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Ascorbate plays a key role in alleviating low-temperature-induced oxidative stress in *Arabidopsis*

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Abstract

Low temperature has a negative impact on plant cells and results in the generation of reactive oxygen species (ROS). In order to study the role of ascorbate under chilling stress, the response of an ascorbate-deficient *Arabidopsis thaliana* mutant *vtc2-1* to low temperature (2°C) was investigated. After chilling stress, *vtc2-1* mutants exhibited oxidative damage. An increase in the H_2O_2 generation and the production of thiobarbituric acid reactive substances (TBARS), and a decrease in chlorophyll content, the maximal photochemical efficiency of PSII (F_v/F_m) and oxidizable P_{700} were also noted. The ratio of ascorbate/dehydroascorbate and reduced glutathione/oxidzed glutathione in the *vtc2-1* mutants were reduced, compared with the wild type (WT) plants. The activities of antioxidant enzymes, such as catalase (CAT) and ascorbate peroxidase (APX), and soluble antioxidants were lower in the *vtc2-1* mutants than those in WT plants. These results suggested that the ascorbate-deficient mutant *vtc2-1* was more sensitive to chilling treatment than WT plants. The low temperature-induced oxidative stress was the major cause of the decrease of PSII and PSI function in the *vtc2-1* mutants. Ascorbate plays a critical role of defense without which the rest of the ROS defense network is unable to react effectively.

Additional key words: *Arabidopsis thaliana*; ascorbate; chilling stress; photoinhibition; reactive oxygen species.

Introduction

Chilling stress is one of major factors that limit the growth, development and yield of plants. Under chilling stress, the photosystem reaction centers are over reduced, which leads to accumulation of ROS in the cell (Foyer 1994), such as hydrogen peroxide (H_2O_2), the superoxide radical ($O_2^{\bullet-}$), the hydroxyl radical and singlet oxygen, leading to oxidative damage (Asada 1999, Shigeoka *et al*. 2002, Mittler 2002, Ishikawa and Shigeoka 2008). To cope with oxidative stress, plants have developed antioxidant mechanisms to protect themselves against ROS. These mechanisms employ antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6), as well as nonenzymatic antioxidants, such as ascorbic acid (AsA), reduced glutathione and phenolic compounds (Asada 1999). Ascorbate is a major antioxidant that ensures protection of plant cells against ROS generated by physiological processes as well as biotic and abiotic stresses, including ozone (Conklin and Barth 2004), UV radiation (Gao and Zhang 2008), low temperatures (Li *et al*. 2010) and high light intensity (Müller-Moulé *et al*. 2004). In addition, ascorbate has been implicated in processes

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Abbreviations: APX – ascorbate peroxidase; AsA – ascorbate; CAT – catalase; Chl – chlorophyll; DHA – dehydroascorbate; DTNB – 5,5-dithiobis-2-nitrobenzoic acid; DTT – dithiothreitol; F_v/F_m – the maximal photochemical efficiency of PSII; EDTA – ethylenediaminetetraacetic acid; GSH – reduced glutathione; GSSG – oxidated glutathione; HL – high light; LD – long day; LL – low light; NADPH – reduced form of nicotinamide adenine dinucleotide phosphate; NBT – nitroblue tetrazolium; NEM – N-ethylmaleimide; PPFD – photosynthetic photon flux density; P_N – the net photosynthetic rate; POD – glutathione peroxidase; ROS – reactive oxygen species; SD – short day; SOD – superoxide dismutase; TBARS – thiobarbituric acid reactive substance; TCA – trichloroacetic acid; WT – wild type.
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including plants growth, programmed cell death, pathogen responses, cell division, cell expansion (Tabata *et al*. 2001) and cell wall metabolism (Smirnoff 1996), hormone responses (Pignocchi and Foyer 2003), flowering (Barth *et al*. 2006) and senescence (Barth *et al*. 2004). The best-described pathway of ascorbate synthesis is known as the Smirnoff/Wheeler or D-mannose/L-galactose pathway, consisting of the formation of ascorbate from guanosine diphosphate-mannose (GDP-Man) with the intermediates GDP-L-galactose (GDP-L-Gal), L-Gal-1-phosphate, L-Gal, and L-galactone-1,4-lactone (Wheeler *et al*. 1998). Recently, Laing *et al*. (2007) and Linster *et al*. (2007) elucidated the last unknown step, the conversion of GDP-L-galactose to L-Gal-1-phosphate, which was identified as the *VTC2* gene in *A. thaliana* (Jander *et al*. 2002). Three other pathways have been described: the L-Gulose pathway (Wolucka and Van Montagu 2003), the D-Galacturonic acid pathway (Agius *et al*. 2003) and the Myo-Inositol pathway (Lorence *et al*. 2004). The GDP-Man pathway, using GDP-L-Gal phosphorylase, is the only physiologically significant source of ascorbate in *A. thaliana* (Dowdle *et al.* 2007). A lot of previous studies showed that the GDP-L-galactose phosphorylase step may play an important role in controlling ascorbate biosynthesis (Kotchoni *et al*. 2009). The *Arabidopsis* mutant *vitamin C2-1*(*vtc2-1*) is ascorbate deficient due to a mutation in the GDP-L-Gal phosphorylase gene (Dowdle *et al*. 2007).

Vtc2-1, a severely AsA deficient, has unusually low levels of ~20% AsA of WT in younger leaves and stem leaves from older plants (Conklin *et al*. 2000). The *vtc2-1* mutant has a point mutation in *At*4g26850, leading to a single amino acid substitution (Jander *et al*. 2002). The *vtc2-1* mutation was found to map to a position on chromosome 4~3 cm centromere distal to CAPS marker WU95, which resides at position 71.70 on the latest *Arabidopsis* recombinant inbred (RI) genetic map (http://genome-www.stanford.edu/*Arabidopsis*/ww/Aug98RImaps/index.html) and ~5 cm centromere proximal to CAPS marker *PRHA* (position 76.17). The *vtc2-1* mutants have lower ascorbate concentration and are smaller than the wild-type (Dowdle 2007). Previous studies have shown that *vtc2-1* mutant is nonphotochemical quenching (NPQ)-deficient (Noctor *et al*. 2000, Smirnoff 2000, Müller-Moulé *et al*. 2002), and it bleaches when transferred from low light (LL) to high light (HL) (Müller-Moulé *et al*. 2003). When the *vtc2* mutant was growing under HL condition, no bleaching occurred in the mutant, but mutant plants had lower maximum photosystem II efficiency and lower oxygen evolution rates (Müller-Moulé *et al*. 2004). It was also reported that AsA deficiency of *vtc2-1* causes significantly early flowering and senescence of *Arabidopsis* under both SD (short day) and LD (long day) (Kotchoni *et al*. 2009). However, the effect of chilling stress on *vtc2-1* mutant plants remains unknown.

