Overlapping Photoprotective Function of Vitamin E and Carotenoids in *Chlamydomonas*1[C][OA]

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Tocopherols (vitamin E) and carotenoids are the two most abundant groups of lipid-soluble antioxidants in the chloroplast. Carotenoids are well known for their roles in protecting against photooxidative stress, whereas the photoprotective functions of tocopherols have only recently been examined experimentally. In addition, little is known about the functional overlap of carotenoids and tocopherols in vivo. To investigate this possible overlap, *Chlamydomonas reinhardtii* strains were engineered to overproduce tocopherols by chloroplast transformation with non-codon-optimized and codon-optimized versions of the homogeniculate phytyltransferase vitamin E2 (VTE2) from *Synechocystis* and by nuclear transformation with VTE2 from *C. reinhardtii*, which resulted in 1.6-fold, 5-fold to 10-fold, and more than 10-fold increases in total tocopherol content, respectively. To test if tocopherol overproduction can compensate for carotenoid deficiency in terms of antioxidant function, the nuclear VTE2 gene from *C. reinhardtii* was overexpressed in the *npq1 lor1* double mutant, which lacks zeaxanthin and lutein. Following transfer to high light, the *npq1 lor1* strains that overaccumulated tocopherols showed increased resistance for up to 2 d and higher efficiency of photosystem II, and they were also much more resistant to other oxidative stresses. These results suggest an overlapping functions of tocopherols and carotenoids in protection against photooxidative stress.

Maintaining a balance between the capture and the use of light energy is essential for the survival of oxygenic photosynthetic organisms. Environmental stresses that disrupt this balance often result in the production of damaging reactive oxygen species (ROS) and eventual cell death. High-light (HL) stress, for example, can lead to the increased formation of ROS such as singlet oxygen (\(1\text{O}_2^*\)), superoxide (\(\text{O}_2^-\)), hydrogen peroxy (\(\text{H}_2\text{O}_2\)), and hydroxyl radicals (Niyogi, 1999; Fryer et al., 2002). ROS can directly damage lipids, proteins, pigments, and nucleic acids in their immediate vicinity if they are not scavenged. Thylakoid membrane lipids are especially susceptible to oxidation because of the abundance of polyunsaturated fatty acids. Oxidation of these lipids by ROS produces lipid hydroperoxides and initiates lipid peroxidation chain reactions, which can eventually destroy the integrity of the chloroplast membrane and cause the death of the organism (Niyogi, 1999).

Oxygenic photosynthetic organisms have evolved multiple mechanisms to cope with the inevitable generation of ROS as by-products of oxidative metabolism. In particular, algae and plants often increase the expression of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase (Rossel et al., 2002), and accumulate antioxidant small molecules, such as ascorbate, glutathione, carotenoids, and tocopherols, to enhance their ROS-scavenging ability in response to photooxidative stress (Demmig-Adams and Adams, 1992; Müller-Moulé et al., 2003). Ascorbate and glutathione are the major hydrophilic antioxidants and are found in cytosolic, mitochondrial, chloroplastic, and nuclear aqueous compartments (Mittler, 2002). Carotenoids and tocopherols are the major lipid-soluble antioxidants and are mainly located in the chloroplast envelope and thylakoid membrane, where photosynthetic light harvesting and electron transport occur.

Carotenoids are a diverse group of \(\text{C}_{40}\) tetrapertene pigments that are synthesized by all photosynthetic organisms as well as by some nonphotosynthetic bacteria and fungi (Walter and Strack, 2011). There are two main classes of naturally occurring carotenoids: carotenes, which are hydrocarbons, such as \(\beta\)-carotene and \(\alpha\)-carotene, and xanthophylls, which are oxygenated derivatives of carotenes, such as zeaxanthin and lutein. In algae and plants, the majority of the carotenoids, together with chlorophylls, bind to...
proteins to form functional pigment-protein complexes embedded in the thylakoid membrane (Havaux, 1998; Baroli and Niyogi, 2000).

Tocopherols, collectively known as vitamin E, are a class of lipid-soluble compounds that are produced exclusively by plants, algae, and some cyanobacteria. Four types of tocopherols, α, β, γ, and δ, are synthesized in nature and differ structurally in the number and position of methyl groups on the chromanol head group. All tocopherols are amphipathic molecules with the polar head group exposed to the membrane surface and a hydrophobic tail incorporated into the membrane. Whereas carotenoids are mainly bound by the thylakoid pigment-protein complexes, tocopherols are free in the lipid matrix of the thylakoid membrane. Tocopherols are synthesized at the inner chloroplast envelope by a pathway that is conserved in cyanobacteria and plants (Fig. 1; DellaPenna and Pogson, 2006; Mène-Saffrane and DellaPenna, 2010). The formation of 2-methyl-6-phytylbenzoquinone (MPBQ) from the condensation of homogentisic acid and a prenyl side chain (phytlyldiphosphate) is the first committed step in the pathway and is catalyzed by homogentisate phytyltransferase, VITAMIN E2 (VTE2), which is a membrane-bound chloroplast enzyme (Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002). MPBQ can be methylated to form 2,3-dimethyl-6-phytylbenzoquinone by MPBQ methyltransferase (VTE3). MPBQ and 2,3-dimethyl-6-phytylbenzoquinone can then be cyclized via tocopherol cyclase (VTE1) to form δ- and γ-tocopherol, respectively. The last enzyme of the pathway, γ-tocopherol methyltransferase (VTE4), methylates δ- and γ-tocopherol to form β- and α-tocopherol, respectively.

