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Hydrogen Sulfide Stimulates Wheat Grain Germination and Counteracts the Effect of Oxidative Damage Caused by Salinity Stress

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Hydrogen sulfide (H_2S) has been recently found to be a gaseous signaling molecule in plants. In this work, we studied the role of H_2S in alleviating salinity stress during wheat grain germination (*Triticum aestivum* L. Yangmai 158). Pretreatment with NaHS, a H_2S donor, during wheat grain imbibition, could significantly attenuate the inhibitory effect of salinity stress on wheat germination. NaHS-pretreated grain showed higher amylase and esterase activities than water control. NaHS pretreatment differentially stimulated the activities of catalase (CAT), guaiacol peroxidase (POD) and ascorbate peroxidase (APX), decreased the level of malondialdehyde (MDA) and reduced NaCl-induced changes in plasma membrane integrity in the radicle tips of seedlings compared with water control. We conclude that H_2S plays an important role in protecting wheat grain from oxidative damage induced by salinity stress.

Keywords: antioxidant enzymes, hydrogen sulfide, salinity stress, wheat *(Triticum aestivum)* grain germination

Introduction

High salinity can inhibit cereal grain germination by affecting diverse physiological processes including osmotic effects, specific-ion toxicity and nutritional disorders (Zhu 2001; 2002). Salt stress can also cause oxidative stress due to the production of reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radicals (Leshem et al. 2007). Excessive ROS can result in oxidative damage to lipids, proteins and DNA. However, plants have developed both enzymatic and non-enzymatic mechanisms to scavenge the overproduction of ROS. Antioxidant en-

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zymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POD) are mobilized for scavenging ROS, thereby avoiding the oxidative stress caused by salt stress (Sekmen et al. 2007).

Hydrogen sulfide (H_2S), like nitric oxide (NO) and carbon monoxide (CO) is a gaseous transmitter in mammalian cells (Wang 2012). Similar to NO and CO, H_2S at low concentrations participates in various biological processes in animal cells (Yang et al. 2008; Wang 2012). In plants, H_2S can be produced from cysteine via a reversible O-acetylserine (thiol) lyase (OAS-TL) reaction and some desulfhydrases (Rausch and Wachter 2005). L-cysteine desulfhydrase expression can be induced upon pathogen attack, suggesting a role of H_2S in pathogen attack (Bloem et al. 2005).

H_2S has been implicated in various physiological processes in plants such as adventitious root organogenesis, stomatal movement, postharvest senescence of fruits and vegetables, flower senescence, and cell autophagy (Zhang et al. 2009; García-Mata and Lamattina 2010; Zhang et al. 2011; Álvarez et al. 2012; Hu et al. 2012; Gao et al. 2013; Lisjak et al. 2013). Evidence also shows that H_2S counteracts multiple abiotic stresses including copper, aluminum, salinity and drought stresses, by activating endogenous antioxidant systems (Zhang et al. 2008; Zhang et al. 2010; Jin et al. 2011; Wang et al. 2012; Jin et al. 2013). However, whether H_2S participates in the antioxidant response to salt stress in wheat is still unknown. In this study, the effects of H_2S pretreatment on wheat grain germination under salinity stress are described and we describe an important role of H_2S in protecting wheat grain from oxidative damage induced by salinity stress.

Materials and Methods

Materials and treatments

Wheat grain used in this research (*Triticum aestivum* L. cv. Yangmai 158) were supplied by the Jiangsu Academy of Agricultural Sciences, Jiangsu Province, China. NaHS (sodium hydrosulfide, Sigma) was used as the H_2S donor (Zhang et al. 2008). Wheat grain were sterilized with 0.1% $HgCl_2$ (w/v) for 3 min and washed extensively with distilled water, and dried with filter paper. To investigate the inhibitory effect of salinity on wheat grain germination, grain were allocated randomly in Petri dishes (9.0 cm diameter × 1.2 cm depth, 50 grain per dish) at 28 ºC and treated with 0, 0.08, 0.16, 0.24, 0.32, 0.4 M NaCl for 48 h. Germination percentage was calculated as a standard that the length of the coleoptile or radicle exceeds 50% of grain length. The length of coleoptiles and radicles and the number of radicles were recorded. Preliminary experiments showed that 0.16 M NaCl caused obvious inhibition of wheat grain germination and this concentration was chosen for further experiments.

