Title: Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in Arabidopsis

Running title: DHAR overexpression enhanced oxidative stress tolerance

Authors: Zinan Wang¹#, Lei Zhang²#, Ying Xiao³, Wansheng Chen³, and Kexuan Tang¹*

¹State Key Laboratory of Genetic Engineering, School of Life Science, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, Fudan University, Shanghai 200433, P. R. China
²Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai, 200433, P. R. China
³Department of pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200003, P. R. China

#: These authors contributed equally to this work.
*Author for correspondence

Tel: +86 21 65642772;
Fax: +86 21 65643922;
E-mail: kxtang1@yahoo.com or kxtang1@163.com or kxtang@fudan.edu.cn

Supported by National Basic Research Program of China (973 Program, 2007CB108805), National Natural Science Foundation of China (30600807, 30900786), Modernization of traditional Chinese medicine foundation (08DZ1971502) and Shanghai Western Development Cooperation Foundation (084358014), Shanghai Science and Technology Committee.
Abstract
Vitamin C (L-ascorbic acid, AsA) has important antioxidant and metabolic functions in both plants and animals. Once used, ascorbic acid can be regenerated from its oxidized form in a reaction catalyzed by dehydroascorbate reductase (DHAR, EC 1.8.5.1). To analyze the physiological role of DHAR catalyzing the reduction of DHA to ascorbate in environmental stress adaptation, we examined whether increase the level of AsA through enhanced AsA recycling would limit the deleterious effects of oxidative stress. A chimeric construct consisting of the double CaMV35S promoter fused to the Myc-dhar gene was introduced into A. thaliana. Transgenic plants were biochemically characterized and tested for responses to oxidative stress with the aim of studying the generality phenomena. Western blot indicated that the dhar-transgene was successfully expressed. In homozygous T₄ transgenic seedlings, DHAR overexpression was increased up to 1.5 to 5.4 fold, which enhanced foliar ascorbic acid levels 2- to 4.25-fold and ratio of AsA/DHA about 3- to 16-fold relative to wild type. In addition, the level of glutathione, the reductant used by DHAR, also increased as did its redox state. When whole plants were treated with high light and high temperature stress or in vitro leaf discs were subjected to 10 μM paraquat, transgenic plants showed a larger AsA pool size, lower membrane damage, a higher level of chlorophyll compared to control. These data suggested that increasing the plant vitamin C content through enhanced ascorbate recycling could limit the deleterious effects of environmental oxidative stress.

Key words: ascorbic acid; dehydroascorbate reductase; glutathione; oxidative stress; redox state
Ascorbic acid (AsA) gains its name from its inherent property as vitamin C. It is required for cardiovascular function, immune cell development, connective tissue, and iron utilization. This compound is also an important enzyme cofactor and it is involved in cell division, expansion and elongation (Smirnoff 1996; Arrigoni et al. 1997; Kato and Esaka 1999, 2000). AsA also plays an important role as an antioxidant or reductant in several pathways including the xanthophyll cycle and regeneration of vitamin E in animals, protects the plant during oxidative damage by scavenging free radicals and reactive oxygen species (Leowus 1999). Moreover, the AsA redox state, controlled by AO activity levels, is mainly responsible for the apoplast capability of transmitting signals related with environmental changes or defence processes (Pignocchi and Foyer 2003).

Oxidation of AsA produces the short-lived radical mono-dehydroascorbate (MDHA), which is converted to AsA by MDHA reductase (MDHAR) or disproportionates nonenzymatically to AsA and dehydroascorbate (DHA) (Asada 1992). DHA undergoes irreversible hydrolysis to 2, 3-diketogulonic acid or is recycled to AsA by dehydroascorbate reductase (DHAR), which uses glutathione (GSH) as the reductant (Chen et al. 2003). Thus, DHAR allows the plant to recycle DHA, thereby recapture AsA before it is lost (Figure 1). Plant DHAR cDNAs were cloned from rice (Urano et al. 2000), spinach (Shimaoka et al. 2000), Arabidopsis (Shimaoka et al. 2000), wheat (Chen et al. 2003) and tomato (Zou et al., 2006).