The protective function of carotenoids and tocopherols is largely based on their antioxidative potential. Some carotenoids (specifically xanthophylls) are involved in quenching singlet chlorophyll (1-Chl*), thereby promoting nonphotochemical quenching (NPQ) to regulate photosynthetic light harvesting (Müller et al., 2001; de Bianchi et al., 2010; Ruban and Johnson, 2010; Jahns and Holzwarth, 2011). In addition to their role in NPQ, carotenoids can function as antioxidants to quench triplet chlorophyll (1-Chl*) and 1O2* and to inhibit lipid peroxidation (Demmig-Adams et al., 1996; Frank and Cogdell, 1996; Baroli and Niyogi, 2000). Quenching of 1-Chl* and 1O2* occurs by a direct transfer of excitation energy to the triplet state of carotenoids and thus requires the close proximity of carotenoids. Experiments with reconstituted light-harvesting complex II pigment-protein complexes have shown that lutein, zeaxanthin, and violaxanthin can quench both 1-Chl* and 1O2* to protect against chlorophyll photobleaching (Croce et al., 1999). Inhibition of lipid peroxidation reactions by carotenoids involves electron transfer or an additional reaction. Evidence for the interaction of lutein or zeaxanthin with free radicals to terminate lipid peroxidation reactions has been reported in artificial lipid membranes (Sujak et al., 1999), but the extent to which this occurs in vivo is unknown.

Similar to carotenoids, the antioxidant functions of tocopherols are thought to include scavenging lipid peroxide radicals to terminate lipid peroxidation chain reactions and physically quenching 1O2* or chemically scavenging 1O2* (Munné-Bosch and Alegre, 2002). Tocopherols scavenge lipid peroxyl radicals by donating an electron from the chromanol head group, and tocopheryl radicals are then formed. Tocopheryl radicals may be recycled back to tocopherol by interacting with other antioxidants such as ascorbate (Smirnoff and Wheeler, 2000) or may be converted to other oxidation products (Kamal-Eldin and Appelqvist, 1996). Direct physical quenching of 1O2* by tocopherols occurs through a charge transfer mechanism, during which 1O2* is deactivated to 3O2. Tocopherols can also react chemically with 1O2* to produce an intermediate hydroperoxydienone that decomposes to form tocopherol quinone and tocopherol quinone-epoxide (Murkovic et al., 1997). Furthermore, it has been shown that tocopherol can react with other reactive species such as alkoyl radicals (Kulás and Ackman, 2001), peroxynitrite (Fredstrom, 2002), and O2− (Fryer, 1992).

There is some evidence suggesting that these two classes of molecules have overlapping antioxidative roles in vivo. The Arabidopsis (Arabidopsis thaliana) npq1 mutant, which lacks zeaxanthin, accumulates more α-tocopherol in young leaves exposed to HL, suggesting that a high amount of tocopherol can compensate for the lack of zeaxanthin (Havaux et al., 2000). Conversely, the Arabidopsis ete1 mutant, which

![Image](35x101 to 274x363)

**Figure 1.** Tocopherol biosynthetic pathways in C. reinhardtii. VTE1, Tocopherol cyclase; VTE2, homogentisate phytyltransferase; VTE3, MPBQ methyltransferase; VTE4, γ-tocopherol methyltransferase.
is tocopherol deficient, accumulates more zeaxanthin in HL than the wild type (Havaux et al., 2005). Similarly, *Synechocystis* tocopherol-deficient mutants are more sensitive to HL treatment in the presence of sublethal levels of norflurazon, an inhibitor of carotenoid synthesis, suggesting that carotenoids and tocopherols have overlapping functions or functionally interact in protecting against lipid peroxidation and HL stress (Maeda et al., 2005). However, in the *Chlamydomonas reinhardtii npq1 lor1* double mutant, which lacks both zeaxanthin and lutein, the level of α-tocopherol is not higher than that of the wild type before HL treatment or during the first 6 h in HL (Ledford et al., 2004). Nevertheless, some suppressors of npq1 lor1 (Baroli et al., 2003) appeared to have elevated tocopherols (K.K. Niyogi, unpublished data). Although the notion of overlapping functions of carotenoids and tocopherols is very intriguing, it has been very difficult to assess how much functional overlap actually exists between these two classes of molecules. The *C. reinhardtii npq1 lor1* double mutant is hypersensitive to \( \text{O}_2^* \) stress induced by exposure to photosensitizers and bleaches when grown under HL conditions (Niyogi et al., 1997), therefore providing a sensitized background that can help to uncover functional overlap in antioxidant activity in vivo.

