1 **Hydrogen sulfide improves the cold stress resistance through the CsARF5-CsDREB3**
2 **module in cucumber**

3
4 **Running title:** *CsARF5* regulates H₂S-mediated cold stress tolerance

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21 **Highlight**

22 Auxin signaling participates in H₂S-mediated cold stress through the CsARF5-CsDREB3 module
23 in cucumber.

24 Abstract

25 Hydrogen sulfide (H₂S) plays a crucial role in regulating cold tolerance. But the synergistic
 26 regulation of H₂S and auxin in the plant response to cold stress has not been reported. In the study,
 27 we found that sodium hydrosulfide (NaHS, an H₂S donor) treatment enhanced the cold tolerance
 28 of cucumber seedlings and increased the level of auxin. *CsARF5*, a cucumber auxin response
 29 factor (ARF) gene was isolated and its role in regulating H₂S-mediated cold stress tolerance was
 30 described. Transgenic cucumber leaves overexpressing *CsARF5* were obtained. Physiological
 31 analysis indicated that overexpression of *CsARF5* enhanced the cold stress tolerance of cucumber
 32 and the regulation of the cold stress response by *CsARF5* depends on H₂S. In addition, molecular
 33 assays showed that *CsARF5* modulated cold stress response by directly activating the expression
 34 of the dehydration-responsive element-binding (DREB)/C-repeat binding factor (CBF) gene
 35 *CsDREB3*, which was identified as a positive regulator of cold stress. Taken together, our results
 36 suggest that *CsARF5* plays an important role in H₂S-mediated cold stress in cucumber. These
 37 results shed light on the molecular mechanism by which H₂S regulates cold stress response by
 38 mediating auxin signaling, and will provide insights for further studies on the molecular
 39 mechanism by which H₂S regulates cold stress.

40

41

42 **Keywords:** ARF, auxin, cold stress, cucumber, DREB, hydrogen sulfide

43 Introduction

44 Cucumber (*Cucumis sativus* L.) is one of the most important economic crops worldwide. The
 45 cultivation and yield of cucumbers in China have ranked among the top in the world for many
 46 years. In China, cucumber is generally grown in greenhouses. Because of the extreme low
 47 temperature conditions, the cucumber in greenhouse is prone to cold injury in winter and early
 48 spring. Cucumbers with cold injury showed inhibited growth, wilted and died in severe cases.
 49 Therefore, it is of great practical significance to study the effects of low temperature stress on
 50 cucumber growth and development and the response mechanism of cucumber to low temperature
 51 stress.

52 Numerous investigations have determined that hydrogen sulfide (H₂S) is an important gas
 53 signaling molecule that plays a key role in the regulation of abiotic stress responses, including
 54 cold tolerance (Shi *et al.*, 2015; Du *et al.*, 2017; Hancock, 2019; Zhang *et al.*, 2021). H₂S is found
 55 to alleviate cold stress tolerance in many plant species, although the mechanisms remain elusive
 56 (Du *et al.*, 2017; Fu *et al.*, 2013; Shi *et al.*, 2013; Nasibi *et al.*, 2020). Several reports show that
 57 H₂S modulates cold stress response possibly through mitogen activated protein kinase (MAPK)
 58 signaling (Du *et al.*, 2017; Ba *et al.*, 2021; Du *et al.*, 2021). In addition to H₂S, phytohormones,
 59 especially auxin, also play a vital positive regulatory role in cold stress response (Shibasaki *et al.*,
 60 2009; Rahman, 2013).

61 Auxin is involved in plant growth and development in various aspects, including cell division
 62 and elongation, tissue patterning, and the response to environmental stimuli (Zhao, 2010; Lv *et al.*,
 63 2019). Since auxin was identified for the first time in 1930s as indole-3-acetic acid, there has been
 64 a major breakthrough in the molecular mechanisms of auxin perception and signal transduction.
 65 Many genetic and biochemical approaches have elucidated that the TRANSPORT INHIBITOR
 66 RESPONSE1 (TIR1) protein functions as the receptor to perceive auxin signaling based on the
 67 reduced auxin response of *tir1* mutations (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005).
 68 Additionally, the SCF^{TIR1} ubiquitin-ligase complex is regarded as a central regulator of auxin
 69 signaling, and it-mediated proteolysis of auxin/indole acetic acid (Aux/IAA) proteins is
 70 responsible for auxin signaling transduction (Gray *et al.*, 2001; Kepinski and Leyser, 2004). In this
 71 signaling pathway, Aux/IAA proteins are direct targets of TIR1. And Aux/IAA proteins directly
 72 interact with auxin response factors (ARFs) to repress their activities (Liscum and Reed, 2002).
 73 Upon exposure to auxin, the F-box protein TIR1 recruits the Aux/IAA proteins for degradation,
 74 which leads to the release of various auxin response factors, including *Small Auxin-up RNAs*
 75 (*SAURs*), *GH3s* and *Aux/IAAs*, and consequently regulates diverse auxin-mediated plant growth
 76 (Guilfoyle and Hagen, 2007; Weijers and Friml, 2009).

77 ARFs are vital transcription factors (TFs) that regulate the expression of auxin response
 78 genes (Guilfoyle and Hagen, 2007; Chandler *et al.*, 2016; Li *et al.*, 2016). To date, 23 and 25 *ARF*
 79 genes have been isolated in *Arabidopsis* and rice, respectively (Guilfoyle and Hagen, 2007; Wang
 80 *et al.*, 2007; Shen *et al.*, 2010). Most ARF members consist of a DNA-binding domain (DBD), a

