHS significantly diminished Cd²⁺-induced H₂O₂ accumulation in leaves to control level.

Lipid peroxidation is one of the first consequences of oxidative damage; MDA is considered as a good indicator of stress-induced cell damage. When plants were treated with 200 μmol/L CdCl₂, the MDA content increased by 200% as compared with the control. In the presence of NaHS, Cd²⁺-induced MDA accumulation in leaves significantly reduced to control level (Fig.3B).

Antioxidant enzymes depress the level of ROS. The effect of Cd²⁺ on CAT and SOD activities, in the presence or absence of NaHS, was determined after 10 days of treatment in Arabidopsis leaves. SOD activity increased about 110% in Cd²⁺-treated plants, while plants pretreated with H₂S and then with Cd also showed increased SOD activity, but only 59% above the control. No changes with respect to the control were observed when plants were treated with H₂S alone (Fig.3C).

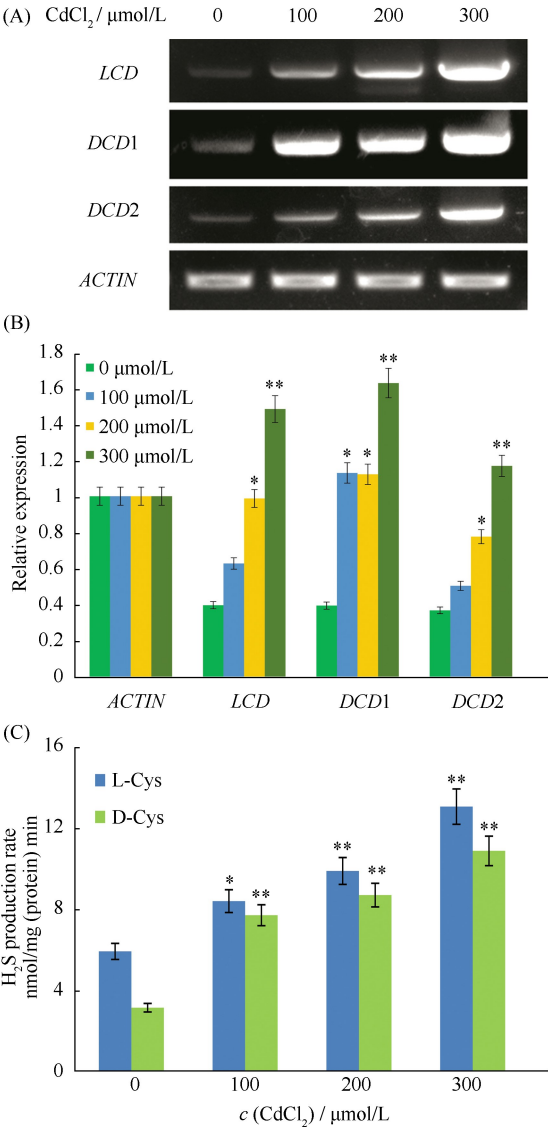


Fig.2 The expression of *CDes* (A, B) and total H₂S production rate (C) in *A.thaliana* treated with different concentrations of Cd²⁺ (A) The expression of *CDes* is increased by Cd stress. The RNA was extracted using leaves from 4-week-old wild-type plants at a fixed time. RT-PCR was performed with 20 cycles for *ACTIN* and 30 cycles for *CDes*. (B) Digitization of *CDes* genes expression by Image J (v2.1.4.7). (C) The total H₂S production rate of 4-week-old plants was measured under different Cd concentrations for 10 days. Values are the means of three different experiments with three replicate measurements, and bars indicate S. E. * Significant differences ($P < 0.05$), ** Significant differences ($P < 0.01$) according to Tukey’s multiple range test.

Plants cope with oxidative stress via a complex system. It is well known that increased H₂O₂ levels can be scavenged by antioxidative enzymes such as CAT. CAT activity in leaves differed significantly in response to the various treatments. As shown in Fig.3D, CAT activity decreased significantly with Cd²⁺ exposure

alone, while combination of H₂S and Cd²⁺ treatment increased CAT activity to the control level. Additionally, H₂S pretreatment alone significantly stimulated CAT activity.

