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Research article

Hydrogen sulfide and proline cooperate to alleviate cadmium stress in foxtail millet seedlings

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ABSTRACT

Hydrogen sulfide (H_2S) and some functional amino acids in crops have been involved in the defense system against heavy-metal pollution. Here we report the relationships and functions of H_2S and proline to cadmium (Cd) stress. Sodium hydrosulfide (NaHS) pretreatment decreased the electrolytic leakage and the malondialdehyde and hydrogen peroxide contents while enhancing photosynthesis in Cd-treated seedlings. Furthermore, pretreatment with NaHS markedly exacerbated Cd-induced alterations in proline content, the activities of proline-5-carboxylate reductase (P5CR) and proline dehydrogenase (PDH), and the transcript levels of P5CR and PDH. When endogenous H_2S was scavenged or inhibited by various H_2S modulators, the Cd-induced increase in endogenous proline was weakened. Combined pretreatment with H_2S and proline was moderately higher in the Cd-stressed growth status, stomata movements and oxidative damage of seedlings compared to a single treatment with H_2S or proline. These results suggest that H_2S and proline cooperate to alleviate Cd-damage in foxtail millet.

1. Introduction

Environmental pollution is particularly serious in many countries that are undergoing rapid population growth, and increased agricultural and industrial practices. The pollution includes heavy metals, industrial waste, sewage runoff, and pesticides. The persistent nature and toxic effects of pollution on the survival and health of plants and animals are gaining consideration (Bharwana et al., 2014). However, plants have developed, over millions of years of evolution, various complex physiological and biochemical processes to effectively respond and adapt to sudden and adverse environmental changes. Plants have evolved self-defense systems that involve several gas molecules, such as nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S), hydrogen (H_2) and ammonia (NH_3) (Wang, 2014).

H_2S, a novel gasotransmitter, has potential regulatory functions for the growth and development of plants, including seed germination (Zhang et al., 2008), root organogenesis (Fang et al., 2014b), photosynthesis (Chen et al., 2011) and flower senescence (Zhang et al., 2011). H_2S also alleviates various biotic and abiotic stresses, including bacterial *Pseudomonas* (*Pst* DC3000) infections, drought, salt, heat and heavy metals (Jin et al., 2011; Fotopoulos et al., 2013; Li et al., 2013; Shi et al., 2013, 2015; Qiao et al., 2015; Ziogas et al., 2015). Under heavy metal stress, H_2S decreases reactive oxygen species (ROS) levels and enhances the antioxidant capacity in barley, wheat and rice treated with 0.4 mM aluminum, 5.0 mM copper and 1.0 mM cadmium (Cd), respectively (Zhang et al., 2008; Chen et al., 2013; Mostofa et al., 2015). H_2S also acts as a downstream molecule of salicylic acid (SA)-transmitted signals to regulate Cd tolerance in *Arabidopsis* and NO-activated H_2S responses to Cd stress in bermudagrass (Shi et al., 2014; Qiao et al., 2015). The H_2S donor sodium hydrosulfide (NaHS) improves the heat tolerance of maize and the acquisition of this heat tolerance involves several amino acids, especially proline (Pro) (Li et al., 2013).

In plants, Pro accumulation occurs in response to many biotic and abiotic stresses. Pro is well-studied and has multiple functions in plants, such as regulating cytoplasmic osmolytes, chelating metal ions and detoxifying ROS (Szabados and Savoure, 2010; Tripathi et al., 2013). A Pro pretreatment mitigates mercury (Hg) toxicity in *Oryza sativa* by reducing ROS levels as hydrogen peroxide (Wang et al., 2009). Pyrroline-5-carboxylate reductase (P5CR) is the critical

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enzyme in Pro biosynthesis. The overexpression of P5CR in transgenic *Arabidopsis* and sweetpotato improves their tolerance to salt stress (Ma et al., 2008; Liu et al., 2014a). Accordingly, stress factors can influence the enzyme activity and transcription of Pro metabolism-related genes (Szabados and Savoure, 2010; Zhang et al., 2014).

Foxtail millet (*Setaria italica* L.), an important cereal crop, is a stress-resistant small millet and wide adaptable to adverse environmental conditions. Thus, it is a model crop for studying metabolism (Zhang et al., 2012). Earlier research using foxtail millet mainly focused on hereditary breeding, including increasing yields, and improving quality and resistance to various stresses. There is little available information regarding Cd pollution. The chromium tolerance in *Setaria italica* is enhanced by H_2S, partially contributing to the calcium (Ca^{2+})-activating antioxidant system (Fang et al., 2014a). However, it was unknown whether H_2S affected Pro metabolism and mitigated Cd damage in foxtail millet. Thus, the objectives of this study were to: (1) determine the effects of H_2S on endogenous Pro accumulation, (2) evaluate the effects of H_2S on Pro metabolism, (3) characterize the role of H_2S in Cd tolerance, and (4) analyze the relationship of H_2S and Pro in response to Cd stress. Our results will provide knowledge regarding the effects of H_2S and Pro on Cd levels.

2. Materials and methods

2.1. Plant materials and treatments

Foxtail millet cultivar seeds, Jingu-21, were used in this study. Seeds were sterilized in a 75% (v/v) ethanol and 6% (v/v) sodium hypochlorite solution and then sown on soaked gauzes in Petri dishes. After germination in the dark at 23 °C, seedlings were kept on a cycle of 16 h of 160 μE $m^{-2}s^{-1}$ light illumination and 8 h of dark at 60% relative humidity. Sterile water (10 ml) in dishes was renewed everyday to keep the gauze moist.