Chen et al (2003) did an excellent work to confirm that overexpression of DHAR in plants would increase the level of ascorbic acid through improved ascorbate recycling. A DHAR cDNA from wheat was isolated and expressed in tobacco and maize, where DHAR expression was increased up to 32- and 100-fold, respectively. The increase in DHAR expression increased foliar and kernel endogenous ascorbic acid levels 2 to 4-fold and significantly increased the ascorbate redox state in both tobacco and maize. At the same time it did not exhibit any disadvantages such as growth impairment, chlorosis, necrosis, or premature senescence. These results demonstrate that the vitamin C content of plants can be elevated by increasing expression of the enzyme responsible for recycling ascorbate.

Naturally, various abiotic stresses, such as UV radiation, ozone, drought and chilling lead to the production of reactive oxygen species (ROS) in plant (Smirnoff 1998). The presence of ROS leads to the induction of antioxidative enzymes, which are ostensibly able to reduce the oxidizing
environment created by ROS (Blokhina et al. 2003). Plants expressing these enzymes are more resistant to paraquat treatment (Van et al. 1994). Increasing the level of AsA through enhanced AsA recycling in tobacco (Nicotiana tabacum) provided greater protection against ozone (Chen and Gallie 2005), H2O2 and other abiotic stress (Kwon et al. 2003).

In the work presented here, we for the first time took Arabidopsis as a model system and introduced homogenous DHAR to investigate whether DHAR overexpression led to increased AsA content and a substantial change in the intracellular redox state, a stimulation of protective effects in environmental stress adaption under various stress including paraquat and high temperature treatment. In particular, we examined some of the chemical details of these enhancements to in-depthly evaluate the mechanism of DHAR function and its application potential in plant biotechnologies.

Results
Plant transformation and molecular characterization of transgenic Arabidopsis
Transforming Arabidopsis with the pHB::dhar binary construct contained both dhar and two selected marker genes (hygromycin and bar) on the same T-DNA fragment and we selected primary transformants (T0) on the basis of resistance to the antibiotic hygromycin (50 μg/ml) and 1% PPT and established transgenic lines by single seed descent. The accumulation of the Myc-DHAR fusion protein in transgenic plants was examined by western blot analysis using an antibody against Myc. A strong cross-reaction signal corresponding to a polypeptide with an expected molecular weight of 33 KD for the Myc-DHAR fusion protein was present in all the six tested plant lines (Figure 2B). As expected, no signal was observed using total protein extracted from WT.

To investigate details on whether the dhar overexpression in Arabidopsis regulated endogenous genes involved in AsA metabolism at transcriptional level, we designed specific primers and carried out QRT-PCR using total RNA from same age and standard cultured WT and dhar transgenic plants (Figure 2C). Densitometry indicated that expression of most candidate genes was not affected by dhar transgene whereas there was marked (range from 2 to 3.5 folds) increase of mdar and dhar expression observed in transgenic lines compared with WT expression. On the
contrary, only ao expression in dhar transgenic plants represented roughly 60% decrease as compared with WT expression.

**Phenotype**
Homozygous T₄ plant seeds were analyzed in order to characterize the global effects of dhar overexpression and physiological differences. WT and transgenic seedlings showed similar phenotype and life cycle (Figure 3A, B). No statistically significant differences ($p > 0.05$) were observed for plant height and number of rosette leaf after tallow and at end of the culture respectively (data not shown). Overexpressing of DHAR did not lead to chlorosis and necrosis or premature senescence.

