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# Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana*

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ABSTRACT

Hydrogen sulfide ($H_2S$) plays a crucial role in human and animal physiology. Its ubiquity and versatile properties have recently caught the attention of plant physiologists and biochemists. Two cysteine desulfhydrases (CDes), L-cysteine desulfhydrase and D-cysteine desulfhydrase, were identified as being mainly responsible for the degradation of cysteine in order to generate $H_2S$. This study investigated the expression regulation of these genes and their relationship to drought tolerance in *Arabidopsis*. First, the expression pattern of *CDes* in *Arabidopsis* was investigated. The expression levels of *CDes* gradually increased in an age-dependent manner. The expression of *CDes* was significantly higher in stems and cauline leaves than in roots, rosette leaves and flowers. Second, the protective effect of $H_2S$ against drought was evaluated. The expression pattern of *CDes* was similar to the drought associated genes induced by dehydration, and $H_2S$ fumigation was found to stimulate further the expression of drought associated genes. Drought also significantly induced increased $H_2S$ production, a process that was reversed by re-watering. In addition, seedlings after treatment with NaHS (a $H_2S$ donor) showed a higher survival rate and displayed a significant reduction in the size of the stomatal aperture compared to the control. These findings provide evidence that $H_2S$, as a gasotransmitter, improves drought resistance in *Arabidopsis*.


## 1. Introduction

Hydrogen sulfide ($H_2S$) has been proposed as the third gasotransmitter, after nitric oxide (NO) and carbon monoxide (CO), in mammals. Along with its ubiquity and versatile properties, $H_2S$ is involved in the regulation of vascular tone, insulin secretion, inflammation, longevity and nociception [1–3]. More recently, $H_2S$ has demonstrated a diverse range of activities in relation to cell survival and death in animals [4]. In the plant kingdom, NO and CO have already been identified as signaling molecules involved in anti-oxidative defense [5–7]. However, whether $H_2S$ serves as a signal molecule in plants remains unclear. In plants, $H_2S$ can be emitted from green cells and $H_2S$ has been reported to be released from the leaves of cucumber, corn, soybeans and other species [8–13].

Two pyridoxal-5′-phosphate (PLP)-dependent enzymes in the trans-sulfuration pathway: cystathionine γ-lyase (CSE, EC 4.4.1.1) and cystathionine β-synthase (CBS, EC 4.2.1.22), have been shown to catalyze production of $H_2S$ in mammalian tissues with L-cysteine as the main substrate [1–3,14]. Recently, two specific desulfhydrases have also been identified and functionally characterized as L-cysteine desulfhydrase (LCD, EC4.4.1.1) and D-cysteine desulfhydrase (DCD, EC4.4.1.15). This demonstrated that $H_2S$ was released mainly by the action of desulfhydrases in plants [15–18]. LCD is the most important enzyme associated with $H_2S$ production in plants and shares a 100% sequence homolog with CSE in mammals.

Expression and activity of LCD were induced upon pathogen attack, suggesting that $H_2S$ release plays an important role in defense against biotic stress in plants [17,19]. Moreover, further findings inferred that $H_2S$ was also involved in the defense mechanisms against abiotic stress in plants. In particular, $H_2S$ released from plants: alleviated injury by $SO_2$ [20,21]; $H_2S$ fumigation improved freezing tolerance in wheat shoots [22] and $H_2S$ treatment enhanced antioxidant response against copper, chromium and other osmotic stresses in a number of species [23]. In addition, $H_2S$ can promote root organogenesis and seed germination in several species [24,25] and also acts as a regulator of flower senescence [26]. However, in contrast to the extensive studies into $H_2S$ activity in animals, the molecular mechanisms underlying the action of

*Abbreviations:* ABA, abscisic acid; CBF, C-repeat binding factor; CBS, cystathionine β-synthase; CDes, cysteine desulfhydrases; CSE, cystathionine γ-lyase; CO, carbon monoxide; DCD, D-cysteine desulfhydrase; DREB, dehydration-responsive element-binding protein; $H_2S$, hydrogen sulfide; LCD, L-cysteine desulfhydrase; MS, Murashige–Skoog; NO, nitric oxide; PLP, pyridoxal-5′-phosphate; RT-PCR, reverse transcriptase polymerase chain reaction; RD, responsive to desiccation.

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endogenous $H_2S$ as a signaling molecule in plant systems is still limited.

