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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

Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Foster, CA, USA) (Liu et al., 2014b). The primers used for q-PCR were as follows: *ACTIN*-F: GGTATGGAGTCGCCTGGAATCC, *ACTIN*-R: GCGGTCAGCAATACCAGGGAAC; *P5CR*-F: TGTTTGGGAGAGATGGCTAC, *P5CR*-R: CAAGAACCGTTTGAGATGC; *PDH*-F: CCTGGGGCTCAAGGTCGT, *PDH*-R: GCATCCGTTGTAGCAGTCGT. All of the above-mentioned primers were designed via Primer Premier 5.0 (Premier, Canada). Annotation was performed using the foxtail millet database (http://foxtailmillet.genomics.org.cn/page/species/index.jsp).

2.7. Statistical analyses

All of the data were expressed as the mean ± standard error (M±SE), and statistical analyses was performed using SPSS version 17.0 (SPSS, IBM, Chicago, IL, USA). The statistical significance between the control and other treatment groups was analyzed using one-way analysis of variance followed by Duncan's test, taking * $p < 0.05$ or ** $p < 0.01$ as a significant difference.

3. Results

3.1. Effects of H_2S on Cd tolerance

To study the effects of H_2S on Cd tolerance in foxtail millet, several indexes of oxidative damage and photosynthesis were measured. As shown in Fig. 1, both the MDA content (Fig. 1A) and electrolyte leakage (Fig. 1B) significantly decreased at a concentration of 50 μM NaHS compared to the control ($p < 0.01$). Similarly, a reduction of 50 μM NaHS on the H_2O_2 content showed a significant difference ($p < 0.05$) (Fig. 1C). This phenomenon was weakened when the concentration of NaHS surpassed 50 μM. In addition, increases in all indexes (Pn, Gs and E) were found with increased NaHS concentrations; however, only a concentration of 50 μM NaHS demonstrated significant results (Fig. 1D, E and 1F). Therefore, 50 μM NaHS was selected for the pretreatment of foxtail millet seedlings in the subsequent experiments.

3.2. Effects of exogenous H_2S on proline under Cd stress

To explore the effects of exogenous H_2S on Pro, the activity and transcription of P5CR and PDH were analyzed in seedlings that underwent different treatments. Fumigating with 50 μM NaHS showed a significant increase in endogenous Pro content ($p < 0.05$). More Pro content was detected in seedlings co-treated with NaHS and Cd (NaHS + Cd) compared to those that received single Cd treatment (Fig. 2A). Furthermore, the increase in P5CR activity and the decrease in PDH activity were observed in NaHS-fumigated seedlings. NaHS + Cd enhanced the degree of Cd-induced alterations in their activities compared with Cd-treated seedlings (Fig. 2B and C). NaHS treatment resulted in increased expression of the P5CR gene and significant downregulation of the PDH gene. Meanwhile, upregulation of P5CR and downregulation of PDH was enhanced in the NaHS + Cd treatment (Fig. 2D and E).

3.3. Effects of proline on oxidative damage

To determine the relationship between endogenous H_2S and Pro in foxtail millet, we analyzed the changes of the Pro content in seedlings pretreated with various H_2S modulators (hypotaurine HT, aminooxyacetic acid AOA, potassium pyruvate PP and hydroxylamine HA, at 1000 μM). Fig. 3A showed that the endogenous H_2S content significantly decreased after treatment with H_2S modulators. Under Cd exposure, pretreating with 50 μM NaHS significantly increased the endogenous Pro levels of the seedlings. By contrast, when endogenous H_2S was inhibited or scavenged by AOA, PP, HA or HT, the Cd-induced increase of endogenous Pro was weakened (Fig. 3B). To verify the mitigative role of Pro in Cd damage, seedlings pretreated with a series of exogenous Pro concentrations were subsequently treated with 5 mM Cd. The results showed 0.1 mM

