Identification of a GLDH-overexpressing Arabidopsis mutant and its responses to high-light stress

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Abstract

Ascorbic acid (AsA) is an important antioxidant protecting plant against environmental stresses. L-galactono-1,4-lactone dehydrogenase (GLDH) is a key enzyme in AsA synthesis pathway. To investigate the roles of AsA in mitigating high light (HL) damage, Arabidopsis GLDH mutants SALK_060087 and SALK_008236 with altered GLDH-expression were screened for homozygotes. No homozygotes were identified from SALK_060087, but most individuals of the SALK_008236 line (GLDH-236OE) were GLDH-overexpressing homozygous mutants accumulating more AsA than wild type (WT). An investigation of the physiological responses to HL demonstrated that the chlorophyll fluorescence parameters were significantly higher in GLDH-236OE than that in WT after 14-d HL. The degradation of photosynthetic pigment in WT was more severe than that in GLDH-236OE. GLDH-236OE accumulated more AsA, anthocyanins, flavonoids, and phenolics, while WT accumulated more reactive oxygen species (ROS) during HL. Our results suggest that GLDH-236OE have lesser sensitivity and higher tolerance to HL due to a higher capacity to eliminate ROS, absorb extra light, and dissipate thermal energy.

Additional key words: abiotic stress; antioxidation; ascorbate; photoinhibition; photoprotection.

Introduction

L-ascorbic acid (AsA) is an abundant metabolite in plants that has many important functions. AsA is one of the most powerful antioxidant molecules for its regenerative nature (Noctor and Foyer 1998). As a vital water-soluble antioxidant molecule, it can directly scavenge ROS in the cell cytoplasm and even in apoplast (Akram et al. 2017). It can also act indirectly via its participation in the xanthophyll cycle and the Mehler reaction (water-water cycle) (Asada 1999, Smirnoff and Wheeler 2000). AsA also participates in the modulation of hormone signaling and acts as a signaling molecule in plant defense responses (Conklin and Barth 2004, Pavet et al. 2005), plays a role in the regeneration of α-tocopherol (vitamin E), helps regulate gene expression (Pastori et al. 2003), and slows down senescence processes (Barth et al. 2006).

The AsA biosynthesis pathway in plants is different from that in animals. Four pathways lead to AsA production in plants, namely, the L-galactose pathway (Wheeler et al. 1998), the D-galacturonic acid pathway (Agius et al. 2003), the L-gulose pathway (Wolucka and Van Montagu 2003), and the myo-inositol pathway (Lorence et al. 2004). The L-galactose pathway is recognized as the main AsA biosynthetic pathway in plants, and L-galactono-1,4-lactone dehydrogenase (GLDH, EC 1.3.2.3) is the final key enzyme in this pathway (Wheeler et al. 1998, Bartoli et al. 2000). The GLDH gene is located on the 3rd chromosome in Arabidopsis thaliana and GLDH enzyme is localized in mitochondria (Schertl et al. 2012). Previous reports showed that GLDH expression affected the accumulation of AsA in plants (Tabata et al. 2001, Liu et al. 2011).

Received 22 May 2018, accepted 15 October 2018.
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Acknowledgements: This work was funded by the National Key R&D Program of China (2017YFC1200105) and the National Natural Science Foundation of China (31570398, 31270287). The study was also supported by the Key Program of the Guangdong Province Natural Science Foundation (2015A030311023, 2017A030313167).
Transgenic tobacco with lower GLDH expression had a lower AsA content and slower cell growth than the wild type (WT) (Tabata et al. 2001). Conversely, transgenic tobacco, in which the GLDH gene was up-regulated, had a significantly higher AsA content and faster cell growth than that of WT. Similarly, a rice mutant with higher GLDH expression had a larger HS pool and seed-setting rate than that of WT (Liu et al. 2011).

In the natural environment, plants are vulnerable to various stresses, such as high light (HL) stress, high temperature stress, osmotic stress, drought stress, heavy metal stress. HS stress is a common stress for plants (Szymańska et al. 2017, Zeng et al. 2017). Demming-Adams and Adams (1992) suggested that light stress results not from HS per se, but rather from an excess of absorbed light beyond that utilized in biosynthesis. Over-absorption of light causes photoinhibition that limits plant photosynthetic activity, development, and productivity (Takahashi and Badger 2011). Under HL conditions, more ROS are produced continuously as by-products of various pathways in chloroplasts and mitochondria if over-absorbed light cannot be dissipated safely (Wituszyńska and Karpiński 2013). When the balance between generation and scavenging of ROS is broken, proteins, lipids, photosynthetic pigments, and other cell component are attacked by ROS, finally leading to functional incapacitation and programmed cell death (PCD) (Petrov et al. 2015).

