

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

Zhirong Li, Jay D. Keasling, and Krishna K. Niyogi*

Howard Hughes Medical Institute and Department of Plant and Microbial Biology (Z.L., K.K.N.) and Department of Chemical and Biomolecular Engineering and Department of Bioengineering (J.D.K.), University of California, Berkeley, California 94720; and Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720 (Z.L., J.D.K., K.K.N.)

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 homogentisate 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 (O_2^-), hydrogen peroxide (H_2O_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 C_{40} tetraterpene 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 β -carotene and α -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

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* Corresponding author; e-mail niyogi@berkeley.edu.

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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-Safrané and DellaPenna, 2010). The formation of 2-methyl-6-phytylbenzoquinone (MPBQ) from the condensation of homogentisic acid and a prenyl side chain (phytyl diphosphate) 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 ($^1\text{Chl}^*$), 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 ($^3\text{Chl}^*$) and $^1\text{O}_2^*$ and to inhibit lipid peroxidation (Demmig-Adams et al., 1996; Frank and Cogdell, 1996; Baroli and Niyogi, 2000). Quenching of $^3\text{Chl}^*$ and $^1\text{O}_2^*$ 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 $^3\text{Chl}^*$ and $^1\text{O}_2^*$ 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 peroxy radicals to terminate lipid peroxidation chain reactions and physically quenching $^1\text{O}_2^*$ or chemically scavenging $^1\text{O}_2^*$ (Munné-Bosch and Alegre, 2002). Tocopherols scavenge lipid peroxy radicals by donating an electron from the chromanol head group, and tocopheroxyl radicals are then formed. Tocopheroxyl 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 $^1\text{O}_2^*$ by tocopherols occurs through a charge transfer mechanism, during which $^1\text{O}_2^*$ is deactivated to $^3\text{O}_2$. Tocopherols can also react chemically with $^1\text{O}_2^*$ 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 alkoxyl radicals (Kulås and Ackman, 2001), peroxytrinitrite (Fredstrom, 2002), and O_2^- (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 *vte1* mutant, which

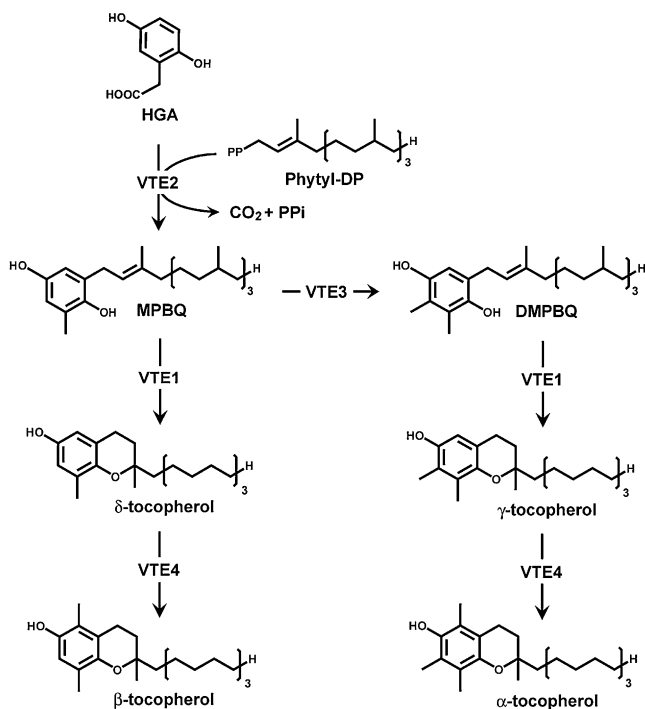


Figure 1. Tocopherol biosynthetic pathways in *C. reinhardtii*. VTE1, Tocopherol cyclase; VTE2, homogentisate phytyltransferase; VTE3, MPBQ methyltransferase; VTE4, γ -tocopherol methyltransferase.

