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Nitric oxide participates in the regulation of ascorbate-glutathione cycle and water physiological characteristics of *Arabidopsis thaliana* by NaHS

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Abstract

We investigated the role of nitric oxide (NO) in the regulation of ascorbate-glutathione (AsA-GSH) cycle and water physiological characteristics of *Arabidopsis thaliana* by sodium hydrosulfide (NaHS). NaHS markedly increased the contents of H_2S, NO, chlorophyll (Chl), and carotenoids, the activity of AsA-GSH cycle, ascorbate/dehydroascorbate ratio, net photosynthetic rate, Chl fluorescence parameters, transpiration rate, stomatal conductance, and relative water content in leaves and the biomass of wild-type *Arabidopsis*. However, NaHS markedly decreased malondialdehyde content and electrolytic leakage. Except H_2S, above NaHS-induced promotions were suppressed by nitrate reductase (NR) inhibitor sodium azide (NaN_3). Application of sodium nitroprusside (SNP) to (NaN_3+NaHS)-treated wild type *Arabidopsis* (NaN_3+SNP+NaHS) reversed above effects of NaN_3+NaHS. However, NaN_3+NaHS and NaN_3+SNP+NaHS had no significant effects on H_2S content. Meanwhile, we proved above results by using NO-associated NR gene mutant *nia1,nia2*. Above results suggested that NO participated in the regulation of AsA-GSH cycle and water physiological characteristics of *Arabidopsis* by NaHS.

Additional key words: donor; gas exchange; Halliwell-Asada pathway; hydrogen sulfide; nitric oxide; nitrate reductase gene mutant.

Introduction

Ascorbate (AsA) is an important metabolite, which plays various physiological functions in plants (Horemans *et al.* 2000). First, AsA is an important antioxidant in cells and helps other antioxidants fulfil their functions (Horemans *et al.* 2000, Shan *et al.* 2018). Second, AsA regulates the division, expansion, and elongation of cells (Potters *et al.* 2002). Third, AsA regulates plant defense and survival by modulating the expression of defense genes and photosynthetic genes, *etc*. (Kiddle *et al.* 2003). Finally, AsA regulates the redox equilibrium of plants by modulating the redox state (Shan *et al.* 2018). Ascorbate-glutathione (AsA-GSH) cycle (Halliwell-Asada pathway) has important roles in maintaining AsA content and the redox equilibrium in plants (Avashthi *et al.* 2018). In this cycle, ascorbate peroxidase (APX) can remove H_2O_2. Monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) can realize the regeneration of AsA. Glutathione reductase (GR) is responsible for the regeneration of GSH (Shan *et al.* 2015). Thus, AsA-GSH cycle has the important role in balancing the redox state of ascorbate in plants, which further regulates plant growth and development. Therefore, we can regulate the redox state of ascorbate through AsA-GSH cycle by using exogenous substances.

Hydrogen sulfide (H_2S) is a key gas signal molecule in plants. Increasing evidence shows that H_2S fulfils important roles in regulating plant growth, development, and other physiological processes (Li *et al.* 2017, Kaya and Ashraf 2019, Mei *et al.* 2019). Nitric oxide (NO) is another key gas signal molecule in plants (Hasanuzzaman *et al.* 2018, Recalde *et al.* 2018, Okant and Kaya 2019). Increasing evidence also showed that NO is involved in

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Abbreviations: APX – ascorbate peroxidase; AsA – ascorbate; AsA/DHA – the ratio of ascorbate to dehydroascorbate; AsA-GSH – ascorbate-glutathione; Car – carotenoids; Chl – chlorophyll; DHAR – dehydroascorbate reductase; E – transpiration rate; EL – electrolyte leakage; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; GR – glutathione reductase; MDA – malondialdehyde; MDHAR – monodehydroascorbate reductase; NR – nitrate reductase; P_N – net photosynthetic rate; Φ_{PSII} – effective quantum yield of PSII; q_N – nonphotochemical quenching; q_P – photochemical quenching; RWC – relative water content; SNP – sodium nitroprusside.

