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BRIEF COMMUNICATION

Hydrogen sulfide is involved in the regulation of ascorbate and glutathione metabolism by jasmonic acid in *Arabidopsis thaliana*

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Abstract

This study investigated the role of hydrogen sulfide (H_2S) in the regulation of the ascorbate (AsA) and glutathione (GSH) metabolism by jasmonic acid (JA) in the leaves of *Arabidopsis thaliana* by using H_2S scavenger hypotaurine (HT) and H_2S synthetic mutant (SALK_041918, designated *Atl-cdes*). The results showed that JA significantly increased the H_2S content, the activities of *L*-cysteine desulfhydrase (*L*-CDes), *D*-cysteine desulfhydrase (*D*-CDes), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), L-galactono-1,4-lactone dehydrogenase (GalLDH) and γ-glutamylcysteine synthetase (γ-ECS), the ratio of AsA to dehydroascorbate (DHA), and decreased the content of malondialdehyde (MDA) and H_2O_2 in the wild type of *A. thaliana*, compared to control. The above effects of JA except the increased activities of *L*-CDes and *D*-CDes were suppressed by addition of HT. However, JA and HT+JA had no significant effects on the ratio of reduced GSH to oxidized GSH (GSSG) in the wild type of *A. thaliana*. Application of HT to the control decreased H_2S content, AsA/DHA ratio, and activities of APX, GR, DHAR, MDHAR, γ-ECS, and GalLDH, but had no effects on MDA content, activities of *L*-CDes and *D*-CDes, and GSH/GSSG ratio. In the H_2S synthetic mutant, JA had no obvious effects on above mentioned parameters except the *D*-CDes activity compared with the control. Our results suggest that JA-induced H_2S, which is a signal that leads to the up-regulation of the AsA and GSH metabolism.

Addtional key words: ascorbate peroxidase, cysteine desulfhydrase, glutathione reductase, H_2S synthetic mutant, hypotaurine.

Ascorbate (AsA) and glutathione (GSH) have important roles in defence against oxidative damage induced by stresses. Ascorbate-glutathione cycle is the regeneration pathway of AsA and GSH through ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer 1998). Through this cycle, AsA and GSH are regenerated and H_2O_2 is scavenged, which, in turn, alleviates oxidative damage induced by reactive oxygen species (ROS). L-galactono-1,4-lactone dehydrogenase (GalLDH) and γ-glutamyl-cysteine synthetase (γ-ECS) are the key enzymes which are responsible for the biosynthesis of AsA and GSH, respectively. The regeneration and biosynthesis pathway of AsA and GSH have important roles in regulating their redox states, which, in turn, regulates the responses of

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbate; DHAR - dehydroascorbate reductase; γ-ECS - γ-glutamylcysteine synthetase; GalLDH - L-galactono-1,4-lactone dehydrogenase; GR - glutathione reductase; GSH - glutathione; HT - hypotaurine; JA - jasmonic acid; MDA - malondiadehyde; MDHAR - monodehydroascorbate reductase; WT - wild type.
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plants to stresses. Thus, it is very important to study the regulatory mechanism of the redox state of AsA and GSH.

Jasmonic acid (JA) is an important plant hormone that plays important roles in regulating stress responses as well as plant growth and development (Radhika *et al*. 2010, Ren and Dai 2012). Increasing evidence shows that JA can enhance the metabolism of AsA and GSH in plants. Our previous studies (Shan *et al*. 2011a, 2015) showed that signal molecules NO and mitogen-activated protein kinase kinase 1/2 (MEK1/2) participate in signal transduction of JA during regulation of the AsA and GSH metabolism.

Hydrogen sulfide is the third gaseous signalling molecule following nitric oxide (NO) and carbon monoxide (CO). In plants, H_2S can promote root organogenesis (Zhang *et al*. 2009a) and seed germination (Zhang *et al*. 2010a). Increasing evidence has proven that exogenous H_2S affects the antioxidative response under heavy metal, drought, and salt stresses (Zhang *et al*. 2008, 2009b, 2010b, Li *et al*. 2012, Wang *et al*. 2012). Our previous study showed that exogenous H_2S could regulate the metabolism of AsA and GSH in wheat leaves under osmotic stress (Shan *et al*. 2011b). It has been reported that H_2S is involved in the JA-induced stomatal closure (Hou *et al*. 2011). However, whether endogenous H_2S participates in the regulation of the AsA and GSH metabolism by JA remains unknown. To fulfil this gap, the current study investigated the content of H_2S, malondialdehyde (MDA), and H_2O_2, and the activities of H_2S synthetases *L*-cysteine desulfhydrase (*L*-CDes) and *D*-cysteine desulfhydrase (*D*-CDes), and the enzymes involved in the ascorbate and glutathione metabolism in the leaves of *Arabidopsis thaliana* treated with JA.

