Journal of Plant Physiology 209 (2017) 31–41

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Overexpression of tomato *SlGGP-LIKE* gene improves tobacco tolerance to methyl viologen-mediated oxidative stress

Dong-Yue Yang [1], Na-Na Ma [1], Kun-Yang Zhuang, Shao-Bo Zhu, Zhong-Ming Liu, Xing-Hong Yang *

College of Life Science, State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an, Shandong 271018, PR China

ARTICLE INFO

Article history:
Received 11 April 2016
Received in revised form 25 October 2016
Accepted 28 October 2016
Available online 25 November 2016

Keywords:
Tobacco
AsA
ROS
GGP
Methyl viologen
Violaxanthin de-epoxidase

ABSTRACT

Ascorbate (AsA) is very important in scavenging reactive oxygen species in plants. AsA can reduce photoinhibition by xanthophyll cycle to dissipate excess excitation energy. GGP is an important enzyme in AsA biosynthesis pathway in higher plants. In this study, we cloned a gene, *SlGGP-LIKE*, that has the same function but different sequence compared with *SlGGP*. The function of *SlGGP-LIKE* gene in response to oxidative stress was investigated using transgenic tobacco plants overexpressed *SlGGP-LIKE* under methyl viologen treatment. After oxidative stress treatment, transgenic tobacco lines exhibited higher levels of reduced AsA content and APX activity than WT plants. Under oxidative stress, transgenic tobacco plants accumulated less ROS and exhibited lower degrees of REC and MDA. Consequently, relatively higher levels of Pn, Fv/Fm, de-epoxidation status of xanthophyll cycle and D1 protein were maintained in transgenic tobacco plants. Hence, overexpression of *SlGGP-LIKE* gene enhances AsA biosynthesis and can alleviate the photoinhibition of PSII under oxidative stress.

1. Introduction

In plants, ROS are generated during the metabolic process in cells, and the generation and scavenging of ROS maintain dynamic balance. When plants subjected to environmental stresses, the accumulation of ROS is rapid and plants have formed mechanisms to detoxify ROS. Ascorbate (AsA) is an essential material in protecting plants from damage of excess ROS that generated during abiotic or biotic stresses (Conklin, 2001; Foyer and Noctor, 2005; Müller-Moulé et al., 2004; Laing et al., 2015). AsA can reduce photoinhibition by the xanthophyll cycle which can promote the conversion of violaxanthin (V) to zeaxanthin (Z) to eliminate excess light energy (Chen and Gallie, 2006; Gallie, 2013; Miyaji et al., 2015). In addition, AsA can also donate electron in the elimination of H_2O_2 catalyzed by APX. In particular, chloroplasts use AsA to eliminate the ROS produced by photosystem II (PSII) for the synthesis of NADPH in the stroma. AsA is drawn into the elimination of excess energy via heat dissipation in the xanthophyll cycle acting as a coenzyme.

The enzyme GGP (GGP: EC 2.7.7.69) is vital important in the Smirnoff-Wheeler pathway (L-galactose pathway) for AsA synthesis (Wheeler et al., 1998). Overexpression of GGP genes can increase the AsA concentrations in plants (Smirnoff, 2000; Laing et al., 2007; Bulley et al., 2009, 2012), thereby indicating that it may balance fluxes between AsA and GDP-L-fucose function as a regulatory role. The *Arabidopsis thaliana vtc2* mutant, which encodes GGP in Arabidopsis, has less AsA content (Linster et al., 2007; Miyaji et al., 2015). Many results indicate that manipulating the gene that regulates the expression of AsA affects the stress tolerance of plants (Kwon et al., 2002; Lim et al., 2007; Li et al., 2010; Wang et al., 2013a,b).

GGP exists in majority of plants, whereas tomato has two GGP genes (*SlGGP* and *SlGGP-LIKE*) which are present in chromosomes 6 and 2, respectively. GGP is vital to plants to maintain the AsA pool, and this gene has been reported in tomato and tobacco (Laing et al., 2007, 2015; Bulley et al., 2009; Wang et al., 2013a,b). However, the

Abbreviations: A, antheraxanthin; Fv/Fm, the maximal photochemical efficiency of PSII; GDP, guanosine 5′-diphosphate; GFP, green fluorescent protein; GGP, GDP-L-galactose phosphorylase; HPLC, high performance liquid chromatography; MDA, malondialdehyde; MV, methyl viologen; PBS, phosphate buffer saline; PSII, photosystem II; $O_2^{\bullet-}$, superoxide anion radical; Pn, net photosynthetic rate; PFD, photon flux density; PVDF, polyvinylidene fluoride; REC, relative electronic conductance; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; V, violaxanthin; VDE, violaxanthin de-epoxidase; WT, wild-type; Z, zeaxanthin; ZEP, zeaxanthin epoxidase.

* Corresponding author at: State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, 61 Dai Zong Street, Tai'an, Shandong 271018, PR China.
E-mail address: xhyang@sdau.edu.cn (X.-H. Yang).
[1] Both these authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jplph.2016.10.013
0176-1617/

function of *GGP-LIKE* is less reported compared with that of *GGP* gene.

In this study, a tomato gene *SlGGP-LIKE* which encoded GDP-L-galactose phosphorylase and located in nucleus, was cloned and transformed into tobacco. The expression of *SlGGP-LIKE* can be induced by chilling stress (4 °C), high salinity, oxidative stress, high light, salicylic acid, methyl jasmonate, and pathogen attack. In our experiments, we used MV to simulate oxidative stress. Under MV treatment, transgenic plants exhibited higher levels of reduced AsA concentration and activity of APX than WT plants, and thus detected less ROS content in transgenic plants. GGP-LIKE is also important in improving the de-epoxidation status of xanthophyll cycle by increasing AsA content under oxidative stress.

2. Theory

ROS are produced inevitably in cell metabolism such as respiration and photosynthetic electronic transmission and will attack the biomacromolecule such as protein, nucleic acid, lipids and induce oxidative damage to cell and tissues if they could not be scavenged efficiently under environmental stresses. In order to analyze the roles of *SlGGP-LIKE* gene under oxidative stress, we obtained *SlGGP-LIKE*-overexpressed transgenic tobacco plants and measured the changes of Pn, REC and MDA in WT and transgenic lines under oxidative stress. The transgenic plants exhibited an elevated endurance against oxidative stress compared with WT plants after MV treatment.

3. Materials and methods

3.1. Plant materials, growth, and treatments

Wild tomato cultivar (*Solanum lycopersium* cv. Zhongshu 6) was used as plant materials.

Seeds were sown on MS agar medium after sterilized and incubated in an incubator at 25 °C (light 16 h/dark 8 h) for 8 d. Young seedlings were planted in sterilized soil and grown in a greenhouse. Eight-week-old tomato seedlings were treated with various stresses. For chilling treatment, the plants were exposed to 4 °C in the incubator. For high light treatment, the plants were exposed to 2000 μmol m^{-2} s^{-1} light from daylight-type microwave sulfur lamps (MSL-1000; Youhe, Ningbo, China). Salinity stress was performed by immersing the whole plants in 200 mM NaCl solution. The plant leaves were sprayed with 100 μM MeJA, 100 μM SA to simulate hormone treatments. For oxidative treatment, the plants were sprayed with 100 μM MV. For pathogen treatment, *Pseudomonas syringae* pv. tomato DC3000 suspensions [1×10^8 cfu (colony forming units) ml^{-1}] were used to infect the leaves. The treated leaves were harvested, frozen in liquid nitrogen, and stored at −80 °C.

3.2. Subcellular localization of SlGGP-LIKE

The full-length cDNA of *SlGGP-LIKE* was cloned and two DNA constructs (p35S-*GFP* and p35S-*SlGGP-LIKE-GFP*) were obtained. *Nicotiana benthamiana* seedlings were injected with the above two constructs and epidermis was examined by dual channel confocal microscopy (LSM510 META; Zeiss, Germany).

3.3. Obtainment of transgenic tobacco

The full-length of *SlGGP-LIKE* gene was cloned with primers GGPL-F and GPL-R. Subsequently, the PCR product was subcloned into the botany expression vector pBI121 with 35S promoter upstream to construct overexpression constructs (pBI-*SlGGP-LIKE*). The recombinant plasmids (pBI121-*SlGGP-LIKE*) were transferred into tobacco plants using *Agrobacterium* strain EHA105-mediated cotyledon infiltration. After a few weeks, 14 independent kanamycin-resistant lines were achieved. These self-pollinated transgenic tobacco plants (T_0 generation) then produce seeds of T_1 generation. The seeds of T_1 generation were planted on Murashige–Skoog (MS) agar medium at 25 °C, a 16-h light/8-h dark photoperiod and photon flux density (PFD) of 600 μmol m^{-2} s^{-1}. After two weeks, seedlings were cultivated into vermiculite and grown in the greenhouse.

3.4. Plant growth

WT seeds of tobacco (*Nicotiana tabacum* L. cv. NC89) and tomato (*Solanum lycopersicum* cv. Zhongshu 6) were germinated on MS agar media, whereas transgenic tobacco seeds were germinated on MS agar media with kanamycin (50 μg mL^{-1}) in tissue culture vessels for a week at 25 °C, then the plants were moved into vermiculite and grown in greenhouse. Eight-week-old tomato and eight-week-old transgenic tobacco plants were applied to oxidative stress and qRT-PCR assays.

