Vitamin E Protects against Photoinhibition and Photooxidative Stress in Arabidopsis thaliana

Michel Havaux, a,1 Françoise Eymery, a Svetlana Porfirova, b Pascal Rey, a and Peter Dörmann b

a Commissariat à l’Energie Atomique/Cadarache, Direction des Sciences du Vivant, Département d’Ecophysiologie Végétale et de Microbiologie, Laboratoire d’Ecophysiologie de la Photosynthèse, F-13108 Saint-Paul-lez-Durance, France
b Max-Planck-Institute of Molecular Plant Physiology, D-14476 Golm, Germany

Vitamin E is considered a major antioxidant in biomembranes, but little evidence exists for this function in plants under photooxidative stress. Leaf discs of two vitamin E mutants, a tocopherol cyclase mutant (vte1) and a homogentisate phytol transferase mutant (vte2), were exposed to high light stress at low temperature, which resulted in bleaching and lipid photodestruction. However, this was not observed in whole plants exposed to long-term high light stress, unless the stress conditions were extreme (very low temperature and very high light), suggesting compensatory mechanisms for vitamin E deficiency under physiological conditions. We identified two such mechanisms: nonphotochemical energy dissipation (NPQ) in photosystem II (PSII) and synthesis of zeaxanthin. Inhibition of NPQ in the double mutant vte1 npq4 led to a marked photoinhibition of PSII, suggesting protection of PSII by tocopherols. vte1 plants accumulated more zeaxanthin in high light than the wild type, and inhibiting zeaxanthin synthesis in the vte1 npq1 double mutant resulted in PSII photoinhibition accompanied by extensive oxidation of lipids and pigments. The single mutants npq1, npq4, vte2, and vte1 showed little sensitivity to the stress treatments. We conclude that, in cooperation with the xanthophyll cycle, vitamin E fulfills at least two different functions in chloroplasts at the two major sites of singlet oxygen production: preserving PSII from photoinactivation and protecting membrane lipids from photooxidation.

INTRODUCTION

Vitamin E is the collective term for a group of amphiphilic lipids, the tocopherols and tocotrienols, which are synthesized exclusively by photosynthetic organisms (for reviews, see Fryer, 1992; Bramley et al., 2000; Wang and Quinn, 2000; Munné-Bosch and Alegre, 2002). Those compounds are composed of a chromanol head group and a prenyl side chain. In membrane lipid bilayers, the hydrophobic prenyl tail associates with lipids, and the polar chromanol head group is exposed to the membrane surface. Tocopherols differ from tocotrienols only in the degree of saturation of their hydrophobic tail, and the α-, β-, γ-, δ-forms of tocopherols and tocotrienols vary only in the number and position of methyl substituents attached to the chromanol ring. α-Tocopherol predominates in leaves of vascular plants, whereas γ-tocopherol is often the major form in seeds of many plant species (Grusak and DellaPenna, 1999).

In vitro experiments in lipid bilayers containing α-tocopherol have demonstrated that this compound has the ability to terminate chain reactions of polyunsaturated fatty acid free radicals generated by lipid oxidation. As a result of hydrogen transfer, alkoxyl and peroxy radicals are reduced to alcohols or hydroperoxides, and chromanoxyl radicals are formed. The chromanoxyl radical is less efficient in propagation of lipid peroxidation; therefore, α-tocopherol acts as an efficient chain-breaking antioxidant. The chromanoxyl radical may be reduced by ascorbate, thereby regenerating α-tocopherol molecule (Kamal-Eldin and Appelqvist, 1996). α-Tocopherol also quenches singlet oxygen (1O2), albeit with a lower rate constant than carotenoids in solution (Di Mascio et al., 1990).

The chain-breaking action during lipid oxidation is assumed to be the most important function of tocopherols in vivo, and vitamin E has long been recognized to be an essential antioxidant in animal cells. Tocopherols have been the subject of numerous studies that have emphasized their importance in the defense of animal or human tissues from a wide range of conditions and diseases that are mediated by oxidative degeneration (Bramley et al., 2000; Ricciarelli et al., 2001). Although tocopherols may be similarly important in plant tissues, their antioxidant activity in plant cells is much less well documented. The involvement of tocopherols in the photoprotection of plants is supported by the strong augmentation of the tocopherol content in plants exposed to adverse environmental conditions that induce oxidative stress (e.g., Delong and Steffen, 1997; Havaux et al., 2000; Munné-Bosch and Alegre, 2000; Fryer et al., 2002; Müller-Moulé et al., 2004). However, up to now, there is no clear demonstration that tocopherol does function as an efficient inhibitor of lipid peroxidation in plants exposed to high light stress. Although there have been several attempts to investigate the function of tocopherols in mutant or transgenic plants.

1 To whom correspondence should be addressed. E-mail michel.havaux@cea.fr; fax 33-4-4225-6265.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Michel Havaux (michel.havaux@cea.fr).

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.105.037036.
affected in tocopherol synthesis, most of these studies did not provide a clear answer regarding the antioxidative activity of tocopherols in photosynthesis. Tocopherol-deficient tobacco (*Nicotiana tabacum*) plants that have a reduced activity of geranylgeranyl reductase were obtained after transformation with an antisense *chip* construct (Tanaka et al., 1999). These plants were found to be more sensitive to high light intensity and to photooxidative stress than the wild type (Havaux et al., 2003). However, because the mutation caused also an accumulation of geranylgeranylated chlorophyll at the expense of (phytlylated) chlorophyll (Tanaka et al., 1999), it cannot be excluded that the photosensitivity of the *chip* antisense plants was due, at least partially, to the chlorophyll alteration, as recently suggested in the corresponding *chip* mutant of the cyanobacterium *Synechocystis PCC6803* (Shpilev et al., 2005). Disruption of the genes encoding homogentisate phytyltransferase or hydroxyphenylpyruvate dioxygenase in *Synechocystis* caused an absence of tocopherols without apparent changes in photosynthesis and growth in low light or in high light (Collakova and Dellapenna, 2001; Dähnhardt et al., 2002). Porfiriva et al. (2002) have isolated an *Arabidopsis thaliana* mutant, named *vte1*, that was deficient in tocopherol cyclase activity, resulting in a complete lack of tocopherols. Again, absence of tocopherol had no large impact on photosynthesis and had minor effects on phototolerance. This lack of deleterious effects of tocopherol deficiency was interpreted as the result of the stress treatments that were not adequate to produce detectable differences between the wild type and the transgenic/mutant lines or by the stimulation of other photoprotective mechanisms that compensated for tocopherol deficiency. A recent study of *vte Arabidopsis* mutants has suggested that biosynthetic precursors of tocopherol could functionally replace tocopherols, thus providing a possible explanation for the photoresistant phenotype of the *vte1* mutant (Sattler et al., 2004). This study emphasized the involvement of tocopherols in seed viability and germination by protecting seed lipids from oxidation (Sattler et al., 2004). Nevertheless, herbicide-induced blocking of tocopherol synthesis in the green alga *Chlamydomonas reinhardtii* exposed to high light was associated with a loss of photosystem II (PSII) activity (Trebst et al., 2002). Based on this observation, it was suggested that α-tocopherol could be specifically involved in the maintenance of the PSII function by scavenging $\cdot$O$_2$ produced at the PSII reaction center.

In this study, we have analyzed two tocopherol-deficient mutants of *Arabidopsis*, the tocopherol cyclase mutant *vte1* and a homogenisate phytyl transferase mutant (*vte2*). Both mutants were isolated previously in a screening of a chemically mutagenized *Arabidopsis* population for plants deficient in neutral lipid synthesis. Leaf discs and whole plants were exposed in the short term (several hours) or in the long term (several days) to photooxidative stress conditions induced by high light intensity combined with low temperature. Experiments with leaf discs demonstrated that tocopherols function as protectors of membrane lipids against peroxidative damage. In whole plants, this antioxidative function was masked by other protective mechanisms that compensate for the lack of vitamin E. Two such mechanisms have been identified here by crossing the *vte1* mutant with *npq* mutants: nonphotochemical energy dissipation (NPQ) and zeaxanthin. NPQ is a measure of photoprotective thermal dissipation of excess energy (Demmig-Adams and Adams, 2000; Müller et al., 2001; Horton and Ruban, 2005). This process is inhibited in the *npq4* mutant that lacks the PsbS protein required for NPQ (Li et al., 2000). Inactivation of the NPQ process in the *vte1 npq4* double mutant showed that tocopherols are involved in the protection of PSII against photoinactivation. The *npq1* mutant is deficient in violaxanthin deepoxidase, the enzyme that converts violaxanthin to zeaxanthin in strong light (Niyogi et al., 1998). The latter xanthophyll has a dual function: it is involved in the formation of NPQ (Niyogi et al., 1998; Demmig-Adams and Adams, 2000) and it has a direct antioxidant function (Havaux and Niyogi, 1999; Havaux et al., 2000; Baroli et al., 2003). Inactivation of zeaxanthin synthesis in the double mutant *vte1 npq1* confirmed that tocopherols also act as general protectors of thylakoid membranes against lipid peroxidation.