Plants have gradually developed different photoprotection mechanisms to avoid the HL-induced photoinhibition during the evolutionary process. The photoprotection mechanisms can be roughly classified into three categories: physical mechanisms, chemical, and self-repairing mechanisms. Physical mechanisms include the movement of leaves and chloroplasts, and irradiance screening by pigment (such as anthocyanins, betalains, carotenoids). Nonphotochemical quenching (NPQ), photochemical quenching, the photorespiratory pathway, ROS-scavenging systems can be classified as chemical mechanisms (Takahashi and Badger 2011). Self-repairing mechanisms refers to the biogenesis and constant repair of ROS-scavenging systems. Physical mechanisms include the movement of leaves and chloroplasts, and irradiance screening by pigment (such as anthocyanins, betalains, carotenoids). Nonphotochemical quenching (NPQ), photochemical quenching, the photorespiratory pathway, ROS-scavenging systems can be classified as chemical mechanisms (Takahashi and Badger 2011). Self-repairing mechanisms refers to the biogenesis and constant repair of ROS-scavenging systems.

**Materials and methods**

**Plant materials and growth conditions:** Seeds of the *Arabidopsis thaliana* WT Columbia (Col) ecotype and T-DNA insertion mutants at the 3' (SALK_060087) and 5' ends (SALK_008236) of the GLDH locus were purchased from ABRC (http://www.arabidopsis.org/) and were germinated on agar plates containing half-strength Murashige and Skoog salts. Ten-day-old plants were transferred into pots containing soil consisting of peat soil:vermiculite (3:1) and were grown in a growth chamber under following conditions: 16-h light/8-h dark, at a light intensity of 100 μmol(photon) m^-2^ s^-1^, and at a constant temperature of 23°C. A homozygous mutant was identified among three-week-old plants.

**Identification of homozygous mutants:** Genomic DNA extracted from *Arabidopsis* leaves according to the method described by Wasseneeger et al. (1994) was used as a PCR template to identify homozygous mutants. The specific primers were designed as follows: LP236, 5'-AGGGTCTCTTGACAGTTCAACAGGT-3'; RP236, 5'-AGACCCCTGATCCAGGGAGCA-3'; LP087, 5'-AGCAGACCCCTGATCCAGGGAGCA-3'; RP087, 5'-AGCCAAGCACGACCAACACT-3'; and LB1, 5'-GGGCGTGAGGCCGTGCTGCAACT-3' (Li et al. 2006). The primers LP236 and RP236 (or LP087 and RP087) were designed to anneal to both sides of the T-DNA insertion site. Therefore, in theory, the target band would be amplified using the primers LP236 and RP236 (or LP087 and RP087) from the genomic DNA of WT plants but not from the genomic DNA of a homozygous GLDH mutant with a T-DNA insertion at the 5' end (or 3' end). Hence, the first round of PCR amplification using the primer pair LP236 and RP236 (or LP087 and RP087) would reveal which plants were homozygous GLDH mutants and where the insertion had occurred. A second round of PCR amplification using the primers LP236/RP236 and LB1 (insertion at the 5' end) or LP087/RP087 and LB1 (insertion at the 3' end) was used to further confirm that the screened homozygous GLDH mutant was due to T-DNA insertion, as the primer LB1 was designed to anneal to the T-DNA sequence. The PCR conditions used were as follows: 3 min at 95°C; cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 90 s; and a final complete extension for 5 min at 72°C.

**RNA extraction and cDNA synthesis:** Total RNA was extracted from mature rosette leaves using TRIZOL reagent (Invitrogen) according to a standard manufacturer's protocol. The RNA was treated with DNase I (Takara) prior to synthesize the first-strand cDNA using an 18-mer oligo (dT) primer and the M-MLV reverse transcriptase kit (Takara).