The seeds of *Arabidopsis thaliana* L. ecotype Col-0 (used as wild-type, WT) were supplied by Arabidopsis Biological Resource Center of USA. The mutant seeds of *A. thaliana* (*Atl-cdes*) based on the same ecotype were supplied by Prof. Xin Liu of Qingdao Agricultural University. *Atl-cdes* is the T-DNA insertion mutant of H_2S synthetase *L*-CDes (Liu *et al*. 2011). In this mutant, the synthetic pathway through *L*-CDes is interrupted. The *A. thaliana* seedlings were grown in half-strength Hoagland's solution for 4 weeks and then transferred to the half-strength Hoagland's solution containing 30 μM JA. A group of plants was pretreated with H_2S scavenger hypotaurine (HT; Peng *et al*. 2016) by soaking roots in half-strength Hoagland's solution containing 20 μM HT for 8 h and then exposed to 30 μM JA for 48 h. Control plants were treated with half-strength Hoagland's solution alone. Also the H_2S synthesis mutant plants were treated by JA for 48 h. Each treatment consisted of five replicates with 30 plants each. Hoagland's solutions were replaced by corresponding fresh solutions every day. The top fully expanded leaves of plants were harvested after 24 and 48 h of JA-treatment, frozen in liquid nitrogen, and stored at -80 °C until analyses. All the experiments were conducted in a greenhouse under a temperature of 22 ± 2 °C, a relative humidity of 70 %, an irradiance of 120 μmol(photons) m^{-2} s^{-1}, and a 16-h photoperiod.

Enzymes were extracted according to Shan and Liang (2010). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). One unit of enzyme was defined as the amount of APX catalyzing the oxidation of 1 μmol of ascorbate per minute Glutathione reductase (GR, EC 1.6.4.2) activity was monitored at 340 nm in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 0.5 mM ethylenediaminetetraacetic acid, 0.5 mM $MgCl_2$, 10 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 of NADPH per minute. Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed at 340 nm (Miyake and Asada 1992). One unit of MDHAR activity was defined as the amount of enzyme that oxidized 1 μmol of NADH per minute. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured at 265 nm in assay solution containing 100 mM Hepes-KOH (pH 7.0), 20 mM GSH, 2 mM dehydroascorbate (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 produced 1 μmol of ascorbate (AsA) per minute. L-galactono-1,4-lactone dehydrogenase (GalLDH, EC 1.3.2.3) was extracted and measured according to Shan and Liang (2010). Frozen samples were ground in a prechilled mortar with ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.4 M sucrose. The extract was passed through 2 layers of miracloth and then centrifuged at 300 *g* for 10 min. The supernatant was centrifuged at 10 000 *g* for 20 min. The sediment was suspended in the above buffer containing 0.4 M sucrose. The assay mixture was composed of enzyme solution, 1.05 mg cm^{-3} cytochrom *c* dissolved in potassium phosphate buffer, and 56 mM L-galactono-1,4-lactone (L-GalL). Before assay, the mixture was incubated at 25 °C for 1 min. The increase in absorbance at 550 nm was followed immediately after the addition of L-Gal. One unit of GalLDH activity was defined as the amount of enzyme required to oxidize 1 nmol of L-Gal per minute. A γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2) was extracted and measured according to Rüeggseger and Brunold (1992). Frozen samples were ground in prechilled mortar with 0.1 M precooled Tris-HCl (pH 8.0). The extract was centrifuged at 20 000 g for 10 min. The supernatant was used for the assay of enzyme activity. The mixture of supernatant, 0.1 M Tris-HCl (pH 8.0), 0.25 mM glutamate, 10 mM ATP, 1 mM dithioerythritol, and 2 mM cysteine reacted at 25 °C for 1 h. Then, phosphorus agent, which consisted of 3 mM H_2SO_4, distilled water, 2.5 % (m/v) ammonium molybdate, and 10 % (m/v) AsA, was added and mixed. The mixture was incubated at 45 °C for 25 min and

cooled at room temperature. Then, the absorbance at 660 nm was measured. One unit of γ-ECS activity was defined as 1 μmol of cysteine-generated PO_4^{3-} per minute determined by the molybdenum blue method. A molar coefficient of 5.6 mM^{-1} cm^{-1} was used for the calculation of enzyme activity. The activities of *L*-cysteine desulfhydrase (*L*-CDes, EC 4.4.1.1) and *D*-cysteine desulfhydrase (*D*-CDes, EC 4.1.99.4) were measured according to the method of Riemenschneider *et al.* (2005). One unit of *L*-CDes and *D*-CDes activities was defined as the formation of 1 nmol of H_2S per minute. The specific activities of all above enzymes were expressed as units per mg of protein. Protein content was determined by the method of Bradford (1976).

