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Hydrogen sulfide is involved in the chilling stress response in *Vitis vinifera* L.

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Abstract

Hydrogen sulfide (H_2S) is an important signaling molecule involved in several stress-resistance processes in plants, such as drought and heavy metal stresses. However, little is known about the roles of H_2S in responses to chilling stress. In this paper, we demonstrated that chilling stress enhance the H_2S levels, the H_2S synthetase (L-/D-cysteine desulfhydrase, L/DCD) activities, and the expression of L/DCD gene in *Vitis vinifera* L. 'F-242'. Furthermore, the seedlings were treated with sodium hydrosulfide (NaHS, a H_2S donor) and hypotaurine (HT, a H_2S scavenger) at 4°C to examine the effects of exogenous H_2S on grape. The results revealed that the high activity of superoxide dismutase and enhanced expression of *VvICE1* and *VvCBF3* genes, but low level of superoxide anion radical, malondialdehyde content and cell membrane permeability were detected after addition of NaHS. In contrast, HT treatment displayed contrary effect under the chilling temperature. Taken together, these data suggested that H_2S might be directly involved in the cold signal transduction pathway of grape.

Keywords: hydrogen sulfide, chilling stress, *Vitis vinifera* L.

Introduction

Hydrogen sulfide (H_2S) is a novel gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO) [1]. Many data indicate that most of the endogenously synthesized H_2S occurred via L-cysteine desulfhydrase (LCD, EC4.4.1.1) and D-cysteine desulfhydrase (DCD, EC4.4.1.15) in high plants [2–5]. Recent studies show that H_2S is involved not only in plant responses to drought and copper stresses but also in tolerance to salinity, heat, cadmium, boron and chromium stresses [6–10]. It is reported that exogenous H_2S can enhance the resistance of plants to drought stress [11–14] and copper stress [15,16] by improving the activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). However, whether endogenous H_2S is involved in plant cold stress response is poorly known.

Signal molecules are involved in perception and transduction of low temperature signal and mediate chilling adaptive responses through physiological processes and transcription factors [17–20]. Recent research indicated that multiple transcription factors and inducers, such as C-repeat-binding factor (CBF), inducers of CBF expression (ICE) and cold-regulated (*COR*) genes, played pivotal roles in the resistance to low temperature stress [21–23]. Many signal molecules can improve plant tolerance to low temperature stress by altering the expression levels of *COR*, *CBFs* and *ICE1* genes. For example, ethylene molecules negatively regulate cold tolerance by repressing expression of *CBFs* [25]. Inositol 1, 4, 5-trisphosphate (IP3) and Ca^{2+} induce *CBFs* and *COR* expression in plant [25]. NO positively regulate the expression of cold related genes *COR15a*, *LT130* and *LTI78* in *Arabidopsis* under low temperature conditions [17]. However, whether H_2S regulates expression of these cold related genes in the response of chilling adaptive is still poorly understood.

Different species of grape exhibit varying level of resistance to cold hardiness, ranging from the cold susceptive grape ('Maoputao', *Vitis quinqanguoari* Rehd. cv. Maoputao) to the high resistant grape 'Zuoshan1' (*Vitis amurensis* Rupr. cv. Zuoshan1) [26]. Even though the many researches about low temperature have been conducted in grape [26], the mechanism of low temperature resistance in grape at molecular level as well as the signal transduction pathways reminds unclear. In this paper, exogenous H_2S was studied in cold-tolerant grapevine 'F-242' in response of cold stress. Our results showed that H_2S was involved in cold stress response in grape by regulating superoxide anion radical content, MDA content, the relative permeability of cell membrane and SOD activity, as well as expression of *VvICE1* and *VvCBF3*.

Material and methods

Plant materials, growth conditions, and treatments

Vitis vinifera L. 'F-242' (tissue culture seedling), a cold resistant variety in Shandong province of China, was maintained

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in ½ MS medium, under a 16 h light/8 h dark photoperiod at 25°C. Four-weeks old seedlings were exposed to 4°C and 25°C for 0, 1, 3, 5, 7, 9, 11 and 22 h, respectively. Fully expanded leaves were harvested to determine cell membrane permeability, content of H_2S, MDA and superoxide anion radical, activities of L-/D-cysteine desulfhydrase and SOD, and the expression levels of *VvLCD* and *VvDCD* genes.

