The Plant Cell, Vol. 17, 3451–3469, December 2005, www.plantcell.org © 2005 American Society of Plant Biologists

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 phytyl 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, where it is found mainly in the envelope and the thylakoid membranes of chloroplasts, 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 peroxyl 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 regenating α-tocopherol molecule (Kamal-Eldin and Appelqvist, 1996). α-Tocopherol also quenches singlet oxygen ($^1O_2$), 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 thylakoid 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 antioxidant 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 *chlP* 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 (phytylated) chlorophyll (Tanaka et al., 1999), it cannot be excluded that the photosensitivity of the *chlP* antisense plants was due, at least partially, to the chlorophyll alteration, as recently suggested in the corresponding *chlP* mutant of the cyanobacterium *Synechocystis* PCC6803 (Shpilyov 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). Porfirova 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 $^1O_2$ 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 homogentisate 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 antioxidant 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). Inhibition 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

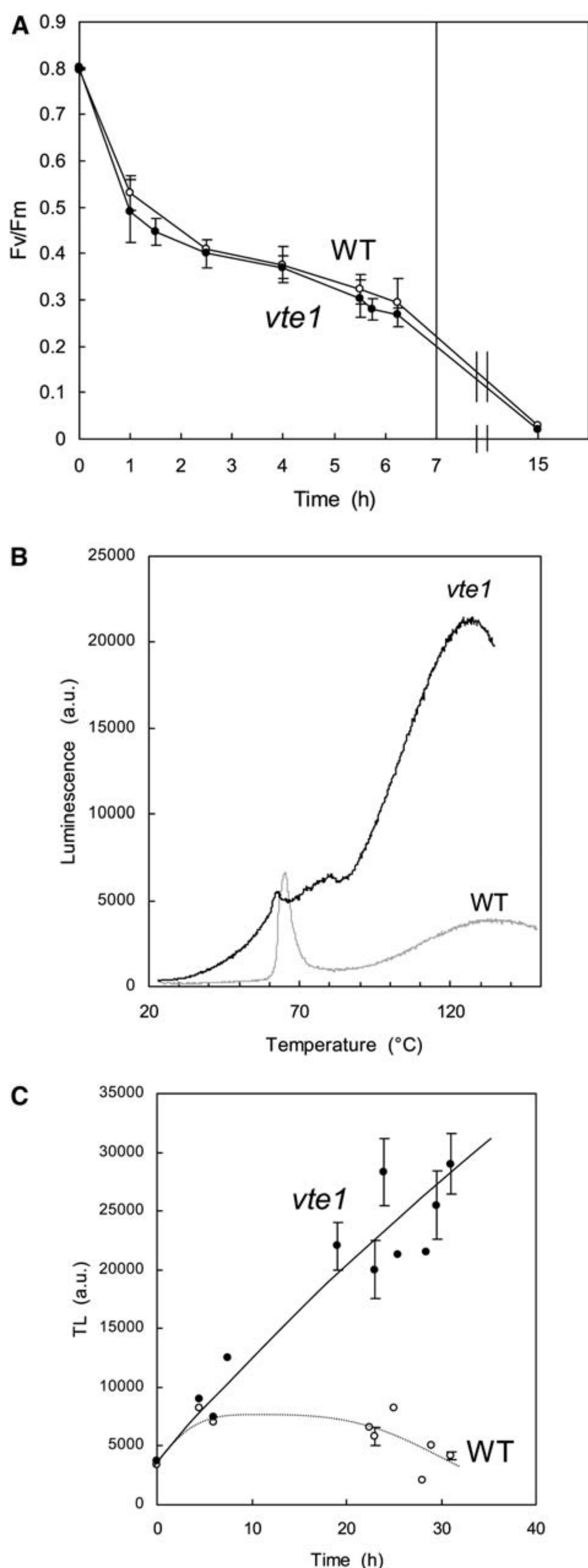


Figure 1. Photoinhibition and Photooxidation of *Arabidopsis* Leaf Discs Exposed to High Light Stress.

Leaf discs (the wild type and *vte1* mutant) were exposed to high light (1000 μmol photons m^{-2} s^{-1}) at 10°C. Open circles, the wild type; closed circles, *vte1*. Data are mean values of three to five separate experiments ± 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 ~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 *vte1* leaf discs exposed for 24 h to the high light stress (1000 μmol photons m^{-2} s^{-1}/10°C), showing the lipid peroxidation–related band peaking at ~130°C. The sharp peak at ~65°C, which tends to disappear with oxidative stress, is specific to unstressed cruciferous plants. The origin of this band is unknown (Havaux, 2003).

decrease in both fatty acid content and chlorophyll level occurred in *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 *Arabidopsis* leaves increases oxidative damage to lipids and degradation of photosynthetic pigments. This conclusion is further supported by the effects of the $^1O_2$ generator eosin on *vte1* leaves. Illumination of *vte1* leaf discs floating on a 5% eosin solution brought about a drastic increase in the 135°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 *Arabidopsis* leaves, at least under harsh and artificial conditions.

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°C, 1100 μmol m^{-2} s^{-1}). As previously observed (Havaux and Kloppstech, 2001), this treatment caused transient photoinhibition of PSII photochemistry in wild-type *Arabidopsis* leaves (Figure 4). Upon transfer to the stress conditions, Fv/Fm decreased, reaching ~0.5 after 2 d. Fv/Fm recovered progressively during the following days, reaching a value of ~0.6 after 7 d, indicating photoacclimation of the photosynthetic apparatus. In *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 *vte1* leaves. Determination of chlorophyll, fatty acid, and MDA (see below) confirmed that tocopherol deficiency in *vte1* leaves did not increase oxidative stress during long-term

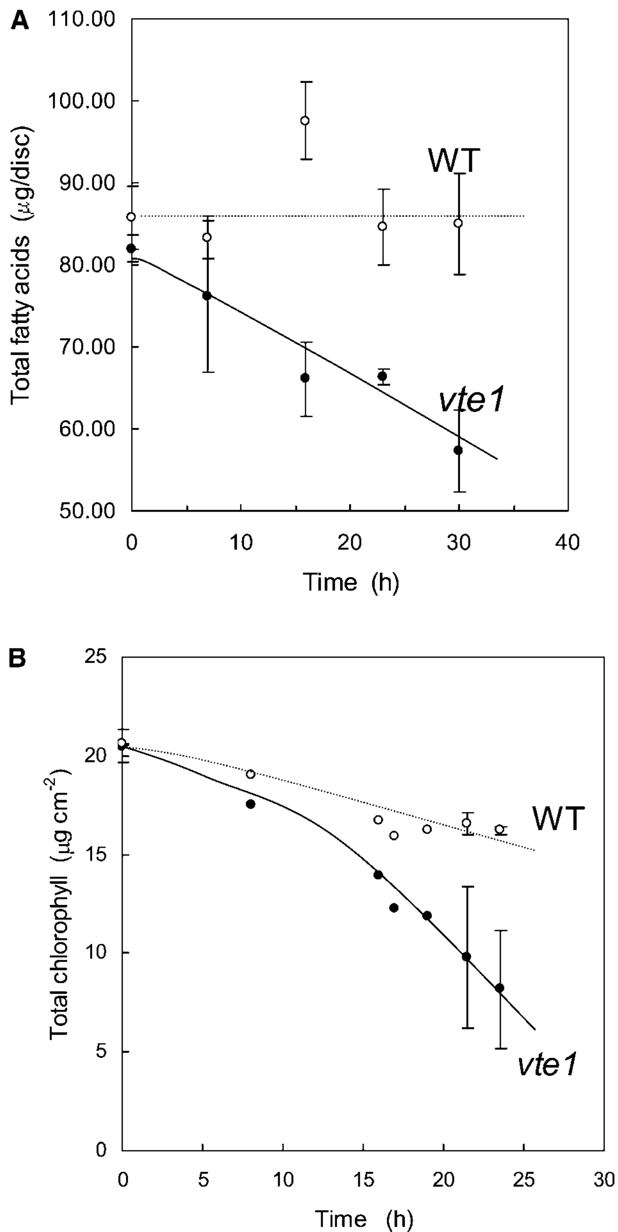


Figure 2. Photodestruction of Fatty Acids and Chlorophylls in *Arabidopsis* Leaf Discs Exposed to High Light Stress.

