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An emphasis of hydrogen sulfide-cysteine cycle on enhancing the tolerance to chromium stress in *Arabidopsis*☆

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ABSTRACT

Increasing attention has been focused on the health of vegetables and grains grown in the contaminated agricultural soil, it is thus meaningful to find ways to reduce the heavy metals (HMs) accumulation in plants. As sulfur is considered to be an essential macronutrient for plant stress defenses, the important role of sulfur assimilation in plants responding to HMs stress has been followed. However, the potential mechanism of the only sulfur-containing gasotransmitter hydrogen sulfide (H_2S) and its main endogenously generated substrate, cysteine (Cys), in plant defense is poorly understood. The physiological and biochemical methods together with qRT-PCR were used to explore the response pattern of H_2S-Cys cycle in plants resisting to chromium (Cr^{6+}) stress. Our results suggested that Cr^{6+} stress inhibited *Arabidopsis* root elongation, increased the H_2S and Cys contents time-dependently, and H_2S production was activated earlier than Cys. Furthermore, H_2S increased Cys accumulation more quickly than Cr^{6+} stress. The qRT-PCR results revealed that H_2S up-regulated the Cys generation-related genes *OASTLa*, *SAT1* and *SAT5* expression levels, and that *SAT1* and *SAT5* expression was elevated for a longer duration. Data suggested that H_2S might regulate Cys metabolism-related genes expression to participate in Cr^{6+}-mediated Cys accumulation. H_2S and Cys relieved the root elongation inhibition caused by Cr^{6+} in *Arabidopsis*. Both H_2S and Cys enhanced glutathione generation and activated phytochelatins (PCs) synthesis by up-regulating *PCS1* and *PCS2* expression levels to fight against Cr^{6+} stress. Besides regulating the expression of PCs synthase encoding genes, H_2S might promote metallothioneins accumulation by significantly increasing the *MT2A* gene expression. Overall, H_2S and H_2S-induced Cys accumulation (H_2S-Cys system) was critical in imparting Cr^{6+} tolerance in *Arabidopsis*. This paper is the first to indicate that gasotransmitter H_2S induced Cys accumulation in *Arabidopsis* Cr^{6+}-stress defense and provides evidence for more extensive studies of the H_2S signaling pathway.

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1. Introduction

Increasing anthropogenic and industrial activities have caused excessive emissions of toxic metals into the environment, which undoubtedly lead to soil contamination (Nriagu and Pacyna, 1988). Chromium (Cr) is the second most abundant inorganic contaminant in agricultural soil, hexavalent chromium (Cr^{6+}) and trivalent chromium (Cr^{3+}) species are the most stable species of chromium (Cr) to occur in the environment (Zhao et al., 2016). Because of its mutagenic and carcinogenic properties, the Cr^{6+} is a serious threat to organisms grown in soil. The contamination of agricultural soil has attracted critical concerns due to the potential adverse ecological effects (Seth et al., 2012). It is thus important to explore the mechanisms contributing to plants stress defense and find ways to reduce the heavy metals (HMs) accumulation in grains.

Excessive HMs adversely affect the growth and development of plants (Jonak et al., 2004). Generally, the overproduction of reactive oxygen species (ROS) is the primary response of plants to HMs. Lipid peroxidation is the most deleterious influence caused by

Abbreviations: ABRC, Arabidopsis Biological Resource Center; AsA, Ascorbic acid; CASC, β-cyanoalanine synthase; CDes, Cys desulfhydrases; Cr, Chromium; Cys, Cysteine; DES, desulfhydrase; GSH, Glutathione; HMs, Heavy metals; H_2S, Hydrogen sulfide; LCD, L-Cys desulfhydrase; MDA, Malondialdehyde; MTs, Metallothioneins; ½ MS, ½ Murashige-Skoog (medium); OAS, O-acetylserine; OASTL, O-acetyl-L-serine (thiol) lyase; PCs, Phytochelatins; PCS, Phytochelatins synthase; qRT-PCR, quantitative real-time PCR; ROS, Reactive oxygen species; SAT, Serine acetyltransferase; SCS, S-sulfocysteine synthase; UBQ, Ubiquitin.

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HMs-induced ROS (Mithofer et al., 2004), and malondialdehyde (MDA), one of the decomposition products of lipid peroxidation, is considered to be an indicator of oxidative damage (Stohs and Bagchi, 1995). Unlike animals, higher plants are sessile and cannot escape from some stimuli, so they have developed strategies for stress avoidance (Xiong et al., 2002), such as activating the antioxidant glutathione (GSH) (Freeman et al., 2004, 2005; Semane et al., 2007) as well as the HMs chelators, phytochelatins (PCs) (Salt and Rauser, 1995; Vatamaniuk et al., 2004) and metallothioneins (MTs) (Hall, 2002; Cobbett and Goldsbrough, 2002).

Sulfur, an essential macronutrient in plants, acts as the functional component of various biochemical compounds, such as cysteine (Cys), GSH, PCs, MTs and hydrogen sulfide (H_2S), all of which play positive roles in plants HMs defense responses (Droux, 2004; Alvarez et al., 2010). H_2S, due to its unpleasant flavor, was previously widely regarded to be a toxic gas (see Lisjak et al., 2013; Jin and Pei, 2015). This changed when H_2S was reported to act as an endogenous neuromodulator in the brain (Abe and Kimura, 1996). Hereafter, H_2S was reported to be the only sulfur-containing gasotransmitter (Wang, 2002, 2012), and its central role in the physiological regulation and disease responses of mammals has been continuously implicated (Yang et al., 2008; Wang, 2012). Reports in plants indicate that the H_2S, with physiological concentration, is not only a crucial player in regulating plants growth and development (Zhang et al., 2008, 2009; Li et al., 2012a, 2012b), including root morphogenesis (Zhang et al., 2009) and flower senescence (Zhang et al., 2011), but is also a critical mediator in plant defense responses and tolerance acquisition (Zhang et al., 2010; Dawood et al., 2012; Li et al., 2012c, 2013; Shen et al., 2013; Shi et al., 2013, 2014; Fang et al., 2014; Cui et al., 2014).