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 $^1\text{O}_2^*$ 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; $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) 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

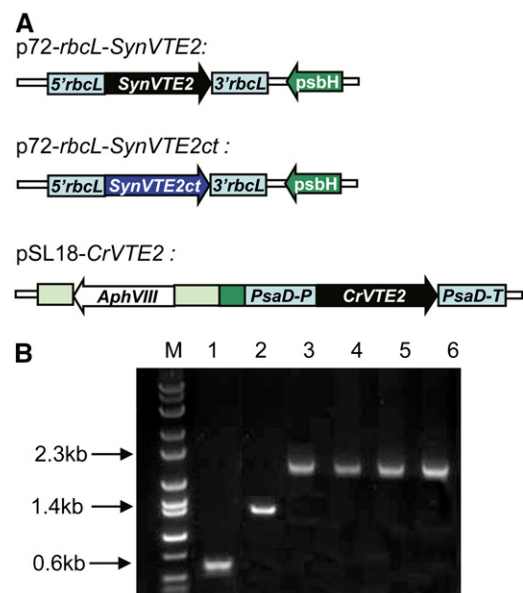


Figure 2. Three VTE2 transformation constructs used. A, Structures of the plasmids p72-*rbcl*-*SynVTE2*, p72-*rbcl*-*SynVTE2ct*, and pSL18-*CrVTE2*. Plasmids p72-*rbcl*-*SynVTE2* and p72-*rbcl*-*SynVTE2ct* are chloroplast transformation constructs, in which *rbcl* flanking sequences were used to drive the constitutive overexpression of *SynVTE2* and *SynVTE2ct* (codon-optimized *SynVTE2*), and the expression cassette was placed next to the *psbH* gene. Plasmid pSL18-*CrVTE2* is a nuclear transformation construct that carries the paromomycin resistance gene (*AphVIII*) and uses the *PSAD* promoter to drive the constitutive overexpression of *CrVTE2*. B, PCR gel showing homoplasmy of chloroplast transformants. Total genomic DNA from the transformants was used as template, and DNA segments containing the insertion site were amplified by PCR. Lane M, DNA ladder; lane 1, *psbH::aadA*; lane 2, *rbcl*; lanes 3 and 4, *rbcl*-*SynVTE2* lines 47 and 62; lanes 5 and 6, *rbcl*-*SynVTE2* lines 11 and 36. [See online article for color version of this figure.]

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 *psbH::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.

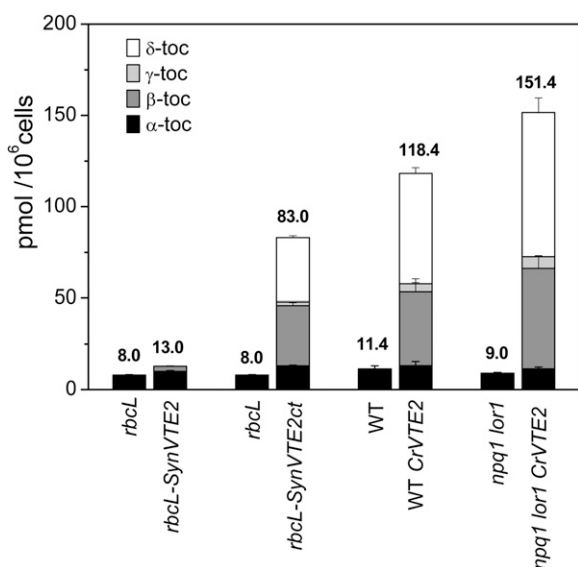


Figure 3. Tocopherol content and composition in different transgenic strains. Cells were grown photoautotrophically in HS medium under LL to a density of 1 to 1.5×10^6 cells mL^{-1} . Cells from 4-mL samples were extracted and analyzed for tocopherols by normal-phase HPLC. Tocopherol levels are expressed as means $\pm$ SD ($n = 3$). 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).

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 paromomycin 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 paromomycin 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 ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were measured to confirm their genotypes (Table I).

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 ± 2.0 , 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

Table 1. Pigment content of the wild type, WT *CrVTE2*, *npq1 lor1*, and *npq1 lor1 CrVTE2*

Pigment measurements were performed before and after transferring LL (50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)-grown cells to HL (500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) for 48 h. Data are normalized to cell number and presented as means $\pm$ SD ($n = 3$). N, Neoxanthin; Lo, linoxanthin; L, lutein; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin.

