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Hydrogen Sulfide is Involved in the Regulation of Ascorbate-glutathione Cycle by Exogenous ABA in Wheat Seedling Leaves under Osmotic Stress

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This study investigated the role of hydrogen sulfide (H_2S) in the regulation of ascorbate-glutathione (AsA-GSH) cycle by exogenous ABA in wheat leaves under osmotic stress. The results showed that osmotic stress significantly increased the activities of ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), the ratio of reduced ascorbate to oxidized ascorbate (AsA/DHA) and reduced glutathione to oxidized glutathione (GSH/GSSG), the malondialdehyde content and electrolyte leakage, and the H_2S content, compared to control. Exogenous ABA significantly increased above indicators under osmotic stress, compared to osmotic stress alone. Above activity increases except MDHAR activity were suppressed by application of H_2S scavenger hypotaurine (HT) and synthesis inhibitor aminooxyacetic acid (AOA). Meanwhile, exogenous ABA significantly decreased malondialdehyde content and electrolyte leakage induced by osmotic stress. Application of HT and AOA reversed above effects of application of exogenous ABA. Application of NaHS can reversed above effects of HT and AOA. Our results suggested that H_2S induced by exogenous ABA is a signal that leads to the up-regulation of AsA-GSH cycle.

Keywords: polyethylene glycol, sodium hydrosulfide, oxidative damage, *Triticum aestivum,* abscisic acid

Introduction

Osmotic stress adversely affects plant growth and productivity (Golldack et al. 2011). Osmotic stress usually causes the overproduction of reactive oxygen species (ROS), which result in oxidative damage to plants (Apel and Hirt 2004). In order to protect themselves from oxidative damage, plants could enhance the ascorbate-glutathione (AsA-GSH) cycle (Shan et al. 2015; Dai et al. 2015). Through this cycle, two important antioxidants ascorbate (AsA) and glutathione (GSH) are regenerated and H_2O_2 is scavenged (Shan and Liang 2010). Thus, the AsA-GSH cycle plays an important role in limiting oxidative damage by maintaining the contents of ascorbate and glutathione and respective redox states in plants.

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Abscisic acid (ABA) is an important plant hormone that plays important roles in regulating stress responses (Gupta et al. 2012; Parvin et al. 2012). Increasing evidence shows that ABA serves an important role in limiting oxidative damage by enhancing AsA-GSH cycle in plants (Zhang et al. 2007). It has been reported that signal molecules NO, H_2O_2, Ca^{2+}-CaM and MAPK participate in the process of ABA signal transduction in regulating the ascorbate-glutathione cycle (Zhang et al. 2007; Hu et al. 2007). Hydrogen sulfide (H_2S) is an important gaseous signaling molecule in plants (Hancock and Whiteman 2014). Zhang et al. (2008) proved that exogenous H_2S affected the antioxidative response against osmotic stress. Our previous study found that exogenous H_2S regulated AsA-GSH cycle in wheat leaves under osmotic stress (Shan et al. 2011). Liu et al. (2011) have reported that endogenous H_2S is involved in the process of ABA-induced stomatal closure. However, whether endogenous H_2S participates in the regulation of AsA-GSH cycle by ABA in plants under osmotic stress remains unknown. To describe antioxidant mechanisms of plants, the role of H_2S in the regulation of AsA-GSH cycle by ABA under osmotic stress deserves further consideration.

This study investigated malondialdehyde (MDA) content, electrolyte leakage, the activities of enzymes in AsA-GSH cycle, the ratios of AsA/DHA and GSH/GSSG in the leaves of Jimai 21 seedlings exposed to osmotic stress induced by 15% polyethylene glycol (PEG)-6000. The specific objective of the study was to describe the role of H_2S in the regulation of AsA-GSH cycle by ABA in wheat plants under osmotic stress by using H_2S scavenger hypotaurine (HT), H_2S synthesis inhibitor aminooxyacetic acid (AOA) and H_2S donor NaHS.