variable middle region and a carboxy-terminal dimerization domain (CTD) (Guilfoyle and Hagen, 2007; Tiwari *et al.*, 2003). The DBD is classified as a plant-specific B3-type and functions to bind to TGTCTC/GAGACA sites (AuxREs) *in vitro* (Ulmasov *et al.*, 1999; Tiwari *et al.*, 2003). The middle region includes two types: activation domain-type (AD) and repression domain-type (RD), which are used as the basis of classification between transcription activators and transcription repressors (Tiwari *et al.*, 2003; Li *et al.*, 2016). Additionally, the CTD domain is responsible for protein-protein interactions by dimerizing with Aux/IAA proteins as well as other ARFs (Kim *et al.*, 1997; Piya *et al.*, 2014). Extensive studies have suggested that ARF proteins are involved in distinct developmental processes. In *Arabidopsis*, numerous ARF genes have been implicated in embryogenesis (ARF5 and ARF17) (Mallory *et al.*, 2005), root growth (ARF7, ARF10, ARF16, and ARF19) (Okushima *et al.*, 2005; Fukaki and Tasaka, 2009; Orosa-Puente *et al.*, 2018; Lee *et al.*, 2019), hypocotyl growth (ARF6, ARF7, ARF8, and ARF19) (Liu *et al.*, 2018; Reed *et al.*, 2018; Wang *et al.*, 2020), shoot regeneration (ARF4 and ARF5) (Zhang *et al.*, 2021), flower development (ARF2, ARF3, ARF6, and ARF8) (Nagpal *et al.*, 2005; Finet *et al.*, 2010), and senescence (ARF1 and ARF2) (Ellis *et al.*, 2005). In the case of rice, genetic studies show that the functions of ARFs are different from the functions of *Arabidopsis*. OsARF1 is involved in root initiation and seed development (Inukai *et al.*, 2005; Attia *et al.*, 2009). OsARF12 regulates root elongation, iron accumulation, and phosphate homeostasis (Qi *et al.*, 2012; Wang *et al.*, 2014). OsARF16 regulates phosphate transport, phosphate starvation and iron deficiency responses (Shen *et al.*, 2013; Shen *et al.*, 2014; Shen *et al.*, 2015). OsARF19 controls leaf angles (Zhang *et al.*, 2015). OsARF11, OsARF12, OsARF16, and OsARF17 are involved in antiviral defence (Qin *et al.*, 2020; Zhang *et al.*, 2020). A recent study showed that OsARF6 regulates rice yield (Qiao *et al.*, 2021). Though a number of ARF members have been functionally characterized in *Arabidopsis* and rice as mentioned earlier, little is known about the functions of *ARF* genes in cucumber.

In this study, we explored the molecular mechanisms by which H₂S regulates cold stress response in cucumber. We found that H₂S treatment could improve cold resistance and auxin content of cucumber. *CsARF5*, a transcriptional regulator in auxin signaling, was responsive to cold stress and H₂S treatments, and overexpression of *CsARF5* improved the cold stress tolerance of cucumber. Further studies indicated that *CsARF5* modulated cold stress response by directly activating the expression of the dehydration-responsive element-binding (DREB)/C-repeat binding factor (CBF) gene *CsDREB3*.

Materials and Methods

Plant material and growth conditions

‘Jinyou 35’ cucumber seedlings were used for cold stress treatment and genetic transformation. After soaking and germinating, the cucumber seeds were sown in nutrition bowls and transferred

118 to a climate chamber with a PFD of 600 $\mu\text{mol m}^{-2} \cdot \text{s}^{-1}$, a 25 °C/16 °C thermo-period, an 11-h
119 photoperiod, and 80% relative humidity.

120

121 ***Vector construction and transient transformation***

122 To generate *CsARF5* and *CsDREB3* overexpression vectors, full-length *CsARF5* and *CsDREB3*
123 were inserted into the pCAMBIA1300 plasmid.

124 For the transient transformation of detached cucumber leaves, cucumber leaves with the same
125 growth conditions were taken and *Agrobacterium tumefaciens* LBA4404 (Weidi, Shanghai, China)
126 with overexpression vector was injected into cucumber leaves through a medical syringe.

127

128 ***Cold stress treatments***

129 To evaluate the effect of H_2S and IAA on the cold resistance of cucumber, cucumber seedlings
130 with two-leaves were foliar sprayed with 1.0 mM Sodium hydrosulfide (NaHS, an H_2S donor;
131 Shanghai Macklin Biochemical Co., Ltd., Shanghai, China), 0.15 mM hypotaurine (HT, a specific
132 scavenger of H_2S ; Sigma-Aldrich, Shanghai, China), and deionized water (H_2O) respectively, or
133 pretreated with 75 μM indole-3-acetic acid (IAA, Solarbio, Beijing, China), 50 μM
134 1-naphthylphthalamic acid (NPA, a polar transport inhibitor of IAA; Shanghai Aladdin
135 Biochemical Technology Co., Ltd., Shanghai, China), or deionized water (H_2O), respectively. 24 h
136 later, half of the treatments were exposed to low temperatures (5 °C), and the other half of the
137 seedlings were placed at normal temperatures as the control. The EL and accumulation of reactive
138 oxygen species (ROS) were determined at 48 h after exposure of seedlings to 5 °C. Leaf samples
139 of seedlings that pretreated with H_2O and 1.0 mM NaHS were collected from 3 plants ($n = 3$) for
140 transcriptome analysis after 6 h of cold stress.

141 To compare the difference in cold tolerance between WT and transgenic cucumber leaves,
142 the pCAMBIA1300 empty vectors and overexpressed *CsARF5* and *CsDREB3* vectors were
143 injected into the first leaf which was just flat of cucumber. 12 h later, WT leaves, *CsDREB3*
144 overexpressing leaves, and some *CsARF5* overexpressing leaves were exposed to 5 °C. Other
145 *CsARF5* overexpressing leaves were treated with HT and then exposed to 5 °C after the water
146 droplets on the leaves were absorbed and dried. The gene expression of *CsDREB3*, *CsCBF1* and
147 *CsCOR* in transgenic cucumber leaves was measured at 3 h after exposure to cold stress. NBT
148 staining, DAB staining and ROS content were detected after cold treatment for 12 h.