Mano *et al*. (2004) have demonstrated that AsA prevents photoinhibition under abiotic stress by donating electrons to the PSII and PSI reaction centers, and thus functions as an emergency donor. When the PSII reaction center with inactive water oxidase complex is under heat stress or UV-B irradiation, PSII reaction centers highly preferred AsA to H_2O as the electron donor (Mano *et al.* 2004). AsA at physiological concentrations in chloroplasts can support a substantial magnitude of electron flux through PSI under the conditions where the electron supply from PSII is suppressed (Foyer and Lelandais 1996, Eskling and Åkerlund 1998). AsA-supported electron flows are likely to function in photoprotection as energy-dissipating cycles. Our previous studies have also indicated that ascorbate played an important role in alleviating photoinhibition of PSI and PSII and enhancing the tolerance of tomato to various abiotic stresses (Li *et al*. 2010). The aim of this study is to provide further insight into the antioxidant process of ascorbate deficient mutants in response to low temperature.

Materials and methods

Plants: The ascorbate-deficient mutant *vtc2-1* has been described by Müller-Moulé (2008). *A. thaliana* ecotype Columbia, the original parental source of the ascorbate-deficient mutant *vtc2-1*, was used as WT plants. All the seeds that were used in the experiment were harvested at the same stage, and stored in the same way and for the same time, which promise that all the seeds have the same vitality. *Arabidopsis* (Col-0/*vtc2-1* mutants) seeds which were surface-sterilized and stratified were sown on 0.7% w/v agar-solidified Murashige and Skoog medium supplemented with 3% w/v sucrose under short-day conditions (8/16 h light/dark cycle, 22/19°C day/night, 200 μmol m^{-2} s^{-1} PPFD and 75% RH). After 10 d, seedlings were transferred to soil and grown under the same conditions. The 4-week-old (from transferred to soil) WT plants and *vtc2-1* mutants were used for subsequent abiotic stress assays. Low temperature treatment was carried out at 2°C (200 μmol m^{-2} s^{-1} PPFD and 75% RH) under the same light periods in an illuminated incubation chamber (*GXZ-260C*, Zhejiang, China). Samples were harvested at the start of the experiment (0 h) and at 12, 24, and 48 h after low temperature (2°C) treatment. Fully developed rosette leaves of WT and mutant plants were harvested, weighed, immediately frozen in liquid nitrogen, and stored at –80°C prior to analysis.

Chilling tolerance assays: For chilling tolerance assays (Fig. 1), 10-d-old seedlings sown on agar plates were transferred to new agar plates and grown for 2 d under the same conditions, and then removed to chilling temperature (2°C) with same photoperiod and RH. After 6 d, the plants were photographed.

Reduced and oxidized AsA: The levels of reduced AsA (AsA) and oxidized AsA (DHA) were determined according to Kampfenkel *et al*. (1995). Leaf tissues (0.5 g) from mutant *vtc2-1* and WT plants were grounded to powder in liquid nitrogen and 2 mL 6% trichloroacetic acid (TCA) was added, then centrifuged at 13 000 × *g* (4°C) for 5 min. The supernatant was assayed for AsA and DHA. The following solutions was used: 0.2 mL sample (6% TCA for blank), 0.6 mL 0.2 mol L^{-1} phosphate buffer (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'-bipyridyl, and 0.4 mL 3% $FeCl_3$. The assay tube was incubated at 42°C for 1 h and the absorbance was read at 525 nm. To assay the total AsA, 0.4 mL of 0.2 mol L^{-1} phosphate buffer (pH 7.4) and 0.2 mL of 10 mmol L^{-1}DTT were added to replace 0.6 mL of 0.2 mol L^{-1} phosphate buffer to reduce DHA to AsA. DHA was calculated as the difference between the total AsA and reduced AsA.

H_2O_2 and TBARS: H_2O_2 concentration of total leaf was measured according to the method as described by Sairam and Srivastava (2002) with slight modifications. Leaf sample (0.2 g) was homogenized with 3 mL of phosphate buffer (50 mmol L^{-1}, pH 6.8). The homogenate was centrifuged at 6,000 × *g* for 25 min. Supernatant was mixed with 1 mL of 0.1% titanium sulfate in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6,000 × *g* for 15 min. The absorbance change at 410 nm was monitored. H_2O_2 level was calculated according to the standard curve plotted with known concentration of H_2O_2.

The level of lipid peroxidation was determined by measuring thiobarbituric acid reactive substance (TBARS) according to the methods of Chen and Gallie (2006). A total of 0.5 g leaf was ground in liquid nitrogen in a 1.5 mL microfuge tube using a micropestle. Then 0.5 mL of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacteic acid and 0.5 mL of buffer (50 mmol L^{-1} Tris-HCl, pH 8.0, 175 mmol L^{-1} NaCl) were added and then heated at 95°C for 25 min. The absorbance of the supernatant was measured at 532 nm, with the A_{600} subtracted to account for nonspecific turbidity.

Histochemical detection and measurement of $O_2^{\bullet-}$: Mature rosettes were harvested at 0 h and at 24 h after low-temperature treatment. $O_2^{\bullet-}$ was visually detected by treating leaves with nitroblue tetrazolium (NBT), as described by Rao and Davis (1999). To detect superoxide anion radicals, 0.5 mg mL^{-1} NBT (*Sigma-Aldrich*) was supplied in 10 mmol L^{-1} potassium phosphate buffer, pH 7.8 (Hückelhoven *et al*. 2000). Subsequently, these stained samples were decolorized by boiling in acetic acid:glycerol:ethanol (1:1:4, v/v/v) solution. After cooling, the samples were extracted at room temperature with fresh ethanol and photographed.

The generation of $O_2^{\bullet-}$ was assayed according to the method of Wang and Luo (1990). Fresh leaves were thoroughly ground in an ice bath in a grinding medium containing 0.05 mol L^{-1} phosphate buffer (pH 7.8). The homogenate was centrifuged at 5000×g for 10 min at 4°C. The supernatant with phosphate buffer (pH 7.8) and 10 mol L^{-1} hydroxylammonium chloride was incubated at 25°C for 20 min, then 17 mmol L^{-1} *p*-aminobenzene sulfonic acid and 7 mM naphthylamine were added, and the mixture was incubated at 25°C for 20 min. Finally, ethyl ether was added into the mixture that was centrifuged at 1,500 × *g* for 5 min. The water phase was used to determine the absorbance at 530 nm. The $O_2^{\bullet-}$ generation was calculated per g of fresh mass of leaves.

Net photosynthetic rate (P_N) and chlorophyll (Chl) *a* fluorescence: Plants were treated at 2°C. P_N was measured with a portable photosynthetic system (*CIRAS-2*, *PP Systems*, Herts, UK) under the condition of concentration of ambient CO_2 (360 µl L^{-1}), a PPFD of 800 µmol m^{-2} s^{-1} and a relative humidity of 80%. Before P_N measurement, plants were kept at 25°C, 200 µmol m^{-2} s^{-1} PPFD for 30 min to induce stomata opening, and they were then illuminated at 25°C and PPFD of 800 µmol m^{-2} s^{-1} for 15 min to be acclimated.

Chl fluorescence was measured at 2°C with a portable fluorometer (*FMS2*, *Hansatech*, Norfolk, UK) according to the method of Van Kooten and Snel (1990). The minimal fluorescence (F_0) with all PSII reaction centers open was determined by modulated light (about 10 µmol m^{-2} s^{-1}) which was low enough not to induce any significant variable fluorescence (F_v). The maximal fluorescence (F_m) with all reaction centers closed was determined by 0.8-s saturating light of 7,000 µmol m^{-2} s^{-1} on a dark-adapted (20 min) leaf. The maximal photochemical efficiency (F_v/F_m) of PSII was expressed as: $F_v/F_m = (F_m - F_o)/F_m$.

