Ethylene signaling plays an important role in UV-B-induced ascorbic acid accumulation in Cucumis Sativus leaves

Peng Liu¹, Qiang Li¹, Heng Wang¹, Tao Lu¹, Yang Li¹, Xiaolei Sui², Hongjun Yu¹, and Weijie Jiang¹

¹Chinese Academy of Agricultural Sciences Institute of Vegetables and Flowers
²China Agricultural University College of Horticulture

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Abstract

UV-B regulates the metabolism of many important substances, such as hormones, ascorbic acid (AsA) and secondary metabolites. However, the mechanism by which UV-B regulates AsA metabolism, especially signal transduction, is largely unclear. Here, we report that UV-B promotes the accumulation of AsA and the production of ethylene in cucumber seedlings. However, when the ethylene signal is blocked, UV-B no longer promotes the accumulation of AsA in cucumber leaves. This indicates that the ethylene signal is a key factor enabling UV-B to regulate AsA levels. The q-PCR results show that UV-B induces the expression of the \textit{CsACO} and \textit{CsACS} genes, which, in turn, promotes the biosynthesis of ethylene; thus, ethylene production is promoted and the ethylene signal is activated. Moreover, the expression of the ethylene response factor \textit{CsERF39} is up-regulated through a series of UV-B signal transduction. Knockout of \textit{CsERF39} and \textit{CsGLDH} results in a reduced AsA pool in cucumber leaves. Furthermore, we identified \textit{CsGLDH} as a direct target for \textit{CsERF39} to regulate AsA biosynthesis, by q-PCR analysis, molecular experiments and genetic data. Therefore, \textit{CsACS}, \textit{CsACO}, \textit{CsERF39} and \textit{CsGLDH} are involved in the regulation of AsA biosynthesis by UV-B through the ethylene signal transcription cascade.
Tao Lu, E-mail: lutao@caas.cn;
Yang Li, E-mail: liyang05@caas.cn

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- Here, we report that UV-B promotes the accumulation of AsA and the production of ethylene in cucumber seedlings. However, when the ethylene signal is blocked, UV-B no longer promotes the accumulation of AsA in cucumber leaves. This indicates that the ethylene signal is a key factor enabling UV-B to regulate AsA levels.
- The q-PCR results show that UV-B induces the expression of the CsACO and CsACS genes, which, in turn, promotes the biosynthesis of ethylene; thus, ethylene production is promoted and the ethylene signal is activated. Moreover, the expression of the ethylene response factor CsERF39 is up-regulated through a series of UV-B signal transduction.
- Knockout of CsERF39 and CsGLDH results in a reduced AsA pool in cucumber leaves. Furthermore, we identified CsGLDH as a direct target for CsERF39 to regulate AsA biosynthesis, by q-PCR analysis, molecular experiments and genetic data. Therefore, CsACS, CsACO, CsERF39 and CsGLDH are involved in the regulation of AsA biosynthesis by UV-B through the ethylene signal transcription cascade.

Keywords: UV-B, CsERF39, CsGLDH, ascorbic acid biosynthesis

1 INTRODUCTION

UV-B, a key component of light affects plant growth and development (Fina et al., 2017; Quan et al., 2018; Yang et al., 2020). In recent years, UV-B radiation has been considered to a lesser extent as a stress factor for plants than in the past, and this is due to a major step forward in our understanding of UV-B regulation in plant cells after the UV-B photoreceptor UVRESISTANCE LOCUS8 (UVR8) was identified in the model plant Arabidopsis thaliana (Yuan et al., 2000; Rizzini et al., 2011). Brown et al. showed that UVR8 can sense specific low-fluence UV-B responses (Brown et al., 2008). In addition to the UVR8-mediated specific low-fluence UV-B pathway, there is a nonspecific high-fluence UV-B mechanism that overlaps with other signal transduction pathways, including those of salicylic acid, jasmonic acid, ethylene and reactive oxygen species (ROS) (Jenkins, 2009). However, the two signal pathways are not mutually exclusive and have an overlap in the UV-B dose (Kilian, 2007; Jenkins, 2009). Therefore, it is possible that two or more signaling pathways can regulate the same UV-B radiation response.