**Semiquantitative PCR:** Semiquantitative PCR was performed to determine the relative GLDH transcript levels in the screened mutant plants using the primers GLDH-KF (5'-GTGTTATATTCCAGGGTGTTGGGGG-3') and GLDH-KR (5'-AGTCTCGGACCCCTATACCTC-3'). UBQ was selected as an internal reference gene and was amplified using the primers 5'-GAGGCTTCACTCTGCTCC-3' (forward) and 5'-CCACAGGTTGCGTATG-3' (reverse).
Quantitative real-time PCR: Quantitative PCR was carried out using a real-time PCR system 7500 (Applied Biosystems) and the SYBR Premix ExTag™ II kit (Takara). The cycling conditions were as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s, and 60°C for 34 s; and 1 cycle to record a melt curve at 95°C for 15 s and 60°C for 1 min. The relative transcript levels of GLDH were analyzed using the primers 5'-TTCTGGGCCTTGACGTGG-3' (forward) and 5'-GGTGCCTGTATGGCTTCTT-3' (reverse). TUB was selected as the control gene and was amplified using the primers 5'-CCAGCCTTGTGATTGGAAC-3' (forward) and 5'-CAAGCTTTCGGAGGTCAAG-3' (reverse). Relative gene transcript levels were calculated using the 2^{ΔΔCt} method (Livak and Schmittgen 2001).

AsA assay: To determine Asc (reduced ascorbic acid) and total AsA contents, 0.04 g of fresh leaves were homogenized in 2 mL of 6% (w/v) trichloroacetic acid (TCA) solution, and the homogenate was centrifuged at 12,000 × g for 10 min. The supernatant was used for Asc determination following the method of Gillespie and Ainsworth (2007). Total AsA was measured in the same way, but the sample was incubated with dithiothreitol prior to measurement.

HL treatments: Seedlings of the identified GLDH overexpression mutant GLDH-236OE and the WT were grown in soil (peat soil:vermiculite = 3:1) in a growth chamber under normal conditions at a light intensity of 100 μmol(photon) m^{-2} s^{-1} for 28 d. The plants were then subjected to HL treatment at 280 μmol(photon) m^{-2} s^{-1} and 7 and 14 d after HL treatment.

Chl pigment assay: Fresh rosette leaf material (0.025 g) was extracted in 5 mL of chilled 80% (v/v) acetone overnight in the dark, and the absorbance at 663, 645, and 470 nm wavelength was measured (UV-Vis 2450 spectrophotometer, Shimadzu, Tokyo, Japan). Chlorophyll (Chl) fluorescence and lipid peroxidation were determined in leaves that were collected from the mutant and WT plants at the beginning of treatment and 7 and 14 d after HL treatment.

Chl fluorescence was measured using a portable pulse-modulated fluorometer (PAM 2100, Walz, Effeltrich, Germany). Plants were dark-adapted for 20 min prior to fluorescence determination. The living rosette leaf was clamped in the leaf chamber for fluorescence determination. Minimal fluorescence yield of the dark-adapted state (F0) was determined by using a weak modulated light [0.04 μmol(photon) m^{-2} s^{-1}]. Maximal fluorescence yield of the dark-adapted state (Fm) was induced by a saturating pulse of light [6,000 μmol(photon) m^{-2} s^{-1}] that was applied over 0.8 s. After exposing to 5 min of continuous actinic light [200 μmol(photon) m^{-2} s^{-1}], the corresponding fluorescence (F) was recorded and maximal fluorescence yield of the light-adapted state (Fm') was monitored using a saturating pulse. The maximal quantum yield of PSII photochemistry (ΦPSII), the electron transport rate (ETR) through PSII, and the photochemical quenching coefficient (qP) were calculated according to Gray et al. (2003) and Schreiber et al. (1986) as follows:

$$F_0/F_m = (F_m - F_0/F_m, \Phi_{PSII} = (F_m' - F)/F_m'; \text{ETR} = \Phi_{PSII} \times \text{PPFD} [200 \mu \text{mol(photon)} \text{m}^{-2}\text{s}^{-1}] \times 0.85 \times 0.5; q_P = 1 - (F - F_0)/(F_m' - F_0').$$

Anthocyanins, flavonoids, and phenolics were extracted from 0.025 g of rosette leaves in 2 mL of methanol:HC1 (99:1, v/v) solution at 4°C in the dark for 24 h. Absorption spectra of the extracts were recorded from 400 to 700 nm. The measurement of anthocyanin content was carried out by following the procedures described by Zhang et al. (2016), while contents of flavonoids and phenolics were determined according to Fukushima and Mazza (2000) with minor modifications. The content of total phenolics and flavonoids were calculated by the absorbance of the extract at 280 and 325 nm divided by the fresh mass (FM), respectively. Spectrophotometric analysis of the methanol extracts was conducted with the spectrophotometer (UV-Vis 2450, Shimadzu, Tokyo, Japan). The antioxidant capacity of each sample was expressed as μmol(DPPH) per g(FM) through a calibration curve established by a DPPH solution series (10–120 μM).

