Roles of a Cysteine Desulfhydrase LCD1 in Regulating Leaf Senescence in Tomato

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Abstract: Hydrogen sulfide (H2S), a novel gasotransmitter in both mammals and plants, plays important roles in plant development and stress responses. Leaf senescence represents the final stage of leaf development. The role of H2S-producing enzyme L-cysteine desulfhydrase in regulating tomato leaf senescence is still unknown. In the present study, the effect of an L-cysteine desulfhydrase LCD1 on leaf senescence in tomato was explored by physiological analysis. LCD1 mutation caused earlier leaf senescence, whereas LCD1 overexpression significantly delayed leaf senescence compared with the wild type in 10-week tomato seedlings. Moreover, LCD1 overexpression was found to delay dark-induced senescence in detached tomato leaves, and the lcd1 mutant showed accelerated senescence. An increasing trend of H2S production was observed in leaves during storage in darkness, while LCD1 deletion reduced H2S production and LCD1 overexpression produced more H2S compared with the wild-type control. Further investigations showed that LCD1 overexpression delayed dark-triggered chlorophyll degradation and reactive oxygen species (ROS) accumulation in detached tomato leaves, and the increase in the expression of chlorophyll degradation genes NON-YELLOW COLORING 1 (NYC1), PAO, PPH, SGR1, and senescence-associated genes (SAGs) during senescence was attenuated by LCD1 overexpression, whereas lcd1 mutants showed enhanced senescence-related parameters. Moreover, a correlation analysis indicated that chlorophyll content was negatively correlated with H2O2 and malondialdehyde (MDA) content, and also negatively correlated with the expression of chlorophyll degradation-related genes and SAGs. Therefore, these findings increase our understanding of the physiological functions of the H2S-generating enzyme LCD1 in regulating leaf senescence in tomato.

Keywords: tomato; hydrogen sulfide; cysteine desulfhydrase; leaf senescence; reactive oxygen species

1. Introduction

Leaf senescence represents the final stage of leaf development, which is a genetically controlled process [1]. As leaves age, the decomposition of chloroplast is initiated, accompanied by the catabolism of macromolecules including nucleic acids, proteins, and lipids. The decomposed nutrients then transfer to other developing organs, such as young leaves and growing fruit [2]. Chloroplasts constitute approximately 70% of the total proteins in green leaves and chlorophyll degradation causes the first visible signs of leaf senescence [3]. Thus, the coordinated degradation of chlorophyll is crucial for the breakdown of chloroplasts. Terrestrial plants utilize two types of chlorophyll species (i.e., chlorophyll a and chlorophyll b) for photosynthesis [4]. Chlorophyll b has to be converted to chlorophyll a before it can be processed into the degradation pathway and NON-YELLOW COLORING 1 (NYC1) catalyzes the reduction of chlorophyll b to 7-hydroxymethyl chlorophyll a [5]. Chlorophyll a
is further decomposed to pheophytin a by Mg-dechelatase NON-YELLOWINGs/STAY-GREENS (NYEs/SGRs) [6]. Pheophytin a is then hydrolyzed by the pheophytinase PPH to generate pheophorbide a which is further catalyzed by oxygenase PAO to produce a red chlorophyll catabolite (RCC) [7]. Moreover, hundreds of senescence-associated genes (SAGs), whose transcripts increase as leaves age [8,9], are also involved in the regulation of leaf senescence.

Plant hormones are major players influencing each stage of leaf senescence. For instance, ethylene, abscisic acid (ABA), and so on promote leaf senescence, while cytokinins (CKs), gibberellic acid (GA), and so on delay leaf senescence [10–12]. Furthermore, leaf senescence-linked events are often associated with the pronounced accumulation of reactive oxygen species (ROS) [13]. Among them, H$_2$O$_2$ is a well-defined inducer of leaf senescence. Recently, it was reported that transcription factor NAC075 delays leaf senescence by deterring ROS accumulation through directly binding the promoter of the antioxidant enzyme gene catalase 2 (CAT) in Arabidopsis [14]. Hydrogen sulfide (H$_2$S) is an important gasotransmitter in both animals and plants [15]. H$_2$S has not only been implicated in seed germination and root development, but can also enhance plant tolerance to various stresses such as heavy metals, drought, salinity, and cold by enhancing the antioxidant system [16–18]. In addition, H$_2$S can extend the shelf life of bananas, grapes, strawberries, tomatoes, and so on [19–22]. The underlining mechanism of H$_2$S in alleviating postharvest senescence may involve the activation of the antioxidant system, the inhibition of ethylene synthesis and the signaling pathway, etc.