To explore possible effect of H_2S on salinity-induced inhibition of grain germination, wheat grain were pretreated with 0.0, 0.6, 1.2, 1.8, 2.4 or 3.0 mM NaHS for 12 h, and subsequently subjected to 0.16 M NaCl for a further 48 h. All solutions were changed every 12 h. The optimal NaHS concentration that alleviated the germination of wheat grain under salinity stress was found by calculating germination percentage.

Grain were pretreated by water (CK) or the optimal concentration of 1.2 mM NaHS for 12 h (pretreatment time), and then exposed to 0.16 M NaCl stress for further 48 h (from 0 to 48 h of stress time). Germinating grain (0.5 g) at different time points were randomly sampled for further analysis. For each treatment, 50 grains were used and the germination percentage represented the mean number of germinated grain from three independent experiments.

Electrophoretic analysis of amylase and esterase

Wheat grain (0.5 g) were homogenized in 5 mL ice-cold phosphate buffer (200 mM, pH 8.3) and the homogenate was centrifuged at 12,000 *g* for 30 min. Then the supernatants were used for electrophoretic analysis of amylase and esterase. Electrophoretic analysis of amylase and esterase were carried out according to the methods described by Zhang et al. (2005) and Deising et al. (1992), respectively.

Assays of CAT, APX and POD activities

Activities of catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and guaiacol peroxidase (POD, EC 1.11.1.7) were assayed according to García-Limones et al. (2002).

Analyses of cell plasma membrane integrity in radicle tips

To study salinity-induced loss of plasma membrane integrity in the radicle tips of wheat, seedlings radicle tips were stained with Evans blue according to the method described by Yamamoto et al. (2001). For water control, intact wheat grain were cultured in water for 72 h (CK). For NaCl stress (NaCl), wheat grain were cultured in water for 36 h, then subjected to 0.16 M NaCl for further 36 h. Grain cultured in water for 24 h and immersed in 1.4 mM NaHS for 12 h were then divided into two equal groups and transferred to water (NaHS) or 0.16 M NaCl stress for 36 h (NaCl + NaHS), respectively. Intact radicles were washed with 0.5 mM $CaCl_2$ (pH 5.6) solution several times, dried with filter papers and immediately immersed in 0.1 mM $CaCl_2$ (pH 5.6) solution containing 0.025% (w/v) Evans blue for 1 h. Stained radicles were then washed three time with sufficient volume of 0.1 mM $CaCl_2$ (pH 5.6) solution and photographed.

Determination of MDA content

The content of MDA was determined by a procedure based on the method of Wang and Yang (2005).

Measurement of reducing sugar in germinating wheat grain

Wheat grain (0.5 g) were homogenized in 5 mL ice-cold phosphate buffer (200 mM, pH 8.3) and the homogenate was centrifuged at 12,000 *g* for 30 min. The content of reducing sugar was assayed according to method of DNS spectrophotometry.

Statistical analysis

Significances were tested by one-way ANOVA, and the results are expressed as the mean values ± SD. Each experiment was repeated for three times. Fisher's least significant differences (LSD) were calculated following a significant ($p < 0.01$ or $p < 0.05$) *t*-test.

Results

Inhibitory effect of salinity on the germination of wheat grain

As shown in Fig. S1* and Table S1, with increasing concentrations of NaCl, germination percentage wheat grain decreased gradually and the germination was totally inhibited with 0.4 M NaCl stress. At 0.16 M NaCl, germination was dramatically reduced to 24% compared with 85% for water control (Table S1, Fig. S1). For instance, coleoptile length at 0.08 M NaCl was almost halved (0.9 cm) in contrast to water control (1.6 cm), indicating high sensitivity of wheat to salinity stress. Meanwhile, radicle growth was also stunted upon 0.08 M NaCl stress.