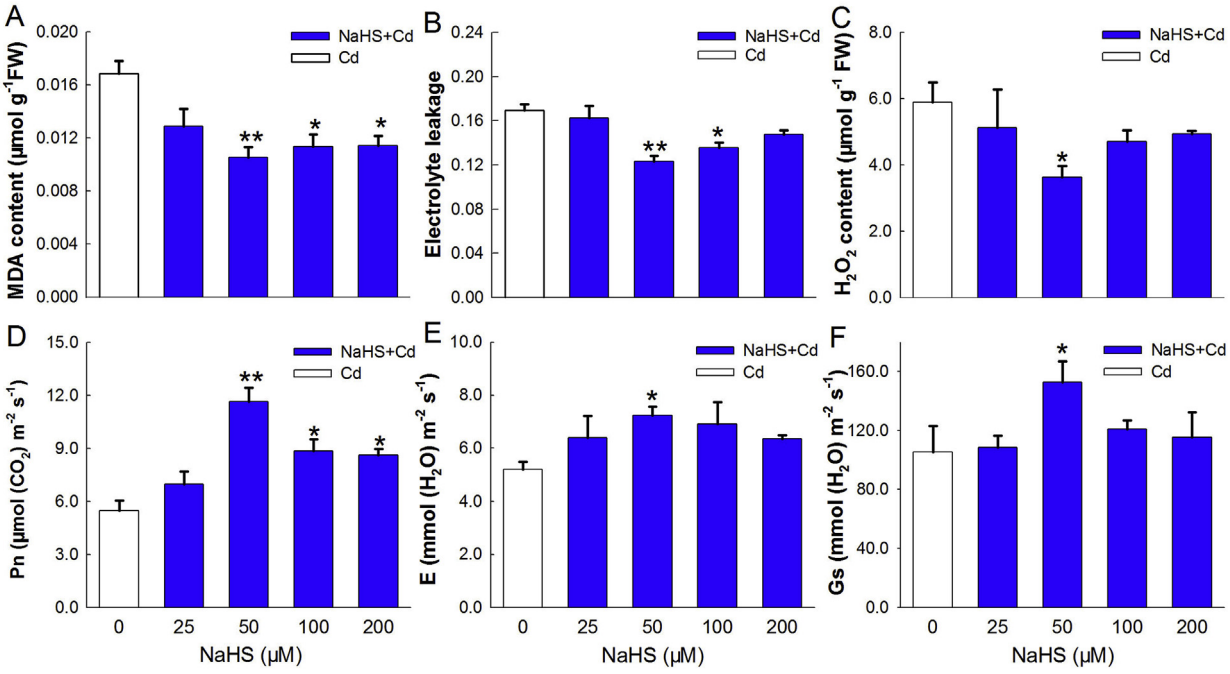

Fig. 1. Effects of H_2S on Cd tolerance. (A) MDA content, (B) electrolytic leakage, (C) H_2O_2 content and (D, E, F) leaf gas exchange parameters in foxtail millet. Five-day-old seedlings were fumigated with different concentrations of NaHS (0, 25, 50, 100 and 200 μM) for 24 h and then exposed to 5 mM $CdCl_2$ solution for 24 h. Data represents the mean ± SE of 30 plants per treatment, with at least three independent repeats, taking * ($p < 0.05$) or ** ($p < 0.01$) as the significant difference in comparison to the control.