H$_2$S is endogenously produced in a precise and regulated manner. Cysteine degradation by cysteine desulphydrases (CDes) to the formation of sulfide, ammonia, and pyruvate was believed to be an important source of H$_2$S [23]. Plant cells contain different CDes localized in the cytoplasm, plastids, and mitochondria [23]. DES1, an O-acetylserine(thiol)lyase homolog with L-cysteine desulphydrase activity, regulates cysteine homeostasis in Arabidopsis [24]. Recently it was reported that the des1 mutant was more sensitive to drought stress and displayed accelerated leaf senescence, while the leaves of OE-DES1 contained adequate chlorophyll levels accompanied by significantly increased drought resistance, suggesting the role of DES1 in regulating leaf senescence [25]. In our previous work, a L-cysteine desulphydrase named LCD1 was found to localize in the nucleus by a potential nuclear localization signal, and an lcd1 mutation caused accelerated fruit ripening compared with the wild type [26]. However, whether and how LCD1 participates in leaf senescence or dark-induced senescence are still unknown. In the present study, we focused on the role of endogenous H$_2$S-producing enzyme LCD1 during leaf senescence. Moreover, a correlation analysis was applied to investigate the potential relations between H$_2$S, ROS, and chlorophyll breakdown.

2. Results

2.1. Role of LCD1 in Regulating Tomato Leaf Senescence

To elucidate the possible involvement of LCD1 in regulating leaf senescence, two previously reported tomato lines, lcd1-7 and lcd1-9, with mutations near the PAM sequence were used as lcd1 mutants, and two lines (hereafter called LCD1-oe and LCD1-oe1) with an increased expression of LCD1 under the control of the CaMV 35S promoter were also used. The overexpression efficacy of five LCD1 overexpression lines was verified by RT-qPCR as shown in Figure S1 and the lines LCD1-oe and LCD1-oe1, which showed a higher LCD1 expression, were used in the present study. To confirm the role of LCD1 in catalyzing H$_2$S production, the H$_2$S producing rates were determined in leaves of lcd1 and LCD1-oe lines. The data in Figure 1B suggest that lcd1 leaves had a lower H$_2$S producing rate compared with the wild type, while LCD1 overexpression induced a significantly higher level of the H$_2$S producing rate. Besides, H$_2$S production was also evaluated by lead acetate H$_2$S detection strips, and the results showed that lcd1 leaves produced less H$_2$S and LCD1 overexpression produced more H$_2$S (Figure 1C). After 10 weeks of growth, the LCD1
mutation caused earlier leaf senescence compared with the wild type. In contrast, LCD1 overexpression significantly delayed leaf senescence (Figure 1D).

Figure 1. Phenotypic characterization of lcd1 mutants and LCD-oe (over-expression) tomatoes. (A) Phenotype of 10-week-old wild-type (WT), lcd1-7, lcd1-9, LCD1-oe, and LCD1-oe1 plants. (B) H$_2$S producing rate in the mature leaves from wild type, lcd1, and LCD1-oe lines of 10 weeks growth. (C) H$_2$S production from the mature leaves of 10-week-old wild type, lcd1, and LCD1-oe lines detected by lead acetate H$_2$S detection strips (Sigma-Aldrich). (D) The leaves of different tomato lines in (A) were detached and photographed. Data are means of three biological replicates ± standard deviation (SD). The symbols * and ** stand for \( p < 0.05 \) and \( p < 0.01 \) as determined by the Student's t-test, respectively.

