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REGULAR ARTICLE

Hydrogen sulfide mediates DNA methylation to enhance osmotic stress tolerance in *Setaria italica* L.

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Abstract

Background and aims DNA methylation is an important form of epigenetic modification. It has a vital role in regulating plant growth and development, and also participates in plant response to various stresses. In recent years, hydrogen sulfide (H_2S) has been shown to have similar functions as DNA methylation, but crosstalk between DNA methylation and H_2S in the acquisition of drought resistance is unclear. In this study, foxtail millet (*Setaria italica* L.), a drought-resistant model crop, was selected as the experimental material to explore the subtle relationship between H_2S and DNA methylation.

Methods The quantitative real-time (qRT)-PCR, bisulfite sequencing PCR (BSP), DNA methyltransferase (DNMT) activity detection and other techniques were used to analyze the differences of millet seedlings under osmotic stress, before and after H_2S treatment.

Results Osmotic stress induced the transcriptional expression and activity of key enzymes in H_2S biosynthesis which regulated the accumulation of endogenous H_2S. Physiological concentration of H_2S (50 μM) can effectively alleviate the decrease of total DNMT activity and transcription level caused by osmotic stress, while the effective inhibitor of H_2S biosynthesis, hydroxylamine (HA), can aggravate this change. Furthermore, transcription factors (TFs) responsive to both 'osmotic stress' and 'H_2S signal' were screened. Six of them were selected to conduct further BSP analysis on seven CpG islands in their promoter regions. The results showed that, with H_2S treatment, two CpG islands were hypermethylated, two were not, and three exhibited insensitivity to H_2S.

Conclusion H_2S signals may improve osmotic stress tolerance of foxtail millet by mediating DNA methylation.

Keywords Hydrogen sulfide · DNA methylation · Osmotic stress · Foxtail millet (*Setaria italica* L.)

Abbreviations

H_2S	Hydrogen sulfide
PEG	Polyethylene glycol
BSP	Bisulfite sequencing PCR
DNMT	DNA methyltransferase
HA	Hydroxylamine
TF	Transcription factor
MET	Methyltransferase

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CMT	Chromomethylase
DRM	Domains rearranged methyltransferase
ABA	Abscisic acid
EDTA	Ethylene diamine tetraacetic acid
CDes	Cysteine desulfhydrases
LCD	L-cysteine desulfhydrase
DCD	D-cysteine desulfhydrase
DES	Desulfhydrase
ROS1	Repressor of Silencing 1
DME	DEMETER
MS	Murashige and Skoog

Introduction

Foxtail millet (*Setaria italica* L.) is an ancient crop from the subfamily Panicoideae. It was domesticated from green foxtail in northern China 8000 years ago (Barton et al. 2009) and was one of the first cultivated small grains. Currently, foxtail millet is extensively cultivated in arid and semiarid regions of Asia, Africa, and the Americas (Lata et al. 2013). According to an estimation by the United Nations Food and Agriculture Organization, China has the largest cultivated area and the highest yield of foxtail millet, accounting for >90% of the total foxtail millet production worldwide. In recent years, with changes of the industrial structure and people's diets in China, its planting area has increased even further.

Foxtail millet has many significant agronomic traits, one of which is its outstanding drought resistance (Fang and Xiong 2015). These natural characteristics render foxtail millet as a useful crop to study drought resistance mechanisms. Drought stress is one of the most important abiotic factors negatively influencing crop productivity (Bodner et al. 2015; Osakabe et al. 2014; Zhu 2016). The annual yield reduction of crops caused by drought in China exceeds the sum of other adverse factors (Zhang et al. 2004). Drought stress poses a sustained challenge to agricultural crop production and urgently needs to be addressed.

Research over the decades has gradually revealed the molecular mechanisms of plant responses to drought stress and, in recent years, an increasing number of studies have shown that plants respond to drought stress by regulating DNA methylation levels and pattern changes. For example, drought causes hypermethylation of specific sequences in the pea root tip genome, resulting in significant increases in genomic DNA methylation level (Labra et al. 2002). In response to drought stress, the level of cytosine methylation in the rice genome also changes remarkably. For example, there was an increase in methylation in roots, with ~50% of the methylation-changed sites not recovering after rehydration, but being passed on to offspring (Wang et al. 2011). Tan (2010) analyzed the changes in DNA methylation after treatments with PEG (Polyethylene glycol) and NaCl and found that the DNA methylation patterns in maize leaves changed significantly. Drought and salt-induced DNA methylation are important regulatory mechanisms enabling maize to cope with drought stress, which mainly results from two regulatory factors, zmPP2C and zmGST (Tan 2010). After drought treatment, the methylation level of *Populus trichocarpa* (*Populus sp.*) also significantly increased, with 1846 TFs associated with changes in methylation level, indicating that this might be an important mechanism behind the response of *Populus* to drought stress (Liang et al. 2014). In short, the modification of DNA methylation is recognized as an important and universal plant drought stress response.

DNA methylation is one of the earliest known epigenetic modification pathways. Primarily, it refers to the controlled process of adding methyl groups to cytosine in the 5-position catalyzed by DNMTs. DNA methylation can cause genetic changes in chromatin structure, DNA conformation, DNA stability, and how the DNA interacts with proteins which further affects the expression of related genes. However, the regulatory mechanism of methyltransferase activity is still poorly understood (Zhu 2016). DNA methylation also provides two prominent contributions to plant defense systems: to protect the genome from invasion by foreign sequences and to regulate gene expression in response to stress (Kumar et al. 2013). According to the characteristics of the catalytic domain, DNMTs with catalytic activity in plants can be divided into three families: methyltransferase (MET), chromomethylase (CMT), and domains rearranged methyltransferase (DRM) (Zhang et al. 2010). MET1 is the dominant regulatory protein for DNA methylation in plants, and it is homologous to DNMT1 in animals and responsible for maintaining methylation in CG sites (Chang et al. 2009). The CMT family of DNMTs is peculiar to plants, and primarily regulates the methylation of symmetric CNG (N = A, T or G) sites (Wada 2005). The members of the third family, DRM, are homologous with DNMT3 of animals

but their catalytic domain order is different. DRM2 is the key protein of this family and modifies cytosine methylation de novo at all three sites, while also participating in maintaining the methylation of cytosine at non-CpG sites (Cao and Jacobsen 2002; Matzke and Birchler 2005; Zhong et al. 2013).

H_2S, the third gas transmitter after nitric oxide and carbon monoxide, is widely involved in the regulation of physiological functions of multiple systems in mammals, and significant research has revealed the physiological mechanisms involved in its activities (García-Mata and Lamattina 2010; Wang 2012; Aroca et al. 2018). In recent years, the physiological functions of H_2S as a signal molecule in plants have been confirmed and widely reported (Romero et al. 2014). Within the physiological concentration range, H_2S can promote seed germination and root morphogenesis, delay flower organ opening and senescence, activate antioxidant enzyme systems, and increase plant stress resistance (Jin and Pei 2015; Wang 2012). Exogenous H_2S appears to be a promising strategy to alleviate damage under abiotic stress conditions in plants (Corpas 2019) .

H_2S can enhance the drought resistance of plants (Li et al. 2016). It has been shown to interact with abscisic acid (ABA) to induce stomatal closure by regulating the activity of guard cell ion channel proteins at both transcriptional and post-translational levels, as well as to mediate ion fluxes (García-Mata and Lamattina 2010; Jin et al. 2011, 2017; Papanatsiou et al. 2015; Wang et al. 2016; Du et al. 2019; Chen et al. 2020; Shen et al. 2020), and simultaneously activate the antioxidant system (Li et al. 2019) to enhance plant drought resistance. An in-depth transcriptome analysis in *Triticum aestivum* L. revealed that H_2S alleviated damage in drought stress through transport systems, plant hormones signal transduction, protein processing pathway, fatty acids and amino acids metabolism (Li et al. 2017).

