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Light- and ascorbate-dependent regulation of VITAMIN C DEFECTIVE 2 protein expression in Arabidopsis

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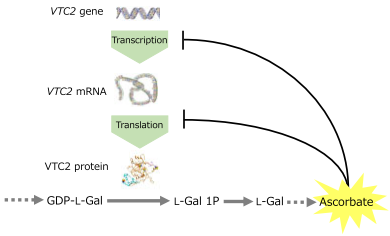
Abstract

Ascorbate accumulation under light conditions is regulated by GDP-L-galactose phosphorylase (GGP), which catalyzes the rate-limiting step in the ascorbate biosynthesis pathway and is encoded by the *Vitamin C Defective 2* (*VTC2*). While the *VTC2* gene expression and the GGP activity in response to light have been extensively studied, the behavior of the VTC2 protein remains unclear. To explore the role of VTC2 protein accumulation in modulating ascorbate biosynthesis, we generated transgenic plants expressing VTC2 fused with a Myc tag, driven by the native *VTC2* promoter, within *vtc2-4* lacking *VTC2*. Under normal light conditions, *VTC2* transcripts, VTC2 protein levels, and GGP activity exhibited an increase during the early light period. These data highlight the importance of VTC2 protein accumulation during the early light phase in determining ascorbate biosynthesis capacity in response to light. Furthermore, our analysis demonstrated that high-ascorbate concentrations suppress VTC2 expression.

Keywords: GDP-L-galactose phosphorylase, light regulation, ascorbate biosynthesis, protein expression, Arabidopsis

Graphical abstract



This study provided evidence that both VTC2 gene expression and protein accumulation are regulated by ascorbate-dependent feedback regulation.

Ascorbate, commonly known as vitamin C, is one of the most abundant antioxidants in plants. It plays a crucial role in various physiological processes, including photosynthesis, phytohormone biosynthesis, and iron uptake (Smirnoff 2018; Smirnoff and Wheeler 2024). Unlike plants, primates, including humans, have lost the ability to synthesize ascorbate and must obtain it through their diet, primarily from fruits and vegetables. Given its essential role in human health, increasing ascorbate content in crops has been a focus of agricultural biotechnology. However, paradoxically, plants engineered to overproduce ascorbate by overexpressing biosynthetic enzymes often exhibit adverse phenotypic traits, such as male sterility (Bulley *et al.* 2012; Deslous *et al.* 2021). Additionally, studies suggest a trade-off between ascorbate content and fruit quality or yield, as domesticated fruit varieties generally have lower ascorbate levels than their wild counterparts (Gest *et al.* 2013). Therefore, understanding the regulatory mechanisms governing ascorbate biosynthesis is critical for plant breeding and crop improvement.

Several biosynthetic pathways for ascorbate have been proposed in plants, with the D-mannose (D-Man)/L-galactose (L-Gal) pathway being the predominant route (Quiñones *et al.* 2024; Smirnoff and Wheeler 2024). This pathway involves eight enzymatic reactions, beginning with converting D-fructose-6-phosphate to D-Man-6-phosphate (D-Man 6P) and culminating in ascorbate synthesis. All enzymes involved in this pathway have been identified through biochemical and genetic studies (Maruta 2022; Smirnoff and Wheeler 2024). Notably, GDP-D-Man and GDP-L-Gal serve dual roles in cell wall synthesis and protein *N*-glycosylation (Qin *et al.* 2008; Gilbert *et al.* 2009). The first committed step of ascorbate biosynthesis is the conversion of GDP-L-Gal to L-Gal 1P, catalyzed by GDP-L-Gal phosphorylase (GGP), which exhibits high catalytic efficiency for this reaction (Linster *et al.* 2007). The gene encoding GGP has been identified as VITAMIN C DEFECTIVE 2 (*VTC2*), with *VTC5* as its minor isoform, based on studies of ascorbate-deficient (*vtc*) mutants in *Arabidopsis thaliana* (Dowdle *et al.* 2007). The lethality of the *vtc2 vtc5* double mutant in the absence of L-Gal supplementation further supports the central role of this pathway in ascorbate biosynthesis (Dowdle 2007).

Ascorbate biosynthesis is influenced by environmental factors such as light, temperature, nitrogen deficiency, and drought, with

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light being particularly well-documented in this process (Conklin *et al.* 2013; Zhang *et al.* 2020; Iwagami *et al.* 2022). Light exposure induces the transcription of several genes in the D-Man/L-Gal pathway, notably *VTC2*, whose expression is also modulated by diurnal rhythms (Dowdle 2007; Yabuta *et al.* 2007; Gao *et al.* 2011). High-light stress enhances *VTC2* transcription, leading to increased GGP enzyme activity (Dowdle 2007; Hamada *et al.* 2023). Overexpression of *VTC2* has been shown to significantly increase ascorbate levels in various plant species (Bulley *et al.* 2009, 2012; Yoshimura *et al.* 2014; Fenech *et al.* 2021), reinforcing the idea that GGP is a rate-limiting enzyme in ascorbate biosynthesis. Kinetic modeling of the D-Man/L-Gal pathway using the Complex Pathway Simulator further supports this conclusion, highlighting GGP as a regulatory hub in ascorbate metabolism (Fenech 2021). Consequently, VTC2 has been extensively studied as a target for modulating ascorbate levels.