Absorbance at 820 nm: Oxidation and reduction of P_{700} were measured at 820 nm with a plant efficiency analyzer sensor (*Hansatech,* Norfolk, UK) as described by Schansker *et al*. (2003). First, the electron transport chain was reduced by a 1-s red light pulse of 1,800 µmol(photon) m^{-2} s^{-1} (phase 1), subsequently oxidized by a 10-s far-red pulse of 200 µmol(photon) m^{-2} s^{-1} (phase 2) and finally reduced again by a 2-s red light pulse (phase 3). The difference between the minimum and the maximum of phase 3 was used to determine the maximum reducible/oxidizable amplitude.

Antioxidant enzyme activities: Antioxidant enzymes were extracted according to Grace and Logan (1996). 0.5 g of fresh sample was frozen in liquid nitrogen and was homogenized with a mortar and pestle in 6 mL ice-cold extraction buffer containing 50 mM L^{-1} phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.3% (v/v) Triton X-100 and 2% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was kept on ice for 10 min, and then centrifuged at 12,000 × *g* for 10 min at 2°C. The supernatant was used immediately for measuring the anti-

oxidant enzyme activities. SOD activity was assayed according to the method of Giannopolitis and Ries (1977). The CAT activity was measured at A_{240} for the H_2O_2 decomposition rate (Aebi 1984). One unit of SOD activity is defined as the amount of enzyme that inhibits the NBT photoreduction by 50% under the condition of the assay. One unit of CAT activity was defined as the amount of enzyme required for oxidation of 1 μmol of H_2O_2 per 1 min.

The homogenization buffer for APX extraction was 50 mM Hepes (pH 7.0) containing 1 mM ascorbate and 1% (v/v) Triton X-100. After centrifugation in a micro-centrifuge at 4°C, supernatants were used to determine enzyme activities. APX activity was measured by monitoring the decrease in absorbance at 290 nm as described by Kwon *et al*. (2002). 1 mL of the assay mixture contained 50 mM Hepes-KOH (potassium hydroxide) (pH 7.0), 0.1 mM EDTA, 0.2 mM H_2O_2, 0.5 mM AsA and enzyme extract. The reaction was initiated by adding H_2O_2. One unit of APX activity is defined as the amount of enzyme that oxidizes 1 μmol AsA per 1 min.

Glutathione: 1 g of leaf discs were homogenized in 5 mM of 10% TCA (w/v) containing 1.5 mM EDTA. After being centrifuged at 14,000 × *g* for 15 min, the supernatant was diluted 1:50 with 5% Na_2HPO_4 (pH 7.5) for neutralization.

Glutathione metabolite pool was measured using the enzymatic recycling assays according to the methods of Anderson *et al*. (1992). 1.5 mL of the sample extract was mixed with equal volume of reaction solution, containing 100 mM phosphate buffer (pH 6.8), 0.3 mM DTNB), 1 unit glutathione reductase, 0.04% BSA, 80 μL 9.0 mM NADPH), and was incubated at 25 °C for 25 min. The change in absorbance at 412 nm of the reaction mixture was measured. For oxidized glutathione GSSG measurement, the sample extract was first incubated with 2-vinylpyridine for 1 h at 25°C in order to eliminate reduced glutathione GSH. After the incubation, reactions and measurements were carried out in the same process. Reduced glutathione was calculated by the difference between total glutathione and GSSG. All values are reported as GSH equivalents according to the standard curve plotted with known concentration of GSH.

Statistical analysis: Data points represent the mean values ± standard deviation (SD) of 5 replications. Statistical significance of differences between WT and transgenic plants in measured parameters was tested by *Student*'s *t*-test. Means were considered to be significantly different when $P<0.05$, which was indicated by asterisks (*) in figures.

Results

Seedling growth under chilling stress: To determine whether *vtc2-1* mutants were more sensitive to chilling stress, seedlings grown on MS medium were transferred to 2°C for 6 d. Before chilling treatment, there was no significant difference between WT and transgenic plants under normal growth conditions (Fig.1*A*). *vtc2-1* seedlings showed more bleaching, long and narrow rosette leaves size, while the WT plants showed strong and thicker leaves after chilling treatment of 6 days, which suggested that mutant plants were more sensitive to the chilling treatment (Fig. 1*B*).

Changes of H_2O_2, $O_2^{\bullet-}$, and TBARS under chilling stress: After chilling stress, there was remarkable increase in the production of H_2O_2 in both WT and mutant plants. The increase in mutant plants was much greater than that in WT plants. NBT staining showed that slight blue marks emerged within both the WT and transgenic plants leaves under normal growth conditions (Fig. 2*A*). After chilling treatment, the blue polymerization product due to $O_2^{\bullet-}$ generation increased in both ofthem, whereas *vtc2-1* leaves exhibited more substantial staining (Fig. 2*A*). The production of $O_2^{\bullet-}$ in WT and *vtc2-1* plants was examined accordingly. The data confirmed the observation better (Fig. 2*B*). The TBARS increased in *vtc2-1* mutants during the co urse of chilling stress. In contrast, the TBARS of WT plants did not change significantly during 12 h of chilling treatment

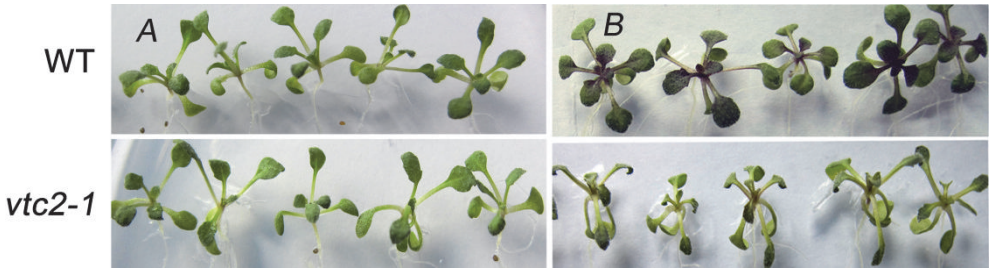

Fig. 1. Performance of WT and *vtc2-1* mutant seedlings under chilling stress. 10-d-old seedlings sown on agar plates were transferred to new agar plates and grown for 2 d (*A*), and then was treated at desired chilling temperature (2°C) with same photoperiod for 6 d (*B*).

(Fig. 3*B*). The TBARS in WT and *vtc2-1* were 7.5 μmol g^{-1} (FM) and 9.7 μmol g^{-1}(FM) after 48-h chilling treatment, which implied that membrane damage in mutants was more serious under chilling stress. There was no significant difference in the generation of H_2O_2 between WT and mutants in normal growth conditions (Fig. 3*C*).

Chl content: Chl diminished during chilling stress in both WT and *vtc2-1* mutant plants, which indicated possible damage to the photosynthetic capacity of the chloroplasts (Fig. 3*A*). After the first 12 h of chilling treatment, both genotypes showed a rapid decrease in Chl content. After 48 h of chilling stress, the WT plants had 1.24 fold as much as Chl content than the *vtc2-1* mutants (P<0.05).

