Plant Physiology and Biochemistry 111 (2017) 179–192

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Hydrogen sulfide alleviates mercury toxicity by sequestering it in roots or regulating reactive oxygen species productions in rice seedlings

Zhen Chen*, Moshun Chen, Ming Jiang

Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation, College of Life Sciences, Taizhou University, No.1139 Shifu Road, Taizhou 318000, People's Republic of China

ARTICLE INFO

Article history:
Received 9 October 2016
Received in revised form
30 November 2016
Accepted 30 November 2016
Available online 1 December 2016

Keywords:
Hg
H_2S
Non-protein thiols
Metallothionein
bZIP60
ROS scavenging

ABSTRACT

Soil mercury (Hg) contamination is a major factor that affects agricultural yield and food security. Hydrogen sulfide (H_2S) plays multifunctional roles in mediating a variety of responses to abiotic stresses. The effects of exogenous H_2S on rice (*Oryza sativa* var 'Nipponbare') growth and metabolism under mercuric chloride ($HgCl_2$) stress were investigated in this study. Either 100 or 200 μM sodium hydrosulfide (NaHS, a donor of H_2S) pretreatment improved the transcription of *bZIP60*, a membrane-associated transcription factor, and then enhanced the expressions of non-protein thiols (NPT) and metallothioneins (OsMT-1) to sequester Hg in roots and thus inhibit Hg transport to shoots. Meanwhile, H_2S promoted seedlings growth significantly even in the presences of Hg and superoxide dismutase (SOD, EC 1.15.1.1) or catalase (CAT, EC 1.11.1.6) inhibitors, diethyldithiocarbamate (DDC) or 3-amino-1,2,4-triazole (AT). H_2S might act as an antioxidant to inhibit or scavenge reactive oxygen species (ROS) productions for maintaining the lower MDA and H_2O_2 levels, and thereby preventing oxidative damages. All these results indicated H_2S effectively alleviated Hg toxicity by sequestering it in roots or by regulating ROS in seedlings and then thus significantly promoted rice growth.

1. Introduction

Mercury (Hg), one of the most toxic heavy metals, has been widely released into environments via natural and anthropogenic sources (Qiu et al., 2008). Hg by-product emission in the world will reach as much as 1.85×10^6 kg in 2020, estimated by Arctic Monitoring and Assessment Programme (AMAP) and United Nations Environment Programme (UNEP) (Kim and Jung, 2012). Natural Hg exists in different chemical forms such as HgS, Hg^0, Hg^+, Hg^{2+} and methyl-Hg or ethyl-Hg (Meng et al., 2011). Most of Hg is easily converted to methyl-Hg and results in neurotoxicity, thereby threatening public health (Linšak et al., 2013). Inorganic Hg (Hg^{2+} or HgII) is the predominant form in soils and easily absorbed by plants, deposits in the different parts and then affects agriculture yield and food security. Hg is biochemically toxic as it binds to sulfhydryl groups (−SH) and leads to disruption of protein structures and functions (Chen et al., 2012). Hg also can trigger the burst of reactive oxygen species (ROS) and cause oxidative damages (Shiyab et al., 2009; Lomonte et al., 2010; Sahu et al., 2012; Malar et al., 2015a; Wang et al., 2015). So it is urgent to find some efficient, economical and safety measures to mitigate the phytotoxicity induced by Hg. Salicylic acid (SA), carbon monoxide (CO) and cysteine have been recommended for alleviating Hg toxicity (Zhou et al., 2009; Meng et al., 2011; Hajeb and Jinap, 2012). Our previous work also showed that exogenous nitric oxide (NO) directly eliminated ROS and thereby prevented oxidative stress caused by Hg (Chen et al., 2015).

Just like CO and NO, gaseous hydrogen sulfide (H_2S) has recently

Abbreviations: APX, ascorbate peroxidase; AT, 3-amino-1,2,4-triazole; BiPs, immunoglobulin heavy-chain binding proteins; bZIPs, basic leucine zipper, membrane-associated transcription factors; CAT, catalase; DDC, diethyldithiocarbamate; DMTU, *N,N'*-dimethylthiourea; Dw, dry weight; Fw, fresh weight; H_2O_2, hydrogen peroxide; H_2S, hydrogen sulfide; Hg, mercury; MDA, malonyldialdehyde; Nox, NADPH oxidases; NPT, non-protein thiols; OsMTs, metallothioneins of *Oryza sativa*; OsPCS1, phytochelatin synthase of *O. sativa*; OsPDILs, protein disulfide isomerase-like proteins of *O. sativa*; OxyR, oxidative stress responding regulator; p66Shc, a 66 kD Src homologous-collagen homologur (Shc) adaptor protein; POD, peroxidase; PT, protein thiols; qPCR, real-time quantitative PCR; SOD, superoxide dismutase; ROS, reactive oxygen species; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid.

* Corresponding author.
E-mail addresses: chenzh@tzc.edu.cn, jennie1980@163.com (Z. Chen).

http://dx.doi.org/10.1016/j.plaphy.2016.11.027
0981-9428/

been identified as a third endogenous signal molecule (Li et al., 2013). In plants, H_2S plays prominent multifunctional roles in mediating various physiological processes and responses to abiotic stresses (Chen et al., 2011; Christou et al., 2013; Dooley et al., 2013; Shi et al., 2013; Fang et al., 2014). Heavy metal stress, one of the major abiotic stresses, affects large terrestrial areas of the world and greatly reduces agricultural productivity. However, H_2S effectively alleviated copper (Cu), boron (B), chromium (Cr), cadmium (Cd), aluminum (Al) and lead (Pb) toxicity (Zhang et al., 2008, 2010; Wang et al., 2010; Li et al., 2012a; Chen et al., 2013; Sun et al., 2013; Ali et al., 2014; Bharwana et al., 2014). For the possible mechanism of detoxification, Wang et al. (2010) suggested that H_2S reverse the effect of B on cell wall related pectin methylesterase (PME) and expansions; Chen et al. (2013) found H_2S ameliorated Al toxicity probably by increasing citrate secretion and transport or enhancing plasma membrane (PM) H^+-ATPase gene expression. In addition, the promotive effects of H_2S were also attributed to the induced activities of antioxidant enzymes or decreased influx and transport of heavy metals (Chen et al., 2013; Sun et al., 2013; Bharwana et al., 2014). On the other hand, increasing studies in animals and plants indicated that H_2S might serve as an essential signal transmitter and activate downstream signal transductions to attenuate the stress of heavy metals (Manna and Jain, 2013; Shi et al., 2014). Li et al. (2012a) considered that there existed a cross-talk between H_2S and NO, which was responsible for the enhanced Cd tolerance of alfalfa, and Shi et al. (2014) confirmed that H_2S acts as a downstream component of NO for Cd detoxification. Furthermore, H_2S might act as a mediator in auxin signaling to launch lateral root formation (Fang et al., 2014), or interact with Ca^{2+} and calmodulin signaling (Li et al., 2012b). Nevertheless, the precise role of the natural detoxicant, H_2S, is still elusive. The metabolic pathways need to be further illuminated. Moreover, no information could be found about the interaction of H_2S and Hg contamination in plants.

In the present study, NaHS, a fast releaser of H_2S, was applied to study the effects of exogenous H_2S on growth of rice seedlings under mercury chloride ($HgCl_2$) stress. And the possible inhibition, reduction or precipitation reactions of H_2S and its bioconversion products on alleviating Hg-induced toxicity were also discussed by using biochemical, physiological and molecular approaches.

2. Materials and methods

2.1. Plant culture and treatment

Seeds of rice (*Oryza sativa* L. var Nipponbare, from State Key Laboratory of Rice Biology, China National Rice Research Institute) were surface sterilized using 10% (v/v) sodium hypochlorite (NaClO) for 25 min, and then rinsed five times with distilled water and soaked at 30 °C in dark. After germination, the seeds were transferred to nylon mesh for hydroponics with half-strength Yoshida's rice culture solution for one week (Yoshida et al., 1976). Then the uniform seedlings were cultured in pails (~10 L) with complete Yoshida's rice nutrient solutions for another 3 weeks. Every 2 seedlings were put in a hole of the homemade lid (8 holes) and total 16 seedlings were set as one group. The culture solutions were changed every 5 days. The photoperiod of the growth chamber was 14 h light (30 °C)/10 h dark (24 °C) with 80% relative humidity (RH).

After that some of seedlings were pretreated for 100 or 200 μM NaHS for 24 h (as 'NaHS pre'), and then transferred to culture solutions with or without 100 μM $HgCl_2$ stress for 3 days. The experiments were duplicated and the treatment without any chemical reagent was set as control. Then growth of seedlings was analyzed and physiological changes were estimated as follows.

2.2. Measurement of Hg concentration

Roots of seedlings treated with Hg or NaHS + Hg were immersed first in 20 mM Na_2-EDTA for 30 min and thoroughly rinsed with deionized water. Then different parts of the seedlings with different treatments were separated respectively, dried at 110 °C for 15 min and then at 70 °C till a constant weight. Samples were prepared in quadruplicate (n = 4). 200 mg Dw (dry weight) of each plant material was immersed in a mixture of HNO_3/HF (6/1, v/v) overnight and digested thoroughly using multi-wave digestion oven. The digestive solution was filtered and diluted (62.5×, 125× or 20,000×) to a suitable concentration. Then the atomic absorption spectrophotometer (AA-7000, Shimadzu, Japan) with hydride generator (HVG-1) was used for detection of Hg concentration (Chen et al., 2015). The standard calibration curve was plotted with 0–80 μg g^{-1} Hg standard solution (from National Certified Reference Materials Center, China) in triplicates. Translocation factor (TF) equals to metal concentration in shoots divided by metal concentration in roots (Malar et al., 2015b).

2.3. Determination of photosynthetic pigments

Chlorophyll *a* (Chla), chlorophyll *b* (Chlb) and carotenoids were determined by spectrophotometry (UV–visible light spectrophotometer T6, Persee, China) (Bharwana et al., 2014). 0.25 g leave sample was dipped overnight in 85% acetone, centrifuged and diluted to the suitable concentration. Then the absorbances of 663, 645 and 440 nm were recorded. The concentrations of Chl a, Chl b and carotenoids were calculated according the reported formulas and expressed as μg g^{-1} fresh weight (Fw).

2.4. Detections of malonyldialdehyde (MDA) and hydrogen peroxide (H_2O_2)

The degrees of lipid peroxidation in rice leaves were assessed by its byproduct MDA in samples according to the previously described method (Chen et al., 2012). H_2O_2 level was determined in terms of the regular method (Jana, 1981; Chen et al., 2015).

2.5. Assays of antioxidant enzymes activities

Enzymes were extracted according to the method described by Guo et al. (2007) with slight modifications. Leaves (0.25 g) were homogenized in 2.5 ml ice-cold extraction buffer containing 50 mM potassium phosphate (pH 7.8), 2% polyvinyl pyrrolidone (PVP, w/v) and 0.2 mM EDTA. The homogenates were centrifuged at 8000 g for 20 min at 4 °C. Then the supernatants were used as crude extracts for further antioxidant enzymes assays.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated by measuring its inhibition of photochemical reduction of nitroblue tetrazolium chloride (NBT) (Beauchamp and Fridovich, 1971). The reaction mixture contained extraction buffer 825 μl, 0.1 mM EDTA 60 μl, 750 μM NBT 600 μl, 26 mM L-methionine 1.5 ml, 0.2 mM riboflavin 60 μl and enzyme extract 15 μl. Reactions were illuminated for 10 min at light intensity of about 200 μmol m^{-2} s^{-1}. Then the absorbance of reaction mixture was measured at 560 nm (Multiskan Spectrum, Thermo, USA). One unit of SOD was defined as the amount of enzyme causing 50% inhibition of initial reduction of NBT under light.