The light-induced transcriptional activation of *VTC2* requires the photosynthetic electron transport (PET) chain. The application of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a PET inhibitor, suppresses *VTC2* induction under light conditions, leading to reduced ascorbate accumulation (Yabuta 2007; Shiroma *et al.* 2019; Sodeyama *et al.* 2021). These findings suggest that yet unidentified chloroplast-derived signals are essential for light-dependent *VTC2* regulation. Beyond transcriptional control, VTC2 expression is also regulated at translational and post-translational levels. The translation of VTC2 is inhibited by high-ascorbate concentrations through an upstream open reading frame (uORF) in the 5′-untranslated region (UTR) of the gene (Laing *et al.* 2015). Deletion of this uORF enhances ascorbate accumulation in several plant species (Li *et al.* 2018; Zhang *et al.* 2018; Deslous 2021). Additionally, recent studies have identified a novel post-translational regulatory mechanism involving the Per-ARNT-Sim (PAS)/Light, Oxygen, or Voltage (LOV) protein (PLP), a blue-light receptor. Yeast two-hybrid assays revealed that PLP interacts with Arabidopsis VTC2 and VTC5 in the dark, but this interaction is disrupted by blue light exposure (Ogura *et al.* 2008). Similar interactions have been observed in tomato and soybean plants (Aarabi *et al.* 2023; Bournonville *et al.* 2023; Zhang *et al.* 2023). *In vitro* studies using recombinant tomato PLP and GGP suggest that darkness enhances the PLP-GGP interaction, inhibiting GGP activity, while blue light disrupts this interaction, leading to the activation of GGP (Bournonville 2023). Consistent with this, PLP mutants exhibit increased ascorbate accumulation and GGP activity (Aarabi 2023; Bournonville 2023; Zhang 2023).

These studies underscore the intricate regulation of VTC2, emphasizing its crucial role in controlling ascorbate biosynthesis (Baldet 2024). However, despite extensive research on *VTC2* transcriptional regulation, few studies have investigated its expression at the protein level. Therefore, it remains unclear whether *VTC2* mRNA abundance directly correlates with protein levels or whether VTC2 protein abundance is necessary for GGP activity. To address this gap, we generated transgenic *vtc2* plants expressing VTC2 fused with a Myc tag at the C-terminus, driven by the native *VTC2* promoter. Our findings reveal that VTC2 protein levels are regulated by both light and ascorbate availability.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Col-0 was utilized as the wild-type plant. The T-DNA insertion line, *vtc2-4* (SAIL_769_H05), was obtained from the Arabidopsis Biological Resource Center. Surfaced-sterilized Arabidopsis seeds were sown on 1/2 MS medium with 0.7% (w/v) agar and 1% sucrose. The plants were subjected to vernalization at 4 °C for 2 days, after which they were transferred to a growth chamber (LH-350S, NK system, Osaka, Japan) maintained at 21 °C for a 16-h light period (approximately 110-120 μmol photons m^{-2} s^{-1}) and an 8-h dark period. For the DCMU treatment, plants were sprayed with a 10 μM DCMU solution containing 0.1% DMSO and incubated under continuous light at 100 μmol photons m^{-2} s^{-1} for 24 h. DCMU is used as the appropriate concentration from our previous studies (Yabuta *et al.* 2007; Shiroma *et al.* 2019). For L-Gal treatment, the shoots of 2-week-old plants were floated on a 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 5.7) with 0.02% polyoxyethylene (20) sorbitan monolaurate (Tween-20), with or without 10 mM L-Gal, under continuous light at 100 μmol photons m^{-2} s^{-1} for 24 h.

Ascorbate assay

Ascorbate contents were quantified according to the method described by Shiroma *et al.* (2019). Plant leaves (50 mg) were frozen in liquid nitrogen and subsequently ground in 500 μL of 2% metaphosphoric acid (MPA). The resultant homogenate was centrifuged at 15 300 *g* for 20 min at 4 °C. The supernatant was then pass through 0.22 μM filters. Total ascorbate and reducing ascorbate levels were determined by treating the samples with or without 35 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), respectively. Dehydroascorbic acid was calculated as the difference between total and reduced ascorbate (without TCEP treatment). These samples were analyzed using an ultra-fast liquid chromatography (UFLC) system (Prominence UFLC, Shimadzu, Kyoto, Japan) equipped with a C18 column (LUNA C18(2), 150 × 4.6 nm, Shimadzu). Ascorbate was detected at 254 nm using a UV detector (SPD-20A, Shimadzu). The mobile phase consisted of 1% MPA, with a flow rate of 0.5 mL min^{-1}.

Quantitative Reverse transcription polymerase chain reaction

Total RNA was extracted using Sepasol®-RNA I Super G (Nakalai Tesque, Osaka, Japan) and subsequently treated with DNase. Complementary DNA (cDNA) was synthesized using ReverTra Ace (TOYOBO, Osaka, Japan) with random primers following the manufacture's direction. A 20 μL reaction mixture was prepared, comprising 10 μL of 2 × GeneAce SYBR® qPCR Mix α (Nippon Gene, Tokyo, Japan), 1 μL of 1μM primer mixture (forward and reverse) and 20 ng of cDNA, with H_2O added to reach a final volume of 20 μL. The reaction was conducted using a LightCycler ®96 System (Roche, Switzerland). The primers employed are detailed in Iwagami *et al.* (2022)