Drought stress is one of several environmental factors greatly limiting crop production and plant distribution worldwide [27]. At present, the defense mechanisms against drought in plants are still poorly understood and the signaling pathways involved remain elusive [28–30]. Drought adaptation in plants is regulated at the transcriptional level by the ABA-dependent and ABA-independent pathways [31]. *CBF4* and *DREB2* are the respective genes associated with these two pathways and they, in turn, regulate *RD29A* gene expression [32].

This preliminary study revealed that the production rate of $H_2S$ was positively correlated with the extent of drought stress in *Arabidopsis thaliana*. This research examined the possible role of $H_2S$ as a gaseous signaling molecule in *Arabidopsis* under drought stress. The potential of $H_2S$ to improve plant resistance to drought was also investigated.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

*A.* thaliana (Columbia ecotype) was used in this study. The growth conditions were described previously [33]. For each experiment, seeds were stratified for 2–4 d at 4 °C and then grown in pots containing a soil:perlite:vermiculite (1:1:1 v/v) mixture that was maintained at 23 °C and 60% relative humidity. They were subjected to a 16 h/8 h (light/dark) photoperiod with light illumination at 160 $\mu E\,m^{-2}\,s^{-1}$, and watered with a ½MS nutritive medium. In order to undertake specific plant developmental measurements, leaves of 2-week (2 w), 4-w and 6-w-old plants were collected. For tissue specific measurements, the roots, stems, rosette leaves, cauline leaves and flowers were collected from 6-w-old plants. All tissues harvested were immediately frozen in liquid nitrogen and stored at −80 °C.

### 2.2. Stress and chemical treatments

For the dehydration treatment, whole plants were removed after 4 w of growth. They were washed gently to remove soil from the roots and left to desiccate on white paper at 22 °C and 60% relative humidity under low light. The whole plants were sampled at 0, 0.3, 0.6, 1, 2, 3 and 6 h, respectively after the start of desiccation. For the fumigation treatment, plants were kept in their pots, placed in a sealed container and were fumigated with 80 μM NaHS. Light and temperature conditions were kept the same as described above. Treated leaves were sampled at 0, 1, 3, 6, 12 and 24 h after the start of fumigation. 4-w-old plants subjected to drought stress, were grown in pots by withholding water for 14 d, and then re-watered until the substrate was saturated. In another experiment, two groups of seedlings were fumigated (6 h duration with 80 μM NaHS) and watered every other day. All tissues harvested were immediately frozen in liquid nitrogen and stored at −80 °C.

### 2.3. Extraction of total RNA and RT-PCR

Total RNA samples were extracted using TRizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All molecular manipulations using reverse transcription (RT)-PCR analyzes were carried out according to standard methods [34]. The appropriate primer pairs were as follows: *LCD* (At3g62130, 1365-bp): 5′-CATGCCATGGCAATGGAGGCGGGAGAGCGGCGCAATG-3′ (forward); and 5′-CGGGATCCCTACAATGCAGGAAGGTTTTGACAAG-3′ (reverse); *DCD* (At1g48420, 1206-bp): 5′-CATGCCATGGCAATGAGAGGACGAAGCTTGACACTCTC-3′ (forward); and 5′-CGGGATCCCTAGAACATTTTCCCAACACCATCTT-3′ (reverse); *DREB2A* (At5g05410, 482-bp): 5′-TATGAAAGGTAAAGGAGGAC-3′ (forward); and 5′-ACAATCC CTTGTCCTTC-3′ (reverse); *DREB2B* (At 3g 11020, 466-bp): 5′-GTATGAAGGGTAAAGGAGG-3′ (forward); and 5′-CCAATACTG CTGCTCAAA-3′ (reverse); *CBF4* (At5g51990, 436-bp): 5′-GGTAAATGGGTTTGTGAAG-3′ (forward); and 5′-TGATT CCAGCCAACTTCC-3′ (reverse); *RD29A* (At5g52310, 459-bp): 5′-TG ACGACGAAGTTACCTAT-3′ (forward); and 5′-ACAGTGGAGC CAA G TGAT-3′ (reverse). Semi-quantitative RT-PCR was carried out to determine the expression level of target genes using *ACTIN* as an internal control. All RT-PCR expression assays were performed and analyzed at least three times in independent experiments.

### 2.4. Determination of $H_2S$ production rate

Total cysteine desulfhydrase activity was ascertained by measuring the production rate of $H_2S$ from L-cysteine [35] and D-cysteine [16]. The Total protein of 4-w-old plants was extracted following the protocol described above [33].