Meanwhile, the plantlets were treated with 0.1 mM HT and NaHS (distilled water as a control) for 1 h, and then measured the cell membrane permeability, MDA content, superoxide anion radical content and SOD activities at their burst time under 4°C and 25°C. In addition, the seedlings treated (spraying) with the same concentration of HT and NaHS were exposed to 4°C for 7 h and 3 d. After that, leaves were harvested and immediately frozen in liquid nitrogen for RNA extraction. The expression levels of *VvICE1* and *VvCBF3* genes were determined by quantitative RT-PCR.

Measurement of H_2S content

Measurement of H_2S content was performed as described by by Liu et al. [27].

L-/D-cysteine desulfhydrase activity measurements

L-/D-cysteine desulfhydrase activity measurements were performed as described by Liu et al. [27].

Superoxide anion radical content measurement

Measurement of superoxide anion radical content was conducted referring to Zhao and Zhou [28] with some modifications. 0.5 g leaves was homogenized with 5 ml 50 mM potassium phosphate buffer at 4°C and centrifuged at 10000 rpm for 10 min. Mixed 0.5 ml 50 mM potassium phosphate buffer 1 ml 1 mM hydroxylamine hydrochloride (Sigma, USA) and 0.5 ml crude extract in reaction tubes, incubated at 25°C for 1 h, then added 1 ml of 17 mmol l^{-1} sulfanilic acid and 1 ml of 7 mmol l^{-1} α-naphthylamine (Sigma, USA) in reaction tubes, incubated at 25°C for 20 min, and the absorbance was read at 530 nm. Converted the photometric value to nitrite content via standard curve of nitrite and hydroxylamine reaction, and the content equaled to half of nitrite was measured.

MDA content measurement

Lipid peroxidation was estimated by concentration of thiobarbituric acid reactive substances (TBARS) [29]. First, 0.1 g leaves was homogenized with 1 ml 10% (w/v) trichloroacetic acid (TCA; Sigma, USA) and the homogenate was centrifuged at 12000 rpm for 10 min. Then 500 µl of the supernatant was mixed with 500 µl 10% (w/v) TCA containing 0.6% (w/v) thiobarbituric acid (TBA; Sigma, USA). The mixture was incubated in boiling water for 15 min, cooled to room temperature, and centrifuged at 12000 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm, and the non-specific absorbance was measured at 600 nm. The MDA content was determined using a molar extinction coefficient of 155 mM^{-1} cm^{-1} [30].

Membrane permeability measurement

Harvested leaves were washed with deionized water and slightly dried with filter paper. Leaf-disc was taken by punching bear, and then incubated in deionized water at 25°C for 1 h. Electrical conductivity (*EC1*) of the extravasation solution was measured using a conductivity meter (YSI model 55). Total ionic strength was determined after heating the solution in a 100°C water bath for 10 min, and the electrical conductivity (*EC2*) was measured after cooling the solution to 25°C as described by Welti et al. [31]. Membrane relative permeability was calculated by the formula $EC1/EC2 \times 100\%$.

SOD activity measurement

Measurement of SOD activity was performed as described by Donahue et al. [32]. First, 0.5 g leaves was homogenized with 5 ml 50 mM potassium phosphate buffer at 4°C and centrifuged at 10000 rpm for 10 min. 200 µl crude extract was mixed with 3 ml 50 mM potassium phosphate buffer in reaction tubes and then illuminated (fluorescent light, 40 W) for 20 min. The absorbance was measured at 560 nm, taken the reaction solution without illumination as a control. A calibration curve with commercial SOD (Sigma, USA) was utilized to calculate SOD activity. One unit of SOD was defined as the amount of enzyme required to cause a 50% reduction of nitrotetrazolium blue chloride.

