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Roles of hydrogen sulfide and nitric oxide in the alleviation of cadmium-induced oxidative damage in alfalfa seedling roots

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Abstract Despite hydrogen sulfide (H_2S) and nitric oxide (NO) are important endogenous signals or bioregulators involved in many vital aspects of plant growth and responses against abiotic stresses, little information was known about their interaction. In the present study, we evaluated the effects of H_2S and NO on alfalfa (*Medicago sativa* L.) plants exposed to cadmium (Cd) stress. Pretreatment with an H_2S donor sodium hydrosulfide (NaHS) and well-known NO donor sodium nitroprusside (SNP) decreased the Cd toxicity. This conclusion was supported by the decreases of lipid peroxidation as well as the amelioration of seedling growth inhibition and Cd accumulation, in comparison with the Cd-stressed alone plants. Total activities and corresponding transcripts of antioxidant enzymes, including superoxide dismutase, peroxidase and ascorbate peroxidase were modulated differentially, thus leading to the alleviation of oxidative damage. Effects of H_2S above were reversed by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), the specific scavenger of NO. By using laser confocal scanning microscope combined with Greiss reagent method, further results showed that NO production increased significantly after the NaHS pretreatment regardless of whether Cd was applied or not, all of which were obviously inhibited by cPTIO. These decreases of NO production were consistent with the exaggerated syndromes associated with Cd toxicity. Together, above results suggested that NO was involved in the NaHS-induced alleviation of Cd toxicity in alfalfa seedlings, and also indicated that there exists a cross-talk between H_2S and NO responsible for the increased abiotic stress tolerance.

Keywords Alfalfa seedling roots · Cd-induced oxidative stress · Hydrogen sulfide · Nitric oxide · Sodium hydrosulfide · Sodium nitroprusside

Introduction

Cadmium (Cd) is toxic to humans, animals and plants. It usually results in plant growth inhibition and even cell death (De Michele et al. 2009). One of the responses of plants to Cd toxicity is oxidative stress that leads to lipid peroxidation of the plasma membrane (Rodríguez-Serrano et al. 2006). Depending on its concentration, Cd stress can either inhibit or stimulate the activities of several antioxidative

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enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) before any visible symptoms of toxicity appear (Milone et al. 2003; Hsu and Kao 2004). Further results suggested that the activation of antioxidative enzymes is, at least partially, beneficial for plant performance against Cd exposure. For example, simultaneous overexpression of both *Cu/Zn–SOD* and *APX* in transgenic tall fescue plants confers increased tolerance to herbicide methyl viologen (MV), hydrogen peroxide (H_2O_2), and some heavy metals, including Cd, copper (Cu) and arsenic (As) exposure (Lee et al. 2007).

In mammalian tissues, hydrogen sulfide (H_2S) is produced endogenously from L-cysteine mainly by two pyridoxal-5′-phosphate-dependent enzymes, cystathionine γ-lyase (CSE) and cystathionine β-synthetase (CBS). Further result illustrated that CSE acts as a plant cysteine-specific desulfhydrase (CDES or DES; EC 4.4.1.1) responsible for H_2S releasing (Papenbrock et al. 2007). It was well known that H_2S is a critical signal in animals, important for various physiological functions, including anti-inflammatory role, the induction of hippocampal long-term potentiation, brain development and blood pressure regulation (Wang 2003; Lefer 2007; Li et al. 2009). In plants, H_2S has been implicated in various development processes, such as adventitious root and lateral root formation (Zhang et al. 2009a; Lin et al. 2012), and seed germination (Zhang et al. 2008; Wang et al. 2012). On the other hand, it has been suggested that H_2S can act as a potential antioxidant in plants upon abiotic stresses by efficiently inducing changes in transcripts of genes encoding pectin methylesterase (PME) (Wang et al. 2010), or total and isozymatic activities of antioxidative enzymes (Zhang et al. 2009b, 2010a, b). These illustrated that H_2S has an active role in modifying plant stress responses. In animals and recently in plants, most H_2S responses are similar to or mediated by nitric oxide (NO) (Ondrias et al. 2008; Zhang et al. 2009a). In fact, NO is another gaseous signal molecule involved in plant response to abiotic stresses (Durner and Klessig 1999; Zhang et al. 2003; Delledonne 2005). In plant kingdoms, NO generation is activated by several hormonal and environmental stimuli (Delledonne 2005; Arasimowicz-Jelonek et al. 2011). Further experiments discovered that the exogenous application of NO could be advantageous against Cd toxicity in *Helianthus annuus* (Laspina et al. 2005), *Oryza sativa* (Hsu and Kao 2004), *Medicago truncatula* (Xu et al. 2010), *Arabidopsis thaliana* and *Solanum nigrum* (Xu et al. 2011). However, contrasting results were also reported. For example, combined transcriptomic, biochemical, pharmacological and genetic analyses, NO was confirmed to contribute to Cd toxicity in *Arabidopsis* seedlings by favoring Cd^{2+} versus Ca^{2+} uptake and by initiating a cellular pathway resembling those activated upon iron deprivation (Besson-Bard et al. 2009).

Although the effects of NO and H_2S on stress physiology have received much attention, little information was known about their molecular mechanisms and possible cross-talk. More recently, by using Greiss reagent method and inhibitor analysis, we provided evidence showing that H_2S enhances plant tolerance against salinity stress, which might have a possible interaction with NO (Wang et al. 2012). In contrast to salinity stress, there has been no detailed study to evaluate the interactions between H_2S and NO in plant responses against Cd toxicity. In this paper, using pharmacological and biochemical approaches, we demonstrated that H_2S and NO pretreatments cause protection against Cd-triggered oxidative damage and toxicity in alfalfa seedling roots. Combined with laser confocal scanning microscope (LSCM) and Greiss reagent method, our results further suggested that there exists a cross-talk between H_2S and NO responsible for the increased abiotic stress tolerance.

Materials and methods

Chemicals

Sodium hydrosulfide (NaHS) and sodium nitroprusside (SNP), purchased from Sigma (St Louis, MO, USA), were used as the H_2S or HS^- and NO donor, respectively (Zhao et al. 2001; Xie et al. 2008; Wang et al. 2010). 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) was used as the specific NO scavenger (Xie et al. 2008; Besson-Bard et al. 2009). NO_2^-/NO_3^- (NO_x^-) and $K_3Fe(CN)_6/K_4Fe(CN)_6$ (Fe) were used as the degradation product of SNP and SNP analogue (Bethke et al. 2006; Xie et al. 2008). 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA), purchased from Sigma (St Louis, MO, USA), was

used as the permeable NO-sensitive fluorophore (Graziano and Lamattina 2007; De Michele et al. 2009). Hypotaurine (HT), which reacts directly with sulphide to form thiotaurine (ThT) (Ortega et al. 2008; García-Mata and Lamattina 2010), was used at 1 mM. DL-propargylglycine (PAG), an inhibitor of CSE/L-CDES (García-Mata and Lamattina 2010), was used at 2 mM.