**Assessment of in vivo / in vitro oxidative stress tolerance**
Morphological differences in response to the *in vitro / in vivo* oxidative stress between transgenic and WT plants were evident. VHb transgene has been shown to improve cellular protection against high light stress and high temperature. The difference in physical appearance was obvious in the whole plants where the WT paradoxically, exhibited evident anthocyanin accumulation (Figure 3C). However the growth defect was not observed in sense dhar lines (Figure 3D). Leaf discs, incubated for 2 days in a 10 μM paraquat solution, WT seedling turned yellow and shrinkage, displayed a profound reduction in chlorophyll (Figure 3E) while the dhar transgenic seedling was greener, healthier, and showed less leaf necrosis (Figure 3F).

Resistance to oxidative stress was assessed by measuring electrolyte leakage and the loss of chlorophyll ($Chl$) before and after oxidative stress. Before treatments, there were no significant differences both in the ion leakage and chlorophyll content between transgenic and WT plants. DHAR transgenic plants showed approximately 46.2% less membrane damage caused by high temperature (40ºC) relative to WT. Transgenic leaf discs exposure to 10 μM paraquat for 2 h showed 23.3% less damage than WT (Figure 4A).

High temperature and exposure to paraquat resulted in a reduction in chlorophyll content. DHAR-OX leaves contained greater chlorophyll (both $Chl$ $a$ and $b$) content than control leaves (Figure 4B). Despite a substantial decrease in $Chl$ $a$ and $b$ content, the $Chl$ $a/b$ ratio remained unaffected by the two kinds of stress treatment (data not shown).

Prior to the paraquat treatment, the stomata of *DHAR-ovx* plants opened wider than those of
the WT (Figure 4C) and the average stomatal aperture as measured in the morning was 40% larger in transgenic leaves compared to the WT (Figure 4D). Although exposure to 10 μM paraquat for 2h was sufficient to induce stomatal closure in both WT and transgenic lines (Figure 4C), no significant difference in the responsive diminished stomatal aperture (Figure 4D).

**Overexpressing dhar increased DHAR activity and maintained a larger AsA pool**

Before stress treatment, the DHAR activity of transgenic lines was \((12.8\pm2.92) \times 10^{-5}\) mol/min/g FW, compared with the WT \((4.1\pm0.8) \times 10^{-5}\) mol/min/g FW (Figure 5A). Transgenic leaves, in which DHAR expression was increased up to 3.1-fold, exhibited a 3.3-fold increases in the level of AsA and a 60% reduction in the level of DHA respectively, thus resulting in an increase in the AsA redox state (AsA/DHA) from 2.8 in WT to 13.6 in transgenic leaves (Figure 5B). In addition to AsA, changes in DHAR expression affected the GSH pool size and redox state. GSH content was \(1.25 \pm 0.17\) µmol/g FW in transgenic plants and \(0.46\pm0.07\) µmol/g FW in WT. Transgenic leaves exhibited a 2.71-fold increases in the level of GSH. Little differences in the level of oxidized glutathione (GSSG) was observed in WT and transgenic leaves (Figure 5B).

Following oxidative stress treatment, the DHAR activity was not significantly different in WT lines, but it was increased approximately 11.7% under high temperature (40°C) culture condition and decreased 15.6% after an acute exposure (2 h) to 10 μM paraquat treatment in transgenic plants (Figure 5A). The foliar level of both AsA and GSH, including their oxides (DHA and GSSG) was increased slightly in WT and dhar transgene plants after two kinds of oxidative stress treatment (Figure 5B). Although total ascorbate content was not found to be significant different between the genotypes, the AsA redox state in transgenic leaves was 8.47 times higher than in the WT plants. As with AsA, oxidative stress treatment resulted in an altered ratio of reduced GHS redox state in both DHAR transgenic and WT plants (Figure 5B).

**Discussion**

Metabolic engineering is a new approach to the understanding and utilization of metabolic processes. Advances of plant biochemical pathways and availability of gene transfer techniques in plant have led to a growing interest in using this technique to redirect metabolic fluxes in plants for industrial purpose. AsA is the major contributor to the value of nutrition, both plant and human, also is an important factor of stress tolerance in agronomical species. The information
gained in recent years about the AsA biosynthesis pathway in plants provide a solid foundation for modern breeding aimed at changing AsA content in crops.

