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REGULAR ARTICLE

H_2S is involved in ABA-mediated stomatal movement through MPK4 to alleviate drought stress in *Arabidopsis thaliana*

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Abstract

Aim Hydrogen sulfide (H_2S) is a gaseous signaling molecule that participates in multiple physiological processes in both animals and plants. Mitogen-activated protein kinase (MAPK) is important signaling molecule that links the growth and developmental signals and environment stimuli to cellular responses. In the current study we explored the relationship between H_2S and MAPK in drought stress resistance in Arabidopsis.

Methods The quantitative real-time (qRT)-PCR, root tip bending experiment and stomatal aperture assay were used in this paper.

Results Drought stress activated both H_2S biosynthesis and gene expression of MAPKs. The increase in MAPK expression was depressed in *lcd/des1*, a double mutant of H_2S synthesis. Then we selected MPK4 as our target and used *mpk4* mutants for further studies. H_2S was able to alleviate the drought stress in wild-type (WT) Arabidopsis but not in *mpk4* mutants. Meanwhile, H_2S-induced stomatal movement was impaired in *mpk4* mutants. We then examined the role of H_2S and MPK4 in stomatal movements in response to abscisic acid (ABA) and hydrogen peroxide (H_2O_2). ABA- and H_2O_2- mediated stomatal movements were impaired in *lcd/des1* and *mpk4* mutants, and H_2S-induced stomatal closure was impaired in *slac1–3* mutants.

Conclusions Our results suggested that MPK4 is important downstream of H_2S in the drought stress response and in stomatal movement, and that the H_2S-MPK4 cascade is involved in ABA-mediated stomatal movement to regulate the drought stress.

Keywords H_2S · MPK4 · Drought stress · Stomata · ABA · H_2O_2

Abbreviations

H_2S	Hydrogen sulfide
DES1	Desulfhydrase 1
LCD	L-cysteine desulfhydrase
MAPK	Mitogen-activated protein kinase
ABA	Abscisic acid
PYR/PYL/RCAR	Pyrabactin Resistance/Pyrabactin resistance-like/Regulatory Component of ABA Receptor
PP2Cs	Protein Phosphatase 2C
SnRK2s	SNF1-Related Protein Kinases type 2

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SLAC1	Slow Anion Channel-Associated 1
H_2O_2	Hydrogen peroxide
1/2 MS	1/2 Murashige and Skoog

Introduction

Hydrogen sulfide (H_2S) which was long considered a toxic gas because of its unpleasant smell is now known to be a gaseous signaling molecule with important physiological roles in both animals and plants. In Arabidopsis, H_2S is enzymatically produced by L-cysteine desulfhydrase (LCD) and desulfhydrase 1 (DES1) via the catalysis of L-cysteine primarily (Álvarez et al. 2010; Papenbrock et al. 2007). In plants, H_2S participates in plant growth and development, hormone responses, and biotic and abiotic stress resistance, including drought stress. H_2S can improve the ability of plants to resist drought stress by inducing stomatal closure (Jin et al. 2011). Stomata are pores that are each surrounded by two guard cells; stomatal movement results from changes in the turgor of the guard cells in response to fluxes in their ionic composition or other osmotically relevant molecules. Jin et al. reported that H_2S is able to mediate the ion fluxes to regulate stomatal movement in Arabidopsis. They used numbers of mutants associated with H_2S production enzymes, and detected the fluxes of H^+, Ca^{2+}, K^+ and Cl^- using a non invasive micro-test technique, and found that H_2S induced a transmembrane K^+ efflux and Ca^{2+} and Cl^- influxes, while not affecting the flow of H^+ (Jin et al. 2017). However the underlying mechanism involved in H_2S-regulated stomatal movement are still need further study.

Mitogen-activated protein kinase (MAPK) is an essential and conserved component of the signaling transduction pathway that converts growth or environment signals into cellular responses in eukaryotes. MAPK cascade comprises MEKK (MAP3K or MPKKK), MEK (MAP2K or MPKK), MPK (MAPK) which each activate the other in a sequential manner through phosphorylation. Phosphorylated MPK could activate downstream signaling molecules, such as enzymes or transcription factors (Šamajová et al. 2013). MAPK is a large family that contains 60 MEKKs, 10 MEKs, and 20 MPKs in Arabidopsis (Ichimura et al. 2002). Given its many members, MAPK has a role in many physiological processes in plant, including growth differentiation, hormonal responses, and biotic and abiotic stress resistance (Rodriguez et al. 2010). Considering so many functional overlaps between H_2S and MAPKs, we studied their relationship in Arabidopsis in previous work and found that H_2S has a positive role in MAPK gene expression especially MPK4, and MPK4 is an important downstream component in H_2S signaling in cold stress response (Du et al. 2017). However, the relationship between H_2S and MAPK in drought stress has not been reported.

Abscisic acid (ABA) is a major phytohormone that participates in seed maturation, plant development, and responses to abiotic stresses. One of the most important functions of ABA is to regulate stomatal movement. The core ABA signaling pathway in stomatal movement has been established. ABA binds with its receptors Pyrabactin Resistance/Pyrabactin resistance-like/Regulatory Component of ABA Receptor (PYR/PYL/RCAR), promoting the bind of PYR/PYL/RCAR with Protein Phosphatase 2C (PP2Cs), leading to the inactivation of PP2Cs. Activated PP2Cs could dephosphorylated hence inactivated SNF1-Related Protein Kinases type 2 (SnRK2s). Therefore, ABA-induced inactivation of PP2Cs results in the activation of SnRK2s. Subsequently, activated SnRK2s could directly activate the S-type anion channels Slow Anion Channel-Associated 1(SLAC1), resulting in the release of anions from the guard cell and inducing stomatal closure. In addition to SLAC1, there are other channels regulated by SnRK2s involved in ABA-mediated stomatal movement such as aluminum-activated anion channels 12 (ALMT12). SnRK2s also regulate the production of hydrogen peroxide (H_2O_2) and Ca^{2+} signaling to activate SLAC1 (Danquah et al. 2014; Zelicourt et al. 2016; Jezek and Blatt 2017). H_2S-mediated stomatal closure involves in ABA signaling. Jin et al. found that the ability of H_2S closing stomata was not influenced in ABA-associated mutants *aba3* and *abi1*, however ABA-induced stomatal closure was impaired in H_2S synthesis mutant *lcd*, indicating that ABA-induced stomatal closure is partially dependent on H_2S (Jin et al. 2013). Whereafter, more studies revealed the function of H_2S in ABA-mediated stomatal movement. H_2S acts downstream of ABI1, a member of the PP2C family (Scuffi et al. 2014), and acts upstream of OST1, a member of the SnRK2 family, to activate the S-type anion channels (Wang et al. 2016). Many members of MAPK are also indicated in the mediation of ABA signaling in guard cells. Guard cell-specific inhibition of MPK3 impaired ABA- and H_2O_2-

regulated stomatal movement (Gudesblat et al. 2007). Double mutants but not single mutants of MPK9 and MPK12 exhibited insensitivity to both ABA- and H_2O_2-induced stomatal closure and inhibited stomatal opening in Arabidopsis (Jammes et al. 2009). However the role of MPK4 in stomatal movement has not been well elucidated in Arabidopsis.

