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Haem Oxygenase-1 is Involved in Hydrogen Sulfide-induced Cucumber Adventitious Root Formation

Yu-Ting Lin · Mei-Yue Li · Wei-Ti Cui · Wei Lu · Wen-Biao Shen

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Abstract Results from our previous study suggested that haem oxygenase-1/carbon monoxide (HO-1/CO) acts as a downstream signal system in the auxin-induced pathway leading to cucumber (*Cucumis sativus*) adventitious root formation. The objective of this study was to test whether HO-1 is also involved in hydrogen sulfide (H_2S)-induced adventitious root formation. Cucumber explants were treated with HO-1 inducer haemin and H_2S donor sodium hydrosulfide (NaHS) in combination with the specific inhibitor of HO-1 zinc protoporphyrin IX (ZnPPIX), and their effects on cucumber adventitious root development in IAA-depleted explants were compared. The results showed that similar to inducible responses of haemin, NaHS brought about the induction of cucumber HO-1 transcripts (*CsHO-1*) and its protein levels, and thereafter adventitious root formation. A further experiment verified that H_2S or HS^- rather than other sulfur-containing components derived from NaHS was ascribed to the stimulation response. The inducible effect is specific for CsHO-1 because ZnPPIX significantly suppressed the above responses, and the inhibitory effects were reversed partially when 30% CO-saturated aqueous solution was added. Molecular evidence further suggested that the NaHS-triggered upregulation of target genes responsible for HO-1/CO-induced adventitious root formation, including *CsDNAJ-1* and *CsCDPK1/5*, was inhibited significantly by ZnPPIX. These decreases were reversed obviously by the addition of CO aqueous solution. However, hypotaurine (HT), the H_2S scavenger, could not influence the haemin- and CO-induced adventitious rooting in IAA-depleted cucumber explants. Together, the above results suggested that HO-1 was involved in H_2S-induced cucumber adventitious root formation.

Keywords Adventitious rooting · Cucumber · Haem oxygenase-1 · Hydrogen sulfide · Molecular mechanism

Y.-T. Lin · M.-Y. Li · W.-T. Cui · W. Lu · W.-B. Shen
College of Life Sciences, Cooperative Demonstration Laboratory of Centrifuge Technique, Nanjing Agricultural University and Beckman Coulter Ltd. Co., Nanjing Agricultural University, Nanjing 210095, China

W.-B. Shen (✉)
College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, Jiangsu Province, People's Republic of China
e-mail: wbshenh@njau.edu.cn

Introduction

Nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) together make up a family of biologically active gases, the so-called gaseous triumvirate in animals (Li and others 2009a). It was also confirmed that cystathionine β-synthase (CBS) and cystathinine γ-lyase (CSE) are two H_2S synthesis enzymes in mammalian tissues (Wang 2003; Lefer 2007). Further results illustrated that CSE acts as a plant L-cysteine-specific desulfhydrase (L-CDES; EC 4.4.1.1) responsible for H_2S release (Papenbrock and others 2007). Similar to animals, plenty of recent evidence suggests that H_2S regulates many aspects of plant growth and development, from seed germination (Zhang and others 2008; Wang and others 2012) to the induction of adventitious root development (Zhang and others 2009a). It has also been implicated in plant responses to abiotic stresses such as salinity, osmotic stress, and drought (Zhang and others 2009b, 2010a; Wang and others 2012), boron and aluminum toxicity (Wang and others 2010; Zhang and others 2010b), and heavy-metal exposure (Zhang and others 2008). However, despite the critical roles of H_2S throughout the plant life cycle and

responses against adverse environments, the molecular mechanism and signal transduction underlying its physiological roles are still poorly understood.

Haem oxygenase (HO; EC 1.14.99.3) catalyzes the oxidative conversion of haem to biliverdin IXα (BV), a well-known antioxidant, with the concomitant release of CO and iron (Fe^{2+}), in animals, plants, and other organisms (Maines 1997; Dulak and Józkowicz 2003; Kikuchi and others 2005; Shekhawat and Verma 2010; Shekhawat and others 2011). BV is subsequently reduced by cytosolic biliverdin reductase to form the potent antioxidant bilirubin (BR) (Baraňano and others 2002). Until now, there were three HO isozymes in mammals: the inducible form HO-1, the constitutively expressed HO-2, and HO-3 isozyme with a very low activity level. In mammals, HO-1/CO controls many diverse functions, including haem degradation and the antioxidant machinery (Ryter and others 2006). In addition to its involvement in phytochrome chromophore biosynthesis (Muramoto and others 1999; Davis and others 2001), plant HO-1 has been shown to have a role in adaptive plant responses against ultraviolet-B irradiation (Yannarelli and others 2006), salinity, and osmotic stresses (Liu and others 2010; Xie and others 2011a, b), as well as in growth and development processes, including lateral root formation (Cao and others 2007) and programmed cell death in aleurone layers (Wu and others 2011). Thus, it was suggested that HO functions in various ways according to the needs of individual species (Kikuchi and others 2005; Shekhawat and others 2011).

