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Suppression of tomato *SlGGP* aggravates methyl viologen-mediated oxidative stress

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Abstract

Ascorbate (AsA) is an important antioxidant that can scavenge reactive oxygen species to protect plant cells against oxidative stress. Guanosine 5'-diphosphate (GDP)-L-galactose phosphorylase (GGP) is a key enzyme in the AsA biosynthetic pathway. To investigate the functions of GGP in AsA synthesis and oxidative stress tolerance in tomato, antisense lines with a reduced expression of *SlGGP* were obtained. Photobleaching after treatment of leaf disks with methyl viologen was more severe in transgenic lines compared to wild type (WT) plants. Moreover, compared with the WT plants, the transgenic plants showed a higher content of hydrogen peroxide, superoxide anion, malondialdehyde, as well as ion leakage, but a lower content of AsA and chlorophylls, ascorbate peroxidase activity, net photosynthetic rate, and maximal photochemical efficiency of photosystem II. Results of real-time quantitative polymerase chain reaction show that suppression of the *SlGGP* gene in the transgenic plants reduced their oxidative stress tolerance.

Additional key words: ascorbate, ascorbate peroxidase, chlorophyll, GDP-L-galactose phosphorylase; malondialdehyde, net photosynthetic rate, photosystem II, *Solanum lycopersicum.*

Introduction

A common feature of plant responses to environmental stress is a burst of reactive oxygen species (ROS) in different cellular compartments, mainly chloroplasts, peroxisomes, and the apoplastic space (Gechev *et al*. 2006, Holuigue *et al*. 2007, Miller *et al*. 2007, Murik *et al*. 2014). For plants, a certain amount of ROS is essential for normal physiological activities, but excessive accumulation of ROS can induce damage to proteins, lipids, sugars, and DNA, and ultimately results in cell death. Plants contain several enzymatic and non-enzymatic antioxidants, which scavenge ROS to protect plant cells.

Ascorbic acid (AsA), as the most abundant water-soluble antioxidant present in plant cells, plays a critical role in detoxification of ROS produced under stress. In addition to these antioxidant functions, AsA has been shown to be involved in a range of important cellular processes including plant development and hormone signalling, cell cycle, cell expansion, senescence, and as cofactor for several important enzymes (Davey *et al*. 2000, Smirnoff *et al*. 2001, Noctor, 2006).

Biosynthesis of AsA occurs through several pathways, including the D-glucosone, D-galacturonate, myo-inositol, and D-mannose/L-galactose (D-Man/L-Gal) pathways (Loewus *et al*. 1990, Wheeler *et al*. 1998, Agius *et al*. 2003, Lorence *et al*. 2004), and is highly regulated in plants (Smirnoff *et al*. 2001, Lorence *et al*. 2004, Hancock and Viola 2005, Zhang *et al*. 2008).

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbate; CAT - catalase; DHA - dehydroascorbate; D-Man/L-Gal - D-mannose/L-galactose; F_v/F_m - variable to maximum chlorophyll fluorescence ratio (maximal photochemical efficiency of photosystem II); GDP - guanosine 5'-diphosphate; GGP - GDP-L-galactose phosphorylase; MDA - malondialdehyde; MV - methyl viologen; $O_2^{\bullet-}$ - superoxide anion radical; PFD - photon flux density; P_N - net photosynthetic rate; PS II - photosystem II; REC - relative electric conductance; ROS - reactive oxygen species; *SlGalLDH* - L-galactono-1,4-lactone dehydrogenase; SOD - superoxide dismutase; WT - wild type.

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Evidence shows that the D-Man/L-Gal pathway is the most important AsA synthesis pathway in plants and guanosine 5'-diphosphate (GDP)-L-galactose phosphorylase (GGP; EC 2.7.7.69) is a key enzyme in this pathway of AsA synthesis in plants (Sasaki-Sekimoto *et al*. 2005, Wolucka *et al*. 2005, Gilbert *et al*. 2009, Zhang *et al*. 2010). This enzyme can convert the substrate GDP-L-galactose to L-Gal-1-phosphate in the L-galactose pathway. The role of GGP in the L-galactose pathway has been clearly established by several studies, *e.g.*, *Arabidopsis* vitamin C 2 gene mutants (*vtc2*) have the lowest relative expression of GGP I and AsA content in these *vtc2* mutants is lower than that in wild type plants (Linster *et al*. 2007). In *SlGME* RNAi lines, expression of GDP-D-mannose 3,5-epimerase (GME) was found to be reduced, which could significantly promote GGP expression (Gilbert *et al*. 2009), whereas overexpression of the tomato *GME* gene could inhibit *GGP* expression (Zhang *et al*. 2010). Genes *GGP* might be a key rate-limiting step for AsA biosynthesis in *Arabidopsis* (Bulley *et al*. 2009), tobacco (Laing *et al*. 2007), and tomato (Zhang *et al*. 2010). Expression of GGP is correlated with AsA accumulation in all tissues of tomato (Wang *et al*. 2013).

Methyl viologen (MV) is herbicide, which acts by inducing cells to produce excess superoxide free radicals leading to a variety of harmful cytological effects. Here, we used MV to simulate oxidative stress to investigate the roles of GGP in tomato AsA biosynthesis and tolerance to oxidative stress.

