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Research article

Physiological response and transcription profiling analysis reveals the role of H_2S in alleviating excess nitrate stress tolerance in tomato roots

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ABSTRACT

Soil secondary salinization caused by excess nitrate addition is one of the major obstacles in greenhouse vegetable production. Excess nitrate inhibited the growth of tomato plants, while application of 100 μM H_2S donor NaHS efficiently increased the plant height, fresh and dry weight of shoot and root, root length, endogenous H_2S contents and L-cysteine desulfhydrases activities. NaHS altered the oxidative status of nitrate-stressed plants as inferred by changes in reactive oxygen species (ROS) accumulation and lipid peroxidation accompanied by regulation of the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX). Besides, NaHS increased the nitric oxide (NO) and total S-nitrosothiols (SNOs) contents, nitrate reductase (NR) activities and decreased the S-nitrosoglutathione reductase (GSNOR) activities under nitrate stress. Furthermore, microarray analysis using the Affymetrix Tomato GeneChip showed that 5349 transcripts were up-regulated and 5536 transcripts were down-regulated under NaHS and excess nitrate stress treatment, compared to the excess nitrate stress alone. The differentially expressed genes ($\log_2$ fold change > 2 or < -2) of up-regulated (213) and down-regulated (271) genes identified were functionally annotated and subsequently classified into 9 functional categories. These categories included metabolism, signal transduction, defence response, transcription factor, protein synthesis and protein fate, transporter, cell wall related, hormone response, cell death, energy and unknown proteins. Our study suggested exogenous NaHS might enhance excess nitrate stress tolerance of tomato plants by modulating ROS and reactive nitrogen species (RNS) signaling and downstream transcriptional adjustment, such as defence response, signal transduction and transcription factors.

1. Introduction

Nitrogen is a crucial plant macronutrient and is needed in the greatest amount of all mineral elements required by plants. A large quantity of nitrogen fertilizer is used for crop production to achieve high yields at a significant economic and environmental cost. In the last half century, the global use of nitrogen fertilizer increased 10-folds in order to increase crop productivity (UNEP, 1999). Plants consumed much less than half of the fertilizers applied (Frink et al., 1999; Socolow, 1999), and the majority of nitrogen fertilizers was lost to the atmosphere or leached into groundwater, lakes and rivers, which causes increasingly severe pollution to the environments. Over-utilization of chemical fertilizer has also caused secondary salinization in Chinese greenhouse. The excessively accumulated anion ion in soil of greenhouse is nitrate (NO_3^-) (Ju et al., 2007; Yang et al., 2010). Nitrate-N contents in 0–60 cm soil layers increased with the increasing age of plastic greenhouse, aggravating soil salinization in the greenhouse of Dianchi basin in China (Zhang et al., 2006). Excess nitrate in agricultural soils, causes a negative impact on crop quality and yield.

Excess nitrate caused oxidative stress that resulted in lipid peroxidation in vegetables. In cucumber roots, excess nitrate was observed to intensify reactive oxygen species (ROS) production and lipid peroxidation (Xu et al., 2012). Similarly, reactive nitrogen species (RNS) have also emerged as key players in a plant's response to a multitude of stresses, including salinity (Corpas et al., 2008). NO, one of the main forms of RNS, can either have a toxic or protective effect against abiotic stress factors, as it additionally alleviates the deleterious effects of ROS (Qiao and Fan, 2008). NO regulation is often associated with the regulation of the activity of the key enzyme of the nitrate assimilation pathway in higher plants, nitrate reductase (NR; EC 1.6.6.1.) (Filippou et al., 2016). The major bioactivity of NO is executed by regulating the activity of proteins through S-nitrosylation, a redox based posttranslational modification mechanism that covalently links an NO group to the reactive Cysteine (Cys) thiol of a protein to form S-nitrosothiols (SNOs) (Astier et al., 2011; Gupta, 2011; Wang et al., 2006). The NO accumulation and total SNOs contents increased after excess nitrate stress

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for 24 h in spinach (Zheng et al., 2016). S-nitrosoglutathione reductase (GSNOR) indirectly controls total cellular S-nitrosylation during the defence response by turning over S-nitrosoglutathione (GSNO), a major cache of NO bioactivity (Malik et al., 2011).