Recent studies have shown that UV-B could promote the expression of ethylene synthesis genes and ethylene accumulation in plants (Nara et al., 2002; He et al., 2011; Pan et al., 2014; Mannucci et al., 2020). In particular, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and the catalysis of ACC oxidase (ACO) are important rate-limiting enzymes for ethylene biosynthesis (Chae et al., 2005; Zabala et al., 2020). The ethylene receptor (ETR) senses ethylene, and, through a series of signal transduction steps, the downstream gene expression is finally regulated by the ethylene response factor (ERF) and combined with the GCC-box, DRE, CE1 and as-1 cis-elements of the promoter region of downstream genes (Wu et al., 2008; Lee et al., 2015). Ethylene regulates many life processes and the physiological metabolism of plants, such as root formation, fruit maturation and ascorbic acid metabolism (Qin et al., 2018; Yu et al., 2019; Li et al., 2020). In Arabidopsis, it has been recently demonstrated that ethylene and ABA antagonistically orchestrate AsA biosynthesis gene VTC2, via the ETHYLENE-INSENSITIVE3 (EIN3) and ABA INSENSITIVE4 (ABI4) transcriptional cascade (Li et al., 2020). Furthermore, in tobacco seedlings overexpressing a tomato ERF protein, namely, JERF3, an ethylene-induced gene, as well as APX1 and APX2, were up-regulated three to eight times compared to wild-type plants (Wuet et al., 2008). This needs further investigation considering whether the change in ethylene content caused by UV-B will affect the AsA content.
AsA is very important for all living eukaryotic cells. AsA is an important antioxidant molecule, acting to scavenge reactive oxygen species that are generated during photosynthesis, etc. (Chen et al., 2004; Li et al., 2010; Steelheart et al., 2020). In addition, AsA content and its redox are involved in photoprotection, the cell cycle and cell expansion (Chen et al., 2006). Meanwhile, AsA is also an important vegetable quality indicator for humans, because humans need to obtain AsA from plants. Several pathways for the biosynthesis of AsA have been identified, including the D-mannose/L-galactose (D-Man/L-Gal), the D-galacturonate, the D-glucosone and the myo-inositol pathways (Wheeler et al., 1998; Davey et al., 1999; Smirnoff et al., 2001; Lorence et al., 2004). However, in plants, the D-Man/L-Gal pathway is the most important, and the genes underlying all the biosynthetic steps have been identified (Smirnoff et al., 2001). Galactono-1, 4-lactone dehydrogenase (GLDH) catalyzes the oxidation of L-GalL to AsA, the final step in the D-Man/L-Gal pathway, and the changes in AsA pool size are closely related to the activity of GLDH (Kuzniak et al., 2004).

Our present study found that $CsGLDH$ responded to UV-B via increased AsA levels (Liu et al., 2019). However, there is no previous study on whether UV-B and ethylene co-regulate AsA metabolism or the mechanism of this regulation. In this study, we clarified the essential nature of ethylene in UV-B-induced AsA accumulation and identified a cucumber ERF/AP2 transcription factor, $CsERF39$, which regulates AsA biosynthesis through the activation of $CsGLDH$ involved in the D-Man/L-Gal pathway. Finally, we then revealed, for the first time, the mechanism by which UV-B signals regulate AsA metabolism at the transcriptional level.

2 Materials and methods

2.1 Plant material and growth conditions

Germinated cucumber (C. sativus L. cv. Chinese long 9930) seeds were transferred to a greenhouse at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (Beijing, China, 39.9N), and when plants had four leaves, they were moved into a controlled chamber under a 14-hour (h) photoperiod, 28/20, 60% relative humidity and 240 $\mu$mol·m$^{-2}$·s$^{-1}$ photon flux density (400-700 nm) supplemented with high-pressure sodium lamps from 6 AM to 8 PM.

2.2 Experimental design

After being placed into the controlled chamber for three days, cucumber seedlings with four leaves were exposed to biologically effective UV-B irradiance, calculated using the generalized plant action spectrum: 0 $\mu$w·cm$^{-2}$ (control), 20 $\mu$w·cm$^{-2}$ (3.33 $\mu$mol·m$^{-2}$·s$^{-1}$) (UV-B), respectively (Liu et al., 2019). Supplemental UV-B was applied using Philips TL20 W/01 RS tubes (311 to 313 nm spectrum peaking, Philips) (Fig. S1) suspended at different distances above the plant canopy.

For the hormone treatments, cucumber seedlings with four leaves were moved into boxes supplemented with or without 10 mM ACC or 1 mg·L$^{-1}$ 1-MCP for 0, 0.5, 1, 2, 4 and 8 h under a light condition. The 1-MCP treatment was carried out 24 hours before sampling. All chemicals used were obtained from Sigma-Aldrich.

For the hormone and UV-B treatment, cucumber seedlings with four leaves were exposed to 0 $\mu$w·cm$^{-2}$ and water (control), 20 $\mu$w·cm$^{-2}$ and water (UV-B), 20 $\mu$w·cm$^{-2}$ and 10 mM ACC (UV-B + ACC) or 20 $\mu$w·cm$^{-2}$ and 1 mg·L$^{-1}$ 1-MCP (UV-B + 1-MCP) for 0, 0.5, 1, 2, 4 and 8 h under a light condition. The 1-MCP treatment was carried out 24 hours before sampling. All chemicals used were obtained from Sigma-Aldrich.

Plant samples were evaluated with three biological replicates. Three fully expanded leaves were collected from each plant, and the leaves of three plants were combined as one replicate. The leaves were immediately frozen in liquid nitrogen and stored at -80 until analysis.

2.3 Analysis of AsA content and ethylene production

AsA content was assayed using the spectrophotometric method described by Besada et al. (1987). According to the methods described by Luo et al. (2014) with a minor modification, we used a syringe to collect 20ml of gas in the box at 0, 0.5, 1, 2, 4 and 8 h after UV-B treatment, and then used a syringe to extract 5ul
of gas and injected it into the gas chromatograph from the injection hole, recording the peak area for each sample. The tasting took approximately 5 minutes. We measured the fresh weight of the seedling leaves in each box and calculated the amount of ethylene production according to the following calculation formula:

\[
\text{Ethylene production} \ (\mu l \cdot g^{-1} \ FW \cdot h^{-1}) = \frac{S \cdot V}{(88939 \cdot FW \cdot \text{Time})}
\]

S: the peak area
V: the box volume
FW: the leaves' fresh weight
Time: the time after treatment

88939: parameter

2.4 Quantification of mRNA levels

Relative expression levels of the genes were evaluated by quantitative real-time PCR (qPCR). Gene-specific primer design and detection, total RNA extraction and detection, cDNA preparation and detection and real-time quantitative RT-PCR detection and analysis were performed as described by Schmittgen et al. (2001). The primer information is listed in Table S1.