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the regulation of plant growth, development, and other physiological processes, such as root growth and stomatal movement (Hao *et al.* 2010, Wang *et al.* 2010, Zhao *et al.* 2010, Sun *et al.* 2017). Many studies showed that AsA-GSH cycle regulates the redox state of ascorbate (Shan *et al.* 2015, Avashthi *et al.* 2018). However, the signal regulation mechanism of AsA-GSH cycle is still unclear. Our previous study showed that H_2S regulated the redox state of ascorbate through AsA-GSH cycle in *Arabidopsis thaliana* (Shan *et al.* 2018). Our previous studies also showed that NO regulated the redox state of ascorbate through AsA-GSH cycle in maize and *Agropyron cristatum* (Shan *et al.* 2012, Shan and Sun 2018). Besides, our previous study showed that H_2S donor sodium hydrosulfide (NaHS) regulated the redox state of ascorbate through AsA-GSH cycle in wheat (Shan *et al.* 2011). However, whether signal molecule NO can participate in the regulation of AsA-GSH cycle in *A. thaliana* by NaHS is still unclear. Thus, it is very important to elucidate the role of NO in NaHS-signaling the redox state of ascorbate through AsA-GSH cycle in *A. thaliana*.

It has been documented that NaHS improved the growth of rice seedlings and submerged macrophytes by regulating water physiological characteristics, including photosynthesis, chlorophyll (Chl) fluorescence, gas exchange, *etc.* (Duan *et al.* 2015, Parveen *et al.* 2017). NO had positive effects on photosynthesis, Chl fluorescence, and gas exchange of *Crambe abyssinica* and tomato (Ahmad *et al.* 2018, Batista *et al.* 2018). However, whether signal molecule NO can participate in the regulation of above water physiological characteristics in *A. thaliana* by NaHS is still unclear. Thus, it is very important to elucidate the role of NO in the regulation of photosynthesis, Chl fluorescence, and gas exchange of *A. thaliana*.

In this study, we investigated the contents of H_2S, NO, Chl, and carotenoids (Car), activities of four enzymes in the AsA-GSH cycle, the ratio of ascorbate to dehydroascorbate (AsA/DHA), leaf relative water content (RWC) and plant biomass, net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and Chl fluorescence parameters, such as maximum photochemical efficiency of PSII (F_v/F_m), photochemical quenching (q_P), nonphotochemical quenching (q_N), and quantum efficiency of PSII photochemistry (Φ_{PSII}), in wild-type *A. thaliana* and NO-associated mutant of nitrate reductase (NR) gene (*nia1,nia2)*. The scope and need of this study was to elucidate the role of NO in the signal process of NaHS in regulating AsA-GSH cycle, photosynthesis, Chl fluorescence and gas exchange of *A. thaliana*, which could provide new knowledge on the action mechanism of NaHS in plants.

Materials and methods

Plant culture and treatment: Seeds of wild-type *Arabidopsis* and mutant *nia1,nia2* were bought from Arabidopsis Biological Resource Center (ABRC) in USA. The ecotype of *Arabidopsis* is Col-0. Ten-day-old seedlings were transferred to half-strength Hoagland's solution. Then, 28-d-old seedlings with uniform height and growth status were selected for our experiments. To study the effect of NaHS, 28-d-old seedlings were transferred to half-strength Hoagland's solution containing 0.2 mM NaHS (*Sigma-Aldrich*, USA) for 7 d. To study the effect of NR inhibitor sodium azide (NaN_3), plants were pretreated with 0.1 mM NaN_3 for 8 h and then exposed to 0.2 mM NaHS for 7 d. To investigate whether the effect of NaN_3 can be reversed by exogenous NO, the plants were pretreated with 0.1 mM NaN_3 for 8 h and then treated by 100 μM NO donor, sodium nitroprusside (SNP), plus 0.2 mM NaHS for 7 d. Control plants were treated by half-strength Hoagland's solution alone. The solutions of SNP, NaN_3, and NaHS were prepared by adding these compounds into half-strength Hoagland's solution, respectively. To further prove the role of NO in the regulation of AsA-GSH cycle, photosynthesis, Chl fluorescence, and gas exchange of *A. thaliana* by NaHS, we investigated the effects of SNP and NaHS on corresponding indicators in NO-associated mutant *nia1,nia2*. The plants of mutant *nia1,nia2* were exposed to 0.2 mM NaHS or 100 μM SNP + 0.2 mM NaHS for 7 d. In each treatment, the number of repetitions was five. After 2 d of treatment, the top fully expanded leaves of seedlings were collected and frozen in liquid nitrogen, and then kept at –80 °C until analyses. All the experiments were operated in a greenhouse. The greenhouse conditions were as follows: the temperature ranged from about 22 ± 2°C, the relative humidity was 70%, and the photoperiod was 16 h with PPFD of 120 μmol(photon) m^{-2} s^{-1}. After treatment for 7 d, plant biomass was measured.

Enzyme activities: After 2 d of treatment for 28-d-old seedlings, 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 activity for above enzymes was expressed as U mg^{-1}(protein). One unit of APX activity was defined as the amount of APX catalyzing the oxidation of 1 μmol(ascorbate) per min. One unit of GR activity was defined as the reduction of 1 μmol(NADPH) per min. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 μmol(NADH) per min. One unit of DHAR activity was defined as the amount of enzyme that produces 1 μmol(AsA) per min. Protein concentration was measured according to Bradford (1976).

AsA/DHA ratio and the contents of H_2S and NO: After 2 d of treatment, AsA/DHA ratio was measured according to Hodges *et al.* (1996). The contents of H_2S and NO were measured according to Zhang *et al.* (2008) and Song *et al.* (2008), respectively. Above three indicators were determined by using *TU-1810* UV-Vis spectrophotometer (*Beijing Purkinje General Instrument Co*., *Ltd*., China).

Chl and Car contents: After 2 d of treatment, the top fully expanded leaves were collected and immediately used to measure the contents of Chl and Car according

to Lichtenthaler and Wellburn (1983). *TU-1810* UV-Vis spectrophotometer (*Beijing Purkinje General Instrument Co*., *Ltd*., China) was used to measure Chl and Car contents.

Chl fluorescence and gas-exchange parameters: After 2 d of treatment, a *Yaxin-1161G* fluorometer (*Yaxin*, China) was used to measure Chl fluorescence parameters from 10:00 to 12:00 h. For dark adaptation, the leaves were covered for 30 min. Then F_v/F_m, q_P, q_N, and Φ_{PSII} were measured and calculated according to Dai and Shan (2019). Measurements were performed in a closed chamber under controlled growth conditions. Minimum fluorescence (F_0) was measured under a weak modulating radiation [0.5 μmol(photon) m^{-2} s^{-1}], and maximum fluorescence (F_m) was induced by a saturating pulse of radiation [2,400 μmol (photon) m^{-2} s^{-1}].

Gas-exchange parameters, such as P_N, E, and g_s were measured by photosynthesis system (*Licor-6400*, USA) at an irradiance of 500 μmol(photon) m^{-2} s^{-1} and a CO_2 concentration of 400 μmol mol^{-1} from 10:00 to 12:00 h.

Plant biomass: After 7 d of treatment, fresh mass (FM) of plants under each treatment was recorded and then oven-dried for 72 h at 80°C. Dry mass (DM) of plants under each treatment was then recorded.

Leaf RWC: After 2 d of treatment, leaf RWC was determined according to Barrs and Weatherley (1962). RWC was calculated using the equation: RWC = [(FM – DM)/(TM – DM)] × 100, where FM, DM, and TM indicate fresh mass, dry mass, and saturated fresh mass, respectively.

Malondialdehyde (MDA) content and electrolyte leakage (EL): After 2 d of treatment, MDA content and EL were measured according to Han *et al.* (2014) and Anjum *et al.* (2015), respectively. MDA content was determined according to the thiobarbituric acid-reactive-substances assay by using *TU-1810* UV-Vis spectrophotometer (*Beijing Purkinje General Instrument Co*., *Ltd*., China). For EL, leaf samples were incubated in 10 ml of distilled water at 25°C for 6 h and then the electrical conductivity was recorded as EC1. Then samples were kept at 90°C for 2 h and the electrical conductivity was recorded as EC2. The electrical conductivity was measured by the conductivity meter (*DDS-307*, *INESA Scientific Instrument Co*., *Ltd*., China). EL was expressed as the percent of EC1 and EC2.

