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Hydrogen sulfide protects wheat seedlings against copper stress by regulating the ascorbate and glutathione metabolism in leaves

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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 copper stress. The results showed that copper stress induced the increases in the activities of APX, DHAR, MDHAR, GR, GalLDH and γ-ECS, and the contents of AsA, GSH, total ascorbate and total glutathione. Pretreatment with sodium hydrosulfide (NaHS), a hydrogen sulfide (H_2S) donor, enhanced the activities of GR, DHAR, GalLDH and γ-ECS, and the contents of AsA, GSH, total ascorbate and total glutathione under copper stress. Meanwhile, pretreatment with NaHS decreased the malondialdehyde content and electrolyte leakage induced by copper stress in plants. Our results suggested that exogenous hydrogen sulfide alleviated oxidative damage by regulating the ascorbate and glutathione metabolism under copper stress, and H_2S has important role for acquisition of copper stress tolerance in wheat seedlings. Therefore, exogenous hydrogen sulfide can be used as a regulator to improve crop tolerance under copper stress.

Keywords: copper 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.

Introduction

Heavy metal stress is one of the major abiotic stresses that causes environmental pollution in recent decades. Copper is required for normal growth of plants. It is an essential redox component participating in a wide variety of processes, including the electron transfer reactions of respiration and photosynthesis or the detoxification of superoxide radicals (Fox and Guerinot, 1998). However, excess copper can induce changes in photosynthetic and respiratory processes, enzyme activities, and DNA and membrane integrity (Hazen et al., 1988; Vinit-Dunand et al., 2002; Alaoui-Sossé et al., 2004; Lombardi and Sebastiani, 2005). Another important feature of copper stress is the induction of oxidative damage to plants by inducing the accumulation of reactive oxygen species (ROS) (Demirevska-Kepova et al., 2004). If not effectively and rapidly removed from plants, ROS can damage a wide range of cellular macromolecules such as lipids, enzymes and DNA (Contreras et al., 2009). 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. It is well known that plants can adjust ascorbate and glutathione contents by modulating the regeneration and biosynthesis of ascorbate and glutathione. L-galactose pathway is the main biosynthetic pathway of ascorbate in plants, L-galactono-1,4-lactone dehydrogenase (GalLDH) is the last enzyme in this way (Wheeler et al., 1998). Gamma-glutamylcysteine synthetase (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, NO and CO have already been identified as signaling molecules involving antioxidative defense (Delledonne, 2005; Huang et al., 2006). Recently, it has also 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 (Zhang et al., 2008, 2009b, 2010b). However, whether H_2S regulates the ascorbate and glutathione metabolism in plants under copper stress remains unknown. In this study, we investigated malondialdehyde

content, electrolyte leakage, the transcript levels and activities of enzymes involved in ascorbate and glutathione metabolism, and the contents of AsA, GSH, total ascorbate and total glutathione in the leaves of wheat seedlings exposed to copper stress induced by 100 µM $CuSO_4$ solution. The aim of the study was to elucidate whether H_2S regulates ascorbate and glutathione metabolism at both molecular and physiological levels under copper stress, and provide new knowledge to antioxidant metabolism in plants under copper stress.

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 copper stress. The sodium hydrosulfide concentrations are 0.4 mM, 0.8 mM, 1.2 mM and 1.6 mM, respectively. Among different NaHS concentrations, application of 0.8 mM NaHS significantly increased the contents of AsA, total ascorbate, GSH and total glutathione under copper stress (Fig 1). These results suggested that 0.8 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 copper stress. Above results also showed that H_2S could significantly affect the contents of AsA, total ascorbate, GSH and total glutathione under copper stress .