Hydrogen sulphide (H_2S), a colorless gas with a strong odor of rotten eggs, has traditionally been considered as a phytotoxin, having deleterious effects on the plant growth and survival. Recently, it is recognized as a potential signaling molecule involving in physiological regulation similar to NO and carbon monoxide (CO) in plants (Chen et al., 2016). H_2S is produced in the mammalian body through the enzymatic activities of cystathionine β -synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) (Yamasaki and Cohen, 2016). In plants, several pyridoxal-5′-phosphate (PLP)-dependent D/L-cysteine desulfhydrases (D/L-CDs) are primarily responsible for H_2S generation (Cheng et al., 2013) and the remove of H_2S is mainly by *O*-acetylserine lyase (Lisjak et al., 2013). H_2S is an important gaseous molecule in various plant developmental processes and plant stress responses. H_2S has been reported to participate in various abiotic response including improve salt (Wang et al., 2012), aluminum (Zhang et al., 2010), mercury toxicity (Chen et al., 2017), cadmium (Sun et al., 2013), drought (Jin et al., 2011; Shan et al., 2011), hypoxia (Cheng et al., 2013), boron (Wang et al., 2010), chromium (Ali et al., 2013), or heat tolerance (Li et al., 2012b). It appears therefore, that instead of thinking of H_2S as a phytotoxin, it needs to be considered as a signaling molecule that interacts with ROS and NO metabolism, as well as having direct effects on the activities of proteins (Lisjak et al., 2013).

Tomato (*Solanum lycopersicum* L.) is an important commercial crop. Despite many studies on the putative functions of exogenous NaHS in abiotic stress resistance, the physiological role and molecular response of NaHS in alleviating nitrate stress tolerance in tomato remain unclear. Therefore, in this study, we analysed the physiological response of tomato under NaHS and excess nitrate stress. Besides, microarray analysis using the Affymetrix Tomato GeneChip was performed to investigate the transcription response of tomato under NaHS and excess nitrate stress.

2. Materials and methods

2.1. Plant materials and treatments

Tomato seeds (*Lycopersicon esculentum* Mill. var. *cerasiforme* Alef. 'Dahongyingtao') were germinated on moisture filter paper in an incubator at 28 °C. After 15 d, batches of 20 plants were transferred to a plastic tank containing 10 L of nutrient solution, including Ca$(NO_3)_2{\cdot}4H_2O$ 590 mg L^{-1}, KNO_3 404 mg L^{-1}, $KH_2PO_4$136 mg·L^{-1}, $MgSO_4{\cdot}7H_2O$ 246 mg L^{-1}, EDTA·Na_2-Fe 40 mg L^{-1}, H_3BO_3 2.86 mg L^{-1}, $MnSO_4{\cdot}4H_2O$ 2.13 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.22 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.08 mg L^{-1}, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.02 mg L^{-1}. For H_2S treatment, 3-week-old tomato plants were treated with the H_2S donor sodium hydrosulfide (NaHS) of 100 μM with or without 100 mM nitrate (KNO_3 and $Ca(NO_3)_2$ provide the same mol of NO_3^-) for 1, 3, 5 d under the same conditions as described earlier. The normal NO_3^- ion concentration of 10 mM was used as a control (CK). All the nutrient solutions were renewed every 2 days. The experiment was carried out under natural conditions in the greenhouse of Kunming University of Science and Technology with an air temperature of 20–28 °C during the day and 13–18 °C during the night. For the microarray analysis, tomato roots were treated with CK, NaHS(S), nitrate (N), nitrate + NaHS(NS) for 3 h. Samples were taken from 3 plants (n = 3) after treatment, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. Plant growth measurements

The plant height and root length were measured directly with ruler at the 1, 3, 5 d. The fresh weight (FW) was directly determined. For dry weight (DW) determination, the shoots and roots were dried at 80 °C for 48 h and weighed. After 5 days of treatment, tomato plants were photographed.

