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BRIEF COMMUNICATION

Effects of exogenous hydrogen sulfide on the redox states of ascorbate and glutathione in maize leaves under salt stress

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Abstract

This study investigated the effects of exogenous hydrogen sulfide (H_2S) on the redox states of ascorbate (AsA) and glutathione (GSH) in maize leaves under NaCl (100 mM) stress. Salt stress increased the activities of ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), γ-glutamylcysteine synthetase (γ-ECS), and *L*-galactono-1,4-lactone dehydrogenase (GalLDH), malondialdehyde content and electrolyte leakage, and reduced the ratios of reduced and oxidised forms of AsA (AsA/DHA) and GSH (GSH/GSSG) compared with control. Pretreatment with NaHS (H_2S donor) further enhanced the activities of the above enzymes except MDHAR and ameliorated the decrease in the ratios of AsA/DHA and GSH/GSSG compared with the salt stress alone. Pretreatment with NaHS significantly reduced the malondialdehyde content and electrolyte leakage induced by the salt stress. Pretreatment with NaHS alone did not affect any of the above mentioned parameters compared with the control. Our results suggest that exogenous H_2S could maintain the redox states of ascorbate and glutathione by up-regulating the ascorbate and glutathione metabolism and thus play an important role for acquisition of salt stress tolerance in maize.

Additional key words: ascorbate peroxidase, electrolyte leakage, glutathione reductase, malondialdehyde, NaCl, *Zea mays*.

Salt stress is one of the main environmental factors that adversely affect plant growth, productivity, and survival (Shi *et al.* 2007, Ferreira-Silva *et al.* 2012). Salt stress usually induces the overproduction of reactive oxygen species (ROS) in plant cells. Plants can protect themselves against oxidative damage by ROS-scavenging systems (Mittler 2002). Ascorbate (AsA) and glutathione (GSH) are two crucial nonenzymatic antioxidants. A fundamental role of AsA and GSH is to protect metabolic processes against H_2O_2 and other ROS. They play important roles in enzyme-catalysed reactions to detoxify H_2O_2. Besides, GSH can also protect proteins against the denaturation caused by oxidation of protein thiol groups (Noctor and Foyer 1998). The functions of AsA and GSH are closely related to their redox states (Kocsy *et al.* 2001) and plants can adjust redox states of AsA and GSH by modulating their regeneration and biosynthesis. *L*-galactose pathway is the main biosynthetic pathway of AsA biosynthesis in plants, *L*-galactono-1,4-lactone dehydrogenase (GalLDH; EC 1.3.2.3) is the last enzyme in L-galactose pathway (Wheeler *et al.* 1998). Gamma-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) is the first enzyme for glutathione biosynthesis (Dringen 2000). AsA-GSH cycle is the pathway for the regeneration of

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; AsA - reduced ascorbic acid; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; GalLDH - L-galactono-1,4-lactone dehydrogenase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; JA - jasmonic acid; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; ROS - reactive oxygen species; γ-ECS - γ-glutamylcysteine synthetase.
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AsA and GSH. In this cycle, ascorbate proxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), and glutathione reductase (GR; EC 1.6.4.2) are the key enzymes (Noctor and Foyer 1998). Thus, the enzymes involved in the biosynthetic and recycling pathways of ascorbate and glutathione play important roles in maintaining the redox states of ascorbate and glutathione in plants.

Hydrogen sulfide (H_2S) is the third gaseous signalling molecule after nitric oxide (NO) and carbon monoxide (CO) in animals (Hosoki *et al.* 1997). In plants, H_2S can promote root organogenesis (Zhang *et al.* 2009a), seed germination (Zhang *et al.* 2010a), and it is involved in responses to heavy metal, salt, drought, and osmotic stresses (Zhang *et al.* 2008, 2009b, 2010b, Li *et al.* 2012, Wang *et al.* 2012). However, whether H_2S participates in the regulation of ascorbate and glutathione metabolism in plants under salt stress remains unknown. Hence, we investigated the effects of exogenous H_2S on the activities of enzymes involved in ascorbate and glutathione metabolism and the ratios of AsA/DHA and GSH/GSSG in maize under NaCl stress.