149

150 ***qRT-PCR analysis***

151 The transcription levels of *CsARF5*, *CsDREB3*, *CsCBF1*, and *CsCOR* were examined using
152 specific primers *CsARF5* (qRT)-F/R, *CsDREB3* (qRT)-F/R, *CsCBF1* (qRT)-F/R, and *CsCOR*
153 (qRT)-F/R, respectively. β -Actin was used as internal reference. All of the primers used are shown
154 in Supplemental Table S1. qRT-PCR was carried out simultaneously with three biological
155 replicates and three technical replicates.

156

157 ***Detection of electrolyte leakage (EL)***

158 EL was detected according to instructions described by Dong et al. (2013) (Dong *et al.*, 2013).
159 Leaf discs (0.2 g) were immersed in 20 ml deionized water and incubated at 25 °C for 3 h. The
160 electrical conductivity (EC1) was estimated using a conductivity meter (DDB-303A, Shanghai,
161 China). The leaf discs were boiled for 10 min, and then cooled to detect EC2. EL was calculated
162 according to the following formula: $EL = EC1/EC2 \times 100$.

163

164 ***IAA content and flavin monooxygenase (FMO) activity assay***

165 IAA content was measured using high-performance liquid chromatography-triple quadrupole mass
166 spectrometry (HPLC–MS, Thermo Fisher Scientific, TSQ Quantum Access) according to the
167 method of Li et al. (2014) with minor modifications by Zhang et al. (2020). In brief, leaf samples
168 were extracted with 80% methanol (containing 30 µg·ml⁻¹ sodium diethyldithiocarbamate) and the
169 supernatant was retained rotary evaporation (Shanghai EYELA, N-1210B). Pigment and phenolic
170 impurities of samples were removed using trichloromethane and polyvinylpyrrolidone
171 (PVPP), respectively. Auxin was further extracted with ethyl acetate and then the ester phase was
172 collected. Finally, rotation drying at 36 °C and drying were dissolved in 1.0 ml mobile phases
173 (methanol: 0.04% acetic acid = 45:55, v/v). The filtrate can then be used directly for HPLC–MS
174 analysis.

175 The activity of FMO was detected using an enzyme-linked immunosorbent assay (ELISA) kit
176 (Jiangsu Meimian Industrial Co. Ltd, Jiangsu, China) as described by Zhang et al. (2020).

177

178 ***Determination of H₂O₂ and superoxide anion (O₂^{•-}) contents***

179 H₂O₂ content was estimated with the H₂O₂ kit (Nanjing Jiancheng Bioengineering Institute,
180 Nanjing, China) according to the instructions. The O₂^{•-} content was detected according to the
181 method of Wang and Luo (1990). Cellular H₂O₂ and O₂^{•-} were fluorescently stained with 2',
182 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, the fluorescent probe of H₂O₂) (MCE, Cat.
183 No. HY-D0940, Shanghai, China) and dihydroethidium (DHE, O₂^{•-} fluorescent probe)
184 (Fluorescence Biotechnology Co. Ltd, Cat. No. 15200, Beijing, China), respectively as described
185 by Galluzzi and Kroemer (2014) and modified by Zhang et al. (2020). In brief, the samples were
186 infiltrated in 20 µM H₂O₂ fluorescent probe at 25 °C under dark conditions for 30 min. Then the
187 tissues were rinsed with HEPES- NaOH buffer (pH 7.5). Under excitation at 488 nm and emission
188 at 522 nm of inverted microscope (Leica DMI8), cellular H₂O₂ showed obvious green fluorescent
189 coloration. For cellular O₂^{•-} measurement, the samples were infiltrated in 10 µM DHE at 37 °C
190 under dark conditions for 30 min. After fixation, the tissues were rinsed with Tris-HCl buffer (pH
191 7.5). O₂^{•-} showed strong red fluorescence under excitation at 490 nm and emission at 520 nm
192 under an inverted microscope (Leica DMI8).

193

194 ***Nitroblue tetrazolium (NBT) and 3, 3-diaminobenzidine (DAB) staining***

195 NBT staining of O₂^{•-} was performed according to the method of Jabs et al. (1996) with minor
196 modifications. The fresh leaves were washed with distilled water, immersed in 0.5 mM NBT in
197 vacuum and stained at 28 °C for 1 h. Then the leaves were boiled in ethanol: lactic acid: glycerol
198 (3:1:1) mixed solution to remove pigments, and O₂^{•-} was visualized in blue-purple coloration.
199 DAB staining of H₂O₂ was carried out as described by Thordal-Christensen et al. (1997). The
200 cleaned fresh leaves were soaked in 1 mM DAB staining solution (pH 3.8) in vacuum and stained
201 at 28 °C for 8 h. Then the leaves were boiled in ethanol: lactic acid: glycerol (3:1:1) mixed solution
202 to remove pigments, and H₂O₂ was visualized in reddish-brown coloration.

204 ***EMSAs***

205 The CsARF5-HIS fusion protein and biotin labelled probes were prepared for EMSAs. The
206 CsARF5-HIS fusion protein was obtained by inducing *Escherichia coli* BL21 (TransGen Biotech,
207 Beijing, China) with isopropyl β-D-thiogalactoside (IPTG). The biotin-labelled probes were
208 synthesized by Sangon Biotech (Shanghai) Co., Ltd. To perform the EMSAs, the fusion protein
209 was mixed with the probe and incubated at 24 °C for 30 min. The protein-probe mixture was
210 separated by nondenatured acrylamide gel electrophoresis.

212 ***Dual luciferase assay***

213 The promoter sequence of *CsDREB3* was amplified and cloned into pGreenII 0800-LUC to
214 generate the reporter construct pCsDREB3-LUC. The effector plasmid was constructed by
215 inserting full-length *CsARF5* into pGreenII 62-SK. Different plasmid combinations were injected
216 into tobacco (*Nicotiana benthamiana*) leaves by *Agrobacterium tumefaciens* LBA4404. The
217 leaves were sprayed with 100 mM luciferin and luminescence was detected after being placed in
218 darkness for 3 min. Fluorescence images were obtained with a live imaging system (Xenogen,
219 USA). The fluorescence activity was determined using a fluorescence activity detection kit
220 (Promega, Madison, WI, USA).

