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Hydrogen sulfide mediated the melatonin induced stoma closure by regulating the K^+ channel in *Arabidopsis thaliana*

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ABSTRACT

The roles of the gasotransmitter hydrogen sulfide (H_2S) and the phytohormone melatonin (MEL) in enhancing plant drought resistance are increasingly understood, but the specific regulatory mechanisms in response to drought remain unclear. This study investigated their relationship in the regulation of stomatal movement in response to drought stress. Under normal and drought stress conditions, exogenous MEL increased the H_2S content, production rate and the transcription level of H_2S production-related encoding genes LCD and DES1. The ability of MEL to increase drought stress tolerance was inhibited significantly in *lcd*, des1 and lcd/des1 mutants, which implied that MEL was partially dependent on H_2S in this process. Exogenous MEL and H_2S increased the transcriptional level of drought related transcription factors *CBF2*, *CBF3*, *RD29A*, *DREB2A* and *DREB2B* in response to drought stress and H_2S and MEL affected the transcription level of K^+ channel-related genes *KCO1*, *GORK*, *AKT1*, *AKT2*, *KAT1*, and *KC1* to regulate stomatal movement. At the post-translational level, H_2S increased the persulfidation levels of KCO1 and AKT1 and reduced their degradation rate. It was also found that endogenous H_2S and MEL content had no effect on the mutants of *kco1*, *kat1*, *akt1* and *gork*. It was concluded that H_2S mediated the MEL induced stoma closure by regulating the K^+ channel to enhance plant drought resistance in Arabidopsis.

1. Introduction

The physiological function of hydrogen sulfide (H_2S) in plants is receiving extensive attention from researchers (Wang, 2002; Pei, 2016). It plays a very important and active role in coping with abiotic stress (Liu et al., 2021) and can respond to it by regulating the transcriptional level of related genes, affecting the post-translational modification of proteins and interacting with other signaling molecules (Zhu et al., 2018; Jiang et al., 2020; Ahmed et al., 2021; Thakur et al., 2021). Among them, the significant role of H_2S in alleviating plant drought stress by regulating stomatal closure has been confirmed (Jin et al., 2011; Du et al., 2019; Zhang et al., 2021; Hsu et al., 2021).

The H_2S has been shown to participate in the post-translational modification of protein cysteine groups to form sulfhydryl (R-SSH) groups (Gotor et al., 2019). Proteomic analysis of *Arabidopsis thaliana* showed that more than 5 % of proteins may be modified by persulfidation. The SnRK2.2 and SnRK2.6/OST1 persulfidation modifications during abscisic acid (ABA) regulated stomatal movement provide important evidence for how H_2S affects the ABA signaling pathway (Chen et al., 2020b). In addition, the persulfidation of proteins involved in other physiological processes in plants has also been gradually revealed (Aroca et al, 2018).

The earliest reports of melatonin (MEL) originated from the discovery of its presence in the pineal gland of cattle by a dermatologist called Lerner (Lerner et al., 1958) and the discovery sparked research into its physiological function in animals. It has been shown that MEL has

Abbreviations: H_2S, Hydrogen sulfide; MEL, Melatonin; AzMC, 7-azido-4-methylcoumarin; DPD, *N,N*-dimethyl-phenylenediamine; LCD, L-cysteine desulfydrase; DES1, L-cysteine desulfhydrase; SNAT, Serotonin N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase; COMT1, Caffeic acid O-methyltransferase; KCO1, Outward rectifying potassium channel protein; GORK, Gated outwardly-rectifying K^+ channel; AKT1, K^+ transporter 1; AKT2, Potassium transport 2; KC1, Potassium channel AKT1-like; CBF2/3, C-repeat/DRE binding factor 2/3; RD29A, Desiccation-responsive protein 29 A; DREB2A/2B, DRE/CRT-binding protein 2 A/2B.

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potent and pleiotropic effects on physiological processes such as regulating circadian rhythms, maintaining body temperature homeostasis, regulating sleep and the immune system and participating in various cellular activities as an important antioxidant (Jan et al., 2009; Sun et al., 2020).

Although this indoleamine molecule has attracted a lot of attention in animals, research in plants started much later (Dubbels et al., 1995; Hattori et al., 1995). The enzymes SNAT, COMT1 and ASMT are related to MEL synthesis in plant (Wang et al., 2022b). In the past two decades, the role of MEL in plants has gradually been revealed and it has been confirmed that it acts as a regulator to promote plant growth and development, by promoting seed germination, enhancing root morphology, regulating the flowering of plants and delaying senescence. It is also a research focus on its role as a stress protectant to improve plant tolerance to adverse environmental conditions (Chen et al., 2019; Arnao and Hernández-Ruiz, 2020; Sun et al., 2020). It can regulate stomatal movement in response to drought stress by interacting with the phytohormone abscisic acid (ABA) (Chen et al., 2020a). It has been confirmed that the MEL receptor CAND2/PMRT1 can regulate stomatal on-off by interacting with H_2O_2 and Ca^{2+} signaling and the specific mechanism of its regulation of stomatal movement has been further investigated (Wei et al., 2018).

Stomatal movement is achieved by the expansion and contraction of guard cells. The activation of ion channels on guard cell membranes and the regulation of ion transporters are the most direct and effective ways to regulate stomatal movement (Kim et al., 2010; García-Mata and Lamattina, 2013). The role of endogenous H_2S in stomatal closure by mediating the influx of K^+ on guard cell membranes has been reported (Papanatsiou et al., 2015; Wang et al., 2016; Jin et al., 2017) and exogenous MEL has a similar regulatory effect (Wei et al., 2018). Studies have already revealed the close interaction of MEL and H_2S in this process, but the understanding of the mechanism is still very limited.

This study provided MEL partially dependent on H_2S to close the stomata by regulating the K^+ channel in *Arabidopsis thaliana* and this process was mediated by persulfidation of ion channel proteins. This study complemented the interaction between H_2S and MEL in regulating stomatal movement and provides a new perspective for plant drought resistance research.

2. Materials and methods

2.1. Plant materials and growth conditions

The materials in this study were Arabidopsis ecotype Columbia-0 (Col-0). The T-DNA insertion mutants of *LCD* (*lcd*, SALK_082099) and *DES1* (*des1*, SALK_205358C) were obtained from the Arabidopsis Biological Resource Center (https://www.arabidopsis.org/abrc/). The *COMT1* (*comt1*, SALK_135290C), *KCO1* (*kco1*, SALK_146903C), *ASMT* (*asmt*, SALK_084490C), *KAT1* (*kat1*, SALK_127506C), *SNAT* (*snat*, SALK_033944C), *AKT1* (*akt1*, SALK_071803C) and *GORK* (*gork*, SALK_082258C) were all obtained from the Arashare (http://www.arashare.cn/). The *lcd/des1* mutant was obtained by the hybridization of *lcd* and *des1*. The primers used in the experiments are listed in Table S1.

Seeds were grown in pots containing a soil/vermiculite/perlite (1/1/1) mixture and plant seedings were cultivated at 160 mE m^{-2} s^{-1} on a 16-h light/8-h dark lighting pattern.

2.2. Experimental treatment of materials

Four-week-old Arabidopsis plants were treated with 0 μM (control plants unstressed (CS)), 80 μM Sodium Hydrosulfide (NaHS) as an H_2S donor, 20 μM MEL, 1 mM Hydroxylamine (HA) as an H_2S synthesis inhibitor, 30 % PEG-8000 (PEG) and H_2S+MEL for 9 h, and then the H_2S content, H_2S production rate and gene expression were measured. For the determination of plant survival rate after drought, four-week-old Arabidopsis seedings were stressed for 10 days. One day after being re-watered, the survival rate of the plants was calculated.