2.2. LCD1 Participates in Dark-Induced Senescence

Leaf senescence is an important phenomenon in the growth and development of plant leaves, and darkness is widely used as a tool to induce senescence in detached leaves. To study the role of LCD1 in dark-induced senescence, the mature leaves without visible senescence of 6-week-old wild type, lcd1 mutant, and LCD1-oe were stored in darkness for 8 days. As shown in Figure 2A, lcd1 showed the obvious syndrome of the leaf yellowing phenotype after 5 and 8 days in dark stress, whereas LCD1 overexpression still maintained the green phenotype. To study the kinetics of tomato leaf H$_2$S production during senescence, H$_2$S production in the leaves at different developmental stages—young leaves (YL), mature leaves (ML), senescent leaves (SL), and late senescent leaves (LS)—was evaluated and the H$_2$S detection strips showed browning with senescence, suggesting
H$_2$S production increased during leaf senescence (Figure S2). Moreover, H$_2$S production in leaves of wild-type (WT), lcd1, and LCD1-oe tomatoes were also determined during dark-induced senescence (Figure 2B). Generally, an increasing trend of H$_2$S production was observed in all samples during storage, while LCD1-oe leaves showed a higher H$_2$S production compared with the wild-type control. In addition, the lcd1 mutant produced a significantly lower level of H$_2$S compared with the wild type. Therefore, it can be concluded that LCD1 deletion caused a lower H$_2$S release and the attenuated H$_2$S release may cause an accelerated senescence in the lcd1 mutant. Overall, the present results indicate that LCD1 plays a negative role in leaf senescence in both developmental and dark-induced senescence.

![Figure 2](image-url)

**Figure 2.** (A) Dark-induced senescence symptoms in detached leaves of 6-week-old wild-type (WT), lcd1, and LCD1-oe tomatoes for up to 8 days. (B) H$_2$S producing rate in detached leaves of 6-week-old wild-type (WT), lcd1, and LCD1-oe tomatoes stored in darkness for up to 8 days. Different letters above the columns stand for significant difference between two values ($p < 0.05$) at the same time point.
2.3. Effect of LCD1 on Dark-Triggered Chlorophyll Degradation and Reactive Oxygen Species Accumulation in Detached Tomato Leaves

Chlorophyll degradation is one of the most significant changes during leaf senescence; thus, chlorophyll contents were determined in wild-type, *lcd1* mutant, and *LCD1-oe* leaves during dark-induced senescence. As shown in Figure 3A, the content of total chlorophyll in the wild type decreased gradually during storage in darkness for 8 days, whereas the content of chlorophyll in the *lcd1* mutant showed an obvious decrease on days 5 and 8 under darkness, and the value on day 8 was about 32.6% of the initial value on day 0. In contrast, *LCD1* overexpression maintained a relatively higher chlorophyll content compared with the wild type and the *lcd1* mutant on days 5 and 8 under darkness. After 8 days in darkness, the chlorophyll content in *LCD1* overexpression decreased to 84.6% of the initial value, suggesting the role of *LCD1* in delaying dark-induced senescence. As shown in Figure 3B, there were minor changes in the chlorophyll content between different groups during storage. Moreover, only a slight decrease in chlorophyll *a* was observed during dark-induced senescence, except for a significant decline found in *lcd1* on day 8. Figure 3C shows the change pattern of chlorophyll *b* content in wild type, *lcd1* mutant, and *LCD1-oe* during dark-induced senescence. With the increase of storage days, the chlorophyll *b* content decreased in each group. At day 0, chlorophyll *b* content in *lcd1* leaves was about 53.4% of that in the *LCD1-oe* group, and decreased to 21.3% on day 8 compared with the value on day 0. Furthermore, the ratio of chlorophyll *a/b* was also evaluated in dark-stored detached leaves of wild-type, *lcd1*, and *LCD1-oe* tomatoes for 0, 2, 5, and 8 days. As shown in Figure 3D, the ratio of chlorophyll *a/b* in WT and *lcd1* mutant leaves increased during storage, while *LCD1* mutation caused the highest ratio compared with other groups. In contrast, the ratio of chlorophyll *a/b* in *LCD1* overexpression almost remained unchanged. The above results indicate that the *lcd1* mutation accelerated dark-induced leaf senescence and *LCD1* overexpression delayed leaf yellowing and chlorophyll degradation.

![Figure 3](image-url)

*Figure 3.* Changes in the contents of (A) total chlorophyll, (B) chlorophyll *a*, (C) chlorophyll *b*, and (D) the ratio of chlorophyll *a/b* in dark-stored detached leaves of 6-week-old wild-type (WT), *lcd1*, and *LCD1-oe* tomatoes for 0, 2, 5, and 8 days. Data are means of three biological replicates ± standard deviation (SD). Different letters above the columns stand for significant difference between two values (*p* < 0.05) at the same time point.
Leaf senescence is usually associated with the excessive accumulation of ROS; therefore, the levels of H$_2$O$_2$ and malondialdehyde (MDA) were monitored in wild-type, lcd1 mutant, and LCD1-oe leaves during dark-induced senescence. As shown in Figure 4A, there was no significant difference in H$_2$O$_2$ content between the different groups on day 0. During the dark-induced senescence, the H$_2$O$_2$ content in each group showed an increasing trend, of which the lcd1 group increased the fastest, followed by the wild-type and LCD1-oe group. However, H$_2$O$_2$ content in the LCD1-oe group increased slowly compared with other groups. As shown in Figure 4B, the change of MDA content among the groups also showed a similar trend to H$_2$O$_2$. The content of MDA in lcd1 leaves during storage was the highest compared with other groups, and the lowest MDA content was observed in LCD1-oe leaves. Therefore, it can be concluded that the overexpression of LCD1 could reduce the accumulation of ROS and MDA in leaves under dark-triggered senescence.