Protective roles of NaHS pretreatment on the germination of wheat grain subjected to salinity stress

NaHS solution was used as an H_2S donor to study the effect of H_2S on germinating wheat grain subjected to 0.16 M NaCl stress. Wheat grains were pretreated for 12 h with different NaHS concentrations (0, 0.6, 1.2, 1.8, 2.4, 3 mM), then exposed to 0.16 M NaCl stress. The germination percentage, coleoptile length, radicle number and length gradually increased to a maximum at 1.2 mM NaHS (Table 1, Fig. S2). However, concentrations of NaHS higher than 1.2 mM conferred no additional protection to wheat grain against NaCl. Thus 1.2 mM NaHS was selected for subsequent experiments.

Table 1. Effects of NaHS pretreatment on wheat grain germination under NaCl stress

Concentration of NaHS (mM)	Germination percentage (%)	Length of coleoptile (cm)	Radicle number (per grain)	Length of radicle (cm)
0.0	25 ± 2d	0.3 ± 0.1a	0.74 ± 0.08d	0.3 ± 0.03b
0.6	46 ± 2bc	0.4 ± 0.1a	1.38 ± 0.06c	0.4 ± 0.07a
1.2	56 ± 2a	0.5 ± 0.2a	1.68 ± 0.06a	0.5 ± 0.07a
1.8	45 ± 1c	0.4 ± 0.2a	1.36 ± 0.04c	0.4 ± 0.05a
2.4	49 ± 1b	0.3 ± 0.1a	1.48 ± 0.04b	0.3 ± 0.04b
3.0	55 ± 2a	0.4 ± 0.1a	1.78 ± 0.06a	0.4 ± 0.03a

Wheat grain were pretreated with 0.0, 0.6, 1.2, 1.8, 2.4 and 3.0 mM NaHS, respectively, for 12 h, and subsequently subjected to 0.16 M NaCl for further 48 h, and then the germination percentage was investigated. Values are the means ± *SD* ($n = 50$). Different letters indicate significant difference ($p < 0.05$) between treatments.

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Effects of NaHS on the activities of amylase and esterase in wheat grain under NaCl stress

To get an insight into the alleviating role of H_2S on salinity-stressed wheat grain germination, the activity of amylase and esterase were investigated by native polyacrylamide gel electrophoresis (PAGE). Figure 1A shows the changes of amylase activity in wheat grain during 48 h of 0.6 M NaCl stress, combined with 12 h water or NaHS pretreatment. During 12 h of water or NaHS pretreatment, NaHS induced significantly higher amylase activity. Under subsequent NaCl stress for 48 h, amylase activity in NaHS-pretreated grain increased steadily, while that of the controls remained.

