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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).

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Abbreviations: 2-ODDs – 2-oxoglutarate-dependent dioxygenases; ABRC – *Arabidopsis* Biological Resource Center; APX – ascorbate peroxidase; AsA – L-ascorbic acid; Asc – reduced ascorbic acid; DPPH – 1, 1-diphenyl-2-picrylhydrazyl; ETR – electron transport rate; F – corresponding fluorescence; F_0 – minimal fluorescence yield of the dark-adapted state; F3H – flavanone 3-hydroxylase; FLS – flavonol synthase; F_m – maximal fluorescence yield of the dark-adapted state; F_m' – maximal fluorescence yield of the light-adapted state; FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry; GLDH – L-galactono-1,4-lactone dehydrogenase; GSH – glutathione; HL – high light; LDOX – leucoanthocyanidin dioxygenase; NBT – nitroblue tetrazolium; NPQ – nonphotochemical quenching; PCD – programmed cell death; q_P – photochemical quenching coefficient; ROS – reactive oxygen species; SOD – superoxide dismutase; TCA – trichloroacetic acid; VDE – violaxanthin de-epoxidase; WT – wild type; Φ_{PSII} – effective quantum yield of PSII photochemistry.

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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 AsA 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. HL stress is a common stress for plants (Szymańska *et al.* 2017, Zeng *et al.* 2017). Demmig-Adams and Adams (1992) suggested that light stress results not from HL *per se*, but rather from an excess of absorbed light beyond that utilized in photosynthesis. 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 the water-splitting PSII complex (Mulo *et al.* 2008). AsA was previously reported to be an important antioxidant component in photoprotection under HL stress (Zechmann *et al.* 2011, Zeng *et al.* 2017). But what specific role does ascorbic acid play in the response to HL stress? Is it just an antioxidant?

In order to explore the effect of AsA on resistance to HL in *Arabidopsis thaliana* by varying the content of endogenous AsA, we purchased two *GLDH* T-DNA insertion mutant lines, SALK_060087 and SALK_008236, from the *Arabidopsis* Biological Resource Center (ABRC). Their identity was also confirmed by using PCR. Then we characterized the expression of the *GLDH* gene and the responses to HL stress in the screened homozygotes.

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 Wassenegger *et al.* (1994) was used as a PCR template to identify homozygous mutants. The specific primers were designed as follows: LP236, 5'-AGGGCTCTGTCAAGGTCAACAGGT-3'; RP236, 5'-AGACCCGATCCAACGGGACGA-3'; LP087, 5'-AGCAGACCCTCGCCAGAGAA-3'; RP087, 5'-AGCAAGACAGCCACAAACCCT-3'; and LB1, 5'-GCGTGGACCGCTTGCTGCAACT-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'-GTGGTATTATTCAGGTTGGTTGGGGC-3') and *GLDH*-KR (5'-AGTCCTGGACCCTATACTTCAC-3'). *UBQ* was selected as an internal reference gene and was amplified using the primers 5'-GACGCTTCATCTCGTCC-3' (forward) and 5'-CCACAGGTTGCGTTAG-3' (reverse).

Quantitative real-time PCR: Quantitative PCR was carried out using a real-time PCR system *7500* (*Applied Biosystems*) and the *SYBR Premix ExTaq^{TM} 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'-TTCTGGGCTTTGACTGTGG-3' (forward) and 5'-GGTGCTGGTATTGCTTCCT-3' (reverse). *TUB* was selected as the control gene and was amplified using the primers 5'-CCAGCTTTGGTGATTTGAAC-3' (forward) and 5'-CAAGCTTTCGGAGGTCAGAG-3' (reverse). Relative gene transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ 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* and 4°C 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} (16-h light/8-h dark, 23°C). 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 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). Chl and carotenoid (Car) concentrations were calculated as described by Lichtenthaler (1987).