Plant material, growth condition and treatments

Commercially available alfalfa (*Medicago sativa* L. cv. Victoria) seeds were surface-sterilized with 5 % NaClO for 10 min, and rinsed extensively in distilled water before being germinated for 1 day at 25 °C in the darkness. Uniform seedlings were then selected and transferred to the plastic chambers and cultured in nutrient medium (quarter-strength Hoagland's solution). Alfalfa seedlings were grown in the illuminating incubator at 25 ± 1 °C, with a light intensity of 200 μmol m^{-2} s^{-1} and 14 h photoperiod. After growing for 5 days, seedlings were then incubated in quarter-strength Hoagland's solution with or without varying concentrations of NaHS, 100 μM SNP, 1 mM HT, 2 mM PAG, 200 μM cPTIO, 100 μM Na_2S, 100 μM $NaHSO_4$, 100 μM Na_2SO_4, 100 μM $NaHSO_3$, 100 μM Na_2SO_3, $K_3Fe(CN)_6/K_4Fe(CN)_6$ (100/100 μM, Fe) and NO_2^-/NO_3^- (100/100 μM, NO_x^-), 100 μM $CdCl_2$ alone, or the combination treatments for 6 h or the indicated times, and/or exposed to 0 or 100 μM $CdCl_2$ or the indicated treatments for another 12 h or the indicated times. Seedlings without chemical treatments were used as the control (Con). The pH for both nutrient medium and treatment solutions was adjusted to 6.0 by using NaOH or HCl, and treatment solutions were renewed each day to maintain constant concentrations. After various treatments, the seedlings were sampled, and growth parameters were determined, root tissues were then used immediately or frozen in liquid nitrogen, and stored at −80 °C for further analysis.

Determination of thiobarbituric acid reactive substances (TBARS) content

Lipid peroxidation was estimated by measuring the amount of TBARS as previously described (Han et al. 2008). About 500 mg fresh tissue was ground in 0.25 % 2-thiobarbituric acid (TBA) in 10 % TCA using a mortar and pestle. After heating at 95 °C for 30 min, the mixture was quickly cooled in an ice bath and centrifuged at 10,000×*g* in a rotor (model Avanti J-25, Beckman) for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The blank was 0.25 % TBA in 10 % TCA. The concentration of lipid peroxides together with oxidatively modified proteins of plants were thus quantified in terms of TBARS amount using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} fresh weight (FW).

Determination of Cd content in plant tissues

Cadmium in root tissues was extracted and measured by graphite furnace atomic absorption spectrophotometry (180-80 Hitachi, Tokyo, Japan) as described by Brune and Dietz (1995).

Histochemical analyses

Histochemical detection of lipid peroxidation was performed with Schiff's reagent as described by Pompella et al. (1987). Histochemical detection of loss of plasma membrane integrity in root apexes was performed with Evans blue described by Yamamoto et al. (2001). All the roots stained with Schiff's reagent or Evans blue were washed extensively, then observed under a light microscope (model Stemi 2000-C; Carl Zeiss, Germany) and photographed on color film (Powershot A620, Canon Photo Film, Japan).

Enzymatic activities assays

Frozen alfalfa seedling roots (approximately 200 mg) were homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 % polyvinylpyrrolidone (PVP) for SOD and guaiacol POD assay, or the combination with the addition of 1 mM ascorbic acid (ASC) in the case of APX assay. The homogenate was centrifuged at 12,000×*g* in a rotor (model Avanti J-25, Beckman) for 20 min at 4 °C and the supernatant was immediately desalted by Sephadex G-25 gel filtration to remove interfering materials and used as the crude enzyme extract.

SOD and guaiacol POD activities were analysed using the methods described in our previous reports

(Han et al. 2008). Total SOD activity was measured on the basis of its ability to reduce nitroblue tetrazolium (NBT) by the superoxide anion generated by the riboflavin system under illumination. One unit of SOD (U) was defined as the amount of the crude enzyme extract required to inhibit the reduction rate of NBT by 50 %. Guaiacol POD was determined by measuring the oxidation of guaiacol (extinction coefficient 26.6 mM^{-1} cm^{-1}) at 470 nm. APX activity was measured as described by Nakano and Asada (1981).

Lipoxygenase (LOX) activity was measured according to the method described by Zhang et al. (2003). Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Real-time quantitative RT-PCR analysis

Total RNA from 100 mg of fresh-weight alfalfa plants was isolated by grinding with mortar and pestle in liquid nitrogen until a fine powder appeared and using Trizol reagent (Invitrogen) according to the manufacturer's instructions. DNA-free total RNA (5 μg) from different treatments was used for first-strand cDNA synthesis in a 20-μl reaction volume containing 2.5 U of avian myeloblastosis virus reverse transcriptase XL (TaKaRa) and 2.5 μM random primer.

Real-time quantitative RT-PCR reactions were performed with Mastercycler® realplex^2 real-time PCR system (Eppendorf, Hamburg, Germany) using the SYBR® *Premix Ex Taq*™ (TaKaRa Bio Inc., China) according to the user manual. The cDNA was amplified using the following primers: for *Cu/Zn–SOD* (accession number AF056621), forward (5′-TAATTGCTGATGCCAACG-3′) and reverse (5′-ACCACAGGCTAATCTTCCAC-3′); for *POD* (accession number X90695), forward (5′-CCTGCTACCCTTCGTCTCT-3′) and reverse (5′-GTCAAAACCATCACCTGCC-3′); for *APX* (accession number DQ122791), forward (5′-GGAACCATCAAGCACCAAG-3′) and reverse (5′-ATCCCAACAGCAACAACCT-3′); and for *EF-2* (accession number DQ122789), forward (5′-CATGTTCAGGTGCTTGTATT-3′) and reverse (5′-GGTTTGTGATGTTGTTCTTC-3′). The PCR program consisted of an initial denaturation and *Taq* activation step of 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 15 s at 50 °C. A melting curve analysis was performed after every PCR reaction to confirm the accuracy of each amplified product. All reactions were set up in triplicate. Relative expression levels were presented as values relative to that of corresponding control sample at the indicated time, after normalization to *EF-2* transcript levels.

