Hydrogen Sulfide Inhibits Cadmium-Induced Cell Death of Cucumber Seedling Root Tips by Protecting Mitochondrial Physiological Function

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Abstract

Hydrogen sulfide (H₂S) can alleviate Cd-induced cell death, but the molecular mechanisms are not clear. To shed light on these mechanisms, cell death induced by 200 μM cadmium chloride in cucumber seedlings root tips was used as a model system. Here, we report that the negative effect of Cd stress in mitochondrial physiological functions include changes in cytochrome c/a, mitochondrial membrane permeability transition pores, and adenosine triphosphatase (ATPase). Moreover, Cd stress led to the release of mitochondrial Ca²⁺ into the cytosol. Exogenous application of sodium hydrosulfide (NaHS, a donor of H₂S) inhibited cell death and maintains mitochondrial function by reducing mitochondrial hydrogen peroxide accumulation, increasing ATPase activity and down-regulating *CsVDAC* and *CsANT* expression. In summary, H₂S suppressed Cd-induced cell death by improving mitochondrial physiological properties.

Keywords Hydrogen sulfide · Cell death · Mitochondrion · ATPase · Ca²⁺ · MPTP

Abbreviations

H ₂ S	Hydrogen sulfide
PCD	Programmed cell death
CdCl ₂	Cadmium chloride
Cyt c/a	Cytochrome c/a
MPTP	Mitochondrial membrane permeability transition pores
ATPase	Adenosine triphosphatase
NaHS	Sodium hydrosulfide
HT	Hypotaurine
H ₂ O ₂	Hydrogen peroxide

VDAC	Voltage-dependent anion channel
ANT	Adenine nucleotide translocator

Introduction

Calcium (Ca²⁺) as a second messenger plays a vital role in plant activity, regulation of gene expression, cell cycle control (Berridge et al. 2003), control of light signaling (Harada et al. 2003), inducing hormone responses (Shintaro et al. 2007), controlling cell growth (Sabine et al. 2007) and trigger plant responses during plant–pathogen interactions (Zhi et al. 2010). The short-term increase of cytosolic calcium

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($[Ca^{2+}]_{Cyt}$) concentration is an early event in a series of biological processes and stress responses (Jörg et al. 2010; Leng 2012). After receiving intracellular or extracellular stimulation, plants activate Ca^{2+} channels and related transporters, induce extracellular Ca^{2+} influx or stored Ca^{2+} release to the cytosol, resulting in an instantaneous rise of Ca^{2+} , which signals a multitude of downstream effects (Sanders et al. 2002). The Ca^{2+} influx and release require different Ca^{2+} ion channels, (i) voltage-dependent Ca^{2+} -permeable channels (VDCCs) which are divided into depolarization-activated Ca^{2+} -permeable channels (DACCs) and hyperpolarization-activated Ca^{2+} -permeable channels (HACCs); (ii) voltage-independent Ca^{2+} -permeable channels (VICCs) (Liu et al. 2018). After the completion of Ca^{2+} signal, in order to maintain a low concentration of Ca^{2+} , plants need to return excess Ca^{2+} to the calcium stores. H^+/Ca^{2+} antiporters and Ca^{2+} -ATPase are the main transport and export protein, which transports Ca^{2+} to extracellular domain or influxes to vacuoles and mitochondria (Boursiac and Harper 2007; Jörg et al. 2010). Mitochondria are not only the energy powerhouse of the cell but also a major hub for cellular Ca^{2+} signaling crucial for cell life and death (Giorgi et al. 2012; Rimessi et al. 2008; Rosario et al. 2012). Mitochondria regulate cell activity by participating in dynamic regulation of Ca^{2+} (Marchi et al. 2018). Mitochondria, as one of the organelles storing Ca^{2+} , play a key role in the intracellular Ca^{2+} signal transmission (Sisalli et al. 2012). Mitochondrial Ca^{2+} is involved in the process of cell death. Jones et al. found that the release of mitochondrial Cyt c into the cytoplasm induced by Ca^{2+} during the differentiation of tubular molecules leads to programmed cell death (PCD) (Jones 2002), and the increase of Ca^{2+} content in tobacco protoplasts induced the opening of mitochondrial membrane permeability, which led to PCD (Lin et al. 2005).