Effects of exogenous H_2S on the transcript levels and the activities of enzymes involved in ascorbate and glutathione metabolism

Fig 2 and 3 showed that the transcript levels and activities of enzymes in ascorbate and glutathione metabolism, including APX, GR, DHAR, MDHAR, γ-ECS and GalLDH, increased under copper stress. In order to determine whether the application of exogenous H_2S could regulate the ascorbate and glutathione metabolism under copper stress, the effects of pretreatment with NaHS on the transcript levels and activities of above enzymes under copper stress were investigated. The results showed that the pretreatment with NaHS significantly increased the transcript levels and activities of GR, DHAR, GalLDH and γ-ECS in stressed leaves. However, pretreatment with NaHS did not affect the transcript levels and activities of APX and MDHAR in stressed leaves. Meanwhile, pretreatment with NaHS alone also significantly increased the transcript levels and activities of GR, DHAR, GalLDH and γ-ECS and did not affect the transcript levels and activities of APX and MDHAR in leaves, compared with control. These results suggested that application of exogenous H_2S could increase the activities of GR, DHAR, GalLDH and γ-ECS under copper stress.

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

To further investigate whether the ascorbate and glutathione metabolism were related to the application of exogenous H_2S under copper stress, the effect of pretreatment with NaHS on the contents of AsA, total ascorbate, GSH, and total glutathione under copper stress were studied. The results showed that the pretreatment with NaHS significantly increased the contents of AsA, GSH, total ascorbate and total glutathione under copper stress (Fig 4). Meanwhile, pretreatment with NaHS alone also significantly increased the contents of AsA, GSH, total ascorbate and total glutathione in leaves, compared with control. Above results suggested once again that the application of exogenous H_2S could affect the ascorbate and glutathione metabolism in wheat seedlings leaves under copper stress.

Effects of exogenous H_2S on the malondialdehyde content and electrolyte leakage

To investigate whether H_2S has important role for copper stress tolerance in wheat seedlings, the effect of pretreatment with NaHS on the malondialdehyde content and electrolyte leakage in leaves under copper stress were studied. The results showed that copper stress significantly increased the malondialdehyde content and electrolyte leakage in leaves. Pretreatment with NaHS significantly decreased the malondialdehyde content and electrolyte leakage induced by copper stress (Fig 5). However, there was no difference in malondialdehyde content and electrolyte leakage between control and pretreatment with exogenous H_2S alone. These results suggested that H_2S has important role for acquisition of copper stress tolerance in wheat seedlings.

Discussion

In our study, pre-treated plants with NaHS had a higher tolerance to Cu toxicity stress. It has been documented that AsA had important role in defensing stresses. The cellular content of AsA can be determined by GalLDH, DHAR, MDHAR and APX. It has been documented that H_2S increased 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 enhance 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 did not affect the activity of APX in wheat under copper stress. Besides, our study also indicated that H_2S increased the activities of DHAR and GalLDH, and the contents of AsA and total ascorbate under copper stress. However, our results showed that H_2S did not affect the MDHAR activity in wheat under copper stress. So, H_2S could signal AsA regeneration by increasing DHAR activity and was involved in the control of AsA synthesis 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 copper 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. Besides, our study also indicated that H_2S increased the activities of γ-ECS and GR under copper stress. NaHS dissociates to Na^+ and HS^- in solution, then HS^- associates with H^+ and produces H_2S (Hosoki et al., 1997). It has been widely used for exogenous H_2S applied in solutions. However, the solutions still contain Na^+ and other sulfur-containing components, such as S^{2-}, SO_4^{2-}, SO_3^{2-}, HSO_4^-, and HSO_3^-. In order to verify H_2S/HS^-, rather than other compounds derived from NaHS, is responsible for the

Table 1. Gene specific primers, accession number, melting temperatures, PCR protocols and size of amplified fragments.