The content of AsA and DHA was measured according to Hodges *et al*. (1996) based on the reduction of ferric to ferrous ion with AsA in acidic solution. The content of GSSG and GSH was measured according to Griffith (1980) based on the enzymatic recycling method and a thiol-masking reagent, 2-vinylpyridine. H_2S was determined by formation of methylene blue from dimethyl-*p*-phenylenediamine in H_2SO_4 as described by (Zhang *et al*. (2008). MDA content was measured by thiobarbituric acid reaction as described by Hodges *et al*. (1999). H_2O_2 content was determined according to Okuda *et al*. (1991).

The whole experiment was repeated five times with 30 seedlings in each repetition. The means were compared by one-way analysis of variance and Duncan's multiple range test at the 5 % level of significance.

After treatment of *Arabidopsis* WT plants with JA for 24 or 48 h, H_2S content in leaves significantly increased and also the activities of APX, GR, DHAR, MDHAR, GalLDH, and γ-ECS, compared to the control (Tables 1 and 2). Compared with JA alone, application of HT + JA significantly reduced H_2S content and the activities of APX, GR, DHAR, MDHAR, GalLDH and γ-ECS after 24 or 48 h. Exogenous JA had no obvious effects on H_2S content and did not increased activities of the above mentioned enzymes in the H_2S synthesis mutant. Meanwhile, application of HT alone significantly reduced H_2S content and the activities of APX, GR, DHAR, MDHAR, GalLDH and γ-ECS after treatment of 24 and 48 h, compared with control. These results suggest that H_2S may be involved in the regulation of the AsA and GSH metabolism by exogenous JA in the leaves of *A. thaliana*.

Compared to the control, JA significantly increased the ratios of AsA/DHA and GSH/GSSG in the leaves of *Arabidopsis* WT plants after 24 and 48 h (Table 1) but had no obvious effects on these ratios in the H_2S synthesis mutant. Meanwhile, application JA + HT or HT alone significantly reduced the ratio of AsA/DHA and had no obvious effect on the ratio of GSH/GSSG, compared with control. These results suggest that the accumulation of H_2S induced by exogenous JA participated in the regulation of AsA/DHA ratio.

Compared with the control, JA significantly reduced the content of MDA and H_2O_2 in the leaves of *Arabidopsis* WT plants after 24 and 48 h (Table 1). Compared with JA alone, application of HT + JA significantly increased the content of MDA and H_2O_2. Exogenous JA had no obvious effects on the content of MDA and H_2O_2 in the H_2S synthesis mutant. Meanwhile,

Table 1. Effects of jasmonic acid (JA) and hypotaurine (HT) on the content of H_2S, malondialdehyde (MDA), and H_2O_2, the ascorbate (AsA)/dehydroascorbate (DHA) and reduced glutathione(GSH)/oxidized glutathione (GSSG) ratios, and activities of cysteine desulfhydrases (*L*-CDes and *D*-CDes) in the leaves of *A. thaliana*. Control - half-strength Hoagland's solution; JA_{WT} - treatment of *Arabidopsis* wild-type with 30 μM JA; HT + JA_{WT} - treatment of *Arabidopsis* wild-type with 20 μM HT + 30 μM JA; JA_{MT} - treatment of H_2S synthetic mutant with 30 μM JA. The plants were pretreated with HT for 8 h, and then exposed to JA for 48 h. Means ± SEs $n = 5$, different small letters stand for significant differences among different treatments at $P < 0.05$. Differences between treatments lasted 24 and 48 h were not significant.