In plants, H_2S can be generated endogenously through enzymatic pathways. The Cys desulfhydrases (CDes) occupy an irreplaceable position in H_2S generation. L-Cys desulfhydrase (LCD) is the most unambiguous CDes in *Arabidopsis*, which mediates L-Cys degradation into H_2S, ammonia and pyruvate (Romero et al., 2013; Jin and Pei, 2015). Interestingly, a novel enzyme was discovered and named DES1, which should be classified into O-acetyl-L-serine (thiol) lyase (OASTL) based on its sequence characteristics (Alvarez et al., 2010). However, the functional analysis revealed that DES1 had a higher affinity to L-Cys and degrades it to generate H_2S (Alvarez et al., 2010; Romero et al., 2013).

Besides being the main substrate for endogenous H_2S production, Cys is the first organosulfur compound of sulfur assimilation in plants and the major donor of reduced sulfur for organic sulfur compounds (Takahashi et al., 2011). Generally, the inorganic sulfate is taken up by plants, and then reduced and assimilated into Cys. Serine acetyltransferase (SAT) and OASTL are indispensable in this process (Harrington and Smith, 1980; Wirtz et al., 2004). SAT physically interacts with OASTL to form the Cys synthase complex, which controls the biosynthesis of Cys appropriately. Firstly, the SAT catalyzes the transfer of acetyl from acetyl-CoA to serine to form the intermediate O-acetylserine (OAS), and the OASTL then catalyzes Cys generation by incorporating the sulfide into OAS (Bonner et al., 2005; Heeg et al., 2008). There are two additional important enzymes in Cys metabolism, CASC1, a β-cyanoalanine synthase, which catalyzes the conversion of Cys and cyanide to H_2S and β-cyanoalanine, and SCS, a S-sulfocysteine synthase, which catalyzes the incorporation of thiosulfate to OAS to form S-sulfocysteine. All of these enzymes work together to maintain the Cys equilibrium (Gotor et al., 2014).

Additionally, Cys acts as a functional precursor for numerous essential biomolecules (Noctor et al., 2012), such as GSH and PCs, both of which play important roles in the acquisition of HMs tolerance in plants. GSH, a sulfur and thiol containing tri-peptide, synthesized by γ-glutamylcysteine synthetase and glutathione synthetase (Wachter and Rausch, 2005; Seth et al., 2012), is an important defender in the organisms fighting against ROS, and it has been reported to eliminate ROS by its own oxidation to glutathione disulfide in a redox signaling pathway. Moreover, GSH regenerates the reduced ascorbic acid (AsA) through the GSH-AsA cycle signaling pathway, which maintains a higher reduced AsA state. Both reduced GSH and AsA act as key regulators of antioxidant defenses (Anjum et al., 2012; Fang et al., 2014). Moreover, GSH is a substrate for PCs synthesis, which is catalyzed by phytochelatins synthase (PCS). As a set of novel HMs-binding peptides, PCs carry toxic HMs to insensitive regions mediated by compartmentalization. Furthermore, the crucial role of PCs in HMs detoxification has been indicated in numerous studies (Cobbett and Goldsbrough, 2002; Seth et al., 2012; Fang et al., 2014).

In the present study, we used physiological and biochemical methods to explore the response mode of the H_2S-Cys system in *Arabidopsis* that responds to chromium (Cr^{6+}) stress. This study proposes a signaling pathway for the gasotransmitter H_2S protecting *Arabidopsis* against Cr^{6+} stress, and it provides some evidence for understanding the mechanism of plant-stress defenses.

2. Materials and methods

2.1. Plant materials and treatments

Arabidopsis thaliana ecotype Col-0 (wild-type, Wt), the *LCD* defective mutant *lcd* (SALK_082099) and the *DES1* defective mutant *des1* (SALK_205358C) were obtained from the Arabidopsis Biological Resource Center (ABRC). The *LCD* over-expression mutant OE-*LCD* (the transgenic line of 35S:*LCD*) and the *DES1* over-expression mutant OE-*DES1* (the transgenic line of 35S:*DES1*) were generated as described previously (Qiao et al., 2015). These seeds were sterilized with 75% ethanol for 50 s and 6% sodium hypochlorite for 8 min under sterile conditions. After rinsed with sterile water three times, the seeds were sown on ½ Murashige-Skoog (½ MS) medium, and then the Petri dishes were sealed with parafilm. These Petri dishes were cultivated under a 16 h/8 h (light/dark) photoperiod with a light illumination of 160 $Em^{-2}s^{-1}$ at 23 °C and 60% relative humidity.

For selecting the Cr^{6+} concentration of stress exposure, ten-day-old *Arabidopsis* seedlings were transferred aseptically to Cr^{6+} containing (0, 100, 200, 300, 400 and 500 μmol/L Cr^{6+}) ½ MS medium.

For the H_2S fumigation pretreatment, the one-week-old seedlings were successively fumigated with H_2S released by NaHS. The NaHS solution-containing tube was placed in the Petri dish mentioned above and the H_2S fumigation concentration was 50 μmol/L. For Cys pretreatment, the Cys was added directly to the ½ MS medium, and the concentration of Cys is 1 mmol/L. After pretreated for 3 d, these seedlings were transferred aseptically to the stress condition of the 300 μmol/L Cr^{6+}-containing (150 μmol/L $K_2Cr_2O_7$) ½MS medium.

2.2. MDA and GSH content assays

The MDA content was determined by the thiobarbituric acid reaction based on published methods (Qiao et al., 2015). The GSH content was measured based on a previously described method (Fang et al., 2014).