**RESULTS**

**Vitamin E Deficiency Increases Photooxidative Stress in Leaf Discs Exposed to Short-Term High Light Stress**

*Arabidopsis* leaf discs, floating on water at 10°C, were exposed to a strong white light with a photon flux density (PFD) of 1000 μmol photons m−2 s−1. This treatment caused a rapid inhibition of PSII photochemistry, with the variable-to-maximal chlorophyll fluorescence ratio (Fv/Fm) decreasing by 50% after ~4 to 5 h (Figure 1A). After 15 h in high light, PSII photochemistry was almost completely inhibited in both lines (Fv/Fm < 0.05). Photooxidative stress was estimated in the light-stressed leaf discs from the extent of lipid peroxidation measured by thermoluminescence. Light-emitting molecules are produced during lipid peroxidation, which can be used as internal markers of oxidative stress, and this light emission can be conveniently measured in plant leaves using the thermoluminescence technique, as a band peaking at ~135°C ( Ducruet, 2003; Havaux, 2003). This high-temperature thermoluminescence (TL) band is attributed to lipid cycloperoxides that are broken down during heating, leading to the formation of carbonyl species in the excited triplet state (Vavilin and Ducruet, 1998). Previous studies have correlated the amplitude of the 135°C TL band with other indexes of lipid peroxidation, such as the lipid hydroperoxide content (Havaux and Niyogi, 1999), the malondialdehyde (MDA) content (Vavilin et al., 1998; Müller-Moulé et al., 2003; Baroli et al., 2004), and the ethane production (Havaux and Niyogi, 1999). Figure 1B shows the TL trace of light-stressed wild-type and *vte1* leaf discs. In *vte1* leaf discs, photoinhibition was followed by a strong increase in the amplitude of the 135°C TL band (Figure 1C), indicating lipid peroxidation and photooxidative damage. By contrast, the luminescence signal emitted by wild-type leaves remained low throughout the stress treatment, indicating that lipid peroxidation was prevented when tocopherols were present. Photooxidation was also monitored by measuring the total fatty acid and chlorophyll contents of the leaf discs. In wild-type leaf discs, fatty acid content remained constant (Figure 2A) and a slight reduction of chlorophyll content (~15% after 23 h in high light) was observed (Figure 2B). By contrast, a strong
decrease in both fatty acid content and chlorophyll level occurred in \textit{vte1} leaves exposed to the same treatment: after 23 h in high light, fatty acids decreased by 25% and chlorophyll was reduced by 60%, confirming that lack of vitamin E in \textit{Arabidopsis} leaves increases oxidative damage to lipids and degradation of photosynthetic pigments. This conclusion is further supported by the effects of the \textsuperscript{1}O\textsubscript{2} generator eosin on \textit{vte1} leaves. Illumination of \textit{vte1} leaf discs floating on a 5\% eosin solution brought about a drastic increase in the 135\textdegree C TL signal amplitude, while the TL signal increase was small in wild-type leaves (Figure 3). Thus, we can conclude from the results presented in Figures 1 to 3 that tocopherols exert an efficient antioxidative activity in \textit{Arabidopsis} leaves, at least under harsh and artificial conditions.

\textbf{Vitamin E Deficiency Does Not Substantially Increase Photooxidative Stress in Whole Plants Exposed in the Long Term to High Light Stress}

Exposure of leaf discs to constant illumination for a long time (up to 30 h), as performed in the experiments shown in Figures 1 to 3, represents unphysiological conditions. The purpose of those in vitro experiments was to reveal the antioxidative action of tocopherol, even if the conditions used were extreme. Under more physiological conditions, the antioxidative function of tocopherol may be masked by alternative protective mechanisms. Whole plants were exposed for several days to high light stress at low temperature (8\textdegree C, 1100 \mu mol m\textsuperscript{2} s\textsuperscript{\textsuperscript{-1}}). As previously observed (Havaux and Kloppstech, 2001), this treatment caused transient photoinhibition of PSII photochemistry in wild-type \textit{Arabidopsis} leaves (Figure 4). Upon transfer to the stress conditions, Fv/Fm decreased, reaching \textasciitilde 0.5 after 2 d. Fv/Fm recovered progressively during the following days, reaching a value of \textasciitilde 0.6 after 7 d, indicating photoacclimation of the photosynthetic apparatus. In \textit{vte1} leaves, PSII photoinhibition was more pronounced, and PSII recovery proceeded at a slower rate relative to wild-type leaves. In contrast with the results obtained in leaf discs, PSII photoinhibition was rather mild and was not accompanied by photooxidation (Figure 4). Lipid peroxidation was not detected by TL measurements either in wild-type leaves or \textit{vte1} leaves. Determination of chlorophyll, fatty acid, and MDA (see below) confirmed that tocopherol deficiency in \textit{vte1} leaves did not increase oxidative stress during long-term

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Photoinhibition and Photooxidation of \textit{Arabidopsis} Leaf Discs Exposed to High Light Stress.}
\end{figure}

Leaf discs (the wild type and \textit{vte1} mutant) were exposed to high light (1000 \mu mol photons m\textsuperscript{2} s\textsuperscript{\textsuperscript{-1}}) at 10\textdegree C. Open circles, the wild type; closed circles, \textit{vte1}. Data are mean values of three to five separate experiments \pm SD, except when there is no error bar (two replicates), a.u., arbitrary units. (A) PSII photoinhibition was measured by the decrease in the Fv/Fm chlorophyll fluorescence ratio. Fv/Fm was \textasciitilde 0 for times >15 h. (B) and (C) Photooxidative stress was estimated from the extent of lipid peroxidation measured by TL. (B) presents TL traces from wild-type and \textit{vte1} leaf discs exposed for 24 h to the high light stress (1000 \mu mol photons m\textsuperscript{2} s\textsuperscript{\textsuperscript{-1}}/10\textdegree C), showing the lipid peroxidation–related band peaking at \textasciitilde 130\textdegree C. The sharp peak at \textasciitilde 65\textdegree C, which tends to disappear with oxidative stress, is specific to unstressed cruciferous plants. The origin of this band is unknown (Havaux, 2003).
exposure of plants to high light stress at low temperature. This is in accordance with previous experiments done at ambient growth temperatures (Porfirova et al., 2002; Kanwischer et al., 2005). For comparison purposes, we have also exposed plants to high light stress at high temperature (30°C). This treatment was found to be less photoinhibitory than high light stress at low temperature, and it did not induce photooxidative stress (Figure 5). Again, photoinhibition was higher in vte1 leaves than in wild-type leaves, but the difference was small. The absence of photooxidative damage in whole vte1 plants exposed to high light and low temperature, in contrast with vte1 leaf discs that suffered lipid peroxidation and pigment bleaching under rather comparable conditions, suggests that vte1 plants are able to compensate for the absence of tocopherol in the long term. We can exclude that the high photosensitivity of vte1 leaf discs compared with wild-type leaf discs was due to the fact that the samples were exposed to continuous light instead of a short-day light regime (8 h). Whole plants were exposed to continuous light of high PFD (1100 μmol m⁻² s⁻¹) at low temperature (6°C), and no lipid peroxidation could be detected, even after 48 h (Table 1). In this experiment, we have examined another tocopherol-deficient mutant, vte2, which is affected in the homogentisate phytyl transferase activity (see below for a detailed description of this mutant). vte2 behaved like vte1.

Compared with whole plants, leaf discs floating on water have a lower photosynthetic activity because CO₂ uptake is restricted; hence, they are exposed to a more acute light stress. Also, temperature of leaf discs floating on chilled water is controlled better than leaf temperature of whole plants, which was observed to be rather variable (from 9 to 14°C depending on the leaf). Due to the rapidity and severity of the light stress imposed on leaf discs, it is likely that compensatory mechanisms for tocopherol deficiency could not develop, thus allowing to reveal the photoprotective function of tocopherols. This was tested by exposing whole plants to very high light (PFD =1500 to 1600 μmol photons m⁻² s⁻¹) at very low temperature (2 to 3°C) (Table 1). This treatment induced a strong photoinhibition of PSII

Figure 2. Photodestruction of Fatty Acids and Chlorophylls in Arabidopsis Leaf Discs Exposed to High Light Stress.