The activity of catalase (CAT, EC 1.11.1.6) was measured according to the procedure described by Aebi (1983). The reaction mixture contained 50 mM potassium phosphate buffer (pH7.2), 10 mM H_2O_2 and 50 μl enzyme extract. The decrease of absorbance at 240 nm was read due to the consumption of H_2O_2 (UV-2401PC, Shimadzu, Japan). And the final CAT activity was calculated using extinction

coefficient 39.4 L $mmol^{-1}$ cm^{-1}.

The peroxidase (POD, EC 1.11.1.7) activity was determined following the reported method (Rao et al., 1997). The reaction mixture (3 ml) consisted of 100 mM phosphate buffer (pH 6.0), 1.68 μl guaiacol, 1.14 μl H_2O_2 and 50 μl enzyme extract. The increase of absorbance at 470 nm was recorded continuously for 2 min (UV-2401PC, Shimadzu, Japan) to monitor the formation of tetraguaiacol. And the extinction coefficient for tetraguaiacol was 26.6 L $mmol^{-1}$ cm^{-1}.

The enzyme ascorbate peroxidase (APX, EC 1.11.1.11) was assayed in terms of the method stated by Nakano and Asada (1981). 3 ml of the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM Na_2-EDTA, 0.5 mM AsA, 0.1 mM H_2O_2 and 100 μl enzyme extract. The decrease of absorbance at 290 nm in one minute was recorded (UV-2401PC, Shimadzu, Japan) to reflect the effect of APX and the extinction coefficient of 2.8 L $mmol^{-1}$ cm^{-1} was used for ascorbate oxidation.

2.6. Quantification of non-protein thiols (NPT)

200 mg leaf or 400 mg root sample was grinded in 2 ml 5% (w/v) sulfosalicylic acid solution with 6.3 mM diethylenetriaminepentaacetic acid (DPTA) under ice-bath for NPT extraction. After centrifugation at 4 °C under 10,000*g* for 10 min, the supernatant was immediately collected and used for assay. 50 μl of the supernatant was mixed with 100 μl 0.5 M K_2HPO_4 solution, and the initial absorbance at 412 nm was measured after 2 min (Multiskan Spectrum, Thermo, USA). Then 5 μl DNTB (5,5′-dithiobis-2-nitrobenzoic acid) solution (6 mM DTNB dissolved in 0.1 M phosphate buffer solution with 5 mM EDTA, pH 7.6) was supplemented and the absorbance at 412 nm was recorded per 5 min. The steadily increased absorbance (at 10 min) was calculated for estimating the concentration of NPT using extinction coefficient of 13.6 L $mmol^{-1}$ cm^{-1} (Mendoza-Cozatl et al., 2008).

2.7. Real-time quantitative PCR assay

The real-time expressions of heavy metal chelator or stress response related protein encoding genes were examined by qPCR experiments. The rice plantlets at two-leaf stage were treated with different reagents: 1) CK, $HgCl_2$ or NaHS 0 μM; 2) Hg, $HgCl_2$ 100 μM treated for 24 h; 3) NaHS pre + Hg, NaHS 200 μM pretreated for 24 h and transferred to $HgCl_2$ 100 μM for 24 h. Total RNAs were isolated from roots of control and treated plants using TRIzol® reagent (Invitrogen, USA) and then treated with 20 U of RNase-free DNase I (TaKaRa, Japan) to remove DNA contamination. cDNA was synthesized from the RNA using PrimerScript™ RT reagent Kit (TaKaRa, Japan) according to the manufacturer's manual. Then qPCRs were carried out using SYBR Premix Ex Taq™ II (DRR081A, TaKaRa, Japan) on a Rotor-Gene Q machine (5 Plex, QIAGEN, German). The parameter was set as follows: 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 57 °C for 30 s, and 72 °C for 39 s. All reactions were done in quadruplicate. Ubiquitin was chosen as the control. PCR primers, including genes encoding Ubiquitin, OsPCS1 (phytochelatin synthase of *O. sativa*), OsMTs (metallothioneins of *O. sativa*), BiPs (immunoglobulin heavy-chain binding proteins), bZIPs (basic leucine zipper, membrane-associated transcription factors), OsPDILs (protein disulfide isomerase-like proteins of *O. sativa*) were listed in table of Table S1.

2.8. Effects of H_2S, antioxidant enzyme inhibitors and ROS scavengers on growth of rice plantlets

For exploring the direct relations of H_2S and ROS, antioxidant enzyme inhibitors and ROS scavengers were applied. The emerge-germinating rice seeds were cultivated on sterilized solid medium (1% agar) in transparent glass bottle supplemented with different chemical reagents: DDC, diethyldithiocarbamate, an inhibitor of Cu/Zn-SOD; AT, 3-amino-1,2,4-triazole, an inhibitor of catalase; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid, a $O_2\cdot^-$ scavenger; DMTU, *N*,*N*′-Dimethylthiourea, a H_2O_2 scavenger. The combinations were as follows: 1) CK, [H_2S 0 μM] + [$HgCl_2$ 0 μM]; 2) [H_2S 100 μM] + [$HgCl_2$ 0 μM/H_2O]; 3) [H_2S 200 μM] + [$HgCl_2$ 0 μM/H_2O]; 4) [H_2S 0 μM/H_2O] +[$HgCl_2$ 100 μM]; 5) [H_2S 100 μM] + [$HgCl_2$ 100 μM]; 6) [H_2S 200 μM] + [$HgCl_2$ 100 μM]; 7) [DDC 1 μM] + [$HgCl_2$ 100 μM]; 8) [DDC 1 μM + H_2S 100 μM] + [$HgCl_2$ 100 μM]; 9) [DDC 1 μM + H_2S 200 μM] + [$HgCl_2$ 100 μM]; 10) [DDC 10 μM] + [$HgCl_2$ 100 μM]; 11) [DDC 10 μM + H_2S 100 μM] + [$HgCl_2$ 100 μM]; 12) [DDC 10 μM + H_2S 200 μM] + [$HgCl_2$ 100 μM]; 13) [Tiron 1 mM] + [$HgCl_2$ 100 μM]; 14) [Tiron 1 mM + H_2S 200 μM] + [$HgCl_2$ 100 μM]; 15) [AT 2 μM] + [$HgCl_2$ 100 μM]; 16) [AT 2 μM + H_2S 100 μM] + [$HgCl_2$ 100 μM]; 17) [AT 2 μM + H_2S 200 μM] + [$HgCl_2$ 100 μM]; 18) [AT 20 μM] + [$HgCl_2$ 100 μM]; 19) [AT 20 μM + H_2S 100 μM] + [$HgCl_2$ 100 μM]; 20) [AT 20 μM + H_2S 200 μM] + [$HgCl_2$ 100 μM]; 21) [DMTU 5 mM] + [$HgCl_2$ 100 μM]; 22) [DMTU 5 mM + H_2S 200 μM] + [$HgCl_2$ 100 μM]. To avoid the direct bind of DDC [$(C_2H_5)_2NCSSNa$] and Hg to form $(C_2H_5)_2NCSHgSNa$, or H_2S and Hg to form HgS crystal, or DMTU ($C_3H_8N_2S$) and Hg to form $C_3H_8N_2SHg$ in media, two means were warranted: before (a4–a22) or after (b4–b22) 100 μM $HgCl_2$ being added into the media for 3 d, the germinant seeds were uniformly transferred to new culture bottles treated with the other reagents mentioned above for another 3 d. Meanwhile, the media supplemented with only water, 100 μM or 200 μM H_2S (a2, a3, H_2S for 3 d then water; or b2, b3, the opposite) were set as controls. The photoperiod of the sterile chamber was 14 h light (28 °C)/10 h dark (22 °C) with 80% RH. After 6 days of treatments, the phenotypes were observed and the shoot and root length were measured.

2.9. Statistical analysis

Values were expressed as mean ± standard deviation (SD) of at least triplicate experiments and measures. Normality and homogeneity of variances were initially checked using SPSS 17.0 software (SPSS, Chicago, IL, USA). If they were homogeneous, significant differences between various treatments were analyzed by one-way analysis of variance (Duncan's new multiple range test). Otherwise, non-parametric statistics of Kruskal Wallis test was selected.

3. Results

3.1. Exogenous H_2S pretreatment promoted seedling growth under Hg stress

To investigate the external and physiological effects of Hg stress and H_2S pretreatment on their growth, rice seedlings were cultured hydroponically until the five-leaf stage. Then reagents for the different treatments were applied (Fig. 1a–f). The seedlings became severely wilted and chlorotic after being exposed to 100 μM Hg stress for 3 d (Fig. 1d). The Fw and Dw were significantly decreased by 27.6 and 25.9%, respectively, in comparison with controls (Fig. 1g and h). However, H_2S-pretreated seedlings exhibited enhanced Hg tolerance and better growth (Fig. 1e and f), and their Fw and Dw notably increased and recovered to control levels (Fig. 1g and h). However, there was no significant difference between H_2S treatment alone and the control, for phenotype or Fw and Dw (Fig. 1b, c and g, h).

