



Research article

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

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fide (H)



H_2S) plays multifunctional roles in mediating a variety of responses to abiotic stresses. The effects of exogenous H_2S on rice (*O. 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.

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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 ($-\text{SH}$) 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 *O. 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.

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been identified as a third endogenous signal molecule (Li et al., 2013). In plants, H₂S 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, H₂S 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 H₂S reverse the effect of B on cell wall related pectin methylesterase (PME) and expansions; Chen et al. (2013) found H₂S 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 H₂S 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 H₂S 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 H₂S and NO, which was responsible for the enhanced Cd tolerance of alfalfa, and Shi et al. (2014) confirmed that H₂S acts as a downstream component of NO for Cd detoxification. Furthermore, H₂S might act as a mediator in auxin signaling to launch lateral root formation (Fang et al., 2014), or interact with Ca²⁺ and calmodulin signaling (Li et al., 2012b). Nevertheless, the precise role of the natural detoxicant, H₂S, is still elusive. The metabolic pathways need to be further illuminated. Moreover, no information could be found about the interaction of H₂S and Hg contamination in plants.

In the present study, NaHS, a fast releaser of H₂S, was applied to study the effects of exogenous H₂S on growth of rice seedlings under mercury chloride (HgCl₂) stress. And the possible inhibition, reduction or precipitation reactions of H₂S 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 material

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 HgCl₂ 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 Na₂-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 HNO₃/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 chlorophyll and carotenoids

Chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b) 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. Determination of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂)

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). H₂O₂ level was determined in terms of the regular method (Jana, 1981; Chen et al., 2015).

2.5. Analysis of antioxidant enzymes

Enzyme activities were estimated as follows: SOD (the)–297.1(method)–241.6(described)–2015.9

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

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 $26.6 \text{ L mmol}^{-1} \text{ cm}^{-1}$.

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 $\text{Na}_2\text{-EDTA}$, 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 $2.8 \text{ L mmol}^{-1} \text{ cm}^{-1}$ was used for ascorbate oxidation.

2.6. *Qua fica* - (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 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 $13.6 \text{ L mmol}^{-1} \text{ cm}^{-1}$ (Mendoza-Cozatl et al., 2008).

2.7. *R a - u a a PCR a a*

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. a a*), OsMTs (metallothioneins of *O. a a*), BiPs (immunoglobulin heavy-chain binding proteins), bZIPs (basic leucine zipper, membrane-associated transcription factors), OsPDILs (protein disulfide isomerase-like proteins of *O. a a*) were listed in table of Table S1.

2.8. *E c H₂S, a a b a ROS* *ca c a*

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 $\text{O}_2\cdot^-$ scavenger; DMTU, *N,N'*-Dimethylthiourea, α -Dimethylornithine

