Ascorbate Deficiency Can Limit Violaxanthin De-Epoxidase Activity in Vivo[1]

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As a response to high light, plants have evolved non-photochemical quenching (NPQ), mechanisms that lead to the dissipation of excess absorbed light energy as heat, thereby minimizing the formation of dangerous oxygen radicals. One component of NPQ is pH dependent and involves the formation of zeaxanthin from violaxanthin. The enzyme responsible for the conversion of violaxanthin to zeaxanthin is violaxanthin de-epoxidase, which is located in the thylakoid lumen, is activated by low pH, and has been shown to use ascorbate (vitamin C) as its reductant in vitro. To investigate the effect of low ascorbate levels on NPQ in vivo, we measured the induction of NPQ in a vitamin C-deficient mutant of Arabidopsis, *vtc2-2*. During exposure to high light (1,500 μmol photons m^{-2} s^{-1}), *vtc2-2* plants initially grown in low light (150 μmol photons m^{-2} s^{-1}) showed lower NPQ than the wild type, but the same quantum efficiency of photosystem II. Crosses between *vtc2-2* and Arabidopsis ecotype Columbia established that the ascorbate deficiency cosegregated with the NPQ phenotype. The conversion of violaxanthin to zeaxanthin induced by high light was slower in *vtc2-2*, and this conversion showed saturation below the wild-type level. Both the NPQ and the pigment phenotype of the mutant could be rescued by feeding ascorbate to leaves, establishing a direct link between ascorbate, zeaxanthin, and NPQ. These experiments suggest that ascorbate availability can limit violaxanthin de-epoxidase activity in vivo, leading to a lower NPQ. The results also demonstrate the interconnectedness of NPQ and antioxidants, both important protection mechanisms in plants.

Excess absorbed light energy can lead to the formation of dangerous oxygen radicals, a problem that plants minimize by dissipating the excess energy as heat. This thermal dissipation process is called nonphotochemical quenching of chlorophyll fluorescence (NPQ). NPQ consists of three mechanisms, one of which is energy dependent, rapidly induced, and rapidly reversible. This energy-dependent quenching is called qE. For the establishment of qE in high light, a proton gradient is required. This gradient causes the protonation of some photosystem II (PS II) proteins and activates the xanthophyll cycle. Both of these elements, the protonation of proteins and the xanthophyll cycle, are required for a maximum qE (for review, see Müller et al., 2001).

The xanthophyll cycle (Fig. 1A) consists of the de-epoxidation of violaxanthin first to antheraxanthin and then to zeaxanthin by VDE. Zeaxanthin and antheraxanthin are the two xanthophyll pigments required for maximum qE (Gilmore and Yamamoto, 1993). In low light, antheraxanthin and zeaxanthin are slowly epoxidized to violaxanthin by the enzyme zeaxanthin epoxidase. The VDE enzyme was first partially purified from spinach (*Spinacia oleracea*; Hager and Perz, 1970) and lettuce (*Lactuca sativa*; Yamamoto and Higashi, 1978) and the gene has since been cloned from lettuce, Arabidopsis, and tobacco (*Nicotiana tabacum*; Bugos and Yamamoto, 1996; Bugos et al., 1998). VDE is localized to the thylakoid lumen and is activated by a pH below 6.5 with a maximum activity at about pH 5.0 in vitro (Hager, 1969; Hager and Holocher, 1994). Also, it has been shown in vitro that VDE requires ascorbate as reductant (Hager, 1969). An Arabidopsis mutant that is lacking VDE activity has been isolated by a screen for plants exhibiting lower NPQ (Niyogi et al., 1998). This VDE mutant, *npq1-2*, shows a 70% reduction in NPQ compared with the wild type. A reduction of the NPQ level is also observed in tobacco antisense VDE plants (Chang et al., 2000).

Ascorbate also has another important photoprotective function because of its antioxidant capacity. Ascorbate is one of the two major soluble antioxidants in chloroplasts (Foyer and Harbinson, 1994) and is thought to reduce oxidized alpha-tocopherol, a major lipid-soluble antioxidant (Beyer, 1994). Ascorbate is a cofactor for the antioxidant enzyme-catalyzed reduction of reactive oxygen species produced by photosystem I (PS I), in the so-called Mehler-peroxidase reaction or water-water cycle (Asada, 1999, Fig. 1B). The Mehler-peroxidase reaction consists of the Mehler reaction, which is the photoreduction of oxygen by PS I to a superoxide anion radical, followed by the dismutation of this superoxide anion radical by superoxide dismutase to