O2•− localization in situ: O2•− localization was conducted following the procedure described by Romero-Puertas et al. (2004) with some modifications. O2•− production was visualized by nitroblue tetrazolium (NBT) staining. Leaves were immersed and vacuum-infiltrated with a solution of 1 mg(NBT) ml^{-1} and 10 mM NaN 3 in 50 mM phosphate buffer (pH 6.4) for 10 min and subsequently incubated at room temperature for 3 h in the dark. After staining, leaves were boiled in 95% ethanol to remove Chl and photographed by digital camera (Canon, Eos-60D, Tokyo, Japan).

Data analysis: All reported data represent the average of five replicates and are expressed as the mean ± standard error (SE). Statistical analysis was performed using IBM SPSS Statistics 19.0 software (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was performed at the 5% level using Tukey’s test. The data were graphed using SigmaPlot 12.5 software (Systat Software, San Jose, CA, USA).

Results

Screening for homozygous mutants with a T-DNA
**insertion of GLDH:** SALK_060087 is the mutant that the T-DNA insertion site was at the 3' end and the open reading frame of GLDH (Fig. 1A). A semi-quantitative PCR analysis of 28-d-old seedlings showed that GLDH expression (SALK_060087) was significantly down-regulated in the heterozygous mutant relative to the WT (Fig. 1B). However, we could not screen any homozygote from SALK_060087 after double round of PCR (Fig. S1, supplement). The SALK_008236 mutant had a T-DNA insertion at the 5' end of the non-coding region (5' UTR) of the GLDH locus (Fig. 1A). Unlike SALK_060087, SALK_008236 were screened out five homozygous after two times of PCR protocols (Fig. 2S, supplement).

Unexpectedly, quantitative PCR analysis of 28-d-old plants showed that the homozygous line, which was screened from the SALK_008236 plants, was a GLDH overexpression mutant (hereafter GLDH-236OE) (Fig. 1C). The relative transcript level of GLDH in GLDH-236OE was 9.4-fold higher than that in the WT. Due to the up-regulation of GLDH, the contents of total AsA were 5.4% higher in GLDH-236OE than that in the WT (Fig. 1D).

Thus, we selected GLDH-236OE, a line with overexpressing GLDH and AsA, as well as WT for experiments. (Figs. 2A; 3SA, supplement). The leaves of the two lines were not significantly affected by HL and appeared healthy green. The two lines showed slight leaf chlorosis after 14-d HL treatment, and the old leaves of WT were partly withered. In addition, leaves of GLDH-236OE and WT appeared red after 14-d of HL treatment. But surprisingly, leaves of GLDH-236OE were redder than that of WT (Fig. 2A).

**Changes in AsA content under HL:** Under normal growth conditions, GLDH-236OE overexpressed GLDH gene and accumulated more AsA (Fig. 1B, C). In order to judge the role of AsA in HL stress, and whether the slight superiority of GLDH-236OE in phenotype was due to the higher AsA concentration or not, we measured the Asc and AsA content of two lines during HL treatment. We showed that Asc and AsA contents of two lines were rising steadily with the extension of treatment time (Fig. 2B, C). GLDH-236OE line maintained the significantly higher AsA content than that of WT during HL treatment (Fig. 2B). In addition, the effective Asc content was significantly higher in GLDH-236OE than that in WT after 14 d of HL treatment (Fig. 2C).