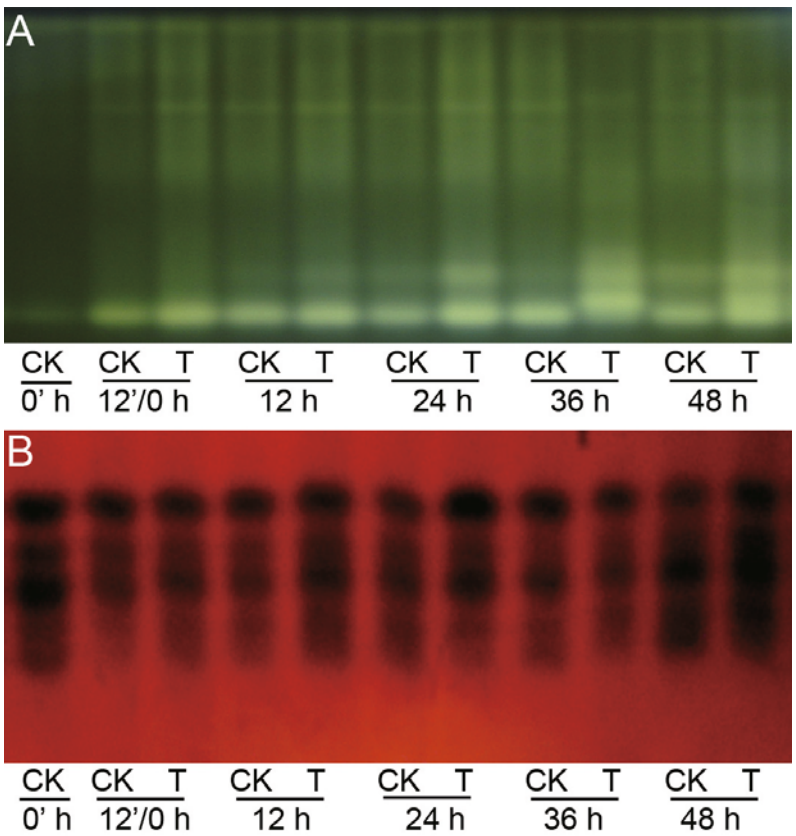

Figure 1. Changes of amylase and esterase activities in salinity-stressed wheat grain pretreated with water as control or 1.2 mM NaHS for 12 h

(A) Native polyacrylamide gel electrophoresis (PAGE) of amylase in wheat grain pretreated with water as control (CK) or 1.2 mM NaHS for 12 h (shown as from 0' h to 12'/0 h of the pretreatment time) prior to 0.16 M NaCl stress for further 48 h (shown as 0, 12, 24, 36, 48 h, respectively)

(B) Native polyacrylamide gel electrophoresis of esterase in wheat grain. The indicated time points are the same to Figure 1A

Figure 1B shows the changes of esterase activity in NaCl-stressed wheat grain with water or NaHS pretreatment. NaHS pretreatment could maintain higher esterase activities than water control especially at 12 h, 24 h and 48 h of salt stress. The above results suggested the role of H_2S in improving the activities of secreted hydrolases in germinating wheat grain under NaCl stress.

Effect of NaHS pretreatment on the activities of antioxidant enzymes in NaCl-stressed wheat grain

To further study the protective role of H_2S on NaCl-stressed grain, activities of antioxidant enzymes were determined with time in NaHS-pretreated grain and controls. Figure 2A illustrates the changes of CAT activity in both treatments. During the pretreatment time, a higher level of CAT activity was observed in water controls than in NaHS-pretreated grain. Thereafter, CAT activity in water pretreated grain decreased gradually until 36 h, whereas that of NaHS pretreatment increased continuously. At 48 h, an obvious increase occurred in both treatments. After NaCl stress for 36 h, CAT activity of NaHS-pretreated grain was significantly higher than that of water controls.

Figure 2B shows that during the pretreatment time, APX activity showed a significant increase in both NaHS pretreatment and water control. Then APX activity in water-pretreated grain kept a relative stable level till 48 h of NaCl stress, whereas that of NaHS pretreatment increased steadily and peaked at 24 h followed by an obvious decrease. At 24 and 48 h of NaCl stress, NaHS pretreatment sustained significantly higher level of APX activity than water control.

Figure 2C illustrates the changes of POD activity in both treatments. During pretreatment time and the first 24 h of NaCl stress, POD activity in water-pretreated control decreased continually and maintained a relatively stable level thereafter. However, POD activity in NaHS-pretreated grain kept stable through the pretreatment time and the first 12 h of NaCl stress. Then the activity in NaHS-pretreated grain increased steadily to a maximum at 48 h. During the entire treatment time, POD activity in NaHS-pretreated grain was maintained at a much higher level than that of water controls.

Effect of H_2S donor NaHS on MDA content in grain and plasma membrane integrity in radicle tips

To estimate membrane lipid breakdown, the content of malondialdehyde (MDA) was determined in wheat grain with treatment time. As illustrated in Figure 3A, MDA content in both NaHS pretreatment and control decreased gradually over the whole duration, except an MDA burst occurred in water controls after exposure to NaCl for 12 h. NaHS pretreatment significantly reduced the accumulation of MDA at 12 h of NaCl stress in wheat grain in contrast to water pretreatment.