3.5. Methyl viologen treatment

Plants were sprayed with 100 μM of MV evenly and deionized water as control, and then the plants were placed into a chamber with PFD of 200 μmol m^{-2} s^{-1} for 24 h continuously. Detached leaves were put into liquid nitrogen quickly and stored at −80 °C until use.

3.6. Germination analysis

To analyze the influence of MV on seed germination, seeds of WT and transgenic tobacco were cultivated on MS agar media with different concentrations of MV (0, 5 and 10 μmol L^{-1}) in Petri dishes. The seeds were subsequently placed in a chamber (GXZ-260C) with PFD of 200 μmol m^{-2} s^{-1}, with a constant temperature of 25 °C and a 16-h light/8-h dark photoperiod for about two weeks. Photographs were then taken, and the germination rate was calculated.

3.7. Determination of reduced, oxidized and total AsA

The reduced AsA (AsA) contents, oxidized AsA (DHA) contents and total ascorbates (AsA + DHA) content were determined according to Li et al. (2010) with a little modification. 0.5 g detached leaves were ground into homogenized with 2 mL 6% TCA and transferred to a centrifugal tube. After centrifuged at 12000 × *g* for 10 min at 4 °C, the supernatant was used to measure the content of AsA and oxidized ascorbic acid (DHA), AsA reaction solution including 0.2 mL extract (blank to 6% TCA instead), 0.6 mL 0.2 M PBS (pH 7.4), 0.2 mL ddH_2O, 1 mL 6% TCA, 0.8 mL 42% H_3PO_4, 0.8 mL 4% 2,2′-bipyridine and 0.4 mL 3% $FeCl_3$. The total AsA was (AsA + DHA) measured with 0.2 mL 10 mM dithiothreitol and 0.4 mL 0.2 M PBS (pH 7.4) instead of 0.6 mL 0.2 M PBS above, and the reaction solution bated 1 h in 42 °C. Absorbance was measured at 525 nm. The content of DHA can be measured by the difference of total AsA content and without DTT AsA content.

3.8. Measurement of H_2O_2 and $O_2^{\bullet -}$

About 0.5 g leaf samples were homogenised with 3 mL of pre-cooled acetone to measure H_2O_2 concentration. After centrifugation at 2000 × *g* for 10 min, 0.1 mL of 5% titanium sulphate and 0.2 mL of $NH_3 \cdot H_2O$ were added into the separated 1 mL supernatant. The mixture was centrifuged again and washed 3–5 times with 1 mL of acetone. Finally, the precipitate was dissolved in

5 mL of 2 M H_2SO_4 and then use acetone volume to 10 mL. The absorbance of the supernatant was measured at 415 nm.

Leaf tissues (0.5 g) were ground and homogenised with 5 mL of extracting solution and the homogenate was centrifuged at 12000 × g at 4 °C for 20 min. Supernatant (0.5 mL), phosphate buffer (0.5 mL, pH 7.8) and hydroxylammonium chloride (1 mM, 1 mL) was placed at 25 °C for 1 h 1 mL of 17 mM *p*-aminobenzene sulfonic acid and 1 mL of 7 mM α-naphthylamine were then added, and then the mixture was placed at 25 °C for another 20 min. Absorbance was measured at 530 nm.

3.9. Measurement of REC and MDA

Diameter of 0.8 cm each of 15 leaf discs were placed into scale test tubes that contained 15 mL of sterile water and shaken for 2 h, and then the initial electric conductance (S1) was measured. The final electric conductance (S2) was measured after the tubes were put into boiling water for 30 min. Blank is deionized water (S0). The REC was evaluated as follows: REC [%] = (S1 − S0)/(S2 − S0) × 100.

Approximately 0.5 g leaf tissues were ground into homogeneous with 8 mL of 10% trichloroacetic acid (TCA), and then centrifuged at 10000 × *g* for 10 min at 4 °C, 2 mL of 0.6% thiobarbituric acid (TBA) reagent was added into 2 mL of the supernatant, boiled for 15 min, cooled with ice and centrifuged at 3000 × *g* for 10 min. Absorbance was measured at 450, 532, and 600 nm.

3.10. Leaf disk experiment under MV treatment and measurement of chlorophyll content

Fifteen leaf disks (0.8 cm, diameter) of WT and transgenic plants were placed into 9 cm Petri dishes, and every dish contained 15 mL solution of various MV concentrations (0, 50, and 100 μM). These Petri dishes were exposed to continuous light for 48 h, with a PFD of 200 μmol m^{-2} s^{-1}. In order to determine chlorophyll content, the leaf disks were put in test tubes with 10 mL of 80% acetone, and the tubes were put in dark conditions for 48 h until leaf colour faded completely. Absorbance was measured at 663, 470, and 646 nm.

3.11. Determination of APX activity

The activity of APX was determined according to the decline of absorbance at 290 nm, as described by Kwon et al. (2002).

3.12. Measurement of Pn and chlorophyll fluorescence

Pn was measured by a portable photosynthetic system (CIRAS-2; PP Systems, Herts, UK) according to the instructions. Before measurement, plants were placed at 25 °C and 100 μmol m^{-2} s^{-1} of PFD for 30 min to make stomatal openings.

According to the protocol, chlorophyll fluorescence was evaluated by using a pulse-activated modulation fluorometer (FMS-2; Hansatech, Cambridge, UK). Plants were acclimated in the dark for 30 min before measurement.

3.13. Quantitative RT-PCR analysis

Quantitative RT-PCR was taken with a Bio-Rad CFX96™ RT-PCR with SYBR real master mix (Tiangen, Beijing, China). *SlEF1a* (acc. No. X144491) and NtUbiquitin (acc. No. U66264) were employed as internal reference genes.

3.14. Analysis of xanthophyll cycle components

Detached leaves were homogenized with 80% cooled acetone, and then centrifuged at 12000 × *g* for 10 min before filtration of the supernatants. The pigment was separated by reversed-phase high-performance liquid chromatography. The de-epoxidation state analysis of the xanthophyll cycle was obtained as (Z + 0.5A)/(V + A + Z).

3.15. Western blot analysis

The ORF sequence of *SlGGP-LIKE* was sub-cloned into pET-30a (+) vector between the BamHI and SalI sites. The recombined vector pET-*SlGGP-LIKE* was transferred into *Escherichia coli* BL21 (DE3). The recombinant *SlGGP-LIKE* protein was obtained from the Abmart (Shanghai, China). The peroxidase-conjugated goat anti-mouse IgG was used as secondary antibody. Total proteins were separated by using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with 10% separating and 4% concentrating gels. Primary and secondary antibodies were used at dilutions of 1:1000 and 1:5000, respectively, in blot analysis.

The extraction methods of thylakoid membrane refer to Zhang et al. (1999). The proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA), probed with D1 antibody. The secondary antibody was peroxidase-conjugated goat antirabbit IgG (Tiangen Biotech, Beijing, China).

3.16. Statistical analysis

Statistically significant differences were tested by Student's *t*-test. The bar represent the ±SD of three repeated experiments, and significant differences were indicated by * and ** in the figures relative to the control at $P < 0.05$ and $P < 0.01$, respectively.

4. Results

4.1. Analysis of the SlGGP-LIKE gene

The full-length cDNA of *SlGGP-LIKE* was obtained from the tomato cDNA library using PCR technology. Fig. 1A shows that the sequence of the open reading frame consisted of 1317 bp nucleotides, which encodes 438 amino acids with a molecular weight of 48 kDa. According to the result of amino acid sequence alignment, the obtained cDNA sequence included the conservative domain- NLS and HIT. NLS represents a nuclear localization sequence. HIT, namely, histidine triad (HIT- HxHxQ), which is a common feature of GDP-$_L$-galactose phosphatase family (Brenner et al., 2002). As shown in Fig. 1B, the isolated cDNA was highly homologous to GDP-$_L$-galactose phosphatase genes of other plants according to phylogenetic analysis, which means this gene coding GDP-$_L$-galactose phosphatase involved in the Smirnoff–Wheeler pathway for AsA synthesis; among them, *StGGP* gene which is isolated from potato was the most homologous to the isolated cDNA; meanwhile, the isolated cDNA showed a close relationship with *NtGGP*. However, the homology between the isolated cDNA on chromosome 2 and another known gene *SlGGP* on chromosome 6 was only 59% (Supplementary Table S2, Fig. S1). These results indicate that an unknown sequence of tomato GDP-$_L$-galactose phosphatase gene which is different from *SlGGP* was successfully cloned, and the gene was named *SlGGP-LIKE*.

4.2. Expression patterns of SlGGP-LIKE

The expression pattern of *SlGGP-LIKE* was detected using qRT-PCR analysis in different tissues of tomato. As shown in Fig. 2A, the expression was highest in leaves and lowest in fruits.