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 (F_0) was determined using a weak modulated light [0.04 μmol(photon) m^{-2} s^{-1}]. Maximal fluorescence yield of the dark-adapted state (F_m) 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 (F_m') was monitored using a saturating pulse. The maximal quantum yield of PSII photochemistry (F_v/F_m), the effective quantum yield of PSII photochemistry (Φ_{PSII}), the electron transport rate (ETR) through PSII, and the photochemical quenching coefficient (q_P) were calculated according to Gray *et al.* (2003) and Schreiber *et al.* (1986) as follows: $F_v/F_m = (F_m - F_0)/F_m$; $\Phi_{PSII} = (F_m' - F)/F_m'$; ETR = Φ_{PSII} × PPFD [200 μmol(photon) m^{-2} s^{-1}] × 0.85 × 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:HCl (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 Fukumoto 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).

Antioxidant capacity assay: According to a previously reported procedure (Zhu *et al.* 2018), total antioxidants were extracted from 0.05 g of fresh leaves after homogenizing in 2 mL of 95% methanol and centrifuging at 4°C, 13,000 × *g* for 10 min. Antioxidant capacity was measured by the organic free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. The supernatant was mixed with coloured DPPH solution. The decrease of coloured DPPH solution absorbance was measured at 525 nm using 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).

$O_2^{\bullet-}$ localization *in situ*: $O_2^{\bullet-}$ localization was conducted following the procedure described by Romero-Puertas *et al.* (2004) with some modifications. $O_2^{\bullet-}$ 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. 1*A*). 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. 1*B*). 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. 1*A*). 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. 1*C*). The relative transcript level of *GLDH* in *GLDH*-236OE was 9-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. 1*C,D*). Thus, we selected *GLDH*-236OE, a line with overexpressing *GLDH* and AsA, as well as WT for experiments.

Changes in plant appearance under HL: The healthy 28-d-old WT and *GLDH*-236OE plants, which were growing under favorable light conditions, were then transferred to HL treatment at 280 µmol(photon) m^{-2} s^{-1}. After the 7-d HL, there was still no significant difference between the two lines with the exception of early flowering *GLDH*-236OE which were similar under normal light conditions (Figs. 2*A*; 3S*A, 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. 2*A*).

Changes in AsA content under HL: Under normal growth conditions, *GLDH*-236OE overexpressed *GLDH* gene and accumulated more AsA (Fig. 1*B*,*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. 2*B*,*C*). *GLDH*-236OE line maintained the significantly higher AsA content than that of WT during HL treatment (Fig. 2*B*). In addition, the effective Asc content was significantly higher in *GLDH*-236OE than that in WT after 14 d of HL treatment (Fig. 2*C*).

