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ORIGINAL ARTICLE

Auxin acts as a downstream signaling molecule involved in hydrogen sulfide-induced chilling tolerance in cucumber

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Abstract

Main conclusion **This report proves a cross talk between H_2S and IAA in cold stress response, which has presented strong evidence that IAA acts as a downstream signal mediating the H_2S-induced stress tolerance in cucumber seedlings.**

Abstract We evaluated changes in endogenous hydrogen sulfide (H_2S) and indole-3-acetic acid (IAA) emission systems, and the interactive effect of exogenous H_2S and IAA on chilling tolerance in cucumber seedlings. The results showed that chilling stress increased the activity and relative mRNA expression of L-/D-cysteine desulfhydrase (L-/D-CD), which in turn induced the accumulation of endogenous H_2S. Similarly, the endogenous IAA system was triggered by chilling stress. We found that 1.0 mM sodium hydrosulfide (NaHS, an H_2S donor) significantly enhanced the activity of flavin monooxygenase (FMO) and relative expression of FMO-like proteins (*YUCCA2*), which in turn elevated endogenous IAA levels in cucumber seedlings. However, IAA had little effects on activities of L-/D-CD and endogenous H_2S levels. H_2S-induced IAA production accompanied by increase in chilling tolerance, as shown by the decrease in stress-induced electrolyte leakage (EL) and reactive oxygen species (ROS) accumulation, and increase in gene expressions and enzyme activities of photosynthesis. 1-naphthylphthalamic acid (NPA, an IAA polar transport inhibitor) declined H_2S-induced chilling tolerance and defense genes' expression. However, scavenging of H_2S had a little effect on IAA-induced chilling tolerance. These results suggest that IAA acting as a downstream signaling molecule is involved in the H_2S-induced chilling tolerance in cucumber seedlings.

Keywords Antioxidant system · Chilling stress · Photosynthesis · Reactive oxygen · Signal pathway

Abbreviations

AsA	Ascorbic acid
Asat	Light-saturated photosynthetic rate
CBF	C-repeat-binding factor
COR	Cold responsive
DHA	Dehydroascorbic acid
EL	Electrolyte leakage
FMO	Flavin monooxygenase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HT	Hydroxylamine
ICE	Inducer of CBF expression
Jmax	Maximum regeneration rate of Ribulose-1, 5-bisphosphate carboxylase/oxygenase
L-/D-CD	L-/D-cysteine desulfhydrase
NPA	1-Naphthylphthalamic acid

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Introduction

Cucumbers (*Cucumis sativus L.*) are sensitive to chilling stress, but they commonly encounter chilling stress, because they are mainly cultivated through the winter in solar greenhouses in northern China (Wu et al. 2016). Chilling stress causes many changes in the biochemical and physiological processes and reactive oxygen species (ROS) homoeostasis of plants (Siddiqui et al. 2011) and, therefore, may be a major limitation to crop productivity. Many applications were proposed to alleviate chilling injury symptoms, such as hydrogen sulfide (H_2S), salicylic acid, and brassinosteroids (Chen et al. 2011b; Luo et al. 2015; Fang et al. 2019). These researches support us to speculate that approaches which will reduce ROS accumulation or sustain the photosynthetic function might be applied to ameliorate chilling injury.

H_2S is part of a suite of small reactive molecules which are known to be involved in cell signaling events in plants (Hancock 2019). It is produced by cells, can move around, including through membranes, and can be removed when no longer needed (Hancock 2019). H_2S participates in various physiological processes in plants, such as plant development, and biotic and abiotic stress resistance through crosstalk with hormones, ROS, and some other molecular signals (Cheng et al. 2013; Scuffi et al. 2014; Jin and Pei 2015; Qiao et al. 2016). Stuiver et al. (1992) investigated cold tolerance in wheat seedlings, and found that H_2S altered the cellular levels of amino acids and sugars. They also measured water-soluble sulfhydryl content which was primarily glutathione and found that this too increased following H_2S fumigation (Hancock 2019). Luo et al. (2015) found that H_2S can alleviate chilling stress in banana through enhancement of the antioxidant system and the activity of Δ_1-pyrroline-5-carboxylate synthetase (P5CS), mainly attributed to the elevation in proline content. More recently, H_2S was proved to increase cold stress resistance through MPK4 in *Arabidopsis*, resulting in a better adaptability to the environment (Du et al. 2017). Our results also showed that sodium hydrosulfide (NaHS), a H_2S donor could alleviate chilling stress by enhancing the antioxidant system in cucumber seedlings, which might have interact with nitric oxide (NO) and Ca^{2+} signaling (Wu et al. 2016, 2017).

Auxin, mainly for indole-3-acetic acid (IAA), has long been recognized as a major regulator of plant growth and development, especially for the regeneration of root systems (Fang et al. 2014). It is also involved in plant responses to abiotic tolerance (Zörb et al. 2013; Ke et al. 2015; Jung et al. 2015; Bashri and Prasad 2016). For example, auxin content is reduced under drought conditions (Wang et al. 2008) and the transcript expression of genes in the biosynthesis of IAA, as well as some auxin-responsive genes including *Aux/IAAs*, is also affected by drought treatment (Jain and Khurana 2009; Park et al. 2013). Shi et al. (2014) found that auxin promoted the drought tolerance through the regulation of root architecture, expression of abscisic acid (ABA)-responsive genes, metabolism of ROS, and metabolic homeostasis. IAA can induce tolerance to sodic alkaline stress in cucumber plants, which depends on its roles in regulating ROS scavenging, antioxidant enzymes activities, Na^+ accumulation, and protecting photosystem II (PSII) from damage, and NO, as a downstream signal, was required during this process (Gong et al. 2014). There have been some reports about the interaction between H_2S and IAA Riemenschneider et al. (2005) found that H_2S, as a signaling molecular, regulated the sulfydryl level, and promoted genesis of adventitious roots in sweet potato and willow plants. In this process, H_2S might play a role in the upstream in IAA signal pathway (Zhang et al. 2009; García-Mata and Lamattina 2010).

To our knowledge, there were no information which has been provided about relationships between H_2S and IAA under abiotic stress in plants. However, some shared physiological functions of H_2S and IAA were found in their regulation of plant tolerance to stress conditions, especially both were involved in ROS signal pathways in plants (Rahman 2013; Li et al. 2015; Sharma et al. 2018; Hancock 2019). Therefore, we presumed that they might have an interactive effect on chilling tolerance of cucumber seedlings. In the present study, we focused on the roles of H_2S and IAA in regulating chilling-induced oxidative stress and photosynthetic carbon assimilation under chilling stress. The objective is to illustrate the mechanism of the positive effects of H_2S and its signaling pathways in the response of cucumbers to chilling stress.

Materials and methods

Plant materials and growth condition

Cucumber (*Cucumis sativus* L. Jinyou 35) seeds (bought from Tianjin Kerun Cucumber Research Institute, Tianjin, China) were soaked in distilled water for 6 h, and then sowed on four layers of moist filter papers in trays with covers and germinated in the dark at 28 °C for 24 h. The germinated seedlings were transferred to a climate chamber with a photon flux density (PFD) of 600 μmol m^{-2} s^{-1}, a 26 °C /18 °C thermo-period, 80% relative humidity, and an 11-h photoperiod. Two leaf stage seedlings were used for subsequent experiments.

Chilling and H_2S or IAA treatment

All experiments were carried out in environmentally controlled growth chambers with white light of an 11-h photoperiod, and a PFD of 100 μmol m^{-2} s^{-1}. For chilling stress

treatment, the seedlings were treated at 5 °C for 120 h. To determine the effect of H_2S and IAA on chilling tolerance of cucumber seedlings, the seedlings with unanimous growth were foliar sprayed with 0 (control), 0.5, 1.0, 1.5, 2.0, and 2.5 mM NaHS respectively, or pre-treated with 0 (control), 25, 50, 75, and 100 μM IAA respectively (10 ml per plant) 24 h before the chilling treatment. Leaf samples were collected immediately at the end of the treatment to determine the electrolyte leakage (EL) and chilling injury index. To analyze interaction between H_2S and IAA, the seedlings were pre-treated with 1.0 mM NaHS, 75 μM IAA, 0.15 mM hydroxylamine (HT, a specific scavenger of H_2S), 50 μM 1-naphthylphthalamic acid (NPA, a polar transport inhibitor of IAA), 0.15 mM HT + 75 μM IAA, 50 μM NPA + 1.0 mM NaHS, or deionized water (control) 24 h before chilling stress. Leaf samples were collected within 120 h after the chilling stress treatment to assay the H_2S- or IAA-emission system, accumulation of ROS, gene expression and the activity of antioxidant enzymes and the key enzymes in Calvin–Benson cycle. The deionized water treatment under chilling stress was marked as the H_2O treatment to distinguish the control at normal condition.

Detection of H_2S content and L-/D-CD activity

H_2S content was determined as described by Sekiya et al. (1982) and modified by Wu et al. (2016). 0.5 g leaves samples were ground into powder and homogenized in 1.0 ml pre-chilled extraction medium (20 mM Tris–HCl buffer, pH 8.0), followed by centrifuged at 15,000 g for 15 min at 4 °C. The homogenate was mixed with 20 mM Tris–HCl buffer (pH 8.0) in a test tube to release H_2S, and H_2S was absorbed in 1% (w/v) zinc acetate (0.5 ml) trap which was located in the bottom of the test tube. After 30 min reaction, 100 μl 30 mM $FeCl_3$ dissolved in 1.2 M HCl was added to the trap, and then 100 μl 20 mM N, *N*-dimethyl-*p*-phenylenediamine-dihydrochloride dissolved in 7.2 M HCl was injected into the trap. The amount of H_2S in the zinc acetate trap was colorimetric determined at 667 nm after incubation at 37 °C for 15 min. L-/D-CD activity was estimated by determining the production rate of H_2S according to Riemenschneider et al. (2005). Cellular H_2S was visualized at the sub-cellular level using the H_2S fluorescent probe 7-Azido-4-Methylcoumarin (AzMC; Sigma) for localization by Mei et al (2017) with minor modification. The leaf discs (0.6 cm in diameter) were infiltrated directly with 20 μM H_2S fluorescent probe in 20 mM HEPES–NaOH buffer (pH 7.5) and incubated at 25 °C in the dark for 30 min. After fixation, the tissues were rinsed three times for 15 min with the HEPES–NaOH buffer. The blue coloration fluorescent was formed in the presence of H_2S and was visible by inverted fluorescence microscope (Leica DMi8; Leica, Wetzlar, Germany).