Many studies suggested that hydrogen sulfide (H_2S) is an important gas signal molecule in plant, just like carbon monoxide (CO) and nitric oxide (NO) (Hancock and Whiteman 2014). L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD) have been reported to be the key enzymes, which generate endogenous H_2S (Anja et al. 2005; Riemenschneider et al. 2010). Moreover, several researches showed that H_2S is involved in plant growth and development, like root development and regulating stomatal closure (Kou et al. 2018; Li et al. 2018a, 2018b; Ma et al. 2019; Chen et al. 2021), and H_2S plays vital role in alleviating heavy metal stress, especially cadmium (Cd) stress (Ahmad et al. 2019; Guan et al. 2018; Zhang et al. 2015a). H_2S has crosstalk with many molecules, like ethylene (Jia et al. 2018) and Ca^{2+} . Fang et al. reported that Ca^{2+} in a H_2S -dependent manner resisted chromium stress in millet and Ca^{2+} /calmodulin2 (CaM2)-mediated Ca^{2+} signal regulated endogenous H_2S content under Cr^{6+} stress, Cr^{6+} promoted the interaction between Ca^{2+} /CAM2 and transcription factor TGA3

to enhance the expression of *LCD* in *Arabidopsis thaliana* (Fang et al. 2014a, 2017). Ca^{2+} activated endogenous H_2S accumulation to improve thermo tolerance in tobacco suspension (Li et al. 2015). In addition, pretreatment with NaHS can improve thermo tolerance of tobacco suspension by regulating intracellular and extracellular Ca^{2+} concentration (Li et al. 2012). Interestingly, H_2S could reduce plant cell death, like hypoxia (Cheng et al. 2013), Cd stress (Zhang et al. 2015a), and GA-triggered PCD in wheat aleurone layers (Xie et al. 2014; Zhang et al. 2015b).

The components of MPTP include voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), and cyclophilin D (Cyp D). ANT is located in the inner membrane of mitochondrion. It catalyzes the energy conversion between ATP and ADP of cytoplasm and mitochondria, which is very important to cells (Javadov et al. 2000). ANT is a main role in cell death. The change of mitochondrial ANT induces the degree of MPTP opening. Such as salt stress induced PCD in tobacco protoplasts (Lin et al. 2006). The VDAC is mitochondrial outer membrane components, and it is a key player in mitochondria-mediated cell death (Kusano et al. 2009). Some studies have shown that the ROS produced by VDAC overexpression was the cause of apoptosis (Huang et al. 2014; Yuan et al. 2008). VDAC and ANT might also elicit mitochondrial Ca^{2+} efflux since it is a part of the MPTP (Bernardi 1999; Crompton et al. 2002).

The change of intracellular Ca^{2+} concentration affects the state of mitochondria and leads to cell death, while H_2S alleviates cell death by reducing oxidative stress. It is noteworthy that it is unclear whether H_2S reduces cell death by affecting the concentration of Ca^{2+} , especially the concentration of mitochondrial Ca^{2+} . In this study, we investigated H_2S and mitochondrial Ca^{2+} in terms of Cd-induced cell death, focusing on effect of H_2S on mitochondrial Ca^{2+} transport.

Materials and Methods

Seeds of cucumber (*Cucumis sativus* ‘Xinchun 4’) were germinated in petri dishes linked with filter papers in darkness at 28 °C in illuminating incubation climate box; all the seeds are divided into four parts. The seeds of the first part germinated in darkness water for 48 h and then cultured in light for 48 h. The second part of the seeds germinated for 48 h and then transferred to 200 μ M $CdCl_2$ solution for stress treatment for 48 h. The third part of the seeds germinated in distilled water for 24 h, then transferred them to petri dish containing 100 μ M NaHS to pretreat for 24 h in the dark, and finally transferred seedlings to another petri dish containing 200 μ M $CdCl_2$ for stress treatment for 48 h. The fourth part of seeds germinated in distilled water for 36 h and then transferred them to 400 μ M hypotaurine (HT, a scavenger

of H_2S) to pretreat for 12 h in the dark and finally replaced with 200 μM CdCl_2 solution for cultivation for 48 h. Light culture was carried out during Cd stress treatment for 48 h, the condition of illuminating incubation climate box was set to 25 ± 1 °C, 12 h photoperiod and photosynthetically active radiation = 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Then, the root length and fresh weight of seedling were measured.