Strain	Light	Total Chlorophyll	Total Carotenoid	N+Lo	L	V	A	Z	V+A+Z	(A+Z)/(V+A+Z)	β -Carotene	α -Carotene
<i>pmol per 10⁶ cells</i>												
Wild type	LL	1,223.6 $\pm$ 25.5	527.4 $\pm$ 10.9	76.3 $\pm$ 1.0	173.2 $\pm$ 4.0	88.6 $\pm$ 1.9	10.6 $\pm$ 0.3	6.0 $\pm$ 0.3	105.2 $\pm$ 2.0	0.16 $\pm$ 0.00	136.7 $\pm$ 3.3	36.1 $\pm$ 1.2
	HL	1,227.7 $\pm$ 77.2	625.8 $\pm$ 53.2	77.73 $\pm$ 8.3	261.4 $\pm$ 21.4	101.7 $\pm$ 10.0	20.7 $\pm$ 1.3	17.2 $\pm$ 1.2	139.6 $\pm$ 11.3	0.27 $\pm$ 0.00	95.5 $\pm$ 8.5	51.6 $\pm$ 3.7
WT <i>CrVTE2</i>	LL	1,708.4 $\pm$ 56.0	768.1 $\pm$ 24.4	111.9 $\pm$ 3.4	240.8 $\pm$ 8.5	124.3 $\pm$ 3.2	12.9 $\pm$ 0.3	7.5 $\pm$ 0.2	144.7 $\pm$ 3.6	0.14 $\pm$ 0.00	224.1 $\pm$ 7.7	46.7 $\pm$ 1.9
	HL	1,809.0 $\pm$ 110.8	915.8 $\pm$ 50.3	121.7 $\pm$ 8.7	372.4 $\pm$ 18.9	122.6 $\pm$ 9.8	29.5 $\pm$ 0.6	23.0 $\pm$ 0.4	175.1 $\pm$ 10.3	0.30 $\pm$ 0.03	162.9 $\pm$ 9.2	83.7 $\pm$ 3.6
<i>npq1 lor1</i>	LL	1,254.6 $\pm$ 32.9	543.8 $\pm$ 4.1	61.0 $\pm$ 0.6	0	204.4 $\pm$ 1.2	15.3 $\pm$ 0.1	24.5 $\pm$ 0.1	244.2 $\pm$ 1.2	0.16 $\pm$ 0.00	208.5 $\pm$ 1.8	30.2 $\pm$ 0.5
	HL	31.1 $\pm$ 0.9	23.0 $\pm$ 2.6	1.9 $\pm$ 0.2	0	12.2 $\pm$ 0.7	1.8 $\pm$ 0.2	2.8 $\pm$ 0.3	16.8 $\pm$ 1.1	0.27 $\pm$ 0.01	3.4 $\pm$ 0.5	0.8 $\pm$ 0.1
<i>npq1 lor1 CrVTE2</i>	LL	1,477.7 $\pm$ 14.1	717.2 $\pm$ 4.5	66.3 $\pm$ 0.7	0	210.0 $\pm$ 1.0	14.0 $\pm$ 0.1	18.9 $\pm$ 0.7	242.9 $\pm$ 0.4	0.14 $\pm$ 0.00	357.2 $\pm$ 3.1	50.7 $\pm$ 0.4
	HL	743.9 $\pm$ 48.6	416.4 $\pm$ 18.7	39.3 $\pm$ 3.2	0	229.4 $\pm$ 10.3	16.7 $\pm$ 0.4	19.6 $\pm$ 0.8	265.7 $\pm$ 10.5	0.14 $\pm$ 0.02	89.7 $\pm$ 8.9	21.7 $\pm$ 3.0

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 photoheterotrophically in different light conditions. As shown in Figure 5, all four strains 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*

