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SHORT COMMUNICATION

Effects of exogenous hydrogen sulfide on the ascorbate and glutathione metabolism in wheat seedlings leaves under water stress

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Abstract This study investigated the effects of exogenous hydrogen sulfide on the ascorbate and glutathione metabolism in wheat seedlings leaves under water stress. The results showed that pretreatment with sodium hydrosulfide (NaHS), hydrogen sulfide donor, increased the activities of ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase and gamma-glutamylcysteine synthetase, and the contents of reduced ascorbic acid, reduced glutathione, total ascorbate and total glutathione under water stress, compared to control and water stress without NaHS. Meanwhile, pretreatment with NaHS decreased the malondialdehyde content and electrolyte leakage induced by water stress in plants, compared to control and water stress without NaHS. Our results suggested that exogenous hydrogen sulfide alleviated oxidative damage by regulating the ascorbate and glutathione metabolism in wheat seedlings under water stress.

Keywords Water stress · Hydrogen sulfide · Ascorbate · Glutathione · Wheat

Abbreviations

APX	Ascorbate peroxidase
GR	Glutathione reductase
MDHAR	Monodehydroascorbate reductase
DHAR	Dehydroascorbate reductase
GalLDH	L-Galactono-1,4-lactone dehydrogenase
γ-ECS	Gamma-glutamylcysteine synthetase
GSH	Reduced glutathione
AsA	Reduced ascorbic acid
NaHS	Sodium hydrosulfide
H_2S	Hydrogen sulfide

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Introduction

Water stress is an environmental factor that adversely affects plant growth, productivity, and survival (Jiang and Zhang 2002). Water stress usually causes oxidative damage to plants by inducing the accumulation of reactive oxygen species (ROS) (Apel and Hirt 2004; Papadakis and Roubelakis-Angelakis 2005). Plants could protect themselves against oxidative damage by antioxidant system including antioxidative enzymes and nonenzymatic compounds (Mittler 2002).

Ascorbate and glutathione are two crucial nonenzymatic compounds involved in defence against oxidative stress (Smirnoff 1996; Schafer and Buettner 2001). It is well known that plants can regulate ascorbate and glutathione contents by modulating the regeneration and biosynthesis of ascorbate and glutathione (Smirnoff 1996; Noctor and Foyer 1998; Schafer and Buettner 2001). L-galactose pathway is the main biosynthetic pathway of ascorbate in plants, GalLDH is the last enzyme in this way (Wheeler et al. 1998). γ-ECS is the first enzyme for glutathione biosynthesis (Dringen 2000). Ascorbate-glutathione cycle is the recycling pathway of ascorbate and glutathione. Thus, the ascorbate–glutathione cycle plays an important role in maintaining the contents of ascorbate and glutathione in plants. In this cycle, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate

reductase (DHAR) and glutathione reductase (GR) are the key enzymes (Noctor and Foyer 1998).

It has been shown that hydrogen sulfide (H_2S) can act as the third gaseous signaling molecule after nitric oxide (NO) and carbon monoxide (CO) in animals (Hosoki et al. 1997). In plants, it has been documented that H_2S can promote root organogenesis (Zhang et al. 2009a) and seed germination (Zhang et al. 2010a). Increasing evidence has proven that H_2S is involved in the antioxidative response against stress conditions, including copper, chromium, aluminum, drought, and osmotic stresses (Zhang et al. 2008, 2009b, 2010b, c, d). However, whether H_2S participates in the regulation of ascorbate and glutathione metabolism in plants remains unknown.

Jimai 21 is a drought-tolerant cultivar and is broadly cultivated in Shandong Province of China. In this study, we investigated malondialdehyde content, electrolyte leakage, the activities of enzymes involved in ascorbate and glutathione metabolism, and the contents of reduced ascorbic acid (AsA), reduced glutathione (GSH), total ascorbate and total glutathione in the leaves of Jimai 21 seedlings exposed to water stress induced by 15% PEG-6000. The aim of the study was to elucidate whether H_2S regulates ascorbate and glutathione metabolism in wheat seedlings leaves under water stress, and provide new knowledge to antioxidant metabolism in plants under water stress.