### 2.5. Assay of stomatal aperture

Four-week-old plants were fumigated for 6 h in their pots as described in Section 2.2. Stomatal aperture measurements were performed on the lower epidermis of rosette leaves. Epidermal peels were floated in 70% ethanol in order to fix the pore structure immediately. After 5 min, stomata were digitized using a NikonDS-Fi1 camera coupled to a Nikon EclipseTi (Nikon, Tokyo, Japan). The whole process was carried out under light at 25 °C. The stomatal aperture was measured using IMAGEJ analysis software (NIH, Bethesda, MD).

### 2.6. Statistical analysis

Each experiment was replicated three times. Values are expressed as means ± SE. Data were analyzed using Statistica (Stat Soft Inc, Tulsa, OK) software. All mean comparisons were carried out using the *t*-test for independent samples. For stomatal opening assays, measurements were subjected to a one-way analysis of variance (ANOVA).

## 3. Results

### 3.1. Drought stress induced more $H_2S$ production in A. thaliana

To explore the effect of drought stress on the CDes/$H_2S$ system, the $H_2S$ production rate was measured in *Arabidopsis*, which, in turn, reflects the total cysteine desulfhydrase activity. The $H_2S$ production rate of two groups of 4-w-old plants was measured under drought conditions for 1 week and then re-watered for 1 and 2 days, respectively. As shown in Fig. 1, $H_2S$ production rate increased considerably under drought conditions (1 w after watering) and decreased dramatically after being re-watered for 1 or 2 days.

### 3.2. Temporal and spatial expressions of CDes

In order to study the expression patterns of *CDes* at the different developmental stages of *Arabidopsis*, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed, using mRNA from the leaves of 2-w, 4-w and 6-w-old plants. The *ACTIN* gene was used as an internal control. The complete coding regions of 1365-bp and 1206-bp were obtained. As shown in the left column of Fig. 2A, the expression patterns of

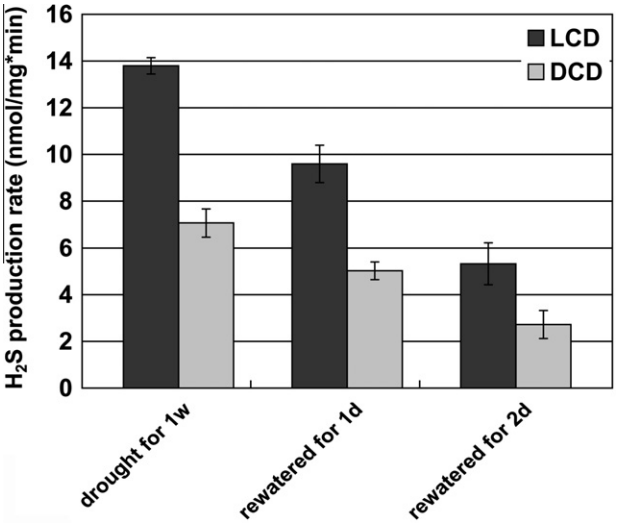


**Fig. 1.** Altered $H_2S$ production rate in *Arabidopsis* under drought and re-watering conditions. The $H_2S$ production rate of 4-w-old plants was measured under conditions of drought for 1 w, and then re-watered for 1 d or 2 d.

*LCD* and *DCD* were similar. Both were expressed at different developmental stages. Between 2 w to 6 w, the expression levels of both *LCD* and *DCD* gradually increased.

Total RNA samples were isolated from different tissues of 6-w-old seedlings, including roots (R), stems (S), rosette leaves (RL), cauline leaves (CL) and flowers (F). These were analyzed by RT-PCR using the *ACTIN* gene as an internal control. As shown in the right column of Fig. 2A, transcripts of *CDes* could be detected in all the tissues of mature plants; the expression pattern of *LCD* was similar to *DCD*. There was, however, considerable variation in the levels of *CDes* expression in different tissues. *CDes* was strongly expressed in stems and cauline leaves but was very weakly expressed in roots, rosette leaves and flowers.

### 3.3. Drought stress induced expressions of both CDes and drought associated genes

As shown in Fig. 2B, expression of *CDes* was strongly induced by drought stress, with a maximum accumulation after 6 h. The mRNAs of the four drought associated genes (*DREB2A*, *DREB2B*, *CBF4* and *RD29A*) accumulated progressively as the dehydration treatment time length increased. *CBF4* was induced at a slower rate than the other genes and the expression levels of all genes began to decline after re-watering. The expression pattern of *CDes* was similar to the drought associated genes induced by dehydration, which suggested that the $H_2S$ level was related to drought resistance in plants.

