

Communication

# Isoenzymatic Pattern of Hydrogen Sulfide (H<sub>2</sub>S)-Generating L-Cysteine Desulphydrase (LCD) in *Arabidopsis thaliana* Seedlings: Effect of Nitric Oxide (NO) and H<sub>2</sub>S

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**Abstract:** In higher plants, hydrogen sulfide (H<sub>2</sub>S) is a recognized signaling molecule that performs multiple regulatory functions. The enzyme L-cysteine desulphydrase (LCD) catalyzes the conversion of L-cysteine (L-Cys) to pyruvate and ammonium with the concomitant generation of H<sub>2</sub>S, and it is considered one of the main sources of H<sub>2</sub>S in plants. Using non-denaturing polyacrylamide gel electrophoresis (PAGE) in combination with a specific assay for LCD activity, this study aims to identify the potential LCD isozymes in wild-type *Arabidopsis thaliana* seedlings of 16 days old grown under in vitro conditions, and to evaluate the potential impact of nitric oxide (NO) and H<sub>2</sub>S on these LCD isozymes. For this purpose, an *Atnoa1* mutant characterized to have a low endogenous NO content as well as the exogenous application of H<sub>2</sub>S were used. Five LCD isozymes were detected, with LCD IV being the isozyme that has the highest activity. However, the LCD V activity was the only one that was positively modulated in the *Atnoa1* mutants and by exogenous H<sub>2</sub>S. To our knowledge, this is the first report showing the different LCD isozymes present in *Arabidopsis* seedlings and how their activity is affected by NO and H<sub>2</sub>S content.

**Keywords:** *Atnoa1*; hydrogen sulfide; L-cysteine desulphydrase activity; posttranslational modifications; sodium hydrosulfide



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## 1. Introduction

Hydrogen sulfide (H<sub>2</sub>S) is a molecule that is endogenously generated in plant cells and, in recent years, it has gained great relevance due to its signaling function under physiological processes such as photosynthesis, seed germination, root growth, fruit ripening, and organ senescence, but also in the mechanism of response against stressful conditions [1–4]. Recently, in the model plant *Arabidopsis thaliana*, an in silico analysis was performed to find out how the H<sub>2</sub>S is generated as part of the sulfate assimilation and in the cysteine (Cys) metabolism [5]. Among the twenty-six identified enzymes involved in the metabolism of H<sub>2</sub>S, five enzymes directly generate H<sub>2</sub>S in plants including the cytosolic L-cysteine desulphydrase (LCD), bifunctional cystathionine γ-lyase/cysteine synthase (DES1), the chloroplastic sulfite reductase (SiR), the mitochondrial bifunctional D-cysteine desulphydrase/1-aminocyclopropane-1-carboxylate deaminase (DCDES1), and D-cysteine desulphydrase 2 (DCDES2) [5–7]. The cytosolic LCD is considered one of the primary sources of H<sub>2</sub>S, which catalyzes the following reaction: L-cysteine + H<sub>2</sub>O → pyruvate + NH<sub>4</sub><sup>+</sup> + H<sub>2</sub>S + H<sup>+</sup>, using pyridoxal 5-phosphate (PLP) as a necessary cofactor [8–11].

Although the enzymatic source of nitric oxide (NO) in plants is still under debate [12], what is well established is that NO and derived species designated as reactive nitrogen

species (RNS) constitute a family of molecules that exert a wide spectrum of functions in the physiology of plants and the mechanisms of responses to environmental stresses [13–21].

The participation of both molecules in plant cells is a research area of great interest, and an increased number of studies indicate that they can act both synergistically and antagonistically in different physiological processes [22,23] and in response to stress [2,24–26]. Furthermore, it has been seen that both NO and H<sub>2</sub>S can regulate protein function through post-translational modifications such as nitration, S-nitrosation, and persulfidation. Thus, some enzymes such as catalase, lipoxygenase, NADP-dependent dehydrogenase, NADP malic enzyme, or peroxidase undergo modifications via both molecules affecting their functions either positively or negatively [27–31].

Based on previous work, where an optimized method in polyacrylamide gels under non-denaturing conditions allowed the identification of different LCD isoenzymes in several species of the genus *Allium* and pepper fruit [8,11], the present study aims to analyze the isozymatic pattern of LCD in *Arabidopsis* seedlings and evaluate if any of these LCD isozymes could be modulated via NO and/or H<sub>2</sub>S.