Because previous experiments were suggestive, we decided to directly test the functional overlap between carotenoids and tocopherols using a new approach. We increased tocopherol content in the *C. reinhardtii npq1 lor1* mutant background through overexpression of *VTE2* to test if elevated tocopherols could compensate for the lack of xanthophylls in terms of their antioxidant functions. The npq1 lor1 strains in which tocopherol was overaccumulated were obtained either by chloroplast transformation of codon-optimized *Synechocystis* VTE2 or nuclear transformation of *C. reinhardtii* VTE2. These new strains were much more resistant to HL or oxidative stress than npq1 lor1, indicating that tocopherols and xanthophylls have overlapping functions in protection against photooxidative stress.

**RESULTS**

**Overexpression of VTE2 by Chloroplast or Nuclear Transformation**

Studies in Arabidopsis and *Synechocystis* sp. PCC6803 have shown that *AtVTE2* and *SynVTE2* encode homogentisate phytyltransferase (Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002) and that VTE2 activity is limiting for tocopherol synthesis in both nonstressed and stressed Arabidopsis leaves (Collakova and DellaPenna, 2003a, 2003b). Because *Synechocystis* sp. PCC6803 and the *C. reinhardtii* chloroplast genome share similar codon usage and GC content, *SynVTE2* from *Synechocystis* sp. PCC6803 was overexpressed in the *C. reinhardtii* chloroplast in an attempt to overproduce tocopherols. The *SynVTE2* gene was amplified from *Synechocystis* sp. PCC6803 genomic DNA and cloned into a chloroplast expression vector, p72-*rbcL* (Bateman and Purton, 2000), containing *rbcL* 5′ and 3′ untranslated regions (Fig. 2A). The resulting overexpression vector (p72-*rbcL-SynVTE2*) was transformed into the chloroplast of *C. reinhardtii psbH::aadA* by biolistic bombardment to insert *SynVTE2* into the neutral site downstream of *psbH* and to rescue the *psbH* disruption mutant to photoautotrophic growth. Primary transformants of *rbcL-SynVTE2* were selected for photoautotrophic growth on minimal medium and then screened for the loss of the spectinomycin resistance phenotype conferred by the *aadA* marker (Bateman and Purton, 2000).

After more than four rounds of streaking on HS medium, the homoplasmic transformant lines were confirmed by PCR using primers flanking the insertion site (Fig. 2B).

Total tocopherol content and composition in cells grown photoautotrophically under low light (LL; 50 μmol photons m \(^{-2}\) s \(^{-1}\)) to a density of 1 to 1.5 × 10^6 cells mL \(^{-1}\) (early exponential phase) were measured by normal-phase HPLC. The best *SynVTE2*-overexpressing

![Figure 2](image-url)
lines contained 1.6-fold higher total tocopherol levels (13.0 ± 0.6 pmol per 10^6 cells) than the control strain (8.0 ± 0.2 pmol per 10^6 cells). Both the control rbcL and rbcL-SynVTE2 strains accumulated predominantly α-tocopherol, although β-tocopherol levels were also elevated to 2.8 pmol per 10^6 cells in rbcL-SynVTE2 (Fig. 3).

The increase in tocopherol content was not as high as expected based on the previous experiments performed in Arabidopsis (Collakova and DellaPenna, 2003b), possibly due to the fact that the codon usage of SynVTE2 was close to but not exactly matching that of the C. reinhardtii chloroplast (Nakamura et al., 2000). In order to obtain higher tocopherol production in C. reinhardtii, a synthetic SynVTE2 gene (SynVTE2ct) whose codon usage was optimized to match that of the C. reinhardtii chloroplast genome was used. The overexpression vector p72-rbcL-SynVTE2ct was constructed by simply replacing SynVTE2 with SynVTE2ct (Fig. 2A), and homoplasmic transformants were identified by PCR after chloroplast transformation into the psl18::aadA mutant (Fig. 2B). In this case, SynVTE2ct overexpression in chloroplast resulted in an accumulation of four different tocopherols, α-, β-, γ-, and δ-tocopherols, and a 5- to 10-fold increase in total tocopherol content relative to the control, primarily because of an increase in β- and δ-tocopherol content (Fig. 3). The control rbcL strain accumulated total tocopherol at 8.0 ± 0.2 pmol per 10^6 cells, whereas the total tocopherol content of rbcL-SynVTE2ct ranged between 38.6 ± 2.0 and 83.0 ± 3.0 pmol per 10^6 cells.