2.5 Analysis of subcellular localization

The full-length cDNA of CsGLDH and CsERF9 without a stop codon was amplified by PCR with a primer pair containing restriction sites of BamHI and KpnI. The purified fragment was digested by BamHI and KpnI and fused to the N terminus of the GFP in the binary vector pCAMBIA 1301 under the control of the CaMV 35S promoter to form a fusion construct, 35S:CsERF39-GFP and 35S:CsERF39-GFP. After sequencing, the fusion construct was mobilized into Agrobacterium tumefaciens strain EHA105 by heat shock. Transient transformation of tobacco leaves was performed as described by Kumar & Kirti (2010), with a minor modification. The abaxial surfaces of tobacco leaves were agroinfiltrated with the bacterial suspension (OD\text{600} = 0.5) and then kept in an incubator for 1–2 days, followed by live cell imaging under an inverted fluorescence microscope (Olympus BX61, Tokyo, Japan). The primer information is listed in Table S1.

2.6 Cucumber transformation

To generate the construct used for CRISPR/Cas9-edited plants of CsERF39 and CsGLDH, the specific single-guide RNA (sgRNA) target sites were selected using the sgRNA design website (http://crispr.hzau.edu.cn/CRISPR2/). The PCR fragment harboring one target site was amplified using two partially overlapping primers and then inserted into the binary CRISPR/Cas9 vector pKSE402 using the BsaI site and T4 Ligase (New England Biolabs) (Hu et al., 2017). The resultant constructs were transferred into the Agrobacterium tumefaciens strain EHA105 and then transformed into cucumber by the Agrobacterium-mediated cotyledon method, as previously described (Hu et al., 2017). The GFP of pKSE402 was used as a reporter to select putative positive buds in T0 transgenic plants. Genomic DNA was extracted from plants using the CTAB method. The primer information is listed in Table S1.

2.7 Yeast one hybrid assay

The full-length CDSs of CsERF39, CsERF39N1 and CsERF39N2 were cloned into the pGADT7 vector (effector). The 2000 bp promoter of CsGLDH upstream of the start codon was cloned into the pABAI vector (reporter). The yeast cells were cultured for 3 days at 30degC on selection medium (SD/-Leu) supplemented with 200 ng*mL^{-1} AbA. The primer information is listed in Table S1.

2.8 Electrophoretic mobility shift assay

EMSA was performed using the Light Shift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s protocol and as described by Wu et al. (2016). For the His-CsERF39 construct, the full-length CDS
of CsERF39 was amplified and inserted into the pET-32a vector to generate the recombinant His-CsERF39 protein. The recombinant protein was introduced into BL21 (DE3) cells and purified with glutathione Sepharose beads (Thermo Scientific, San Jose, CA, USA). The oligonucleotide probe of the CsGLDH promoter was labeled using the Light Shift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s instructions. The binding activity of the protein was analyzed using an oligonucleotide containing the DRE core AGCCGAC motif and DRE1 ACCGAGA present in the CsGLDH promoter, labeled using the biotin 3’ and 5’ End DNA Labeling Kit (Thermo Fisher Scientific). The primer information is listed in Table S1.

2.9 Dual-Luciferase reporter (DLR) assay

To detect the transient transcriptional activity of CsGLDH promoters, the full-length CDS of CsERF39 was fused with the cp516 vector to generate effector constructs; the promoter of CsGLDH (-2000bp) was cloned into the vector cp516-LUC to generate the reporter constructs. The REN gene under the control of cauliflower mosaic virus 35S promoter in the cp516-LUC vector was used as the internal control. The prepared vectors were introduced into agrobacterium strain EHA105 and then co-infiltrated in N. Benthamian leaves. After incubation for 48 h, the firefly LUC and REN activities were assayed using the Dual-Luciferase(r) reporter assay reagents (Promega, Madison, USA) (Hellens et al., 2005). The ratio of LUC to REN was calculated as the final transcriptional activity of the corresponding combination. The primer information is listed in Table S1.

2.10 Statistical analysis

Statistical analysis was performed using Excel and SPSS 20.0 software. Comparisons between pairs of groups were performed using Student’s t-test at the $P \leq 0.05$ level.

