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The Ascorbate-deficient *vtc-1* *Arabidopsis* Mutant Shows Altered ABA Accumulation in Leaves and Chloroplasts

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Abstract

Abscisic acid (ABA) accumulation has been analyzed in irrigated and water-stressed wild-type and the *vtc-1* mutant of *Arabidopsis thaliana*, which shows an ascorbate deficiency in leaves of approximately 60%. The amounts of ABA increased progressively up to 2.3-fold in water-stressed wild-type plants, whereas levels were kept at low levels in the irrigated plants. In contrast, initial increases followed by a sharp decrease of abscisic acid levels were observed in water-stressed *vtc-1* mutants. Furthermore, the levels of this phytohormone increased up to fivefold in irrigated mutants. This differential accumulation of ABA in the mutant strongly correlated with the ascorbate redox state, but not with ascorbate levels. Changes in ABA levels in leaves paralleled those of chloroplasts. Immunolocalization studies showed a differential ABA accumulation in chloroplasts of *vtc-1* mutants, which displayed the highest ABA labeling in irrigated plants. Our results indicate an altered pattern of ABA accumulation in the *vtc-1* mutant compared to the wild type, under both irrigated conditions and water-stress conditions, which is strongly dependent on the ascorbate redox state.

Key words: Ascorbate (AA); Ascorbate redox state; Abscisic acid (ABA); ABA immunolocalization; *vtc-1* mutant; Water stress

Introduction

Ascorbate (AA) has numerous and diverse functions in plants. It is the most abundant low-M_r antioxidant in the plant cell; it serves as the major contributor to the cellular redox state (Conklin and Bar 2004); it participates in the mechanisms of plant responses to stress and in the regulation of mitosis and cell expansion (Noctor and Foyer 1998; Smirnoff and Wheeler 2000); it is a co-substrate for a large number of key enzymes (Arrigoni and De Tullio 2000) and it is involved in a complex phytohormone-mediated signaling network (Pastori and others 2003; Conklin and Barth 2004; Wolucka and others 2005). Ascorbate is required as a co-substrate for the activity of 2-oxoacid-dependent dioxygenases, a class of enzymes that regulate the synthesis of hydroxyproline-containing proteins and hormones (Arrigoni and De Tullio 2000, 2002; De Tullio and Arrigoni 2004). Indeed, AA modulates the content

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of several signaling molecules, such as abscisic acid (ABA) and gibberellins (GAs) (Schwartz and others 1997; Pastori and others 2003), ethylene (McGarvey and Christoffersen 1992) and salicylic acid (SA) (Barth and others 2004).

Plants control different aspects of their growth and development and respond to a number of environmental stresses, such as drought, cold and high temperatures, or salt stress by modulating endogenous ABA levels (Shinozaki and Yamaguchi-Shinozaki 1999; Schwartz and others 2003). Unlike other phytohormones, the endogenous concentrations of ABA can increase by more than 10-fold in water stress conditions (Zeevaart 1980). In higher plants, the ABA biosynthesis pathway involves the oxidative cleavage of 9-cis-epoxycarotenoids, which is catalyzed by 9-cis-epoxicarotenoid dioxygenase (NCED) and is considered a key regulatory step in the ABA biosynthetic pathway (Zeevaart and Creelman 1988; Li and Walton 1990; Parry and others 1990; Quin and Zeevaart 1999; Sun-Young and others 2004). Although the role of AA in the regulation of ABA biosynthesis is complex, it has been shown that AA is specifically required as a cofactor for the activity of the NCED enzyme (Arrigoni and De Tulio 2000). Pastori and others (2003) reported that transcripts encoding NCED are upregulated in the *vtc-1 Arabidopsis* mutant. Furthermore, it has been suggested that the rise in NCED transcript compensates for AA deficiency in this mutant, and an increase in ABA levels has been reported in these plants (Conklin and others 1996; Veljovic-Jovanovic and others 2001).