2.3. Determination of H_2S content and production rate

The determination of H_2S content and production rate was by the methylene blue method (Siegel, 1965; Zhao et al., 2001). Hao et al. (2020) had described the measurement method of H_2S content, where the PBS-ground plant material was placed in an Erlenmeyer flask, with a 1.5 centrifuge tube containing zinc acetate. After the addition of hydrochloric acid, the reaction occurred at room temperature for 30 min, so the zinc acetate could fully absorb the H_2S in the samples and generate a stable intermediate product. The reaction was terminated in darkness for 15 min after the addition of $FeCl_3$ and *N,N*-dimethyl-phenylenediamine (DPD). Finally, the absorbance value was measured at 670 nm.

The method of measuring the H_2S production rate has been described (Du et al., 2019). Total protein was obtained by centrifugation of the PBS-ground plant material.

As with the determination of the content, a 1.5 centrifuge tube containing zinc acetate was placed in the Erlenmeyer flask. After adding the reaction mixture containing the enzymatic reaction substrate L-Cys, the flask was placed in a constant temperature shaker at 115 rpm for 15 min, so the zinc acetate could fully absorb the H_2S released by the reaction between the enzyme and the substrate. The $FeCl_3$ and DPD were then added and the reaction was conducted in the dark for 15 min. Finally, the absorbance was measured at 670 nm.

2.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from plant samples was extracted with RNAiso Plus (TaKaRa, Shiga, Japan) and reversed transcribed into cDNA using 5 ×All-In-One MasterMix (ABM, Canada). The specific primers used in qRT-PCR experiments were designed by the software Primer 5.0. (Wang et al., 2022b) Gene expression was detected using SYBR Green PCR kit (QuantiNova, Germany) and the qRT-PCR has been described by Jin et al. (2011).

2.5. Stomatal aperture measurement

The lower epidermal strips of Arabidopsis rosette leaves were used to observe the stomatal apertures after different treatments. Epidermal strips were incubated in an epidermal strip buffer of 10 mM morpholino ethane sulfonic acid (MES) and 50 mM KCl under plant culture lamp for 2 h (160 mE m^{-2} s^{-1}). The samples were then treated with different treatment solutions for 4 h, then the stomata were observed and counted with a BX53F optical microscope (Olympus, Japan).

2.6. Detection of H_2S contents by probes

The probe 4-(diethylamino) salicylaldehyde can specifically detect the content of H_2S in plant samples (Liu et al., 2013). The probe was kindly provided by Jing Liu of the Shanxi University. This probe was selected to detect H_2S content in 10-day-old Arabidopsis leaves and four-week-old Arabidopsis lower epidermal strips. The probe was diluted to 10 μM in epidermal strip buffer for sample treatment. The probe-added samples were protected from light for 2 h, then the samples were washed three times with epidermal strip buffer to remove excess probe for 30 min each. The cleaned samples were made into simple mounts, placed under a fluorescence microscope, and the H_2S probe fluorescence 205 was observed using ultraviolet light. Another H_2S-specific probe, 7-azido-4-methylcoumarin (AzMC), was also used to detect H_2S content in 10-day-old Arabidopsis seedlings as shown in Fig. S1. The AzMc probes has been described in a previous study (Wang et al., 2022a).

2.7. Molecular cloning and expression vector construction

The methods were described previously (Wang et al., 2022a). Using the method of homologous recombination, the coding sequences of *AKT1*, *KAT1* and *KCO1* were integrated into the downstream of the His6 tag in the pET28a expression vector using the Ligation-free cloning system (ABM, Canada) and then the sequenced vector was transformed into *Escherichia coli* BL21 strain to induce the target protein.

2.8. Recombinant protein expression and detection

Details of protein expression have been described (Wang et al., 2022b). The *Escherichia coli* BL21 were incubated in 100 mL LB liquid medium containing 50 μg/mL kanamycin at 37 °C until the OD600 was 0.6–0.8. Then, 0.3 mM isopropyl β-D-1-thiogalactropyranoside was added, and the fusion proteins were incubated 8 h at 37 °C (AKT1, KCO1) and 10 h at 28 °C (KAT1), respectively. Then, the purified proteins were eluted with 200 mM imidazole using Ni-NTA-chelation chromatography (Novagen, China). The supernatant was collected and subjected to polyacrylamide gel electrophoresis and the purification was observed with Coomassie brilliant blue (Diamond, China) staining as seen in Fig. S2.

2.9. Biotin-Switch Assay for determining persulfidation

The Biotin-Switch Assay (BSA) was used to detect the persulfidation level of proteins (Mustafa et al., 2009). The recombinant proteins were first treated with different concentrations of NaHS for persulfidation modification. The treated proteins were obtained by precipitation with the organic solvent acetone at − 20 °C. The HEN buffer (250 mM Hepes-NaOH, pH 7.7, 1 mM EDTA and 0.1 mM neocuproine) was used to dissolve the precipitated protein and methyl thiosulfonate (MMTS) was added to block the free sulfhydryl groups in the recombinant proteins. Blocked proteins were subjected to a second cryoprecipitation and resolubilization. Biotin was then added to conduct the labeling reaction to specifically label the modified sulfhydryl group and during the reaction, the sample was placed in a rotator at 37 °C for 3 h. The protein concentrations of the reaction samples were measured, after ensuring that they were consistent, by adding 5 × SDS (Sodium dodecyl sulfate) loading buffer in a boiling water bath for 10 min. The samples after SDS-PAGE electrophoresis were detected by Western Blot and the primary antibody used in the detection was the biotin antibody (Sigama, USA). The color reaction of nitro blue tetrazolium/5-bromo-4-chloro-3-indole phosphate was used to detect whether the proteins could be modified by NaHS and the software ImageJ was used to quantify the degree of protein persulfidation.

2.10. Cell-free protein degradation assay

The cell-free protein degradation assay was described previously (Zhou et al., 2022). Ten-day-old Arabidopsis seedlings were selected to extract their total protein. Plant samples were ground in liquid nitrogen and homogenized in a degradation buffer made up of 25 mM pH 7.4 Tris-HCl, Triton X-100, one mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 0.2 % 5 mM ATP and 1 mM PMSF (Phenylmethylsulfonyl fluoride). The specific operation process was to mix total protein and recombinant protein into a reaction system at 25 °C with a total volume of 100 μL with reaction times of 0, 3, 6, 9, 12 and 24 h. The addition of 5× SDS loading buffer stopped the reaction after the time was up. Samples were detected with an anti-His antibody (BBI, China) during western blot analysis.

2.11. Statistical analysis

The experimental results of three technical repetitions were expressed as the mean ± SE. The SPSS 17.0 (SPSS, IBM, Chicago, IL, USA) was used to analyze the data. Tukey's test ($P < 0.05$) was used to evaluate the significance of difference between the treatment means. An asterisk (*) and different letters indicate significant differences ($P < 0.05$).