2.4. Effect of LCD1 on the Expressions of Genes Related to Chlorophyll Degradation in Detached Tomato Leaves

Chlorophyll degradation marks the senescence stage of leaves. In order to explore the molecular mechanism of the differences in chlorophyll content of lcd1, LCD1-oe, and wild-type leaves during dark-induced senescence, the expression levels of key genes NYC1, PAO, PPH, and SGR1 in the chlorophyll degradation pathway were analyzed by RT-qPCR. The present data showed NYC1 was transcriptionally induced during dark-induced senescence in all groups (Figure 5A). In accordance with the early senescence phenotype of the lcd1 mutant and late senescence in LCD1-oe leaves, the expression of NYC1 was significantly higher in the lcd1 mutant and was less expressed in LCD1-oe leaves during dark storage. Three other genes—PAO (Figure 5B), PPH (Figure 5C), and SGR1 (Figure 5D)—were also analyzed at the transcriptional level in wild-type, lcd1 mutant, and LCD1-oe leaves during dark-induced senescence and similar changes to that of the NYC1 expression were observed. The higher expression of PAO, PPH, and SGR1 in lcd1 and lower expression in LCD1-oe again supported the role of LCD1 in delaying leaf senescence. The results suggest that LCD1 may delay the chlorophyll degradation by down-regulating the transcription of key genes in the chlorophyll degradation pathway.

2.5. Effect of LCD1 on the Expressions of SAGs in Detached Tomato Leaves

To further analyze the senescence-alleviating role of LCD1, we conducted an RT-qPCR analysis to evaluate the expression patterns of senescence-associated genes (SAGs) in lcd1, LCD1-oe, and wild-type leaves during dark-induced senescence. As shown in Figure 6, SAG12, SAG15, and SAG113 were transcriptionally induced during dark-induced senescence. Compared with SAG15 and SAG113, SAG12 showed more fold changes during leaf senescence, which was 109.6 times in the wild type on day 8 compared with day 0.
In accordance with the early senescence phenotype of the *lcd1* mutant and late senescence in *LCD1-oe* leaves, the expression of the three SAGs was significantly higher in the *lcd1* mutant and was less expressed in *LCD1-oe* leaves during dark storage, especially on day 8.

2.5. Effect of LCD1 on the expressions of SAGs in Detached Tomato Leaves

To further analyze the senescence-alleviating role of LCD1, we conducted an RT-qPCR analysis to evaluate the expression patterns of senescence-associated genes (SAGs) in *lcd1*, *LCD1-oe*, and wild-type leaves during dark-induced senescence. As shown in Figure 6,

![Figure 6A](image)

SAG12, SAG15, and SAG113 were transcriptionally induced during dark-induced senescence. Compared with SAG15 and SAG113, SAG12 showed more fold changes during leaf senescence, which was 109.6 times in the wild type on day 8 compared with day 0 (Figure 6A). In accordance with the early senescence phenotype of the *lcd1* mutant and late senescence in *LCD1-oe* leaves, the expression of the three SAGs was significantly higher in the *lcd1* mutant and was less expressed in *LCD1-oe* leaves during dark storage, especially on day 8.

Figure 5. Changes in the gene expressions of chlorophyll degradation related genes: (A) *NYC1*, (B) *PAO*, (C) *PPH*, and (D) *SGR1* in detached leaves of 6-week-old wild-type (WT), *lcd1*, and *LCD1-oe* tomatoes stored in darkness for 0, 2, 5, and 8 days. Data are means of three biological replicates ± standard deviation (SD). Different letters above the columns stand for significant difference between two values (*p* < 0.05) at the same time point.