Materials and Methods

Plant culture and treatment

Wheat (*Triticum aestivum* L., cv. Jimai 21) seeds were sown in plastic trays filled with a sand/vermiculite matter mix (2:1, v/v) and grown in a greenhouse. Culture conditions: 25/15 °C (day/night) temperature and 500 μmol m^{-2} s^{-1} light intensity with a 12 h photoperiod. The seedlings were watered with half-strength Hoagland's solution every day. Seedlings of uniform height were selected when the third leaf was fully expanded. The roots of selected plants were washed and placed in beakers containing 50 ml 15% (w/v) PEG solution for 24 and 48 h under above conditions after soaking in half-strength Hoagland's solution for 12 h. The beakers were wrapped with aluminium foil to keep roots in dark. To study the effect of ABA, the roots of plants were soaked in 100 μM ABA solution for 8 h and then exposed to PEG solution for 24 and 48 h. To study the effects of HT and AOA, the roots of plants were soaked in 20 μM HT or 0.3 mM AOA or 100 μM ABA + 20 μM HT or 100 μM ABA + 0.3 mM AOA solution for 8 h and then exposed to PEG solution for 24 and 48 h. To investigate whether the effects of HT and AOA can be reversed by exogenous H_2S, the roots of plants were soaked in 20 μM HT or 0.3 mM AOA or 100 μM ABA + 20 μM HT or 100 μM ABA + 0.3 mM AOA solution for 8 h and then exposed to NaHS (H_2S donor) + 15% PEG or half-strength Hoagland's solution for

24 and 48 h. Above solutions used to treat plants were prepared by adding corresponding substances into half-strength Hoagland's solution. Control plants were treated with half-strength Hoagland's solution alone. Following 24 and 48 h treatment, the third fully-expanded leaf of wheat seedlings was collected and immediately frozen in liquid nitrogen, and stored at –80 °C until analyses.

The extraction and assay of enzymes in AsA-GSH cycle

Enzymes were extracted according to Shan and Liang (2010). The activities of ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) were measured according to Nakano and Asada (1981), Grace and Logan (1996), Miyake and Asada (1992) and Dalton et al. (1986), respectively. The specific enzyme activity for all the above enzymes was expressed as units mg^{-1} protein. Protein concentration was measured according to Bradford (1976).

Determination of AsA, DHA, GSSG and GSH

The contents of AsA, DHA, GSSG and GSH were measured according to Hodges et al. (1996) and Griffith (1980), respectively. AsA/DHA was expressed as the ratio between the content of AsA and the content of DHA. GSH/GSSG was expressed as the ratio between the content of GSH and the content of GSSG.

Determination of H_2S, MDA and electrolyte leakage

H_2S content was determined by formation of methylene blue from dimethyl-*p*-phenylenediamine in H_2SO_4 as described previously (Zhang et al. 2008). MDA content was measured according to Hodges et al. (1999). Electrolyte leakage was determined according to Zhao et al. (2004).

Statistical analysis

Data presented were the mean values of five times with four seedlings each time. Statistical assays were carried out by one-way analysis of variance and Duncan's multiple range test at the 5% level of significance.

Results

Effect of exogenous ABA, HT, AOA and NaHS on H_2S content

Osmotic stress led to an increase in H_2S content, compared with control (Fig. 1). After 24 and 48 h of treatment, osmotic stress increased H_2S content by 123.3% and 80%, respectively. Exogenous ABA significantly induced the production of H_2S in stressed leaves,

compared with osmotic stress alone. After 24 and 48 h of treatment, exogenous ABA increased H_2S content by 50% and 46.7% under osmotic stress, respectively. ABA-pretreatment alone also increased H_2S content, compared with the control. Pretreatments with HT and AOA significantly lowered the accumulation of H_2S during osmotic stress when applied alone or in conjunction with ABA. The addition of NaHS reversed the effects of HT and AOA on the accumulation of H_2S during osmotic stress when applied alone or in conjunction with ABA.