3 RESULTS

3.1 CsGLDH is involved in UV-B modulation of AsA biosynthesis

Previous reports showed that the expression of CsGLDH (Csa4M236360.1) and CsMIOX (Csa2M000640) involved in the AsA biosynthesis pathway was induced by 20 μw·cm$^{-2}$ UV-B (Liu et al., 2019). GLDH is a key enzyme in the L-galactose pathway, the most important pathway for AsA synthesis. Therefore, we speculated that UV-B induces the expression of CsGLDH and increases the content of ascorbic acid. To prove our hypothesis, we used the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated system 9) system to knock out CsGLDH, as previously described (Hu et al., 2017). Two null mutants, Csgldh #1 (a homozygous allele with a 5 bp deletion) and Csgldh #2 (a homozygous allele with an 8 bp deletion), and two mutants generated a premature stop codon, resulting in a truncated protein of 125 amino acids and 92 amino acids, respectively (Fig. 1A-B). Meanwhile, we used the constructed 35s:CsGLDH expression vector to infect cucumber with Agrobacterium to obtain CsGLDH -overexpressing plants OX-1 and OX-2 (Fig. 1C-D). Compared with the wild-type (WT) plants, the content of AsA and T-AsA was decreased in Csgldh #1 and Csgldh #2 mutants’ leaves at the four-true-leaf stage (Fig. 1E-F). In contrast, the content of AsA and T-AsA was increased in OX-1 and OX-2 plants’ leaves at the four-true-leaf stage (Fig. 1G-H). Interestingly, compared with WT, the leaf width and leaf length of the OX-1 and OX-2 plants were significantly increased, and the plant height was also increased. The overall performance of the OX-1 and OX-2 plants was strong, but the Csgldh #1 and Csgldh #2 mutants were weak (Fig. 1K-L). Furthermore, the content of AsA and T-AsA in WT cucumber leaves was significantly increased, but the content of AsA and T-AsA in Csgldh #1 and Csgldh #2 cucumber leaves was not changed at 4 h of 20 μw·cm$^{-2}$ UV-B exposure (Fig. 1J). These results suggest that CsGLDH regulates AsA metabolism in cucumber leaves and CsGLDH participates in the UV-B regulation of AsA metabolism in cucumber leaves.

3.2 CsERF39 directly promotes CsGLDH expression

To elucidate the molecular mechanisms of UV-B’s regulation of CsGLDH expression and AsA metabolism, a Y1H assay was carried out to screen a Cucumis sativus cDNA library.
A total of fifty putative CsGLDH-interacting candidates were obtained (Fig. S2). After sequencing, 18 positive proteins were identified, and XP.004147220.1 was annotated as an ERF-like transcription factor; the gene was designated as CsERF39 in the Cucurbitaceae Genome Database (Table S2). To further identify CsERF39, a BLAST search against the NCBI database demonstrated that CsERF39 displayed 95.33% sequence similarity with a dehydration-responsive element-binding protein, DREB3-like (XP.008458661.1) of Cucumis melo. Sequence analysis indicated that it contained 214 amino acid residues, with a calculated molecular mass of 27.081 Kd, a conserved AP2 domain and an isoelectric point of 8.84. Multiple alignments showed that CsERF39 shares a conserved AP2 region (Fig. 2A).

To identify the subcellular localization of CsERF39, the full-length open reading frame (ORF) without the stop codon of CsERF39 was fused to the 5’ terminus of the green fluorescent protein (GFP) gene in pCAMBIA super 1300 driven by the CaMV 35S promoter. The fusion plasmid was infiltrated into tobacco epidermal cells, followed by the monitoring of green fluorescence with a microscope. The fluorescence of the CsERF39-fused GFP protein was only found in the nucleus, as well as perfectly merged with the DAPI (exclusive nucleus dye) regime (Fig. 2B), suggesting that CsERF39 is a nuclear protein.

Real-time quantitative PCR was used to analyze the expression profiles of CsERF39 in different tissues of cucumber. We found that CsERF39 was expressed more highly in female flowers, 7 days post-flowering fruits and tendrils, and it showed lower expression in male flowers (Fig. 2C). In addition, the expression of CsERF39 was induced by UV-B, and the expression level increased significantly at 0.5 hours after UV-B treatment; moreover, the expression level reached the peak value at 8 hours, approximately five times higher than the control (Fig. 2D).

The Y1H assay was further carried out to verify the interaction between CsERF39 and the promoter of CsGLDH. All of the yeast cells, the negative control and the reporter containing thepro - CsGLDH -effector (CsERF39) could grow normally on the SD/-Leu medium. However, when 200 µg*mL^-1 Aureobasidin A (AbA) was added, only those transformed with effector-reporter could grow normally (Fig. 3A). Furthermore, sequence analysis showed that CsERF39 has an open reading frame (ORF) of 645 bp, encoding a 214-aa protein with an AP2 (aa 44–102) domain (Fig. 3B). The CsGLDH promoter was recognized and bound by different CsERF39 truncations, all of which contained the AP2 domain (full-length CsERF39 and CsERF39N2); however, the CsERF39N1 (aa 1–43) fragment did not recognize or bind to the cis-element in yeast cells (Fig. 3C).

Previous reports showed that AP2/ERF proteins can recognize and bind to a DNA sequence with GCC-box (GCCGCC) and DRE / CRT (Dehydration-responsive element,DRE/ CRT :CCGAC) cis-elements, which allows them to regulate downstream genes. Bioinformatics prediction demonstrated that two putative DRE / CRT cis-elements were identified in the promoter of CsGLDH (Fig. 4A). To further verify whether CsERF39 could specifically bind to the promoters of CsGLDH in vitro, an electrophoretic mobility shift assay (EMSA) was conducted using purified CsERF39-6xHis-TrxA fusion proteins. A specific protein–DNA complex was strongly detected when the DRE core AGCCGAC-containing oligonucleotide was synthesized and labeled as a probe. The formation of these complexes was not detected when a 100x cold probe with the same sequence was added. Meanwhile, no specific protein–DNA complex was detected when the DRE1 ACCGAGA-containing oligonucleotide synthesized and labeled as a probe (Fig. 4B). The specificity of this competition confirms that CsERF39 recognized and bound specifically to the AGCCGAC motif but not the ACCGAGA motif within the CsGLDH promoter.