However, our understanding of the mechanism of H_2S signals in drought resistance of plants is far from complete. Although it is widely accepted that epigenetic modification is involved in plant response to stress, whether H_2S induces DNA methylation is unknown. By using foxtail millet seedlings exposed to osmotic stress, we investigated the changes in DNA methylation and gene expression in response to H_2S application and hydroxylamine, an inhibitor of H_2S biosynthesis.

Materials and methods

Plant materials and growth conditions

Two kinds of plant materials, foxtail millet and Arabidopsis, were employed in this research. Seeds of foxtail millet (*Setaria italica* L.) were provided by the Millet Research Institute, Shanxi Academy of Agricultural Science, Taiyuan, China. The soaked seeds were sown in two types of containers. One is a plastic seedling tray (50 holes per tray, diameter of each hole is 5 cm × 5 cm, 35 seeds per hole) containing a soil: perlite: vermiculite (1:1:1) mixture, and the other is a 12 cm-diameter Petri dish with six layers of wetted gauze (approximately 130 seeds per dish). It is very important to strictly control the water content of soil (20 mL/hole, to saturation) and gauze (20 mL/Petri dish) before sowing. Within 10 days thereafter, the soil does not need to be watered because the initial water supply in the soil is sufficient to meet the growth needs of foxtail millet seedlings for 10 days. Conversely, the Petri dish needs to be watered regularly and quantitatively every day to ensure the normal growth of the seedlings. After 2 days of cultivation in darkness at 23 °C, with 60% relative humidity, the seed samples were then kept in 16/8 h light/dark at 160 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 10 days.

Seeds of *Arabidopsis thaliana* (WT, Columbia-0), T-DNA insertion mutants of *LCD* (L-cysteine desulfhydrase) (*lcd*, SALK_082099), *DES1* (Desulfhydrase 1) *(des1*, SALK_205358C), were obtained from the ABRC (http://www.arabidopsis.org/abrc). *OE-LCD* (transgenic lines of 35S::*LCD*) and *OE-DES1* (transgenic lines of 35S::*DES1*) were constructed in our lab. After stratification at 4 °C, seeds were sterilized in 75% ethanol for 30 s and 6% sodium hypochlorite for 10 min under sterile conditions. After washing with sterile water three times, seeds were grown on the nylon mesh on the surface of 1/2 MS (Murashige and Skoog) medium (1.0% agar, 1.0% sucrose, pH 6.0) for three weeks. Then, the Petri dishes were placed vertically and cultured under the same growth conditions as foxtail millet.

Experimental treatments of materials

The seedling phenotype was recorded by digital camera. At the same time, the survival rate of the foxtail millet seedlings on the water control day (0 d) and the day after

rehydration was counted using the method of Jin et al. (2013). The relative water content of millet and Arabidopsis seedlings on the osmotic stress was measured with reference to Han et al. (2019). For the osmotic stress experiment, 30% PEG8000 aqueous solution (−1.1 MPa) was added to gauze directly to treat 10-day-old foxtail millet seedlings, while for Arabidopsis, it was conducted on PEG stress medium (for detailed preparation methods, see Verslues et al. 2006). For HA treatment, 50% HA aqueous solution was added to the soil or the 30% PEG8000 and the final concentration of HA was 1.5 mM. HA is an effective plant endogenous H_2S synthesis inhibitor (Li et al. 2013; Qiao et al. 2015; Tian et al. 2016). It mainly prevents the endogenous H_2S synthesis in plants by inhibiting the activity of LCD enzymes (Asimakopoulou et al. 2013; Jin et al. 2011, 2017). For the H_2S treatment, the foxtail millet seedlings were kept in seedling trays or Petri dishes with PEG treatment simultaneously placed in a sealed glass container and then fumigated with 50 μM NaHS (a widely recognized H_2S donor). Previous studies have shown that the concentration of H_2S detected in plants ranges from 1 to 100 μM, which is similar to the levels found in animals and humans (Yang et al. 2008; García-Mata and Lamattina 2010, Jin et al. 2011). Our preliminary experimental results showed that the survival of plants was prolonged after fumigation with 50 μM NaHS. Therefore, 50 μM NaHS was selected for further experiments. All manipulations were carried out as described previously (Jin et al. 2013).

When PEG was used alone, foxtail millet seedlings were sampled at 0, 6, 9, 12 and 24 h for the detection of physiological indicators and gene expression analysis. When PEG and NaHS (or HA) were used simultaneously, the treated time was set to 9 h, including the following three groups: (1) PEG: 30% PEG8000; (2) PEG + H_2S: 30% PEG8000 + 50 μM H_2S; (3) PEG + HA: 30% PEG8000 + 1.5 mM HA (Fig. S1). Arabidopsis seedlings were treated on PEG stress medium for 9 h (Fig. S2) for physiological indicator detection and gene expression analysis.

Measurement of endogenous H_2S content and LCD activity

Determination of H_2S content

The H_2S content in the seedlings was measured using the methylene blue method (Tian et al. 2016). 0.2 g seedlings were homogenized in 2 mL of extraction buffer: 50 mM PBS buffer, pH 6.8, containing 0.2 M AsA and 0.1 M ethylene diamine tetraacetic acid (EDTA); 1 mL of HCl (1 M) was then added to the mixture. H_2S was collected in a trap containing 0.5 mL of 1% (*w*/*v*) zinc acetate. After 30 min of reaction, 0.25 mL of dimethyl-phenylenediamine and ferric ammonium sulfate were added to the trap. The absorbance of the mixture was examined at 667 nm.

Determination of activity of L-cysteine desulfhydrase

As previously described (Du et al. 2019; Liu et al. 2019), the total activity of LCD was measured.

Total protein was extracted as follows: 0.3 g fresh seedlings were ground in 1 mL pre-cold PBS buffer (50 mM, pH 7.0) on ice and the homogenate was centrifuged (4 °C, 10,000×*g* for 10 min). Then, the supernatant was applied to determine the LCD activity using the methylene blue method. The reaction was performed in a 25 mL flask containing reaction mixture (500 mM, Tris-HCl, pH = 9.0; 10 mM L-cysteine; 50 mM DTT and the supernatant) and a 1.5 mL test tube containing 0.5 mL 1% zinc acetate as a trapping solution. The flasks were transferred to a table concentrator (37 °C) to initiate the reaction. 15 min later, the test tubes were taken 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 mM in 1.2 M HCl) were added into the test tubes which were then placed in darkness for 15 min. The absorbance was measured at 670 nm.

Extraction of total RNA and qRT-PCR

Total RNA was extracted from 0.1 g of whole-plant foxtail millet using a RNAprep Pure Plant Kit P432 (TIANGEN, Beijing, China), and cDNA was generated using All-In-One RT MaterMix (abm, Nanjing, China). With the above cDNA as the template, qRT-PCR was performed after some adjustments according to Shen (2013). Meanwhile, the gen *Actin* (Millet_GLEAN_10003390) was used as the internal control. The primers used for qRT-PCR are listed in Table S1.

Detection of DNMT activity

Total DNMT protein was extracted using an extraction kit for plant nuclear proteins and organelle proteins (Beijing biolab technology co. LTD, www.baiaolaibo.

com, Plant Nucleoprotein / Organelle Protein Extraction Kit, HR0131–1). Subsequently, a EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric; EPIGENTEK, Base Catalog # P-3009) was used to measure the DNMT activity of differently treated materials.

The basic principles of DNMTs activity detection are as follows. In this assay, a universal DNMT substrate is stably coated onto microplate wells. DNMT enzymes transfer methyl group to cytosine from Adomet to methylate DNA substrate and the methylated DNA can be recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be measured through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The activity of DNMT enzymes is proportional to the optical density intensity measured.