2.3. Measurement of the endogenous H_2S content

To determine the regulation of Cr^{6+} stress on endogenous H_2S generation, the content of endogenous H_2S was measured according to previously described methods (Qiao et al., 2015).

2.4. Measurement of the endogenous Cys content

Cys can react specifically with acid ninhydrin to form a pink product, which has a maximum absorbance at 560 nm. The reaction is highly sensitive for Cys determination. Thus, the Cys content was determined according to this method with some modifications (Gaitonde, 1967).

2.5. Extraction of total RNA and qRT-PCR

The total RNA was extracted using TRizol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, an oligo(dT) primer was used to synthesize complementary DNA (cDNA), and quantitative real-time PCR (qRT-PCR) was performed according to previously published methods (Shen et al., 2013). All of the molecular techniques were carried out according to standard methods. The gene *UBQUITIN4* (*UBQ4*, At5g20620) was used as the internal control. The primers used for qRT-PCR are listed in Table 1. Each experiment was repeated independently with three biological replicates.

2.6. Statistical analysis

Each experiment was carried out with three biological replicates. The results were expressed as the means ± SE. The data were analyzed using SPSS (version 17, IBM SPSS, Chicago, IL, USA), and error bars were calculated based on Tukey's multiple range test ($P < 0.05$).

3. Results

3.1. The roots elongation was suppressed by Cr^{6+} stress in Arabidopsis

Ten-day-old *Arabidopsis* seedlings were transferred aseptically to Cr^{6+}-containing ½ MS medium, and the lengths of the primary roots were measured 5 d later. Cr^{6+} stress led to toxic symptoms and repressed the elongation of *Arabidopsis* roots in a concentration-dependent manner. The root elongation was significantly inhibited ($P < 0.05$) in 300 μmol/L Cr^{6+} stressed plants, which exhibited approximately 50% inhibition (Fig. 1). Thus, the 300 μmol/L Cr^{6+} was chosen in further stress-based tests.

Table 1
List of all genes for qRT-PCR in the manuscript.

Gene	Accession number	Primer pairs
UBQ4	At5g20620	5′ GGGCACTCAAGTATCTTGTTAGC 3′ 5′ TGCTGCCCAACATCAGGTT 3′
OASTLa	At4g14880	5′ TATTCCCACAAGAAGACC 3′ 5′ GCCAGTTGAAAGTGCTAT 3′
OASTLb	At2g43750	5′ AGCACTTTCCGTGGGTTC 3′ 5′ GAGACGACTGGTCCTGAG 3′
OASTLc	At3g59760	5′ AAACGCAGGTTATTGGTG 3′ 5′ TTGCTTTGCGGTTTCTAT 3′
SAT1	At1g55920	5′ ACCACCACCGACCCTGAT 3′ 5′ GTGACCTTGGGAGGAACA 3′
SAT5	AT5G56760.1	5′ AAGATTGGTGCAGGTGCTA 3′ 5′ TCCGAGATGAATGAAGTATG 3′
CASC1	At3g61440	5′ GCCACCGTTGAGTATGTT 3′ 5′ CCTGAGATTTGGGAAGAT 3′
SCS	At3g03630	5′ GCTGACTGCTGCTACTGC 3′ 5′ TTTCTGGAGACAACCCTG 3′
MT3	AT3G15353	5′ ATGTCAAGCAACTGCGGAAG 3′ 5′ TTAGTTGGGGCAGCAAGTGCA3′
MT2A	AT3G09390	5′ ATGTCTTGCTGTGGAGGAAAC 3′ 5′ TCACTTGCAGGTGCAAGGATC 3′
PCS1	AT5G44070.1	5′ TGGAGTTGTGGTGCGTGAT 3′ 5′ GAAGCAAAGTTGGGAGGGA3′
PCS2	AT1G03980	5′ CTTCTGTTGGCTTTACCTC 3′ 5′ GTTGTGTTTGATTAGGCAGG 3′

3.2. The H_2S-Cys system was activated to respond to Cr^{6+} stress in Arabidopsis

3.2.1. H_2S was elevated by Cr^{6+} stress and acted as a trigger in the subsequent Cys accumulation

To explore the response pattern of the H_2S-Cys system to Cr^{6+} stress, the time-course analyses of endogenous H_2S and Cys contents were determined subsequently. As shown in Fig. 2a, both H_2S and Cys were raised by varying degrees with the extension of the Cr^{6+}-stress period. Interestingly, the increase of Cys lagged behind that of H_2S, but was maintained at a high level for longer period than H_2S. The Cys accumulation was triggered after 6 h of Cr^{6+} stress and had a longer duration, while H_2S exhibited a substantial increase after 3 h of Cr^{6+} exposure, followed by a 3 h persistent peak from 6 h to 9 h (Fig. 2a). Further data suggested that H_2S fumigation boosted the Cys content immediately, and the Cys content reached its peak after 3 h of H_2S treatment. Importantly, the Cys activation mediated by H_2S fumigation was much earlier than that mediated by Cr^{6+} stress (Fig. 2b). These results suggested that H_2S increased during Cr^{6+} stress and that this rise might play an important role in subsequent Cys accumulation.

3.2.2. Cys metabolic enzyme-encoding genes expression levels were regulated by the gasotransmitter H_2S in Arabidopsis

To investigate the effects of H_2S on Cys generation, the expression patterns of genes responsible for Cys metabolism during H_2S fumigation were analyzed. H_2S significantly up-regulated *OASTLa*, *SAT1* and *SAT5* expression, *SAT1* and *SAT5* were induced for a longer duration, while the expression of *OASTLb*, *OASTLc*, *CASC1* and *SCS* were barely affected by H_2S fumigation (Fig. 2c).