According to qRT-PCR results, we analyzed the expression levels of *SlGGP-LIKE* under chilling (4 °C), high light (2000 μmol m^{-2} s^{-1}), salt (200 mM NaCl), oxidative (100 μM MV), SA (100 μM M), MeJA (100 μM) and biotic (*Pst*. DC3000) stresses. All of the tested stresses induced the expression of *SlGGP-LIKE* to different extents. Under

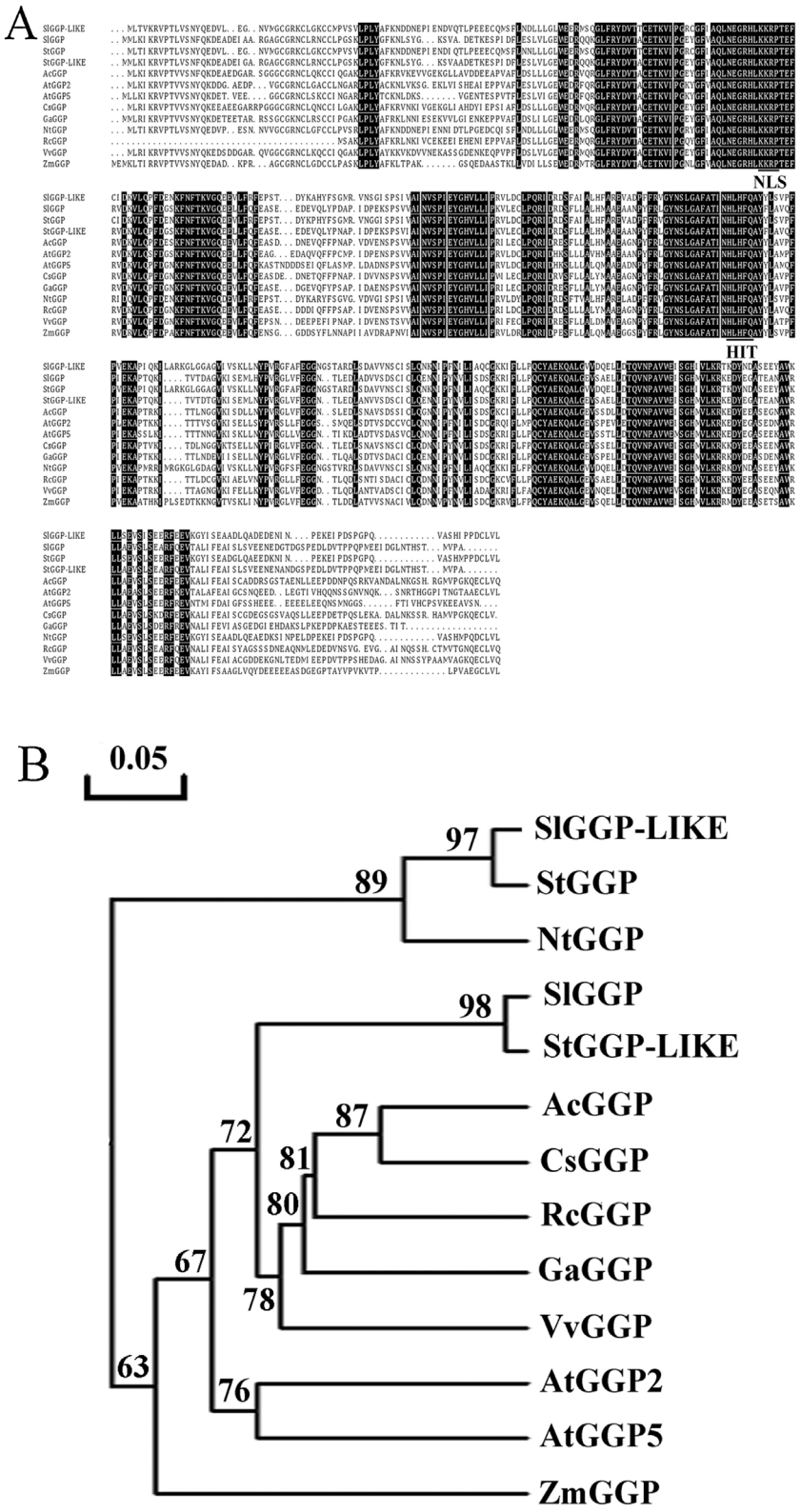

Fig. 1. Alignments of the deduced amino acid sequence of *SlGGP-LIKE* from different species. (A) Multiple alignment of the deduced amino acid sequences of *SlGGP-LIKE* with GGP genes from AcGGP (EF379384.1), *Actinidia chinensis*; AtGGP2 (At4g26850.1), *Arabidopsis thaliana*; AtGGP5 (AT5G55120.1), *Arabidopsis thaliana*; CsGGP (AGI78464.1), *Camellia sinensis*; GaGGP (KHG00569.1), *Gossypium arboretum*; NtGGP (AII99836.1), *Nicotiana tabacum*; RcGGP (EEF32929.1), *Ricinus communis*; SlGGP (JQ517313.1), *Solanum lycopersicum*; SlGGP-LIKE (XM_004231938.2), *Solanum lycopersicum*; StGGP (JN000933.1), *Solanum tuberosum*; StGGP-LIKE (JN000934.1), *Solanum tuberosum*; VvGGP (XP_002278339.1), *Vitis vinifera*; ZmGGP (EU966173.1), *Zea mays*. The conserved motifs (NLS and HIT) are indicated by dot underline in the amino acid sequence of *SlGGP-LIKE*. Identical and similar amino acid residues are shaded black, and dashes indicate the gaps introduced to optimise alignment. (B) Phylogenetic analysis of *SlGGP-LIKE* protein with other GGP proteins.

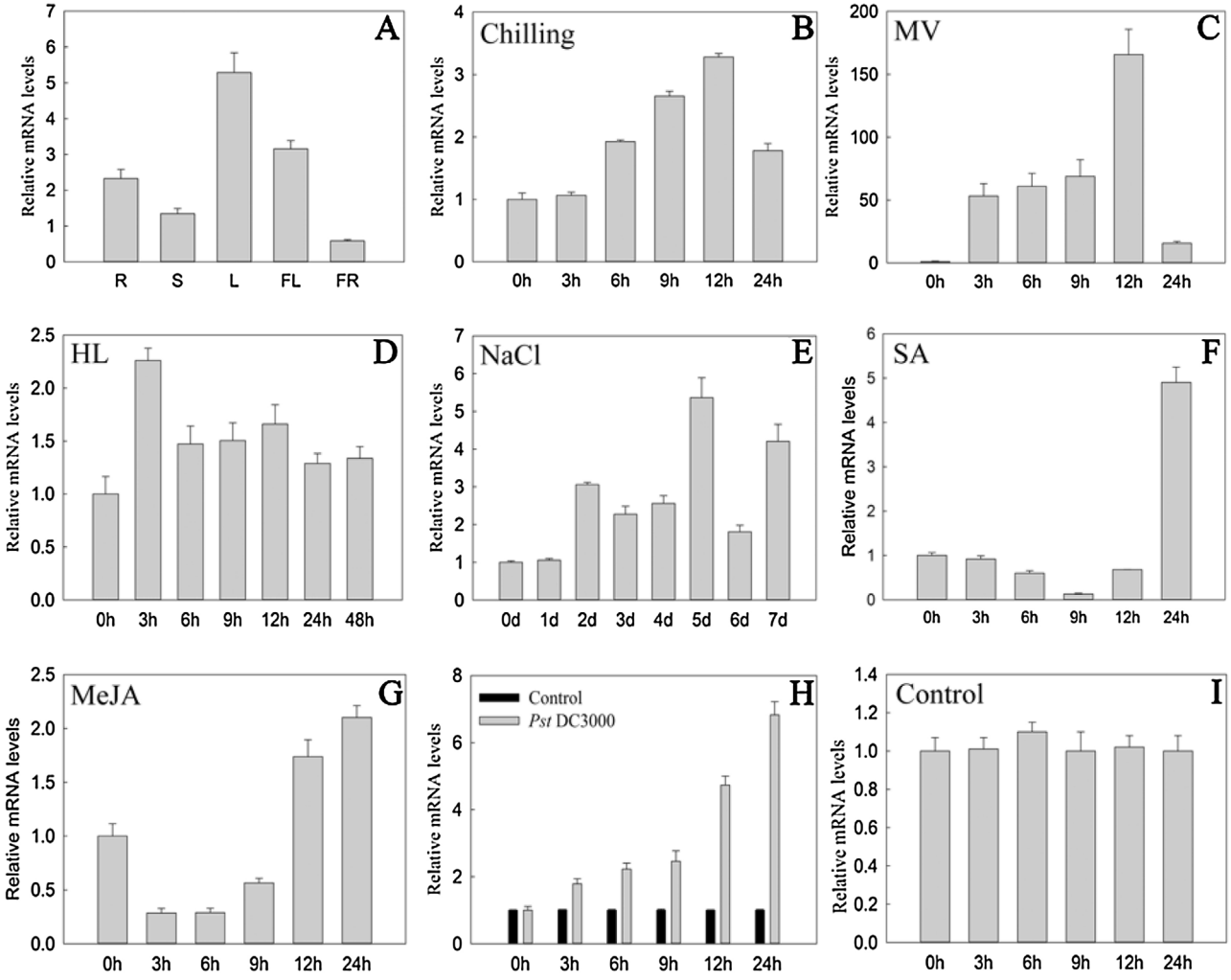

Fig. 2. Analysis of the expression patterns of *SlGGP-LIKE* in tomato. (A–H) (A) Expression of *SlGGP-LIKE* in the roots, stems, leaves, flowers, and fruits. Responses of *SlGGP-LIKE* to chilling (B), 100 μM MV (C), high light (2000 μmol m^{-2} s^{-1}) (C), 200 mM NaCl (E), 100 μM SA (F), 100 μM MeJA (F) and *Pst*. DC3000. The transcript level of *SlGGP-LIKE* was normalized to EF-1α expression. Error bars represent the SD of triplicate experiments.

chilling stress and MV treatments, the expression of *SlGGP-LIKE* was induced to a peak of the transcripts at 12 h and then decreased (Fig. 2B, C). High light stress induced the expression during the first 3 h, and the transcripts decreased to a stable level until 48 h (Fig. 2D). The transcriptional levels reached the maximum levels after treated with NaCl for 5 days and then decreased (Fig. 2E). After SA and MeJA treatment, the expression levels both reached maximum at 24 h (Fig. 2F, G). Biotic stress induced a sustained increase of gene expression and reached a maximum after 24 h (Fig. 2H). These results suggested that *SlGGP-LIKE* is involved in plant responses to various stresses.