Plasmid construction and generation of Arabidopsis transgenic lines

The *VTC2* promoter region, extending 2056 bp upstream from the + ATG site, was amplified from the Col-0 genome using the following primer sets: VTC2pro F (5′-aggtgtattcacaaagttactttatagaaaattag-3′) and VTC2pro R (5′-tctcaaaaaagacaaatttcgaagagc-3′) with KOD -Plus- Neo polymerase (Toyobo). PCR amplification incorporated a dA at the 3′ end using 10 × A-attachment mix (Toyobo). The fragment with the dA was cloned into the pDONR vector, and then sequence was verified by DNA sequencing. The liner vector was amplified from VTC2pro/pDONR using the following primer sets: pDONR R (5′- AGTGTCTACCCAGCTTTCTTG -3′) and VTC2pro R. VTC2 CDS was amplified from cDNA of Col-0 as template, using following primer sets: VTC2pro-VTC2CDS infu

F (5′-ttgtctttttttgagaATGTTGAAAATCAAAAGAGTTCCGACC-3′) and VTC2pro-VTC2CDS infu R (5′-AGCTGGGTAGACACTCTGAAGGACAAG-3′). The amplification was performed with KOD -Plus- Neo polymerase (Toyobo). The liner vector derived from VTC2pro/pDONR and the VTC2 CDS were assembled using the In-Fusion HD Cloning Kit (Takara Bio, USA), and the sequence was confirmed by DNA sequencing. The resulting pDONR/VTC2pro-VTC2 construct, as an entry clone, was subjected to an LR reaction with pGWB16 to generate pGWB16/VTC2pro-VTC2. The LR reaction was carried out using Gateway™ LR Clonase™ II Enzyme mix (Invitrogen, MS, USA) according to the manufacturer's instructions. The resulting pGWB16/VTC2pro-VTC2 construct was introduced into *vtc2-4* *via* the *Agrobacterium*-mediated floral drip method (Clough and Bent 1998). Transgenic T1 plants were selected using 25 μg/mL hygromycin and 100 μg/mL cefotaxime. Homozygous T3 lines were established and used for analysis.

Western blotting

Arabidopsis plants (50 mg) were frozen in liquid nitrogen and homogenized in 0.5 mL of 50 mM Tris-HCl buffer (pH 7.5) supplemented with 1 mM dithiothreitol (DTT), 1 mM EDTA, and 1 mM phenylmethanesul fluoride (PMSF). The homogenate was centrifuged at 15 300 *g* for 15 min at 4 °C. The supernatant was transferred to a fresh tube. The protein concentration of the supernatant was quantified using the Bradford Protein Assay Kit (Takara, Japan). The protein solution was mixed with Sodium Dodecyl Sulfate – Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) loading buffer containing 125 mM Tris-HCl (pH6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 0.015% (w/v) bromophenol blue, and 1% (w/w) 2-mercaptoethanol, and then heated at 95 °C for 5 min. Proteins (2 μg per lane) were separated by 10% (w/v) SDS-PAGE and subsequently electro-transferred onto a polyvinylidene fluoride (PVDF) membrane. Following transfer, the membrane was blocked with Skim Blocker (Wako, Japan) and then probed with a primary antibody against Myc (Anti-Myc-tag mAb-HRP-DirecT, M192-7, 1:10 000, MBL, Tokyo, Japan). Protein bands were visualized using the ImmunoStar LD (Wako). Signal intensities of protein bands corresponding to VTC2:: Myc and the large subunit of Rubisco (RBCL; as indicated by the predominant Coomassie Brilliant Blue-stained band) were quantified using ImageJ software. Western blot signal intensities (VTC2:: Myc) were normalized against the corresponding CBB-stained band intensities (RBCL) and subsequently analyzed.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence in Arabidopsis leaves was measured using a Closed FluorCam 800MF (Photon Systems Instruments, Drásov, Czech Republic). Prior to and following DCMU treatment, plants were dark-adapted for 30 min before chlorophyll fluorescence measurements were conducted. The actinic light was calibrated to 120 μmol photons m^{-2} s^{-1}. The quantum yields of photosynthesis II (ΦPS II) were calculated according to the method described by Genty *et al.* (1989).

GGP enzyme assay

GGP enzyme activity was measured following the methodology described by Ishida *et al.* (2024). GGP activity was evaluated based on the Pi-dependent degradation of GDP-D-glucose. Arabidopsis plants (100 mg) were frozen in liquid nitrogen, then ground and homogenized in 1 mL of extraction buffer comprising 50 mM Tris-HCl (pH 7.5), 10% (w/v) glycerol, 2 mM DTT, 10 mM $MgCl_2$, 1 mM aminocaproic acid, 1 mM benzamidine, and 1 mM PMSF. The homogenate was centrifuged at 15 300 × *g* for 20 min at 4 °C. The supernatant was transferred to a concentrated ultrafiltration tube (Amicon ultra centrifugal filter 10 kDa, NMWL) and further centrifuged at 15 300 × *g* for 20 min at 4 °C to obtain a concentrated extract for the assay. The extract (20 μL) was combined with 80 μL of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 100 μM GDP-D-glucose, with or without 5 mM sodium phosphate, and incubated for 1 h at 26 °C. The reaction was terminated by boiling for 5 min, and the mixture was filtered through 0.22 μm filters (Merck Millipore). The samples were analyzed using an ultra-fast liquid chromatography (UFLC) system (Prominence UFLC, Shimadzu) equipped with a C-18 column (LUNA C18(2), Column 150 × 4.6 nm, Shimadzu). The mobile phase consisted of 50 mM phosphate buffer (pH 6.7), 5 mM tetrabutylammonium dihydrogen phosphate, 5 mg L^{-1} EDTA, and 3.5% (v/v) methanol, with a flow rate of 1 mL min^{-1}. GDP-D-glucose was detected at 254 nm using the SPD-20A UV-Vis detector (Shimadzu). Pi-independent degradation was subtracted from the Pi-dependent substrate decrease to determine GDP-D-glucose degradation activity.