Quantitative RT-PCR

Total RNA of grape leaves was extracted with CTAB method as presented by Iandolino et al [33]. First-strand cDNA was synthesized from 4 ng DNase I (TaKaRa, Japan) -treated total RNA using M-MLV reverse transcription kit (TaKaRa, Japan). Quantitative RT-PCR was performed using MyiQ real-time PCR detection system (Bio-Rad, USA) with the presence of SYBR Green I (BioWhittaker Molecular Applications) in the amplification mixture according to the manufacturer's protocols. Specific primer pairs were designed for *VvLCD* (forward primer 5′-CACTACGCAAACGGAGCACA-3′, reverse primer 5′-TCATTATTCACCGCCATCACC-3′), *VvDCD* (forward primer 5′-CGATAGGAGGCATCCAAAGC-3′, reverse primer 5′-TCCAATTAAACGTTCAACCAAGA-3′), *VvICE1* (forward primer 5′- GGTGGAAGTAACTCAAATGC-3′, reverse primer 5′- GCTCCTTCAAGTACTCAATAGC-3′), *VvCBF3* (forward primer 5'- CAATAGATGGGTATGCGAAGTG -3', reverse primer 5′-CTGTATGTCCCTAGCTGAAGATGAC-3′) and *VvACTIN* (forward primer 5′-TACGAGCAAGAGCTGGAAA-3′, reverse primer 5′-AATGAGAGATGGCTGGAAGAG-3′). Amplification of actin transcripts served as the internal standard. The data were analyzed using MyiQ software (Bio-Rad).

Data processing and statistical analysis

The DPS data processing system was used to carry out the significance analysis of the data. P value <0.05 was considered statistically significant.

Results

Chilling treatment increased endogenous H_2S level and activity of L-/D-cysteine desulfhydrase in grape leaves

Endogenous H_2S content and the activity of VvLCD and VvDCD were detected in grape leaves in order to investigate H_2S function in response to chilling temperature. When the grape seedlings were exposed to 4°C, H_2S content did not change within the first three hours of cold treatment, but increased and reached the peak 5 h after the start of cold treatment, then deceased sharply to the normal level ($P < 0.05$; Fig. 1a). Additionally, the activity of VvL/DCD was measured at the same treatment condition to determine if the enzyme was involved in H_2S accumulation. Similarly to the accumulation pattern of H_2S, the activity of VvLCD and VvDCD were enhanced and