NO production determined by using Greiss reagent

NO production was determined using the method described by Zhou et al. (2005) with slight modifications. Samples were ground in a mortar and pestle in 3 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 4 % zinc diacetate). The homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was collected. The pellet was washed by 1 ml of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal was added. After vortex and filtration, the filtrate was leached and collected. The mixture of 1 ml of filtrate and 1 ml of the Greiss reagent was incubated at room temperature for 30 min. Meanwhile, identical filtrate which was pretreated with cPTIO, the specific scavenger of NO, for 15 min, was used as blanks. Absorbance was assayed at 540 nm. NO content was calculated by comparison to a standard curve of $NaNO_2$.

Visualization of endogenous NO by LSCM

Endogenous NO was imaged using the positive fluorescent probe DAF-FM DA. Roots were loaded with 10 μM DAF-FM DA (Graziano and Lamattina 2007; De Michele et al. 2009) in 20 mM HEPES buffer (pH 7.4) for 30 min, washed three times in fresh buffer for 15 min. Then root tips were examined by a TCS-SP2 confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany; excitation with the 488 nm, and emission using a 500–530 nm). Pictures without the addition of probe were also taken as a blank. Data are presented as the mean of fluorescence intensity relative to the control samples. Experiments were repeated six times, and similar results were obtained. All manipulations were performed in the dark at 25 ± 1 °C.

Statistical analysis

Values are means ± SE of at least three different experiments with at least three replicated measurements. Differences among treatments were analysed

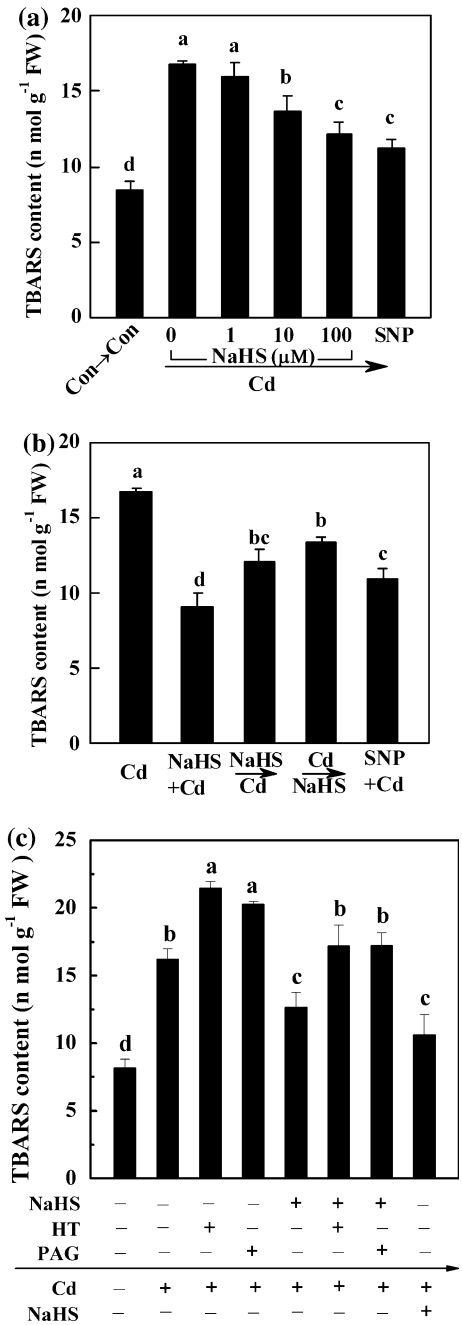

Fig. 1 Effects of NaHS, SNP, HT and PAG on TBARS concentration in alfalfa seedling roots upon Cd exposure. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution containing increasing concentrations of NaHS or 100 μM SNP for 6 h, followed by another 12 h incubation in 100 μM $CdCl_2$ (**a**), or incubating the roots in solution supplemented with 100 μM $CdCl_2$, 100 μM $CdCl_2$ and 100 μM NaHS or 100 μM SNP together for 12 h, or incubating the roots in 100 μM NaHS for 6 h followed by another 12 h incubation in 100 μM $CdCl_2$, or incubating the roots in 100 μM $CdCl_2$ for 12 h followed by another 6 h incubation in 100 μM NaHS (**b**), or incubating the roots in solution supplemented with 100 μM NaHS, 1 mM HT, 2 mM PAG alone, or the combination treatments for 6 h, followed by 100 μM $CdCl_2$ with or without 100 μM NaHS for another 12 h (**c**). Values are the mean ± SE of at least three independent experiments. *Bars* with different *letters* were significantly different in comparison with control or Cd stressed alone samples at $P < 0.05$ according to Duncan's multiple range test

by one-way ANOVA, taking $P < 0.05$ as significant according to Duncan's multiple range test.

Results

NaHS and SNP alleviated Cd-induced lipid peroxidation

To examine the sensitivity of alfalfa seedlings to Cd, the effects of NaHS, an H_2S donor, and SNP (a well-known NO donor) on TBARS content, an indicator of lipid peroxidation, of alfalfa seedling roots were investigated. In comparison with the control sample, TBARS content was increased after 12 h exposure of alfalfa seedlings to 100 μM $CdCl_2$ ($P < 0.05$; Fig. 1a). However, pretreatment with the increasing concentrations of NaHS caused a significant decrease in levels of TBARS at 12 h after the Cd treatment in a dose-dependent manner. A NaHS or SNP concentration of 100 μM, which decreased TBARS by approx. 28 or 33 %, respectively, was used to investigate the link between H_2S and NO in Cd toxicity throughout the study.

In the subsequent experiment, we further examined the effect of pretreatment of alfalfa seedling roots with Cd and NaHS individually on TBARS content and compared this with the effect of treatment of roots with Cd and NaHS simultaneously on lipid peroxidation. As shown in Fig. 1b, a similar effect of NaHS on the alleviation of Cd-induced lipid peroxidation was observed regardless of whether NaHS and Cd were present separately. Similar alleviation of lipid peroxidation induced by SNP was also confirmed, in comparison with that of the NaHS pretreatment. By contrast, the combination of Cd and NaHS together brought about the maximal decrease of TBARS content, suggesting the possibility that the alleviation of Cd-induced oxidative stress by NaHS together with