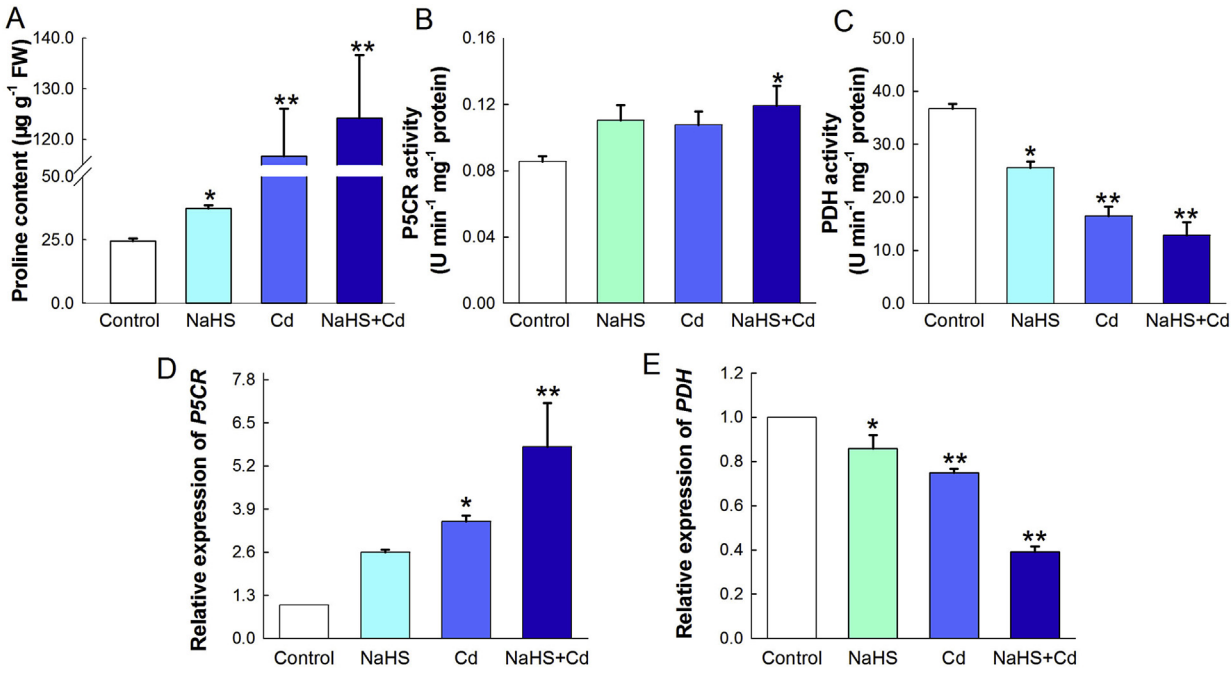

Fig. 2. Effects of exogenous H_2S on Proline under Cd stress. (A) Proline content, the activities of (B) P5C reductase and (C) PDH, and the gene expression levels of (D) *P5CR* and (E) *PDH* for five-day-old seedlings that underwent different treatments. Twelve dishes (30 plants/each dish) were exposed to four different treatments (3 dishes/each treatment): control: non-treated; NaHS: fumigated with 50 μM NaHS for 24 h; Cd: treated with 5 mM Cd for 24 h; and NaHS + Cd: seedlings fumigated with 50 μM NaHS for 24 h and then treated with 5 mM Cd for 24 h. Data represents the mean ± SE of the 30 plants per treatment with at least three independent repeats, and taking * ($p < 0.05$) or ** ($p < 0.01$) as the significant difference between the control and treatment groups.

Pro significantly inhibited the increased tendency of MDA, EL and H_2O_2 ($p < 0.05$) compared to Cd-treated seedlings (Fig. 3C, D and 3E). Furthermore, increases in all indexes (Pn, Gs and E) were significant with 0.1 mM and 1 mM pro concentrations (Fig. 3F, G and 3H). Therefore, we chose 0.1 mM Pro as the pretreatment concentration and found that Pro played a mitigative role in Cd-damage.

3.4. Effects to Cd tolerance

To evaluate the role of H_2S in the certainly growth will be effected by Cd, and probably as a result of oxidative damage. we measured the growth, stomatal movements and H_2O_2 accumulation in seedlings that underwent different treatments (Fig. 4). The results showed that the single NaHS treatment raised the chlorophyll content of foxtail millet seedlings compared to the control. Moreover, this content was decreased by 37% with Cd treatment. However, when seedlings received the NaHS + Cd treatment, the chlorophyll content decreased to the control level and the toxic symptoms of the leaves were mitigated (Fig. 4A and B). As seen in Fig. 4D, NaHS caused the stomata to open, and the stomatal close was demonstrated by the Cd treatment; NaHS pretreatment weakened the Cd-induced stomatal ability to close and therefore the stomata were not defective (Fig. 4C and D). Compared to the control, Cd exposure resulted in a 226% greater accumulation of H_2O_2 and more reddish-brown spot formation with DAB staining, while weaken H_2O_2 accumulation and reduced spot formation were observed under the NaHS + Cd treatment or Pro + Cd treatment, respectively (Fig. 4E and F). Specifically, the combined treatment with H_2S and Pro was a more effective alleviation than a single Pro or H_2S pretreatment under Cd stress (Fig. 4).