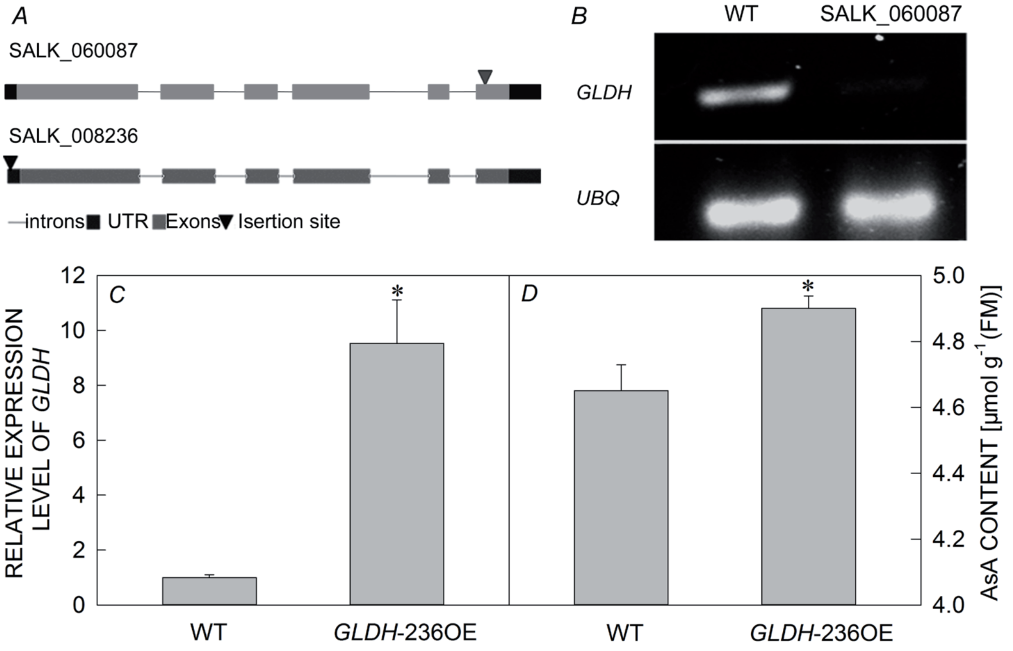

Fig. 1. (*A*) Schematic diagram of T-DNA insertion site at 3' end of *GLDH* locus in SALK_060087 and 5' T-DNA insertion site in *GHLD* locus in SALK_008236. (*B*) Semi-quantitative PCR of *GLDH* gene based on *UBQ*. Relative transcript levels of *GLDH* based on *TUB* (*C*), and AsA (total ascorbic acid) content (*D*) in leaves of *GLDH*-236OE compared with WT. 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).

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. 3*C*). The content of Chl *a*,

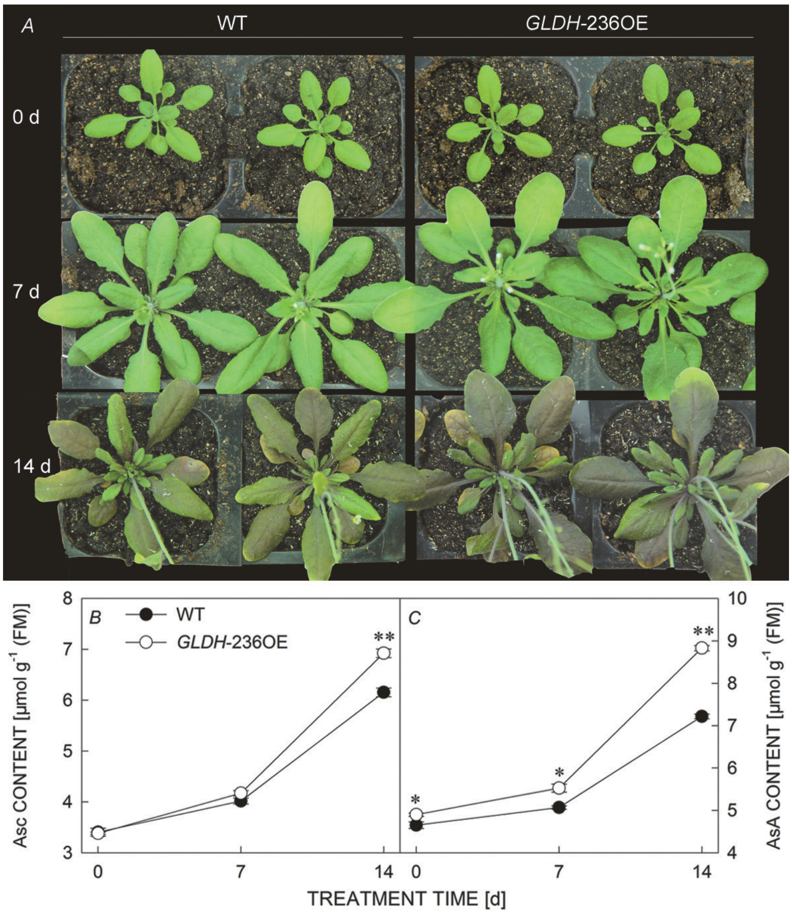

Fig. 2. Different phenotype responses (*A*), Asc (reduced ascorbic acid, *B*) and AsA (L-ascorbic acid, *C*) content in WT and *GLDH*-236OE under HL [280 μmol(photon) m^{-2} s^{-1}] stress. Phenotype on 0-d, 7-d, 14-d HL are shown in (*A*) from the first row to the third row. WT and *GLDH*-236OE are 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, $*P<0.05$; $**P<0.01$).