Materials and methods

Plants and treatments: Antisense transgenic plants (lines A1, A3, and A5 with a reduced expression of *SlGGP*) were generated and identified as described by Wang *et al*. (2013). Surface-sterilized seeds of tomato (*Solanum lycopersicum* Mill. cv. Zhongshu 6) and T_3 antisense transgenic plants were grown on Murashige and Skoog medium with kanamycin (50 μg cm^{-3}) in closed glass bottles and placed in a chamber with a 16-h photoperiod, a photon flux density (PFD) of 100 μmol m^{-2} s^{-1}, and a temperature of 25 °C for 7 d. Sprouted seedlings were transplanted into plastic pots (15 cm in diameter, 10 cm high, three plants per pot) filled with *Vermiculite* moistened with Hoagland nutrient solution (with an adequate irrigation to avoid water stress) and grown in a greenhouse at a 14-h photoperiod, a PFD of 800 - 1000 μmol m^{-2} s^{-1}, day/night temperatures of 25 - 30/15 - 20 °C, and a relative humidity of 60 %. After two months, 100 μM MV was sprayed on leaves, and whole plants were exposed to a continuous irradiance of 200 μmol m^{-2} s^{-1} in an incubation chamber (*GXZ-260C*, *Jiangnan,* Ningbo, China) at 25/20 °C and a 50 - 60 % relative humidity. Plant leaves were harvested at 0, 12, and 24 h after the MV treatment. The stressed leaves were collected from about 3 - 4 seedlings per treatment, immediately frozen in liquid nitrogen, and stored at -80 °C. All biochemical and physiological measurements were carried out on the youngest fully expanded leaves.

Leaf disk assay and determination of chlorophyll content: To investigate the effects of MV treatment on wild type (WT) and transgenic plants, 15 leaf disks (Ø 0.8 cm) from each line were placed into Petri dish containing 20 cm^3 of MV at different concentrations (0, 50, 100 μM), after which they were placed under vacuum for 30 s, and then under continuous irradiance (200 μmol m^{-2} s^{-1}) at 25 °C for 24 h. The leaf disks were then photographed to record changes in disk colour.

Chlorophyll *a* and *b* content were determined according to Hemavathi *et al*. (2010). Methyl viologen-treated leaf disks were put in a test tube containing 10 cm^3 of 80 % (v/v) acetone and then placed at room temperature in the dark until the leaf colour faded completely. Absorbance was measured at 663 and 645 nm using a spectrophotometer *UV-1780*, (*Shimadzu*, Shanghai, China)

Determination of reduced, oxidized, and total AsA: The wild type and transgenic tomato plants (two-month-old) were allowed to grow under 25 °C for 24 h after a 100 μM MV treatment. Their AsA (reduced), oxidized AsA (dehydroascorbate, DHA), and the total AsA (AsA+DHA) content were determined as described by Li *et al*. (2010). Detached leaves (0.5 g) were ground and homogenized with 2 cm^3 of 6 % (m/v) trichloroacetic acid, then the homogenate was centrifuged at 12 000 *g* and 4 °C for 10 min, and the supernatant was used. For determination of AsA, a reaction solution included 0.2 cm^3 of the extract, 0.6 cm^3 of 0.2 M phosphate buffer saline (PBS; pH 7.4), 0.2 cm^3 of double distilled H_2O, 1 cm^3 of 6 % trichloroacetic acid, 0.8 cm^3 of 42 % (m/v) H_3PO_4, 0.8 cm^3 of 4 % (m/v) 2,2'-bipyridine, and 0.4 cm^3 of 3 % (m/v) $FeCl_3$. The total AsA content (AsA+DHA) was measured in 0.2 cm^3 of 10 mM dithiothreitol and 0.4 cm^3 of 0.2 M PBS (pH 7.4) instead of 0.6 cm^3 of 0.2 M PBS as mentioned above. Absorbance was measured at 525 nm after mixing the reaction solution and incubating it at 42 °C for 1 h. Content of DHA was then determined as the difference in the total AsA+DHA content and AsA content.

Measurement of electrolyte leakage: Twenty leaf discs (Ø 0.8 cm) were put into 15 cm^3 of distilled water, then placed under vacuum for 30 min, and surged for 3 h to measure the initial electric conductance (S1). The leaf pieces were then heated in boiling water for 30 min and cooled to room temperature to determine the final electric

conductance (S2). The relative electric conductance (REC) was calculated as: REC = (S1/S2) × 100 %.

Detection of superoxide anion and H_2O_2: Leaves (0.5 g) were transferred to centrifuge tubes after grinding them in liquid nitrogen. After adding 3 cm^3 of cold 50 mM PBS (pH 6.8) to the tubes, the samples were centrifuged at 6000 *g* for 15 min; 3 cm^3 of the supernatant and 1 cm^3 of 0.1 % (m/v) titanium sulfate in 20 % (m/v) H_2SO_4 were put into a new tube, mixed, and centrifuged at 6 000 *g* for 15 min. Absorbance was measured at 410 nm. Content of H_2O_2 was calculated according to a standard curve plotted with known concentrations of H_2O_2.

For superoxide anion radical ($O_2^{\cdot-}$) assay, about 0.5 g of leaves were ground to powder in liquid nitrogen and transferred into a tube with 5 cm^3 of 50 mM PBS (pH 7.8). The homogenate was centrifuged at 12 000 *g* for 20 min and the supernatant was mixed with 0.5 cm^3 of 50 mM PBS and 1 cm^3 of 1 mM hydroxylammonium chloride and heated at 25 °C for 20 min. After adding 17 mM *p*-aminobenzene sulfonic acid and 7 mM α-naphthylamine, the mixture was incubated at 25 °C for 20 min. Absorbance was measured at 530 nm. Content of $O_2^{\cdot-}$ was calculated according to a standard curve plotted with known concentrations of NO_2^-.

