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Vitamin C deficiency enhances the high light sensitivity of a xanthophyll cycle mutant of *Arabidopsis thaliana*

P Müller-Moulé and KK Niyogi.

Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA, fax +1-510-642-4995, pmueller@uclink4.berkeley.edu

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Introduction

Plants have evolved several mechanisms to protect themselves from excess absorbed light energy. One of them is the harmless dissipation of light energy as heat, measured as nonphotochemical quenching of chlorophyll fluorescence (NPQ). NPQ requires the build-up of a proton gradient across the thylakoid membrane, the presence of the PsbS protein, and conversion of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) through the xanthophyll cycle. The *Arabidopsis thaliana* mutant *npq1* is deficient in the enzyme violaxanthin de-epoxidase (VDE) that is responsible for the conversion of V to A and Z and therefore lacks Z in high light and is deficient in NPQ (Niyogi et al., 1998). The *npq1* mutant is more sensitive to photoinhibition and lipid peroxidation than the wild type when transferred from low light (LL) to high light (HL), but it is able to survive in HL (Havaux and Niyogi, 1999).

Other important protective mechanisms against excess light are antioxidants, such as ascorbate, glutathione, and tocopherols, and antioxidant enzymes, which scavenge or quench dangerous reactive oxygen species (ROS). The *Arabidopsis* mutant *vtc2* is ascorbate (vitamin C) deficient and ozone sensitive. To investigate the role of antioxidant metabolism in the acclimation of *npq1* to HL, *vtc2* was crossed to the *npq1* mutant to produce the *vtc2npq1* double mutant.

Materials and methods

Arabidopsis thaliana plants (ecotype Columbia, Col-0) were grown for 6 weeks in low light (LL; 150 mol photons m^{-2} s^{-1}) with a 10 hr light (20°C) / 14 hr dark (18°C) photoperiod and then transferred to high light (HL; 1500 mol photons m^{-2} s^{-1}). The mutants used for the experiment were *npq1-2* and *vtc2-1* and the double mutant *vtc2-1 npq1-2*. Samples for measurement of lipid peroxidation, chlorophyll fluorescence, ascorbate, and -tocopherol were taken in LL, after 1hr HL, 4 hr HL, 1 d HL, 2 d HL, 3 d HL, 4 d HL, 5 d HL, and after 6 d LL (control). Only fully expanded mature leaves were sampled. Severely bleached leaves were taken only if they were not dried out yet. Data shown in graphs are the averages and standard errors of four to nine samples from three independent experiments.

For fluorescence measurements, an FMS2 instrument (Hansatech, King's Lynn, UK) was used. F_v/F_m values were measured after 15 min dark adaptation.

For pigment and tocopherol measurements, leaf disc samples were immediately frozen in liquid nitrogen. The frozen disc was ground to a fine powder and extracted with 150 l of 100% acetone by vortexing for 1 min. The extract was centrifuged for 20 s, and the supernatant was saved. Another 150 l of 100% acetone was added to the pellet and mixed thoroughly by pipetting. The extract was centrifuged again, and the supernatants were pooled. 15 l of the filtered supernatant (0.2 m nylon filter) was subjected to HPLC and separated on a Waters Spherisorb S5 ODS1 4.6 X 250 mm cartridge column at 30∝C. HPLC analysis was performed using a modification of the method of García-Plazaola and Becerril (1999). Pigments were eluted with a linear gradient from 100% solvent A (acetonitrile:methanol:0.1 M Tris-HCl pH 8.0; 84:2:14) to 100% solvent B (methanol:ethyl acetate; 68:32) for 15 min, followed by 3 min of solvent B. The solvent flow rate was 1.2 ml/min. Pigments were

detected by absorbance at 445 nm with a reference at 550 nm. Tocopherols were detected by fluorescence (excitation at 295 nm, emission at 325 nm). Concentrations were determined using standard curves of purified pigments (VKI, Hørsholm, Denmark) at known concentrations.

Total ascorbate was determined by a spectrophotometric method using the absorption at 265 nm by reduced ascorbate (Conklin et al., 1996), and data were expressed on a fresh weight (FW) basis.

Lipid peroxidation was determined as the level of thiobarbituric acid derivatives (Hodges et al., 1999) and data were also expressed on a FW basis.

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Results

Plants were transferred to HL after 6 weeks of growth in LL. At this age, wild type and the *npq1* mutant were the same size, whereas *vtc2* and *vtc2npq1*, which only have about 15- 30% of wild-type ascorbate levels, were smaller in size and also had fewer leaves. Upon transfer from LL to HL, the *vtc2npq1* double mutant showed obvious bleaching after only 1 d in HL. After 2 d all mature leaves of the double mutant were bleached (Fig. 1). Bleaching occurred more slowly in the *vtc2* mutant, and the *npq1* mutant and wild type did not exhibit any obvious bleaching during the entire experiment (5 d).