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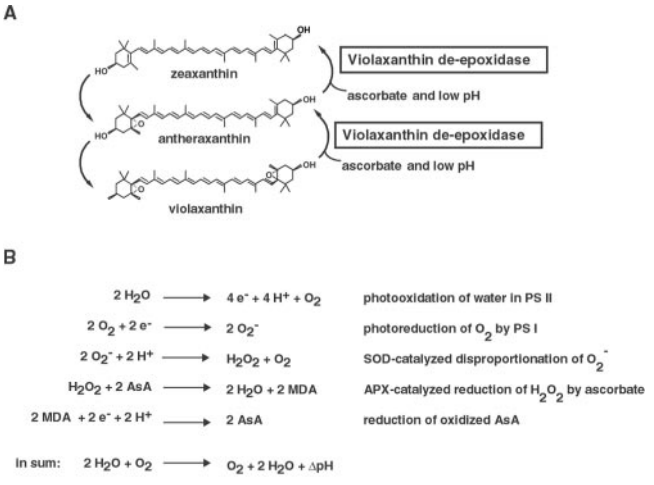

Figure 1. A, The xanthophyll cycle consists of the conversion of violaxanthin to antheraxanthin and zeaxanthin in high light. Ascorbate and a low pH are the requirements for the violaxanthin de-epoxidase (VDE), the enzyme catalyzing the conversion. In low light, zeaxanthin and antheraxanthin are epoxidized to violaxanthin. B, Mehler-peroxidase reaction or water-water cycle. The pseudocyclic electron transport from water to water generates a proton gradient. APX, Ascorbate peroxidase; AsA, ascorbate; MDA, monodehydroascorbate; SOD, superoxide dismutase. B, Based on Asada (1999).

hydrogen peroxide and oxygen. Hydrogen peroxide then is reduced by ascorbate peroxidase (APX) to water, followed by the regeneration of ascorbate by direct reduction of monodehydroascorbate by PS I or by the NADPH-dependent monodehydroascorbate reductase. The Mehler-peroxidase reaction results in electron flow from PS II to PS I with no net oxygen evolution. The proton gradient generated by this "pseudocyclic" electron flow has been shown to be important for zeaxanthin formation and qE in intact chloroplasts under conditions in which CO_2 fixation was limiting (Neubauer and Yamamoto, 1992). On the other hand, the Mehler-peroxidase reaction is also competing with VDE for ascorbate. It has been shown that the Mehler-peroxidase reaction can limit qE by decreasing ascorbate availability for the VDE reaction in isolated thylakoids (Neubauer and Yamamoto, 1994).

We used a vitamin C-deficient Arabidopsis mutant, called *vtc2-2*, hereafter called *vtc2*, to determine how ascorbate availability affects NPQ in vivo. This mutant was isolated previously by a screen for ozone sensitive mutants (Conklin et al., 2000; P.L. Conklin and R.L. Last, unpublished data). We observed a decrease in the extent of NPQ in this mutant, and similar experiments with *vtc2* and other *vtc* mutants have also shown a lowering of NPQ (Noctor et al., 2000; Smirnoff, 2000). Whereas these previous studies did not investigate the basis of the effect on NPQ, here we present detailed evidence that the lowered NPQ in *vtc2* is because of ascorbate limitation of VDE activity in vivo. We show that the ascorbate and NPQ phenotypes of *vtc2* cosegregate genetically, and that both the de-epoxidation and NPQ defects in the mutant can be rescued by ascorbate feeding.

Table I. *Total ascorbate content of mature leaves*

Data shown are the averages of three measurements with SDs.

Genotype	Ascorbate
	μmol (g fresh wt)$^{-1}$
VTC2/VTC2	7.26 ± 0.31
vtc2/vtc2	1.83 ± 0.65
vtc2/VTC2	7.18 ± 0.89

RESULTS

vtc2 Has Less Ascorbate Than Arabidopsis Ecotype Columbia (Col-0)

Under short-day conditions, the *vtc2* mutant had only 25% ± 9% of the wild-type ascorbate level (Table I). In previous measurements (mature leaves from 6-week-old, long day-grown *vtc2* plants), *vtc2* had about 10% of wild-type ascorbate levels (Conklin et al., 2000). The F_1 generation of a cross between the *vtc2* mutant and Col-0 showed the same level of ascorbate as Col-0. Therefore, the *vtc2-2* allele is recessive for ascorbate deficiency as has been shown previously (Conklin et al., 2000).

vtc2 Has Less NPQ Than Col-0

When illuminated with 1,500 μmol photons m^{-2} s^{-1}, Col-0 showed a rapid establishment of NPQ to a value of 2.2 within 10 min, whereas the mutant showed a slower rate of NPQ induction. Even after 10 min, *vtc2* had an NPQ value of less than 2.0 (Fig. 2). In addition, when measuring NPQ at different light intensities (from 25–2,150 μmol photons m^{-2} s^{-1}), *vtc2* consistently had lower NPQ than Col-0 at light intensities above that of the original growth conditions (Fig. 3). The difference in NPQ level did not