Therefore in the current study, we examined the relationship of H_2S and MAPK especially MPK4 in response to drought stress, focusing on the involvement of H_2S and MPK4 in stomatal movement regulated by ABA and H_2O_2 in Arabidopsis.

Materials and methods

Plant material and growth conditions

lcd/des1 double mutants of Columbia background (Col), generously provided by Shaowu Xue of Huazhong Agriculture University, were formed by hybridization of *lcd* (SALK-082099) and *des1* (SALK-205358C). T-DNA insertion mutant *mpk4* of Columbia background and *Ds* insertion mutant *mpk4* of Landsberg background (Ler) were kindly provided by John Mundy of Copenhagen University. These mutants has been described elsewhere (Du et al. 2017). *slac1–3* mutants (SALK-099139) of Col background (Vahisalu et al. 2008) were also provided by Shaowu Xue. The primers used in genotyping are listed in Table S1. Seeds were grown in pots containing a soil:perlite:vermiculite (1:1:1 v:v) mixture or on the plates containing 1/2 Murashige and Skoog (1/2 MS) medium after being sterilized with 75% ethyl alcohol and 6% NaClO. The plants were grown under 23 °C, 60% relative humidity, 16/8 h (light/dark) photoperiod and 160 $\mu Emm^{-2}\ s^{-1}$ light illumination.

PEG treatment

To determine the gene expression level after PEG treatment, 4-week-old seedlings grown in pots containing a soil:perlite:vermiculite (1:1:1 v:v) mixture were used. 20 ml 0.4 g/ml PEG8000 (−1.92 MPa) were applied to the seedlings for indicated time (3 h, 6 h, 9 h), and seedlings of 0 h group were applied with 20 ml diluted water. To perform the root tip bending experiment, the 1/2 MS plates with 0.4 g/ml PEG8000 or not were used in which 20 ml 1/2 MS fluid medium containing 0.4 g/ml PEG8000 or not were add on the normal 1/2 MS plates, and the fluid medium were removed when the plates were used.

Determination of gene expression level

The leaves of 4-week-old seedlings grown in pots containing a soil:perlite:vermiculite (1:1:1 v:v) mixture were used. RNA was extracted using RNAiso Plus (TaKaRa, Shiga, Japan), and cDNA was generated using All-In-One RT MasterMix (abm, Nanjing, China). Quantitative real-time (qRT)-PCR was executed using cDNA as a template and the methods described elsewhere (Shen et al. 2013). The primers used are listed in the Table S1, and *UBQ* was used as an internal control.

Measurement of endogenous H_2S production rate and content

The H_2S production rate was measured as described previously with some modifications (Zhao et al. 2001). Briefly, the leaves from 4-week-old seedlings grown in pots containing a soil:perlite:vermiculite (1:1:1 v:v) mixture were homogenized in pre-cold 50 mM phosphate buffer (pH = 7) and centrifuged at 12000 rpm for 10 min. The supernatants were then collected for use in later experiments. The reaction was performed in a 25 ml flask containing reaction mixture (100 mM Tris-HCl pH = 9, 10 mM L-cysteine, 2.5 mM DTT and the supernatant) and a test tube (1.5 ml) containing 0.5 ml 1% zinc acetate as a trapping solution. The flasks were transferred to a table concentrator at 37 °C to initiate the reaction. After 15 min of incubation at 37 °C, the test tubes were removed from flasks and 0.1 ml *N*,*N*-dimethyl-p-phenylenediamine sulfate (20 mM in 7.2 M HCl) and 0.1 ml $FeCl_3$ (30 m M in 1.2 M HCl) were added into the test tubes; the test tubes were placed in the dark. The absorbance was measured at 670 nm after 15 min. The leaves from 4-week-old seedlings grown in pots containing a soil:perlite:vermiculite (1:1:1 v:v) mixture were used for H_2S content measurement, and the method was described previously (Du et al. 2017).

Root tip bending experiment

7-day-old seedlings grown on 1/2 MS plates were used in a root tip bending study. The seedlings were transferred to 1/2 MS plates with 0.4 g/ml PEG8000 or not.

The hook length of roots was observed and measured after 2 days.

Stomatal aperture assay

The leaves from 4-week-old seedlings grown in pots containing a soil:perlite:vermiculite (1:1:1 v:v) mixture were used for stomata observation. To measure the degree of the stomatal closure, the leaves were soaked into epidermal strip buffer (containing 10 mM MES and 50 mM KCl, pH 6.15) and placed under light for 2 h. Then NaHS (H_2S donor), ABA, or H_2O_2 were applied and U0126 (an inhibitor of the MEK2 → MPK4 pathway) were added 30 min prior to NaHS application. After 3 h, the abaxial epidermis was peeled from leaves, and the epidermal strip was used for stomatal aperture observation.

To measure the inhibition of stomatal opening, the leaves were soaked into epidermal strip buffer and placed in the dark for 2 h. Then, NaHS, ABA, or H_2O_2 were applied and the leaves were placed under a light. After 2 h, the abaxial epidermis was peeled from leaves, and the epidermal strip was used for stomatal aperture observation.

Statistical analysis

The data were expressed as the mean ± standard error (SE). The statistically significant differences were analyzed with SPSS version 17.0. Statistical analyses of H_2S production rate, H_2S content, the expression level of genes, root bending experiment were performed using one-way analysis of variance (ANOVA) followed by Duncan's test or students' *t*-test as indicated in the figure legends. Statistical analyses of stomatal aperture assay were performed using two-way ANOVA. If no interaction occurred between genotypes and treatments, the one-way ANOVA followed by Duncan's test was performed, and if interaction occurred, simple effect analysis was performed. Asterisks, plus signs and different letters represented significant differences. At least three independent experiments were performed for every test.