Further indications of the relationship between AA and ABA are those related to the AA redox state. For example, the H_2O_2-mediated, ABA-induced stomatal closure was impaired in dehydroascorbate reductase (DHAR)-overexpressing transgenic tobacco plants with an increased ascorbate-to-dehydroascorbate ratio (Chen and Gallie 2004).

The *vtc-1* mutant provides an excellent system in which to evaluate the effects of physiologically relevant changes in AA and its redox state on endogenous ABA concentrations in plants. The *vtc-1* mutant was isolated by its sensitivity to ozone, but it also showed high sensitivity to other abiotic stress factors like sulfur dioxide, UV-B radiation and freezing (Conklin and others 1996), or water stress (Munné-Bosch and Alegre 2002).

The *vtc-1* mutant of *Arabidopsis* has been used to examine the interplay between AA and lipophilic antioxidant defenses in chloroplasts of water-stressed (WS) plants (Munné-Bosch and Alegre 2002). In this work, we showed that the AA levels and xanthopyll composition differed between wild-type and mutant plants under both irrigated conditions and water-stress conditions. Furthermore, Pastori and others (2003) reported the ABA content to be significantly higher in *vtc-1* mutants than in wild-type plants under irrigated conditions, but no data have been provided thus far either on ABA changes during development or under water stress conditions in ascorbate-deficient plants.

Here, we report for the first time the effects of water stress, plant development, and the AA redox state on the endogenous levels of ABA in the *vtc-1 Arabidopsis* mutants.

Materials and Methods

Plant Material and Growth Conditions

Seedlings of *Arabidopsis thaliana* Columbia (Col) ecotype, wild-type (Col-0) and the AA-deficient mutant *vtc-1* (Conklin and others 1996) 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 was started, and two water regimes were imposed for 3 weeks on both Col-0 and *vtc-1* plants (1) watered with Hoagland's solution (Hoagland and Arnon 1950) at saturation (irrigated [IR] (plants) and (2) subjected to progressive substrate-water-deficit (WS plants).

Samples for ABA, AA, and relative leaf water content (RWC) analysis were taken once a week at the beginning of the light period. Rosettes of wild-type and mutant plants were harvested, weighed, immediately frozen in liquid N_2, and stored at –80°C until ABA and AA analysis. Leaf cell ultrastructure was observed 3 weeks after the beginning of the experiment.

Plant Water Status Measurement

Plant water status was determined by measuring the relative leaf water content (RWC) of leaves as 100× (FW – DW)/(TW – DW), where FW is the fresh matter, TW is the turgid matter after re-hydrating the leaves for 24 h at 4°C in darkness, and DW is the dry matter after oven-drying the leaves for 24 h at 80°C.

Analyses of Reduced and Oxidized Ascorbate

The extraction and HPLC analysis of reduced and oxidized ascorbate were carried out essentially as described by Munné-Bosch and Alegre (2003). In

short, leaves were repeatedly extracted (four times) with ice-cold extraction buffer (40% [v/v] methanol, 0.75% [w/v] *m*-phosphoric acid, 16.7 mM oxalic acid, 0.127 mM diethylenetriaminepentaacetic acid). Following extraction, 0.1 ml of the pooled supernatants were transferred to 0.9 ml of the mobile phase (24.25 mM Na-acetate/acetic acid, pH 4.8; 0.1 mM diethylenetriaminepentaacetic acid; 0.015% [w/v] *m*-phosphoric acid; 0.04% [w/v] octylamine; 15% [v/v] methanol) for determination of reduced ascorbate. For determination of total ascorbate (reduced plus oxidized) 0.1 ml of the pooled supernatants were incubated for 10 min at room temperature in darkness with 0.25 ml of 2% (w/v) dithiothreitol and 0.5 ml of 200 mM $NaHCO_3$. The reaction was stopped by adding 0.25 ml of 2% (v/v) sulfuric acid and 0.8 ml of the mobile phase. Ascorbate was isocratically separated on a Spherisorb ODS C_8 column (Teknokroma, St. Cugat, Spain) by using the mobile phase (24.25 mM Na-acetate/acetic acid, pH 4.8; 0.1 mM diethylenetriaminepentaacetic acid; 0.015% [w/v] *m*-phosphoric acid; 0.04% [w/v] octylamine; 15% [v/v] methanol) at a flow rate of 0.8 ml min^{-1}. Detection was carried out at 255 nm (diode array detector 1000S, Applied Biosystems, Foster City, CA, USA). Ascorbate was identified by its characteristic spectrum and by co-elution with an authentic standard from Sigma (Steinheim, Germany).