Total fatty acid ([A]; in micrograms per leaf disk of 1.2 cm in diameter) and total chlorophyll ([B]; in μg cm⁻²) in leaf discs (the wild type and vte1 mutant) exposed to high light stress (1000 μmol photons m⁻² s⁻¹) at 10°C. Open circles, wild type; closed circles, vte1. Data are mean values of three separate experiments ± SD, except when there is no error bar (two replicates).

Figure 3. Singlet Oxygen Toxicity in Arabidopsis Leaf Discs.

Leaf discs (the wild type and vte1 mutant) floating on eosin were illuminated with white light of PFD 200 μmol m⁻² s⁻¹. Oxidative stress (lipid peroxidation) was monitored by TL. Open circles, wild type; closed circles, vte1. Each experimental point corresponds to a different sample. These data are representative of two independent experiments.
within 1 d in both wild-type and vte1 leaves. Lipid peroxidation could be detected by TL in vte1 plants exposed for 1 d to this very strong light stress, and some leaves started to bleach. By contrast, photooxidative stress was detected in wild-type plants after 2 d only. Therefore, the antioxidative function of tocopherols occurs also in whole plants, and not only in leaf discs, provided

Figure 4. Photoinhibition and Photooxidation of Arabidopsis Plants Exposed to High Light Stress at Low Temperature. PSII photoinhibition was measured by the decrease in the chlorophyll fluorescence ratio Fv/Fm, and photooxidation (lipid peroxidation) was measured by TL in wild-type and vte1 mutant plants (open and closed circles, respectively) exposed to high light stress at low temperature (8°C/1000 μmol m⁻² s⁻¹; photoperiod, 8 h). Data are mean values of a minimum of 10 measurements (Fv/Fm) or three measurements (TL). This experiment was done three times with qualitatively similar results. a, significantly different with P < 0.05 (t test).

Figure 5. Photoinhibition and Photooxidation of Arabidopsis Plants Exposed to High Light Stress at High Temperature. PSII photoinhibition was measured by the decrease in the chlorophyll fluorescence ratio Fv/Fm, and photooxidation (lipid peroxidation) was measured by TL in wild-type and vte1 mutant plants (open and closed circles, respectively) exposed to high light stress at high temperature (30°C/1300 μmol m⁻² s⁻¹; photoperiod, 8 h). Data are mean values of a minimum of 10 measurements (Fv/Fm) or three measurements (TL) ± SD, except when there is no error bar (two replicates). This experiment was done three times with qualitatively similar results. a, significantly different values with P < 0.001 (t test).
Ascorbate and glutathione in the reduced form did not vary appreciably with the growth conditions or the tocopherol content of the leaves. Carotenoids are, with tocopherols, the major lipid-soluble antioxidants of the thylakoid membranes. While \( \beta \)-carotene and neoxanthin decreased in high light, the lutein level and the pool size of the xanthophyll-cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin) increased in wild-type leaves (Table 3). Under control conditions (low light, 23°C), no differences were found for carotenoid content and composition, confirming previous experiments done with wild-type and \( \text{vte}1 \) plants grown at ambient temperatures (Kanwischer et al., 2005). In \( \text{vte}1 \), only the xanthophyll-cycle pool increased with light stress. As expected, violaxanthin was partially deepoxidized into antheraxanthin and zeaxanthin in high-light-grown leaves. However, zeaxanthin accumulation was higher in \( \text{vte}1 \) leaves relative to wild-type leaves (210 versus 112 ng cm\(^{-2} \)). This phenomenon was amplified at high temperature (1300 \( \mu \)mol m\(^{-2} \) s\(^{-1} \) at 30°C), with the zeaxanthin level reaching 680 ng cm\(^{-2} \). This accumulation was not observed for the other carotenoids. Figure 6A shows the time course of zeaxanthin accumulation in wild-type and \( \text{vte}1 \) plants during acclimation to high light at low (8°C) or that the stress conditions are harsh enough. Again, \( \text{vte}2 \) behaved like \( \text{vte}1 \).

In the following paragraphs, we have examined possible mechanisms involved in the acclimation of \( \text{vte}1 \) plants to high light (1000 to 1100 \( \mu \)mol photons m\(^{-2} \) s\(^{-1} \) at 6°C) and extreme light stress (1500 to 1600 \( \mu \)mol m\(^{-2} \) s\(^{-1} \) at 2 to 3°C, photoperiod 8 h). Data are mean values ± SD. Number of repetitions (n): 6 to 10 for Fv/Fm and 3 for the TL measurements, except when indicated otherwise.

### Table 1. Photoinhibition (as Measured by the Decrease in the Chlorophyll Fluorescence Parameter Fv/Fm) and Photooxidation (as Measured by the Amplitude of the Lipid Peroxidation–Related TL Band Peaking at \( \sim 135° \)) of Leaves Taken from Plants Exposed to Continuous High Light or to an Extreme Light Stress

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fv/Fm</th>
<th>TL (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>( \text{vte}1 )</td>
</tr>
<tr>
<td>Unstressed leaves</td>
<td>0.78 ± 0.02</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>Continuous light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.53 ± 0.12</td>
<td>0.42 ± 0.17</td>
</tr>
<tr>
<td>48 h</td>
<td>0.48 ± 0.14</td>
<td>0.34 ± 0.18</td>
</tr>
<tr>
<td>Very high light stress</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>0.37 ± 0.07</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>2 d</td>
<td>0.38 ± 0.07</td>
<td>0.32 ± 0.06</td>
</tr>
</tbody>
</table>

Continuous high light (1100 \( \mu \)mol photons m\(^{-2} \) s\(^{-1} \) at 6°C) and extreme light stress (1500 to 1600 \( \mu \)mol m\(^{-2} \) s\(^{-1} \) at 2 to 3°C, photoperiod 8 h). Data are mean values ± SD. Number of repetitions (n): 6 to 10 for Fv/Fm and 3 for the TL measurements, except when indicated otherwise.

\(^a\) Significantly different (\( \text{t} \) test, P < 0.05).

### Acclimation of \( \text{vte}1 \) Plants to High Light Stress Is Associated with a Selective Accumulation of the Carotenoid Zeaxanthin

We have examined different antioxidative mechanisms involved in the protection of thylakoid lipids and proteins. Table 2 shows the concentration of the soluble antioxidants, ascorbate, and glutathione in \( \text{Arabidopsis} \) plants grown under control conditions or in high light at low temperature (7 d). Under control conditions, \( \text{vte}1 \) leaves contained less ascorbate and slightly more glutathione than wild-type leaves. Growth in high light induced a strong accumulation of both compounds. While the glutathione increase was similar in wild-type and \( \text{vte}1 \) plants, less ascorbate accumulated in \( \text{vte}1 \) relative to the wild type. The percentage of ascorbate and glutathione in the reduced form did not vary appreciably with the growth conditions or the tocopherol content of the leaves.

### Table 2. Ascorbate and Glutathione Concentration (in \( \mu \)g cm\(^{-2} \)) in \( \text{Arabidopsis} \) Leaves (Wild Type and \( \text{vte}1 \)) before and after High Light Stress (7 d at 1100 \( \mu \)mol m\(^{-2} \) s\(^{-1} \)) at 8°C

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Before Stress</th>
<th>After Stress</th>
<th>Before Stress</th>
<th>After Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>( \text{vte}1 )</td>
<td>Wild Type</td>
<td>( \text{vte}1 )</td>
</tr>
<tr>
<td>Ascorbate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>16.8 ± 1.2</td>
<td>12.4 ± 0.1</td>
<td>25.7 ± 6.8</td>
<td>28.9 ± 3.5</td>
</tr>
<tr>
<td>Total</td>
<td>21.0 ± 2.4</td>
<td>14.8 ± 0.6</td>
<td>41.2 ± 3.0</td>
<td>33.5 ± 0.6</td>
</tr>
<tr>
<td>Reduction (%)</td>
<td>80</td>
<td>83</td>
<td>87</td>
<td>86</td>
</tr>
<tr>
<td>Glutathione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>1.2 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>4.4 ± 0.3</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>Total</td>
<td>1.7 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>6.2 ± 0.3</td>
<td>7.1 ± 0.6</td>
</tr>
<tr>
<td>Reduction (%)</td>
<td>72</td>
<td>76</td>
<td>71</td>
<td>64</td>
</tr>
</tbody>
</table>