In parallel, the putative VTE2 from C. reinhardtii (CrVTE2) was constitutively overexpressed in the C. reinhardtii nucleus as an alternative approach to increase tocopherol content. The VTE2 gene was amplified by PCR from C. reinhardtii genomic DNA and cloned into vector pSL18 under the control of the PSAD promoter and terminator linked to a paromycin resistance cassette (Depège et al., 2003; Pollock et al., 2003; Fig. 2A). The nuclear overexpression vector pSL18-CrVTE2 was transformed into both the wild type and the npq1 lor1 mutant background, and the corresponding transformants WT CrVTE2 and npq1 lor1 CrVTE2 were selected for paromycin resistance. When grown photoautotrophically to a density of 1 to 1.5 × 10^6 cells mL^{-1}, the best CrVTE2-overexpressing line in either the wild type or the npq1 lor1 background (WT CrVTE2 line 73 and npq1 lor1 CrVTE2 line 36) had more than a 10-fold increase in total tocopherol content relative to the controls (Fig. 3). The control wild type and npq1 lor1 strain accumulated total tocopherols at 11.4 ± 2.0 and 9.0 ± 0.4 pmol per 10^6 cells, respectively, whereas total tocopherol levels of WT CrVTE2 line 73 and npq1 lor1 line 36 were 118.4 ± 13.0 and 151.4 ± 16.4 pmol per 10^6 cells, respectively. Like chloroplast transformants containing rbcL-SynVTE2ct, the tocopherol composition changed significantly in both WT CrVTE2-73 and npq1 lor1 CrVTE2-36. Both strains contained α-, β-, γ-, and δ-tocopherols, and the increase of total tocopherol content was largely due to the accumulation of β- and δ-tocopherol (Fig. 3). Because nuclear transformants CrVTE2-73 and npq1 lor1 CrVTE2-36 had the highest percentage increase in total tocopherol content and their respective controls (the wild type and npq1 lor1) were in a near-isogenic background, these four strains were selected for the subsequent analysis. The pigment composition and content of the four strains grown in LL (50 μmol photons m^{-2} s^{-1}) were measured to confirm their genotypes (Table I).

**Figure 3.** Tocopherol content and composition in different transgenic strains. Cells were grown photoautotrophically in HS medium under LL to a density of 1 to 1.5 × 10^6 cells mL^{-1}. Cells from 4-mL samples were extracted and analyzed for tocopherols by normal-phase HPLC. Tocopherol levels are expressed as means ± so (n = 3). Total tocopherol levels in pmol per 10^6 cells are indicated above the error bar for each strain. Statistical significance for total tocopherol levels of all transgenic strains relative to controls was P < 0.001 (t test).

**Tocopherol Accumulation Is Dependent on the Age of the Culture**

It has been shown previously that a moderate increase in leaf tocopherol content is associated with aging in a variety of plants (Rise et al., 1989; Molina-Torres and Martinez, 1991; Tramontano et al., 1992; Lichtenthaler, 2007). To examine whether a similar age-dependent accumulation of tocopherol occurs in C. reinhardtii, wild-type, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2 cells were grown photoautotrophically under LL for up to 15 d and were assayed for their tocopherol content and composition during the continuous growth. Total tocopherol levels steadily increased in all four strains under the conditions tested (Fig. 4). In 3-d-old cultures, the total tocopherol contents in the wild type, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2 were 6.5 ± 0.2, 57.0 ± 3.0, 5.4 ± 0.4, and 101.6 ± 9.4 pmol per 10^6 cells, respectively. In 15-d-old cultures, the total tocopherol content in the four strains
increased to 47.4 ± 2.0, 267.6 ± 22.4, 35.0 ± 0.6, and 337.2 ± 25.2 pmol per 10^6 cells, respectively. Whereas both wild-type and npq1 lor1 cells accumulated predominantly α-tocopherol, the major tocopherols accumulated in both WT CrVTE2 and npq1 lor1 CrVTE2 shifted from δ- and β-tocopherol in relatively younger cultures to α- and β-tocopherols in older cultures (Fig. 4).

**Tocopherol Overproduction Partially Protects npq1 lor1 from HL Stress**

Due to the lack of both zeaxanthin and lutein in HL, the npq1 lor1 double mutant is impaired at multiple levels of photoprotection, including quenching \(^{1}\text{Chl}^*\) (i.e. NPQ), quenching \(^{3}\text{Chl}^*\), and quenching \(^{1}\text{O}_2^*\) (Niyogi et al., 1997; Baroli et al., 2004). During HL exposure, the deficiency in these photoprotective mechanisms is expected to increase ROS levels in thylakoids and to cause severe photooxidative damage inside the cells of npq1 lor1. To test if tocopherol overproduction can compensate for carotenoid deficiency to protect against HL stress, wild-type, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2 cells were grown phototrophically in different light conditions. As shown in Figure 5, all four stains were able to grow under continuous LL. When exposed to HL on plates for 48 h, the wild type and WT CrVTE2 were able to grow, whereas the npq1 lor1 double mutant underwent photobleaching and died, as reported previously (Niyogi et al., 1997; Baroli et al., 2004). In contrast, npq1 lor1 CrVTE2