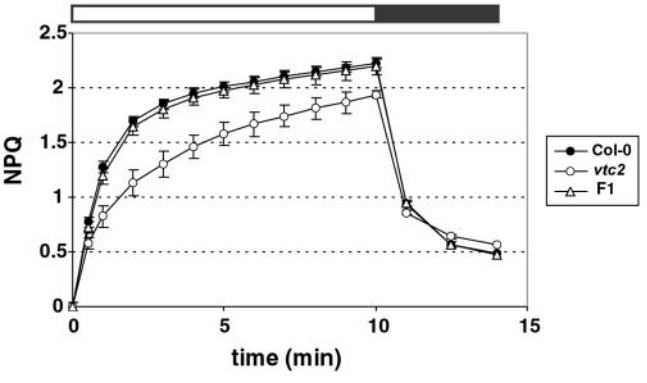

Figure 2. NPQ phenotype of homozygous and heterozygous *vtc2* mutants. NPQ was measured during 10 min of illumination with 1,500 μmol photons m^{-2} s^{-1} followed by 4 min of darkness. Data shown are averages of eight measurements with SEs.

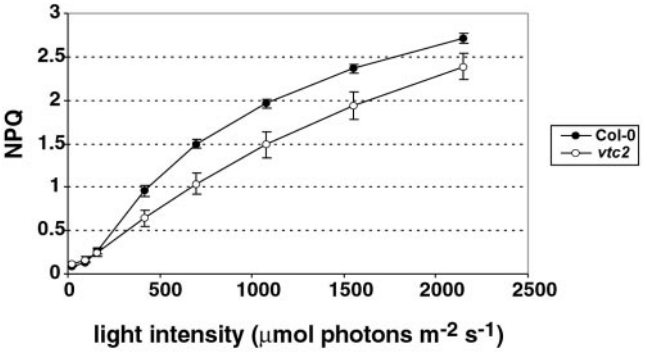

Figure 3. Light response curves for NPQ. Data shown are the averages of six measurements with SEs.

seem to change with increasing light intensities, but rather stayed constant.

vtc2 Is an *npq* Mutant

The *vtc2* mutation is the cause of the lowered NPQ. The *vtc2* mutant was crossed to Col-0, and the ascorbate and NPQ phenotypes were measured in the resulting F_1 and F_2 generations. The F_1 generation of this cross had wild-type NPQ levels (Fig. 2), showing that the *vtc2-2* allele is also recessive for the NPQ phenotype. When screening F_2 plants for ascorbate and NPQ deficiency, the ascorbate and NPQ phenotypes cosegregated (Table II). Although there was a continuum in NPQ levels reached after 4 min in high light in both the wild type and the mutant, no ascorbate-deficient plant showed NPQ above 1.6, and no plant with wild-type ascorbate level showed NPQ below 1.6. Furthermore, when averaging the NPQ values obtained from all plants with wild-type ascorbate levels and those from ascorbate-deficient plants, there was a significant difference between the two groups, with an average NPQ of 2.0 ± 0.2 for the wild-type ascorbate level plants versus an average NPQ of 1.3 ± 0.2 for the ascorbate-deficient plants. Out of 44 F_2 plants, nine were identified as homozygous *vtc2*/*vtc2* mutants, which is not significantly different from the expected number (11, or 25%) for a single recessive nuclear mutation.

Table II. *Cosegregation of ascorbate and NPQ phenotypes*

Two-week-old plants were screened with the nitroblue tetrazolium squish assay for ascorbate and then screened two weeks later for NPQ.