Measurement of superoxide dismutase, catalase, and ascorbate peroxidase activities: Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to the method described by Li *et al*. (2003) with slight modifications. Extraction was identical to that used in the $O_2^{\cdot-}$ assay. To measure SOD activity, the supernatant with 13 mM L-methionine, 75 μM nitroblue tetrazolium, 10 μM EDTA-Na_2 and 2 μM riboflavin were used. The mixture was allowed to react at a PFD of 100 μmol m^{-2} s^{-1} for 20 - 30 min and absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme that inhibited the nitroblue tetrazolium photoreduction reaction by 50 %. For catalase (CAT, EC 1.11.1.6) activity determination, the supernatant was mixed with 10 mM H_2O_2 and absorbance was measured at 240 nm. One unit of CAT activity was defined as converting 1 mmol of H_2O_2 per min. Activity of ascorbate peroxidase (APX, EC 1.11.1.11**)** was measured by monitoring decrease in absorbance at 290 nm as described by Kwon *et al*. (2002). One unit of APX activity was defined as the amount of the enzyme that oxidized 1 μmol of AsA per min.

Measurement of net photosynthetic rate and chlorophyll fluorescence: Net photosynthetic rate (P_N) was measured with a portable photosynthetic system (*CIRAS-2*, *PP Systems*, Herts, UK) under CO_2 concentration of 360 μmol mol^{-1}, a PFD of 800 μmol m^{-2} s^{-1}, a temperature of 25 °C, and a relative humidity of 80 %. In order to induce stomata to open before P_N measurement, the plants treated with MV were kept at 25 °C and a PFD of 200 μmol m^2 s^1 for 30 min, and then the PFD was increased to 800 μmol m^{-2} s^{-1} for 15 min to allow the plants to acclimate.

Chlorophyll fluorescence was measured with a portable fluorometer (*FMS2*, *Hansatech*, Norfolk, UK) according to the method described by Van Kooten and Snel (1990). An initial fluorescence [F_o, fluorescence when all reaction centres of photosystem II (PS II) are open] was determined under a PFD of 10 μmol m^{-2} s^{-1}, which was low enough not to induce any significant variable fluorescence (F_v). The maximal fluorescence (F_m, fluorescence when all PS II reaction centers are closed) was determined by exposing a dark-adapted (for 15 min) leaf to a 0.8 s saturating PFD of 7 000 μmol m^{-2} s^{-1}. The maximal photochemical efficiency of PS II (F_v/F_m) was calculated using the formula: $F_v/F_m = (F_m - F_o)/F_m$.

Real time quantitative PCR analysis: The total RNA was extracted from detached leaves using *RNA-prep* plant kits (*Tiangen Biotech*, Beijing, China). The DNase-treated RNA was reverse-transcribed using *M-MLV* reverse transcriptase (*Tiangen Biotech*). Real time PCR was performed on a *CFX96*™ system (*BioRad*, Hercules, USA) using a *SYBR* real PCR master mix (*Tiangen Biotech*) according to the supplier's protocols. The PCR thermal cycling conditions were as follows: denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 5 s,

Table 1. A list of primers used in real time quantitative PCR analysis.

Primer	Forward 5'-3'	Reverse 5'-3'
EF1α	GGAACTTGAGAAGGAGCCTAAG	CAACACCAACAGCAACAGTCT
GPI	TGCTCTTCAAAAGCGTGTCC	CGGCAATAAGTGCTCTGTCA
PMI	TACATTGTGGTGGAACGAGGA	ACCCCATTTGGCAAGAACAG
PMM	TTTACCCTCCATTACATTGCTGA	TCTTCTTGACTACAGTTTCTCCCA
GMP	AAACCTGAAATCGTGATGTGAGA	TGAAGAAGAGGAGAACTGGAAAC
GME1	AATCCGACTTCCGTGAGCC	CTGAGTTGCGACCACGGAC
GME2	CCATCACATTCCAGGACCAGA	CGTAATCCTCAACCCATCCTT
GGP	CAAACCCACAGCCACAAT	GTTACACAACCCTTACGAC
GLDH	AGGTCTGCAGGCAAGGCT	GGCATGAAGATGATCACTATCG

58 °C for 10 s, and 72 °C for 20 s. *EF-1α* (GenBank accession No. X144491) was used as reference gene. We measured expression of genes participating in AsA biosynthesis. Primer sequences are listed in Table 1.

Statistical analysis: Mean values ± SDs of at least three replicates are presented. Statistical significances of differences of measured parameters between the WT and transgenic plants were analyzed by Student's *t*-test at $\alpha = 0.05$ using *SigmaPlot 12.5* and *SPSS v. 13.0* (Chicago, IL, USA).

Results

Leaf disks were floated on MV solutions (0, 50, and 100 μM) under continuous irradiance at 25 °C for 24 h. All of the leaf disks without MV remained green. However, with the increase in MV concentration, necrosis was observed in all the disks, and the degree of necrosis was more severe in the transgenic plants (Fig. 1*A*). No significant difference in chlorophyll content was observed in the plants without the MV treatment. When MV concentration increased to 50 μM, chlorophyll content in the WT, A1, A3, and A5 transgenic plants decreased by 69.3, 77.1, 77.0, and 77.5 %, respectively. Under the 100 μM MV treatment, chlorophyll content in the WT plants decreased by 73.9 %, whereas decreases of 88.1, 84.9, and 85.3 % were observed in the A1, A3, and A5 plants (Fig. 1*B*). These results suggest that the inhibition of *SlGGP* reduced resistance of tomato to the oxidative stress.