Results

Response of VTC2 protein expression to growth light

To elucidate the expression profile of VTC2 protein under light conditions, we generated *vtc2-4* complementation lines expressing VTC2 fused with a c-Myc tag under the control of the native *VTC2* promoter. The promoter region used included 2 kbp upstream of the transcription start site, along with the 5′-UTR. From more than 10 independent lines, we selected two representative lines (*vtc2*/VTC2pro:: VTC2-Myc #1 and #2) for further analysis. As a negative control, *vtc2-4* mutants transformed with an empty vector (*vtc2*/EV) were used.

Plants were grown under long-day conditions (16 h light at 110-120 μmol photons m^{-2} s^{-1}) for 2 weeks. Before light exposure (0 h, at the end of the dark period), the ascorbate levels in Lines #1 and #2 were 114% and 143% of the wild type, respectively, confirming that the loss of VTC2 was successfully complemented in these lines. In contrast, *vtc2*/EV exhibited ascorbate deficiency. Using these transgenic lines, we examined changes in ascorbate levels, *VTC2* transcription, and VTC2 protein expression during the light phase. As shown in Figure 1a, ascorbate levels increased in response to light in the wild type and the transgenic lines, except for *vtc2*/EV. In the wild type, *VTC2* transcript levels displayed a biphasic pattern, peaking at 4 and 16 h after light exposure, with an intermediate decline at 8 h. A similar trend was observed in the transgenic lines, though their *VTC2* transcript levels before light exposure were more than twice as high as in the wild type, resulting in a less distinct increase at the 4-h time point. GGP activity in the wild type peaked at 4 h after light exposure and gradually declined thereafter, which did not directly correlate with *VTC2* transcription levels. In the transgenic lines, GGP activity was already higher before light exposure (0 h) than in the wild type, consistent with their elevated *VTC2* transcript levels. As a result, the light-induced increase in GGP activity was less apparent in these lines. This suggests that nighttime suppression of *VTC2* expression was attenuated in the transgenic lines, potentially due to regulatory elements outside the *VTC2* promoter region used in this study, such as the 3′-UTR or terminator region. Nevertheless, GGP activity in the transgenic lines increased 4 h after light exposure and reached levels comparable to those of the wild type at the same