DNA extraction and bisulfite sequencing

Total genomic DNA was isolated from pooled seedlings collected from control and treatment groups using a plant genomic DNA kit (TIANGEN, DP305). DNA quantity was assessed spectrophotometrically, and integrity and purity were checked with 1.2% agarose gel electrophoresis (Fang et al. 2017)

A DNA Bisulfite Conversion Kit (DP215) was used to convert DNA cytosine, and the conversion efficiency of cytosine (Table S3) was detected by a Biq Analyzer (Bock et al. 2005) CpG island prediction and related primer design was performed using MethPrimer software (Li and Dahiya 2002) in conjunction with Primer 5.0 (BSP primers, see Table S2). After PCR amplification (as shown in kit TaKaRa EpiTaqTM HS (for bisulfite-treated DNA) R110A), the purified products were ligated into pMD™19-T (TaKaRa), and at least ten positive clones were picked after transformation for sequencing.

Statistical analysis

For each sample and assay, three biological and three technical repeats were performed. The results were expressed as the mean ± standard error (SE). Data were analyzed using SPSS (version 17, IBM SPSS, Chicago, IL, USA), and error bars were made according to Tukey's multiple range test ($P < 0.05$).

Results

H_2S improved the drought tolerance of foxtail millet seedlings

Ten-day-old foxtail millet seedlings cultivated in plastic seedlings trays were subjected to drought stress for 13 days by completely withholding water during this period. As shown in Fig. 1a, wilting occurred primarily in 'CK' and 'HA' samples, with the latter even worse. Obviously, the seedlings treated by H_2S were greener and were more turgid than those of the other two groups, whose leaves were severely wilting after 13 days withholding water. Moreover, after 13 days, almost all 'CK' samples lodged and all 'HA' samples were nearly dead, while most of the H_2S-treated seedlings survived. After re-watering, the surviving seedlings in the 'CK' and 'H_2S' groups gradually returned to normal, while most seedlings in the 'HA' group died. Figure 1b shows that the survival rate of each group on the water control day (0 d) was quite consistent, about 100%. The survival rate after rehydration significantly differed between treatments: 9% in the HA group, 54% in the control group and as high as 98% in the H_2S group.

Osmotic stress stimulation of H_2S emission

To investigate the effect of osmotic stress (30% PEG8000 $_{\Psi} = -1.1$ MPa) on endogenous H_2S level, we assayed the H_2S content, the LCD activity, and the gene expression patterns of *LCD1*, *LCD2*, *DCD1* (D-cysteine desulfhydrase), *DCD2* and *DES1* (genes encoding H_2S-generating enzymes in foxtail millet) in 10-day-old seedlings exposed to PEG8000 for different lengths of time (0, 6, 9, 12, and 24 h). H_2S content and LCD activity peaked after 9 h of 30% PEG8000 treatment (Fig. 2a, b).

Additionally, the expression of H_2S-synthesis related genes was induced to varying degrees by PEG treatment. Transcript levels of *LCD1*, *LCD2*, and *DCD2* in seedlings began to significantly increase after exposure to PEG8000 for 6 h and peaked at 9 h, when levels were 78-, 33- and 13-fold more than the control, respectively. By contrast, *DCD1* and *DES1* showed a positive response within a lag of only a few hours. Subsequently, the expression of all genes gradually declined to the same or lower levels as the control over the following 12-h period (Fig. 2c).

Significant changes in DNA methylation levels of foxtail millet under osmotic stress

The level of methylation in plants is maintained by both methylases and demethylases (Manoharlal et al. 2018). Thus, we analyzed the expression of genes encoding the main methylases (*MET1a*, *MET1b*, *CMT1a*, *CMT1b*, *CMT2*, *DRM1*, and *DRM2*) and demethylases (*DME* (DEMETER) and *ROS1* (Repressor of Silencing 1)) in foxtail millet (Fig. 3a, c). During the early stage of PEG treatment (0–9 h), the transcription level of key DNMTs increased significantly over the extension of stress time. The expression of *MET1a*, *CMT1a*, *CMT2*, and *DRM1* reached a significant level within 6 h of PEG treatment; after 3 h of further treatment, the expression levels of *MET1b*, *CMT1b*, and *DRM2* were also significantly increased; the expression levels of all genes tended to decrease after 9 h of exposure. Among them, *MET1a*, *CMT1a*, *CMT2*, and *DRM1* could respond to PEG stimulation quickly, followed by *MET1b*, *CMT1b*, and *DRM2*. By contrast, the expression of *CMT1b* continued to increase until peaking at 12 h. Reflecting a more lasting response to PEG treatment, *DRM2* showed a stepwise upward trend during the PEG treatment, peaking at 9 h, then returning to the control level at 12 h, but then increasing significantly following prolonged PEG treatment. Similarly, under PEG treatment the expression levels of *DME* and *ROS1* showed similar changes to the other DNA-methylase-encoding genes, with expression levels first increasing and then decreasing. In addition, we also tested the total enzyme activity of DNMTs under PEG treatment (Fig. 3b). The total enzyme activity was inhibited during the early stage of PEG treatment, but increased gradually as the PEG treatment continued.

Response of DNA methylation level to regulation of H_2S signaling

Many studies have shown that the appropriate concentration of H_2S can enhance the ability of plants to resist abiotic stress. However, it is unclear what role H_2S has in regulating the epigenetic modification of DNA methylation. Therefore, we also explored whether 'H_2S-DNA methylation' is relevant in plant stress resistance.

We first analyzed the expression of the major DNMT-related genes in Arabidopsis *lcd* (*LCD* mutant) and *OE-LCD* (transgenic lines of 35S::*LCD*). Compared with WT (wild-type), the expression level of the DNMT-related genes in the *lcd* mutant was significantly inhibited. However, their transcription levels recovered remarkably in the *OE-LCD* (Fig. 4a), which suggests

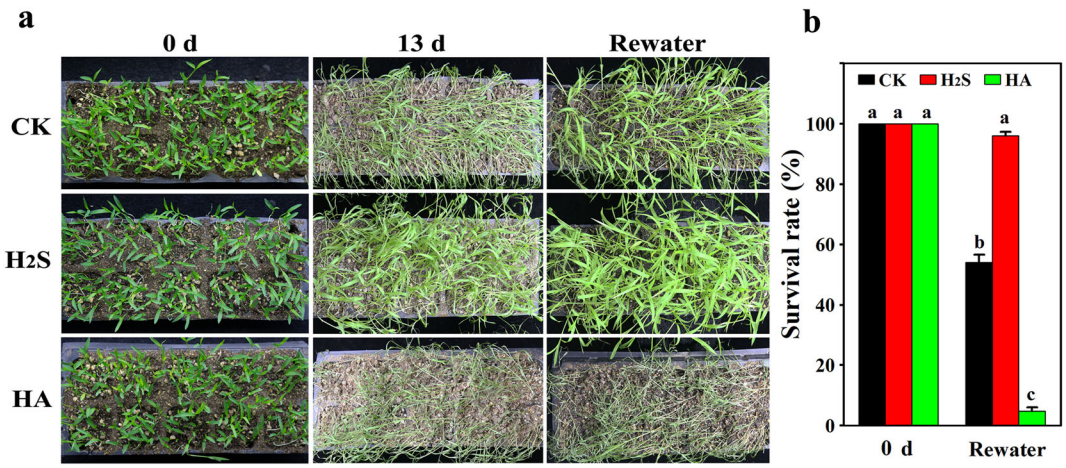

Fig. 1 H_2S improved the drought tolerance of foxtail millet seedlings. **a** H_2S protected foxtail millet from drought stress. The 10-day-old foxtail millet seedlings with the same growth status and conditions were divided into three groups for the following treatment: (i) CK group: 20 mL water was added to each hole. (ii) H_2S group: added 20 mL of water to each hole and, simultaneously, fumigated with 50 μM NaHS. (iii) HA group: added 20 ml of 1.5 mM HA water solution to each hole. After that, water control continued for 13 days until the seedlings exhibited typical drought stress phenotypes (wilting, lodging, death, etc.) and then watered to saturation. During water control, the H_2S group was fumigated every other day (50 μM NaHS) for 9 h each time. During the fumigation, all three groups were independently enclosed. **b** The survival rate was quantified by counting the percentage of living plants relative to total plants in the three groups after re-watering for 1 d. Data are mean ± SE of three independent repeats. Bars with different letters show significant differences compared with each control (one-way ANOVA, $P < 0.05$)

that H_2S signaling is closely related to DNA methylation.