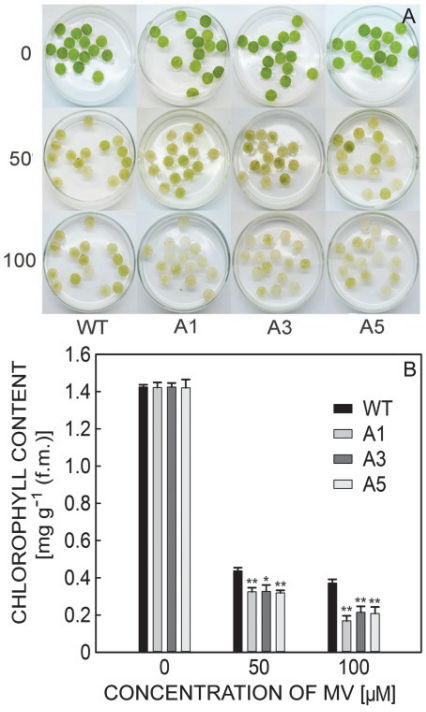

Fig. 1. Effect of methyl viologen (MV) on appearance of leaf disks of wild type (WT) and A1, A3, and A5 transgenic lines (*A*) and their chlorophyll content (*B*). The leaf disks were floated on 0, 50, and 100 μM MV solutions for 24 h. Significant differences compared to the WT are marked by *asterisks*.

Under the control conditions (without MV), content of H_2O_2 in the WT and transgenic plants exhibited almost no difference (Fig. 2*A*). However, after 12 h of the 100 μM MV treatment, content of H_2O_2 in the WT, A1, A3, and A5 plants increased by 0.57, 0.72, 0.61, and 0.92 μmol g^{-1}(f.m.), and after 24 h by 0.99, 1.45, 1.20, and 1.88 μmol g^{-1}(f.m.), respectively. Changes in $O_2^{\cdot-}$ were similar to changes in H_2O_2 (Fig. 2*B*). After 12 h of the MV treatment, content of $O_2^{\cdot-}$ in the WT, A1, A3, and A5 plants increased by 40.4, 49.5, 49.5, and 52.3 %, and after 24 h by further 55.2, 63.9, 61.5, and 62.4 %, respectively. These results indicate that suppressing *SlGGP* reduced scavenging ROS resulting in oxidative stress.

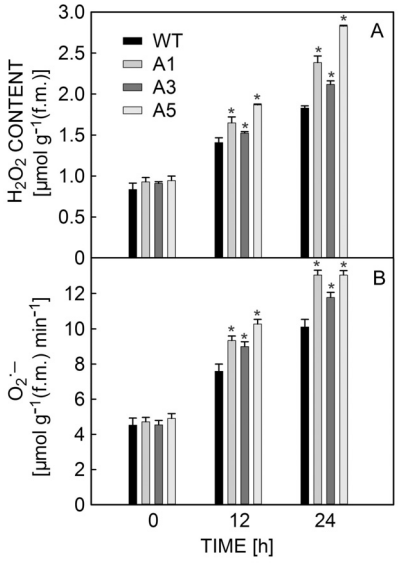

Fig. 2. Changes in H_2O_2 content (*A*) and $O_2^{\cdot-}$ accumulation (*B*) in wild type (WT) and A1, A2, and A3 transgenic plants under 100 μM MV treatment for 0, 12, and 24 h. Means ± SDs of three measurements of each of the three plants. Significant differences compared to the WT are marked by *asterisks*.

Under the control conditions, the transgenic and WT plants had similar ion leakage (measured as REC) and MDA content (Fig. 3*A*,*B*). However, the MV-induced oxidative stress resulted in a more leakage in the transgenic plants than in the WT plants. In addition, content of MDA increased by 301, 310, 383, and 211 % in the A1, A3, A5, and WT plants after the 24-h MV

treatment, respectively, and REC increased by about 71.1, 87.6, 80.1, and 81.7 % in the WT, A1, A3, and A5 lines, respectively. Therefore, the oxidative stress destroyed biological membrane systems to some extent.

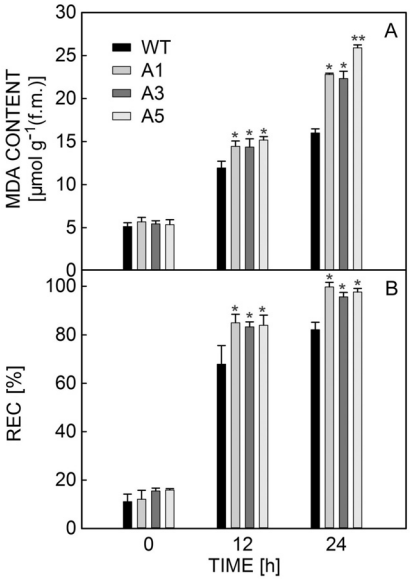

Fig. 3. Changes in malondialdehyde (MDA) content (*A*) and relative electric conductance (REC, *B*) in wild type (WT) and A1, A3, and A5 transgenic plants under 100 μM MV treatment for 0, 12, and 24 h. Means ± SDs; three measurements were taken for each of the three plants. Significant differences compared to the WT are marked by *asterisks*.

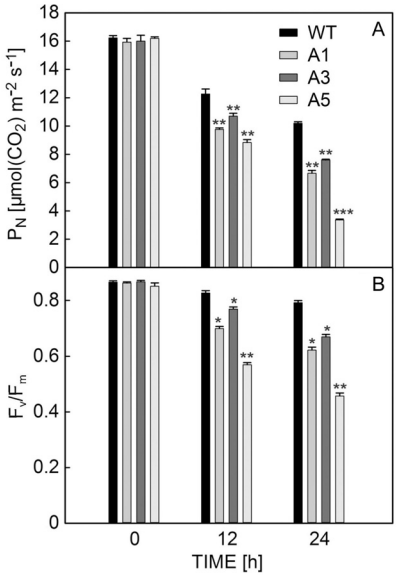

Fig. 4 Changes in net photosynthetic rate (P_N, *A*) and variable to maximum chlorophyll fluorescence ratio (F_v/F_m, *B*) in wild type (WT) and A1, A3, and A5 transgenic plants under 100 μM MV treatment for 0, 12, and 24 h. Means ± SDs of three measurements of each of the three plants. Significant differences compared to the WT are marked by *asterisks*.