Determination of IAA content and FMO activity

The IAA content was determined using high-performance liquid chromatography-triple quadrupole mass spectrometry (HPLC–MS, Thermo Fisher Scientific, TSQ Quantum Access) as described by Li et al. (2014). 0.3 g of each freeze-dried leaf sample was ground with 5 ml of 80% methanol [containing 30 μg ml^{-1} sodium diethyldithiocarbamate (DDTC)] and extracted by the ultrasonic method for 20 min, followed by standing overnight in darkness at 4 °C for 16 h. The homogenates were centrifuged at 7155*g* for 10 min at 4 °C. The resulting supernatants were dried in vacuo at 38 °C using a rotary evaporator (Shanghai EYELA, N-1210B). Solid residues were dissolved in 0.4 mM chloroform, and standing for 30 min to remove pigment, and then purified further by polyvinylpolypyrrolidone (PVPP), followed by centrifugation at 7155 g for 10 min. Afterwards, the supernatants were re-extracted three times with ethyl acetate for chromatography, and the ester phase was dried in vacuo at 36 °C using a rotary evaporator. Solid residue was dissolved in 1.0 ml mobile phases (methanol: 0.04% acetic acid = 45:55, v/v), and the filtrate was used for the HPLC–MS analysis.

The separation was performed on a Hypersil Gold C_{18} column (Thermo Fisher Scientific, 100 × 2.1 mm, 1.9 μm) using methanol and 0.04% acetic acid as mobile phases, at column temperature of 30 °C, and flow velocity of 0.3 ml min^{-1}. The fractions indicating the presence of IAA on the HPLC analysis were further characterized and quantified by MS using triple quadrupole mass spectrometry with electro-spray ionization (ESI) in positive mode. The optimum conditions for MS were 110 °C source temperature, 350 °C desolvation temperature, capillary pressure of 28 kV, flow rates of the desolvent, and cone hole gas (N_2) of 320 l h^{-1} and 50 l h^{-1}, respectively. The standard curve was generated using the IAA standard sample (Sigma-Aldrich), and the IAA content in cucumber leaves was calculated according to the peak area.

Flavin monooxygenase (FMO) activity was measured using an enzyme-linked immunosorbent assay (ELISA) kit of FMO (Jiangsu Meimian Industrial Co. Ltd, Jiangsu, China) according to the instructions. In brief, 50 μl standards, 50 μl samples, and 100 μl horseradish peroxidase (HRP) labeled detection antibody were added into the Microelisa Strip-plate of FMO1 antibody, and incubated at 37 °C for 60 min. Then, each well was aspirated and washed, repeating the process for five times, and any remaining wash solution was removed by aspirating or decanting. Afterwards, the chromogen solution was added to each well, and incubated at 37 °C for 15 min**.** The reaction was terminated by the addition of a sulfuric acid solution and the absorbance was measured using a microplate reader at 450 nm, and the FMO activity was calculated through standard curve.

Measurement of EL and chilling injury index

EL was estimated as described by Dong et al. (2013). 0.2 g of each sample was incubated at 25 °C in 25 ml test tubes containing 20 ml deionized water. Electrical conductivity of the bathing solution was measured at 3 h (EC_1) of incubation using a conductivity meter (Shanghai, China). The samples were then autoclaved (100 °C) for 10 min and electrical conductivity (EC_2) of bathing solution was measured after cooling. EL was calculated from the following equation: $E = EC_1/EC_2 \times 100$.

For chilling injury index examination, the seedlings ware graded according to the standard described by Semeniuk et al. (1986), and the chilling injury index was calculated using the following equation: chilling injury index = Σ(plants of different grade × grade)/[total plants × 5 (the maximum grade)].

Quantification, histochemical analysis of ROS

Hydrogen peroxide (H_2O_2) was extracted from 0.5 g leaf samples and determined with the H_2O_2 kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instruction. The superoxide anion ($O_2^{\cdot -}$) production rate was measured as described by Wang (1990). Cellular H_2O_2 was visualized at the sub-cellular level using the H_2O_2 fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (MCE, Cat. No. HY-D0940, Shanghai, China) for localization described by Galluzzi and Kroemer (2014). The leaf discs (0.6 cm in diameter) were infiltrated directly with 20 µM H_2O_2 fluorescent probe in 20 mM HEPES–NaOH buffer (pH 7.5) and incubated at 25 °C in the dark for 30 min. After fixation, the tissues were rinsed three times for 15 min with the HEPES–NaOH buffer. The green coloration fluorescent is formed in the presence of H_2O_2 and are visible by inverted fluorescence microscope (Leica DMi8). Cellular superoxide anion ($O_2^{\cdot -}$) was also measured with dihydroethidium (DHE) (Fluorescence Biotechnology Co. Ltd, Cat. No. 15200, Beijing, China) according to Galluzzi and Kroemer's description. The leaf discs (0.6 cm in diameter) were infiltrated directly with 10 µM DHE in 10 mM Tris–Hcl buffer (pH 7.5) and incubated at 37 °C in the dark for 30 min. After fixation, the tissues were rinsed two times for 15 min with the Tris–HCl buffer. The coloration fluorescent was formed in the presence of H_2O_2 (excitation at 488 nm and emission at 522 nm), and $O_2^{\cdot -}$ (excitation at 490 nm and emission at 520 nm) were visualized by inverted fluorescence microscope (Leica DMi8; Leica, Germany).

Activity of antioxidant enzymes assay

0.5 g of each leaf sample was ground with 3 ml of ice-cold 50 mM phosphate buffer (pH 7.8) containing 0.2 mM EDTA, 2% [w/v] PVP and 1% Triton X-100. The homogenates were centrifuged at 12,000*g* for 20 min at 4 °C. The resulting supernatants were used to determine the activities of antioxidant enzymes (Cho and Park 2000). The superoxide dismutase (SOD) activity was determined according to Beyer and Fridovich's (1987), and inhibiting 50% photochemical reduction was used as one unit of enzyme activity. The peroxidase (POD) activity was assayed using the method of Omran (1980), and the activity was expressed by the absorbance changes at 470 nm within 1 min. The ascorbate peroxidase (APX) activity was measured as described by Nakano and Asada (1987); the activity was expressed by the changes in absorbance at 290 nm in 1 min. The glutathione reductase (GR) activity was detected according to the method of Foyer and Halliwell (1976), and the activity was expressed by absorbance changes at 340 nm in 1 min.

Detection of the glutathione and ascorbic acid contents

The reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were measured using the glutathione content kit (Nanjing Jiancheng Bioengineering Institute) according to the instruction. The ascorbic acid (AsA) and dehydroascorbic acid (DHA) contents were determined as described by Law et al. (1983).

Measurements of gas-exchange parameters and photosynthetic enzymes' activity

The gas-exchange parameters were measured for the second apical leaves using a portable photosynthetic system (Ciras-3, PP-systems International, Hitchin, Hertfordshire, UK). Constant photon flux density (PFD, 800 µmol m^{-2} s^{-1}), CO_2 concentration (360–380 mg l^{-1}), and leaf temperature (25 ± 1 °C) were maintained throughout all measurements. Each measurement was repeated three-to-five times. We assessed the response curves of photosynthetic rate (Pn) to PFD and Pn to CO_2 concentration, and estimated the maximum regeneration rate of Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Jmax), and the light-saturated photosynthetic rate (Asat), respectively.

Ribulose-1,5-bisphosphatecarboxylase (RuBPCase) activity was measured using an ELISA kit of plant ribulose-1,5-bisphosphate carboxylase/oxygenase (Shanghai Bangyi Biological Technology, Shanghai, China) according to the instructions. Activities of fructose bisphosphatase (FBPase), fructose-1,6-bisphosphate aldolase (FBA), and transketolase (TK) were detected as described by Bi et al. (2015), and

sedoheptulose-1,7-bisphosphatase (SBPase) activity was measured according to Rao and Terry (1989).

RNA extraction and gene expression analysis

Total RNA from cucumber leaves were extracted using an RNA extraction kit (Trizol; Tiangen, Beijing, China) and reverse transcribed using the PrimeScript® RT Master Mix Perfect Real Time (TaKaRa, Dalian, China) according to the instructions. The relative expression of FMO-like proteins (*YUCCA2*), *L-/D-CD*, genes of anti-oxidation and Calvin–Benson cycle enzymes, and cold stress response genes in cucumber seedlings were analyzed by real-time quantitative PCR (RT-q-PCR) using the TransStart® TipTop Green q-PCR SuperMix (Cwbio, Beijing, China), according to the instructions. The β-actin gene of cucumber (Gene ID: Solyc11g005330) was used as an internal reference gene. The primers were designed and synthesized by BGItech, and are shown in Table 1. Amplification was performed with a Light Cycler® 480II system (Roche, Penzberg, Germany).

Statistical analysis

The experimental design was a completely randomized block design. The data are presented as the mean ± the standard deviation (SD) of 3–5 replicates. Analysis of variance (ANOVA) was conducted using the Microsoft Excel

Table 1 Primers sequences

Gene	Primer name	Accession number	Primer pairs
LCD	LCD1	XM_004144690	5′-GGTTCGTCTGGCTGTGATTGATC -3′
	LCD2		5′-GGACCTCCTGGAATACAAGAAAGC -3′
DCD	DCD1	XM_004137337	5′-GTCCTGGGCCTCACACCTTAAT -3′
	DCD2		5′-CACGACAGTGATTGCTTTGGATGC -3′
YUCCA2	YUCCA1	Csa020745	5′-TTCTGTCAACTTCCCAACTTCCCTT -3′
	YUCCA2		5′-GGAAGTTGGGAAGTTGACAGAATTG -3′
SOD	SOD1	NM_001280768	5′-GGAAAGATGTGAAGGCTGTGG -3′
	SOD2		5′-GCACCATGTTGTTTTCCAGCAG -3′
POD	POD1	XM_004151830	5′-GGTTTCTATGCCAAAAGCTGCCC-3′
	POD2		5′-CAGCTTGGTTGTTTGAGGTGGAG-3′
APX	APX1	NM_001280706	5′-GTGCTACCCTGTTGTGAGTG -3′
	APX2		5′-AACAGCGATGTCAAGGCCAT -3′
GR	GR1	NM_001308836	5′-TGATGAGGCTTTGAGTTTAGAGGAG -3′
	GR2		5′-AACTTTGGCACCCATACCATTC -3′
rbcL	rbcL1	EF208123	5′-GCTATGGAATCGAGCCTGTTG-3′
	rbcL2		5′-CCAAATACATTACCCACAATGGAAG-3′
rbcS	rbcS1	EF208124	5′-CGCATTCATCAGGGTTATTGG-3′
	rbcS2		5′-AAGAGTAGAACTTGGGGCTTGTAGG-3′
FBP	FBP1	XM_004140842	5′-ATGATGTGTACAATTGTGAAAG-3′
	FBP2		5′-TTGATAAAGACTTCATTTGAGA-3′
SBP	SBP1	NM_001280729	5′-GTGTCCTCCTCATACTTGGGTTG-3′
	SBP2		5′-GAATGCTGGGAAGAAAGATTGG-3′
FBA	FBA1	XM_011653692	5′-GCAGAGTGAGGAGGAAGCAAC-3′
	FBA2		5′-CCAAACGAGAAAGATAACGACC-3′
TK	TK1	XP_004145002	5′-ACGATGAGGTCATGAAG-3′
	TK2		5′-ACGATGAGGTCATGAAG-3′
CBF1	CBF1-1	XM_004140746	5′-ATGGCTTCATATTGCTCTGAG-3′
	CBF1-2		5′-ATGCCTCAAGTCAATTGCTTG-3′
ICE	ICE1	XM_011653285	5′-CGCATCGAGTTGGCTCTGGTG-3′
	ICE2		5′-GTCCTCATCGCCGTTCATCTTCC-3′
COR	COR1	XM_011659051	5′-CACTTTGAGAGGACATTTGATG-3′
	COR2		5′-AGAAGCTCCAATTTTGACTTG-3′
Actin	Actin1	DQ115883	5′-CCACGAAACTACTTACAACTCCATC -3′
	Actin2		5′-GGGCTGTGATTTCCTTGCTC -3′