4.3. Subcellular localization of SlGGP-LIKE gene

Based on the result of the amino acid sequence analysis, *SlGGP-LIKE* contains a nuclear localization signal sequence (NLS-KKRP). To confirm the prediction, we carried out transient expression experiments by using the *N. benthamiana* wih the recombinant vectors expressing 35S:GFP and 35S:SlGGP-LIKE-GFP fusion protein (Fig. 3). As shown in Fig. 3B, the 35S:GFP fusion protein distributed GFP signals evenly in the nucleus and cytoplasm, while the SlGGP-LIKE-GFP fusion protein was only found in the nucleus. These results proved that *SlGGP-LIKE* is localized in the nucleus.

4.4. Identification of transgenic plants

Fourteen individual kanamycin-resistant T_1 plants were evaluated with PCR. Approximately 1500-bp PCR product was acquired in transgenic plants, whereas no PCR product was observed in WT plants. Three independent T_1 lines showed high expression at mRNA level detected by qRT-PCR (Fig. 4A). Western blot analysis was performed to identify *SlGGP-LIKE* expression at the protein level. The results indicated that *SlGGP-LIKE* gene was successfully overexpressed in transgenic tobacco plants (Fig. 4B). OE-1, OE-3 and OE-6 lines which represented different expression levels were chosen for further analysis.

4.5. Overexpression of SlGGP-LIKE increased ascorbate content

To verify the function of the *SlGGP-LIKE* gene, we measured the reduced AsA, oxidized AsA (DHA), total AsA and AsA/DHA of all plants. The contents of reduced AsA increased to 1.51, 2.13 and 2.02 fold in OE-1, OE-3 and OE-6, respectively, compared with WT plants (Fig. 5A). Under oxidative stress, the degrees of reduced AsA in plants were all increased, and the ratios of AsA/DHA enhanced to 1.84, 2.31 and 1.99 fold in OE-1, OE-3 and OE-6, respectively, compared with WT plants (Fig. 5D). However, the total AsA con-

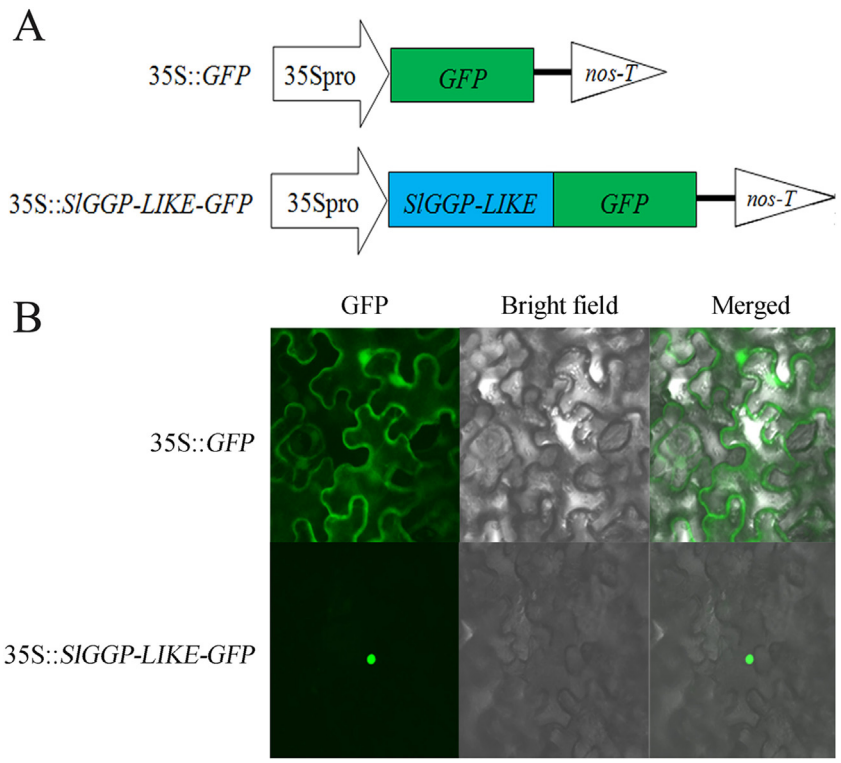

Fig. 3. Subcellular targeting of *SlGGP-LIKE* in *Nicotiana benthamiana*. (A) Schematic of the GFP fusion recombinant vector. (B) Transient expression of 35S:GFP and 35S: SlGGP-LIKE-GFP. Epidermis was examined using dual-channel confocal microscopy (LSM510 META; Zeiss, Germany).

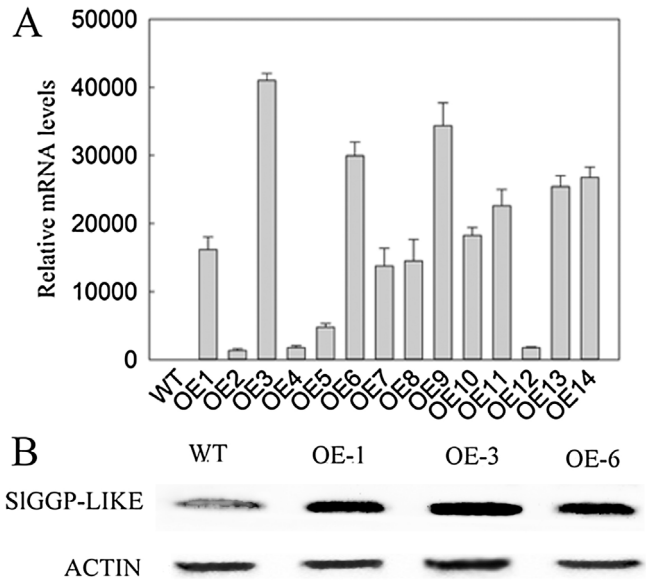

Fig. 4. Identification of transgenic plants via qRT-PCR and Western blot. (A) Transcript levels of *SlGGP-LIKE* in WT plants and different transgenic lines analysed by qRT-PCR. Transcript level of *SlGGP-LIKE* was normalised to NtUbiquitin expression. Error bars represent the SD of triplicate experiments. (B) SlGGP-LIKE protein was overexpressed in transgenic plants. β-Actin was used as the reference protein.

tent in transgenic plants did not change obviously, whereas that of WT plants increased to approximately 2.32-fold after treatment for 24 h. Therefore, overexpression of *SlGGP-LIKE* gene enhanced the reduced AsA content and enabled the transgenic tobacco plants to maintain higher levels of AsA content, which can improve plant resistance to oxidative stress effectively.

4.6. Growth analysis experiment under oxidative stress

Seeds of T_2 generation of transgenic lines and WT were grown on MS media contained different concentrations of MV (0, 5 and 10 μM) simultaneously and placed in the same conditions to investigate the function of *SlGGP-LIKE* in oxidative tolerance. After two weeks, all seeds grown on MS media without MV apparently did not show any significant differences (Fig. 6A). However, with increasing MV concentration, WT seedlings were suppressed in growth and leaves showed visible light bleaching phenomenon significantly. On the contrary, the growth of transgenic plants did not significantly change until the concentration of MV increased to 10 μM, which led to a slight light bleaching on leaves. Consistently, with increasing MV concentration from 5 μM to 10 μM, the germination rate of WT seeds decreased from 25% to 14% whereas the germination rate of the transgenic plants was slightly affected (Fig. 6B). There were no differences the growth between WT and transgenic plants at 25 °C for two months (Fig. 6C). But once the plants were treated with 100 μM of MV and placed in a continuous light incubation chamber at 25 °C for 12 h, WT plants showed evident wilting, while the transgenic plants still exhibited well growth. The fresh weight of WT plants decreased evidently compared with transgenic plants under oxidative stresses (Fig. 6D).