### 3.4. $H_2S$ stimulated expression of drought associated genes

To determine the influence of $H_2S$ at physiologically relevant concentrations on drought associated genes, the expression of drought associated genes was studied under normal growth conditions after treatment with NaHS. As expected, maximum accumulation following NaHS treatment occurred after 3 h (Fig. 2C). The expression pattern of drought associated genes induced by NaHS fumigation was similar to that found with the dehydration treatment.

### 3.5. $H_2S$ protected Arabidopsis from drought stress

Four week old *Arabidopsis* plants were subjected to drought stress for 2 w by completely withholding water during this period.

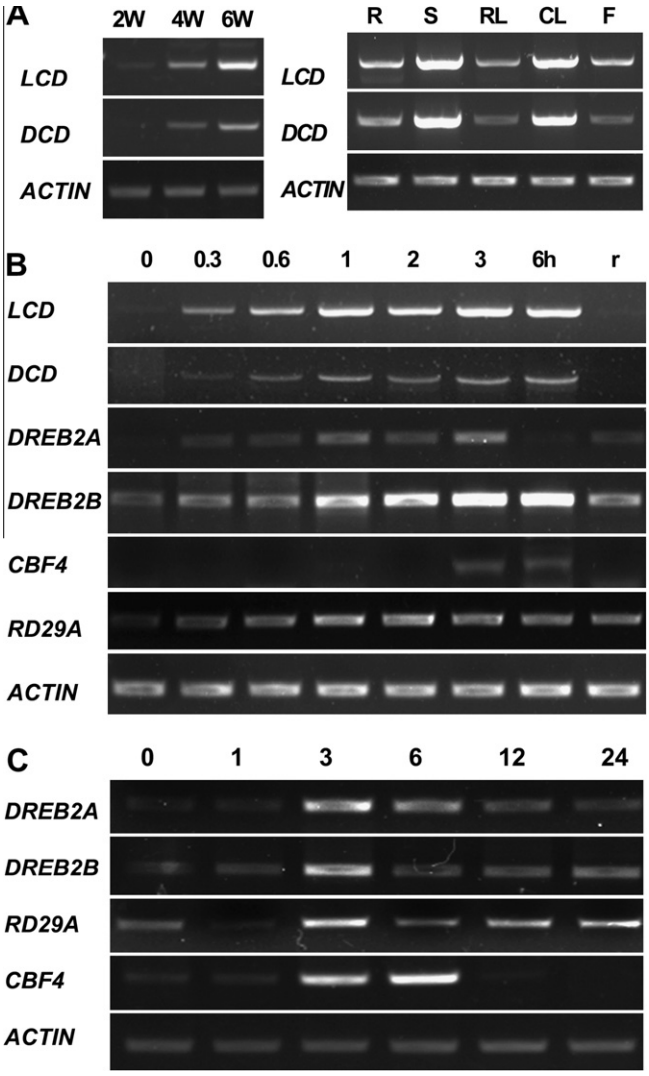


**Fig. 2.** Expression analysis of *CDes* and drought associated genes. (A) Temporal and spatial expression pattern of *CD* genes. Left column: Total RNA samples were isolated from leaves of 2-w-old, 4-w-old and 6-w-old plants; right column: total RNA samples were isolated from different tissues of 6-w-old plants, including roots (R), stems (S), rosette leaves (RL), cauline leaves (CL) and flowers (F). (B) Expression of *CD* genes, *DREB2*, *CBF4* and *RD29A* were induced by drought. Total RNA was extracted from 4-w-old plants treated with dehydration for 0, 0.3, 0.6, 1, 2, 3, 6 h and re-watered (r) for 6 h. The accession numbers of the corresponding genes are as follows: *LCD* (At3g62130), *DCD* (At1g48420), *DREB2A* (At5g05410), *DREB2B* (At3g11020), *CBF4* (At5g51990), *RD29A* (At5g52310). (C) Expression patterns of *DREB2*, *CBF4* and *RD29A* during NaHS fumigation. 4-w-old seedlings were treated with 80 μM NaHS. Leaf samples were collected after having been treated for 0, 1, 3, 6, 12 and 24 h, respectively. The expression level of each gene was detected by RT-PCR, with *ACTIN* as an internal control.