Results

Drought stress induced H_2S biosynthesis

To assess the function of H_2S in Arabidopsis seedlings under drought stress, we used PEG8000 (0.4 g/ml, −1.92 MPa) to mimic the drought stress and determined the gene expression level of *LCD* and *DES1* (genes encoding H_2S production enzymes), the H_2S production rate and H_2S content in the seedlings exposed to PEG8000 for different lengths of time. The expression levels of *LCD* and *DES1* were increased after 3 h of PEG8000 treatment (Fig. 1a). H_2S production rate and H_2S content increased after 6 h of PEG8000 treatment (Fig. 1b, c).

Drought-induced MAPK gene expression was depressed in *lcd/des1* mutants

We then explored the role of MAPK in the drought stress response. We selected MEKK1, MEK1, MEK2, MPK3, MPK4, and MPK6 as targets given that they are the most well-studied MAPK members and are known to participate in the stress response. We determined the expression levels of MAPKs in the WT seedlings subjected to PEG8000 treatment. The gene expression of MAPKs were induced after PEG8000 treatment for different length of time (Fig. 1d). We then determined the role of H_2S in PEG8000-induced gene expression of MAPKs by treating homozygous *lcd/des1* double mutants (Fig. S1a-b) with PEG8000 for different lengths of time. After PEG8000 treatment, decreases in the expression of MAPKs in *lcd/des1* mutants were recorded (Fig. 1e).

H_2S alleviating drought stress was impaired in *mpk4* mutants

In a previous study, we found that MPK4 is an important downstream component of H_2S (Du et al. 2017) and, thus, we chose MPK4 as a target for the following experiments. To further investigate the relationship between MPK4 and H_2S in the drought stress response, the root tip bending experiments were performed with WT and *mpk4* mutants. We got two kinds of *mpk4* mutant, Col background and Ler background. Homozygous *mpk4* seedlings of Col background need to be distinguished from heterozygous *mpk4* seedlings of Col background because homozygous *mpk4* mutants of Col background is too weak to produce seeds (Fig. S2). Homozygous *mpk4* of Ler background is able to produce seeds even though the seedlings of homozygous *mpk4* is weaker than WT (Fig. S3). So we used homozygous *mpk4* of Ler background (Fig. S1c) in root bending experiment. We used NaHS (H_2S donor) to fumigate the seedlings of WT and *mpk4* mutants and

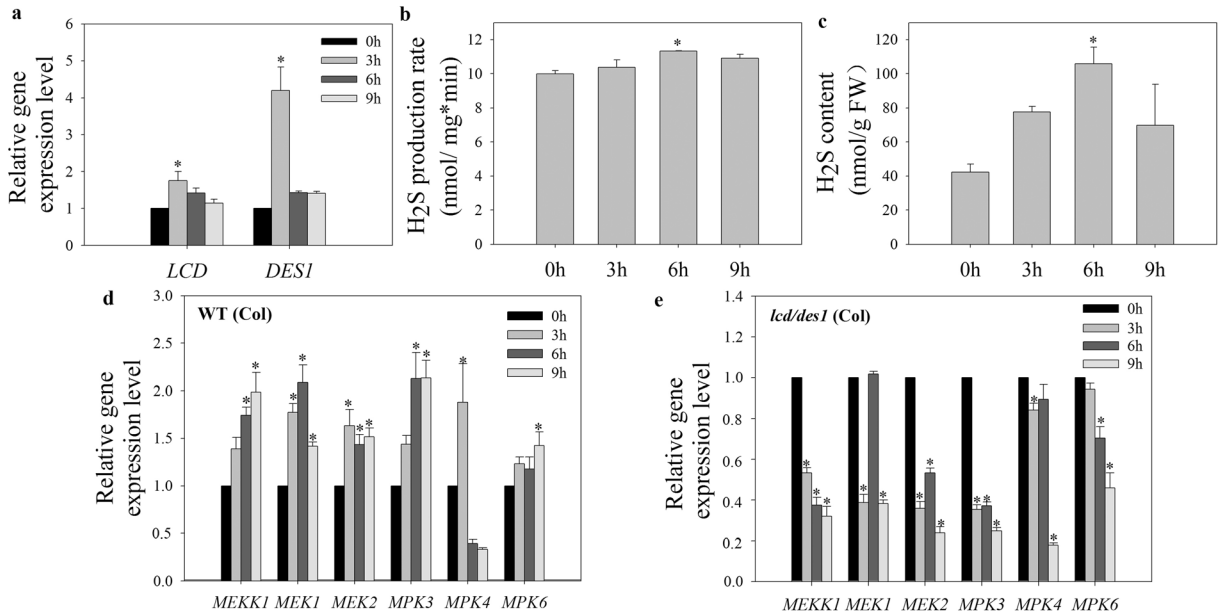

Fig. 1 **a–c** The effect of PEG8000 on H_2S biosynthesis in Arabidopsis. The expression level of *LCD* and *DES1* (**a**), H_2S production rate (**b**), and H_2S content (**c**) in WT after PEG8000 treatment. 4-week-old seedlings of WT of Col background treating with 0.4 g/ml PEG8000 for different time (0, 3, 6, 9 h) were used. Data are Mean ± SE and three independent experiments were repeated, the asterisk indicated significant differences compared with each control (one way ANOVA, * $P < 0.05$). **d**, **e** The effect of PEG8000 on the expression of MAPKs in WT (**d**) and *lcd/des1* (**e**). 4-week-old seedlings of WT and *lcd/des1* of Col background treating with 0.4 g/ml PEG8000 for different time (0, 3, 6, 9 h) were used. Data are Mean ± SE and three independent experiments were repeated, the asterisk indicated significant differences compared with each control (one way ANOVA, * $P < 0.05$)

then exposed the seedlings to PEG8000. The hooks of each root were observed and quantified. In WT, the inhibition of root length caused by PEG8000 was significantly rescued by NaHS (Fig. 2a). However, NaHS was unable to alleviate this damage caused by PEG8000 in *mpk4* mutants (Fig. 2b).

Stomatal movement in response to H_2S was impaired in *mpk4* mutants

Regulating stomatal closure is an important way that H_2S alleviates drought stress (Jin et al. 2011); therefore, we explored the function of MPK4 in stomatal movement regulated by H_2S. First, we administered U0126, the inhibitor of MEK2 → MPK4 pathway, to test the function of H_2S in closing stomata in WT of Ler background. NaHS treatment and U0126 treatment closed the stomata respectively, whereas administering U0126 before NaHS attenuated the stomatal closing compared with NaHS treatment alone (Fig. 3a). To further verify the function of MPK4, we treated WT and *mpk4* mutants of Ler background with NaHS and observed the stomatal apertures. We used *mpk4* mutants of Ler background in stomatal aperture assay because *mpk4* mutants of Col background is dwarf and its leaves are too small to observe stomata (Fig. S2). As shown in Fig. 3b, NaHS treatment closed the stomata in WT, but did not affect the stomatal aperture in *mpk4* mutants. We also studied the process of H_2S inhibition stomatal opening. In WT, NaHS inhibited the opening of stomata. Whereas the inhibition of NaHS to stomatal opening displayed small in *mpk4* mutants compared with that in WT (Fig. 3c).