Our results have demonstrated that the vitamin C content of Arabidopsis thaliana can be increased by overexpressing DHAR. The increase in ascorbate was exciting, it suggested that although AsA is the major antioxidant contributing to the redox status of the cell and that its synthesis is subject to feedback inhibition by the pool size of ascorbate (Pallanca and Smirnoff 2000), its content can be increased by genetic engineering. Accompanied by 3.1-fold increase in DHAR activity, a 3.3-fold increase in the level of AsA and a 60% reduction in the level of DHA were observed after introduction of homogeneous DHAR cDNA into Arabidopsis thaliana. Because GSH is used as the reductant by DHAR, the levels of reduced (GSH) and oxidized (GSSG) glutathione were also determined. An increase in the level and redox state of glutathione was observed in DHAR-overexpressing plants. Increased glutathione redox state plays an important role in maintaining their viability during storage (Chen and Gallie 2006).

The cellular concentration of AsA is determined by the rate of its synthesis and decay. If not salvaged by DHAR, DHA is rapidly hydrolyzed into 2, 3-diketogulonic acid, finally decayed into CO₂, H₂O₂ and L-thearate (Green and Fry 2005). The overexpression of DHAR in transgenic plants would increase the likelihood that DHA is converted to AsA before decayed, that would lead to the increase in AsA in transgenic plants. Therefore, the enhanced rescue of DHA from its decay pathway may be the reason that the AsA concentration in DHAR-overexpressing plants increased. The increased AsA accompanied the increased DHAR activity suggests that the amount of endogenous DHAR may be limiting. It might enable plants to control their intracellular redox state, particularly in response to developmental cues or changes in their external environment (Chen et al. 2003).

Through its AsA recycling function, DHAR affects the level of foliar ROS and photosynthetic activity during leaf development and as a consequence, influences the rate of plant growth and leaf aging (Creissen et al. 1999). Tobacco overexpressed γ-ECS exhibited chlorosis and necrosis. Although DHAR-overexpressing plants exhibit elevated levels of ascorbate and glutathione, they differ from γ-ECS-overexpressing plants and did not exhibite chlorosis and necrosis (Chen et al. 2003). In our study, the life cycle and the rate of plant growth was similar with the control. It suggested that these growth defects were not correlated with the increase in glutathione but rather with a decrease in glutathione redox state.
Oxidative stresses are encountered in all living organisms. Oxidative damage to proteins, nucleic acid can occur when the concentration of reactive species exceeds the capacity of the cell’s mechanisms for elimination (Farr and Kogoma 1991). Naturally, various abiotic stresses, such as UV radiation, ozone, drought and chilling lead to the production of ROS in plant (Smirnoff 1998). The presence of ROS leads to the induction of antioxidative enzymes, which are ostensibly able to reduce the oxidizing environment created by ROS (Blokhina et al. 2003).

Plants expressing these enzymes are more resistant to paraquat treatment (Van et al. 1994). The positive effects of DHAR expression in plant genetic engineering have been previously described (Chen and Gallie 2005; Huang et al. 2005; Ratajczak and Pukacka 2006; Ushimaru et al. 2006; Yoshida et al. 2006), which leads resistance to ozone, salt and desiccation tolerance. To evaluate the potential of DHAR function as concerning the response to oxidative stress with a genetic engineering based approach, we have tested DHAR transgenic Arabidopsis plants under in vitro and in vivo stress conditions.

In this study we analyzed the effects of DHAR transgene on the oxidative stress protection in the presence of ROS in Arabidopsis in vivo and in vitro. DHAR transgene has been shown to improve cellular protection against high light and high temperature stress. The difference in physical appearance was obvious in the transgenic plants for the increased AsA played a key photoprotective role in protecting plant against light-dependent photo-oxidative cell damage (Smirnoff 1996).