2.6. Correlation Analysis of Different Leaf Physiological Indexes and Senescence-Related Gene Expression

The correlation among total chlorophyll, chlorophyll a, chlorophyll b, chlorophyll a/b, H$_2$O$_2$, and MDA content and the gene expression of *NYC1*, *PAO*, *PPH*, *SGR1*, *SAG12*, *SAG15*, and *SAG113* was analyzed to investigate the potential relations among the indexes. As shown in Figure 7, chlorophyll content was negatively correlated with H$_2$O$_2$, MDA content, and with the expression of chlorophyll degradation-related genes *NYC1*, *PAO*, *PPH*, and *SGR1* and senescence-related genes *SAG12*, *SAG15*, and *SAG113*. Moreover, total chlorophyll and chlorophyll b showed a higher negative correlation to ROS content and senescence-related gene expression in comparison to chlorophyll a. The contents of H$_2$O$_2$ and MDA were positively correlated with the expression levels of *NYC1*, *PAO*, *PPH*, *SGR1*, *SAG12*, *SAG15*, and *SAG113*. Among them, the expressions of *PPH* and *NYC1* were highly positively correlated (*r* = 0.966), and the total chlorophyll content was highly negatively correlated with the H$_2$O$_2$ content (*r* = −0.882). Moreover, chlorophyll a/b also showed a significant positive correlation with H$_2$O$_2$ and MDA content. Through these analyses, the positive correlation between H$_2$O$_2$/MDA content and senescence-related gene expressions indicates that they may act synergistically to accelerate the senescence process of leaves.
Figure 6. Changes in the gene expressions of senescence-related genes: (A) SAG12, (B) SAG15, and (C) SAG113 in detached leaves of 6-week-old wild-type (WT), lcad1, and LCD1-oe tomatoes stored in darkness for 0, 2, 5, and 8 days. Data are means of three biological replicates ± standard deviation (SD). Different letters above the columns stand for significant difference between two values (p < 0.05) at the same time point.

2.7. Principal Component Analysis of Different Leaf Physiological Indexes and Senescence-Related Gene Expression

The principal component analysis (PCA) was performed based on the data of chlorophyll, H$_2$O$_2$, and MDA content and the expressions of chlorophyll degradation-related genes and SAGs. As shown in Figure 8, PC1 and 2 contributed to 81.1% and 10.4% of the variability of the data, respectively. It can be seen that lcad1 2 d and lcad1 5 d clustered together, and lcad1 8 d was distributed separately from other groups. The variety showing the highest positive load value in the direction of PC1 was LCD1-oe 0 d and the variety that showed the lowest load value in the direction of PC2 was lcad1 5 d. Therefore, it could be concluded that a decrease in the endogenous H$_2$S content in the lcad1 mutant caused significant changes during dark-induced senescence compared with other groups.
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leaves of 6-week-old wild-type, 

Figure 7. Correlation analysis among the parameters of chlorophyll, chlorophyll a, chlorophyll b, chlorophyll a/b, H2O2, malondialdehyde (MDA), and gene expressions of NYC1, PAO, PPH, SGR1, SAG12, SAG15, and SAG113 in detached leaves of 6-week-old wild-type, lcd1, and LCD1-oe tomatoes stored in darkness for 0, 2, 5, and 8 days.

Figure 8. Principal component analysis based on the parameters of chlorophyll, chlorophyll a, chlorophyll b, H2O2, malondialdehyde (MDA), and gene expressions of NYC1, PAO, PPH, SGR1, SAG12, SAG15, and SAG113 in detached leaves of 6-week-old wild-type, lcd1, and LCD1-oe tomatoes stored in darkness for 0, 2, 5, and 8 days.
3. Discussion

H₂S, a multifunctional signaling molecule in plants, was found to alleviate the postharvest senescence of broccoli, banana, grape, and tomato [19–22,27]. Furthermore, H₂S is implicated in suppressing the chlorophyll degradation of detached leaves of Arabidopsis by regulating a dark-dependent reaction [13]. DES1, an O-acetylserine(thiol)lyase homolog with L-cysteine desulphydrase activity, regulates cysteine homeostasis in Arabidopsis [24]. Recently it was reported that a des1 mutant displayed accelerated leaf senescence, while the leaves of OE-DES1 contained adequate chlorophyll levels, suggesting the role of DES1 in regulating leaf senescence [25]. In our previous work, mutation of an L-Cys desulphydrase named LCD1 caused accelerated fruit ripening compared with the wild type [26]. In the present study, to elucidate the possible involvement of LCD1 in regulating leaf senescence, two previously reported tomato lines, lcd1-7 and lcd1-9, with mutations near the PAM sequence were used as lcd1 mutants, and two lines (hereafter called LCD1-oe and LCD1-oe1) with an increased expression of LCD1 under the control of the CaMV 35S promoter were also used. To confirm the role of LCD1 in catalyzing H₂S production, the H₂S producing rates were determined in leaves of the lcd1 and LCD1-oe1 lines; lcd1 leaves showed a lower H₂S producing rate compared with the wild type, while LCD1 overexpression induced a significantly higher level of the H₂S producing rate, suggesting the efficiency of LCD1 in producing H₂S.