Ascorbate Level	NPQ > 1.6	NPQ < 1.6
Wild type	35	0
Ascorbate deficient	0	9

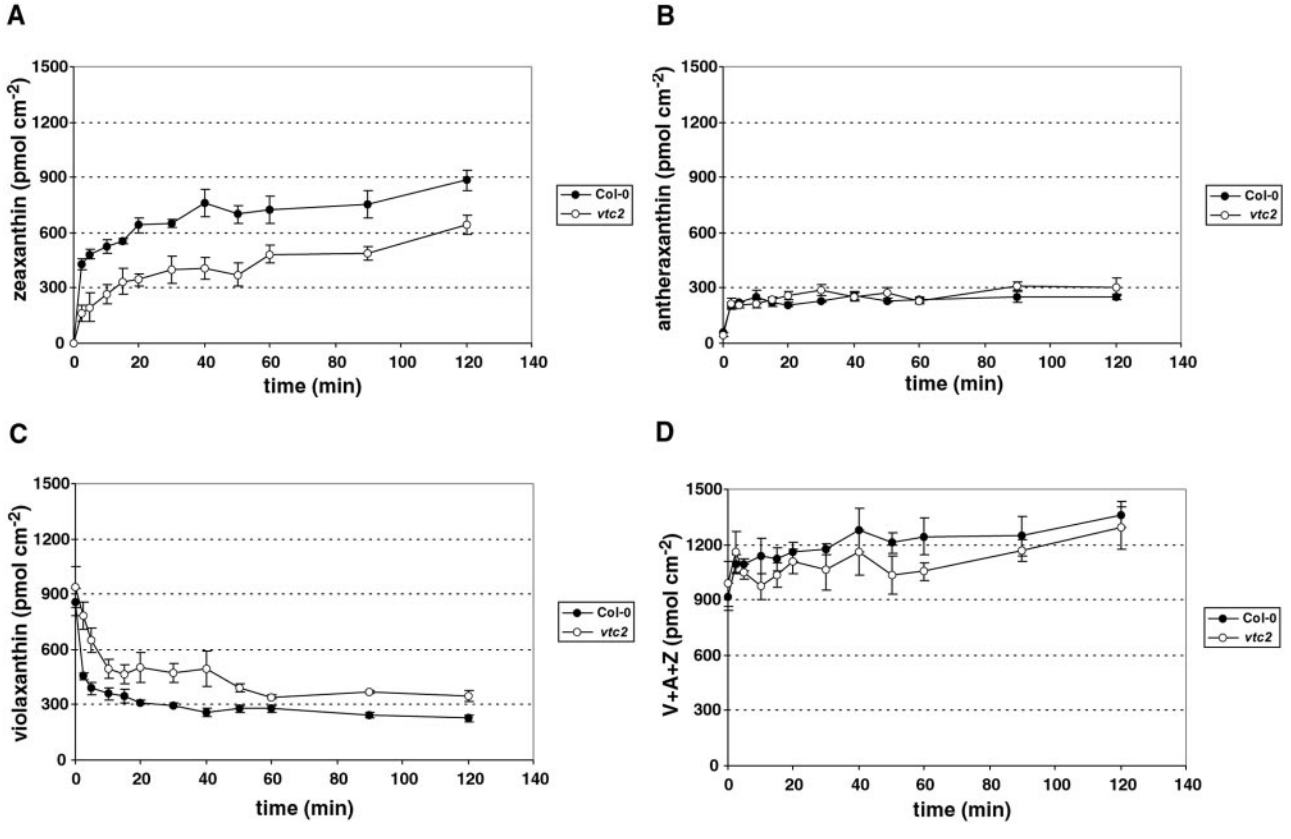

Figure 4. Changes in xanthophyll pigments during illumination. A, Zeaxanthin. B, Antheraxanthin. C, Violaxanthin. D, Xanthophyll cycle pool. Data shown are averages of five measurements with SEs.

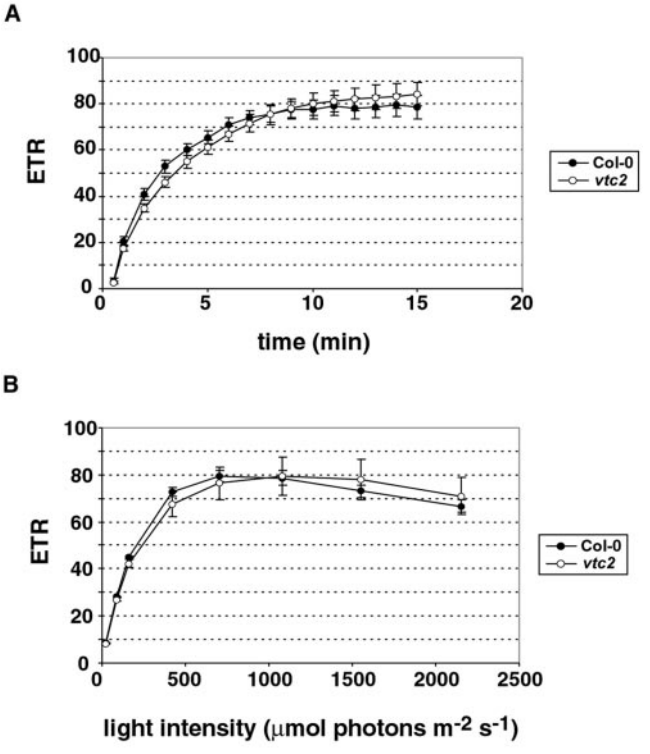

Figure 5. ETR in *vtc2*. A, ETR versus illumination time. B, Light response curve for ETR. Data shown are averages of 10 (A) or six (B) measurements with SEs.