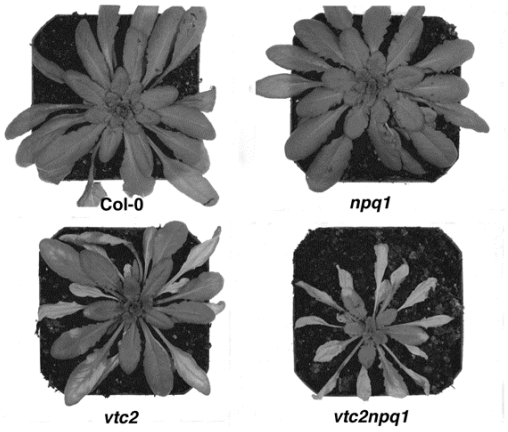

Fig. 1. Plants after 2.5 days in HL

The bleaching in *vtc2npq1* and *vtc2* was paralleled by an increase in lipid peroxidation (Fig.2). The *vtc2npq1* mutant showed lipid peroxidation after only 1 d, whereas *vtc2* showed lipid peroxidation after 2 d. The double mutant exhibited twice as much lipid peroxidation as *vtc2*. Wild type and *npq1* showed some lipid peroxidation after 5 d, although the *npq1* mutant seemed to show lipid peroxidation earlier than the wild type.

In the first 4 hr all genotypes showed a fast decrease in F_v/F_m, a chlorophyll fluorescence parameter that is commonly used to follow photoinhibition (Fig. 3). F_v/F_m continued to decrease drastically in the *vtc2npq1* mutant during the first day of HL, and also substantially in the *vtc2* mutant. In contrast, the decline in F_v/F_m was stabilized in the *npq1* mutant and the wild type, which did not show any further decrease in F_v/F_m after the first day of HL.

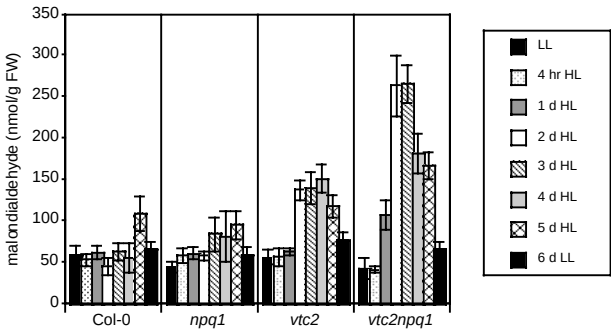

Fig. 2. Lipid peroxidation

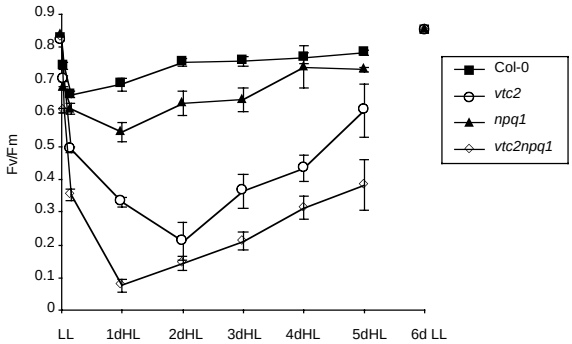

Fig. 3. F_v/F_m

The *vtc2* and *vtc2npq1* mutants had only about 15% of the ascorbate that wild type and *npq1* had in LL (Fig. 4A). The ascorbate content of wild type and *npq1* started to increase after 1 d in HL from about 2 mol (g FW)$^{-1}$ in LL to about 8 mol (g FW)$^{-1}$ after 5 d in HL. *vtc2* and *vtc2npq1* also showed a slight increase in ascorbate content from about 0.4 mol (g FW)$^{-1}$ to about 0.8 mol (g FW)$^{-1}$. All mutants and the wild type also exhibited an increase in -tocopherol content after only 4 hr in HL (Fig. 4B). -tocopherol levels increased in wild type and the *npq1* mutant to a 7-fold higher level than in LL, but only to a 3-fold higher level in *vtc2* and *vtc2npq1*. All genotypes also showed a progressive increase in xanthophyll pool size (V+A+Z) (data not shown).