## 2. Materials and Methods

### 2.1. *Arabidopsis* Material and Growth Conditions

The *Arabidopsis thaliana* ecotype Columbia wild type (Wt) and the *Atnoa1* (*Arabidopsis* NO-associated 1) mutant, which has a deficit in the production of endogenous nitric oxide (NO), were used in this study. Seeds were surface sterilized as previously described [32]. Briefly, seeds were incubated for 5 min in 70% (*v/v*) ethanol solution containing 0.1% (*w/v*) SDS, then placed for 20 min in sterile water containing 20% (*v/v*) bleach and 0.1% (*w/v*) SDS, and washed four times in sterile water. The seeds were sown for 2 days at 4 °C in the dark for vernalization. Then, the seeds were placed on Petri dishes 90 mm in diameter containing a growth medium composed of 4.32 g/L commercial Murashige and Skoog (MS) medium (Sigma-Aldrich, Madrid, Spain) with a pH of 5.5, containing 1% (*w/v*) sucrose and 0.8% (*w/v*) phyto-agar. For the experiments with sodium hydrosulfide (NaHS) as H<sub>2</sub>S donor, a solution of 200 µM NaHS (final concentration) in sterile water was added to the Petri dishes [33]. All procedures were carried out in a laminar flow cabinet. Then, seedlings were grown for 16 days under long-day conditions (16 h light at 22 °C and 8 h dark at 18 °C), and a light intensity of 100 µE m<sup>−2</sup>·s<sup>−1</sup>.

### 2.2. Morphological Analyses of *Arabidopsis thaliana* Root System

Fresh weight and primary root length were evaluated in sixty *A. thaliana* Wt and *Atnoa1* seedlings per treatment and expressed in mg per seedling and mm (mean ± SE), respectively. The Petri dishes containing the seedlings were scanned at 1200 dpi with Epson Perfection 2450 using Vuescan 9.0.94 software and the primary root length was analyzed with RootNav image analysis tool (University of Nottingham, Ver. 1.8.1, 64-bit).

### 2.3. Crude Extracts of Plant Tissues and Protein Assay

*Arabidopsis* seedlings of 16 days were collected and frozen in liquid nitrogen. Then, they were ground in a mortar with a pestle. The powder was suspended in a homogenizing medium (ratio 1:3, *w/v*) containing 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 (*v/v*) Triton X-100 and 10% (*v/v*) glycerol, and homogenates were centrifuged at 27,000× *g* for 20 min at 4 °C. The supernatants were used for the assays.

Protein content in the samples was determined using the Bio-Rad Protein Assay (Hercules, CA, USA), with bovine serum albumin as standard.

### 2.4. In-Gel Profile of L-Cysteine Desulphydrase (LCD) Activity

*Arabidopsis* samples were separated non-denaturing polyacrylamide gel electrophoresis (PAGE) on 8% acrylamide gels. After the electrophoresis, to visualize the LCD (EC 4.4.1.28) activity bands, the gels were incubated in the dark in a staining buffer containing Tris-HCl 100 mM, pH 7.5, L-cysteine 20 mM, lead acetate 0.4 mM, pyridoxal 50-phosphate hydrate

50  $\mu\text{M}$  and dithiothreitol 5 mM, until the appearance of brown bands over a colorless background [10,11]. Two internal controls were accomplished to check the specificity of the LCD activity; one was to incubate the gel in the absence of the substrate L-cysteine or the cofactor pyridoxal 5'-phosphate. In both cases, no band activity was detected.

### 2.5. Statistical Analysis

It was carried out using SPP program in which a one-way ANOVA was applied accompanied by Tukey's post hoc test with a  $p$  level of 0.05.

## 3. Results and Discussion

The involvement of  $\text{H}_2\text{S}$  in numerous physiological plant processes as well as in response to diverse types of environmental stresses is a subject of an increasing number of studies [2,3]. In plant cells, there are different enzymatic sources of  $\text{H}_2\text{S}$ , and cytosolic L-cysteine desulphydrase (LCD) is one of them [5]. Using Arabidopsis seedlings, this work has the goal to identify the presence of LCD isoenzymes and if they could be modulated by NO or  $\text{H}_2\text{S}$ .