Five days later, seedlings were pretreated with H_2S. For H_2S fumigation, NaHS dissolved into water was used to provide H_2S, seedlings were kept in their own Petri dishes placed in a sealed glass container and then fumigated with different NaHS concentrations for 24 h, and all of the manipulations were performed using the method described by Jin (Jin et al., 2011). For treatment with H_2S modulators with 24 h, the water in the Petri dishes was replaced with different chemical solutions (the inhibitors of H_2S biosynthesis: aminooxyacetic acid (AOA), potassium pyruvate (PP), hydroxylamine (HA) and the scavenger of H_2S: hypotaurine (HT), at 1000 μM). For Pro pretreatment for 24 h, the water in the Petri dishes was substituted with different Pro concentrations. Our pre-experiments revealed that treatment with a 5 mM cadmium chloride ($CdCl_2$) solution for 24 h could significantly inhibit growth. Therefore, After 24 h pretreatment, all the chemical reagents were sucked out from Petri dishes then this concentration of $CdCl_2$ solution was used to treat the seedlings. The seedlings were treated according to the following descriptions: 1) control check (Ck), 2) NaHS, 3) Cd, 4) NaHS + Cd, 5) NaHS + Pro + Cd, 6) Pro + Cd and 7) Pro. All of the agents ($CdCl_2$, NaHS, Pro, HT, AOA, PP and HA, Sigma-Aldrich, Shanghai) used in this study were of analytical pure (A.P.) grade. Thirty plants per dish were arranged according to the different treatments in the growth chamber with three replicates for each treatment.

2.2. Physiological index assays

Electrolyte leakage (EL) was calculated on the basis of the ratios of initial to final conductivity. The treated-leaves of foxtail millet were immersed in deionized water at room temperature for 12 h. The initial conductivity was then measured and the final conductivity was measured after the leaves were boiled for 30 min (Liu et al., 2008).

Malondialdehyde (MDA), an important indicator of the lipid peroxidation level, was spectrophotometrically measured according to a reported method (Schmedes and Hølmer, 1989). The fresh plant sample (0.2 g) was homogenized with trichloroacetic acid (TCA), and the mixture was centrifuged at 1662×*g* for 5 min at 20 °C. An equal amount of thiobarbituric acid (TBA) was added to the supernatant. The mixture was boiled for 30 min and cooled, and the absorbance was measured at 450 nm, 532 nm and 600 nm.

The H_2O_2 content assay was carried out with the potassium iodide (KI) method (Fang et al., 2014a). Histochemical detection of H_2O_2 used 3,3′-diaminobenzidine (DAB) as the chromogenic substrate. After staining with DAB, the leaves (~2 cm long) were washed extensively and boiled with 95% ethanol for 10 min and then photographed on color film (EOS 70D, Canon Photo Film, Tokyo, Japan).

2.3. Leaf gas exchange and total chlorophyll content assays

The net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (E) were measured using a portable photosynthesis system (SY-1020, Shiyakeji, Shijiazhuang). The light intensity, leaf temperature, and CO_2 concentration inside the leaf chamber were kept constant at 2000 μmol $m^{-2}s^{-1}$, 23 ± 0.5 °C, and 300 ± 5 μM, respectively. The total chlorophyll content was measured by detecting the absorbance at 663 and 645 nm in an 80% acetone extract after the different treatments (Qiao et al., 2015).

2.4. Stomata examination

To immediately fix the pore structure, the leaves were floated in 70% ethanol for 5 min. The upper epidermis of the leaves was scraped with a sharp knife to remove mesophyll,and then the leaves were placed on a surface-sterilized microscope slide and covered with a cover slip. Microscopic images of the stomata on the lower epidermis were obtained using a microscope equipped with a digital camera and the stomatal aperture was measured (Olympus BX51, Japan).

2.5. Measurement of endogenous H_2S and pro content

The H_2S content in the seedlings was measured using the methylene blue method (Shi et al., 2015). The leaves (200 mg) were homogenized in 2 mL of extraction buffer (50 mM phosphate buffer, pH 6.8, 0.2 M ascorbic acid and 0.1 M ethylene diamine tetraacetic acid (EDTA)); then, 1 ml of HCl (1 M) was added to the mixture. H_2S was collected in a trap containing 0.5 mL of 1% (w/v) zinc acetate. After 30 min of reaction time, 0.25 mL of dimethyl-p-phenylenediamine and ferric ammonium sulphate were added to the trap. The absorbance of the mixture was examined at 667 nm. The proline content in the seedlings was measured using the ninhydrin method (Bates et al., 1973).

2.6. Analysis of enzyme activities and corresponding gene transcription

Activities of $\triangle^1$-pyrroline-5-carboxylate reductase (P5CR) and proline dehydrogenase (PDH) were measured using the described methods (Tripathi et al., 2013).

Total RNA was extracted from seedlings with TRIzol Reagent (TaKaRa, Tokyo, Japan), and cDNA was synthesized using M-MLV reverse transcriptase (TransGen Biotech, Beijing, China). The transcript levels of the genes (P5CR and PDH) were detected using an