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Susceptibility of an ascorbate-deficient mutant of *Arabidopsis* to high-light stress

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Abstract

Ascorbate is an important antioxidant involved in both enzymatic and nonenzymatic reactions in plant cells. To reveal the function of ascorbate associated with defense against photo-oxidative damage, responses of the ascorbate-deficient mutant *vtc2-1* of *Arabidopsis thaliana* to high-light stress were investigated. After high-light treatment at 1,600 μmol(photon) m^{-2} s^{-1} for 8 h, the *vtc2-1* mutant exhibited visible photo-oxidative damage. The total ascorbate content was lower, whereas accumulation of H_2O_2 was higher in the *vtc2-1* mutant than that in the wild type. The chlorophyll (Chl) content and PSII Chl fluorescence parameters, such as maximal quantum yield of PSII photochemistry, yield, and electron transport rate, in *vtc2-1* mutant decreased more than that in the wild type, whereas the nonphotochemical quenching coefficient increased more in the wild type than that in *vtc2-1* mutant. Therefore, the *vtc2-1* mutant was more sensitive to high-light stress than the wild type. Accumulation of reactive oxygen species was mainly responsible for the damage of PSII in the *vtc2-1* mutant under high light. The results indicate that ascorbate plays a critical role in maintaining normal photosynthetic function in plants under high-light stress.

Additional key words: *Arabidopsis thaliana*, ascorbic acid, high-light stress, chlorophyll fluorescence, reactive oxygen species.

Introduction

Photosynthesis is very sensitive to environmental stresses, and the decline of photosynthetic capacity constrains growth and development of plants and reduces crop production (Ding *et al.* 2012). Abiotic stress is an important limiting factor for sustainable development of agriculture. It leads to premature ageing of plant leaves and causes food production losses (Sun *et al*. 2013). High-light stress is one of the major factors that limit the growth, development, and yield of plants. Under high light, the energy absorbed by plants is much more than they can use, resulting in accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), and consequent photoinhibition of PSII reaction centers occurs (Pekker *et al*. 2002, Asada 2006, Ishikawa and Shigeoka 2008, Locato *et al*. 2008). ROS can act in stress-signaling in plants, but the accumulation of ROS can also lead to plant cells suffering oxidative stress (Finkel and Holbrook 2000). Under normal conditions, the production and removal of free ROS are dynamically balanced in plants. Environmental stresses can

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Abbreviations: APX – ascorbate peroxidase; AsA – ascorbic acid; AsA-GSH cycle – ascorbate–glutathione cycle; CAT – catalase; Chl – chlorophyll; DAB – diaminobenzidine; ETR – electron transport rate; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_m'– maximal fluorescence yield of the light-adapted state; F_s – steady-state fluorescence yield; F_v – variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; NPQ – nonphotochemical quenching; ORF – open reading frame; q_N – nonphotochemical quenching coefficient; q_P – photochemical quenching coefficient; RH – relative humidity; ROS – reactive oxygen species; SOD – superoxide dismutase; TCA – trichloroacetic acid; Φ_{PSII} – effective quantum yield of PSII photochemistry.
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trigger the production of ROS beyond the limits of a plant's ROS-scavenging ability, and the excess ROS attacks biological macromolecules and cell membranes, even leading to cell death in plants. To cope with overproduction of ROS, plants have developed an antioxidative system during the process of evolution (Adams *et al*. 1995), which consists of 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), and nonenzymatic antioxidants, such as ascorbic acid (AsA), α-tocopherol, reduced glutathione, and phenolic compounds (Alscher 1997, Smirnoff 1998).

Ascorbate is an important antioxidant in plants, which can directly eliminate ROS, such as singlet oxygen, superoxide radical, and hydroxyl radical, or eliminate ROS through the ascorbate–glutathione cycle (AsA-GSH cycle), thereby protecting plant cells from damage due to oxidative stress and maintaining normal metabolism. Ascorbate also acts as a signal molecule for plant responses to stress conditions (Pignocchi and Foyer 2003), which ensures protection of plant cells against ROS generated by physiological processes under 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 *et al*. 2004). So far, there are at least four biosynthetic pathways for ascorbate in plants: the Smirnoff-Wheeler pathway (Wheeler *et al*. 1998), galacturonic acid pathway (Agius *et al*. 2003), gulonic way (Wolucka *et al*. 2003), and inositol pathway (Lorence *et al*. 2004). The Smirnoff-Wheeler pathway, which is the first among the four to be discovered, has been suggested to be the major pathway for ascorbate synthesis in plants (Gallie 2013). The VTC2 enzyme, which can function as GDP-L-Gal phosphorylase and L-Gal-hexose-1-phosphate guanylyltransferase to produce L-Gal-1-phosphate, is the key enzyme in the Smirnoff-Wheeler pathway (Laing *et al*. 2007, Linster *et al*. 2007, Wolucka and Van 2007, Müller 2008) The *VTC2* gene is located on the 4th chromosome in *Arabidopsis thaliana* (Conklin *et al*. 2000), and VTC2 protein is present in green tissues and is localized in both the cytosol and the nucleus (Müller 2008).