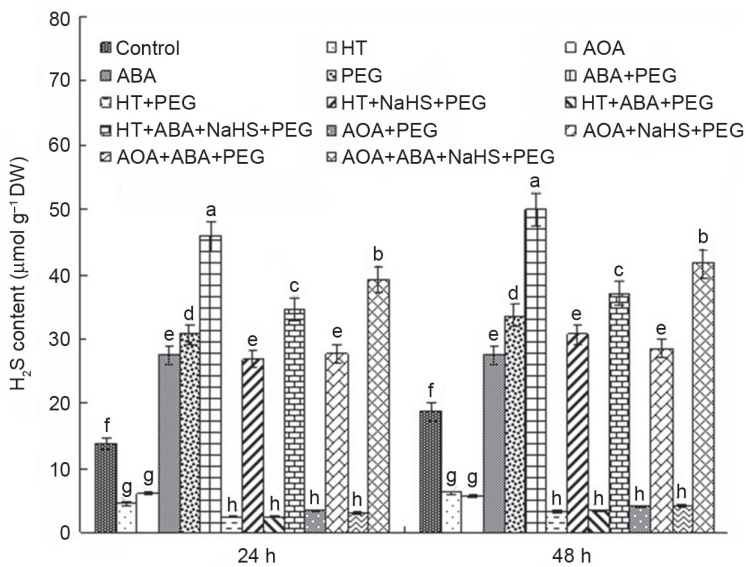

Figure 1. Effects of exogenous ABA, HT, AOA and NaHS on H_2S content in leaves of PEG-induced osmotic stressed leaves. The plants were treated as follows: Control, half-strength Hoagland's solution; HT, 20 μM HT; AOA, 0.3 mM AOA; ABA, 100 μM ABA; PEG, 15% PEG; ABA+PEG, 100 μM ABA+15% PEG; HT+PEG, 20 μM HT+15% PEG; HT+NaHS+PEG, 20 μM HT+0.3 mM NaHS+15% PEG; HT+ABA+PEG, 20 μM HT+100 μM ABA+15% PEG; HT+ABA+NaHS+PEG, 20 μM HT+100 μM ABA+0.3 mM NaHS+15% PEG; AOA+PEG, 0.3 mM AOA+15% PEG; AOA+NaHS+PEG, 0.3 mM AOA+0.3 mM NaHS+15% PEG; AOA+ABA+PEG, 0.3 mM AOA+100 μM ABA+15% PEG; AOA+ABA+NaHS+PEG, 0.3 mM AOA+100 μM ABA+0.3 mM NaHS+15% PEG. The plants were pretreated with ABA or HT or AOA or ABA+HT or ABA+AOA for 8 h, and then exposed to 15% PEG or NaHS+15% PEG for 24 and 48 h. Values represent mean±standard deviations (SD), small letters stand for significant difference among different treatments at $P<0.05$

Effects of exogenous ABA, HT, AOA and NaHS on the activities of enzymes in AsA-GSH cycle

Osmotic stress significantly increased the activities of APX, GR, DHAR and MDHAR, compared with control (Fig. 2). After 24 h of treatment, osmotic stress increased the activities of APX, GR, DHAR and MDHAR by 133.3%, 158.3%, 130.8% and 100%, respectively. After 48 h of treatment, osmotic stress increased the activities of APX, GR,

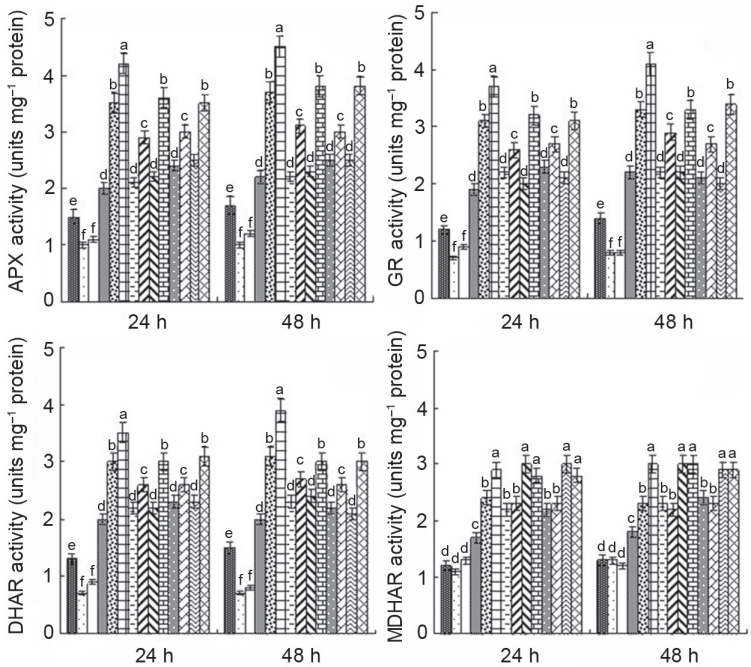

Figure 2. Effects of exogenous ABA, HT, AOA and NaHS on the activities of APX (A), GR (B), DHAR (C) and MDHAR (D) in PEG-induced osmotic stressed leaves. The plants were treated as described in the legend of Fig. 1

DHAR and MDHAR by 117.6%, 135.7%, 100% and 76.9%, respectively. Treatment with ABA before applying PEG significantly increased the activities of above enzymes, compared with osmotic stress alone. After 24 h of treatment, treatment with ABA before applying PEG increased the activities of APX, GR, DHAR and MDHAR by 20%, 19.3%, 16.7% and 20.8%, respectively. After 48 h of treatment, treatment with ABA before applying PEG increased the activities of APX, GR, DHAR and MDHAR by 21.6%, 24.2%, 30% and 17.4%, respectively. ABA-pretreatment alone also increased the activities of APX, GR, DHAR and MDHAR, compared with the control. HT and AOA significantly reduced the activities of APX, GR, DHAR but not MDHAR during osmotic stress with or without ABA. The addition of NaHS reversed above effects of HT and AOA on the activities of APX, GR and DHAR.