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).

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 CO_2 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

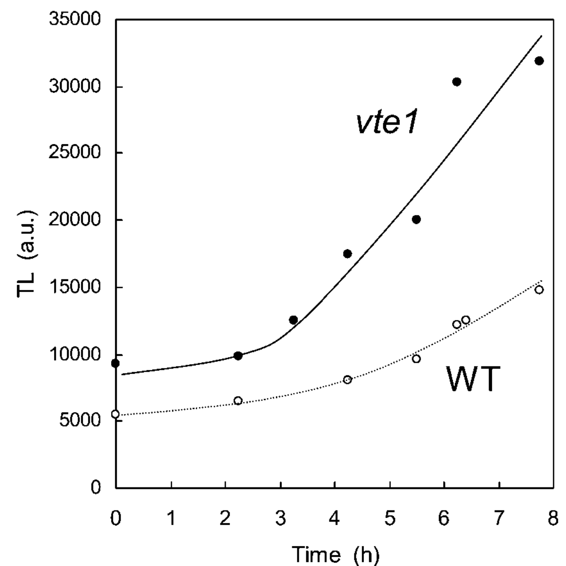
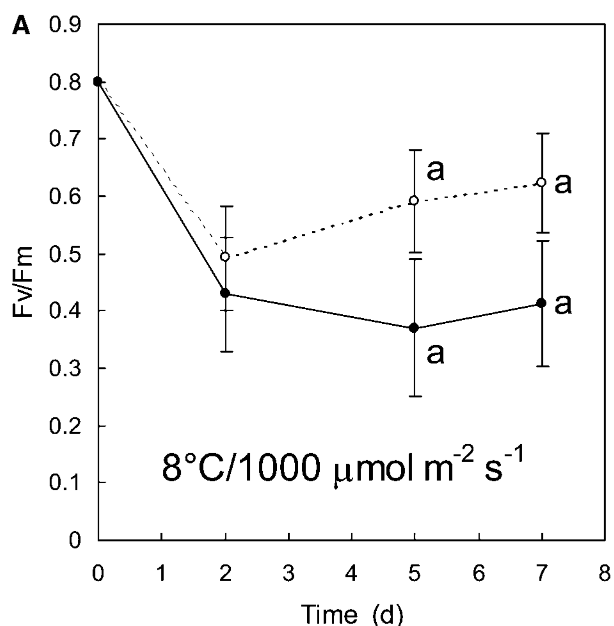


Figure 3. Singlet Oxygen Toxicity in *Arabidopsis* Leaf Discs.

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.



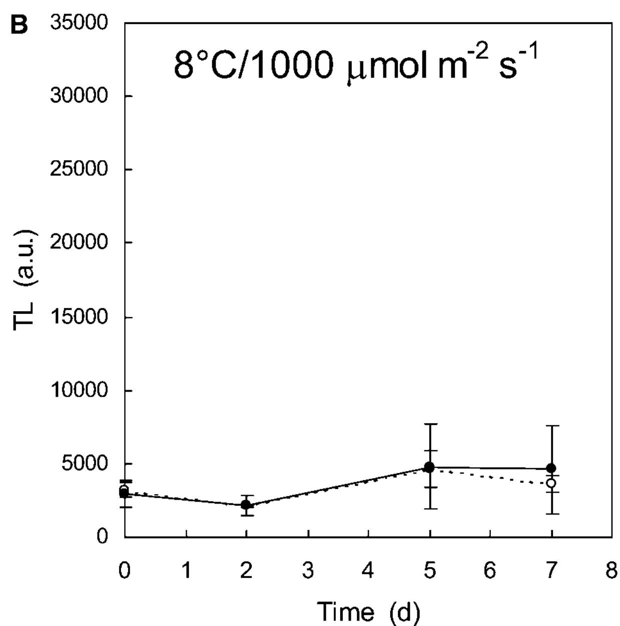
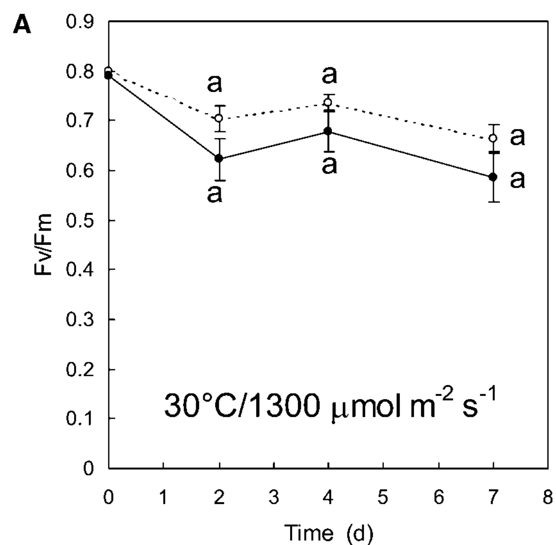


Figure 4. Photoinhibition and Photooxidation of *Arabidopsis* Plants Exposed to High Light Stress at Low Temperature.

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 photons 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).

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



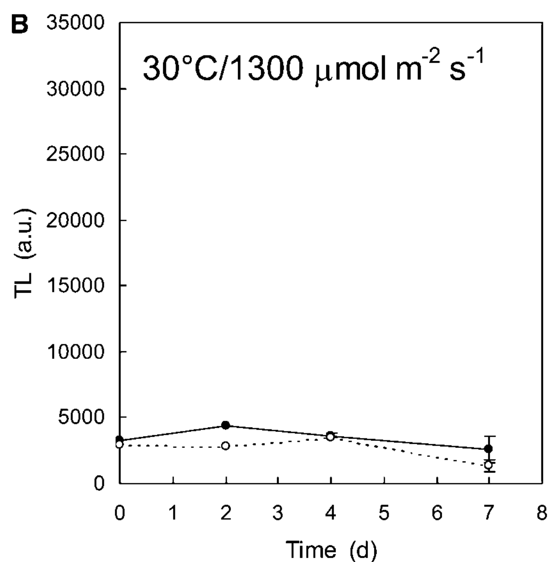


Figure 5. Photoinhibition and Photooxidation of *Arabidopsis* Plants Exposed to High Light Stress at High Temperature.

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 photons 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).

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 ~135°C) of Leaves Taken from Plants Exposed to Continuous High Light or to an Extreme Light Stress

	Fv/Fm			TL (a.u.)		
Treatment	Wild Type	*vte1*	*vte2*	Wild Type	*vte1*	*vte2*
Unstressed leaves	0.78 ± 0.02	0.79 ± 0.01	0.79 ± 0.01	6,100 ± 250	6,900 ± 730	6,750 ± 640
Continuous light						
24 h	0.53 ± 0.12	0.42 ± 0.17	0.34 ± 0.08	7,600 ± 1,490	6,400 ± 860	8,700 (*n* = 2)
48 h	0.48 ± 0.14	0.34 ± 0.18	0.47 ± 0.15	6,700 ± 710	8,260 ± 1,270	9,000 (*n* = 2)
Very high light stress						
1 d	0.37 ± 0.07	0.30 ± 0.08	0.34 ± 0.08	4700 ± 300[a]	27,660 ± 3,180[a]	22,000 (*n* = 2)
2 d	0.38 ± 0.07	0.32 ± 0.06	0.32 ± 0.10	20,600 ± 940	20,800 ± 3,400	28,000 (*n* = 2)

Continuous high light (1100 μmol photons m^{-2} s^{-1} at 6°C) and extreme light stress (1500 to 1600 μmol 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 (*t* test, P < 0.05).

that the stress conditions are harsh enough. Again, *vte2* behaved like *vte1*.

In the following paragraphs, we have examined possible mechanisms involved in the acclimation of *vte1* plants to high light (1000 to 1100 μmol photons m^{-2} s^{-1}) and low temperature (6 to 7°C).