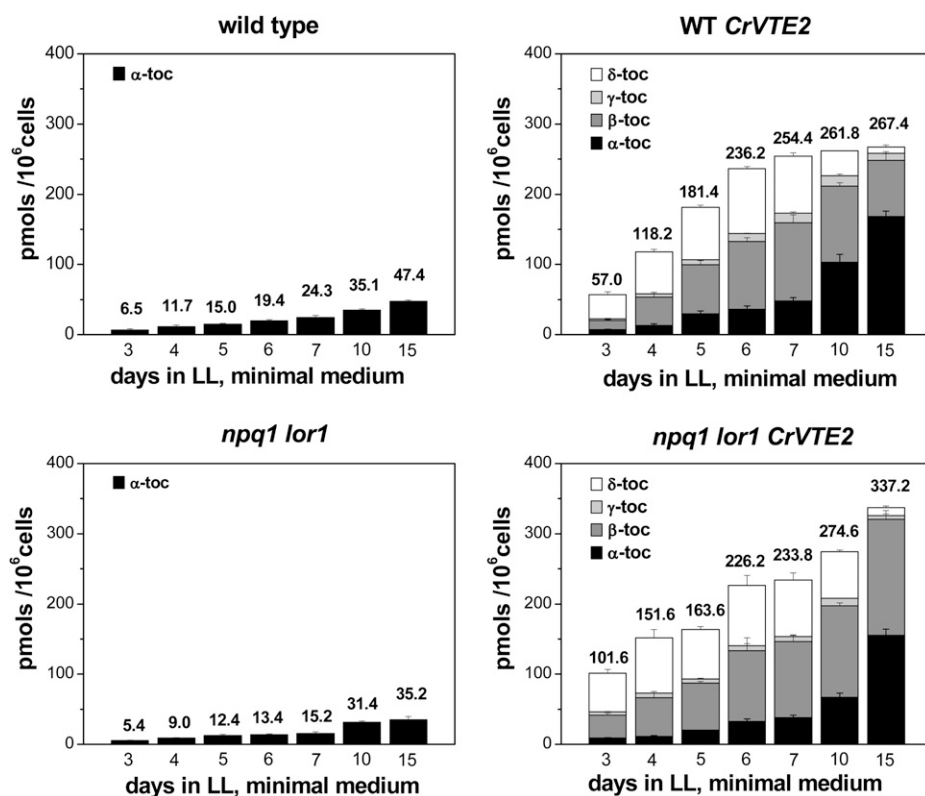


Figure 4. Tocopherol accumulation kinetics in the wild type, WT *CrVTE2*, *npq1 lor1*, and *npq1 lor1 CrVTE2*. Cells were grown photoautotrophically in HS medium under LL for 15 d. Cells from 2- to 6-mL samples harvested at the indicated time points were extracted, and individual tocopherols were separated and quantified by normal-phase HPLC. Tocopherol levels are shown as means $\pm$ 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 photochemical efficiency of PSII in the dark-adapted state, measured as the variable-to-maximal 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$)-grown cells to HL (1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) 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 $^1\text{O}_2^*$ 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 ROS.

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 *SynVTE2* (*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-*

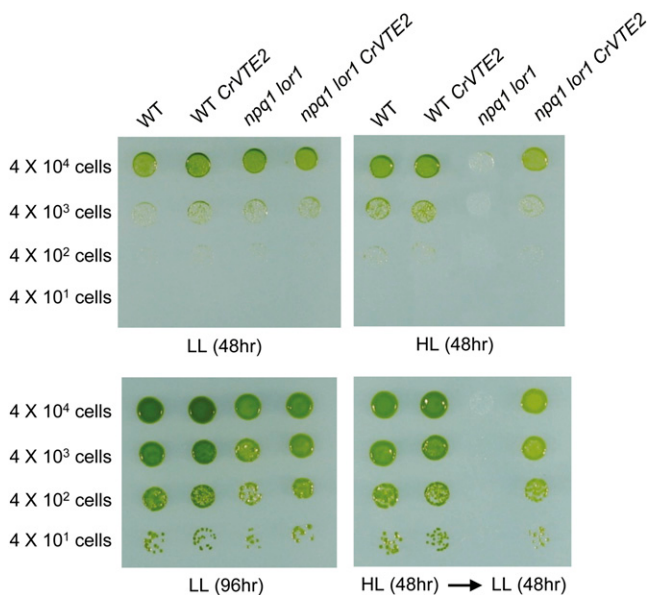


Figure 5. Growth phenotypes of *C. reinhardtii* strains under different light conditions. Photoheterotrophic growth on TAP agar medium was assayed in air at 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LL) and at 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (HL). Serial dilutions of cells were spotted on plates, and the plates were incubated under the indicated light conditions. WT, Wild type.