Detection of Cell Death

Evans blue was used for cell death detection. The seedling root tips (2 cm) treated in different treatments were excised and stained with 0.1% (w/v) Evans blue for 15 min and washed them with water for 10 min and then taken pictures with fluorescence microscope (Revolve RVL-100-G, ECHO, USA). Finally, the stained samples were extracted with 1 mL 80% ethanol and bathed in water at 50 °C for 15 min and then centrifuged at 10,000 g for 10 min; the absorbance was measured at 600 nm (Zhang et al. 2015a).

Detection of Endogenous H_2S Content and Mitochondrial H_2O_2 Content

According to the method of determining H_2S content by Fang et al. (Fang et al. 2014b), 0.2 g root sample was added with 5 mL 50 mM phosphate buffer solution (0.2 M ascorbic acid (AsA), 0.1 M EDTA, and 0.5 mL 1 M HCl, PH 6.8) was added to homogenate, the released H_2S was caught by 1% (w/v) zinc acetate. After 30 min, 0.3 mL 5 mM dimethyl-p-phenylenediamine dissolved in 3.5 mM H_2SO_4 added to the mixing and then 0.3 mL of 50 mM ferric ammonium sulfate was added. After 15 min of reaction, the value of absorption at 667 nm was detected.

According to the method of He et al. (He et al. 2018), 1 mL of the isolated mitochondrial extract was taken and put into the test tube; 1 mL of 0.1 M phosphoric acid buffer (pH 7.0) and 2 mL KI were added, mixed, and shaken well and allowed to stand for 20 min at room temperature and then the change of absorption value at 390 nm was measured.

Mitochondrial Extraction from Root Tip

Mitochondrion was isolated from root tips of 4-d-old cucumber seedlings using a kit (Bestbio, BB-3611-1, China) and following the manufacture's instruction. 1 g root sample was added with 3 mL extract to grind on ice. The sample was homogenized at 4 °C, centrifuged at 100 g for 1 min, collected supernatant and discarded sediment. The filtrate was centrifuged at 700 g for 10 min and the supernatant was collected. Supernatant 11,000 g was centrifuged for 20 min and the supernatant was discarded. 1.5 mL of mitochondrial preservation solution was added to the precipitation and resuspended and then suspension at 11,000 g was

centrifuged for 15 min, the supernatant was discarded, and the precipitation was mitochondria. The isolated mitochondria were resuspended with suspension and stored in refrigerator at 4 °C for further experiments.

Determination of Mitochondrial Membrane Permeability Transition Pore (MPTP) and Cyt c/a

The method of determination is referred to He et al. (He et al. 2018). The isolated mitochondria were suspended in buffer solution (220 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM sodium succinate, pH 7.2) with a protein concentration of 0.3 mg/mL at 20 °C for 2 min, and then, UV light photometer was used to detect the absorbance value of 540 nm.

The isolated mitochondria were suspended with 0.2% BSA with a protein concentration of 0.5 mg/mL, and then, the absorption values of 550 nm and 630 nm were detected. The absorption values of the two wavelengths were Cyt c/a.

Determination of Ca^{2+} in Mitochondria

Mitochondrial extract (1.5 mL) added 5 mL concentrated nitric acid and mixed solution was cultured at 25 °C for 7 days, and then, 1% LaCl_3 was added to make the volume 10 mL. The absorption value was measured by flame atomic spectrophotometer, and the concentration was calculated according to the Ca^{2+} standard curve (Li et al. 2018a, 2018b).