Data are mean values of three separate experiments ± SD. Leaf specific weight: wild-type control = 20.89 mg fresh weight cm\(^{-2} \); wild-type stress = 20.12 mg cm\(^{-2} \); \( \text{vte}1 \) control = 20.55 mg cm\(^{-2} \); \( \text{vte}1 \) stress = 20.83 mg cm\(^{-2} \).
Table 3. Carotenoid Content (in ng cm⁻²) in Wild-Type and vte1 Arabidopsis Leaves before and after High Light Stress at Low Temperature (7 d at 1100 μmol m⁻² s⁻¹ and 8°C) or at High Temperature (4 d at 1300 μmol m⁻² s⁻¹ and 30°C)

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Control</th>
<th>High Light at Low Temperature</th>
<th>High Light at High Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>vte1</td>
<td>Wild Type</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>731 ± 29</td>
<td>752 ± 71</td>
<td>660 ± 37</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>461 ± 15</td>
<td>539 ± 85</td>
<td>473 ± 43</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>271 ± 55</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>112 ± 27</td>
</tr>
<tr>
<td>Lutein</td>
<td>2065 ± 69</td>
<td>2220 ± 207</td>
<td>2424 ± 266</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>996 ± 57</td>
<td>1136 ± 112</td>
<td>756 ± 106</td>
</tr>
<tr>
<td>(A+Z)/(A+Z+V)</td>
<td>0 ± 0</td>
<td>0.45 ± 0.01</td>
<td>0.51 ± 0.02</td>
</tr>
</tbody>
</table>

Data are mean values of three to five separate experiments ± s.e. n.d., not detected.

a,b Different from wild-type values under similar conditions using the Student’s t test with P < 0.12 and P < 0.01, respectively.

high temperature (30°C). Compared with wild-type leaves, vte1 leaves accumulated high amounts of zeaxanthin in both conditions, and this accumulation was particularly marked when light stress was combined with high temperature. Interestingly, the latter condition was also found to induce the strongest accumulation of tocopherols in wild-type leaves (Figure 6B), much above the level reached in high light at low temperature. Thus, a high level of tocopherols in wild-type leaves corresponded to a high level of zeaxanthin in vte1 (Figure 6A). All the zeaxanthin molecules that accumulated in high-light-treated wild-type plants were converted into violaxanthin during the night (data not shown). By contrast, in vte1 plants, a substantial fraction (∼20%) of the xanthophyll-cycle pigment pool remained permanently in the form of zeaxanthin or antheraxanthin, even after 16 h in the dark.

Zeaxanthin is involved in the photoinduced increase in thermal energy dissipation—a phenomenon that can be measured as a nonphotochemical quenching of chlorophyll fluorescence (NPQ) (Demmig-Adams and Adams, 2000; Müller et al., 2001; Horton and Ruban, 2005). NPQ is a photoprotective mechanism that lowers energy delivery to the PSII centers. As shown in Figure 7, NPQ was noticeably increased in high-light-treated vte1 leaves relative to wild-type leaves. It should be noticed that NPQ increased in vte1 leaves exposed to high light at high temperature (30°C) was smaller than NPQ in leaves exposed to high light at low temperature (6°C), although zeaxanthin content was noticeably higher in the former plants. Clearly, zeaxanthin concentration is not the only factor that determines the extent of NPQ.

A number of chloroplastic antioxidant proteins were analyzed by protein gel blotting. Peroxiredoxins (PRXs) are peroxidases with a catalytic center containing a cysteiny1 residue that reduces diverse peroxides, including lipid hydroperoxides (Dietz, 2003). Therefore, one may speculate that increased oxidation of membrane lipids in the absence of tocopherols will induce the synthesis of these enzymes involved in the detoxification of lipid hydroperoxides. We analyzed the abundance of the three chloroplastic PRXs (Figure 8). The level of BAS1 2-cys PRX and PRXII-E was not affected by the growth conditions or by the vte1 mutation. By contrast, an accumulation of PRXQ was observed during growth in high light at low temperature. Although vte1 leaves seemed to contain significantly more PRXQ than wild-type leaves in low light conditions, the level reached in high light was not very different. The catalytic center of PRXs is regenerated by electron donors, such as thioredoxins. In chloroplasts, the thioredoxin CDSP32 has been shown to interact with PRXQ and BAS1 2-cys PRX (Broin and Rey, 2003; Rey et al., 2005). Wild-type and vte1 leaves contained similar amounts of CDSP32, both under control and high light conditions. Methionine sulfoxide reductase (Msr) is also a target of CDSP32 (Rey et al., 2005). By catalyzing reduction of Met sulfoxides back to Met, Msrs can repair proteins damaged by oxidative stress (Stadtman et al., 2002; Vieira Dos Santos et al., 2005). We analyzed the abundance of three chloroplastic Msrs, and we did not find significant difference between vte1 and the wild type, although their abundance changed with the light conditions. Msr-A increased during light stress with the oxidized form increasing more than the reduced form. Both Msr-B1 and Msr-B2 increased during high light stress, as previously observed (Vieira Dos Santos et al., 2005).

In conclusion, neither the soluble antioxidants nor the antioxidant proteins investigated here were selectively induced in vte1 grown in high light relative to the wild type. Thus, the most striking difference found between the wild type and vte1 is a marked accumulation of the xanthophyll zeaxanthin in the mutant exposed to high light.

Isolation of a vte2 Mutant of Arabidopsis

vte1 disrupts tocopherol cyclase activity, thus inhibiting tocopherol synthesis and leading to the accumulation of the pathway intermediate dimethyl phytyl benzoquinone (DMPBQ) (Porfirova et al., 2002; Sattler et al., 2004). It has been suggested that the redox-active DMPBQ can functionally compensate for the absence of tocopherols in seeds of vte1. This could explain the lack of photosensitive phenotype when vte1 plants are exposed to high light. However, vte1 leaf discs were very sensitive to high light compared with wild-type leaf discs. Therefore, the antioxidant activity of DMPBQ might compensate for tocopherol deficiency in vte1 leaf discs only to a minor extent. We have isolated a vte2 mutant that is deficient in homogentisate phytyl trans-ferase activity and does not accumulate detectable amounts of
DMPBQ as determined by gas chromatography–mass spectrometry (data not shown).

Although overall morphology of the vte2 mutant was not different than the wild type, growth was slightly inhibited. No tocopherol was measured in vte2 leaves in low light, but vte2 leaves contained a small amount of tocopherols, ~6% of the tocopherol amount accumulated in wild-type leaves when grown in high light at low temperature, indicating that the mutated enzyme had a small residual activity (Table 4). Despite the strong reduction of tocopherols and the absence of DMPBQ, the vte2 mutant plants were found as tolerant to photooxidative stress as...
vte1 plants. Lipid peroxidation, PSII photochemical efficiency, and chlorophyll content were similar in high-light-grown vte1 and vte2 plants (Table 4). As shown above (Table 1), vte2 and vte1 behaved similarly under harsh environmental conditions (continuous light or very high light at very low temperature). Thus, absence of DMPBQ in the presence of very low amounts of tocopherols did not lead to photooxidative stress. We also exposed vte2 leaf discs (plants raised under control conditions and, hence, completely deficient in tocopherols; Table 4) to the high light stress used in the experiments shown in Figure 1. This treatment induced pronounced lipid peroxidation and oxidative stress that were comparable to vte1. For instance, 24-h exposure of vte2 leaf discs to high light at low temperature induced a lipid peroxidation-related TL band at 135°C with an amplitude of 20,892 ± 4,250 (a.u.), which is very similar to the signal amplitude measured in vte1 (19,700 ± 4,175; see also Figure 1C). Thus, under the light stress conditions used in this study, protection of the photosynthetic system by DMPBQ appeared to be limited. At this point, we cannot exclude the possibility that small amounts of tocopherol (below detection limit) might attenuate the effects of oxidative stress in the vte2 mutant. However, the strong photooxidation phenotype observed for vte1 leaf discs demonstrates that tocopherols are required to protect the photosynthetic apparatus from oxidative stress and that DMPBQ cannot compensate for this deficiency.