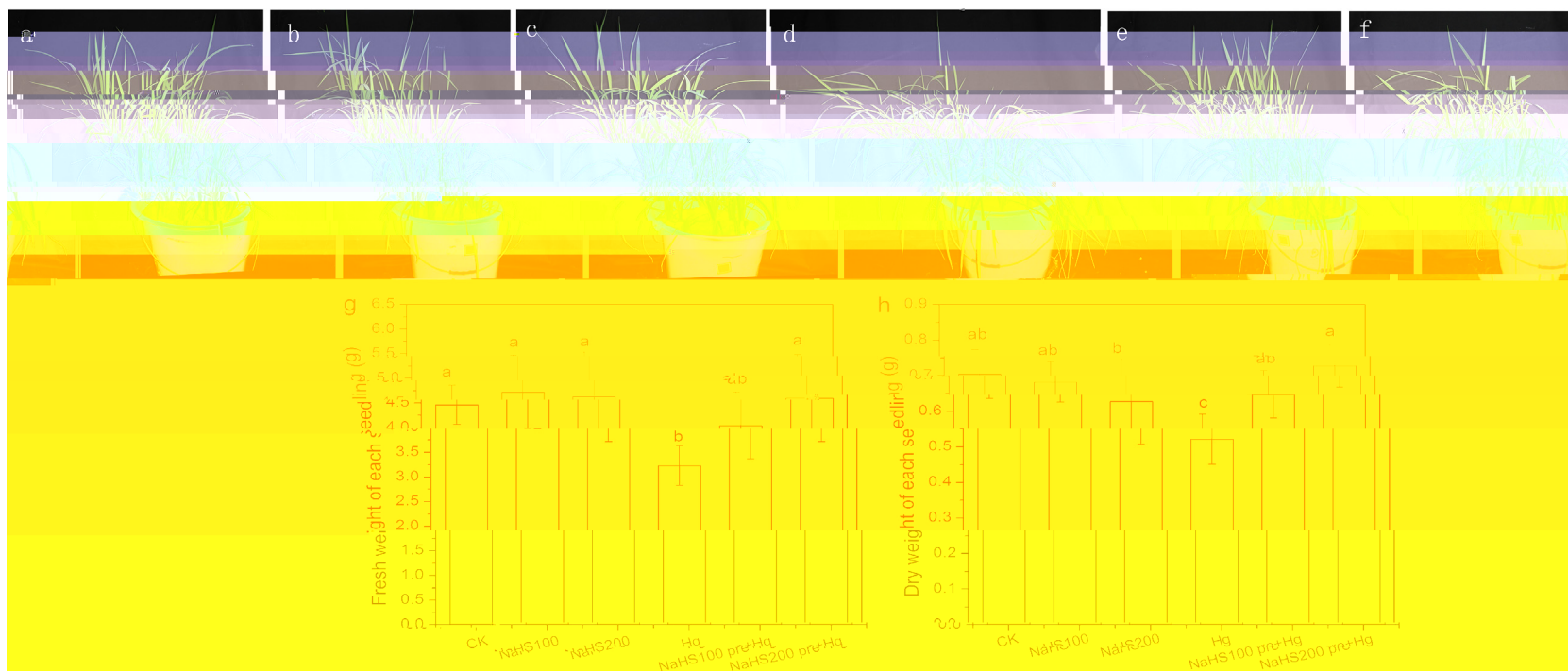


Fig. 1. Effects of NaHS pretreatments on growth of rice seedlings at five-leaf stage. a, NaHS and HgCl_2 0 μM ; b, NaHS 100 μM ; c, NaHS 200 μM ; d, HgCl_2 100 μM ; e, NaHS 100 μM pre + HgCl_2 100 μM ; f, NaHS 200 μM pre + HgCl_2 100 μM ; g, h, fresh and dry weight of each seedling. 'NaHS pre' means 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 \pm SD (n = 8). Values with different lowercase mean the significant difference at $P < 0.05$ level.

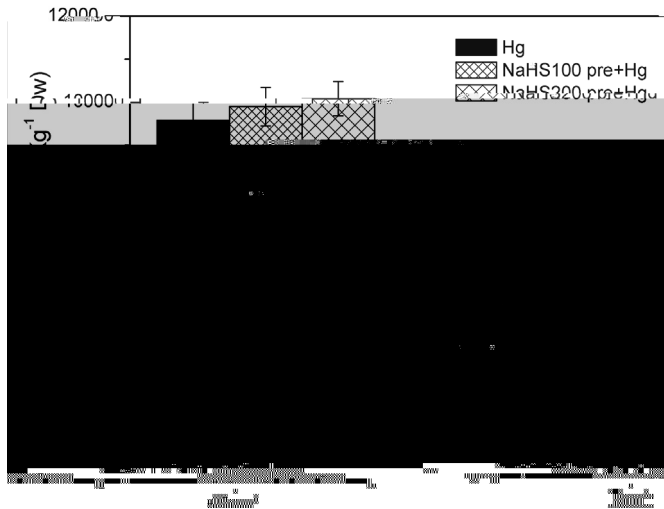


Fig. 2. Effects of H₂S (NaHS 100 or 200 µM) on Hg accumulations in different parts of rice seedlings at five-leaf stage with 100 µM HgCl₂ treatments for 3 d. Data are presented as mean ± SD (n = 4). The significant level of the difference between Hg and NaHS pre + Hg treatment is indicated by ** (P < 0.01).

3.2. E ν H₂S a a H c b
a

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 H₂S in leaves may be partly attributed to inhibited Hg transport.

3.3. E ν H₂S a -c a c ν
ν H

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 *O PCS1*, *O MT-1*, *O MT-2*, *B P-1*, *B P-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, H₂S had different impacts on expression of these genes. The expression of *O MT-1* dramatically increased with application of exogenous H₂S 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 *O PCS1*, *O MT-2*, *B P-1*, *bZIP17*, *bZIP28* and *PDIL1-3* were not strengthened by H₂S but significantly reduced, as compared to Hg treatment alone. Moreover, expressions of *B P-2*, *PDIL1-1*, *PDIL5-1*, *PDIL5-2* and *PDIL5-3* sharply declined to the levels even lower than controls, indicating the depressive effects of H₂S on them (Fig. 4). Additionally, expressions of *O MT-3*, *PDIL2-3* and *PDIL5-4* were unchanged by the addition of Hg or H₂S (Fig. 4).