Effects of exogenous ABA, HT, AOA and NaHS on the redox state of AsA and GSH

Osmotic stress significantly decreased the ratios of AsA/DHA and GSH/GSSG, compared with control (Fig. 3). After 48 h of treatment, osmotic stress decreased the ratios of AsA/DHA and GSH/GSSG by 36.8% and 35%, respectively. After 48 h of treatment, osmotic stress decreased the ratios of AsA/DHA and GSH/GSSG by 45% and 42.8%, respectively.

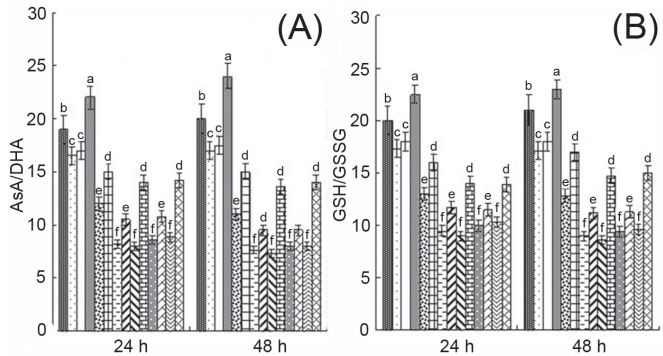

Figure 3. Effects of exogenous ABA, HT, AOA and NaHS on the ratios of AsA/DHA (A) and GSH/GSSG (B) in PEG-induced osmotic stressed leaves. The plants were treated as described in the legend of Fig. 1

Treatment with ABA before applying PEG significantly increased AsA/DHA and GSH/GSSG ratios, compared with osmotic stress alone. After 24 h of treatment, treatment with ABA before applying PEG increased the ratios of AsA/DHA and GSH/GSSG by 25% and 23.1%, respectively. After 48 h of treatment, treatment with ABA before applying PEG increased the ratios of AsA/DHA and GSH/GSSG by 36.4% and 41.6%, respectively. ABA-pretreatment alone also increased the ratios of AsA/DHA and GSH/GSSG, compared with the control. HT and AOA significantly reduced AsA/DHA and GSH/GSSG ratios induced by osmotic stress with or without ABA. The addition of NaHS reversed above effects of HT and AOA on AsA/DHA and GSH/GSSG ratios induced by osmotic stress with or without ABA.

Effects of exogenous ABA, HT, AOA and NaHS on MDA content and electrolyte leakage

Osmotic stress led to increases in MDA content and electrolyte leakage, compared with control (Fig. 4). After 24 h of treatment, osmotic stress increased MDA content and electrolyte leakage by 147.8% and 200%, respectively. After 48 h of treatment, osmotic stress increased MDA content and electrolyte leakage by 143.6% and 125%, respectively. Treatment with ABA before applying PEG significantly reduced the MDA content and electrolyte leakage, compared with osmotic stress alone. After 24 h of treatment, treatment with ABA before applying PEG decreased MDA content and electrolyte leakage by 26.3% and 37.5%, respectively. After 48 h of treatment, treatment with ABA before applying PEG decreased MDA content and electrolyte leakage by 40.3% and 33.3%, respectively. ABA-pretreatment alone had no obvious effect on MDA content and electrolyte leakage, compared with the control. Pretreatments with HT and AOA significantly increased the MDA content and electrolyte leakage of stressed leaves, compared with osmotic stress with or without ABA. The addition of NaHS reversed above effects of HT and AOA on MDA content and electrolyte leakage induced by osmotic stress with or without ABA.