The *vtc2-1* mutant has a point mutation in *At*4g26850, leading to a single amino acid substitution in hexose 1-phosphate guanylyltransferase (Jander *et al*. 2002). Thus, it is deficient in the synthesis of ascorbate (Dowdle 2007). Early flowering and senescence are the main phenotypic traits of the *vtc2-1* mutant (Kotchoni *et al*. 2009). Since the mutant is deficient in the synthesis of ascorbate, we hypothesized that the *vtc2-1* mutant is more susceptible to high-light stress. The aim of this study is to provide an insight into the role of ascorbate as an antioxidant in defense against photo-oxidative damage in plant cells.

Materials and methods

Plant materials and growth conditions: The ascorbate-deficient mutant *vtc2-1* of *A. thaliana* was constructed from the Columbia ecotype as reported by Müller-Moulé (2008). Seeds of *vtc2-1* and wild-type (Col), which were used in the experiment, were harvested at the same time and stored in the same conditions, ensuring that they had the same vitality. The seeds were surface-sterilized, sown on 0.9% (w/v) agar-solidified Murashige and Skoog medium supplemented with 3% (w/v) sucrose, and grown under an 8 h/16 h light/dark cycle, at 25°C, 80 μmol(photon) m^{-2} s^{-1}, and 60% relative humidity (RH). After being grown for 10 d, the seedlings were transferred to soil and continuously grown under the same light and humidity conditions. Four weeks after transfer to soil, plants were subjected to 1,600 μmol(photon) m^{-2} s^{-1} of a high-light treatment for 8 h. Samples were taken from the *vtc2-1* and Col plants before (0 h) and after the treatment for physiological and biochemical analyses.

RNA extraction and quantitative real-time PCR: Total RNA was extracted from mature rosette leaves using the *RNA Plant isolation Mini* kit (*Sangon Biotech*). The RNA was treated with desoxyribonuclease I (*Takara*, Japan) prior to reverse transcription. The first strand of cDNA was synthesized from the 1 μg of total RNA using oligo$(dT)_{18}$ primer and M-MLV reverse transcriptase (*Takara*, Japan). The cDNA was used as template for quantitative PCR. Quantitative PCR was performed using a real-time PCR device (*7500 Sequence Detection system*, *Applied Biosystems*, Japan) and the *SYBR Premix* test kit (*SYBR Premix ExTaq^{TM} II*, *Takara*). Two μL of 1:10-diluted cDNA was used as a template in a 20 μL reaction system for quantitative PCR. The amplified reactions used the following primers:
*VTC*2-F (5'-TTCGCTATGATGTCACTGCCTG-3') and *VTC*2-R (5'-GCAACGAAACCATACTTCCCC-3'). The *α-tubulin* gene was used as a reference gene that was amplified by the primers:
5'-CCAGCTTTGGTGATTTGAAC-3' (forward), and 5'-CAAGCTTTCGGAGGTCAGAG-3' (reverse) (Endo *et al*. 2005).

H_2O_2 histochemical analyses: Rosette leaves were harvested at 0 h and at 8 h after treatment. H_2O_2 in the leaves was stained by 0.5 mg(diaminobenzidine, DAB) mL^{-1} in 10 mM potassium phosphate buffer (pH 7.0) under vacuum, as described by Romero *et al*. (2004). After staining, samples were bleached by a 75% methyl alcohol (v/v) solution to remove pigments, and then photographed.

Ascorbate content assay: For determination of total ascorbic acid, 0.04 g of leaf sample was homogenized in 2 mL of 6% (w/v) trichloroacetic acid (TCA) solution and centrifuged at 13,000 × *g* and 4°C for 5 min. The super-

natant was used for total ascorbic acid measurement as described by Gillespie and Ainsworth (2007).