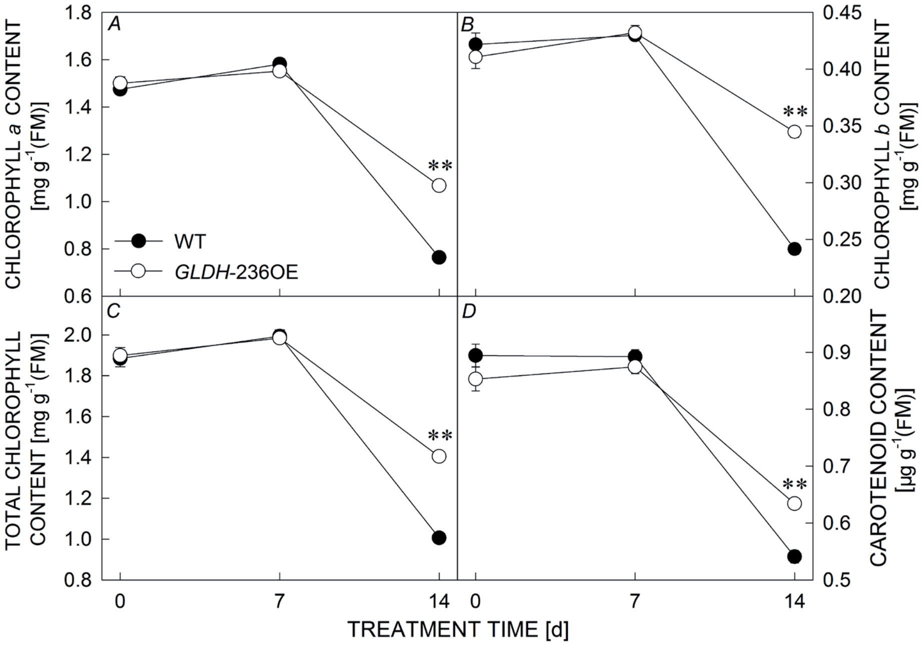

Fig. 3. Changes of chlorophyll *a* (*A*), chlorophyll *b* (*B*), total chlorophylls (*C*), and carotenoid (*D*) content in WT and *GLDH*-236OE under HL [280 μmol(photon) m^{-2} s^{-1}] stress. Values are means ± SE ($n = 5$). The *Student*'s *t*-test analysis indicates a significant difference (compared 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, Φ_{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, Φ_{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. 5*A*). 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. 5*B*). The contents of flavonoids and total phenolics also increased after HL stress (Fig. 5*C*,*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. 5*C*,*D*).