Figure 1 shows the appearance of Arabidopsis Wt and *Atnoa1* seedlings grown in the absence and the presence of 200  $\mu\text{M}$  NaHS (Figure 1a,b), as well as the analysis of their biomass (Figure 1c) and primary root length (Figure 1d) after 16 days. A visual examination allows observing that the green cotyledons of the *Atnoa1* seedlings had smaller size and a yellowish color in comparison to Wt seedlings. This aspect should be associated with the NO deficiency in this mutant, which is likely related to cotyledon organogenesis and chloroplast formation. A similar situation occurred in previous assays with these mutants and the cyclic GTPase family, where smaller yellowish and pale green leaves were observed [34,35]. On the other hand, through comparing the color of the leaves from the mutant seedlings treated with  $\text{H}_2\text{S}$  (*Atnoa1* plus NaHS) with the control mutant seedlings, it is observed that the treated plantlets are greener than the untreated ones. Therefore, it is suggested that the treated seedlings have a lower loss of green color, similar to what occurs in pak choi plants (*Brassica rapa* subsp. *chinensis*) fumigated with  $\text{H}_2\text{S}$  [36].

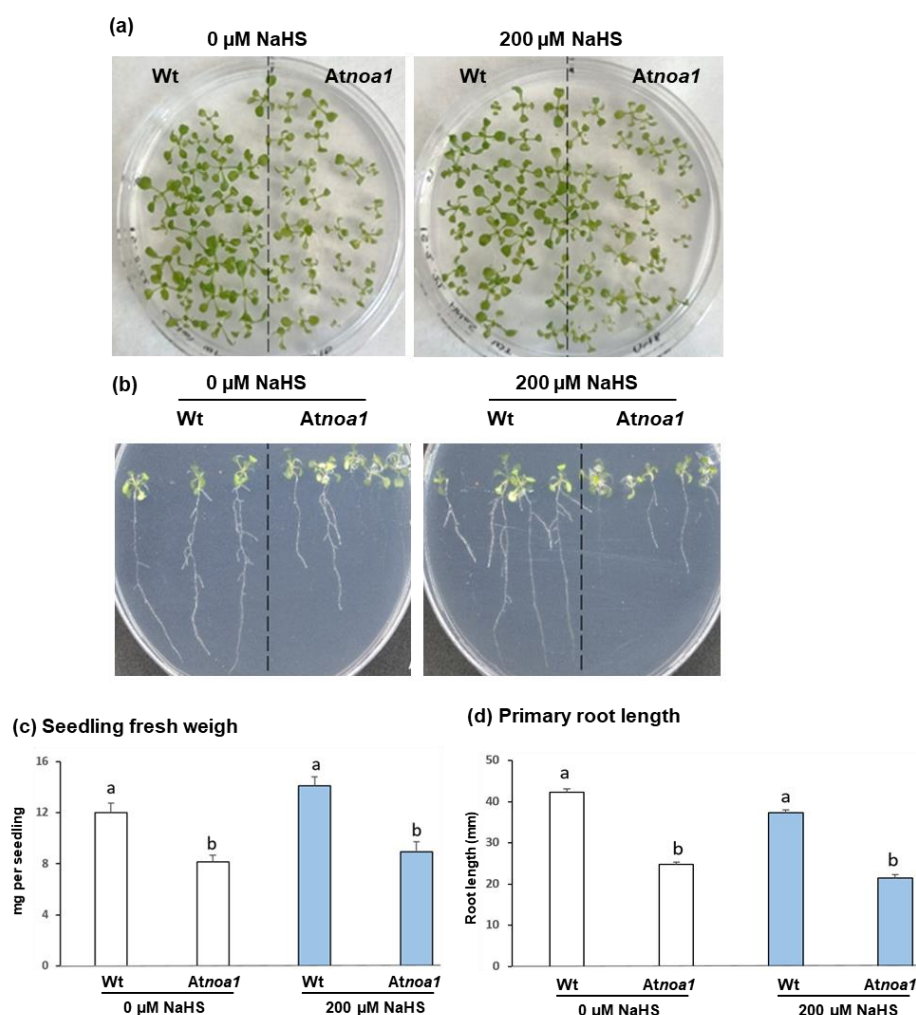
When seedlings were grown in Petri dishes placed in a vertical position (Figure 1b,d), it was possible to study if there were changes in the root phenotype between Wt and *Atnoa1* seedlings untreated and treated with  $\text{H}_2\text{S}$ . The analysis of the length of the primary root of untreated seedlings allowed to observe that Wt seedlings were 42% higher than that of *Atnoa1* mutants. Finally, in  $\text{H}_2\text{S}$ -treated plants, the primary root length in Wt seedlings was 51% higher than that determined in the *Atnoa1* mutants.