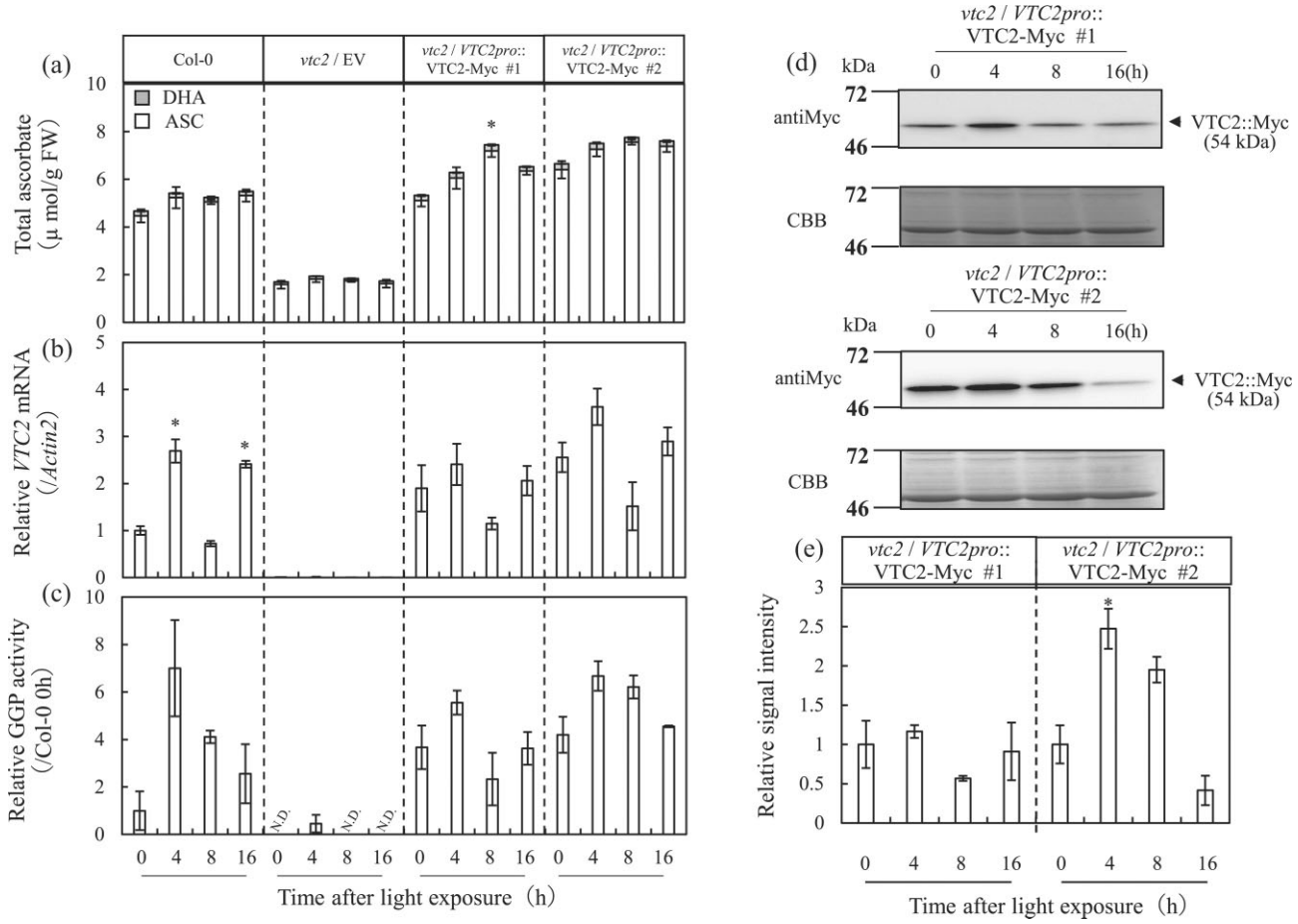

Figure 1. Analysis of ascorbate content and VTC2 expression under normal long-day conditions. The plants were grown on 1/2 MS medium with 1% sucrose and 0.7% w/v agar for 2 weeks under long-day conditions at 110-120 μmol photons $m^{-2}s^{-1}$, then harvested after dark following the time course and analyzed. Error bar means ± SE from 3 biological replicates. A statistically significant difference (Dunnett test): *$P < 0.05$ versus values before light. (a) Total ascorbate content. (b) The transcription levels of *VTC2* were normalized by before light in WT. (c) Relative GGP activities were normalized by before light in WT. N.D.: Not Detected. (d) Western blot of VTC2. Coomassie brilliant blue R (CBB) staining was used as a loading control. (e) Relative signal intensity of western blot analysis. The signals were normalized by the value of CBB-stained proteins.

time point, suggesting that *VTC2* expression and activity remained light-responsive in the transgenic lines.

To further investigate this response, we examined VTC2 protein levels in the transgenic lines. Western blot analysis using an anti-MYC antibody successfully detected VTC2:: Myc protein in both lines. As shown in Figure 1d, VTC2:: Myc protein levels increased at 4 h after light exposure and gradually declined thereafter, reflecting the pattern observed for GGP activity. Quantification of Western blot signals (Figure 1e) revealed that Line #2 exhibited a particularly clear light-responsive pattern, whereas Line #1 showed a more gradual response, likely due to its lower *VTC2* transcript levels and GGP activity compared to Line #2. These findings collectively indicate that the light responsiveness of GGP activity is regulated at both the transcriptional and protein levels of VTC2.

Inhibition of photosynthesis suppresses the accumulation of VTC2 transcripts and proteins

Previous studies have shown that PET inhibitors suppress *VTC2* transcription under illumination, suggesting that photosynthesis is required for light-dependent *VTC2* activation (Yabuta 2007). To examine whether photosynthesis influences VTC2 protein expression, we used DCMU, a potent PET inhibitor. Following the dark period of the long-day cycle, plants were treated with 0 or 10 μM DCMU and subsequently exposed to continuous light at 100 μmol photons m^{-2} s^{-1} for 24 h. DCMU treatment effectively suppressed the quantum yield of PSII (ΦPSII) in all genotypes tested (Figure S1). Compared to the mock control, DCMU treatment significantly reduced *VTC2* transcription levels in both the wild type and transgenic lines, leading to a partial decrease in ascorbate levels (Figure 2a and b). Consistent with this transcriptional suppression, VTC2 protein levels also decreased following DCMU treatment. Although the reduction in protein levels was less pronounced than the transcriptional suppression, it correlated well with the observed decrease in ascorbate levels. These results support the notion that photosynthesis is required for the light-induced accumulation of VTC2 transcripts and proteins.

High ascorbate pool size suppresses VTC2 protein expression

Laing *et al.* (2015) proposed that elevated ascorbate levels inhibit VTC2 translation via uORF in its 5′-UTR. To investigate whether this mechanism affects VTC2 protein levels, we examined VTC2 expression under high-ascorbate conditions. Transgenic plants were incubated in a 10 mM L-Gal solution or a mock control for 24 h under continuous light at 100 μmol photons m^{-2} s^{-1}. As shown in Figures 3a and S2a, ascorbate levels increased in a time-dependent manner following L-Gal treatment. After 24 h, ascorbate levels were 3.8-fold and 3.4-fold higher in complementation