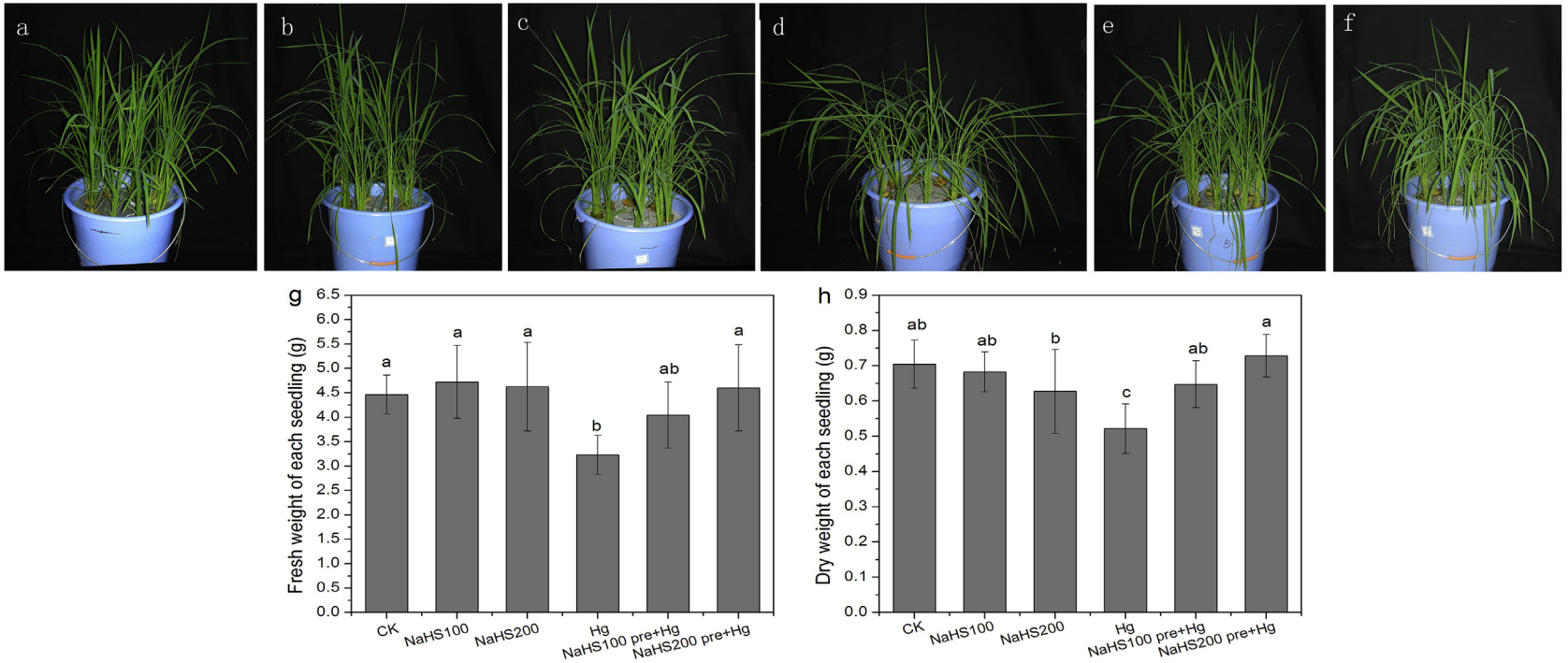

Fig. 1. Effects of NaHS pretreatments on growth of rice seedlings at five-leaf stage. a, NaHS and $HgCl_2$ 0 μM; b, NaHS 100 μM; c, NaHS 200 μM; d, $HgCl_2$ 100 μM; e, NaHS 100 μM pre + $HgCl_2$ 100 μM; f, NaHS 200 μM pre + $HgCl_2$ 100 μM; g, h, fresh and dry weight of each seedling. 'NaHS pre' means that seedlings were pretreated with 100 or 200 μM NaHS for 24 h and then transferred to culture solution with 100 μM $HgCl_2$ treatment for 3 d (the same as follows). The upper inner diameter of pail is 27 cm. Data are presented as mean ± SD (n = 8). Values with different lowercase mean the significant difference at $P < 0.05$ level.

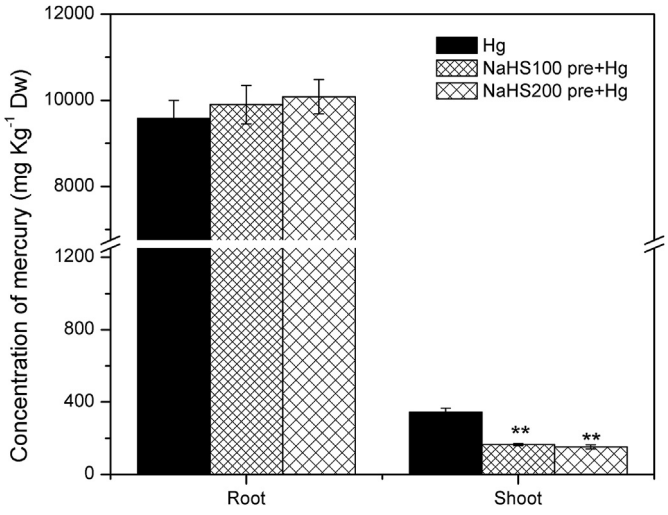

Fig. 2. Effects of H_2S (NaHS 100 or 200 μM) on Hg accumulations in different parts of rice seedlings at five-leaf stage with 100 μM $HgCl_2$ treatments for 3 d. Data are presented as mean ± SD (n = 4). The significant level of the difference between Hg and NaHS pre + Hg treatment is indicated by ** ($P < 0.01$).

3.2. Exogenous H_2S alleviated Hg toxicity by preventing its transport to shoots

Standard curve regression equation for Hg detection was $y = 0.009x + 0.060$, $R^2 = 0.985$. Relative standard deviations (RSD) for Hg contents in samples were ~0.04% (roots) or 1.0–2.0% (shoots). The analysis of uptake and movement of Hg in different parts of rice seedlings showed that Hg mainly accumulated in the roots. The 100 or 200 μM NaHS pretreatment did not inhibit the absorption of Hg by roots, but did impede Hg transport to shoots (Fig. 2). TF of Hg treatment alone was 0.036, while TFs of 100 or 200 μM NaHS + Hg were 0.017 and 0.015. So the concentrations of Hg in shoots of seedlings treated with 100 or 200 μM NaHS + Hg were significantly ($P < 0.01$) lower than that of Hg treatment alone, with decreases of 51.99 and 55.81%, respectively (Fig. 2). Thus, the ameliorative effect from H_2S in leaves may be partly attributed to inhibited Hg transport.

3.3. Exogenous H_2S elevated levels of thiol-containing compounds to sequester Hg in roots

In the absence of Hg ions, NPT concentrations in roots and leaves remained at low levels (Fig. 3). With Hg exposure, NPT synthesis elevated dramatically, and continued to increase even further in roots of seedlings pretreated with NaHS (Fig. 3). In contrast, in leaves, NPT did not continue to increase further with the pretreatment of NaHS, parallel to the relatively low Hg concentrations (Figs. 2 and 3). Moreover, NaHS alone promoted NPT synthesis in roots (Fig. 3).

The transcript changes of heavy metal chelators (thiol-containing compounds), chaperones and transcription factors were also determined. Hg exposure stimulated the expression of *OsPCS1*, *OsMT-1*, *OsMT-2*, *BiP-1*, *BiP-2*, *bZIP17*, *bZIP28*, *bZIP60*, *PDIL1-1*, *PDIL1-3*, *PDIL5-1*, *PDIL5-2* and *PDIL5-3* (Fig. 4). Thus, the increment in bZIPs transcription factors could ensure the increase in chelators (PCs and MTs) that could sequester Hg in roots and consequently alleviate toxicity. In addition, active repairs with BiPs and PDILs of the impaired proteins caused by Hg could ameliorate plant growth. Nevertheless, H_2S had different impacts on expression of these genes. The expression of *OsMT-1* dramatically increased with application of exogenous H_2S and reached 6.89-fold of the level in seedlings treated with Hg alone, and might be stimulated by the relevant enhanced bZIP60 (Fig. 4). However, transcriptions of *OsPCS1*, *OsMT-2*, *BiP-1*, *bZIP17*, *bZIP28* and *PDIL1-3* were not strengthened by H_2S but significantly reduced, as compared to Hg treatment alone. Moreover, expressions of *BiP-2*, *PDIL1-1*, *PDIL5-1*, *PDIL5-2* and *PDIL5-3* sharply declined to the levers even lower than controls, indicating the depressive effects of H_2S on them (Fig. 4). Additionally, expressions of *OsMT-3*, *PDIL2-3* and *PDIL5-4* were unchanged by the addition of Hg or H_2S (Fig. 4).

3.4. Effects of H_2S on photosynthetic pigment contents in leaves under Hg stress

For comprehensive investigation, stress-related responses in leaves were also determined. Initially, Hg toxicity caused sharp declines in photosynthetic pigments, including Chla, Chlb and carotenoids (Fig. 5a–c). However, the 100 or 200 μM H_2S pretreatment had no obvious effects on the synthesis of photosynthetic pigments (Fig. 5a–c).

3.5. Exogenous H_2S decreased the levels of MDA and H_2O_2 in leaves caused by Hg stress

NaHS treatments alone did not induce excessive MDA production in leaves of rice seedlings and there was no significant difference between NaHS treatment and the control (Fig. 6a). However, exposure to Hg ions triggered a sharp increase in MDA content, up to 2.36-fold of controls. The MDA levels were lowered by NaHS pretreatments and recovered almost to control level (Fig. 6a).

Furthermore, H_2O_2, a ROS and also a cellular messenger, was not induced by NaHS treatment but was stimulated by Hg stress. The generation of H_2O_2 reached a peak when seedlings were exposed to the $HgCl_2$ solution. In contrast, if seedlings were pretreated with NaHS for 24 h, they generated much less H_2O_2 (Fig. 6b). The low oxidative damage demonstrated the protective effect of H_2S in leaves.

3.6. Effects of H_2S on antioxidant enzyme activities

When subjected to Hg stress, the SOD, POD and APX in leaves of rice seedlings were all greatly activated and reached peak levels to scavenge $O_2\cdot^-$ and H_2O_2 in time (Fig. 7a, c, d). For control, 100 and 200 μM NaHS treatments, the SOD activities were 26.67, 37.99 and 39.49 U mg^{-1} protein, respectively. However, this markedly increased to 56.55 U mg^{-1} protein with addition of Hg stress. However, it should be noted that H_2S application did not up-regulate SOD activity and SOD activity recovered to 41.83 or even 36.54 U mg^{-1} protein for treatments of 100 or 200 μM NaHS + Hg (Fig. 7a). There was a similar trend for POD and APX activities (Fig. 7c and d). However, CAT activity slightly went up under Hg stress and also not improved by H_2S.

3.7. Cross-talk of H_2S and ROS in rice seedlings

ROS scavengers and antioxidant enzymes inhibitors were used to verify the protection from H_2S against oxidative damages. The 100 or 200 μM H_2S pretreatment or after-treatment both increased the root and shoot lengths and promoted growth of rice seedlings under Hg stress (Fig. 8a4–a6, b4–b6). The 1 and 10 μM DDC aggravated the Hg toxicity by inhibiting SOD activity, while the damage was significantly attenuated by H_2S (Fig. 8a7–a12, b7–b12) and the root length markedly increased. However, DDC at higher concentration of 1 mM might induce enough damage that H_2S could not notably rescue the growth (data not shown). Pretreatment of 1 mM tiron did not effectively eliminate subsequent

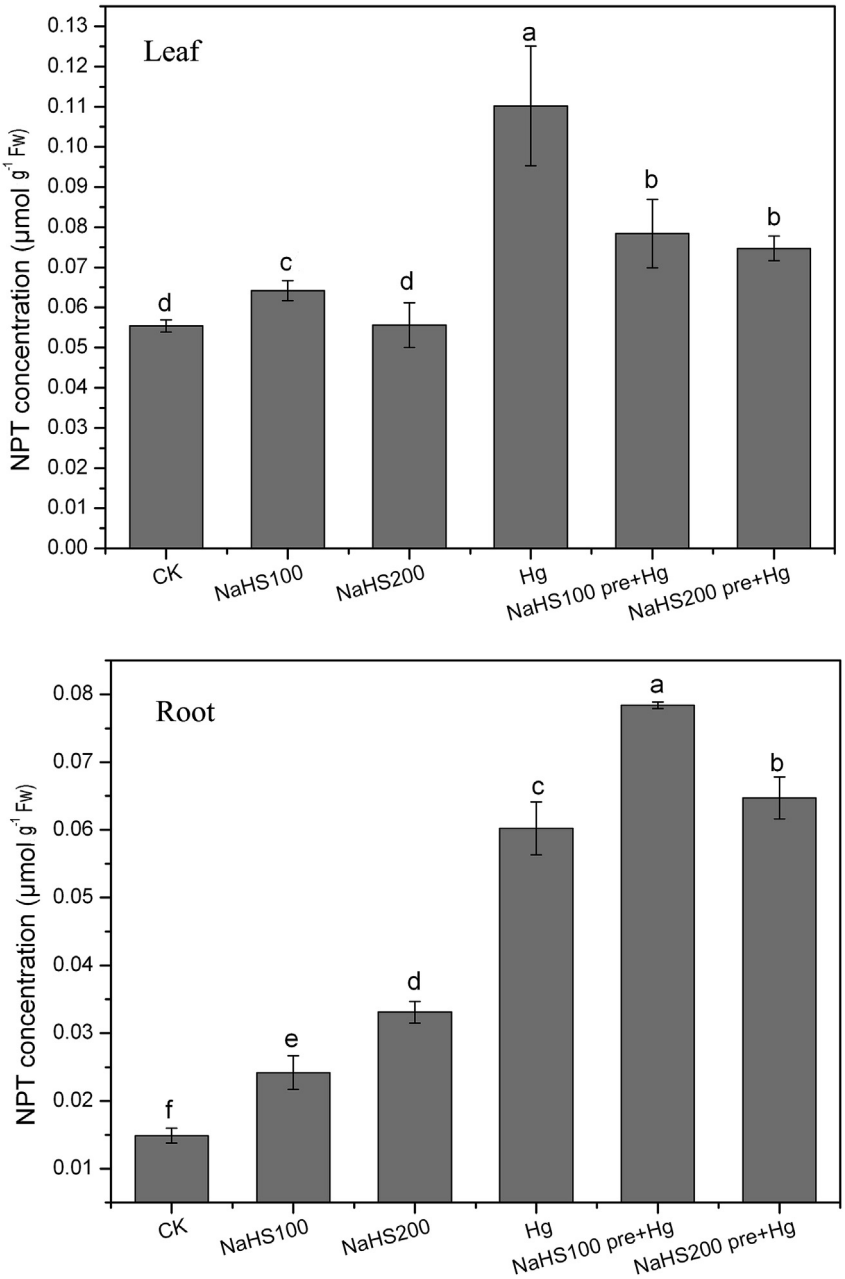

Fig. 3. Effects of H_2S (NaHS 100 or 200 μM) pretreatment on non-protein thiol (NTP) contents in leaves and roots of rice seedlings at five-leaf stage with or without 100 μM $HgCl_2$ treatments for 3 days. Data are presented as mean ± SD (n = 4). Values with different lowercase mean the significant difference at $P < 0.05$ level.

generation of $O_2\cdot^-$ caused by Hg, but if H_2S was added with tiron, the root length significantly increased and reached the same value as NaHS alone did (Fig. 8a13 and a14). Oppositely, after-treatment of tiron could better scavenge the $O_2\cdot^-$ caused by Hg treatment and attenuate the need of H_2S (Fig. 8b13 and b14). Furthermore, 2 and 20 μM AT seriously inhibited CAT activity and plant growth under Hg stress (Fig. 8a15, a18, b15 and b18). H_2S notably eliminated H_2O_2 stress caused by AT and Hg, and significantly promoted growth (Fig. 8a15–a20, b15–b20). Pretreatment or after-treatment of the H_2O_2 scavenger, DMTU, greatly eliminated H_2O_2 under Hg stress and significantly increased the root and shoot length (Fig. 8a21 and b21). When H_2O_2 was effectively controlled by DMTU, the action of H_2S activated by H_2O_2 might be diminished (Fig. 8a22 and b22).

4. Discussion

Hg is one persistent and hazardous heavy metal contaminant threatening food safety and human health (Linšak et al., 2013). Hg toxicity in plants occurs when Hg ions are transported from soil to roots and further move to different plant parts, consequently affecting various physiological processes (Chen et al., 2012; Malar et al., 2015a; Wang et al., 2015). Efficient approaches for detoxification of Hg are greatly needed. External application of natural or synthetic compounds has been identified as a powerful tool for helping plants to adapt environmental stresses in recent years (Sun et al., 2013). H_2S is emerging as a potential messenger molecule involved in modulation of physiological processes in both animals and plants (Dooley et al., 2013; Bruce King, 2013; Kabil et al., 2014).

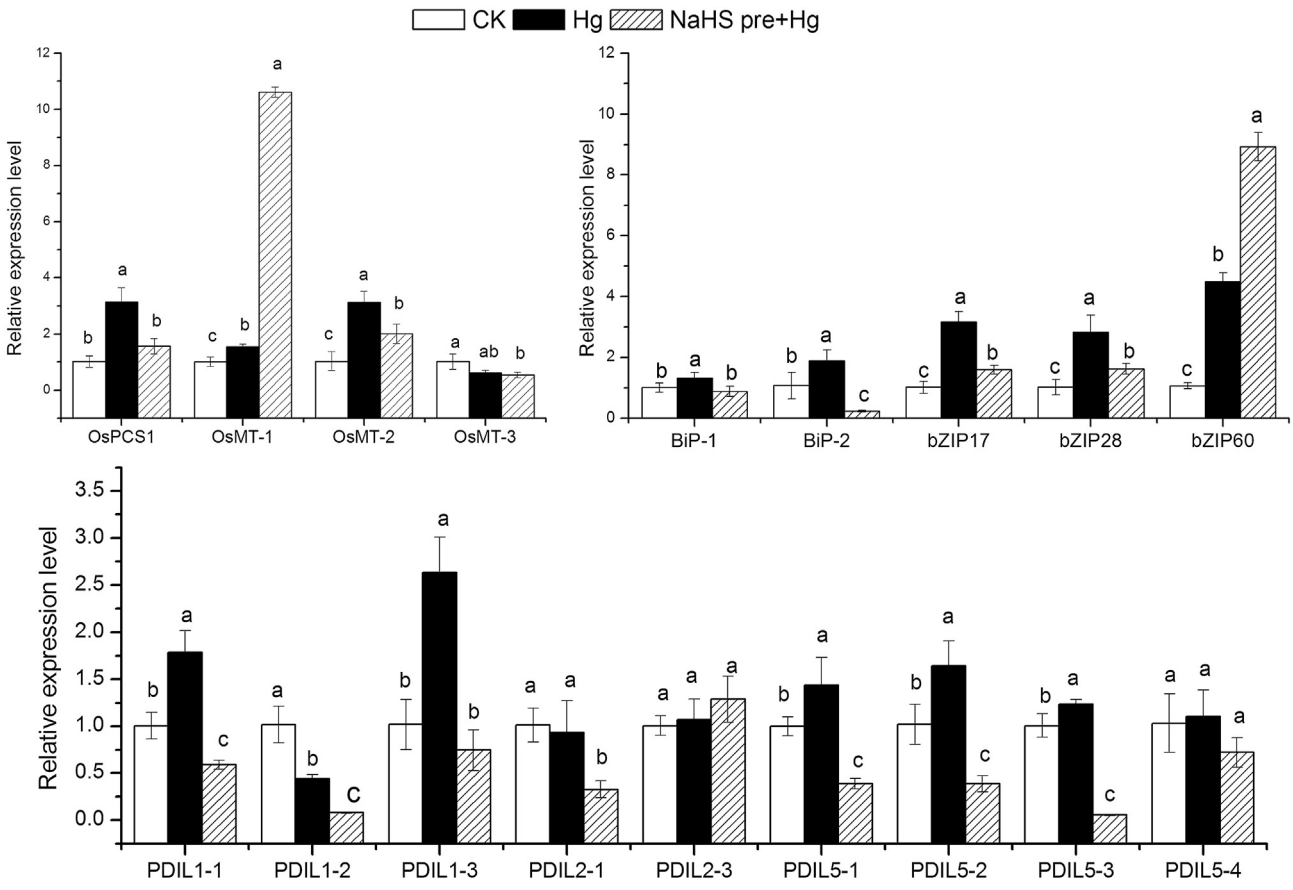

Fig. 4. Gene expression analyses of the selected heavy metal chelators, chaperones and transcription factors associated proteins by real-time qPCR in roots of rice seedlings at two-leaf stage. CK, $HgCl_2$ and NaHS 0 μM; Hg, $HgCl_2$ 100 μM treated for 24 h; NaHS pre + Hg, NaHS 200 μM pretreated for 24 h and then $HgCl_2$ 100 μM treated for 24 h. Data are presented as mean ± SD (n = 4). Values with different lowercase mean the significant difference at $P < 0.05$ level. [OsPCS1, phytochelatins synthase; MTs, metallothioneins; BiPs, immunoglobulin heavy-chain binding proteins, chaperones; bZIPs, basic leucine zipper proteins, membrane-associated transcription factors; PDILs, protein disulfide isomerase-like proteins, also function as chaperones].

In the present study, the symptoms of damage in rice seedlings caused by Hg were markedly relieved by pretreatment of 100 or 200 μM NaHS, indicating that H_2S could mediate the alleviation of Hg toxicity and help plants better withstand this stress (Fig. 1). Pharmacological and molecular indices were also determined to gain insight into the beneficial and precise roles of H_2S. The results showed that H_2S could sequester Hg ions in roots, inhibit Hg transport to shoots or regulate ROS productions to prevent oxidative stress in rice seedlings (Figs. 2, 3 and 8).

In many plant species, root accumulated the highest amounts of heavy metals and lesser in stem, petiole and leaf for its direct exposure in soil or solution (Ali et al., 2014; Bharwana et al., 2014). While some hyperaccumulation plants, such as *Eichhornia crassipes* (Mart.), *Jatropha curcas* L., and *Scirpus grossus* L.f. were found TF values >1 (Malar et al., 2015b). In this study, Hg was mainly accumulated in rice roots and H_2S did not inhibit its absorption (Fig. 2). Wang et al. (2010) reported H_2S might play a regulatory role on PME in roots under B toxicity but only marginally inhibited B concentrations in the first 4 h. After 8 h, there were no differences in B concentrations in roots with or without NaHS treatments. Sun et al. (2013) observed H_2S decreased Cd accumulation in *Populus euphratica* Oliv. cytoplasm and Cd influx across PM. The authors thought the inhibition of PM calcium channels contributed to the reduced Cd influx. But the mechanism for preventing heavy metals transport by intercellular H_2S is still unclear. Hg has a high affinity for −SH and consequently disturbs the non-protected S-containing proteins. PDILs can catalyze protein disulfide bonds formation, reduction or isomerization. Our previous work has proved that overexpression of MTH1745, a PDIL, did not inhibit Hg uptake but really reduced Hg transport to shoot (Chen et al., 2012). Meaningfully, H_2S also restrained Hg transport and alleviated Hg toxicity in leaves (Figs. 2 and 6).