3.4. E c H₂S c c a
ν H

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 H₂S pretreatment had no obvious effects on the synthesis of photosynthetic pigments (Fig. 5a–c).

3.5. E ν H₂S c a MDA a H₂O₂ a
cav b H

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, H₂O₂, a ROS and also a cellular messenger, was not induced by NaHS treatment but was stimulated by Hg stress. The generation of H₂O₂ reached a peak when seedlings were exposed to the HgCl₂ solution. In contrast, if seedlings were pretreated with NaHS for 24 h, they generated much less H₂O₂ (Fig. 6b). The low oxidative damage demonstrated the protective effect of H₂S in leaves.

3.6. E c H₂S a a ac

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 O₂^{·-} and H₂O₂ 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 H₂S 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 H₂S.

3.7. C - a H₂S a ROS c

ROS scavengers and antioxidant enzymes inhibitors were used to verify the protection from H₂S against oxidative damages. The 100 or 200 µM H₂S 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 H₂S (Fig. 8a7–a12, b7–b12) and the root length markedly increased. However, DDC at higher concentration of 1 mM might induce enough damage that H₂S could not notably rescue the growth (data not shown). Pretreatment of 1 mM tiron did not effectively eliminate subsequent

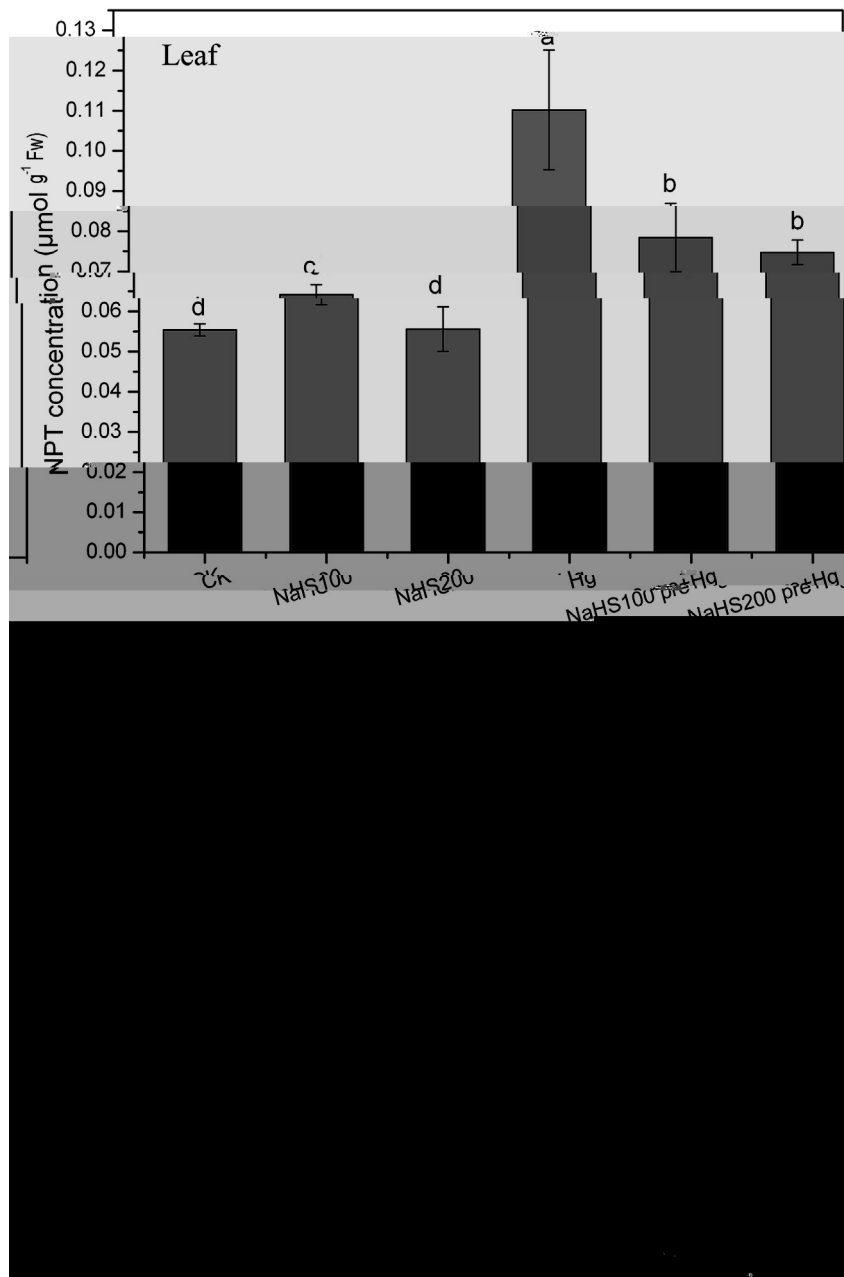


Fig. 3. Effects of H₂S (NaHS 100 or 200 µM) pretreatment on non-protein thiol (NPT) contents in leaves and roots of rice seedlings at five-leaf stage with or without 100 µM HgCl₂ treatments for 3 days. Data are presented as mean ± SD (n = 4). Values with different lowercase mean the significant difference at P < 0.05 level.