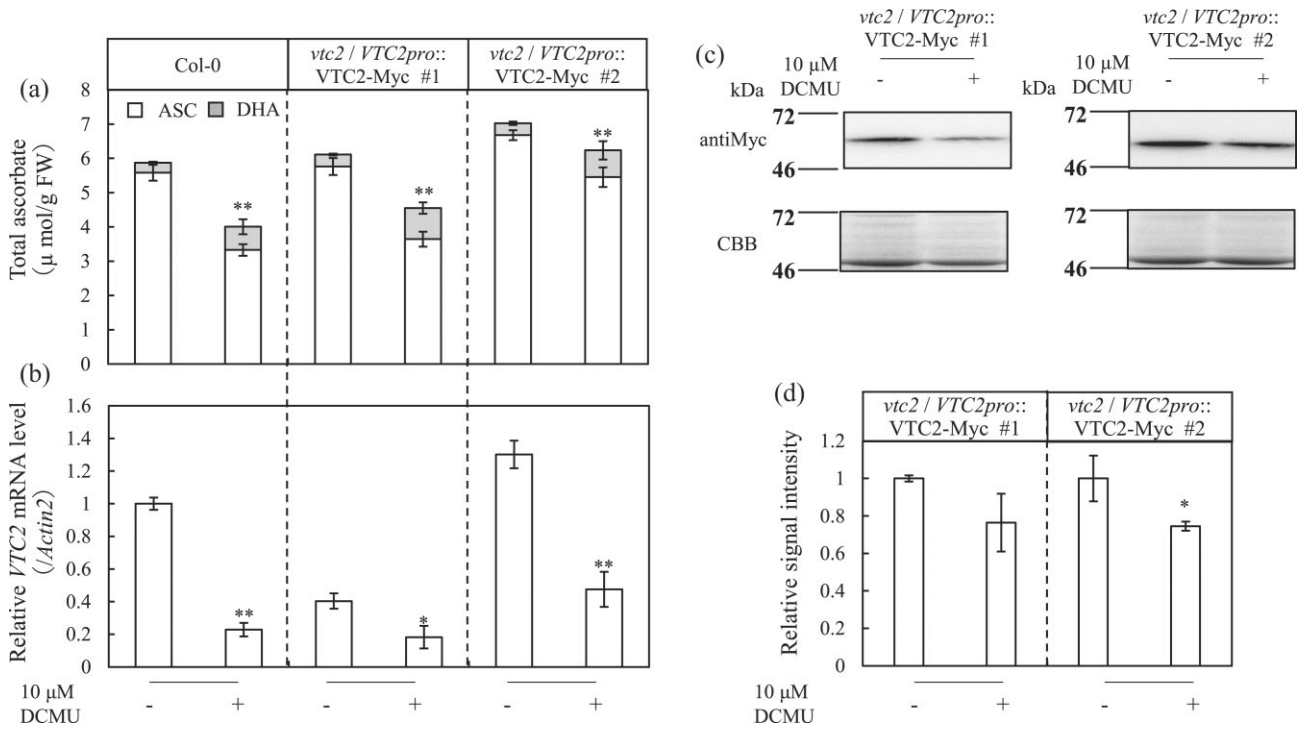

Figure 2. Effect of DCMU treatment for ascorbate biosynthesis under continuous light. The plants were grown on 1/2 MS medium with 1% sucrose and 0.7% w/v agar for 2 weeks under long-day conditions at 110-120 μmol photons $m^{-2}s^{-1}$. The plants treated 0.1% DMSO with or without 10 μM DCMU for 24 h under continuous normal light at 100 μmol photons $m^{-2}s^{-1}$. Error bar means ± SE from at least 3 biological replicates. A statistically significant difference (student's *t*-test): *$P < 0.05$, **$P < 0.01$ versus values mock control. (a) Total ascorbate content. (b) The transcription levels of *VTC2* were normalized by mock control in WT. (c) Western blot of VTC2. CBB staining was used as a loading control. (d) Relative signal intensity of western blot analysis. The signals were normalized by the value of CBB-stained proteins.

lines #1 and #2, respectively, compared to the mock controls. We then analyzed *VTC2* transcript and protein levels as well as GGP activity before and after the 24-h treatment. Due to continuous light exposure, *VTC2* transcript levels increased in both the mock and L-Gal treatments. However, in contrast to the transcript increase, VTC2 protein levels and GGP activity did not increase with mock treatment, deviating from their typical light-responsive pattern observed under a normal diurnal cycle (Figure 1). The reason for this discrepancy remains unclear, but it suggests that VTC2 protein expression may be subject to circadian regulation. Importantly, VTC2 protein levels and GGP activity tended to be lower after L-Gal treatment compared to the mock control (Figure 3c-e), supporting the hypothesis of feedback inhibition of VTC2 translation by ascorbate. However, due to high variability in the data, this difference was not statistically significant. To further investigate this phenomenon, we included two additional independent lines (#3 and #14) in the experiment (Figure S2). In these lines, VTC2 transcription was induced by continuous light, but VTC2 protein levels and GGP activity remained unchanged. Notably, L-Gal treatment led to a clear suppression of VTC2 protein levels, reflecting the lower *VTC2* transcription levels and GGP activity observed under high-ascorbate conditions.

Discussion

This study aimed to investigate the response of VTC2 protein levels to light and ascorbate. To achieve this, we generated *vtc2* complementation lines expressing a VTC2:: Myc fusion protein under the control of the native *VTC2* promoter. In two independent lines (#1 and #2), the ascorbate deficiency caused by the *vtc2* mutation was fully complemented by VTC2:: Myc expression. Western blot analysis using an anti-Myc antibody successfully detected VTC2:: Myc protein, confirming that these complemented lines were suitable for our study.