Acclimation of *vte1* 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 *Arabidopsis* plants grown under control conditions or in high light at low temperature (7 d). Under control conditions, *vte1* 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 *vte1* plants, less ascorbate accumulated in *vte1* 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.

Carotenoids are, with tocopherols, the major lipid-soluble antioxidants of the thylakoid membranes. While β-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 *vte1* plants grown at ambient temperatures (Kanwischer et al., 2005). In *vte1*, 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 *vte1* leaves relative to wild-type leaves (210 versus 112 ng cm^{-2}). This phenomenon was amplified at high temperature (1300 μmol 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 *vte1* plants during acclimation to high light at low (8°C) or

Table 2. Ascorbate and Glutathione Concentration (in μg cm^{-2}) in *Arabidopsis* Leaves (Wild Type and *vte1*) before and after High Light Stress (7 d at 1100 μmol m^{-2} s^{-1}) at 8°C

	Before Stress		After Stress	
Antioxidants	Wild Type	*vte1*	Wild Type	*vte1*
Ascorbate				
Reduced	16.8 ± 1.2	12.4 ± 0.1	35.7 ± 6.8	28.9 ± 3.5
Total	21.0 ± 2.4	14.8 ± 0.6	41.2 ± 3.0	33.5 ± 0.6
Reduction (%)	80	83	87	86
Glutathione				
Reduced	1.2 ± 0.2	1.9 ± 0.2	4.4 ± 0.3	4.6 ± 0.6
Total	1.7 ± 0.3	2.5 ± 0.1	6.2 ± 0.3	7.1 ± 0.6
Reduction (%)	72	76	71	64

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}; *vte1* control = 20.55 mg cm^{-2}; *vte1* 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)

	Control		High Light at Low Temperature		High Light at High Temperature	
Carotenoids	Wild Type	*vte1*	Wild Type	*vte1*	Wild Type	*vte1*
Neoxanthin	731 ± 29	752 ± 71	680 ± 37	659 ± 44	709 ± 93	664 ± 82
Violaxanthin	461 ± 15	539 ± 85	473 ± 43	392 ± 150	437 ± 119	144 ± 5
Antheraxanthin	n.d.	n.d.	271 ± 55	192 ± 45	224 ± 44	181 ± 49
Zeaxanthin	n.d.	n.d.	112 ± 27	210 ± 53[a]	171 ± 40	680 ± 66[b]
Lutein	2065 ± 69	2220 ± 207	2424 ± 266	1981 ± 341	2191 ± 338	2098 ± 213
β-Carotene	996 ± 57	1136 ± 112	756 ± 106	685 ± 193	872 ± 90	776 ± 63
(A+Z)/(A+Z+V)	0	0	0.45 ± 0.01	0.51 ± 0.02	0.47 ± 0.05	0.85 ± 0.01

Data are mean values of three to five separate experiments ± SD. 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 reconverted 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 in 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 antioxidative proteins were analyzed by protein gel blotting. Peroxiredoxins (PRXs) are peroxidases with a catalytic center containing a cysteinyl 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 CDPS32, 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 antioxidative 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 transferase activity and does not accumulate detectable amounts of

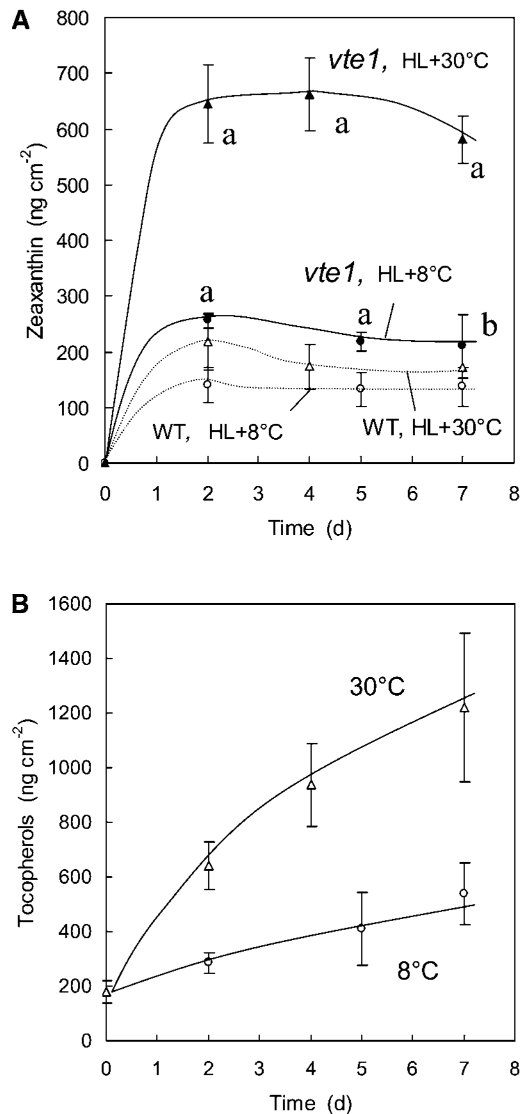


Figure 6. Accumulation of Zeaxanthin and Tocopherols in Wild-Type and *vte1* Leaves Exposed to High Light Stress at Low or High Temperature.

(A) Zeanthin in wild-type and *vte1* leaves exposed to high light stress at low or high temperature (1000 μmol photons m^{-2} s^{-1} at 8°C [circles] or 1300 μmol photons m^{-2} s^{-1} at 30°C [triangles], respectively).

(B) Tocopherols in wild-type and *vte1* leaves exposed to high light stress at low or high temperature (1000 μmol photons m^{-2} s^{-1} at 8°C [circles] or 1300 μmol photons m^{-2} s^{-1} at 30°C [triangles], respectively).

Samples were taken after 2-h illumination, and the measured concentrations of zeaxanthin corresponded to the steady state level of this xanthophyll. Closed symbols, *vte1*; open symbols, wild type. Tocopherol was not detected in *vte1* under any conditions. Data are mean values of three to five separate experiments ± SD. a, significantly different from wild-type values under similar conditions using *t* test with P < 0.01. b, different from wild-type value under similar conditions in a *t* test with P < 0.12. HL, high light.

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

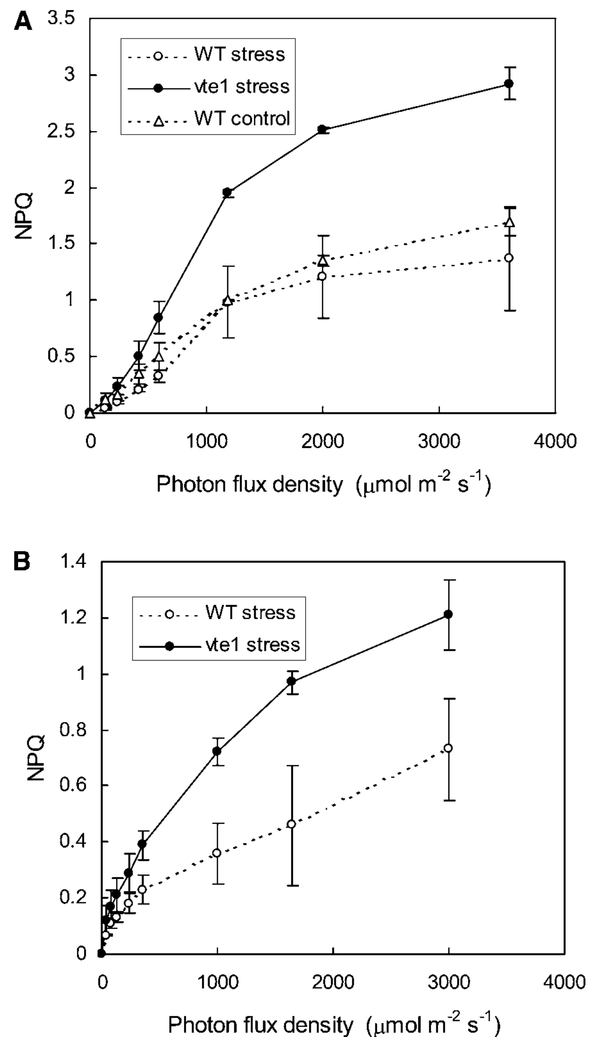


Figure 7. NPQ in Wild-Type and *vte1 Arabidopsis* Leaves after High Light Stress at Low Temperature or after High Light at High Temperature.