3. Results

3.1. Exogenous MEL increased the H_2S content, production rate and the transcription level of H_2S synthase-encoding genes LCD and DES1

In the previous process of exploring the relationship between MEL and H_2S, the four-week-old Arabidopsis plants were treated with MEL for 0, 1, 3, 6 and 9 h and it was found that exogenous MEL increased the H_2S content and production rate with increasing treatment time as seen in Fig. 1a and b, so based on this result, 9 h was chosen as the processing time. The H_2S content, production rate and the transcription level of H_2S synthase-encoding genes *LCD* and *DES1* were measured after MEL treatment under normal and drought stress. The results showed that MEL could significantly increase the H_2S content and production rate and the transcription of *LCD* and *DES1* under normal and drought treatments as shown in Fig. 1c–f. These results suggested that exogenous MEL could promote the production of endogenous H_2S.

3.2. MEL closed stomata by increasing H_2S content under drought stress

To further study the relationship between MEL and H_2S in drought stress, Arabidopsis leaves and lower epidermal strips were treated with H_2S-specific probes 4-(Diethylamino) salicylaldehyde. It was shown in Fig. 2a that after MEL treatment, the H_2S content increased under both normal conditions and drought stress. Another H_2S-specific probe, AzMC, had the same results as shown in Fig. S1.

The stomatal aperture was measured under different treatments. The results showed that exogenous MEL significantly closed stomata under normal and drought stress as seen in Fig. 2b and c. In epidermal strips after probe 4-(Diethylamino) salicylaldehyde incubation, it was found that the fluorescence was concentrated in the guard cells of the stomata, with the highest content in PEG+MEL as shown in Fig. 2c. These results suggested that the H_2S generated after MEL treatment triggered stomatal closure.

3.3. MEL partially dependent on H_2S to close the stomata

The role that H_2S played in MEL closing the stomata was examined by measuring the stomatal aperture in Col, *lcd*, *des1* and *lcd/des1* after MEL treatment. Compared with the Col, the ability of MEL to close the stomata was impaired in *lcd* and *des1*. This phenotype was more prominent in *lcd/des1* as seen in Fig. 3a and b. After HA+MEL treatment, the ability of MEL to close the stomata was impaired compared with MEL treatment as shown in Fig. 3c. These results indicated that H_2S participated in the closure of stomata by MEL.

3.4. MEL improved drought resistance in Arabidopsis

To further confirm the role of MEL and H_2S in resisting drought stress. Four-week-old Arabidopsis was used for phenotypic observation after 10 days of drought in Fig. 4a. One day after rewatering, the survival rate was counted and the results showed that the survival rates of plants after MEL treatment were significantly increased compared with that of Col as seen in Fig. 4b. However, the ability of MEL to improve drought tolerance was weakened in the mutants, especially in the mutant *lcd/des1* as shown in Fig. 4a and b. This result further confirmed that H_2S was required for MEL to improve plant drought tolerance.

3.5. MEL and H_2S enhanced drought resistance in plants by increasing the expression of the specific transcription factors

To determine the effect of MEL and H_2S on drought-related

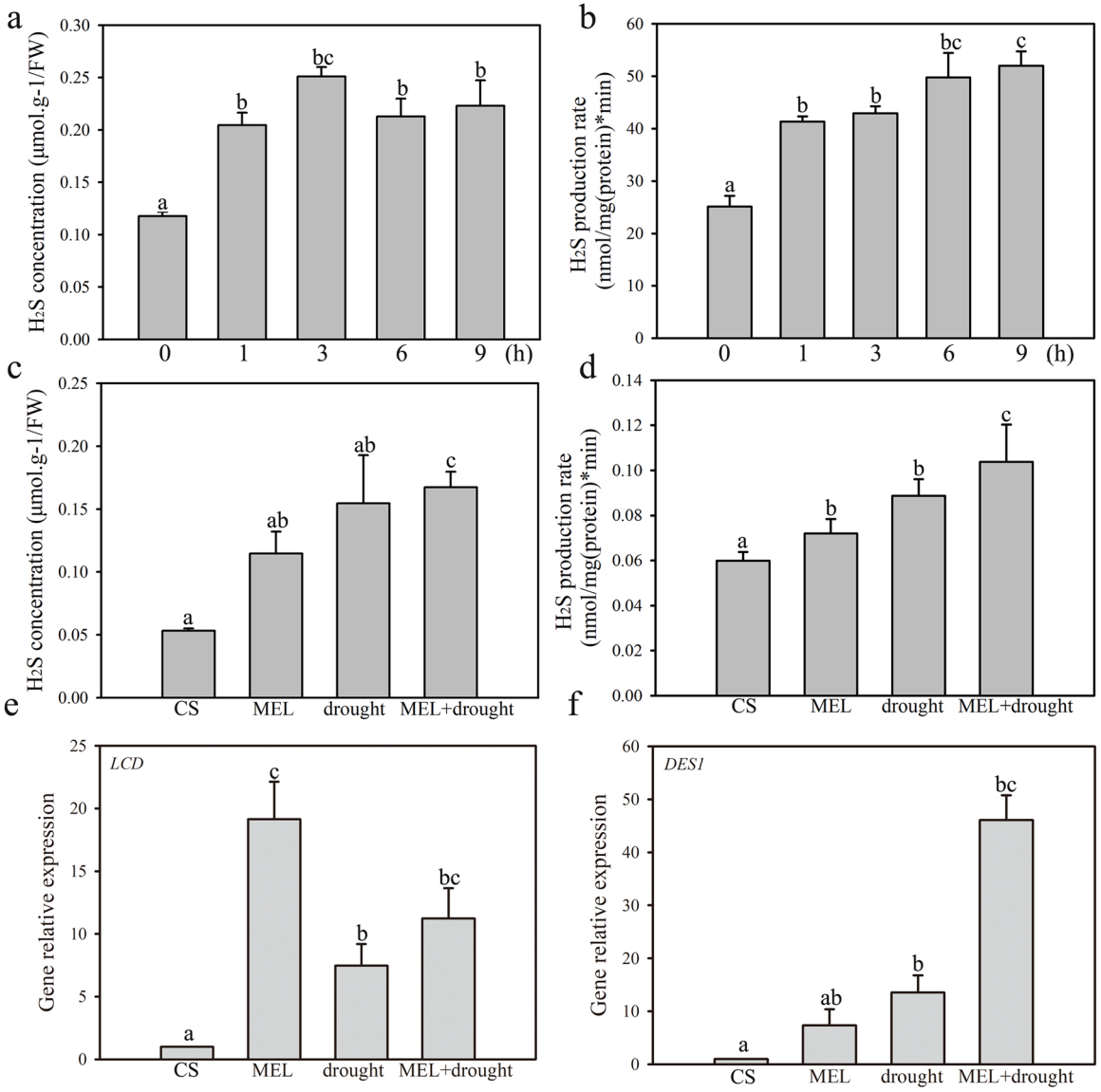

Fig. 1. The effect of MEL on H_2S biosynthesis under the drought condition in Arabidopsis. The H_2S concentration (a) and production rate (b) were measured after treatment with MEL for 0, 1, 3, 6 and 9 h. The H_2S concentration (c) and production rate (d) were measured under different treatments. The expression of H_2S synthase-encoding genes LCD (e) and DES1 (f) was detected under different treatments. Error bars indicate the standard error of three biological repeats and different letters are significantly different among treatments ($P < 0.05$).

transcription factors, the expression of *CBF2*, *CBF3*, *RD29A*, *DREB2A* and *DREB2B* were detected in Col, *lcd*, *des1* and *lcd/des1* under normal and drought conditions, as shown in Fig. 5a–e. The results showed that the expression of drought-related transcription factors in *lcd*, *des1* and *lcd/des1* were lower than that of the Col under the drought conditions. Exogenous MEL further increased the expression of these genes, but this increase was not obvious in *lcd/des1* as seen in Fig. 5. The results suggested that MEL was partially dependent on H_2S in response to drought by regulating the transcriptional levels of drought-related transcription factors.