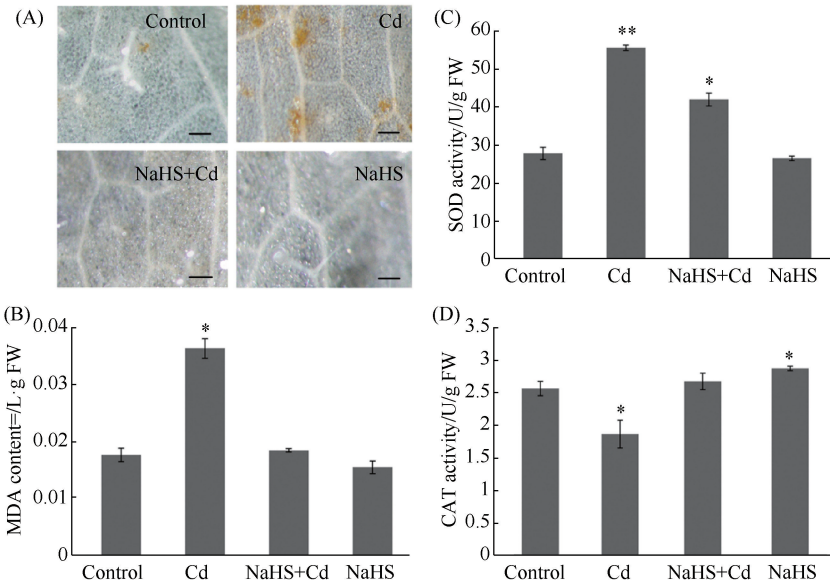


Fig.3 H₂S alleviates cadmium toxicity in *Arabidopsis* Plants of 4-week-old were treated with various chemicals for 24 hours (A) Effects of 50 μmol/L NaHS or/and 200 μmol/L CdCl₂ on H₂O₂ content in leaves. Bars = 20 μm. (B) Effects of 50 μmol/L NaHS or/and 200 μmol/L CdCl₂ on MDA content in leaves. (C) Effect of NaHS pretreatment on SOD activity under Cd²⁺ stress. One unit of SOD is the amount of enzyme that inhibited the reduction of NBT by 50% under the assay condition. (D) Effect of Cd and NaHS pretreatment on CAT activity. One unit of CAT is the amount of enzyme that oxidized 1 nmol of H₂O₂ min⁻¹ under the assay condition

2.4 GSH-GR system was induced by H₂S under Cd²⁺ stress

Reduced glutathione (GSH) and oxidized glutathione

(GSSG) are the products of sulfur metabolism. The former has been documented as a major antioxidant, which has positive biological functions in plant responses to heavy