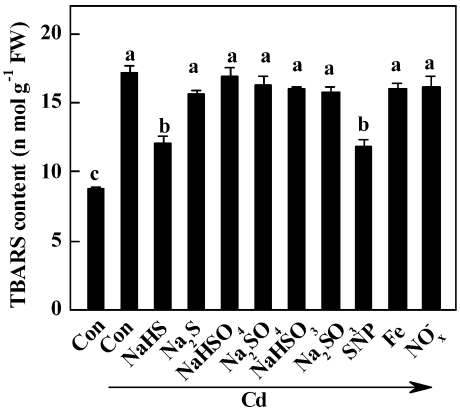

Fig. 2 H_2S or HS^- and NO, but not other compounds derived from NaHS or SNP contributed to the alleviation of lipid peroxidation in alfalfa seedling roots upon Cd exposure. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution containing 100 μM NaHS, 100 μM Na_2S, 100 μM $NaHSO_4$, 100 μM Na_2SO_4, 100 μM $NaHSO_3$, 100 μM Na_2SO_3, 100 μM SNP, $K_3Fe(CN)_6/K_4Fe(CN)_6$ (100/100 μM, Fe) and NO_2^-/NO_3^- (100/100 μM, NO_x^-) for 6 h, and then exposed to 100 μM $CdCl_2$ for another 12 h. Sample without chemicals was the control (Con). Values are the mean ± SE of three independent experiments. *Bars* denoted by the same *letter* did not differ significantly at $P < 0.05$ according to Duncan's multiple range test

Cd could be derived from the chelation or direct reaction of Cd by NaHS. As expected (Wilson et al. 1978; Rao and El-Rahaiby 1985), we also observed yellow crystals of CdS formed after the addition of the increasing concentrations of NaHS + $CdCl_2$ (Online Resource, Fig. S1). Furthermore, effects of a H_2S scavenger, hypotaurine (HT), or a synthetic inhibitor of H_2S, PAG, on NaHS- and/or Cd-induced responses was analyzed. As shown in Fig. 1c, the addition of HT or PAG together with NaHS was able to block the alleviation of Cd-induced lipid peroxidation triggered by NaHS, also indicating that the reduced response of NaHS treatment in the presence of HT or PAG might be the result of the decrease of endogenous H_2S production in alfalfa seedling roots. On the other hand, HT or PAG pretreatment bought about the aggravation of lipid peroxidation caused by Cd exposure.

H_2S and NO rather than other derivatives caused the alleviation of lipid peroxidation

In order to distinguish the role of H_2S from that of other sulphur-containing derivatives and sodium, some sulphur- and sodium-containing chemicals including NaHS, Na_2S, $NaHSO_4$, Na_2SO_4, $NaHSO_3$ and Na_2SO_3 were used. As expected, Na^+ or other sulphur-containing chemicals, which were used as controls of NaHS, did not show as great a decrease in TBARS content as NaHS did (Fig. 2). Together, we speculated that H_2S or HS^-, rather than other compounds directly or indirectly derived from the decomposing of NaHS, were responsible for the promotive effects of NaHS on the amelioration of oxidative damage induced by Cd exposure.

Unlike the cytoprotective role of SNP, we also noticed that in comparison with the sample under Cd stress alone, no significant difference in TBARS content was observed when $K_3Fe(CN)_6/K_4Fe(CN)_6$ (Fe) or NO_2^-/NO_3^- (NO_x^-), the degradation product of SNP and SNP analogue, was applied exogenously. These results suggested that NO rather than other derivatives caused the alleviation of lipid peroxidation triggered by SNP.

Histochemical staining

The effect of NaHS on the alleviation of Cd-induced oxidative damage was investigated by histochemical staining. The alfalfa seedling roots treated with Cd alone were stained extensively with Schiff's reagent (Fig. 3a) and Evans blue (Fig. 3b), whereas those pretreated with NaHS and SNP had only slight staining. No additive effects of NaHS and SNP were discovered. To further assess whether endogenous NO was involved in NaHS-triggered response, the specific NO scavenger cPTIO was applied. As shown in Fig. 3, the addition of cPTIO notably prevented NaHS-triggered responses. Meanwhile, pretreatment with NaHS, SNP or cPTIO alone did not change the staining patterns observed in comparison with the control samples. All the results were consistent with the changes in TBARS formation and LOX activity (Fig. 3c), an ubiquitous enzyme responsible for the formation of hydroperoxides and oxy-free radicals (Gardner 1991). Thus, these results further suggested that the application of H_2S donor NaHS and NO donor SNP exhibited the protection against Cd-induced oxidative damage in the root tissues of alfalfa seedlings.

Cd accumulation was attenuated by NaHS and SNP

Cd treatment caused a rapid uptake of Cd in the root tissues during the initial 24 h. Afterwards, a gradual

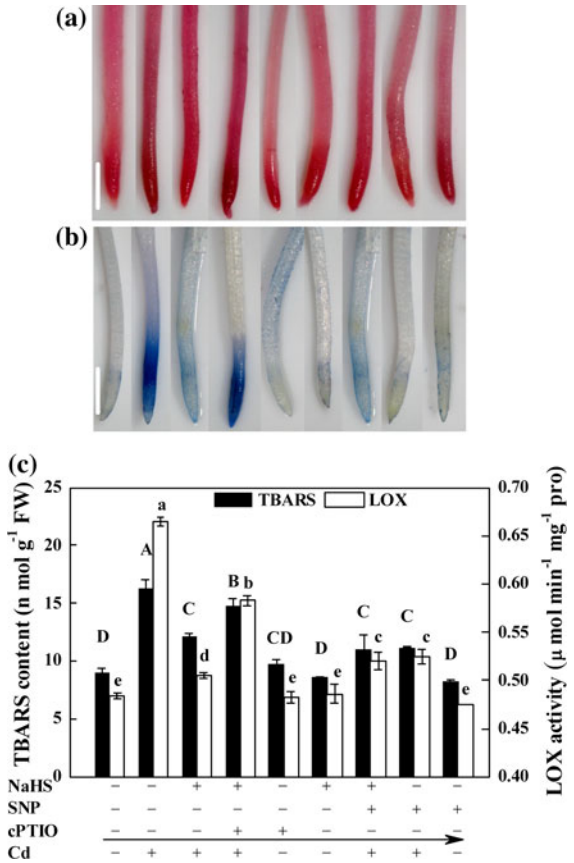

Fig. 3 Effects of pretreatment with NaHS, SNP and cPTIO on the histochemical localization of lipid peroxidation (**a**) and loss of plasma membrane integrity (**b**), LOX activity and TBARS content (**c**), in alfalfa seedling roots upon Cd exposure. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution containing 100 μM NaHS, 100 μM SNP, 200 μM cPTIO alone, or the combination treatments for 6 h, and then exposed to 0 or 100 μM $CdCl_2$ for another 12 h. Afterwards, seedling roots were stained with Schiff's reagent (**a**) or Evans blue (**b**), and the root tips were immediately photographed under a light microscope. *Bars* 1 mm. LOX activity and TBARS content (**c**) were also determined in the root tissues of alfalfa seedlings. Values are the mean ± SE of three independent experiments. Within each set of experiments, *bars* denoted by the same *letter* did not differ significantly at $P < 0.05$ according to Duncan's multiple range test