(A) NPQ in wild-type and *vte1 Arabidopsis* leaves after high light stress at low temperature (6°C and 1100 μmol m^{-2} s^{-1} for 6 d).

(B) NPQ in wild-type and *vte1 Arabidopsis* leaves after high light at high temperature (30°C and 1500 μmol m^{-2} s^{-1}).

Data are mean values of three separate experiments ± SD. NPQ in unstressed wild-type leaves is also shown in **(A)**. NPQ in unstressed *vte1* leaves (data not shown) was very similar to NPQ in wild-type leaves.

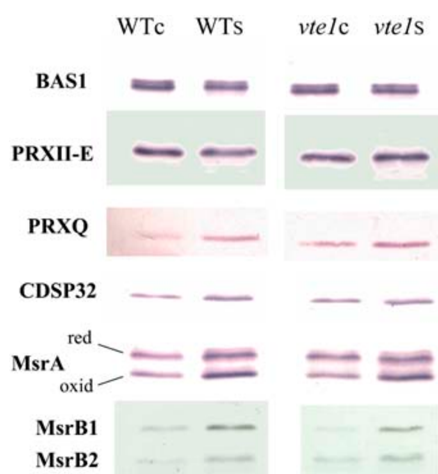


Figure 8. Protein Gel Blots of a Series of Enzymes Involved in the Protection of Thylakoid Membrane Lipids or Proteins against Oxidation.

The following proteins were analyzed: PRXs PRXQ, BAS1, and PRXE II, the thioredoxin CDSP32, and the Msrs MsrA, MsrB1, and MsrB2. Control leaves (WTc and *vte1*c) are compared with leaves stressed for 7 d in high light at low temperature (WTs and *vte1*s). Amounts of soluble protein loaded were as follows: PRXQ, 25 μg; BAS1, 4 μg; PRXEII-E, 4 μg; CDSP32, 25 μg; MsrBs and MsrA, 25 μg.

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*.

PSII photoinhibition is related to the rapid turnover of the PSII reaction center protein D1 and to a net degradation of this protein in high light (Aro et al., 1993). In Figure 12, the abundance of the D1 protein was measured by protein gel blotting in wild-type,

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*

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

Data are mean values of three to seven measurements ± SD. n.d., not detected.

Figure 9. Wild-Type, *vte1*, *npq1*, and *vte1 npq1* Plants Grown for 7 d in High Light at Low Temperature.

vte1, *npq1*, and *npq1 vte1* leaves before and after growth for 7 d in high light at low temperature. The D1 concentration in wild-type and *npq1* leaves did not decrease significantly during high light stress, and this is consistent with the Fv/Fm fluorescence data. The D1 abundance decreased slightly in the *vte1* single mutant (~15%), while the decrease was strong in the *vte1 npq1* double mutant (−50%). Statistical analysis of the data (Student's *t* test) indicated that the D1 decrease in *vte1 npq1* relative to the wild type was significant at the 1% level, while the decrease in *vte1* was significant at the 20% level only. Again, the D1 data are consistent with the Fv/Fm data (Figure 10A).

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 photoinhition 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 photoinhibition, 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). As shown in Figures 10 and 11, *vte1 npq1* suffers extensive photooxidation in high light at low temperature. By striking contrast, *vte1 npq4* behaved like the wild type regarding lipid peroxidation and oxidative stress. The *vte1 npq4* leaves did not bleach and the amplitude of the 135°C TL band was low, close to the TL signal amplitude measured in the wild type (Figures 13B and 13C). Thus, overexcitation of the PSII reaction centers resulting from the NPQ inhibition caused a pronounced photoinhibition of PSII when tocopherols were absent, pointing to a specific function of vitamin E in the photoprotection of PSII. However, in the zeaxanthin-containing *vte1 npq4*, the strong photoinhibition of the PSII centers was transitory and was not followed by photooxidation. Photooxidative stress became obvious only when zeaxanthin was also absent, confirming that vitamin E acts also as a protector of membrane lipids against photooxidation along with the xanthophyll zeaxanthin.

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 CO_2 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 $^1O_2$ 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, $^1O_2$ is produced from chlorophyll excitation in the light-harvesting complexes or the PSII reaction centers, whereas exogenously added eosin is expected to generate $^1O_2$ throughout the thylakoid membrane (Knox and Dodge, 1985). Consequently, the protective effect of vitamin E is not restricted to $^1O_2$ scavenging in the vicinity of the PSII centers, as recently suggested (Trebst et al., 2002), but it must also involve a general protection of

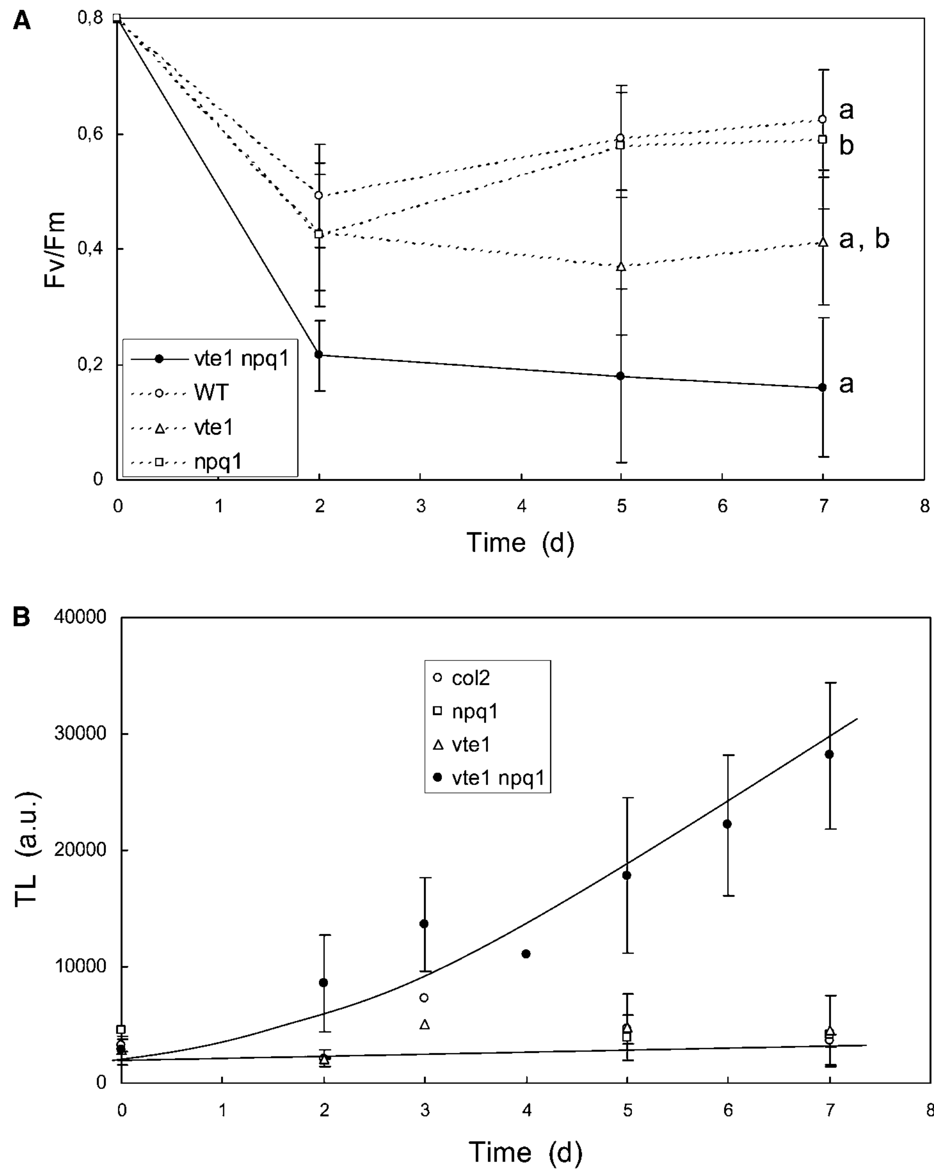


Figure 10. Photoinhibition and Photooxidation of Wild-Type, *vte1*, *npq1*, or *npq1 vte1* Leaves during Growth of Whole Plants in High Light at Low Temperature.