**The Double Mutant vte1 npq1, Deficient in Both Zeaxanthin and Vitamin E, Is Highly Sensitive to Photooxidative Stress**

Accumulation of zeaxanthin when tocopherols are absent (Havaux et al., 2003; this study) and vice versa (Havaux et al., 2000) suggests that both compounds have overlapping functions. Therefore, we have examined the consequences of the simultaneous suppression of tocopherol and zeaxanthin on the chloroplast photostability. To this end, the vte1 mutant was crossed with the zeaxanthin-deficient npq1 mutant to generate a double mutant, npq1 vte1, that lacks both zeaxanthin and tocopherols. This double mutant was found to be very sensitive to high light stress. When exposed to high light at low temperature, most of the mature leaves of npq1 vte1 bleached in contrast with wild-type, npq1, or vte1 leaves, which did not exhibit visual symptoms of oxidative stress (Figure 9). Chlorophyll fluorescence and TL measurements confirmed the photosensitivity of the double mutant: PSII was strongly photoinhibited (Figure 10A) and lipid peroxidation was enhanced (Figure 10B) in the double mutant. We have also analyzed the level of molecules that are sensitive targets of photooxidative stress (fatty acids and chlorophylls) or that are produced during oxidative stress (MDA). As shown in Figure 11, MDA accumulated in vte1 npq1 leaves after 7 d in high light, confirming the occurrence of lipid peroxidation. Also, fatty acids and chlorophylls were decreased in npq1 vte1 leaves. These changes were not observed or were much more subtle in the wild type and in the single mutants vte1 and npq1.

As a result of these experiments, we have analyzed the consequences of the simultaneous suppression of tocopherol and zeaxanthin on the chloroplast photostability. To this end, the vte1 mutant was crossed with the zeaxanthin-deficient npq1 mutant to generate a double mutant, npq1 vte1, that lacks both zeaxanthin and tocopherols. This double mutant was found to be very sensitive to high light stress. When exposed to high light at low temperature, most of the mature leaves of npq1 vte1 bleached in contrast with wild-type, npq1, or vte1 leaves, which did not exhibit visual symptoms of oxidative stress (Figure 9). Chlorophyll fluorescence and TL measurements confirmed the photosensitivity of the double mutant: PSII was strongly photoinhibited (Figure 10A) and lipid peroxidation was enhanced (Figure 10B) in the double mutant. We have also analyzed the level of molecules that are sensitive targets of photooxidative stress (fatty acids and chlorophylls) or that are produced during oxidative stress (MDA). As shown in Figure 11, MDA accumulated in vte1 npq1 leaves after 7 d in high light, confirming the occurrence of lipid peroxidation. Also, fatty acids and chlorophylls were decreased in npq1 vte1 leaves. These changes were not observed or were much more subtle in the wild type and in the single mutants vte1 and npq1.

**Table 4.** Responses of the vte2 Arabidopsis Mutant to High Light Stress at Low Temperature (1100 μmol m⁻² s⁻¹ at 7°C for 5 d) Compared with the Wild Type and vte1

<table>
<thead>
<tr>
<th></th>
<th>Fv/Fm</th>
<th>TL (a.u.)</th>
<th>Tocopherols (ng cm⁻²)</th>
<th>Total Chlorophyll (μg cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.79 ± 0.01</td>
<td>3860 ± 460</td>
<td>135 ± 14</td>
<td>27.90 ± 3.04</td>
</tr>
<tr>
<td>Stressed vte1</td>
<td>0.65 ± 0.11</td>
<td>4250 ± 1250</td>
<td>515 ± 131</td>
<td>21.51 ± 1.88</td>
</tr>
<tr>
<td><strong>vte2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.80 ± 0.01</td>
<td>3600 ± 1230</td>
<td>n.d.</td>
<td>24.33 ± 1.49</td>
</tr>
<tr>
<td>Stressed</td>
<td>0.59 ± 0.15</td>
<td>3250 ± 750</td>
<td>n.d.</td>
<td>21.17 ± 1.21</td>
</tr>
<tr>
<td><strong>vte1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.79 ± 0.01</td>
<td>4800</td>
<td>n.d.</td>
<td>21.25 ± 2.02</td>
</tr>
<tr>
<td>Stressed</td>
<td>0.63 ± 0.18</td>
<td>5125 ± 2132</td>
<td>26 ± 2</td>
<td>21.55 ± 0.95</td>
</tr>
</tbody>
</table>

Data are mean values of three to seven measurements ± sd. n.d., not detected.
The Photosensitivity of the vte1 npq1 Double Mutant Is Only Partially Due to the Inhibition of NPQ

As a consequence of the zeaxanthin deficiency, the npq1 mutant is also affected in NPQ (Niyogi et al., 1998), a photoprotective mechanism that reduces light absorption in PSII by increasing thermal energy dissipation in the light-harvesting system during high light stress (Müller et al., 2001; Holt et al., 2005; Horton and Ruban, 2005). Therefore, the double mutant vte1 npq1 has a low NPQ activity relative to the wild type and vte1, and we cannot exclude that this difference was responsible, at least partially, for the increased photosensitivity of the double mutant. Therefore, we crossed vte1 with the npq4 mutant. The latter mutant does not contain the PSII protein PsbS that is required for NPQ (Li et al., 2000). vte1 npq4 and vte1 npq1 are both affected in NPQ but differ from each other by the presence or absence of zeaxanthin, respectively. The wild type, vte1 npq1, and vte1 npq4 were exposed to high light at low temperature. PSII photooxidation was strongly enhanced in vte1 npq4 relative to the wild type (Figure 13A). During the first 2 d, vte1 npq4 was as sensitive as vte1 npq1 to photooxidation, with Fv/Fm decreasing to ~0.28. Subsequently, PSII slightly recovered in vte1 npq4 but not in vte1 npq1. As previously shown, npq4 behaved like npq1, with Fv/Fm decreasing moderately to ~0.45 after 2 d in high light and recovering subsequently like the wild type when exposed to high light stress at low temperature (Havaux and Kloppstech, 2001).

DISCUSSION

Vitamin E Functions as a General Protector of Thylakoid Membranes against Photooxidation

This study has clearly established that vitamin E exerts an antioxidant activity in plant cells. Indeed, tocopherol deficiency in vte1 Arabidopsis leaves was associated with a photosensitive phenotype compared with wild-type leaves. When vte1 leaf discs were suddenly exposed to an intense white light (~1000 μmol photons m⁻² s⁻¹) under conditions that lowered the photosynthetic activity (namely, low leaf temperature and reduced CO₂ uptake at the lower leaf surface), chlorophylls and lipids were rapidly photooxidized, but no such alterations were observed in tocopherol-containing wild-type leaf discs. Also, tocopherol deficiency enhanced lipid peroxidation when O₂ was directly generated in the leaves by the photosensitizing dye eosin. Our results are consistent with a previous study of chlP transgenic tobacco plants affected in geranylgeranyl reductase, showing a correlation between tocopherol content and lipid peroxidation in high light (Havaux et al., 2003). However, because the chlP mutation also caused an accumulation of geranylgeranylated chlorophyll at the expense of (phytylated) chlorophyll (Tanaka et al., 1999), unambiguous and definite conclusions on the photoprotective function of tocopherols could not be drawn in the previous study.

The increased sensitivity of vte1 leaf discs to photooxidation did not result from a lower stability of the photosystems since the rate of PSII inhibition during illumination of leaf discs was similar in vte1 and the wild type. Also, experiments with the photosensitizing dye eosin showed that photooxidation in vte1 resulted from an increase in the intrinsic sensitivity of the thylakoid membranes to active forms of oxygen. During high light stress, O₂ is produced from chlorophyll excitation in the light-harvesting complexes or the PSII reaction centers, whereas exogenously added eosin is expected to generate O₂ throughout the thylakoid membrane (Knox and Dodge, 1985). Consequently, the protective effect of vitamin E is not restricted to O₂ scavenging in the vicinity of the PSII centers, as recently suggested (Trebst et al., 2002), but it must also involve a general protection of
thylakoid membranes by tocopherol molecules localized in the lipid phase. vte1 npq1 plants, but not vte1 npq4 plants, suffered from extensive oxidative damage in high light, although PSII was strongly photoinhibited in both double mutants. Thus, photooxidative stress in vte1 npq1 did not result directly from the loss of PSII activity, confirming that tocopherols exert an antioxidant activity distinct from their role in the protection of PSII against photodestruction.

Germination and early seedling development were impaired in the tocopherol-deficient vte2 affected in homogentisate phytol transferase, but not in vte1 (Sattler et al., 2004). The main biochemical difference between vte1 and vte2 is that the former one accumulates the biosynthetic precursor of tocopherol, DMPBQ. It was hypothesized that DMPBQ could functionally replace tocopherols, so that it would be difficult to reveal the functions of tocopherols in mutants that accumulate tocopherol precursors, such as vte1. It is clear from our results on leaf discs that, in mature leaves exposed to photooxidative stress, DMPBQ does not, or only partially, substitute for tocopherols in photoprotection. Moreover, the vte2 mutant was not found to be more sensitive to growth in high light at low temperature than vte1, despite the fact that it did not accumulate biosynthetic

**Figure 10.** Photoinhibition and Photooxidation of Wild-Type, vte1, npq1, or npq1 vte1 Leaves during Growth of Whole Plants in High Light at Low Temperature.