Chl content: Leaf sample (0.1 g) was extracted in 80% (v/v) acetone, and the absorbance at 663.6 and 646.6 nm wavelength was measured (*UV-Vis 2450* spectrophotometer, *Shimadzu*, Tokyo, Japan). Chl *a* and *b* concentrations were calculated as: Chl $a = 13.71A_{663.6} - 2.85A_{646.6}$ and Chl $b = 22.39A_{646.6} - 5.42A_{663.6}$ (Porra *et al*. 1989).

Chl fluorescence: The parameters (F_0, F_m, F_s, and F_m') of rosette leaves were measured using a *PAM-2100* (*Walz*, Effeltrich, Germany) according to the method described by Genty *et al*. (1989). The PSII primary photochemical efficiencies for dark adaptation (F_v/F_m) and light adaptation (Φ_{PSII}) and the coefficients of photochemical quenching (q_P) and nonphotochemical quenching (NPQ) were calculated as follows:
$F_v/F_m = (F_m - F_0)/F_m$; Yield = $\Phi_{PSII} = (F_m' - F_s)/F_m'$; $q_P = (F_m' - F_s)/(F_m' - F_0')$; and $q_N = 1 - (F_m' - F_0')/(F_m - F_0)$; $F_0' = F_0/(F_v/F_m + F_0/F_m')$.
The actinic light used in the measurement of these parameters was 150 μmol(photon) m^{-2} s^{-1}.

Statistical analysis: Data are shown as means ± SE. *Student*'s *t*-test ($P<0.05$) was conducted with *SPSS v. 12.0*. Figures were drawn by *Sigmaplot v. 12.0*.

Results

Phenotype under high light: There was no significant difference of phenotype between Col and *vtc2-1* after growth in normal conditions for four weeks. After high-light treatment at 1,600 μmol(photon) m^{-2} s^{-1} for 8 h, leaves of Col appeared normal looking. By contrast, visible damage was observed in *vtc2-1*. The leaves of *vtc2-1* turned yellow and appeared dehydrated (Fig. 1*A*). Therefore, *vtc2-1* was more sensitive to high-light treatment than Col.

Accumulation of H_2O_2 under high-light treatment: H_2O_2 was detected with DAB staining in the leaves of both Col and *vtc2-1* (Fig. 1*B*). More brown spots were observed in leaves of *vtc2-1* after high-light treatment (Fig. 1*B*), indicating that accumulation of H_2O_2 was greater in *vtc2-1* than Col.

***VTC*2 mRNA expression**: The *VTC*2 gene of *Arabidopsis* has an ORF of 2,640 bp encoding a protein of 431 amino acids, which is similar to *Oryza sativa* (Os12g0190000). The mRNA level of *VTC*2 gene was 14% lower than that of Col (Fig. 2*A*).

Total ascorbic acid content: The changes in total ascorbic after treatment are shown in Fig. 2*B*. The content of total ascorbic acid in *vtc2-1* was 24% significantly lower than that in Col. Both Col and *vtc2-1* showed a decrease in total ascorbic acid after the high-light treatment. However, the total ascorbic acid was greater in Col than that in *vtc2-1*, either before or after the high-light treatment.

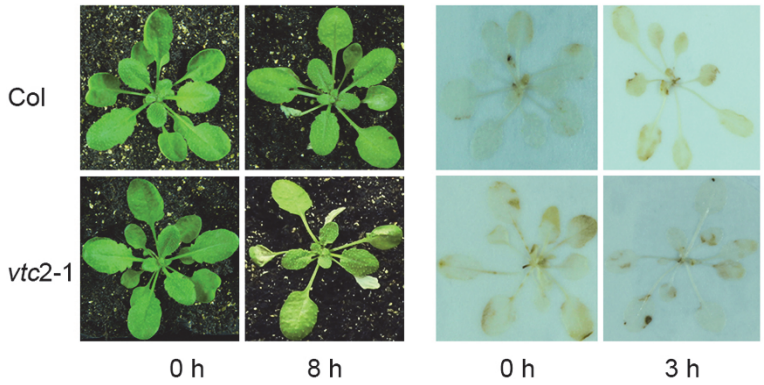

Fig. 1. Changes in phenotype (*A*) and accumulation of H_2O_2 in rosette leaves (*B*) of *vtc2-1* and Col after high-light treatment at 1,600 μmol(photon) m^{-2} s^{-1}.