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).

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 form their role in the protection of PSII against photodestruction.

Germination and early seedling development were impaired in the tocopherol-deficient *vte2* affected in homogentisate phytyl 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 form our results on leaf discs that, in mature leaves exposed to photooxidative stress, DMPBQ does not, or only partially, substitutes 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

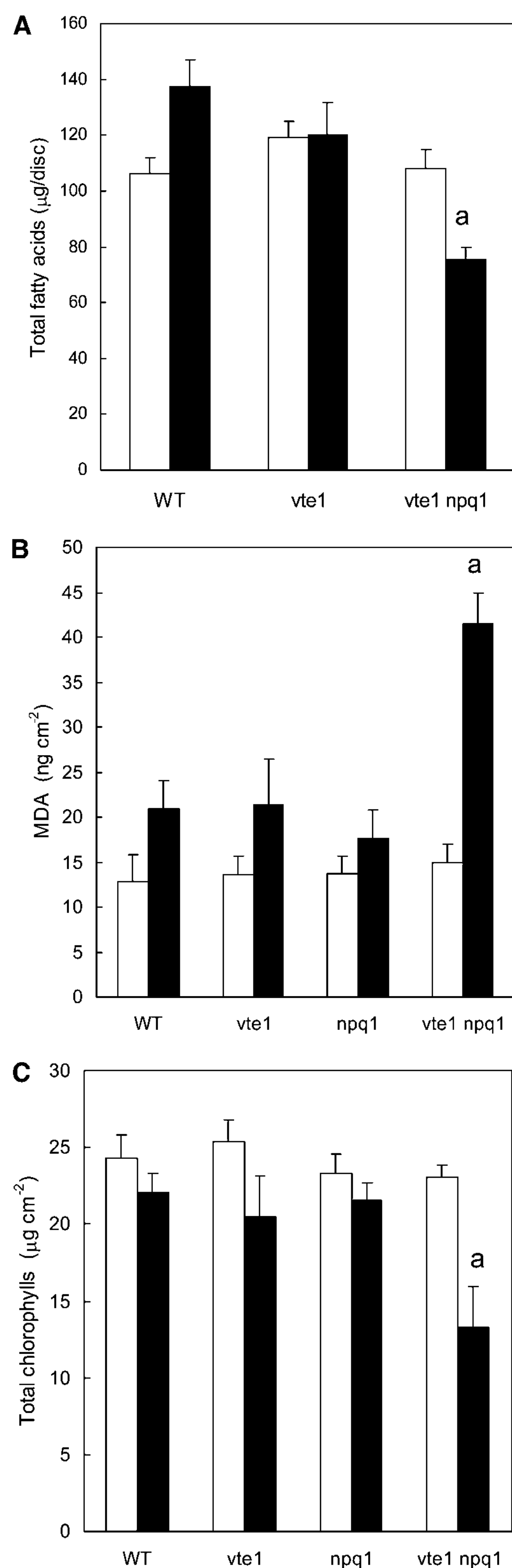


Figure 11. Total Fatty Acid, MDA, and Total Chlorophyll in Leaves of Wild-Type, *vte1*, *npq1*, and *vte1 npq1* Plants Grown for 7 d in High Light at Low Temperature.

(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)

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, DMPBQ 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 CO_2 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 $^1O_2$ produced within PSII is a probable intermediate in the triggering of the degradation of this protein (Hideg et al., 1998). $^1O_2$ is generated in PSII by interaction

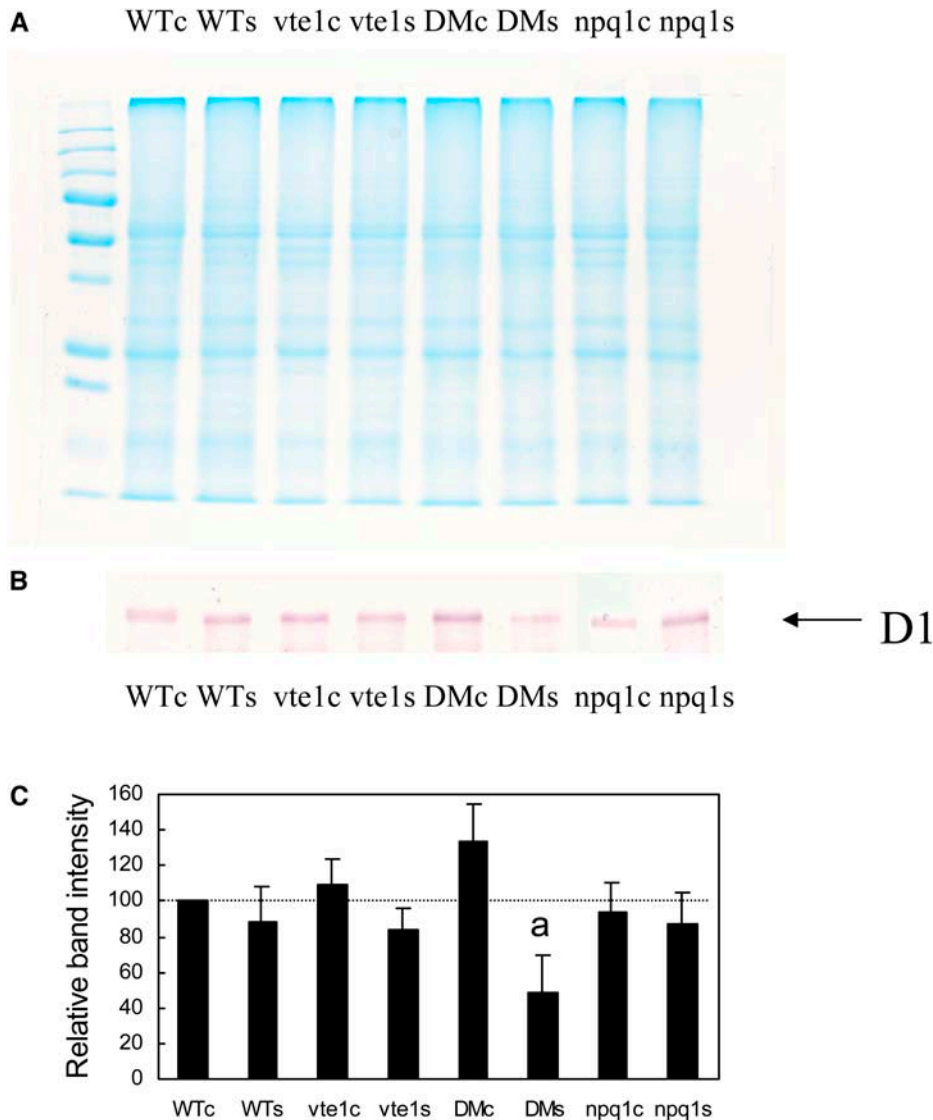


Figure 12. Protein Gel Blots of the PSII Reaction Center Protein D1 in Wild-Type, *vte1*, *vte1 npq1*, and *npq1* Leaves Grown for 0 or 7 d in High Light at Low Temperature.