3.6. MEL and H_2S closed the stomata by regulating the expression of K^+ channel-related genes

Drought stress drives the closure of stomata by activating the K^+ channel and its mediated K^+ outflow and KCO1 and GORK participate in the formation of K^+_{out} channels in the plasma membrane of stoma guard cells, whereas AKT1, AKT2, KAT1 and KC1 participate in the formation of the K^+_{in} channel.

The results showed that exogenous MEL and H_2S increased the transcription levels of *KCO1* in Fig. 6a and *GORK* in Fig. 6b under normal and drought conditions. The transcription levels of *AKT1*, *AKT2*, *KAT1* and *KC1* were significantly reduced under different treatments, as seen in Fig. 6c–f. Drought+MEL+H_2S had the greatest effect on the transcript level. The results indicated that exogenous MEL and H_2S closed the stomata by promoting K^+_{out} related genes and reducing the expression of K^+_{in} related genes.

The effects of endogenous MEL and H_2S on the transcriptional levels of K^+ channel-related genes were investigated next. The expression levels of K^+ channel related genes were measured in Col, *snat*, *comt1*, *asmt*, *lcd*, *des1* and *lcd/des1* plants. This result was consistent with the exogenous MEL and H_2S treatments in Figs. 6 and 7. The contents of H_2S and MEL were determined Col, *kco1*, *kat1*, *akt1* and *gork* mutants and the results showed that the mutants had no effect on the contents of H_2S and MEL as seen in S3.

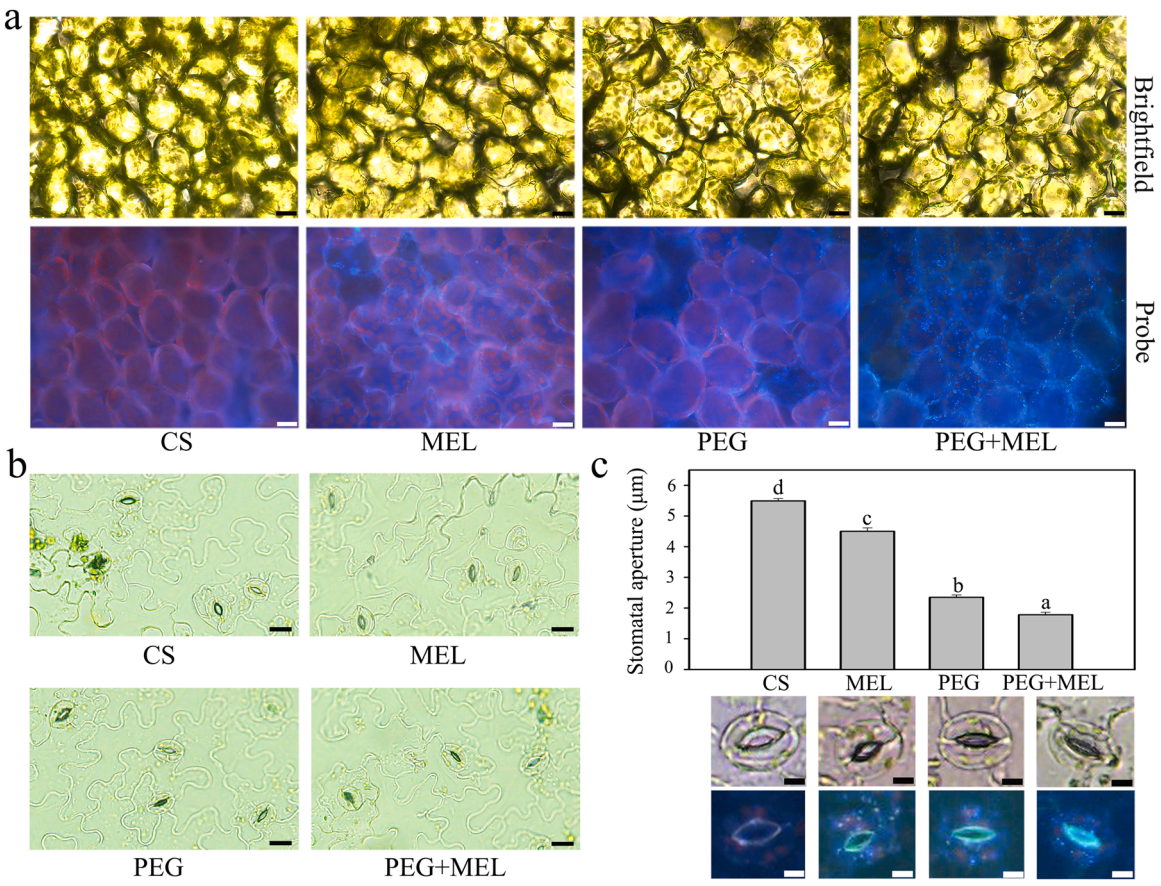

Fig. 2. The effect of H_2S content and stomatal aperture under different treatments. (a) The H_2S fluorescence intensity in the leaves (bars = 20 μm). (b) Effect on stomatal aperture under different treatments. The samples were incubated with 20 μM MEL and 30 % PEG for 4 h before stomatal aperture measurement. The stomate were observed under an optical microscope at 200 × magnification (bars = 10 μm). (c) The H_2S fluorescence intensity in the lower epidermis (bars = 5 μm) and stomatal aperture under different treatments. Data was measured from at least 60 guard cells. The error bars indicate the standard error and the different letters or an asterisk (*) are significantly different among treatments (*, $P < 0.05$).

3.7. H_2S induced the persulfidation level of KCO1 and AKT1 and inhibits their degradation

The BSA method was used to detect whether K^+ channel related proteins could be persulfidated by H_2S. The results showed KCO1 and AKT1 were persulfidated by NaHS, but not KAT1 in Fig. 8a–d and the increase in persulfidation levels of KCO1 and AKT1 was dependent on the NaHS concentration.

Cell-free protein degradation assay was used to detect the degradation of persulfidation proteins (Zhou et al., 2022). It was apparent from the results that the KCO1 and AKT1 protein levels were gradually decreased, which was further rescued by NaHS in Fig. 8g–i. These results indicated that the stability of KCO1 and AKT1 were related to its persulfidation.

4. Discussion

The production of endogenous H_2S and MEL in plants can be activated by various signaling molecules, hormones and abiotic stresses (Aroca et al., 2018; Sun et al., 2020; Liu et al., 2021). It has been reported that exogenous H_2S can promote the production of MEL (Wang et al., 2022b). This study confirmed that exogenous MEL also promoted the production of endogenous H_2S, especially under drought stress as showed in Fig. 1. It has been demonstrated that MEL and H_2S separately improve the drought tolerance of plants by closing stomata (Wei et al., 2018; Jin et al., 2017). The mutants *lcd*, *des1* and *lcd/des1* were introduced to study the relationship between H_2S and MEL, which showed that H_2S was necessary for MEL to close stomata, especially the phenotype of *lcd/des1* plants as showed in Fig. 3. This phenomenon was also reflected in the study of the survival rate of plants with varied materials after drought in Fig. 4 and the results suggested that MEL was partially dependent on H_2S to regulate stomatal closure.