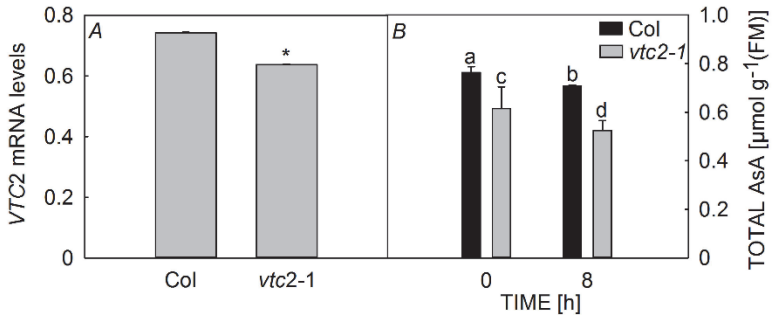

Fig. 2. Relative expression of *VTC*2 (*A*) and changes in total AsA content in leaves of *vtc2-1* and Col (*B*). Relative expression of *VTC*2 was calculated using the REST program. * – *Student*'s *t*-test, $P<0.05$. Data are means ± SE.

Chl content: The Chl content, particularly that of Chl *b*, decreased in leaves of *vtc2-1* under high light, indicating that *vtc2-1* was very susceptible to high-light stress. In contrast, Chl in leaves of Col showed little change after the high-light treatment (Table 1). After treatment, Chl *a*+*b* in Col was 1.14 fold of that in *vtc2-1*.

Chl fluorescence parameters: Under favorable growth conditions, *vtc2-1* and Col had similar F_v/F_m, yield, ETR, and q_P (Fig. 3*A–C,E*). The high-light treatment resulted in decreased F_v/F_m and yield in rosette leaves of both *vtc2-1* and Col. However, Col demonstrated a slower decrease in these parameters than *vtc2-1* (Fig. 3*A,B*). After 3 h of the high-light treatment, the percentage decline in F_v/F_m was 33 and 77% in Col and *vtc2-1*, respectively; and that in yield was 49 and 92% in Col and *vtc2-1*, respectively; the percentage increase in q_P was 11 and 1% in Col and *vtc2-1*, respectively (Fig. 3*E*). q_N showed a reverse changing trend compared to other Chl fluorescence parameters (Fig. 3*D*): q_N of *vtc2-1* showed no significant change after 3 h of the high-light treatment, but it was markedly increased in Col (Fig. 3*D*).

Discussion

Ascorbate (AsA) is a small molecular compound in plants and animals that mainly acts as an antioxidant. It plays a critical role in scavenging ROS to improve photosynthesis, growth, and development of plants, and is correlated with resistance of plants to stress. A higher AsA content in plant cells can enhance plant resistance to alkali stress, cold stress, and heat stress (Conklin and Barth 2004, Ishikawa *et al*. 2006), suggesting that regulation of AsA metabolism is a feasible way of breeding for plant resistance to environmental stress.

Table 1. Effects of high-light stress on chlorophyll content [mg g^{-1}(FM)]. Data are means ± SE ($n = 4$). Different *lowercase letters* show significant differences at $P<0.05$. *Student*'s *t*-test was used to calculate statistical significance.

Genotype	Treatment	Chl *a*	Chl *b*	Chl (*a*+*b*)	Chl *a*/*b*
Col	CK	14.29 ± 0.32^a	5.15 ± 0.12^a	19.81 ± 0.31^a	2.89 ± 0.04^a
	high-light stress	15.56 ± 0.45^a	5.29 ± 0.13^a	21.42 ± 0.98^a	2.84 ± 0.01^a
vtc2-1	CK	14.56 ± 0.13^b	5.00 ± 0.04^a	20.34 ± 0.17^a	2.92 ± 0.01^c
	high-light stress	14.17 ± 0.40^b	4.34 ± 0.13^b	18.72 ± 0.62^b	3.20 ± 0.01^a