The Conversion of Violaxanthin to Zeaxanthin Is Slower in *vtc2* Than in Col-0

The conversion of violaxanthin to zeaxanthin was slower in the mutant and did not reach the same saturation level as in the wild type (Fig. 4, A and C). In Col-0, 73% of violaxanthin was converted to zeaxanthin after 2 h, whereas in the mutant, only 63% of violaxanthin was de-epoxidized. In the first 2.5 min, 47% of the total violaxanthin was de-epoxidized in Col-0, whereas only 17% of the total violaxanthin in *vtc2* was converted to zeaxanthin, showing the much faster rate of conversion in the wild type compared with the mutant. The antheraxanthin levels in both Col-0 and *vtc2* increased in the first 2.5 min and then stabilized at the same steady-state value (Fig. 4B), showing that this intermediate pigment did not accumulate in the mutant. The zeaxanthin level in the mutant was substantially lower than in the wild type (Fig. 4A). The wild type had 884 ± 57 pmol zeaxanthin cm^{-2}, and *vtc2* had only 643 ± 55 pmol cm^{-2}. Thus, the mutant had about 30% less zeaxanthin than the wild type even after 2 h in high light. Comparing the first 2.5 min, the initial rate of zeaxanthin synthesis in the mutant was only 40% of the wild-type rate. The total xanthophyll pool consisting of violaxanthin, zeaxanthin, and antheraxanthin was similar in wild type and *vtc2* before and also during the treatment (Fig. 4D), as were the chlorophyll levels (data not shown).

Col-0 and *vtc2* Have the Same Electron Transport Rate (ETR)

Ascorbate is both a reductant for VDE, and it also plays an important role in the Mehler-peroxidase pathway that might be involved in generating a sufficient pH gradient for activation of VDE. Because of this dual function of ascorbate, we compared the ETR in wild type and the mutant. If ascorbate was limiting for the Mehler-peroxidase pathway, a decrease in ETR in the mutant compared with the wild type could be expected. Even though the NPQ levels were quite different in Col-0 and *vtc2*, the ETRs were the same except for the first 3 min in which *vtc2* had a slightly lower rate (Fig. 5A). The ETR increased in both Col-0 and *vtc2* at the beginning of the illumination (during the induction of photosynthesis) and reached saturation after 10 min of high light. Also, *vtc2* and Col-0 had the same ETR when comparing different light intensities (Fig. 5B). Both genotypes showed a maximum ETR at light intensities around 1,000 μmol photons m^{-2} s^{-1}, followed by a decrease in the rate at higher intensities.

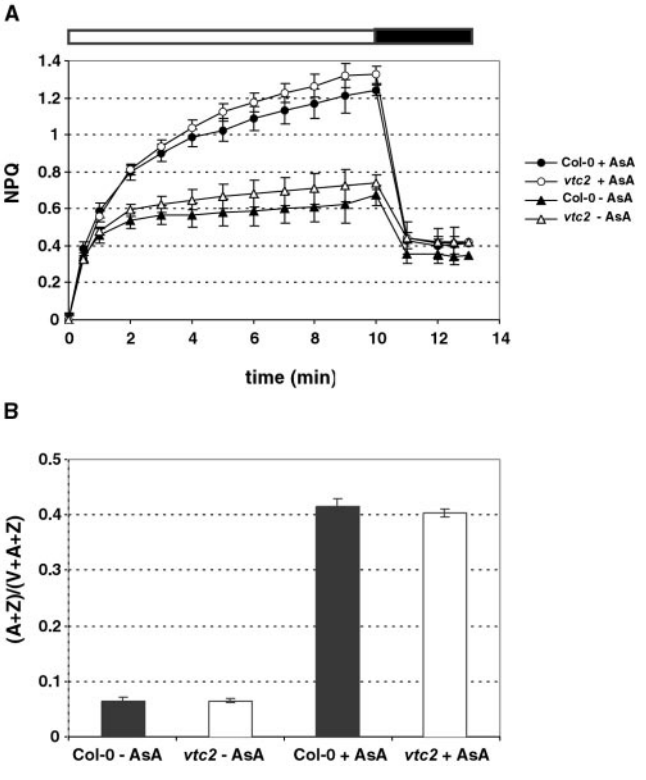

Figure 6. Thylakoid treatment with ascorbate. A, NPQ of isolated wild-type and *vtc2* thylakoids with added buffer (−AsA) or with added ascorbate (+AsA) during 10 min of illumination and 3 min of darkness. B, De-epoxidation state of the thylakoids after illumination. Data shown are averages of seven measurements with SEs.