(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 μg. Data are mean values of four separate experiments ± SD. DM, *vte1 npq1*; c, control; s, high light.

between the reaction center pigment in the triplet excited state (^{3}P680*) and molecular oxygen—a reaction that is facilitated by the fact the β-carotenes in PSII do not quench ^{3}P680* (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 $^1O_2$ concentration was increased in PSII and that tocopherol is important to control D1 degradation by scavenging $^1O_2$ 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 $^1O_2$ 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 β-carotene molecules (Palozza and Krinsky, 1992; Böhm et al., 1997). We measured a strong reduction of the β-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 $^1O_2$ can inhibit the de novo synthesis of the D1 protein (Nishiyama

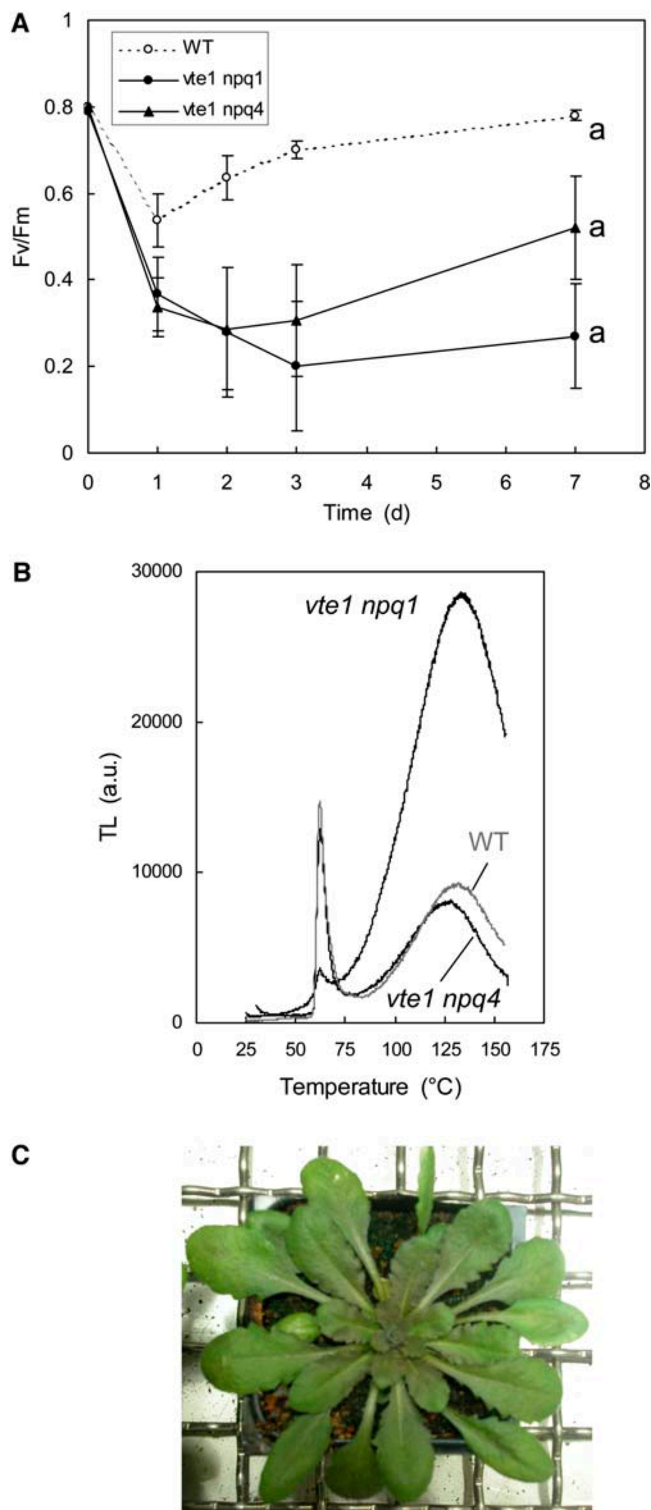


Figure 13. Photoinhibition and Photooxidation of Wild-Type, *vte1 npq1*, and *vte1 npq4* Leaves during Growth of Whole Plants in High Light at Low Temperature.

(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.

(C) *vte1 npq4* plants after 7 d in high light at low temperature.

et al., 2004). Therefore, $^1O_2$ 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 $^1O_2$ 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 $^1O_2$ 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 photooxidation 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

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 mecanism 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 deepoxidase 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 (Krinsky 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 (Krinsky et al., 2003; Alves-Rodrigues and Shao, 2004; Chitchumroonchokchai 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 α-tocopherol has been shown to exert a synergistic protection against lipid peroxidation in vitro (Palozza and Krinsky, 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 $^1O_2$. 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 $^1O_2$ and correspond to the existence of two major sites of $^1O_2$ 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* PCC6803 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 $^1O_2$ in high amounts (Nishiyama et al., 2004). The network of antioxidative mechanisms was probably overwhelmed by this massive $^1O_2$ 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 $^1O_2$ 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 (Porfirova 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 phytyl transferase 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* [Porfirova et al., 2002], *vte4* [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 homogentisate phytyltransferase (HPT1) on chromosome 2 of *Arabidopsis*. Therefore, the genomic sequence of *HPT1* was amplified from the mutant genome by PCR using the oligonucleotides PD321 (5′-CCG-TAAATGCCGATTCCTCCCTTGTCTAA-3′) and PD322 (5′-CCCAGGCAG-TTACGTACAGTTTCAAGACA-3′). Sequencing of the *HPT1* gene from the *vte2* mutant revealed the existence of a single base exchange from C to T at position 811 of the *HPT1* open reading frame. This apparent point mutation results in a Leu-to-Phe exchange at position 271 of the amino acid sequence. Presumably, the replacement of Leu with a bulky aromatic amino acid (Phe) affects protein conformation and strongly reduces HPT1 enzyme activity.

Stress Conditions

Light stress was imposed by transferring plants aged 45 d to a growth chamber at 8/6°C (day/night air 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 33 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 a 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 (Fm − Fo)/Fm = Fv/Fm 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 chromatographic column was a reverse phase C18 column (Waters Nova Pak; 60 A, 4 μm, 3.9 × 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; 60 A, 4 μm, 3.9 × 300 mm) protected by a Bondapak C18 guard column, as previously described (Havaux et al., 2003). The chromatography system consisted of a Waters 600E system controller and a Waters 996 photodiode srray detector. Pure carotenoids used for calibration were bought from Extrasynthèse 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 (Devaraj 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 (Ducruet, 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 thiobarbituric acid (TBA). After heating at 95°C for 20 min and centrifugation, the MDA-$(TBA)_2$ 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 KH_2PO_4-KOH, pH 7.0, and

35% methanol. The time of chromatography was 12 min, with a flow rate of 0.8 mL min^{-1}, an injected volume of 120 μL, and detection at visible light (532 nm). The average retention time of the MDA-$(TBA)_2$ adduct was 8 min. The levels of MDA were calculated using tetraethoxy-propane (Sigma-Aldrich) as a standard.

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 phenylmethylsulfonylfluoride, and 50 mM β-mercaptoethanol and centrifuged at 30,000*g* 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* CDSP32 thioredoxin 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 (Bréhelin 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; *NPQ1*, At1g08550; *NPQ4*, 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 Msr-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

Adams, W.W., Zarter, C.R., Ebbert, V., and Demmig-Adams, B. (2004). Photoprotective strategies of overwintering evergreens. Bioscience **54,** 41–49.

Alves-Rodrigues, A., and Shao, A. (2004). The science behind lutein. Toxicol. Lett. **150,** 57–83.

Aro, E.-M., Virgin, I., and Andersson, B. (1993). Photoinhibition of photosystem II. Inactivation, protein damage and turnover. Biochim. Biophys. Acta **1143,** 113–134.

Baroli, I., Do, A.D., Yamane, T., and Niyogi, K.K. (2003). Zeaxanthin accumulation in the absence of a functional xanthophyll cycle protects *Chlamydomonas reinhardtii* from photooxidative stress. Plant Cell **15,** 1–18.

Baroli, I., Gutman, B.L., Ledford, H.K., Shin, J.W., Chin, B.L., Havaux, M., and Niyogi, K.K. (2004). Photo-oxidative stress in a xanthophyll-deficient mutant of Chlamydomonas. J. Biol. Chem. **279,** 6337–6344.

Bergmüller, E., Porfirova, S., and Dörmann, P. (2003). Characterization of an Arabidopsis mutant deficient in gamma-tocopherol methyltransferase. Plant Mol. Biol. **52,** 1181–1190.

Böhm, F., Edge, R., Land, E.J., McGarvey, D.J., and Truscott, T.G. (1997). Carotenoids enhance vitamin E antioxidant efficiency. J. Am. Chem. Soc. **119,** 621–622.