generation of O₂^{·-} caused by Hg, but if H₂S 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 O₂^{·-} caused by Hg treatment and attenuate the need of H₂S (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). H₂S notably eliminated H₂O₂ stress caused by AT and Hg, and significantly promoted growth (Fig. 8a15–a20, b15–b20). Pretreatment or after-treatment of the H₂O₂ scavenger, DMTU, greatly eliminated H₂O₂ under Hg stress and significantly increased the root and shoot length (Fig. 8a21 and b21). When H₂O₂ was effectively controlled by DMTU, the action of H₂S activated by H₂O₂ 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). H₂S 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).

In the present study, the symptoms of damage in rice seedlings caused by Hg were markedly relieved by pretreatment of 100 or 200 μ M NaHS, indicating that H₂S 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 H₂S. The results showed that H₂S 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 *Erica arborea* (Mart.), *Jatropha curcas* L., and *Scorpiocarpus* L.f. were found TF values >1 (Malar et al., 2015b). In this study, Hg was mainly accumulated in rice roots and H₂S did not inhibit its absorption (Fig. 2). Wang et al. (2010) reported H₂S 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 H₂S decreased Cd accumulation in *Panicum virgatum* 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 H₂S 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, H₂S 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, H₂S is endogenously generated from cysteine and homocysteine via desulfhydrases, including L-cysteine desulfhydrases (LCD) and D-cysteine desulfhydrase (DCD) (Jin et al., 2011). And H₂S is quickly oxidated to persulfide (hydrodisulfide)



... 5. Effects of H₂S (NaHS 100 or 200 μM) pretreatment on photosynthetic pigments contents in leaves of rice seedlings at five-leaf stage with or without 100 μM HgCl₂ 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.