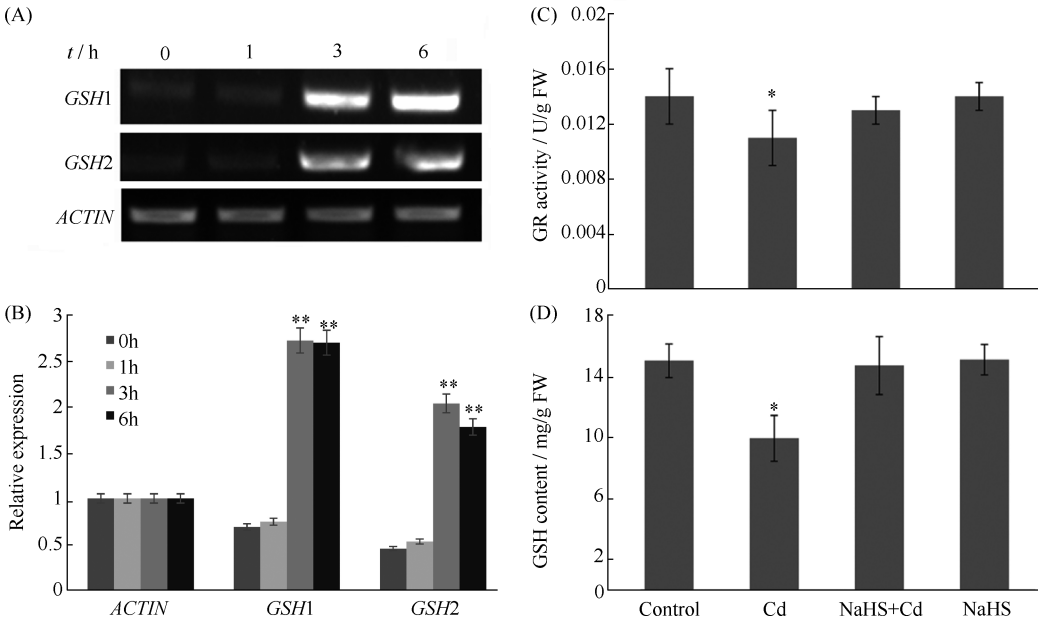


Fig.4 Effect of H₂S on GSH-GR system under Cd²⁺ stress (A) The expression levels of *GSH1* and *GSH2* in NaHS (50 μmol/L)-treated leaves were detected by RT-PCR. Tissue of 4-old-leaf was collected at set times (0, 1, 3 and 6 hours) after treatment. (B) Digitization of *GSH* genes expression by Image J (v2.1.4.7). (C) Effect of Cd and NaHS pretreatment on GR activity in leaves. One unit of GR is the amount of enzyme that oxidized 1 mmol/L of NADPH/ min under the assay condition. (D) Effect of Cd and NaHS pretreatment on glutathione content in leaves.

metal stress and oxidative stress, and the latter can be restored to GSH by glutathione reductase (GR). GSH is synthesized via two enzymes, GSH1 (E.C. 6.3.2.2) and GSH2 (E.C. 6.3.2.3)^[3].

Here, pharmacological experiments were carried out to test whether H₂S affects the GSH synthesis pathway. To determine the treatment time, the expression of *GSH1* and *GSH2* was measured in the presence of NaHS using RT-PCR. After NaHS treatment, the level of these two transcripts was upregulated significantly with a concomitant maximum accumulation at 6 hours (Fig. 4 A, B). GSH-GR system in leaves differed significantly in response to the various treatments. As shown in Fig. 4C, Cd²⁺ stress considerably reduced GR activity. Although H₂S treatment alone did not modify GR activity in *Arabidopsis* leaves, H₂S pretreatment restored GR activity to control values. At the same time, GSH content decreased significantly with CdCl₂ exposure and the addition of H₂S markedly reversed to the control level (Fig. 4D).

2.5 Regulation of *IRT1* and *AtHMA4* expression by cadmium and H₂S treatment

The effect of Cd²⁺ on plants was reflected by the transcription levels of the heavy metal related genes,

including transporters from the extracellular to intracellular (*IRT1* and *HMA4*) and from cytoplasm to vacuole (*MRP3* and *CAX2*). Compared with control in 4-week-old leaves, *IRT1* and *AtHMA4* were upregulated considerably with Cd²⁺ treatment. In addition, the expression levels of *MRP3* and *CAX2* were little higher than those of control (Fig. 5A left and 5B). As we mentioned before, endogenous H₂S was induced by Cd²⁺ treatment. Therefore, the expression of these transporters was measured with exogenous H₂S supplementation. Interestingly, the expression of *IRT1* and *HMA4* was found to be effectively declined, while the expression of *MRP3* and *CAX2* was induced remarkably (Fig. 5A right and 5B).

2.6 Cd²⁺ influx was inhibited and Cd concentration was reduced by H₂S treatment

Ion balance inside the cell is closely related to plant adaptation to environmental stress. Therefore, we compared the Cd contents of leaves and roots in *Arabidopsis* seedlings with or without NaHS pretreatment under Cd stress. Fig. 5C showed that after the pretreatment of NaHS, Cd concentration decreased by 15% and 38.4% in roots and leaves, respectively, when compared with Cd-treated seedlings.