PSII photoinhibition as measured by the Fv/Fm (A) and photooxidative stress measured by thermoluminescence (B). Open circles, wild type; closed circles, vte1 npq1; triangles, vte1; squares, npq1. Number of replicates was 10 for (A) and 3 for (B). a and b, significantly different with P < 0.001 and P < 0.003, respectively (t test).
precursors and its tocopherol content was reduced to trace level. Moreover, vte2 leaf discs exhibited a similar sensitivity to short-term photostress. Also, introducing the vte1 mutation into the npq1 mutant background enhanced photooxidative stress in leaves during long-term photostress. The light-sensitive phenotype of vte1 npq1 confirms that DMBPQ cannot replace tocopherols during oxidative stress under these conditions since otherwise vte1 npq1 would behave like npq1, in contrast with our observations (Figure 7). Thus, DMBPQ can functionally replace tocopherols in some functions (e.g., during germination; Sattler et al., 2004) but not as a protector of thylakoid membranes against oxidation induced by high light stress.

Vitamin E Mitigates Photoinactivation of PSII in High Light

The photosensitive phenotype of vte1 revealed in leaf discs exposed to a short-term light stress was not observed when light stress was imposed on whole vte1 plants. Although an increased photoinhibition of PSII was observed in vte1 relative to the wild type, no increase in lipid peroxidation and oxidative stress could be detected. This is consistent with previous studies of mutant or transgenic plants affected in tocopherol synthesis, including the vte1 Arabidopsis mutant (Porfirova et al., 2002), that have failed to show appreciable changes in tolerance to photooxidative stress. A small increase in the susceptibility of PSII to photoinhibition was observed, however, in tocopherol-deficient chlP transgenic tobacco leaves using chlorophyll fluorescence measurements (Grasses et al., 2001). The lack of deleterious effects of tocopherol deficiency on the thylakoid resistance to lipid peroxidation likely resulted from the stress treatment that was not severe enough to produce detectable differences between the wild type and the mutant strain. Tocopherols are part of a network of antioxidants that could be functionally redundant (Demmig-Adams and Adams, 2002), and it is necessary to overwhelm this network to see the impact of tocopherol deficiency on the thylakoid membrane photostability. This was probably the case in the short-term experiments on leaf discs. Although the temperature and light conditions used for the short-term and long-term photostresses were comparable, photoinhibition was much less severe in whole plants than in leaf discs. This likely resulted from a better control of leaf temperature and a strong restriction of the CO2 availability in leaf discs floating on chilled water relative to whole plants. Photoinhibition of PSII proceeded rather slowly in plants (compared with leaf discs), allowing us to reveal a difference between vte1 and the wild type regarding PSII photoinhibition.

Photoinhibition is related to the rapid turnover of the D1 protein (Aro et al., 1993), and 1O2 produced within PSII is a probable intermediate in the triggering of the degradation of this protein (Hideg et al., 1998). 1O2 is generated in PSII by interaction

**Figure 11.** Total Fatty Acid, MDA, and Total Chlorophyll in Leaves of Wild-Type, vte1, npq1, and vte1 npq1 Plants Grown for 7 d in High Light at Low Temperature.

(A) Total fatty acid.
(B) MDA.
(C) Total chlorophyll.

Open bars, before stress; closed bars, after stress. The fatty acid content was not measured in npq1 leaves. Data are mean values of three separate experiments ± SD. a, significantly different from all the other mean values with P < 0.03 (t test)
between the reaction center pigment in the triplet excited state (\(3P_{680}^*\)) and molecular oxygen—a reaction that is facilitated by the fact the \(\beta\)-carotenes in PSII do not quench \(3P_{680}^*\) (Telfer, 2002). In high light, the rate of translation and assembly of new D1 protein may no longer compensate for the degradation rate, leading to net PSII degradation. Thus, the increased photoinhibition observed here in tocopherol-deficient plants suggests that \(^1\text{O}_2\) concentration was increased in PSII and that tocopherol is important to control D1 degradation by scavenging \(^1\text{O}_2\) produced in PSII, as previously proposed by Trebst et al. (2002, 2004). This also implies that a fraction of the tocopherol pool must be located very close to PSII for efficient quenching of \(^1\text{O}_2\) at its site of production and for prevention of D1 oxidation. It is also possible that one of the functions of tocopherols in PSII is to protect the \(\beta\)-carotene molecules (Palozza and Krinsky, 1992; Böhm et al., 1997). We measured a strong reduction of the \(\beta\)-carotene concentration in high-light-treated \(vte1\ npq1\) plants (data not shown), and this is consistent with this idea. It has been shown recently in the cyanobacterium Synechocystis that \(^1\text{O}_2\) can inhibit the de novo synthesis of the D1 protein (Nishiyama

Figure 12. Protein Gel Blots of the PSII Reaction Center Protein D1 in Wild-Type, \(vte1\), \(vte1\ npq1\), and \(npq1\) Leaves Grown for 0 or 7 d in High Light at Low Temperature.

(A) A Coomassie blue–stained gel showing equal protein loading.
(B) Protein gel blot of the PSII reaction center protein D1.
(C) Relative intensity of the D1 bands measured in four different protein gel blots. a, significantly different from control value, \(t\) test with \(P < 0.01\). Amount of thylakoid membrane proteins loaded for the D1 blot was 4.5 \(\mu\)g. Data are mean values of four separate experiments ± sd. DM, \(vte1\ npq1\); c, control; s, high light.
Therefore, $^{1}\text{O}_2$ quenching by tocopherols may be important for the repair of photodamage to PSII. Protection of PSII by tocopherols was very obvious in the double mutants vte1 npq4 and vte1 npq1, which are impaired in the NPQ process. NPQ corresponds to an increased dissipation of absorbed light energy in the form of heat (Müller et al., 2001; Horton and Ruban, 2005). This process protects PSII from photoinhibition by increasing energy dissipation in the chlorophyll antennae and hence decreasing energy delivery to the PSII reaction centers. Overexcitation of the PSII centers due to the absence of NPQ led to a dramatic photoinhibition of PSII in vte1 npq4 and vte1 npq1 but not in the single mutants npq1 and npq4. We observed also an increased degradation of D1 in vte1 npq1 relative to vte1 or npq1. This demonstrates that PSII is less resistant to photoinhibition when tocopherols are missing, confirming that tocopherols play a specific role in the protection of PSII. However, at least under the high light stress conditions used in this study, NPQ seems to be efficient enough to avoid excessive production of $^{1}\text{O}_2$ in the PSII reaction centers, thus limiting PSII photoinhibition in vte1 despite the complete absence of tocopherols. Moreover, we observed that NPQ was stimulated in vte1 leaves during high light stress.

D1 degradation can also be viewed as a protective mechanism that can serve to lower the production of reactive oxygen species when thermal energy dissipation and scavenging of reactive oxygen species are overwhelmed (Öquist and Huner, 2003; Adams et al., 2004). This view is consistent with the findings that $^{1}\text{O}_2$ suppresses D1 synthesis (Nishiyama et al., 2004) and that oxidative modification of chloroplast proteins is genetically programmed rather than being a result of simple damage (Wagner et al., 2004). Thus, we cannot exclude that the sustained low value of Fv/Fm in vte1 is also a compensation for tocopherol deficiency.

**Zeaxanthin and Vitamin E Have Overlapping Functions**

Under the stress conditions used in this study, PSII photoinhibition induced by the lack of vitamin E was not followed by substantial photioxidation in whole plants, unless tocopherols and zeaxanthin were simultaneously absent. This indicates that those lipid-soluble antioxidants cooperate, either directly or indirectly. In agreement with this idea, zeaxanthin-deficient npq1 leaves were previously shown to accumulate higher levels of α-tocopherol than the wild type (Havaux and Niyogi, 1999; Havaux et al., 2000), and, in this study, tocopherol-deficient vte1 leaves contained more zeaxanthin than wild-type leaves in high light. Selective accumulation of zeaxanthin was also found in ChlP transgenic tobacco plants, which have only half of the tocopherol level of the wild type (Havaux et al., 2003). Accumulation of zeaxanthin when vitamin E is lacking and vice versa suggests that both compounds have overlapping functions, allowing one

---

**Figure 13.** Photoinhibition and Photooxidation of Wild-Type, vte1 npq1, and vte1 npq4 Leaves during Growth of Whole Plants in High Light at Low Temperature.