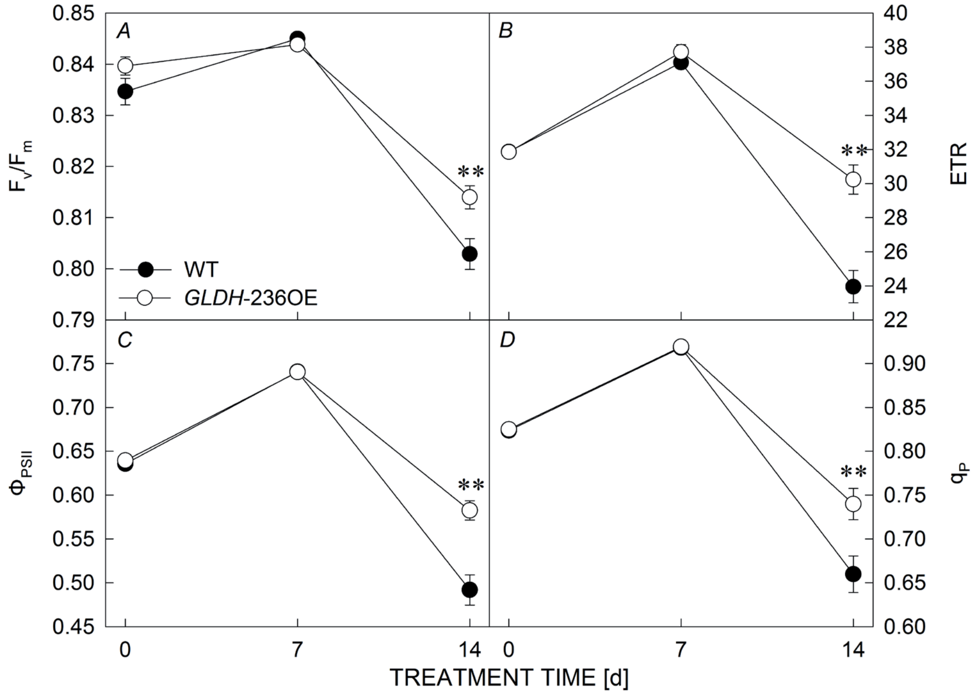

Fig. 4. Changes of chlorophyll fluorescence parameters in WT and *GLDH*-236OE under HL [280 μmol(photon) m^{-2} s^{-1}] stress. (*A*) F_v/F_m – maximal quantum yield of PSII photochemistry. (*B*) ETR – electron transport rate. (*C*) Φ_{PSII} – effective quantum yield of PSII photochemistry. (*D*) q_P – photochemical quenching coefficient. Values are means ± SE (*n* = 5). The *Student*'s *t*-test analysis indicates a significant difference (compared with the WT, $**P<0.01$).

Antioxidant capacity and localization of $O_2^{\bullet -}$ 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. 6*A*). 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. 6*A*). We located $O_2^{\bullet -}$ 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. 6*B*).

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. 1*C*), resulting in higher AsA contents in *GLDH*-236OE plants than that in the WT (Fig. 1*D*). 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

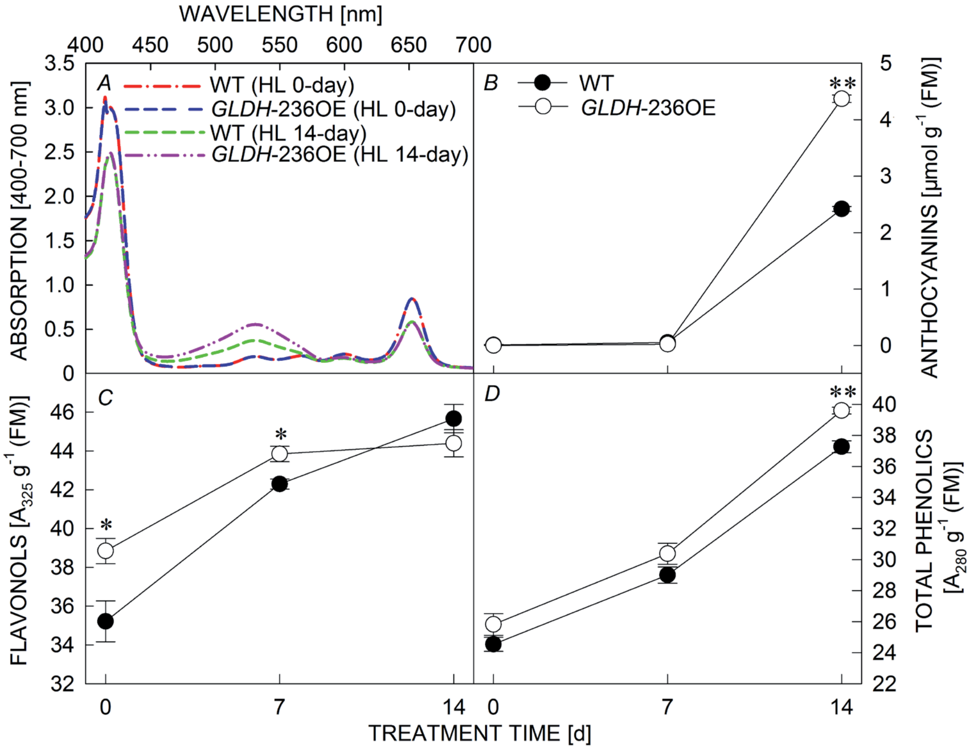

Fig. 5. Changes of absorption spectra of methanol-HCl extracts (*A*), anthocyanins content (*B*), flavonols (*C*), and total phenolics (*D*) in *GLDH*-236OE and WT under HL [280 μmol(photon) m^{-2} s^{-1}] stress for 14 d. Values are means ± SE ($n = 5$). The *Student*'s *t*-test analysis indicates a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