Seeds of maize (*Zea mays* L.) cv. Xindan29 were sown in plastic trays filled with a sand/*Vermiculite* mix (1:1, v/v) and grown in a greenhouse under temperature of 25 - 30 °C, relative humidity of 70 %, irradiance of 500 μmol m^{-2} s^{-1} (photosynthetically active radiation), and a 10-h photoperiod. The seedlings were watered with distilled water every day. Fourteen days old seedlings of uniform height were selected for all experiments.

Roots of plants were washed thoroughly with tap water, placed in distilled water for 8 h, and then placed in beakers containing 100 cm^3 of 100 mM NaCl solution for 48 h at 25 °C and a continuous irradiance of 500 μmol m^{-2} s^{-1}. The beakers were wrapped with aluminum foil to keep the roots in dark. In order to study the effect of H_2S, a group of plants were pretreated with 0.6 mM NaHS for 8 h and then exposed to 100 mM NaCl or distilled water (control) for 48 h under the same conditions as described above. After 24 or 48 h, the top full expanded leaves were collected, frozen in liquid nitrogen, and then kept at -80 °C until used for analyses. Five seedlings were used for each analysis.

APX, GR, DHAR, and MDHAR were extracted by grounding each sample (0.5 g) into fine powder in liquid nitrogen and then homogenising the fine powder in 6 cm^3 of 50 mM KH_2PO_4 (pH 7.5) containing 0.1 mM ethylenediaminetetraacetic acid, 0.3 % (v/v) *Triton X-100*, and 1 % (m/v) polyvinylpolypyrrolidone, with an extra addition of 1 mM AsA for APX assay (Grace and Logan 1996). The extract was centrifuged at 13 000 *g* and 2 °C for 15 min and the supernatant was used for assays. The activities of APX, GR, MDHAR, and DHAR were assayed according to Nakano and Asada (1981), Grace and Logan (1996), Miyake and Asada (1992), and Dalton *et al.* (1986), respectively. One unit of APX activity was defined as the amount of APX catalyzing the oxidation of 1 μmol of ascorbate per min. One unit of GR activity was defined as the reduction of 1 μmol of NADPH per min. One unit of DHAR activity was defined as the amount of enzyme that produces 1 μmol of AsA per min. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 μmol of NADH per min.

GalLDH was extracted and measured by the method of Tabata *et al.* (2001). γ-ECS was extracted and measured by the method of Rüeggseger and Brunold (1992). One unit of GalLDH activity was defined as the amount of extract required to oxidize 1 nmol of *L*-Gal (equivalent to the formation of 2 nmol of reduced Cyt *c*) per min. One unit of γ-ECS activity was defined as 1 μmol of cysteine-dependently generated PO_4^{3-} per min. AsA and DHA were measured according to Hodges *et al.* (1996). GSSG and GSH were measured according to Griffith (1980). Malondialdehyde (MDA) content and electrolyte leakage were measured according to Hodges *et al.* (1999) and Zhao *et al.* (2004), respectively. Protein content was assayed according to Bradford (1976).

The experimental design was a randomized complete block design with five replications. Means were compared by one-way *ANOVA* and Duncan's multiple range test at the 5 % level of significance.

There were no obvious changes in the activities of APX, GR, DHAR, MDHAR, GalLDH, and γ-ECS in control plants after 24 and 48 h. Compared with the controls, NaCl stress (24 or 48 h) significantly increased the activities of the above mentioned enzymes. Pretreatment with H_2S significantly increased the activities of APX, GR, DHAR, GalLDH, and γ-ECS compared with NaCl alone. However, pretreatment with exogenous H_2S alone did not affect the activities of the enzymes in ascorbate and glutathione metabolism compared with the controls (Table 1).

There were also no obvious changes in the ratios of AsA/DHA and GSH/GSSG in control leaves after 24 and 48 h. Compared with the control, the salt stress significantly decreased the ratios of AsA/DHA and GSH/GSSG after 24 or 48 h, however, less after pretreatment with H_2S. The pretreatment with H_2S alone did not affect these ratios (Table 1).

To investigate whether H_2S has an important role for salt stress tolerance in maize leaves, the effects of pretreatment with exogenous H_2S on electrolyte leakage and MDA content under salt stress were studied. The results show that there was no difference between the controls and pretreatment with H_2S alone in the electrolyte leakage and MDA content after 24 and 48 h of the treatment. The addition of NaCl significantly increased the electrolyte leakage and MDA content compared to the controls but less after the pretreatment with H_2S (Table 1).