We found that *VTC2* transcription in the wild type followed a biphasic pattern, with peaks at 4 and 16 h after light exposure. Interestingly, GGP activity exhibited a single peak at 4 h, followed by a gradual decline. These findings suggest that the second transcriptional peak of *VTC2* does not contribute to GGP activity, highlighting a potential disconnect between *VTC2* mRNA levels and enzyme activity. In the complemented lines, *VTC2* transcription and GGP activity exhibited trends similar to those of the wild type, though the changes were more gradual. This attenuation was likely due to the elevated *VTC2* transcription and GGP activity before light exposure, suggesting that the nighttime suppression of VTC2 expression was partially disrupted in these lines. It is reasonable to postulate that suppressor *cis*-elements located beyond the promoter region utilized in this study, or within the introns or the 3′-UTR region, may exert significant repressive effects on *VTC2* expression during the dark phase. Notably, in potato, methylation events have been detected within both the intron and promoter regions of *VTC2* gene, potentially contributing to this regulatory mechanism (Boutsika *et al.* 2023). Despite this, the fundamental light-responsive pattern of *VTC2* transcription and the transient increase in GGP activity at 4 h remained largely consistent with the wild type. Importantly, VTC2 protein levels increased slightly 4 h after light exposure, followed by a gradual decrease. This response was particularly pronounced in Line #2, where the difference was statistically significant. Furthermore, VTC2 protein dynamics resembled those of GGP activity more closely than *VTC2*

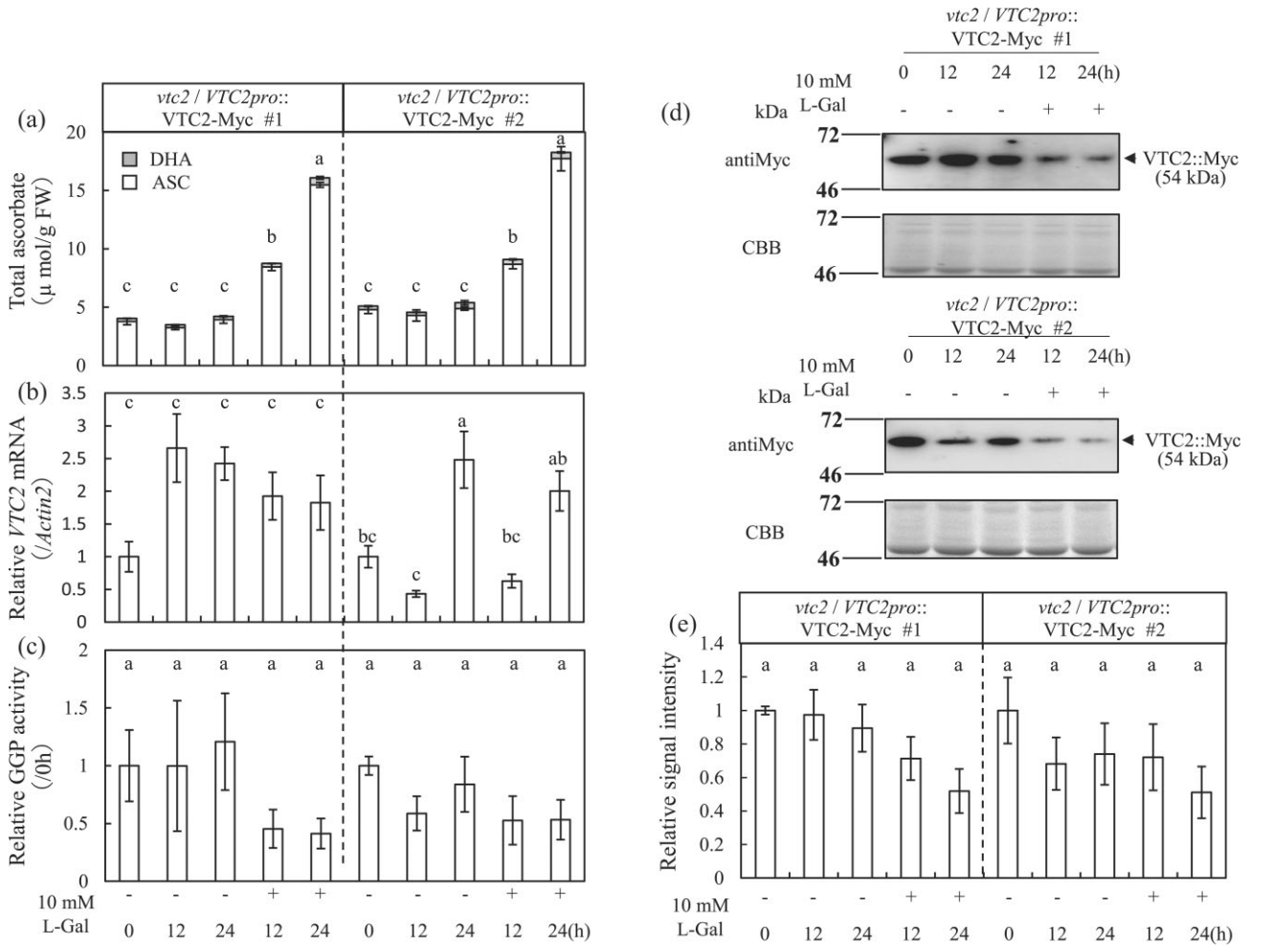

Figure 3. Effect of L-Gal treatment for *VTC2* transcripts and VTC2 protein levels under continuous light. The plants were grown on 1/2 MS medium with 1% sucrose and 0.7% w/v agar for 2 weeks under long-day conditions at 110-120 μmol photons m^{-2} s^{-1}. The shoots were harvested and floated on the 10 mM MES buffer (pH 5.7) containing polyoxyethylene (20) sorbitan monolaurate with or without 10 mM L-Gal, then transferred continuous normal light at 100 μmol photons m^{-2} s^{-1} for 24 h. Error bar means ± SE from 3 biological replicates. Different letters indicate statistically significant differences. ($P < 0.05$, Tukey-Kramer test). (a) Total ascorbate content. (b) Relative transcription levels of *VTC2* were normalized by before treatment. (c) Relative GGP activities were normalized by before treatment. (d) Western blot of VTC2. CBB staining was used as a loading control. (e) Relative signal intensity of western blot analysis. The signals were normalized by the value of CBB-stained proteins.