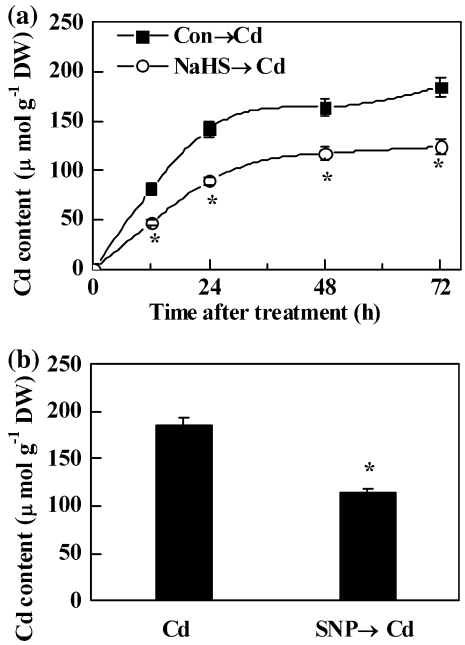

Fig. 4 Time-course analysis of the H_2S donor NaHS (**a**) and effect of SNP treatments (**b**) on Cd concentration in root tissues of alfalfa plants upon Cd exposure. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution containing 100 μM NaHS, or 100 μM SNP for 6 h, and then followed by another 72 h incubation in 100 μM $CdCl_2$. Sample without chemical pretreatment was the control (Con). Values are the mean ± SE of three independent experiments. *Asterisks* indicate that mean values are significantly different at $P < 0.05$ according to Duncan's multiple range test

accumulation of Cd uptake was observed (Fig. 4a). By contrast, the application of NaHS slowed down the accumulation rate of Cd. For example, at 24 and 72 h of treatment, the Cd content in NaHS-pretreated root tissues was 37 and 33 % lower than that of Cd-stressed alone sample. Comparatively, SNP also brought about a prominent reduction (38 % approximately) in Cd content at 72 h of treatment (Fig. 4b).

Seedling growth inhibition were attenuated by NaHS and SNP, but reversed by cPTIO

Exposure of plants to heavy metal ions often results in growth inhibition. In the following test, the FW of 10 alfalfa seedling plants upon Cd stress for 72 h decreased by 34 % as compared with the chemical-free control sample (Fig. 5). In contrast, a significant improvement of seed growth inhibition was observed when NaHS or SNP was applied, respectively ($P < 0.05$), and the cytoprotective of NaHS was reversed significantly by the addition of cPTIO. In addition, no additive effects of NaHS and SNP were observed. Only a slight but not obvious decrease in seedling growth occurred in the plants pretreated with cPTIO

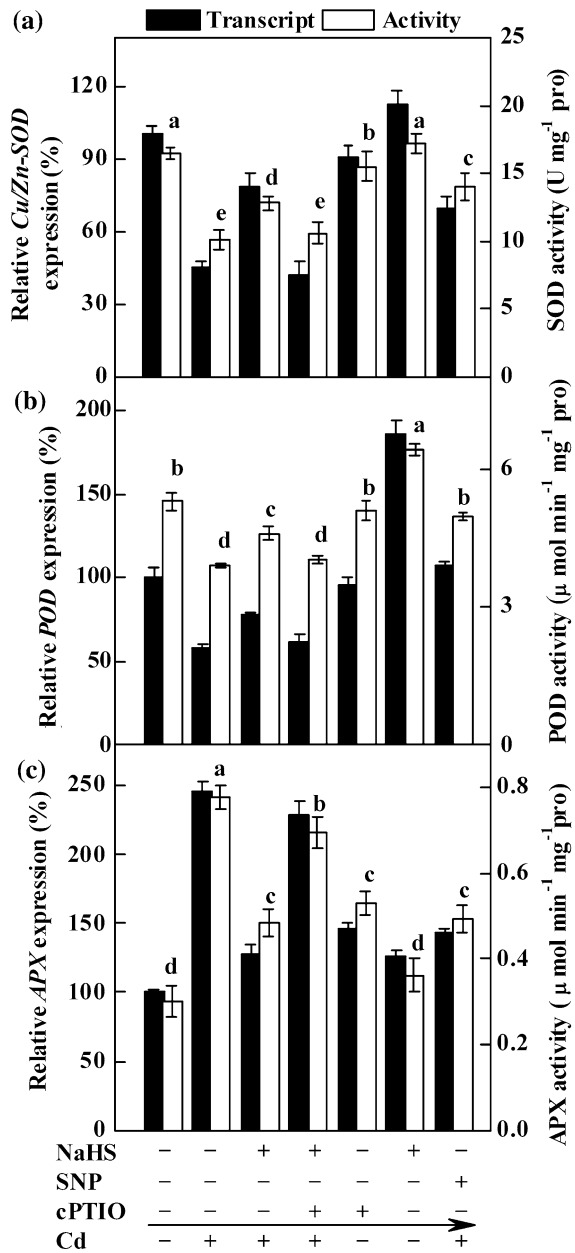

Fig. 6 Effects of pretreatment with NaHS, SNP and cPTIO on the expression and activities of superoxide dismutase (SOD, **a**), guaiacol peroxidase (POD, **b**) and ascorbate peroxidase (APX, **c**) in alfalfa seedling roots upon cadmium exposure. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution containing 100 μM NaHS, 100 μM SNP, 200 μM cPTIO alone, or the combination treatments for 6 h, and then exposed to 0 or 100 μM $CdCl_2$ for another 12 h. The corresponding mRNA expression was analysed by real-time quantitative RT-PCR. The relative abundance of the corresponding genes is presented as values relative to the control sample. Meanwhile, the corresponding enzyme activities were determined. Values are the mean ± SE of three independent experiments. *Bars* denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test

alone, although the significant increases appeared in NaHS or SNP-treated alone samples.