Materials and methods

Plant material, growth conditions, and treatments

Wheat (*Triticum aestivum* L., Jimai 21) seeds were supplied by Shandong Academy of Agricultural Sciences and sown in plastic trays filled with a sand/vermiculite matter mix (2:1, v/v) and grown in a greenhouse under a day/night temperature of 25/15°C, 500 μmol m^{-2} s^{-1} photosynthetic active radiation and a 12-h photoperiod. The seedlings were watered with half-strength Hoagland's solution every day. When the third leaf was fully expanded, the seedlings of uniform height were selected for all experiments.

The plants taken out of trays were studied. The roots of plants were washed softly and thoroughly. After placing in distilled water for 12 h, the roots were placed in beakers containing 100 ml 15% (W/V) PEG solution and wrapped with aluminium foil for 24 h at 25°C with a continuous light intensity of 500 μmol m^{-2} s^{-1}. In order to study the effect of hydrogen sulfide, a group of plants were pretreated with 1 mM sodium hydrosulfide (NaHS, H_2S donor, Sigma, USA) for 12 h and then exposed to water stress for 24 h under the same conditions as described above. The control plants were treated with distilled water alone under the same conditions as the above groups. After treatment of 0, 4, 8, 12, and 24 h, the third leaf of wheat seedlings was collected and frozen in liquid nitrogen, and then kept at –80°C until used for analyses.

Analysis of APX, GR, DHAR, MDHAR

Enzymes were extracted according to Grace and Logan (1996) with some modifications. Each frozen sample (0.5 g) was ground into a fine powder in liquid N_2 with a mortar and pestle. Fine powder was homogenized in 6 ml 50 mM KH_2PO_4 (pH 7.5) containing 0.1 mM ethylenediaminetetraacetic acid, 0.3% (v/v) Triton X-100, and 1% (w/v) soluble polyvinylpolypyrrolidone, with the addition of 1 mM AsA in the case of the APX assay. The extract was immediately centrifuged at 13,000×*g* for 15 min at 2°C. The supernatant was then used immediately for measuring the following enzymes.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by monitoring the decrease in absorbance at 290 nm (Nakano and Asada 1981). The assay mixture (2.5 ml) contained 50 mM phosphate buffer (pH 7.3), 0.1 mM ethylenediaminetetraacetic acid, 1 mM H_2O_2, 10 mM AsA and enzyme extract. The reaction was initiated by adding H_2O_2. One unit of enzyme was defined as the amount of APX catalyzing the oxidation of 1 μmol ascorbate per minute. A molar coefficient of 2.8 mM^{-1} cm^{-1} was used for the calculation of enzyme activity (Nakano and Asada 1981).

Glutathione reductase (GR, EC 1.6.4.2) activity was monitored at 340 nm in 3 ml reaction mixture containing 100 mM Tris–HCl (pH 8.0), 0.5 mM ethylenediaminetetraacetic acid, 0.5 mM $MgCl_2$, 0.5 mM oxidized glutathione (GSSG), 1 mM NADPH and enzyme extract. The reaction was initiated by adding NADPH (Grace and Logan 1996). One unit of GR activity was defined as the reduction of 1 μmol NADPH per minute. A molar coefficient of 6.2 mM^{-1} cm^{-1} was used for the caculation of enzyme activity (Rao et al. 1996).

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed at 340 nm in 3 ml reaction mixture containing 50 mM Hepes–KOH (pH 7.6), 1 mM NADH, 2.5 mM AsA, 2.5 units AsA oxidase (EC 1.10.3.3) and enzyme extract. The reaction was initiated by adding AsA oxidase (Miyake and Asada 1992). One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 μmol NADH per minute. A molar coefficient of 6.2 mM^{-1} cm^{-1} was used for the caculation of enzyme activity (Shanker et al. 2004).

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured at 265 nm in 3 ml assay solution

containing 100 mM Hepes–KOH (pH 7.0), 20 mM GSH, 2 mM DHA. The reaction was initiated by adding DHA (Dalton et al. 1986). One unit of DHAR activity was defined as the amount of enzyme that produces 1 μmol AsA per minute. A molar coefficient of 14.6 mM^{-1} cm^{-1} was used for the calculation of enzyme activity (Hossain and Asada 1984).

The specific enzyme activity for all the above enzymes was expressed as Units mg^{-1} protein.

Analysis of GalLDH and γ-ECS

L-Galactono-1,4-lactone dehydrogenase (GalLDH, EC 1.3.2.3) was extracted and measured by the method of Tabata et al. (2001). Gamma-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2) was extracted and measured by the method of Rüeggseger and Brunold (1992).