4. Discussion

Hydrogen sulfide (H_2S) is a newly regarded gaseous transmitter in plants that is involved in several developmental and biochemical processes, as well as biotic and abiotic stress responses (Fotopoulos et al., 2013; Jin and Pei, 2015). NaHS has been widely used as an H_2S donor in plant research. In this study, a NaHS treatment was shown to alleviate Cd damage by enhancing photosynthesis and reducing oxidative damage. Meanwhile, NaHS promoted photosynthesis, the chlorophyll content, and growth (Fig. 1D–F and Fig. 4A and B). These findings were consistent with those reported in the literature. For instance, treatment with 100 μM NaHS induces seedling growth and chlorophyll accumulation in *Spinacia oleracea* seedlings mainly by motivating the mRNA transcription of the gene encoding the RuBISCO large subunit (*RBCL*) and small subunit (*RBCS*), and by enhancing the activity of RuBISCO (Chen et al., 2011). NaHS pretreatment raises the chlorophyll content of *Arabidopsis* seedlings under Cd stress (Qiao et al., 2015). H_2S rescues the loss of photosynthetic pigments (Chl a, Chl b, total Chl and carotenoids) in rice under 1.0 mM Cd stress (Mostofa et al., 2015). Thus, all the findings presented here indicates that NaHS appears to positively effect leaf photosynthesis.

NaHS inhibited the Cd-induced increase of MDA, EL and H_2O_2 (Fig. 1A–C). We further confirmed that a pretreatment with an H_2S donor suppressed the H_2O_2 accumulation in leaves according to an *in vivo* staining method (Fig. 4F). Meanwhile, a similar case showed that H_2S plays a highly beneficial role in plants exposed to aluminum, chromium or copper stress by suppressing heavy metal-induced H_2O_2 production and lipid peroxidation in barley roots, foxtail millet leaves and wheat seed, respectively (Zhang et al., 2008; Chen et al., 2013; Fang et al., 2014a). Therefore, H_2S is involved in the physiological response to heavy metal stress. Interestingly, Lisjak et al. suggested that H_2S does not work as a signaling molecule in regulating the levels and effects of ROS, but also acts as a modulator to ensure that the over-accumulation of such compounds is not causing plant damage (Lisjak et al., 2013).