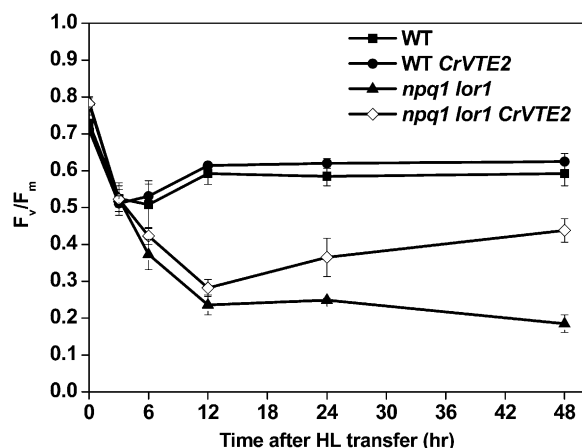


Figure 6. PSII efficiency (F_v/F_m) of *C. reinhardtii* strains during HL exposure. Cells were grown in 100 mL of minimal medium (HS) under continuous 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LL) to the early exponential phase (1 to 1.5×10^6 cells mL^{-1}) before transfer to 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (HL). The chlorophyll fluorescence parameter F_v/F_m , representing the maximal photosynthetic activity of PSII in the dark-adapted state, was measured as a function of time in HL. WT, Wild type.

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.,

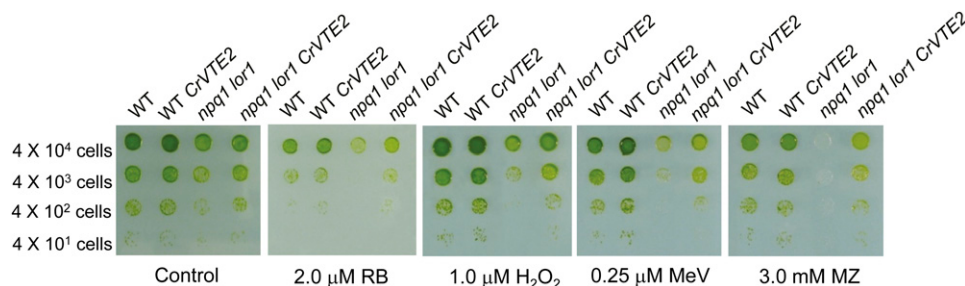


Figure 7. Growth phenotypes of *C. reinhardtii* strains under other oxidative stress conditions. Serial dilutions of cells were spotted on TAP agar medium containing the indicated pro-oxidants at concentrations that were sublethal for the wild-type strain. The plates were incubated for 4 d at 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LL). MeV, Methyl viologen; MZ, metronidazole; RB, Rose Bengal; WT, wild type.

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; Porfirova 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 photoinhibition 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., 2006, 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 $^1\text{O}_2^*$, O_2^- , and H_2O_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 $^1\text{O}_2^*$, O_2^- , and H_2O_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 $^1\text{Chl}^*$ and $^3\text{Chl}^*$. 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+ (*mt+*) and a near-isogenic strain of *npq1 lor1* (Sirikhachornkit et al., 2009), are in the 137c genetic background (Dent et al., 2005). The *psbH::aadA* disruption strain (O'Connor et al., 1998) was kindly provided by Saul Purton (University College London).