Abscisic Acid Analysis

Abscisic acid determination was carried out as described by López-Carbonell and Jauregui (2005). Briefly, leaves were repeatedly extracted with acetone/water/acetic acid (80:19:1, v/v) at –20°C and centrifuged at 18,000 rpm, at 4°C, for 15 min. The supernatants were collected and the pellets were re-extracted with 3 ml of the extraction solvent. The second group of extracts was centrifuged, and the supernatants were combined and dried completely under a nitrogen stream. Dried samples were reconstituted in 200 µl of acetonitrile/water/acetic acid (90:10:0.05, v/v), centrifuged (10,000 rpm, 10 min), filtered through a 0.45-µm polytetrafluoroethylene (PTFE) filter (Waters, Milford, MA, USA), and injected into the LC-MS/MS system. Quantification was carried out using the standard addition method, by spiking control plant samples with ABA solutions (ranging from 10 to 200 ng/ml).

The high-performance liquid chromatography (HPLC) system (PerkinElmer Series 200, USA) was equipped with a ultraviolet light (UV) detector. For the analysis of the extracts, a Luna C_{18} Phenomenex (Torrance, CA, USA) column (50 × 2.1 mm, 3.5 µm) was used. Mass spectroscopy (MS) and MS/MS analysis were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada). Product ion scan experiments of *m/z* 263 ion yielded the *m/z* 153. Multiple reaction monitoring (MRM) acquisition was performed by monitoring the 263/153 transition.

Immunocytochemical and Ultrastructural Analysis

Ultrastructural studies were carried out essentially according to the method of López-Carbonell and others (1994). Electron micrographs were obtained with a Jeol 1010 transmission electron microscope at an accelerating voltage of 80 kV. For each water treatment, an average of 95 randomly chosen cells were examined.

Abscisic acid was immunolocalized with specific monoclonal antibodies against (±) 2-*cis*-4 *trans* ABA (Agdia, Elkhart, Indiana, USA), basically as described by Pastor and others (1999). Percentage cross reactivities from tracer displacement curves at 50% displacement on a molar basis was determined for the monoclonal antibody, and it resulted in 100% for 2-*cis* (S)-ABA and 0 or less than 0.1% for other ABA metabolites. Cross-sections 1–2 mm wide from IR and WS plants were fixed at reduced pressure in 2% aqueous solution of water-soluble 1-(3-dimethylaminopropyl)-3 ethyl carbodiimide (EDC, Sigma-Aldrich, USA) for 30 min at room temperature and then post-fixed in EDC for 2 h at 4°C. After washing with 0.1 M cacodylate (pH 7.3), samples were progressively cryoprotected by infusing in 2.3 M sucrose 0.1 M cacodylate (pH 7.3) as described by Raposo and others (1997). They were then frozen and stored in liquid nitrogen at –196°C. Cryosubstitution was performed in 0.5% uranyl acetate in methanol at – 90°C for 48 h, and then the temperature was increased to –35°C, at 5°C per hour. After several rinses in methanol, samples were infiltrated in Lowicryl K4M for 6 days and polymerized at – 35°C with UV lamps.