Both H_2S and MPK4 were involved in ABA-mediated stomatal movement

H_2S is an important component of ABA-dependent stomatal movement (Jin et al. 2013; Scuffi et al. 2014). To understand the mechanism of the H_2S and MPK4 response to drought stress, the roles of H_2S and MPK4 in ABA-mediated stomatal movement were explored. First, the involvement of H_2S in ABA-mediated

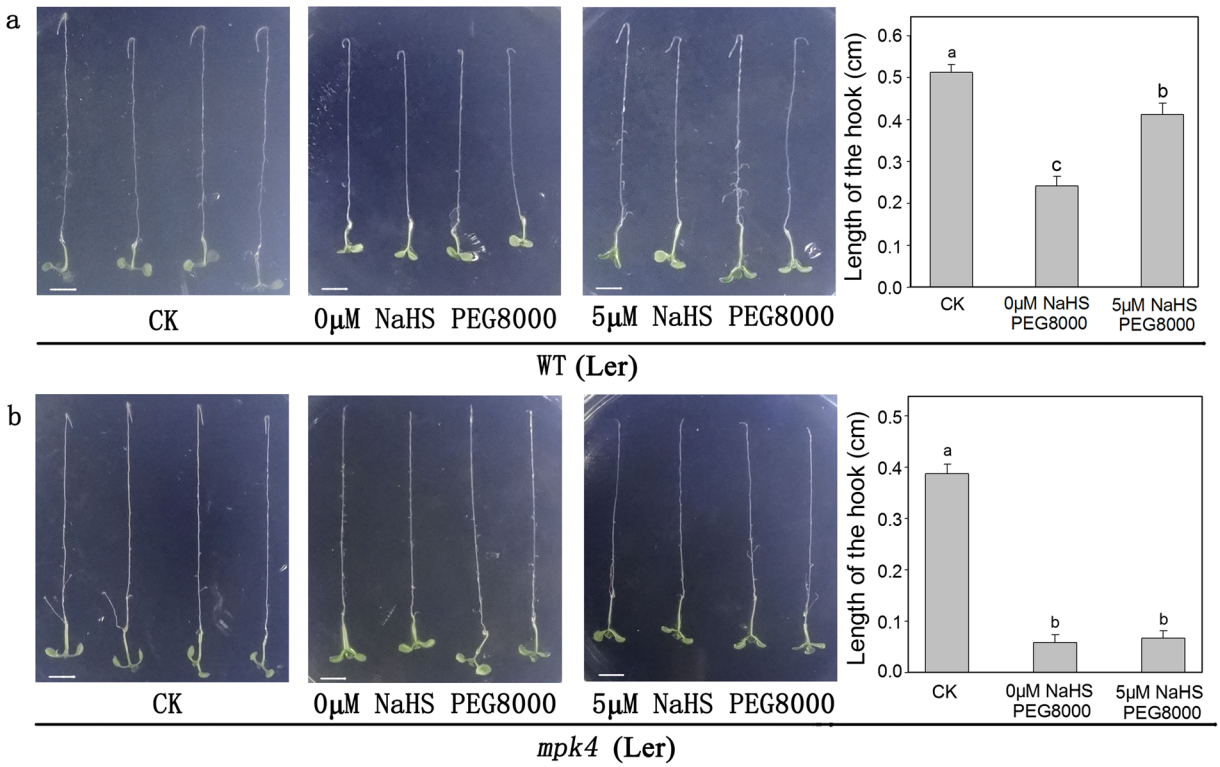

Fig. 2 The alleviation effect of H_2S in WT (**a**) and *mpk4* (**b**) under PEG8000. The 7-day-old seedlings of WT and *mpk4* of Ler background grown on 1/2 MS plates were used. The seedlings were fumigated by 5 μM NaHS for 12 h or not, then transferred to the 1/2 MS plates with 0.4 g/ml PEG8000 or not. The hook length of each root were observed and measured 2 d later, 10 hook were measured for each treatment. Data are Mean ± SE and three independent experiments were repeated, different letters indicated significant differences among treatments (one way ANOVA, $P < 0.05$). Scale bar: 0.5 cm

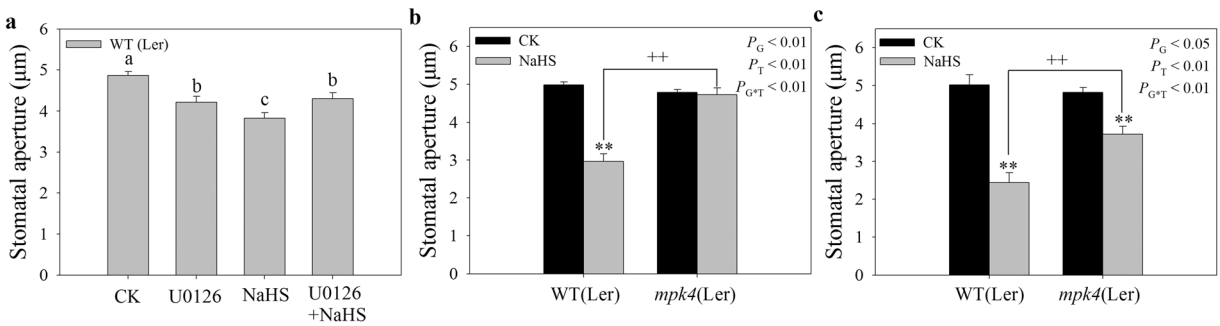

Fig. 3 The effect of H_2S on stomatal movement in WT and *mpk4*. **a** The stomatal apeture of WT after U0126 and NaHS treatment. ($n = 3$ independent experiments, 60 stomatal aperture for each treatment.) The 4-week-old seedlings of WT of Ler background were used. The leaves were treated with 10 μM U0126 for 0.5 h and 50 μM NaHS for 3 h or not before observing the stomatal aperture. **b** H_2S-induced stomatal closure in WT and *mpk4*. ($n = 3$ independent experiments, 60 stomatal aperture for each treatment.) **c** H_2S-inhibited stomatal opening in WT and *mpk4*. ($n = 4$ independent experiments, 80 stomatal aperture for CK, 120 stomatal aperture for NaHS treatment.) The 4-week-old seedlings of WT and *mpk4* of Ler background were used, and stomatal aperture of the leaves were observed after 50 μM NaHS treatment for 3 h (**b**) or 2 h (**c**) or not. Data are Mean ± SE, different letters in a indicated significant differences among treatments (one way ANOVA, $P < 0.05$), the asterisks and plus signs in b and c indicated significant differences compared with each CK and WT treated with NaHS, respectively (two way ANOVA, ** and ++ $P < 0.01$). The P values in the top right corner of figures indicate the effect of genotypes (WT and *mpk4*), treatment (CK and NaHS) and their interaction on stomatal aperture. G:genotype, T:treatment