Bramley, P.M., Elmadfa, I., Kafatos, A., Kelly, F.J., Manios, Y., Roxborough, H.E., Schuch, W., Sheehy, P.J.A., and Wagner, K.-H. (2000). Vitamin E. J. Sci. Food Agric. **80,** 913–938.

Bréhelin, C., Meyer, E.H., de Souris, J.P., Bonnard, G., and Meyer, Y. (2003). Resemblance and dissemblance of Arabidopsis type II peroxiredoxins: Similar sequences for divergent gene expression, protein localization, and activity. Plant Physiol. **132,** 2045–2057.

Broin, M., Cuiné, S., Eymery, F., and Rey, P. (2002). The plastidic 2-cysteine peroxiredoxin is a target for a thioredoxin involved in the protection of the photosynthetic apparatus against oxidative damage. Plant Cell **14,** 1417–1432.

Broin, M., and Rey, P. (2003). Potato plants lacking the CDSP32 plastidic thioredoxin exhibit over-oxidation of the BAS1 2-cys peroxiredoxin and increased lipid peroxidation in thylakoids under photooxidative stress. Plant Physiol. **132,** 1335–1343.

Browse, J., McCourt, P.J., and Somerville, C.R. (1986). Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal. Biochem. **152,** 141–145.

Carrier, P., Baryla, A., and Havaux, M. (2003). Cadmium distribution and microlocalization in oilseed rape (*Brassica napus*) after long-term growth on cadmium-contamined soil. Planta **216,** 939–950.

Chitchumroonchokchai, C., Bomser, J.A., Glamm, J.E., and Failla, M.L. (2004). Xanthophylls and α-tocopherol decrease UVB-induced lipid peroxidation and stress signaling in human lens epithelial cells. J. Nutr. **134,** 3225–3232.

Collakova, E., and DellaPenna, D. (2001). Isolation and functional analysis of homogentisate phytyltransferase from *Synechocystis* sp. PCC 6803 and Arabidopsis. Plant Physiol. **127,** 1113–1124.

Dähnhardt, D., Falk, J., Appel, J., van der Kooij, T.A.W., Schulz-Friedrich, R., and Krupinska, K. (2002). The hydroxyphenylpyruvate dioxygenase from *Synechocystis* sp. PCC 6803 is not required for plastoquinone biosynthesis. FEBS Lett. **523,** 177–181.

Delong, J.M., and Steffen, K.L. (1997). Photosynthetic function, lipid peroxidation and α-tocopherol content in spinach leaves during exposure to UV-B radiation. Can. J. Plant Sci. **77,** 453–459.

Demmig-Adams, B., and Adams III, W.W. (2000). Harvesting sunlight safely. Nature **403,** 371–374.

Demmig-Adams, B., and Adams III, W.W. (2002). Antioxidants in photosynthesis and human nutrition. Science **298,** 2149–2153.

Devaraj, B., Usa, M., and Inaba, H. (1997). Biophotons: Ultraweak light emission from living systems. Curr. Opin. Solid State Mater. Sci. **2,** 188–193.

Dietz, K.-J. (2003). Plant peroxiredoxins. Annu. Rev. Plant Biol. **54,** 93–107.

Di Mascio, P., Devasagayam, T.P., Kaiser, S., and Sies, H. (1990). Carotenoids, tocopherols, and thiols as biological singlet molecular oxygen quenchers. Biochem. Soc. Trans. **18,** 1054–1056.

Ducruet, J.M. (2003). Chlorophyll thermoluminescence of leaf discs: Simple instruments and progress in signal interpretation open the way to new ecophysiological indicators. J. Exp. Bot. **54,** 2419–2430.

Fryer, M.J. (1992). The antioxidant effects of thylakoid vitamin E (α-tocopherol). Plant Cell Environ. **15,** 381–392.

Fryer, M.J., Andrews, J.R., Oxborough, K., Mullineaux, P.M., and Baker, N.R. (2002). Relationship between CO_2 assimilation, photosynthetic electron transport, and active O-2 metabolism in leaves of maize in the field during periods of low temperature. Plant Physiol. **116,** 571–580.

Grasses, T., Grimm, B., Koroleva, O., and Jahns, P. (2001). Loss of α-tocopherol in tobacco plants with decreased geranylgeranyl reductase activity does not modify photosynthesis in optimal growth conditions but increases sensitivity to high-light stress. Planta **213,** 620–628.

Grusak, M.A., and DellaPenna, D. (1999). Improving the nutrient composition of plants to enhance human nutrition and health. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50,** 133–161.

Havaux, M. (1998). Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci. **3,** 147–151.

Havaux, M. (2003). Spontaneous and thermoinduced photon emission: New methods to detect and quantify oxidative stress in plants. Trends Plant Sci. **8,** 409–413.

Havaux, M., Bonfils, J.-P., Lütz, C., and Niyogi, K.K. (2000). Photodamage of the photosynthetic apparatus and its dependence on the leaf developmental stage in the *npq1* Arabidopsis mutant deficient in the xanthophyll cycle enzyme violaxanthin de-epoxidase. Plant Physiol. **124,** 273–284.

Havaux, M., and Kloppstech, K. (2001). The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in *Arabidopsis npq* and *tt* mutants. Planta **213,** 953–966.

Havaux, M., Lütz, C., and Grimm, B. (2003). Chloroplast membrane photostability in *chlP* transgenic tobacco plants deficient in tocopherols. Plant Physiol. **132,** 300–310.

Havaux, M., and Niyogi, K.K. (1999). The violaxanthin cycle protects plants from photo-oxidative damage by more than one mechanism. Proc. Natl. Acad. Sci. USA **96,** 8762–8767.

Hideg, E., Kalai, T., Hideg, K., and Vass, I. (1998). Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. Biochemistry **37,** 11405–11411.

Hieber, A.D., Kawabata, O., and Yamamoto, H.Y. (2004). Significance of the lipid phase in the dynamics and functions of the xanthophyll cycle as revealed by PsbS overexpression in tobacco and in-vitro de-epoxidation in monogalactosyldiacylglycerol micelles. Plant Cell Physiol. **45,** 92–102.

Holt, N.E., Zigmantas, D., Valkunas, L., Li, X.P., Niyogi, K.K., and Fleming, G.R. (2005). Carotenoid cation formation and the regulation of photosynthetic light harvesting. Science **307,** 433–436.

Horton, P., and Ruban, A. (2005). Molecular design of the photosystem II light-harvesting antenna: Photosynthesis and photoprotection. J. Exp. Bot. **56,** 365–373.

Kamal-Eldin, A., and Appelqvist, L.A. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids **31,** 671–701.

Kanwischer, M., Porfirova, S., Bergmüller, E., and Dörmann, P. (2005). Alterations in tocopherol cyclase (VTE1) activity in transgenic and mutant plants of *Arabidopsis* affect tocopherol content, tocopherol composition and oxidative stress. Plant Physiol. **137,** 713–723.

Knox, J.P., and Dodge, A.D. (1985). The photodynamic action of eosin, a singlet-oxygen generator. Planta **164,** 22–34.

Krinsky, N.I., Landrum, J.T., and Bone, R.A. (2003). Biological mechanisms of the protective role of lutein and zeaxanthin in the eye. Annu. Rev. Nutr. **23,** 171–201.

Li, X.-P., Björkman, O., Shih, C., Grossman, A.R., Rosenquist, M., Jansson, S., and Niyogi, K.K. (2000). A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature **403,** 391–395.

Lykkesfeldt, J. (2000). Determination of ascorbic acid and dehydroascorbic acid in biological samples by high-performance liquid chromatography using subtraction methods: Reliable reduction with Tris[2-carboxyethyl]phosphine hydrochloride. Anal. Biochem. **282,** 89–93.

Macko, S., Wehner, A., and Jahns, P. (2002). Comparison of violaxanthin de-epoxidation from the stroma and lumen sides of isolated thylakoid membranes from Arabidopsis: Implications for the mechanism of de-epoxidation. Planta **219,** 309–314.