Statistical analysis: The data presented was the mean of five replicates. Means were compared by one-way analysis of variance (*ANOVA*) and *Duncan*'s multiple range test at the 5% level of significance. The program *SAS 9.1* (*SAS Institute*, Cary, NC, USA) was used for the statistical analysis.

Results

H_2S and NO: NaHS markedly increased the contents of H_2S and NO, compared with the control (Table 1). Pretreatment with NaN_3 plus NaHS markedly decreased NO content, compared with NaHS alone. However, pretreatment with NaN_3 plus NaHS had no obvious effect on H_2S content, compared with NaHS alone. Compared with NaN_3+NaHS, NaN_3+SNP+NaHS markedly increased NO content and had no obvious effect on H_2S content. Meanwhile, application of NaHS had no obvious effect on NO content in *nia1,nia2* mutant. The application of SNP+NaHS improved NO content in the leaves of *nia1,nia2* mutant. These results indicated that NaHS induced NO production through NR.

AsA/DHA ratio and the activities of enzymes in AsA-GSH cycle: NaHS markedly increased AsA/DHA ratio and the activities of APX, GR, DHAR, and MDHAR, compared with the control (Tables 1, 2). Pretreatment with NaN_3 plus NaHS markedly inhibited the activities of above enzymes and reduced AsA/DHA ratio, compared with

Table 1. Effects of NaHS, NaN_3, and SNP on AsA/DHA ratio and the contents of H_2S and NO in wild-type *Arabidopsis* and effects of NaHS and SNP on above indicators in *Arabidopsis* mutant *nia1,nia2*. Control – treatment of wild-type *Arabidopsis* with half-strength Hoagland's solution; NaHS – treatment of wild-type *Arabidopsis* with 0.2 mM NaHS; NaN_3+NaHS – treatment of wild-type *Arabidopsis* with 0.1 mM NaN_3 + 0.2 mM NaHS; NaN_3+SNP+NaHS – treatment of wild-type *Arabidopsis* with 0.1 mM NaN_3 + 100 μM SNP + 0.2 mM NaHS; *nia1,nia2* – treatment of *Arabidopsis* mutant *nia1,nia2* with half-strength Hoagland's solution; *nia1,nia2*+NaHS – treatment of *Arabidopsis* mutant *nia1,nia2* with 0.2 mM NaHS; *nia1,nia2*+SNP+NaHS – treatment of *Arabidopsis* mutant *nia1,nia2* with 100 μM SNP + 0.2 mM NaHS. The plants were pretreated with half-strength Hoagland's solution or NaN_3 for 8 h and then exposed to half-strength Hoagland's solution or NaHS or SNP+NaHS for 2 d. Values represent mean ± standard deviations (SD), *different letters* stand for significant difference between different treatments at $P<0.05$.

Treatment	H_2S [nmol g^{-1}(FM)]	NO [μmol g^{-1}(protein)]	AsA/DHA
Control	1.96 ± 0.23^b	1.44 ± 0.16^b	19.2 ± 2.11^b
NaHS	2.72 ± 0.35^a	1.90 ± 0.23^a	22.0 ± 2.65^a
NaN_3+NaHS	2.65 ± 0.30^a	1.13 ± 0.14^c	16.8 ± 1.93^c
NaN_3+SNP+NaHS	2.77 ± 0.28^a	1.80 ± 0.24^a	21.5 ± 2.25^a
nia1,nia2	2.06 ± 0.25^b	1.16 ± 0.12^c	17.0 ± 1.92^c
nia1,nia2+NaHS	2.60 ± 0.29^a	1.22 ± 0.15^c	17.4 ± 1.87^c
nia1,nia2+SNP+NaHS	2.70 ± 0.33^a	1.85 ± 0.22^a	21.8 ± 2.40^a

Table 2. Effects of NaHS, NaN_3, and SNP on the activities of enzymes in AsA-GSH cycle in wild-type *Arabidopsis* and effects of NaHS and SNP on above indicators in *Arabidopsis* mutant *nia1,nia2*. For explanation of treatments *see* Table 1. The plants were pretreated with half-strength Hoagland's solution or NaN_3 for 8 h and then exposed to half-strength Hoagland's solution or NaHS or SNP+NaHS for 2 d. Values represent mean ± standard deviations (SD), *different letters* stand for significant difference between different treatments at $P<0.05$.