stomatal closing and opening was confirmed using *lcd/des1* mutants. ABA induced stomatal closing (Fig. 4a) and inhibited stomatal opening (Fig. 4b) in WT. However, ABA- induced stomatal closing and inhibited stomatal opening were diminished in the *lcd/des1* mutants compared with that in WT. We then examined whether MPK4 participates in the stomatal movement in response to ABA. WT and *mpk4* mutants of Ler background were used. As shown in Fig. 4c, ABA was able to close the stomata in WT. But in *mpk4* mutants the stomatal closing caused by ABA was impaired compared with that in WT. ABA inhibited the stomatal opening in WT, while this function also was impaired in *mpk4* mutants (Fig. 4d).

Both H_2S and MPK4 were involved in H_2O_2-mediated stomatal movement

H_2O_2 is involved in ABA-regulated stomatal movement (Danquah et al. 2014). To better understand the mechanism of H_2S and MPK4 in ABA signaling, we studied whether both H_2S and MPK4 participate in stomatal movement in response to H_2O_2. As shown in Fig. 5a, b, compared with WT, *lcd/des1* mutants were significantly impaired in H_2O_2- induced stomatal closure and inhibited stomatal opening. *mpk4* mutants of Ler background were also significantly impaired in H_2O_2- induced stomatal closure and inhibited stomatal opening compared with WT (Fig. 5c, d).

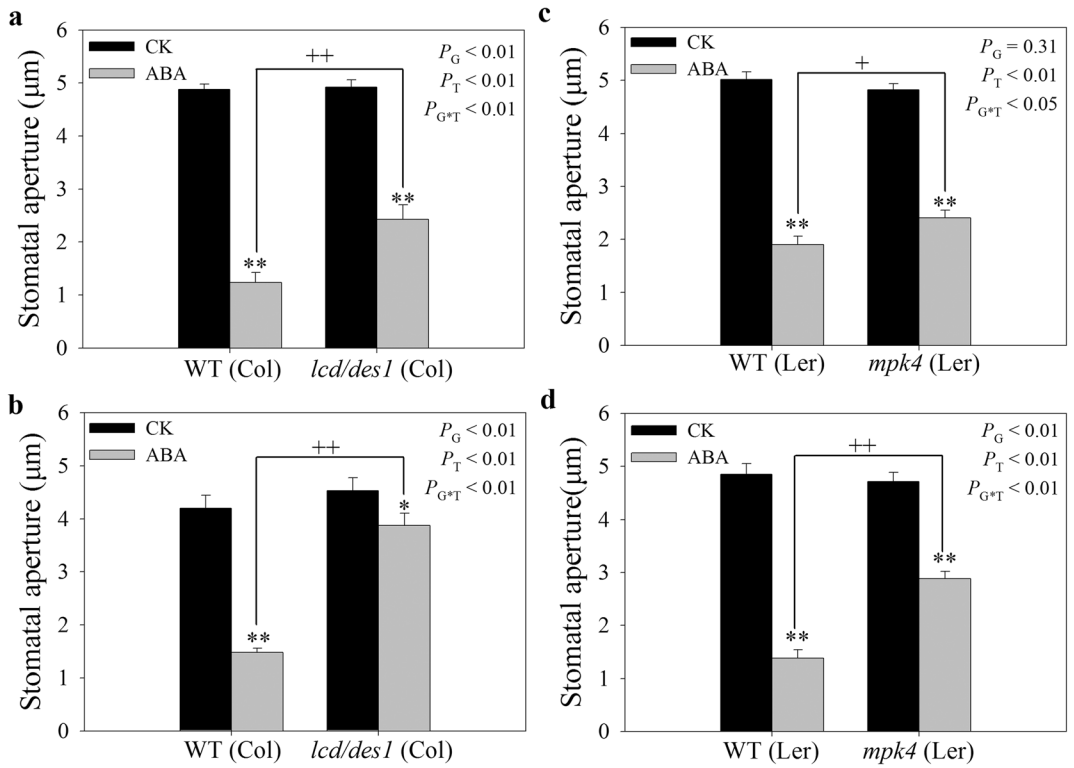

Fig. 4 The effect of ABA on stomatal movement in WT, *lcd/des1* and *mpk4*. **a** ABA-induced stomatal closure in WT and *lcd/des1*. ($n = 3$ independent experiments, 60 stomatal aperture for each treatment.) **b** ABA-inhibited stomatal opening in WT and *lcd/des1*. ($n = 3$ independent experiments, 60 stomatal aperture for CK, 90 stomatal aperture for ABA treatment.) The 4-week-old seedlings of WT and *lcd/des1* of Col background were used, and stomatal aperture of the leaves were observed after 20 μM ABA treatment for 3 h (**a**) or 2 h (**b**) or not. **c** ABA-induced stomatal closure in WT and *mpk4*. ($n = 3$ independent experiments, 60 stomatal aperture for each treatment.) **d** ABA-inhibited stomatal opening in WT and *mpk4*. ($n = 3$ independent experiments, 60 stomatal aperture for CK, 75 stomatal aperture for ABA treatment.) The 4-week-old seedlings of WT and *mpk4* of Ler background were used, and stomatal aperture of the leaves were observed after 20 μM ABA treatment for 3 h (**c**) or 2 h (**d**) or not. Data are Mean ± SE, the asterisks and plus signs indicated significant differeces compared with each CK and WT treated with ABA, respectively (two way ANOVA, * and + $P < 0.05$, ** and ++ $P < 0.01$). The P values in the top right corner of figures indicated the effect of genotypes (WT and *lcd/des1* or *mpk4*), treatment (CK and ABA) and their interaction on stomatal aperture. G:genotype, T:treatment

