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Interplay between ascorbic acid and lipophilic antioxidant defences in chloroplasts of water-stressed *Arabidopsis* plants

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Abstract **The effects of low ascorbic acid (Asc) on lipophilic antioxidant defences and lipid peroxidation in chloroplasts were evaluated in the *vtc-1* mutant of *Arabidopsis thaliana*, which had an Asc deficiency in chloroplasts of ca. 60%. Although low Asc did not cause oxidative stress in optimal growth conditions, it increased malondialdehyde levels in chloroplasts by ca. 60%, and reduced α-tocopherol and β-carotene by ca. 85% and 40%, respectively, in water-stressed (WS) mutants. These results are indicative of the interplay between Asc and lipophilic antioxidants in chloroplasts of WS plants in vivo, and show that Asc contributes to the protection of thylakoid membrane lipids from oxidation in stressed plants.** © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antioxidant; Ascorbic acid; α-Tocopherol; Carotenoid; Chloroplast; *vtc-1* mutant

1. Introduction

L-Ascorbic acid (Asc; vitamin C) functions as an antioxidant, an enzyme cofactor, and a precursor for oxalate and tartrate synthesis in plants. It participates in a variety of processes, including photosynthesis, photoprotection, the cell cycle, cell-wall growth and cell expansion, synthesis of ethylene, gibberellins, anthocyanins, and hydroxyproline, and resistance to environmental stresses [1,2]. Asc is synthesized partly in the cytosol and the mitochondria, and it is also found in the apoplast, vacuoles, peroxisomes and chloroplasts [2,3].

Under water deficit, the formation of active oxygen species (AOS) increases and the antioxidant system protects the cell by controlling the intracellular AOS concentration [4]. Asc functions as an antioxidant in chloroplasts [5–7]. Asc detoxifies H_2O_2, which is formed by dismutation of $O_2^{\bullet-}$. Asc functions coordinately with glutathione and several enzymatic antioxidants to counteract $O_2^{\bullet-}$, which is formed by direct reduction of molecular oxygen (O_2) in photosystem I (Mehler reaction). Asc is also believed to detoxify $^1O_2$ [1,8] and $OH^{\bullet}$ [9]. It acts as a cofactor for violaxanthin de-epoxidase in vitro [10,11] and can limit violaxanthin de-epoxidase activity in vivo [12]. Besides, it is assumed to protect α-tocopherol (α-Toc) and to recycle α-Toc from its α-tocopheroxyl radical [2,13] but in vivo evidence of this in plants has not been provided so far.

The *vtc-1* (formerly *soz*) mutant of *Arabidopsis*, which has low GDP-mannose pyrophosphorylase activity and an Asc deficiency in leaves of ca. 70% [14,15], was used in this study to evaluate the interplay between Asc and lipophilic antioxidant defences in chloroplasts of water-stressed (WS) plants in vivo.

2. Materials and methods

2.1. Plant material and water deficit treatment

Seedlings of *Arabidopsis thaliana* Columbia (Col) ecotype, wild-type (Col-0) and the Asc-deficient mutant (*vtc-1*) [14] were grown in pots containing a mixture of peat/perlite/vermiculite (1:1:1, v/v/v) in a constant-environment chamber (8-h photoperiod, 90–110 μmol quanta m^{-2} s^{-1}, air temperature between 21 and 23°C). After 8 weeks of growth, the experiment started and two water regimes were imposed for 3 weeks on both Col-0 and *vtc-1* plants: (i) plants watered with Hoagland's solution [16] at saturation (irrigated (IR) plants), and (ii) plants not watered at all (WS plants).

2.2. Plant water status and leaf area measurements

Plant water status was determined by measuring the relative leaf water content (RWC) of leaves 1 h before the light period as described [17]. Leaf area was measured with a flatbed scanner (model GT-5000, Epson, Nagano, Japan) and analyzed with an image-processing program.

2.3. Isolation of chloroplasts

For chloroplast isolation, leaves were collected 1 h before the light period and were immediately subjected to cell fractionation as described [18]. The identity and purity of Percoll-purified chloroplasts were determined by assaying amounts and activities of appropriate markers, and confirmed further by microscopic observation as described [18]. Chloroplasts-enriched fractions did not show detectable activities of the markers assayed, i.e. NADPH-cytochrome *c* reductase, latent IDPase, vanadate-sensitive ATPase, and cytochrome *c* oxidase, which are markers of the endoplasmic reticulum, Golgi apparatus, plasma membrane, and mitochondrion, respectively. The chloroplast intactness was determined by the ferricyanide reducing assay [19]. The intactness of chloroplasts exceeded 80% in preparations from both IR and WS plants.