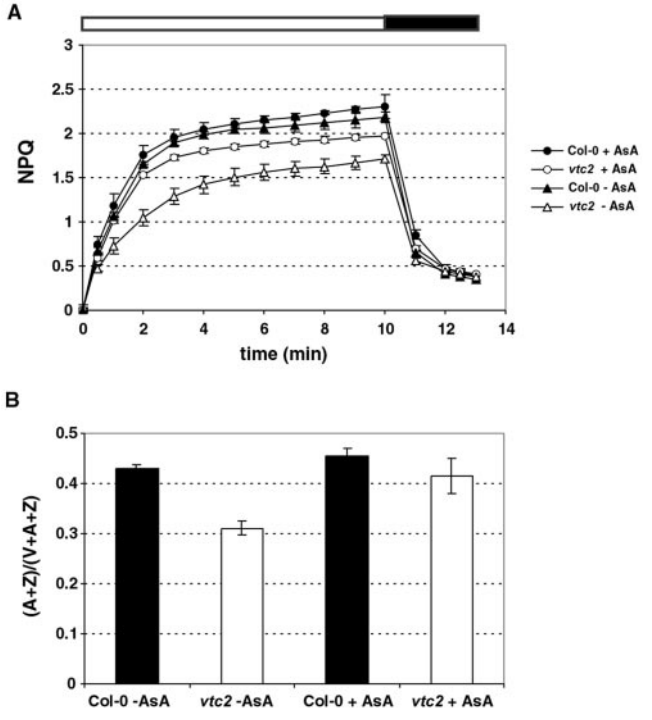

Figure 7. Ascorbate feeding to detached leaves. A, NPQ of wild-type and mutant leaves after feeding with ascorbate (+AsA) or water (−AsA) during 10 min of illumination and 3 min of darkness. B, De-epoxidation state after illumination. Data shown are averages of seven measurements with SEs.

Ascorbate Feeding Restores NPQ and Violaxanthin De-Epoxidation in Isolated Thylakoids and Detached Leaves

Thylakoids were isolated with buffer that did not contain any ascorbate. Ascorbate is typically added to buffers to stabilize thylakoids because the naturally occurring ascorbate in the thylakoids generally is lost during the isolation. This was apparent when measuring NPQ in thylakoids isolated without addition of ascorbate, in which NPQ only reached a value of approximately 0.7 in both Col-0 and *vtc2* (Fig. 6A). After adding ascorbate, both genotypes showed a 2-fold increase in NPQ. A corresponding increase was seen in the de-epoxidation state (Fig. 6B) of Col-0 and *vtc2* thylakoids. The thylakoids had equally low de-epoxidation states when no ascorbate was present, but each showed a very large increase in de-epoxidation state when ascorbate was added. Again, in either case there was never a difference in the levels of NPQ or de-epoxidation state between Col-0 or *vtc2*, arguing that there are no inherent differences between Col-0 and *vtc2* thylakoids except for the ascorbate level.

The lower NPQ phenotype could also be partially rescued in detached leaves by feeding with 10 mM ascorbate. Col-0 leaves reached a NPQ level of 2.3 when fed with ascorbate, which was slightly higher than for the control leaves (2.2, Fig. 7A). The leaves of the *vtc2* mutants fed with ascorbate, on the other hand, showed a large increase in the level of NPQ compared with those fed with water. Whereas control leaves had a maximum NPQ of 1.7, ascorbate-fed leaves had an NPQ level of 2.0. This rescue effect could also be seen in the de-epoxidation state of leaf discs taken immediately after the end of the NPQ measurement. Whereas the de-epoxidation state of Col-0 leaves only changed slightly, there was a significant increase in the de-epoxidation state in *vtc2* to nearly wild-type levels (Fig. 7B).

DISCUSSION

NPQ Deficiency in *vtc2* Is Caused by Ascorbate Limitation

Several lines of evidence suggest that the ascorbate content within the chloroplasts of the *vtc2* mutant is reduced in comparison with the wild type. First, NPQ and the xanthophyll cycle are thylakoid-localized processes, so the ascorbate content within the chloroplast must be affected. Second, it has been estimated that chloroplasts contain 20% to 40% of the ascorbate found in a mesophyll cell (Foyer et al., 1983). However, if we were to assume that the ascorbate content in chloroplasts was not affected in *vtc2*, then all other compartments would completely lack ascorbate. Because ascorbate is required among other things for cell cycle function (Smirnoff, 1996), this seems highly unlikely. Therefore, it can be concluded that the ascorbate content of chloroplasts must be affected in the *vtc2* mutant.

The *vtc2* mutant is not only affected in its ascorbate content (Table I), but also in its ability to perform maximum NPQ (Fig. 2). Using genetic cosegregation analysis, it was established that the inability to perform maximum NPQ is caused by the mutant's deficiency in ascorbate (Table II). In a similar but less extensive experiment, *vtc2* and three other *vtc* mutants all showed less NPQ than the wild type during a very short light treatment (Smirnoff, 2000). None of the mutants were affected in qP. In another experiment, these results were confirmed for *vtc1* (Noctor et al., 2000). The fact that all ascorbate-deficient mutants tested had less NPQ indicates that ascorbate is likely to be the cause of the NPQ phenotype.