As a signaling molecule, H_2S has been largely studied in animals

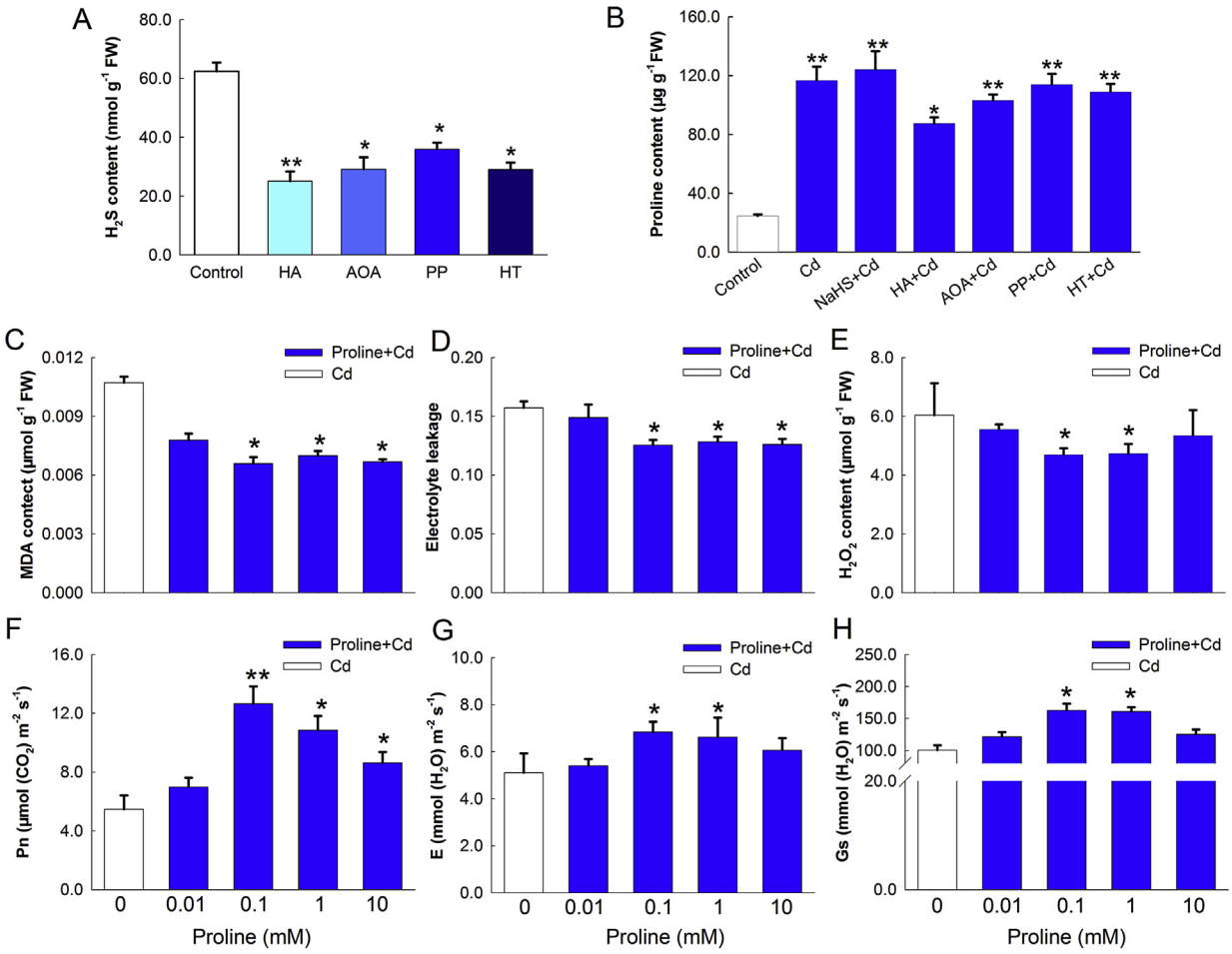

Fig. 3. Effects of Proline on oxidative damage. (A) Endogenous H_2S contents of five-day-old seedlings treated with H_2S modulators (HT, AOA, PP and HA at 1000 μM) for 24 h. (B) Contents of endogenous proline of the five-day-old seedlings pretreated with H_2S modulators (NaHS at 50 μM; HT, AOA, PP and HA at 1000 μM) for 24 h, and then treated with 5 mM Cd for 24 h (C) MDA content, (D) Electrolytic leakage, (E)H_2O_2 content and (F, G, H) leaf gas exchange parameters of the five-day-old seedlings pretreated with proline with a gradual increase in the concentration (0, 0.01, 0.1, 1 and 10 mM) for 24 h, and then treated with 5 mM Cd for 24 h. Data represents the mean ± SE for 30 plants per treatment with at least three independent repeats, taking * ($p < 0.05$) or ** ($p < 0.01$) as the significant difference between the control and treatment groups.

and received increasing attention in plants in recent decades (Lisjak et al., 2013; Farrugia and Szurszewski, 2014; Wang, 2014; Jin and Pei, 2015). In the present work, H_2S promoted Pro accumulation in the presence or absence of excessive Cd (Fig. 2A), which acts as a heavy metal chelator to alleviate heavy metal stress, an osmoprotectant, and a ROS quencher (Szabados and Savoure, 2010; Tripathi et al., 2013). Compared with the control, the increased P5CR activity and decreased proline dehydrogenase activity in Cd-stressed seedlings was enhanced by NaHS (Fig. 2B and C). Li et al. reported that NaHS could enhance P5CS activity while lowering proline dehydrogenase activity and induce endogenous Pro accumulation in maize seedlings (Li et al., 2013). NaHS maintained the functionality of the photosynthetic apparatus under PEG osmotic stress through the up-regulation of the photosystem II oxygen-evolving complex protein, RuBISCO large subunit and glutamate glyoxylate aminotransferase (Ziogas et al., 2015). Furthermore, the transcript levels of two key enzymes, P5CR and proline dehydrogenase, were induced in NaHS-pretreated seedlings in comparison with in untreated ones. The NaHS pretreatment up-regulated the expression of the gene encoding the citrate transporter, leading to increased citrate secretion in barley seedlings and protecting plants against aluminum toxicity (Chen et al., 2013). Hence, H_2S may play key roles in the integral regulation of metabolism with regard to protein and nucleic acid levels.

The roles of H_2S in plants *in vivo* were determined by a pharmacology experiment using an H_2S donor (NaHS), H_2S scavenger (HT) and inhibitors of H_2S biosynthesis (HA, AOA, PP) in previous studies (Shi et al., 2013, 2014; Qiao et al., 2015). The Cd-induced increase in the Pro content was regulated by H_2S via the addition of the H_2S donor, scavenger and inhibitors in plants (Fig. 3A and B). The above results illustrate that H_2S affects Pro accumulation, enhancing its protective capabilities during Cd stress. Exogenous treatments of H_2S modulators specifically affected the endogenous H_2S, and this pharmacology experiment could make up for the insufficient status of crop mutant resources (Shi et al., 2014; Fang et al., 2014a).