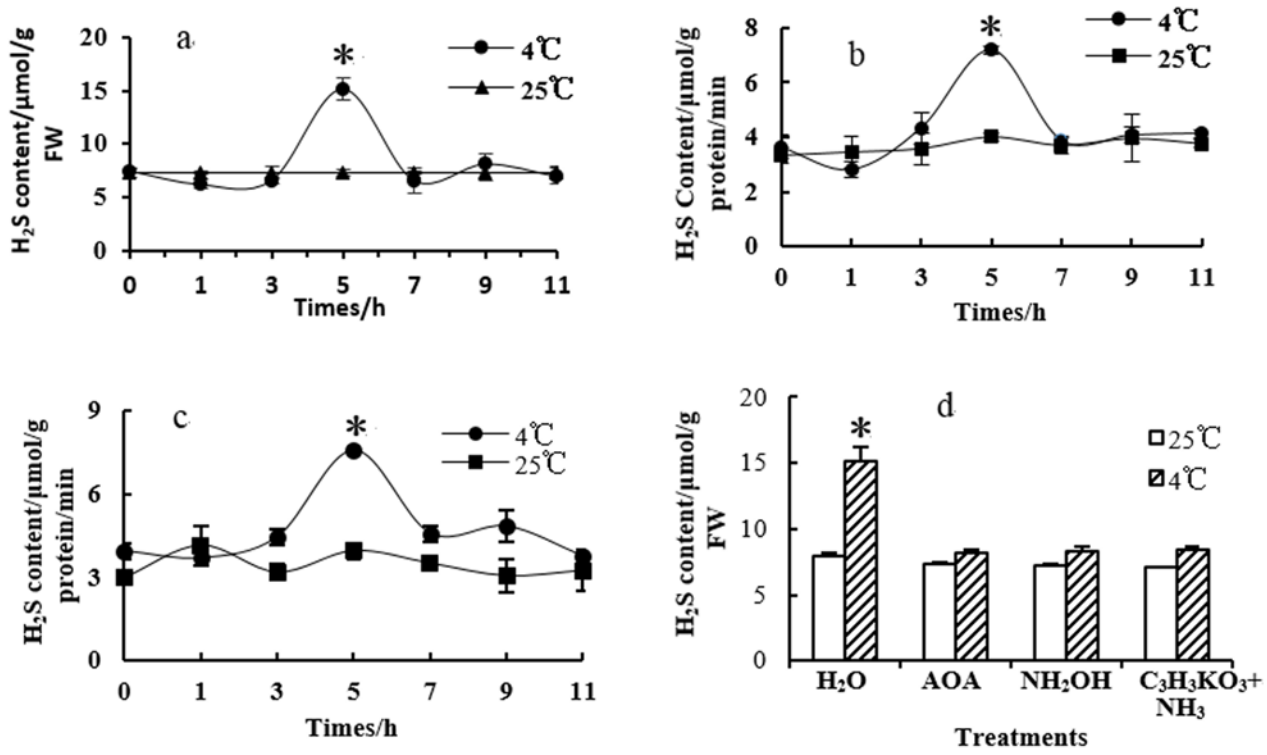

Fig. 1 Detection of endogenous H_2S contentand L/DCD activity in 'F-242' leaves under chilling stress. The values represent the average of three independent samples. **a** Effects of chilling on H_2S content. **b** Effects of chilling on LCD activity. **c** Effects of chilling on DCD activity. **d** Effects of H_2S synthesis inhibitors (AOA, NH_2OH and $C_3H_3KO_3$+NH_3) on chilling-induced H_2S content. Error bars indicate ±*SE*. * indicates significant differences at $P < 0.05$ (Student's *t*-test).

reached a peak 5 h after the start of 4°C treatment ($P < 0.05$) (Fig. 1b,c). Furthermore, H_2S accumulation was inhibited by L-/D-cysteine desulfhydrase production [aminooxy acetic acid (AOA), NH_2OH or potassium pyruvate ($C_3H_3KO_3$) + NH_3; $P < 0.05$; Fig. 1d]. These results indicated that chilling temperature induce the H_2S accumulation probably by L-/D-cysteine desulfhydrase.

Expression of L/DCD genes was induced by cold stress in grape leaves

Primers for *VvLCD* (forward primer 5′-TCTAGAATGGCTTCCAGACTCCTCC-3′, reverse primer 5′-GGTACCTTAATGCAACATTTCATCTCTGTGT-3′) and *VvDCD* (forward primer 5′-GTCGACATGGAGGCAACCAAGCAACT-3′, reverse primer 5′-GGTACCTTAGTAGAACATTTTTCCAATACCATC-3′) genes were designed based on the mRNA sequence of *Vitis vinifera* L. PN40024 (accession number: XM_003635001 and XM_002263322) and the targeted genes were cloned in grape 'F-242'. In contrast with PN40024, *VvLCD* showed some differences in nucleotide sequences ($27^{C\text{-}T}$, $666^{T\text{-}G}$, $943^{C\text{-}A}$, $1038^{G\text{-}T}$, $1143^{G\text{-}A}$; Fig. 2a) but amino acid sequences was conserved (Fig. 2b). Meanwhile, *VvDCD* presented some changes in nucleotide sequences ($42^{G\text{-}A}$, $156^{C\text{-}T}$, $456^{A\text{-}G}$, $537^{A\text{-}G}$, $734^{T\text{-}A}$, $907^{G\text{-}A}$, $1059^{A\text{-}G}$, $1067^{G\text{-}A}$, $1088^{G\text{-}A}$, $1096^{A\text{-}G}$, $1113^{C\text{-}A}$, $1120^{A\text{-}G}$; Fig. 3a) and amino acid sequences ($245^{L\text{-}H}$, $303^{V\text{-}I}$, $356^{G\text{-}E}$, $363^{C\text{-}Y}$, $366^{N\text{-}D}$, $374^{K\text{-}E}$; Fig. 3b). In addition, *VvLCD* and *VvDCD* were submitted to NCBI, and accession numbers are KC505216 and KC505217.

Furthermore, expression level of *VvLCD* and *VvDCD* were analyzed by quantitative RT-PCR. Expression pattern were similar between *VvLCD* and *VvDCD*, which reached their peak at 3 h and 5 h, respectively, then gradually declined to almost normal expression levels at 11 h cold treatment (Fig. 4). These results were in good agreement the profiles of H_2S accumulation and corresponding H_2S synthetase activity (Fig. 1). In conclusion, there is a hypothesis that H_2S is involved in the chilling signaling pathway of grapevine 'F-242'.