Cd²⁺-sensitive microprobes were used to detect the

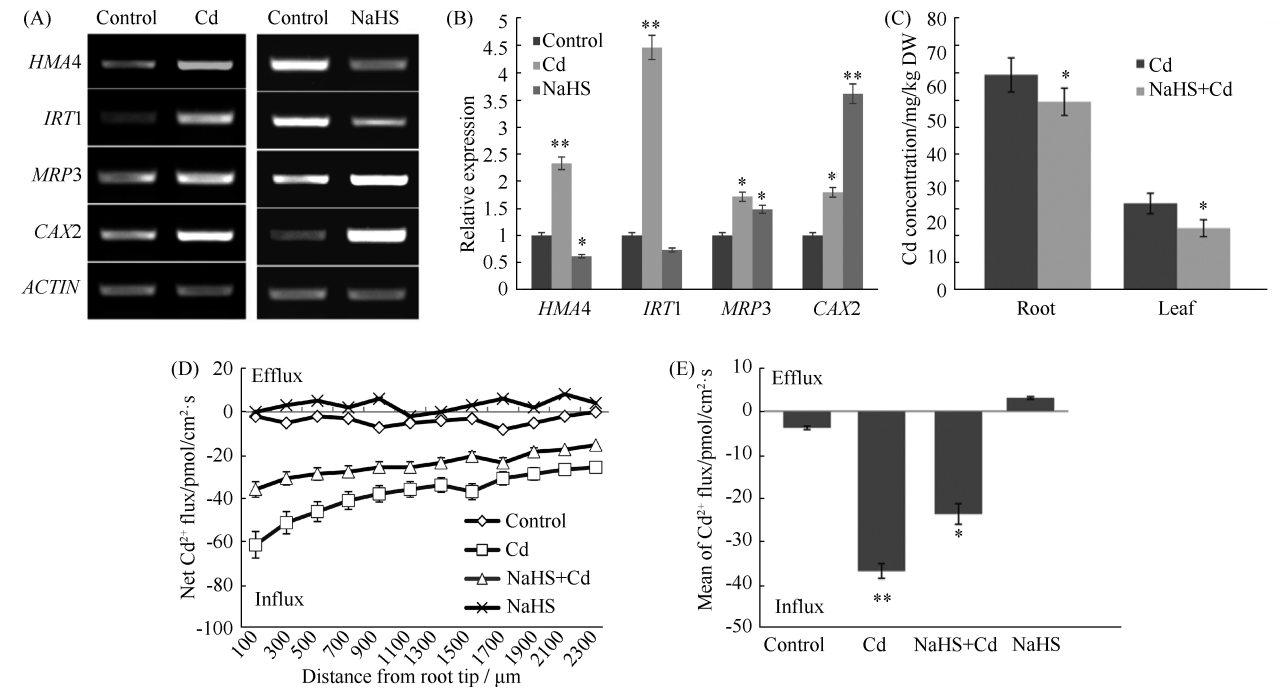


Fig.5 Effect of H₂S on Cd²⁺ transport in transcriptional level and physiological level (A) Expression levels of *HMA4*, *IRT1*, *MRP3* and *CAX2* in 4-week-old leaves were induced by Cd and NaHS treatment. (B) Digitization of *HMA4*, *IRT1*, *MRP3* and *CAX2* expressions by Image J (v2. 1.4. 7). (C) Accumulation of Cd in root and leaf tissue of 4-week-old *Arabidopsis* under different treatments, expressed in mg/kg dry weight. (D) Net Cd²⁺ fluxes in *Arabidopsis* roots were measured along root axis, 100-2300 μm from the apex, at intervals of 200-300 μm. Cd²⁺ fluxes were measured along the surface of pelleted hyphae over a recording period of 30 minutes. (E) Mean fluxes of Cd²⁺ within the measuring periods. Each point is the mean of 4-5 individual plants, and bars represent the standard error of the mean. Significant difference at *P* < 0.05 between treatments according to Tukey's multiple range test