Measurement of protein concentration

Protein concentration was measured using bovine serum albumin as standard according to the method of Bradford (1976).

Analysis of AsA, GSH, total ascorbate and total glutathione

AsA and DHA were measured according to Hodges et al. (1996). For each sample, DHA was estimated from the difference between total ascorbate and AsA. Total glutathione, GSSG and GSH were measured according to Griffith (1980). For each sample, GSH was estimated from the difference between total glutathione and GSSG.

Measurement of malondialdehyde content and electrolyte leakage

Malondialdehyde content was measured by thiobarbituric acid (TBA) reaction as described by Hodges et al. (1999). Electrolyte leakage was determined according to Zhao et al. (2004). The electrolyte leakage was expressed as the relative ion leakage, a percentage of the total conductivity after boiling.

Statistical analysis

The whole experiment repeated six times with five seedlings each time. The results presented were the mean of six times. Means were compared by one-way analysis of variance and Duncan's multiple range test at the 5% level of significance.

Results

The selection of suitable NaHS treatment concentration

In order to select a suitable NaHS treatment concentration, we investigated the effects of different NaHS concentrations on the contents of AsA, total ascorbate, GSH and total glutathione under water stress. The NaHS concentrations are 0.5, 1.0, 1.5 and 2.0 mM, respectively. The results showed that water stress without NaHS and application of 0.5, 1.0 and 1.5 mM NaHS under water stress significantly increased the contents of AsA, total ascorbate, GSH and total glutathione, compared to control. However, there was no significant difference between control and application of 2.0 mM NaHS under water stress. There was also no significant difference between water stress without NaHS and application of 0.5 and 1.5 mM NaHS under water stress. Application of 1 mM NaHS significantly increased the contents of AsA, total ascorbate, GSH and total glutathione under water stress, compared to control, other NaHS concentrations and water stress without NaHS (Fig. 1). These results suggested that 1 mM NaHS was a suitable concentration to study the effect of exogenous H_2S on the ascorbate and glutathione metabolism in wheat seedlings leaves under water stress. Above results also showed that H_2S could significantly affect the contents of AsA, total ascorbate, GSH and total glutathione under water stress.

Effects of exogenous H_2S on the activities of enzymes in ascorbate and glutathione metabolism under water stress

As shown in Fig. 2, there were no obvious changes in the activities of APX, GR, DHAR, MDHAR, γ-ECS and GalLDH under control from 0 to 24 h of treatment. There was no significant difference among different treatments at 0 h of treatment. From 4 to 24 h of treatment, there was significant difference among different treatments. Water stress significantly increased the activities of APX, GR, DHAR, MDHAR, γ-ECS and GalLDH from 4 to 24 h of treatment, compared to control. After 24 h of treatment, the activities of APX, GR, DHAR, MDHAR, γ-ECS and GalLDH increased by 46.1, 54.8, 62.5, 70, 38.8, and 56.2%, respectively. In order to determine whether the application of exogenous H_2S could regulate the ascorbate and glutathione metabolism under water stress, the effects of pretreatment with NaHS on the activities of above enzymes under water stress were investigated. The results showed that pretreatment with NaHS significantly increased the activities of APX, GR, DHAR, and γ-ECS in stressed leaves from 4 to 24 h of treatment, compared to water stress. After 24 h of treatment, the activities of APX, GR, DHAR and γ-ECS increased by 31.5, 33.3, 26.9, and