The ABA plays a very important role in regulating stomatal movement. It can induce stomatal closure and inhibit its opening under drought stress (Kim et al., 2010; Chen et al., 2020a; Hus et al., 2021), which could activate the expression of many transcription factors in plants (Jin et al., 2011; Mao et al., 2015; Kumar et al., 2017; Takahashi et al., 2020). Transcription factors that respond to drought stress can be divided into two categories as ABA-dependent and ABA-independent (Zhang et al., 2004). Several studies on ABA, H_2S and MEL in drought tolerance have been reported. Li et al., (2015) found that MEL down-regulated the ABA biosynthetic gene MdNCED3 (Iuchi et al., 2001), and upregulated the catabolic genes MdCYP707A1 and MdCYP707A2, thereby reducing ABA content and maintaining stomatal closure under drought stress (2015). García-Mata and Lamattina revealed the role of H_2S in ABA dependent stomatal closure. Exogenous application of H_2S scavenger and the absence of endogenous H_2S synthase could partially block ABA dependent stomatal closure (García-Mata and Lamattina 2010). The role of H_2S in regulating the relationship between stoma and ABA has also been confirmed (Hsu et al., 2021; Scuffi et al., 2014). It was found that the expression of synthetic ABA and DES1 in guard cells could synergistically promote stomatal closure in response to drought stress (Zhang et al., 2021). In this study, several representative transcription factors including ABA-dependent *CBF2* and *CBF3*, ABA-independent *DREB2A* and *DREB2B*, and downstream *RD29A* were selected to study the effects of H_2S and MEL on their transcriptional levels. These results

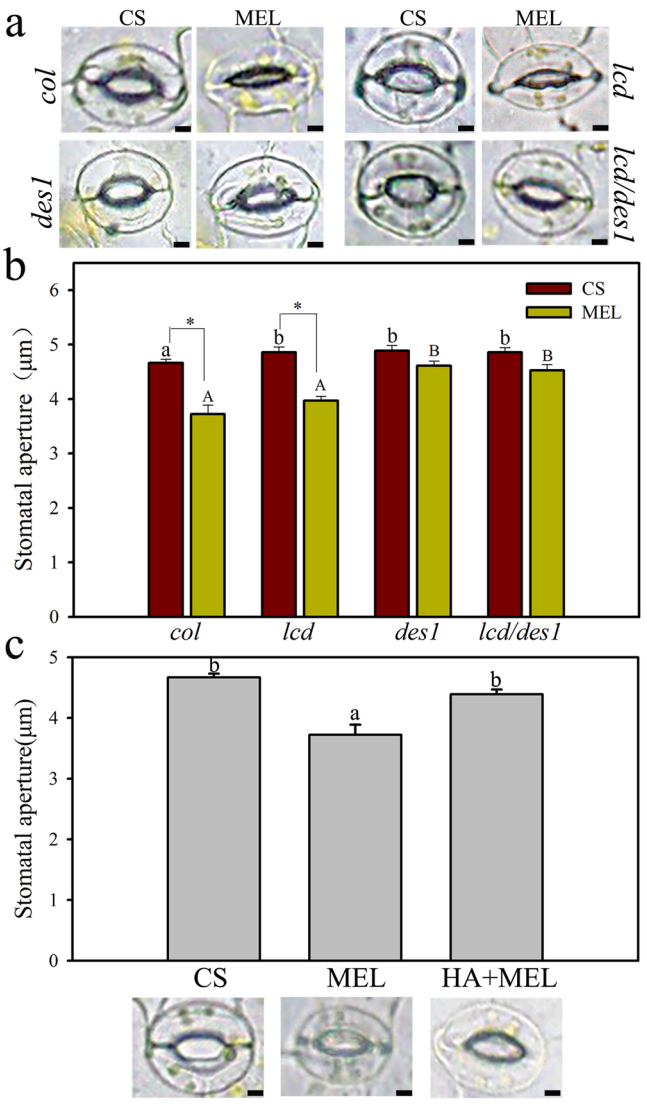

Fig. 3. The effect of stomatal aperture of Col, lcd, des1 and lcd/des1 under different treatments. The samples were incubated with 20 μM MEL and one mM HA for 4 h before stomatal pore measurement. (a) The effect of H_2S content on the closure of stomata by MEL (bars = 3 μm). (b, c) Stomatal aperture for the treatments was measured from at least 60 guard cells. The experiment was repeated at least three times. The error bars indicate the standard error and the different letters or an asterisk (*) are significantly different among treatments (*, $P < 0.05$).

suggested that H_2S and MEL cooperated with ABA in the process of regulating the expression of drought transcription factors and improving plant drought tolerance.

The activity of ion channels in guard cells is crucial to the control of stomatal movement. Stomatal movement can be regulated by activating anion and K^+_{out} integrating channels and inhibiting K^+_{in} channels (Kim et al., 2010). At present, several K^+ related genes that have been cloned, including AtKC*1*, *KT1*, *AKT1*, *KAT1/2*, *GORK*, *SKOR*, *KCO*, etc. Among them, the main role of AKT1 is to help plants absorb K^+, and the role of KAT1 is to transmit signals during stomatal movement (Liam and Julia, 2004). GORK controls the process of K^+ flowing out of guard cells through KCO channels (Ache et al., 2000). It has been found that when the activity of GORK was inhibited, the stomatal closing function of plants was inhibited and becomed more sensitive to drought stress (Hosy et al., 2003). Some results also showed that ABA could inhibit K^+_{in} and increase the activity of the K^+_{out} encoding gene *GORK* in promoting stomatal closure (Sato et al., 2009; Corratgé-Faillie et al., 2017). The results of the H_2S probe 4-(Diethylamino) salicylaldehyde incubation in this study showed that both MEL and drought stress could increase H_2S content in guard cells, as seen in Fig. 2. It is worth mentioning that MEL further enhanced the accumulation of H_2S in guard cells under drought stress, and these results suggested that H_2S and MEL are closely related in regulation of stomatal movement.

It has been reported that MEL and H_2S regulate stomata by affecting the flow direction of K^+ in the K^+ channel. Previous studies by this group have shown that the flow rate of K^+ in guard cells is closely related to the content of endogenous H_2S (Jin et al., 2017) and MEL could regulate stomatal closure by inducing K^+ efflux and Ca^{2+} influx in stomatal guard cells under drought stress (Wei et al., 2018).

The results in Fig. 6 showed that exogenous MEL and H_2S could increase the expression of K^+_{out} encoding genes *KCO1* and *GORK*, and reduce the K^+_{in} encoding genes *AKT1*, *AKT2*, *KAT1* and *KC1* under normal and drought stress conditions. This phenomenon is consistent with previous findings (Kim et al., 2010). The effect of Drought+MEL+H_2S on the regulation of K^+ channel-encoding gene transcription levels was extremely significant as seen in Fig. 6. This result is highly consistent with the previous observation of stomatal pore size (Wang et al., 2022b). That is to say, the process of MEL and H_2S synergistically regulating stomatal movement could be achieved by regulating K^+ channels.

In a further study of the response of MEL and H_2S to K^+ channels, mutants of MEL and H_2S-producing enzymes were introduced for in vivo studies, which indicated that the effects of endogenous MEL and H_2S on the transcriptional level of K^+ channel-related genes were very significant. The results in vivo and in vitro were also highly consistent (Figs. 6,7). The K^+_{in} and K^+_{out} encoding genes were selected for positive and negative arguments and some other channel-related encoding genes were also selected for research. The results showed that in addition to K^+ channels, H_2S and MEL also induced stomatal closure through the regulation of Ca^{2+}, anion channels, and H^+-ATPase in Fig. S4. The presence of H_2S has been reported to induce stomatal closure by inhibiting K^+ influx and activating anion channels (Papanatsiou et al., 2015; Blatt, 2000), so the various ion channels present in the cytoplasmic membrane obviously do not function independently. How H_2S and MEL participate in the interaction of the various ions still needs further study.