Parameters	Time [h]	Control	HT	JA_{WT}	HT + JA_{WT}	JA_{MT}
H_2S	24	6.62 ± 0.71b	2.15 ± 0.30c	14.58 ± 1.35a	7.22 ± 0.68b	6.59 ± 0.52b
[nmol g^{-1}(f.m.)]	48	7.34 ± 0.84b	2.44 ± 0.26c	11.29 ± 1.11a	6.54 ± 0.74b	6.90 ± 0.80b
AsA/DHA	24	18.51 ± 2.13b	16.87 ± 1.81c	20.74 ± 1.75a	17.96 ± 1.71b	18.06 ± 1.94b
	48	20.03 ± 2.01b	18.00 ± 2.06c	22.95 ± 2.33a	19.73 ± 2.51b	19.80 ± 2.37b
GSH/GSSG	24	20.09 ± 1.95a	19.81 ± 2.13a	20.46 ± 2.03a	19.87 ± 1.78a	20.30 ± 2.51a
	48	22.17 ± 2.46a	21.79 ± 2.37a	22.55 ± 2.97a	21.64 ± 2.16a	22.07 ± 2.22a
MDA	24	2.19 ± 0.24b	2.88 ± 0.35a	1.67 ± 0.15c	2.11 ± 0.20b	2.08 ± 0.23b
[nmol g^{-1}(f.m.)]	48	2.41 ± 0.28b	3.10 ± 0.33a	1.80 ± 0.18c	2.32 ± 0.25b	2.38 ± 0.21b
H_2O_2	24	6.07 ± 0.72b	6.97 ± 0.81a	4.64 ± 0.51c	5.97 ± 0.53b	6.12 ± 0.55b
[μmol g^{-1}(f.m.)]	48	6.53 ± 0.66b	7.23 ± 0.71a	4.59 ± 0.53c	6.35 ± 0.47ab	6.37 ± 0.78b
L-CDes	24	13.96 ± 1.56b	14.83 ± 1.42b	33.57 ± 4.11a	35.09 ± 4.72a	0.00 ± 0.00c
[U mg^{-1}(protein)]	48	15.00 ± 1.72b	14.15 ± 1.51b	29.35 ± 2.75a	30.64 ± 3.83a	0.00 ± 0.00c
D-CDes	24	7.25 ± 1.03b	7.40 ± 0.97b	16.18 ± 1.73a	16.43 ± 1.57a	17.54 ± 1.77a
[U mg^{-1}(protein)]	48	6.81 ± 0.94b	7.03 ± 0.85b	18.00 ± 1.80a	17.55 ± 1.84a	18.32 ± 1.95a

Table 2. Effects of jasmonic acid (JA) and hypotaurine (HT) on the activities of ascorbate peroxidase (APX), gutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), γ-glutamylcysteine synthetase (γ-ECS), and L-galactono-1,4-lactone dehydrogenase (GalLDH) in the leaves of *A. thaliana*. Control - half-strength Hoagland's solution; JA_{WT} - treatment of *Arabidopsis* wild-type with 30 μM JA; HT + JA_{WT} - treatment of *Arabidopsis* wild-type with 20 μM HT + 30 μM JA; JA_{MT} - treatment of H_2S synthetic mutant with 30 μM JA. The plants were pretreated with HT for 8 h, and then exposed to JA for 48 h. Means ± SEs $n = 5$, different small letters stand for significant differences among different treatments at $P < 0.05$. Differences between treatments lasted 24 and 48 h were not significant.

Parameters	Time [h]	Control	HT	JA_{WT}	HT + JA_{WT}	JA_{MT}
APX	24	0.93 ± 0.10c	0.45 ± 0.05d	2.42 ± 0.26a	1.50 ± 0.13b	1.02 ± 0.09c
[U mg^{-1} (protein)]	48	0.75 ± 0.06c	0.37 ± 0.05d	2.00 ± 0.22a	1.25 ± 0.15b	0.81 ± 0.08c
GR	24	2.44 ± 0.27b	1.03 ± 0.15c	3.16 ± 0.34a	2.30 ± 0.22b	2.19 ± 0.24b
[U mg^{-1} (protein)]	48	2.25 ± 0.25b	0.79 ± 0.09c	3.50 ± 0.31a	2.25 ± 0.22b	2.40 ± 0.21b
DHAR	24	0.62 ± 0.07b	0.28 ± 0.04c	1.24 ± 0.11a	0.71 ± 0.09b	0.57 ± 0.05b
[U mg^{-1} (protein)]	48	0.50 ± 0.04b	0.23 ± 0.03c	1.00 ± 0.11a	0.60 ± 0.06b	0.54 ± 0.04b
MDHAR	24	0.87 ± 0.09c	0.39 ± 0.05d	1.60 ± 0.14a	1.26 ± 0.12b	1.00 ± 0.11c
[U mg^{-1} (protein)]	48	1.00 ± 0.11b	0.34 ± 0.03c	1.65 ± 0.18a	1.15 ± 0.11b	1.10 ± 0.12b
γ-ECS	24	2.88 ± 0.30c	1.08 ± 0.15d	5.12 ± 0.55a	3.84 ± 0.41b	3.00 ± 0.25c
[U mg^{-1} (protein)]	48	3.00 ± 0.27c	1.32 ± 0.21d	5.53 ± 0.61a	4.22 ± 0.46b	3.28 ± 0.35c
GalLDH	24	1.00 ± 0.12b	0.44 ± 0.06c	1.93 ± 0.23a	1.13 ± 0.13b	1.20 ± 0.15b
[U mg^{-1} (protein)]	48	1.07 ± 0.12b	0.53 ± 0.05c	1.70 ± 0.18a	1.22 ± 0.11b	1.12 ± 0.11b

application of HT alone significantly increased the content of MDA and H_2O_2, compared with the control. These results suggested that H_2S has important role in regulating the redox potential in the cells.