Changes of SOD, POD and APX activities and transcripts

Analysis of two antioxidant enzymes revealed that activities of SOD and POD in alfalfa seedling roots decreased after 12 h of Cd stress, being 39 and 26 % lower than the chemical-free control samples,

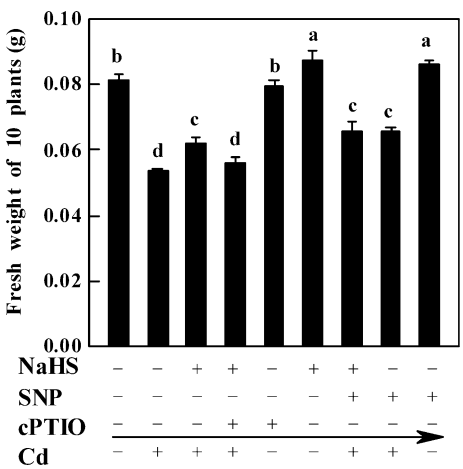

Fig. 5 Effects of pretreatment with NaHS, SNP and cPTIO on the Cd-induced inhibition of 10 alfalfa seedling growth for 72 h. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution containing 100 μM NaHS, 100 μM SNP, 200 μM cPTIO alone, or the combination treatments for 6 h, and then exposed to 100 μM $CdCl_2$ for another 72 h. Sample without chemicals was the control (Con). Values are means ± SE of three different experiments with four replicated measurements. *Bars* denoted by the same *letter* did not differ significantly at $P < 0.05$ according to Duncan's multiple range test

respectively (Fig. 6a, b). Whereas, when NaHS or SNP was applied with Cd separately, it significantly alleviated the effect of Cd on SOD and POD activities, being 27 and 18 %, 38 and 29 % higher, respectively, than those with the Cd treatment alone. Similar increased tendency was observed in the transcript levels of *Cu/Zn–SOD* and *POD*. Pretreatment with NaHS alone led to significant increases in SOD and POD activities as well as the corresponding transcripts. Comparatively, the treatment in combined

with cPTIO produced an obvious decrease tendency in the mRNA and activity levels compared with the values obtained when NaHS was added together with Cd. In addition, cPTIO alone only produced the down-regulation of *Cu/Zn–SOD* mRNA or SOD activity, and no significant difference was observed in *POD* transcripts and the corresponding activity.

Figure 6c showed that mRNA and activity of APX increased after Cd exposure for 12 h, being 146 and 157 % higher, than those of the Cd-free control plants. The increased activity and transcript of APX caused by Cd stress were notably reduced by the addition of NaHS and SNP, separately, and the responses of NaHS could be blocked by the addition of cPTIO. Additionally, cPTIO and NaHS alone resulted in the up-regulation of *APX* transcripts, and APX activity was only increased significantly in cPTIO treatment.

NaHS- and SNP-triggered NO production were sensitive to cPTIO

Results of Fig. 7 showed that NO concentration determined by using Greiss reagent in alfalfa seedling roots, continuously increased during the 12 h treatment period upon Cd exposure, in comparison with the control samples. The pretreatment with NaHS or SNP for 6 h in culture solution enhanced the NO contents in the root tissues, both of which mimicked a physiological response triggered by Cd treatment at 12 h. However, the addition of Cd to the NaHS- or SNP-treated plants at the end of the pretreatment period resulted in slight increases in the 3 h of Cd treatment, followed by gradual decline in NO concentrations until 12 h. For example, after 12 h, the NO content in NaHS- and SNP-pretreated samples was 45 and 51 % lower than that of the Cd stressed alone sample.

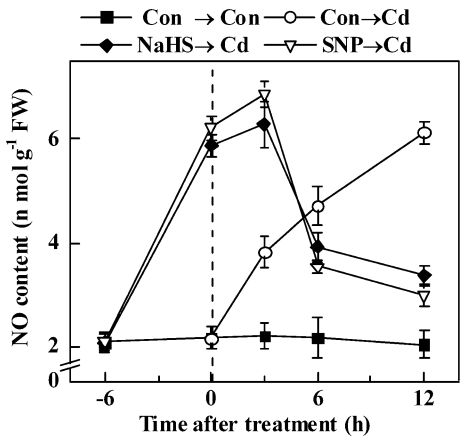

Fig. 7 Changes in endogenous concentrations of NO in alfalfa seedling roots under Cd stress. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution with or without 100 μM NaHS, or 100 μM SNP for pretreatment for 6 h. The seedlings were then grown for 12 h, either exposed to 100 μM $CdCl_2$ or under Cd-free control conditions (Con) for another 12 h. NO contents of alfalfa seedling roots were detected by using Greiss reagent at the indicated time points. Values are the mean ± SE of three independent experiments

To examine the results above, we further compared DAD-FM DA florescence in seedling root tips of alfalfa plans in the presence or absence of Cd exposure. DAF-FM DA is membrane permeable and fluoresces in the presence of oxidized NO (NO^+ and N_2O_3) and is used widely to monitor NO levels in plants (Chen et al. 2011; Van Ree et al. 2011). To verify the specificity of DAF-FM DA fluorescence for NO-derived compounds, we compared the fluorescence detected in the presence of cPTIO, an NO specific scavenger. As shown in Fig. 8b, increased DAF-FM DA fluorescence was discovered in alfalfa seedling roots upon Cd stress treatment for 3 h, in comparison with the control sample (Besson-Bard et al. 2009; De Michele et al. 2009). Similarly to the previous results (Fig. 7), either NaHS or SNP application was able to induce DAF-FM DA fluorescence, regardless of the Cd applied or the different detection points (Fig. 8a, b). Figure 8c and d also quantifies the fluorescence levels and revealed that cPTIO remarkably reduces, at least partially, the DAF-FM DA fluorescence, consistent with the interpretation that some, if not most, of the DAF-FM DA fluorescence is caused by NO. We also observed that the combination of NaHS plus Cd treatment led to less NO fluorescence compared to the NaHS pretreatment followed by Cd stress (especially) or Cd treatment alone samples (Online Resource, Fig. S2). Combining with former results (Online Resource, Fig. S1), it was further suggested the possible chelation or direct reaction of Cd by NaHS.

Discussion

Recent studies have demonstrated that H_2S is produced in trace amounts by various animal tissues and organisms (Baskar and Bian 2011). It was also produced in plant leaf tissues in response to L-cysteine