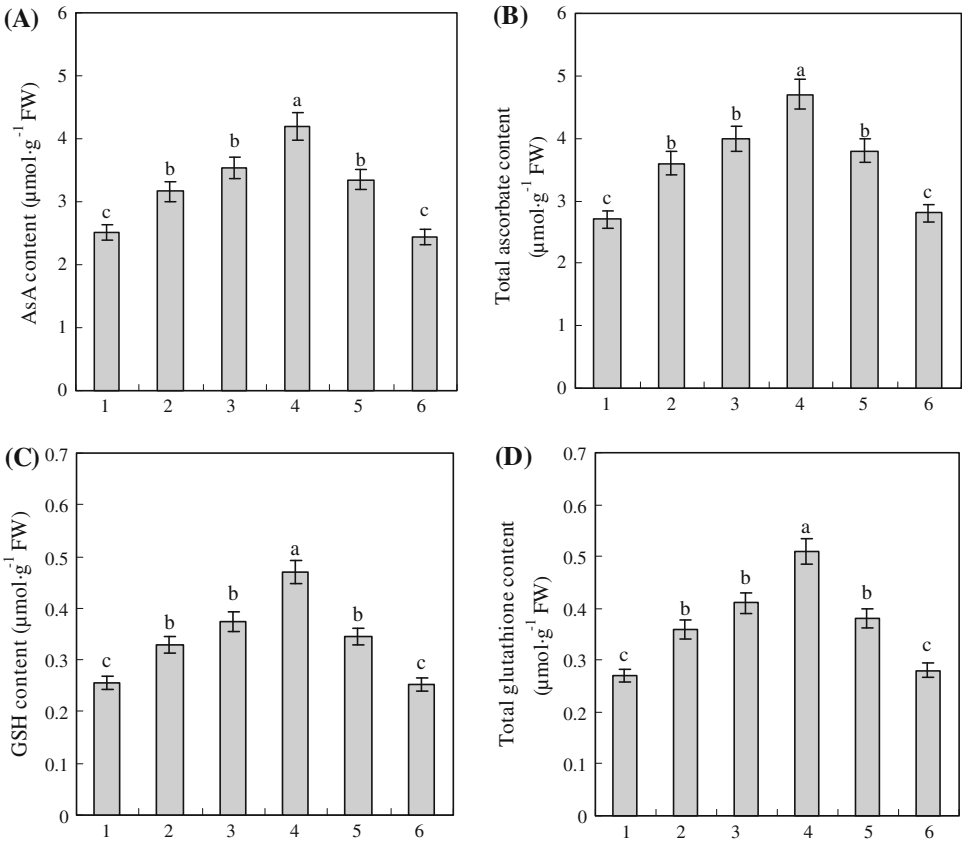

Fig. 1 Effects of different NaHS concentrations on the contents of AsA (**a**), total ascorbate (**b**), GSH (**c**), and total glutathione (**d**) in leaves of wheat seedlings. The plants were treated as follows: *1* distilled water, *2* 15%PEG, *3* 0.5 mM NaHS + 15%PEG, *4* 1 mM NaHS + 15%PEG, *5* 1.5 mM NaHS + 15%PEG, *6* 2 mM NaHS + 15%PEG. The plants were pretreated with NaHS for 12 h, and then exposed to 15% PEG for 12 h

20%, respectively. However, pretreatment with NaHS did not affect the activities of MDHAR and GalLDH in stressed leaves, compared to water stress. These results suggested that application of exogenous H_2S could regulate the ascorbate and glutathione metabolism by increasing the activities of APX, GR, DHAR, and γ-ECS under water stress.

Effects of exogenous H_2S on the contents of AsA, total ascorbate, GSH, and total glutathione under water stress

To further investigate whether the ascorbate and glutathione metabolism were related to the application of exogenous H_2S under water stress, the effects of pretreatment with NaHS on the contents of AsA, total ascorbate, GSH, and total glutathione under water stress were studied. The results showed that there were no obvious changes in the contents of AsA, total ascorbate, GSH, and total glutathione under control from 0 to 24 h of treatment (Fig. 3). There was no significant difference among different treatments at 0 h of treatment. From 4 to 24 h of treatment, there was significant difference among different treatments. Water stress significantly increased the contents of AsA, total ascorbate, GSH, and total glutathione from 4 to 24 h of treatment, compared to control. After 24 h of treatment, the contents of AsA, total ascorbate, GSH, and total glutathione increased by 45.9, 56, 66.6, and 73.9%, respectively. Pretreatment with NaHS significantly increased the contents of AsA, GSH, total ascorbate and total glutathione under water stress from 4 to 24 h of treatment, compared to water stress. After 24 h of treatment, the contents of AsA, total ascorbate, GSH, and total glutathione increased by 16.7, 15.4, 29, and 27.5%, respectively. Above results suggested once again that the application of exogenous H_2S could regulate the ascorbate and glutathione metabolism in wheat seedlings leaves under water stress.

