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Endogenous hydrogen sulfide enhances salt tolerance by coupling the reestablishment of redox homeostasis and preventing salt-induced K^+ loss in seedlings of *Medicago sativa*

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ABSTRACT

Despite the external application of hydrogen sulfide (H_2S) conferring plant tolerance against various environmental cues, the physiological significance of L-cysteine desulfhydrase (L-DES)-associated endogenous H_2S production involved in salt-stress signaling was poorly understood. To address this gap, the participation of *in planta* changes of H_2S homeostasis involved in alfalfa salt tolerance was investigated. The increasing concentration of NaCl (from 50 to 300 mM) progressively caused the induction of total L-DES activity and the increase of endogenous H_2S production. NaCl-triggered toxicity symptoms (175 mM), including seedling growth inhibition and lipid peroxidation, were alleviated by sodium hydrosulfide (NaHS; 100 μM), a H_2S donor, whereas aggravated by an inhibitor of L-DES or a H_2S scavenger. A weaker or negative response was observed in lower or higher dose of NaHS. Further results showed that endogenous L-DES-related H_2S modulated several genes/activities of antioxidant defence enzymes, and also regulated the contents of antioxidant compounds, thus counterbalancing the NaCl-induced lipid peroxidation. Moreover, H_2S maintained K^+/Na^+ homeostasis by preventing the NaCl-triggered K^+ efflux, which might be result form the impairment of *SKOR* expression. Together, our findings indicated that endogenous H_2S homeostasis enhance salt tolerance by coupling the reestablishment of redox balance and restraining K^+ efflux in alfalfa seedlings.

1. Introduction

It is well known that salt stress is a major environmental factor that leads to significant inhibition of plant growth and decrease of productivity [1,2]. Normally, the excess salinity imposes numerous negative influences on plant cells, including osmotic stress, and the overproduction of reactive oxygen species (ROS) as well as lipid peroxidation [3–6]. Salt stress also results in the over-accumulation of sodium ions (Na^+) in plants cells, which competitively inhibits the uptake of potassium (K^+), thus leading to a deficiency of K^+ [4,7]. Increasing evidence suggests that the prevention of Na^+-triggered K^+ leakage, thereby restraining the decreased ratio of K^+/Na^+, is critical for plant salt tolerance [8–10]. Potassium channels or transporters are responsible for the K^+ transport into or out of root cells [11]. Several reports showed that the outward-rectifying K^+ channels, such as shaker-like K^+ outward-rectifying channel (SKOR) and guard cell outward rectifying K^+ channel (GORK), are involved in K^+ efflux. *SKOR* is expressed in the root stele and responsible

Abbreviations: AsA, ascorbic acid; CAT, catalase; CHES, 2-(N-cyclohexylamino)ethane-sulphonic acid; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; PAG, DL-propargylglycine; DTT, dithiothreitol; EDX, energy-dispersive X-ray detector; GORK, guard cell outward rectifying K^+ channel; GSH, glutathione; GR, glutathione reductase; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; H_2S, hydrogen sulfide; hGSH, homoglutathione; hGSSGh, oxidized homoglutathione; HT, hypotaurine; ICP-OES, inductively coupled plasma-optical emission spectrometer; IRK, inward-rectifying K^+ channel; L-DES, L-cysteine desulfhydrase; mBBr, monobromobimane; MDHAR, monodehydroascorbate reductase; NaHS, sodium hydrosulfide; NEM, N-ethylmaleimide; NMT, non-invasive micro-test technology; NO, nitric oxide; ROS, reactive oxygen species; SKOR, Shaker-like K^+ outward-rectifying channel; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; UPLC, ultra performance liquid chromatography.

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for K^+ release to the xylem [12], while GORK is found to regulate K^+ release in guard cell and stomatal closure [13]. Meanwhile, inward-rectifying K^+ channels (IRK) have also been identified as mechanisms for long-term net K^+-selective influx, and may reduce Na^+ toxicity [14].

On the other hand, several lines of evidence have shown that plant tolerance against salt stress is closely related to the maintenance of cellular redox balance [1,4]. Redox-regulation systems could regulate the cascades of uncontrolled oxidation, thus protecting plant cells from oxidative damage and maintaining endogenous ROS homeostasis [15]. Plant redox-regulation machinery was composed of several non-enzymatic antioxidants (glutathione, GSH; ascorbic acid, AsA, etc.) and the efficient antioxidant defence systems (catalase, CAT; superoxide dismutase, SOD, etc.) [16,17]. Particularly, homoglutathione (hGSH) is more abundant than GSH in legumes, in which glycine is substituted by alanine [18]. GSH/hGSH pool is regarded as an important parameter that reflects the redox state of cells, which is subsequently vital for plant tolerance to heavy metals-triggered oxidative stress [19]. The recycling pathways of GSH and AsA include several key enzymes, such as ascorbate peroxidase, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [15].