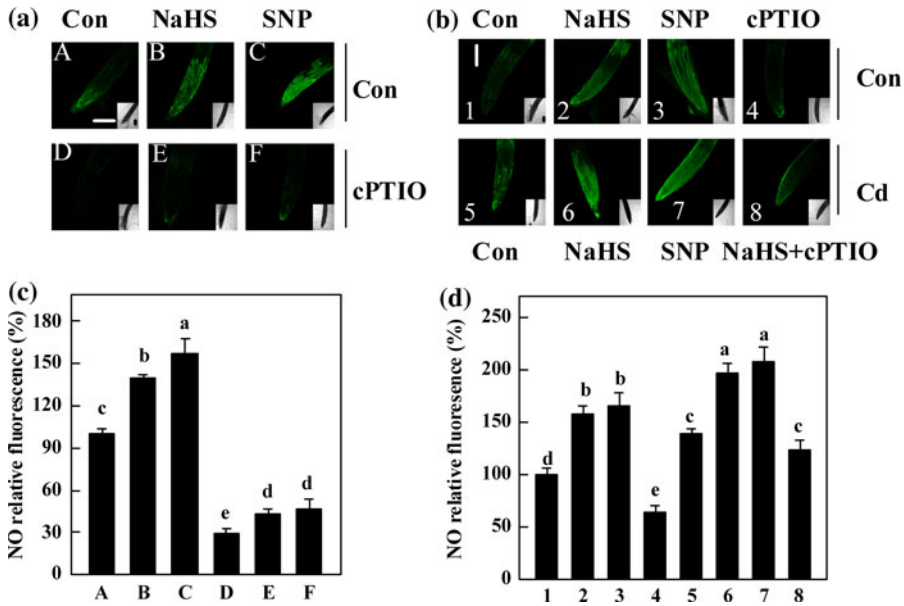

Fig. 8 Effects of NaHS, SNP and cPTIO on NO production in alfalfa seedling roots with or without Cd exposure. Seedling roots were incubated in quarter-strength Hoagland's solution for 5 days, then transferred to the same solution containing 100 μM NaHS, 100 μM SNP, 200 μM cPTIO alone, or the combination treatments for 6 h (**a**), or then exposed to 0 or 100 μM $CdCl_2$ for another 3 h (**b**). Sample without chemicals was the control (Con). NO production in alfalfa seedling root tips was detected by fluorescence probe DAF-FM DA, after different treatments under fluorescence and bright field microscopy (TCS-SP2 confocal laser scanning microscope; Leica Lasertechnik GmbH). Photographs showing the identical results, as well as the corresponding bright-field images, were taken. *Bars* 200 μm. **c**, **d** Mean relative DAF-FM DA fluorescence densities corresponding to (**a**) and (**b**), respectively, were given, taking control (*lanes A* or *lane 1*) as 100 %. *Lines A–F* represent different treatments as follows: *A* Cd-free treatment; *B* NaHS treatment; *C* SNP treatment; *D* cPTIO treatment; *E* NaHS plus cPTIO treatment; *F* SNP plus cPTIO treatment (**a**, **c**). Additionally, *lines 1–8* also represent various treatments as follows: *1* Cd-free pretreatment followed by Cd-free treatment; *2* NaHS pretreatment followed by Cd-free treatment; *3* SNP pretreatment followed by Cd-free treatment; *4* cPTIO pretreatment followed by Cd-free treatment; *5* Cd-free pretreatment followed by Cd treatment; *6* NaHS pretreatment followed by Cd treatment; *7* SNP pretreatment followed by Cd treatment; *8* NaHS + cPTIO pretreatment followed by Cd treatment (**b**, **d**). Values are the mean ± SE of six independent experiments. *Bars* denoted by the same *letter* did not differ significantly at $P < 0.05$ according to Duncan's multiple range test

supplement (Sekiya et al. 1982) or sulfur dioxide fumigation conditions (Hällgren and Fredriksson 1982). Besides NO and carbon monoxide (CO), it is now known that H_2S is the third physiologically active molecule both in animals and plants with versatile functions (Calvert et al. 2010; García-Mata and Lamattina 2010). In this report, we present evidence of the beneficial effect of H_2S and NO on the alleviation of Cd toxicity. Additionally, NO might be a component of the H_2S-indued cytoprotective roles against Cd stress, remarkably similar to those found in animals (Ondrias et al. 2008).

H_2S and NO alleviate Cd toxicity

It has been shown that the exogenous H_2S, in the form of the H_2S-releasing compound, NaHS, mitigates syndromes associated with boron toxicity (Wang et al. 2010), aluminum toxicity (Zhang et al. 2010b), and copper stress (Zhang et al. 2008). The ameliorating effects of NaHS on plants suffering from above abiotic stresses have been attributed to the up-regulation of antioxidative enzymes, thus resulting in the protection against oxidative damage. In this experiment, the dose-dependent ameliorating effects of NaHS, an H_2S donor, on lipid peroxidation shown as the TBARS content in Cd-stressed alfalfa seedling roots were observed, which mimicked the cytoprotective role of SNP (Fig. 1a). These results were consistent with the observations in animals (Kimura and Kimura 2004; Yonezawa et al. 2007). For example, NaHS could strongly protect gastric mucosal epithelial cells against H_2O_2-induced oxidative damage through the stimulation of mitogen-activated protein

kinase (MAPK) signaling pathways (Yonezawa et al. 2007). Cytoprotective roles of NaHS have been also found in plants upon salinity (Wang et al. 2012), osmotic and drought stresses (Zhang et al. 2009b, 2010a; Jin et al. 2011). We also deduced that the inhibition of endogenous H_2S via the addition of HT or PAG might be related to the blocking response of the alleviation of Cd-induced lipid peroxidation caused by NaHS (Fig. 1c).

It was well known that NaHS dissolves in water and dissociates to produce Na^+ and HS^-, which associates with H^+ to form H_2S. In the subsequent test, other sulphur-containing compounds such as S^{2-}, HSO_4^-, SO_4^{2-}, HSO_3^-, SO_3^{2-} and Na^+ were used as the controls of NaHS. And, we found that these compounds failed to exhibit the above cytoprotective response triggered by NaHS (Fig. 2), suggesting that NaHS-mediated response is H_2S- or HS^--dependent. Such ameliorating effects were confirmed by the histochemical staining for the detection of peroxidation of lipids and injury of plasma membrane integrity in root apexes (Fig. 3a, b), and LOX activities (Fig. 3c).

As expected (Laspina et al. 2005; Xu et al. 2010, 2011), analogous cytoprotective responses (Figs. 1, 2, 3) were observed when SNP was used as an NO donor, compared with no significant changes trigged by its control chemicals (Fig. 2). Our results further illustrated that, besides exhibiting an obvious reversing effect on the plant growth inhibition caused by Cd (Fig. 5), NaHS and SNP pretreatments were able to suppress Cd uptake (Fig. 4). A similar role for SNP treatment was reported previously (Xu et al. 2010). However, the mechanism behind the declining effects of Cd uptake induced by NaHS or SNP was still unclear.