A transient expression assay was then used to confirm the activation of CsERF39 through the CsGLDH promoter. The CsGLDH promoter was fused to the CP516-LUC vector to generate the reporter construct. The coding region of CsERF39 was inserted into the pCAMBIA3301 vector to generate the effector plasmid. The transient expression assay was then performed to confirm the interaction between CsERF39 and the promoter. The reporter construct (the CsGLDH promoter) and the effector (CsERF39) were co-transformed into tobacco leaves, and the relative LUC activity was determined by the Luciferase (LUC)/Renilla (REN) ratio. As shown in Figure 4C, the LUC activity was higher in the presence of both the effector and reporter constructs than in the control, indicating that CsERF39 activated the promoter of CsGLDH.
These results suggest that the AP2 domain of CsERF39 is critical for its recognition and interaction with the AGCCGAC motif, and CsERF39 acts as a transcriptional activator of CsGLDH.

3.3 Knockout of CsERF39 results in decreased CsGLDH expression, AsA and T-AsA levels in cucumber leaves

To investigate the function of CsERF39 in cucumber, we used the CRISPR/Cas9 system to knock out CsERF39, as previously described (Hu et al., 2017). Two null mutants, Cserf39#1 (a homozygous allele with a 1 bp deletion) and Cserf39#2 (a homozygous allele with a 2 bp deletion), were obtained (Fig. 5A). The two null mutants generated a premature stop codon, resulting in a truncated protein of 58 amino acids and 79 amino acids, respectively (Fig. 5B). Compared with the WT plants, the expression of CsGLDH and the levels of AsA and T-AsA in the cucumber leaves were reduced in both Cserf39 #1 and Cserf39 #2 at the four-true-leaf stage (Fig. 5C-E). These results suggest that CsERF39 serves the functions of CsGLDH expression and AsA metabolism regulation in cucumber leaves.

3.4 Ethylene signaling is involved in UV-B regulation of AsA metabolism

CsERF39 is a member of the ethylene-responsive factor family. Plants can sense ethylene signals through ethylene receptors (ETRs), and, through signal cascades, downstream genes are finally regulated by the ethylene response factor (ERF) transcription factor, and a series of ethylene responses occur. Moreover, studies have reported that AsA synthesis is regulated by ethylene (Yu et al., 2019). Therefore, we speculated that ethylene signaling is involved in the UV-B regulation of AsA metabolism.

To further verify that ethylene signaling is involved in the UV-B regulation of AsA accumulation, we analyzed the effect of UV-B on ethylene production. After UV-B treatment, the ethylene production of cucumber leaves increased rapidly, and the subsequent decrease was consistent with the ethylene levels of the control, and then increased and then decreased, showing undulating changes (Fig. 6F). ACS and ACO are the key genes for ethylene synthesis and their expression levels can indicate the amount of ethylene production to a certain extent. Here, we analyzed the expression of CsACS and CsACO, and one CsACS (CsaV3_6G000890.1) and four CsACO (CsaV3_1G040170.1, CsaV3_3G016420.1, CsaV3_3G012300.1, CsaV3_3G012300.1) were found to be induced by UV-B (Fig. 6A-E). These results suggest that UV-B promotes the synthesis of endogenous ethylene in cucumber leaves.

In the present research, we analyzed the response of AsA accumulation in cucumber leaves under normal light or UV-B and the blocking or activation of the ethylene signal. Compared to normal light, when plants were subjected to 20 μW·cm⁻² UV-B, the AsA and T-AsA concentrations increased (Fig. 7A, D). Moreover, when they were subjected to 10 μM ACC, the content of AsA and T-AsA increased, and the content of AsA and T-AsA decreased when subjected to 1 mg·L⁻¹ 1-MCP (Fig. 7B, E). These results indicate that UV-B and ethylene positively regulate the AsA and T-AsA accumulation in cucumber at the seedling stage. To further study whether the ethylene signal is involved in UV-B-induced AsA accumulation, we analyzed the response of AsA accumulation while blocking and activating the ethylene signal during UV-B treatment. Compared with the control, when plants were subjected to 1 mg·L⁻¹ 1-MCP and 20 μW·cm⁻², the content of AsA increased, but the content of T-AsA did not change. In contrast, when single 20 μW·cm⁻² UV-B or 10 μM ACC and 20 μW·cm⁻² UV-B were applied, the content of AsA and T-AsA increased. Moreover, compared to single UV-B treatment, the content of AsA and T-AsA was not changed when we applied 10 μM ACC and 20 μW·cm⁻² UV-B (Fig. 7C, F). The results indicate that UV-B requires the participation of the ethylene signal to promote the accumulation of AsA.