222 ***Statistical analysis***

223 All experiments were performed at least in triplicate, and the results are expressed as the mean ±
224 standard deviation (SD) of three replicates. The data were analyzed statistically with DPS software.
225 Duncan's multiple range test was used to compare differences among treatments, and *P* < 0.05
226 was considered statistically significant.

229 ***Accession numbers***

230 CsARF5 (CsaV3_3G045690), CsDREB3 (CsaV3_2G030880), CsCBF1 (XM_004140746),
231 CsCOR (XM_011659051).

232

233

234 **Results**

235 ***NaHS improves cold tolerance in cucumber seedlings***

236 In a previous study, we demonstrated that NaHS could improve the cold tolerance of cucumber
237 seedlings in a concentration-dependent manner, and 1.0 mM NaHS treatment showed a very
238 significant difference compared with the control (Zhang *et al.*, 2020). Here, we found that 1.0 mM
239 NaHS significantly reduced cold stress injury, accumulation of H₂O₂ and O₂^{•-}, as well as EL in
240 cucumber seedlings after exposure to 5°C for 48 h. However, 0.15 mM H₂S scavenger HT
241 increased cold stress injury, H₂O₂, O₂^{•-} and EL, compare with the deionized water (H₂O, as a
242 comparison) -treated seedlings (Fig. 1A-F). We also noticed that the mRNA abundance of
243 *CsCBF1* and *CsCOR* in NaHS treated-seedlings increased by 1.38- fold and 3.14- fold,
244 respectively under cold stress, but no obvious differences were observed between H₂O and HT
245 treatments (Fig. 1G, H). Therefore, we further confirmed that H₂S improves cold tolerance in
246 cucumber.

247

248 ***NaHS treatment affects auxin signaling***

249 To explore the molecular mechanism by which H₂S improves cold resistance in cucumber, we
250 performed transcriptome analysis of cucumber seedlings treated with NaHS and H₂O. A total of
251 1952 cucumber genes were analyzed from transcriptome data (Fig. 2A). Among these cucumber
252 genes, 118 genes were downregulated and 54 genes were upregulated (Fig. 2A). We further
253 analyzed the upregulated genes (Fig. 2B). One of the genes that caught our attention was an auxin
254 response gene (accession number: CsaV3_3G045690, Fig. 2B). NCBI database comparison found
255 that it was the *CsARF5* gene.

256

257 To study the effect of NaHS on auxin signaling, we estimated the change in auxin content in
258 cucumber seedlings treated with NaHS or HT. 1.0 mM NaHS markedly increased endogenous
259 IAA accumulation and FMO (a key enzyme in auxin synthesis) activity. However, HT treatment
260 revealed lower or similar IAA content and FMO activity compared with H₂O treatment (Fig. 3A,
261 B). In addition, NaHS treatment also significantly upregulated the mRNA levels of *CsARF5* and
262 *CsDREB3*, while HT-treated seedlings showed no marked differences in the relative mRNA
263 expression of *CsARF5* and *CsDREB3* relative to the H₂O treatment (Fig. 3C, D). These data
264 indicate that H₂S affects auxin signaling in cucumber seedlings under cold-stress.

265

266 ***IAA treatment improves cold resistance of cucumber seedlings***

267 Our previous study proved that 75 μM IAA enhances the cold tolerance of cucumber seedlings
268 (Zhang *et al.*, 2020). Here, we found that 75 μM IAA significantly reduced the EL, and
269 accumulation of H₂O₂ and O₂^{•-} caused by cold stress, while 50 μM 1-naphthylphthalamic acid

(NPA, a polar transport inhibitor) treatment showed no remarkable difference relative to H₂O treatment under cold stress (Fig. 4A-F). As an auxin response factor, the relative mRNA expression of *CsARF5* was up-regulated in IAA-treated seedlings under cold stress. However, no remarkable difference was found in the mRNA expression of *CsARF5* between the NPA and H₂O treatments (Fig. 4G). The mRNA expression levels of *CsCBF1* and *CsCOR* were significantly upregulated in IAA-treated seedlings, but downregulated or not influenced in HT-treated seedlings compared with H₂O-treated seedlings when exposed to cold stress (Fig. 4H, I). The latest results are in keeping with our earlier findings, so we further confirm that IAA enhances cold tolerance in cucumber.

CsARF5 positively regulates cold stress tolerance of cucumber

We wondered whether the auxin response gene *CsARF5* is involved in the H₂S-mediated cold stress response. qRT-PCR results showed that cold stress increased the mRNA abundance of the *CsARF5* gene, and the expression reached a peak after seedlings were exposed to cold for 3 h, and then decreased gradually (Fig. 5A). Compared with the control and HT treatments, NaHS treatment further increased the expression of *CsARF5* (Fig. 5B). These results demonstrate that *CsARF5* is responsive to cold stress and H₂S treatment.

To further explore the role of *CsARF5* in the response to cold stress in cucumber, we obtained *CsARF5* transgenic cucumber leaves through *Agrobacterium*-mediated transient genetic transformation (Supplemental Fig. 1A). Then, we observed the accumulation of ROS in transgenic leaves after exposure to cold stress for 12 h, using NBT and DAB staining. The results showed that the accumulation of ROS in cucumber leaves overexpressing with *CsARF5* (*CsARF5*) was less than that of the empty vector control (WT) under cold stress (Fig. 5C-D). In addition, the contents of O₂^{•-} and H₂O₂ were measured with biochemical analysis, and the results were in agreement with the NBT and DAB staining images (Fig. 5E-F). qRT-PCR results revealed that *CsARF5* overexpression upregulated the mRNA level of cold stress-responsive genes *CsDREB3*, *CsCBF1*, and *CsCOR* (Fig. 5G-I). These data suggest that *CsARF5* is a positive regulator of cold stress response.