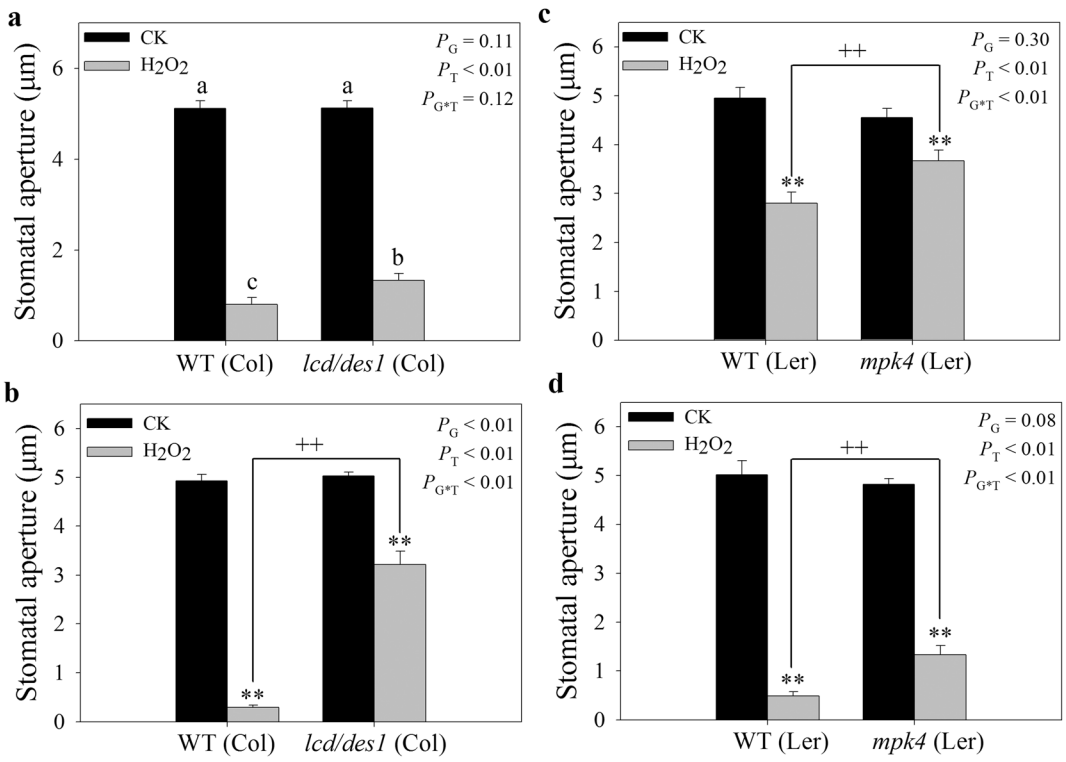

Fig. 5 The effect of H_2O_2 on stomatal movement in WT, *lcd/des1* and *mpk4*. **a** H_2O_2-induced stomatal closure in WT and *lcd/des1*. ($n = 3$ independent experiments, 60 stomatal aperture for CK, 100 stomatal aperture for H_2O_2 treatment.) **b** H_2O_2-inhibited stomatal opening in WT and *lcd/des1*. ($n = 4$ independent experiments, 80 stomatal aperture for CK, 96 stomatal aperture for H_2O_2 treatment.) The 4-week-old seedlings of WT and *lcd/des1* of Col background were used, and stomatal aperture of the leaves were observed after 500 μM H_2O_2 treatment for 3 h (**a**) or 2 h (**b**) or not. **c** H_2O_2-induced stomatal closure in WT and *mpk4*. ($n = 3$ independent experiments, 60 stomatal aperture for CK, 90 stomatal aperture for H_2O_2 treatment.) **d** H_2O_2-inhibited stomatal opening in WT and *mpk4* (n = 4 independent experiments, 80 stomatal aperture for CK, 120 stomatal aperture for H_2O_2 treatment.) The 4-week-old seedlings of WT and *mpk4* of Ler background were used, and stomatal aperture of the leaves were observed after 500 μM H_2O_2 treatment for 3 h (**c**) or 2 h (**d**) or not. Data are Mean ± SE, different letters in a indicated significant differences (one way ANOVA, $P < 0.05$), the asterisks and plus signs in b, c and d indicated significant differeces compared with each CK and WT treated with H_2O_2, respectively (two way ANOVA, ** and ++ $P < 0.01$). The P values in the top right corner of figures indicate the effect of genotypes (WT and *lcd/des1* or *mpk4*), treatment (CK and H_2O_2) and their interaction on stomatal aperture. G:genotype, T:treatment

Stomatal movement in response to H_2S was impaired in *slac1–3* mutants

SLAC1 is a S-type anion channel that responds to ABA signaling (Vahisalu et al. 2008). Therefore, we studied the effect of H_2S and MPK4 on the gene expression of *SLAC1* using *lcd/des1* mutants and *mpk4* mutants (Fig. S1d) of Col background. The expression level of *SLAC1* increased in *lcd/des1* mutants and did not change significantly in *mpk4* mutants compared with that in WT (Fig. 6a, b). We then examined the effect of H_2S on stomatal closure in homozygous *slac1–3* mutants (Fig. S1e). NaHS was able to induce stomatal closure in WT, however NaHS inducing stomatal closing was impaired in *slac1–3* mutants compared with that in WT (Fig. 6c).

Discussion

H_2S and MAPK have positive roles in drought response

The H_2S and MAPK pathway is activated by various abiotic stresses (Rodriguez et al. 2010; Šamajová et al. 2013; Shi et al. 2015). Our results showed that H_2S production was induced after drought stress (Fig. 1a–c). Likewise, the expression level of MAPKs, including *MEKK1*, *MEK1*, *MEK2*, *MPK3*, *MPK4*, and *MPK6*,