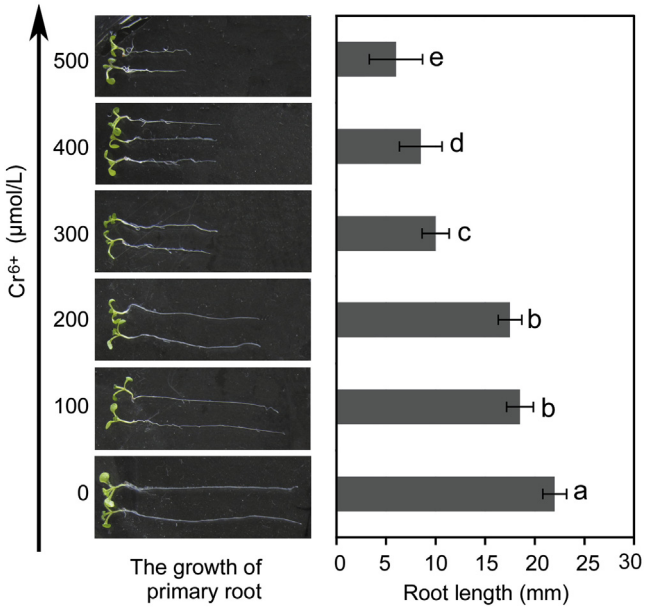

Fig. 1. The negative effects of Cr^{6+} stress on *Arabidopsis* seedlings. (a) Phenotype of root growth in *Arabidopsis* seedling stressed by Cr^{6+}. (b) The root lengths of seedlings stressed by Cr^{6+}. Ten-day-old *Arabidopsis* seedlings were transferred aseptically to Cr^{6+} containing ½ MS medium (0, 100, 200, 300, 400 and 500 μmol/L Cr^{6+}). After 5 d of stress exposure, the growth phenotype of the seedlings and the lengths of the primary roots were recorded. Data are means ± SE of three biological repeats, error bars indicate error standard and bars with different letters are different ($P < 0.05$).

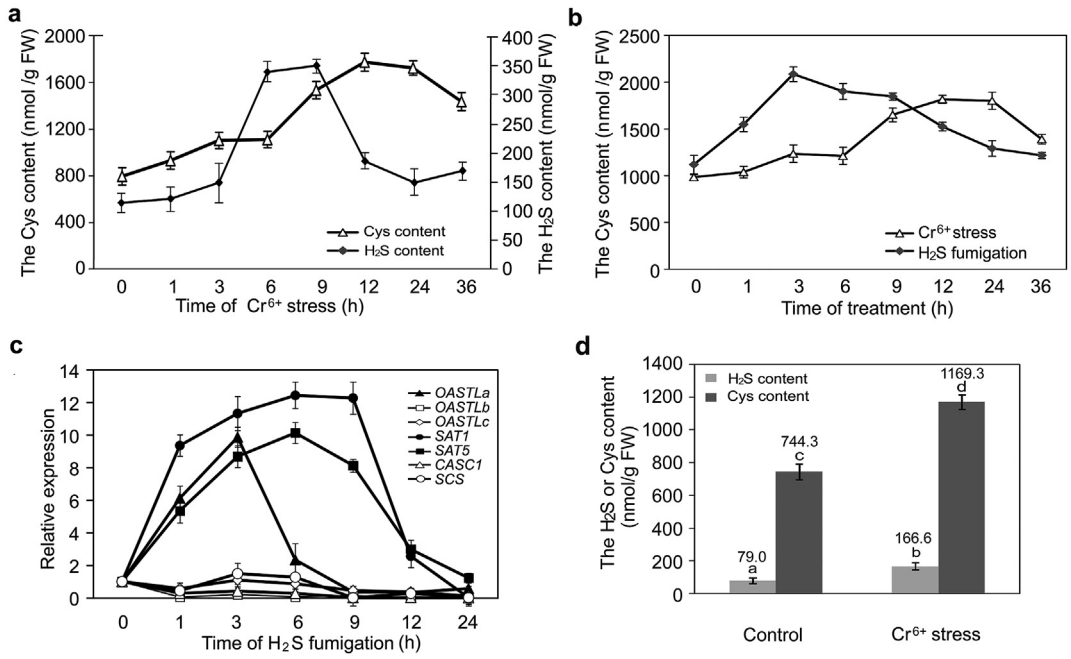

Fig. 2. The response pattern of the H_2S-Cys system in *Arabidopsis* to Cr^{6+} stress. (a) The effects of Cr^{6+} stress on the H_2S and Cys contents. (b) The regulation of the Cys content induced by H_2S fumigation and Cr^{6+} stress. (c) The expression patterns of Cys metabolism-related genes in seedlings fumigated with H_2S. Ten-day-old seedlings exposed to different treatments were used to conduct the time-course analyses of the H_2S and Cys contents, and the expression levels of Cys metabolism-related genes. (d) The contents of H_2S and Cys in Cr^{6+}-stressed *Arabidopsis*. Ten-day-old seedlings were stressed with or without 300 μmol/L Cr^{6+} (150 μmol/L $K_2Cr_2O_7$). After 5 d of stress exposure, the H_2S and Cys contents were detected. Data are means ± SE of three biological repeats, error bars indicate error standard and bars with different letters are different ($P < 0.05$).

3.2.3. The correlation analysis of H_2S-generation-induced Cys depletion and the Cr^{6+}-induced Cys accumulation

As mentioned above, both H_2S and Cys can be activated by Cr^{6+}-stress and H_2S seemed to be an important mediator in Cr^{6+}-induced Cys increase. Since Cys is the main substrate for H_2S generation, the process of H_2S-generation-caused Cys depletion and the H_2S mediated Cys accumulation in Cr^{6+} stressed *Arabidopsis* was explored. As shown in Fig. 2d, during Cr^{6+} stress, the content of H_2S increased from 79.0 nmol/g to 166.6 nmol/g and the content of Cys increased from 744.3 nmol/g to 1169.3 nmol/g (Fig. 2d). Accordingly, just a few Cys was consumed by Cr^{6+} stress-induced H_2S generation, and this H_2S-generation-caused Cys depletion did not affect the Cr^{6+} stress-mediated Cys increase.