Radicle tips from grains of each treatment were stained with Evans blue to detect the effect of H_2S on plasma membrane permeability in NaCl-stressed wheat radicles. There was no obvious difference between NaHS-treatment and water control (Fig. 3B), suggesting that 1.2 mM NaHS showed no negative effect to radicle membrane integrity. However, after exposure to NaCl stress, NaHS-pretreatment showed less staining intensity, indicating

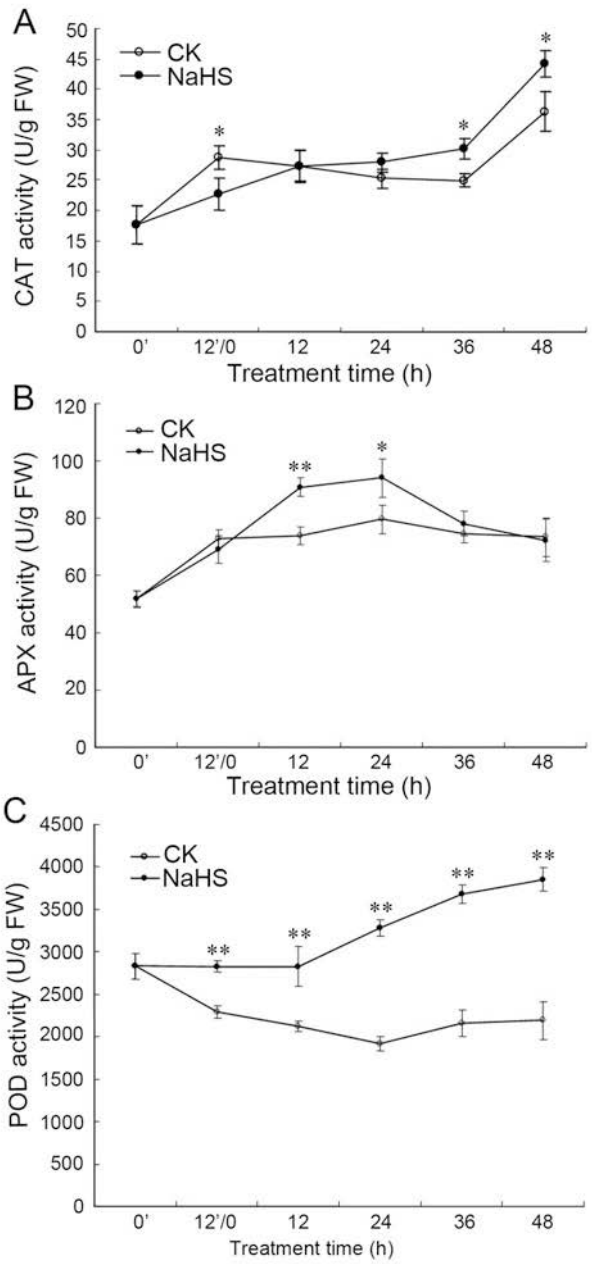

Figure 2. Effect of NaHS pretreatment on the activities of catalase (CAT) (A), ascorbate peroxidase (APX) (B), and peroxidase (POD) (C) in germinating wheat grain under 0.16 M NaCl stress. Wheat grain were pretreated with water (CK) or NaHS (NaHS) for 12 h (shown as from 0' h to 12'/0 h) and further treated with 1.2 mM NaCl for 12, 24, 36, 48 h, respectively