2.4. Asc determination

The amounts of Asc and dehydroascorbic acid (Dha) were measured in leaves and chloroplasts by high-performance liquid chromatography (HPLC) as described [20], except that detection was carried out at 255 nm (Diode array detector 1000S, Applied Biosystems, Foster City, CA, USA). Asc was identified by its characteristic spec-

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Abbreviations: AOS, active oxygen species; Asc, ascorbic acid; Asc_t, total ascorbic acid (reduced plus oxidized); α-Toc, α-tocopherol; β-Car, β-carotene; Chl, chlorophyll a+b; Dha, dehydroascorbic acid; DPS, de-epoxidation state of the xanthophyll cycle; Fv/Fm, maximum efficiency of photosystem II photochemistry; IR, irrigated plants; MDA, malondialdehyde; RWC, relative leaf water content; WS, water-stressed plants

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trum and by coelution with an authentic standard from Sigma (Steinheim, Germany).

2.5. Estimation of lipid oxidation

The extent of lipid oxidation was estimated by measuring the amount of malondialdehyde (MDA) in chloroplasts by HPLC as described [21], except that 80:20 (v/v) ethanol/water containing 1 ppm butylated hydroxytoluene was used for extraction, and a Hypersyl ODS-5 μm column (250×4.6 mm, Teknokroma, St. Cugat, Spain) was used for HPLC analysis. The $(TBA)_2$–MDA adduct was quantified by its absorbance at 537 nm (Diode array detector 1000S, Applied Biosystems), and was identified by its characteristic spectra and by coelution with an authentic standard. 1,1,3,3-Tetraethoxypropane (Sigma, Steinheim, Germany), which was used as a standard, is stoichiometrically converted into MDA during the acid-heating step of the assay.

2.6. Pigment determination

For measurement of pigments, leaves were collected 1 h before the light period, frozen in liquid nitrogen and stored at −80°C until analysis. The extraction and HPLC analysis of pigments were carried out as described [17]. Compounds were identified by their characteristic spectra and by coelution with chlorophyll and carotenoid standards, which were obtained from Fluka (Buchs, Switzerland) and Hoffman-La Roche (Basel, Switzerland).

2.7. α-Toc determination

For measurement of α-Toc, leaves were collected 1 h before the light period, frozen in liquid nitrogen and stored at −80°C until analysis. The extraction and HPLC analysis of α-Toc were carried out as described [17]. α-Toc was quantified through its absorbance at 285 nm (Diode array detector 1000S, Applied Biosystems). α-Toc was identified by its characteristic spectrum and by coelution with an authentic standard from Sigma (Steinheim, Germany).

2.8. Chlorophyll fluorescence measurements

The maximum efficiency of photosystem II photochemistry (Fv/Fm) was calculated from chlorophyll fluorescence data, which were obtained from leaves 1 h before the light period (after 15 h of dark adaptation) with a portable fluorimeter mini-PAM (Walz, Effeltrich, Germany), by using the equations described by Genty et al. [22].

3. Results

The extent of Asc deficiency in the mutant depended on plant age, and varied between 50 and 93% throughout the experiment (Fig. 1). RWC of WS plants decreased as water deficit progressed, and was below 70% in both Col-0 and *vtc-1* plants after 3 weeks of stress (Fig. 1). Leaf area increased progressively throughout the experiment in IR plants, but less in *vtc-1* than in Col-0. Growth was almost completely inhibited in both Col-0 and *vtc-1* after 1 week of water deficit. Asc increased progressively in the mutants grown under optimal conditions, but not in Col-0. Besides, Asc was lower in