Cells were grown photoheterotrophically 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or in the dark. For tocopherol analysis, cells were grown in 100 mL of HS liquid culture under continuous LL (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) 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 $\mu\text{mol photons}$

$\text{m}^{-2} \text{s}^{-1}$), 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 1.5×10^6 cells mL^{-1} . Cells were counted using a hemacytometer and concentrated to a density of 1×10^7 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 H_2O_2 (Fisher) were prepared freshly before pouring onto plates, and plates were prepared 1 d 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-*rbcl* (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' *Bsp*HI site and a 3' *Bam*HI site immediately outside the coding region. The *SynVTE2* *Bsp*HI forward primer sequence was 5'-CATGTCATGATTATGGCAACTATC-CAAG-3' and the *SynVTE2* *Bam*HI reverse primer sequence was 5'-CGCGGATCCCTAAAAAATAGTATTAGAAA-3'. The 0.9-kb PCR products were cloned into pCR-Blunt II-TOPO vector (Invitrogen) to generate plasmid p*SynVTE2* and verified by sequencing. The plasmid p*SynVTE2* was digested with *Bsp*HI and *Bam*HI, and the resulting fragment was ligated into p72-*rbcl* vector digested with *Nco*I and *Bam*HI to generate plasmid p72-*rbcl*-*SynVTE2*.

The *SynVTE2ct* gene was codon optimized for the *C. reinhardtii* chloroplast (GENEART). The synthetic *SynVTE2ct* gene was designed to contain a *Nco*I site right at the start codon and a *Pst*I site right after the stop codon and then cloned using these enzymes into p72-*rbcl* vector to generate plasmid p72-*rbcl*-*SynVTE2ct*.

C. reinhardtii chloroplast transformation of p72-*rbcl*-*SynVTE2* or p72-*rbcl*-*SynVTE2ct* into recipient strain *psbH::aadA* was carried out using the PDS-1000/He biolistic particle-delivery system (Bio-Rad) as described previously (Boynton and Gillham, 1993), except that a rupture disk of 1,100 pounds per square inch instead of one 1,300 pounds per square inch was used, and the replating step after bombardment was omitted. After transformation, the plates were incubated at 25°C in dim light (10 μmol photons $\text{m}^{-2} \text{s}^{-1}$) overnight and then shifted to LL to select for photoautotrophic growth. The transformant colonies were restreaked four or more times on HS plates under LL until they were homoplasmic. To confirm the homoplasmicity of the chloroplast genome in transformants, DNA segments containing the insertion site were amplified by PCR using primers Zhirong20 (5'-AATCCTGAGAGG-GAATGCAC-3') and Zhirong21 (5'-TTATCCCGGAGGGAAGTAGG-3').

Construction of Nuclear Transformation Vectors and Nuclear Transformation

Nuclear transformation vector pSL18 (Pollock et al., 2003) was obtained from Stephane D. Lemaire (Université Paris-Sud). Genomic DNA containing the open reading frame (protein identifier 345347; Joint Genome Institute Chlamy version 4.0) that encodes a putative *C. reinhardtii* homogentisate phytyltransferase (*CrVTE2*) was amplified by PCR from *C. reinhardtii* genomic DNA using primers designed to engineer a 5' *Eco*RI site at the beginning and a 3' *Xba*I site at the end. The *CrVTE2* *Eco*RI forward primer sequence was 5'-CCGGAATTCCTCCGGTCTAAACATAGGATATCATAG-3' and the *CrVTE2* *Xba*I reverse primer sequence was 5'-ACGAGATCTGTCCGTAGTG-ATGATAGTGGGTG-3'. The resulting 4-kb PCR products were cloned into pCR-Blunt II-TOPO vector (Invitrogen) to generate plasmid p*CrVTE2* and verified by sequencing. The plasmid p*CrVTE2* was digested with *Eco*RI and *Xba*I and cloned into pSL18 vector to generate plasmid pSL18-*CrVTE2*.

Nuclear transformation of pSL18-*CrVTE2* into *C. reinhardtii* wild type or *npq1 lor1* double mutant was performed using the glass bead method as described previously (Dent et al., 2005). One microgram of the *Kpn*I-linearized pSL18-*CrVTE2* was used for each transformation. Transformants were selected on TAP plates containing 10 μg mL^{-1} paromomycin (Sigma).

Tocopherol Analysis and Quantification

Cell densities were determined using a Coulter Counter (Multisizer 3; Beckman Coulter). Cells from 2- to 6-mL samples were harvested by centrif-

ugation (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- $\times$ 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 1.5×10^6 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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