Diversity of protein functions is inseparable from the post-translational modification of proteins and H_2S regulates the structure and activity of proteins through persulfidation of proteins (Aroca rt al, 2018; Shen et al., 2020). It has been confirmed that H_2S induces the catalytic action of DES1 in guard cells and promotes the sulfhydrylation of SnRK2.6/OST1 at Cys131 and Cys137, resulting in the exposure of these two Cys on the surface of the protein. The sulfhydrylated SnRK2.6/OST1 interacted with ABF2 and communicates the signal to close stomata (Chen et al., 2020b). The ABA-activated OST1 can phosphorylate KAT1 and inhibit K^+_{in} during stomatal closure (Sato et al., 2009). The results from this study indicated that H_2S was able to increase the sulfhydrylation levels and protein stability of KCO1 and AKT1 as shown in Fig. 8 and it regulates stomatal movement by enhancing the sulfhydrylation levels and reducing its degradation rate of KCO1 and AKT1. Our previous research confirmed that endogenous H_2S promotes K^+ to close stomata by affecting K^+ channel activity (Jin et al., 2017). In summary, we comprehensively expounded how H_2S regulates stomatal movement by affecting K^+ channels by combining the transcriptional levels of K^+ channel related genes, the in vitro sulfhydrylation levels of recombinant proteins, and the stability of proteins.

5. Conclusions

H_2S and MEL have been gradually discovered to enhance plant drought resistance by closing stomata, but the specific regulatory mechanisms remain unclear. Here, this study focused on exploring the interaction between H_2S and MEL in response to drought, then

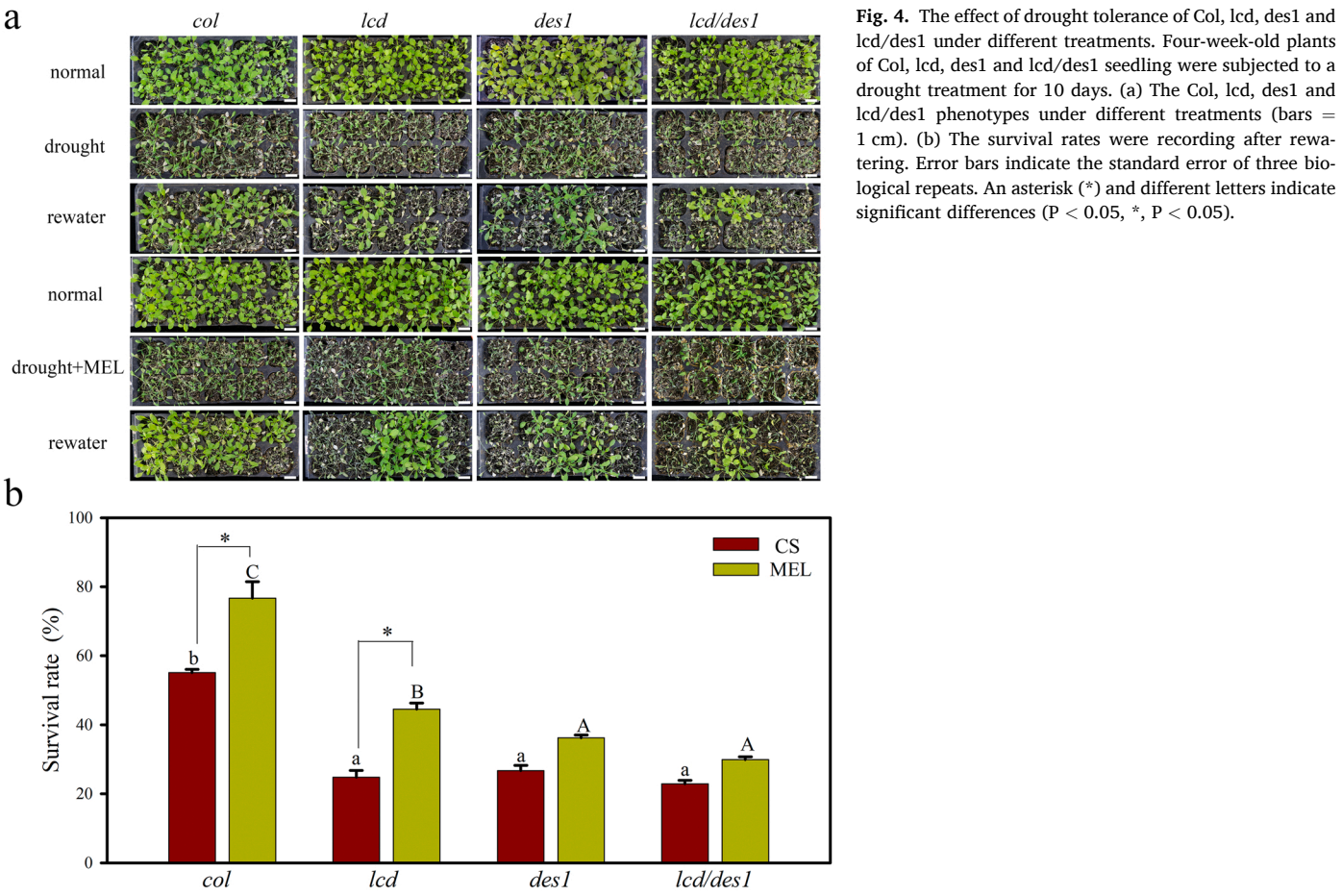

Fig. 4. The effect of drought tolerance of Col, lcd, des1 and lcd/des1 under different treatments. Four-week-old plants of Col, lcd, des1 and lcd/des1 seedling were subjected to a drought treatment for 10 days. (a) The Col, lcd, des1 and lcd/des1 phenotypes under different treatments (bars = 1 cm). (b) The survival rates were recording after rewatering. Error bars indicate the standard error of three biological repeats. An asterisk (*) and different letters indicate significant differences ($P < 0.05$, *, $P < 0.05$).