HT treatment affects CsARF5-mediated cold stress tolerance

Considering that H₂S induces the expression of *CsARF5*, and that *CsARF5* positively regulates cold stress resistance, we wondered about the role of *CsARF5* in H₂S-mediated cold stress. The H₂S scavenger HT was applied to *CsARF5*-overexpressing cucumber leaves to observe ROS accumulation. The NBT and DAB staining results showed that the application of HT alleviated the *CsARF5*-decreased ROS accumulation in detached cucumber leaves (Fig. 6A-B). The biochemical analysis for O₂^{•-} and H₂O₂ was consistent with the NBT and DAB staining results (Fig. 6C-D). qRT-PCR results showed that the application of HT inhibited the promotion of *CsDREB3*,

CsCBF1, and *CsCOR* mRNA expression levels caused by *CsARF5* (Fig. 6E-G). These results suggest that the regulation of the cold stress response by *CsARF5* depends on H_2S .

Overexpression of CsDREB3 enhances cold stress tolerance of cucumber

DREB/CBF TFs play essential roles in the regulation of the plant cold stress response (Zhou *et al.*, 2010; Lata and Prasad, 2011). *CsDREB3* was induced by NaHS (Fig. 2B), which prompted us to explore whether *CsDREB3* was involved in the cold stress response in cucumber. As shown in Fig. 7A, cold stress induced the expression of the *CsDREB3* gene, and the expression reached a peak at 3 h, and then decreased gradually. Compared with the control and HT treatments, NaHS treatment further increased the expression of *CsDREB3* (Fig. 7B).

To investigate the role of *CsDREB3* in the response to cold stress in cucumber, *CsDREB3* transient transgenic cucumber leaves were obtained (Supplemental Fig. 1B). NBT and DAB staining results displayed that overexpression of *CsDREB3* decreased ROS accumulation after cold stress treatment (Fig. 7C-D). In addition, $O_2^{\cdot -}$ and H_2O_2 detection results also revealed that the accumulation of $O_2^{\cdot -}$ and H_2O_2 was significantly lower in leaves of overexpressing *CsDREB3* than in WT leaves (Fig. 7E-F). qRT-PCR results suggested that overexpression of *CsDREB3* increased the expression of *CsCBF1* and *CsCOR* (Fig. 7G-H). These data demonstrate that *CsDREB3* positively regulates the cold tolerance of cucumber.

CsARF5 directly activates the expression of CsDREB3

CsARF5 acts as an auxin response factor and can bind to the AuxRE motif in the promoters of target genes (Guilfoyle and Hagen, 2007). Considering the similar expression patterns of *CsARF5* and *CsDREB3* in cold stress and H_2S treatment, as well as the key role of DREB/CBF TFs in the regulation of the cold stress response, we hypothesized that *CsARF5* might be involved in the response to cold stress by mediating the expression of *CsDREB3*. Then, we analyzed the sequence of the *CsDREB3* gene promoter region and found a putative AuxRE motif (Fig. 8A). Fortunately, the directly binding between *CsARF5* protein and the promoter of *CsDREB3* was detected by electromobility shift assay (EMSA) (Fig. 8B). To test how *CsARF5* regulated the expression of *CsDREB3*, dual luciferase assays in tobacco leaves were performed. The *CsARF5* effector construct was expressed under the 35S promoter and the promoter of *CsDREB3* was fused to the Luc gene as a reporter (Fig. 8C). The results showed that co-expression of 35Spro:*CsARF5* with *CsDREB3*pro: Luc led to an obvious increase in luminescence intensity (Fig. 8D-E), while the binding site was mutated, and the activation was abolished (Fig. 8D-E). These results suggest that *CsARF5* trans- activates the expression of *CsDREB3* in cucumber.

Discussion

H₂S, as a gaseous signaling molecule, plays a crucial role in plant relevance to various stress conditions, such as low temperature, salt, drought, and heavy metals (Huang et al., 2021; Zhang et al., 2021). Exogenous application of the H₂S donor NaHS can effectively improve plant growth and stress response (Guo et al., 2016). In this study, we found that NaHS treatment improved cold-tolerance of cucumber seedlings (Fig. 1), suggesting that the response of H₂S to cold stress is consistent in different species. H₂S and phytohormones have synergistic effects on the H₂S-mediated plant stress response. For example, H₂S interacts with ABA and ethylene and is involved in the plant response to drought stress and in the regulation of stomatal closure (Jin *et al.*, 2013; Hou *et al.*, 2013). SA may play a key role in H₂S-alleviated heavy metal stress and low temperature stress (Li *et al.*, 2015; Qiao *et al.*, 2015). Here, we found that NaHS treatment promoted IAA synthesis and upregulated the expression of auxin-responsive genes (Fig. 2 and Fig. 3), indicating that H₂S may crosstalk with auxin to regulate the cold stress response.

Several plant hormones, such as jasmonic acid, salicylic acid, and ethylene, have been shown to play key roles in the response to cold stress (Miura and Tada, 2014; Kazan, 2015; Hu *et al.*, 2017). However, the role of auxin under cold stress is limited. Auxin is a crucial phytohormone that is involved in a variety of plant physiological and developmental processes including the regulation of the cold stress response (Zhao, 2018; Blakeslee *et al.*, 2019). Previous investigations have determined that cold stress promotes auxin biosynthesis or changes auxin gradient distribution, thus affecting the root gravity response in *Arabidopsis*, rice, and poplar (Shibasaki *et al.*, 2009; Popko *et al.*, 2010; Du *et al.*, 2013; Rahman, 2013). However, there are few studies on the effects of auxin to the cold stress response of plants. In this study, we found that the application of auxin improved cold resistance, whereas the application of the polar transport inhibitor NPA slightly reduced the cold resistance of cucumber seedlings (Fig. 4), demonstrating that auxin is a positive regulator of the cold stress response.