Ultrathin sections were obtained and mounted on nickel grids. Immunolabeling was performed by first placing the grid in drops of 0.10 M PBS (pH 7.4) containing 1% bovine serum albumin (BSA) for 15 min. The grids were then transferred to a drop of the monoclonal anti-ABA antibody diluted 1/10 (v/v) in PBS for 2 h. After rinsing four times with PBS, they were floated on a 1/25 (v/v) dilution of goat anti-mouse (IgG Au 10 nm, Sigma) in PBS for 1 h. Finally, they were rinsed three times in PBS and

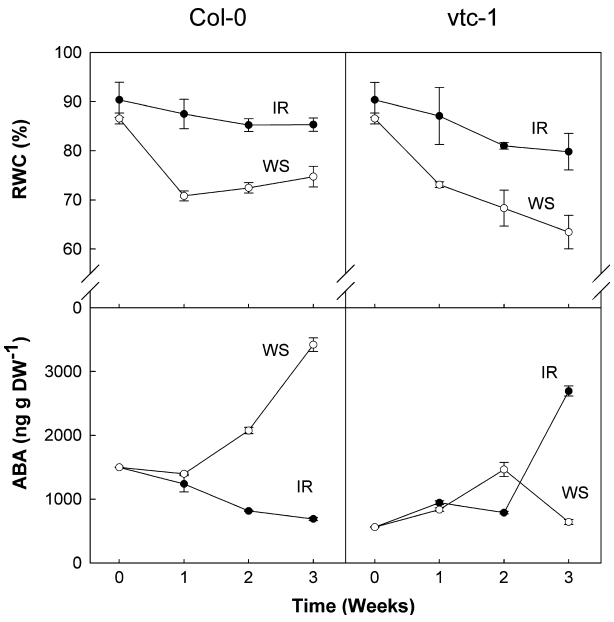

Figure 1. Changes in the relative leaf water content (RWC) and abscisic acid (ABA) accumulation in leaves of irrigated (IR) and water-stressed (WS) wild type (Col-0) and *vtc-1* mutants of *Arabidopsis*. Water stress was imposed on plants by withholding water for 3 weeks. Data correspond to the mean ± SE of three independent measurements.

four times in a ''jet-wash'' with water. To establish the immunolabeling specificity, controls included the omission of the primary antibody.

The grids were subsequently stained with uranyl acetate and Reynold's lead citrate and examined under a Jeol 1010 transmission electron microscope at an accelerating voltage of 80 kV. The density of labeling was calculated and expressed as the number of gold particles per μm^2. The amount of labeling over a specified area was estimated by scanning the photographic enlargements and calculated as the number of gold particles per unit of surface. For each water treatment, an average of 25 randomly chosen mesophyll cells were examined.

Results

The response of ABA accumulation to water stress differs between wild-type and ascorbate-deficient plants

The RWC of water-stressed plants decreased as water deficit progressed and was approximately 75% and 64% in Col-0 and *vtc-1* plants, respectively, after 3 weeks of stress (Figure 1). Abscisic acid levels ranged between 700 and 1500 ng g DW^{-1} in irrigated Col-0 plants (Figure 1). In response to

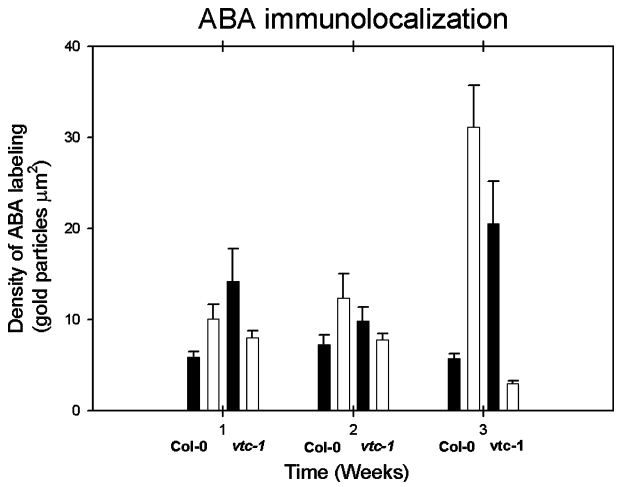

Figure 2. Density of ABA labeling in chloroplasts of mesophyll cells of irrigated (IR, closed bars) and water-stressed (WS, open bars) wild type (Col-0) and *vtc-1* mutants of *Arabidopsis*. Water stress was imposed on plants by withholding water for 3 weeks. Data correspond to the mean ± SE of 15 independent measurements and represent gold particles μm^{-2}. For the control, pre-immune serum was used.