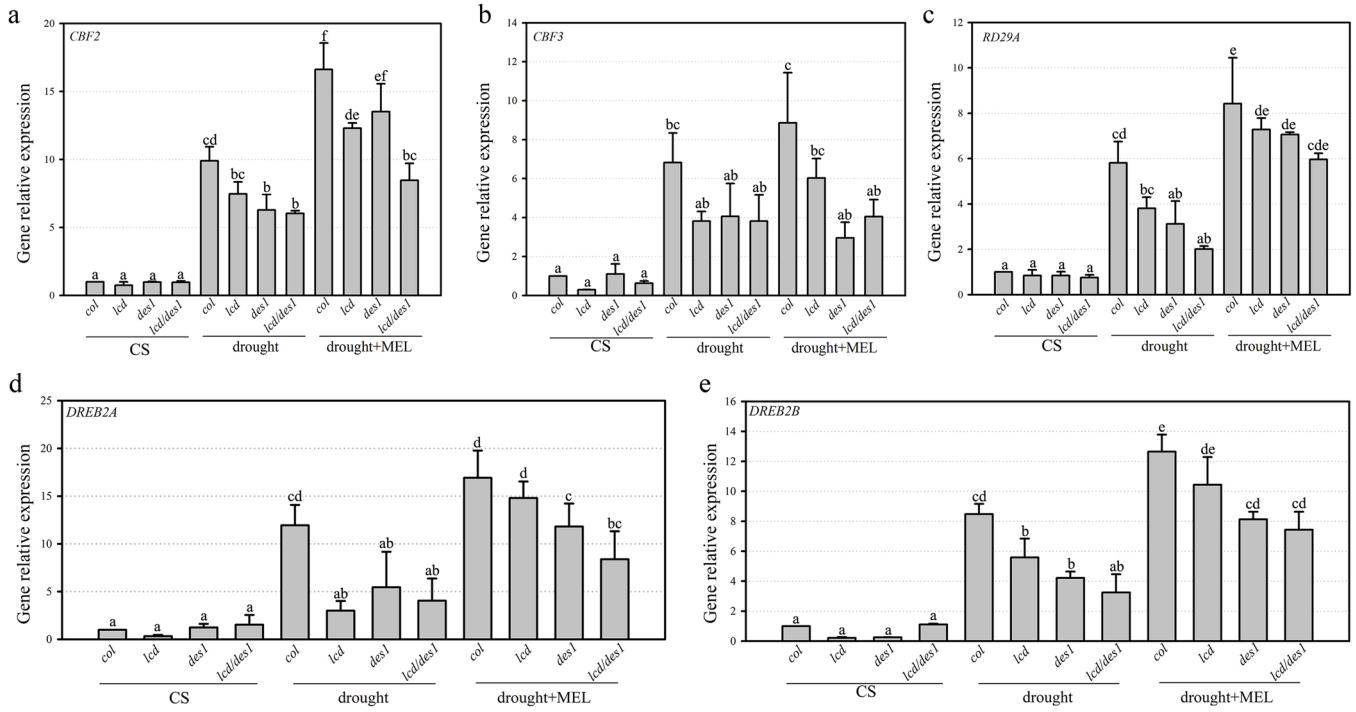

Fig. 5. The transcriptional levels of drought-related transcription factors in Col, lcd, des1and lcd/des1 plants under the different treatments. The expression levels of the drought-related transcription factors CBF2 (a), CBF3 (b), RD29A (c), DREB2A (d) and DREB2B (e) in Col, lcd, des1 and lcd/des1 phenotypes under different treatments.

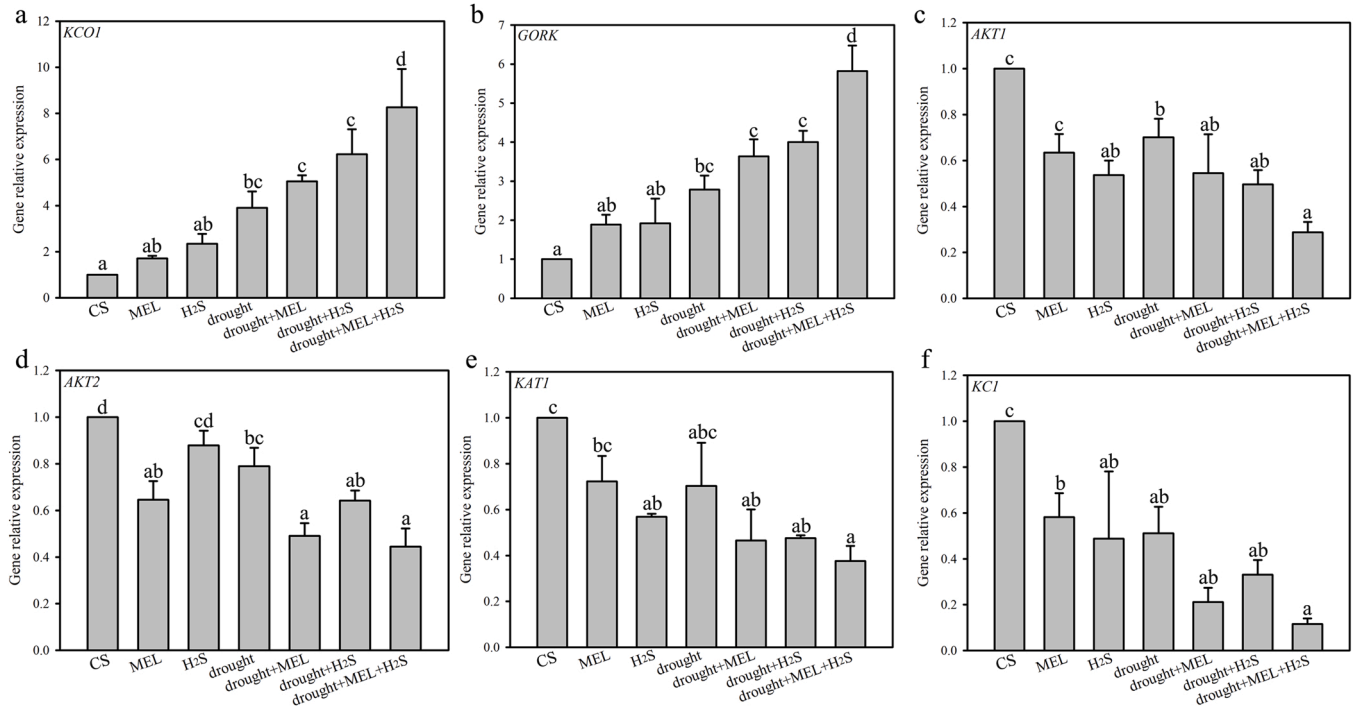

Fig. 6. The responses to K^+ channel related genes at the transcriptional levels under the different treatments. The expression levels of the K^+ channel related genes (a) KCO1, (b) GORK, (c) AKT1, (d) AKT2, (e) KAT1 and (f) KC1 under the different treatments.