Plants have developed various mechanisms to counteract heavy metal toxicity. One of these is chelating heavy metal ions with intracellular thiol-containing compounds, including NPT, e.g. cysteine, glutathione (GSH) and phytochelatins (PCs), and protein thiols (PT), containing metallothioneins (MTs) and thioredoxin (Trx) (Kotrba et al., 2009). In plants, H_2S is endogenously generated from cysteine and homocysteine via desulfhydrases, including L-cysteine desulfhydrases (LCD) and D-cysteine desulfhydrase (DCD) (Jin et al., 2011). And H_2S is quickly oxidated to persulfide (hydrodisulfides), sulfite, thiosulfate and sulfate, thus maintaining a low steady H_2S concentration (Kabil et al., 2014). These oxidation products may then contribute to the formation of GSH via various catabolic pathways (see figure of pathways for sulfide biogenesis and clearance, Kabil et al., 2014). Cysteine is the precursor of H_2S, GSH, PCs, MTs and other sulfur-rich proteins, and a high level of H_2S will be beneficial for synthesis of NPT and PT, which play prominent roles in heavy metal detoxification. Shi et al. (2014) showed that various concentrations of Cd greatly increased endogenous H_2S levels. Additionally, researches have verified that exogenous root application of NaHS can notably elevate H_2S and GSH levels

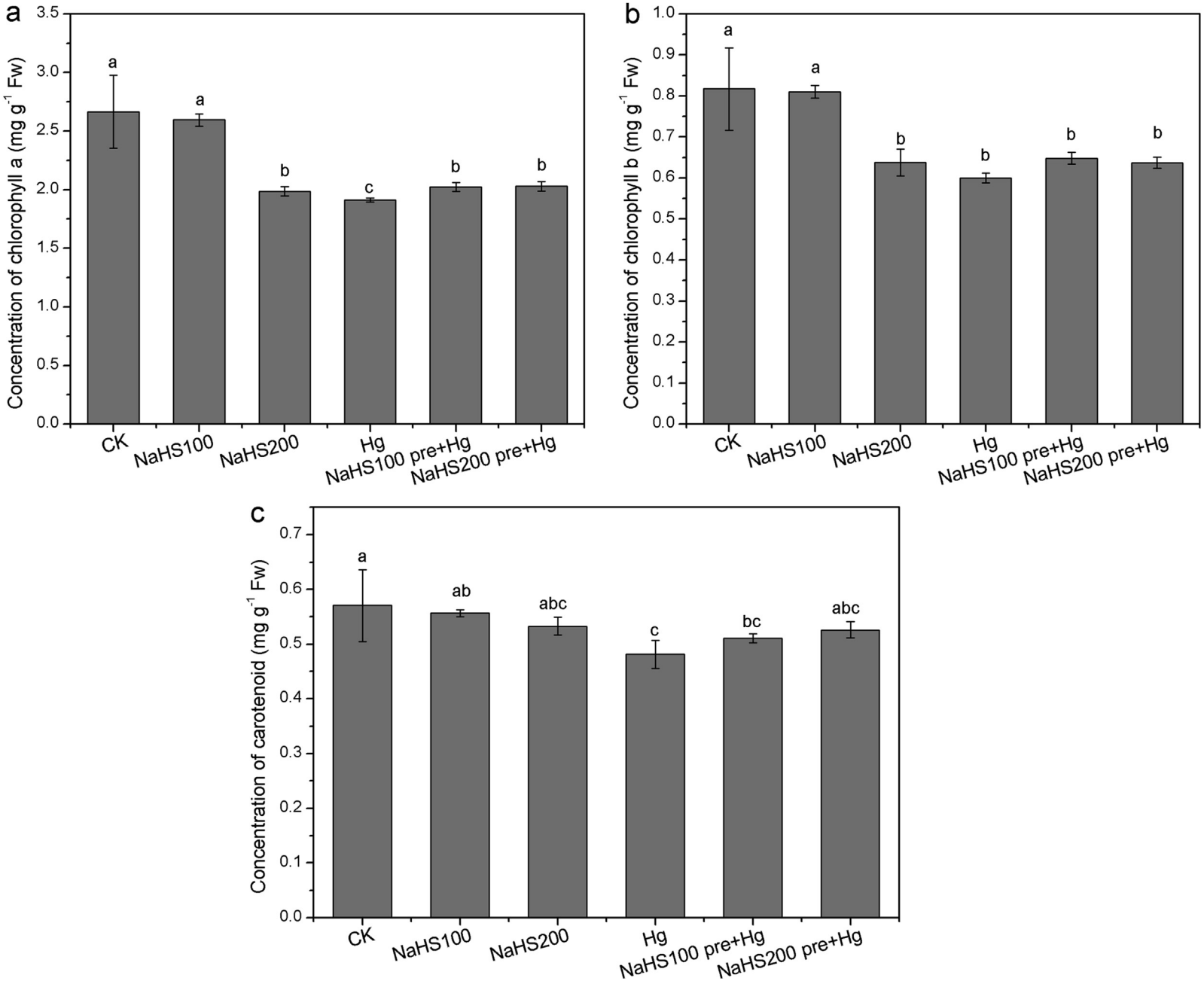

Fig. 5. Effects of H_2S (NaHS 100 or 200 μM) pretreatment on photosynthetic pigments contents in leaves of rice seedlings at five-leaf stage with or without 100 μM $HgCl_2$ treatments for 3 d. a, chlorophyll *a*; b, chlorophyll *b*; c, carotenoids. Data are presented as mean ± SD (n = 3). Values with different lowercase mean the significant difference at $P < 0.05$ level.

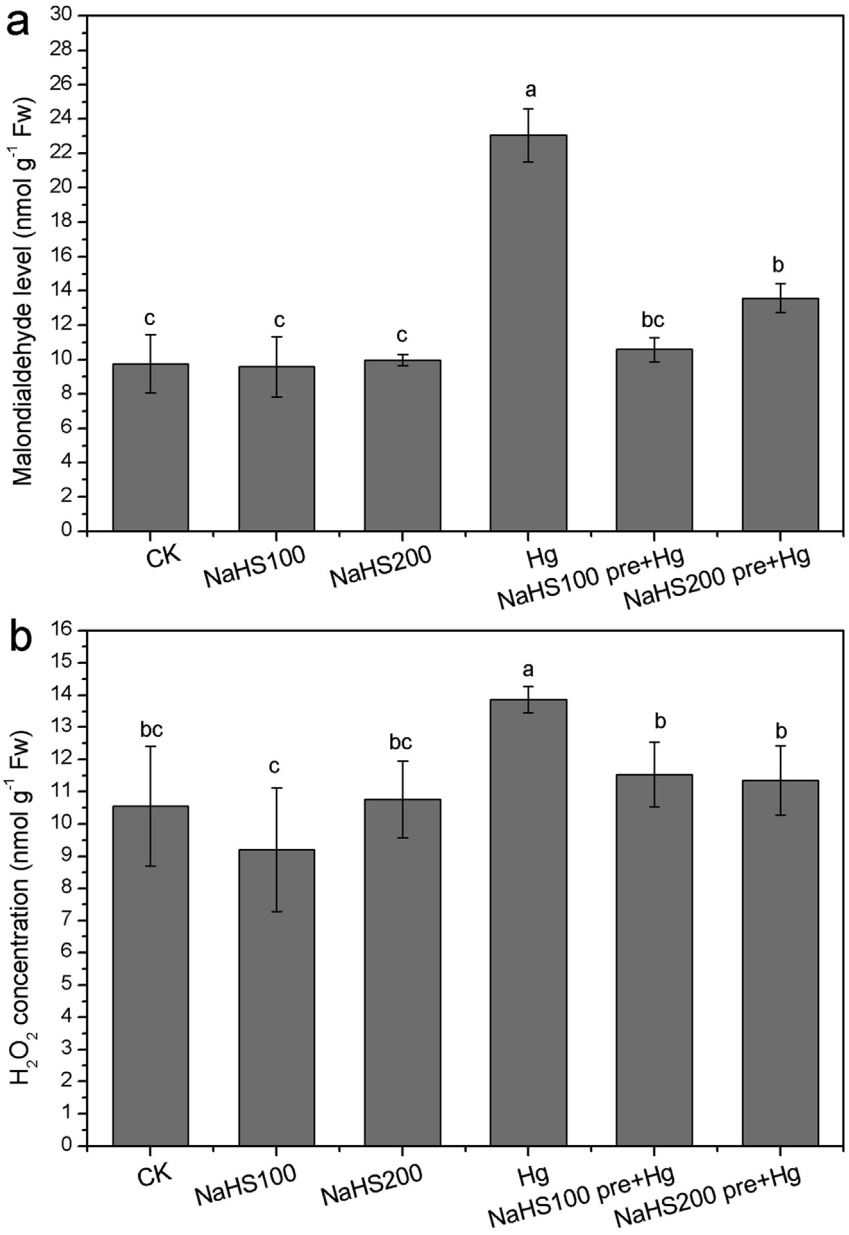

Fig. 6. Effects of H_2S (NaHS 100 or 200 μM) pretreatment on malondialdehyde (a) and H_2O_2 (b) levels in leaves of rice seedlings at five-leaf stage cultured hydroponically with or without 100 μM $HgCl_2$ treatments for 3 d. Data are presented as mean ± SD (n = 3). Values with different lowercase mean the significant difference at $P < 0.05$ level.

irrespective of the conditions of control or stresses (Chen et al., 2011; Christou et al., 2013; Shan et al., 2011, 2012). It was estimated that approximately 40% of the H_2S was converted to GSH (Li et al., 2012b). GSH, the major component of NPT, is the largest intracellular thiol pool for ROS scavenging and also for Hg chelating to form $Hg(GSH)_2$, a non-toxic form of Hg (Chen et al., 2012). Likewise, in our study, H_2S application greatly strengthened the synthesis of NPT to fix Hg in roots (Fig. 3), thereby resulting in reduced transport to shoots (Fig. 2).

In addition, MTs are cysteine-rich proteins that play important roles in metal detoxification by binding metal ions with thiol groups of their cysteine residues (Kotrba et al., 2009). Several MT isoforms have been found and classified into four types according to the arrangement of cysteine residues in plants (Nezhad et al., 2013). Previous research revealed that MT isoforms had different coordination numbers of different heavy metals. The ability of OsMTI-1b to bind different metals was in the order of Cd^{2+}/Ni^{2+} > Zn^{2+} > Cu^{2+}, while OsMTI-1a preferred to bind Zn^{2+} (Nezhad et al., 2013). Similarly, three *Vicia faba* L. MT isoforms and three *Prosopis juliflora* (Sw.) D.C. MT isoforms were found to have greater binding affinity for Cd^{2+} than for Zn^{2+} and Cu^{2+} (Foley et al., 1997; Usha et al., 2009). In the present study, the binding ability of OsMT isoforms for Hg was determined for the first time and the relationship between H_2S and MTs was also analyzed. Hg exposure significantly increased *OsMT-1* and *OsMT-2* expression by 53 and 202%, respectively, while it had no impact on *OsMT-3*, in comparison with control. Application of H_2S sharply enhanced the expression of *OsMT-1*, as much as 6.89-fold compared with Hg treatment alone, but did not increase the level of *OsMT-2* and *OsMT-3*. These data suggest the H_2S susceptibility and Hg preference to OsMT-1. Similar to MTs, PCs are capable of efficient sequestration of Hg to form a Hg–thiolate complex. PCs are enzymatically