To explore the relationship between H_2S and DNA methylation, we treated 10-day-old foxtail millet seedlings with 50 μM H_2S and 1.5 mM HA. The transcription level of DNMT-encoding genes significantly changed under different H_2S treatment conditions (Fig. 4b). Among them, *CMT1a* was the most active, and responded to the regulation of H_2S signaling in the same way as *MET1a, MET1b* and *CMT1b*. That is, when an exogenous physiological concentration of H_2S was applied, the expression levels of these four members increased significantly. However, when HA was used to inhibit the production of endogenous H_2S, the expression patterns of these four genes differed. *MET1b* and *CMT1b* remained at the same level, whereas *MET1a* and *CMT1a* increased to some extent. *CMT2, DRM1*, and *DRM2,* showed the same response trend as that of the *A. thaliana* mutant tested with H_2S. When the synthesis of endogenous H_2S was inhibited, the expression of DNMT-related genes was significantly reduced. When supplemented exogenously with a physiological concentration of H_2S, the transcription level was markedly increased, indicating a positive correlation between DNA methylation level and H_2S content.

To study the effect of H_2S signaling on DNA methylation level, we also examined the activity of DNMTs in different plant tissues and in response to different H_2S levels in the same tissues. Due to the absence of H_2S, DNMT enzyme activity in both Arabidopsis mutants *lcd* and *des1* was significantly inhibited but was effectively restored (*OE-LCD*) or even greatly improved (*OE-DES1*) in overexpressed mutants (Fig. 4c). A similar phenomenon was observed in foxtail millet. Osmotic

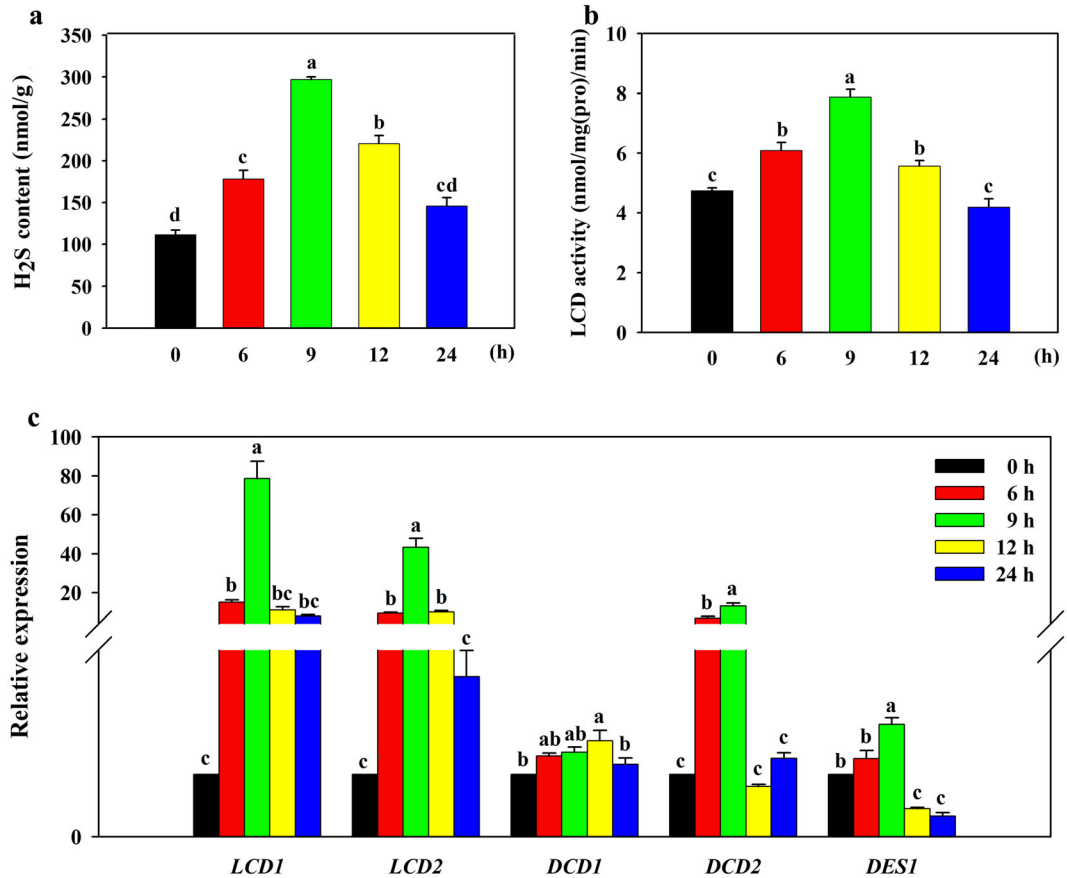

Fig. 2 The effect of PEG8000 on H_2S biosynthesis in foxtail millet. **a** H_2S content. **b** LCD activity. **c** The expressions of H_2S synthase-encoding genes (*LCD1*, *LCD2*, *DCD1*, *DCD2* and *DES1*). Ten-day-old foxtail millet seedlings were treated with 30% PEG8000. Whole seedlings were sampled at 0, 6, 9, 12 and 24 h post treatment. Tubulin was used as the control. Data are mean ± SE of three independent repeats. Bars with different letters show significant differences compared with each control (one-way ANOVA, $P < 0.05$)

stress effectively inhibited the activity of DNMTs, and the application of HA further intensified the inhibition of the enzyme activity. However, the external application of a physiological concentration of H_2S effectively alleviated the inhibitory effect of osmotic stress on DNMTs (Fig. 4d).

Methylation status of cytosine C in the promoter region of drought-resistant TFs

Drought-related genes in plants can be divided into two categories according to their functions. The first group encodes proteins with a direct protective role in the drought resistance of plants and are functional genes. The second group includes numerous TFs and encodes protein factors with a regulatory role in signal transduction and stress expression. Thus, we selected several representatives of drought-resistant TFs from the reported plants and obtained candidate genes for drought resistance in foxtail millet through homology comparison (Table S4).

Expression patterns of drought-resistant candidate genes under different H_2S treatment conditions were analyzed with qRT-PCR. Based on the results (Fig. 5), four genes (*AREB1*, *NAC5*, *DREB2A*, and *ZIP44*) were selected as representative drought-resistant genes that