As a master regulator of auxin signaling, ARF TFs have been functionally characterized in *Arabidopsis* and rice (Guilfoyle and Hagen, 2007; Wang *et al.*, 2007; Shen *et al.*, 2010). An increasing number of studies have indicated that ARFs regulate multiple plant developmental processes such as root growth (Orosa-Puente *et al.*, 2018; Lee *et al.*, 2019), flower development (Nagpal *et al.*, 2005; Finet *et al.*, 2010), senescence (Ellis *et al.*, 2005), and stress response (He *et al.*, 2005; Rahman, 2013). Among these processes, the auxin-regulated cold stress response has become a major focus of biotechnology in cucumber with the task of identifying the cold resistance genes and improving the yield of cucumber. In this study, an ARF TF, CsARF5, was isolated from cucumber and its expression was induced by NaHS and cold stress treatments, suggesting that CsARF5 may be involved in H₂S-mediated cold tolerance (Fig. 5A-B).

To investigate the functions of CsARF5, transgenic cucumber leaves overexpressing *CsARF5* were generated. As hypothesized, the physiology and genetic analysis indicated that the overexpression of *CsARF5* decreased the accumulation of ROS and increased the expression level of cold stress-responsive genes, thus improving the cold stress resistance of cucumber (Fig. 5).

These results suggest that CsARF5 positively regulates the cold stress tolerance of cucumber. Meanwhile, the H₂S scavenger HT was applied to *CsARF5*-overexpressing cucumber leaves to study the role of *CsARF5* in H₂S-mediated cold stress. The results showed that HT inhibited the cold stress resistance increased by *CsARF5* (Fig. 6), indicating that the regulation of the cold stress response by *CsARF5* depends on H₂S.

DREB/CBF proteins play important roles in the regulation of the plant cold stress response (Zhou *et al.*, 2010; Lata and Prasad, 2011). In *Arabidopsis*, DREB1A, DREB2C, CBF1, CBF2, and CBF3 are involved in the cold stress response (Liu *et al.*, 1998; Gilmour *et al.*, 1998; Lee *et al.*, 2010; Lata and Prasad, 2011). In rice, OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1F, OsDREB2B, and OsDREBL are responsive to cold stress treatment (Chen *et al.*, 2003; Dubouzet *et al.*, 2003; Wang *et al.*, 2008; Matsukura *et al.*, 2010). Here, a cucumber *DREB* genes, namely *CsDREB3*, were induced by NaHS and cold stress treatments, and overexpression of *CsDREB3* significantly enhanced the cold stress tolerance of cucumber (Fig. 7), revealing that *CsDREB3* is a positive regulator of cold stress.

ARF5 is known to function as a transcriptional activator (Guilfoyle and Hagen, 2007). The remarkable expression of *CsDREB3* in *CsARF5* transgenic leaves prompted us to consider whether *CsARF5* can directly regulate *CsDREB3* expression (Fig. 5G). The putative AuxRE elements that were recognized by *CsARF5* were searched in the promoter region of *CsDREB3*. Fortunately, one AuxRE site was found, and the results of EMSAs and dual luciferase assays provided evidence to show that *CsARF5* could bind to the promoter of *CsDREB3* and activate its expression (Fig. 8).

Based on previous studies in model plant species and our results in this study, a working model of *CsARF5* regulating the H₂S-mediated cold stress response was proposed (Fig. 9). CsIAA proteins interact with *CsARF5* and interfere with the transcriptional regulation of *CsDREB3* by *CsARF5*. When H₂S signaling was detected, auxin levels in cucumber were increased. *CsARF5* is freed from the CsIAA protein complex. Then, *CsARF5* specifically activated the expression of *CsDREB3* to improve the cold tolerance of cucumber. Our study elucidates the molecular mechanism by which H₂S regulates the cold stress response in cucumber by mediating auxin signaling, which will provide insights for further studies on the molecular mechanisms by which H₂S regulates cold stress. A better understanding of the function and signal transduction of *CsARF5* in cucumber is helpful to regulate the resistance of cucumber to low temperature stress, to obtain high-quality fruit.

Data Availability

All data generated or analyzed during this study are included in this published article.

420 Acknowledgements

421 This work was supported by grants from The National Science Foundation of China (31572170),
422 The National Key Research and Development Program of China (2018YFD1000800), The Major
423 Science and Technology Innovation of Shandong Province in China (2019JZZY010715), and The
424 Special Fund of Vegetable Industrial Technology System of Shandong Province (SDAIT-05-10).

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427 Conflicts of interests

428 All authors agreed with the final manuscript and have no conflicts of interest.

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431 Author contributions

432 Xi-Zhen Ai and Xiao-Wei Zhang designed the experiment. Xiao-Wei Zhang performed the
433 research and analyzed the data. Xin Fu, Feng-Jiao Liu, Ya-Nan Wang and Huan-Gai Bi worked
434 together with Xiao-Wei Zhang to accomplish the experiment. Xi-Zhen Ai and Xiao-Wei Zhang
435 wrote the paper.

436

437

438 Supplemental data

439 Supplemental Tables

440 **Supplemental Table 1.** Primers used for gene expression analysis and vector construction.

441 **Supplemental Table 2.** The promoter sequence of CsDREB3.

442 Supplemental Figures

443 **Supplemental Figure 1.** Identification of transient transgenic cucumber leaves.