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**Figure 4.** Tocopherol accumulation kinetics in the wild type, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2. Cells were grown phototrophically in HS medium under LL for 15 d. Cells from 2- to 6-mL samples harvested at the indicated time points were extracted, and individual tocopherols were separated and quantified by normal-phase HPLC. Tocopherol levels are shown as means ± sd (n = 3). Total tocopherol levels in pmol per 10^6 cells are indicated above the error bar for each strain.
cells were able to stay green and survive in HL for up to 48 h. After shifting the plates back to LL for another 48 h, npq1 lor1 CrVTE2 completely resumed its growth, and the difference between npq1 lor1 and npq1 lor1 CrVTE2 became even more apparent (Fig. 5).

One important antioxidant function of tocopherol is thought to be in protecting the photosynthetic membrane from photooxidation and helping to provide an optimal environment for the photosynthetic machinery (Fryer, 1992; Munné-Bosch and Alegre, 2002).

To determine if tocopherol accumulation can function to protect PSII from photoinhibition under HL stress, the maximum chlorophyll fluorescence ratio ($F_v/F_m$), was determined in the wild type, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2 at various times after transfer of LL-grown liquid cultures to HL. All four strains exhibited similar $F_v/F_m$ values before shifting to HL (Fig. 6). In the wild type, the $F_v/F_m$ value initially decreased to 0.52 at 3 h, recovered to approximately 0.6 at 12 h, and then stabilized for the rest of the treatment. A similar trend was observed in WT CrVTE2, except that it consistently had a slightly higher $F_v/F_m$ value than the wild type after 6 h. However, in the npq1 lor1 double mutant, $F_v/F_m$ dramatically decreased to the lowest value of approximately 0.2 at 48 h. The $F_v/F_m$ value of npq1 lor1 CrVTE2 decreased in the first 12 h to 0.3, but it was able to recover to an intermediate level between that of the wild type and npq1 lor1 at 48 h.

The pigment composition and content of the four strains were measured before and after transferring LL (50 μmol photons m$^{-2}$ s$^{-1}$)-grown cells to HL (1,000 μmol photons m$^{-2}$ s$^{-1}$) for 48 h (Table I). Both the wild type and WT CrVTE2 maintained their total chlorophyll content and increased their carotenoid levels slightly. In contrast, the npq1 lor1 strain showed a dramatic decrease in both total chlorophyll and carotenoids levels, consistent with obvious bleaching of the cells after transfer to HL for 48 h, whereas npq1 lor1 CrVTE2 was able to maintain intermediate levels of its total chlorophyll and carotenoids after HL transfer.

**Tocopherol Overproduction Protects npq1 lor1 from Other Oxidative Stresses**

To examine if tocopherol overaccumulation can help to protect from other oxidative stresses, we examined the sensitivity of the wild type, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2 to different ROS induced by various pro-oxidants in LL. Concentrations of pro-oxidants were selected that were sublethal for the wild type. When exposed to the $1O_2$* generator Rose Bengal, to $H_2O_2$, and to O$_2^-$ generators methyl viologen and metronidazole, the npq1 lor1 double mutant was more sensitive than the wild type and WT CrVTE2 (Fig. 7). The addition of Rose Bengal, $H_2O_2$, or methyl viologen to agar plates severely retarded the growth of npq1 lor1, whereas npq1 lor1 completely bleached in the presence of metronidazole. In contrast, npq1 lor1 CrVTE2 showed resistance to all the conditions tested, suggesting that tocopherols can function to protect against multiple ROSs.