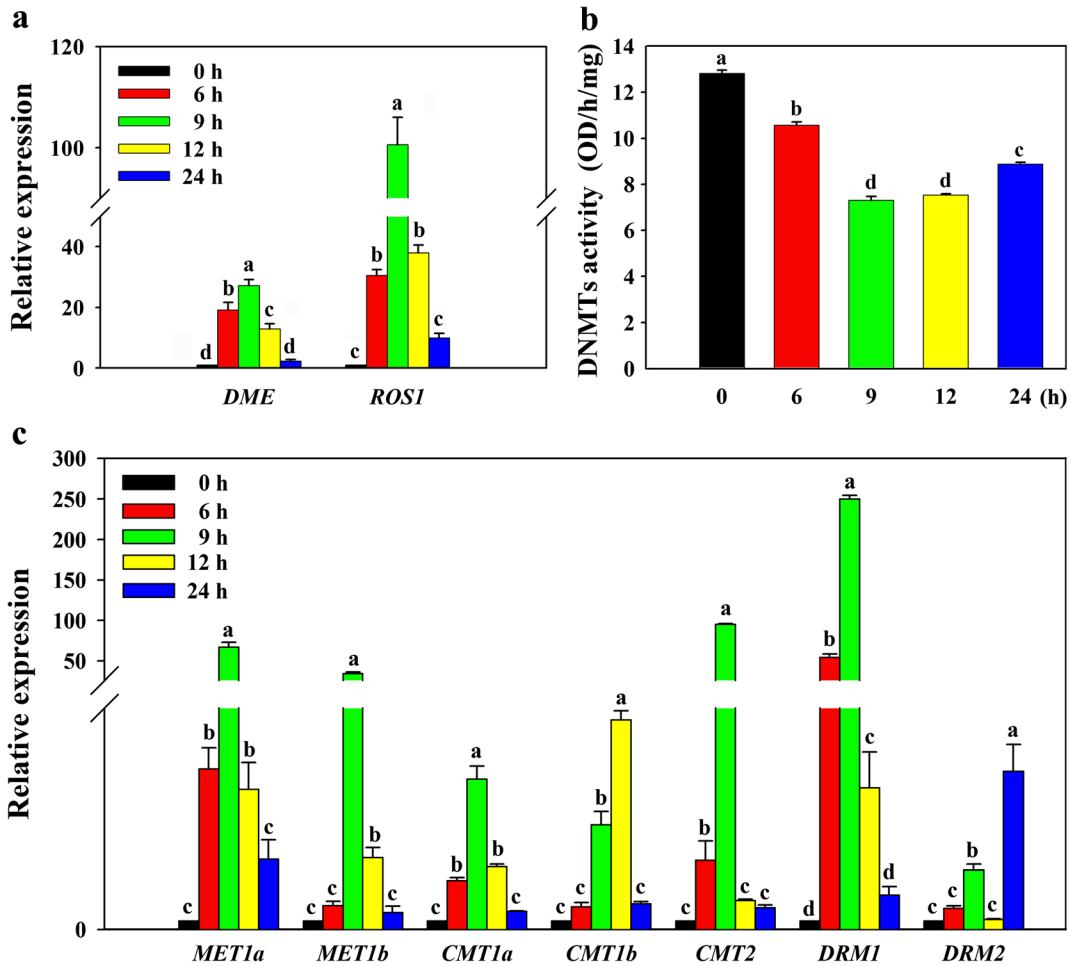

Fig. 3 Effects of PEG8000 on DNA methylation in foxtail millet. **a** Relative transcript levels of demethylases (*DME* and *ROS1*). **b** Detection of total enzyme activity of DNA methyltransferases. **c** Relative transcript levels of methyltransferases *(MET1a*, *MET1b*, *CMT1a*, *CMT1b*, *CMT2*, *DRM1*, and *DRM2*). Ten-day-old foxtail millet seedlings were treated with 30% PEG8000. Whole seedlings were sampled at 0, 6, 9, 12 and 24 h post treatment. Tubulin was used as the control. Data are mean ± SE of three independent repeats. Bars with different letters show significant differences compared with each control (one-way ANOVA, $P < 0.05$)

positively respond to H_2S regulation for further methylation analysis. Compared with the control 'CK' seedlings, the expression level of the selected TFs significantly changed under H_2S treatment. The categories of these TFs and their patterns of response to H_2S regulation also varied.

With 'CK' as the control group and under the treatment of 'HA', the transcription level of *AREB1* (Fig. 5a) remained unchanged, the expression level of *NAC5* (Fig. 5c) decreased significantly, whereas the expression levels of *ZIP44* and *DREB2A* (Fig. 5d, e) increased significantly. However, under the treatment of 'H_2S' with 'HA' as the control, the expression levels of *AREB1* and *NAC5* significantly increased, whereas that of *ZIP44* and *DREB2A* significantly decreased. Compared with 'CK', the expression levels of *AREB1* and *DREB2A* were always above the 'CK' level when treated with 'H_2S', whereas those of *NAC5* and *ZIP44* were lower than those in 'CK' group.

DNA methylation level in the promoter regions of drought-resistant genes before and after H_2S treatment

Promoter sequences of foxtail millet drought-resistant genes in GenBank are shown in Table S5. Methprimer

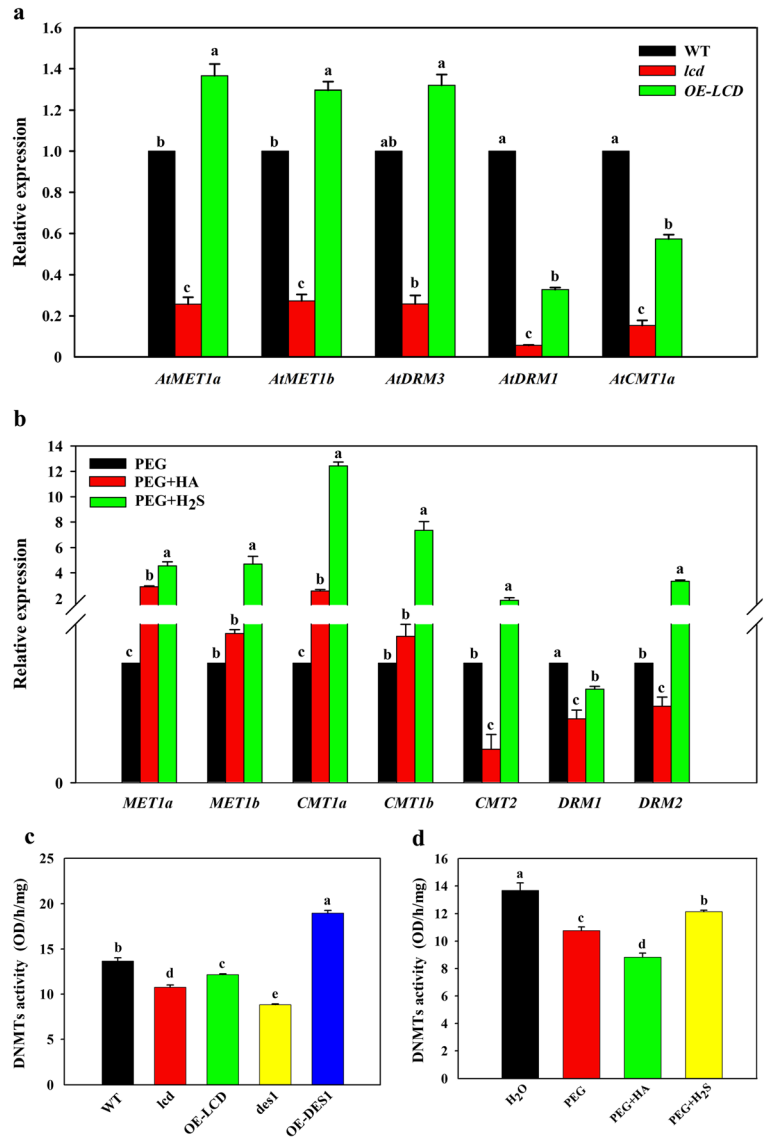

Fig. 4 Effects of H_2S on the DNA methylation level of foxtail millet and Arabidopsis. **a, b** Expression analysis of DNA methylation-related genes in Arabidopsis (*AtMET1a*, *AtMET1b*, *AtCMT1a*, *AtDRM1*, and *AtDRM3*) and foxtail millet (*MET1a*, *MET1b*, *CMT1a*, *CMT1b*, *CMT2*, *DRM1*, and *DRM2*). **c, d** Determination of the total enzyme activity of DNMTs in Arabidopsis and foxtail millet. Three-week-old Arabidopsis seedlings (WT, H_2S-deficient, and overexpressed mutants) cultured in 1/2 MS medium were tested for DNMT total enzyme activity by whole-plant sampling. WT-type was used as a control. Ten-day-old foxtail millet seedlings were treated with 30% PEG8000-bound 50 μM H_2S (or 1.5 mM HA) for 9 h, and then the whole plant was sampled to detect DNMT activity. 30% PEG single treatment was used as a control. Data are mean ± SE of three independent repeats. Bars with different letters are significantly different ($P < 0.05$)

was used to predict the CpG islands of each promoter region (Table S5), one of which was selected as the detection object through analysis. The DNA methylation status of the target CpG island cytosine C before and after H_2S treatment was analyzed via BSP and a statistical analysis was performed (Figs. 6, 7).