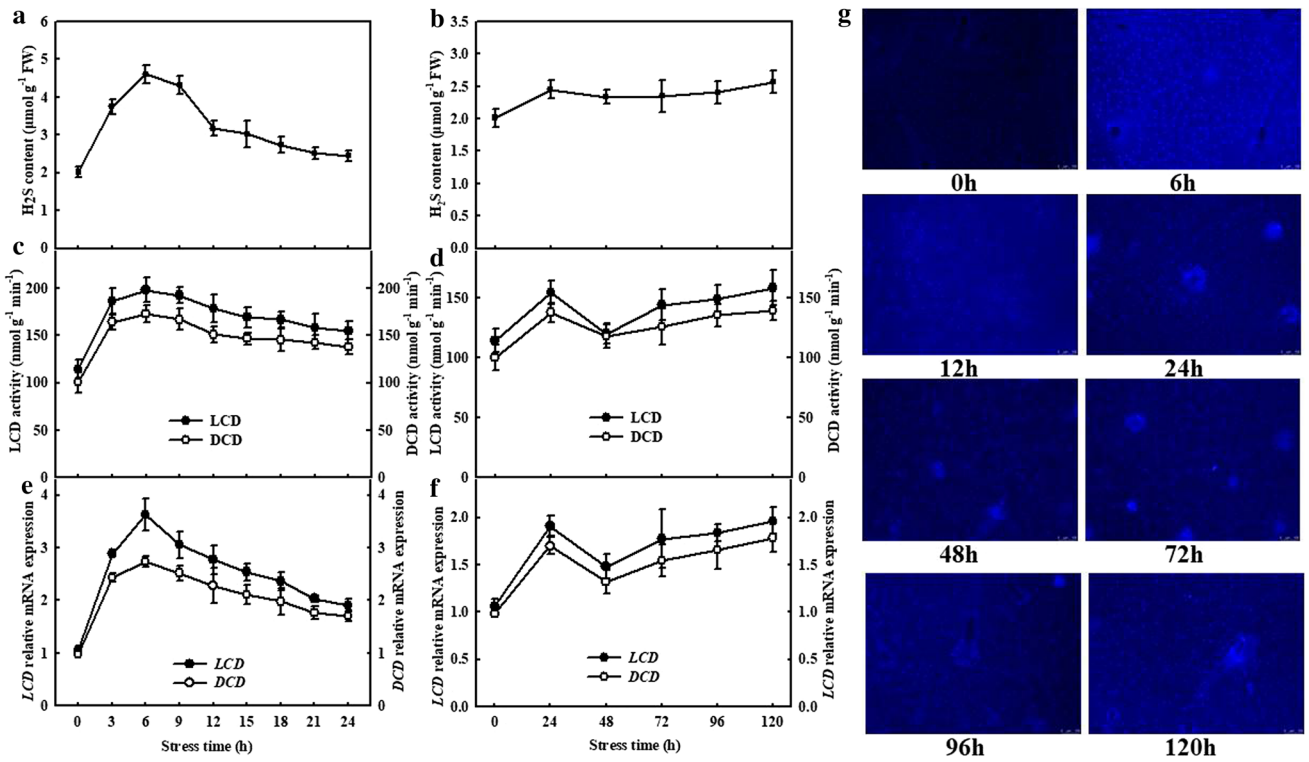

Fig. 1 Response of the H_2S-emission system to chilling stress in short term (within 24 h) and long term (up to 120 h) in cucumber seedlings. **a**, **b** Change of H_2S content during short- and long-term chilling stress, respectively. **c**, **d** Changes of LCD and DCD activities during short- and long-term chilling stress, respectively. The second leaves of cucumber seedlings were sampled for the H_2S concentration and LCD/DCD activities assay. **e**, **f** Changes of relative expression of *LCD* and *DCD* during short- and long-term chilling stress, respectively. Total RNA was separately isolated from the same tissues for the LCD/DCD activities' determination, and subjected to real-time PCR analysis. **g** H_2S inverted fluorescence microscope imaging. Seedlings were treated at 5 °C for 120 h, and sampled every 3 h within the first 24 h, and every day in the subsequently days. All values shown are mean ± SD ($n = 3$)

software. Duncan's multiple range test (DMRT) was applied to analyze differences among treatments.

Results

The response of H_2S and IAA to chilling stress in cucumber seedlings

To explore the changes of endogenous H_2S under chilling stress, we determined the endogenous H_2S content, activities and relative mRNA expressions of LCD and DCD in two-leaf-old cucumber seedlings under chilling stress for 120 h. Chilling stress led to a significant increase in H_2S in cucumber seedlings at the first 6 h but subsequently declined within 48 h (Fig. 1a, g). Afterwards, the H_2S content enhanced gradually by stress time increasing (Fig. 1b, g). The activities (Fig. 1c, d) and relative expressions (Fig. 1e, f) of *LCD* and *DCD* were also increased by chilling stress, and the increase was the most significant after 6 h at 5 °C and followed by a decrease. 48 h later, L-D-CD activities and relative expressions of *LCD* and *DCD* showed the increasing tendency as well (Fig. 1d, f). These data are in agreement with Wu et al. (2016), and demonstrated that H_2S can be induced by chilling stress.

Figure 2 revealed that chilling stress induced greater IAA production in cucumber seedlings. The FMO (the key enzyme for IAA biosynthesis) activity and relative expression of *YUCCA2* also increased by chilling stress in a time-dependent manner. These up-regulated responses were remarkable after 9 h of chilling stress during the first 24 h (Fig. 2a, c). In the subsequent chilling days, the IAA content, FMO activity, and relative expression of *YUCCA2* increased gradually (Fig. 2b, d). These data indicated that the endogenous IAA was involved in the response to chilling stress.

Exogenous H_2S- and IAA-induced chilling tolerance in cucumber seedlings

The two leaf stage cucumber seedlings pre-treated with 0, 0.5, 1.0, 1.5, 2.0, or 2.5 mM NaHS were exposed to chilling stress at 5 °C for 72 h. As shown in Fig. 3, NaHS reduced EL and chilling injury index of cucumber seedlings under chilling stress. The seedlings pre-treated with 1.0 mM NaHS

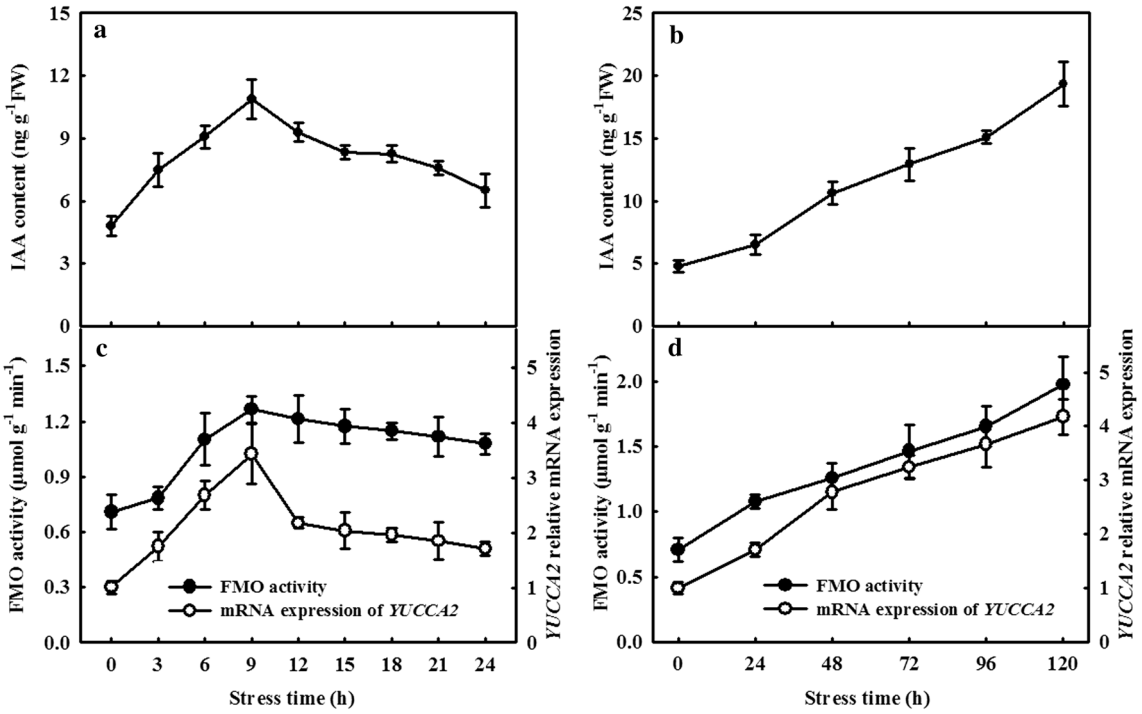

Fig. 2 Response of IAA content, FMO activity, and relative expression of *YUCCA2* to chilling stress in short term (within 24 h) and long term (up to 120 h) in cucumber seedlings. **a**, **b** Change of IAA content during short- and long-term chilling stress, respectively. **c**, **d** Changes of FMO activity and relative mRNA expression of *YUCCA2* during short- and long-term chilling stress, respectively. The second leaves of cucumber seedlings were sampled for the IAA content and FMO activities assay. Total RNA was separately isolated from the same tissues for the FMO activity determination, and subjected to real-time PCR analysis. Seedlings were treated at 5 °C for 120 h, and sampled every 3 h within the first 24 h, and every day in the subsequently days. All values shown are mean ± SD ($n = 3$)

showed the lowest EL and chilling injury index among the six treatments ($P < 0.05$). Similarly, seedlings were foliar sprayed with different concentrations of IAA and subjected to chilling stress. We found that IAA also decreased the EL and chilling injury index of stressed seedlings. Among the five concentrations (0, 25, 50, 75, 100, or 125 μM), the concentration of IAA at 75 μM showed a very significant difference compared with the untreated seedlings ($P < 0.05$). These results illustrated that NaHS and IAA could improve the chilling tolerance of cucumber seedlings in a concentration-dependent manner. Therefore, 1.0 mM NaHS and 75 μM IAA were used in further experiments.

H_2S- and IAA-induced chilling tolerance is associated with increased scavenging activity of ROS

1 mM NaHS and 75 μM IAA significantly alleviated the injury symptoms caused by chilling stress (Fig. 4a), but the alleviation effect of NaHS on chilling injury was blocked by 50 μM NPA. The injury symptom was also aggravated in seedlings treated with 0.15 mM HT + 75 μM IAA than that in IAA-treated seedlings under chilling stress, but to a lesser extent than that in H_2O-treated seedlings. No significant differences were found in injury symptoms among seedlings treated with HT, NPA, and H_2O.