Adventitious rooting is part of postembryonic root development and involves the development of a meristematic tissue after removal of the primary root system. Evidence has confirmed that auxin plays a central role in the induction of adventitious root formation and may interact with other endogenous factors or environmental stimuli. For example, calcium and calcium-dependent protein kinases (CDPK) are downstream messengers in the signaling pathway triggered by auxin and NO to promote adventitious rooting (Lanteri and others 2006). Our previous report found that HO-1/CO presented a new signal system with significant impact on auxin-induced adventitious root development by the modulation of one DNAJ-like gene (*CsDNAJ-1*) and two CDPK genes (*CsCDPK1* and *CsCDPK5*; Xuan and others 2008). In view of the fact that H_2S was able to induce adventitious root development involving auxin signaling (Zhang and others 2009b), the possibility of cross talk between endogenous HO-1/CO and H_2S during adventitious rooting still remains to be examined.

The objective of this work was to investigate the relationship between HO-1- and H_2S-induced cucumber adventitious root formation. IAA-depleted cucumber explants were treated with the H_2S donor NaHS, HO-1 inducer haemin, and the specific HO-1 inhibitor zinc protoporphyrin IX (ZnPPIX) with or without the by-products of HO-1, such as CO, BR, and Fe^{2+}. Phenotypes of adventitious root development, the expression of cucumber HO-1 (*CsHO-1*; Li and others 2011) and its protein level, as well as the target genes of HO-1/CO-induced adventitious root formation, including *CsDNAJ-1* and *CsCDPK1/5* (Xuan and others 2008), were determined and compared. Additionally, effects of hypotaurine (HT), the H_2S scavenger (García-Mata and Lamattina 2010), on NaHS- and CO-induced cucumber adventitious rooting were investigated.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Sodium hydrosulfide (NaHS) and haemin were used as the H_2S or HS^- donor and HO-1 inducer, respectively (Xuan and others 2008; Wang and others 2010). Naphthaleneacetic acid (NAA) was used at the indicated concentrations. N-1-naphthylphthalamic acid (NPA) from Chem Service (West Chester, PA, USA), was regarded as the auxin transport inhibitor at 10 μM (Xuan and others 2008). Zinc protoporphyrin IX (ZnPPIX), a specific inhibitor of HO-1 (Xuan and others 2008; Cao and others 2011), was used at 10 μM. Hypotaurine (HT), which reacts directly with sulfide to form thiotaurine (ThT), was used at 200 μM (Ortega and others 2008; García-Mata and Lamattina 2010). Both bilirubin (BR) and $FeSO_4 \cdot 7H_2O$ (Fe^{2+}), were used at 10 μM.

CO Aqueous Solution Preparation

The preparation of CO aqueous solution was carried out according to the method described in our previous report (Xuan and others 2008). The saturated stock solution (100% saturation) was diluted immediately with distilled water to the concentration required with a maximal inducible response (30% saturation [v/v]).

Plant Material and Growth Conditions

Cucumber seeds (*Cucumis sativus* 'Lufeng') were kindly supplied by Jiangsu Agricultural Institutes, Jiangsu Province, China. Selected identical seeds were germinated in Petri dishes on filter papers imbibed in distilled water, then transferred to an illuminating incubator and maintained at 25 ± 1°C for 5 days with a 14-h photoperiod at 200 μmol m^{-2} s^{-1} intensity. Cucumber seedlings were used either intact (IAA nondepleted) or decapitated by excising the apical bud immediately above the cotyledons and incubated in the presence of 10 μM NPA

(IAA-depleted) for 48 h, before removing the primary root. Cucumber explants were then maintained under the same conditions of temperature and photoperiod for another 90 h or the indicated time points in the presence of different media as indicated.

Explant Treatments

After primary roots were removed, every eight cucumber explants were put into a Petri dish containing 10 ml of distilled water, varying concentrations of NaHS or NAA, 10 μM haemin, 30% CO aqueous solution, 10 μM ZnP-PIX, 10 μM BR, 10 μM $FeSO_4 \cdot 7H_2O$, or 200 μM HT, either alone or in combination, and kept at 25 ± 1°C for 90 h or different time periods according to the experimental design. Previous studies showed that the concentrations and the time of treatments with these chemicals are suitable for investigating the role of HO-1/CO in root developmental signaling (Pagnussat and others 2002, 2003, 2004; Xuan and others 2008; de Montaigu and others 2010; Cao and others 2011). Finally, excised cucumber hypocotyls (5-mm-long segments of the hypocotyl base, where the adventitious root develops; Lanteri and others 2006) were used for the following determination.

Western Blot Analysis for CsHO-1

Rabbit polyclonal antibody was made against mature cucumber HO-1 expression in *E. coli* (Li and others 2011). Sixty micrograms of protein from homogenates was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% acrylamide-resolving gel (Mini-PROTEAN® II System, Bio-Rad, Hercules, CA, USA). Separated proteins were then transferred to PVDF membranes and nonspecific binding of antibodies was blocked with 5% nonfat dried milk in PBS (pH 7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibody raised against cucumber HO-1 (CsHO-1) diluted 1:3,000 in PBS buffer. Immune complexes were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. The color was developed with a solution containing 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

Semiquantitative RT-PCR Analysis

Total RNA was isolated from 100 mg (fresh weight) of excised cucumber hypocotyls by grinding with mortar and pestle in liquid nitrogen until a fine powder appeared and using Trizol reagent (Invitrogen, Carlsbad, CA) 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 AMV reverse transcriptase XL (TaKaRa-Bio, Shiga, Japan) and 2.5 μM random primer. PCR reactions were performed using 2 μl of a twofold dilution of the cDNA, 10 pmol of each oligonucleotide primer, and 1 U of Taq polymerase (TaKaRa-Bio) in a 20-μl reaction volume.