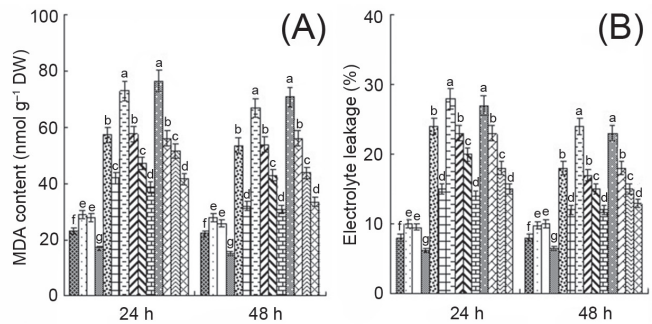

Figure 4. Effects of exogenous ABA, HT, AOA and NaHS on MDA content (A) and electrolyte leakage (B) of PEG-induced osmotic stressed leaves. The plants were treated as described in the legend of Fig. 1

Discussion

Many studies showed that osmotic stress induced oxidative damage in plants. In the current study, higher levels of lipid peroxidation, as indicated by MDA content and electrolyte leakage, was observed in wheat leaves in response to osmotic stress. To cope with the oxidative stress induced by osmotic stress, the activity of the AsA-GSH cycle in wheat leaves was up-regulated. It has been reported that exogenous ABA will up-regulate the activity of the AsA-GSH cycle in maize leaves through APX and GR activity, which alleviates the oxidative stress induced by osmotic stress (Zhang et al. 2007; Hu et al. 2007). In the present study, it was also found that exogenous ABA enhanced the activities of APX and GR under osmotic stress. Besides, this study found that exogenous ABA could increase the activities of DHAR and MDHAR involved in the AsA-GSH cycle in wheat leaves under osmotic stress.

AsA and GSH are major redox compounds in plants. It has been reported that the changes in the ratios of AsA/DHA and GSH/GSSG are more important than the individual changes in AsA and GSH content in response to oxidative stress (Kocsy et al. 2001). Our results showed that osmotic stress significantly decreased the ratios of AsA/DHA and GSH/GSSG. Osmotic stress plus ABA significantly increased AsA/DHA and GSH/GSSG ratios, compared with osmotic stress alone. Besides, our results indicated that signal molecular H_2S participated in the up-regulation of AsA/DHA and GSH/GSSG ratios by ABA under osmotic stress. Liu et al. (2011) reported that endogenous H_2S is involved in the process of ABA-induced stomatal closure. Present study showed that H_2S induced by exogenous ABA and osmotic stress was involved in the regulation of AsA-GSH cycle in wheat leaves through APX, GR and DHAR, which resulted in the increases in the ratios of AsA/DHA and GSH/GSSG under osmotic stress without or plus ABA. Christou et al. (2013) reported that H_2S could maintain high ascorbate and glutathione redox states through APX and GR in the AsA-GSH cycle under osmotic stress, which was consistent with our results. These results suggested that H_2S served an important role in ABA signaling and in the regulation of AsA-GSH cycle of plants.

It has been shown that osmotic stress and exogenous ABA can induce the production of H_2S and that endogenous H_2S is involved in the process of ABA-induced stomatal closure (Liu et al. 2011; Wang et al. 2012). Similar results were found in the present study, exogenous ABA and osmotic stress also could induce the production of endogenous H_2S. Besides, the results of our present study found that H_2S induced by exogenous ABA and osmotic stress was involved in the regulation of AsA-GSH cycle in wheat leaves through APX, GR and DHAR, which, in turn, increased the ratios of AsA/DHA and GSH/GSSG under osmotic stress alone and osmotic stress plus ABA. These results suggested that H_2S served an important role in ABA signaling and in the regulation of AsA-GSH cycle of plants.

As a stress-signaling molecule, ABA serves important roles in defending against oxidative stress in plant cells (Ding et al. 2009). Hu et al. (2008) has reported that NO, MAPK and H_2O_2 are all involved in the regulation of AsA-GSH cycle by ABA under osmotic stress. In the present study, H_2S was also involved in the regulation of AsA-GSH cycle by ABA under osmotic stress. However, whether there is relationship between NO, MAPK, H_2O_2 and H_2S in the regulation of AsA-GSH cycle in ABA signaling remains unknown. Further studies should investigate the relationship between NO, MAPK, H_2O_2 and H_2S in the regulation of AsA-GSH cycle in ABA signaling to provide more knowledge for the antioxidant metabolism in plants under osmotic stress.

In conclusion, the results clearly suggest that exogenous ABA and osmotic stress-induced H_2S accumulation participates in the regulation of AsA-GSH cycle, which, in turn, enhances the antioxidant ability and protects wheat seedling against oxidative stress induced by osmotic stress. These results provide new knowledge to the antioxidant metabolism in plants under osmotic stress.

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