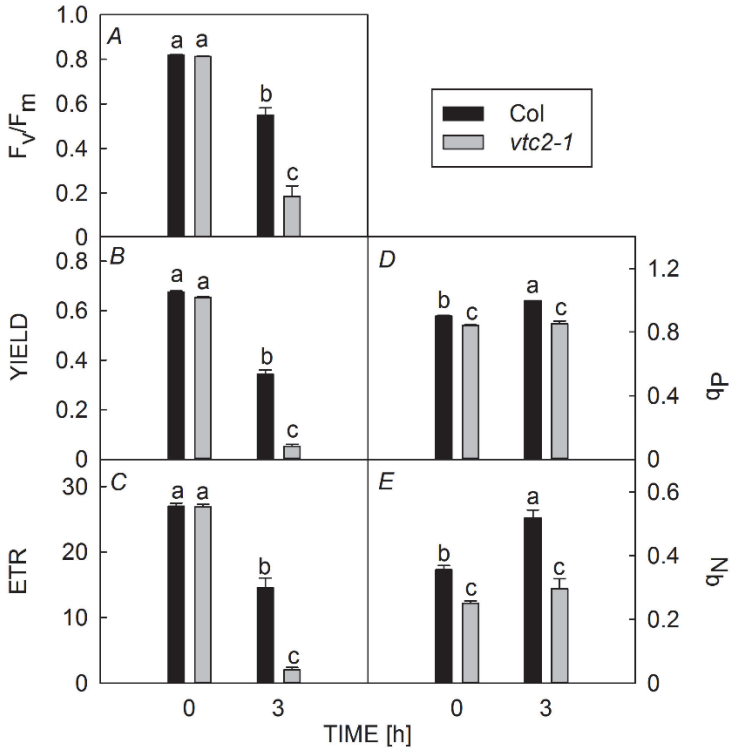

Fig. 3. Changes in maximum efficiency of PSII photochemistry (F_v/F_m) (*A*), yield (*B*), electron transport rate (ETR) (*C*), photochemical quenching coefficient (q_P) (*D*), and nonphotochemical quenching coefficient (q_N) (*E*) between Col and *vtc2-1* after high-light treatment at 1,600 μmol(photon) m^{-2} s^{-1}. The measurement photon flux density was 150 μmol m^{-2} s^{-1}. Means and SE were calculated from three replicates. *Student*'s *t*-test was used to calculate statistical significance.

AsA scavenges ROS independently or through acting as a cofactor of antioxidative enzymes, or the AsA-GSH cycle. A previous study showed that exogenous AsA protected plant leaves against photoinhibition in ginger under strong light (Huang *et al*. 2008), and protected biological membranes by preventing lipid peroxidation (Gallie 2013). Under ozone stress, the application of exogenous AsA alleviated the oxidative degradation of carotenoids in rice (Xie *et al*. 2009). Thus, the function of AsA is very critical in defense against environmental stresses.

This study showed that the ascorbic acid-deficient mutant plant was more susceptible to high-light stress. Our result is consistent with that of Li *et al*. (2010). The *VTC2* mRNA level in *vtc2-1* was reduced by 14% compared with Col (Fig. 2*A*), likely due to nonsense-mediated mRNA decay (Conti and Izaurralde 2005). Lower *VTC2* mRNA levels contributed to lower amounts of AsA in *vtc2-1*. Under high-light treatment, higher amounts of H_2O_2 accumulated in *vtc2-1* (Fig. 1*B*) was responsible for the faster decline of Chl content and F_v/F_m, yield, and ETR. In contrast to *vtc2-1*, Col had higher Chl content and F_v/F_m, yield, and ETR after the high-light treatment, suggesting that, under high light, maintaining normal amounts of AsA is critical for protection of the photosynthetic apparatus. After high-light treatment, the damage to photosynthetic apparatus reduced q_P capacity in *vtc2-1*, aggravating photodamage to its photosynthetic apparatus. This is consistent with previous reports that the *vtc2-1* mutant had lower nonphotochemical quenching capacity (Noctor *et al*. 2000, Smirnoff 2000, Müller *et al*. 2002). By contrast, Col exhibited a strong capability for dissipating excessive light energy in the form of heat, thereby ameliorating photodamage to its photosynthetic apparatus. The q_N exhibited in Col under high light is consistent with that exhibited in Col under drought stress (Ding *et al*. 2012).

In conclusion, the ascorbate-deficient mutant *vtc2-1* is more sensitive to high-light stress than Col plants, due to the accumulation of ROS and deficiency in the capacity to dissipate excess energy. Our data provide evidence that ascorbate plays an important role in protecting photosynthetic apparatus form high-light damage.

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