The cDNA was amplified using the following primers: for *CsHO-1* (accession No. HQ198046.1), forward 5′-GGA GTCACCTATGCTCGTTA-3′ and reverse 5′-CTTTCGC CCAATCATTCTAC-3′, amplifying a 118-bp fragment; and for *CsActin* (accession No. AB010922.1), forward 5′-AGATGACGCAGATAATGTTT-3′ and reverse 5′-AT-CACCAGAATCCAGCAC-3′, amplifying a 119-bp fragment. To standardize the results, the relative abundance of *CsActin* was determined and used as the internal standard.

The cycle numbers of the PCR were adjusted for each gene to obtain visible bands on agarose gels. Aliquots of the PCR reactions were loaded on 1.5% agarose gels with the use of ethidium bromide (EB). Specific amplification products of the expected size were observed and their identities were confirmed by sequencing.

Real-time RT-PCR Analysis

Real-time quantification RT-PCR reactions were performed using a Mastercycler® ep *realplex* real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR® *Premix Ex Taq*™ (TaKaRa-Bio) according to the manufacturer's instructions. The cDNA was amplified using the following primers: for *CsActin* (accession No. AB010922.1), forward 5′-AGATGACGCAGATAATGTTT-3′ and reverse 5′-ATC ACCAGAATCCAGCAC-3′; for *CsHO-1* (accession No. HQ198046.1), forward 5′-GGAGTCACCTATGCTCGTT A-3′ and reverse 5′-CTTTCGCCCAATCATTCTAC-3′; for *CsDNAJ-1* (accession No. X67695), forward 5′-CGACACT GTTACTGGGGACA-3′ and reverse 5′-GACGAGAGACA AGGTATGCT-3′; for *CsCDPK1* (accession No. AJ312239), forward 5′-GTAAGACCATCCCCAAG-3′ and reverse 5′-CTCTCCACCCTCACAAA-3′; and for *CsCDPK5* (accession No. AY027885), forward 5′-TTCTGGCTCGTCC CTTTTC-3′ and reverse 5′-CCTGTTTCGTTTCCTTGTG-3′. Relative expression levels were presented as values relative to that of the corresponding control sample at the indicated time, after normalization to *CsActin* transcript levels.

Data Analysis

Where indicated, results were expressed as the mean value ± SE of at least three independent experiments ($n = 16$). Statistical analysis was performed using SPSS 8.0 software (SPSS, Inc., Chicago, IL, USA). For statistical

analysis, Duncan's multiple-range test ($p < 0.05$) was chosen.

Results

IAA Depletion-induced Inhibition of Adventitious Root Formation is Reversed by H_2S and Haemin

As expected, we observed that in comparison with the nondepleted treatment, the application of an inhibitor of basipetal auxin efflux, naphthylphthalamic acid (NPA; 10 μM, IAA depletion), was able to prevent adventitious root formation (Fig. 1a). To investigate the possible role of H_2S in signal transduction leading to adventitious root formation, we added the H_2S donor sodium hydrosulfide (NaHS) to IAA-depleted cucumber explants and checked the effect on the NPA-induced process. Two parameters, the number and the length of adventitious roots per explant, were measured. As shown in Fig. 1, NaHS at concentrations lower than 1 μM had no apparent effect on adventitious root formation, whereas a concentration-dependent restoration was observed when NaHS concentration was at least 1 μM. The promotion of adventitious root formation was maximal at 10 μM NaHS ($p < 0.05$), whereas a higher concentration of the donor was less effective. As was reported previously (Xuan and others 2008), the HO-1 inducer haemin at a concentration of 10 μM displayed the maximal inducible effect on adventitious root development (some data not shown).

To verify the specific role of H_2S in the restoration of adventitious root formation from IAA depletion-induced adventitious root inhibition in cucumber, 10 μM Na_2S, Na_2SO_4, $NaHSO_4$, Na_2SO_3, and $NaHSO_3$ were used as the controls for Na^+- and sulfur-containing compounds. However, we observed that these chemicals were unable to exhibit similar inducible responses (Fig. 2). Therefore, combined with the former results shown in Fig. 1, we illustrated that H_2S or HS^-, rather than other compounds directly or indirectly derived from the decomposing of NaHS, was responsible for the inducible effects of NaHS on the restoration of adventitious root formation in IAA-depleted cucumber explants.

Auxin and NaHS Rescue Adventitious Root Formation from IAA Depletion-induced Inhibition

As expected, 50 nM NAA was required for IAA-depleted cucumber explants to attain similar adventitious root development observed in nondepleted seedlings (Fig. 3). Interestingly, 10 nM NAA was able to obtain comparable numbers of adventitious roots when supplemented with