Effects of exogenous H_2S on the malondialdehyde content and electrolyte leakage under water stress

As shown in Fig. 4, there were no obvious changes in the malondialdehyde content and electrolyte leakage under control from 0 to 24 h of treatment. There was no significant difference among different treatments at 0 h of treatment. From 4 to 24 h of treatment, there was significant difference among different treatments. Water stress significantly increased the malondialdehyde content and electrolyte leakage in leaves from 4 to 24 h of treatment, compared to control. After 24 h of treatment, the malondialdehyde content and electrolyte leakage increased by 2.6- and 2.2-fold, respectively. To investigate whether H_2S

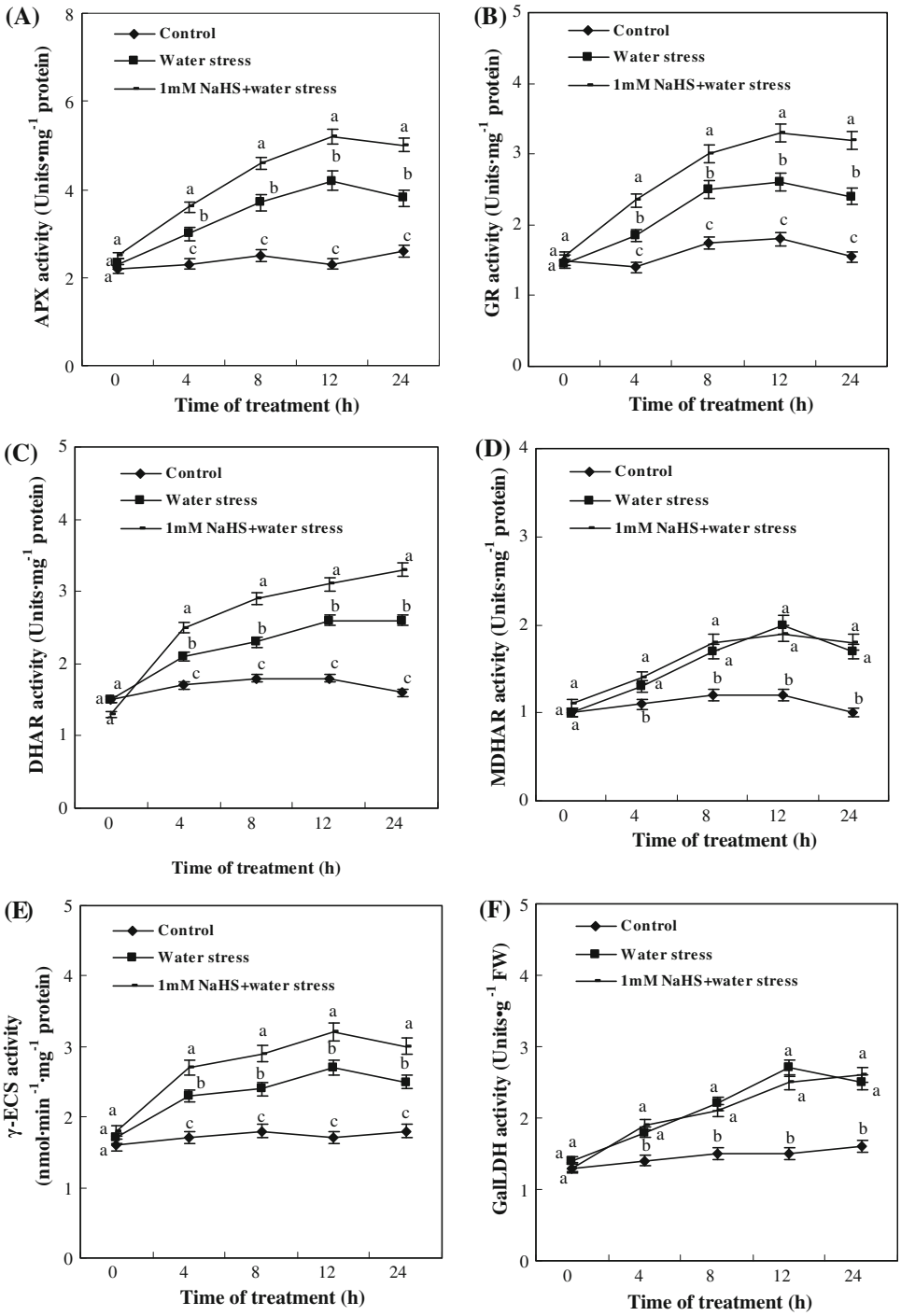

Fig. 2 Effects of exogenous hydrogen sulfide on the activities of enzymes involved in ascorbate and glutathione metabolism. The plants were pretreated with 1 mM NaHS for 12 h, and then exposed to 15%PEG for 0, 4, 8, 12 and 24 h