Histochemical observations with inverted fluorescence microscope indicated that H_2O_2 and $O_2^{\cdot-}$ generations were obviously increased in chilling stressed seedlings (Fig. 4b, c). As a result of excess ROS accumulation, lipid peroxidation, which can be known from higher EL, was significantly induced in stressed seedlings (Fig. 4d). Application of NaHS or IAA significantly declined accumulation of H_2O_2 and $O_2^{\cdot-}$, and resulted in lower EL. The decrease in accumulation of H_2O_2 and $O_2^{\cdot-}$ and EL caused by NaHS in stressed seedlings was blocked by NPA, whereas IAA-induced decrease in H_2O_2, $O_2^{\cdot-}$, and EL under chilling stress was not affected by HT. In addition, biochemical analysis for H_2O_2 and superoxide anion $O_2^{\cdot-}$ was in agreement with the fluorescence image.

Chilling stress led to an increase in the activities of superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) in cucumber seedlings (Fig. 5a–d). Both NaHS and IAA treatments showed higher activity of the above enzymes than the H_2O treatment during chilling stress ($P < 0.05$). NPA distinctly repressed NaHS–induced antioxidant activities ($P < 0.05$), whereas HT had little effect on IAA–induced antioxidant

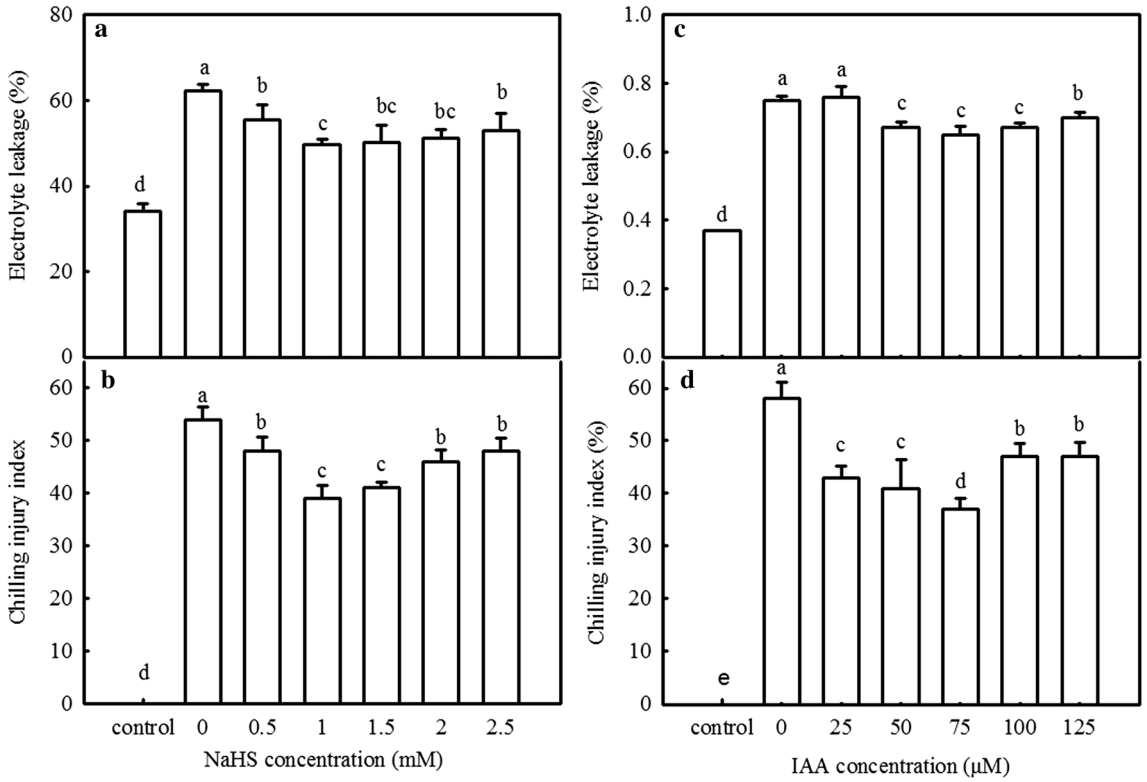

Fig. 3 Effects of NaHS and IAA on chilling tolerance of cucumber seedlings under chilling stress. **a**, **c** Electrolyte leakage. **b**, **d** Chilling injury index. Parts of the two leaf stage cucumber seedlings were foliage sprayed with 0, 0.5, 1.0, 1.5, 2.0, or 2.5 mM NaHS (H_2S donor) solution, respectively. Other parts of seedlings were pre-treated by the same method with 0, 25, 50, 75, 100, or 125 μM IAA solution, respectively, for 24 h, and then, seedlings were exposed to 5 °C for 72 h. All values shown are mean ± SD ($n = 3$). **a**–**e** Mean values are significantly different among samples ($P < 0.05$)

activities. Real-time PCR showed that the relative mRNA expressions of *SOD, POD, APX,* and *GR* were increased by 2.94-, 2.72-, 7.42-, and 0.45-fold, respectively when the seedlings were exposed to 5 °C for 48 h (Fig. 5e–h). They were up-regulated by NaHS or IAA, while down-regulated by HT and NPA ($P < 0.05$). NPA + NaHS-treated seedlings displayed obviously lower relative mRNA expressions of *SOD, POD, APX,* and *GR* than NaHS-treated seedlings ($P < 0.05$), indicating that NPA weakened the positive effect of NaHS in protecting the membrane against oxidative damage caused by chilling stress. However, seedlings treated with HT + IAA showed little differences in relative mRNA expressions of *SOD, POD, APX,* and *GR* compared with IAA-treated seedling under chilling stress.

Since NaHS and IAA significantly increased the activities and gene expression of APX and GR which is vital in ascorbic acid–glutathione recycling, we further determined inter conversions between reduced and oxidized AsA and GSH. AsA + DHA and GSH + GSSG contents were not greatly affected by chilling stress (Fig. 6a, d). However, compared with H_2O treatment, the AsA + DHA content in NaHS and IAA treatments following 48 h stress was increased by 9.5% and 13.1%, respectively. There was no remarkable difference in AsA + DHA content among HT, NPA, HT + IAA, NPA + NaHS, and H_2O treatments. NaHS-, IAA-, HT + NAA-, and NPA + NaHS-treated seedlings also showed obviously higher GSH + GSSG content relative to H_2O-treated seedlings under chilling stress ($P < 0.05$). No differences were observed in GSH + GSSG content among HT-, NPA-, and H_2O-treated seedlings. AsA and GSH contents decreased significantly in H_2O-treated seedlings relative to the control ($P < 0.05$) after 48 h at 5 °C (Fig. 6b, e). NaHS- and IAA-treated tissues had obviously higher AsA and GSH contents than those of the H_2O-treated seedlings ($P < 0.05$). Application of NPA, but not HT, dramatically inhibited there generation of AsA and GSH under chilling stress ($P < 0.05$). Similar trends were also shown in the ratios of AsA/DHA and GSH/GSSG (Fig. 6c, f). These data suggest that either NaHS or IAA can scavenge ROS in cucumber seedlings, and IAA acts as a downstream signal of NaHS to relieve oxidative damage caused by chilling stress.

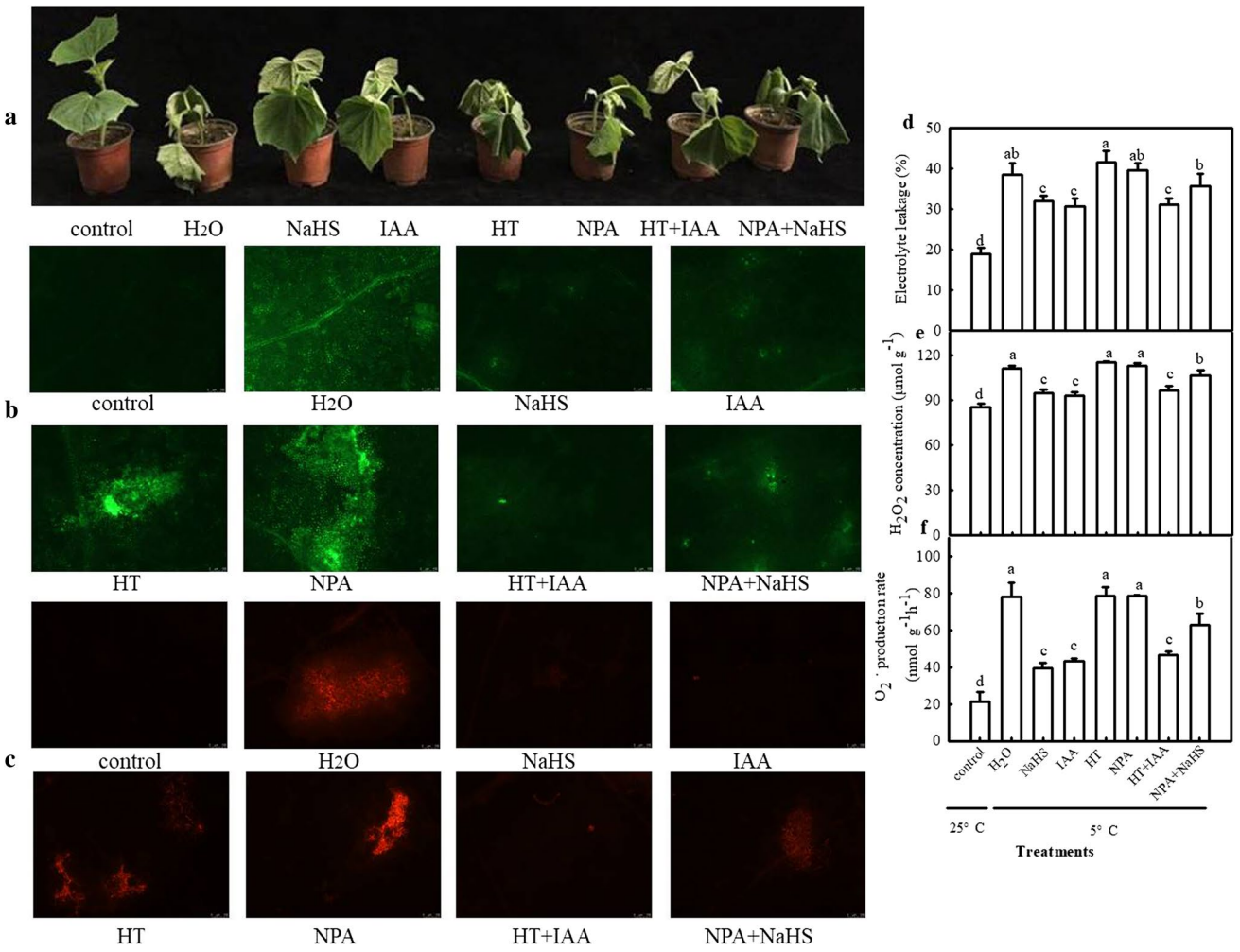

Fig. 4 Interactive effects of NaHS and IAA on ROS and electrolyte leakage of cucumber seedlings under chilling stress. **a** Phenotype of seedling. **b** H_2O_2 inverted fluorescence microscope imaging. **c** $O_2^{\cdot-}$ inverted fluorescence microscope imaging. **d** Electrolyte leakage. **e** H_2O_2 accumulation. **f** $O_2^{\cdot-}$production rate. Two leaf stage seedlings were foliar sprayed with 1.0 mM NaHS (H_2S donor), 0.15 mM HT (H_2S scavenger), 75 μM IAA, 50 μM NPA (a polar transport inhibitor of IAA), or distilled water (control) for 24 h. Then the seedlings were exposed to 5 °C for 48 h. All values shown are mean ± SD ($n = 5$). **a**–**e** Mean values are significantly different among samples ($P < 0.05$)

H_2S and IAA improved photosynthesis in cucumber seedlings under chilling stress

The Pn of cucumber seedlings was significantly reduced ($P < 0.05$) by chilling stress (Fig. 7). The decrease in Pn in cucumber seedlings following 48 h stress was 83.6%, 52.1%, 55.8%, 83.9%, 84.6%, 57.9%, and 78.1% in H_2O, NaHS, IAA, HT, NPA, HT + IAA, and NPA + NaHS treatment, respectively. The seedlings treated with NaHS and IAA showed significantly higher Jmax and Asat relative to H_2O-treated seedlings following exposure to chilling stress for 48 h (Fig. 7b, c). The increase in Jmax and Asat in NaHS-treated seedlings under chilling stress was blocked by NPA, while the IAA-induced higher Jmax and Asat in stressed seedlings was not remarkably affected by HT.