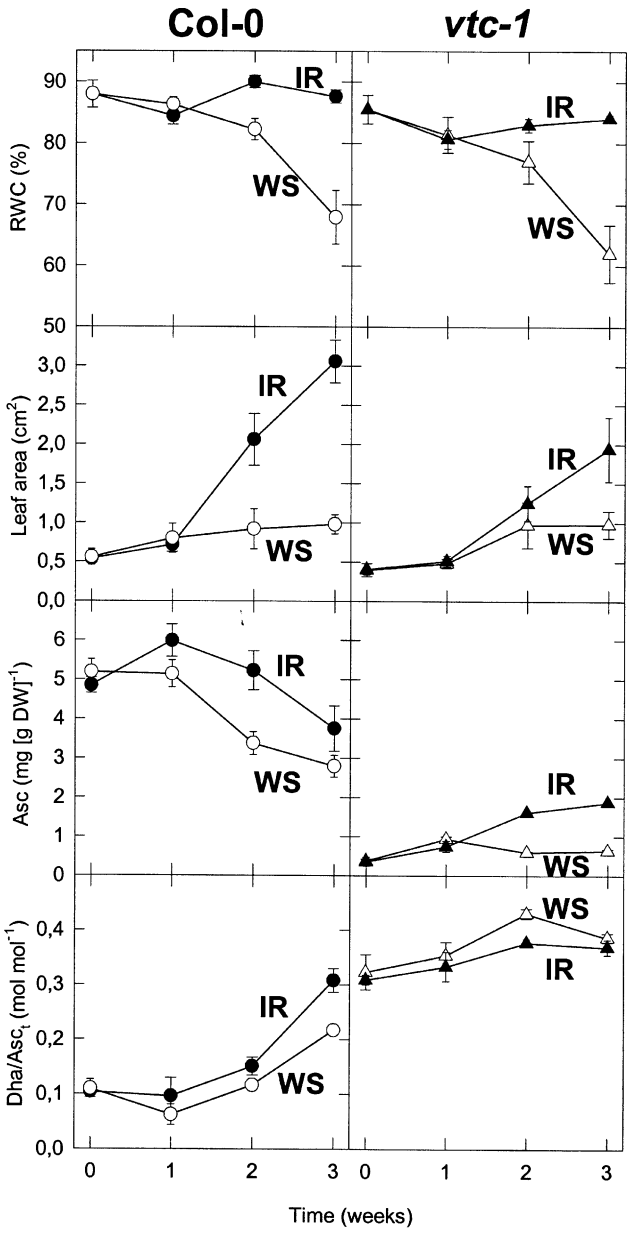

Fig. 1. Changes in the RWC, leaf area, Asc levels and the ratio Dha/Asc_t in leaves of IR and WS wild-type (Col-0) and Asc-deficient *vtc-1* mutants (*vtc-1*) of *Arabidopsis*. Water stress was induced by withholding water for 3 weeks. Data correspond to the mean ± S.E.M. of six independent measurements made on leaves collected 1 h before the light period.

Table 1
Xanthophyll composition, DPS, and Fv/Fm ratio in the wild-type (Col-0) and the Asc-deficient *vtc-1* mutant (*vtc-1*) of *Arabidopsis* under IR conditions and after 3 weeks of water deficit (WS)

	Col-0		*vtc-1*	
	IR	WS	IR	WS
V/Chl	80.0 ± 16.2	33.4 ± 9.9[b]	82.7 ± 16.3	98.6 ± 15.1[a]
A/Chl	4.2 ± 0.2	4.1 ± 0.4	2.5 ± 0.2[a]	2.6 ± 0.1[a]
L/Chl	561.2 ± 66.7	576.1 ± 39.1	660.1 ± 34.6[a]	692.9 ± 20.2[a]
N/Chl	132.1 ± 17.2	106.0 ± 18.1	123.5 ± 24.1	118.8 ± 11.6
DPS	0.025 ± 0.003	0.055 ± 0.002	0.014 ± 0.003[a]	0.012 ± 0.003[a]
Fv/Fm	0.80 ± 0.01	0.77 ± 0.01[b]	0.80 ± 0.01	0.76 ± 0.02[b]

Data, which are given in mmol (mol Chl)$^{-1}$ for xanthophylls, correspond to the mean ± S.E.M. of six independent measurements made on leaves collected 1 h before the light period. [a] and [b] indicate statistical difference (Student's *t*-test, $P \leq 0.05$) between *vtc-1* and Col-0, and between WS and IR plants, respectively. V, violaxanthin; A, antheraxanthin; L, lutein; N, neoxanthin. Zeaxanthin was not detected in either plant.

WS than in IR Col-0 and *vtc-1* plants after 2 weeks of water deficit. The ratio of Dha to total Asc (Dha/Asc_t) increased as plants aged, the mutants showing Dha/Asc_t ratios three-fold higher at the beginning of the experiment. Leaf area was positively correlated with the amounts of Asc in *vtc-1* but not in Col-0 ($r^2 = 0.773$, slope = 0.917; and $r^2 = 0.021$, slope = −0.173 for *vtc*-1 and Col-0, respectively).