Determination of Ca^{2+} -ATPase, H^+ -ATPase, and Mg^{2+} -ATPase Activity

Ca^{2+} -ATPase, H^+ -ATPase, and Mg^{2+} -ATPase activity were measured according to Blumwald's method (Blumwald and Poole 1987). H^+ -ATPase reaction system includes 30 mmol/L HEPES-Tris, 50 mmol/L KCl, 30 mmol/L MgSO_4 , 0.1 mmol/L ammonium molybdate, 0.1 mmol/L Na_3VO_4 , and 100 μL membrane proteins and then adds 50 μL 3 mmol/L ATP-Tris for incubation at 37 °C for 30 min. Reaction system was added with 50 μL 55% three chloroacetic acid to terminate the reaction. Finally, the reaction solution was added with 2.5 mL phosphorus-free protectant and 250 μL 6.47 mmol/L NaOH to continue the reaction for 40 min. The absorbance was measured at 550 nm.

Ca^{2+} -ATPase activity is determined according to H^+ -ATP. In the reaction system, the activity difference caused by adding or not adding 50 μL 3 mmol/L $\text{Ca}(\text{NO}_3)_2$ was taken as the Ca^{2+} -ATPase activity. Similarly, the activity difference caused by adding or not adding 30 mmol/L MgSO_4 was taken as the Mg^{2+} -ATPase activity.

The Activity of Caspase-3-Like

Caspase-3-like assay kit (solarbio, BC3830) was used according to the manufacture's instruction. To measure the activity of caspase-3-like, assays were performed on 96-well microtitre plates by incubating 35 μ L extraction solution and 65 μ L reaction buffer [5 μ L caspase-3 substrate (DEVD-pNA, 2 mM)]. Lysates were incubated at 37 °C for 4 h. The mixture was measured with an ELISA reader (CMax Plus, Molecular Devices, USA) at 405 nm.

Determination of CsVDAC and CsANT Expression by Quantitative RT-PCR

Total RNA was extracted from peanut root tips after different treatments for 24 h with an RNAiso plus kit (TaKaRa Inc., Japan) according to the manufacturer's instructions. The cDNA was amplified using the following primers shown in Table 1.

Statistical Analysis

All the experiments in this study were repeated three times, and the results shown are the mean $\pm$ SE of three independent experiments. Data analysis was used for Duncan's multiple test ($P < 0.05$) using SPSS 19.0 software (IBM SPSS, Chicago, USA).

Results

H₂S Alleviated the Inhibition of Cd Stress on Root Length and Fresh Weight

Observations about the effect of different treatments on the root length and fresh weight are shown in Fig. 1A–B. Treatment with 200 μ M Cd caused significant decrease of 48.85% and 19.32% in root length and fresh weight, respectively,

Table 1 Primers used for qRT-PCR assays

Gene name	Primer name	Sequence (5'–3')
<i>Actin</i>	Actin-F	TGGACTCTGGTGATGGTGTTA
	Actin-R	AAAAGGTCGGTAGGGAGTAAC
<i>VDAC2</i>	VDAC2-F	GACATCGGCAAGAAAGC
	VDAC2-R	TCACTGCGGTTGAGGTAA
<i>VDAC4</i>	VDAC4-F	GTTTAGGTGGTGATGTGGG
	VDAC4-R	CTTTGTCGGAGAATCGTGT
<i>ANT1</i>	ANT1-F	GCTCTTGTTGGCTCAT
	ANT1-R	CTTGACACCTTCGTTCT
<i>ANT2</i>	ANT2-F	GGAGGGTTTCGGTTCAT
	ANT2-R	CATTTGCCAATCGGGTA