Chilling stress led to significant decrease in activities of RuBPCase, FBPase, SBPase, and TK (Fig. 8a, b, c, e) in cucumber seedlings ($P < 0.05$). However, the FBA activity was enhanced by chilling stress (Fig. 8d). NaHS and IAA significantly increased the activities of the above enzymes in stressed seedlings. Application of NPA distinctly repressed NaHS-treated activities of Calvin–Benson cycle enzymes, but HT had little effect on IAA-induced increase in activities of the key enzymes in Calvin–Benson cycle. In addition,

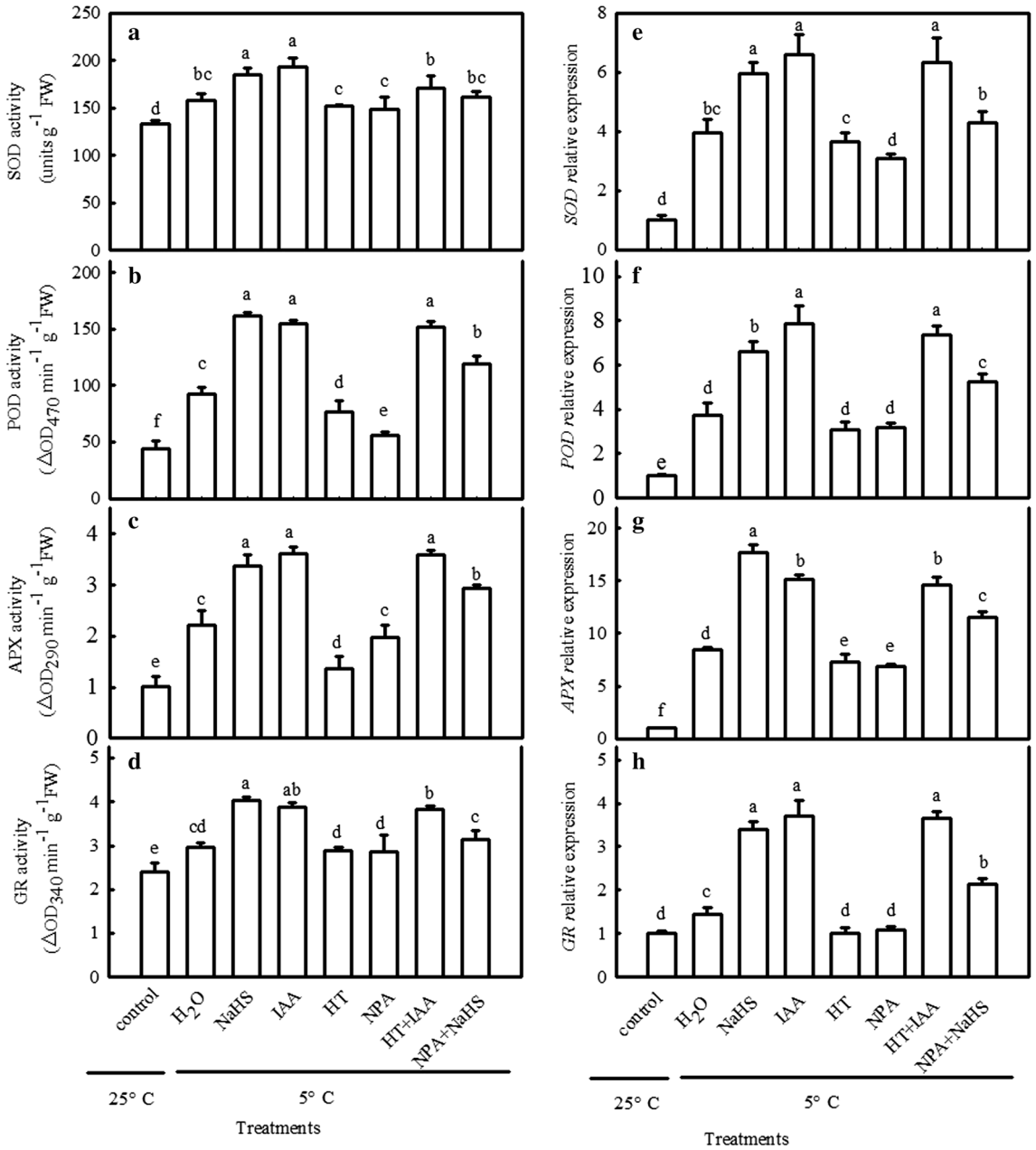

Fig. 5 Interactive effects of NaHS and IAA on activities and relative mRNA expression of antioxidant enzymes in cucumber seedlings under chilling stress. Two leaf stage seedlings grown in solar greenhouse were foliar sprayed with 1.0 mM NaHS (H_2S donor), 0.15 mM HT (H_2S scavenger), 75 μM IAA, 50 μM NPA (a polar transport inhibitor of IAA), or distilled water (control) for 24 h. Then the seedlings were exposed to 5 °C for 48 h. **a**–**d** Activities of superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR). The second leaf was sampled for the activities assay. **e**–**h** Relative mRNA expression of *SOD*, *POD*, *APX*, and *GR*. Total RNA was separately isolated from the same tissues for the activities determination, and subjected to real-time PCR analysis. All values shown are mean ± SD ($n = 3$). **a**–**f** Mean values are significantly different among samples ($P < 0.05$)

the relative mRNA expression of *rbcL, rbcS, FBP, SBP, FBA,* and *TK* in cucumber seedlings showed a good agreement with the activities of RuBPCase, FBPase, SBPase, FBA, and TK, respectively, under chilling stress (Fig. 9). All above results suggested that either H_2S or IAA could relieve the effect of photosynthetic capacity caused by chilling stress. Adding NPA reduced H_2S-induced positive regulation in photosynthesis. However, the application of HT had little effect on IAA-induced regulation in photosynthesis.

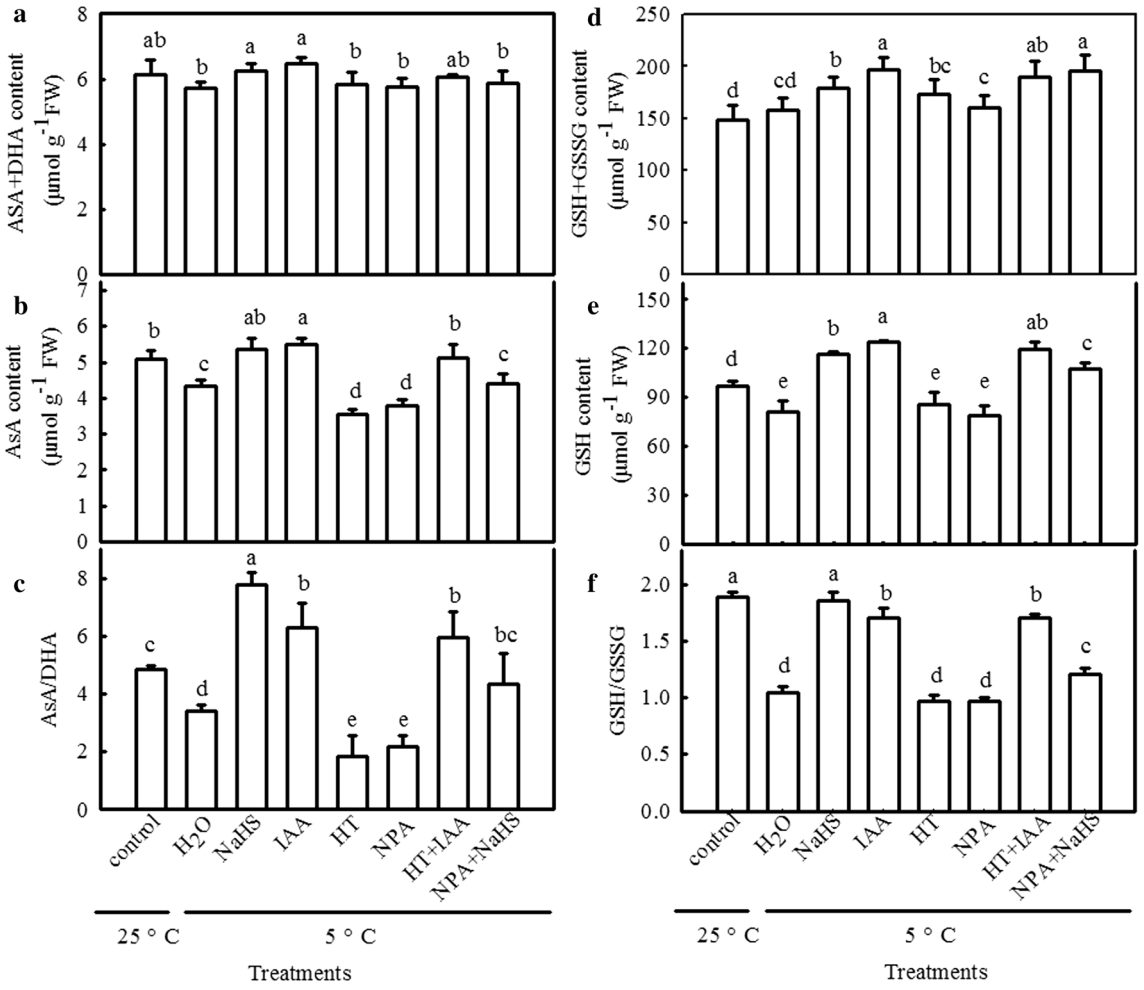

Fig. 6 Interactive effects of NaHS and IAA on AsA, DHA, GSH, and GSSG contents in cucumber seedlings under chilling stress. **a** AsA + DHA. **b** AsA. **c** ASA/DHA ratio. **d** GSH + GSSG. **e** GSH. **f** GSH/GSSG ratio. Two leaf stage seedlings grown in solar greenhouse were foliar sprayed with 1.0 mM NaHS (H_2S donor), 0.15 mM HT (H_2S scavenger), 75 μM IAA, 50 μM NPA (a polar transport inhibitor of IAA), 0.15 mM HT + 75 μM IAA, 50 μM NPA + 1.0 mM NaHS, or deionized water (control) for 24 h, Then, the seedlings were exposed to 5 °C for 48 h. All values shown are mean ± SD ($n = 5$). **a**–**e** Mean values are significantly different among samples ($P < 0.05$)

The interaction between H_2S and IAA in cucumber seedlings

Because both NaHS and IAA reduce chilling stress, we examined the effects of NaHS on the IAA biosynthesis as well as the effects of IAA on the accumulation of H_2S in cucumber seedlings. It was found that 1.0 mM NaHS improved FMO activity in cucumber seedlings ($P < 0.05$), and the improvement was remarkable after 6 h of treatment (Fig. 10). However, 0.15 mM HT treatment showed little variation in FMO activity compared with the control. IAA was also increased by NaHS, but no distinct difference in IAA content was observed between HT and the control seedlings. Figure 11 showed that IAA-treated seedlings displayed no remarkably variation in the activities of L-/D-CD, or H_2S accumulation, and no significant differences were found in the L-/D-CD activities and H_2S content between IAA treatment and the control. These data suggest that H_2S can improve the endogenous IAA by activating FMO activity in cucumber seedlings, whereas, IAA had little effect on H_2S accumulation.