We were also able to find any significant differences in oxidative stress defense imposed by paraquat between DHAR transgenic lines and WT leaf discs. When exposed to the 10% paraquat for 2 h, although typical oxidative stress symptoms could be seen, DHAR transgene lines showed distinctive leaf morphology compared with the control. Cell leakage and total chl content were less affected in DHAR-expressing cultures compared with WT. To examine whether the level of AsA had a similar effect on guard cell responsiveness following exposure to paraquat, the stomatal aperture was measured before and immediately after paraquat treatment. Prior to the paraquat treatment, the average stomatal aperture was significant larger in the DHAR transgenic leaves relative to the control. But little difference was observed between WT and trasgenic lines following exposure to 2h of 10% paraquat. These findings are in good agreement with previous
measurements (Chen and Gallie 2005; Chen and Gallie 2006). Our results have clearly demonstrated that overexpress DHAR properly works for the protection against oxidative stress in plants, as reflected in relieving oxidative stress and protect it from oxidative damage both in vivo and in vitro.

Although chemical synthesis is the major method to produce vitamin C, the natural product is welcome in the market. Increasing in AsA in plants gives convenience in industrial production. And also, such an increase would require decreased dietary emphasis of foodstuffs rich in ascorbate. Overexpressing DHAR to increase AsA represents a simple strategy of metabolic engineering.

For future developing the full potential of metabolic engineering it is thus necessary to increase our detail knowledge about plant AsA metabolism on the level of intermediates, enzymes and genes. On the physiology of the pathway, the flues through the pathway are controlled to a greater extent at the level of enzymes and intracellular and intercellular transport. Fluxes are not only determined by gene expression, but also by post-translational regulation of enzyme activity and enzyme and metabolite compartmentation and transport. Classic biochemistry will continue to play an important role in determining cellular regulatory features and in measuring flux and intermediate concentrations to provide essential metabolic context. In addition, developmental processes, stress responses and environmental conditions are all known to markedly affect the AsA content of individual species. A better understanding of the mechanisms internal and external to the plant that control AsA accumulation in plants could lead to strategies for increasing the AsA content of crops. Unraveling plant AsA metabolic pathway with this level of understanding is the challenging way to successful applications in fields such as molecular farming, healthy food, functional food, and plant resistance.

Materials and methods

Construction of expression cassette, transformation, and growth conditions of plants

Arabidopsis seeds were sterilized with 20% bleach and were put in a 4 ºC refrigerator for 3 to 5 d, germinated on MS medium and then transferred to soil and grown in long-day light (16 hr light/ 8 hr dark) at 22ºC at a fluence rate of 100 μmol·s⁻¹·m⁻² of white light produced by cool-white fluorescent lamps.

Full-length Arabidopsis (Columbia ecotype) DHAR cDNA was isolated from seedling total
RNA by one-step RNA PCR. The 6×Myc tag was cloned into Bam HI and Spe I sites of pBluescript SK+ (pBS; Stratagene). The dhar gene was amplified from the pMD-dhar construct using PCR (forward primer, 5′-CCGAATTCAAGATGGCTCTGGAAATCTGUG-3′; reverse primer, 5′-AACTGCAGAGGGTACCTTGGGAGCCC-3′) with an introduced EcoRI I site at the first ATG and was cloned into EcoRI I and Pst I sites of pBS-6×Myc. After confirmation by sequencing, the fragment encoding dhar-myc was excised with EcoRI I and Spe I and subsequently inserted into PHB binary vector (supplied by Prof. H. Yang, SIPPE, CAS) containing a hygromycin resistant gene and anti-herbicide gene (BAR) inside the T-DNA for the selection of transformants.

The pHB::dhar construct (Figure 2A) was transferred into Agrobacterium tumefaciens GV3101 (rifampicin resistant) by triparental mating and the floral-dip method was used to conduct transformation of Arabidopsis, Columbia ecotype (Clough and Bent 1998). T0 seeds were screened on 15 cm Murashige–Skoog (MS) basal plates supplemented with 50 μg/ml hygromycin. Then, 30 to 50 independent lines expressing transgene were transferred to soil and screened on 1% PPT. Polymerase chain reaction (PCR) and protein gel blot analysis were performed on 8 to 16 T3 independent transgenic lines to check for homozygosity (T4). Six independent dhar-overexpressing homozygous T4 lines (4, 5, 12, 17, 26, 33) were random selected for final analysis. Chemical components were measured in plants 18-20 days old.