3.3. The H_2S-Cys system plays a vital role in Arabidopsis resisting to Cr^{6+} stress

3.3.1. Cys enhanced Arabidopsis tolerance to Cr^{6+} stress

Cys-pretreated seedlings exhibited obvious mitigating symptoms and Cys primed the plants' tolerance to Cr^{6+} stress. Cys alleviated the Cr^{6+} stress-induced inhibition of roots elongation and lipid peroxidation dose-dependently (Fig. 3a and b). The most significant positive effects were found in plants pretreated with 1 mmol/L Cys, which even restored the inhibited roots to the control level, and these plants showed minimal lipid peroxidation (Fig. 3a and b). Moreover, Cys caused a substantial increase in the GSH content during Cr^{6+} stress (Fig. 3c). This increase occupied a critical position in plants defense against oxidative damage. The 1 mmol/L Cys treatment was strong enough to mediate the acquisition of Cr^{6+} tolerance in *Arabidopsis*; therefore, 1 mmol/L Cys was used in the subsequent experiment.

3.3.2. Both H_2S and Cys eased Cr^{6+} stress-induced inhibition of root elongation in Arabidopsis

One-week-old seedlings, pretreated with 50 μmol/L H_2S or 1 mmol/L Cys for 3 d, were transferred to ½ MS medium containing 300 μmol/L Cr^{6+} for the stress exposure. Simultaneously, these Petri dishes were placed upside down for 5 d causing new growth to bend. The lengths of the "hooks" were subsequently observed in the root tip bending experiment. The Cr^{6+} stress strongly inhibited the roots bending growth, and H_2S-generation defective mutants *lcd* and *des1* exhibited more sensitivity to this stress (Fig. 3d). The inhibitory effect of Cr^{6+} on the roots growth was mitigated by a H_2S or Cys pretreatment. Moreover, H_2S mitigated the Cr^{6+}-induced root growth inhibition in Wt, *lcd* and *des1* mutants. Similarly, Cys also exhibited this protective effect and almost alleviated the inhibition of mutants root growth to the same level as Wt during Cr^{6+} stress, which might indicate that the H_2S decrease did not impede the positive effects of Cys. The H_2S plus Cys combined pretreatment correspondingly showed twice the protective effects (Fig. 3d).

3.3.3. The H_2S-Cys system alleviated Cr^{6+} stress-induced oxidative damage in Arabidopsis by GSH synthesis regulation

As shown in Fig. 4a, Cr^{6+} stress caused lipid peroxidation and MDA accumulation, while both H_2S and Cys pretreatments relieved the Cr^{6+} stress-induced MDA accumulation by varying degrees. The H_2S plus Cys combined pretreatment showed even more pronounced positive effects. Further results demonstrated that Cr^{6+} stress, H_2S fumigation or the Cys treatment substantively activated GSH production. Additionally, the GSH content in H_2S, Cys and H_2S + Cys pretreated plants that were subsequently exposed to Cr^{6+} stress was much higher than in Cr^{6+} stressed plants that were not pretreated. H_2S and Cys caused the Cr^{6+}-induced GSH increase to be more significant (Fig. 4a).