**A** PSII photoinhibition as measured by the Fv/Fm chlorophyll fluorescence parameter. Data are mean values of 8 to 10 measurements ± SD. Open circles, wild type; closed circles, vte1 npq1; closed triangles, vte1 npq4. a, significantly different with $P < 0.001$ (t test).

**B** Lipid peroxidation, as measured by the high-temperature TL band, in vte1 npq1 and vte1 npq4 leaves after 7 d in high light at low temperature. vte1 npq4 plants after 7 d in high light at low temperature.
antioxidant to substitute for the other one. Interestingly, a recent study has shown that, in cyanobacteria, carotenoids and tocopherols can functionally interact (Maeda et al., 2005).

Studies of xanthophyll mutants of Arabidopsis and Chlamydomonas have shown that zeaxanthin plays a critical role in photoprotection and that part of the protection exerted by this xanthophyll occurs via a mechanism distinct from NPQ (Havaux and Niyogi, 1999; Havaux et al., 2000; Baroli et al., 2003). Zeaxanthin has been shown to act as an antioxidant that stabilizes membrane lipids and protects membranes from peroxidative damage (Havaux, 1998). Since this effect corresponds to the function typically attributed to vitamin E both in plant and animal membranes, the functional redundancy found in this study between zeaxanthin and vitamin E is not very surprising.

Operation of the xanthophyll cycle has been assumed to involve binding and release of violaxanthin from pigment binding complexes (Morosinotto et al., 2002, 2003), diffusion of the xanthophyll molecules within the thylakoid membrane (Macko et al., 2002), and conversion of violaxanthin to zeaxanthin by the deinoxidase enzyme in the lipid (monogalactosyldiacylglycerol) phase (Hieber et al., 2004). Therefore, the zeaxanthin molecules formed in the xanthophyll cycle are initially present in the thylakoid lipid phase or at the interface lipid light-harvesting complex where they could supplement the antioxidative action of vitamin E (Havaux, 1998), so that removal of both compounds in vte1 npq1 resulted in almost complete deprivation of lipophilic antioxidants from the thylakoid membrane.

It is interesting to note that zeaxanthin and vitamin E are present in high amounts in different animal and human organs, especially the eye (Krisnky et al., 2003). The outer layers of the human retina containing photoreceptor outer segments and retina pigment epithelium are particularly susceptible to photooxidative damage due to the presence of endogenous photosensitizers, high fluxes of incident light, high oxygen tension, and high concentrations of polyunsaturated fatty acids. All these features are shared with the photosynthetic apparatus of plants. Beside vitamin E and zeaxanthin, the eye also contains substantial amounts of lutein (Krisnky et al., 2003; Alves-Rodrigues and Shao, 2004; Chichumroonchokchai et al., 2004), and in plants, this xanthophyll is the major carotenoid present in the light-harvesting antennae of the photosystems (see Table 2). Seemingly, the light-harvesting systems of plants and animals use the same combination of lipid-soluble antioxidants for their photoprotection.

Combination of carotenoid, especially zeaxanthin, and \( \alpha \)-tocopherol has been shown to exert a synergistic protection against lipid peroxidation in vitro (Palozza and Krisnky, 1992; Böhm et al., 1997; Wrona et al., 2003, 2004). This effect was explained in terms of prevention of carotenoid consumption by effective scavenging of free radicals by tocopherol, therefore allowing zeaxanthin to effectively quench the primary oxidant \( \text{1O}_2 \). Although this synergism can participate in the high tolerance of the wild type to photooxidative stress, it is unlikely that it was the main factor involved in the photosensitivity of vte1 npq1 since neither vte1 nor npq1 was particularly susceptible to lipid peroxidation. Elimination of both zeaxanthin and vitamin E was necessary to obtain a photosensitive phenotype, indicating functional overlapping rather than functional synergism.

### Conclusion: Photoprotection of the Photosynthetic Apparatus by Vitamin E Involves Two Different Mechanisms

By analyzing a set of single and double mutants affected in tocopherol production or in photosynthesis, this study has shown that vitamin E plays at least two different, but related, roles in chloroplasts: it protects PSII from photoinhibition and thylakoid membranes from photooxidative damage. One can assume that both mechanisms are related to the capacity of tocopherols to quench \( \text{1O}_2 \) and correspond to the existence of two major sites of \( \text{1O}_2 \) production in the chloroplast, namely, the PSII reaction centers and the light-harvesting antennae. It is also clear from this study that both functions are integrated in a network of antioxidative and photoprotective mechanisms. It was necessary to inhibit other photoprotective mechanisms to reveal a clear photosensitive phenotype when vitamin E was absent. Alternatively, it was necessary to use drastic stress conditions (such as exposure of leaf discs to continuous high light at low temperature for up to 30 h or treatment of whole plants to very high light at very low temperature) in order to observe lipid peroxidation in the absence of tocopherols. Similarly, a recent study of tocopherol-deficient mutants of the cyanobacterium Synechocystis PCC 6803 has shown that tocopherol deficiency does not lead to a photosensitive phenotype unless artificial generators of reactive oxygen species are used (Maeda et al., 2005). Under extreme light stress conditions (leaf disk experiments), photoinhibition of PSII was very fast, leading to an almost complete destruction of the PSII activity within 15 h (Figure 1). Nonfunctional PSII is known to generate \( \text{1O}_2 \) in high amounts (Nishiyama et al., 2004). The network of antioxidative mechanisms was probably overwhelmed by this massive \( \text{1O}_2 \) production, so that lack of tocopherols in the thylakoids was problematic, resulting in pronounced lipid peroxidation. Under more physiological conditions when NPQ and zeaxanthin (and possibly other complementary mechanisms) were operating, PSII photoinhibition was limited, thus preventing lipid peroxidation and allowing us to observe the protective function of tocopherols against PSII inactivation.

One of the outcomes of this study is that tocopherols play a specific role in the maintenance of the PSII function, as previously hypothesized by Trebst and coworkers in Chlamydomonas cells treated with herbicides (Trebst et al., 2002), and that this function supplements the photoprotective function of NPQ. While the NPQ-deficient npq4 mutant and the tocopherol-deficient vte1 mutant exhibited a rather moderate sensitivity to PSII inhibition in high light, dramatic loss of PSII activity was observed in high light in the vte1 npq4 double mutant deficient in both NPQ and vitamin E. Thus, NPQ and \( \text{1O}_2 \) quenching by tocopherols within PSII are two important aspects of PSII photostability that cooperate to preserve PSII function under conditions of excess light energy.

### METHODS

**Plant Material**

Arabidopsis thaliana plants (ecotype Columbia) were grown on compost in a phytotron under controlled environmental conditions: day/night...
temperature, 22/18°C; PFD, 250 μmol photons m⁻² s⁻¹; photoperiod, 8 h; relative air humidity, 60%. Wild-type Arabidopsis was compared with the following mutants: the vitamin E-deficient vte1 mutant (Porfiriova et al., 2002), the zeaxanthin-deficient npq1 mutant (Niyogi et al., 1998), the PsbS-deficient npq4 mutant (Li et al., 2000), and the double mutants vte1 npq1 and vte1 npq4. We have isolated another tocopherol mutant, vte2. vte1 and vte2 affect the tocopherol biosynthesis pathway at two different steps. vte1 disrupts tocopherol cyclase activity, while vte2 disrupts homogentisate pyrlyltransferase activity. The npq1 and npq4 mutants were obtained from K. Niyogi (University of California, Berkeley, CA). Each npq mutant was crossed with vte1 to produce F1 plants. In the progeny of these F1 plants, we analyzed various individuals using chlorophyll fluorometry and HPLC. This allowed the identification of vte1 npq1 and vte1 npq4 double mutants.

Isolation of the vte2 Mutant

Screening of a chemically mutagenized (ethyl methanesulfonate) Arabidopsis population for plants deficient in neutral lipid synthesis resulted in the isolation of several tocopherol deficient lines (i.e., vte1 [Porfiriova et al., 2002], vte2 [Bergmüller et al., 2003], and hpt1 [vte2; this study]). The vte2 mutant was three times backcrossed to the wild type (Columbia) to reduce the number of background mutations. Mapping of the mutation indicated that it is closely linked to the gene At2g18950 encoding the npq4 mutant was crossed with vte1 to produce F1 plants. In the progeny of these F1 plants, we analyzed various individuals using chlorophyll fluorometry and HPLC. This allowed the identification of vte1 npq1 and vte1 npq4 double mutants.