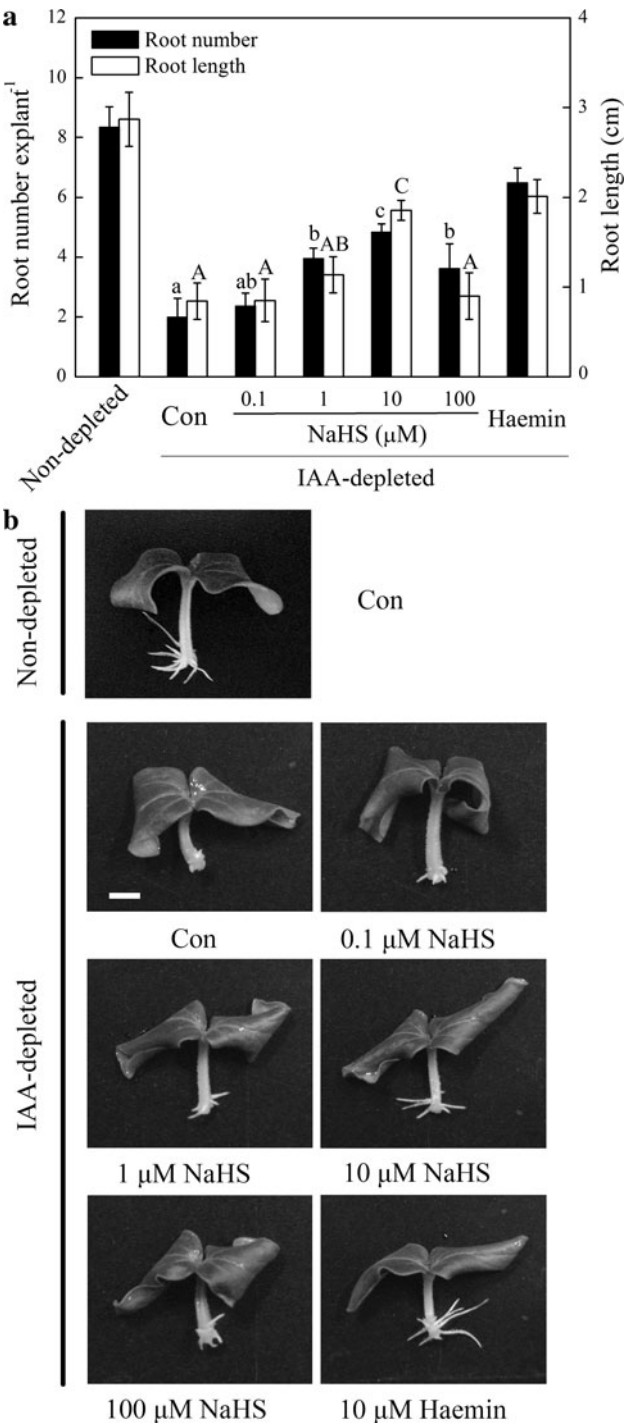

Fig. 1 Hydrogen sulfide (H_2S) donor sodium hydrosulfide (NaHS) and haemin alleviate the IAA depletion-induced inhibition of adventitious root development in cucumber. Explants with or without auxin depletion pretreatment were further incubated in water (Con), NaHS at the indicated concentrations, and 10 μM haemin for 90 h. **a** The IAA depletion-induced inhibition of adventitious root formation is reversed by H_2S and haemin. **b** Photographs were also taken. *Scale bar* 0.5 cm. Mean and SE were calculated from at least three independent experiments ($n = 16$). Within each set of experiments, bars with different letters were significantly different in comparison with water treatment (Con) at $p < 0.05$ according to Duncan's multiple-range test

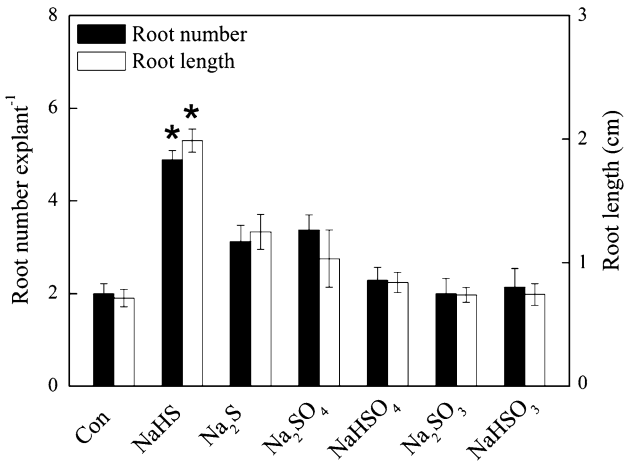

Fig. 2 Hydrogen sulfide (H_2S) or HS^-, but not other compounds derived from sodium hydrosulfide (NaHS), alleviate IAA depletion-induced adventitious rooting inhibition in cucumber. Explants with auxin depletion pretreatment were further incubated with water (Con), 10 μM NaHS, 10 μM Na_2S, 10 μM Na_2SO_4, 10 μM $NaHSO_4$, 10 μM Na_2SO_3, or 10 μM $NaHSO_3$ for 90 h. Mean and SE were calculated from at least three independent experiments ($n = 16$). Within each set of experiments, *bars with asterisks* were significantly different in comparison with water treatment (Con) at $p < 0.05$ according to Duncan's multiple-range test