Changes of antioxidant content under chilling stress: Fig. 4 showed the level of total AsA, reduced AsA, and

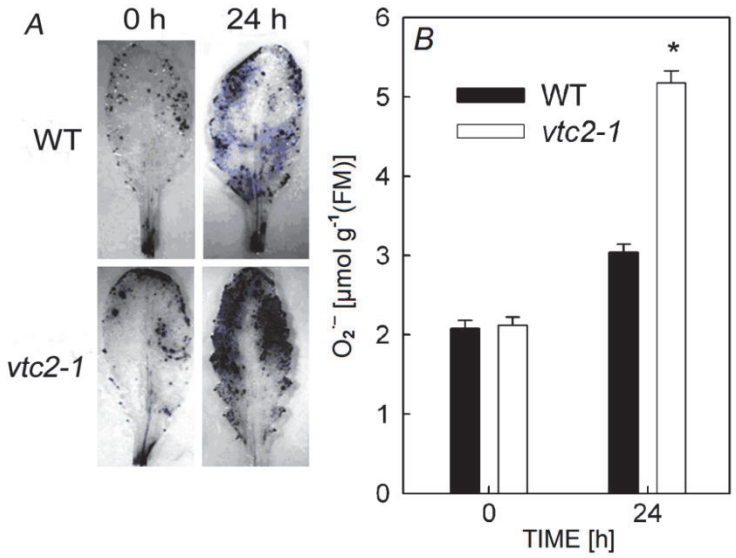

Fig. 2. $O_2^{\bullet-}$ staining (*A*) and $O_2^{\bullet-}$ (*B*) generation of *Arabidopsis* leaves after chilling stress. Plants were grown at normal growth conditions (25°C) and treatment at 2°C for 24 h.

the ratio of AsA/DHA in *Arabidopsis* plants. The *vtc2-1* mutants exhibited a very low level of total AsA and reduced AsA. The level of total AsA and reduced AsA in the WT plants were 2.32 times and 2.37 times higher than that of *vtc2-1* mutants before chilling stress, respectively. Both genotypes showed an increase in total ascorbate and reduced ascorbate under chilling stress, but the AsA and total AsA of the two genotypes decreased separately after 12 h (Fig. 4*A*,*C*). The redox status of AsA (AsA/DHA) decreased in WT plants and *vtc2-1* mutants after chilling stress, but the ascorbate pool in the *vtc2-1* mutants was more oxidized after chilling treatment and always remained at lower deoxidized levels (ranges from 4.50 to 1.13), relative to the WT plants (ranges from 5.01 to 1.45; Fig. 4*E*).

The total glutathione levels in the WT plants increased under chilling stress, whereas the reduced GSH and the ratio of reduced GSH to GSSG showed an increase during the first day of chilling treatment, after which they decreased (Fig. 4*B*,*D*,*F*). The *vtc2-1* mutants also showed an increase in total glutathione and the same change of the reduced GSH and the ratio of GSH to GSSG (Fig. 4*B*,*D*,*F*). The ratio of GSH to GSSG of *vtc2-1* mutants was obviously lower than that of WT plants at 12, 24, and 48 h (P<0.05).

Activities of antioxidant enzymes under chilling stress: After chilling treatment, the activities of SOD in mutant plants and WT plants were all induced. The activity of SOD in mutant plants was higher than that in WT plants, but the differences were not significant (P>0.05) (Fig. 5*A*). Compared to their levels at the start of the experiment (0 h), the increased activity was noted in WT (1.24 fold) and *vtc2-1*(1.37 fold) after 24-h treatment. After 48-h chilling treatment, the activity of SOD in mutant plants [5.6 U g^{-1} (FM)] was slightly lower than that of WT plants [5.9 U g^{-1} (FM)].

Chilling treatment induced significant increase in the activity of APX, but there was no remarkable distinction in the activity of APX between WT and mutant plants under optimal growth conditions and after 12- and 24-h chilling treatment (P>0.05). After treatment for 48 h, APX activity in the WT plants was 1.37 fold higher than that in *vtc2-1*(P<0.05) (Fig. 5*B*).

WT plants maintained slightly higher CAT activity under optimal growth conditions (P>0.05). Under chilling stress, the activity of CAT increased in the WT plants while decreased in *vtc2-1* mutants. After chilling stress for 24 and 48 h, the CAT activity in WT was obviously higher than that in *vtc2-1* mutants (P<0.05) (Fig. 5*C*).

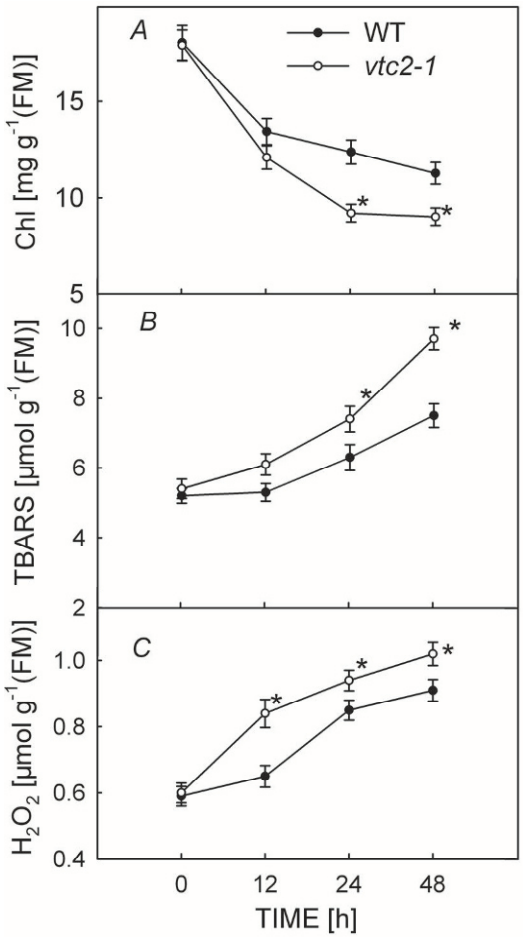

Fig. 3. Changes in chlorophyll (Chl) content (*A*), TBARS (*B*), and H_2O_2 (*C*) in WT and *vtc2-1* mutants of *Arabidopsis* under chilling stress (2°C). Chl content (*A*), TBARS (*B*), and H_2O_2 (*C*) were measured at 0, 12, 24, and 48 h. The data presented are the mean values ± SD of five individual experiments. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT ($P<0.05$).

Responses of P_N and F_v/F_m to chilling stress in WT and mutant plants: As treated with 2°C temperature, P_N of the wild type and mutants both decreased markedly. The decreases of P_N in mutants were always distinctly more serious than those of WT plants (Fig. 6*A*). During chilling stress, photoinhibition of PSII in wild type and mutant seedling leaves was estimated by measuring maximal photochemical efficiency of PSII (F_v/F_m). F_v/F_m analysis showed a similar profile with P_N (Fig. 6*B*).

Responses of oxidizable P_{700} to chilling stress in WT and mutant plants: The oxidizable P_{700} decreased significantly both in WT and *vtc2-1* plants under chilling stress and the decrease in mutants was more rapid. Moreover, when plants were transferred to normal growth conditions, the recovery of oxidizable P_{700} in *vtc2-1* plant was evidently delayed as compared with WT. After 36-h recovery, the oxidizable P_{700} could recover 93.2% in *vtc2-1*, while in WT plants it was 98.2%, respectively (Fig. 7).