Leaf senescence is a highly programmed degeneration process during the final stage of leaf development. To study the role of H₂S-producing enzyme LCD1 in regulating natural leaf senescence, we compared 10-week old tomatoes of lcd1 mutants, and the two lines of LCD1-oe with the enhanced expression of LCD1, and found that lcd1 developed more senescence syndrome while LCD1 overexpression maintained more functional leaves.

Prolonged darkness is often used to initiate rapid and synchronous senescence in detached leaves [13]. The roles of LCD1 on dark-induced senescence were evaluated in tomato leaves. The lcd1 mutant leaves showed an obvious syndrome of the leaf yellowing phenotype after 5 and 8 days in dark stress, whereas LCD1 overexpression still maintained the green phenotype (Figure 2A). All this evidence suggests the role of H₂S in alleviating the dark-induced senescence of detached leaves. In accordance with the phenotype of accelerated senescence in the lcd1 mutant and delayed senescence in LCD1-oe leaves, chlorophyll decreased significantly in lcd1, but the decrease was attenuated in LCD1-oe leaves. Interestingly, we found that chlorophyll b may contribute more to the decrease in total chlorophyll compared with chlorophyll a (Figure 3). By analyzing the ratio of chlorophyll a/b during leaf storage, it was found that the ratio in LCD1 deletion increased significantly, implying that more chlorophyll b was decomposed to chlorophyll a in the lcd1 mutant. Moreover, the ratio in LCD1 overexpression almost remained unchanged, suggesting the significant impact of H₂S content on the ratio of chlorophyll a/b. Then, the expression levels of key genes NYCI, PAO, PPH, and SGR1 in the chlorophyll degradation pathway were analyzed by RT-qPCR. It was found that NYCI, PAO, PPH, and SGR1 transcript abundance increased during darkness in all groups, especially in lcd1 mutant leaves, whereas this response was significantly inhibited by LCD1 overexpression (Figure 5). Senescence-associated gene (SAG) 12, 15, and 113 are widely used as molecular markers for leaf senescence [28] and their transcriptions were also analyzed in detached tomato leaves. In agreement with the phenotype, significant increases in SAGs expression were observed in all groups, especially in lcd1 mutant leaves, and the increase was greatly attenuated by LCD1 overexpression (Figure 6). The results suggest that LCD1 may delay the chlorophyll degradation by down-regulating the transcription of key genes in the chlorophyll degradation pathway and SAGs.

Leaf senescence is often associated with the pronounced accumulation of ROS [13]. Recently, it was reported that transcription factor NAC075 delays leaf senescence by deterring ROS accumulation through directly activating the expression of the antioxidant enzyme gene catalase 2 (CAT) in Arabidopsis [14]. To unveil the relations between H₂S and ROS metabolism in leaf senescence, H₂O₂ and MDA contents were determined during leaf
senescence in darkness. During the dark-induced senescence, the H$_2$O$_2$ and MDA content in each group showed an increasing trend, but the overexpression of LCD1 could reduce the accumulation of ROS and MDA in leaves under dark-triggered senescence. Furthermore, the correlation analysis indicated that the ROS and MDA content showed a higher negative correlation to total chlorophyll and chlorophyll b in comparison to chlorophyll a. The contents of H$_2$O$_2$ and MDA were positively correlated with the expression levels of NYC1, PAO, PPH, SGR1, SAG12, SAG15, and SAG113. The positive correlation between H$_2$O$_2$ and MDA content and senescence-related genes indicates that they may act synergistically to accelerate the senescence process of leaves, whereas LCD1 overexpression delayed leaf senescence by inhibiting ROS accumulation and senescence-related gene expressions. Interestingly, increasing H$_2$S production was observed in natural senescence leaves (Figure S2).