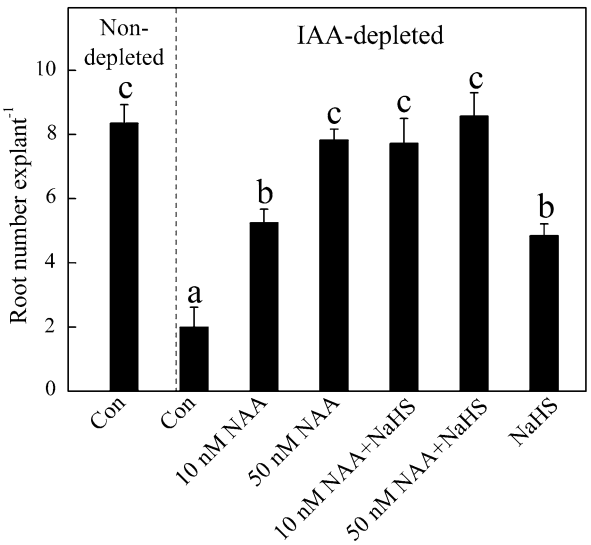

Fig. 3 The inhibition of adventitious root development in IAA-depleted cucumber seedlings was rescued by auxin and NaHS. Explants with or without auxin depletion pretreatment were further incubated with either water (Con), 10 or 50 nM NAA, 10 μM NaHS alone, or a combination treatment for 90 h. Mean and SE were calculated from at least three independent experiments ($n = 16$). *Bars* with different letters were significantly different in comparison with water treatment (Con) in the IAA-depleted condition at $p < 0.05$ according to Duncan's multiple-range test

10 μM NaHS. These results clearly indicated a possible interrelationship between IAA and H_2S during adventitious root formation.

Induction of *CsHO-1* Transcript and Its Protein in Response to H_2S

The reversing effect of NaHS on the inhibition of adventitious root formation in IAA-depleted cucumber explants led us to assess whether induction of HO-1 in cucumber is associated with the above response. In the following experiment, IAA-depleted cucumber explants were tested for HO accumulation using semiquantitative RT-PCR and Western blot analysis. The results in Fig. 4a show that NaHS induced *CsHO-1* expression in a time-dependent manner. Semiquantitative RT-PCR revealed that in comparison with the corresponding control samples, the treatment with NaHS for 6 and 12 h brought about the highest induction of *CsHO-1* expression (63.6 and 45.2%, respectively), and the level of *CsActin* was unaffected throughout the experimental periods. Furthermore, a close correlation was found between transcript levels and the amount of protein. For example, as shown in Fig. 4b, CsHO-1 protein increased 2.20- and 1.47-fold in cucumber explants treated with NaHS for 12 and 24 h, respectively. We also noticed that the enhancement of *CsHO-1* gene expression apparently preceded adventitious root formation.

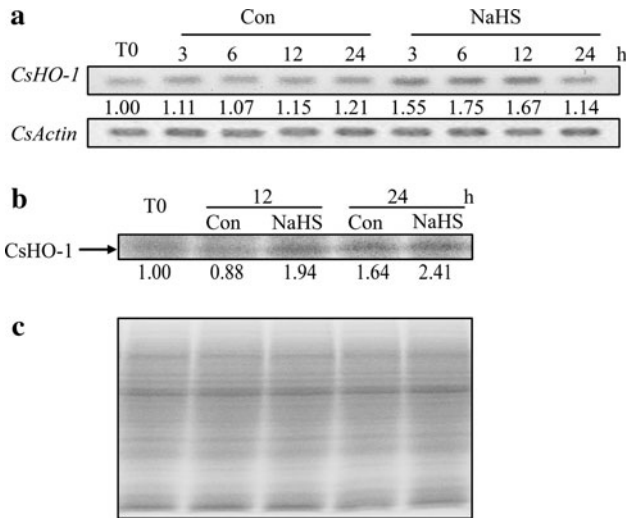

Fig. 4 Time-course changes of cucumber *CsHO-1* transcripts and protein levels in response to NaHS in IAA-depleted explants. Explants with auxin depletion pretreatment were incubated with water (Con) and 10 μM NaHS for 24 h. **a** *CsHO-1* transcript was then analyzed by semiquantitative RT-PCR. The number below the band indicates relative abundance of the corresponding gene with respect to the loading control *CsActin*. **b** CsHO-1 protein expression was determined by Western blot. The number below the band illustrates the relative abundance of the CsHO-1 protein compared with that of the T0 sample (100%). **c** Coomassie Brilliant Blue-stained gels were present to show that equal amounts of proteins were loaded. The blot was representative of three blots with a similar tendency

H_2S-triggered Responses Were Sensitive to the Specific Scavenger of HO-1 ZnPPIX and CO

To confirm the role of HO-1 in the NaHS-induced responses, we also adopted a pharmacological approach by using the specific inhibitor of HO-1, ZnPPIX. As expected, the addition of haemin brought about not only the induction of adventitious roots (Fig. 5), but also the enhancement of *CsHO-1* gene expression in cucumber explants (Fig. 6a). Furthermore, we discovered that the NaHS-induced adventitious root formation and *CsHO-1* upregulation were markedly reduced by treatment with ZnPPIX ($p < 0.05$) and were substantially recovered by 30% saturation of CO aqueous solution. By contrast, similar to the IAA-depleted cucumber explants (Con), there was no effect of Fe^{2+} or BR on adventitious root development when treated with NaHS plus ZnPPIX. These results suggested that changes in endogenous HO-1 are likely to be involved in NaHS-induced restoration of adventitious root development, and that CO, one of the by-products of HO-1, plays a crucial role in the process. We also confirmed that the application of CO aqueous solution alone remarkably increased adventitious rooting (Fig. 5) (Xuan and others 2008) as well as the induction of *CsHO-1* transcript (Fig. 6a). By contrast, no significant difference was observed after the addition of Fe^{2+} or BR in comparison with the control sample (Fig. 5). A slight but not significant decrease in adventitious root number (Fig. 5) and *CsHO-1* transcripts (Fig. 6a) was found in the ZnPPIX-treated IAA-depleted cucumber explants.