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660 **Figure legends**

661 **Figure 1. Effects of NaHS and HT on the cold tolerance of cucumber seedlings.**

662 (A) Phenotypic characterization of cucumber seedlings treated with NaHS or HT under cold stress
663 (5 °C for 48 h). Water treatment was used as the control. H₂O: water treatment; NaHS: NaHS
664 (hydrogen sulfide provider) treatment; HT: HT (hydrogen sulfide scavenger) treatment. Control:
665 cucumber seedlings before cold stress treatment; Cold stress: cucumber seedlings after cold stress
666 treatment. Each treatment contained 5-10 cucumber seedlings. The experiments were repeated
667 three times with similar results. A typical picture is shown here. (B) The EL results of cucumber
668 seedlings before (control) and after cold stress for 48 h. (C-D) Inverted fluorescence microscopy
669 imaging of H₂O₂ and O₂⁻ levels in cucumber seedling leaves before (control) and after cold stress
670 treatment for 48 h. (E-F) Detection of H₂O₂ and O₂⁻ content of cucumber seedlings before
671 (control) and after cold stress treatment for 48 h. (G-H) Expression of *CsCBF1* and *CsCOR* genes
672 in cucumber seedlings treated with H₂O, NaHS or HT under cold stress treatment for 6 h.
673 qRT-PCR was performed simultaneously with three biological replicates and three technical
674 replicates. The value of the water treatment was used as the reference and was set to 1. Error bars
675 denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on
676 Duncan's multiple range tests.

678 **Figure 2. RNA- seq analysis of cucumber seedlings with or without NaHS treatment.**

679 (A) Normalized heat map showing the changes in gene expression after NaHS treatment at 5 °C
680 for 6 h. All experiments were performed in triplicate. (B) Normalized heat map of upregulated
681 gene expression after NaHS treatment at 5 °C for 6 h. All experiments were performed in

triplicate.

Figure 3. NaHS treatment affects IAA level in cucumber.

(A) The IAA content of cucumber seedlings treated with NaHS or HT under cold stress treatment for 24 h. (B) The FMO activity of cucumber seedlings treated with NaHS or HT under cold stress treatment for 24 h. (C) Expression of the *CsARF5* gene in cucumber seedlings treated with NaHS or HT under cold stress treatment for 6 h. (D) Expression of the *CsDREB3* gene in cucumber seedlings treated with NaHS or HT under cold stress treatment for 6 h. qRT-PCR was performed simultaneously with three biological replicates and three technical replicates. The value of 0 h was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

Figure 4. Effects of IAA and NPA on the cold resistance of cucumber seedlings.

(A) Phenotypic characterization of cucumber seedlings treated with IAA or NPA under cold stress (5 °C for 48 h). Water treatment was used as the control. H₂O: water treatment; IAA: indole-3-acetic acid treatment; NPA: NPA (a polar transport inhibitor) treatment. Control: cucumber seedlings before cold stress treatment; Cold stress: cucumber seedlings after cold stress treatment. Each treatment contained 5-10 cucumber seedlings. The experiments were repeated three times with similar results. A typical picture is shown here. (B) The EL results of cucumber seedlings before (control) and after cold stress treatment for 48 h. (C-D) Inverted fluorescence microscope imaging of H₂O₂ and O₂^{•-} levels in cucumber seedling leaves before (control) and after cold stress treatment for 48 h. (E-F) Detection of H₂O₂ and O₂^{•-} accumulation of cucumber seedlings before (control) and after cold stress treatment for 48 h. (G-I) Expression of *CsARF5*, *CsCBF1* and *CsCOR* genes in cucumber seedlings treated with H₂O, IAA or NPA under cold stress for 6 h. qRT-PCR was performed simultaneously with three biological replicates and three technical replicates. The value of the water treatment was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

Figure 5. Overexpression of *CsARF5* improves cold tolerance of cucumber.

(A) Expression of the *CsARF5* gene in cucumber seedlings under cold stress treatment for 12 h. (B) Expression of the *CsARF5* gene in cucumber seedlings treated with NaHS or HT under cold stress treatment for 12 h. (C-D) NBT and DAB staining of empty vector control (WT) and *CsARF5* transient transgenic cucumber leaves treated with cold stress for 12 h. Each genotype contained 5-10 cucumber leaves. The experiments were repeated three times with similar results. A typical picture is shown here. (E-F) Detection of O₂^{•-} and H₂O₂ contents of transgenic cucumber leaves before (control) and after cold stress treatment for 12 h. (G-I) Expression of *CsDREB3*, *CsCBF1* and *CsCOR* genes in transgenic cucumber leaves under cold stress for 3 h. qRT-PCR was

performed simultaneously with three biological replicates and three technical replicates. The value of WT was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

Figure 6. Effect of HT on the cold resistance of *CsARF5* transgenic cucumber.

(A-B) NBT and DAB staining of empty vector control (WT), *CsARF5* transient transgenic cucumber leaves (*CsARF5*) and *CsARF5* sprayed with HT (*CsARF5*+HT) treated with cold stress for 12 h. Each genotype contained 5-10 cucumber leaves. The experiments were repeated three times with similar results. A typical picture is shown here. (C-D) Detection of $O_2^{\cdot-}$ and H_2O_2 contents of transgenic cucumber leaves before (control) and after cold stress or HT treatment for 12 h. (E-G) Expression of *CsDREB3*, *CsCBF1* and *CsCOR* genes in transgenic cucumber leaves under cold stress for 3 h. qRT-PCR was performed simultaneously with three biological replicates and three technical replicates. The value of WT was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

Figure 7. Overexpression of *CsDREB3* improves the cold tolerance of cucumber.

(A) Expression of the *CsDREB3* gene in cucumber seedlings under cold stress treatment for 12 h. (B) Expression of the *CsDREB3* gene in cucumber seedlings treated with NaHS or HT under cold stress treatment for 12 h. (C-D) NBT and DAB staining of empty vector control (WT) and *CsDREB3* transient transgenic cucumber leaves treated with cold stress for 12 h. Each genotype contained 5-10 cucumber leaves. The experiments were repeated three times with similar results. A typical picture is shown here. (E-F) Detection of $O_2^{\cdot-}$ and H_2O_2 contents of transgenic cucumber leaves before (control) and after cold stress treatment for 12 h. (G-H) Expression of *CsCBF1* and *CsCOR* genes in transgenic cucumber leaves under cold stress for 3 h. qRT-PCR was performed simultaneously with three biological replicates and three technical replicates. The value of WT was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

Figure 8. *CsARF5* directly modulates the expression of *CsDREB3*.