In a more detailed recent study, the *vtc1* mutant showed only a slight decrease in NPQ (Veljovic-Jovanovic et al., 2001). The authors concluded that ascorbate was not limiting for the de-epoxidation of violaxanthin or that ascorbate was preferentially accumulated in the chloroplast. In light of our finding that ascorbate deficiency does limit VDE activity and reduces NPQ in *vtc2*, it seems likely that the two mutants are differentially affected by the lower ascorbate content. It is still unknown which gene, if biosynthetic or regulatory, is affected in *vtc2*, whereas the defect in *vtc1* is in GDP-Man pyrophosphorylase required for synthesis of ascorbate. It is

also possible that the compartmentalization of ascorbate is different in these two mutants.

Lowered De-Epoxidation State and NPQ Can Be Rescued by Feeding with Ascorbate

Another line of evidence for the reduced ascorbate content in the chloroplasts and for this limitation causing the lowered NPQ comes from the results of our measurements of the de-epoxidation state and NPQ in isolated thylakoids. When thylakoids were isolated without ascorbate in the isolation buffer, internal ascorbate was lost, leading to a low de-epoxidation state and NPQ in both the mutant and the wild type. This low-NPQ phenotype is consistent with measurements of NPQ in maize (*Zea mays*) chloroplasts that had been isolated without ascorbate (Ivanov and Edwards, 2000). After adding the same amount of ascorbate to the isolated thylakoids, both mutant and wild type had higher and similar de-epoxidation states and NPQ levels (Fig. 6). This suggests that the observed difference in whole plants is not caused by an inherent difference between the thylakoids other than the ascorbate content. By feeding ascorbate to detached leaves, we also showed that the difference in de-epoxidation state and NPQ between wild type and mutant could be decreased drastically (Fig. 7), again confirming that ascorbate deficiency is causing the difference in the NPQ phenotype.

Reduced Amount of Ascorbate Likely Has a Direct Effect on VDE

If we consider the ascorbate level in the chloroplast to be affected, then there are two ways in which the reduced ascorbate availability could affect the xanthophyll cycle activity (Fig. 4), the NPQ level (Figs. 2 and 3), and the rate of the VDE enzyme. First, there could be a direct effect on the xanthophyll cycle by reduced availability of reductant for VDE. Second, the Mehler-peroxidase pathway (water-water cycle) could be affected, which would result in a lowered proton gradient across the thylakoid membrane. This in turn might affect the activity of VDE, which has an optimum activity at pH 5 (Hager, 1969). It also could affect the degree of PS II protein protonation, which is also required for qE.

Our results indicate that reduced ascorbate content is affecting the xanthophyll cycle directly. Direct substrate limitation of ascorbate on VDE activity has been shown by stimulating ascorbate oxidation by APX in vitro (Neubauer and Yamamoto, 1994). After adding hydrogen peroxide that is metabolized by APX, VDE activity was transiently inhibited and qP was increased. In addition, the K_m (0.36 mM) of APX was found to be lower than the K_m (3.1 mM) of VDE (Neubauer and Yamamoto, 1994), making it likely that APX would be a better competitor for ascorbate than VDE. In a more recent study, it has been argued that the K_m of VDE, which is strongly pH dependent, could be expressed as a single K_m of 0.1 mM for the acid form of ascorbate (Bratt et al., 1995). Even though in this case VDE would have a higher affinity for ascorbate than APX, there is at least 30 times more APX than VDE in thylakoids. It has been estimated that there is one thylakoid-associated APX protein per 0.2 to 0.7 electron transport chains (Miyake and Asada, 1992), whereas there is only one VDE protein per 20 to 100 electron transport chains (Arvidsson et al., 1996). In addition, the different locations of APX and VDE favor APX because APX in the stroma could still take up the available ascorbate before it could reach VDE in the lumen. A possible direct substrate limitation on VDE in vivo even in the wild type could be seen in the feeding experiment. After feeding ascorbate, the NPQ increased from a value of 2.2 (−AsA) to 2.3 (+AsA). This was also accompanied by a small increase in the de-epoxidation ratio (Fig. 7). Similarly, an increase in zeaxanthin content has been reported after infiltrating detached maize leaves with ascorbate (Leipner et al., 2000). Thus, it is conceivable that ascorbate limitation of VDE and NPQ could occur in wild-type plants under certain oxidative stress conditions that affect the redox state of the ascorbate pool, perhaps via APX activity.