transcript levels. Our findings partially contrast with those reported for *Glycine max* VTC2b (GmVTC2b) by Zhang *et al.* (2023), who observed that protein levels of GmVTC2b exhibited two distinct peaks following light exposure. This discrepancy between Arabidopsis and soybean may stem from species-specific differences, growth conditions, plant age, or other experimental factors. Further research is necessary to elucidate the mechanisms underlying VTC2 regulation in different plant species therefore.

The light-induced transcriptional activation of *VTC2* is suppressed by PET inhibitors (Yabuta 2007), implying that photosynthesis plays a key role in its regulation. However, the impact of photosynthesis inhibition on VTC2 protein levels had not been previously examined. In our study, DCMU treatment suppressed VTC2 protein accumulation under light exposure, consistent with the observed reduction in *VTC2* transcript levels. This implies that a plastid-derived signal, potentially the redox state of the plastoquinone pool or reactive oxygen species, facilitates the transcriptional activation of *VTC2*, culminating in protein accumulation. Further investigation is required to elucidate the precise nature of this signaling molecule. Since DCMU also inhibits *VTC2* transcription in immature green tomato fruits and *Physcomitrella patens* (Badejo *et al.* 2012; Sodeyama 2021), this regulatory mechanism appears to be evolutionarily conserved across plant species. The *VTC2* 5′-UTR contains an uORF, which has been proposed to mediate translational repression in response to high ascorbate levels. Previous studies have demonstrated that removing this uORF *via* mutation or genome editing enhances VTC2 translation and increases the ascorbate pool size in multiple plant species, including tobacco, tomato, and lettuce (Li 2018; Zhang 2018; Deslous 2021). To investigate whether high ascorbate levels suppress VTC2 protein expression, we treated transgenic plants with L-Gal, which elevated the ascorbate pool size by 3.4- to 3.8-fold, exceeding the 2.5-fold increase reported in tobacco VTC2 overexpression studies (Laing 2015). This provided a sufficiently high-ascorbate concentration to potentially trigger feedback inhibition of VTC2 translation. Following L-Gal treatment, VTC2 protein levels and GGP activity exhibited a slight downward trend, supporting the hypothesis of ascorbate-dependent feedback regulation. However, due to high variability in Western blot quantification, the effect was relatively minor and not statistically significant. To further clarify this, we included two additional independent transgenic lines. These additional data reinforced the observation that L-Gal treatment suppressed VTC2 protein levels and GGP activity, aligning with the proposed model of ascorbate-mediated translational repression.

Notably, L-Gal treatment also reduced *VTC2* transcript levels in the complemented lines, raising the question of whether the decrease in VTC2 protein was due to uORF-mediated translational inhibition or transcriptional downregulation. Since this study did not directly assess the role of the uORF, we cannot conclusively determine which mechanism predominated. Previous reports suggest that uORF translation regulate downstream main ORF by causing ribosome stalling and/or triggering nonsense-mediated decay (NMD) of the mRNA. Smirnoff and Wheeler (2024) proposed that ribosome stalling at the VTC2 uORF could lead to *VTC2* mRNA degradation *via* NMD. This idea is supported by findings showing increased *VTC2* transcript levels in Arabidopsis UP frameshift mutants, which lack an NMD system (Kurihara *et al.* 2009). Such a mechanism may explain the observed suppression of *VTC2* transcription following L-Gal treatment. However, evidence supporting ascorbate-dependent activation of the uORF system remains limited, having been demonstrated only in transient VTC2 overexpression experiments in tobacco. Further studies are required to determine whether this mechanism operates broadly across plant species.

Our findings demonstrate that VTC2 protein levels are regulated by both photosynthesis-dependent activation and ascorbate-dependent suppression. In most conditions, VTC2 protein dynamics closely corresponded with transcriptional changes, underscoring the importance of transcriptional regulation. However, the second peak in *VTC2* transcriptional response to light did not translate to increased VTC2 protein levels or GGP activity, suggesting the presence of additional regulatory mechanisms at the post-transcriptional level. Since VTC2/GGP is a rate-limiting enzyme in ascorbate biosynthesis, further elucidation of its regulatory mechanisms will contribute to a deeper understanding of plant stress responses and may facilitate the development of crops with enhanced ascorbate content. The complemented lines generated in this study are expected to serve as valuable tools for advancing research on VTC2 regulation at multiple levels.

Supplementary martial

Supplementary martial is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contribution

T.I. and Y.T. conceived the study. Material preparation, data collection, and analysis were carried out by Y.T. All authors interpreted the data. The first draft of the manuscript was written by Y.T., T.M., and T.I. revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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