Visible damage on leaf disks is a commonly standard in the determination of oxidative stress tolerance (Yoshimura et al., 2004). Serious necrosis was found in the leaf discs of WT plants, whereas only partial necrosis was detected on the leaf discs of the transgenic plants after treated with MV (Fig. 6E). Leaf disks of WT and transgenic lines still sustained green with sterile water treatment. There was no difference in all plants without MV treatment, but the content of chlorophyll was decreased less in transgenic plants than that in WT plants after MV treatment for 24 h (Fig. 6F). All results suggested that the transgenic plants suffered minor injury compared with WT plants, indicating that overexpression of *SlGGP-LIKE* improved the resistance ability of tobacco to oxida-

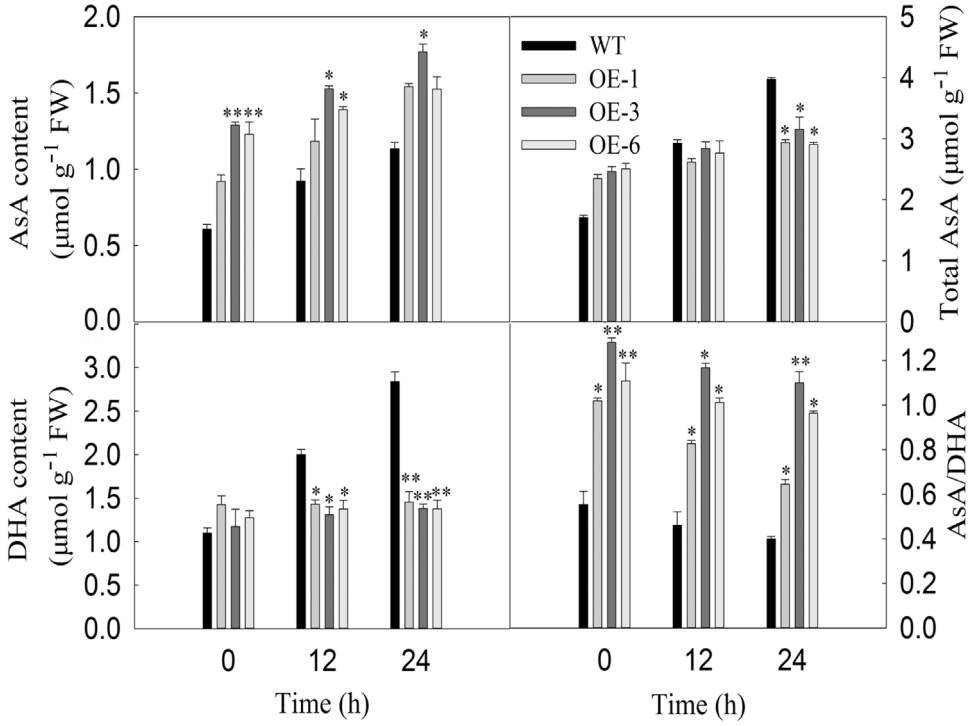

Fig. 5. Contents of ascorbate (AsA), oxidised AsA (DHA), total AsA and the ratio of AsA/DHA in WT plants and different transgenic lines under oxidative stress. Error bars represent the SD of triplicate experiments. *P < 0.05; **P < 0.01.

tive stress and alleviated the inhibition of plant growth induced by MV.

4.7. *Overexpression of* SlGGP-LIKE *protected the biological membrane under oxidative stress*

Under normal growth conditions, the contents of H_2O_2 and $O_2^{\bullet-}$ in WT and transgenic plants showed little difference (Fig. 7). The contents of H_2O_2 increased much higher in WT plants compared with transgenic lines after treatment. After 24 h of MV treatment, the levels of H_2O_2 in WT, OE-1, and OE-3 and OE-6 plants were enhanced 2.22, 1.52, 1.32, and 1.61 fold, respectively, compared with plants without treatment (Fig. 7A). Similarly, after treated with MV for 24 h, the levels of $O_2^{\bullet-}$ in WT OE-1, OE-3 and OE-6 plants were increased 3, 2.71, 2.42, and 1.97fold, respectively (Fig. 7B). These observations suggested that MV treatment resulted in a higher ROS accumulation in WT plants compared with transgenic plants. The damage degree of membrane lipids was measured by MDA, and electrolyte leakage was determined by REC. After MV treatment for 24 h, the levels of MDA in WT, OE-1, OE-3 and OE-6 enhanced approximately 6, 4, 3, and 3.2fold, respectively (Fig. 8A); correspondingly, their RECs increased to 70%, 61%, 53%, and 58% (Fig. 8B). Changes of MDA and REC suggested WT plants suffered more severe injury of biological membrane systems than that of transgenic plants. This result is probably due to WT plants, which have accumulated larger amount of ROS than transgenic plants under oxidative stress. Overexpression of *SlGGP-LIKE* can significantly increase the AsA content, which can protect biological membrane systems via the timely removal of ROS under oxidative stress and therefore improves the tolerance of transgenic plants.

4.8. *Overexpression of* SlGGP-LIKE *maintained high activities of* APX *under oxidative stress*

There was almost no difference about the activities of APX without treatment (Fig. 9). After treated with MV for 24 h, the APX activities in plants were all decreased. The activities of APX in OE-1, OE-3 and OE-6 were 54.69, 67.11, and 55.89 μmol g^{-1} FW min^{-1}, respectively, but only 29.19 μmol g^{-1} FW min^{-1} in WT plants. Because AsA is necessary to keep the APX activity, thus, under oxidative stress, transgenic tobacco plants can maintain relatively higher levels of APX activity, which may be associated with the higher levels of AsA content.

4.9. *Overexpression of* SlGGP-LIKE *alleviated the photoinhibition of* PSII *under oxidative stress*

After MV treatment for 24 h, the value of Pn and Fv/Fm of all plants were analyzed. There were no obvious differences of Pn and Fv/Fm under normal conditions (Fig. 10A). With the extension of treatment time, Pn and Fv/Fm showed a gradual downward trend. After 24 h of treatment, the value of Pn in plants markedly decreased by about 69.34%, 39.39%, 69.28% and 85% in OE-1, OE-3, OE-6 and WT plants, respectively. The Fv/Fm decreased by 69.34%, 39.39%, 69.28% and 85% in OE-1, OE-3, OE-6 and WT plants, respectively (Fig. 10B). These results could be due to the damage effects of the excessive ROS accumulation to the photosynthetic reaction centre caused by MV in WT plants.

4.10. *Overexpression of* SlGGP-LIKE *improved the degree of de-epoxidation under oxidative stress*

AsA is a coenzyme of VDE which participated in xanthophylls cycle, so the degree of de-epoxidation was measured. In transgenic plants the ratio of Z/(V + A + Z) was about fivefold higher than that in WT plants without treatment (Fig. 11A). After treatment, the contents of zeaxanthin in transgenic plants was evidently decreased but was still significantly higher than that in WT plants. In all plants, the ratio of (Z + 0.5A)/(V + A + Z) decreased after stress, but the ratio of in transgenic plants was still higher than that in WT (Fig. 11B). The results indicated that *SlGGP-LIKE* overexpression can