water-stress, the ABA levels increased gradually, by up to 2.3-fold after 3 weeks of water deficit. Stress also increased ABA levels in *vtc-1* mutant plants, but only during the first 2 weeks, in which ABA levels were near twofold higher than those obtained at the beginning of the experiment. As water deficit progressed further (3 weeks of stress), ABA levels decreased and were fourfold lower in the mutant than in the wild type.

Abscisic acid levels in the leaves of Col-0 and *vtc-1* plants were associated with ABA accumulation in chloroplasts as determined by immunocytochemistry (Figure 2). In irrigated conditions, ABA levels in chloroplasts were maintained fairly constant throughout the study in the wild type, showing ABA labeling at a density of approximately 6 gold particles μm^{-2}. In water stress conditions, ABA labeling increased progressively in chloroplasts of these plants, reaching a density of 30 gold particles μm^{-2}. Although no significant differences in ABA labeling in chloroplasts of *vtc-1* plants were observed during the first 2 weeks of water deficit; this decreased sharply to a density of 4 gold particles μm^{-2} after 3 weeks of water stress. At this point, ABA labeling in chloroplasts of the mutant was sevenfold lower than in the wild type. Water stress affected chloroplast ultrastructure both in wild-type and mutant plants (Figure 3). Well-organized chloroplasts were observed in irrigated plants of both plant groups, whereas dilated chloroplasts and thylakoids, as well

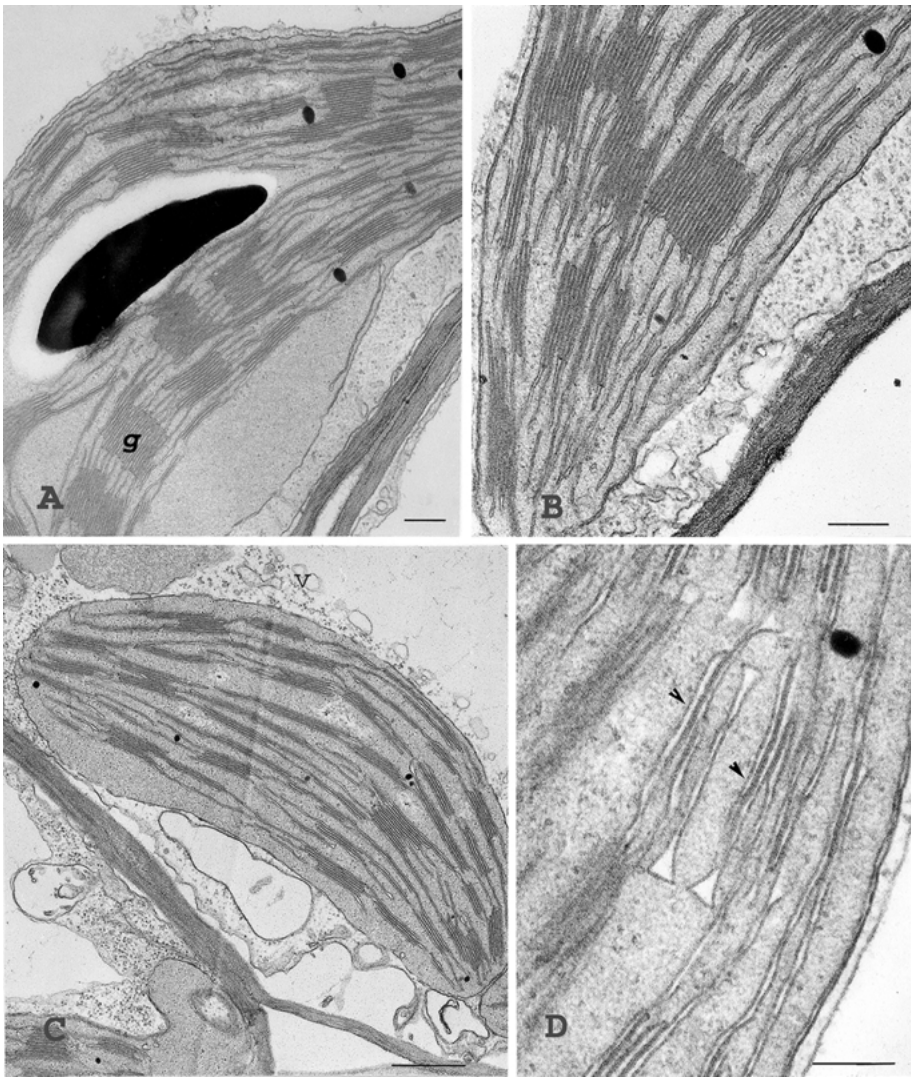

Figure 3. Comparison of chloroplast ultrastructure between irrigated (IR) and water-stressed (WS) wild type (Col-0) and *vtc-1* mutants of *Arabidopsis*. **A, B.** IR Col-0 and *vtc-1* plants, respectively. Note the presence of grana in chloroplasts. **C.** WS Col-0 plants. Dilated thylakoids, less packed and partially disorganized grana were observed. **D.** Chloroplast of WS *vtc-1* plants showed extensive changes. Thylakoids from chloroplasts showed a marked reduction in stacked membranes, alteration of stromatic thylakoids and loss of stroma density. Water stress was imposed on plants by withholding water for 3 weeks. Arrowheads indicate dilated thylakoids; g, grana. Scale bars: 0.2 μm.

as less packed and partially disorganized grana, were detected in water-stressed Col-0 and *vtc-1* plants.

Age-dependent ABA Increases in Ascorbate-deficient Plants

The experiment started when plants were 8 weeks old and continued for 3 more weeks, and then ended when plants were 11 weeks olds. No significant differences were observed throughout the experiment, either in leaf ABA content or in ABA labeling in chloroplasts of irrigated wild-type plants (Figures 1 and 2).

However, ABA levels in irrigated *vtc-1* mutants increased as plants aged. During the first 2 weeks of the experiment, there were no significant differences in ABA content between Col-0 and *vtc-1* plants. Nevertheless, ABA levels increased up to threefold in 11-weeks-old *vtc-1* irrigated plants (Figure 1).

Again, ABA labeling did not differ between Col-0 and *vtc-1* irrigated plants during the first 2 weeks. However, in the third week, ABA labeling in IR *vtc-1* plants was fourfold higher than in IR Col-0 plants (Figure 2).

ABA increases in ascorbate-deficient plants correlated with the ascorbate redox state

The *vtc-1* mutant exhibited an AA deficiency of approximately 60% compared to the wild type in both irrigated and water-stressed plants. Ascorbate levels ranged between 5–6 mg g DW^{-1} and between 2–3 mg g DW^{-1} throughout the experiment in wild-type and ascorbate-deficient plants, respectively (data not shown). To get insights into the relation among ABA, ascorbate levels, and ascorbate redox state, these parameters were measured simultaneously in *vtc-1* mutants (Figure 4). The levels of DHA/AA_t increased as plants aged; in *vtc-1* irrigated