Treatment	APX [U mg^{-1}(protein)]	GR [U mg^{-1}(protein)]	DHAR [U mg^{-1}(protein)]	MDHAR [U mg^{-1}(protein)]
Control	2.16 ± 0.26^{b}	1.62 ± 0.19^{b}	1.16 ± 0.16^{b}	0.92 ± 0.11^{b}
NaHS	2.55 ± 0.24^{a}	1.97 ± 0.26^{a}	1.55 ± 0.18^{a}	1.16 ± 0.14^{a}
NaN_3+NaHS	1.83 ± 0.22^{c}	1.36 ± 0.16^{c}	0.90 ± 0.12^{c}	0.70 ± 0.08^{c}
NaN_3+SNP+NaHS	2.65 ± 0.30^{a}	1.90 ± 0.22^{a}	1.61 ± 0.22^{a}	1.22 ± 0.17^{a}
nia1,nia2	1.80 ± 0.21^{c}	1.40 ± 0.17^{c}	0.98 ± 0.14^{c}	0.74 ± 0.09^{c}
nia1,nia2+NaHS	1.91 ± 0.25^{c}	1.46 ± 0.20^{c}	1.05 ± 0.14^{c}	0.80 ± 0.10^{c}
nia1,nia2+SNP+NaHS	2.60 ± 0.29^{a}	1.86 ± 0.22^{a}	1.55 ± 0.20^{a}	1.15 ± 0.16^{a}

Table 3. Effects of NaHS, NaN_3, and SNP on chlorophyll fluorescence parameters and photosynthetic pigments in wild-type *Arabidopsis* and effects of NaHS and SNP on above indicators in *Arabidopsis* mutant *nia1,nia2*. For explanation of treatments *see* Table 1. The plants were pretreated with half-strength Hoagland's solution or NaN_3 for 8 h and then exposed to half-strength Hoagland's solution or NaHS or SNP + NaHS for 2 d. Values represent mean ± standard deviations (SD), *different letters* stand for significant difference between different treatments at $P<0.05$.

Treatment	Chl [mg g^{-1}(FM)]	Car [mg g^{-1}(FM)]	F_v/F_m	q_P	q_N	Φ_{PSII}
Control	2.02 ± 0.25^{b}	0.47 ± 0.07^{b}	0.69 ± 0.08^{b}	0.73 ± 0.09^{b}	0.37 ± 0.05^{b}	0.41 ± 0.05^{b}
NaHS	2.30 ± 0.31^{a}	0.58 ± 0.09^{a}	0.84 ± 0.11^{a}	0.86 ± 0.09^{a}	0.45 ± 0.06^{a}	0.50 ± 0.07^{a}
NaN_3+NaHS	1.80 ± 0.21^{c}	0.39 ± 0.06^{c}	0.55 ± 0.07^{c}	0.63 ± 0.08^{c}	0.30 ± 0.05^{c}	0.34 ± 0.04^{c}
NaN_3+SNP+NaHS	2.23 ± 0.26^{a}	0.54 ± 0.07^{a}	0.80 ± 0.09^{a}	0.86 ± 012^{a}	0.40 ± 0.07^{a}	0.49 ± 0.06^{a}
nia1,nia2	1.82 ± 0.23^{c}	0.40 ± 0.06^{c}	0.57 ± 0.08^{c}	0.60 ± 0.07^{c}	0.31 ± 0.05^{c}	0.34 ± 0.05^{c}
nia1,nia2+NaHS	1.90 ± 0.22^{bc}	0.43 ± 0.06^{bc}	0.60 ± 0.08^{c}	0.63 ± 0.07^{c}	0.34 ± 0.04^{bc}	0.37 ± 0.05^{bc}
nia1,nia2+SNP+NaHS	2.26 ± 0.27^{a}	0.54 ± 0.08^{a}	0.79 ± 0.10^{a}	0.83 ± 0.10^{a}	0.43 ± 0.06^{a}	0.48 ± 0.07^{a}

Table 4. Effects of NaHS, NaN3 and SNP on water physiological indicators in wild-type *Arabidopsis* and effects of NaHS and SNP on above indicators in *Arabidopsis* mutant *nia1,nia2*. For explanation of treatments *see* Table 1. The plants were pretreated with half-strength Hoagland's solution or NaN_3 for 8 h and then exposed to half-strength Hoagland's solution or NaHS or SNP + NaHS for 2 d. Values represent mean ± standard deviations (SD), *different letters* stand for significant difference between different treatments at $P<0.05$.

Treatment	E [mmol(H_2O) m^{-2} s^{-1}]	g_s [mmol(H_2O) m^{-2} s^{-1}]	P_N [μmol(CO_2) m^{-2} s^{-1}]	RWC [%]
Control	1.24 ± 0.12^{b}	29.85 ± 3.38^{b}	7.12 ± 0.90^{b}	88.90 ± 9.21^{ab}
NaHS	1.58 ± 0.20^{a}	36.13 ± 4.32^{a}	8.75 ± 1.03^{a}	91.17 ± 9.77^{a}
NaN_3+NaHS	1.03 ± 0.12^{c}	25.57 ± 2.46^{c}	5.50 ± 0.66^{c}	84.50 ± 8.20^{b}
NaN_3+SNP+NaHS	1.50 ± 0.20^{a}	34.77 ± 4.15^{a}	8.36 ± 0.77^{a}	90.10 ± 8.88^{a}
nia1,nia2	1.06 ± 0.13^{c}	26.54 ± 3.09^{c}	6.10 ± 0.84^{c}	85.00 ± 7.57^{b}
nia1,nia2+NaHS	1.10 ± 0.11^{c}	27.10 ± 3.22^{c}	6.34 ± 0.73^{c}	85.30 ± 9.04^{b}
nia1,nia2+SNP+NaHS	1.53 ± 0.18^{a}	34.10 ± 3.84^{a}	8.11 ± 0.95^{a}	90.50 ± 9.54^{a}

NaHS alone. Compared with NaN_3+NaHS, NaN_3+SNP+NaHS significantly increased above indicators, which reversed the effects of NaN_3+NaHS. NaHS had no obvious effects on AsA/DHA ratio and the activities of above enzymes of AsA-GSH cycle in mutant *nia1,nia2*. However, application of SNP+NaHS improved above indicators in this mutant. These results indicated that signal molecule NO participated in the regulation of AsA-GSH cycle by NaHS through APX, GR, DHAR, and MDHAR, which further improved the AsA/DHA ratio.

Photosynthetic pigments and P_N: NaHS markedly increased P_N and the contents of Chl and Car, compared with the control (Tables 3, 4). Pretreatment with NaN_3 plus NaHS markedly reduced above parameters, compared with NaHS alone. Compared with NaN_3+NaHS, NaN_3+SNP+NaHS significantly increased above parameters, which reversed the effects of NaN_3+NaHS.

Table 5. Effects of NaHS, NaN_3, and SNP on MDA content, EL, plant biomass of wild-type *Arabidopsis* and effects of NaHS and SNP on above indicators in *Arabidopsis* mutant *nia1,nia2*. For explanation of treatments *see* Table 1. The plants were pretreated with half-strength Hoagland's solution or NaN_3 for 8 h and then exposed to half-strength Hoagland's solution or NaHS or SNP + NaHS for 7 d. MDA content and EL were measured after 2 d of treatment. Plant biomass was measured after 7 d of treatment. Values represent mean ± standard deviations (SD), *different letters* stand for significant difference between different treatments at $P<0.05$.

Treatment	MDA [nmol g^{-1}(FM)]	EL (%)	Plant biomass [mg(FM) per plant]
Control	4.8 ± 0.52^b	7.7 ± 0.81^b	81.0 ± 7.36^b
NaHS	4.0 ± 0.44^c	6.0 ± 0.57^c	95.8 ± 9.08^a
NaN_3+NaHS	5.5 ± 0.60^a	9.1 ± 0.88^a	65.5 ± 7.13^c
NaN_3+SNP+NaHS	4.2 ± 0.39^c	6.3 ± 0.70^c	93.6 ± 9.75^a
nia1,nia2	5.8 ± 0.63^a	8.8 ± 0.79^a	68.0 ± 6.66^c
nia1,nia2+NaHS	5.5 ± 0.59^a	8.5 ± 0.83^a	70.9 ± 7.77^c
nia1,nia2+SNP+NaHS	4.3 ± 0.50^c	6.5 ± 0.65^c	94.4 ± 8.59^a

Application of NaHS had no effect on P_N and the contents of Chl and Car in mutant *nia1,nia2*, whereas SNP+NaHS application improved these parameters. The present results indicated that signal molecule NO participated in the regulation of photosynthetic pigments and P_N by NaHS.

Chl fluorescence parameters: NaHS markedly increased Chl fluorescence parameters F_v/F_m, q_P, q_N, and Φ_{PSII}, compared with the control (Table 3). Pretreatment with NaN_3 plus NaHS markedly reduced above parameters, compared with NaHS alone. Compared to NaN_3+NaHS, NaN_3+SNP+NaHS significantly increased Chl fluorescence parameters, which reversed the effects of NaN_3+NaHS. No obvious effects of NaHS application were found in *nia1,nia2* mutant, whereas SNP+NaHS improved its Chl fluorescence parameters. These results indicated that signal molecule NO is involved in the regulation of Chl fluorescence parameters by NaHS.

***E*, g_s and RWC**: NaHS markedly increased E and g_s, compared with the control (Table 4). Pretreatment with NaN_3 plus NaHS markedly reduced above parameters, compared with NaHS alone. Compared with NaN_3+NaHS, NaN_3+SNP+NaHS significantly increased above indicators, which reversed the effects of NaN_3+NaHS. NaHS increased RWC, compared with the control (Table 4). However, there was no significant difference between NaHS and the control. Pretreatment with NaN_3 plus NaHS markedly reduced RWC, compared with NaHS alone. Compared with NaN_3+NaHS, NaN_3+SNP+NaHS significantly increased RWC, which reversed the effects of NaN_3+NaHS. In *nia1,nia2* mutant, NaHS application did not change E, g_s, and RWC, while SNP+NaHS improved these parameters. The present results indicated that signal molecule NO participated in the regulation of E, g_s, and RWC by NaHS.

MDA, EL and plant biomass: NaHS markedly decreased MDA and EL, whereas notably increased plant biomass, compared with the control (Table 5). Pretreatment with NaN_3 plus NaHS markedly increased MDA and EL and reduced plant biomass, compared with NaHS alone. Compared with NaN_3+NaHS, NaN_3+SNP+NaHS significantly decreased MDA and EL and increased plant biomass, which reversed the effects of NaN_3+NaHS. NaHS had no obvious effects on above indicators in mutant *nia1,nia2*. However, SNP+NaHS application improved these indicators in the mutant. The present results indicated that signal molecule NO participated in the regulation of MDA, EL, and plant growth by NaHS.

Discussion

Previous studies showed that exogenous H_2S had important effects on AsA-GSH cycle in plants, especially in stressed plants (Shan *et al.* 2011, Li *et al.* 2017, Kaya and Ashraf 2019). Our previous study showed that exogenous H_2S enhanced AsA-GSH cycle of wheat seedlings by regulating APX, GR, and DHAR (Shan *et al.* 2011). For *Arabidopsis*, our previous study showed that endogenous H_2S was involved in JA-regulated AsA-GSH cycle through APX, GR, DHAR, and MDHAR (Shan *et al.* 2018). In the present study, we found that exogenous H_2S enhanced AsA-GSH cycle through APX, GR, DHAR, and MDHAR in *Arabidopsis* leaves. Thus, our previous and present results clearly indicated that exogenous and endogenous H_2S had the same effects on AsA-GSH cycle in *Arabidopsis.* Many studies showed that the biological function of H_2S depended on its concentration (Shan *et al.* 2011). However, H_2S concentration in plants is usually very low under normal growth conditions, which cannot probably regulate physiological and biochemical processes and plant growth and development well. Applying exogenous H_2S can enhance H_2S amount in plants under normal growth conditions, which can further positively regulate physiological and biochemical processes, including plant growth and development. NO is another important gas signal molecule in plants (Batista *et al.* 2018, Hasanuzzaman *et al.* 2018, Okant and Kaya 2019). Ahmad *et al.* (2018) and our previous study showed that exogenous NO enhanced AsA-GSH cycle through APX, GR, DHAR, and MDHAR (Shan and Sun 2018, Shan *et al.* 2012). In the present study, our findings showed that endogenous and exogenous NO also had the same role in regulating AsA-GSH cycle. However, there is still no report about the role of NO in the regulation

of AsA-GSH cycle by NaHS in plants. In this study, we found that NaHS induced NO production, which further enhanced AsA-GSH cycle in *Arabidopsis.* By this way, NO participated in the regulation of AsA/DHA ratio by NaHS in *Arabidopsis.* Therefore, our present findings demonstrated that gas signal molecule NO was involved in the regulation of AsA/DHA ratio by NaHS through AsA-GSH cycle in *Arabidopsis.*

Plant growth has a close relationship with the photosynthesis. Moreover, P_N is closely related to contents of photosynthetic pigments. Parveen et al. (2017) showed that low concentration of NaHS improved the photosynthesis and the growth of submerged macrophytes. Duan *et al.* (2015) showed that suitable concentration of NaHS improved P_N and Chl contents of rice seedlings. In this study, our findings showed that NaHS also improved P_N and Chl content in *Arabidopsis.* Besides, we also found that NaHS enhanced Car content in *Arabidopsis.* Our present results clearly indicated that NaHS improved the photosynthesis by enhancing P_N and increasing the contents of photosynthetic pigments. Meanwhile, we found that gas signal molecule NO was involved in the regulation of the photosynthesis by NaHS in *Arabidopsis*.

The decreases in Chl fluorescence parameters, F_v/F_m and Φ_{PSII}, indicated that inhibition of photosynthesis and reduction of photochemical activity of PSII occurred. The higher q_P implies higher PSII electron transfer activity. q_N is indicative of a high photoprotective capacity for plants (Kumar and Prasad 2015). In this study, we found that NaHS significantly increased F_v/F_m, Φ_{PSII}, q_P, and q_N, which indicated that NaHS improved the electron transport, photochemical activity of PSII, and photoprotective capacity. Besides, we found that gas signal molecule NO was involved in the regulation of above Chl fluorescence parameters in *Arabidopsis* by NaHS. Combined with the results from the regulation of AsA-GSH cycle by NaHS, we deduced that NaHS improved PSII function by maintaining the redox status through AsA-GSH cycle.

The water status of plants, which was indicated by RWC, had important effects on the E and g_s. It has been documented that suitable concentration of NaHS markedly improved E and g_s of rice seedlings (Duan *et al.* 2015). In this study, our findings showed that NaHS markedly improved leaf RWC of *Arabidopsis*. We also found that NaHS markedly improved E and g_s in *Arabidopsis*, which was consistent with the results of Duan *et al.* (2015). Besides, we found that gas signal molecule NO was involved in the regulation of above water physiological parameters of *Arabidopsis* by NaHS.

Many studies showed that NaHS improved plant growth (Parveen *et al.* 2017, Chen *et al.* 2018). Chen *et al.* (2018) showed that NaHS ameliorated seed germination and seedling growth of cauliflower under lead stress. In this study, we also showed NaHS improved the growth of *Arabidopsis*. Meanwhile, our present study showed that NaHS markedly decreased MDA content and EL in *Arabidopsis* leaves. Besides, we found that gas signal molecule NO was involved in the regulation of above parameters of *Arabidopsis* by NaHS.

In this study, all the plants of different treatments were grown under normal water conditions. However, we found that there were significant differences in leaf RWC between different treatments, which indicated that different treatments had significant effects on the water conditions of *Arabidopsis*. Meanwhile, our results showed that there were significant differences in leaf MDA and EL between different treatments. So, our present results implied that the most fully expanded leaves of plants under some treatments were subjected to water stress. What is the reason for this result? Under the same normal water conditions, NaHS-treated plants had higher E, g_s, and RWC than that of control plants. Thus, this difference may be due to the effects of NaHS on the water absorption and transport in plants. However, the physiological and molecular mechanisms of NaHS-regulated water absorption and transport of plants is still unclear. Therefore, it could be very interesting to investigate the effects of NaHS on water absorption and transport of plants, which could provide more information for the action mechanism of NaHS in regulating water regime of *Arabidopsis.*

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