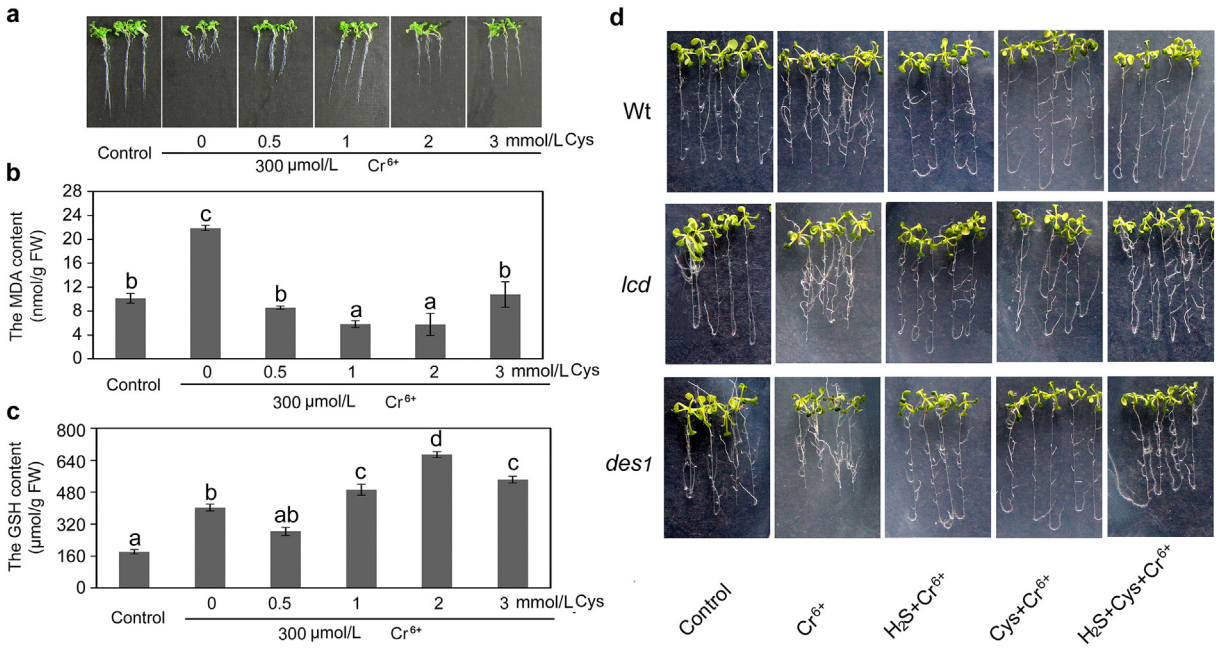

Fig. 3. The positive effects of H_2S and Cys on *Arabidopsis* seedlings stressed with Cr^{6+}. (a) The mitigating effects of Cys on the Cr^{6+}-induced inhibition of root elongation, (b)&(c) The effects of Cys on the Cr^{6+}-induced MDA and GSH contents. The one-week-old seedlings were pretreated with Cys (0, 0.5, 1, 2, and 3 mmol/L) for 3 d, then these seedlings were transferred to ½ MS medium containing 300 μmol/L Cr^{6+} (150 μmol/L $K_2Cr_2O_7$) for the stress exposure. After 5 d of stress exposure, the growth phenotype, as well as the contents of MDA and GSH, were recorded. Data are means ± SE of three biological repeats, error bars indicate error standard and bars with different letters are significantly different ($P < 0.05$). (d) The protective effects of H_2S and Cys on Cr^{6+}-induced inhibition of root elongation in Wt and mutants. The one-week-old seedlings were pretreated with H_2S, Cys or H_2S + Cys for 3 d, then transferred to ½ MS medium containing 300 μmol/L Cr^{6+} (150 μmol/L $K_2Cr_2O_7$) for the stress exposure. After 5 d of growing upside down, the lengths of the "hooks" were subsequently observed.

3.3.4. The H_2S-Cys system enhanced Arabidopsis tolerance to Cr^{6+} stress by regulating HMs chelators synthesis

As mentioned above, both H_2S and Cys played important roles in *Arabidopsis* response to Cr^{6+} stress. During Cr^{6+} stress, the HMs chelators synthase-encoding genes *MT2A*, *PCS1* and *PCS2* expression were significantly up-regulated in the *LCD* and *DES1* over-expression mutants, OE-*LCD* and OE-*DES1*, compared with in Wt, while their expression have no obvious differences between Wt and the H_2S-generation-defective mutants, *lcd* and *des1*. However, the *MT3* expression has no obvious change in both over-expression (OE-*LCD* and OE-*DES1*) and H_2S-generation-defective mutants (*lcd* and *des1*) (Fig. 4b).

The results suggested that Cr^{6+}-stressed plants activated HMs chelators synthesis by up-regulating *PCS1*, *PCS2*, *MT3* and *MT2A* expression levels (Fig. 4c). Interestingly, H_2S, Cys or H_2S + Cys pretreatments strengthened the Cr^{6+} stress-mediated up-regulation of *PCS1* and *PCS2* expression levels, while the increased *MT3* expression mediated by Cr^{6+} stress was weakened by H_2S, Cys or H_2S + Cys pretreatments. The increased *MT2A* expression mediated by Cr^{6+} stress was reinforced by the H_2S pretreatment but reduced by Cys or H_2S + Cys pretreatments (Fig. 4c).

4. Discussion

The critical role of sulfur metabolism in plants HMs-induced stress responses has been emphasized (Takahashi, 2010), and the superiority of the exclusive sulfur-containing gasotransmitter H_2S in protecting plants and suppressing HMs uptake and accumulation in plants has been reported, but the knowledge of the underlying mechanism still needs more researches to reveal (Zhang et al., 2010; Dawood et al., 2012; Li et al., 2012c, 2013; Shen et al., 2013; Shi et al., 2013, 2014; Fang et al., 2014; Cui et al., 2014). Notably, the significant organosulfur compound Cys is not only the precursor of some resistant molecules but is also the main substrate for endogenous H_2S generation in plants (Papenbrock et al., 2007; Takahashi et al., 2011). The response pattern of the H_2S-Cys system, as well as the crosstalk between H_2S and Cys in plant defenses, has rarely been studied.

Both H_2S and Cys contents increased significantly in Cr^{6+}-stressed *Arabidopsis*, which led us to determine the amount of Cys consumed by generating H_2S. In other words, the process of H_2S-generation-induced Cys depletion and the Cr^{6+}-induced Cys accumulation is a worthwhile avenue of investigation. As previously reported, Cys releases H_2S based on a one to one stoichiometric ratio (Harrington and Smith, 1980; Jin and Pei, 2015). Data in this study indicated that the Cr^{6+}-induced H_2S-generation consumed 87.6 nmol/g Cys, which only accounts for 7.5% of the Cys in Cr^{6+}-stressed plants (1169.3 nmol/g) (Fig. 2d). Additionally, the Cys consumed by H_2S generation does not affect the Cr^{6+} stress-mediated Cys increase.

Moreover, H_2S fumigation activated the Cys increase more quickly than Cr^{6+} stress (Fig. 2b). Thus, Cr^{6+} stress stimulated the H_2S generation, and afterwards H_2S acted as a reactive gasotransmitter to promote further Cys accumulation. Undoubtedly, Cys metabolism can be regulated by H_2S fumigation. The expression levels of the Cys synthesis-related genes *OASTLa*, *SAT1* and *SAT5* were up-regulated significantly by H_2S treatment, and both *SAT1* and *SAT5* exhibited a longer duration of high expression (Fig. 2c). SAT transfers the acetyl moiety from acetyl-CoA to serine producing OAS, and then OAS-TL combines a sulfide with OAS to form Cys (Bonner et al., 2005; Heeg et al., 2008). H_2S might strengthen the synthesis of the intermediate OAS mainly by inducing the high expression levels of *SAT1* and *SAT5*, promoting a Cys increase.