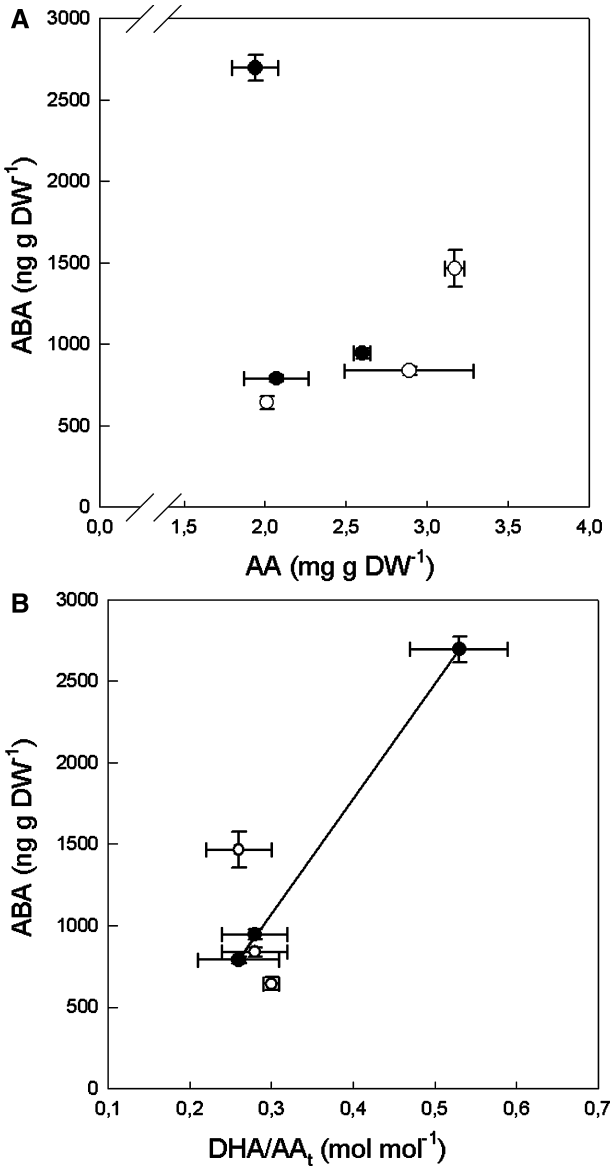

Figure 4. **A.** Relationship between abscisic acid (ABA) and ascorbate (AA) levels in *vtc-1* plants. **B.** Relationship between ABA and ascorbate redox state (DHA/AA_t) in *vtc-1* mutants of *Arabidopsis*. Values correspond to irrigated (closed circles) and water stressed (open circles) plants from 1 to 3 weeks of the experiment. Data correspond to the mean ± SE of three independent measurements.

plants the highest values of DHA/AA_t (0.53 mol mol^{-1}) were attained 3 weeks into the experiment (Table 1). This value was twofold higher than observed 2 weeks into the experiment (0.26 mol mol^{-1}) and correspond to the lowest AA level (1.94 mg g DW^{-1}). Although low correlation was obtained between ABA and AA levels ($r^2 = 0.354$; Figure 4A), the highest ABA levels (2.695 ng g DW^{-1}) were associated with the highest ascorbate redox state (DHA/AA_t, 0.53 mol mol^{-1}), showing a strong positive correlation between ABA and DHA/AA_t ($r^2 = 0.999$; Figure 4B) in irrigated *vtc-1* plants.

Discussion

Phytohormones, reactive oxygen species and antioxidants interact in the control of plant metabolism, morphology, and development (Foyer and Noctor 2003, 2005; Jiang and Feldman 2005). The redox state of AA is an indicator of the cell redox state, and its level in the apoplast is considered an important modulator of redox-sensitive proteins, thus controlling the biosynthesis of signaling molecules as ABA (Barth and others 2004). Also, it has been described that other phytohormones such as methyl jasmonate may regulate the biosynthesis of AA and thus its redox state (Wolucka and others 2005).