Discussion

Chilling stress has been shown to induce ROS generation, resulting in oxidative damage (Asada 1999, Shigeoka *et al*. 2002, Mittler 2002, Ishikawa and Shigeoka 2008). In our study, *vtc2-1* mutants produced more TBARS, H_2O_2, and $O^{2\bullet-}$ than WT plants under chilling stress (Fig. 3*B,C*; Fig. 2), indicating that more oxidative stress took place in the *vtc2-1* mutants. Ascorbate is the most abundant low-molecular mass antioxidant in plant cells (Noctor and Foyer 1998, Smirnoff and Wheeler 2000) and plays a primary antioxidant role in complex antioxidation processes (Conklin 2001). Ascorbate has the capacity to directly eliminate ROS, including singlet oxygen, superoxide, and hydroxyl radicals (Padh 1990). Ascorbate is also an important cosubstrate of many enzymes, such as APX and AsA-dependent dioxygenases (Arrigoni and Tullio 2002). Under chilling treatment, the content of total ascorbate, reduced ascorbate and the ratio of reduced ascorbate to dehydroascorbate in mutants were lower than that in WT plants (Fig. 4*A,C,E*), which indicated that the regeneration of reduced ascorbate is impacted in the *vtc2-1* mutant. Several different studies have been conducted to prove that the increase in oxidative stress is due to the lack of ascorbate by feeding ascorbate to leaves. Conklin *et al*. (1996) showed that pretreatment of leaves with ascorbate or L-galactono-1,4-lactone caused elevated levels of ascorbate in both *vtc1* and WT plants, and restored *vtc1* resistance to O_3. Furthermore, Müller-Moulé *et al*. (2002) found that pigment in the xanthophyll cycle of the *vtc2-2* mutant could be rescued by feeding ascorbate to leaves. An increase in oxidative stress after treatment with short time UV-B stress is due to the lack of ascorbate has also been demonstrated (Gao and Zhang 2008). Our data strongly supported the hypothesis that the deficiency of ascorbate causes increased plant sensitivity to chilling stress.

The acceleration of ROS generation under environmental stresses could enhance the extent of photoinhibition by inactivating the elongation factor of D1 protein and inhibit the repair of PSII (Yang *et al*. 2007). AsA can alleviate photoinhibition of PSII not only by donating electrons to violaxanthin de-epoxidase to form zeaxanthin that dissipates excess excited energy but also by providing electrons to APX for detoxification of H_2O_2. Moreover, it has been shown that AsA can donate electrons to PSII in thylakoids to protect PSII from photoinhibition when electron donation from water is

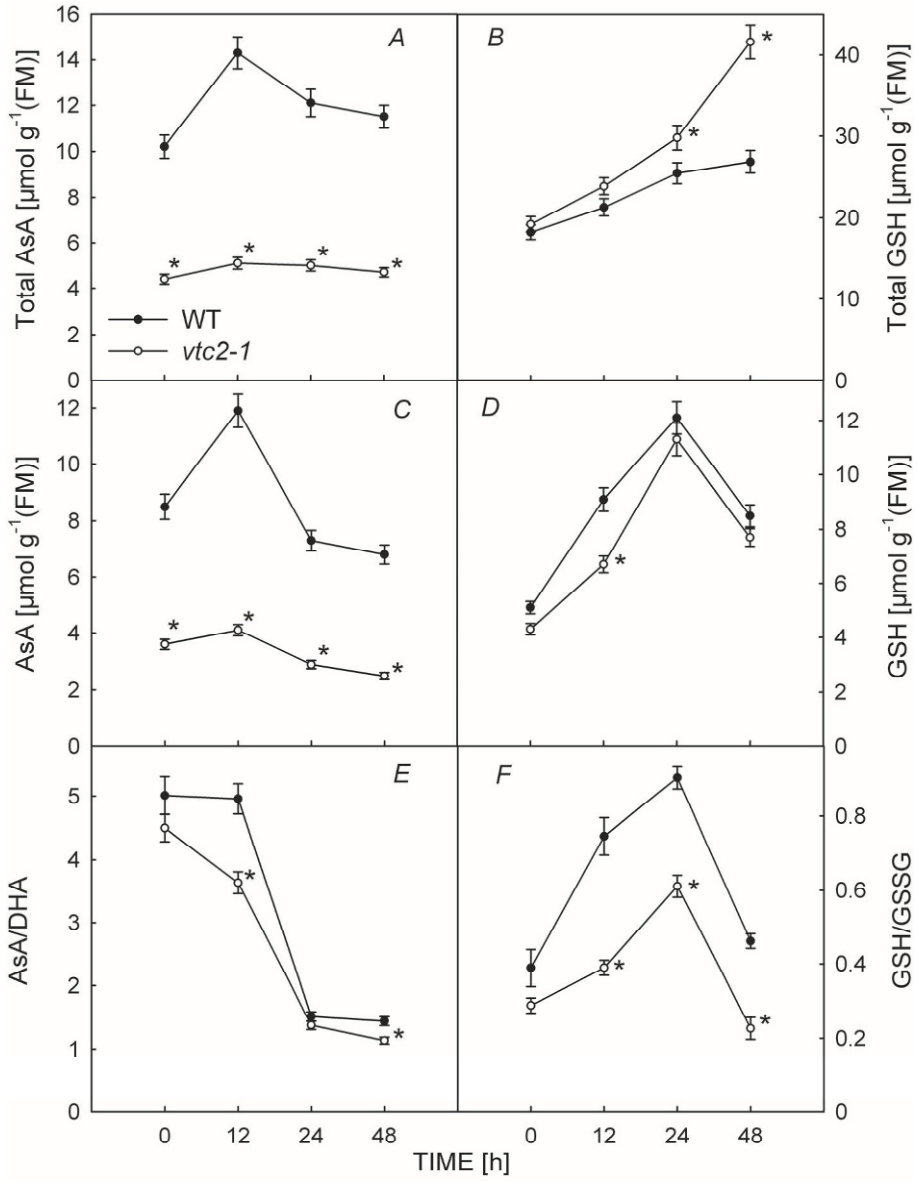

Fig. 4. Changes in total AsA (*A*), total GSH (*B*), AsA (*C*), GSH (*D*), the ASA/DHA ratio (*E*), and GSH/GSSG (*F*) in WT and *vtc2-1* mutants of *Arabidopsis* under chilling stress (2°C). The content of total AsA (*A*), total GSH (*B*), AsA (*C*), GSH (*D*), the ASA/DHA ratio (*E*), and GSH/GSSG (*F*) were measured at 0, 12, 24, and 48 h. The data presented are the mean values ± SD of five individual experiments. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT ($P<0.05$).

disturbed as a result of heat stress (Mano *et al*. 1997). Chilling stress induced production of H_2O_2 is associated with the damage and degradation of PSII (Li *et al*. 2010). F_v/F_m decreased much more in the *vtc2-1* mutant than in the WT under chilling treatment (Fig. 6*B*). These results suggested that the low level of reduced AsA of *vtc2-1* mutants could not detoxify ROS effectively, which further induced the damage to membrane lipids and influenced the repair of PSII. The irradiance-dependent xanthophyll cycle plays an important role in the protection of plants under environmental stress. Ascorbate is a cofactor of violaxanthin de-epoxidase, an enzyme that converts violaxanthin to zeaxanthin when plants were exposed to abiotic stress. The absence of AsA has been shown to be a limiting factor for the xanthophyll cycle.The increase in excitation pressure results in further damage to PSII and shows that F_v/F_m decreases faster in *vtc2-1* mutants. Huang *et al*. (2005) has reported that the ability of ascorbate to restore F_v/F_m to the control level in the *vtc1* mutant after treatment with NaCl for 48 h. Therefore, ascorbate plays a crucial role in both scavenging ROS produced in photosynthesis and dissipating excess energy.