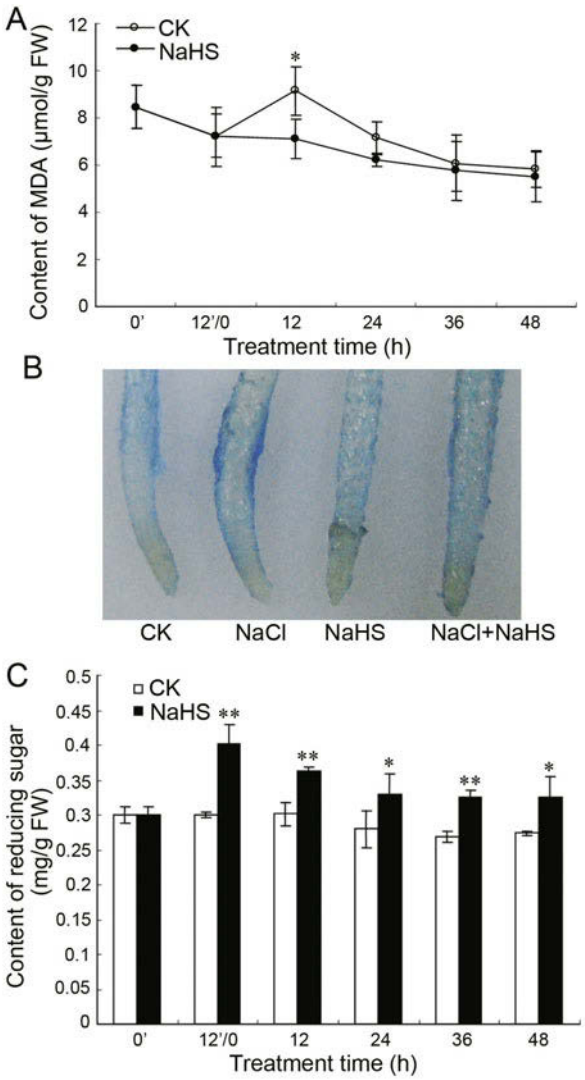

Figure 3. Effects of NaHS pretreatment on the content of malondialdehyde (MDA) (A), plasma membrane integrity (B) and reducing sugar (C) in wheat grain under NaCl stress.
The time points (0' h, 12'/0 h, 12 h, 24 h, 36 h, 48 h) represented as the same mentioned in Fig. 2

For the plasma membrane integrity in radicle tips (B), intact wheat grain were cultured in water for 72 h (CK). For NaCl stress (NaCl), wheat grain were cultured in water for 36 h, then subjected to 0.16 M NaCl for further 36 h. Grain were cultured in water for 24 h and immersed in 1.2 mM NaHS for 12 h, then divided into two equal groups and transferred to water (NaHS) or 0.16 M NaCl stress (NaCl + NaHS) for 36 h, respectively. Then the radicle tips (about 2 cm) were sampled for Evans blue staining to analyze plasma membrane integrity. (C) shows effect of NaHS pretreatment on the content of reducing sugar in wheat grain under NaCl stress. The time points (0' h, 12'/0 h, 12 h, 24 h, 36 h, 48 h) represented as the same mentioned in Fig. 2

less plasma membrane damage compared to pure NaCl stress. These results indicated that H_2S apparently alleviated membrane damage induced by NaCl stress.

Effect of H_2S donor NaHS on reducing sugar contents in grain

Sugars that contain aldehyde groups are classified as reducing sugars. Figure 3C shows that after 12 h of pretreatment time, reducing sugar contents in NaHS-pretreated grain showed a significant increase, while that of water control was maintained at a stable level equal to that of dry grain. After exposure to the NaCl stress, contents of reducing sugar decreased slightly in both treatments until 48 h. During entire treatment time, reducing sugar content in NaHS-pretreated grain was maintained at a significantly higher level than that of water controls.

Discussion

A high concentration of salt in the soil is known to be injurious to plant growth and may lead to three major types of stress, namely ionic, osmotic, and oxidative stress (Zhu 2002). The results reported in this paper show clearly that NaCl inhibits wheat grain germination and retards coleoptile and radicle growth. Similar trends have been found in several plant species subjected to salinity stress (Wang et al. 2012). The novel aspect of our work is that pretreatment of wheat grains with NaHS as an exogenous H_2S donor for 12 h could alleviate subsequent salinity stress, suggesting a potential strategy of NaHS application in mitigating salinity stress in wheat.