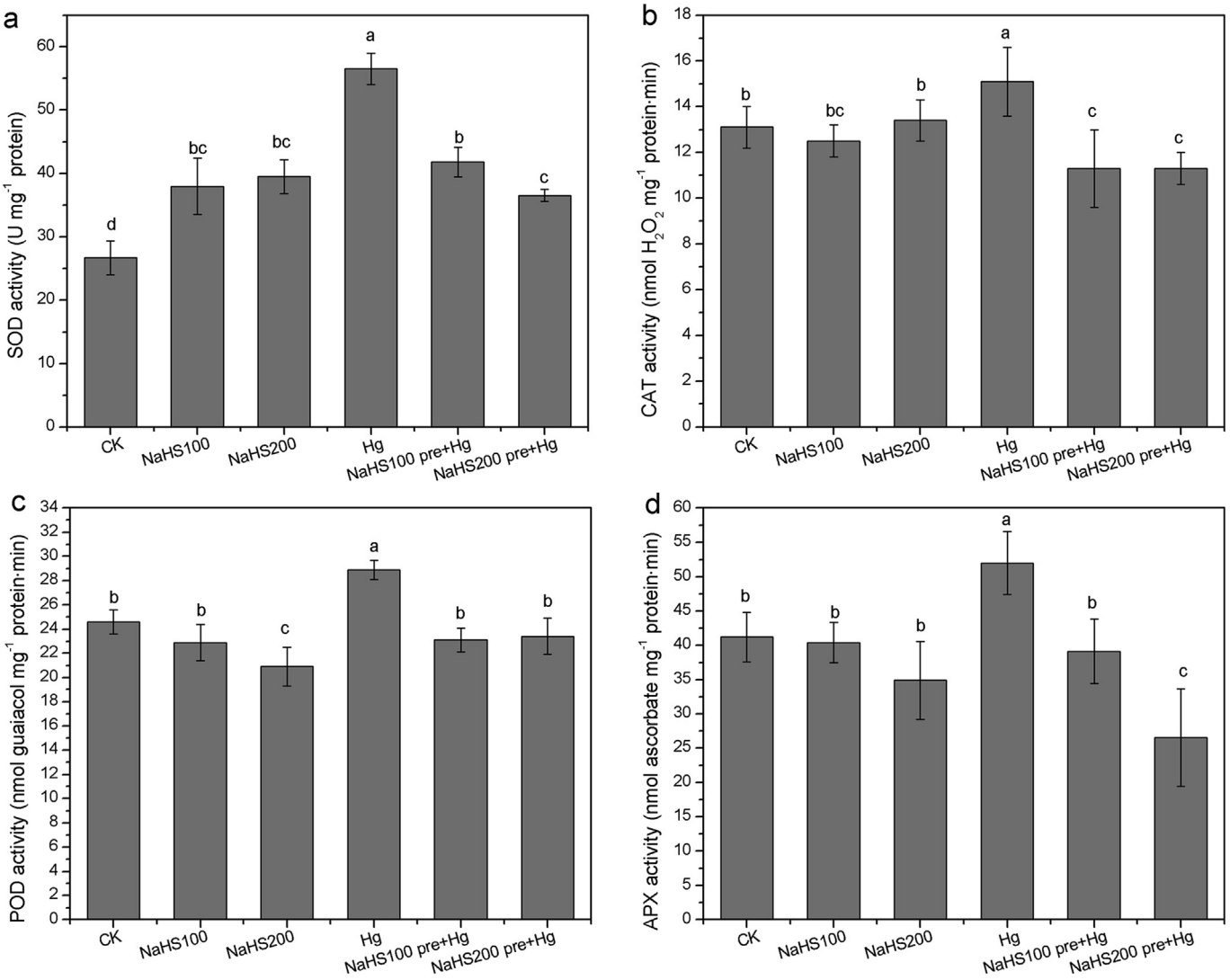

Fig. 7. Activities analysis of antioxidant enzymes, including SOD (a), CAT (b), POD (d), and APX (d), in leaves of rice seedlings at five-leaf stage under Hg or NaHS pre + Hg treatments. Data are presented as mean ± SD (n = 4). Values with different lowercase mean the significant difference at $P < 0.05$ level.

synthesized by PC synthases (PCS) from GSH or its homologs (Kotrba et al., 2009). However, *OsPCS1* was activated by Hg treatment alone but not strengthened by H_2S. Since about 81.5% of H_2S would dissociate into H^+, HS^- and S^{2-}, while 18.5% remain as undissociated molecules in plant cells (Al-Magableh et al., 2014; Xie et al., 2016), no rise of *OsPCS1* might mean that S bonded with Hg to directly form non-toxic HgS sediment.

Furthermore, the expressions of these stress response genes in the nucleus may be induced by the transduction signals of bZIP proteins (E et al., 2014). There have been 89 bZIP encoding genes discovered in rice − OsbZIP60 is candidate for the ER stress sensor transducer and may also be involved in drought and heat tolerance (E et al., 2014). *OsbZIP17*, *OsbZIP28* and *OsbZIP60* were all induced by Hg stress to trigger protective protein synthesis. H_2S amplified the transcription of *OsbZIP60*, the prominent signal factor (Fig. 4). An analogous phenomenon was also observed in *Arabidopsis thaliana* (L.) Heynh. (Lu and Christopher, 2008).

Reactive oxygen free radicals induced by Hg often cause damages of thiol oxidation and disulfide bond cross-linking. Nevertheless, PDILs and BiPs, both chaperones, are involved in preventing or repairing impaired proteins and relieving ER stress (Lu and Christopher, 2008; Han et al., 2012). Our previous experiments demonstrated that overexpression of a PDIL protein enhanced Hg tolerance in rice (Chen et al., 2012). BiPs and some PDILs were activated by Hg stress but kept at low levels with H_2S supplementation (Fig. 4). So the deposition of Hg by H_2S and its derivants mitigated the damage, and correspondingly resulted in the reduced expression of protective genes. These confirmed the reduced damage and the beneficial and protective effects of H_2S.

In leaves, other detoxifications also exist due to the abundant metabolisms. Hg stress caused evident pigment loss and H_2S did not much delay this degradation (Fig. 5). This was consistent with the findings by Zhang et al. (2009) that spraying with 100–800 μM NaHS had no extra protective effect on chlorophyll concentrations. In contrast, Bharwana et al. (2014) reported that 200 μM H_2S obviously ameliorated the decrease in Chla, Chlb and carotenoids caused by Pb stress. Moreover, Chen et al. (2011) found 100 μM H_2S resulted in great increases in chlorophyll content and photosynthetic rate in *Spinacia oleracea* L. leaves while higher concentrations H_2S inhibited photosynthesis. Nonetheless, the functional mechanisms of H_2S at low concentrations in plant photosynthesis remained unclear until now. Our investigation indicated that the

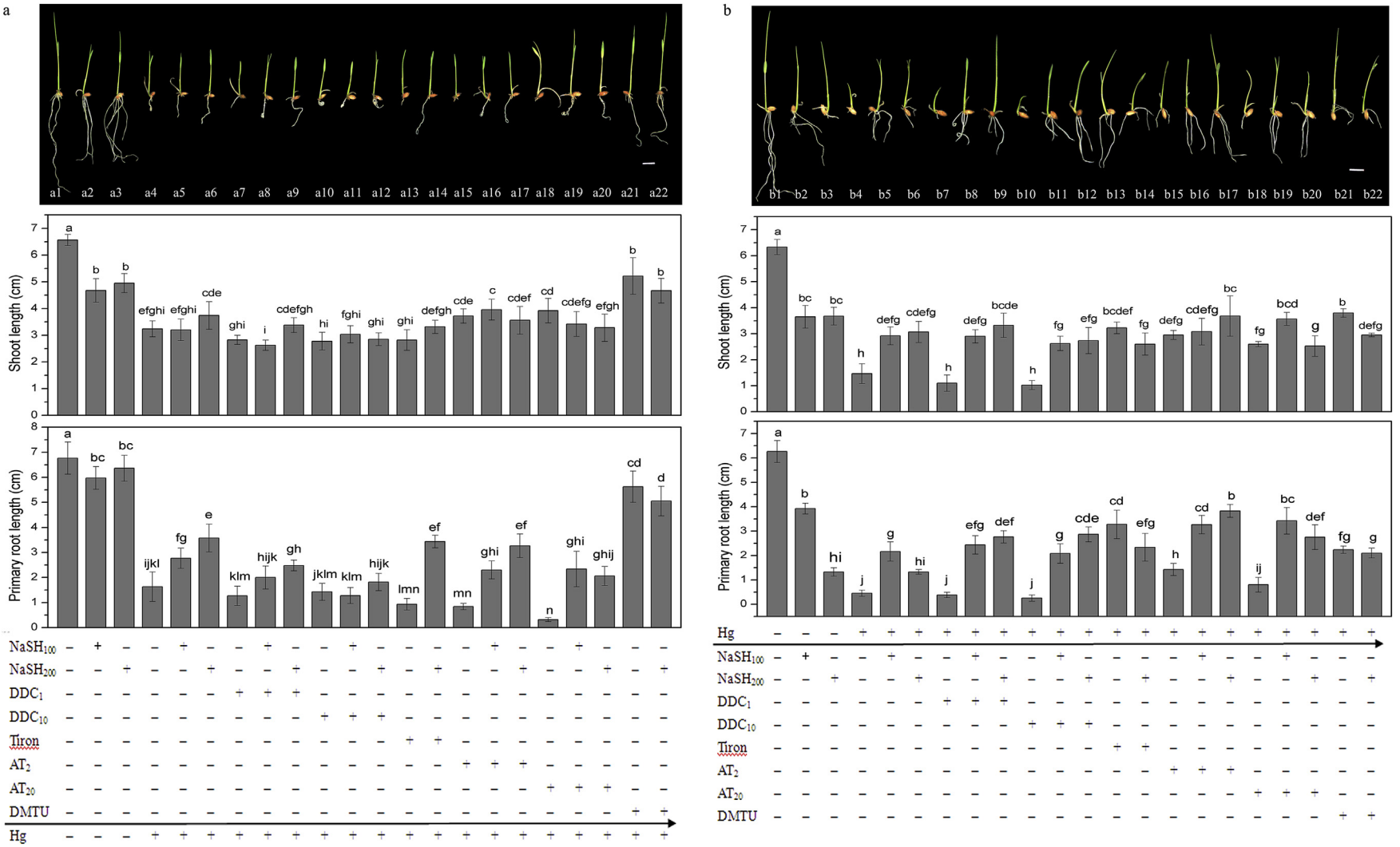

Fig. 8. Effects of H_2S, antioxidant enzyme inhibitors and ROS scavengers on growth of rice plantlets after seeds germination under Hg stress by means of agar culturing in sterilized bottles. 'a' means Hg aftertreatment while 'b' means Hg pretreatment. The long arrow means seelings were pretreated with the above reagents for 3 d and then followed the bellows in new media for another 3 d. Data are presented as mean ± SD (n = 10). Values with different lowercase mean the significant difference at $P < 0.05$ level.

decreased photosynthetic pigments may be explained by displacement of essential metal ions from biomolecules in photosynthetic pigments by Hg cations. The 100 or 200 μM H_2S could not counteract chlorophyll loss caused by Hg displacement in rice seedlings.

Since the initial toxic effect of Hg is characterized by oxidative damage, the antioxidant mechanisms in plants have been widely researched (Zhou et al., 2009; Lomonte et al., 2010; Sahu et al., 2012; Malar et al., 2015a,b). Hg induced a sharp generation of $O_2\cdot^-$ and H_2O_2, resulting in the increase of MDA and lipid peroxidation of biomembranes. The antioxidant systems of enzymatic and non-enzymatic antioxidants were launched immediately to abolish the ROS. Consistent with the preceding, increased H_2O_2 and MDA levels and accordingly enhanced activities of antioxidant enzymes (SOD, CAT, POD and APX) for ROS scavenging under $HgCl_2$ stress were also observed in our study (Figs. 6 and 7). In contrast, the Hg-induced accumulation of H_2O_2 was markedly reduced by the presence of NaHS, the H_2S donor (Fig. 6b). This coincides with other discoveries in plants related to other metals like Cu, Al, Cd, and Pb (Zhang et al., 2008; Chen et al., 2013; Bharwana et al., 2014; Shi et al., 2014; Mostofa et al., 2015). However, it is noteworthy that the activities of antioxidant enzymes were not enhanced by H_2S (Fig. 7). The same phenomena were also found by Chen et al. (2013) at 200 μM NaHS pre-application plus Al stress, the decreased SOD, POD and APX activities matched the reduced damage to membranes and lower H_2O_2 content. The authors suggested that H_2S diminish the need for antioxidant enzymes by maintaining low levels of $O_2\cdot^-$ and H_2O_2 in plant tissues. However, there was no explanation for how these occurred. All these findings suggested that 100 μM $HgCl_2$ could exert severe oxidative damage on rice seedlings while exogenous H_2S prevented free radicals stress not via antioxidant enzymes in this condition. So we speculated that H_2S might act as a ROS inhibitor or scavenger, similarly to NO (Singh et al., 2009; Saxena and Shekhawat, 2013; Al-Magableh et al., 2014; Chen et al., 2015) or as a reductant (Kabil et al., 2014), and thereby result in accelerated detoxification. This was definitively verified by applications of antioxidant enzyme inhibitors and ROS scavengers.