has important role for water stress tolerance in wheat seedlings, the effects of pretreatment with NaHS on the malondialdehyde content and electrolyte leakage in leaves under water stress were studied. The results showed that pretreatment with NaHS significantly decreased the malondialdehyde content and electrolyte leakage induced by water stress from 4 to 24 h of treatment, compared to water stress. After 24 h of treatment, the malondialdehyde content and electrolyte leakage decreased by 32.2 and 32.5%, respectively. These results suggested that H_2S has important role for acquisition of water stress tolerance in wheat seedlings.

Discussion

AsA is an important compound of plant antioxidant system and a major redox compound in plants. The cellular content

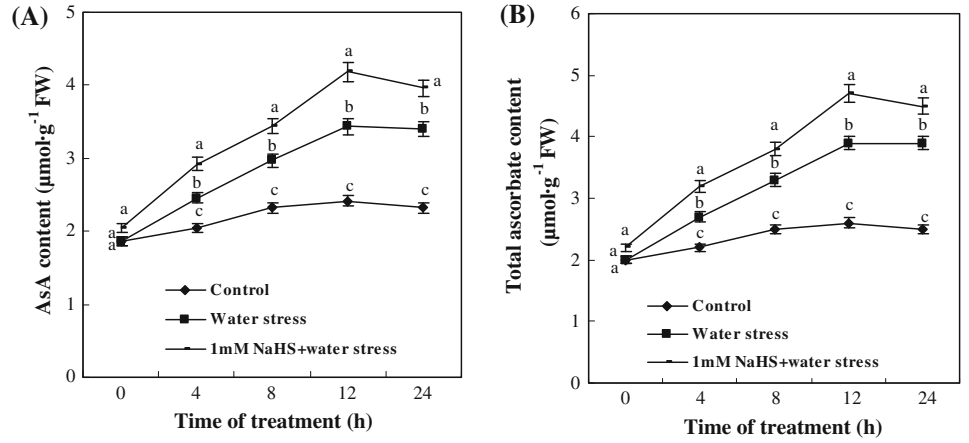

Fig. 3 Effects of exogenous hydrogen sulfide on the contents of AsA (**a**), total ascorbate (**b**), GSH (**c**), and total glutathione (**d**) in leaves. The plants were pretreated with 1 mM NaHS for 12 h, and then exposed to 15%PEG for 0, 4, 8, 12 and 24 h

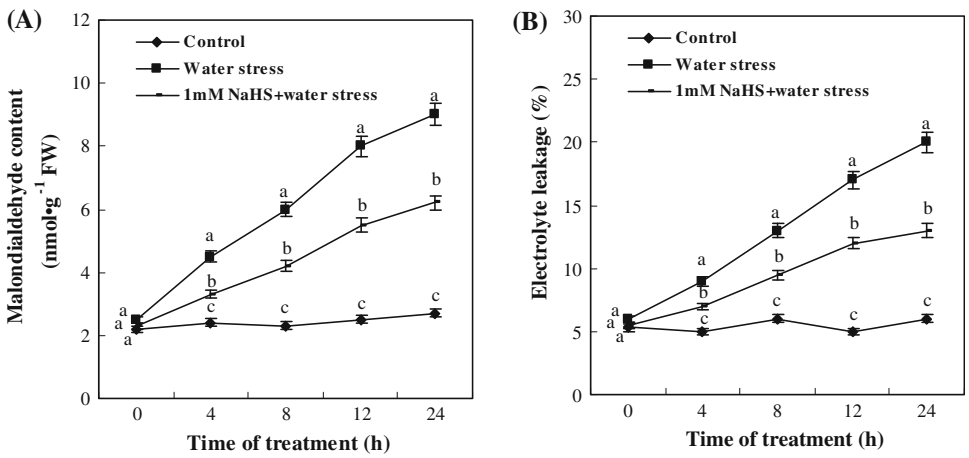

Fig. 4 Effects of exogenous hydrogen sulfide on the malondialdehyde content and electrolyte leakage of wheat seedlings leaves. The plants were pretreated with 1 mM NaHS for 12 h, and then exposed to 15%PEG for 0, 4, 8, 12 and 24 h