Asc deficiency in the leaves of *vtc-1* was associated with decreased levels of Asc in chloroplasts, which were 60% lower in *vtc-1* than in Col-0. Besides, Asc in chloroplasts was, in turn, more oxidized in *vtc-1* than in Col-0 in both IR and WS plants (Fig. 2). MDA levels in chloroplasts were constant throughout the experiment in IR and WS plants in Col-0, but they increased 1.9-fold in chloroplasts of WS mutants (Fig. 2). However, Fv/Fm was not significantly altered in the mutants, compared with the wild-type (Table 1), and chlorophyll a+b (Chl) contents remained unchanged throughout the experiment at ca. 12 mg (g dry weight)$^{-1}$ in both Col-0 and *vtc-1* (data not shown). In optimal growth conditions, Col-0 and

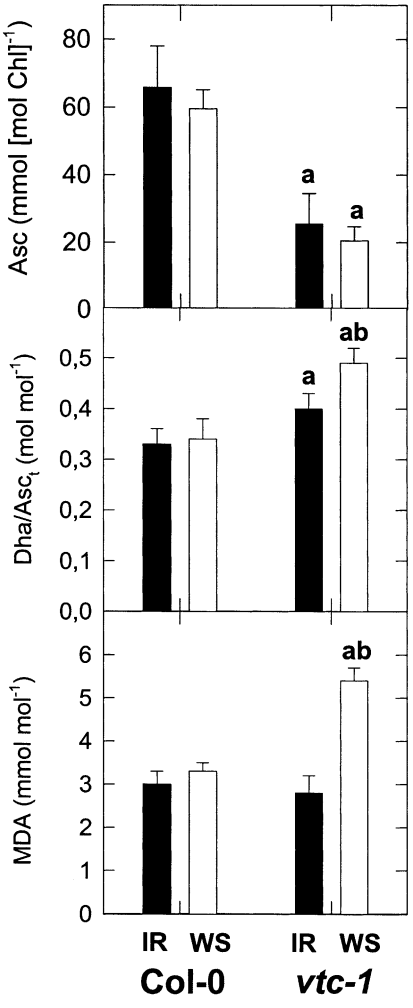

Fig. 2. Asc levels, the ratio Dha/Asc_t and MDA levels in leaf chloroplasts of the wild-type (Col-0) and the Asc-deficient *vtc-1* mutant (*vtc-1*) of *Arabidopsis* under IR conditions and after 3 weeks of water deficit (WS). Data correspond to the mean ± S.E.M. of three independent measurements. [a] and [b] indicate statistical difference (Student's *t*-test, $P \leq 0.05$) between *vtc-1* and Col-0, and between WS and IR plants, respectively. Chloroplasts were obtained as described in Section 2.

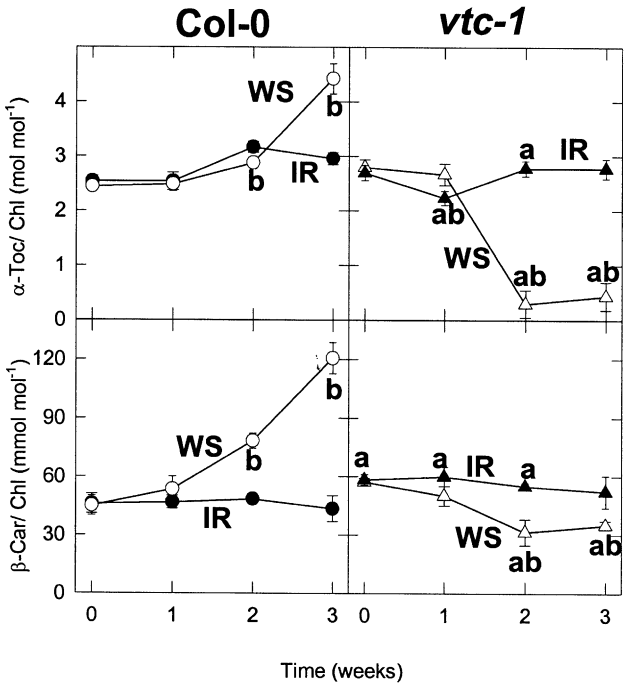

Fig. 3. Changes in the lipophilic antioxidants, α-Toc and β-Car per unit of Chl in leaves of IR and WS wild-type (Col-0) and Asc-deficient *vtc-1* mutants (*vtc-1*) of *Arabidopsis*. After 2 weeks of water deficit, the *vtc-1* mutants showed a severe loss of lipophilic antioxidant defences. Data correspond to the mean ± S.E.M. of six independent measurements made on leaves collected 1 h before the light period. [a] and [b] indicate statistical difference (Student's *t*-test, $P \leq 0.05$) between *vtc-1* and Col-0, and between WS and IR plants, respectively.

mutants showed similar α-Toc, violaxanthin and neoxanthin contents per unit of Chl, but the mutants showed slightly higher β-carotene (β-Car) and lutein contents, and lower antheraxanthin, which caused lower de-epoxidation states of the xanthophyll cycle (DPS) than Col-0 (Table 1, Fig. 3). Although violaxanthin was significantly reduced in WS Col-0 plants, neither Col-0 nor *vtc-1* plants showed increases in de-epoxidized xanthophylls under stress (Table 1). Under water deficit, the α-Toc/Chl ratio increased in Col-0, whereas strong reductions (by ca. 85%) were observed between the first and the second week of water deficit in *vtc-1*. β-Car changed in parallel with α-Toc. β-Car levels per unit of Chl showed a 60% increase in Col-0, whereas the levels decreased by ca. 40% in the mutant under water deficit (Fig. 3).