(A) Schematic diagram showing the *CsDREB3* promoter probe used for EMSAs. AuxRE indicates the *CsARF5* binding site. Mutated probe (*CsDREB3*-mut) in which the 5'-GAGACA-3' motif was replaced by 5'-GGGAAA-3'. (B) EMSAs showing that *CsARF5* binds to the *MdDREB3* promoter. "-" indicates the absence of corresponding proteins or probes. "+" indicates the presence of corresponding proteins or probes. "5x", "25x" and "50x" indicate increased probe concentrations. The experiments were repeated three times with similar results. A typical picture is shown here. (C) Schematic representation of the LUC reporter vector containing the *CsDREB3* promoter and

757 the effector vectors expressing *CsARF5* under the control of the 35S promoter. **(D)** Dual luciferase
758 tests in tobacco leaves showing that *CsARF5* activates *CsDREB3* transcription. Empty vector:
759 pGreenII 62-SK + pGreenII 0800-LUC; pCsDREB3: pGreenII 62-SK + pCsDREB3-pGreenII
760 0800-LUC; *CsARF5* + pCsDREB3: *CsARF5*-pGreenII 62-SK + pCsDREB3-pGreenII 0800-LUC;
761 *CsARF5* + pCsDREB3(Mut): *CsARF5*-pGreenII 62-SK + pCsDREB3(Mut)-pGreenII 0800-LUC.
762 Mutated promoter sequence (pCsDREB3-Mut) in which the 5'-GAGACA-3' motif was replaced
763 by 5'-GGGAAA-3'. The experiments were repeated three times with similar results. A typical
764 picture is shown here. **(E)** LUC/REN activity detection to verify that *CsARF5* activates the
765 transcription of *CsDREB3*. Empty vector was used as the reference and set to 1. Error bars denote
766 standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's
767 multiple range tests. All experiments were performed three times with similar results, and
768 representative data from one repetition are shown.
769

770 **Figure 9. A regulatory model elucidates that H₂S mediates the cold stress response in**
771 **cucumber through auxin signaling.**

772 In the absence of H₂S, IAA repressor proteins inhibit the expression of *CsARF5*, which in turn
773 inhibits the cold stress response mediated by the *CsARF5*-*CsDREB3* module. In the presence of
774 H₂S, IAA repressor proteins release *CsARF5*, which promotes the cold stress response by
775 activating *CsDREB3* expression.
776

Figure 1. Effects of NaHS and HT on the cold tolerance of cucumber seedlings.

(A) Phenotypic characterization of cucumber seedlings treated with NaHS or HT under cold stress (5 °C for 48 h). Water treatment was used as the control. H₂O: water treatment; NaHS: NaHS (hydrogen sulfide provider) treatment; HT: HT (hydrogen sulfide scavenger) treatment. Control: cucumber seedlings before cold stress treatment; Cold stress: cucumber seedlings after cold stress treatment. Each treatment contained 5-10 cucumber seedlings. The experiments were repeated three times with similar results. A typical picture is shown here. **(B)** The EL results of cucumber seedlings before (control) and after cold stress for 48 h. **(C-D)** Inverted fluorescence microscopy imaging of H₂O₂ and O₂⁻ levels in cucumber seedling leaves before (control) and after cold stress treatment for 48 h. **(E-F)** Detection of H₂O₂ and O₂⁻ content of cucumber seedlings before (control) and after cold stress treatment for 48 h. **(G-H)** Expression of *CsCBF1* and *CsCOR* genes in cucumber seedlings treated with H₂O, NaHS or HT under cold stress treatment for 6 h. qRT-PCR was performed simultaneously with three biological replicates and three technical replicates. The value of the water treatment was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

Figure 2. RNA- seq analysis of cucumber seedlings with or without NaHS treatment.

(A) Normalized heat map showing the changes in gene expression after NaHS treatment at 5 °C for 6 h. All experiments were performed in triplicate. **(B)**

Normalized heat map of upregulated gene expression after NaHS treatment at 5 °C for 6 h. All experiments were performed in triplicate.

Figure 3. NaHS treatment affects IAA level in cucumber.

(A) The IAA content of cucumber seedlings treated with NaHS or HT under cold stress treatment for 24 h. **(B)** The FMO activity of cucumber seedlings treated with NaHS or HT under cold stress treatment for 24 h. **(C)** Expression of the *CsARF5* gene in cucumber seedlings treated with NaHS or HT under cold stress treatment for 6 h. **(D)** Expression of the *CsDREB3* gene in cucumber seedlings treated with NaHS or HT under cold stress treatment for 6 h. qRT-PCR was performed simultaneously with three biological replicates and three technical replicates. The value of 0 h was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

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(A) Expression of the *CsDREB3* gene in cucumber seedlings under cold stress treatment for 12 h. (B) Expression of the *CsDREB3* gene in cucumber seedlings treated with NaHS or HT under cold stress treatment for 12 h. (C-D) NBT and DAB staining of empty vector control (WT) and *CsDREB3* transient transgenic cucumber leaves treated with cold stress for 12 h. Each genotype contained 5-10 cucumber leaves. The experiments were repeated three times with similar results. A typical picture is shown here.

(E-F) Detection of O_2^- and H_2O_2 contents of transgenic cucumber leaves before (control) and after cold stress treatment for 12 h. (G-H) Expression of *CsCBF1* and *CsCOR* genes in transgenic cucumber leaves under cold stress for 3 h. qRT-PCR was performed simultaneously with three biological replicates and three technical replicates. The value of WT was used as the reference and was set to 1. Error bars denote standard deviations. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range tests.

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- H₂S

+ H₂S

Cold stress

Cold stress

Figure 9. A regulatory model elucidates that H₂S mediates the cold stress response in cucumber through auxin signaling.

In the absence of H₂S, IAA repressor proteins inhibit the expression of CsARF5, which in turn inhibits the cold stress response mediated by the CsARF5-*CsDREB3* module. In the presence of H₂S, IAA repressor proteins release CsARF5, which promotes the cold stress response by activating *CsDREB3* expression.