**Changes in photosynthetic pigments under HL:** In the initial phase of HL treatment (from 1st day to 7th day of HL treatment), there was no significant difference between two lines in the total Chl content which was consistent with the phenotype. The total Chl content sharply decreased after 14-d HL treatment; it declined more severely in WT than that of GLDH-236OE (Fig. 3C). The content of Chl a,
Fig. 2. Different phenotype responses (A), Asc (reduced ascorbic acid), B) and AsA (L-ascorbic acid, C) content in WT and GLDH-236OE under HL [280 μmol(photon) m⁻² s⁻¹] stress. Phenotype on 0-d, 7-d, 14-d HL are shown in (A) from the first row to the third row. WT and GLDH-236OE are the first two plants on the left and right, respectively. Values are means ± SE (n = 5). The Student's t-test analysis indicates a significant difference (compared with the WT, *P<0.05; **P<0.01).

Fig. 3. Changes of chlorophyll a (A), chlorophyll b (B), total chlorophylls (C), and carotenoid (D) content in WT and GLDH-236OE under HL [280 μmol(photon) m⁻² s⁻¹] stress. Values are means ± SE (n = 5). The Student's t-test analysis indicates a significant difference (compared with the WT, **P<0.01).
Chl b, and Car showed the similar pattern as that of total Chl (Fig. 3).

**Changes in Chl fluorescence under HL:** Under normal growth conditions, WT and GLDH-236OE had similar $F_v/F_m$, ETR, $\Phi_{PSII}$, and $q_P$. On the 7th day of the HL treatment, the values of Chl fluorescence parameters were the same in two lines and were higher than that under normal growth conditions. After 14-d HL stress, subsequent HL stress resulted in decreased $F_v/F_m$, $\Phi_{PSII}$, ETR, and $q_P$ in rosette leaves of both WT and GLDH-236OE, but GLDH-236OE showed the higher values than that of WT (Fig. 4).

**Changes in anthocyanins, flavonoids, and total phenolics contents under HL:** Since the rosette leaves turned red, we extracted the leaves with methanol-HCl and scanned the spectra of extracts (Fig. 5A). There was an absorption peak around 530 nm, which was the typical absorption peak for anthocyanins. We estimated the content of anthocyanins, flavonoids, and total phenolics of two lines. The results showed that both lines accumulated anthocyanins after 14-d HL treatment, and GLDH-236OE accumulated more anthocyanins than WT (Fig. 5B). The contents of flavonoids and total phenolics also increased after HL stress (Fig. 5C, D). Compared with WT, GLDH-236OE showed significantly higher flavonoid content at the initial phase of HL treatment while maintained a significantly higher total phenolic content during HL stress (Fig. 5C, D).

**Antioxidant capacity and localization of O$_2^•-$ compounds:** Considering a different content of antioxidants (AsA, anthocyanins, flavonoids, phenolics, and Car), we determined antioxidant capacity. Our results showed that antioxidant capacity increased during HL stress in both lines (Fig. 6A). There was no statistical difference between their antioxidant capacities at the initial phase of HL treatment, but GLDH-236OE exhibited significantly greater antioxidant capacity than that of WT after 14 d of treatment (Fig. 6A). We located O$_2^•-$ by its reaction with NBT. We showed that there was slightly bluer formazan precipitation in leaves after HL treatment than that under normal growth conditions. Compared to GLDH-236OE, the leaf of WT accumulated more of formazan precipitation (blue-colored product) near petiole after 14 d of HL treatment (Fig. 6B).

**Discussion**

GLDH-236OE is a homozygote mutant with GLDH-overexpression: Surprisingly, the homozygote (designated GLDH-236OE), which was isolated from the SALK 008236 line, was a mutant overexpressing GLDH. The relative gene transcript level of GLDH in GLDH-236OE was significantly higher than that in the WT (Fig. 1C), resulting in higher AsA contents in GLDH-236OE plants than that in the WT (Fig. 1D). Why was the expression of GLDH gene up-regulated in GLDH-236OE, contrary to our expectations? The results of promoter analysis may provide a clue. The regulation of GLDH gene expression may be associated with the core sequence...
(GGTTAA) of the GT cis element (GT-1) (Mehrotra and Panwar 2009). GT-1 was originally characterized in the promoter of the rbcS-3A gene in pea and was later identified in the promoters of various plant genes (such as the rice phyA gene) (Dehesh et al. 1990). Previous studies showed that removal of the GT-1 site from the RCA promoter increased the expression of a GUS reporter gene in roots by 100-fold (Orozco and Ogren 1993). The GT-1 site in the 5' UTR of the GLDH gene may be bound by a transcription factor that inhibits its expression. The T-DNA insertion would abolish the GT-1 site, thereby increasing the transcription of the GLDH gene.