We hypothesize that endogenous H$_2$S production was activated to counteract the effect of increasing ROS in senescence leaves. In our previous reports, exogenous H$_2$S fumigation delayed the postharvest senescence of broccoli in a dose-dependent manner and H$_2$S maintained higher levels of chlorophyll, carotenoids, anthocyanin, and ascorbate, suggesting the role of H$_2$S in delaying the postharvest senescence of broccoli [21]. Moreover, H$_2$S treatment effectively alleviates ethylene-induced banana peel yellowing and fruit softening [22]. The above results suggest that H$_2$S is an effective signal in delaying the postharvest senescence of fruits and vegetables. In the present research, an increasing trend of H$_2$S production was observed during leaf senescence, suggesting that H$_2$S generation may be activated in response to leaf senescence as ROS metabolites (H$_2$O$_2$ and MDA) accumulate during dark-induced senescence. Leaf senescence, once initiated, cannot be stopped. Though more H$_2$S is produced during leaf senescence, leaves still undergo senescence during storage in darkness. Moreover, compared with the early senescence phenotype of the lcd1 mutant, LCD1 overexpression induced more H$_2$S production and showed a delayed leaf senescence, clearly suggesting the role of H$_2$S in delaying leaf senescence. In all, the data suggest that senescent leaves produced more H$_2$S, but reduction in H$_2$S production of the lcd1 mutant caused early senescence in both natural and dark-induced senescence. Moreover, the principal component analysis (PCA) in Figure 8 shows that lcd1 2 d and lcd1 5 d clustered together, and lcd1 8 d was distributed separately from other groups, suggesting that the decreased endogenous H$_2$S content in lcd1 caused significant changes during dark-induced senescence compared with other groups.

4. Conclusions

In the present study, the role of a cysteine desulphydrase LCD1 in regulating leaf senescence in tomato was explored. The LCD1 mutation caused an earlier leaf senescence, whereas LCD1 overexpression significantly delayed leaf senescence compared with the wild type in 10-week tomato seedlings. Furthermore, LCD1 was found to play a negative role in dark-induced senescence in detached tomato leaves. Further investigations showed that LCD1 delayed dark-triggered chlorophyll degradation and ROS accumulation in detached tomato leaves, and the increase in chlorophyll degradation and SAGs related gene expression was attenuated by LCD1 overexpression. Moreover, a correlation analysis indicated that chlorophyll content was negatively correlated with H$_2$O$_2$ and MDA content, and also negatively correlated with the expression of chlorophyll degradation-related genes NYC1, PAO, PPH, and SGR1 and senescence-related genes SAG12, SAG15, and SAG113. Therefore, these findings increase our understanding of the physiological functions of the H$_2$S-generating enzyme LCD1 in regulating leaf senescence in tomato.

5. Materials and Methods

5.1. Plant Material and Growth Conditions

*Solanum lycopersicum* cv. “Micro-Tom” was used as the control in this study. The mutants *lcd1*–7, which contained a T residue inserted near the PAM sequence, and *lcd1*–9, which had a deletion of G near the PAM as previously reported were used as *lcd1* mutants [26]. The coding sequence of tomato cysteine desulphydrase LCD1 (LOC101258894) was ob-
tained from NCBI (http://www.ncbi.nlm.nih.gov/, accessed on 11 September 2018) and the primers including the restriction enzyme sites (LCD1-F: CGCGGATCC TAATCCTAAAT GGAACCGGC; LCD1-R: CCGCTCGAG TTCTGAGTGAAGCATTTAC, the underlines stand for BamHI and XhoI sites, respectively) were used to amplified the coding sequence of LCD1. Then, the coding sequence of LCD1 was inserted into the pBI121 vector at the sites of BamHI and XhoI and transformed into wild-type tomato by Agrobacterium tumefaciens, which contained the recombinant LCD1-pBI121. The efficiency of LCD1 overexpression was verified by RT-qPCR. The seeds of tomatoes were grown in a nutrient soil:vermiculite (3:1, v/v) in growth pots 10 cm in diameter in an environment-controlled growth room (23 ± 2 °C; 50–70% relative humidity, RH) under 16 h light/8 h dark and 250 mol/m²/s light.