Effects of H_2S and IAA on the expression of cold stress response genes in cucumber seedlings under chilling stress

Currently, the best understood cold acclimation signaling pathway is the ICE1–CBF–COR transcriptional cascade (Shi et al. 2015). Therefore, we examined the effect of H_2S and IAA on the relative mRNA expression of C-repeat-binding factor (*CBF1*), inducer of CBF expression (*ICE*), and cold-responsive (*COR*) gene in cucumber seedlings under chilling

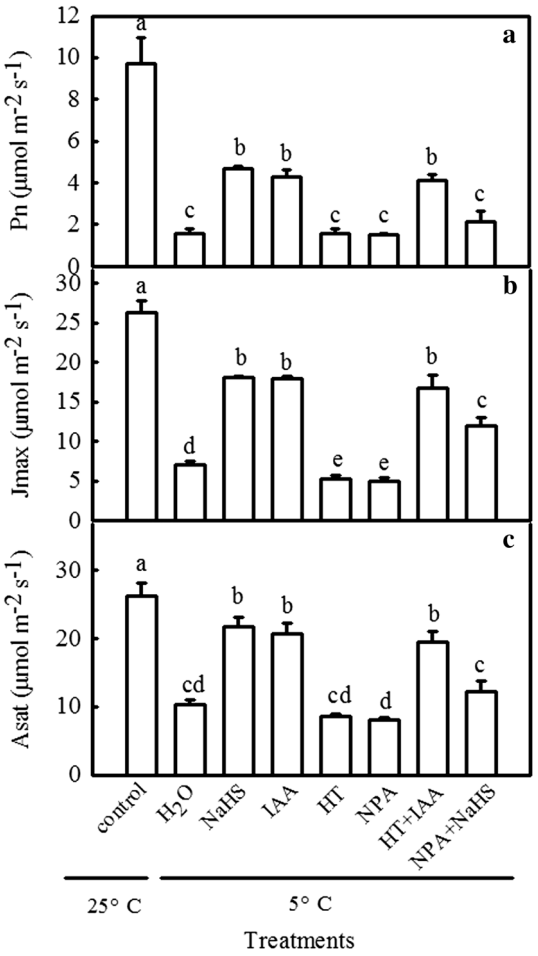

Fig. 7 Interactive effects of NaHS and IAA on the gas-exchange parameters of cucumber seedlings under chilling stress. **a** Photosynthetic rate. **b** Maximum regeneration rate of Rubisco. **c** Light-saturated CO_2 assimilation rate. Two leaf stage seedlings grown in solar greenhouse were foliar sprayed with 1.0 mMNaHS (H_2S donor), 0.15 mM HT (H_2S scavenger), 75 μM IAA, 50 μM NPA (a polar transport inhibitor of IAA), 0.15 mM HT + 75 μM IAA, 50 μM NPA + 1.0 mM NaHS, or deionized water (control) for 24 h Then, the seedlings were exposed to 5 °C for 48 h. All values shown are mean ± SD ($n = 5$). **a**–**e** Mean values are significantly different among samples ($P < 0.05$)

stress. Chilling stress induced the expression of *ICE*, *CBF1,* and *COR* in cucumber seedlings (Fig. 12). Both NaHS and IAA treatments showed higher transcriptional level of these genes than the H_2O treatment during chilling stress ($P < 0.05$). Treatment with NPA significantly weakened the effects of NaHS on activating *ICE–CBF1–COR* signaling pathway. However, application of HT had little influence on the function of IAA in regulating these genes' expression.

Discussion

Previous studies indicated that exogenous H_2S (Jin et al. 2011; Wang et al. 2012; Luo et al. 2015; Zhang et al. 2015) and IAA (Zörb et al. 2013; Gong et al. 2014; Ke et al. 2015; Jung et al. 2015; Bashri and Prasad 2016) treatments could enhance stress tolerance in a diversity of plant species. However, none studies about their interaction in any stresses were performed. The present study showed that both the endogenous H_2S emission system and IAA signaling were activated by chilling stress in cucumber seedlings (Figs. 1, 2). Chilling stress inhibited photosynthesis and serious oxidative damage in cucumber seedlings, but this deleterious effect could be alleviated by exogenous NaHS or IAA (Figs. 3, 4). Adding NPA reduced H_2S-driven alleviation of oxidative damage by chilling stress. However, IAA- induced chilling stress tolerance did not affected by HT. By examining physiological strategies, antioxidant system, photosynthetic parameters, and the ICE–CBF–COR pathway, we speculate that IAA, as a downstream signal, plays a vital role in H_2S-induced chilling tolerance in cucumber seedlings. Although the *YUCCA2* expression and IAA content significantly increased in cucumber seedlings at 120 h long-term chilling stress, the growth was significantly inhibited (data not shown). This implied that the growth reduction of cucumber seedlings was mainly due to the decrease in photosynthetic capacity caused by chilling stress (Figs. 7, 8, 9). In addition, Shibasaki et al. (2009) reported that cold stress induced the ectopic accumulation of auxin in the root meristem of *Arabidopsis*, and this accumulation of auxin in the meristem was due to the inhibition of basipetal polar transport of auxin. Rahman et al. (2007) considered that the accumulation of IAA in the *Arabidopsis thaliana* root inhibited elongation by affecting the residence time of the cells in the elongation zone. Based on these previous findings, we speculate that IAA accumulation in the leaves may be due to the inhibition of polar transport of IAA in long-term chilling stress. The increased concentration of IAA may also inhibit growth of cucumber seedlings; however, it still needs further verification through much more experiments.

LCD and DCD are the most important enzymes involved in the respective decomposition of L-cysteine and D-cysteine into H_2S in plants (Harrington and Smith 1980; Nagasawa et al. 1985). Fang et al. (2014) considered that H_2S production rate directly reflected the intensity of enzymatic activity of H_2S-generating proteins, and it would take time for transcribed mRNA to be translated into protein following posttranslational modification to have complete activity. Therefore, H_2S production rate significantly reduced at 36 h and 48 h of Cr^{6+} exposure, indicative of a delayed response to the reduced gene expression of LCD and DCD at 24 h of Cr^{6+} exposure (Fang et al. 2014). In the present study, the

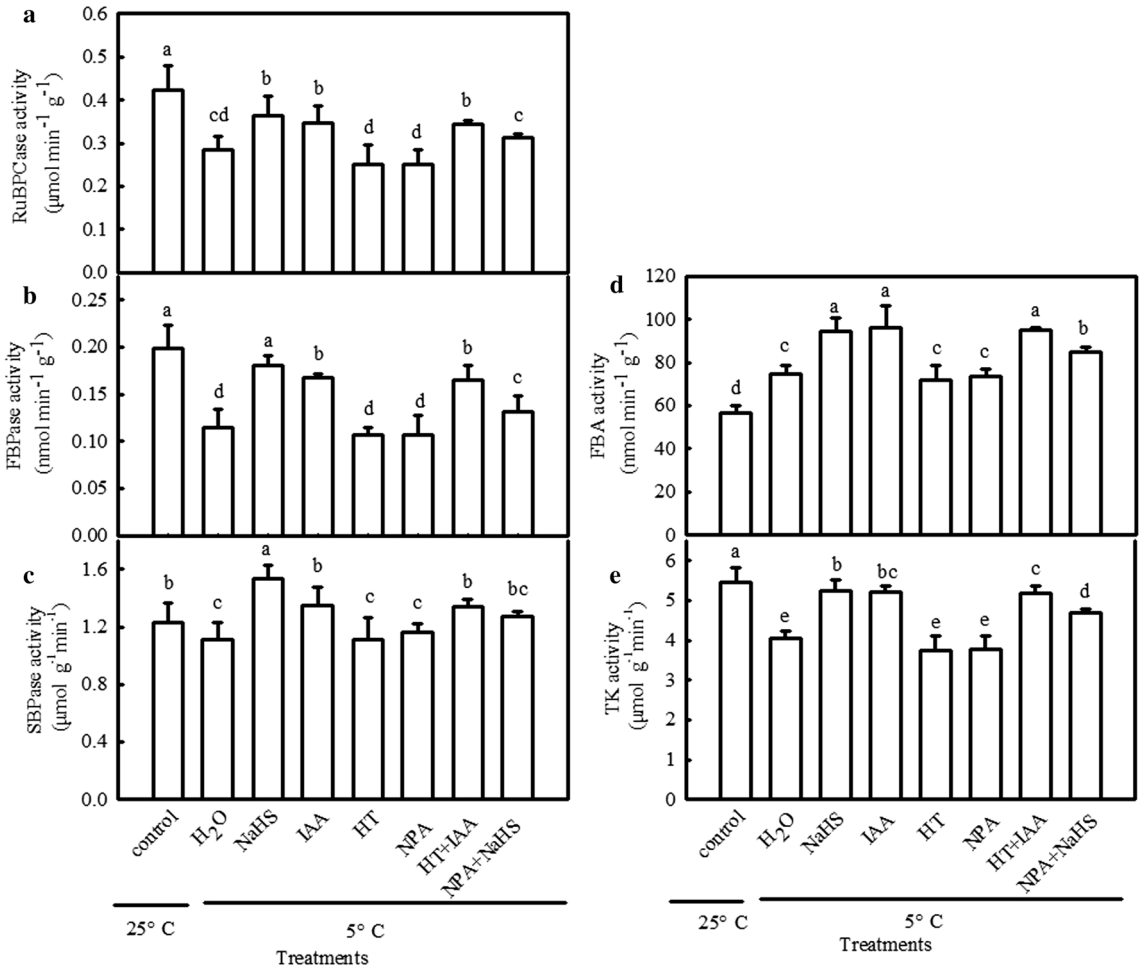

Fig. 8 Interactive effects of NaHS and IAA on the key enzymes in Calvin–Benson cycle of cucumber seedlings under chilling stress. Two leaf stage seedlings grown in solar greenhouse were foliar sprayed with 1.0 mM NaHS (H_2S donor), 0.15 mM HT (H_2S scavenger), 75 μM IAA, 50 μM NPA (a polar transport inhibitor of IAA), 0.15 mM HT + 75 μM IAA, 50 μM NPA + 1.0 mM NaHS, or deionized water (control) for 24 h. Then, the seedlings were exposed to 5 °C for 48 h. All values shown are mean ± SD ($n = 5$). **a**–**e** Mean values are significantly different among samples ($P < 0.05$)