The *AREB1*-IS2 island contained 59 cytosine C sites. After H_2S treatment, 34 underwent demethylation changes, 7 sites showed methylation modification, and

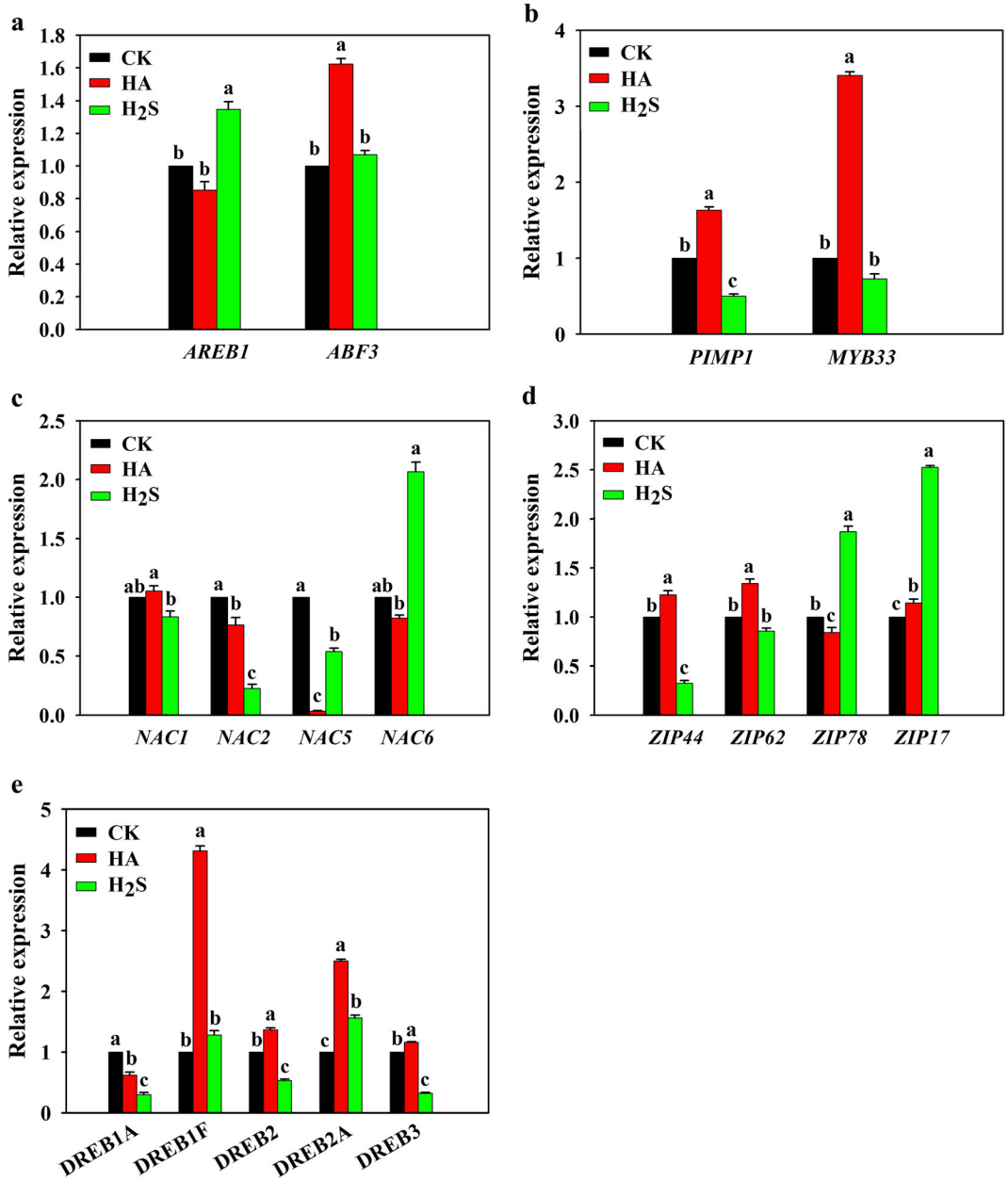

Fig. 5 The expression of drought-resistant TFs in foxtail millet under different levels of H_2S treatment. **a** *AREB/ABFs*. **b** *MYBs*. **c** *NACs*. **d** *ZIPs*. **e** *AP2/ERFs*. Ten-day-old foxtail millet seedlings treated with 30% PEG8000 only (marked as CK), 30% PEG8000 + 50 μM H_2S (marked as H_2S), and 30% PEG8000 + 1.5 mM HA (marked as HA) for 9 h, and then the whole plant was sampled to detect the expression levels of the drought-resistant TFs. Data are mean ± SE of three independent repeats. Bars with different letters are significantly different ($P < 0.05$)

the remaining 18 sites remained unchanged. The overall methylation levels decreased from 78.7% to 63.2% (Fig. 6a). In view of the obvious advantage of demethylation, cytosine sites with a decrease of >40% were selected for comparative analysis (Fig. 6b). The *DREB2A*-IS3 island contained a total of 33 cytosine C sites. H_2S treatment caused the demethylation of 10 sites and methylation of 5 sites (Fig. 6d), resulting in an overall decrease of 7.8 percentage points in methylation level (Fig. 6c). All of the 15 cytosine C sites with changed methylation status were then used for comparative analysis (Fig. 6d). In addition, the overall methylation levels of *ZIP44*-IS2 and *NAC5*-IS3 islands were increased. After H_2S treatment, 22 of the 30 cytosine C