Many studies have proved that salt stress can induce oxidative damage in plants (Fatehi *et al.* 2012, Sorkheh *et al.* 2012). In our study, enhanced lipid peroxidation, as

Table 1. Effects of salt stress (100 mM NaCl) and H_2S (0.6 mM NaHS) pretreatment on the activities of enzymes involved in ascorbate and glutathione metabolism, the ratios of AsA/DHA and GSH/GSSG, malondialdehyde content, and electrolyte leakage in maize leaves measured after 24 or 48 h. Means ± SE, n = 5. Different letters within the same row indicate statistically significant differences according to Duncan's test ($P < 0.05$).

Parameters	Time [h]	Control	NaHS	NaCl	NaHS + NaCl
APX [U mg^{-1}(protein)]	24	1.50 ± 0.10c	1.60 ± 0.10c	2.30 ± 0.15b	3.00 ± 0.19a
	48	1.30 ± 0.08c	1.50 ± 0.11c	2.50 ± 0.17b	3.30 ± 0.20a
GR [U mg^{-1}(protein)]	24	1.70 ± 0.11c	1.60 ± 0.11c	2.40 ± 0.14b	3.30 ± 0.18a
	48	1.60 ± 0.09c	1.60 ± 0.08c	2.30 ± 0.12b	3.20 ± 0.20a
DHAR [U mg^{-1}(protein)]	24	2.50 ± 0.15c	2.30 ± 0.12c	3.65 ± 0.24b	5.00 ± 0.41a
	48	2.70 ± 0.17c	2.50 ± 0.14c	3.85 ± 0.28b	4.90 ± 0.38a
MDHAR [U mg^{-1}(protein)]	24	0.80 ± 0.05b	0.90 ± 0.07b	1.50 ± 0.10a	1.70 ± 0.10a
	48	0.70 ± 0.05b	0.90 ± 0.06b	1.70 ± 0.11a	1.60 ± 0.09a
γ-ECS [U mg^{-1}(protein)]	24	1.50 ± 0.09c	1.60 ± 0.12c	2.30 ± 0.14b	3.20 ± 0.19a
	48	1.60 ± 0.10c	1.50 ± 0.11c	2.50 ± 0.15b	3.50 ± 0.23a
GalLDH [U g^{-1}(f.m.)]	24	0.90 ± 0.06c	1.00 ± 0.07c	1.60 ± 0.12b	2.40 ± 0.16a
	48	1.00 ± 0.08c	1.10 ± 0.09c	1.70 ± 0.12b	2.30 ± 0.14a
AsA/DHA	24	19.9 ± 1.43a	19.3 ± 1.36a	13.5 ± 1.04c	16.5 ± 1.18b
	48	19.5 ± 1.38a	19.5 ± 1.41a	13.0 ± 1.01c	16.7 ± 1.13b
GSH/GSSG	24	21.0 ± 1.58a	20.7 ± 1.52a	12.0 ± 0.99c	16.8 ± 1.16b
	48	20.5 ± 1.42a	20.5 ± 1.51a	12.3 ± 1.03c	17.0 ± 1.20b
MDA content [nmol g^{-1}(f.m.)]	24	7.50 ± 0.49c	7.20 ± 0.45c	14.0 ± 1.11a	10.0 ± 0.81b
	48	8.00 ± 0.61c	7.50 ± 0.50c	19.0 ± 1.33a	13.0 ± 1.06b
Electrolyte leakage [%]	24	8.60 ± 0.68c	8.50 ± 0.65c	15.0 ± 1.14a	11.0 ± 0.91b
	48	9.00 ± 0.73c	8.50 ± 0.64c	19.0 ± 1.34a	14.0 ± 1.15b

indicated by the increased MDA content, and the electrolyte leakage were observed in the maize leaves in response to the 100 mM NaCl treatment. This result suggests that salt stress also induced oxidative stress in maize. To cope with it, the activities of antioxidants were up-regulated.