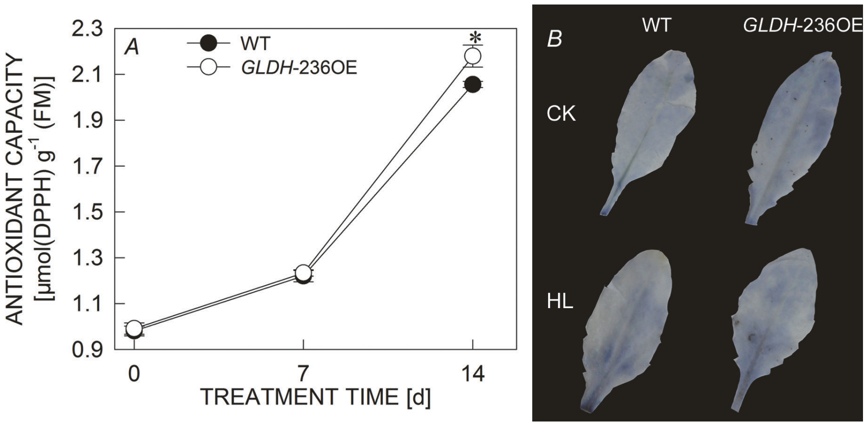

Fig. 6. Changes in antioxidant capacity (*A*) and the accumulation of $O_2^{\bullet-}$ in rosette leaves (*B*) of WT (the first column on the left) and *GLDH*-236OE (the first column on the right) under CK [100 μmol(photon) m^{-2} s^{-1}] and HL [280 μmol(photon) m^{-2} s^{-1}] stress for 14 d. Values are means ± SE ($n = 5$). The *Student*'s *t*-test analysis indicates a significant difference (compared with the WT, $^*P<0.05$).

(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. 2*A*). 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 are essential structural components of the photosynthetic antenna and reaction center complexes (Bartley 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, Φ_{PSII}, and q_P, are commonly used to measure photosynthetic function in study on resistance to stress (Fracheboud and Leipner 2003). Our results showed that F_v/F_m, ETR, Φ_{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.

The Asc and AsA contents of *GLDH*-236OE and WT increased after HL stress (Fig. 2*B*,*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. 2*B*,*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. 6*A*).

Under normal conditions, chloroplasts are the major source of ROS in plants due to $O_2^{\bullet-}$ yielded in Mehler reaction (Neill and Gould 2003). Under HL, more $O_2^{\bullet-}$ was generated and then protonated to form of hydroperoxyl radical, or dismutated by superoxide dismutase (SOD, EC 1.15.1.1) to H_2O_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.* 2012). Here, $O_2^{\bullet-}$ was located in leaves as visualized by a reaction of NBT with $O_2^{\bullet-}$ (Romero-Puertas *et al.* 2004). NBT staining results showed that the content of $O_2^{\bullet-}$ slightly increased after HL stress, and *GLDH*-236OE accumulated somewhat less $O_2^{\bullet-}$ than WT (Fig. 6*B*). ROS directly attack organelles and cause plant cells to suffer oxidative stress, finally causing the PCD of the cell (Wituszyńska and Karpiński 2013). AsA is not only a part of enzymatic antioxidant defense system but also plays an important role in the nonenzymatic antioxidant system, which increased its efficiency and contribution to ROS neutralization and detoxification (Akram *et al.* 2017). Nakano and Asada (1981) considered that ROS detoxification in chloroplasts was mainly attributed to the ascorbate-glutathione (AsA-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, as the coenzyme 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 scavenge HL-induced ROS. But apart from antioxidation, anthocyanins can also absorb part of the light energy as light attenuation. As apparent from Fig. 5*A*, 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. 2*A*, 5*A*). 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 ther inactivation by reducing over-oxidized Fe^{IV} 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_060087. 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 *GLDH*-236OE 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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