Surprisingly, *MT2A*, *PCS1* and *PCS2* expression levels were significantly up-regulated in Cr^{6+}-stressed OE-*LCD* and OE-*DES1* mutants, but were hardly changed in Cr^{6+}-stressed *lcd* and *des1*

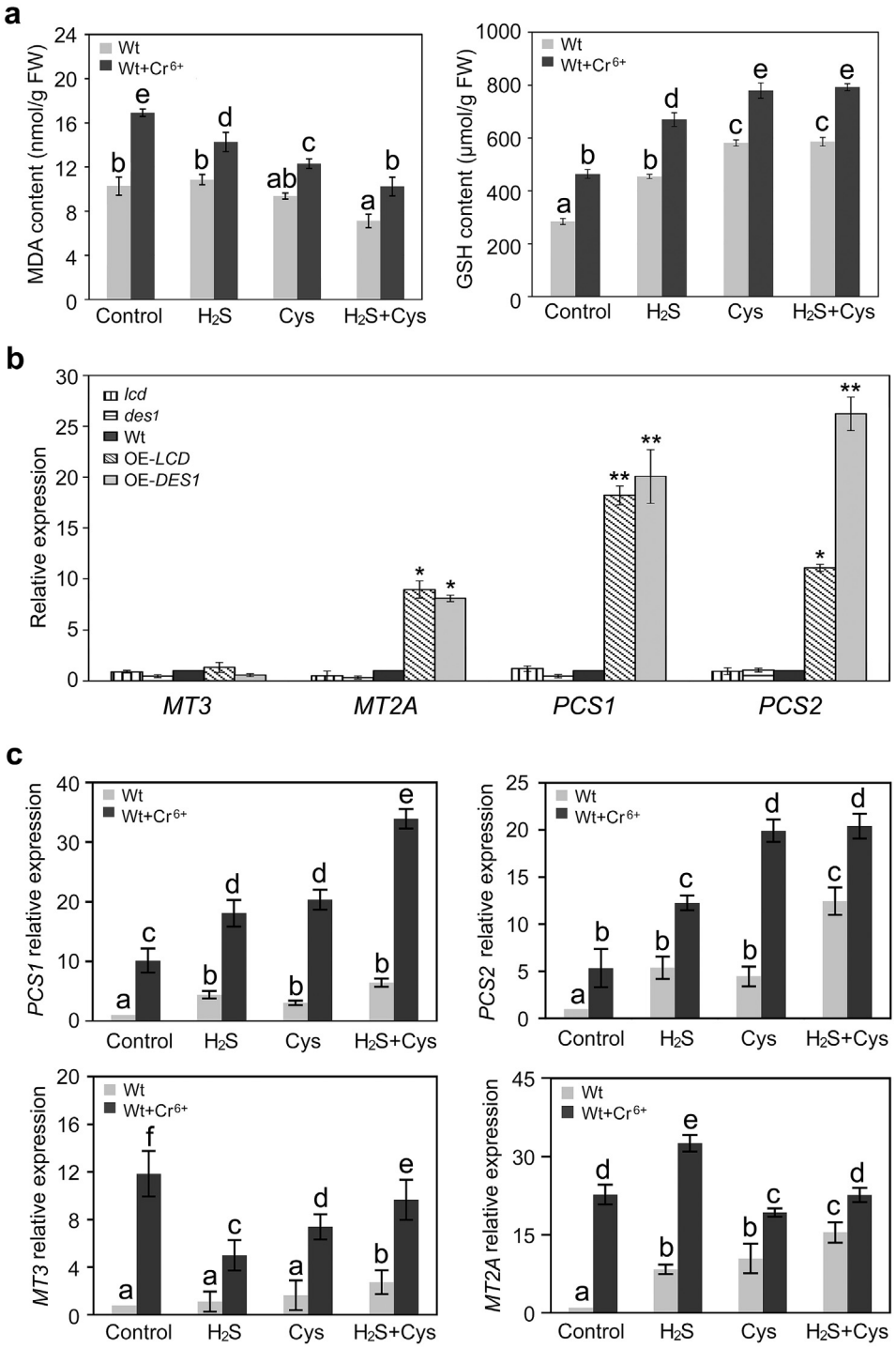

Fig. 4. The regulation of H_2S and Cys on the antioxidant GSH as well as the chelators PCs and MTs during Cr^{6+} stress in *Arabidopsis* seedlings. (a) The effects of H_2S and Cys on the MDA and GSH contents in seedlings with or without Cr^{6+} stress. The one-week-old seedlings were pretreated with H_2S, Cys or H_2S + Cys for 3 d, then transferred to ½ MS medium containing 300 μmol/L Cr^{6+} (150 μmol/L $K_2Cr_2O_7$) for 5 d of stress, and the MDA and GSH contents were measured. (b) The expression levels of *MT3*, *MT2A*, *PCS1* and *PCS2* genes in Wt, OE-*LCD*, OE-*DES1*, *lcd* and *des1* mutants during Cr^{6+} stress. Ten-day-old Wt and mutants seedlings were transferred to ½ MS medium containing 300 μmol/L Cr^{6+} (150 μmol/L $K_2Cr_2O_7$) for 12 h of stress, and the *MT3*, *MT2A*, *PCS1* and *PCS2* gene expression levels were subsequently measured. (c) The regulation of H_2S and Cys on *PCS1*, *PCS2*, *MT3* and *MT2A* expression levels in seedlings with or without Cr^{6+} stress. The one-week-old seedlings were pretreated with H_2S, Cys or H_2S + Cys for 3 d, then transferred to ½ MS medium containing 300 μmol/L Cr^{6+} (150 μmol/L $K_2Cr_2O_7$) for 12 h of stress exposure, and the *MT3*, *MT2A*, *PCS1* and *PCS2* expression levels were subsequently measured. Data are means ± SE of three biological repeats, error bars indicate error standard and bars with different letters are different ($P < 0.05$), bars with * are different ($P < 0.05$) and ** are significantly different ($P < 0.01$).