**DISCUSSION**

**Engineering Tocopherol Overproduction in C. reinhardtii by Overexpressing VTE2**

Overexpression of AtVTE2 increased total tocopherol levels up to 5- and 2-fold in Arabidopsis leaves and seeds, respectively, indicating that VTE2 activity is limiting in tocopherol biosynthesis (Collakova and DellaPenna, 2003a, 2003b). Therefore, the VTE2 gene was chosen as a target for metabolic engineering to overproduce tocopherol in C. reinhardtii. Tocopherol-overproducing strains were obtained by either chloroplast or nuclear transformation. Overexpressing codon-optimized SynVTE2ct (SynVTE2ct) in the chloroplast led to a 5- to 10-fold increase in total tocopherol content, whereas overexpressing putative CrVTE2 in the nucleus increased total tocopherol levels more than 10-fold (Fig. 3). It has been shown previously that C. reinhardtii chloroplast transformation results in very high transgenic expression levels in many cases (Franklin and Mayfield, 2004). However, the nuclear transformation of putative CrVTE2 was superior to chloroplast transformation of SynVTE2ct in an effort to obtain tocopherol-overproducing strains. One possible explanation is that tocopherol biosynthetic enzymes may interact to form specific complexes, possibly for efficient metabolic channeling, and the native C. rein-
hardtii VTE2 enzyme might be better than exogenous *Synechocystis* VTE2 in forming such a complex. Overexpression of putative *CrVTE2* resulted in the accumulation of tocopherol in *C. reinhardtii* (Figs. 3 and 4), confirming that the putative *CrVTE2* gene does encode homogentisate phytyltransferase and is involved in tocopherol synthesis. Overexpression of *CrVTE2* also resulted in a shift in tocopherol composition. The wild type and npq1 lor1 accumulated predominantly α-tocopherol, whereas WT CrVTE2 and npq1 lor1 CrVTE2 contained increased levels of α-, β-, γ-, and δ-tocopherols (Figs. 3 and 4). When overexpressing VTE2 to alleviate the VTE2 limitation in the tocopherol pathway, it is possible that other enzymes or their substrates become limiting in α-tocopherol synthesis. A limitation in VTE3 activity would lead to the increased levels of β- and δ-tocopherol, whereas a limitation in VTE4 activity would result in the accumulation of γ- and δ-tocopherol. The accumulation of β-, γ-, and δ-tocopherols in CrVTE2-overexpressing strains indicates that both VTE3 and VTE4 activities might limit α-tocopherol synthesis. It would be interesting to co-overexpress VTE2, VTE3, and VTE4 to test this hypothesis and possibly convert all other forms to α-tocopherol.

Altering flux through a metabolic pathway often has some effects on biochemically related pathways, especially when these pathways share some common substrates. For example, geranylgeranyl pyrophosphate is a common substrate for the carotenoid and tocopherol biosynthetic pathways. Mutation of phytoene synthase in *C. reinhardtii* results in increased levels of α-tocopherol, possibly due to an increased availability of geranylgeranyl pyrophosphate for tocopherol biosynthesis (McCarthy et al., 2004). Since the prenylation steps of the tocopherol and plastoquinone biosynthesis pathways require the same aromatic head group, homogentisic acid, it is conceivable that VTE2 overexpression might affect plastoquinone biosynthesis. Although we did not directly measure plastoquinone levels in tocopherol-overproducing strains, plastoquinone biosynthesis appeared to be uninhibited, because no obvious photoautotrophic growth phenotype was observed relative to the wild type. It has been reported that overexpression of Arabidopsis homogentisate solanesyltransferase (*AtHST*), which is involved in plastoquinone synthesis, led to an increase in both plastoquinone and tocopherol levels in wild-type Arabidopsis (Sadre et al., 2006; Venkatesh et al., 2006). Furthermore, tocopherol and plastoquinone levels were reduced below detection in the Arabidopsis *pds2* mutant, which contains a lesion in the *AtHST* gene (Norris et al., 1995; Tian et al., 2007). There seem to be interactions between the tocopherol and plastoquinone synthesis pathways, but it is likely not a competition in both Arabidopsis and *Chlamydomonas*.

**Tocopherols and Carotenoids Have Functional Overlap in Protecting against Photooxidative Stress**

Tocopherols are only synthesized by photosynthetic organisms, and the majority of tocopherols are localized in photosynthetic membranes (Lichtenthaler et al., 1995). Tocopherols and carotenoids protect against photooxidative damage by quenching singlet oxygen and excited singlet states of chlorophyll. Tocopherols are only synthesized by photosynthetic organisms, and the majority of tocopherols are localized in photosynthetic membranes (Lichtenthaler et al., 1995). Tocopherols and carotenoids protect against photooxidative damage by quenching singlet oxygen and excited singlet states of chlorophyll.
1981; Maeda and DellaPenna, 2007). Tocopherol levels increase dramatically during a variety of abiotic stresses, including HL stress (Havaux et al., 2000; Collakova and DellaPenna, 2003b; Müller-Moulé et al., 2003; Ledford et al., 2004; Golan et al., 2006), so it has long been assumed that tocopherols are involved in the protection of pigments and proteins of the photosynthetic apparatus and of thylakoid lipids against oxidative degradation. The isolation of mutants disrupting tocopherol synthesis has allowed several groups to initiate studies of tocopherol functions in plants. It was shown that tocopherol does have some antioxidant functions in plants, such as limiting non-enzymatic lipid peroxidation during seed dormancy, germination, and early seedling development (Sattler et al., 2004b, 2006) and protection from oxidative stress induced by heavy metals (Collin et al., 2008). Surprisingly, tocopherol-deficient mutants in both Arabidopsis and Synechocystis were indistinguishable from the wild type under optimal laboratory growth conditions (Collakova and DellaPenna, 2001; Porfiriova et al., 2002; Sattler et al., 2003, 2004a; Maeda et al., 2005, 2006; Sakuragi et al., 2006) and during HL stress (Dähnhardt et al., 2002; Maeda et al., 2005, 2006). Only during exposure to HL stress in combination with low temperature (2°C–8°C), Arabidopsis vte1 and vte2 mutants exhibited higher degrees of PSII photo-inhibition and lipid peroxidation than the wild type in leaf discs but not in whole plants (Havaux et al., 2005). However, subsequent studies on vte1 and vte2 mutants revealed that tocopherols play important roles in the development of transfer cell walls and the maintenance of photoassimilate export capacity during low-temperature acclimation, a response that is independent of light level (Maeda et al., 2005, 2008). These data collectively do not provide unequivocal support for the assumed role of tocopherols in protecting photosynthetic organisms from HL stress, and the lack of deleterious effects of tocopherol deficiency under HL could be due to compensation by other photoprotective mechanisms. Synechocystis tocopherol-deficient mutants are not more sensitive to HL treatment unless carotenoid synthesis is also inhibited, suggesting that tocopherols and carotenoids have overlapping functions in protecting against lipid peroxidation and HL stress (Maeda et al., 2005).