Expression Profiles of *CsDNAJ-1* and *CsCDPK1/5*

In a subsequent experiment, molecular evidence also showed that similar to the responses of haemin (Xuan and others 2008), NaHS and CO were able to induce higher expression of the *CsDNAJ-1* and *CsCDPK1/5* genes after 12 h of treatment (Fig. 6b), and these expressions were consistent with the number of adventitious roots observed after another 78 h of treatment (Figs. 1, 5). By contrast, the

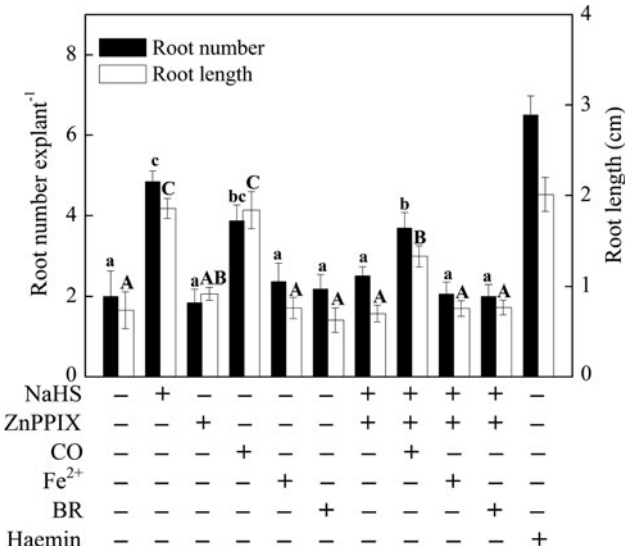

Fig. 5 Effects of ZnPPIX, CO, Fe^{2+}, BR, and haemin on NaHS-induced adventitious rooting. IAA-depleted explants were preincubated with water, the H_2S donor NaHS (10 μM), the HO-1 inducer haemin (10 μM), the specific HO-1 inhibitor ZnPPIX (10 μM), and three catalytic by-products of HO-1 [CO aqueous solution (30% saturation), Fe^{2+} ($FeSO_4 \cdot 7H_2O$, 10 μM), BR (10 μM) alone or in combination treatments] for 90 h. Then, adventitious root number and length were recorded. Mean and SE were calculated from at least three independent experiments ($n = 16$). Within each set of experiments, bars with different letters were significantly different in comparison with water treatment (Con) at $p < 0.05$ according to Duncan's multiple-range test

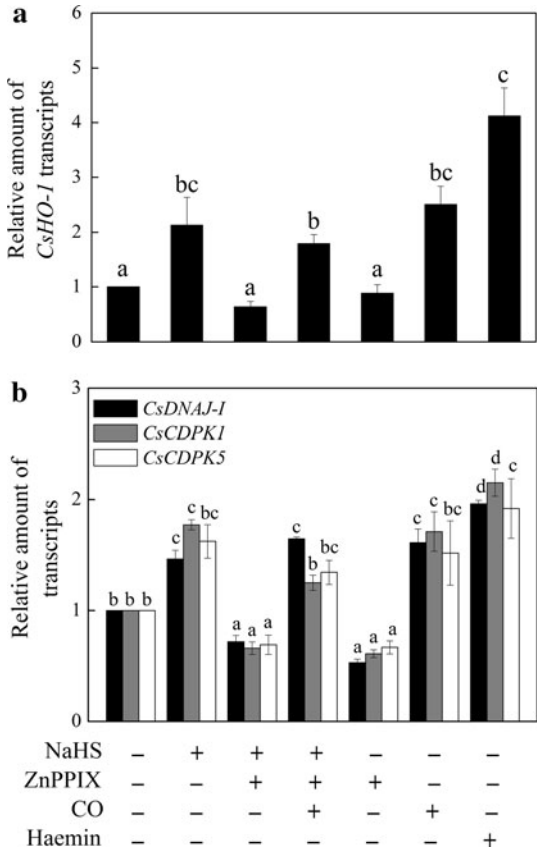

Fig. 6 Effects of NaHS, ZnPPIX, CO, and haemin on the expression profiles of *CsHO-1*, *CsDNAJ-1*, and *CsCDPK1/5*. IAA-depleted cucumber explants were preincubated with water, NaHS (10 μM), ZnPPIX (10 μM), CO aqueous solution (30% saturation), and haemin (10 μM) alone or in combination treatments for 6 h (**a**) and 12 h (**b**). Then, the corresponding gene expression was analyzed by real-time RT-PCR. The expression levels of the genes were presented as values relative to the control (water treatment). Within each set of experiments, *bars* with different letters were significantly different in comparison with the control at $p < 0.05$ according to Duncan's multiple-range test

NaHS-induced expression of these genes was prevented when HO-1 was inhibited by ZnPPIX. These findings provided preliminary evidence and suggested that endogenous HO-1 and its products modulated the expression of *CsDNAJ-1* and *CsCDPK1/5* genes, which were also involved in NaHS-induced adventitious root formation. Further results confirmed the restoration effects of CO aqueous solution on the ZnPPIX-induced inhibition of *CsDNAJ-1* and *CsCDPK1/5* genes, further strengthening the hypothesis that CO produced by CsHO-1 might be responsible for NaHS-induced adventitious root formation.