Gene and accession no.	Primer sequences	Melting temperatures	PCR protocols	Size of amplified fragments (bp)
APX (EF555121)	Forward AAAGCGAAGCATCCAAAG Reverse CAGAGGGTCACGAGTCCA	53.6 53.5	94°C/2 min; 94°C/30 s, 53°C/30 s, 72°C/2 min, 28 cycles; 72°C/10 min	297
GR (AK332288)	Forward GTTCAAAGCAAAGGCACA Reverse ATTCGTTACATCACCCACA	52.1 50.9	94°C/2 min; 94°C/30 s, 55°C/30 s, 72°C/2 min, 30 cycles; 72°C/10 min	542
DHAR (AY074784)	Forward ACGGTGGTGATGGCAAAT Reverse GCGACCTGGAGATGGTAGAG	56.1 56.9	94°C/2 min; 94°C/30 s, 53°C/30 s, 72°C/2 min, 28 cycles; 72°C/10 min	301
MDHAR (BT009096)	Forward TTCTACGAGGGTTACTACGC Reverse GGGAAAGTCGCCACATCA	52.6 56.7	94°C/2 min; 94°C/30 s, 55°C/30 s, 72°C/2 min, 28 cycles; 72°C/10 min	278
GalLDH (BT009545)	Forward ATCGGCAGTCGTTCTCAG Reverse GGCAATCTCGCACCAGTA	53.4 54.5	94°C/2 min; 94°C/30 s, 55°C/30 s, 72°C/2 min, 27 cycles; 72°C/10 min	319
γ-ECS (AY864064)	Forward TCAAGCAGGGAAAGCAAA Reverse AAGGTCCACCATCAGCAC	54.7 52.6	94°C/2 min; 94°C/30 s, 55°C/30 s, 72°C/2 min, 32 cycles; 72°C/10 min	735
Actin (AB181991)	Forward CTATCCTTCGTTTGGACCTT Reverse CGGGACCAGACTCATCGT	53.7 55.5	94°C/2 min; 94°C/30 s, 55°C/30 s, 72°C/2 min, 30 cycles; 72°C/10 min	582

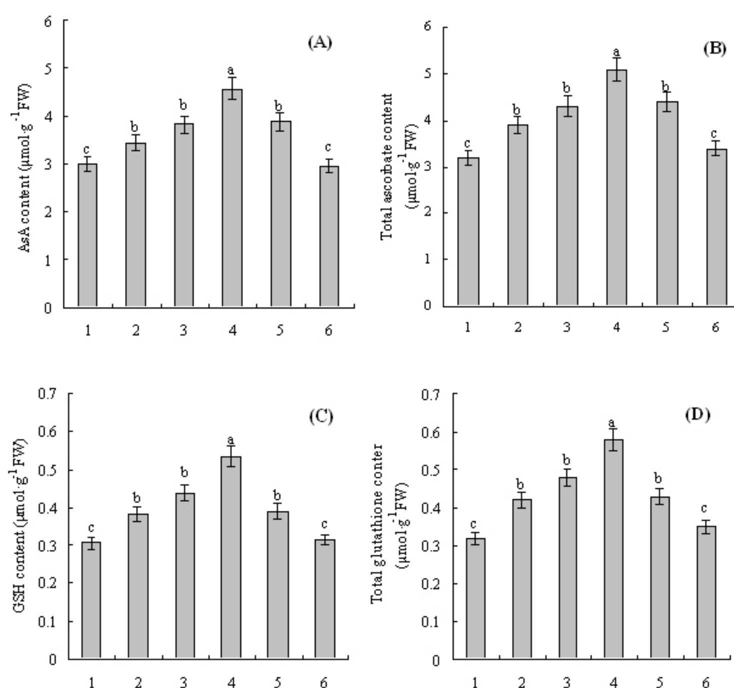

Fig 1. Effects of different NaHS concentrations on the contents of AsA (A), total ascorbate (B), GSH (C), and total glutathione (D) in leaves of wheat seedlings under copper stress. The plants were treated as follows: 1, distilled water; 2, 100 µM $CuSO_4$; 3, 0.4 mM NaHS + 100 µM $CuSO_4$; 4, 0.8 mM NaHS + 100 µM $CuSO_4$; 5, 1.2 mM NaHS + 100 µM $CuSO_4$; 6, 1.6 mM NaHS + 100 µM $CuSO_4$. The plants were pretreated with NaHS for 12 h, and then exposed to 100 µM $CuSO_4$ for 24 h.

regulation of the ascorbate and glutathione metabolism in wheat seedlings under copper stress, Na_2S, Na_2SO_4, Na_2SO_3, $NaHSO_4$, $NaHSO_3$, and NaAC (0.8 mM) were used as the controls of NaHS. We found that above sulfur-containing components and Na^+ were not able to increase the contents of AsA, GSH, total ascorbate and total glutathione under copper stress as NaHS did (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 copper stress.