4. Discussion

The *vtc-1* (formerly *soz*) mutant of *Arabidopsis* was first isolated for its sensitivity to ozone, and it was shown to be more sensitive to SO_2 and UV-B radiation than the wild-type [14]. Here we show that Asc deficiency increases the sensitivity of these mutants to water deficit-induced oxidative stress. Asc deficiency in the mutant ranged between 50 and 93% in leaves depending on plant age and was around 60% in chloroplasts. Previous reports [23–26] have shown that Asc_t in chloroplasts accounts for ca. 7–30% of that found in leaves in several species. Asc_t in chloroplasts was between 4 and 6% of that found in leaves in both Col-0 and *vtc-1 Arabidopsis* plants. Plants were grown in low light (90–110 μmol quanta m^{-2} s^{-1}),

which may explain the low amounts of Asc_t found in chloroplasts [25,27].

Although mutants showed lower DPS, and their chloroplasts contained more β-Car and lutein, lower Asc and higher Dha/Asc_t ratios than those of Col-0 under optimal growth conditions, both Col-0 and *vtc-1* showed similar Fv/Fm ratios and MDA levels in chloroplasts. According to a previous report [28], this indicates that *vtc-1* mutants withstand Asc deficiency and avoid oxidative stress under optimal growth conditions. Although the biological significance of increased Dha/Asc_t ratios is controversial [7,29,30], our results suggest that higher Dha/Asc_t ratios do not necessarily indicate oxidative stress in plants.

The Fv/Fm ratio remained above 0.76 in both Col-0 and *vtc-1* throughout the experiment, which suggests no photoinhibitory damage to the photosynthetic apparatus in either plant. The parallel degradation in β-Car and α-Toc and enhanced lipid peroxidation in chloroplasts of WS mutants demonstrates the significance of Asc on the antioxidant defence system and is indicative of the interplay between hydrophilic and lipophilic antioxidants in chloroplasts. Asc has been shown to protect α-Toc and recycle α-Toc from its α-tocopheroxyl radical in vitro [31,32], and in human erythrocytes [33], but not in plants. Our study provides evidence consistent with the protective effects of Asc on α-Toc in plants in vivo. The degradation of lipophilic antioxidants (i.e. α-Toc and β-Car) and increased lipid peroxidation indicate oxidative stress in chloroplasts of WS *vtc-1* plants, and it is compatible with an enhanced production of $^1O_2$ and/or $OH^\bullet$ under stress.

Asc is found in the thylakoid lumen and stroma of chloroplasts [6], while α-Toc and β-Car are found in the lipid matrix, and associated with protein domains, respectively, in the thylakoid membrane [13,36], which makes a protective effect of Asc on α-Toc and β-Car oxidation likely by scavenging and/or preventing the formation of $OH^\bullet$, but to a lesser extent by scavenging $^1O_2$, which is formed in the thylakoid membrane [34,35]. Alternatively, $^1O_2$ and $OH^\bullet$ may increase peroxidation of thylakoid membrane lipids, and the resulting lipid peroxyl radicals increase oxidation of α-Toc, β-Car and propagate lipid peroxidation. Asc in chloroplasts may protect α-Toc by recycling α-tocopheroxyl radicals to α-Toc in the thylakoid membrane interface. In the mutants, deficiency in Asc may limit the recycling of α-tocopheroxyl radicals to α-Toc, which may, in turn, increase oxidation of β-Car and thylakoid membrane lipids when $^1O_2$ and $OH^\bullet$ increase under water deficit.

In conclusion, we have demonstrated that low Asc in the *vtc-1* mutants of *Arabidopsis* increases oxidative stress in chloroplasts of WS plants. As stress progresses, Asc becomes indispensable for the correct functioning of antioxidant defences in chloroplasts. The interplay between Asc and lipophilic antioxidants seems to be essential for the protection of thylakoid membrane lipids from oxidation in WS *Arabidopsis* plants.

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