Fig. 1 Effects of H₂S on root length and fresh weight of cucumber seedlings under Cd stress. CK: distilled water; Cd: 200 μ M; NaHS + Cd: pretreatment with 100 μ M NaHS for 24 h and then added 200 μ M CdCl₂; HT + Cd: pretreatment with 400 μ M HT for 12 h and then added 200 μ M CdCl₂. Determination of root length (A) and fresh weight (B) after 48 h. The results shown are means $\pm$ SE ($n = 15$) of three independent experiments. Different letters indicate statistically significant difference ($P < 0.05$; Duncan's multiple test)

reference to CK. Pretreatment with HT enhanced Cd toxicity and reduced root length by 51.86% and fresh weight by 22.67%, respectively. However, exogenously supplied NaHS alleviated toxic effect of Cd by increasing the root length and fresh weight by 28.32% and 14.78%, respectively, in comparison to Cd-alone-treated seedlings. In comparison to HT + Cd-treated plants, treatment of NaHS + Cd led to 36.34% and 19.76% increase in root length and fresh weight. These results showed that H₂S could alleviate the inhibition of root length and fresh weight under Cd stress.

H₂S Promoted Root Length and Fresh Weight by Reducing Root Tip Cell Death

To investigate whether the inhibition of Cd on root length and fresh weight was caused by the cell death of root tip, we measured degree of cell death by Evans blue. Fig. 2A shows that staining degree of root tips cell death under different treatments, roots treated with Cd and HT showed a deeper

Fig. 2 Effects of different treatments on cell death in cucumber seedlings root. **A** Roots stained with Evans blue observed under a light microscope. **B** Quantitative analysis of root cell death in cucumber seedlings. The results shown are means $\pm$ SE of three independent experiments. Different letters indicate statistically significant difference ($P < 0.05$; Duncan's multiple test). FW fresh weight

staining level compared with CK and NaHS + Cd. Likewise, roots staining extract showed similar phenomena (Fig. 2B). In order to quantify the root tips cell death under different treatments, we measure the OD value of Evans blue staining. The results showed that Cd stress significantly increased cell death, especially HT + Cd. Compared with CK, Cd and HT + Cd treatments increased by 151.14% and 177.12%,

respectively. Pretreatment with 100 μ M NaHS significantly reduced cell death by 17.47%, 25.21% compared with Cd and HT + Cd, repetitively. These results demonstrated that H₂S could alleviate Cd-induced cell death, while the elimination of H₂S (HT) aggravated cell death.

Change of H₂S and Mitochondrial H₂O₂ Content of Cucumber Seedlings Root Tips at Different Treatments Under Cd Stress

To examine whether H₂S and mitochondrial H₂O₂ are involved in Cd-induced PCD, the effects of Cd and H₂S on mitochondrial H₂S and H₂O₂ were investigated. The measurement results are shown in Fig. 3. After 48 h with Cd alone, endogenous H₂S and mitochondrial H₂O₂ content significantly raised by 40.00% and 96.78% compared with CK. Under treatments of H₂S scavenger HT, mitochondrial H₂O₂ content was higher level than other treatments. On the contrary, endogenous H₂S content was lower level than other treatments. However, pretreatment with 0.1 mM NaHS not only increased endogenous H₂S content by 76.84% and 26.32% (CK and Cd) but also decreased mitochondrial H₂O₂ content by 22.31% and 36.49% compared with Cd and HT + Cd, respectively. These results indicated that the decrease of endogenous H₂S aggravated the oxidation of mitochondrion; NaHS pretreatment could protect mitochondrion from oxidative damage by increasing endogenous H₂S level and reducing mitochondrial H₂O₂ content.

Effect of H₂S on Mitochondrial Ca²⁺ Concentration and Mitochondrion Ca²⁺-ATPase, H⁺-ATPase, and Mg²⁺-ATPase Activities Under Cd Stress

To investigate whether increased cytosolic Ca²⁺ levels in cucumber seedling root tips are due to the release of mitochondrial Ca²⁺ into cytoplasm, mitochondrial Ca²⁺ concentration was measured. Cd and HT + Cd significantly