Fig. 2B shows that gene expression was higher after plants had been subjected to drought for 6 h, together with fumigation for 6 h every other day, and then re-watered for 1 d. As shown in Fig. 3A, wilting occurred primarily in control samples. NaHS treated plants were greener and showed more turgidity than the control plants where leaves were seriously wilting after 7 d of withholding water. Moreover, after two weeks, most of the control plants had died, whereas most treated plants had survived. After re-watering, the surviving seedlings from the two groups gradually returned to normal. Fig. 3B shows that the rate of survival was about 95% for NaHS treated plants, which was significantly higher than for the control plants at 62%. These results indicated that $H_2S$ protected plants from drought stress.

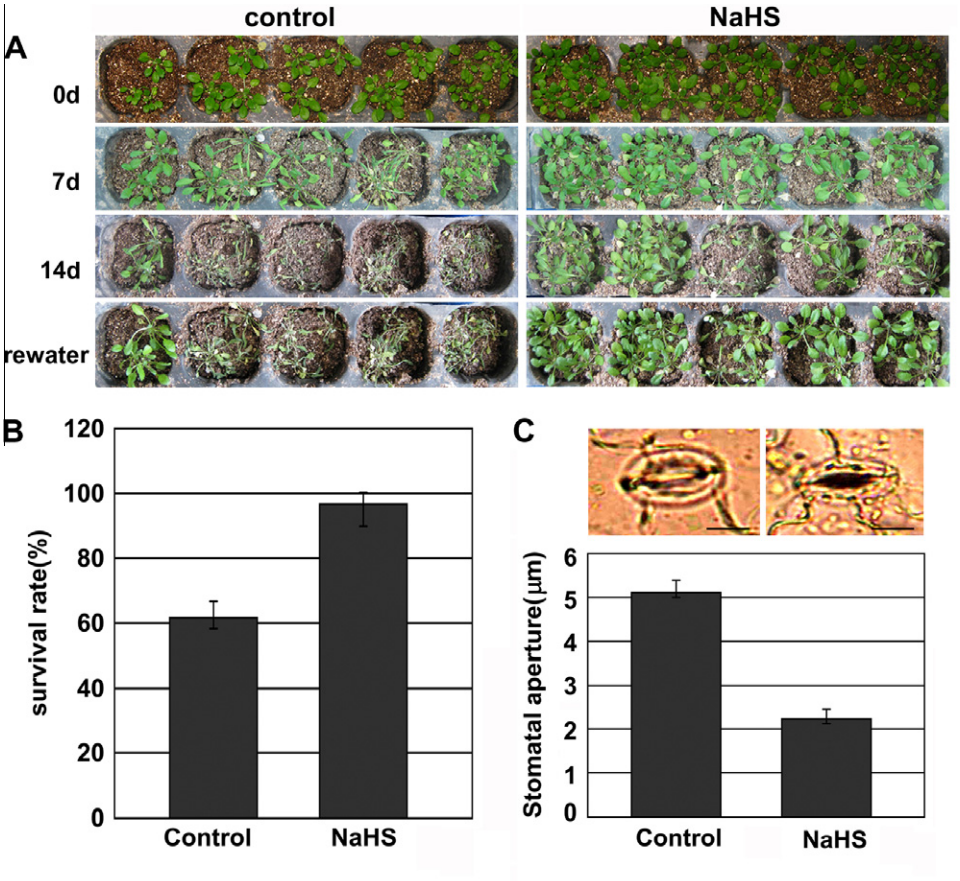


**Fig. 3.** Hydrogen sulfide improves drought resistance by inducing stoma closing. (A) $H_2S$ protected *Arabidopsis* from drought stress. Four-week-old plants were subjected to drought stress for 14 d. Water were completely withheld during this period. Two groups of seedlings were fumigated with 80 μM NaHS and watered respectively every other day. Fumigation was maintained for 6 h at a time. Finally, all seedlings were re-watered for 1 d. (B) Survival was quantified by counting the percentage of living plants relative to total plants in the two groups after re-watering for 1 d. (C) Effect of $H_2S$ on stomatal opening. Photographs above chart: open and closed stomata observed under an optical microscope (200×) (bars = 20 μm). Chart below: stomatal aperture measurements were performed on the lower epidermis of the control and NaHS treated leaves. Values were expressed as means ± SE and represent the mean of 20–30 stomata from at least three independent experiments.

### 3.6. $H_2S$ induces stomatal closure

Stomata are pores present in plant aerial tissues and are surrounded by a pair of guard cells. These specialized cells receive and integrate a great number of external and internal stimuli to accurately respond to plant physiological requirements. To determine the effect of $H_2S$ on stomatal movement, leaves from both NaHS fumigated and control plants were used for the stomatal aperture study. The macroscopic observations were supported by microscopic examination. As shown in Fig. 3C, leaves treated with NaHS showed a significant reduction in the stomatal aperture size compared to the control samples. This suggests that $H_2S$ plays a role in plant stomatal closure as a novel component of guard cell signaling.