Materials and methods

Plant material, growth conditions and treatments

Wheat (*Triticum aestivum* L., Luomai 9133) seeds were supplied by Luoyang 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-hour 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 rinsing in distilled water for 12 h, the roots were placed in beakers wrapped with aluminium foil containing 100 ml 100 µM $CuSO_4$ (Sigma, USA) solution for 24 h at 25 °C with a continuous light intensity of 500 μmol m^{-2} s^{-1}. In order to select a suitable concentration of sodium hydrosulfide (NaHS, H_2S donor,Sigma, USA), effects of pretreatments with 0, 0.4, 0.8, 1.2 and 1.6 mM NaHS on the contents of AsA, total ascorbate, GSH and total glutathione in wheat seedlings were investigated under the same conditions as described above, respectively. In order to study the effect of hydrogen sulfide

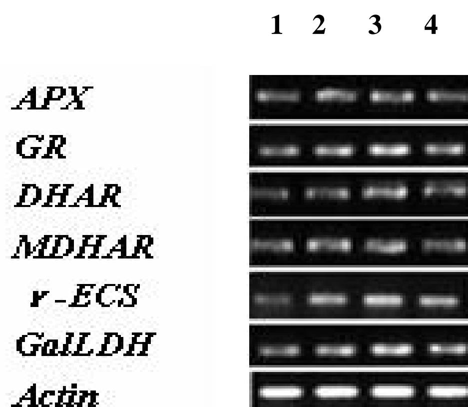

Fig 2. Effects of exogenous hydrogen sulfide on the transcript levels of enzymes involved in ascorbate and glutathione metabolism. The plants were treated as follows: 1, distilled water; 2, 100 µM $CuSO_4$; 3, 0.8 mM NaHS + 100 µM $CuSO_4$; 4, distilled water + 0.8 mM NaHS. The plants were pretreated with 0.8 mM NaHS for 12 h, and then exposed to 100 µM $CuSO_4$ or distilled water for 24 h.

on the ascorbate and glutathione metabolism, a group of plants were pretreated with 0.8 mM NaHS (suitable concentration selected from above five concentrations) for 12 h and then exposed to copper stress for 24 h. The control plants were treated with distilled water alone under the same conditions as the above groups. After treatment of 0 h, 4 h, 8 h, 12 h, 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.

Isolation of Total RNA and Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from leaves by using TRNzol Total RNA Reagent (TIANGEN) according to the instruction supplied by the manufacturer. Approximately 3 µg of total RNA were reverse transcribed using oligo (dT) primer and TIANScript RT Kit (TIANGEN). cDNA was amplified by PCR using primers designed according to the genes of following enzymes in *Triticum aestivum* (Table 1). To standardize the results, the relative abundance of *Actin* was also determined and used as the internal standard.

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) insoluble polyvinylpolypyrrolidone, with the addition of 1 mM AsA in the case of the APX assay. The extract was immediately centrifuged at 13000×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. 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 calculation of enzyme activity. 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 calculation of enzyme activity. 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. 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 Rüegsegger 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.