Hypotaurine (HT), the H_2S Scavenger, Does Not Influence Haemin- and CO-induced Adventitious Rooting

To further rule out the possibility that H_2S might be involved in HO-1-induced adventitious root formation, the effect of a H_2S scavenger, hypotaurine (HT), on haemin- and CO-induced response was analyzed. As shown in Fig. 7 (left), HT could not inhibit haemin- and CO-induced adventitious root formation in IAA-depleted cucumber explants. By contrast, HT was able to reverse adventitious root development triggered by 10 μM NaHS, which was consistent with the interpretation that NaHS-induced bioactivity is caused by H_2S. Additionally, no significant difference was observed in the sample treated with HT alone compared to the control explants in the IAA-depleted condition. On the other hand, in nondepleted cucumber explants, HT treatment alone brought about an obvious decrease of adventitious root formation, which was partially reversed by the addition of NaHS (Fig. 7, right). We also noticed that the application of NaHS alone, at the concentration of 10 μM, could bring about a slight but insignificant increase in adventitious rooting in nondepleted explants compared to the chemical-free control sample.

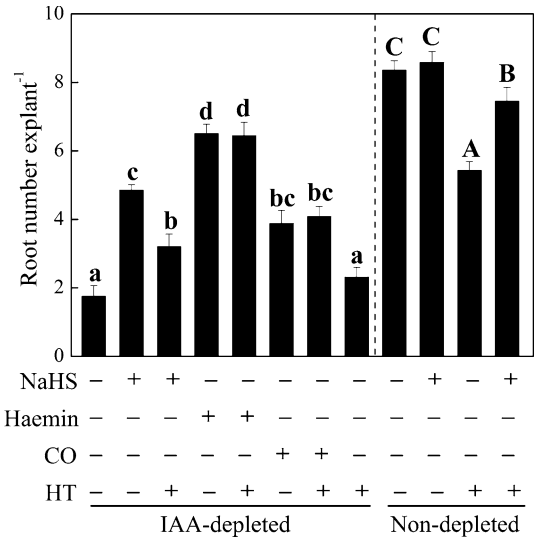

Fig. 7 Effects of hypotaurine (HT) on NaHS-, haemin-, or CO-induced adventitious rooting. Explants with or without auxin depletion pretreatment were further incubated in water, NaHS (10 μM), haemin (10 μM), CO (30% saturation), and HT (200 μM) alone or in combination of treatments for 90 h. Then, adventitious root numbers were recorded. Mean and SE were calculated from at least three independent experiments ($n = 16$). Within each set of experiments, *bars* with different letters were different significantly at $p < 0.05$ according to Duncan's multiple-range test

Discussion

Although H_2S has been known as a toxic gas for a long time and can also be endogenously generated in both animals and plants (Hällgren and Fredriksson 1982; Sekiya and others 1982; Wang 2003; García-Mata and Lamattina 2010), it was recently found that H_2S has important physiological functions in plants. For example, H_2S was implicated in stomatal aperture regulation (García-Mata and Lamattina 2010; Lisjak and others 2010) and abiotic stress responses (Zhang and others 2009b; Wang and others 2012). In this study, we confirmed that application of NaHS, the well-known H_2S and HS^- donor in animals and plants (Zhao and others 2001; Wang and others 2010), induced adventitious root development (Fig. 1; Li and others 2011), which was previously demonstrated in sweet potato, willow, and soybean explants (Zhang and others 2009a). In view of the fact that NaHS dissolves in water and is dissociated to produce Na^+ and HS^-, and HS^- associated with H^+ can produce H_2S, a further test was carried out by using other chemicals such as S^{2-}, SO_4^{2-}, HSO_4^-, SO_3^{2-}, HSO_3^-, and Na^+ as controls for NaHS. As shown in Fig. 2, none of the above chemicals was found to exhibit an inducible effect similar to that of NaHS in triggering adventitious root formation. We further suggest that H_2S or HS^-, but not the other compounds derived from NaHS, was responsible for the induction of adventitious roots in cucumber explants. The results of Fig. 3 also suggested the possible interrelationship between auxin and H_2S during adventitious root formation.

Further data suggested a linear signal transduction cascade involving upregulation of HO-1 gene expression in H_2S-induced cucumber adventitious root formation. There are several results supporting these conclusions. First, NaHS-induced adventitious root development was blocked in the presence of the HO-1 inhibitor ZnPPIX (Fig. 5). The result was consistent with the observations that NaHS induced the expression of *CsHO-1* transcripts and its protein levels in a time-dependent manner (Fig. 4). Moreover, the increase in *CsHO-1* transcripts in NaHS-treated cucumber explants was suppressed by the addition of

ZnPPIX (Fig. 6a). When exogenous 30% CO-saturated aqueous solution was added together with ZnPPIX, the number and length of adventitious roots as well as *CsHO-1* transcripts inhibited by ZnPPIX treatment were significantly relieved (Figs. 5, 6a). In addition, that the inhibition of endogenous H_2S formation by HT could not influence haemin- and CO-induced adventitious root formation (Fig. 7) indicated that the reduced effect of NaHS treatment in the presence of HT might be the result of the decrease of endogenous H_2S production in cucumber explants. The above data supported the hypothesis that HO-1 might mediate H_2S-induced adventitious root formation in cucumber explants.