Cd^{2+} response to NaHS treatment in roots. In the absence of Cd^{2+} , roots exhibited a stable Cd^{2+} efflux ($-9.8 \sim 0.5 \text{ pmol} \cdot \text{cm}^{-2} \text{ s}^{-1}$) along the root axis; NaHS treatment alone showed the same trend as control, with a little higher efflux ($-0.3 \sim 10.5 \text{ pmol} \cdot \text{cm}^{-2} \text{ s}^{-1}$) (Fig.5D). Both the mean of flux rate kept at the same level (Fig.5E). After exposure to CdCl_2 (200 $\mu\text{mol/L}$, 30 minutes), steady-state recordings on roots showed that the pattern of Cd^{2+} flux differed from control, with the decrease of influx along the root axis (Fig. 5D). The mean of the Cd^{2+} flow rates was negative during the measurement and increased 8.4-fold in response to Cd^{2+} treatment compared with control (Fig.5E). Similarly, with the pretreatment of NaHS, Cd^{2+} exposure still increased the net influx rate compared with control or NaHS treatment (Fig. 5D). But the mean value was significantly lower than that with Cd^{2+} treatment alone, which implied that H_2S effectively restricted influx of Ca^{2+} or induced net efflux (Fig.5E).

3 Discussion

Heavy metals are involved in the direct and indirect generation of ROS in the following ways: (1) direct transfer of electron in single electron reduction; (2) disturbance of metabolic pathways resulting in an increase in the rate of ROS formation; (3) inactivation and down regulation of the enzymes of the antioxidative defense system and (4) depletion of low molecular weight antioxidants^[30]. Under stress, high ROS could result in oxidative damage. Plants have evolved a complex antioxidant system to avoid the harmful effects of ROS. It is well known that Cd^{2+} causes oxidative stress in *Arabidopsis* plants. The ROS produced in leaf cells are removed by complex enzymes such as CAT, APX, glutathione peroxidases (GPX), SOD and GR of antioxidant systems^[16]. However, there is still limited information on the mechanisms by which Cd^{2+} produces oxidative damage. Our data showed that at concentrations below 200 $\mu\text{mol/L}$ CdCl_2 , SOD activity was elevated over the control, and we can assume that the plant antioxidant machinery was effectively struggling against the stress induced by CdCl_2 . However, the increase in MDA content and GSH depletion, together with the reduction in CAT and GR activities, provide evidence of oxidative damage at the cellular level. The high SOD and low CAT activities observed under Cd stress could have contributed to H_2O_2 accumulation. When plants were pretreated with NaHS prior to metal exposure, SOD activity was reduced compared with Cd^{2+} -treated plants, and GR and CAT activities returned to control levels. This result agrees with those of a previous report^[31].

In plants, GSH is the major antioxidant. It can detoxify ROS by direct scavenging or by acting as a

cofactor in the enzymatic reaction that involve APX and GR enzymes. In this study, GSH in *Arabidopsis* leaves was reduced by Cd, while H_2S was able to efficiently counteract GSH depletion. In fact, Cd^{2+} forms stable complexes with thiol groups such as GSH and phytochelatins^[32]. Considering that in NaHS pretreated plants, CAT activity and GSH content returned to control values, it suggested that H_2O_2 formation was controlled by H_2S .

The role of H_2S as a gasotransmitter in plants remains unclear, however, H_2S emission has already been observed in many species. H_2S synthase-like activity has been measured in various plant extracts. H_2S can be generated mainly from cysteine desulfhydrases according to available references. Published data do not suggest a causal relationship between heavy metal toxicity and endogenous H_2S content in plants. In this study, we analyzed the expression of *CDes* in *Arabidopsis* treated with different concentrations of Cd. The results showed that the expression of *CDes* was increased by Cd stress. The rate of H_2S production was gradually increased with increasing CdCl_2 concentration from 0 to 300 $\mu\text{mol/L}$. This demonstrated the close relationship between H_2S and Cd stress. However, the effects of other heavy metals on endogenous H_2S content in different plant species and tissues will be a focus of continuous research and controversy. Because of the toxic characteristics of H_2S at high concentrations, it is very important to grasp its physiological concentrations in vivo. More recently, it was reported that the H_2S content in different plants in vivo was maintained at a stable level, from 0.177 to 0.708 $\mu\text{mol g}^{-1} \text{ FW}$ ^[33]. Additionally, NaHS was usually used as a donor of H_2S to treat experimental materials, which is toxic in *Vicia faba* guard cells at concentrations $\geq 500 \mu\text{mol/L}$, and cell viability was similar to control at concentrations $\leq 100 \mu\text{mol/L}$ ^[34]. Therefore, a preliminary experiment was conducted in this concentration range. Our data clearly showed that plants treated with 50 $\mu\text{mol/L}$ NaHS survived for a longer period. Similarly, several reports showed that around 50 $\mu\text{mol/L}$ of H_2S pretreatment or fumigation reduce Cd content in CdCl_2 -stressed *Arabidopsis* and foxtail millet seedlings^[24-27].