With the aim to identify the presence of different LCD isozymes, in-gel analyses were performed using 16-day-old Arabidopsis seedlings. Figure 2a displays the identification of five LCD isozymes designated AtLCD I to AtLCD V, according to their increasing electrophoretic mobility in the gel. AtLCD IV was the most prominent isozyme whereas AtLCD II was the least abundant. As shown in the figure, both NO and  $\text{H}_2\text{S}$  influenced the zymogram, with AtLCD V being the most affected isozyme.

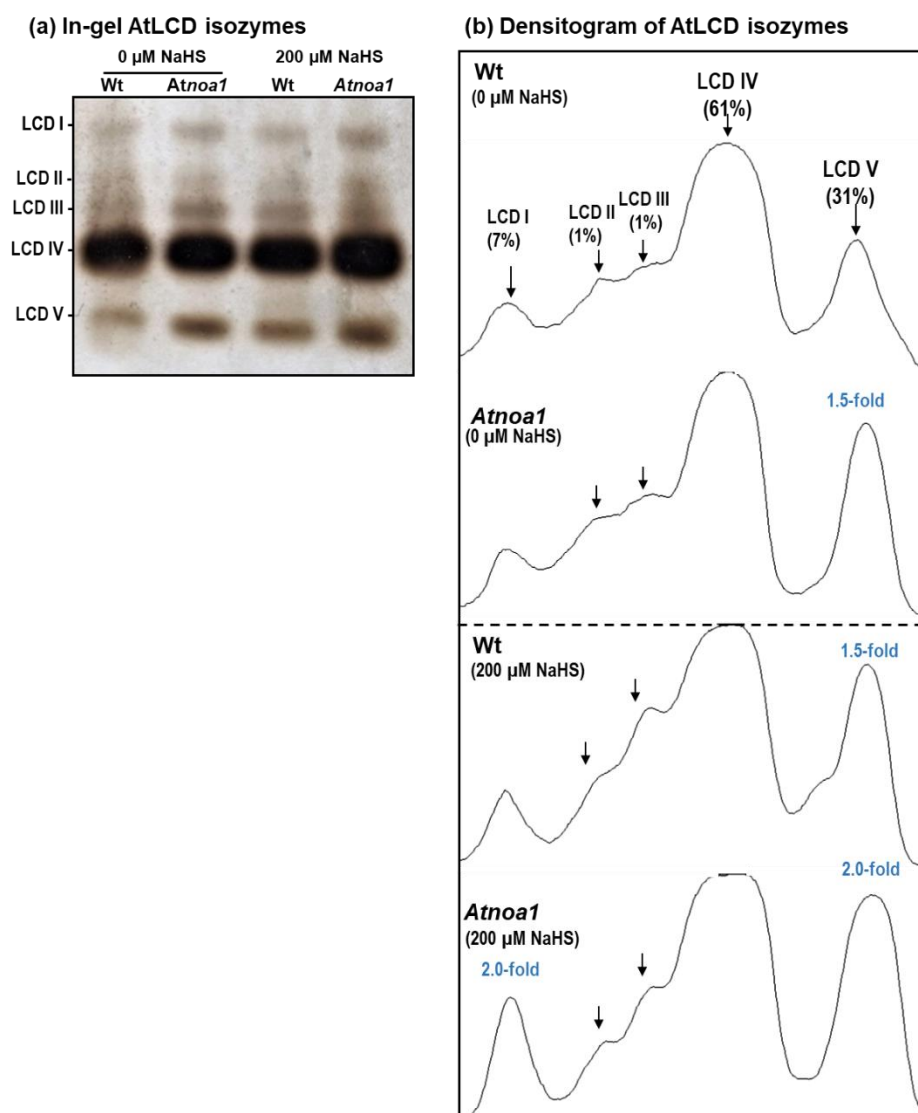
Figure 2b depicts the total relative activity quantification considering all the LCD isozymes present in the Wt seedling (control). Thus, AtLCD IV represented 61% of the total activity, followed by AtLCD V with 31%, and AtLCD I with 7%, whereas AtLCD II and AtLCD III represented less than 1% each. Among the different isozymes, AtLCD V is the isozyme that underwent the greatest change in comparison to the Wt seedlings not supplemented with NaHS (control). Thus, it is observed that the *Atnoa1* mutant increases 1.5-fold, and in seedlings supplemented with 200  $\mu\text{M}$  NaHS, the increase in Wt is also 1.5-fold while in *Atnoa1* it is 2-fold. On the other hand, the AtLCD I in *Atnoa1* seedlings grown in a medium with 200 NaHS increases 3-fold in comparison to the control Wt seedlings grown without NaHS and 2-fold to the Wt seedlings supplemented with 200  $\mu\text{M}$  NaHS.