**Western Analysis**

Protein gel blot analysis was performed as described previously with minor modifications (Yang et al. 2000). **Aliquots of 50 μg total soluble proteins per sample**, determined with the DC Protein Assay Kit (Bio-Rad, Hercules,CA), was fractionated on a 10% SDS-PAGE mini-gel and blotted to a polyvinylidene difluoride membrane (Amersham). The blots were probed with the primary antibody -Myc (α-Myc, Santa Cruz Biotechnology) (diluted in PBST (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, and 0.1% Tween 20)), washed in PBST three times, reacted with goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000, Amersham), washed, and exposed to x-ray film using the enhanced chemiluminescence method according to the manufacturer’s instructions (Amersham).

**Real-time quantitative analysis**
Total RNA was extracted from 5-week-old both transgenic *dhar* leaf tissue and wild type controls (WT) cultured in standard conditions with Plant RNA Mini Kit (Watson, China) and treated with RNase-free DNase (Promega). The quality and concentration of RNA samples were examined by agarose gel electrophoresis and spectrophotometer analysis. Total RNA was reversely transcribed by using AMV reserve transcriptase (Takara, Japan) to generate cDNA. All the gene-specific primers were designed according to the conserved region of the corresponding sequences of *A. thaliana*. Partial of polyubiquitin gene was amplified with primers (5′-ACCCTCACGGGAAGACCATC-3′ and 5′-ACCACGGAGACGGAGGACAAG-3′) as a control. The Real-time PCR was performed according to manufacturer’s instruction (Takara, Japan) under the following condition: 1 min pre-denaturation at 95°C, 1 cycle; 10s denaturation at 95°C, 30 s annealing at 56°C, 15 s collection fluorescence at 72°C, 40 cycles. The products of real-time quantitative PCR were run on 1.5% agarose gel electrophoresis and showed an equal-sized band as predicted. Quantification of the gene expression was done with comparative CT method. Each data represents the average of three experiments.

**Oxidative stress tolerance assay and stomatal aperture measurements**

4-week-old plants grown in normal condition were subjected to high-light (1, 000 μmol·s⁻¹·m⁻²) and high-temperature (40°C) for 1 week as *in vivo* oxidative stress. *In vitro* oxidative stress experiments were performed as described previously (Wang et al. 2009) with minor modification. Leaf discs from 3- to 4- week-old plants were incubated for 2 days in 10 μM paraquat (Sigma-Aldrich) solution or in water at 22°C with constant light. Mature stomata of epidermal strips were used for stomatal aperture measurements as described (Mao et al. 2005).

**Measurement of chlorophyll content and electrolyte leakage**

After *in vitro* and *in vivo* oxidative stress treatment, 1 g of young leaves from transgenic and WT Arabidopsis lines were collected for the presence of chlorophyll *a* and *b* and total protein by spectral determination. Immediately after sampling, leaves were frozen in liquid N₂ and stored at -80°C. Subsequently, frozen leaf samples were ground in an ice-cold mortar and pigments were extracted with 5 ml of 80% (v/v) acetone. The homogenates were centrifuged at 2500 rpm for 10 min at 4°C. The extraction procedures were carried out under low light intensity. *Chl a, b* were determined by measuring the absorbance of the supernatant at 663 and 646 nm respectively.
(Wellburn 1994). Electrolyte leakage was determined using an osmometer (DDS-11A, SuoShen, China). Values were given as a percentage: for each sample 100% was determined by boiling the sample for 3 min leading to the complete release of electrolytes. Electrolyte leakage was given as the ratio of the amounts of electrolyte with and without stress treatment. Each analysis was repeated ten plants per treatment and three times per plant.