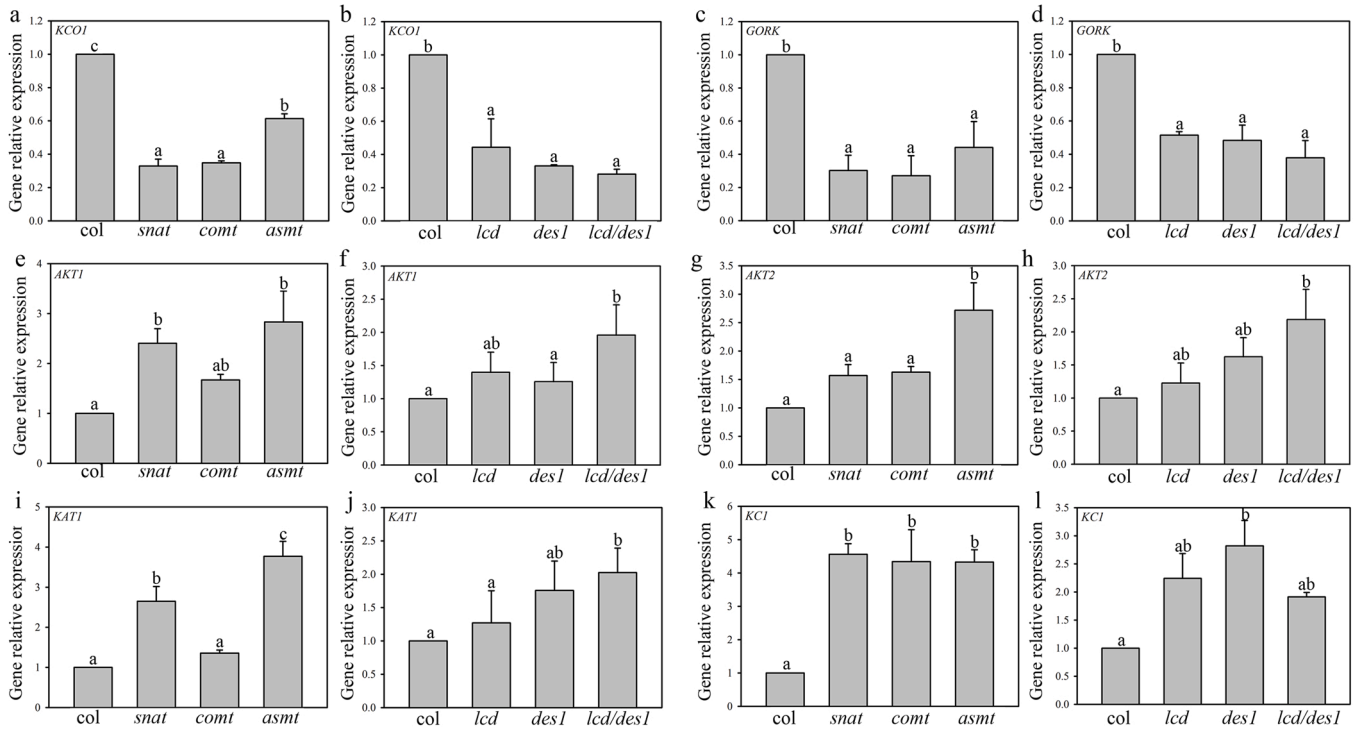

Fig. 7. The responses to K^+ channel related genes at the transcriptional levels in Col, snat, comt1, asmt, lcd, des1 and lcd/des1 plants. The expression levels of the K^+ channel related genes (a,b) KCO1, (c,d) GORK, (e,f) AKT1, (g,h) AKT2, (i,j) KAT1 and (k,l) KC1 in Col, snat, comt1, asmt, lcd, des1 and lcd/des1 plants.

confirmed their effects on transcription levels of K^+ channel-related genes and post-translational modifications of K^+ associated proteins, and revealed how H_2S and MEL modulate the molecular mechanisms of stomatal closure by responding to K^+ channels. In detail, MEL-induced stomata closure was partially dependent on H_2S in vitro and in vivo. MEL and H_2S regulated stomatal movement by regulating the transcription level of K^+ channel-related genes. At the post-translational level, H_2S induced the KCO1 and AKT1 persulfidation to maintain protein stability. It was concluded that H_2S was involved in the MEL induced stoma closure by affecting the conformation and stability of KCO1 and AKT1. This study contributes to a better understanding of how plants enhance drought tolerance by closing stomata.

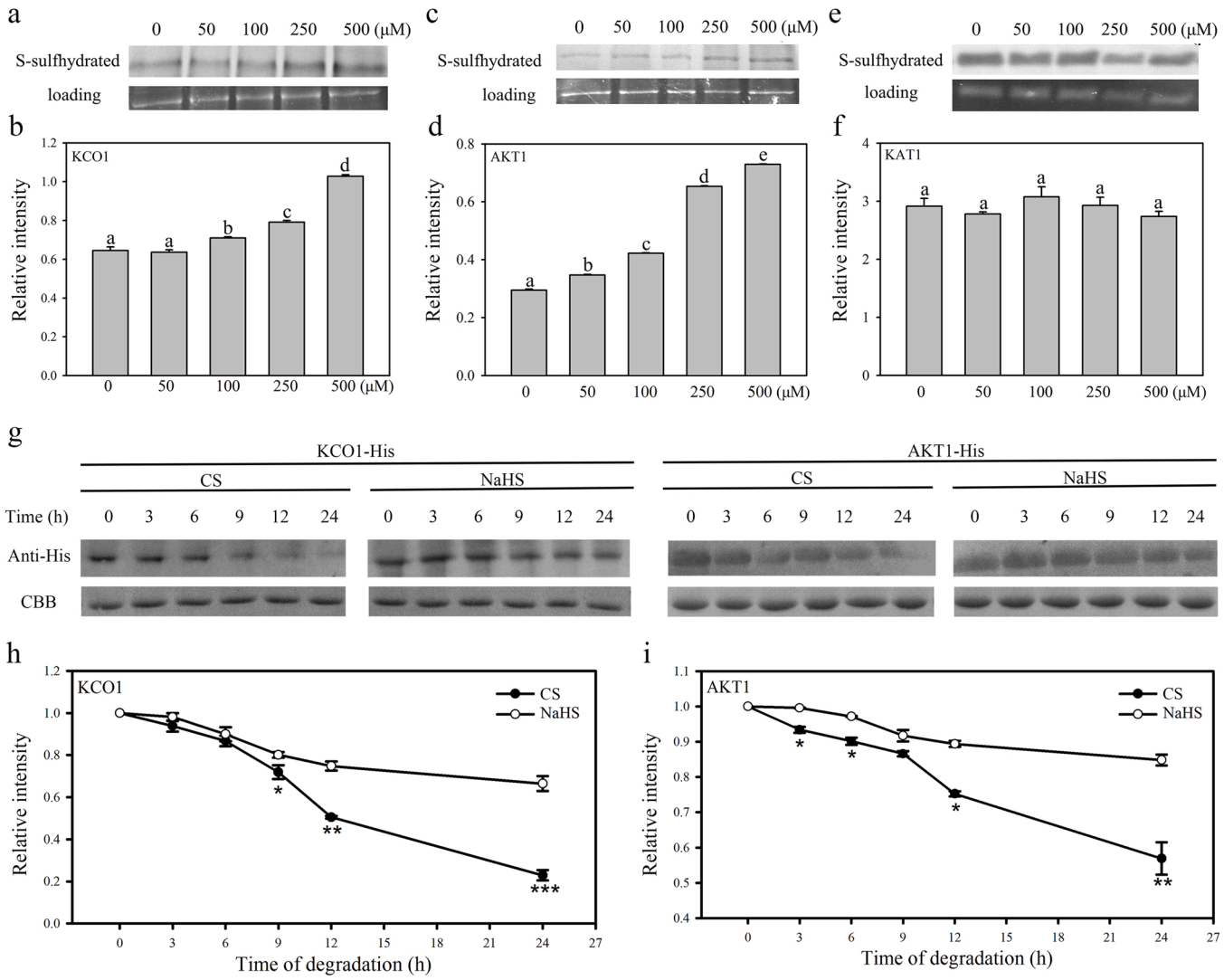

Fig. 8. The effect of H_2S on persulfidation and degradation of K^+ channel related proteins. The effect of H_2S on persulfidation of (a,b) KCO1, (c,d) AKT1 and (e,f) KAT1. Persulfidated KCO1 and AKT1 proteins were detected with biotin antibody. Quantification analysis was performed using ImageJ. The effect of H_2S on degradation of KCO1 (g), AKT1 (h). Purified recombinant proteins were treated with NaHS for one hour and then incubated with total proteins at 25 °C for different times. Proteins without NaHS treatment were set as control. Persulfidated KCO1-His and AKT1-His proteins were detected with anti-His antibody. Recombinant proteins that did not react with total protein were used as internal control. Error bars indicate the standard error of three biological repeats. Different letters and asterisk (*) are significantly different among treatments (*, $P < 0.05$).

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The authors do not have permission to share data.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2022.105125.

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