It has been reported that PSI was more sensitive to chilling stress than PSII under low irradiance (Li *et al*. 2004). Cucumber leaves decreased in the activity of PSI by 70–80% when treated at 5°C in an irradiance of 100 µmol m^{-2} s^{-1} (Terashima *et al*. 1994), which indicated that PSI was the basic site of photoinhibition during chilling stress under low irradiance. Under chilling stress, the oxidizable P_{700} decreased significantly both in *vtc2-1* and WT plants (Fig. 7), but the decrease of oxidizable P_{700} in mutant plants was faster and the recovery was slower. The lower level of AsA in mutant plants could

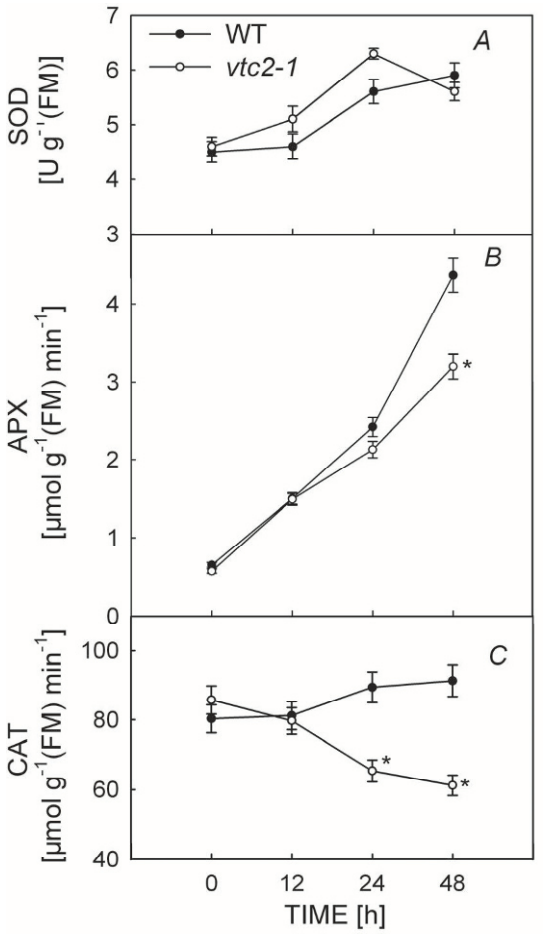

Fig. 5. The activities of superoxide dismutase (SOD) (*A*), ascorbate peroxidase (APX) (*B*) and catalase (CAT)(*C*) in WT and *vtc2-1* plants under chilling stress. The data presented are the mean values ± SD of five individual experiments. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT ($P<0.05$).

not detoxify H_2O_2 more efficiently and increased the photodamage (Terashima *et al*. 1998) to PSI compared with WT plants.

The cellular redox state, consisting of a delicate balance between ROS production and ROS scavenging, must be strictly controlled under biotic or abiotic stress (Mittler *et al*. 2004). The degree of damage caused by chilling stress should be strongly dependent on the efficiency of chilling-induced mechanisms of protection and repair, such as the activation of antioxidant defenses. The ascorbate-glutathione pathway plays an important role in the detoxification of ROS in vascular plants (Foyer and Noctor 2005). Ascorbate and glutathione are differently influenced by abiotic stress, and most data suggested that ROS has more impact on the GSH-GSSG ratio than on the redox status of ascorbate pool. The catalase deficiency in *Arabidopsis* drived glutathione oxidation while ascorbate remained highly reduced (Mhamdi *et al*. 2010). The AsA level in plants is tightly controlled by the level of its synthesis, recycling, degradation and transportation. The AsA regeneration plays an important role in the stress response and adaptation (Stevens *et al*. 2008, Yin *et al*. 2010). As an AsA-deficient mutant, the regeneration of AsA is more important in *vtc2-1* under chilling stress. It is generally believed that the regeneration of AsA from its primary oxidation product MDHA (monodehydroascorbate) is an essential process and obviously indispensable if the ROS-scavenging capability of AsA is to be maintained (Sano and Asada 1994). MDHAR (monodehydroascorbate reductase) catalyses the reduction of the MDHA to AsA, using NAD(P)H as an electron donor. Our results showed that under chilling stress WT plants had higher reduced GSH and higher ratio of reduced GSH to GSSG than *vtc2-1* mutants only after 12-h chilling stress (Fig. 4*B*, *D*,*E*). It was proved that insufficient NADPH prevents the

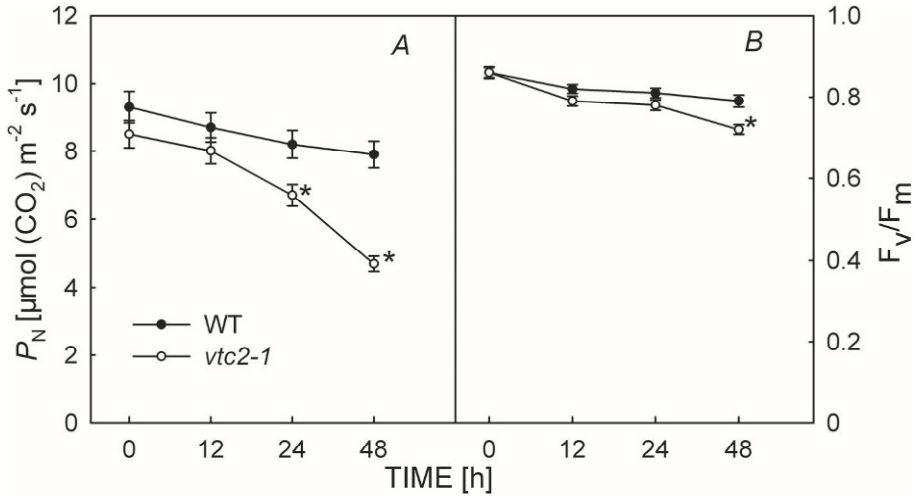

Fig. 6. Effect of chilling stress (2°C) on P_N (*A*), F_v/F_m (*B*) in WT and *vtc2-1* mutant. Before P_N measurement, plants were kept about 30 min at 25°C and at the PPFD of 200 µmol m^{-2} s^{-1} to induce stomata opening, and then illuminated about 15 min at the PPFD of 800 µmol m^{-2} s^{-1} (*A*). During chilling stress, plants were adapted in darkness for 20 min before F_v/F_m measurement and F_v/F_m was measured at 2°C (*B*). The data presented are the mean values ± SD of five individual experiments. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT ($P<0.05$).