The previous results showed that CsGLDH and CsERF39 were involved in the regulation of UV-B during AsA metabolism in cucumber leaves. Are CsGLDH and CsERF39 also regulated by ethylene signaling? We analyzed the effects of the ethylene signal and UV-B on CsGLDH and CsERF39 expression in cucumber leaves. Consistent with the previous results, CsGLDH and CsERF39 could respond rapidly to 20 μW·cm⁻² UV-B treatment, the expression level was significantly up-regulated after UV-B treatment for 0.5 h, and the expression level was the highest at 8 h UV-B exposure (Fig. 8A, D). Furthermore, 10 μM ACC treatment promoted the expression of CsGLDH and CsERF39, and the expression of CsGLDH and CsERF39 was the
highest at 2 h and 1 h of ACC treatment, with values approximately 4 and 4.5 times those of the control, respectively. Both UV-B and ACC could promote the expression of CsGLDH and CsERF39 in cucumber leaves. In contrast, the expression of CsGLDH and CsERF39 was reduced by 1 mg·L⁻¹ 1-MCP (Fig. 8B, E). However, when the ethylene signal was blocked, the expression of CsGLDH and CsERF39 was not changed by UV-B (Fig. 8C, F). These results suggest that UV-B activated ethylene signaling by regulating ethylene biosynthesis, and it regulated CsGLDH and CsERF39 expression.

4 DISCUSSION

4.1 Ethylene signaling is required for UV-B regulation of AsA metabolism in cucumber leaves.

Plants perceive UV-B through a high-intensity UV-B-nonspecific signaling pathway and a low-intensity UV-B-dependent UVR8-specific signaling pathway (Liu et al., 2015). These two signaling pathways do not exist in isolation, and plants may also utilize nonspecific and specific signaling pathways (Jenkins, 2009). Plants show combined responses to frequently changing UV-B radiation levels in natural environments (Lang-Mladek et al., 2012; Jenkins, 2014; Tossi et al., 2019). For example, when plants are stimulated by acute UV-B, nonspecific signaling pathways will be activated, and, over time, plants will adapt to UV-B and complete plant photomorphogenesis through specific signaling pathways (Jenkins, 2014). Nonspecific signaling pathways require the participation of other signaling substances, such as DNA damage signaling (Pandey et al., 2019), ROS signaling (Leeet et al., 2021) and hormone signaling, including ethylene, salicylic acid, and jasmonic acid (Caputo et al., 2006; Heet et al., 2011; Jenkins, 2014). However, the signaling pathway involved in the UV-B regulation of cucumber AsA metabolism has not yet been elucidated.

Different intensities of UV-B can stimulate ethylene biosynthesis in plants, and many previous studies have focused on the effects of high-intensity UV-B on Arabidopsis (Ge et al., 2020) and tobacco (Nara and Takeuchi, 2002), etc. In this study, it was found that 20 μW·cm⁻² UV-B treatment could significantly increase the expression of CsACS and CsACO, key genes in ethylene biosynthesis, as well as ethylene biosynthesis itself in cucumber leaves (Fig. 6), which was consistent with previous findings in other plants (Jia et al. et al., 2018; Ge et al., 2020). Promoter analysis found that there are many light-responsive elements involved in the activation of these ethylene synthesis genes, which also confirmed that these genes can respond to light (Table S4). Therefore, UV-B may regulate ethylene synthesis key genes CsACS and CsACO via the low-intensity UV-B signaling pathway, thereby promoting ethylene synthesis in cucumber leaves. It has been reported that ethylene signaling can regulate AsA metabolism in Arabidopsis (Yu et al., 2019; Song et al., 2019). Therefore, we speculated that UV-B-induced ethylene synthesis is likely to act as a signal involved in the regulation of AsA metabolism in cucumber leaves. To test this idea, we exogenously applied the ethylene precursor ACC and the ethylene signal blocker 1-MCP on the basis of UV-B treatment, and found that when the ethylene signal was blocked, UV-B lost its effect on AsA metabolism in cucumber leaves (Fig. 7C, F). The results indicate that the ethylene signal is a necessary factor for UV-B to regulate AsA metabolism in cucumber leaves. We reveal for the first time that ethylene signaling is involved in the UV-B regulation of AsA metabolism in cucumber.

4.2 CsERF39 is a key transcription factor of AsA metabolism regulation by UV-B in cucumber leaves.

Ethylene signals can be sensed by ethylene receptors (ETRs), and, through signal cascades, downstream genes are finally regulated by the ethylene response factor (ERF) transcription factor, resulting in a series of ethylene responses (Chang, 2003; Chen et al., 2005; Binder et al., 2007). In the present research, we found that CsERF39 is a member of the ethylene response factor family (Fig S4), and the expression of CsERF39 is a response to UV-B and ethylene signals (Fig. 2D, Fig. 8D-F). Furthermore, we concluded that CsERF39 belongs to the DREB subfamily (Hu and Liu, 2011), so the CsERF39 transcription factor can bind to the DRE element on the promoter, as shown in previous studies (Stockinger et al., 1997). Therefore, we analyzed the CsGLDH promoter and found that there are two cis-acting elements upstream of the CsERF39 transcription factor, which are the 107-113-bp DRE1 (ACCGAGA) and 77-83-bp DRE core (AGCCGAC), respectively (Fig. 4A). The EMSA results showed that CsERF39 could bind to the 77-83-bp DRE core.
In addition, the Y1H assay results showed the interaction between the AP2 domain (aa 44–72) of CsERF39 and the promoter of CsGLDH in vivo (Fig. 3). Importantly, the results of the Dual-Luciferase reporter assay showed that the CsERF39 transcription factor positively regulates the expression of the CsGLDH gene (Fig. 4C), which is also consistent with the spatiotemporal expression analysis results obtained for the CsERF39 and CsGLDH genes to a certain extent (Fig. 2C, Fig. S3C). The subcellular localization results showed that the CsERF39 protein acts on the nucleus (Fig. 2B), which indicates that the CsERF39 transcription factor plays a transcriptional regulatory function in the nucleus.