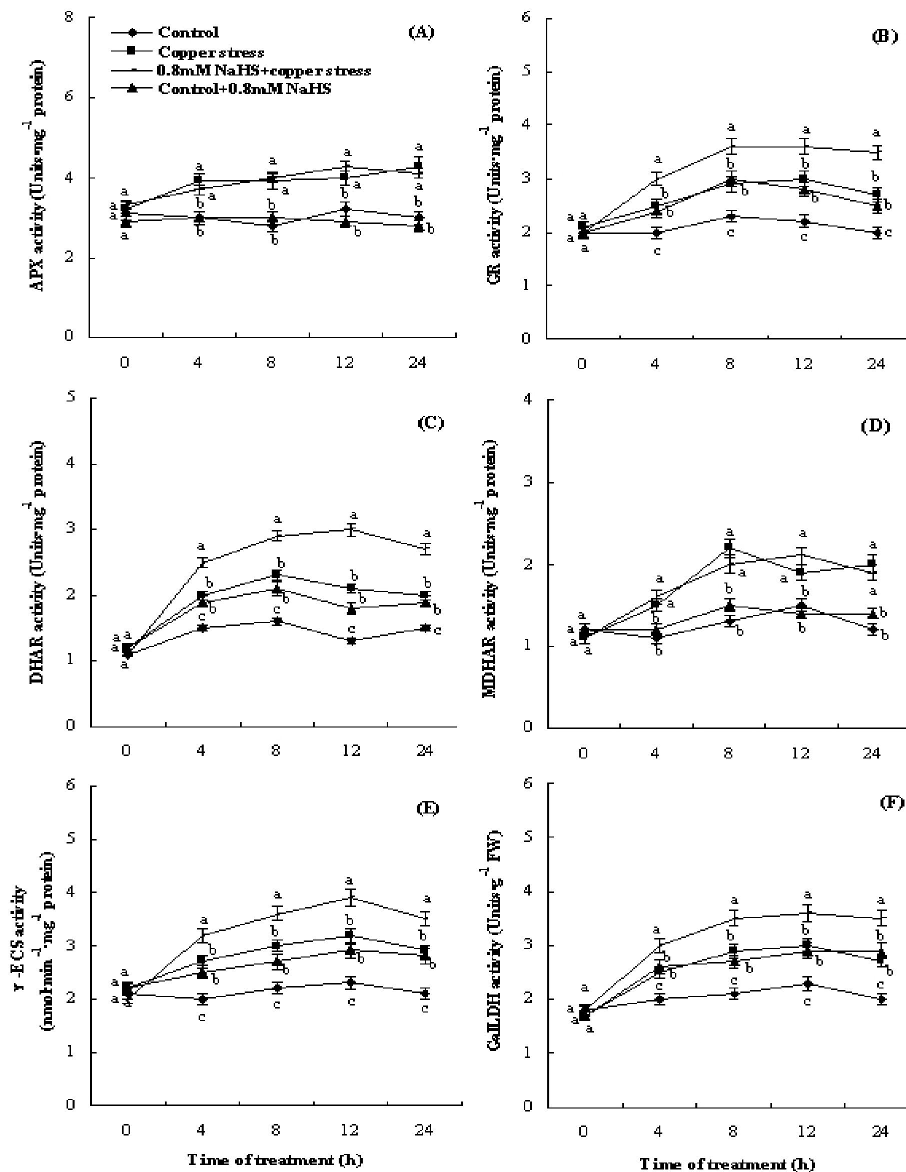

Fig 3. Effects of exogenous hydrogen sulfide on the activities of enzymes involved in ascorbate and glutathione metabolism. The plants were pretreated with 0.8 mM NaHS for 12 h, and then exposed to 100 µM $CuSO_4$ or distilled water for 0 h, 4 h, 8 h, 12 h and 24 h.

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 experimental design was a randomized complete block design with six replications. The results presented were the mean of six replications. Means were compared by one-way analysis of variance and Duncan's multiple range test at the 5% level of significance.