The expression levels of the H_2S synthesis-related genes *LCD* and *DCD1/2* were up-regulated significantly under Cd^{2+} stress (Fig.2 A). At the same time, H_2S production rate was significantly stimulated in a dose-dependent manner not only based on L-cysteine but also D-cysteine (Fig.2B). These results suggested that the H_2S production system was triggered by Cd^{2+} in order to response to the Cd^{2+} stress. The expression levels of Cd^{2+} transport-related genes were regulated by Cd^{2+} or H_2S by the opposite trends (Fig.5A), showing that compartmentalization and efflux of Cd^{2+} in plant

cells might be the mechanism of H_2S in heavy metal resistance. It is necessary to verify further using the related genetic mutants in the future.

Root is the primary organ that plants deploy to accumulate most of the heavy metals to which they are exposed. In this study, we used NaHS as the donor of H_2S to pretreat the seedlings before Cd exposure. Then, we intensively researched the special role of H_2S on Cd^{2+} flux in primary roots through NMT, whose data show the real-time Cd^{2+} flux changes *in vivo*. Our results indicated that Cd^{2+} can rapidly accumulate in *Arabidopsis* roots especially through root hair and inhibit the growth and dry matter accumulation (Fig.1, 5C and 5D), suggesting that Cd^{2+} is easily absorbed and highly toxic. As expected, the Cd^{2+} influx was blocked in the presence of NaHS, indicating that the Cd^{2+} influx was significantly inhibited by H_2S . This is the direct evidence of H_2S participating in Cd^{2+} resistance. These findings agree with the expression of heavy metal related transporters (Fig. 5A) and Cd content in leaves and roots (Fig.5B). Similarly, it was reported that ABA pretreatment reduces Cd^{2+} content in CdCl_2 -stressed rice seedlings and popular cells^[35]. H_2S interacts with abscisic acid to regulate stomatal movement in response to drought stress^[36]. Interaction between H_2S and with salicylic acid responding to Cd stress was revealed previously^[24]. All these findings implied that there are inextricable links between H_2S and plant hormones in performing its biological functions.

Additionally, H_2S works through an underlying mechanism called sulfhydration in mammals^[37] and in higher plants^[38]. H_2S might be directly acting on protein cysteine residues that contain S-H bonds, converting them into S-S-H, reversibly regulating the functions of plant proteins. Therefore, whether the change in the transporter activity is due to the posttranslational modification of H_2S needs to be determined.

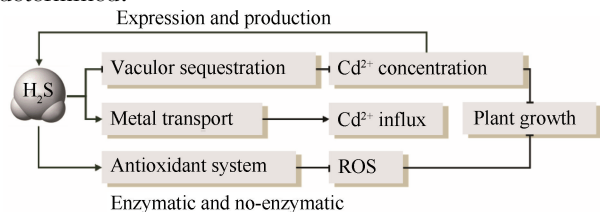


Fig.6 A schematic model of H_2S alleviation of Cd^{2+} toxicity in *Arabidopsis* Arrows indicate a promotion effect, and blunts indicate a repression effect.

Based on the data described above, a signal pathway model was developed and depicted in Fig.6. Cd stress triggered the production of endogenous H_2S in plants not only in transcriptional level but also in

physiological level. Pretreated by physiological concentration of H_2S before Cd exposure, could prevent an increase in the contents of H_2O_2 and MDA, by regulating antioxidant enzyme activities (such as CAT, SOD and GR) and GSH content. H_2S also seems to be involved in the expression of Cd^{2+} -transporter genes during accumulation. Inhibiting Cd^{2+} uptake and enhancing Cd^{2+} compartmentalization are the main defense strategies that H_2S use to prevent Cd toxicity in plant cells.

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