To assess the degrees of injury of the photosynthetic system caused by MV in the WT and transgenic plants, P_N and F_v/F_m were assayed. There were no significant differences in P_N and F_v/F_m between the WT and transgenic plants under the control conditions (Fig. 4*A*,*B*). Although P_N and F_v/F_m of the WT and transgenic plants decreased markedly during the MV treatment, the decreases in P_N and F_v/F_m were more obvious in the transgenic plants than in the WT plants. After 24 h of the treatment, P_N of the WT, A1, A3, and A5 decreased to 10.2, 6.6, 7.6 and 3.4 μmol(CO_2) m^{-2} s^{-1}, respectively, (Fig. 4*A*), and F_v/F_m was reduced by 0.08, 27.8, 22.7, and 46.3 % in the WT, A1, A3, and A5 plants, respectively (Fig. 4*B*). These results may suggest some damage of the photosynthetic apparatus under the MV stress.

Since antioxidative enzymes, such as SOD, CAT, and APX play important roles in plant response to oxidative stress, we analyzed their activities under the MV treatment. There were almost no differences in activities of SOD, CAT, and APX between the transgenic lines and the WT plants under the control conditions (Fig. 5). Nevertheless, after the treatment with 100 μM MV for

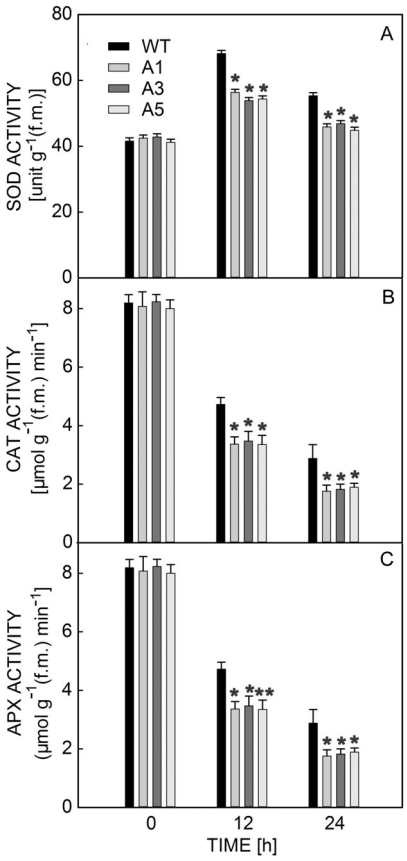

Fig. 5 Changes in SOD (*A*), CAT (*B*) and APX (*C*) activities in wild type (WT) and A1, A3, and A5 transgenic plants after 100 μM MV treatment for 0, 12, and 24 h. Means ± SDs of five measurements of each of the five plants. Significant differences compared to the WT ($P < 0.05$) are marked by *asterisks*.

24 h, SOD activities increased both in the WT and in the transgenic lines, but more in the WT plants than in the transgenic plants, and CAT activity decreased more in transgenic lines (Fig. 5*A*,*B*). When exposed to the 100 μM MV treatment for 24 h, APX activity decreased by about 64.8, 78.3, 77.9, and 76.4 % in the WT, A1, A3 and A5 plants, respectively (Fig. 5*C*). These results suggest that reduction in activities of CAT and APX is one of the reasons behind accumulation of ROS in the transgenic plants.

Since AsA is an important non-enzymatic antioxidant, we measured the amounts of AsA (reuced), DHA, and the total AsA and calculated the reduced AsA/DHA ratio in leaves after the MV treatment (Fig. 6). With the increase in time of the MV treatment, content of AsA in all the plants decreased. After the treatment with 100 μM MV for 24 h, content of AsA in the WT, A1, A3, and A5 plants was reduced to 0.41, 0.30, 0.33, and 0.29 μmol g^{-1}(f.m.), respectively. Nevertheless, content of DHA increased by 0.9, 0.6, 0.5 and 0.7 μmol g^{-1}(f.m.), respectively. Compared with the transgenic seedlings, DHA content of the WT seedlings markedly increased, which indicates that there was less AsA reacting with H_2O_2 to produce DHA and H_2O in the transgenic lines. The AsA/DHA ratio decreased more substantially in the transgenic plants than in the WT plants when exposed to the MV stress. The ratio decreased by 76.4, 77.2, and 79.2 % in the A1, A3, and A5 transgenic plants compared with 75.3 % in the WT plants after 24 h of the MV treatment. On the other hand, the total AsA content increased by 0.6 μmol g^{-1}(f.m.) in the WT, whereas in the A1, A3, and A5 lines by 0.39, 0.38, and 0.39 μmol g^{-1}(f.m.), respectively. As a result, the transgenic plants had a lower tolerance toward the oxidative stress.

In order to investigate the involvement of *SlGGP* in regulation of gene expression under the MV stress, we performed real time quantitative PCR analysis for genes involved in the AsA biosynthesis pathway (Fig. 7). After the MV treatment for 24 h, a relative mRNA content of the most genes upstream in the AsA biosynthesis pathway decreased, but most genes upstream in the AsA biosynthesis pathway, including glucose-6-phosphate isomerase (*SlGPI*), phosphomannose isomerase (*SlPMI*), GDP-d-mannose pyrophosphorylase (*SlGMP*), and GDP-D-mannose 3,5-epimerase (*SlGME1*, and *SlGME2*), exhibited an obvious increase in transcript abundance in the transgenic lines compared with the WT plants. On the other hand, L-galactono-1,4-lactone dehydrogenase (*SlGalLDH*), which lies at the bottom of the AsA biosynthesis pathway, was little affected.