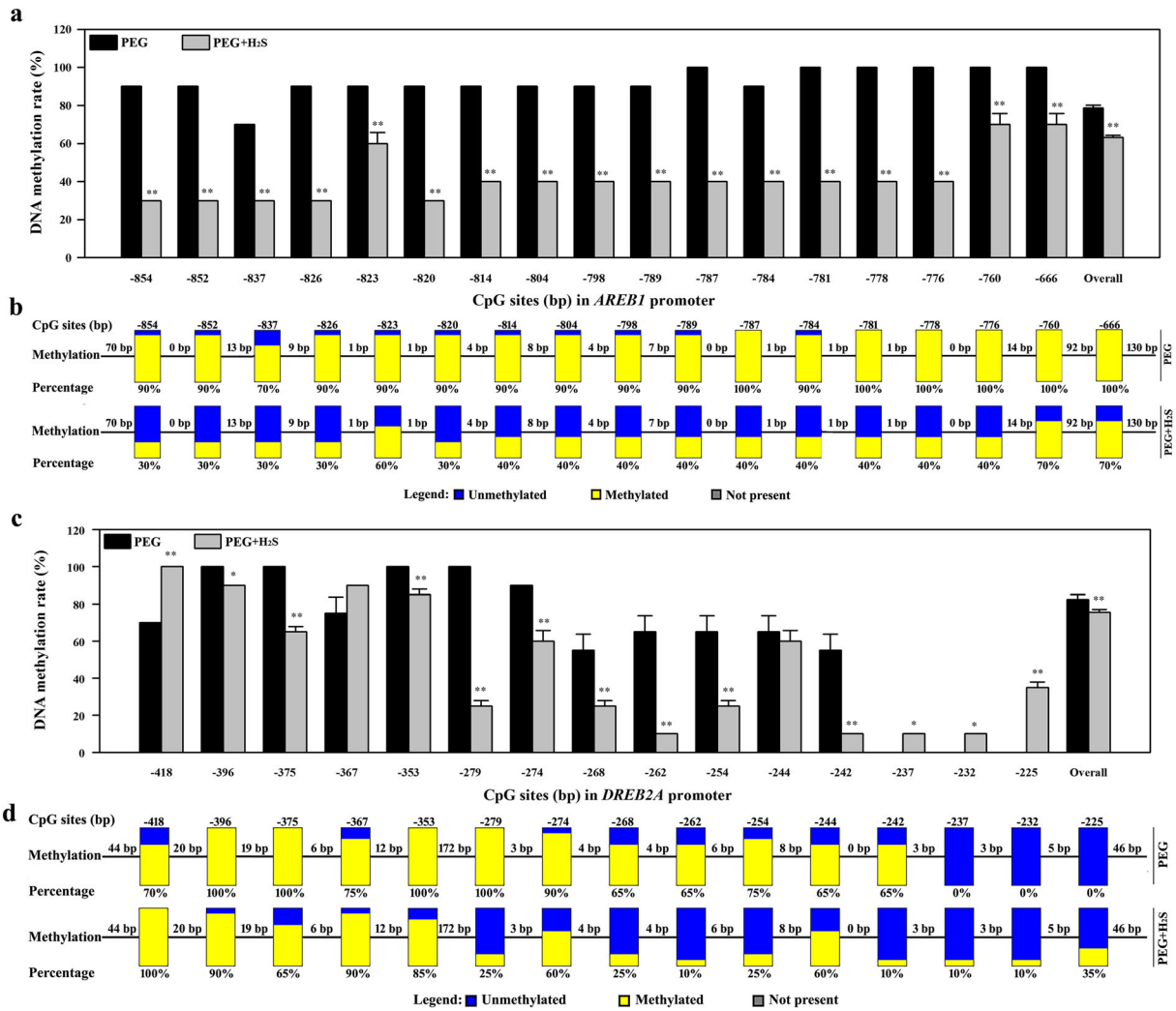

Fig. 6 The ratio of methylated cytosine in the promoter region of *AREB1* and *DREB2A* was down-regulated by H_2S. **a, c** The proportion of cytosine methylated in the total cytosine of each CpG island before and after H_2S treatment. "Overall" represents the percentage of cytosine methylated in the total cytosine of all CpG islands in the promoter region. The abscissa represents the corresponding base sites of each CpG island in the promoter region. **b, d** The methylation status of cytosine in each CpG island in the promoter regions of *AREB1* and *DREB2A* where the blue region represents non-methylated cytosine and the yellow region represents methylated cytosine. The number above the box indicates the corresponding base site of the CpG island in the promoter region, and the percentage below the box indicates the ratio of methylated cytosine to total cytosine. The number above the midline represents the number of bases of the adjacent CpG. Control group: PEG (30% PEG8000); treatment group: PEG + H_2S (30% PEG8000 + 50 μM H_2S). PEG8000 and H_2S were used simultaneously to treat 10-day-old foxtail millet seedlings for 9 h; data are given as the mean ± SD

sites of *ZIP44*-IS2 underwent hypermethylation, whereas only 2 sites underwent demethylation. Given that the overall methylation level of *ZIP44*-IS2 increased from 43.9% to 71.2% (Fig. 7a), we selected only 16 cytosine sites with an increase of >40% for comparative analysis (Fig. 7b). However, in *NAC5*-IS3, 10 of the 24 C sites were methylated and 1 was demethylated. The overall level of *NAC5*-IS3 methylation increased from 82.5% to 97.9% (Fig. 7c). Considering the relatively few cytosine C change sites, all the 11 H_2S regulated change sites were statistically analyzed, and the cytosine methylation status of each site is shown in Fig. 7d.

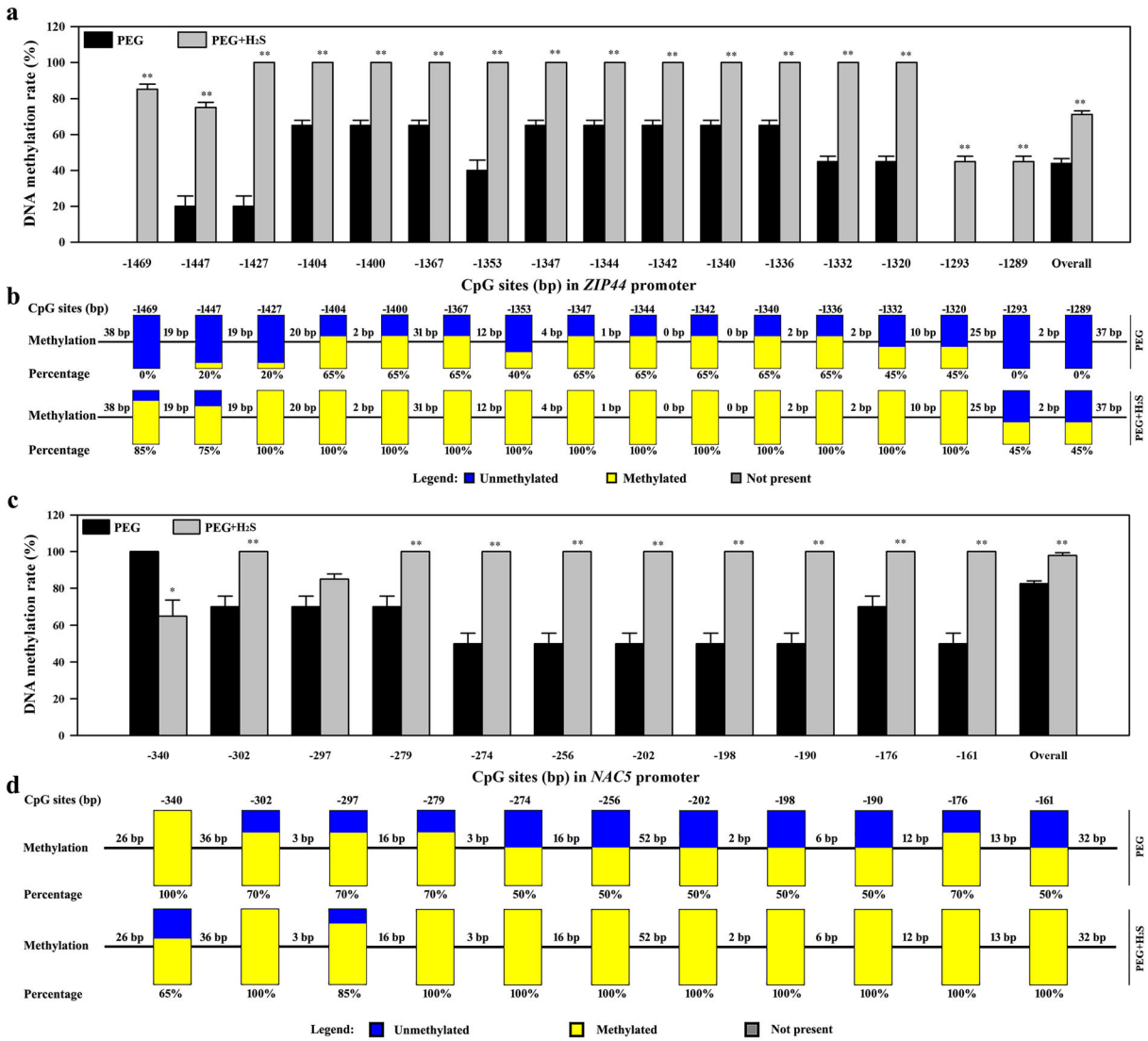

Fig. 7 The ratio of methylated cytosine in the promoter region of *ZIP44* and *NAC5* was up-regulated by H_2S. **a, c** The proportion of cytosine methylated in the total cytosine of each CpG island before and after H_2S treatment. "Overall" represents the percentage of cytosine methylated in the total cytosine of all CpG islands in the promoter region. The abscissa represents the corresponding base sites of each CpG island in the promoter region. **b, d** The methylation status of cytosine in each CpG island in the promoter regions of *ZIP44* and *NAC5* where the blue region represents non-methylated cytosine and the yellow region represents methylated cytosine. The number above the box indicates the corresponding base site of CpG island in the promoter region, and the percentage below the box indicates the ratio of methylated cytosine to total cytosine. The number above the midline represents the number of bases of the adjacent CpG. Control group: PEG (30% PEG8000); treatment group: PEG + H_2S (30% PEG8000 + 50 μM H_2S). PEG8000 and H_2S were used simultaneously to treat 10-day-old foxtail millet seedlings for 9 h; data are given as the mean ± SD