In animals, HO-1 is an early response gene that can be induced by a variety of stress factors, including haem, metalloporphyrins, heavy metals, cytokines, oxidative stress, and oxidized lipids (Maines 1997; Dulak and Józkowicz 2003; Kikuchi and others 2005; Leffler and others 2006). It was further suggested that HO-1 functions as a defensive system against oxidative stress, because BV or BR produced locally in the animal body may act as physiological antioxidants (Stocker and others 1987; Kikuchi and others 2005). CO at low concentrations has anti-inflammatory effects involving the mitogen-activated protein kinase (MAPK) pathway (Otterbein and others 2000). However, involvement of the MAPK cascade has not yet been discovered in the plant HO/CO signaling system. Given the abundance of haem as an oxygen carrier in animals, it is more likely that there are major differences in the regulatory mechanisms of HO-1/CO between animals and plants (Shekhawat and others 2011). In fact, H_2S and CO are important intra- and intercellular messengers, and both confer cytoprotective and immunomodulatory effects (Li and others 2009b; Mancuso and others 2010; Baskar and Bian 2011). Previous results showed that NaHS elevated the CO level and upregulated HO-1 expression in rats; by contrast, hydroxylamine, an inhibitor of CBS, reduced CO levels and downregulated HO-1 expression (Han and others 2006). Exogenous H_2S could alleviate the elevation of pulmonary arterial pressure. Plasma CO levels and the expression of HO-1 protein and mRNA in pulmonary arteries were significantly increased. However, the addition of DL-propargylglycine (PAG), an inhibitor of CSE, decreased the plasma H_2S content and worsened hypoxic pulmonary hypertension (HPH) (Zhang and others 2004). In agreement with these results, we illustrated that the addition of NaHS resulted in the induction of *CsHO-1* transcripts and its protein level in cucumber explants in a time-dependent manner (Fig. 4). Certainly, CsHO-1 induction would be beneficial for adventitious root formation by enhancing the release of CO, a signal molecule responsible for adventitious root development in mung bean (Xu and others 2006) and cucumber explants (Xuan and others 2008). We also deduced that the inhibition of endogenous H_2S via the addition of HT and thus the decreased adventitious root development in nondepleted cucumber explants (Fig. 7) might be related to the down-regulation of *CsHO-1* gene expression.

The DNAJ-like gene was upregulated during root initiation and formation triggered by auxin, suggesting a phase-specific modulation during the cell cycle in G2/M (Frugis and others 1999). The CDPK gene was also regarded as the target gene of auxin- or NO-induced adventitious root formation (Kumar and others 2004; Lanteri and others 2006). Our previous study further suggested that *CsDNAJ-1* and *CsCDPK1/5* might be the target genes of adventitious root development triggered by HO-1/CO (Xuan and others 2008). Therefore, the above link prompted us to further investigate the HO-1-mediated molecular mechanisms leading to adventitious root development triggered by NaHS. Results of Fig. 6b show that NaHS induced higher expression of *CsDNAJ-1* and *CsCDPK1/5* genes at 12 h of treatment, and these expression levels were consistent with the number and length of adventitious roots after another 78 h of treatment (Fig. 1). Further results confirmed that besides the significant inhibition of adventitious root formation and *CsHO-1* transcripts (Figs. 5, 6a), the application of the potent HO-1 inhibitor ZnPPIX not only blocked the induction of adventitious root development conferred by NaHS (Fig. 5), it also downregulated the NaHS-induced transcription of *CsDNAJ-1* and *CsCDPK1/5* genes in cucumber explants, compared to the control sample (Fig. 6). By contrast, these ZnPPIX responses could be reversed by the application of 30% CO-saturated aqueous solution. Therefore, we suggest that *CsDNAJ-1*

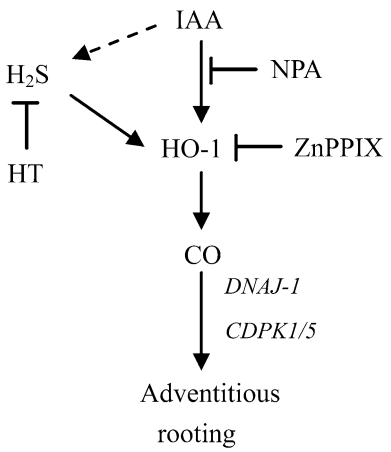

Fig. 8 Schematic representation of the signaling pathway involving auxin, hydrogen sulfide (H_2S), and the HO-1/CO system during the adventitious rooting process in cucumber. The above pathway might be mediated by the expression of *DNAJ-1* and *CDPK1/5* genes. *Dashed line* denotes indirect or still undescribed pathway. T bars, inhibition

and *CsCDPK1/5* genes, which have been proposed to be involved in the HO-1/CO-mediated auxin-induced adventitious root formation (Xuan and others 2008), might be also the target genes for the HO-1-mediated H_2S-induced adventitious root development in cucumber plants (Fig. 8).

In conclusion, our results give the first indication, to our knowledge, that HO-1 functions as a downstream component in H_2S-induced adventitious root formation by the modulation of expression of *DNAJ-1* and *CDPK1/5* genes, although whether auxin could induce H_2S release and its detailed mechanisms or roles should be elucidated in the near future (Fig. 8). Certainly, our finding opens a wide field of signal transduction pathways for every recently reported H_2S effect in plant biology.

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