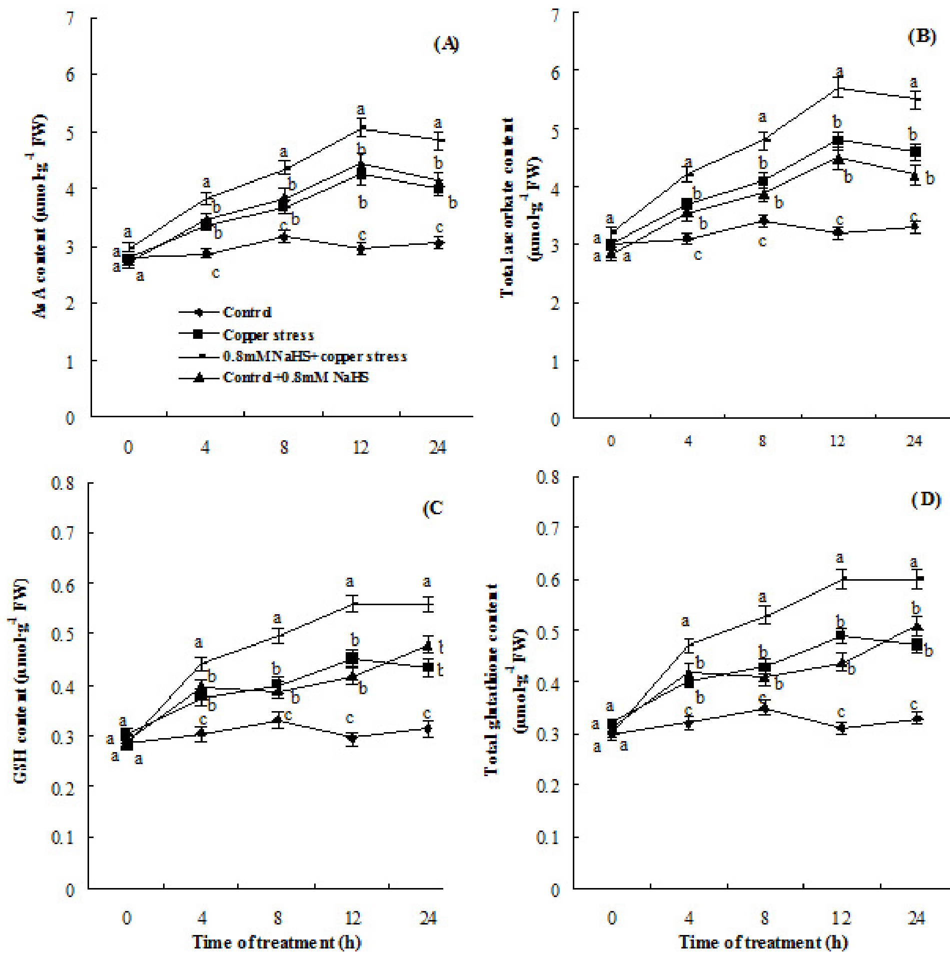

Fig 4. Effects of exogenous hydrogen sulfide on the contents of AsA (A), total ascorbate (B), GSH (C), and total glutathione (D) in leaves. The plants were pretreated with 0.8 mM NaHS for 12 h, and then exposed to 100 µM $CuSO_4$ or distilled water for 0 h, 4 h, 8 h, 12 h and 24 h.

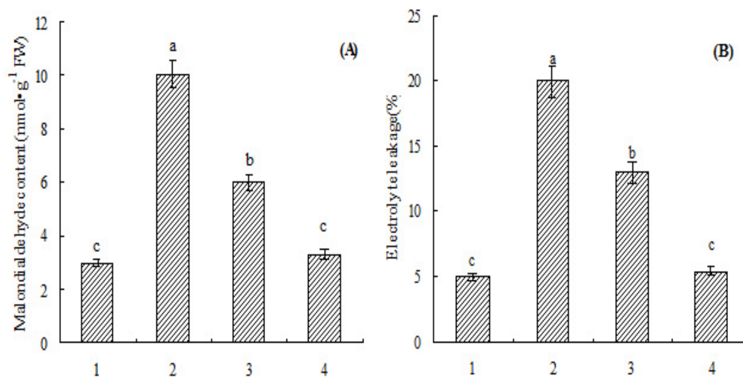

Fig 5. Effects of exogenous hydrogen sulfide on the malondialdehyde content and electrolyte leakage of wheat seedlings leaves. The plants were treated as follows: 1, distilled water; 2, 100 µM $CuSO_4$; 3, 0.8 mM NaHS + 100 µM $CuSO_4$; 4, distilled water + 0.8 mM NaHS. The plants were pretreated with 0.8 mM NaHS for 12 h, and then exposed to 100 µM $CuSO_4$ or distilled water for 24 h.

Conclusion

Our results clearly suggest that exogenous H_2S regulates the ascorbate and glutathione metabolism by increasing the activities of GR, DHAR, GalLDH, 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 copper stress. These results provide new knowledge to the antioxidant metabolism in plants under copper stress conditions.

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