Our results show that the ratio of AsA/DHA decreased under the salt stress that was consistent with a previous study (Ferreira-Silva *et al.* 2012). The redox state of ascorbate is controlled by enzymes involved in its biosynthetic and recycling pathways. In the present study, the salt stress increased the activities of APX, DHAR, and MDHAR. Similarly, it has been reported that salt stress increases APX activity in lentil and mungbean (Bandeoğlu *et al.* 2004, Nazar *et al.* 2011) and DHAR and MDHAR activities in pea (Hernández *et al.* 1999, 2000). However, Ferreira-Silva *et al* (2012) reported that salt stress decreases APX activity in cashew leaves. Talukdar (2012) reported that salt stress does not affect MDHAR activity in *Lathyrus sativus*. These discrepancies may be due to the use of different plant species. Besides, we also found that the salt stress increased the GalLDH activity in the maize leaves. Many studies showed that H_2S, as a signal molecule, can modulate antioxidant response in plants under stresses (Wang *et al.* 2010, Chen *et al.* 2013). It has been reported that pretreatment with H_2S increases APX activity in alfalfa under salt stress (Wang *et al.* 2012). In the present study, we found that the pretreatment with H_2S increased the APX activity under the NaCl stress that is consistent with a previous study (Wang *et al.* 2012). Besides, H_2S increased the activities of DHAR and GalLDH, and the ratio of AsA/DHA in the maize leaves under the salt stress. On the other hand, the MDHAR activity was not increased. These results suggest that the pretreatment with H_2S could increase AsA/DHA through the stimulation of the biosynthetic and recycling pathways of AsA.

The redox state of glutathione can be maintained by γ-ECS and GR which are involved in the biosynthetic and recycling pathways of glutathione, respectively. Increase in GR activity in response to salinity stress in pea plants has been reported (Hernández *et al.* 1999, 2000). Mittova *et al.* (2003) reported that salt stress up-regulates γ-ECS protein in tomato. In the present study, the salt stress up-regulated the activities of γ-ECS and GR in the maize leaves which is consistent with previous studies (Hernández *et al.* 1999, 2000, Mittova *et al.* 2003). The NaCl stress decreased the ratio of GSH/GSSG in the maize leaves. However, Ferreira-Silva *et al* (2012) reported that salt stress increases the ratio of GSH/GSSG in cashew. In the present study, H_2S increased the activities of γ-ECS and GR, and the ratio of GSH/GSSG under the salt stress. Therefore, the pretreatment with H_2S could maintain the redox state of GSH under the salt stress.

Signal molecules, such as Ca^{2+}, H_2O_2, NO, abscisic

acid (ABA), and jasmonic acid (JA), can regulate ascorbate and glutathione metabolism and have important roles in responses to stresses (Li *et al.* 1998, Jiang and Zhang 2003, Wendehenne *et al.* 2004, Arasimowicz and Floryszak-Wieczorek 2007, Ai *et al.* 2008, Hu *et al.* 2008). In the present study, we found that H_2S may regulate the ascorbate and glutathione metabolism and has an important role in defencing oxidative stress in maize leaves. Wang *et al.* (2012) reported that NO is involved in the signal transduction of H_2S in regulating antioxidant response to salt stress in alfalfa. It has also been reported that ABA and JA can induce the production of H_2S and H_2O_2 in the process of stomatal closure (Hou *et al.* 2011, Liu *et al.* 2011). However, the signal transduction of H_2S in regulating ascorbate and glutathione metabolism remains unclear. So, it will be very interesting to investigate the relationship between H_2S and above signal molecules in regulating ascorbate and glutathione metabolism in plants.

NaHS in solutions has been widely used for production of H_2S (Hosoki *et al.* 1997). However, these solutions also contain Na^+ and other sulfur-containing components. In order to verify that H_2S/HS^- rather than other compounds derived from NaHS are responsible for the regulation of the ascorbate and glutathione metabolism in the maize seedlings under the salt stress, Na_2S, Na_2SO_4, Na_2SO_3, $NaHSO_4$, $NaHSO_3$, and NaAC (0.6 mM) were used as the controls of NaHS. The results show that above mentioned sulfur-containing components and Na^+ were not responsible for the increases in the ratios of AsA/DHA and GSH/GSSG under the salt stress (data not shown). These results suggest that H_2S or HS^- derived from NaHS play an important role in regulating the ascorbate and glutathione metabolism under salt stress.

In conclusion, our results clearly suggest that exogenous H_2S regulated the ascorbate and glutathione metabolism, which, in turn, enhanced the antioxidant ability and protected the maize seedlings against oxidative stress induced by the salt stress. These results also suggest that H_2S could be used as a regulator of salt tolerance of maize.

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