**Enzyme assays**
Leaves were ground in extraction buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM MgCl2), after centrifuged at 13, 000 rpm for 5 minutes, the soluble protein in the leaves was obtained. DHAR was assayed as described (Bradford 1976; Hossain and Asada 1984) in 50 mM K2HPO4/KH2PO4, pH 6.5, 0.5 mM DHA, 1 mM GSH (equal loading of total protein), and its activity was followed by an increase in absorbance at 265 nm.

**AsA, DHA, GSH, and Oxidized Glutathione (GSSG) measurements**
Leaves were ground in 2.5 M HClO4 and after a 10 min centrifugation at 13, 000 rpm, two volumes of 1.25 M Na2CO3 were added to the supernatant. 100 μl of the mixture after centrifugation was added to 895 μl of 100 mM K2HPO4-KH2PO4, pH 5.6. After 0.25 unit of ascorbate oxidase (AO) was added, AsA was determined by the change in absorbance at 265 nm. When DHA was reduced to AsA (100 mM K2HPO4/KH2PO4 at pH 6.5, 2 mM GSH, and 0.1 μg of AtDHAR protein, incubated at 25°C for 20 min) before measuring AsA, the total amount of reduced and oxidized ascorbic acid (i.e., AsA and DHA) could be determined. The amount of DHA was determined as the difference between these two assays above. GSH and GSSG were determined as described (Griffith 1980).

**Statistical analysis**
The statistic significance of the genes expression level, responses to oxidative stress, chlorophylls, electrolyte leakage, enzyme activity and metabolites content among various transgenic plants and wild type controls were calculated using Statgraphics® Plus 5.0 (Statistical Graphics Corp.) and error bars on graphs indicated standard error (SE) calculated from at least ten replicated samples.

**Acknowledgements**
The authors are very grateful to Professor Hongquan Yang (Shanghai JiaoTong University, China) for the generous gift of pBS-MYC vector and for assistance with Arabidopsis transformation. The authors also gratefully acknowledge to Dr. Peng Zou (National University of Singapore, Singapore) for assistance in HPLC analyses.

References


Figure legends

Figure 1. **Generalized scheme of AsA biosynthetic network in plants.** Oxidation of AsA produces monodehydroascorbate (MDHA) by the enzymes ascorbate peroxidase (APX) and ascorbate oxidase (AO), which is converted to AsA by MDHAR or disproportionates nonenzymatically to AsA and dehydroascorbate (DHA). DHA spontaneously hydrolyzes to 2, 3-diketogulonic acid unless salvaged by DHAR, which uses GSH as the reductant. Oxidized glutathione (GSSG) is reduced by glutathione reductase (GR).

Figure 2. **Over-expression of Myc-Tagged DHAR in Arabidopsis.** (A) Schematic diagrams displaying constructs expressing Myc-tagged DHAR. (B) Western blot showing expression of Myc-AtDHAR by using α-Myc antibody in non-transgenic (WT) and six independent *AtDHAR-ovx Arabidopsis* lines (No. 12, 33, 4, 5, 26, 17). (C) Quantitative reverse transcriptase (QRT)-PCR was performed on 5-week-old plant from six independent *AtDHAR-ovx Arabidopsis* lines (same line No. as used in protein gel blot analysis) and WT to analysis the expression level of endogenous genes involved in plant vitamin C biosynthetic pathway. Polyubiquitin was used as the internal control. ao: ascorbate oxidase; ap: ascorbate peroxidase; dhar: dehydroascorbate reductase; galdh: L-galactose dehydrogenase; galppase: L-galactose 1-P phosphatase; gdp-gal: GDP-galactosephosphorylases; gdpme: GDP-D-mannose-3, 5-epimerase; gdpmppase: GDP-D-mannose pyrophosphorylase; gldh: L-galactono-1, 4-lactone dehydrogenase; mdar: MDA reductase.