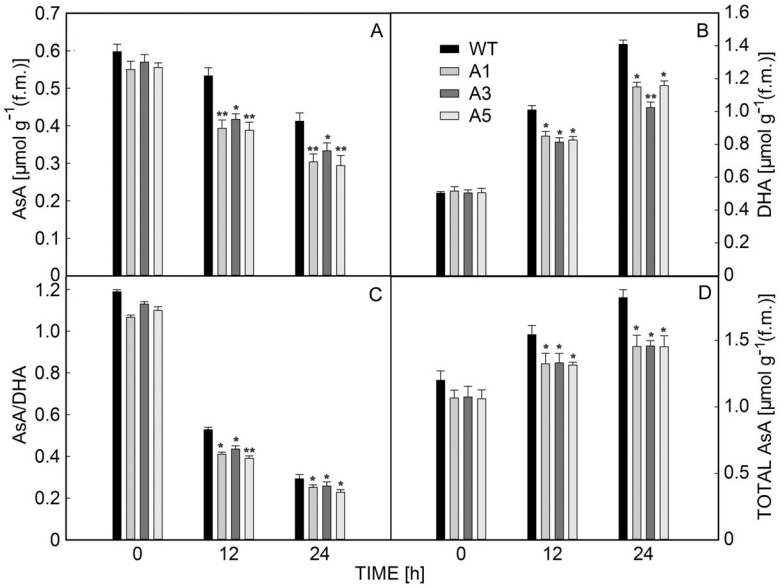

Fig. 6. Effect of 100 μM MV treatment for 0, 12, and 24 h on ascorbate (AsA, *A*), dehydroascorbate (DHA, *B*), AsA/DHA (*C*), and the total AsA (*D*) in transgenic and wild type (WT) plants. Means ± SDs from three measurements for each of the three plants. Significant differences compared to the WT are marked by *asterisks*.

Discussion

Ascorbate is the most abundant soluble antioxidant that plays a key role in photoprotection in chloroplasts (Smirnoff 2000). Previous study about AsA-deficient mutation indicated that AsA contributes to plant tolerance to oxidative stress (Conklin *et al*. 1996, Munné-Bosch and Alegre 2002, Huang *et al*. 2005, Li *et al*. 2010). In the presence of H_2O_2, AsA can be transformed into monodehydroascorbate by intracellular APX, and then

DHA is formed by a non-enzymatic reaction. Biosynthesis and regeneration of ASA are of vital importance to plants for detoxification free radicals generated under stressful conditions.

GDP-L-galactose phosphorylase catalyzes conversion of GDP-L-galactose to L-galactose-1-phosphate in the first committed step of Smirnoff-Wheeler pathway for plant AsA synthesis (Wheeler *et al*. 1998). The protein product of GGP is broadly conserved in plants and animals. The GGP might be a key rate-limiting enzyme for AsA biosynthesis in *Arabidopsis* (Bulley *et al*. 2009), tobacco (Laing *et al*. 2007), and apple (Mellidou *et al*. 2012). In our previous study (Wang *et al*. 2013), *SlGGP* antisense transgenic tomato lines were found to maintain only 50 - 75 % of ascorbate content found in the WT and produce more DHA under chilling stress (Wang *et al*. 2013). In this study, the AsA content of the transgenic plants under the MV stress was significantly reduced compared to the WT plants (Fig. 6). These results indicate that GGP played an important role in maintaining the AsA pool and AsA redox state under the stress conditions.

Methyl viologen is thought to be a very effective electron acceptor and catalyzes photo-reduction of O_2,

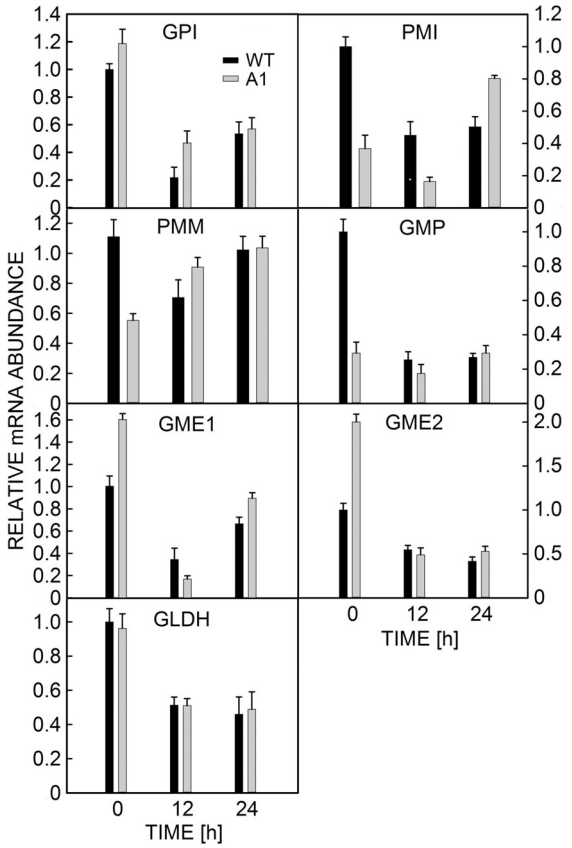

Fig. 7. Relative transcript abundances of ascorbate biosynthesis-related genes in A1, A3, and A5 transgenic lines and wild type (WT) plants after 100 μM MV treatment for 0, 12, and 24 h. Data obtained by real-time qPCR were normalized against tomato elongation factor (*SlEF1α*) expression and are expressed as the percentage of the control. Means ± SEs (n = 3) from a representative experiment.

thereby accelerating production of $O_2^{\cdot-}$ and H_2O_2 (Cornic *et al*. 2000). A visible damage of leaf disks under MV treatment is commonly used for resistance analysis in plants (Yoshimura *et al*. 2004). In our study, leaf disk experiments show that the antisense transgenic plants bleached more easily than the WT plants under the same MV concentrations (Fig. 1*A*). In addition, chlorophyll content was correlated with the degree of necrosis (Fig. 1*B*). These results suggest that the high AsA content helped maintain chloroplast activity through timely and effective removal of ROS, and this mechanism underlied the protective effect of GGP under the oxidative stress.