Maeda, H., Sakuragi, Y., Bryant, D.A., and Dellapenna, D. (2005). Tocopherols protect Synechocystis sp. strain PCC 6803 from lipid peroxidation. Plant Physiol. **138,** 1422–1435.

Morosinotto, T., Baronio, R., and Bassi, R. (2002). Dynamics of chromophore binding to Lhc proteins in vivo and in vitro during operation of xanthophyll cycle. J. Biol. Chem. **277,** 36913–36920.

Morosinotto, T., Caffari, S., Dall'Osto, L., and Bassi, R. (2003). Mechanistic aspects of the xanthophyll dynamics in higher plant thylakoids. Physiol. Plant. **119,** 347–354.

Müller, P., Li, X.P., and Niyogi, K.K. (2001). Non-photochemical quenching. A response to excess light energy. Plant Physiol. **125,** 1558–1566.

Müller-Moulé, P., Golan, T., and Niyogi, K.K. (2004). Ascorbate-deficient mutants of Arabidopsis grow in high light despite chronic photooxidative stress. Plant Physiol. **134,** 1163–1172.

Müller-Moulé, P., Havaux, M., and Niyogi, K.K. (2003). Zeaxanthin deficiency enhances the high light sensitivity of an ascorbate-deficient mutant of Arabidopsis. Plant Physiol. **133,** 748–760.

Munné-Bosch, S., and Alegre, L. (2000). Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. Planta **210,** 925–931.

Munné-Bosch, S., and Alegre, L. (2002). The function of tocopherols and tocotrienols in plants. Crit. Rev. Plant Sci. **21,** 31–57.

Nishiyama, Y., Allakhverdiev, S.I., Yamamoto, H., Hayashi, H., and Murata, N. (2004). Singlet oxygen inhibits the repair of photosystem II by suppressing the translation elongation of the D1 protein in *Synechocystis* sp. PCC6803. Biochemistry **43,** 11321–11330.

Niyogi, K.K., Grossman, A.R., and Björkman, O. (1998). Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. Plant Cell **10,** 1121–1134.

Öquist, G., and Huner, N. P. (2003). Photosynthesis of overwintering evergreen plants. Annu. Rev. Plant Biol. **54,** 329–55.

Palozza, P., and Krinsky, N.I. (1992). β-Carotene and α-tocopherol are synergistic antioxidants. Arch. Biochem. Biophys. **297,** 184–187.

Porfirova, S., Bergmüller, E., Tropf, S., Lemke, R., and Dörman, P. (2002). Isolation of an *Arabidopsis* mutant lacking vitamin E and identification of a cyclase essential for all tocopherol biosynthesis. Proc. Natl. Acad. Sci. USA **99,** 12495–12500.

Rey, P., Cuiné, S., Eymery, F., Garin, J., Court, M., Jacquot, J.P., Rouhier, N., and Broin, M. (2005). Analysis of the proteins targeted by CDSP32, a plastidic thioredoxin participating in oxidative stress responses. Plant J. **41,** 31–42.

Ricciarelli, R., Zungg, J.-M., and Azzi, A. (2001). Vitamin E: Protective role of a Janus molecule. FASEB J. **15,** 2314–2325.

Rouhier, N., et al. (2004). Poplar peroxiredoxin Q. A thioredoxin-linked chloroplast antioxidant functional in pathogen defense. Plant Physiol. **134,** 1027–1038.

Sanchez-Mata, M.C., Camara-Hurtado, M., Diez-Marques, C., and Torija-Isasa, M.E. (2000). Comparison of high-performance liquid chromatography and spectrofluorimetry for vitamin C analysis of green beans (*Phaseolus vulgaris* L.). Eur. Food Res. Technol. **210,** 220–225.

Sattler, S.E., Gilliland, L.U., Magallanes-Lunback, M., Pollard, M., and DellaPenna, D. (2004). Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. Plant Cell **16,** 1419–1432.

Shpilyov, A., Zinchenko, V.V., Shestakov, S.V., Grimm, B., and Lokstein, H. (2005). Inactivation of the geranylgeranyl reducatse (*ChlP*) gene in the cyanobacterium *Synechocystis* sp PCC 6803. Biochim. Biophys. Acta **1706,** 195–203.

Stadtman, E.R., Moskowitz, J., Berlett, B.S., and Levine, R.L. (2002). Cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism. Mol. Cell. Biochem. **234/235,** 3–9.

Tanaka, E., Oster, U., Kruse, E., Rüdiger, W., and Grimm, B. (1999). Reduced activity of geranylgeranyl reductase leads to loss of chlorophyll and tocopherol and to partially geranylgeranylated chlorophyll in transgenic tobacco plants expressing antisense RNA for geranylgeranyl reductase. Plant Physiol. **120,** 695–704.

Telfer, A. (2002). What is β-carotene doing in the photosystem II reaction center? Philos. Trans. R. Soc. Lond. B Biol. Sci. **357,** 1431–1440.

Trebst, A., Depka, B., and Höllander-Czytko, H. (2002). A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in *Chlamydomonas reinhardtii.* FEBS Lett. **516,** 156–160.

Trebst, A., Depka, B., Jäger, J., and Oettmeier, W. (2004). Reversal of the inhibition of photosynthesis by herbicides affecting hydroxyphenylpyruvate dioxygenase by plastoquinone and tocopheryl derivatives in *Chlamydomonas reinhardtii.* Pest Manag. Sci. **60,** 669–674.

Vavilin, D.V., and Ducruet, J.-M. (1998). The origin of 115–130°C thermoluminescence bands in chlorophyll containing material. Photochem. Photobiol. **68,** 191–198.

Vavilin, D.V., Ducruet, J.-M., Matorin, D.N., Venediktov, P.S., and Rubin, A.B. (1998). Membrane lipid peroxidation, cell viability and photosystem II activity in the green alga *Chlorella pyrenoidosa* subjected to various stress conditions. J. Photochem. Photobiol. B Biol. **42,** 233–239.

Vieira Dos Santos, C., Cuiné, S., Rouhier, N., and Rey, P. (2005). The Arabidopsis plastidic methionine sulfoxide reductase B proteins. Sequence and activity characteristics, comparison of the expression with plastidic methionine sulfoxide reductase A, and induction by photooxidative stress. Plant Physiol. **138,** 909–922.

Wagner, D., Przybyla, D., Op den Camp, R., Kim, C., Landgraf, F., Lee, K.P., Wursch, M., Laloi, C., Nater, M., Hideg, E., and Apel, K. (2004). The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana.* Science **306,** 1183–1185.

Wang, X., and Quinn, P.J. (2000). The location and function of vitamin E in membranes. Mol. Membr. Biol. **17,** 143–156.

Wrona, M., Korytowski, W., Rozanowska, M., Sarna, T., and Truscott, T.G. (2003). Cooperation of antioxidants in protection against photosensitized oxidation. Free Radic. Biol. Med. **35,** 1319–1329.

Wrona, M., Rozanowska, M., and Sarna, T. (2004). Zeaxanthin in combination with ascorbic acid or alpha-tocopherol protects ARPE-19 cells against photosensitized peroxidation of lipids. Free Radic. Biol. Med. **36,** 1094–1101.

Vitamin E Protects against Photoinhibition and Photooxidative Stress in *Arabidopsis thaliana*
Michel Havaux, Françoise Eymery, Svetlana Porfirova, Pascal Rey and Peter Dörmann
Plant Cell 2005;17;3451-3469; originally published online October 28, 2005;
DOI 10.1105/tpc.105.037036

This information is current as of September 23, 2019

References	This article cites 75 articles, 25 of which can be accessed free at: /content/17/12/3451.full.html#ref-list-1
Permissions	https://www.copyright.com/ccc/openurl.do?sid=pd_hw1532298X&issn=1532298X&WT.mc_id=pd_hw1532298X
eTOCs	Sign up for eTOCs at: http://www.plantcell.org/cgi/alerts/ctmain
CiteTrack Alerts	Sign up for CiteTrack Alerts at: http://www.plantcell.org/cgi/alerts/ctmain
Subscription Information	Subscription Information for *The Plant Cell* and *Plant Physiology* is available at: http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY