Overlapping photoprotective function of vitamin E and carotenoids in

*Chlamydomonas*

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ABSTRACT

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 photo-oxidative 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 homogentisate phytyltransferase (*VTE2*) from *Synechocystis*, and by nuclear transformation with *VTE2* from *C. reinhardtii*, which resulted in a 1.6-fold, 5- to 10-fold, and more than 10-fold increase 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 days and higher efficiency of photosystem II, and they were also much more resistant to other oxidative stresses. These results suggest overlapping function of tocopherols and carotenoids in protection against photo-oxidative stress.
INTRODUCTION

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 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 peroxide ($\text{H}_2\text{O}_2$), and hydroxyl radicals ($\text{OH}'$) (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 (PUFAs). 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 byproducts of oxidative metabolism. In particular, algae and plants often increase the expression of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) (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 photo-oxidative 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 non-photosynthetic 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 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 (Figure 1) (DellaPenna and Pogson, 2006; Méné Saffrané and DellaPenna, 2010). The formation of 2-methyl-6-phytylbenzoquinone (MPBQ) from the condensation of homogentisic acid (HGA) and a prenyl side chain (phytlyl diphosphate; PDP) is the first committed step in the pathway and is catalyzed by homogentisate phytyltransferase (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 (DMBPQ) by MPBQ methyltransferase (VTE3). MPBQ and DMBPQ 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 (1Chl*), thereby promoting nonphotochemical quenching (NPQ) to regulate photosynthetic light harvesting (Müller et al., 2001; de Bianchi et al.,
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 LHC 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 chomanol 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 (KamalEldin 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 (Kulas and Ackman, 2001), peroxynitrite (Fredstrom, 2002), and $\text{O}_2^-$ (Fryer, 1992).

There is some evidence suggesting that these two classes of molecules have overlapping antioxidative roles in vivo. The Arabidopsis 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 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 function or functionally interact in protecting against lipid
peroxidation and HL stress (Maeda et al., 2005). However, in the C. reinhardtii npq1 lor1
double mutant, which lacks both zeaxanthin and lutein, the level of α-tocopherol is not
higher than that of 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.N., unpublished data). Although the notion of overlapping
function 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 $^{1}O_2^*$ stress induced by exposure to
photosensitizers and bleaches when grown under high-light 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 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 photo-oxidative 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 non-stressed 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 (Figure 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 (Figure 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 1-1.5 × 10⁶ cells/ml (early exponential phase) were measured by normal-phase HPLC. The best SynVTE2 overexpressing lines contained 1.6-fold higher total tocopherol levels (13.0±0.6 pmol/10⁶ cells) than the control strain (8.0±0.2 pmol/10⁶ cells). Both the control rbcL and rbcL-SynVTE2 strains accumulated predominantly α-tocopherol, although β-tocopherol levels were also elevated to 2.8 pmol/10⁶ cells in rbcL-SynVTE2 (Figure 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 (Figure 2A), and homoplasmic transformants were identified by PCR after chloroplast transformation into the psbH::aadA mutant (Figure 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 (Figure 3). The control rbcL strain accumulated total tocopherol at 8.0±0.2 pmol/10^6 cells, whereas total tocopherol content of rbcL-SynVTE2ct ranged between 38.6±2.0 and 83.0±3.0 pmol/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 PSAD promoter and terminator linked to a paromomycin resistance cassette (Depege et al., 2003; Pollock et al., 2003) (Figure 2A). The nuclear overexpression vector pSL18-CrVTE2 was transformed into both the wild type and the npq1 loriI mutant background, and the corresponding transformants WT CrVTE2 and npq1 loriI CrVTE2 were selected for paromomycin resistance. When grown photoautotrophically to a density of 1-1.5 × 10^6 cells/ml, the best CrVTE2-overexpressing line in either wild type or npq1 loriI background (WT CrVTE2 line 73 and npq1 loriI CrVTE2 line 36) had more than a 10-fold increase in total tocopherol content relative to the controls (Figure 3). The control wild type and npq1 loriI strain accumulated total tocopherols at 11.4±2.0 and 9.0±0.4 pmol/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/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 (Figure 3). Because nuclear transformants CrVTE2-73 and npq1 lor1 CrVTE2-36 had the highest percentage increase in total tocopherol content and their respective controls (wild type and npq1 lor1) were in 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 1).

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; Molinatrones 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 were grown photoautotrophically under LL for up to 15 days, 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 (Figure 4). In 3-day-old cultures, the total tocopherol contents in wild type, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2 were 6.6±2.0, 57.2±3.0, 5.4±0.4, and 101.0±9.4 pmol/10^6 cells, respectively. In 15-day-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/10^6 cells, respectively. Whereas both wild type and npq1 lor1 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 (Figure 4).
Tocopherol Overproduction Partially Protects npq1 lor1 from HL Stress

Due to the lack of both zeaxanthin and lutein in high light, 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 high-light exposure, the deficiency in these photoprotective mechanisms is expected to increase ROS levels in thylakoids and to cause severe photo-oxidative 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 were grown photoheterotrophically 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 hours, wild type and WT CrVTE2 were able to grow, whereas the npq1 lor1 double mutant underwent photo-bleaching and died as previously reported (Niyogi et al, 1997; Baroli et al, 2004). In contrast, npq1 lor1 CrVTE2 cells were able to stay green and survive in HL for up to 48 hours. After shifting the plates back to LL for another 48 hours, npq1 lor1 CrVTE2 completely resumed its growth, and the difference between npq1 lor1 and npq1 lor1 CrVTE2 became even more apparent (Figure 5).