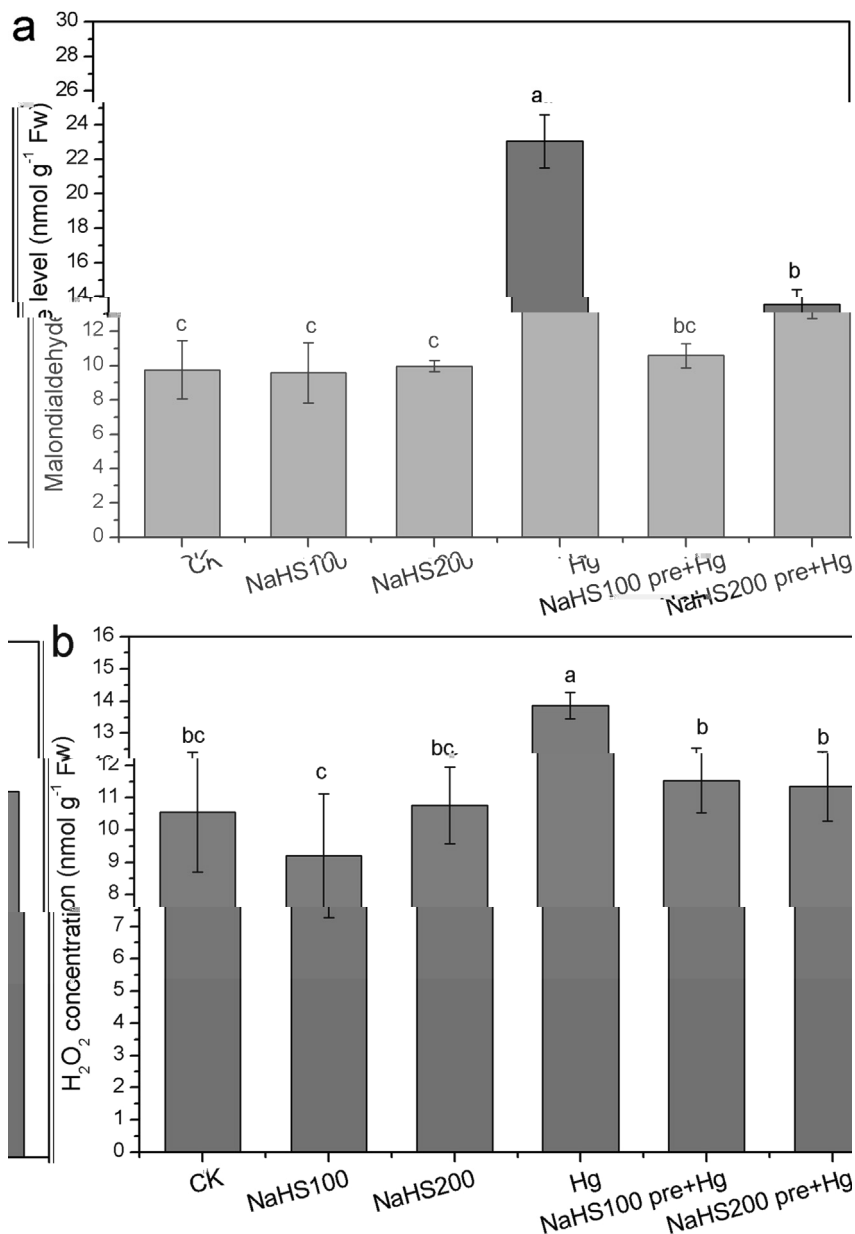


Fig. 6. Effects of H₂S (NaHS 100 or 200 μM) pretreatment on malondialdehyde (a) and H₂O₂ (b) levels in leaves of rice seedlings at five-leaf stage cultured hydroponically with or without 100 μM HgCl₂ treatments for 3 d. Data are presented as mean ± SD (n = 3). Values with different lowercase mean the 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 H₂S 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, H₂S 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 Cd²⁺/Ni²⁺ > Zn²⁺ > Cu²⁺, while OsMTI-1a preferred to bind Zn²⁺ (Nezhad et al., 2013). Similarly, three *Vca aba* L. MT isoforms and three *Pvufl a* (Sw.) D.C. MT isoforms were found to have greater binding affinity for Cd²⁺ than for Zn²⁺ and Cu²⁺ (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 H₂S and MTs was also analyzed. Hg exposure significantly increased *O MT-1* and *O MT-2* expression by 53 and 202%, respectively, while it had no impact on *O MT-3*, in comparison with control. Application of H₂S sharply enhanced the expression of *O MT-1*, as much as 6.89-fold compared with Hg treatment alone, but did not increase the level of *O MT-2* and *O MT-3*. These data suggest the H₂S 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