As one way to get further insights into the functional overlap between tocopherols and carotenoids, we tested directly if tocopherol overproduction can compensate for carotenoid deficiency by comparing the growth phenotype of npq1 lor1 with npq1 lor1 CrVTE2 under photooxidative stress. The npq1 lor1 double mutant provides an ideal background to study the antioxidant functions of tocopherols, because of its supersensitivity to photooxidative stress. As reported previously (Baroli et al., 2004), npq1 lor1 quickly bleached and died under HL (Fig. 5), was severely inhibited in PSII efficiency (Fig. 6), and was very sensitive to O$_2^*$, O$_2^+$, and H$_2$O$_2$ (Fig. 7). In contrast, npq1 lor1 CrVTE2 survived and stayed green in HL for up to 48 h (Fig. 5), indicating that elevated tocopherols can function to protect npq1 lor1 against HL stress. A higher PSII efficiency was observed in npq1 lor1 CrVTE2 when cultures were shifted to HL (Fig. 6), consistent with the suggestion that tocopherols help to protect PSII from photoinhibition (Trebst et al., 2002; Inoue et al., 2011). Altogether, these results strongly suggest that tocopherols and carotenoids have overlapping photoprotective functions in vivo.

Phenotypes similar to those of npq1 lor1 CrVTE2 were recently reported for a vte3 npq1 lor1 triple mutant of C. reinhardtii that accumulates β-tocopherol instead of α-tocopherol (Sirikhachornkit et al., 2009). Because npq1 lor1 CrVTE2 accumulated a significant amount of β-tocopherol, it is likely that a change in the composition of the tocopherol pool in npq1 lor1 CrVTE2 contributes to its ability to withstand HL. However, the npq1 lor1 CrVTE2 strain also showed additional phenotypes, such as resistance to multiple ROS including O$_2^*$, O$_2^+$, and H$_2$O$_2$ (Fig. 7), suggesting that an increased content of tocopherols is also important in conferring resistance to photooxidative stress.

Although it is clear that elevated tocopherols can improve the oxidative stress tolerance of npq1 lor1, it is also evident that the tocopherols were not able to replace entirely the function of the missing xanthophylls in this strain background. For example, a similar decline in F$_{v}$/F$_{m}$ was observed in npq1 lor1 and npq1 lor1 CrVTE2 during the first 12 h in HL, whereas the decrease in F$_{v}$/F$_{m}$ lasted only 3 h in the wild type and WT CrVTE2 (Fig. 6). This observation suggests that elevated tocopherols are not able to prevent the further decrease in PSII activity that occurs in the xanthophyll-deficient strain during HL stress. This might be related to a different localization of tocopherols, which are found in the lipid phase of the membrane and in plastoglobules, compared with xanthophylls, which are bound mainly to light-harvesting antenna proteins, where they have additional functions in quenching Chl* and Chl*. The difference between npq1 lor1 and npq1 lor1 CrVTE2 became evident after 12 h in HL (Fig. 6), suggesting that having extra tocopherols might have a positive effect on processes related to HL acclimation.

**MATERIALS AND METHODS**

**Strains and Growth Conditions**

The *Chlamydomonas reinhardtii* strains used in this work, wild-type 4A+ (mts-) and a near-isogenic strain of npq1 lor1 (Sirikhachornkit et al., 2009), are in the 137c genetic background (Dent et al., 2005). The psbH-and A disruption strain (O’Connor et al., 1998) was kindly provided by Saul Purton (University College London).

Cells were grown phototrophically in Tris-acetate-phosphate (TAP) medium or photoautotrophically in high-salt minimal (HS) medium (Harris, 1989). Strains were maintained on TAP agar medium either at very low light (10 μmol photons m$^{-2}$ s$^{-1}$) or in the dark. For tocopherol analysis, cells were grown in 100 mL of HS liquid culture under continuous LL (50 μmol photons m$^{-2}$ s$^{-1}$) for 15 d.