The present study indicates that the combination of H_2S and Pro has a role in reducing Cd phytotoxicity and that H_2S is a potential conductor, which acts through the stimulation of non-enzymatic (Pro) antioxidant machinery in response to Cd stress. A feasible mechanism of H_2S-induced Cd stress tolerance in foxtail millet is that H_2S not only affects Pro metabolism, but it also underlies physiological and biochemical mechanisms involved in the antioxidant enzymatic system (Shi et al., 2013), ascorbate–glutathione (AsA-GSH) cycle (Fang et al., 2014a), heavy metal chelators (PCs and MTs) (Fang et al., 2016) and others. Therefore, we focused on the ameliorative effects of H_2S on stress in plants and found that H_2S induced the attenuation of Cd-associated negative effects, perhaps

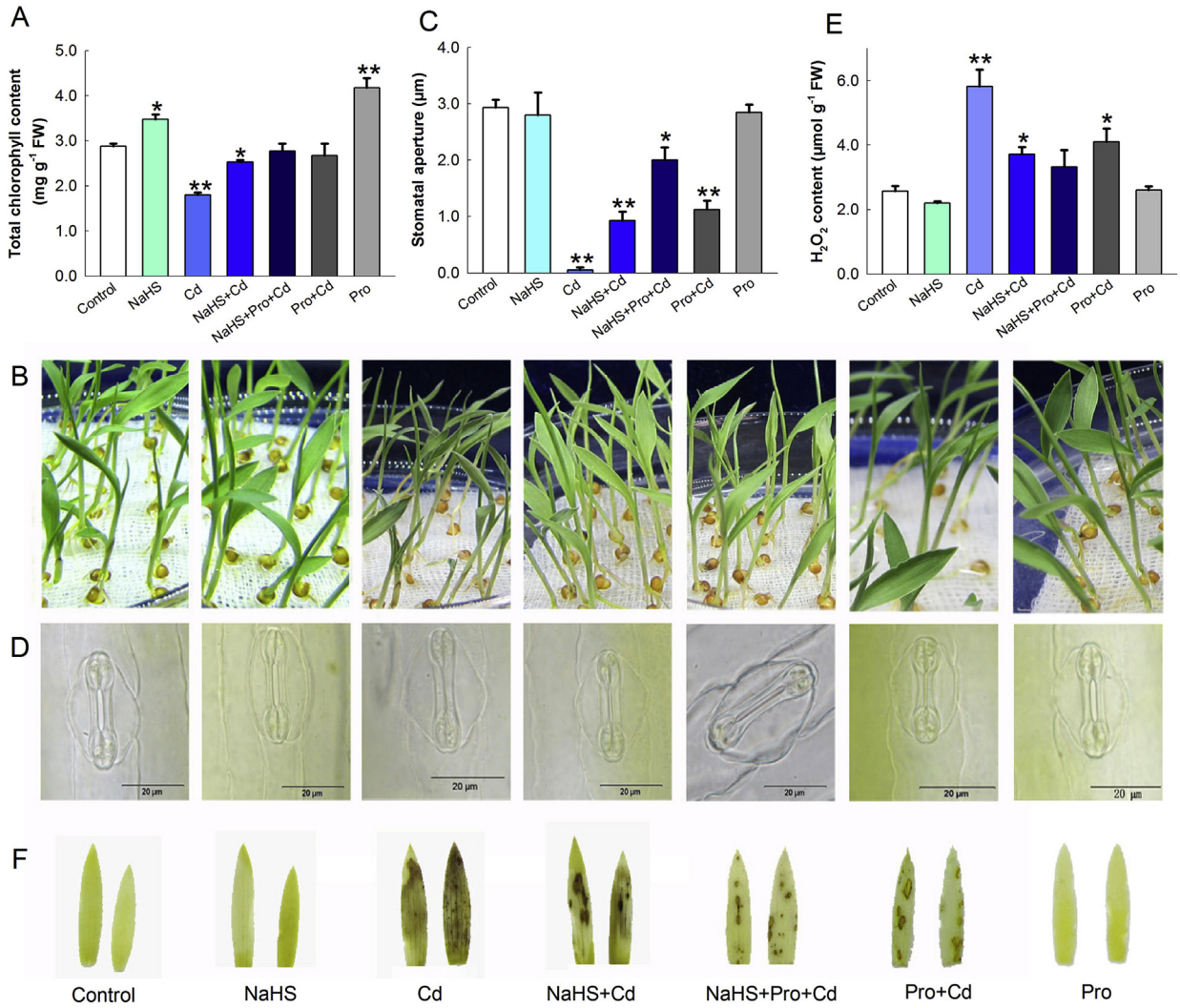

Fig. 4. Cooperative effects of H_2S and Proline to Cd tolerance. (A) Chlorophyll content, (B) symptoms of growth, (C) stomatal aperture, (D) stomatal opening, (E) H_2O_2 content and (F) DAB staining of the leaves of the five-day-old seedlings that underwent different treatments. Twenty-one dishes (30 plants/each dish) were exposed to seven different treatments (3 dishes/each treatment): control: non-treated; NaHS: fumigated with 50 μM NaHS for 24 h; Cd: treated with 5 mM Cd for 24 h; NaHS + Cd: seedlings fumigated with 50 μM NaHS for 24 h and then treated with 5 mM Cd for 24 h; NaHS + Pro + Cd: seedlings co-treated with 50 μM NaHS and 0.1 mM proline for 24 h and then treated with 5 mM Cd for 24 h; Pro + Cd: seedlings pretreated with 0.1 mM proline for 24 h and then treated with 5 mM Cd for 24 h, and Pro: seedlings treated with 0.1 mM proline for 24 h. Data represents the mean ± SE of 30 plants per treatment with at least three independent repeats, and taking * ($P < 0.05$) or ** ($P < 0.01$) as the significant difference between the control and treatment groups.

through working together with multiple signals.

Cd-induced oxidative damage through changes in the ROS levels in plants was reported previous in the literature (Tripathi et al., 2013; Mostofa et al., 2015). In accordance with several studies, a significant decrease was found in MDA, EL and H_2O_2 after the application of exogenous Pro. Xu et al. reported that a Pro pretreatment reduced the ROS level, improving Cd tolerance in *Solanum nigrum* (Xu et