Different stresses can lead to increased accumulation of ROS such as superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals (Ishikawa and Shigeoka 2008). The ROS-scavenging capacity of chloroplasts depends on both non-enzymatic antioxidants and enzymatic antioxidants. After the MV treatment, we observed a significant decrease in activities of CAT and APX (Fig. 5). Oxidative stress often affects the stability of biological membrane systems that are related to normal physiological functions. The extent of lipid peroxidation is determined by MDA content and leakage of electrolytes (REC). Consistent with the idea that AsA

content is critical for stress tolerance, the antisense transgenic plants showed a higher content of $O_2^{\cdot-}$, H_2O_2, MDA, and a higher REC under the MV treatment (Figs. 2 and 3), which indicates that the antisense transgenic plants accumulated more ROS. Thus, inhibition of *GGP* gene expression led to a decreased capacity to scavenge ROS in the plants. These results indicate that GGP plays a major role in protecting plants against oxidative stress by driving the L-Gal pathway of AsA synthesis to maintain AsA pool and AsA redox state.

Ascorbate functions as electron donor in H_2O_2 detoxification, and a high content of AsA in chloroplasts helps maintain APX activity. A high AsA content and APX activity in chloroplasts can directly scavenge free radicals and so protect PS II. Consistent with this, the WT plants could sustain a higher P_N and F_v/F_m while antisense plants had a more decreased P_N and F_v/F_m under the MV stress (Fig. 4). Thus, an efficient biosynthesis of AsA may remove ROS to protect PS II against oxidative stress by alleviating photodamage and enhancing repair rate.

In previous study, up-regulation of *SlGMP* and *SlGGP* genes has been observed in *SlGME* transgenic tomato plants (Gilbert *et al*. 2009). Similarly, a significant increase in transcript abundances of *SlGME2* and *SlGGP1* downstream of *SlGMP* was found in *SlGMP2/3*-KD plants (Zhang *et al*. 2013). In contrast, the last two steps of the pathway, catalyzed by L-galactose dehydrogenase (SlGDH) and by SlGalLDH, were unaffected. Similar results have been observed with *SlGGP* antisense tomato plants under chilling stress: the transcript abundance of the genes of the upper part of the AsA biosynthesis pathway are remarkably increased, including *GME1* and *GME2*, and the last gene of the pathway, namely *SlGalLDH*, is unaffected (Wang *et al*. 2013). Interestingly, we found that, under the oxidative stress induced by MV, the suppression of the *SlGGP* gene in tomato led to a significant increase in transcript abundance of the upper part of the ascorbate biosynthetic pathway (Fig. 7), maybe because of biological processes to maintain a relatively stable ascorbate content in plants. Thus, our findings suggest that the *SlGGP* gene plays an important role in ascorbate biosynthesis in tomato.

In summary, the reduction of ascorbate content resulted in the changes of the antioxidative enzyme activities and in accumulation of ROS in the tomato, which aggravated PS II damage. Our findings indicate that the suppression of *SlGGP* reduced tolerance to the oxidative stress in the tomato.

References

Agius, F., Gonzalez-Lamothe, R., Caballero, J.L., Munoz-Blanco, J., Botella, M.A., Valpuesta, V.: Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. - Nat. Biotechnol. **21**: 177-181, 2003.

Bulley, S.M., Rassam, M., Hoser, D., Otto, W., Schüneman, N., Wright, M., MacRae, E., Gleave, A., Laing, W.: 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, 2009.

Conklin, P., Williams, E., Last, R.: Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. - Proc. natl. Acad. Sci. USA **93**: 9970-9974, 1996.

Cornic, G., Bukhov, N.G., Wiese, C., Bligny, R., Heber, U.: 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, 2000.

Davey, M., Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I., Strain, J., Favell, D., Fletcher, J.: Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. - J. Sci. Food Agr. **80**: 825-860, 2000.

Gechev, T.S., Van, Breusegem. F., Stone, J.M., Denev, I., Laloi, C.: Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. - Bioassays **28**: 1091-1101, 2006.

Gilbert, L., Alhagdow, M., Nunes-Nesi, A., Quemener, B., Guillon, F., Bouchet, B., Faurobert, M., Gouble, B., Page, D., Garcia, V.: The GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell wall biosynthesis in tomato. - Plant J. **60**: 499-508, 2009.

Hancock, R.D., Viola, R.: Biosynthesis and catabolism of L-ascorbic acid in plants. - Crit. Rev. Plant Sci. **24**: 167-188, 2005.

Holuigue, L., Salinas, P., Blanco, F., Garreton, V.: Salicylic acid and reactive oxygen species in the activation of stress defense genes. - In: Hayat, S., Ahmad, A. (ed.): Salicylic Acid – a Plant Hormone. Pp. 197-246. Springer, Dordrecht 2007.