Stress Conditions

Light stress was imposed by transferring plants aged 45 d to a growth chamber at 8/6°C (day/night temperature) and under a PFD of 1000 to 1100 μmol photon m⁻² s⁻¹ (photoperiod, 8 h) or at 30/25°C (day/night) under a PFD of 1300 to 1400 μmol photon m⁻² s⁻¹ (photoperiod, 8 h). Leaf temperature, measured with an infrared thermometer, was 10 to 12°C or 35 to 34°C. For comparison purposes, one series of experiments (Table 1) was performed under more extreme conditions: 1500 to 1600 μmol m⁻² s⁻¹, 2 to 3°C/6°C (day/night temperature), and an 8-h photoperiod. Leaf samples were always taken at the beginning of the photoperiod, after 2 h of illumination. Light stress was also imposed on leaf discs of 16 mm in diameter. The discs were punched out from mature leaves. Leaf discs floating on water were exposed to white light (PFD, 1000 μmol m⁻² s⁻¹) produced by 150-W metal halide lamps equipped with infrared suppressor filters. Leaf temperature was maintained constant at 10°C. Eosin treatments of leaf discs were done as previously described (Havaux et al., 2000). In brief, leaf discs of 12 mm in diameter, floating on an aqueous solution of eosin Y (5%), were illuminated with white light at a PFD of 200 μmol photon m⁻² s⁻¹. Temperature of the eosin solution was maintained constant at 22°C.

Fluorometry

Chlorophyll fluorescence emission from leaf discs or leaves attached to the plant was measured with a PAM-2000 modulated fluorometer (Walz) as previously described (Havaux et al., 2003). The maximal quantum yield of PSII photochemistry was measured in dark-adapted samples by the

\[
\text{Fm} - \text{Fo} = \text{Fv}/\text{Fm} \quad \text{ratio, where Fo is the initial fluorescence level and Fm is the maximal fluorescence level. Fm was measured with an 800-ms pulse of intense white light, and Fo was measured with a 2-s pulse of far-red light. NPQ was measured as (Fm/Fm') - 1 where Fm' is the maximal fluorescence level in the light.}
\]

Determination of Soluble Antioxidants

Glutathione was determined by HPLC, as described in detail elsewhere (Carrier et al., 2003). Ascorbate was determined by HPLC using the method described by Sanchez-Mata et al. (2000) with some modifications. Leaves (~50 mg) were ground in 750 μL of chilled 4.5% metaphosphoric acid. The extract was centrifuged, and the supernatant was filtered through a 0.22-μm nylon filter. Six microliters of the filtered extract were injected into the HPLC system (Waters 600E controller/996 photodiode array detector). The chromophoric column was a reverse phase C18 column (Waters Nova Pak; 80 Å, 4 μm 3.9 x 300 mm) protected by a guard column (Bondapak C18). The chromatographic conditions of analysis were as follows: distilled water acidified, pH 2.5, with sulfuric acid as the mobile phase, flow rate of 0.65 mL min⁻¹, and UV detection at 245 nm. Total ascorbate was determined after reduction with Tris-carboxyethylphosphine as described by Lykkesfeldt (2000): 40 μL of 50 mM Tris-carboxyethylphosphine was added to 200 μL of the extract. The mixture was left at 25°C in the dark for 4 h before HPLC analysis.

Determination of Lipid-Soluble Antioxidants

Carotenoids and tocopherols were extracted in 400 μL of pure methanol. After centrifugation and filtration of the extracts, photosynthetic pigments were separated by HPLC with a reverse phase C18 column (Waters Nova Pak; 80 Å, 4 μm 3.9 x 300 mm) protected by a Bondapak C18 guard column, as previously described (Havaux et al., 2003). The chromatographic system consisted of a Waters 600E system controller and a Waters 996 photodiode array detector. Pure carotenoids used for calibration were bought from Extrasynthese or were prepared by thin-layer chromatography with n-hexane/isopropanol (100:10; v/v) as solvent system. Tocopherols were detected with a Waters 474 scanning fluorescence detector. Excitation wavelength was 295 nm, and emission wavelength was 340 nm. Tocopherol standards were obtained from Sigma-Aldrich.

Biophysical Determination of Lipid Peroxidation

Light-emitting species (triplet carbonyls and singlet oxygen) are produced during lipid peroxidation (Devraj et al., 1997; Havaux, 2003). In plants, the lipid peroxidation–related luminescence can be measured by TL as a strong band peaking at high temperature, ~135°C (Ducrot, 2003; Havaux, 2003). TL measurements were performed on leaf discs (diameter, 1 cm) with a custom-built apparatus, as previously described (Havaux, 2003). The leaf sample was slowly heated from 25 to 150°C at a rate of 6°C min⁻¹.

Biochemical Determination of Lipid Peroxidation

Lipid peroxidation was assessed indirectly by measuring MDA using HPLC. MDA is a three-carbon, low molecular weight aldehyde that is produced from radical attack on polyunsaturated fatty acids. The sample (3 leaf discs of 12 mm in diameter) was ground in 1 mL of chilled ethanol:water (80:20; v/v). After centrifugation, 750 μL of the supernatant was mixed with 750 μL of the following reaction mixture: 20% trichloroacetic acid, 0.01% butylated hydroxytoluene, 0.65% and thioharbituric acid (TBA). After heating at 95°C for 20 min and centrifugation, the MDA-TBA₂ adduct was separated and quantified by HPLC. The analytical column and the HPLC apparatus were similar to those used for pigment analyses. The elution buffer was 65% 50 mM H₂PO₄-KOH, pH 7.0, and
Lipid Analyses

Fatty acid determinations were done according to Browse et al. (1986). Lipids were extracted from one or two leaf discs (diameter, 12 mm) in 1 M HCl in methanol. Pentadecanoic acid (15:0) was used as internal standard. After incubation at 80°C for 30 min, the reaction mixture was cooled to room temperature and 1 mL 0.9% NaCl and 1 mL hexane were added. After vortexing, the hexane phase was removed and concentrated in a nitrogen gas stream. Fatty acid methyl esters were separated and quantified by gas chromatography with a flame ionization detector (Browse et al., 1986).

Protein Extraction, SDS-PAGE, and Protein Gel Blotting Experiments

Leaf samples were ground in liquid nitrogen, and the powder was resuspended in 50 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl-fluoride, and 50 mM β-mercaptoethanol and centrifuged at 30,000g for 15 min at 4°C. Soluble proteins were precipitated at −20°C by adding 3 volumes of acetone to the supernatant. The protein content was determined using a method based on bicinchoninic acid (BC assay reagent; Interchim). Proteins were separated using SDS-PAGE (13%, w/v gel) and electrotransferred onto a nitrocellulose membrane (Pall Corporation). Polyclonal antibodies raised against Arabidopsis CDS32 thiol-oxidoredoxin and BAS1 PRX were produced as described by Broin et al. (2002) and used at a dilution of 1:800 and 1:20,000, respectively. The sera raised against poplar PRXQ (Rouhier et al., 2004) and Arabidopsis type-II-E PRX (Bréhelien et al., 2003) were used diluted 1:800 and 1:20,000, respectively. Serum against PSII D1 subunit was used at a dilution of 1:100. Bound antibodies were detected using an anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1:10,000. The D1 antibody was obtained from Agrisera. The protein gel blots were scanned, and the band intensities were measured with E-Capt software (Vilber Lourmat).

Accession Numbers

Arabidopsis Genome Initiative locus numbers for the genes described in this work are as follows: VTE1, At4g32770; VTE2, At2g18950; NPO1, At1g08550; NPO4, At1g44575.

ACKNOWLEDGMENTS

High light treatments of plants were done in the growth chambers of the Groupe de Recherches Appliquées en Phytotechnologie at the Commissariat à l’Energie Atomique/Cadarache. We thank the staff of this laboratory for their help. We would like also to thank S. Cuiné (Commissariat à l’Energie Atomique/Cadarache) for help with HPLC analyses. We are grateful to N. Rouhier (Institut National de la Recherche Agronomique, Université Henri Poincaré, Nancy I, France) for providing us with the sera raised against Arabidopsis PRXQ and Mr-A and Y. Meyer (Centre National de la Recherche Scientifique, Université de Perpignan, France) for providing us with the Arabidopsis PRXII-E antibody.

Received August 11, 2005; revised September 12, 2005; accepted October 6, 2005; published October 28, 2005.

REFERENCES


Vitamin E Protects against Photoinhibition and Photooxidative Stress in Arabidopsis thaliana
Michel Havaux, Françoise Eymery, Svetlana Porfirova, Pascal Rey and Peter Dörmann
Plant Cell; originally published online October 28, 2005;
DOI 10.1105/tpc.105.037036

This information is current as of July 8, 2017