## 4. Discussion

In animals, endogenous $H_2S$ can be formed primarily from cysteine by PLP-dependent enzymes, including CSE and CBS [1–3,14]. However, the mechanism for $H_2S$ generation in higher plants remains unclear. So far, several candidate proteins, that exist in plants, have been confirmed to be involved in $H_2S$ release, such as LCD, DCD, *O*-acetyl-L-serine(thiol) lyase (OAS-TL) and NFS/NifS. LCD is the most important enzyme involved in the decomposition of L-cysteine into $H_2S$, which was first reported by Harrington and Smith [36]. Subsequently, DCD was identified as the second main enzyme, which catalyzed D-cysteine to $H_2S$ [37]. Nifs/NFS, also identified in *Arabidopsis*, process L-cysteine desulfhydrase activity and are involved in Fe–S cluster biosynthesis [38]. OAS-TL catalyzes the incorporation of sulfide at the β-position of *O*-acetyl-L-serine (OAS) leading to the formation of cysteine and is also able to release $H_2S$ in a side reaction [39]. Among these enzymes, LCD found in plants, is homologous with CSE, which is the most important $H_2S$ producing enzyme in animals. In shoots of *Brassica napus* and *Arabidopsis*, DCD activity was about half as great as LCD activity [16]. These results showed that the activity of LCD was about 2–3 times higher than that of DCD (Fig. 1). From this result, both LCD and DCD concentrations were chosen as indicators of desulfhydrase production in order to determine the functions of $H_2S$.

$H_2S$, the third gasotransmitter, can be produced in a variety of cells, tissues, organs and systems. Usually the physiological concentration of $H_2S$ in mammals is about 50 μM. It may be above 100 μM under certain pathological conditions. In blood, the $H_2S$ level is 30–50 μM [3,4,40]. In plants, techniques have been developed to measure $H_2S$ concentration but these methods are still imperfect just as they are in animal research. Rennenberg et al. [13] used a large 2.6-L gas exchange cuvette to measure volatile sulfur compounds via gas chromatography and a connected flame photometric detector (this has now been replaced with a sulfide hydrogen microsensor), equipment that was originally used to detect $H_2S$ in bacteria and liquids but in this case was used to detect the $H_2S$ concentration in plants [36,41,42]. In general, different

experimental approaches have their advantages and disadvantages. Technical problems need to be solved before the equipment can be used routinely with a high degree of reproducibility in plants [39]. The results obtained via different approaches show significant differences, let alone the inherent differences between different plants or tissues. Therefore, based on the data reported, no unanimous conclusions can be drawn.

In past studies the concentration of $H_2S$ detected in plants was from 1 to 100 μM, which is similar to the levels found in animals and humans. NaHS starts to be toxic for *V. faba* guard cells at concentrations $\geqslant$ 500 μM, and cell viability was similar to the control plants at concentrations $\leqslant$ 100 μM [43]. Therefore, these preliminary experiments were conducted using this concentration range and these data clearly showed that plants fumigated with 80 μM $H_2S$ survived for a longer period of time than expected from the 100 μM results obtained from the previous studies.

The signaling transduction events in drought stress have been comprehensively investigated [31,44]. In contrast to ion homeostasis, plant adaptation to drought is, to a large extent, under transcriptional control; some processes are regulated by abscisic acid (ABA), while others are ABA-independent [31]. *DREB2* and *CBF4* play a major role in drought-regulated gene expression; the drought induction of *DREB2* is ABA-independent while the dehydration induced expression of *CBF4* is controlled by ABA. In addition, *RD29A* plays a central role in both ABA-dependent and ABA-independent pathways by providing binding sites for *ABRE* and *DREB2* [45]. The expression pattern of these drought associated genes was consistent with *LCD* and *DCD,* which are the main enzymes involved in the endogenous production of $H_2S$ in plants. The responsiveness of the *CDes* gene to dehydration implies that its role in the transduction of drought stress signals was not only similar to that of *DREB2* and *RD29A* in *Arabidopsis*, which occurred through an ABA-independent pathway, but also similar to the responsiveness of *CBF4* which occurs through an ABA-dependent pathway. In other words, enhanced $H_2S$ production in plants may contribute to improved resistance to drought stress by affecting both ABA-dependent and ABA-independent pathways.

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