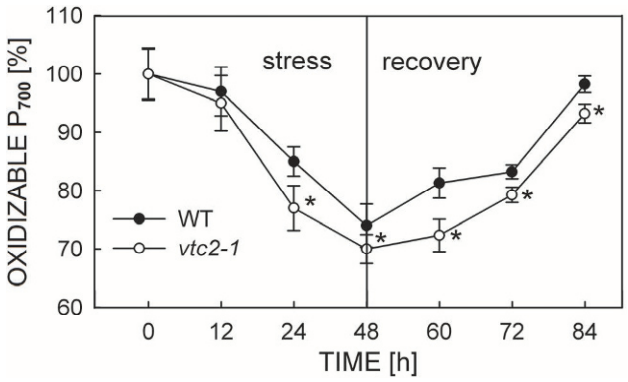

Fig. 7. Effect of chilling stress on oxidizable P_{700} in WT and *vtc2-1* mutant. The oxidizable P_{700} was measured during chilling stress (2°C) and recovery (25°C). The data presented are the mean values ± SD of 5 individual experiments. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT ($P<0.05$).

glutathione reduction state which is necessary for the activation of *PR1* (pathogenesis-related gene) gene expression (Mou *et al*. 2003). The consumption of NAD(P)H for AsA regeneration may further restrain the AsA-GSH cycle and may affect the regeneration of GSH in *vtc2-1* mutants. Furthermore, in the ascorbate-glutathione pathway, DHA (dehydroascorbate) can be regenerated by GSH, either chemically or *via* DHAR (dehydroascorbate reductase). In conclusion, all of the two regeneration pathway of ascorbate will affect the radox status of glutathione pool.

Ascorbate is responsible for keeping prosthetic metal ions in their reduced form to maintain other enzymes activity (Conklin 2001). SOD is an important scavenger of superoxide radicals of organism, and it is able to effectively prevent the damage of superoxide radicals to organism. It is the principal enzyme in the process of ROS elimination when responding to all kinds of stress. Under chilling stress, SOD activity slightly increased, but there were no obvious differences between WT and *vtc2-1* mutants except with treatment for 24 h. Rizhsky *et al*. (2002) reported that a deficiency in APX resulted in the induction of CAT and SOD, suggesting that these enzymes were induced to compensate for APX suppression. In addition, we showed that AsA deficiency results in the slight induction of SOD. The $O_2^{\cdot-}$ generation in *vtc2-1* was higher than WT plants (Fig.2) although the higher activity of SOD in *vtc2-1*, which indicated serious destruction on AsA-deficient mutants of chilling stress. The generation of $O_2^{\cdot-}$ undoubtedly can destroy membrane, and as a result a higher content TBARS emerged (Fig.3). So it is understandable why the activity of CAT decreased in *vtc2-1* mutants while it increased in WT plants. The decrease in *vtc2-1* mutants may be due to the destruction of the peroxisomes. The decrease of CAT activity induced an uneffective elimination of H_2O_2 and consequently a higher level of H_2O_2 may in turn have more damage on plants. APX activity increased both in *vtc2-1* mutants and WT plants under chilling treatment, but it was lower in the mutants. As a co-substrate of APX, the absence of AsA may be a limiting factor for the APX under chillling stress.

In conclusion, the ascorbate-deficient mutant *vtc2-1* is more sensitive to chilling treatment than WT plants. The aggravated oxidative stress and deficiency in excess energy dissipation were the major causes of the decrease of PSII and PSI functions in the *vtc2-1* mutants. Ascorbate maybe plays a critical role of defense without which the rest of the ROS defense network can be unable to react. Our data provide further evidence that ascorbate plays an important antioxidant role for chilling stress.

References

Aebi, H. Catalase *in vitro*. – Methods Enzymol. **105**: 121-126, 1984.

Agius, F., Gonzalez-Lamothe, R., Caballero, J.L. *et al*.: Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. – Nature Biotechnol. **21**: 177-181, 2003.

Anderson, J.V., Chevone, B., Hess, J.L.: Seasonal variation in the antioxidant system of eastern white pine needles. – Plant Physiol. **98**: 501-508, 1992.

Arrigoni, O., De, Tullio, M.C.: Ascorbic acid: much more than just an antioxidant. – Biochim. Biophys. Acta. **1569**: 1-9, 2002.

Asada, K.: The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. – Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**: 601-639, 1999.

Barth, C., Moeder, W., Klessig, D.F. Conklin, P.L.: The timing of senescence and response to pathogens is altered in the ascorbate-deficient *Arabidopsis* mutant vitamin *c-1*. – Plant Physiol. **134**: 1784-1792, 2004.

Barth, C., Tullio, M. D., Conklin, P. L.: The role of ascorbic acid in the control of flowering time and the onset of senescence. – J. Exp. Bot. **57**: 1657-1665, 2006.

Chen, Z., Gallie, D.R.: Dehydroascorbate reductase affects leaf growth, development and function. – Plant Physiol. **142**: 775-787, 2006.

Conklin, P.L.: Recent advances in the role and biosynthesis of ascorbic acid in plants. – Plant Cell Environ. **24**: 383-394, 2001.

Conklin, P.L., Barth, C.: 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, 2004.

Conklin, P.L., Saracco, S.A., Norris S.R., Last, R.L.: Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants. – Genetics **154**: 847-856, 2000.

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

Dowdle, J., Ishikawa, T., Gatzek, S., Rolinski, S., Smirnoff, N.: Two genes in *Arabidopsis thaliana* encoding GDP-L-galac-

tose phosphorylase are required for ascorbate biosynthesis and seedling viability. – Plant J. **52**: 673-685, 2007.

Eskling, M., Åkerlund, H.E.: Changes in the quantities of violaxanthin de-epoxidase, xanthophylls and ascorbate in spinach upon shift from low to high light. – Photosynth. Res. **57**: 41-50, 1998.

Foyer, C.H., Descourvieres, P. Kunert K.J.: Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. – Plant Cell Environ. **17**: 507-523, 1994.

Foyer, C.H., Lelandais, M.: A comparison of the relative rates of transport of ascorbate and glucose across the thylakoid, chloroplast and plasma membranes of pea leaf mesophyll cells. – J. Plant Physiol. **148**: 391-398, 1996.

Foyer, C.H, Noctor, G.: Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. – Plant Cell **17**: 1866-1875, 2005.

Gao, Q., Zhang, L.X.: Ultraviolet-B-induced oxidative stress and antioxidant defense system responses in ascorbate-deficient *vtc1* mutants of *Arabidopsis thaliana*. – J. Plant Physiol. **165**: 138-148, 2008.

Giannopolitis, C.N., Ries, S.K.: Superoxide dismutases: I. Occurrence in higher plants. – Plant Physiol. **59**: 309-314, 1977.

Grace, S.C., Logan, B.A.: Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. – Plant Physiol. **112**: 1631-1640, 1996.

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.

Hückelhoven, R., Fodor, J., Trujillo, M., Kogel, K.H.: Barley Mla and Rar mutants compromised in the hypersensitive cell death response against *Blumeria graminis* f. sp Hordei are modified in their ability to accumulate reactive oxygen intermediates at sites of fungal invasion. – Planta **212**: 16-24, 2000.

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.

Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M., Last, R.L.: *Arabidopsis* map-based cloning in the post-genome era. – Plant Physiol. **129**: 440-450, 2002.

Kampfenkel, K., Van M.M., Inzé D.: Extraction and determination of ascorbate and dehydroascorbate from plant tissue. – Anal. Biochem. **225**: 165-167, 1995.

Kotchoni, S.O., Larrimore, K.E., Mukherjee, M., Kempinski, C.F., Barth, C.: Alterations in the endogenous ascorbic acid content affect flowering time in *Arabidopsis*. – Plant Physiol. **149**: 803-815, 2009.

Kwon, S.Y., Jeong, Y.J., Lee, H.S. *et al*.: 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, X.G., Duan, W., Meng, Q.W., Zou, Q., Zhao, S.J.: The function of chloroplastic NAD(P)H dehydrogenase in tobacco during chilling stress under low irradiance. – Plant Cell Physiol. **45**: 103-108, 2004.

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.

Li F., Wu Q.Y., Sun Y.L., Wang L.Y., Yang X.H., Meng Q.W.: Overexpression of chloroplastic monodehydroascorbate reductase enhanced tolerance to temperature and methyl viologen-mediated oxidative stresses. – Physiol. Plant. **139**: 421-434, 2010.

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

Mano, J., Hideg, E., Asada, K.: Ascorbate in thylakoid lumen functions as an alternative electron donor to photosystem II and photosystem I. – Arch. Biochem. Biophys. **429**:71-80, 2004.

Mano, J., Ushimaru, T., Asada, K.: Ascorbate in thylakoid lumen as an endogenous electron donor to photosystem II: Protection of thylakoids from photoinhibition and regeneration of ascorbate in stroma by dehydroascorbate reductase. – Photosynth. Res. **53**: 197-204, 1997.

Mittler, R.: Oxidative stress, antioxidants and stress tolerance. – Trends Plant Sci. **9**: 405-410, 2002.

Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F.: Reactive oxygen gene network of plants. – Trends Plant Sci. **9**: 490-498, 2004.

Mhamdi, A., Hager, J., Chaouch, S. *et al*.: *Arabidopsis* GLUTATHIONE REDUCTASE1 plays a crucial role in leaf responses to intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways. – Plant Physiol. **153**: 1144-1160, 2010.

Mou, Z., Fan, W.H., Dong, X.N.: Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. – Cell **113**: 935-944, 2003.

Müller-Moulé, P.: An expression analysis of the ascorbate biosynthesis enzyme VTC2. – Plant Mol. Biol. **68**:31- 41, 2008.

Müller-Moulé, P., Conklin, P.L., Niyogi, K.K.: Ascorbate deficiency can limit violaxanthin de-epoxidase activity *in vivo*. – Plant Physiol. **128**: 970-977, 2002.

Müller-Moulé, P., Havaux, M., Niyogi, K.K.: Zeaxanthin deficiency enhances the high light sensitivity of an ascorbate deficient mutant of *Arabidopsis*. – Plant Physiol. **133**: 748-760, 2003.

Müller-Moulé, P., Talila, G., Niyogi, K.K.: Ascorbate-deficient mutants of *Arabidopsis* grow in high light despite chronic photooxidative stress. – Plant Physiol. **134**: 1163-1172, 2004.

Noctor, G., Foyer, C.H.: Ascorbate and glutathione: keeping active oxygen under control.– Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**: 249-279,1998.

Noctor, G., Veljovic-Jovanovic, S., Foyer, C.H.: Peroxide processing in photosynthesis: antioxidant coupling and redox signalling. – Philos. T. Roy. Soc. B **355**: 1465-1475, 2000.

Padh, H.: Cellular functions of ascorbic acid. – Biochem. Cell Biol. **68**: 1166-73, 1990.

Pignocchi, C., Foyer, C.H.: Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. – Curr. Opin. Plant Biol. **6**: 379-389, 2003.

Rao, M.V., Davis, K.R.: Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic

acid. – Plant J. **17**:603-14, 1999.

Rizhsky, L., Hallak-Herr, E., Van Breusegem, F., Rachmilevitch, S., Barr, J.E., Rodermel, S., Inzé, D., Mittler, R.: Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. – Plant J. **32**: 329-342, 2002.

Sairam, P.K., Srivastava, G.C.: Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. – Plant Sci. **162**: 897-904, 2002.

Sano, S., Asada, K.: cDNA cloning of monodehydroascorbate radical reductase from cucumber: a high degree of homology in terms of amino acid sequence between this enzyme and bacterial flavoenzymes. – Plant Cell Physiol. **35**: 425-437, 1994.

Schansker, G., Srivastava, A., Govindjee, Strasser, R.J.: Characterization of the 820-nm transmission signal paralleling the chlorophyll a fluorescence rise (OJIP) in pea leaves. – Funct. Plant Biol. **30**: 785-796, 2003.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., Yoshimura, K.: Regulation and function of ascorbate peroxidase isoenzymes. – J. Exp. Bot. **53**: 1305-1319, 2002.

Smirnoff, N.: The function and metabolism of ascorbic acid in plants. – Ann. Bot. **78**: 661-669, 1996.

Smirnoff, N.: Ascorbate biosynthesis and function in photoprotection. – Philos. T. Roy. .Soc. B: **355**: 1455-1464, 2000.

Smirnoff, N., Wheeler, G.L.: Ascorbic acid in plants biosynthesis and function. – Crit. Rev. Biochem. Mol. Biol. **35**: 291-314, 2000.

Stevens, R., Page, D., Gouble, B., Garchery, C., Zamir, D., Causse, M.: Tomato fruit ascorbic acid content is linked with monodehydroascorbate reductase activity and tolerance to chilling stress. – Plant Cell Environ. **31**: 1086-1096, 2008.

Tabata, K., Oba, K., Suzuki, K., Esaka, M.: Generation and properties of ascorbic acid-deficient transgenic tobacco cells expressing antise RNA for L-galactone dehydrogenase. – Plant J. **27**:139-148, 2001.

Terashima, I., Funayama, S., Sonoike, K.: The site of photoinhibition in leaves of *Cucumis sativus* L. at low temperatures is photosystem I, not system II. – Planta **193**: 300-306, 1994.

Terashima, I., Noguchi, K., Itoh-Nemoto, T. *et al.*: The cause of PSI photoinhibition at low temperatures in leaves of *Cucumis sativus*, a chilling sensitive plant. – Physiol. Plant. **103**: 295-303, 1998.

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

Wang, A.G., Luo, G.H.: Quantitative relation between the reaction of hydroxylamine and superoxide anion radicals in plants. – Plant Physiol. **6**: 55-7, 1990.

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

Wolucka, B.A. Van Montagu, M.: GDP-mannose 3′,5′- epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosynthesis of vitamin C in plants. – J. Biol. Chem. **278**: 47483-47490, 2003.

Yang, X.H., Wen, X.G., Gong, H.M., Lu, Q.T., Yang, Z.P., Tang, Y.L., Liang, Z., Lu, C.M.: Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. – Planta **225**: 719-733, 2007.

Yin, L., Wang, S.W., Eltayeb, A.E., Uddin, Md. I., Yamamoto, Y., Tsuji, W., Takeuchi, Y., Tanaka, K.: Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. – Planta **231**: 609-621, 2010.