2.3. Determination of the endogenous H_2S contents and LCD activities

H_2S contents in the roots of tomato plants were measured by the formation of methylene blue from dimethyl-*p*-phenylenediamine in H_2SO_4, according to a previous method of Li et al. (2012a). Roots (0.2 g) were ground and extracted in 10 mL phosphate buffered saline (pH 6.8, 50 mM) containing 0.1 mM EDTA and 0.2 mM ascorbic acid. The homogenate was mixed in a test tube containing 100 mM phosphate buffered saline (pH 7.4), 10 mM L-cysteine and 2 mM phosphopyridoxal at room temperature, and the released H_2S was absorbed in a zinc acetate trap, a small glass tube containing zinc acetate, fixed in the bottom of the test tube. After 30 min reaction, 0.3 mL 5 mM dimethyl-p-phenylenediamine dissolved in 3.5 mM H_2SO_4 was added to the trap followed by injection of 0.3 mL 50 mM ferric ammonium sulfate in 100 mM H_2SO_4. The amount of H_2S in the zinc acetate trap was determined colorimetrically at 667 nm after incubation for 15 min at room temperature. Calibration curve was made according to above methods and H_2S content was expressed as nmol g^{-1} FW.

The activity of LCD was determined according to the method described by Li et al. (2012a). Roots (0.3 g) were ground with a mortar and pestle in liquid nitrogen and the soluble proteins were extracted by adding 10 mL 20 mM Tris-HCl, pH 8.0. LCD activity was determined by the release of H_2S from L-cysteine in the presence of dithiothreitol (DTT). The assay contained in a total volume of 1 mL: 0.8 mM L-cysteine, 2.5 mM DTT, 100 mM Tris-HCl, pH 9.0, and 10 μg protein solution. The reaction was initiated by the addition of L-cysteine; after incubation for 15 min at 37 °C the reaction was terminated by adding 100 μL of 30 mM $FeCl_3$ dissolved in 1.2 M HCl and 100 μL 20 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 M HCl. The amount of H_2S was determined colorimetrically at 667 nm after incubation for 15 min at room temperature. Blanks were prepared by the same procedures and known concentrations of Na_2S used in a standard curve, and activity of LCD was expressed as nmol g^{-1} FW min^{-1}.

2.4. The endogenous ROS accumulation assays

The ROS accumulation in roots was visualised by loading the tissue with 20 μM 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; Calbiochem, La Jolla, CA, USA) in 20 mM HEPES buffer, pH 7.8, for 30 min, then washing the roots three-times in the same buffer for 15 min (Mazel et al., 2004). ROS accumulation in roots were examined using an epifluorescence microscope (model DMI6000B; Leica, Solms, Germany).

2.5. Determination of H_2O_2 contents and lipid peroxidation

H_2O_2 contents were estimated according to Li et al. (2012a). Roots were ground with a mortar and pestle in liquid nitrogen and endogenous H_2O_2 was extracted by adding phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine (inhibitor of catalase). The homogenate was centrifuged at 10,000 × *g* for 20 min. To determine H_2O_2 content, the extracted solution was mixed with 0.1% titanium sulfate in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 10,000 *g* for 20 min. The absorbance was measured at 410 nm H_2O_2 content was counted using extinction coefficient 0.28 μM^{-1} cm^{-1} and expressed as μmol g^{-1} FW.

Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) using the thiobarbituric acid reaction method (Madhava Rao and Sresty, 2000). 0.2 g roots were homogenized in 5 mL of 10% (w/v) trichloroacetic acid (TCA) containing 0.25% (w/v) TBA. The mixture was incubated in a water bath at 95 °C for 30 min and the reaction was terminated in an ice bath. The mixture was centrifuged at