Huang, C.H., He, W.L., Guo, J.K., Chang, X.X., Su, P.X., Zhang, L.X.: Increased sensitivity to salt stress in an ascorbate-deficient *Arabidopsis* mutant. - J. exp. Bot. **56**: 3041-3049, 2005.

Ishikawa, T., Shigeoka, S.: Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. - Biosci. Biotechnol. Biochem. **72**: 1143-1154, 2008.

Kwon, S.Y., Jeong, Y.J., Lee, H.S., Kim, J.S., Cho, K.Y., Allen, R.D., Kwark, S.S.: 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, 2002.

Laing, W.A., Wright, M.A., Cooney, J., Bulley, S.M.: The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L -galactose guanyltransferase, increases leaf ascorbate content. - Proc. nat. Acad. Sci. USA **104**: 9534-9539, 2007.

Li, F., Wu, Q.Y., Sun, Y.L., Wang, L.Y., Yang, X.H., Meng, Q.W.: Overexpression of chloroplastic monodehydro-ascorbate reductase enhanced tolerance to temperature and

methylviologen-mediated oxidative stresses. - Physiol. Plant. **139**: 421-434, 2010.

Li, X.G.., Meng, Q.W., Jiang, G..Q., Zou, Q.: The susceptibility of cucumber and sweet pepper to chilling under low irradiance is related to energy dissipation and water-water cycle. - Photosynthetica **41**: 259-265, 2003.

Linster, C.L., Gomez, T.A., Christensen, K.C., Adler, L.N., Young, B.D., Brenner, C., Clarke, S.G.: *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, 2007.

Loewus, M.W., Bedgar, D.L., Saito, K., Loewus, F.L.: Conversion of L-sorbonone to L-ascorbic acid by a NADP-dependent dehydrogenase in bean and spinach leaf. - Plant Physiol. **94**: 1492-1495, 1990.

Lorence, A., Chevone, B.I., Mendes, P., Nessler, C.L.: Myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. - Plant Physiol. **134**: 1200-1205, 2004.

Mellidou, I., Chagne, D., Laing, W.A., Keulemans, J., Davey, M.W.: Allelic variation in paralogs of GDP-L-galactose phosphorylase is a major determinant of vitamin C concentrations in apple fruit. - Plant Physiol. **160**: 1613-1629, 2012.

Miller, G., Suzuki, N., Rizhsky, L., Hegie, A., Koussevitzky, S., Mittler, R.: Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. - Plant Physiol. **144**: 1777-1785, 2007.

Munné-Bosch, S., Alegre, L.: Interplay between ascorbic acid and lipophilic antioxidant defences in chloroplasts of water-stressed *Arabidopsis* plants. - FEBS Lett. **524**: 145-148, 2002.

Murik, O., Elboher, A., Kaplan, A.: Dehydroascorbate: a possible surveillance molecule of oxidative stress and programmed cell death in the green alga *Chlamydomonas reinhardtii*. - New Phytol. **202**: 471-484, 2014.

Noctor, G.: Metabolic signalling in defence and stress: the central roles of soluble redox couples. - Plant Cell Environ. **29**: 409-425, 2006.

Sasaki-Sekimoto, Y., Taki, N., Obayashi, T., Aono, M., Matsumoto, F., Sakurai, N., Ohta, H.: Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in *Arabidopsis*. - Plant J. **44**: 653-668, 2005.

Smirnoff, N., Conklin, P.L., Loewus, F.A.: Biosynthesis of ascorbic acid in plants: a renaissance. - Annu. Rev. Plant Physiol. Plant mol. Biol. **52**: 437-467, 2001.

Smirnoff, N.: Ascorbate biosynthesis and function in photoprotection. - Phil.. Trans. roy. Soc. London B. Biol. Sci. **355**: 1455-1464, 2000.

Van Kooten, O., Snel, J.F.H.: The use of chlorophyll fluorescence nomenclature in plant stress physiology. - Photosynth. Res. **25**: 147-150, 1990.

Wang, L.Y., Li, D., Deng, Y.S., Lv, W., Meng, Q.W.: Antisense-mediated depletion of tomato GDP-L-galactose phosphorylase increases susceptibility to chilling stress. - J. Plant Physiol. **170**: 303-314, 2013.

Wheeler, G., Jones, M., Smirnoff, N.: The biosynthetic pathway of vitamin C in higher plants. - Nature **393**: 365-369, 1998.

Wolucka, B.A., Goossens, A., Inze, D.: Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. - J. exp. Bot. **56**: 2527-2538, 2005.

Yoshimura, K., Miyao, K., Gaber, A., Takeda, T., Kanaboshi, H., Miyasaka, H., Shigeoka, S.: Enhancement of stress tolerance in transgenic tobacco plants overexpressing *Chlamydomonas* glutathione peroxidase in chloroplasts or cytosol. - Plant J. **37**: 21-33, 2004.

Zhang, C., Ouyang, B., Yang, C., Zhang, X., Liu, H., Zhang, Y., Zhang, J., Li, H., Ye, Z.: Reducing AsA leads to leaf lesion and defence response in knock-down of the AsA biosynthetic enzyme GDP-D-mannose pyrophosphorylase gene in tomato plant. - PLoS ONE **8**: e61987, 2013.

Zhang, C.J., Liu, J.X., Zhang, Y.Y., Cai, X.F., Gong, P.J., Zhang, J.H., Wang, T.T., Li, H.X., Ye, Z.B.: Over-expression of *SlGMEs* leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. - Plant Cell Rep. **30**: 389-398, 2010.

Zhang, W., Gruszewski, H.A., Chevone, B.I., Nessler, C.L.: An *Arabidopsis* purple acid phosphatase with phytase activity increases foliar ascorbate. - Plant Physiol. **146**: 431-440, 2008.