Effects of NaHS and HT on the levels of superoxide anion radical, MDA, the relative permeability of cell membrane and SOD activity in grape leaves

Superoxide anion radical content, MDA levels, the relative permeability of cell membrane and SOD activity were identified in grape seedlings treated with NaHS and HT. The data revealed that the superoxide anion radical content (Fig. 5a), MDA content (Fig. 5b), the relative permeability of cell membrane (Fig. 5c) and SOD activity (Fig. 5d) were all significantly increased at 3 h, 5 h, 11 h and 3 h, respectively, in the grape leaves after 4°C treatment. While a decline occurred for superoxide anion radical, MDA and the relative permeability of the plasma membrane in the seedlings pretreated with NaHS (Fig. 5e–g). As was expected, the seedlings pretreated with HT presented the contrary results (Fig. 5e–g). However, SOD activity showed opposite changes, with the increase of SOD by NaHS addition and decreased by HT pretreatment (Fig. 5h).

Thus, it was suggested that H_2S could reduce the level of superoxide anion radical and MDA, and improve SOD activity and the plasma membrane stability of grape leaves under low temperature condition.

Effects of NaHS and HT on transcription levels of *VvICE1* and *VvCBF3*

To explore the possible targets of H_2S during chilling acclimation, transcription levels of cold-responsive genes *VvICE1* and *VvCBF3* were analyzed in 4°C acclimated 'F-242' seedlings that were pretreated with NaHS and HT. At normal temperature

Fig. 2 Nucleotide (**a**) and amino acid (**b**) differences of VvLCD between 'PN40024' and 'F-242'.

Fig. 3 Nucleotide (**a**) and amino acid (**b**) differences of VvDCD between 'PN40024' and 'F-242'.

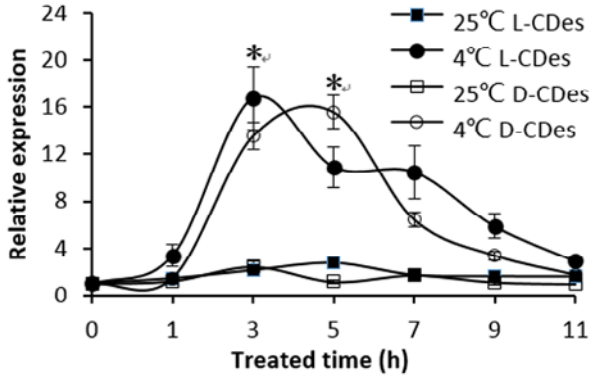

Fig. 4 Expression patterns of *VvLCD* and *VvDCD* in 'F-242' under chilling stress. The relative expression of *VvLCD* and *VvDCD* was quantified in comparison with the *VvACTIN* using quantitative RT-PCR with gene-specific primers (see "Material and methods"). The values represent the average of three independent samples. Error bars indicate ±*SE*. * indicates significant differences at $P < 0.05$ (Student's *t*-test).

(25°C), expression of *VvICE1* and *VvCBF3* in 'F-242' were at low levels whether NaHS and HT were applied or not (Fig. 6). However, cold treatment significantly enhanced expression levels of *VvICE1* and *VvCBF3*, which were further induced by the application of NaHS, but suppressed by HT (Fig. 6). Thus, there is a hypothesis that H_2S play an important role in grape responses to cold stress by modulating *VvICE1* and *VvCBF3* expression.

Discussion

Recent studies in plants revealed that H_2S plays multiple roles in the modulation of various physiological processes [3], such as copper, cadmium, drought, salinity and heat et al. [6–10]. In addition, our results indicate that the content of H_2S was rapidly increased by low temperature. All the results reveal that H_2S may be an important signaling molecule in plant responses to abiotic stresses.

In mammals, H_2S is produced from L-cysteine by at least four separate pathways, such as cystathionine β synthetase (CBS, EC 4.2.1.22), cystathionine γ lyase (CSE, EC 4.4.1.1), cysteine aminotransferase (CAT, EC 2.6.1.3) and cysteine lyase (CL, EC 4.4.1.10) [34], and CBS and CSE consistently demonstrated to produce H_2S in mammalian tissues [1,35]. Similar to animals, the homologs of CBS (LCD) and CSE (DCD) also are found in plants, and they are reported mainly responsible for generating H_2S [4,36]. *LCD* (*At3g62130*) and *DCD* (*At1g48420*) genes have been isolated from *Arabidopsis* [2,37]. Meanwhile, the homolog genes of *CDes* also were isolated from *B. napus* and *O. sativa* [38]. In our study, we isolated *LCD* and *DCD* genes from 'F-242'. Moreover, we also find the transcription levels and enzyme activities of LCD and DCD were induced by 4°C acclimation. However, inhibitors of LCD and DCD pathways could inhibit the accumulation of H_2S. Therefore, we conclude that *VvLCD* and *VvDCD* genes contribute to the major production of H_2S in grape at low temperature.