H_2S concentration, L-/D-CD activities, and relative mRNA expression of *LCD* and *DCD* significantly increased during the first 6 h of chilling stress, followed by a decrease (Fig. 1). These data are not consistent with Fang et al. (2014), but in agreement with Wu et al. (2016) who reported that H_2S content in cucumber seedlings followed the same trend as the activity and gene expression of LCD and DCD under chilling stress. The possible reason for the different results is that the H_2S emission system enable quick response to chilling stress in cucumber, a most cold-sensitive plant. The IAA content in cucumber seedlings also presented the same trends as FMO activity and relative expression of *YUCCA2*, which increased from 0 to 9 h under chilling stress, and subsequently decreased (Fig. 2). Based on these results, we further suggest that IAA may as a downstream signal involved in H_2S-induced chilling tolerance in cucumber seedlings.

It is generally considered that dysfunction of cell membrane and excess production of ROS are two primary events involved in chilling injury development (Ben-Amor et al. 1999; Chongchatuporn et al. 2013). Previous studies have proven that H_2S and IAA act as antioxidative signaling molecules to cope with abiotic stress through reduction of ROS biosynthesis and promotion of antioxidative enzyme activity (Iglesias et al. 2010; Zhang et al. 2010; Christou et al. 2013). We demonstrated that chilling stress induced the burst of H_2O_2, $O_2^{\cdot-}$, and increased EL, and then caused ROS-associated damage in the cucumber seedlings. Application of NaHS and IAA significantly decreased ROS-induced lipid peroxidation in cucumber seedlings under chilling stress (Figs. 3, 4). Moreover, NaHS and IAA also increased activities of ROS scavenging enzymes of SOD, POD, APX, and GR under chilling stress (Figs. 5, 6). This may better protect the membrane of cellular and sub-cellular structures.

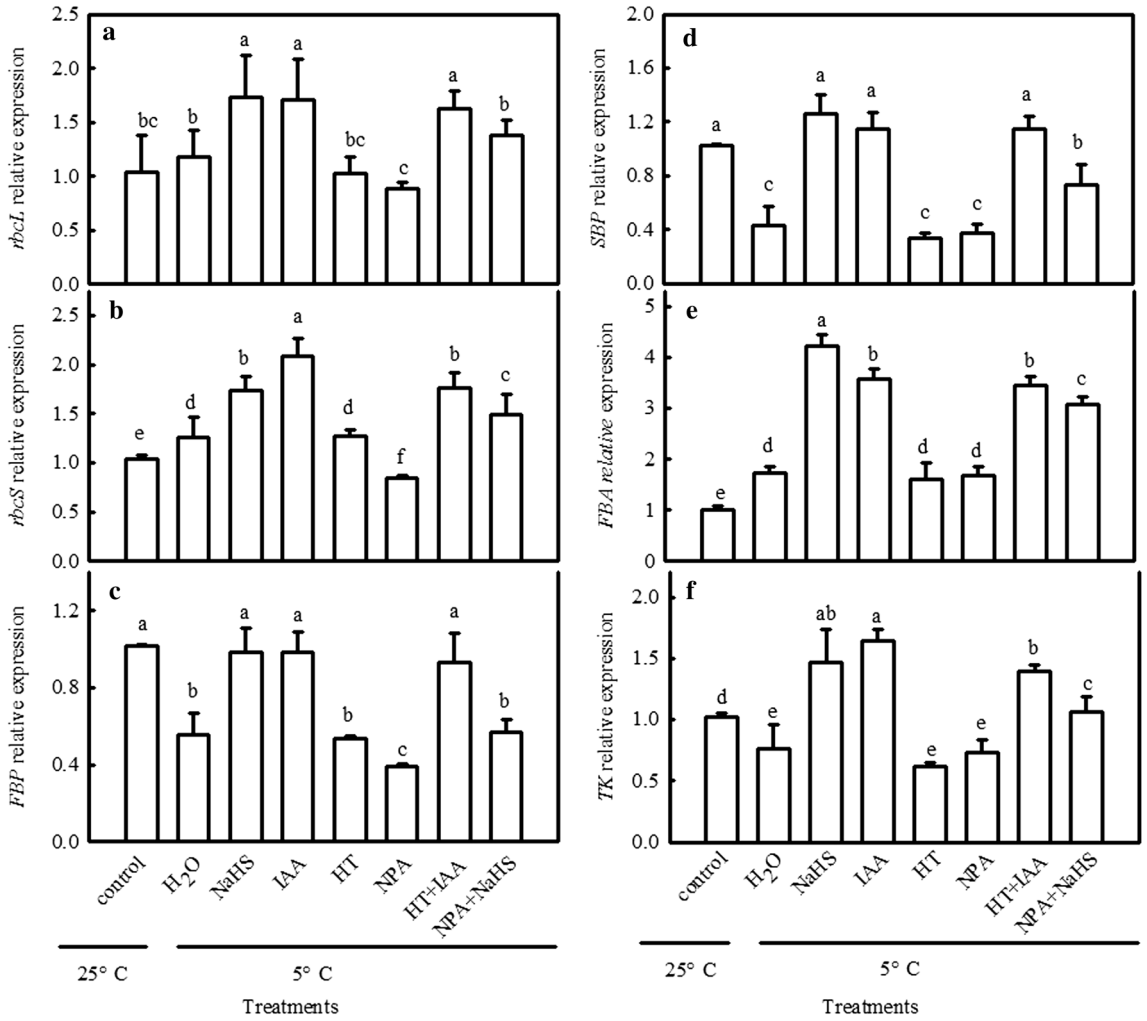

Fig. 9 Interactive effects of NaHS and IAA on the relative expression of the key enzymes in Calvin-cycle of cucumber seedlings under chilling stress. **a** *rbcL*. **b** *rbcS*. **c** *FBP*. **d** *SBP*. **e** *FBA*. **f** *TK*. Two leaf stage seedlings grown in solar greenhouse were foliar sprayed with 1.0 mM NaHS (H_2S donor), 0.15 mM HT (H_2S scavenger), 75 µM IAA, 50 µM NPA (IAA biosynthesis inhibitor), 0.15 mM HT + 75 µM IAA, 50 µM NPA + 1.0 mM NaHS, or deionized water (control) for 24 h, Then, the seedlings were exposed to 5 °C for 48 h. All values shown are mean ± SD ($n = 5$). **a**–**f** Mean values are significantly different among samples ($P < 0.05$)

AsA and GSH are molecules with a regulatory role that participate in the redox signaling of the plant cell under abiotic stress conditions (Fotopoulos et al. 2010). There functions are closely related to the redox states (Kocsy et al. 2001), and plants can adjust redox states of AsA and GSH by modulating their regeneration and biosynthesis. AsA and GSH are linked through the AsA–GSH cycle. During this cycle, AsA is converted to the unstable radical monodehydroascorbate (MDHA) which rapidly disproportionates to yield dehydroascorbate (DHA) (Smirnoff 2000). The latter is converted back to reduced ascorbate with the usage of reduced glutathione (GSH), acting as the electron donor in this reduction (Fotopoulos et al. 2010). Previous studies revealed that a high ratio of GSH/GSSG and/or AsA/DHA may be the key element for efficient protection against abiotic stress-induced accumulation of ROS (Szalai et al. 2009). Levitt (1962) demonstrated that a higher GSH/GSSG ratio in the freezing-tolerant genotypes may keep the sulphydryl groups of the proteins in the reduced form, thus decreasing the possibility of intermolecular disulphide bridge formation when the plants are exposed to freezing temperatures. Fotopoulos et al. (2010) also considered the maintenance of high AsA/DHA and GSH/GSSG ratios as very important to ensure that AsA and GSH can function appropriately in the AsA–GSH cycle and other physiological processes during chilling stress. Here, we found that chilling stress decreased AsA, GSH, AsA/DHA, and GSH/GSSG in cucumber seedlings, although no differences were found in AsA + DHA and GSH + GSSG contents between H_2O treatment and the control. However, NaHS- and IAA-treated seedlings significantly increased in AsA + DHA, GSH + GSSG, AsA, and GSH, especially in AsA/DHA and GSH/GSSG ratios under

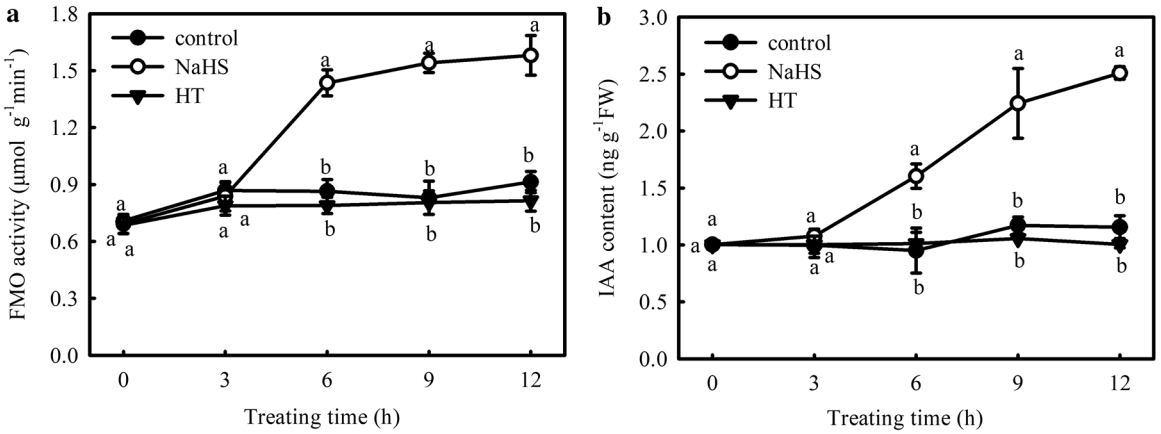

Fig. 10 Effects of NaHS on the FMO activity (**a**) and IAA content (**b**) in cucumber seedlings under normal culture conditions. Cucumber seedlings grown in solar greenhouse were foliar sprayed with 1.0 mM NaHS (H_2S donor), 0.15 mM HT (a specific scavenger of H_2S), or distilled water (control), respectively, for 12 h, and then determined the FMO activity and IAA content in cucumber seedlings. All values shown are mean ± SD ($n = 3$). a and b Mean values are significantly different among samples ($P < 0.05$)