DDC can lead to dose-dependent inhibition of Cu/Zn-SOD activity in vivo through ligaturing and removing Cu (II) ions from its active site (Lushchak et al., 2005). Thus $O_2\cdot^-$ was not rapidly dismutated into H_2O_2 under the combination of Hg and DDC stress, so the growth was severely inhibited (Fig. 8). Surprisingly, H_2S greatly promoted root formation and elongation even in the presence of Hg plus DDC, indicating that H_2S might inhibit $O_2\cdot^-$ production. It could also be proved by the fact Tiron pretreatment had no obvious effect on Hg stress while NaHS do (Fig. 8). Tiron, an $O_2\cdot^-$ scavenger, can provide H to $O_2\cdot^-$ or the alcoxyl radical (Sharma and Mishra, 2006; Fang et al., 2012). On the other hand, the promotive effect of H_2S after-treatment was better than pretreatment (Fig. 8a7–a12, b7–b12), meaning that H_2S also acted as a downstream signal or reductant of $O_2\cdot^-$ to scavenge it. Moreover, with treatments of AT 2 and 20 μM AT, a specific inhibitor of catalase (Han et al., 2009), the H_2O_2 could not be immediately decomposed by CAT and the root elongation significantly retarded. However, under these conditions, H_2S presence participated in effective decomposition of H_2O_2 and thereby promoted root extension. DMTU, specific traps for H_2O_2, have been reported to prevent oxidative and DNA damage caused by Al or H_2O_2 (Chen et al., 2009; Murali Achary and Panda, 2010). It was also observed in the present study that DMTU could effectively scavenge the ROS caused by Hg stress and markedly promoted growth. All these data confirmed that H_2S could act as antioxidant and prevent oxidative stress by regulating $O_2\cdot^-$ and H_2O_2 productions.

H_2S and HS^- are potent reductants that are theoretically capable of clearing free radicals by single electron 'e' or hydrogen atom 'H' transfer (Olson, 2012). So it can detoxicate $O_2\cdot^-$ and H_2O_2 directly and its reductive properties is comparable to that of GSH and cysteine (Li and Lancaster, 2013). But this direct role is still in debate for its physiology concentration in vivo at submicromolar range is not paralleled with its antioxidant effect (Olson, 2012; Xie et al., 2016). Li and Lancaster (2013) mentioned some demonstrations had been attempted for the direct reaction of H_2S with either H_2O_2 or $O_2\cdot^-$, but there were some caveats and misunderstandings. Xie et al. (2016) suggested that H_2S may act as a trigger to start the antioxidant process. Therefore, location and precise quantification of H_2S require to be improved urgently. Alternatively, H_2S can suppress activity of NADPH oxidases (Nox), the only enzymes with the primary function of generating $O_2\cdot^-$ and H_2O_2, by S-sulfhydration or phosphorylation of p66Shc [a 66 kD Src homologous-collagen homologur (Shc) adaptor protein] and then inhibit H_2O_2 production (Xu et al., 2011; Al-Magableh et al., 2014; Li et al., 2016; Mistry et al., 2016; Xie et al., 2016). Moreover, there is a competition for thiols from H_2O_2, glutathione, NO and H_2S. Therefore, when the thiols-containing proteins are being modified by H_2S, they are no longer accessible to be modified by NO and H_2O_2 and avoiding the oxidative damages (Hancock and Whiteman, 2016). Additionally, H_2S increases nonenzymatic antioxidants, such as, GSH and Trx, to scavenge ROS directly (Bian et al., 2016). Or H_2S confers an enhanced ability to combat oxidative stress by activating OxyR, the master regulator mediating the cellular response to H_2O_2 (Wu et al., 2015). So we speculate that chemical property of H_2S enables it to act as a direct or indirect regulator of ROS and so prevents the oxidative damage caused by Hg ions.

5. Conclusion

In conclusion, NaHS (the H_2S donor) pretreatment increased expressions and contents of prominent thiol-containing compounds, including NPT and OsMT-1, to sequester Hg in roots of rice seedlings under Hg stress and inhibit Hg transport to shoots. Meanwhile, H_2S increased the level of transcription factor bZIP60 to trigger the expression of these stress response genes. Furthermore, H_2S could inhibit or scavenge peroxy radicals ($O_2\cdot^-$ and H_2O_2) even in the presence of SOD or CAT inhibitors (DDC and AT) and Hg supplementation, thus maintaining MDA and H_2O_2 at low levels and weakening the need for antioxidant enzymes. Therefore, H_2S effectively alleviated the Hg toxicity and significantly promoted growth of rice seedlings.

Author contributions

Z.C. experiment design and performance, result analysis and discussion, manuscript writing and revising; M.S.C. experiment performance, manuscript editing; M.J. molecular experiment analysis and manuscript revising.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

This work was supported by Taizhou University Scientific Research Project (2014PY024), Zhejiang Provincial Natural Science Foundation of China (3100246), Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation Open Projects (EEC2014-08), and Taizhou Municipal Innovation Team and Key Laboratory Independently Design and Technology Projects (1403ky06).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.11.027.

References

Aebi, H.E., 1983. Catalase. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, 3. Verlag Chemie, Weinheim, Germany, pp. 273–285.

Ali, B., Song, W.J., Hu, W.Z., Luo, X.N., Gill, R.A., Wang, J., Zhou, W.J., 2014. Hydrogen sulfide alleviates lead-induced photosynthetic and ultrastructural changes in oilseed rape. Ecotoxicol. Environ. Saf. 102, 25–33.

Al-Magableh, M.R., Kemp-Harper, B.K., Ng, H.H., Miller, A.A., Hart, J.L., 2014. Hydrogen sulfide protects endothelial nitric oxide function under conditions of acute oxidative stress in vitro. Schmiedeb. Arch. Pharmacol. 387, 67–74.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Bharwana, S.A., Ali, S., Farooq, M.A., Ali, B., Iqbal, N., Abbas, F., Ahmad, M.S.A., 2014. Hydrogen sulfide ameliorates lead-induced morphological, photosynthetic, oxidative damages and biochemical changes in cotton. Environ. Sci. Pollut. Res. 21, 717–731.

Bian, J.S., Olson, K.R., Zhu, Y.C., 2016. Hydrogen sulfide: biogenesis, physiology, and pathology. Oxid. Med. Cell. Longev. 2016, 6549625.

Bruce King, S., 2013. Potential biological chemistry of hydrogen sulfide (H_2S) with the nitrogen oxides. Free Radic. Biol. Med. 55, 1–7.

Chen, J., Wang, W.H., Wu, F.H., You, C.Y., Liu, T.W., Dong, X.J., He, J.X., Zheng, H.L., 2013. Hydrogen sulfide alleviates aluminum toxicity in barley seedlings. Plant Soil 362, 301–318.

Chen, J., Wu, F.H., Wang, W.H., Zheng, C.J., Lin, G.H., Dong, X.J., He, J.X., Pei, Z.M., Zheng, H.L., 2011. Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in *Spinacia oleracea* seedlings. J. Exp. Bot. 62, 4481–4493.

Chen, X.Y., Ding, X., Xu, S., Wang, R., Xuan, W., Cao, Z.Y., Chen, J., Wu, H.H., Ye, M.B., Shen, W.B., 2009. Endogenous hydrogen peroxide plays a positive role in the upregulation of heme oxygenase and acclimination to oxidative stress in wheat seedling leaves. J. Integr. Plant Biol. 51, 951–960.

Chen, Z., Pan, Y.H., Wang, S.S., Ding, Y.F., Yang, W.J., Zhu, C., 2012. Overexpression of a protein disulfide isomerase-like protein from *Methanothermobacter thermoautotrophicum* enhances mercury tolerance in transgenic rice. Plant Sci. 197, 10–20.

Chen, Z., Zhang, L., Zhu, C., 2015. Exogenous nitric oxide mediates alleviation of mercury toxicity by promoting auxin transport in roots or preventing oxidative stress in leaves of rice seedlings. Acta. Physiol. Plant 37, 194.

Christou, A., Manganaris, G.A., Papadopoulos, I., Fotopoulos, V., 2013. Hydrogen sulfide induces systemic tolerance to salinity and non-ionic osmotic stress in strawberry plants through modification of reactive species biosynthesis and transcriptional regulation of multiple defence pathways. J. Exp. Bot. 64, 1953–1966.

Dooley, F.D., Nair, S.P., Ward, P.D., 2013. Increased growth and germination success in plants following hydrogen sulfide administration. PLoS. One 8, e62048.

E, Z.G., Zhang, Y.P., Zhou, J.H., Wang, L., 2014. Mini review roles of the bZIP gene family in rice. Genet. Mol. Res. 13, 3025–3036.

Fang, T., Cao, Z., Li, J., Shen, W., Huang, L., 2014. Auxin-induced hydrogen sulfide generation is involved in lateral root formation in tomato. Plant Physiol. Biochem. 76, 44–51.

Fang, Y., Hu, X.H., Jia, Z.G., Xu, M.H., Guo, Z.Y., Gao, F.H., 2012. Tiron protects against UVB-induced senescence-like characteristics in human dermal fibroblasts by the inhibition of superoxide anion production and glutathione depletion. Australas. J. Dermatol. 53, 172–180.

Foley, R.C., Liang, Z.M., Singh, K.B., 1997. Analysis of type1 metallothionein cDNAs in *Vicia faba*. Plant Mol. Biol. 33, 583–591.

Guo, B., Liang, Y.C., Zhu, Y.G., Zhao, F.J., 2007. Role of salicylic acid in alleviating oxidative damage in rice roots (*Oryza sativa*) subjected to cadmium stress. Environ. Pollut. 147, 743–749.

Hajeb, P., Jinap, S., 2012. Reduction of mercury from mackerel fillet using combined solution of cysteine, EDTA, and sodium chloride. J. Agric. Food. Chem. 60, 6069–6076.

Han, X.H., Wang, Y.H., Liu, X., Jiang, L., Ren, Y.L., Liu, F., Peng, C., Li, J.J., Jin, X.M., Wu, F.Q., Wang, J.L., Guo, X.P., Zhang, X., Cheng, Z.J., Wan, J.M., 2012. The failure to express a protein disulphide isomerase-like protein results in a floury endosperm and an endoplasmic reticulum stress response in rice. J. Exp. Bot. 63, 121–130.

Han, Y.H., Moon, H.J., You, B.R., Kim, S.Z., Kim, S.H., Park, W.H., 2009. The effects of buthionine sulfoximine, diethyldithiocarbamate or 3-amino-1,2,4-triazole on propyl gallate-treated HeLa cells in relation to cell growth, reactive oxygen species and glutathione. Int. J. Mol. Med. 24, 261–268.

Hancock, J.T., Whiteman, M., 2016. Hydrogen sulfide signaling: interactions with nitric oxide and reactive oxygen species. Ann. N.Y. Acad. Sci. 1365, 5–14.

Jana, S.C.M., 1981. Glycolate metabolism of three submerged aquatic angiosperms during aging. Aquat. Bot. 12, 345–354.

Jin, Z.P., Shen, J.J., Qiao, Z.J., Yang, G.D., Wang, R., Pei, Y.X., 2011. Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana*. Biochem. Biophys. Res. Co. 414, 481–486.

Kabil, O., Motl, N., Banerjee, R., 2014. H_2S and its role in redox signaling. BBA-Proteins Proteom. 1844, 1355–1366.