One important antioxidant function of tocopherol is thought to be in protecting the photosynthetic membrane from photo-oxidation 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 high-light 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 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 (Figure 6). In the wild type, the \(F_v/F_m\) value initially decreased to 0.52 at 3 hours, recovered to ~0.6 at 12 hours, 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 hours. However, in
the npq1 lor1 double mutant, $F_v/F_m$ dramatically decreased to the lowest value of ~0.2 at 48 hours. The $F_v/F_m$ value of npq1 lor1 CrVTE2 decreased in the first 12 hours to 0.3, but it was able to recover to an intermediate level between that of the wild type and npq1 lor1 at 48 hours.

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 high light (HL, 1000 μmol photons m$^{-2}$ s$^{-1}$) for 48 hours (Table 1). 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 hours, 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 sensitivity of 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$_2$O$_2$, and to O$_2^-$ generators methyl viologen and metronidazole, the npq1 lor1 double mutant was more sensitive than the wild type and WT CrVTE2 (Figure 7). The addition of rose bengal, H$_2$O$_2$, 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 five-fold and two-fold in Arabidopsis leaves and seed, 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 (Figure 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. reinhardtii VTE2 enzyme might be better than exogenous Synechocystis VTE2 in forming such a complex.

Overexpression of putative CrVTE2 resulted in accumulation of tocopherol in C. reinhardtii (Figure 3 and Figure 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. 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 (Figure 3 and Figure 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 (GGPP) 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 increased availability of GGPP for tocopherol biosynthesis (McCarthy et al., 2004). Since the prenylation steps of the tocopherol and plastoquinone biosynthesis pathways require the same aromatic head group, HGA, 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 Photo-oxidative Stress

Tocopherols are only synthesized by photosynthetic organisms, and the majority of tocopherols are localized in photosynthetic membranes (Lichtenthaler et al., 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 thylakoids 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 nonenzymatic lipid peroxidation during seed dormancy, germination and early seedling development (Sattler et al., 2004b; Sattler et al., 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 wild type under optimal laboratory growth conditions (Collakova and DellaPenna, 2001; Porfirova et al., 2002; Sattler et al., 2003; Sattler et al., 2004a; Maeda et al., 2005; Maeda et al., 2006; Sakuragi et al., 2006) and during HL stress (Dähnhardt et al., 2002; Maeda et al., 2005; Maeda et al., 2006). Only during exposure to HL stress in combination with low temperature (2-8°C), Arabidopsis vte1 and vte2 mutants exhibited higher degrees of PSII photoinhibition and lipid peroxidation than 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 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 the 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 function 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 photo-oxidative stress. The npq1 lor1 double mutant provides an ideal background to study the antioxidant functions of tocopherols because of its supersensitivity to photo-oxidative stress. As previously reported (Baroli et al., 2004), npq1 lor1 quickly bleached and died under HL (Figure 5), was severely inhibited in PSII efficiency (Figure 6), and was very sensitive to $^1$O$_2^*$, O$_2^-$ and H$_2$O$_2$ (Figure 7). In contrast, npq1 lor1 CrVTE2 survived and stayed green in HL for up to 48 hours (Figure 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 (Figure 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 $^1$O$_2^*$, O$_2^-$ and H$_2$O$_2$ (Figure 7), suggesting that increased content of tocopherols is also important in conferring resistance to photo-oxidative 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 Fv/Fm was observed in npq1 lor1 and npq1 lor1 CrVTE2 during the first 12 hours in HL, whereas the decrease in Fv/Fm lasted only 3 hours in WT and WT CrVTE2 (Figure 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 to 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 hours in HL (Figure 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 *C. 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 medium (TAP) or photoautotrophically in 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 HS liquid culture under continuous low light (LL; 50 μmol photons m⁻² s⁻¹) for 15 days.

To test the effect of high light (HL) on growth in liquid cultures, cells were grown in 100 ml HS medium under continuous LL to the early exponential phase (1-1.5 × 10⁶ cells/ml) before being transferred to HL (500 μmol photons 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 HS medium under LL until they reached a density of 1-1.5 × 10⁶ cells/ml. Cells were counted using a hemacytometer, and concentrated to a density of 1 × 10⁷ cells/ml. Series dilutions of the concentrated culture were prepared, and 4 μl of these dilutions were spotted onto TAP agar plates with or without pro-oxidant. Rose Bengal (Sigma), methyl viologen (Sigma), metronidazole (Sigma) and hydrogen peroxide (Fisher) were prepared freshly before pouring plates, and plates were prepared 1 day prior to use. The plates were incubated in LL for 4 days with the exception of HL plates, which were incubated for 2 days 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 (University College London). The open reading frame (ORF) *slr1736*
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′-CATGTCATGATTATGGCAACTATCCAAG-3′ and the SynVTE2_BamHI reverse primer sequence was 5′-CGCGGATCCCTAAAAATAGTATTAGAAA-3′. The 0.9-kilobase (kb) PCR products were cloned into pCR-BluntII-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-rbcL vector digested with NcoI and BamHI to generate plasmid p72-rbcL-SynVTE2.