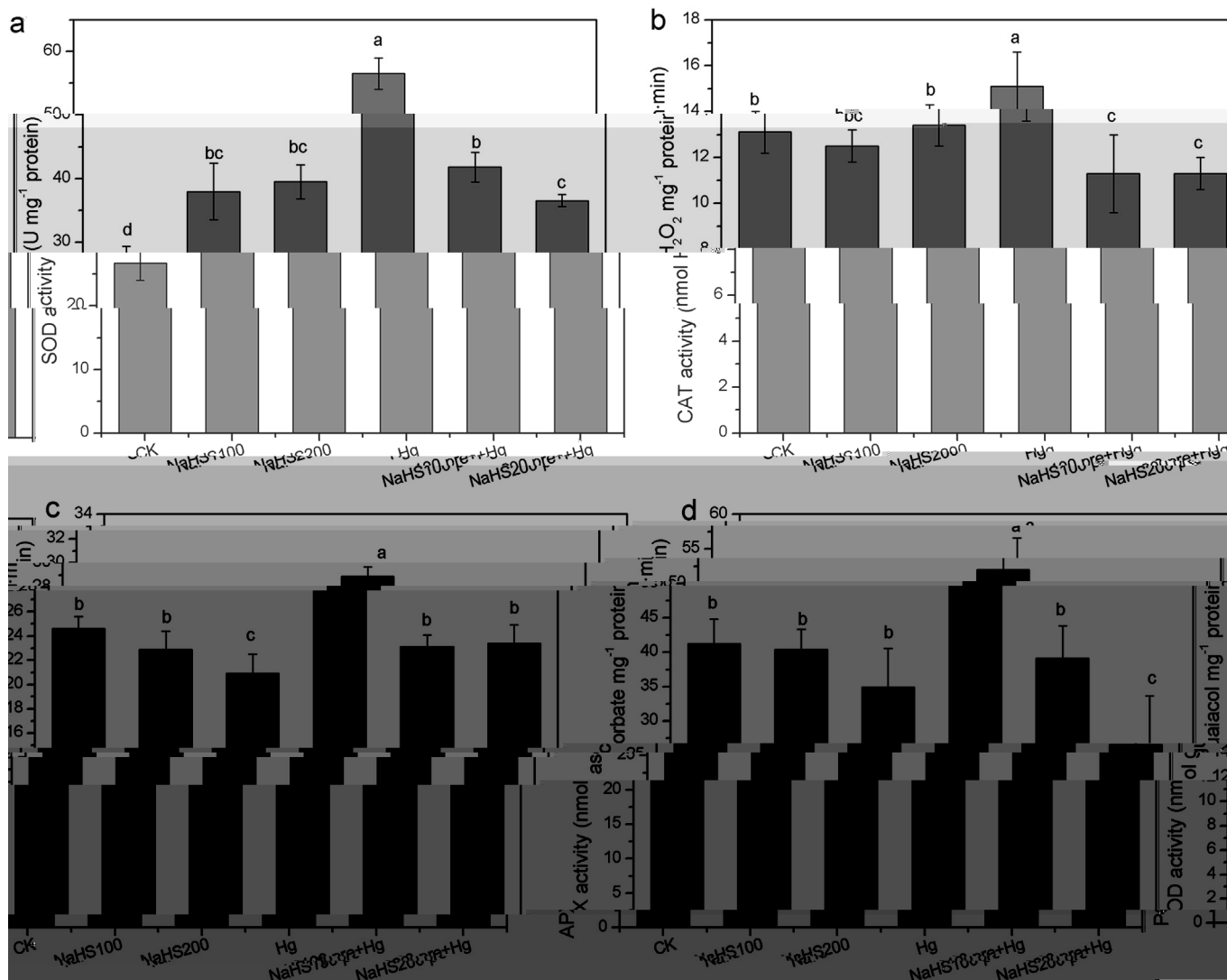


Fig. 7. Activities analysis of antioxidant enzymes, including SOD (a), CAT (b), POD (c), and APX (d), in leaves of rice seedlings at five-leaf stage under Hg or NaHS pre + Hg treatments. Data are presented as mean \pm SD ($n = 4$). Values with different lowercase mean the significant difference at $P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