There is currently growing evidence of the intimate interaction between  $\text{H}_2\text{S}$  and NO in different physiological processes such as root development, stomatal movement, and

plant cell death [37,38] as well as in response to environmental stress. For example, in maize (*Zea mays* L.) seedlings exposed to chromium stress, the exogenous application of NO and H<sub>2</sub>S protect plasma membrane integrity through upregulating H<sup>+</sup>-ATPase and glyoxalase pathways and through keeping optimal GSH content [39]. Furthermore, they also alleviate chromium toxicity through stimulating the antioxidant system and interfering with the Cr accumulation in roots, since it seems that these gasotransmitters stimulate the biosynthesis of phytochelatins [40]. In many cases, these molecules act via modulating the metabolism of reactive oxygen species (ROS) both in antioxidant systems and in ROS-generating enzymes [2,27,41,42]. Regulation is usually performed through posttranslational modifications (PTMs) such as nitration, S-nitrosation, and persulfidation [28–31,43]. The best-known examples are the antioxidant enzymes that scavenge H<sub>2</sub>O<sub>2</sub>, such as catalase and ascorbate peroxidase (APX). While catalase is inhibited via nitration, S-nitrosation, and persulfidation [7,27], APX is positively modulated via S-nitrosation and persulfidation but inhibited via nitration [44–46]. Recently, it has been shown that DES1 undergoes persulfidation, which regulates its activity [42], and also, the pepper cytosolic LCD is downregulated via nitration [10]. Taken together, all these data support a complex network between the metabolism of NO, H<sub>2</sub>S, and ROS, which might drive plant physiology under diverse conditions.



**Figure 1.** Effect of the H<sub>2</sub>S donor NaHS on Arabidopsis seedling growth. (a) Appearance of 16-day-old Arabidopsis Wt and *Atnoa1* seedlings grown in a horizontal position in MS medium supplemented and non-supplemented with 200  $\mu\text{M}$  NaHS. (b) Appearance of 16-day-old Arabidopsis Wt and *Atnoa1* seedlings grown in a vertical position in the same conditions of panel (a). (c) Arabidopsis seedling fresh weight (mg per seedling). (d) Primary root length per plant. Different letters indicate significant differences ( $p < 0.05$ ).



**Figure 2.** (a) In-gel isozyme profile of L-cysteine desulfhydrase (LCD) activity in 16-day-old *Arabidopsis thaliana* Wt and *Atnoa1* seedlings grown in MS medium supplemented and not supplemented with 200  $\mu$ M NaHS. Protein samples (40  $\mu$ g protein per lane) were separated using non-denaturing polyacrylamide gel electrophoresis on 8% acrylamide gels. LCD activity was detected using lead acetate staining (see Section 2 for details). (b) Densitometric analysis of LCD isozymes and their relative quantification (%) was made with the ImageJ 1.45 program. The numbers in parentheses indicate the fold increase in activity compared to the corresponding isozyme detected in Wt no supplemented with 200  $\mu$ M NaHS.

#### 4. Conclusions

The enzyme L-cysteine desulfhydrase is recognized as one of the main sources of  $H_2S$  in the cytosol of plant cells. To our knowledge, there are very few works that have analyzed the existence of different LCD isoenzymes in plants [8,10], so the existence of up to five LCD isoenzymes in *Arabidopsis* seedlings provides a piece of knowledge about this enzymatic system which can be very useful for future work. Furthermore, among the identified enzymes, LCD V seems to be positively modulated by low NO levels as well as by  $H_2S$  itself, demonstrating the close relationship between both molecules in the sulfur metabolic pathway. Very recently, it has been reported that the LCD activity of pepper fruits undergoes a double regulation by NO, since S-nitrosation has a positive effect on the activity whereas the nitration triggered its inhibition [10].



**Author Contributions:** J.D.L.O.-S. and M.A.M.-V. performed experiments. F.J.C. and J.M.P. designed the work and drove and coordinated the tasks. F.J.C. wrote the first draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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