Kim, H.S., Jung, M.C., 2012. Mercury contamination in agricultural soils from abandoned metal mines classified by geology and mineralization. Environ. Geochem. Health 34, 55–69.

Kotrba, P., Najmanova, J., Macek, T., Ruml, T., Mackova, M., 2009. Genetically modified plants in phytoremediation of heavy metal and metalloid soil and sediment pollution. Biotechnol. Adv. 27, 799–810.

Li, L., Wang, Y.Q., Shen, W.B., 2012a. Roles of hydrogen sulfide and nitric oxide in the alleviation of cadmium-induced oxidative damage in alfalfa seedling roots. Biometals 25, 617–631.

Li, L.N., Li, M.X., Li, Y.Y., Sun, W.M., Wang, Y.H., Bai, S.Z., Li, H.X., Wu, B., Yang, G.D., Wang, R., Wu, L.Y., Li, H.Z., Xu, C.G., 2016. Exogenous H_2S contributes to recovery of ischemic post-conditioning-induced cardioprotection by decrease of ROS level via down-regulation of NF-κB and JAK2-STAT3 pathways in the aging cardiomyocytes. Cell. Biosci. 6, 26.

Li, Q., Lancaster Jr., J.R., 2013. Chemical foundations of hydrogen sulfide biology. Nitric Oxide 35, 21–34.

Li, Z.G., Gong, M., Xie, H., Yang, L., Li, J., 2012b. Hydrogen sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (*Nicotiana tabacum* L) suspension cultured cells and involvement of Ca^{2+} and calmodulin. Plant Sci. 185, 185–189.

Li, Z.G., Yang, S.Z., Long, W.B., Yang, G.X., Shen, Z.Z., 2013. Hydrogen sulphide may be a novel downstream signal molecule in nitric oxide-induced heat tolerance of maize (*Zea mays* L.) seedlings. Plant Cell. Environ. 36, 1564–1572.

Linšak, Ž., Linšak, D.T., Špirić, Z., Srebočan, E., Glad, M., Milin, Č., 2013. Effects of mercury on glutathione and glutathione-dependent enzymes in hares (*Lepus europaeus* Pallas). J. Environ. Sci. Health A 48, 1325–1332.

Lomonte, C., Sgherri, C., Baker, A.J.M., Kolev, S.D., Navari-Izzo, F., 2010. Antioxidative response of *Atriplex codonocarpa* to mercury. Environ. Exp. Bot. 69, 9–16.

Lu, D.P., Christopher, D.A., 2008. Endoplasmic reticulum stress activates the expression of a sub-group of protein disulfide isomerase genes and AtbZIP60 modulates the response in *Arabidopsis thaliana*. Mol. Genet. Genomics 280, 199–210.

Lushchak, V., Semchyshyn, H., Lushchak, O., Mandryk, S., 2005. Diethyldithiocarbamate inhibits in vivo Cu,Zn-superoxide dismutase and perturbs free radical processes in the yeast *Saccharomyces cerevisiae* cells. Biochem. Biophys. Res. Co. 338, 1739–1744.

Malar, S., Sahi, S.V., Favas, P.J.C., Venkatachalam, P., 2015a. Assessment of mercury heavy metal toxicity-induced physiochemical and molecular changes in *Sesbania grandiflora* L. Int. J. Environ. Sci. Technol. 12, 3273–3282.

Malar, S., Sahi, S.V., Favas, P.J.C., Venkatachalam, P., 2015b. Mercury heavy-metal-induced physiochemical changes and genotoxic alterations in water hyacinths [*Eichhornia crassipes* (Mart.)]. Environ. Sci. Pollut. Res. 22, 4597–4608.

Manna, P., Jain, S.K., 2013. L-cysteine and hydrogen sulfide increase PIP3 and AMPK/PPARg expression and decrease ROS and vascular inflammation markers in high glucose treated human U937 monocytes. J. Cell. Biochem. 114, 2334–2345.

Mendoza-Cozatl, D.G., Butko, E., Springer, F., Torpey, J.W., Komives, E.A., Kehr, J., Schroeder, J.I., 2008. Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*, A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. Plant J. 54, 249–259.

Meng, D.K., Chen, J., Yang, Z.M., 2011. Enhancement of tolerance of Indian mustard (*Brassica juncea*) to mercury by carbon monoxide. J. Hazard. Mater 186, 1823–1929.

Mistry, R.K., Murray, T.V.A., Prysyazhna, O., Martin, D., Burgoyne, J.R., Santos, C., Eaton, P., Shah, A.M., Brewer, A.C., 2016. Transcriptional regulation of cystathionine-γ-Lyase in endothelial cells by NADPH oxidase 4-dependent signaling. J. Biol. Chem. 291, 1774–1788.

Mostofa, M.G., Rahman, A., Ansary, M.M.U., Watanabe, A., Fujita, M., Tran, L.S.P., 2015. Hydrogen sulfide modulates cadmium-induced physiological and biochemical responses to alleviate cadmium toxicity in rice. Sci. Rep. 5, 14078.

Murali Achary, V.M., Panda, B.B., 2010. Aluminium-induced DNA damage and adaptive response to genotoxic stress in plant cells are mediated through reactive oxygen intermediates. Mutagenesis 25, 201–209.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell. Physiol. 22, 867–880.

Nezhad, R.M., Shahpiri, A., Mirlohi, A., 2013. Heterologous expression and metal-binding characterization of a type 1 metallothionein isoform (OsMTI-1b) from rice (*Oryza sativa*). Protein J. 32, 131–137.

Olson, K.R., 2012. A practical look at the chemistry and biology of hydrogen sulfide. Antioxid. Redox. Sign 17, 32–44.

Qiu, G.L., Feng, X.B., Li, P., Wang, S.F., Li, G.H., Shang, L.H., Fu, X.W., 2008. Methylmercury accumulation in rice (*Oryza sativa* L.) grown at abandoned mercury mines in Guizhou, China. J. Agric. Food. Chem. 56, 2465–2468.

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., Watkins, C.B., 1997. Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes. Salicylic acid-mediated oxidative damage requires H_2O_2. Plant Physiol. 115, 137–149.

Sahu, G.K., Upadhyay, S., Sahoo, B.B., 2012. Mercury induced phytotoxicity and oxidative stress in wheat (*Triticum aestivum* L.) plants. Physiol. Mol. Biol. Plants 18, 21–31.

Saxena, I., Shekhawat, G.S., 2013. Nitric oxide (NO) in alleviation of heavy metal induced phytotoxicity and its role in protein nitration. Nitric Oxide 32, 13–20.

Shan, C.J., Dai, H.P., Sun, Y.F., 2012. Hydrogen sulfide protects wheat seedlings

against copper stress by regulating the ascorbate and glutathione metabolism in leaves. Aust. J. Crop. Sci. 6, 248–254.
Shan, C.J., Zhang, S.L., Li, D.F., Zhao, Y.Z., Tian, X.L., Zhao, X.L., Wu, Y.X., Wei, X.Y., Liu, R.Q., 2011. Effects of exogenous hydrogen sulfide on the ascorbate and glutathione metabolism in wheat seedlings leaves under water stress. Acta. Physiol. Plant 33, 2533–2540.
Sharma, P., Mishra, K.P., 2006. Aluminum-induced maternal and developmental toxicity and oxidative stress in rat brain: response to combined administration of tiron and glutathione. Reprod. Toxicol. 21, 313–321.
Shi, H.T., Ye, T.T., Chan, Z.L., 2013. Exogenous application of hydrogen sulfide donor sodium hydrosulfide enhanced multiple abiotic stress tolerance in bermudagrass (*Cynodon dactylon* (L). Pers.). Plant Physiol. Biochem. 71, 226–234.
Shi, H.T., Ye, T.T., Chan, Z.L., 2014. Nitric oxide-activated hydrogen sulfide is essential for cadmium stress response in bermudagrass (*Cynodon dactylon* (L). Pers.). Plant Physiol. Biochem. 74, 99–107.
Shiyab, S., Chen, J., Han, F.X., Monts, D.L., Matta, F.B., Gu, M.M., Su, Y., Masad, M.A., 2009. Mercury-induced oxidative stress in Indian mustard (*Brassica juncea* L.). Environ. Toxicol. 24, 462–471.
Singh, H.P., Kaur, S., Batish, D.R., Sharma, V.P., Sharma, N., Kohli, R.K., 2009. Nitric oxide alleviates arsenic toxicity by reducing oxidative damage in the roots of *Oryza sativa* (rice). Nitric Oxide 20, 289–297.
Sun, J., Wang, R.G., Zhang, X., Yu, Y.C., Zhao, R., Li, Z.Y., Chen, S.L., 2013. Hydrogen sulfide alleviates cadmium toxicity through regulations of cadmium transport across the plasma and vacuolar membranes in *Populus euphratica* cells. Plant Physiol. Biochem. 65, 67–74.
Usha, B., Venkataraman, G., Parida, A., 2009. Heavy metal and abiotic stress inducible metallothionein isoforms from *Prosopis juliflora* (SW) D.C. show differences in binding to heavy metals in vitro. Mol. Genet. Genomics 281, 99–108.
Wang, B.L., Shi, L., Li, Y.X., Zhang, W.H., 2010. Boron toxicity is alleviated by hydrogen sulfide in cucumber (*Cucumis sativus* L.) seedlings. Planta 231, 1301–1309.
Wang, Z.Q., Li, G.Z., Gong, Q.Q., Li, G.X., Zheng, S.J., 2015. OsTCTP, encoding a translationally controlled tumor protein, plays an important role in mercury tolerance in rice. BMC. Plant Biol. 15, 123.
Wu, G.F., Wan, F., Fu, H.H., Li, N., Gao, H.C., 2015. A matter of timing: contrasting effects of hydrogen sulfide on oxidative stress response in *Shewanella oneidensis*. J. Bacteriol. 197, 3563–3572.
Xie, Z.Z., Liu, Y., Bian, J.S., 2016. Hydrogen sulfide and cellular redox homeostasis. Oxid. Med. Cell. Longev. 2016, 6043038.
Xu, Z.S., Wang, X.Y., Xiao, D.M., Hu, L.F., Lu, M., Wu, Z.Y., Bian, J.S., 2011. Hydrogen sulfide protects MC3T3-E1 osteoblastic cells against H_2O_2-induced oxidative damage-implications for the treatment of osteoporosis. Free Radic. Biol. Med. 50, 1314–1323.
Yoshida, S., Forno, D.A., Cock, J.H., Gomez, K.A., 1976. Laboratory Manual for Physiological Studies of Rice. International Rice Research Institute, Los Banōs, Philippines.
Zhang, H., Hu, L.Y., Hu, K.D., He, Y.D., Wang, S.H., Luo, J.P., 2008. Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. J. Integr. Plant Biol. 50, 1518–1529.
Zhang, H., Hu, L.Y., Li, P., Hu, K.D., Jiang, C.X., Luo, J.P., 2010. Hydrogen sulfide alleviated chromium toxicity in wheat. Biol. Plant 54, 743–747.
Zhang, H., Ye, Y.K., Wang, S.H., Luo, J.P., Tang, J., Ma, D.F., 2009. Hydrogen sulfide counteracts chlorophyll loss in sweetpotato seedling leaves and alleviates oxidative damage against osmotic stress. Plant Growth Regul. 58, 243–250.
Zhou, Z.S., Guo, K., Elbaz, A.A., Yang, Z.M., 2009. Salicylic acid alleviates mercury toxicity by preventing oxidative stress in roots of *Medicago sativa*. Environ. Exp. Bot. 65, 27–34.