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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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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.

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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