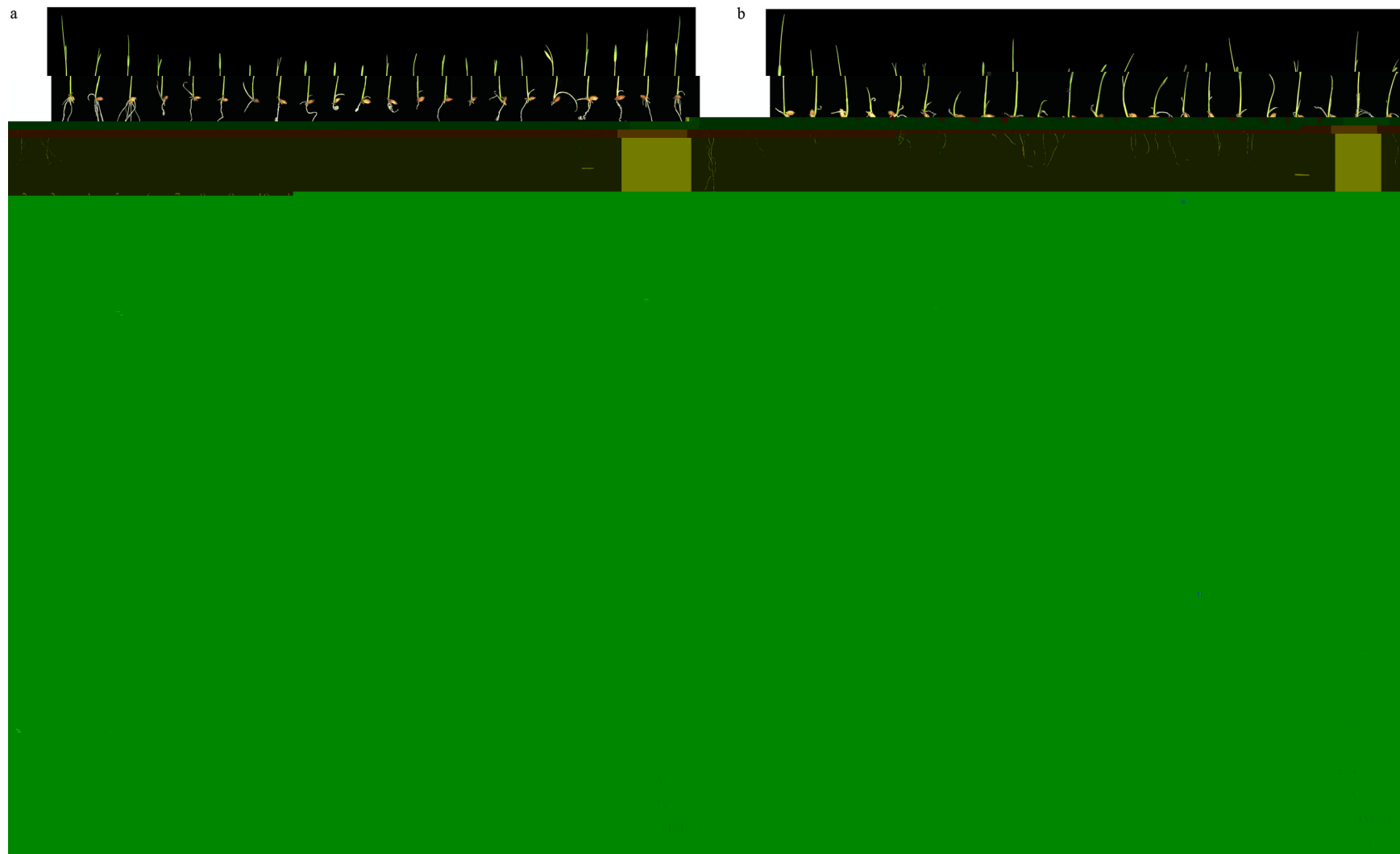
synthesized by PC synthases (PCS) from GSH or its homologs (Kotrba et al., 2009). However, *O* PCS1 was activated by Hg treatment alone but not strengthened by H₂S. Since about 81.5% of H₂S would dissociate into H⁺, HS⁻ and S²⁻, while 18.5% remain as undissociated molecules in plant cells (Al-Magableh et al., 2014; Xie et al., 2016), no rise of *O* PCS1 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). *O* bZIP17, *O* bZIP28 and *O* bZIP60 were all induced by Hg stress to trigger protective protein synthesis. H₂S amplified the transcription of *O* bZIP60, the prominent signal factor (Fig. 4). An analogous phenomenon was also observed in *A. baumannii* (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 H₂S supplementation (Fig. 4). So the deposition of Hg by H₂S and its derivatives 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 H₂S.

In leaves, other detoxifications also exist due to the abundant metabolisms. Hg stress caused evident pigment loss and H₂S did not much delay this degradation (Fig. 5). This was consistent with the findings by Zhang et al. (2009) that spraying with 100–800 μ M NaHS had no extra protective effect on chlorophyll concentrations. In contrast, Bharwana et al. (2014) reported that 200 μ M H₂S obviously ameliorated the decrease in Chla, Chlb and carotenoids caused by Pb stress. Moreover, Chen et al. (2011) found 100 μ M H₂S resulted in great increases in chlorophyll content and photosynthetic rate in *S. acuminata* L. leaves while higher concentrations H₂S inhibited photosynthesis. Nonetheless, the functional mechanisms of H₂S at low concentrations in plant photosynthesis remained unclear until now. Our investigation indicated that the



... 8. Effects of H₂S, 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 seedlings 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 \pm 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 $\text{O}_2\cdot^-$ 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 $\text{O}_2\cdot^-$ 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 Shekhar, 2015; Magablehe et al., 2015) and

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

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