H_2S had been known for centuries as a potentially toxic gas. In recent years, accumulating evidence has demonstrated that H_2S acts as a signaling molecule in animals (Wang 2012). It has now been shown that H_2S can be formed endogenously in plants from sulfite or cysteine by the actions of sulfite reductase and desulfhydrases, respectively (Rausch and Wachter 2005). Given the fact that H_2S is endogenously produced in plants, it is important to examine its potential physiological roles. Recently, H_2S was shown to participate in abiotic stress resistance (Zhang et al. 2008, 2010; Jin et al. 2011, 2013), in plant defense against pathogen attack (Bloem et al. 2004), stomatal movement (García-Mata and Lamattina 2010; Lisjak et al. 2010), adventitious root organogenesis (Zhang et al. 2009), and autophagy (Aìlvarez et al. 2012). In our present study, pretreatment with 1.2 mM NaHS for 12 h significantly improved wheat grain germination under NaCl stress. Starch endosperm and esters are the major part of wheat grain, so their mobilization by amylase and esterase is essential for wheat grain germination. NaHS pretreatment enabled higher activities of amylase and esterase in NaCl-stressed wheat grain compared with water pretreatment controls (Fig. 1). In NaHS-pretreated grain, the content of reducing sugar was higher than that of control, indicating that higher amylase activity induced by H_2S could stimulate the mobilization of starch reservoir in wheat grain.

Overproduction of ROS, i.e. superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radical is an important consequence of salinity stress (Zhu 2002; Leshem et al. 2007). To cope with ROS accumulation, plants mobilize antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) to scav-

enge ROS and avoid oxidative damage (Sekmen et al. 2007). In our present study, salinity treatment resulted in MDA overproduction at 12 h of NaCl stress, suggesting that oxidative stress induced by salinity contributed to excessive lipid peroxidation. In contrast, NaHS pretreatment significantly reduced the accumulation of MDA (Fig. 3A). Evans blue staining indicated that H_2S could serve to protect against NaCl-induced loss of plasma membrane integrity in the radicle tips (Fig. 3B). To further understand the protective role of H_2S to wheat grain under salt stress, we examined the activities of several antioxidant enzymes. We observed that NaHS pretreatment generally enhanced CAT, APX, POD activities in wheat grain under NaCl stress. The increase in ROS scavenging enzymes may help to alleviate lipid oxidation as shown by much lower amounts of MDA content in NaHS-pretreated wheat. Taken together, this data strongly supports the idea that H_2S promotes wheat grain germination under salt stress by activating antioxidant system. Consistent to our study, H_2S has been implicated in antioxidant activation in many plants subjected to various stresses (Zhang et al. 2008, 2010; Jin et al. 2011, 2013; Christou et al. 2013). Besides, we previously show that concentrations of NaHS up to 1.4 mM showed no negative effect on wheat germination, suggesting role of H_2S is not due to cross-tolerance (Zhang et al. 2008).

Our evidence adds to the concept that H_2S acts as an important signal molecule in abiotic stress tolerance. NaHS pretreatment could significantly promote the germination percentage and root growth, enhance the activities of amylase and esterase and the content of reducing sugar and ameliorate the NaCl-induced damage of plasma membrane integrity. Meanwhile, increase in the activities of CAT, POD and APX suggested that H_2S exerted its protective effect through the activation of some antioxidant enzymes. However, the underlying mechanism of H_2S as a signal molecule is still obscure. Similar to the mechanism that NO physiologically S-nitrosylates diverse proteins, in animals, endogenous H_2S was found to regulate proteins through protein S-sulfhydration (that is, converts cysteine –SH groups to –SSH) (Mustafa et al. 2009). Thus, research of protein modification by H_2S might help to improve our understanding of the functional mechanisms of H_2S.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1.* Inhibitory effect of NaCl on wheat grain germination

Electronic Supplementary *Figure S1.* Inhibitory effect of NaCl stress on wheat grain germination. Wheat grain were cultured under 0.00, 0.08, 0.16, 0.24, 0.32 and 0.40 M NaCl stress for 72 h, then photographed

Electronic Supplementary *Figure S2.* H_2S donor NaHS promoted wheat grain germination under NaCl stress. Wheat grain were pretreated with 0.0, 0.6, 1.2, 1.8, 2.4, 3.0 mM NaHS, respectively, for 10 h, and subsequently subjected to 0.16 M NaCl stress for further 48 h, then photographed