To confirm the functions of H_2S in grape response to chilling stress, we analyzed superoxide anion radical content, MDA

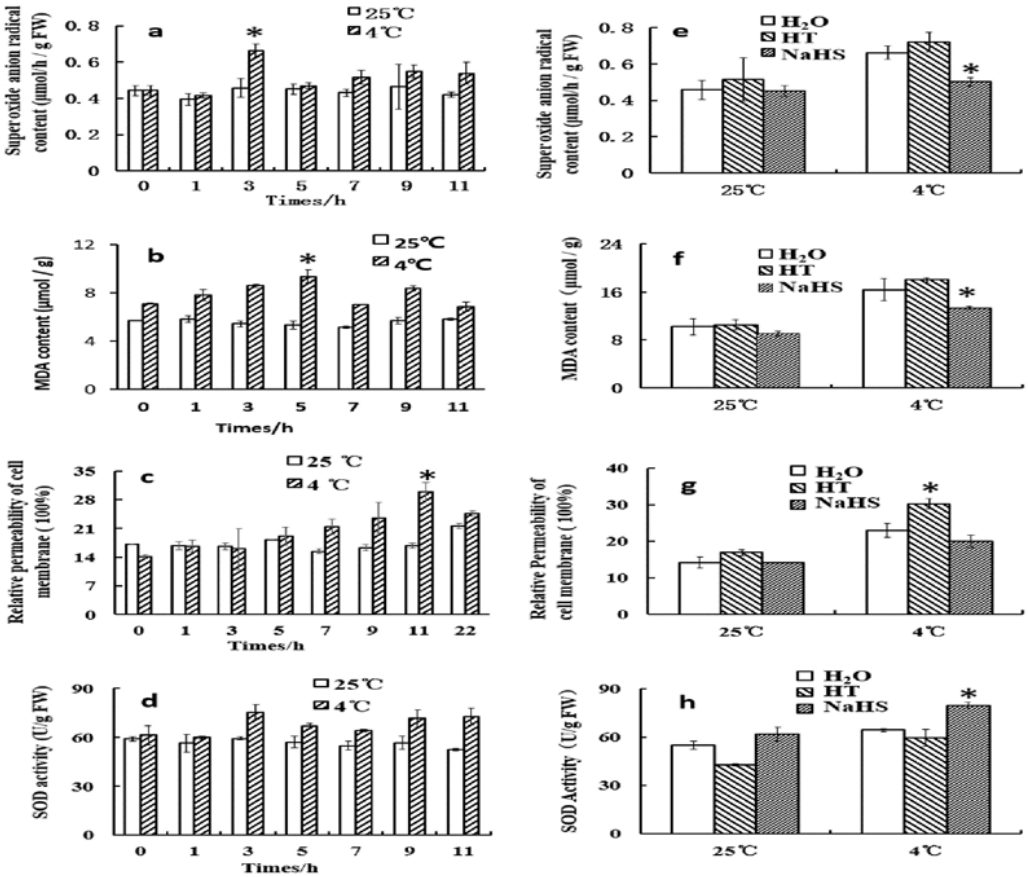

Fig. 5 Effects of low temperature and H_2S on several parameters involved in cold tolerance in *V. vinifera* L. 'F-242'. The values represent the average of three independent samples. **a** Effect of low temperature on superoxide anion radical content. **b** Effect of low temperature on MDA content. **c** Effect of temperature on the relative permeability of cell membrane. **d** Effect of low temperature on SOD activity. **e** Effect of NaHS and HT on superoxide anion radical content. **f** Effect of NaHS and HT on MDA content. **g** Effect of NaHS and HT on the relative permeability of cell membrane. **h** Effect of NaHS and HT on SOD activity. Error bars indicate ±*SE*. * indicates significant differences at $P < 0.05$ (Student's *t*-test).