(GLDH-236OE has higher resistance to HL stress: Seedlings of Arabidopsis wild type and GLDH-236OE (28-d-old) were subjected to HL [280 µmol(photon) m⁻² s⁻¹] to test their physiological and biochemical responses to HL stress. After exposure to HL for 14 d, some leaves were somewhat etiolated and even became brown in WT (Fig. 2A). WT showed slightly weaker resistance to HL stress than GLDH-236OE. Chls are important pigment in photosynthesis, which perform essential processes of harvesting light energy and driving electron transfer in the reaction centers (Fromme et al. 2003). Thus, the Chl content reflects the ability of leaves to absorb and utilize light. Car

Fig. 5. Changes of absorption spectra of methanol-HCl extracts (A), anthocyanins content (B), flavonols (C), and total phenolics (D) in GLDH-236OE and WT under HL [280 µmol(photon) m⁻² s⁻¹] stress for 14 d. Values are means ± SE (n = 5). The Student’s t-test analysis indicates a significant difference (compared with the WT, *P<0.05; **P<0.01).

Fig. 6. Changes in antioxidant capacity (A) and the accumulation of O₂⁻ in rosette leaves (B) of WT (the first column on the left) and GLDH-236OE (the first column on the right) under CK [100 µmol(photon) m⁻² s⁻¹] and HL [280 µmol(photon) m⁻² s⁻¹] stress for 14 d. Values are means ± SE (n = 5). The Student’s t-test analysis indicates a significant difference (compared with the WT, *P<0.05).
are essential structural components of the photosynthetic antenna and reaction center complexes (Barley and Scolnik 1995). Chls and Car of WT degraded more than that of GLDH-236OE after 14-d HL treatment (Fig. 3). It indicated that photosynthesis of WT was more severely impacted by HL than that of GLDH-236OE. The Chl fluorescence parameters, including \( F_{v}/F_{m} \), ETR, \( \Phi_{psii} \), and \( q_{p} \), are commonly used to measure photosynthetic function in study on resistance to stress (Frachbrode and Leipner 2003). Our results showed that \( F_{v}/F_{m} \), ETR, \( \Phi_{psii} \), and \( q_{p} \) of two lines decreased after 14 d of HL treatment, which reflected the damaged photosynthetic apparatus under HL (Fig. 4). But a reduction of Chl fluorescence parameters in GLDH-236OE was significantly lower than that of WT (Fig. 4). The results suggested that GLDH-236OE had the greater number of open PSII reaction center traps, higher electron transport activity, and higher photochemical dissipation capacity. In short, GLDH-236OE showed a lesser sensitivity and higher tolerance to HL stress.

The Asc and AsA contents of GLDH-236OE and WT increased after HL stress (Fig. 2B,C), which was consistent with a previous study (Zechmann et al. 2011). After 14-d HL treatment, the Asc and AsA contents were significantly higher in GLDH-236OE than that of WT, which reflected that the level of oxidation in WT was higher than that of GLDH-236OE (Fig. 2B,C). Apart from AsA, HL also induced the accumulation of anthocyanins, flavonoids, and phenolics on a similar time scale (Fig. 5), which are vital antioxidants and photoprotective substances (Zhu et al. 2018). Interestingly, GLDH-236OE accumulated significantly more anthocyanins and phenolics than WT during the HL treatment. The antioxidant capacity of GLDH-236OE was higher than that of WT due to higher antioxidant content (Fig. 6A).