Fig. 3 Change of H₂S and H₂O₂ content of cucumber seedlings root tips at different treatments. CK: distilled water; Cd: 200 μ M CdCl₂; NaHS + Cd: 100 μ M NaHS pretreatment for 24 h + Cd; HT + Cd: 400 μ M HT pretreatment for 12 h + Cd. H₂O₂ content in mitochondria of root tip cells and H₂S content in roots was determined under Cd stress for 48 h. The results are means $\pm$ SE of three independent experiments. Different letters show significant differences ($P < 0.05$; Duncan's multiple test). FW fresh weight

decreased mitochondrial Ca^{2+} levels in root tips (Fig. 4A). NaHS + Cd significantly alleviated the decrease of mitochondrial Ca^{2+} concentration. Compared with Cd alone, pretreatment with 100 μM NaHS increased by 22.64%. Compared with HT + Cd, NaHS + Cd raised mitochondrial Ca^{2+} level by 44.73%.

To investigate whether H_2S could maintain mitochondrial chemical potential and stabilize membrane structure, we measured mitochondrion Ca^{2+} -ATPase activity, H^+ -ATPase activity, and mitochondrion Mg^{2+} -ATP activity. Under Cd stress, both mitochondrion Ca^{2+} -ATPase activity (Fig. 4B), H^+ -ATPase activity (Fig. 4C), and Mg^{2+} -ATPase activity (Fig. 4D) decreased significantly, especially under HT + Cd. Compared with Cd alone, pretreatment with NaHS increases mitochondrion Ca^{2+} -ATPase activity (32.12%), H^+ -ATPase activity (24.72%), and mitochondrion Mg^{2+} -ATPase activity (18.25%). These data suggested that H_2S could inhibit the destruction of mitochondrial chemical potential and membrane stability by Cd stress and reduce the efflux of mitochondrial Ca^{2+} .

Effect of H_2S on Mitochondrial Cyt c/a, MPTP, and Caspase-3-Like Activity Under Cd Stress

As shown in Fig. 5A–B, Cd stress markedly decreased Cyt c content and the OD value of MPTP by 28.42% and 37.17% compared with CK. Moreover, after application of H_2S scavenger HT, Cyt c/a and MPTP showed lower than CK and Cd alone. Under NaHS pretreatment, Cyt c/a and MPTP were 25.20% and 34.90% higher than Cd stress. Likewise, pretreatment with NaHS increased by 48.16% and 67.93% compared with HT + Cd. We measured caspase-3-like activity (Fig. 5C). The results showed that caspase-3-like activity of Cd stress and HT + Cd were 2.10 and 2.76 times higher than CK, respectively. Pretreatment with 0.1 mM NaHS significantly decreased caspase-3-like activity compared with Cd and HT + Cd treatments. The above results suggested that H_2S inhibited the opening of MPTP, release of mitochondrial Cyt c and reduced caspase-3-like activity under Cd stress.

Fig. 4 Change of mitochondrion Ca^{2+} concentration, mitochondrion Ca^{2+} -ATPase activity, H^+ -ATPase activity, and mitochondrion Mg^{2+} -ATPase activity with different treatments under Cd stress. CK: distilled water; Cd: 200 μM CdCl_2 ; NaHS + Cd: 100 μM NaHS pretreatment for 24 h + Cd; HT + Cd: 400 μM HT pretreatment for

12 h + Cd. After 48 h, mitochondrion Ca^{2+} concentration (A), mitochondrion Ca^{2+} -ATPase activity (B), H^+ -ATPase activity (C), and mitochondrion Mg^{2+} -ATPase activity (D) were determined. The results are means $\pm$ SE of three independent experiments. Different letters show significant differences ($P < 0.05$; Duncan's multiple test)

Fig. 5 Effects of different treatments on mitochondrion Cyt c/a, MPTP, and caspase-3-like activity under Cd stress. 2-day-old cucumber seedlings were treated by different treatments for 48 h and then mitochondrion Cyt c/a (A), MPTP (B), and caspase-3-like (C) activ-

ity were determined. The results are means $\pm$ SE of three independent experiments. Different letters show significant differences ($P < 0.05$; Duncan's multiple test)