Moreover, UV-B treatment rapidly increased CsERF39 expression and maintained it at a high level, which was consistent with the expression pattern of CsGLDH under UV-B treatment (Fig. 8A, D) and the expression pattern of CsERF39 under the ethylene signal and UV-B signal consistent with the expression pattern of CsGLDH (Fig. 8), the two genes that are co-expressed. These results all indicate that UV-B regulates the expression of CsERF39, which in turn regulates the expression of CsGLDH, and regulates AsA metabolism in cucumber leaves.

Both biotic and abiotic stresses can cause changes in ethylene metabolism (Gravino et al., 2015; Yu et al., 2017; Wang et al., 2021), so our discovery of the UV-B regulation of AsA metabolism by ethylene may also be applicable to plants responding to these biotic stresses by regulating AsA metabolism, but this view still needs to be further explored.

4.3 **CsGLDH is a key gene for UV-B regulation of AsA metabolism in cucumber leaves**

L-galactose-1,4 lactone dehydrogenase (GLDH) is a key enzyme for AsA metabolism (Mapson et al., 1958). As a key enzyme gene involved in AsA metabolism, CsGLDH plays an important role in the regulation of AsA metabolism in cucumber leaves. When CsGLDH was knocked out, the content of AsA and T-AsA in cucumber leaves was significantly reduced. On the other hand, when CsGLDH was highly expressed, the content of AsA and T-AsA in cucumber leaves was also significantly increased, which is consistent with results obtained in tomato (Alhagdow et al., 2007). Interestingly, the mutant plants displayed weak performance, which may have been caused by the reduced levels of TCA cycle intermediates (Alhagdow et al., 2007) (Fig. 1). In addition, spatiotemporal expression analysis showed that the expression of CsGLDH was highly consistent with the content of AsA and T-AsA in various tissues and organs (Fig. S3C-E), which also confirmed this view.

The laboratory previously found that UV-B promotes the expression of CsGLDH in cucumber leaves (Liu et al., 2019), so it was speculated that UV-B regulates AsA metabolism by regulating the expression of CsGLDH. To test this speculation, we used Csgldh #1, Csgldh #2 and WT cucumber as materials, and subjected them to UV-B treatment. AsA and T-AsA content in Csgldh #1 and Csgldh #2 leaves did not respond to UV-B treatment (Fig. 1). It can be concluded that CsGLDH is involved in the regulation of AsA metabolism by UV-B.

The regulation of cucumber AsA metabolism by UV-B requires the participation of ethylene signaling. Does UV-B require the participation of ethylene signaling in the regulation of CsGLDH expression? To explore this question, we analyzed the changes in CsGLDH expression under ethylene signaling. The results showed that CsGLDH expression responded to UV-B and ethylene signals, and both UV-B and ACC could promote the expression of CsGLDH in cucumber leaves, while UV-B lost its promoting effect on CsGLDH expression when the ethylene signals were blocked by 1-MCP (Fig. 8A-C). Therefore, we infer that UV-B and ethylene signals regulate AsA metabolism in cucumber leaves by regulating the expression of CsGLDH. It can be seen that CsGLDH is an essential biological factor for the regulation of AsA metabolism by UV-B and ethylene signals.

Finally, the mechanism of the transcription factor–key enzyme response pathway in the UV-B regulation of cucumber AsA metabolism was revealed: after cucumber leaves sense UV-B signals, the key ethylene synthesis genes, CsACSs and CsACOs, are up-regulated, the ethylene content in leaves is increased, and the downstream response factor CsERF39 in the ethylene signal transduction pathway is up-regulated. CsERF39 directly binds to the 77-83-bp DRE core (AGCCGAC) element on the CsGLDH promoter by the
AP2 domain, promoting the expression of CsGLDH and increasing the AsA and T-AsA content in cucumber leaves (Fig. 9). This study provides a new understanding of the mechanism by which UV-B regulates ascorbic acid metabolism in plants, and also provides a theoretical basis and practical guidance for improving the resistance and quality of protected vegetables by supplementary light technology.

5 CONCLUSIONS

UV-B induces the expression of ethylene synthesis key genes CsACS and CsACO in cucumber leaves, thereby promoting endogenous ethylene synthesis in cucumber leaves. The ethylene signal is activated to promote the expression of the ethylene-responsive factor CsERF39. The CsERF39 transcription factor binds to the 77-83-bp DRE core (AGCCGAC) on the CsGLDH promoter by the AP2 domain from the 44th to the 102nd amino acid to promote the expression of CsGLDH, and this increases the AsA and T-AsA content in cucumber leaves (Fig. 9).

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WJ, HY and XS planned and designed the research. PL, QL, HW, TL and YL performed experiments, conducted fieldwork, analysed data etc. PL, QL and HW wrote the manuscript. PL, QL and HW contributed equally.