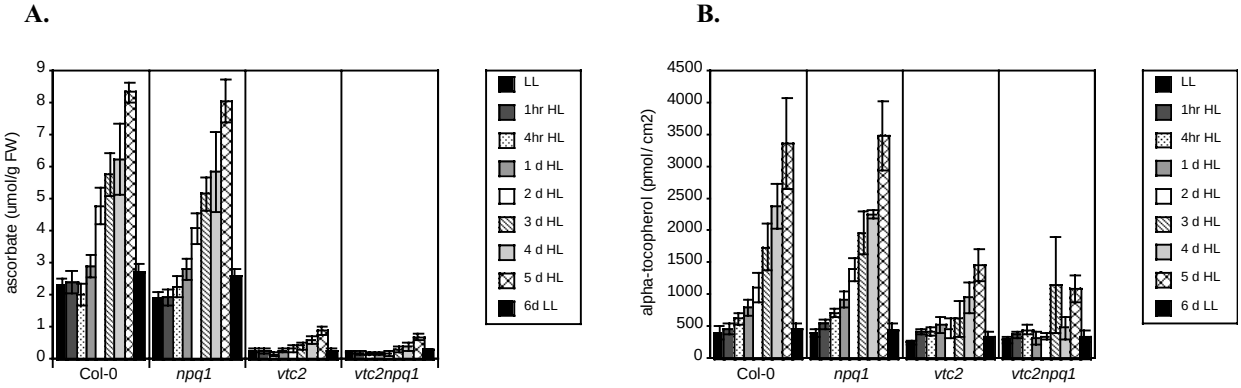

Fig. 4. A. Ascorbate **B.** Tocopherol levels

Discussion

Wild-type *Arabidopsis* plants grown in LL did not have any problems acclimating to HL conditions; they showed a transient decrease in F_v/F_m (Fig. 3) and a small increase in lipid peroxidation (Fig. 2), but otherwise no visible damage. The *npq1* mutant behaved very similarly to the wild type, but was more photoinhibited and showed lipid peroxidation slightly earlier than the wild type. This result is somewhat different from a previous result, where the *npq1* mutant showed more pronounced lipid peroxidation than the wild type when transferred to HL (Havaux and Niyogi, 1999). In contrast to the previous experiment, the photoperiod in this experiment was not changed after transfer to HL, and plants were treated with a 10-hr light period instead of a 15-hr light period. The difference in photon exposure (mol photons m^{-2} s^{-1} times the duration of light) is a likely reason why the *npq1* mutant acclimated better to HL in this experiment.

The lack of ascorbate has a more drastic effect on the ability of *Arabidopsis* plants to tolerate HL than the lack of Z, because the *vtc2* mutant showed much more bleaching (Fig. 1), lipid peroxidation (Fig. 2) and photoinhibition (Fig. 3) than the *npq1* mutant. Ascorbate has several different functions: it functions as an antioxidant itself and also supports other antioxidants (-tocopherol) as well as antioxidant enzymes (ascorbate peroxidase, APX). Ascorbate can function as electron acceptor for PS I in the so-called water-water cycle, thereby increasing electron transport rates. It is required for the production of Z in the xanthophyll cycle and plays an important role in modulating the cell cycle by stimulating cell division and by accelerating cell expansion and cell elongation. (Horemans et al., 2000). It is also required for cell wall synthesis.

The HL sensitivity of the ascorbate mutants seems not to be due to the lack of Z, because the *npq1* mutant did not show any bleaching in this experiment, whereas *vtc2* did. It seems unlikely that the cell cycle plays a direct role in acclimation, at least in multicellular plants, and the electron transport in *vtc2* does not seem to be affected (unpublished results). It therefore seems most likely that the HL sensitivity of the ascorbate mutants is due to the role of ascorbate as antioxidant. Plants generally respond to an increase in light intensity and the subsequent rise in ROS by increasing the ascorbate and the xanthophyll cycle pool size (Logan et al., 1998), and in some species the -tocopherol content (Leipner et al., 1997,

Munné-Bosch and Alegre, 2000), as was the case in this experiment (Fig. 4). It seems likely that the lack of ascorbate in the ascorbate-deficient mutants gave them less protection against the increased level of ROS, thereby increasing the damage observed in these plants. It will be interesting to assay APX activity and test the HL sensitivity of an APX-deficient mutant to see if the HL sensitivity is due to the lack of the general antioxidant protection by ascorbate or due to the more specific protection by APX. More damage was apparent in the *vtc2npq1* mutant, where the lack of Z, which might also function as an antioxidant or in membrane stabilization (Havaux and Niyogi, 1999), accelerated and intensified the bleaching as compared to the *vtc2* mutant.

Both *vtc2* and *vtc2npq1* were unable to increase their -tocopherol level as much as wild type (Fig. 4A). This could be either due to the photooxidation of tocopherols or to the inability of the ascorbate-deficient mutants to efficiently regenerate oxidized -tocopherol. An inability to regenerate -tocopherol would make the plants even more susceptible to HL or other stresses that produce ROS in the thylakoid membrane. This hypothesis could be tested using a mutant deficient in tocopherols.

In conclusion, this experiment shows the importance of ascorbate for the acclimation to HL in both wild type and the *npq1* mutant of *Arabidopsis.*

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