In order to elucidate the role of H_2S synthetases *L*-CDes and *D*-CDes in regulating the content of H_2S, the effects of different treatments on the activities of *L*-CDes and *D*-CDes were studied. The results showed that JA significantly increased the activities of *L*-CDes and *D*-CDes in the leaves of *Arabidopsis* WT plants after 24 and 48 h, compared with the control (Table 1). Compared with JA alone, application of HT + JA had no obvious effects on the activities of *L*-CDes and *D*-CDes. Meanwhile, application of HT alone had no obvious effects on the activities of *L*-CDes and *D*-CDes, compared with control. Exogenous JA also increased *D*-CDes activity in the H_2S synthesis mutant. However, *L*-CDes activity was not detected in this mutant. These results suggested that HT has no obvious effect on the activities of H_2S synthetases, and JA could regulate the content of H_2S through regulation of *L*-CDes and *D*-CDes.

For the same treatment, there were no significant differences in H_2S content, the activities of *L*-CDes, *D*-CDes, APX, GR, DHAR, MDHAR, GalLDH and γ-ECS, the ratios of AsA/DHA and GSH/GSSG, and the content of MDA and H_2O_2 between 24 and 48 h.

H_2S has important roles in improving drought, heat, tolerance and flooding tolerance (Ziogas *et al*. 2015, Chen *et al*. 2016, Li *et al.* 2016, Peng *et al*. 2016). Especially, H_2S could regulate antioxidant responses in plants (Chen *et al*. 2013). For example, Ziogas *et al*. (2015) reported that H_2S could alleviate the damage induced by drought stress in citrus through antioxidant responses indicated by decreased ion leakage and lipid peroxidation in plants. It has been documented that H_2S could regulate the redox states of AsA and GSH by affecting their biosynthesis and regeneration (Zhang *et al*. 2010b, Shan *et al*. 2011b, Wang *et al.* 2012). Our previous studies (Shan and Liang 2010, Shan *et al*. 2011a) showed that JA also regulated the redox state of AsA and GSH by affecting their biosynthesis and regeneration. It has been shown that endogenous H_2S is involved in the JA-induced stomatal closure (Hou *et al*. 2011). In the present study, we found that exogenous JA increased H_2S content which was involved in the regulation of the AsA and GSH metabolism through increased APX, DHAR, MDHAR, and GalLDH activities which, in turn, increased the ratio of AsA/DHA. Besides, we found that JA-induced H_2S had significant effects on the activities of GR and γ-ECS, but had no obvious effect on the GSH/GSSG ratio. What is the reason for this phenomenon? This may be due to that there exist other regulatory factors, such as other enzymes in the GSH metabolism, including other enzymes in GSH biosynthesis, glutathione peroxidase (GPX) and glutathione S-transferase (GST). These results suggested that H_2S has an important role in JA signalling in regulating the metabolism of AsA and GSH and the ratio of AsA/DHA in plants, which was the new finding in comparison with the already published data.

L-CDes and *D*-CDes are two enzymes known to produce H_2S in plants (Yamasaki and Cohen 2016). It was not surprising that the *Alt-cdes* mutant had no *L*-CDes activity, but it had higher *D*-CDes activity in response to JA, compared with control. Meanwhile, JA

also significantly increased the activities of *L*-CDes and *D*-CDes in WT plants. Thus, we have strong evidence that the activities of *L*-CDes and *D*-CDes were induced by JA among the other enzymes that produce H_2S in *Arabidopsis*, which may be also a new finding.

As a signal molecule, JA has important role in modulating the redox state of the cells through the AsA and GSH metabolism. However, the relationship between H_2S, NO, and MEK1/2 in the regulation of the AsA and GSH metabolism during JA signalling remains unclear. Therefore, it might be very interesting to investigate the relationships between JA, H_2S, H_2O_2, NO, and MEK1/2 in the regulation of the AsA and GSH metabolism in detail.

In conclusion, the results clearly suggest that exogenous JA-induced H_2S accumulation participated in the regulation of the AsA and GSH metabolism, which, in turn, maintains the redox state in the cells.

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