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Reference


Figure Legends

**Figure 1** *CsGLDH* participates in the UV-B regulation of AsA metabolism.

(A), Base sequence of *CsGLDH* knockout plants obtained by CRISPR/Cas9. (B), Amino acid sequence of *CsGLDH* knockout plants. (C), Positive plants obtained by agarose gel electrophoresis. +: positive control;
-1: template is the negative control of water; -2: template is the negative control of WT. (D), CsGLDH expression levels of CsGLDH-overexpressing plants. AsA (E) and T-AsA (F) contents in leaves of WT and CsGLDH knockout plants. AsA (G) and T-AsA (H) contents in leaves of WT and CsGLDH-overexpressing plants. The effect of UV-B on AsA (I) and T-AsA (J) contents in leaves of WT and CsGLDH knockout plants. CsGLDH transgenic cucumber lines at 21 days post germination.

**Figure 2** CsERF39 expression in response to UV-B exposure.

(A), Schematic diagram of the gene structure of CsERF39. The blue box represents the AP2 domain of 58 amino acids and Arabic numerals represent the position of the base pair sequence. (B), Subcellular localization indicating CsERF39-GFP fusion protein located in the nucleus of N. benthamiana leaves; 4,6-diamidino-2-phenylindole (DAPI) nuclear staining is used as nuclear marker. (C), Expression analyses of CsERF39 in different cucumber organs. R, root; S, shoot; L, leaf; MF, male flower; FF, female flower; 1DPF, one-day post-flowering fruit; 7DPF, seven-day post-flowering fruit; T, tendril. (D). The effect of UV-B on CsERF39 expression in cucumber leaves. Different letters in figure indicate significant differences ($p < 0.05$).

**Figure 3** CsERF39 fragment binds to CsGLDH promoter in yeast.

(A), Growth of yeast cells (with or without dilutions) of bait–prey and negative control (bait/pGADT7) co-transformations on selective medium (SD/-Leu) without (left) or with (right) 200 ng/ml AbA. (B), Schematic diagrams of CsERF39. (C), Growth of yeast cells (with or without dilutions) of different bait–prey co-transformations on selective medium (SD/-Leu) without (left) or with (right) 200 ng/ml AbA.

**Figure 4** CsERF39 binds to CsGLDH promoter fragments and acts as a transcriptional activator.

(A), Schematic diagrams of CsGLDH promoter. DRE1: ACCGACA, DRE core: AGCCGAC. (B), Identification of the His-CsERF39 protein binding to the cis-element of the CsGLDH promoter in electrophoretic mobility shift assay. The non-labeled fragment was used as a cold probe; -: absence; +: presence. (C), Transient expression assay of the promoter activity, shown as a ratio of LUC to REN, of N. benthamiana leaves co-transformed with reporter and the effector constructed using CsERF39 or not.

**Figure 5** Knockout of CsERF39 decreased AsA synthesis.

(A), Base sequence of CsERF39 knockout plants obtained by CRISPR/Cas9. (B), Amino acid sequence of CsERF39 knockout plants. (C), The CsGLDH expression in CsERF39 knockout plants. (D), The AsA content in CsERF39 knockout plants. (E), The T-AsA content in CsERF39 knockout plants. Different letters in figure indicate significant differences ($p < 0.05$).

**Figure 6** The effect of UV-B on the expression of key genes in ethylene synthesis and ethylene production in cucumber leaves

(A), The effect of UV-B on the expression of CsACS1 CsaV3_6G000890.1 in cucumber leaves. (B), The effect of UV-B on the expression of CsACS CsaV3_1G040170.1 in cucumber leaves. (C), The effect of UV-B on the expression of CsACS CsaV3_3G016420.1 in cucumber leaves. (D), The effect of UV-B on the expression of CsACO CsaV3_3G012300.1 in cucumber leaves. (E), The effect of UV-B on the expression of CsACO CsaV3_3G012230.1 in cucumber leaves. (F), The effect of UV-B on ethylene production in cucumber leaves. * in figure indicates significant differences ($p < 0.05$).

**Figure 7** The effect of UV-B and ethylene on AsA and T-AsA content in cucumber leaves.

(A), The effect of UV-B on AsA in cucumber leaves. (B), The effect of ACC and 1-MCP on AsA in cucumber leaves. (C), The effect of UV-B and ethylene signals on AsA in cucumber leaves. (D), The effect of UV-B on T-AsA in cucumber leaves. (E), The effect of ACC and 1-MCP on T-AsA in cucumber leaves. (F), The effect of UV-B and ethylene signals on T-AsA in cucumber leaves. Different letters in figure indicate significant differences ($p < 0.05$).
Figure 8 The effect of UV-B and ethylene on CsGLDH and CsERF39 expression in cucumber leaves

(A), The effect of UV-B on CsGLDH expression in cucumber leaves. (B), The effect of ACC and 1-MCP on CsGLDH expression in cucumber leaves. (C), The effect of UV-B and ethylene signals on CsGLDH expression in cucumber leaves. (D), The effect of UV-B on CsERF39 expression in cucumber leaves. (E), The effect of ACC and 1-MCP on CsERF39 expression in cucumber leaves. (F), The effect of UV-B and ethylene signals on CsERF39 expression in cucumber leaves. Different letters in figure indicate significant differences ($p < 0.05$).

Figure 9 The model of UV-B regulation of AsA metabolism in cucumber leaves.

![Figure 1](image1.png)

![Figure 2](image2.png)
Fig. 3

Fig. 4

Fig. 5
Fig. 6

Fig. 7

Fig. 8
Fig. 9