Several studies have been performed to elucidate the regulation of ABA increase in wild-type and several *Arabidopsis* mutants growing in optimal or stress conditions (Rock and Zeevaart 1991; Iuchi and others 2001; Tian and others 2004). It has been shown that ABA content is significantly higher in the AA-deficient *vtc-1 Arabidopsis* mutant than in wild-type plants under irrigated conditions, and this ABA increase has been attributed to a differential gene expression in *vtc-1* with respect to wild-type plants (Pastori and others 2003; Conklin and Barth 2004). Furthermore, these mutants are more sensitive to water stress and show a higher AA redox state than wild-type plants (Munné-Bosch and Alegre 2002). However, knowledge regarding ABA accumulation in *vtc-1* water-stressed plants or its role in water stress responses is lacking. Here we report for the first time that plant water status, plant developmental stage, and the ascorbate redox state modulate ABA levels in these mutants.

The present study demonstrates an altered ABA accumulation in leaves and chloroplasts of the *vtc-1* mutants, which are deficient in ascorbate. The mutant showed lower ABA levels than the wild type under water deficit. Abscisic acid levels increased similarly in both plant groups after 2 weeks of water deficit, but they decreased sharply after 3 weeks of stress in the mutant only. After 3 weeks of water deficit, ABA levels fell to the lowest values in the mutant, reaching values fivefold lower than in wild-type plants. It is likely that at this point, the mutants lost their capacity to close stomata, because RWC values were 20% lower in the mutant than in the wild type. Therefore, the low levels of ABA observed in these mutants exposed to prolonged water deficit can make them more susceptible to stress.

Table 1. Ascorbate (AA) and Ascorbate Redox State (DHA/AA_1) in *vtc-I* mutants of *Arabidopsis* under Irrigated (IR) and Progressive Weeks of Water Stress (WS)

	AA (mg g DW^{-1})		DHA/AA_t (mol mol^{-1})	
	IR	WS	IR	WS
1^{rst} week	2.60 ± 0.05	2.89 ± 0.40	0.28 ± 0.04	0.28 ± 0.04
2^{nd} week	2.07 ± 0.20	3.17 ± 0.06	0.26 ± 0.05	0.26 ± 0.04
3^{rd} week	1.94 ± 0.14	2.01 ± 0.02	0.53 ± 0.06	0.30 ± 0.01

An enhanced accumulation of ABA was observed in an age-dependent manner in the mutant, and this correlated with a rise in the ratio of DHA/AA_t. This increase in the ascorbate redox state in the irrigated *vtc-1* mutant at the final harvest point may have occurred because analyses have been conducted on rosettes at advanced developmental stages. No major differences in ABA levels were observed between Col-0 and *vtc-1* irrigated plants throughout the 2 first weeks of the experiment, whereas a strong increase in ABA levels was observed between the second and third weeks in the irrigated mutants only. This increase in ABA was associated with a rise in the AA redox state, and both ABA and DHA/AA_t were positively correlated throughout the experiment in the mutants.

The biosynthesis of ABA is rapidly induced in drought-stressed leaves and this stress-induced abscisic acid accumulation is derived from the carotenoid precursors, violaxanthin and neoxanthin (Quin and Zeevaart 1999). We previously analyzed the carotenoid composition of the wild type and the ascorbate-deficient mutant in water-stressed plants, and we found a decrease by 58% and 20% in the levels of violaxanthin and neoxanthin, respectively (Munné-Bosch and Alegre 2002). This may account for the increase observed in ABA levels in water-stressed plants and supports the role of the NECD enzyme in ABA biosynthesis. However, the amounts of each of these xanthophylls exceed the amounts required for ABA biosynthesis. Thus, it is difficult to correlate the rise in ABA to a very small decrease in the amount of any carotenoid present (Milborrow 2001).

We may conclude that (1) the ascorbate-deficient *vtc-1* mutants of *Arabidopsis* show an altered accumulation of ABA in leaves and chloroplasts and (2) the ascorbate redox state correlates with, and may therefore play a central role in controlling, endogenous ABA levels in these mutants. Further studies are needed to better understand the interplay between ascorbate and phytohormones in plants, and more specifically to determine the mechanisms involved in the interaction between ABA accumulation and the AA redox state in the *vtc-1 Arabidopsis* mutants.

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