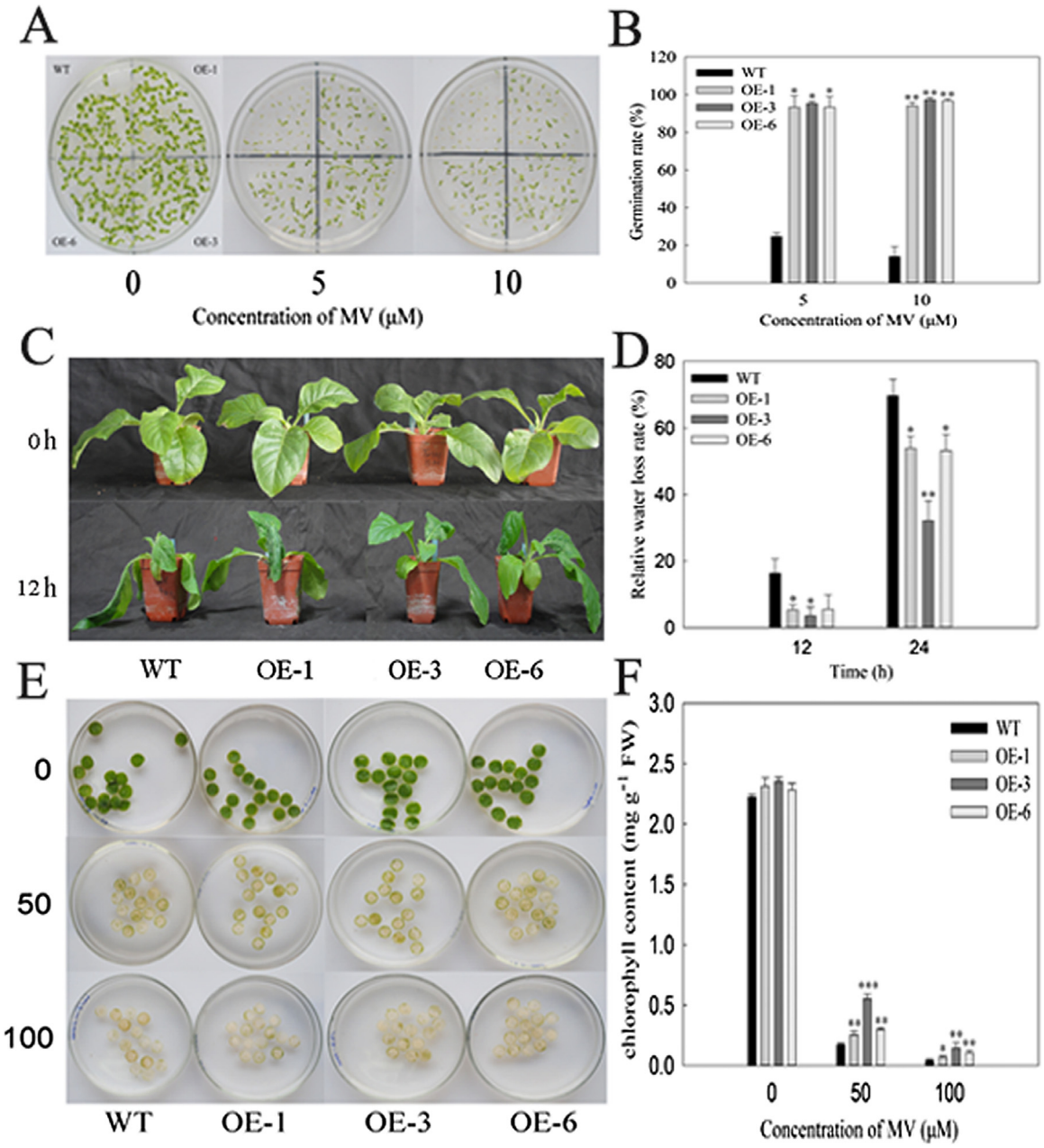

Fig. 6. Growth analysis and leaf disc damage of transgenic tobacco with *SlGGP-LIKE* overexpression under oxidative stress. (A) Seed germination of WT plants and different transgenic lines in the MS medium that contained 0, 5 and 10 μM MV for 14 days, and then photographed. (B) Germination rate under oxidative stress. (C) Phenotype of WT and different transgenic seedlings under normal conditions and oxidative stress. (D) Relative water loss rate under oxidative stress. (E) Phenotypic differences in the leaf disks between WT plants and different transgenic lines after 48 h of MV treatment under continuous light conditions. (F) Chlorophyll content of WT plants and different transgenic lines. Error bars represent the SD of triplicate experiments. *P < 0.05; **P < 0.01.

cause relatively higher levels of zeaxanthin in tobacco before and after oxidative stress.

4.11. Overexpression of SlGGP-LIKE *maintained high level of D1 protein content under oxidative treatment*

When plants are subjected to photoinhibition, D1 protein is the primary target. Protein gel blot analysis was used to detect whether the D1 protein changed during MV treatment. No differences were observed under normal conditions (Fig. 12). After MV treatment for 12 h, the content of D1 protein reduced in all plants, but a less decline showed in transgenic plants compared with WT plants. Hence, the overexpression of *SlGGP-LIKE* decreased the photodamage to PSII in transgenic plants, which may be caused by the high level of APX activity.

5. Discussion

Our previous research indicated that cytosolic and nucleus localisation of *SlGGP* gene in transgenic tobacco increased tolerance to chilling stress (Wang et al., 2014). Tomato and potato are highly homologous. The presence of two genes encoding GGP in potato shows the possibility that another gene encoding GGP protein may also exist in tomato. Basing on the above findings, we isolated another *GGP* gene encoding GGP from tomato leaves, which was named *SlGGP-LIKE* (XM_004231938.2). We found that the gene contained the conserved sequence of GGP family, named HIT,

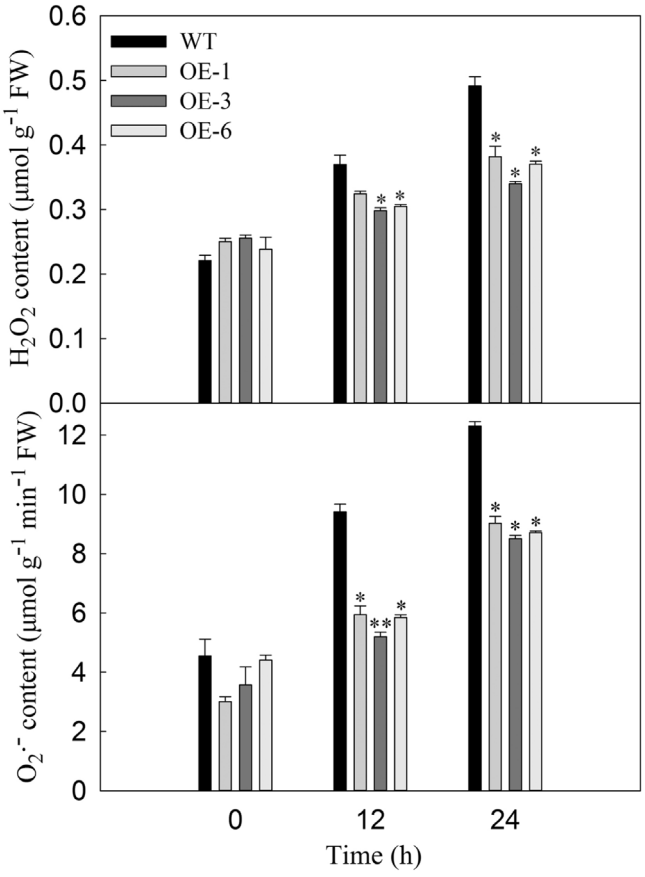

Fig. 7. (A) H_2O_2 and (B) $O_2^{\bullet-}$ contents of WT plants and different transgenic lines under oxidative stress. Error bars represent the SD of triplicate experiments. *P < 0.05; **P < 0.01.

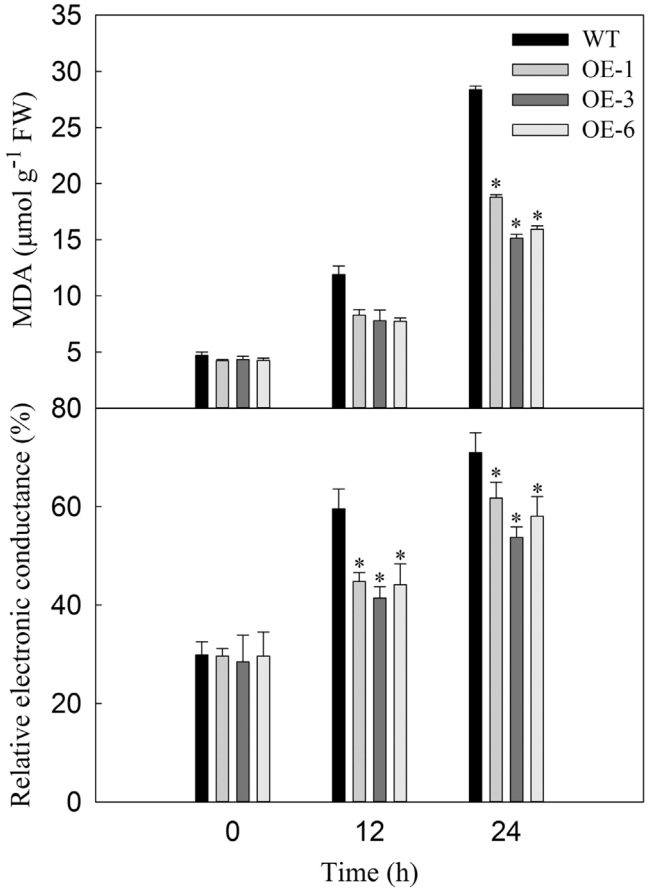

Fig. 8. (A) MDA content and (B) relative electronic conductance of WT plants and different transgenic lines under oxidative stress. Error bars represent the SD of triplicate experiments. *P < 0.05; **P < 0.01.

by using sequence comparison (Fig. 1A). The homology of *SlGGP* and *SlGGP-LIKE* is about 59% (Supplementary Fig. S1). Furthermore, *SlGGP* is located in cytosolic and nucleus, while *SlGGP-LIKE* is located in nucleus (Fig. 3), which means we have obtained a new gene involved in the Smirnoff–Wheeler pathway. The homology of *SlGGP-LIKE* and *StGGP* is the highest (Fig. 1B). The expression of *SlGGP-LIKE* was detected in all tissues. Biotic and abiotic stresses can significantly induce the expression of *SlGGP-LIKE* (Fig. 2). These results suggested that *SlGGP-LIKE* exerts roles in biotic and abiotic stresses. In order to study the physiological function of this gene, transgenic tobacco plants were obtained. These transgenic lines exhibited high levels in mRNA and protein than those of WT, which indicated that *SlGGP-LIKE* had over expressed in transgenic tobacco plants (Fig. 4). The transgenic plants exhibited significantly higher degree of reduced AsA and AsA/DHA ratio compared with WT plants (Fig. 5). Therefore, overexpression of *SlGGP-LIKE* can increase the AsA level efficiently.

AsA is the most abundant soluble antioxidant and acts as coenzyme in the metabolic reactions in living organisms (Smirnoiff, 2000; Conklin and Barth, 2004; Davey et al., 2000). Previous studies about GGP are mainly concentrated in *Arabidopsis* (Munné-Bosch and Alegre, 2002; Laing et al., 2015). Only a few studies have been carried out on tomato. Furthermore, little is known about a physiological role of *SlGGP-LIKE* gene. In this study, transgenic plants possess higher germination rate and fresh weight than those of WT plants under stressful conditions, and the transgenic plants possessing the highest AsA content showed the lowest oxidative damage (Fig. 6). Serious necrosis appeared in the leaf disk of WT plants, while, only partial necrosis was found in transgenic plants leaves (Fig. 6E), and transgenic lines showed the highest chlorophyll content (Fig. 6F). These results indicate that high levels of AsA were beneficial to chloroplast development and plant growth under oxidative stress.