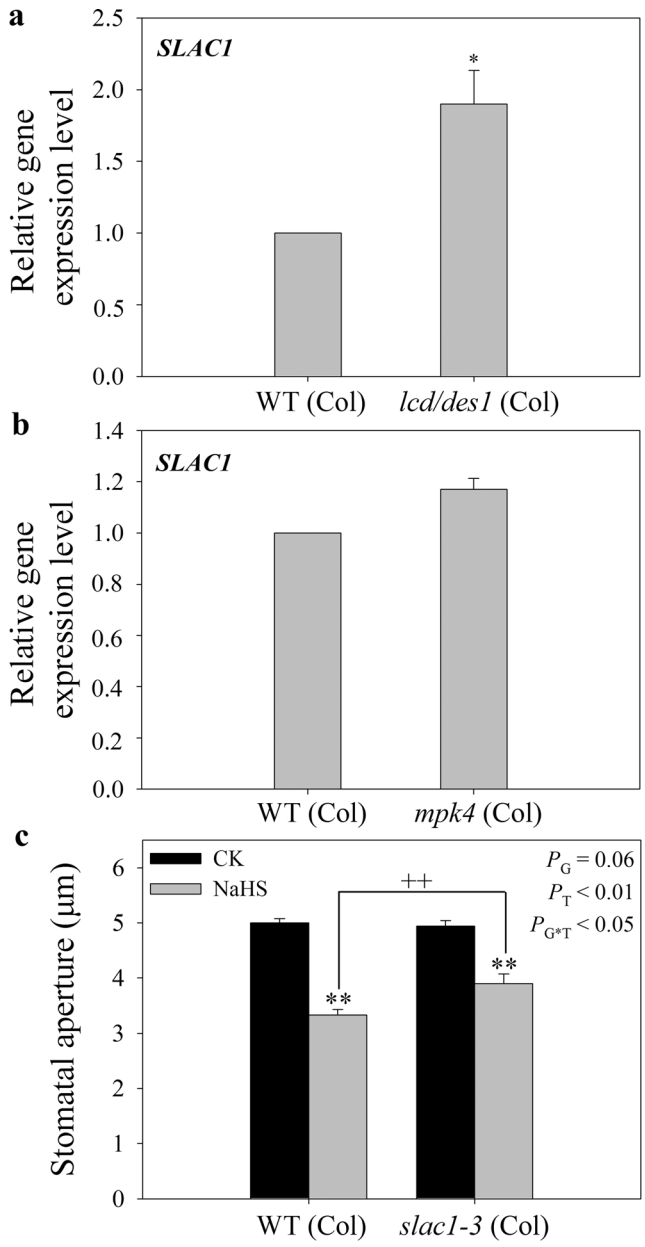

Fig. 6 The effect of H_2S and MPK4 on SLAC1. The expression of *SLAC1* in *lcd/des1* (**a**) and *mpk4* (**b**). The 4-week-old seedlings of WT, *lcd/des1* and *mpk4* of Col background were used. Data are Mean ± SE and three independent experiments were repeated, the asterisk indicated significant differences (Students' *t*-test, * $P < 0.05$). **c** H_2S-induced stomatal closure in WT and *slac1–3*. (n = 3 independent experiments, 60 stomatal aperture for CK, 90 stomatal aperture for NaHS treatment.) The 4-week-old seedlings of WT and *slac1–3* of Col background were used, and stomatal aperture of the leaves were observed after 50 μM NaHS treatment for 3 h or not. Data are Mean ± SE, the asterisks and plus signs indicated significant differeces compared with each CK and WT treated with NaHS, respectively (two way ANOVA, ** and ++ $P < 0.01$). The *P* values in the top right corner of figures indicate the effect of genotypes (WT and *slac1–3*), treatment (CK and NaHS) and their interaction on stomatal aperture. G:genotype, T:treatment

also increased after drought stress (Fig. 1d). These results showed that both H_2S and MAPK are activated in response to drought stress, and that they have a positive role in the drought response. The kinase activity of MEKK1, MEK1, MPK3, MPK4, and MPK6 increased after drought stress (Rodriguez et al. 2010; Šamajová et al. 2013), and the mRNA abundance of *MEKK1* and *MPK3* increased after drought treatment (Mizoguchi et al. 1996). Our results showed that the gene expression of not only *MEKK1* and *MPK3*, but also *MEK1*, *MEK2*, *MPK4*, and *MPK6* were induced by drought stress, indicating that these MAPKs are regulated not only at the post-translation level, but also at the transcription level during drought stress. However, Ichimura et al. reported that the mRNA abundance of *MPK4* and *MPK6* was not influenced by drought stress (Ichimura et al. 2000). This discrepancy in results could be caused by the different drought treatments used.

MPK4 is an important downstream component in the alleviation of drought stress by H_2S

Given that both H_2S and MAPK were activated by drought stress, we explored the involvement of MAPK in the mechanism whereby H_2S alleviates drought stress. Figure 1e showed that H_2S was indispensable in the MAPK gene expression induced by drought. Our previous study indicated that MPK4 is a critical component downstream of H_2S signaling involved in the cold stress response (Du et al. 2017). Therefore, we targeted MPK4 in the following experiment. Figure 2 showed that MPK4 was required in the alleviation of drought stress by H_2S. These two results together reveal that MAPKs, MPK4 in particular, are important downstream components in H_2S signaling in the drought stress response. On the other hand, H_2S also plays important roles in drought-induced expression of other MAPK members including MEKK1, MEK1, MEK2, MPK3, and MPK6 (Fig. 1e). Thus, it is worth studying the functions of these MAPKs in H_2S-alleviated drought stress.

Role of MPK4 in H_2S-mediated stomatal movement

MPK4 is highly expressed in guard cells in Arabidopsis (Petersen et al. 2000), implying an important function of MPK4 in stomatal movement. Here, we showed that U0126 alone could induce stomatal closure in WT plants (Fig. 3a), indicating the important role of the MEK2 → MPK4 cascade in the process. In addition, stomatal movement in response to H_2S was impaired after applying U0126 and in *mpk4* mutants (Fig. 3), suggesting that MPK4 has an important role in stomatal movement regulated by H_2S. Further studies are required to determine whether other MAPK members, such as MPK3, MPK6, MPK9, and MPK12, also participate in stomatal movement in response to H_2S.

A H_2S-MPK4 cascade is involved in stomatal movement regulated by ABA and H_2O_2 in Arabidopsis

H_2S is known to participate in the ABA-induced stomatal closure (Jin et al. 2013). Here we used *lcd/des1*, the double mutants of H_2S production enzymes, to confirm that H_2S participated in both stomatal closure and inhibition of stomatal opening caused by ABA (Fig. 4a, b).

Few studies have focused on the relationship between H_2S and H_2O_2 in stomatal movement. However, a recent paper showed that H_2S was upstream of H_2O_2 in the process of stomatal closure and that H_2S could increase H_2O_2 production through NADPH oxidases and phospholipase D (Scuffi et al. 2018). By contrast, our results showed that the stomatal movement in response to H_2O_2 was impaired in the *lcd/des1* mutants (Fig. 5a, b), suggesting that H_2S is an important downstream component in the mediation of stomatal movement by H_2O_2. The reason for these discrepancies might be that different materials were used in the two studies (*des1* versus *lcd/des1* mutants).

There are many studies of the involvement of MAPK members in stomatal movement, such as MPK3, MPK9, and MPK12. However, the role of MPK4 in stomatal movement has remained ambiguous, especially in Arabidopsis. In *Nicotiana tabacum*, silencing of *MPK4* did not alter the ABA sensitivity of guard cells (Marten et al. 2008). However, silencing *MPK4* in *Nicotiana attenuata* impaired both ABA- and H_2O_2- induced stomatal closure (Hettenhausen et al. 2012). These divergent results suggest the different role of MPK4 in different species. Our results showed that MPK4 is required in stomatal movement in response to both ABA and H_2O_2 in Arabidopsis (Figs. 4c, d and 5c, d), as also reported for MPK3, MPK9, and MPK12.