Discussion

H_2S signaling is involved in the process of foxtail millet seedlings coping with drought stress

H_2S in plants is mainly generated by degradation of Cys through an enzyme-catalyzed reaction (Papenbrock et al. 2007). In Arabidopsis, the main enzyme identified with definite functions is CDes (Cysteine desulfhydrases). The members of CDes include LCD (At3g62130), DCD1 (At1g48420), DCD2 (At3g26115) and DES1 (At5g28030) (Jin and Pei 2015). In this study, endogenous H_2S was found to be involved in the response of foxtail millet seedlings to drought stress (Fig. 1). Osmotic stress activated the production of endogenous H_2S in foxtail millet (Fig. 2a, b) and upregulated the expression of *LCD1*, *LCD2*, *DCD1*, *DCD2*, and *DES1* in a time-dependent manner. However, they showed different response speeds to PEG treatment: *LCD1, LCD2* and *DCD2* responded relatively quickly, followed by *DES1*, whereas *DCD1* was the slowest (Fig. 2c).

There are two possible reasons for this finding. Firstly, different H_2S-producing enzymes of foxtail millet have different sensitivities to osmotic stress, resulting in the different regulation efficiencies detected; Secondly, L-Cys may be the dominant form of Cys in millet cells, which makes the enzyme with L-Cys as the substrate to become the main force of endogenous H_2S production and extremely active. In contrast, the slowest response of DCD1 may be due to its preference for D-Cys.

DNA methylation is an important regulatory process in the response of foxtail millet to osmotic stress

Plants can adapt to drought environments by genomic DNA methylation and/or demethylation (Zhang et al. 2018). In the current study, osmotic stress significantly changed the expression levels of major DNMT- and demethylase-coding genes in foxtail millet, and the response of each gene to osmotic stress was also different. Moreover, the total enzyme activity of DNMTs depended on the duration of PEG treatment. With the extension of stress time, the total enzyme activity changed from an inhibited to an activated state, showing a strong response to PEG treatment. In the process, the response patterns of DNMTs differed at the transcriptional and translation levels. The most probable reason is that the DNMT contains various types of enzymes with different activities and the total DNMT activity includes the activities of those various types of DNMT enzymes.

H_2S signaling is closely related to DNA methylation

It is common for H_2S signaling to modify DNA methylation. Different plant tissues (Arabidopsis and foxtail millet) showed dose-dependent effects of DNA methylation on signal H_2S at both the transcription level and protein level (Fig. 4), although there were some differences in response patterns. Such differences have rarely been reported in plants. Compared with Arabidopsis at different H_2S levels, foxtail millet showed a broader range of transcript levels of DNMTs; this might reflect one of the intrinsic reasons for its drought tolerance.

Foxtail millet has a strong osmotic stress tolerance and DNA methylation is one of the many ways it responds to osmotic stress. When responding to PEG treatment, foxtail millet activates different pathways simultaneously. In foxtail millet, H_2S signaling activates the DNA methylation drought-resistant pathway, including the upregulation of *MET1b* and *CMT1b*. By contrast, for *MET1a* and *CMT1a*, H_2S signaling acts to maintain their transcription levels (Fig. 4 b). In addition, the relationship between H_2S and DNA methylation has been explained more directly by using the existing H_2S-producing mutants of Arabidopsis (Fig. 4a).

Thus, by combining the results of our three experiments, it can be concluded that H_2S, osmotic stress, and DNA methylation are closely related. This provides a theoretical basis for the establishment of a new mechanism of drought resistance in foxtail millet, namely, that H_2S enhances the drought resistance of foxtail millet by mediating DNA methylation (Fig. 8).

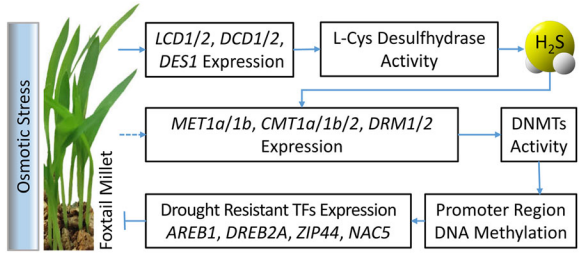

Fig. 8 Proposed model of H_2S mediated DNA methylation to enhance osmotic stress tolerance in foxtail millet. Arrow end: activation; Blunt end: inactivation

The subtle relationship between DNA methylation and gene expression

Gene expression is closely related to methylation in promoter regions (Boyes and Bird 1991). Abiotic stress can cause changes in the level of DNA methylation in plants, activate the expression of stress-related genes and the activity of transposons, and regulate the switching of promoter regions to enhance plant adaptation and resistance to stress (Khan et al. 2016).

Under osmotic stress condition, H_2S signaling induced a gain of hypermethylation in the *ZIP44* and *NAC5* promoters and a loss in the *AREB1* and *DREB2A* promoters, as reflected by the pattern of transcription. The overall methylation level of *ZIP44*-IS2 and *NAC5*-IS3 increased after H_2S treatment, with the reverse seen with *ZIP44* and *NAC5*. The lower methylation levels within *AREB1*-IS2 and *DREB2A*-IS3 were also in contrast to the higher levels of transcription in the H_2S treatment group compared with the control group. Unexpectedly, both *ZIP44*-IS3 and *DREB3*-IS4 were significantly downregulated by H_2S signaling, whereas the DNA methylation levels of the H_2S treatment group were largely unaffected (Table S6).

These results suggest that H_2S-mediated changes in DNA methylation levels are responsible for the induced expression of drought-resistant genes, reflecting the diverse effect of methylation on gene expression. Demethylation of genes can induce the synthesis of resistance-related proteins to enhance plant stress resistance. However, DNA methylation, especially hypermethylation in the promoter region, can prevent transcriptional activators from binding to the promoter by affecting the conformation of the promoter region, and instead recruit transcriptional suppressor genes to that region. When the methylated CpG-binding protein binds to the hypermethylated promoter, it initiates transcription inhibition, thus affecting the expression of downstream related genes and ultimately improving the ability of the plant to resist stress. Moreover, some of the H_2S-mediated alterations in cytosine methylation occurred at sites located within known *cis*-elements, such as ABRE in *ZIP44*-IS2 (C18) and the CGTCA-motif in *AREB1*-IS2 (C24, C25; Table S7). However, whether and how changes in methylation levels at these sites affect the expression of downstream genes remains to be investigated.

Mechanism of H_2S enhancement of the osmotic stress resistance of foxtail millet

Our results showed that H_2S induced significant changes in the transcription level of drought-resistant TFs in foxtail millet by mediating DNA methylation in promoter regions to cope with osmotic stress. In addition, the transcription level of *ZIP44* decreased significantly after H_2S treatment, which correlated with the hypermethylation of *ZIP44*-IS2. However, the DNA methylation level of *ZIP44*-IS3 was unaffected by H_2S treatment. This suggests that different CpG islands in the *ZIP44* promoter region have different abilities to respond to H_2S regulation, whereas H_2S can modify the cytosine C methylation site. In addition, *DREB3* expression was strongly inhibited by H_2S, whereas its DNA methylation level remained unchanged. This also indicates that, although these drought-resistant TFs are actively involved in the process of osmotic stress, different genes act in different ways.

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Compliance with ethical standards

Conflict of interests The authors declared no conflict of interests.

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