Pharmacological and genetic evidence indicated that the activation of antioxidative enzymes are beneficial for plant growth upon Cd stress, because adequate capacity of antioxidative enzymes and other antioxidant metabolites may help in the removal of excess reactive oxygen species (ROS) and inhibit lipid peroxidation (Lee et al. 2007; Sharma and Dietz 2009). Previous studies have shown that NaHS could activate or up-regulate the antioxidant enzymatic activities and/or the corresponding transcripts in salinity-stressed plants (Wang et al. 2012). Meanwhile, thiol redox modification modulated by H_2S was observed in *Spinacia oleracea* and *Arabidopsis thaliana* under normal growth conditions (Riemenschneider et al. 2005; Chen et al. 2011). In agreement with these discoveries, our experimental results showed that upon 100 μM Cd stress, the induction and activation of *Cu/Zn–SOD* and *POD* transcripts, and total SOD and POD activities (Fig. 6a, b), were observed in NaHS- or SNP-pretreated samples. These results match the partially preventing oxidative injury to membranes, as evaluated by the TBARS contents and LOX activities (Fig. 3c), in alfalfa seedling roots. Such effects were confirmed by the histochemical staining for the detection of peroxidation of lipid and the injury of plasma membrane integrity in root apexes (Fig. 3a, b).

By contrast, the Cd-induced increases in the transcript level and the total activity of APX (Fig. 6c), were consistent with previous results in rice (Chao et al. 2010) and alfalfa seedlings (Fu et al. 2011), although the opposite effect was observed in pea plants subjected to higher doses of Cd treatment (Hana et al. 2008). Different responses of APX and POD were also discovered in maize leaf (Krantev et al. 2008), suggesting that the induction or suppression of antioxidant enzyme activities in heavy metal-stressed plants might be dependent on the concentration of heavy metals and exposure times, and even different plant species (Sharma and Dietz 2009). Interestingly, above responses conferred by Cd stress were significantly reversed by the pretreatment with NaHS or SNP, respectively (Fig. 6c), which was consistent with the alleviation of Cd toxicity (Figs. 1, 4, 5). Above phenomena are difficult to reconcile with the fact that, on the contrary, *des1* mutants of Arabidopsis, which should have reduced levels of H_2S production, have an increased tolerance to Cd exposure. Meanwhile, the APX activity was shown to qualitatively increase in gel band intensity over wild-type levels in both two *des1* mutants (Álvarez et al. 2010). To account for these discrepancies, it might be suggested that, the deleterious effects versus the beneficial effects of H_2S might be related to the dynamic of its synthesis, that is, the level of produced H_2S but also the subcellular site of its production and the rate of its diffusion. Similar phenomenon of NO and corresponding discussion were previously illustrated in plant biology (Besson-Bard et al. 2009; Arasimowicz-Jelonek et al. 2011). Furthermore, it was postulated that H_2S- or NO-activated antioxidative enzymes protected membrane lipids against peroxidation by blocking LOX activity (Fig. 3; Zhang et al. 2008, 2010b). It was possible that

H_2S or NO prevents ROS-mediated cytotoxicity of the plasma membrane of root tissues (Fig. 3), thus slowing Cd^{2+} permeability into the seedling cells and ameliorating the Cd-induced seedling growth inhibition.

A possible link between H_2S and NO in plant responses against Cd stress

In animals, it is interesting that many of the novel properties of H_2S have strong analogies or are mediated with the well-established biological activities elicited by NO, another gaseous molecule (Mancuso et al. 2010; Yong et al. 2011). For example, lower concentrations of H_2S greatly enhanced the smooth muscle relaxation induced by NO in the thoracic aorta, suggesting that H_2S may regulate smooth muscle tone in synergy with NO (Hosoki et al. 1997). Jeong et al. (2006) further discovered that although NaHS by itself showed no effect on the NO production, it augmented IL-1 β-induced NO production in a concentration-dependent fashion. Most importantly, this effect was apparently associated with the increased inducible NO synthase (iNOS) expression. Thus, it was speculated that H_2S and NO in animals could form a unique interactive network (Pae et al. 2009). Interestingly, NO can regulate endogenous production of H_2S in cultured vascular smooth muscle cells by increasing *CSE* and *CBS* gene expression (Zhao et al. 2001). Unlike in animals, plant NO synthase (NOS) enzymes or genes have not yet been cloned. For example, Arabidopsis *Nitric Oxide Associated 1* (*NOA1*, previously named *Atnos1*) has a primary role in chloroplast function and that its effects on the accumulation of NO are likely to be indirect (Van Ree et al. 2011). Thus, nitrate reductase (NR) is one proposed enzymatic source of NO in plants, and NO has been confirmed to act as a messenger in plant developmental processes, stress tolerance, and defence responses (Delledonne 2005; Van Ree et al. 2011).

Similar to the H_2S responses, NO donor SNP exhibited the similar cytoprotective roles against Cd stress in alfalfa seedlings (Figs. 1, 2, 3, 4, 5, 6). These results were consistent with the observations, showing that exogenously supplied NO could attenuate heavy metal toxicity, probably due to its ability to act as antioxidant scavenging ROS (Hsu and Kao 2004; Xu et al. 2010; Arasimowicz-Jelonek et al. 2011) or by modulating Ca^{2+} channels and transporters which might be involved in the corresponding signaling cascade (Besson-Bard et al. 2009). In further investigations, by using LSCM combined with Greiss reagent method, it was proved that NO production, triggered by NaHS (Figs. 7, 8), might serve as a signal for the alleviation of Cd toxicity, because the alleviation of Cd-induced oxidative damage (Figs. 1, 3), seedling growth inhibition (Fig. 5), antioxidant enzyme activities and corresponding transcripts (Fig. 6) could be blocked by the application of cPTIO, the specific scavenger of NO (Fig. 8). Thus, decreases of NO production were consistent with the exaggeration syndromes associated with Cd toxicity. We further suggested that endogenous NO production, at least partially, serve as a gaseous signal in H_2S-induced cytoprotective roles, although the possibility of NO acting as the upstream component of H_2S signaling could not be easily ruled out. Certainly, the potential source of NO triggered by NaHS treatment should be investigated. Unlike those in animals (Hosoki et al. 1997; Jeong et al. 2006), however, no synergistic effects of H_2S plus NO were observed in lipid peroxidation and LOX activities (Fig. 3), as well as in seedling growth parameters (Fig. 5).

Collectively, we found that both H_2S and NO attenuated Cd toxicity in alfalfa seedlings by the alleviation of antioxidative damage. Most importantly, the seedling growth inhibition and Cd accumulation in seedling roots were also relieved. Subsequent results discovered that above H_2S-mediated responses were, at least partially, mediated by NO, another gaseous reactive molecule with a pivotal signaling role in many developmental and response processes (Durner and Klessig 1999; Besson-Bard et al. 2008). Further genetic approaches to manipulate endogenous production of H_2S and NO may help to decisively establish physiological importance of H_2S and NO in the plant responses against abiotic stresses.

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