H₂S Downregulates Expression of MPTP-Related Genes to Prevent Loss of Ca²⁺

Furthermore, *CsVDAC2*, *CsVDAC4*, *CsANT1*, and *CsANT2* gene expression was as a molecular probe to investigate the molecular mechanism of H₂S regulating MPTP. Compared with CK, Cd and HT + Cd treatments were able to induce high expression of *CsVDAC2*, *CsVDAC4*, *CsANT1*, and *CsANT2*. These gene expressions were matched with the opening of MPTP. It was worth noting that NaHS pretreatment down-regulated these gene expressions by 24.63%, 32.15%, 27.87%, and 21.65%, respectively. These results showed that H₂S down-regulated *CsVDAC* and *CsANT* expression to inhibit the opening of MPTP.

Discussion

H₂S is a signal molecule involved in regulating physiological processes of animals and plants (García-Mata and Lamattina 2010; Li et al. 2011). In addition, H₂S is involved in heavy metal stress (Ahmad et al. 2019; Mostofa et al. 2015; Qian et al. 2014). Our study showed that Cd

treatment significantly inhibited root elongation and fresh weight of cucumber seedlings (Fig. 1). Moreover, Cd stress led to root tips cell death, while pretreatment with 0.1 mM NaHS increased root length and fresh weight by reducing cell death (Figs. 1, 2). These findings were consistent with those reported in the literature. Zhang et al. reported that 5 mM CdCl₂ seriously affected the root growth of cabbage seedlings and markedly caused root tips cell death. When seedlings were treated with 5 μ M for 24 h, the inhibition effect of Cd stress was significantly reduced (Zhang et al. 2015a). In rice seedlings, 0.5 mM CdCl₂ significantly decreased plant height, fresh weight, and dry weight; Evans blue staining showed that Cd stress caused cell death. NaHS treatment (0.1 mM) could alleviate Cd toxicity to maintain growth (Mostofa et al. 2015). Thus, all findings presented here indicate that NaHS appears to positively effect in plant growth.

The accumulation of endogenous H₂S is the response of plants to stress. In this study, Cd stress led to the accumulation of endogenous H₂S content that was higher than CK. Pretreatment with NaHS strengthened this accumulation effect (Fig. 3). High-concentration heavy metal ions can stimulate a ROS burst (Dai et al. 2016; Qian et al. 2014; Zhang et al. 2015a), which is one of the inducers of cell

death (Jabs 1999). Figure 3 shows that mitochondria H_2O_2 accumulation was stimulated by Cd stress. NaHS + Cd treatment significantly decreased this accumulation, which was consistent with other reports (Huang et al. 2016). Mitochondria are generally considered to be an important site of ROS-mediated PCD events; the release of Cyt c and collapse of MPTP are also reported to be one of the vital events in PCD (He et al. 2018; Yao et al. 2004). Mitochondria are involved in cell apoptosis of plants (Rurek 2014); because of the increased mitochondrial membrane permeability and decreased membrane potential, mitochondrial Ca^{2+} and Cyt c were released into the cytoplasm, causing an increase in cytosolic Ca^{2+} levels. We also confirmed that Cd induced the release of mitochondria Ca^{2+} (Fig. 4A) and decreased mitochondria Cyt c/a (Fig. 5A). Li et al. also found that allelochemicals induced cell death in maize root tip, which was released from mitochondria with Cyt c and Ca^{2+} (Li et al. 2018a, 2018b). Cyt c release has been seen also in plant cells under-going opening of MPTP and subsequent PCD under the influence of biotic and abiotic stress conditions (Panda et al. 2008). We noticed that the OD value of MPTP also decreased by Cd stress (Fig. 5B). This is consistent with He et al. who reported that aluminum caused Cyt c/a and OD

value of MPTP in peanut root (He et al. 2018). In our study, Cd stress induced up-regulation of *VDAC* and *ANT* expression that indicated the opening of MPTP. NaHS pretreatment significantly down-regulated *VDAC* and *ANT* expression to inhibit the opening of MPTP compared with Cd, while scavenger HT treatment exacerbates this condition (Fig. 6). Similarly, in a rat model of ischemia-reperfusion, H_2S prevents cardiomyocyte apoptosis by inhibiting the opening of MPTP (Yao et al. 2010; Zhang et al. 2013). These results suggested that H_2S as an antioxidant protected mitochondrial function from Cd toxicity.