mutants (Fig. 4b). These results indicated that there might be other intricate signals ameliorating the negative influence of the H_2S decrease on the expression levels of these genes. However, Cr^{6+} stress did not affect the *MT3* expression level in over-expression or knock-down mutants, which were the same as in Cr^{6+}-stressed Wt (Fig. 4b). Moreover, the exogenous H_2S fumigation took the Cr^{6+}-

induced expression of *PCS1*, *PCS2* and *MT2A* to a higher level, but reduced the *MT3* expression level (Fig. 4c). Overall, H_2S enhanced the Cr^{6+} tolerance by increasing *MT2A*, *PCS1* and *PCS2* expression levels, but not *MT3*, to accumulate HMs chelators PCs and MTs. Interestingly, H_2S has different mechanism for regulating the two MTs encoding genes *MT2A* and *MT3* in Cr^{6+}-stressed plants. *MT2A* was reported to have a tight correlation with HMs tolerance acquisition (Murphy et al., 1997), therefore gasotransmitter H_2S up-regulated the *MT2A* transcription to help plants resist to Cr^{6+} stress. However, neither H_2S increase nor H_2S decrease markedly affected *MT3* expression, this could be explained either by the diverse response pattern of different MTs encoding genes to H_2S and Cr^{6+} stress, or by the existence of some posttranscriptional regulations. There is no doubt that elucidating the complicated regulatory network of H_2S demands more profound and advanced researches.

The Cys pretreatment strengthened the Cr^{6+}-increased *PCS1* and *PCS2* expression but mitigated *MT2A* and *MT3* expression (Fig. 4c), which indicated that Cys helped plants fight against Cr^{6+} stress mainly through PCs accumulation, but not MTs. These data revealed the different regulatory mechanisms between MTs and PCs, which are the two most important HMs chelators. Given their similarity in being cysteine rich, PCs are catalytically synthesized by PCS while MTs are proteins encoded by the *MT* genes (Cobbett and Goldsbrough, 2002). This appears to be consistent with their different regulatory mechanisms.

Cys is well known as a precursor of GSH, which stores and transports Cys using the γ-glutamyl cycle (Seth et al., 2012). As the principal sulphydryl-containing polypeptide, GSH is involved in numerous physiological metabolic reactions during plant HMs-stress responses. GSH is not only the major antioxidant involved in balancing cellular redox homeostasis, but is also the defender of HMs detoxification by promoting PCs generation (Wachter and Rausch, 2005; Anjum et al., 2012). Correspondingly, exogenous H_2S and Cys pretreatments strengthened the Cr^{6+}-mediate GSH elevation (Fig. 4a). This suggested that H_2S and Cys enhance Cr^{6+} tolerance by facilitating the accumulation of GSH, which then acts as a precursor of PCs to accumulate PCs and increase HMs chelation. Additionally, GSH participated in both the direct and indirect control of ROS, protecting plants against HMs-induced oxidative stress.

A signal pathway model is proposed based on the evidence demonstrated in this study (Fig. 5). This paper presents the interaction between H_2S and Cys, indicating that H_2S is activated much earlier than Cys in plant responses to Cr^{6+} stress (Fig. 2a). When exposed to Cr^{6+} stress, *Arabidopsis* appeared to elevate H_2S, which then acted as a gasotransmitter to improve Cys accumulation by regulating the transcription levels of the Cys synthesis-related genes. H_2S induced the significant up-regulation of *SAT1*, *SAT5* and *OASTLa* expression levels, and the high expression levels of *SAT1* and *SAT5* were maintained for a longer period (Fig. 2c). Consequently, H_2S might play a significant role in catalyzing the formation of OAS by regulating the expression of SAT encoding genes, and SAT-catalyzed OAS generation may be the key step in H_2S-mediated Cys increase. The H_2S-Cys signaling participated in complex physiological processes to protect plants against Cr^{6+} stress. On one hand, H_2S and Cys activated the generation of GSH (Fig. 4a), which not only fought against the excessive ROS caused by Cr^{6+} stress but also acted as a precursor to promote PCs generation. On the other hand, H_2S and Cys significantly increased the PCs by up-regulating *PCS1* and *PCS2* expression. In addition to regulating the expression of PCs synthase encoding genes, H_2S markedly increased *MT2A* expression, but not *MT3*, to facilitated MTs elevation (Fig. 4c). *MT2A* has been reported to have a tight correlation with HMs tolerance acquisition (Murphy et al., 1997), therefore H_2S up-regulated the *MT2A* transcription to achieve its protective function. Cys mitigated Cr^{6+}-increased *MT2A* and *MT3* expression (Fig. 4c), which indicated that Cys helped plants fight against Cr^{6+} stress mainly through PCs accumulation, but not by enhancing *MT2A* and *MT3* expression. In summary, HMs chelators, PCs and MTs, were activated to defend against Cr^{6+}stress, while their regulatory mechanisms appeared to be different. The emphasis of H_2S in regulating chelators generation might differ from that of Cys.

Comprehensively, *Arabidopsis* activated the H_2S-Cys system to survive Cr^{6+} stress, mainly by regulating the generation of the antioxidant GSH and promoting HMs chelators accumulation.

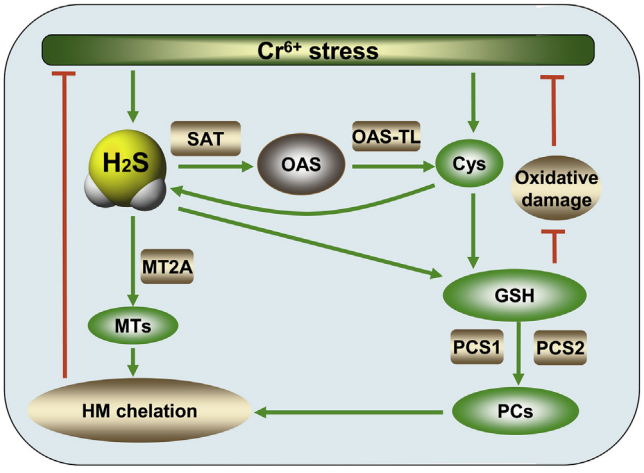

Fig. 5. The response patterns of the H_2S-Cys system in *Arabidopsis* to Cr^{6+} stress. Arrows indicate enhanced effects and hyphens indicate suppressed effects.

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