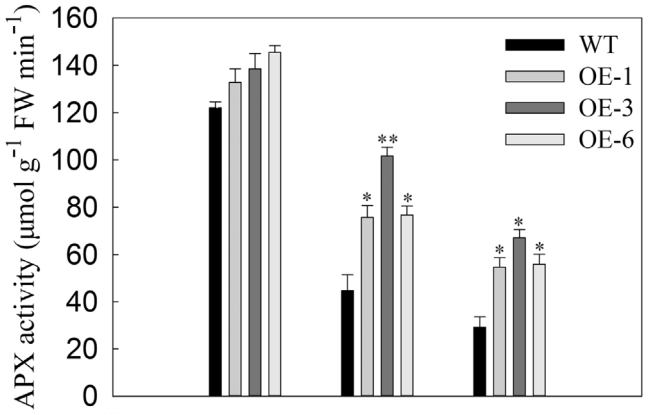

Fig. 9. Enzyme activity of APX in WT plants and different transgenic lines under oxidative stress. Error bars represent the SD of triplicate experiments. *P < 0.05; **P < 0.01.

ROS acts as an intermediary involved in the injured plant in various environment stresses (Lim et al., 2007). MV can increase the production of $O_2^{\bullet-}$ and H_2O_2 via catalyzing the photoreduction of O_2 (Cornic et al., 2000; Mano et al., 2001). Plants are damaged after MV treatment because of the excessive H_2O_2 accumulation, and the main target of oxidative stress is chloroplast APX. Under oxidative stress, AsA in chloroplast can provide electron to APX

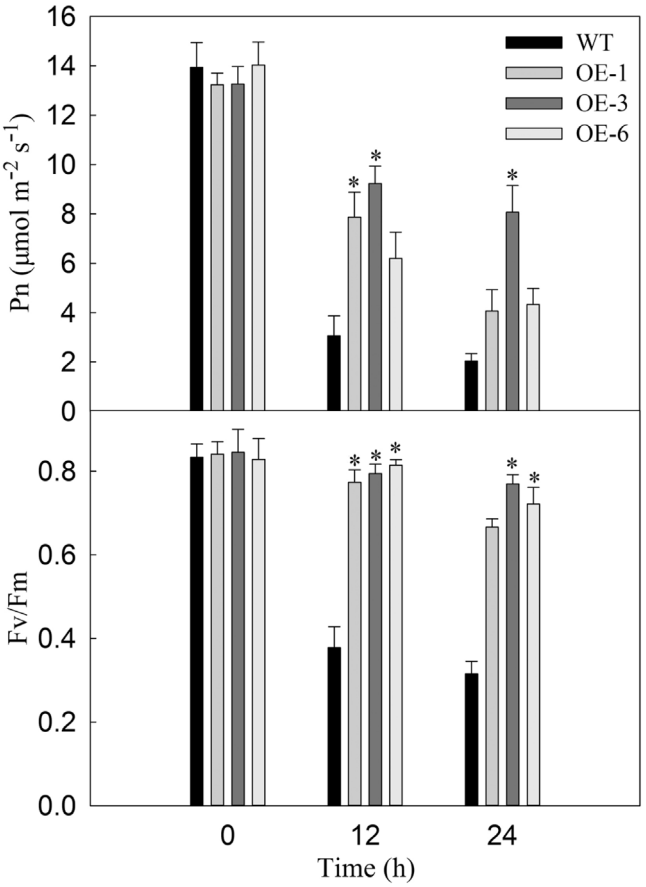

Fig. 10. Changes of Pn and Fv/Fm in WT plants and different transgenic lines before and after oxidative stress. (A) Changes in the Pn of WT plants and transgenic plants. (B) Changes of Fv/Fm in WT plants and transgenic plants. Error bars represent the SD of triplicate experiments. *P < 0.05; **P < 0.01.

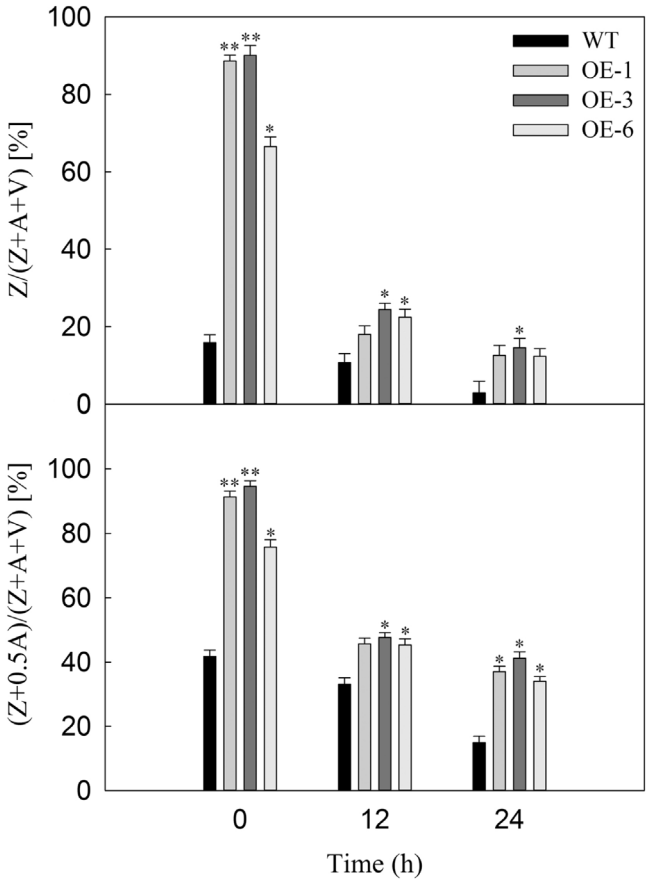

Fig. 11. Changes of the ratio of zeaxanthin (Z) in the xanthophyll cycle pigments Z/(V + A + Z) (A) and de-epoxidation ratio of xanthophyll cycle pigments (Z + 0.5A)/(V + A + Z) under oxidative stress (B). Error bars represent the SD of triplicate experiments. *P < 0.05; **P < 0.01.

for H_2O_2 detoxification. In this study, transgenic plants exhibited increased endurance to oxidative stress than WT plants, which may due to higher levels of reduced AsA content and AsA/DHA ratio in transgenic plants (Fig. 5); such higher levels ensure that transgenic plants can maintain higher APX ability (Fig. 9) to remove ROS (Fig. 7), further alleviate the injury to membrane systems (Fig. 8) and alleviate oxidative stress effectively.

Zeaxanthin is vital important in alleviating excess light energy existed on PSII reaction centres via the transfer of excess light energy into heat (Niyogi et al., 1998; Nilkens et al., 2010). AsA, as a coenzyme of VDE that can convert violaxanthin to antheraxanthin and then zeaxanthin at a low rate, is important in the xanthophyll cycle. After MV treatment, the recycling pathway of AsA was affected because the electron transport chain was blocking. Overexpression lines had higher AsA content, thus the product and de-epoxidation ratio of xanthophyll cycle can maintain relatively higher levels under oxidative stress (Fig. 11). Hence, ROS may be removed effectively, thereby protecting the chloroplast membranes from oxidative stress (Niinemets et al., 2003; Havaux et al., 2000, 2007).

Accelerating ROS accumulation under various stresses could increase the degree of photoinhibition by damaging D1 protein. This ROS generation could impair the ability of PSII to repair the photodamage (Yang et al., 2007). Under oxidative conditions, AsA existing in chloroplast can help decrease photoinhibition of PSII via acting as an electron-donor of APX for H_2O_2 detoxification (Smirnoff, 2000; Horemans et al., 2000). According to the results of Pn and Fv/Fm, the higher levels of AsA and APX activities in chloroplasts could scavenge ROS rapidly, furthermore increased the rate of PSII repair to maintain relative higher levels of Pn and Fv/Fm in transgenic plants (Fig. 10). D1 protein is the main target, when plants suffer photoinhibition (Andersson and Aro, 2001;

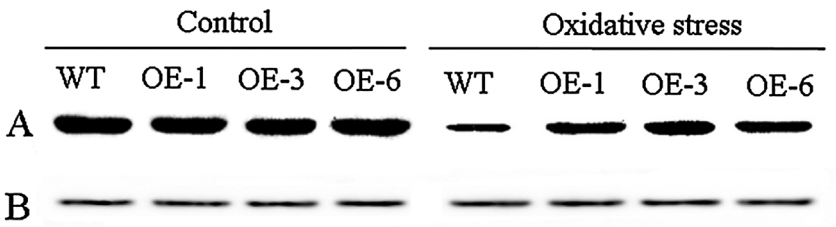

Fig. 12. Western blot analysis of the D1 protein in WT plants and different transgenic lines under normal conditions and after MV treatment for 12 h. (A) Thylakoid membrane proteins were separated by SDS–PAGE and probed with D1 antibody. (B) The same thylakoid membrane proteins were separated by SDS-PAGE and stained with β-actin antibody to qualify as a loading control for Western blot.