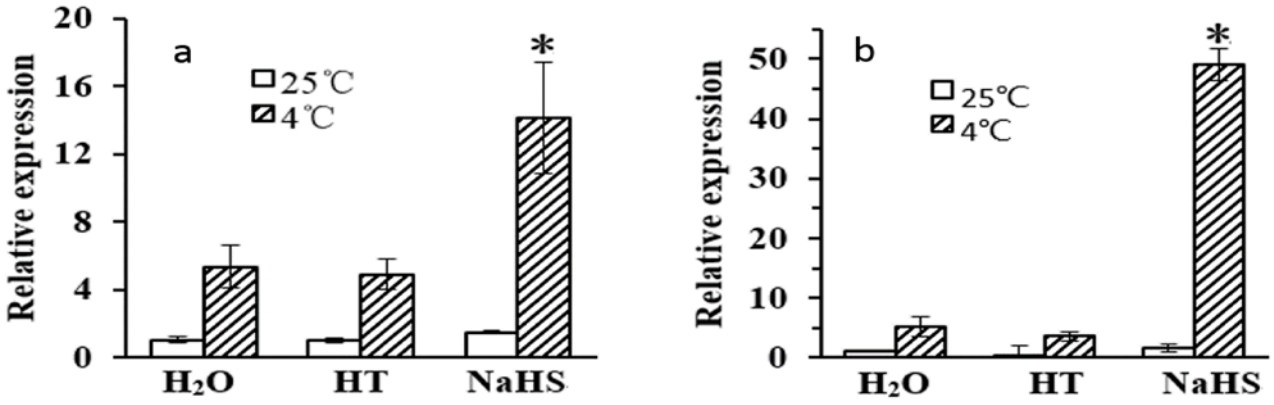

Fig. 6 Effects of HT and NaHS on *VvICE1* (**a**) and *VvCBF3* (**b**) expression patterns under chilling stress in *V. vinifera* L. 'F-242'. The relative expression of *VvICE1* and *VvCBF3* was quantified in comparison with the *VvACTIN* using quantitative RT-PCR with gene-specific primers (see "Material and methods"). The values represent the average of three independent samples. Error bars indicate ±*SE*. * indicates significant differences at $P < 0.05$ (Student's *t*-test).

content, the relative permeability of the plasma membranes and SOD activity in NaHS- and HT- pretreated grape seedlings under low temperature. The results demonstrated that H_2S participated in the response of grape to chilling by protecting membrane integrity and enhancing SOD activities related to cold resistance. Meanwhile, the study of Li et al. shows that spraying NaHS improves heat tolerance in maize by alleviating the increase of electrolyte leakage and MDA [39]. In addition, Zhang and his colleagues indicate that spraying NaHS delays excessive accumulation of MDA and reactive oxygen species, but enhance SOD activity against cooper stress and drought-induced oxidative stress in wheat seeds and soybean seedlings [11,15]. All these findings suggest that H_2S possibly use the similar mechanism to resist various stresses in different plants.

Transcription factors play important roles in chilling stress response in plants. For instance, the expression levels of *ICE1* and *CBF3* can be enhanced in different freezing temperatures [40]. Ectopically expression of *AtCBFs* and *AtICE1* in different plant species can enhance chilling tolerance [41,42], and ectopically expression of *CBFs* from other plants is able to enhance the freezing tolerance of transgenic *Arabidopsis* [20,43]. In the present work, it is shown that the transcription levels of *VvICE1* and *VvCBF3* were induced greatly and reached their peak at 7 h and 3 d, respectively, under low temperature in 'F-242' (data not shown). In addition, NaHS significantly induced *VvICE1* and *VvCBF3* at low temperature. It indicates that H_2S is involved in grapevine chilling stress response by modulating *VvICE1* and *VvCBF3* transcription. Recently, it is shown that *DREB1/CBF3* and *RD29A* (also known as *COR78* or *LTI78*, a target gene of *CBF3*) can be induced by both cold and drought in plants [44–46]. In addition, H_2S up-regulates the expression of *RD29A* to improve drought resistance in *Arabidopsis* [13]. We guess from these results that, in plants, the genes regulated by H_2S could participate not only in drought stress but also in cold stress.

To further investigate the physiological function and regulatory mechanism of H_2S in response to low temperature, some work could be conducted, such as grape genetic transformation, RNAi technology to generate the *VvL-CD* and *VvD-CD* mutants and transgenic grape plants for the genetic controls of H_2S responses to low temperature in the future studies.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: conceived and designed the study: PF, XL; performed the experiments: PF, WW; analyzed the experimental data: PF, XL, WW, LH; wrote the paper: PF, WW; reviewed and edited the manuscript: PF, XL.

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