Figure 3. **Plant physical appearances.** *AtDHAR*-overexpression is not detrimental to growth of transgenic plants (A) compared to wild type (B) which were grown under normal culture conditions for ≈21 days; *AtDHAR-ovx* plants exhibit less damage following subject to high temperature stress (D) and exposure to 10 μM paraquat (F) compared to wild type (C and E) respectively.
Figure 4. Biochemical characterizations of WT and six independent AtDHAR-ovx lines (No. 12, 33, 4, 5, 26, 17). (A) Analysis of cellular damage measured as ion leakage before and after in vivo / in vitro oxidative stress treated. (B) DHAR transgenic plants maintain greater Chl content following treated with two kinds of oxidative stress. (C) Images of stomata of the WT and DHAR-ovx plants from the abaxial epidermis of fully expanded leaves prior to and immediately following a 2-h exposure to 10 μM paraquat (Scale bars in this images represent 10 μm); (D) The stomatal aperture (width/length) was determined from the abaxial epidermis of fully expanded WT and trasgenic leaves prior to and immediately following a 2-h exposure to 10 μM paraquat. Three replicated samples analyzed for each line. This experiment was repeated three times and similar results were obtained. *: Significant differences in mean conductivities between DHAR transgenic and WT plants determined by t-test (P=0.05)

Figure 5. Enzyme and chemical analysis in non-transgenic control (WT) and six independent AtDHAR-ovx plants (OX) (No. 12, 33, 4, 5, 26, 17) prior to and following subject to high temperature stress and exposure to 10 μM paraquat.. (A) Foliar levels of DHAR activities; (B) ascorbate and glutathione content. Values are mean ± SE of three replicates. *: Significant differences in mean activities between DHAR transgenic and WT plants determined by t-test (P=0.05)
Figure 1

D-Glucose $\xrightleftharpoons{HK}^{}$ D-Glucose-6-P $\xrightarrow{PGI}^{}$ D-Fructose-6-P $\xrightarrow{PMI}^{}$ D-Mannose-6-P

GDP-L-Galactose $\xrightarrow{GDPME}^{}$ GDP-D-Mannose $\xrightarrow{GDPME Pase}^{}$ D-Mannose-1-P

L-Gal-1-P $\xrightarrow{L-Gal Pase}^{}$ L-Galacto-1,4-lactone $\xrightarrow{L-Gal DH}^{}$ L-Galactose $\xrightarrow{ADLT}^{}$ L-Galactono-1,4-lactone

CH$_2$OH $\xrightarrow{CH_2OH}^{}$ CH$_2$OH $\xrightarrow{CytCOX}^{}$ CytCed

NAD $\xrightarrow{NAD}^{}$ NADH $\xrightarrow{CytCed}^{}$ L-Ascorbic acid

L-Gala $\xrightarrow{APX}^{}$ Monodehydro-L-ascorbic acid

MDHAR $\xrightarrow{L-Gal A}^{}$ Dehydro-L-ascorbic acid

DHAR $\xrightarrow{(GR)}^{}$ GSSG $\xrightarrow{}^{}$ NADPH+H$^+$

GSSG $\xrightarrow{}^{}$ GSH $\xrightarrow{}^{}$ NADP$^+$

non-enzymatic disproportionation

Spontaneous hydrolysis

2,3-diketo-gulonic acid
Figure 2

A

2 × 35S  1  642 rbcs polyA

B

KDa

WT  12  33  4  5  26  17

Myc-AtDHAR

C

Expression level

WT  DHAR-OVX

gdpmpase  gdpine  galpase  gdp-gal  gldh  gldh  indar  dhar  ap  ap
Figure 5

A

Before stress 40 °C 10 μM paraquat, for 2h

DHAR μmol AsA/min/g FW

WT OX WT OX WT OX

B

AsA  DHA

0 4 8 12 16

0 2 4 6 8

μmol/g FW

WT OX 10 μM paraquat for 2h

40 °C WT OX

Before stress WT OX

WT OX

0 0.5 1 1.5 2 2.5

μmol/g FW

GSH GSSG