Under normal conditions, chloroplasts are the major source of ROS in plants due to \( O_{2}^{-} \) yielded in Mehler reaction (Neill and Gould 2003). Under HL, more \( O_{2}^{-} \) was generated and then protonated to form hydroperoxyl radical, or dismutated by superoxide dismutase (SOD, EC 1.15.1.1) to H\(_2\)O\(_2\), so various forms of ROS occurred in the cell cytoplasm (Yamasaki et al. 1997, Zhang et al. 2018). ROS production is enhanced under HL or by other abiotic stresses (Suzuki et al. 2018). ROS directly attack organelles causing the PCD of the cell (Wituszyńska and Karpiński 2013). AsA is not only a part of enzymatic antioxidant GSH) system directly involved in the Mehler reaction. Additionally, AsA has been suggested as the coenzyme of ascorbate peroxidase (APX, EC 1.11.1.11) to scavenge ROS (Foyer and Halliwell 1976, Akram et al. 2017). Based on the above, AsA mitigates the damage of HL-induced ROS to photosynthetic apparatus. In addition to chloroplasts, AsA is also involved in ROS detoxification in peroxisomes, mitochondria, cell cytosol, and even apoplast (Plöchl et al. 2000, Mittler et al. 2004, Noctor et al. 2014). Apart from ROS neutralization and detoxification, the function of violaxanthin de-epoxidase (VDE) in the xanthophyll cycle, AsA participates in nonphotochemical quenching (NPQ) to dissipate excess light (Müller-Moulé et al. 2002). What is more, besides AsA, GLDH-236OE showed the advantage of accumulating more anthocyanins, flavonoids, and phenolics during HL stress. Higher accumulation of antioxidants means a higher antioxidant capacity to scavange HL-induced ROS. But apart from antioxidation, anthocyanins can also absorb part of the light energy as light attenuation. As apparent from Fig. 5A, the absorption spectra (400–700 nm) of methanol-HCl extracts from leaves showed that anthocyanins could absorb light of specific wavelengths around 530 nm, which are green wavelengths. Anthocyanins can mask most of green light but less blue and red light which are the main lights used in photosynthesis (Neill and Gould 2003). So anthocyanins screen excess light but have a slight effect on photosynthesis-effective light. GLDH-236OE, thereby, had the higher tolerance to HL for a higher capacity of scavenging ROS and shielding excess light.

The more AsA, the more anthocyanins: Leaves of GLDH-236OE and WT turned red after HL stress as in previous studies (Kim et al. 2017). The absorption spectra (400–700 nm) of methanol-HCl extracts from leaves proved that the red material in leaves was anthocyanin (Figs. 2A, 5A). But GLDH-236OE over-accumulated anthocyanins compared with WT under HL stress. There are many developmental and environmental factors affecting the accumulation of anthocyanins (Xu et al. 2017). But why GLDH-236OE, which had the higher AsA content, accumulated more anthocyanins? Previously, the ascorbate-deficient mutants vtc1, vtc2, and vtc3 accumulated less anthocyanins than that of WT during HL acclimation (Page et al. 2012), which implied the potential link between anthocyanins and ascorbic acid. Here, we attempted to envisage the reason for the higher amount of anthocyanins in GLDH gene-overexpressing line. On the one hand, AsA might stabilize enzymes involved in anthocyanins biosynthesis to promote the synthesis of anthocyanins. There are three 2-oxoglutarate-dependent dioxygenases (2-ODDs) in the anthocyanin biosynthetic pathway: flavanone 3-hydroxylase (F3H, EC 1.14.11.9), flavonol synthase (FLS, EC 1.14.11.23), and leucoanthocyanidin dioxygenase (LDOX, EC 1.14.11.19) (Pelletier 1997). 2-ODDs generally require AsA to prevent their inactivation by reducing over-oxidized Fe\(^{III}\) to Fe\(^{II}\) in the active site (Page et al. 2012). Thus, more AsA maintains efficient activity of anthocyanin synthase. On the other hand, AsA stabilizes anthocyanins. Anthocyanins are generally transported and kept in the vacuole after synthesizing in the cytoplasm (Klein et al. 1996). More ASA in the vacuole relieves oxidative degradation.
of anthocyanins under stress. In order to evaluate the possibility mentioned above, future experiments should address how AsA induces biosynthesis of anthocyanins. A pure GLDH gene-overexpression line which GLDH was driven by the CaMV 35S promoter should be constructed for our further research, but not just the mysterious T-DNA insertion line, GLDH-236OE.

In conclusion, there was no homozygote identified from SALK_008236. In contrast, another T-DNA insertion line, SALK_008236, could be screening out homozygote, which is the AsA-up-regulated mutant. The HL-treatment experiment suggested that AsA plays an important role in the mitigation of HL-induced photooxidative damage. AsA over-accumulating line, SALK_008236OE plants had lesser sensitivity and higher tolerance to HL due to its higher capacity of scavenging ROS, shielding against excessive light and dissipating it as thermal energy. However, the deeper mechanism of endogenous AsA alleviating photooxidative damage, and the mysterious link between AsA and anthocyanins still need to be elucidated.

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