5.2. Determination of H₂S Producing Rate and H₂S Detection in Tomato Leaves

The H₂S producing rate was measured as described previously [29]. Tomato leaves at 1 g were ground to a fine powder in liquid nitrogen and homogenized in 9 mL of 20 mM Tris-HCl, pH 8.0. After centrifugation at 12,000 × g for 15 min, the protein content of the supernatant was sampled and the H₂S producing rate was detected by monitoring the release of H₂S from L-cysteine in the presence of dithiothreitol (DTT). The assay was performed in a total volume of 1 mL comprising 0.8 mM L-cysteine, 2.5 mM DTT, 100 mM Tris-HCl, pH 8.0, and 100 μL of protein solution. The reaction was incubated for 15 min at 37 °C, and terminated by adding 100 μL of 30 mM FeCl₃ dissolved in 1.2 N HCl and 100 μL of 20 mM N,N-dimethyl-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of methylene blue was determined at 670 nm.

The end-point detection of H₂S production from tomato leaves by lead acetate strips (cat. number WHA2602501A, Sigma, Darmstadt, Germany) were performed as previously described [30]. One gram of fresh tomato leaves was ground to a fine powder in liquid nitrogen and then homogenized in 10 mL of Phosphate Buffered Saline (pH 6.8) supplemented with 10 mM L-cysteine and 10 μM pyridoxal-5′-phosphate (PLP), and then the mixture was placed in petri dishes. The H₂S detection strips were attached to the inner part of the upper lid of the petri dishes and incubated at 37 °C for 2–5 h until lead sulfide darkening of the strip occurred.

5.3. Dark Treatment of Tomato Leaves

For dark-induced leaf senescence experiments, detached mature leaves from 6-week-old wild-type, lcd1, and LCD1-oe transgenic plants were placed on filter papers which were moistened by 2 mL of sterile water in petri dishes with a 9 cm diameter and the adaxial side facing upwards. The petri dishes which contained 5–6 detached leaves were kept in darkness at 23 °C for 8 days. The leaves were sampled and rapidly frozen in liquid nitrogen and stored at −80 °C until analysis.

5.4. Determination of Chlorophyll Content

Tomato leaves at 2 ± 0.01 g were homogenized in liquid nitrogen and subsequently extracted ethanol and 80% (v/v) acetone solution in a ratio of 1:1 (v/v) according to the method in [31]. The absorbance of the supernatant was read at 663 and 645 nm. The experiments were repeated three biological times, and the chlorophyll levels were expressed as mg/g fresh weight (FW).

5.5. Determination of H₂O₂ and Malondialdehyde (MDA) Content in Tomato Leaves

The contents of H₂O₂ and malondialdehyde (MDA) were assayed as described by Ge et al. [22] and Hu et al. [27]. For the determination of H₂O₂ content, 2.0 ± 0.01 g of tomato leaves were homogenized in 3 mL of precooled acetone, and centrifuged at 12,000 × g for 30 min. The content of H₂O₂ was measured by determining the absorbance value at 508 nm. For the determination of MDA content, 2.0 ± 0.01 g of tomato leaf powder was homogenized with 5% trichloroacetic acid, and the supernatant was obtained by
centrifugation at 12,000× g for 30 min. The absorbance values were measured at 600 nm, 532 nm, and 450 nm, respectively. The experiments were repeated three times, and the contents of H$_2$O$_2$ and MDA were expressed as µmol/g fresh weight (FW).

5.6. Gene Expression Analysis

Total RNA from 0.1 g of tomato leaves was extracted using an Extraction Kit (Tiangen, Beijing, China) and cDNA was synthesized by a reverse transcription kit (PrimeScript RT Master Mix; Takara, Kyoto, Japan). Then the cDNA products were used for gene expression analysis by quantitative polymerase chain reaction (qPCR) performed using a Bio-Rad IQ5 (Hercules, CA, USA). The specific primers used for qPCR were designed based on the coding sequence of the genes as shown in the SGN database (https://solgenomics.net/, accessed on 12 April 2021). *Tubulin* gene expression in control tomato plants was used for the normalization of the data. The experiments were repeated in three technical replicates.

5.7. Data Analysis

The statistical analysis of data was based on Student’s t-tests. Significant differences were evaluated using multiple pair wise t-test comparisons at p < 0.05. The correlation among the contents of chlorophyll, H$_2$O$_2$, MDA, and the expression of chlorophyll degradation related genes and SAGs in tomato leaves and the principal component analysis (PCA) of the data above were analyzed by the tools on the OmicShare platform (https://www.omicshare.com/, accessed on 20 November 2021).

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