To test the effect of HL on growth in liquid cultures, cells were grown in 100 mL of HS medium under continuous LL to the early exponential phase (1 to 1.5 × 10$^6$ cells mL$^{-1}$) before being transferred to HL (500 μmol photons m$^{-2}$ s$^{-1}$) for 7 d.
m⁻² s⁻¹), as described previously (Baroli et al., 2004). To test the effect of HL and pro-oxidants on growth on agar plates, cells were grown in 100 mL of HS medium under LL until they reached a density of 1 to 1.5 × 10⁶ cells mL⁻¹. Cells were counted using a hemacytometer and concentrated to a density of 1 × 10⁶ cells mL⁻¹. Series dilutions of the concentrated culture were prepared, and 4 μL of these dilutions was spotted onto TAP agar plates with or without pro-oxidant. Rose Bengal (Sigma), methyl viologen (Sigma), metronidazole (Sigma), and H₂O₂ (Fisher) were prepared freshly before pouring onto plates, and plates were prepared 1 day prior to use. The plates were incubated in LL for 4 d with the exception of HL plates, which were incubated for 2 d at HL before being shifted back to LL.

Construction of Chloroplast Transformation Vectors and Chloroplast Transformation

Chloroplast transformation vector p72-rchl (Bateman and Purton, 2000) was obtained from Saul Purton. The open reading frame slr1736 (BA000022) encoding SynVTE2 was amplified by PCR from Synechocystis sp. PCC 6803 genomic DNA using primers designed to engineer a 5’ BspHI site and a 3’ BamHI site immediately outside the coding region. The SynVTE2_BspHI forward primer sequence was 5’-CATGTCATGATTTGGCAACATCTC-GAAG-3’ and the SynVTE2_BamHI reverse primer sequence was 5’-CGCGGATCCCTAAAAATAGTATTAGAAA-3’. The 0.9-kb PCR products were cloned into pCR-Blunt II-TOPO vector (Invitrogen) to generate plasmid pSynVTE2 and verified by sequencing. The plasmid pSynVTE2 was digested with BspHI and BamHI, and the resulting fragment was ligated into p72-rchl vector digested with NcoI and BamHI to generate plasmid p72-rchl-SynVTE2.

The SynVTE2ct gene was codon optimized for the C. reinhardtii chloroplast (GENEART). The synthetic SynVTE2ct gene was designed to contain an NcoI site right at the start codon and a PsI site right after the stop codon and then cloned using these enzymes into p72-rchl vector to generate plasmid p72-rchl-SynVTE2ct. C. reinhardtii chloroplast transformation of p72-rchl-SynVTE2 or p72-rchl-SynVTE2ct into recipient strain Cbl/HaadA was carried out using the PDS-1000/He particle-delivery system (Bio-Rad) as described previously (Boynton and Gillham, 1993), except that a rupture disk of 1,800 psi was used, and the medium under LL until they were homoplasmic. To confirm the homoplasmicity of the transformant colonies were restreaked four or more times on HS plates under LL and plates were prepared 1 d prior to use. The plates were incubated in LL for 3 min) and immediately frozen in liquid nitrogen.

Uptake (3,200g, 4°C, 3 min) and immediately frozen in liquid nitrogen. Tocopherols were extracted by vortexing in 200 μL of acetone for 1 min, and the acetone extracts were evaporated using a vacufuge concentrator (Eppendorf) and redissolved in 200 μL of hexane. The hexane extracts were filtered through a 2-μm filter, and 25 μL of the filtered extract was subjected to normal-phase HPLC on a 4.6 × 250-mm Luna 5-μm silica column (Phenomenex) at 42°C using a method described previously (Shintani et al., 2002). Tocopherols were detected via fluorescence with excitation at 295 nm and emission at 325 nm. The concentrations of different forms of tocopherol were determined using standard curves of the purified compounds (α-, γ-, and δ-tocopherol [Sigma] and β-tocopherol [Matreya]) at known concentrations and were normalized to cell number.

Pigment Analysis and Quantification

HPLC analysis of carotenoids and chlorophylls was done as described previously (Müller-Moulé et al., 2002). A total of three independent samples were measured. Carotenoids were quantified using standard curves of purified pigments (Sigma and Danish Hydraulic Institute) and normalized to cell number.

Chlorophyll Fluorescence Measurement

To measure chlorophyll fluorescence parameters, cells were grown in liquid HS medium to 1 to 1.5 × 10⁶ cells mL⁻¹. Culture aliquots of 5 to 8 mL were collected at the indicated time points after transfer to HL. Cells were deposited onto 25-mm (diameter), 12-μm (pore size) nitrocellulose filters by filtration and were dark adapted in a moist petri dish for 20 min prior to measurement. Chlorophyll fluorescence was measured using a pulse-amplitude modulation fluorometer (FMS2; Hansatech) as described previously (Baroli et al., 2004).

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