Like nitric oxide (NO) and carbon monoxide, hydrogen sulfide (H_2S), a colorless gas with foul odor of rotten eggs, is known as the gaseous transmitter in mammalian studies [20,21]. For example, H_2S has been reported to participate in several physiological processes, such as blood pressure regulation and neuronal excitability [22,23]. Meanwhile, anti-inflammatory, vaso-relaxant and neuron-protective functions of H_2S in mammalian studies have also been described [24–26]. Due to its association with adaptive responses against multiple stress conditions, H_2S has further received increasing attention by plant researchers. It has been documented that H_2S is involved in the anti-oxidative response against numerous environmental stimuli, including copper, aluminum, heat, drought, and osmotic stresses [27–31]. H_2S was generated mainly by the desulfhydrases in higher plants. Hereinto, two specific desulfhydrases have been identified as the key enzymes involved in plant H_2S biosynthesis, namely L-cysteine desulfhydrase (L-DES, EC4.4.1.1; in particular) and D-cysteine desulfhydrase (D-DES, EC4.4.1.15) [32–35]. The transcript abundance of *L-DES* or total enzyme activity of L-DES was induced/increased by drought stress, salicylic acid, abscisic acid, etc. [36,37]. On the other hand, the transcripts encoding L-DES have not been cloned in *Medicago sativa*. Mutation of *Arabidopsis* L-DES (*DES1*) gene disrupts the cytosolic H_2S generation and strongly affects plant metabolism [38]. However, the physiological functions of L-DES and its generated H_2S involved in the plant salt tolerance are still unknown.

Previous studies revealed that exogenous sodium hydrosulfide (NaHS, a H_2S or HS^- donor) promotes alfalfa seed germination upon salt stress by reducing oxidative damage, which might have a possible interaction with NO [39]. However, the biological significance of endogenous H_2S and its related enzymatic source in the modulation of salt tolerance in alfalfa seedling remain obscure. In this study, by using the inhibitor of L-DES and a scavenger of H_2S as well as checking the kinetics changes of endogenous H_2S production, our aim was to characterize the impact of NaCl on endogenous H_2S homeostasis and subsequent NaCl toxicity symptoms, with particular emphasis on the temporal signature of this event. Meanwhile, the physiological and biochemical function of endogenous H_2S homeostasis that acts as a critical bio-modulator to improve plant tolerance against salt stress was also examined. Our results support the hypothesis that H_2S could serve as an endogenous regulon to enhance salt tolerance by helping reestablishment of the redox balance including the improvement of hGSH/hGSSGh in particular, as well as preventing the K^+ leakage in alfalfa seedling roots.

2. Materials and methods

2.1. Plant materials, growth conditions and treatments

Alfalfa (*M. sativa* L. cv. Biaogan, $2n = 4x = 32$, semi-dormant genotype, fall dormancy rating of 6) seeds were surface-sterilized with 5% NaClO for 10 min, rinsed extensively in distilled water and germinated for 1 day at 25 °C in the darkness. Identical seeds were then selected and transferred to the plastic chambers and cultured in quarter-strength Hoagland's solution (pH 6.0) for another 4 days in the growth incubator (25 ± 1 °C, 14 h photoperiod with a light intensity of 200 μmol m^{-2} s^{-1}).

For physiological assays, 5-day-old alfalfa seedlings were grown hydroponically in plastic chambers with quarter-strength Hoagland's solution in the presence or absence of varying concentrations of NaCl, NaHS (a H_2S or HS^- donor), Na_2SO_4 (100 μM), $NaHSO_3$ (100 μM), $NaHSO_4$ (100 μM), CH_3COONa (100 μM), DL-propargylglycine (PAG; 2 mM; an inhibitor of L-DES), or hypotaurine (HT; 10 mM; a scavenger of H_2S) alone for 6 h or the indicated times as presented in figure legends, respectively, followed by the exposure to NaCl stress at the indicated concentrations for the indicated times as presented in figure legends. The concentrations of these chemicals were determined in pilot experiments from which significant responses were obtained. The sample without chemicals was regarded as control (Con). The pH of nutrient medium or treatment solutions was adjusted to 6.0. At the indicated time points, the mature zone or intact alfalfa seedling roots were chosen for used immediately or quick-frozen in liquid nitrogen, and stored at −80 °C for further analysis.

2.2. Determination of fresh weight, primary root elongation and dry weight

The primary root elongation was determined according to the method described previously [6]. Fresh weight and dry weight were also measured.

2.3. Determination of endogenous H_2S content, total activities of L-DES and D-DES

Endogenous H_2S content was determined by the formation of methylene blue from dimethyl-*p*-phenylenediamine in H_2SO_4 according to the method described previously [27]. Root samples (0.2 g) were extracted in 1 ml of phosphate buffer solution (pH 6.8, 50 mM) containing 0.1 M EDTA and 0.2 M AsA. The supernatant was mixed with 0.5 ml of 1 M HCl in a test tube to release H_2S, and H_2S was absorbed in 1% (w/v) zinc acetate (0.5 ml) trap which is located in the bottom of the test tube. After 30 min reaction, 0.3 ml 5 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 3.5 mM H_2SO_4 was injected into the trap. Then 0.3 ml of 50 mM ferric ammonium sulphate in 100 mM H_2SO_4 was injected into the trap. The amount of H_2S in zinc acetate traps was determined spectrophometrically at 670 nm, after leaving the mixture for 15 min at room temperature. Solutions with different concentrations of Na_2S were prepared, treated in the same way as the assay samples, and were used for the quantification of H_2S.

Total L-DES activity was determined according to previous method with some modifications [37]. Soluble proteins were extracted by adding 1 ml of 20 mM Tris–HCl (pH 8.0) to 0.2 g of samples. Centrifuged at 12,000 × *g* for 15 min, the protein content of the supernatant was adjusted to 100 μg ml^{-1} to obtain equal amounts of protein in each assay sample. Total L-DES activity was determined by the release of H_2S from L-cysteine in the presence