Pokorska et al., 2009). Western blot results indicated that the content of D1 protein in all plants decreased with MV treatment, but the decline was more obvious in WT plants (Fig. 12) because the overexpression of *SlGGP-LIKE* enhanced the AsA content and leading to a small number of ROS accumulations in transgenic plants. These results suggest that overexpression of *SlGGP-LIKE* in tobacco enhanced the content of reduced AsA, which can donate electron to detoxify ROS. Higher levels of AsA and APX activities can ensure to scavenge ROS efficiently, therefore enhance the tolerance of plants to various stresses.

6. Conclusions

In conclusion, we cloned a *GGP-LIKE* gene, which responded to MV stress. Overexpression of *SlGGP-LIKE* enhanced the oxidative tolerance of tobacco plants by sustaining higher APX activity and reduced the excessive accumulation of ROS, thus can reduce damage to photosystem.

Acknowledgements

This work was supported by the State Key Basic Research and Development Plan of China (2015CB150105), the Natural Science Foundation of China (31171474, 31371553, 31470341), the Doctor Foundation of Shandong (2014BSB01031).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2016.10.013.

References

Andersson, B., Aro, E.M., 2001. Photodamage and D1 protein turnover in photosystem II. In: Andersson, B., Aro, E.M. (Eds.), Regulation of Photosynthesis, 11. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 377–393.
Brenner, C., Fhit, Hint, Gal, T., 2002. Function, structure, evolution, and mechanism of three branches of the histidine triad superfamily of nucleotide hydrolases and transferases. Biochemistry 41, 9003–9014.
Bulley, S.M., Rassam, M., Hoser, D., Otto, W., Schüneman, N., Wright, M., MacRae, E., Gleave, A., Laing, W., 2009. Gene expression studies in kiwifruit and gene over-expression in Arabidopsis indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis. J. Exp. Bot. 60, 765–778.
Bulley, S.M., Wright, M., Rommens, C., Yan, H., Rassam, M., Wang, K.L., Andre, C., Brewster, D., Karunairetnam, S., Allan, A.C., Laing, W.A., 2012. Enhancing ascorbate in fruits and tubers through over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase. Plant Biotechnol. J. 10, 390–397.
Chen, Z., Gallie, D.R., 2006. Dehydroascorbate reductase affects leaf growth, development and function. Plant Physiol. 142, 775–787.
Conklin, P.L., Barth, C., 2004. Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ. 27, 959–970.
Conklin, P.L., 2001. Recent advances in the role and biosynthesis of ascorbic acid in plants. Plant Cell Environ. 24, 383–394.
Cornic, G., Bukhov, N.G., Wiese, C., Bligny, R., Heber, U., 2000. Flexible coupling between light-dependent electron and vectorial proton transport in illuminated leaves of C3 plants; role of photosystem I dependent proton pumping. Planta 210, 468–477.
Davey, M., Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I., Strain, J., Favell, D., Fletcher, J., 2000. Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. J. Sci. Food Agric. 80, 825–860.
Foyer, C.H., Nocdox, G., 2005. Redox homeostasis and antioxidant signalling: a metabolic interface between stress perception and physiological responses. Plant Cell 17, 1866–1875.
Gallie, D.R., 2013. The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. J. Exp. Bot. 64, 433–443.
Havaux, M., Bonfils, J.P., Lŭtz, C., Niyogi, K.K., 2000. Photodamage of the photosynthetic apparatus and its dependence on the leaf developmental stage in the npq1 Arabidopsis mutant deficient in the xanthophyll cycle enzyme violaxanthin de-epoxidase. Plant Physiol. 124, 273–284.
Havaux, M., Dall'osto, L., Bassi, R., 2007. Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in Arabidopsis leaves and functions independent of binding to PSII antennae. Plant Physiol. 145, 1506–1520.
Horemans, N., Foyer, C.H., Potters, G., Asard, H., 2000. Ascorbate function and associated transport system in plants. Plant Physiol. Biochem. 38, 531–540.
Kwon, S.Y., Jeong, Y.J., Lee, H.S., Kim, J.S., Cho, K.Y., Allen, R.D., Kwark, S.S., 2002. Enhanced tolerance of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. Plant Cell Environ. 25, 873–882.
Laing, W.A., Wright, M.A., Cooney, J., Bulley, S.M., 2007. The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. Proc. Natl. Acad. Sci. U. S. A. 104, 9534–9539.
Laing, W.A., Martínez-Sánchez, M., Wright, M.A., et al., 2015. An upstream open reading frame is essential for feedback regulation of ascorbate biosynthesis in arabidopsis. Plant Cell 27, 772–786.
Li, F., Wu, Q.Y., Sun, Y.L., Wang, L.Y., Yang, X.H., Meng, Q.W., 2010. Overexpression of chloroplastic monodehydroascorbate reductase enhanced tolerance to temperature and methyl viologen-mediated oxidative stresses. Physiol. Plant. 139, 421–434.
Lim, S., Kim, Y.H., Kim, S.H., Kwon, S.Y., Lee, H.S., Kim, J.S., et al., 2007. Enhanced tolerance of transgenic sweet potato plants that express both CuZnSOD and APX in chloroplasts to methyl viologen-mediated oxidative stress and chilling. Mol. Breed. 19, 227–239.
Linster, C.L., Gomez, T.A., Christensen, K.C., Adler, L.N., Young, B.D., Brenner, C., Clarke, S.G., 2007. Arabidopsis VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. J. Biol. Chem. 282, 18879–18885.
Müller-Moulé, P., Talila, G., Niyogi, K.K., 2004. Ascorbate-deficient mutants of Arabidopsis grow in high light despite chronic photooxidative stress. Plant Physiol. 134, 1163–1172.
Mano, R., Ohno, C., Domae, Y., Asada, K., 2001. Chloroplastic ascorbate peroxidase is the primary target of methylviologen-induced photooxidative stress in spinach leaves: its relevance to monodehydroascorbate radical detected with in vivo ES. Biochim. Biophy. Acta 1504, 275–287.
Miyaji, T., Kuromori, T., Takeuchi, Y., Yamaji, N., Yokosho, K., Shimazawa, A., 2015. AtPHT4;4 is a chloroplast-localized ascorbate transporter in Arabidopsis. Nat. Commun. 6, 5928.
Munné-Bosch, S., Alegre, L., 2002. Interplay between ascorbic acid and lipophilic antioxidant defences in chloroplasts of water-stressed Arabidopsis plants. FEBS Lett. 524, 145–148.
Niinemets, Ü., Kollist, H., Garcia-Plazaola, J.A., Hernández, A., Becerril, J.M., 2003. Do the capacity and kinetics for modification of xanthophyll cycle pool size depend on growth irradiance in temperate trees. Plant Cell Environ. 26, 1787–1801.
Nilkens, M., Kress, E., Lambrev, P.H., Miloslavina, Y., Müller, M., Holzwarth, A.R., Jahns, P., 2010. Identification of a slowly inducible zeaxanthin-dependent component of nophotochemical quenching of chlorophyll fluorescence generated under steady state conditions in Arabidopsis. Biochim. Biophys. Acta 1797, 466–475.
Niyogi, K.K., Grossman, A.R., Björkman, O., 1998. Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. Plant Cell 10, 1121–1134.
Pokorska, B., Zienkiewicz, M., Powikrowska, M., Drozak, A., Romanowska, E., 2009. Differential turnover of the photosystem II reaction centre D1 protein in mesophyll and bundle sheath chloroplasts of maize. Biochim. Biophys. Acta 1787, 1161–1169.
Smirnoff, N., 2000. Ascorbate biosynthesis and function in photoprotection: philos. Trans. R. Soc. Lond B Biol. Sci. 355, 1455–1464.
Wang, J., Yu, Y., Zhang, Z., Quan, R., Zhang, H., Ma, L., Deng, X.W., Huang, R., 2013a. Arabidopsis CSN5B interacts with VTC1 and modulates ascorbic acid synthesis. Plant Cell 25, 625–636.
Wang, L.Y., Li, D., Deng, Y.S., Lv, W., Meng, Q.W., 2013b. Antisense-mediated depletion of tomato GDP-L-galactose phosphorylase gene increases susceptibility to chilling stress. J. Plant Physiol. 170, 303–314.
Wang, L.Y., Xia, M., Yang, D.Y., Ma, N.N., Wang, G.D., Meng, Q.W., 2014. Overexpression of tomato GDP-L-galactose phosphorylase gene in tobacco improves tolerance to chilling stress. Plant Cell Rep. 33, 1441–1451.
Wheeler, G.L., Jones, M.A., Smirnoff, N., 1998. The biosynthetic pathway of vitamin C in higher plants. Nature 393, 365–369.
Yang, X.H., Wen, X.G., Gong, H.M., Lu, Q.T., Yang, Z.P., Tang, Y.L., Liang, Z., Lu, C.M., 2007. Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. Planta 225, 719–733.
Yoshimura, K., Miyao, K., Gaber, A., Takeda, T., Kanaboshi, H., Miyasaka, H., Shigeoka, S., 2004. Enhancement of stress tolerance in transgenic tobacco plants overexpressing Chlamydomonas glutathione peroxidase in chloroplastsor cytosol. Plant J. 37, 21–33.
Zhang, L.X., Paakkarinen, V., van Wijk, K.J., Aro, E.M., 1999. Cotranslational assembly of the D1 protein into photosystem II. J. Biol. Chem. 274, 16062–16067.