ATP is the energy to maintain plant life activities, and H^+ -ATPase, Ca^{2+} -ATPase, and Mg^{2+} -ATPase located on plasma membrane is an important ATPase. They play a key role in maintaining the chemical potential inside and outside the membrane and stabilizing the function of plasma membrane. Ca^{2+} -ATPase is an important regulator of Ca^{2+} transport in plants, which can maintain the gradient of Ca^{2+} concentration intracellular and extracellular (Ferrol and Bennett 1996). Similar studies are reported (Ahn et al. 2001; Zhan et al. 2014; Zhao et al. 2008). Our study demonstrated that Cd stress decreased H^+ -ATPase, Ca^{2+} -ATPase, and Mg^{2+} -ATPase activity (Fig. 4B–D),

Fig. 6 Effects of different treatments on the expression of *CsVDC2*, *CsVDC4*, *CsANT1*, and *CsANT2*. After 24 h treatment, the relative expressions of *CsVDC2* (A), *CsVDC4* (B), *CsANT1* (C), and *CsANT2* (D) were detected by real-time fluorescence quantitative

analysis under different treatments. The results are means $\pm$ SE of three independent experiments. Different letters show significant differences ($P < 0.05$; Duncan's multiple test)

Fig. 7 Schematic diagram for the effect of H_2S on cell death induced by Cd. The Figure shows that $\text{H}_2\text{S}/\text{H}_2\text{O}_2$ may mediate Cd-induced PCD via mitochondria-dependent pathway as a mixed signal. The yellow and blue circles represent Cyt c and Ca^{2+} , respectively. HT hypotaurine, a scavenger of H_2S ; MPTP mitochondrial permeability transition pore; VDAC voltage-dependent anion channel; ANT adenine nucleotide translocator

but pretreatment with NaHS maintained mitochondrial ATPase activity and inhibited mitochondrial Ca^{2+} efflux into cytoplasm (Fig. 4A). When Ca^{2+} -ATPase was activated, mitochondrial Ca^{2+} exchanged with cytoplasmic Ca^{2+} to maintain balance. Cd stress decreased Ca^{2+} -ATPase enzyme activity and increased mitochondrial membrane permeability, breaking this balance and led to $[\text{Ca}^{2+}]_{\text{cyt}}$ overload. Dawood and Li have similar results to ours (Dawood et al. 2012; Juan et al. 2018). These results showed that H_2S could maintain mitochondria ATP activity to limit mitochondria Ca^{2+} efflux and $[\text{Ca}^{2+}]_{\text{cyt}}$ overload.

Cd can cause plant chlorosis, growth inhibition, and cell death due to its toxicity; the typical PCD upon Cd treatment were observed in cell suspensions of tobacco (Kuthanova et al. 2008; Ma et al. 2010), tomato (Elena et al. 2005; Gallego et al. 2012), and *Arabidopsis* (De Michele et al. 2009). Our results confirmed that Cd induced the occurrence of cell death caspase-3-like enzyme activation (Fig. 5C). Caspase activity plays an important role in the process of PCD and caspase-3 is the executor of PCD (Li and Xing 2010). Cd treatment induced the increase of caspase-3-like activity significantly (Fig. 5C). Exogenous NaHS pretreatment reduced the occurrence of PCD, while HT (H_2S scavenger) could accelerate the process of cell death.

Conclusion

Taken together, Cd-induced cell death in cucumber root tips was mitochondria-dependent cell death. Cd stress damaged mitochondrial physiological functions by mitochondrial H_2O_2 accumulation and the opening of MPTP to release Cyt c and Ca^{2+} . H_2S prevented cell death of Cd-induced by improving mitochondrial physiological properties and a potential schematic diagram is shown in Fig. 7.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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