of AsA can be determined by GalLDH, DHAR, MDHAR and APX, which are the enzymes for glutathione biosynthetic and recycling pathway, respectively. It has been documented that H_2S increases the activity of APX in the root tip of *Pisum sativum* (Li et al. 2010). Zhang et al. (2010a, b, d) also reported that H_2S could enchance the activity of APX in wheat under chromium, aluminum, and osmotic stress. However, Zhang et al. (2008) also reported that H_2S did not affect the activity of APX in wheat under copper stress. In the present study, we found that H_2S also increased the activity of APX in wheat under water stress. Besides, our study also indicated that H_2S increased the DHAR activity and the contents of AsA and total ascorbate under water stress. However, our results showed that H_2S did not affect the activities of MDHAR and GalLDH in wheat under water stress. Therefore, H_2S could signal AsA regeneration by increasing DHAR activity and was involved in the control of AsA synthesis but not through GalLDH regulation.

GSH is another important compound of plant antioxidant system. The cellular content of GSH can be determined by γ-ECS and GR, which are the enzymes for glutathione biosynthetic and recycling pathway, respectively. The results of our study showed that H_2S may regulate the glutathione metabolism by increasing the activities of γ-ECS and GR, and the contents of GSH and total glutathione under water stress. It has been reported that H_2S induced the accumulation of GSH and total glutathione in rice suspension cell (Ma 2007), which was consistent with our experimental results.

For the activities of APX, GR, DHAR and γ-ECS, and the contents of AsA, total ascorbate, GSH and total glutathione, our results showed that the longer time of treatment, the more significant separation among the treatments. So, the longer time of treatment, the more significant decreases in the malondialdehyde content and electrolyte leakage induced by pretreatment with NaHS under water stress, compared to water stress without NaHS. But for the activities of MDHAR and GalLDH, there was no significant separation between pretreatment with NaHS under water stress and water stress without NaHS from 0 to 24 h of treatment. Above results suggested that there were timing effects of NaHS on the activities of above enzymes except for MDHAR and GalLDH, the ontents of AsA, total ascorbate, GSH, total glutathione and malondialdehyde, as well as the electrolyte leakage.

More studies have provided evidence that many signal molecules in plants, such as Ca^{2+}, hydrogen peroxide (H_2O_2), NO, abscisic acid (ABA) and jasmonic acid (JA), could regulate the ascorbate and glutathione metabolism and have important roles in defencing oxidative stress in plant cells (Li et al. 1998; Jiang and Zhang 2002, 2003; Wendehenne et al. 2004; Arasimowicz and Floryszak-Wieczorek 2007; Hu et al. 2008; Ai et al. 2008). In the present study, we found that H_2S may also regulate the ascorbate and glutathione metabolism and has important role in defencing oxidative stress in wheat seedlings leaves. However, the signal transduction of H_2S in regulating the ascorbate and glutathione metabolism remains unkown. Therefore, it is very interesting to investigate the relationship between H_2S and above signal molecules in plants. This part work will provide more new knowledge to the antioxidant metabolism in plants under water stress.

NaHS has been widely used for exogenous H_2S applied in solutions (Hosoki et al. 1997). However, the solutions still contain Na^+ and other sulfur-containing components. In order to verify H_2S/HS^-, rather than other compounds derived from NaHS, is responsible for the regulation of the ascorbate and glutathione metabolism in wheat seedlings under water stress, Na_2S, Na_2SO_4, Na_2SO_3, $NaHSO_4$, $NaHSO_3$, and NaAC (1 mM) were used as the controls of NaHS. The results showed that above sulfur-containing components and Na^+ were not responsible for the increases in the contents of AsA, GSH, total ascorbate and total glutathione under water stress (data not shown). These results suggested that H_2S or HS^-, rather than other compounds derived from NaHS, plays an important role in regulating the ascorbate and glutathione metabolism under water stress.

In conclusion, our results clearly suggest that exogenous H_2S regulates the ascorbate and glutathione metabolism by increasing the activities of APX, GR, DHAR, γ-ECS and the contents of AsA, GSH, total ascorbate and total glutathione, which, in turn, enhances the antioxidant ability and protects wheat seedlings against oxidative stress induced by water stress. These results provide new knowledge to the antioxidant metabolism in plants under water stress conditions.

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