The Mehler-peroxidase pathway has been implied to promote electron transport, especially during the first few minutes of photosynthetic induction after a dark-light transition when CO_2 assimilation is limited (Schreiber and Neubauer, 1990; Polle, 1996). In *vtc2*, a small difference in ETR was apparent in the first 3 min of illumination with high light (Fig. 4A), implying there might be a slight limitation of the Mehler-peroxidase reaction that could also have an effect on NPQ during the first few minutes. In favor of this hypothesis, there was no significant difference between the ETRs of the mutant and the wild type after feeding ascorbate (data not shown). We are in the process of isolating APX-deficient mutants so that we can test the influence of the Mehler-peroxidase pathway on NPQ and VDE in more detail.

After the induction period, however, there was very little difference in steady-state ETR between the *vtc2* mutant and wild type regardless of whether it was measured at a constant light intensity for 15 min or at different light intensities (Fig. 4). This implies that the Mehler-peroxidase reaction is not significantly affected in *vtc2*, or that it does not play a major role in electron transport and hence in the establishment of the proton gradient in vivo. The experiments that have shown that this reaction mediates the formation of zeaxanthin and qE have been done in isolated chloroplasts in which carbon fixation had been inhibited (Neubauer and Yamamoto, 1992). In conclusion, it seems most likely that the effect of reduced ascorbate content is as a direct substrate limitation on VDE activity in vivo.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis Col-0 and the vitamin C-deficient mutant, *vtc2-2*, also Col-0 ecotype, were grown in soil for 6 weeks in controlled growth conditions of 10 h light, 22°C/14 h dark, 18°C, with a light intensity of 150 μmol photons m^{-2} s^{-1}. The *vtc2* mutant was isolated in a screen for ozone-sensitive plants (Conklin et al., 2000; P.L. Conklin and R.L. Last, unpublished data). For the cosegregation analysis, plants were grown for 2 weeks on minimal plant nutrient agar plates (Haughn and Somerville, 1986) and then transferred to soil.

Thylakoid Preparation

Thylakoids for the NPQ measurements were prepared as in Gilmore et al. (1998) except that ascorbate was omitted.

Fluorescence Measurements

Standard modulated chlorophyll fluorescence measurements were done with overnight dark-adapted plants using an FMS2 instrument (Hansatech, King's Lynn, UK). Dark-adapted plants (overnight) or thylakoids were subjected to a saturating light pulse and then illuminated (1,500 μmol photons m^{-2} s^{-1}) for 10 min followed by a 4-min dark recovery period. For light response curves, plants were illuminated for 5-min periods, each with increasing light intensities. NPQ was calculated as $(F_m' - F_m)/F_m'$ and ΦPS II as $(F_m' - F_s)/F_m'$, where F_m' is maximum PS II fluorescence in the light-adapted state, F_m is maximum PS II fluorescence in the dark-adapted state, and F_s is steady-state fluorescence. ETR was calculated as photosynthetically active radiation × 0.5 × 0.84 × ΦPS II. To measure NPQ in isolated thylakoids, the thylakoids were diluted to 50 μM chlorophyll in reaction buffer lacking ascorbate (Gilmore et al., 1998), and either ascorbate (30 mM final concentration) or more buffer was added.

Pigments

Plants were illuminated with high light (1,500 μmol photons m^{-2} s^{-1}) for several minutes to 2 h; leaf disc samples were taken and immediately frozen in liquid nitrogen. The frozen disc was ground to a fine powder and extracted with 150 μL of 100% (v/v) acetone by vortexing for 1 min. The extract was centrifuged for 20 s, and the supernatant was saved. Another 150 μL of 100% (v/v) acetone was added to the pellet and mixed thoroughly. The extract was centrifuged again, and the supernatants were pooled. Fifteen microliters of the filtered (0.2-μm nylon filter) supernatant was subjected to HPLC and separated on a Spherisorb S5 ODS1 4.6- × 250-mm cartridge column (Waters, Milford, MA) 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% (v/v) solvent A (acetonitrile: methanol:0.1 M Tris-HCl, pH 8.0; 84:2:14 [v/v]) to 100% (v/v) solvent B (methanol:ethyl acetate, 68:32 [v/v]) for 15 min, followed by 3 min of solvent B. The solvent flow rate was 1.2 mL min^{-1}. Pigments were detected by A_{445} with a reference at 550 nm by a diode array detector.

Ascorbate

Total ascorbate was determined by a spectrophotometric method using the UV absorption at 265 nm by reduced ascorbate (Conklin et al., 1996). To test ascorbate deficiency qualitatively, the nitroblue tetrazolium squish test was used as described previously (Conklin et al., 2000).

Ascorbate Feeding

Leaves were detached at the petiole with a razor blade under water to prevent embolism. The detached leaves were placed into 1 mL of 10 mM ascorbic acid or water (control) and incubated for 160 min in the dark.

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