The activation of S-type anion channels by H_2S requires OST1, a member of the SnR2K family (Wang et al. 2016). H_2S actives the production of H_2O_2 through NADPH oxidases and phospholipase D and acts as upstream of H_2O_2 in stomatal closure (Scuffi et al. 2018). In the current study, we showed that H_2S acts as downstream of H_2O_2 and upstream of MPK4 in stomatal movement (Figs. 5a, b and 3). However, the precise point of involvement of H_2S in ABA signaling in stomatal movement is unclear. We speculate that H_2S interacts with the molecules in the pathway of ABA-induced stomatal movement both downstream and upstream, and that there might be more than one target of H_2S in ABA-induced stomatal movement (Fig. 7), and further studies are required to determine the underlying mechanisms involved.

SLAC1 is required in H_2S-induced stomatal closure

S-type anion channels are responsible for ABA-mediated stomatal closure. SLAC1 is an important S-type anion channel, and SLAC1 mutants showed very strong insensitivity to ABA-mediated stomatal closure (Vahisalu et al. 2008). H_2S can active S-type anion currents via SLAC1 to induce stomatal closure (Wang

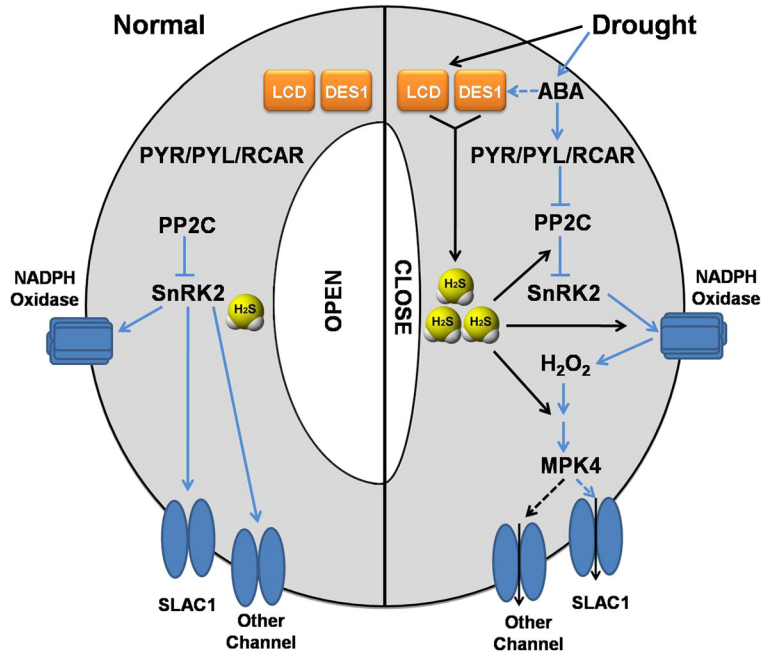

Fig. 7 A schematic model of the role of H_2S in ABA-induced stomatal closure. ABA is absent in guard cells under normal conditions. PYR/PYL/RCAR, the receptors of ABA, remain inactive and PP2Cs bind and inhibit the activity of SnRK2s, which leads to the inactivation of NADPH oxidases and SLAC1or other ion channels and therefore causes the opening of stomata. However, under drought conditions, the ABA signal in guard cells is activated. ABA binds to PYR/PYL/RCAR and inhibits the activity of PP2Cs, leading to the activation of SnRK2s. Therefore, SnRK2s can activate the NADPH oxidases to promote the production of H_2O_2 or SnRK2s can directly activate SLAC1and other ion channels (not shown in the figure). H_2O_2 regulates the stomatal movement through MPK4 (Fig. 5c, d). However, whether other MAPK members upstream of MPK4 such as MEK2 are involved in this process remains elusive. On the other hand, drought stress increase the biosynthesis of H_2S through LCD and DES1 simultaneously (Fig. 1a–c). The production of H_2S also regulated by ABA through increasing the expression level of *DES1*(Scuffi et al. 2014). H_2S plays a crucial role in ABA-mediated stomatal movement. H_2S functions downstream of PP2Cs (Scuffi et al. 2014) and upstream of SnRK2s (Wang et al. 2016). H_2S is able to enhance the production of H_2O_2 through NADPH oxidases (Scuffi et al. 2018). H_2S serving as downstream of H_2O_2 and upstream of MPK4 functions in stomatal movement (Figs. 5a, b and 3). H_2S-MPK4 cascade can induce stomatal closure in SLAC1- dependent or independent way (Fig. 6c). Arrow-headed lines indicate activation; bar-headed lines indicate inhibition; dotted arrows indicate putative effect

et al. 2016). Here, we showed that H_2S-induced stomatal closure was impaired in *slac1–3* mutants (Fig. 6c), providing additional evidence for the role of SLAC1 in H_2S-induced stomatal closure, and confirming that H_2S is involved in ABA signaling resulting in stomatal movement. However the effect of H_2S on stomatal closure was not completely blocked in *slcac1–3* mutants (Fig. 6c) indicating that H_2S could also close stomata through other ion channels other than SLAC1. Salicylic acid (SA), one of the plant hormones, is able to induce stomatal closure in an ABA-independent pathway (Miura and Tada 2014). It has been reported that H_2S and SA are tightly correlated in the Cd stress response (Qiao et al. 2015). It is highly possible that H_2S is not only involved in ABA-induced stomatal closure but also in SA-induced stomatal closure. The expression level of SLAC1 was increased in *lcd/des1* mutants (Fig. 6a), showing that H_2S has a negative effect on *SLAC1* gene expression, which might be a compensation effect. The expression level of *SLAC1* did not change in *mpk4* mutants (Fig. 6b), suggesting that MPK4 does not regulate the SLAC1 at the transcription level. However, further studies are required to determine whether H_2S and MPK4 regulate SLAC1 at another level, such as post-transcriptionally or post-translationally.

Overall, our results show that MPK4 is an important downstream molecule involved in H_2S-mediated stomatal movement to alleviate drought stress, and that the H_2S-MPK4 cascade is involved in ABA signaling pathway resulting in stomatal movement (Fig. 7). However, further work is required to determine the underlying mechanism of H_2S regulating stomatal movement

through MPK4, and how H_2S coordinates the numerous signaling component (H_2O_2, Ca^{2+}, NO, MAPK, etc) in ABA-mediated stomatal movement.

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