al., 2009). Additionally, an exogenous Pro application was found to reduce lipid peroxidation in algal (*Chlorella vulgaris*) cells after exposure to heavy metals, including copper, chromium, nickel and zinc (Mehta and Gaur, 1999). A Pro pretreatment also alleviated mercury toxicity in rice (*O. sativa*) by scavenging ROS, such as H_2O_2 (Wang et al., 2009). Both growth inhibition and the stomatal ability to close were weakened and H_2O_2 accumulation was reduced, after a combined pretreatment with NaHS and Pro (Fig. 4). Therefore, H_2S could be correlated with free Pro in response to Cd stress. H_2S and Pro appear to cooperate to alleviate Cd stress. This cooperation has been regarded as a feasible method of detoxification, H_2S is involved in heavy metal detoxification and may act as a mediator or a regulator (Jin and Pei, 2015). However, a more detailed regulatory mechanism between H_2S and Pro needs to be evaluated.

The present study indicated that Pro increased significantly in the Cd-treated foxtail millet seedlings and that an H_2S application enhanced the Pro levels. Therefore, we proposed a possible cooperative relationship between H_2S and Pro in response to Cd stress. Our results provided novel insights into the heavy metal detoxification mechanism in crops. Previous studies showed that H_2S extensively interacts with other molecules in plants, such as NO (Shi et al., 2014; Ziogas et al., 2015; Molassiotis et al., 2016), ROS (Lisjak et al., 2013),Ca^{2+} (Fang et al., 2014a), SA (Qiao et al., 2015), auxin (Fang et al., 2014b) and abscisic acid (ABA) (Jin and Pei, 2015). These interactions result in a complex signaling network in plant biology. Further studies of the regulatory roles of H_2S in Pro detoxification are currently underway. However, additional studies are needed to better understand these results. The relationship

between H_2S and Pro in heavy metal detoxification and metal uptake will be investigated in our next study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.10.006.

Authors' contributions

Banhua Tian and Yanxi Pei designed the experiments and wrote the manuscript.

Banhua Tian and Zengjie Qiao performed the experiments.

Banhua Tian, Zengjie Qiao, Liping Zhang and Hua Li analyzed data.

All authors read and approved the final manuscript.

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