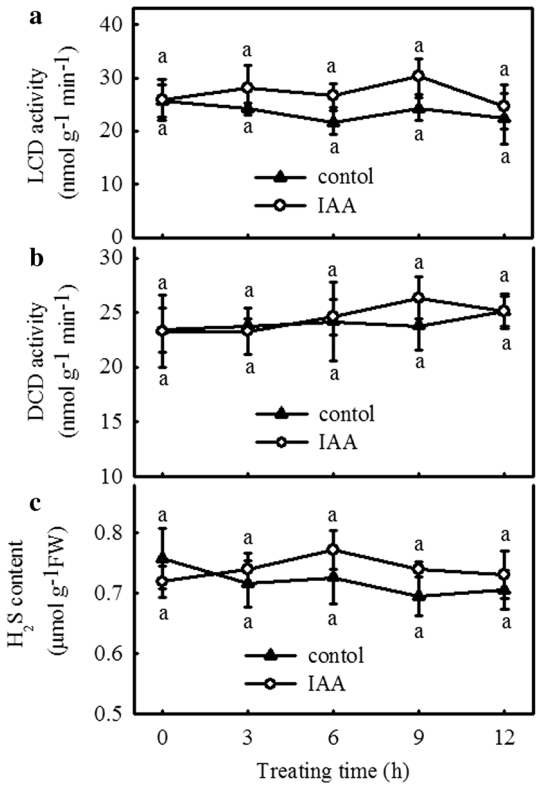

Fig. 11 Effects of IAA on the H_2S-emission system in cucumber seedlings under normal culture conditions. **a** LCD activity. **b** DCD activity. **c** H_2S content. Cucumber seedlings grown in solar greenhouse were foliar sprayed with 75 μM IAA or distilled water (control) for 12 h, and then determined the LCD/DCD activities and H_2S content in cucumber seedlings. All values shown are mean ± SD ($n = 3$). **a**–**d** Mean values are not significantly different among samples ($P < 0.05$)

chilling stress. These data suggest that chilling stress seriously affects the balance of redox couple in plants, resulting in excessive accumulation of DHA and GSSG. NaHS and IAA could maintain the redox states of ascorbate and glutathione by up-regulating the AsA/DHA, GSH/GSSG ratios and thus play an important role in H_2S-induced chilling tolerance. Our results are in agreement with the previous results (Li et al. 2011; Zhang et al. 2011). To elucidate the importance of H_2S and IAA-induced ROS scavenging under chilling stress, HT and NPA were also added. The improvement of NaHS on antioxidant capacity was significantly diminished by NPA, but HT did not affect IAA-induced ROS scavenging ability during chilling stress. Therefore, we suggest that the effect of H_2S depends on the IAA signaling pathway. Although the activation of antioxidant by H_2S and IAA have been reported in many plants under various abiotic stresses, little was known about the interaction of H_2S and IAA in regulating ROS metabolism under chilling stress. From the present study, we confirmed that IAA is involved in H_2S-induced protection under chilling stress, especially in defense against oxidative stress.

In recent years, a few works on the function of H_2S in plant photosynthesis have been published. Chen et al. (2011a, b) found that NaHS increased the chlorophyll content in spinach leaves and markedly enhanced the grana lamellae stacking in the functional chloroplasts. The optimal concentration of NaHS also improved the light saturation point (LSP), maximum net photosynthetic rate (P_{max}), carboxylation efficiency (CE), and maximal photochemical efficiency of photosystem II (Fv/Fm), as well as the activity of RuBCase and the protein expression of *rbcL*, but decreased the light compensation point (LCP) and dark respiration (Rd). A similar result had been illustrated that application of exogenous H_2S has a protective role

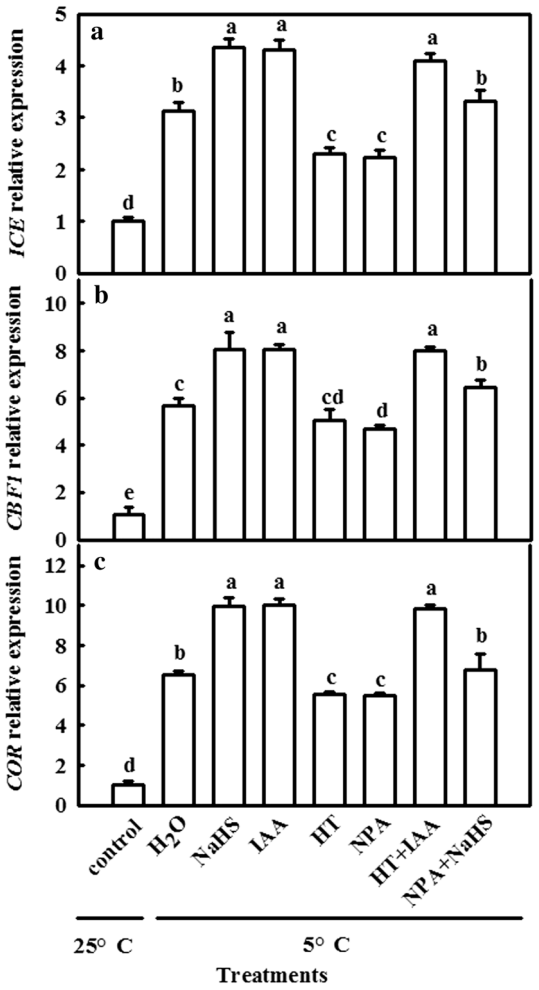

Fig. 12 Interactive effects of NaHS and IAA on relative expression of cold-responsive genes under chilling stress. **a** *ICE*. **b** *CBF1*. **c** *COR*. Two leaf stage seedlings grown in solar greenhouse were foliar sprayed with 1.0 mM NaHS (H_2S donor), 0.15 mM HT (H_2S scavenger), 75 μM IAA, 50 μM NPA (a polar transport inhibitor of IAA), 0.15 mM HT + 75 μM IAA, 50 μM NPA + 1.0 mM NaHS, or deionized water (control) for 24 h. Then, the seedlings were exposed to 5 °C for 48 h. All values shown are mean ± SD ($n = 3$). **a**–**e** Mean values are significantly different among samples ($P < 0.05$)

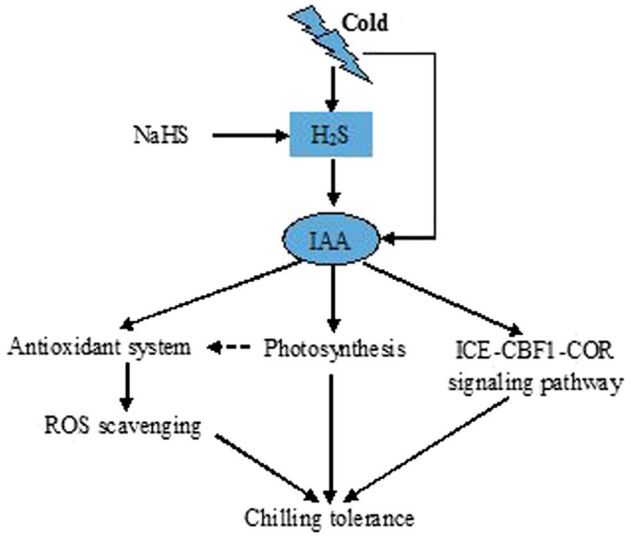

Fig. 13 Simplified schematic model between H_2S and IAA signaling in plants responding to chilling stress. Arrows (→) indicate positive effects. *H_2S* hydrogen sulfide, *IAA* indole-3-acetic acid

on photosynthetic parameters, antioxidant enzyme activities, and ultrastructural changes in *Brassica napus* under high Cd stress conditions (Ali et al. 2014). IAA could not only regulate plant growth, but also involve in the photosynthesis under abiotic stresses. Singh and Prasad (2015) found that exogenous IAA alleviated Cd-induced toxicity on growth performance by improving the structural and functional attributes of photosynthetic apparatus, i.e., pigment contents and photosynthetic activity. Work on the role of H_2S and IAA in enhancing photosynthetic capacity under abiotic stresses has been published, but no information was provided about the interaction of H_2S and IAA in regulating of activities and relative expression of enzymes in Calvin–Benson cycle under chilling stress. Here, we observed that NaHS and IAA enhanced Pn, Jmax, and Asat significantly (Fig. 7), as well as the activities and relative expression of the key enzymes in Calvin–Benson cycle, including RuBPCase, FBPase, SBPase, FBA, and TK (Figs. 8, 9), compared with the H_2O-treated seedlings under chilling stress. NPA weakened the protective effects of NaHS on photosynthetic parameters against chilling stress, but HT had revealed little ability to interfere with the function of IAA under stress condition. These results demonstrated that H_2S and IAA could protect the photosynthetic apparatus by improvement of the activities and gene expression of key enzymes in Calvin–Benson cycle, and strengthen the photosynthetic carbon assimilation capacity. IAA may act as a downstream signal involved in H_2S-induced protection of the photosynthetic apparatus.

In higher plants, the known major cold signaling pathway is the CBF-mediated transcriptional regulatory cascade, which is essential for the induction of a set of COR genes (Shi et al. 2015). Growing evidence indicates that hormonal components play important roles in regulating plant cold tolerance by either CBF-dependent or CBF-independent pathways (Shi et al. 2015). For example, Hu et al. (2013) showed that repressors of jasmonate acid (JA) signaling—the basic helix–loop–helix (bHLH)-interacting proteins JAZ1/4—interact with ICE1/2 and repress ICE1 transcriptional activity, thereby modulating CBF expression and cold tolerance. Our data showed that chilling stress

increased the transcription levels of *ICE, CBF1,* and *COR* in cucumber seedlings (Fig. 12). Application of NaHS and IAA markedly up-regulated expression of *ICE, CBF1* and *COR*. This implied that H_2S and IAA are involved in the ICE–CBF1–COR signal transduction pathway. Moreover, taking into account our observations following application of NPA and HT, the result indicated that IAA, as a downstream signal for the protective effects induced by H_2S, may be required in the *ICE–CBF1–COR* signal pathway.

Conclusion

In summary, based on the results obtained in the present studies, NaHS and IAA alleviated the negative effects of chilling stress on growth and photosynthesis by preventing excessive accumulation of ROS, and activating antioxidant enzyme activity, AsA–GSH detoxification capacity, gene expression of Calvin–Benson cycle, and defense response signal pathway (Fig. 13). All the investigated parameters clearly indicate a cross talk between H_2S and IAA, which have presented strong evidence that IAA acting as downstream signal mediates the H_2S-induced stress tolerance in cucumber seedlings. This process creates a new signaling pathway for improving stress tolerance in plant. Further studies using advanced molecular techniques and mutant analyses are required to better explore the detailed mechanisms and interaction of H_2S- and IAA-induced stress tolerance in plants.

Author contribution statement XWZ performed most part of the experiment, analyzed the data, and completed the first draft. XZA and HGB designed the experimental plan and edited the manuscript. FJL, JZ, and FDL worked together with XWZ to accomplish the experiment.

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Compliance with ethical standards

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

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