The SynVTE2ct gene was codon-optimized for the *C. reinhardtii* chloroplast (GENEART AG, Germany). The synthetic SynVTE2ct gene was designed to contain an NcoI site right at the start codon and a PstI 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, CA, USA) as previously described (Boynton and Gillham, 1993), except that a 1100-psi instead of 1300-psi rupture disk 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 m⁻² 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′-AATCCTGAGAGGGAATGCAC-3′) and Zhirong21 (5′-TTATCCCGAGGGGAAATGG-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 ORF (Protein ID:345347,
JGI Chlamy v4.0) that encodes a putative *C. reinhardtii* homogentisate phytlytransferase (CrVTE2) was amplified by PCR from *C. reinhardtii* genomic DNA, using primers designed to engineer a 5’-EcoRI site at the beginning and a 3’-XbaI site at the end. The CrVTE2_EcoRI forward primer sequence was 5’-CCGGAA TTCCCGGTCTAAACATAGGATATCAG-3’, and the CrVTE2_XbaI reverse primer sequence was 5’-ACGAGATCTGTCCGTAGTGATGATGATGATGATGATG-3’. The resulting 4-kb PCR products were cloned into pCR-BluntII-TOPO vector (Invitrogen) to generate plasmid pCrVTE2 and verified by sequencing. The plasmid pCrVTE2 was digested with EcoRI and XbaI 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 μg of the KpnI-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, Miami, FL). Cells from 2 to 6 ml samples were harvested by centrifugation (3200 × g, 4˚C, 3 min) and immediately frozen in liquid nitrogen. Tocopherols were extracted by vortexing in 200 μl acetone for 1 min, and the acetone extracts were evaporated using a vacufuge concentrator (Eppendorf) and redissolved in 200 μl 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-X 250-mm Luna 5 um silica column (Phenomenex, Torrance, CA) 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, δ-tocopherol (Sigma), β-tocopherol (Matreya)) at known concentrations and were normalized to cell number.
Pigment Analysis and Quantification

HPLC analysis of carotenoids and Chls was done as previously described (Müller-Moulé et al., 2002). A total of three independent samples were measured. Carotenoids were quantified using standard curves of purified pigments (VKI, Hørsholm, Denmark) and normalized to cell numbers.

Chlorophyll Fluorescence Measurement

To measure chlorophyll fluorescence parameters, cells were grown in liquid HS medium to 1-1.5 × 10⁶ cells/ml. Culture aliquots of 5-8 ml were collected at 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, King’s Lynn, UK) as previously described (Baroli et al., 2004).

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We thank Saul Purton for the p72-rbcL plasmid and psbH::aadA disruption strain and Stephane D. Lemaire for the pSL18 plasmid. We also thank Peggy Lemaux for sharing the PDS-1000/He Biolistic particle delivery system.

LITERATURE CITED


Jahns P, Holzwarth AR (2011) The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. Biochimica et Biophysica Acta (BBA) In press


Chlamydomonas mutants lacking PSII-H and a site-directed mutant lacking the
phosphorylation site. Biochimica Et Biophysica Acta-Bioenergetics 1364: 63-72
is required for optimal photosynthesis in the green alga Chlamydomonas reinhardtii in a
low-CO2 atmosphere. Plant Physiology 133: 1854-1861
Arabidopsis mutant lacking vitamin E and identification of a cyclase essential for all
tocopherol biosynthesis. Proceedings of the National Academy of Sciences of the
United States of America 99: 12495-12500
Rise M, Cojocaru M, Gottlieb HE, Goldschmidt EE (1989) Accumulation of
alpha-tocopherol in senescing organs as related to chlorophyll degradation. Plant
Physiology 89: 1028-1030
high light in Arabidopsis. Plant Physiology 130: 1109-1120
Archives of Biochemistry and Biophysics 504: 78-85
prenyltransferases involved in plastoquinone-9 and tocochromanol biosynthesis. Febs
Letters 580: 5357-5362
Sakuragi Y, Maeda H, DellaPenna D, Bryant DA (2006) alpha-tocopherol plays a role in
photosynthesis and macronutrient homeostasis of the cyanobacterium Synechocystis sp
PCC 6803 that is independent of its antioxidant function. Plant Physiology 141:
508-521
Sattler SE, Cahoon EB, Coughlan SJ, DellaPenna D (2003) Characterization of
tocopherol cyclases from higher plants and cyanobacteria. Evolutionary implications for
tocopherol synthesis and function. Plant Physiology 132: 2184-2195
Sattler SE, Cheng ZG, DellaPenna D (2004a) From Arabidopsis to agriculture:
engineering improved Vitamin E content in soybean. Trends in Plant Science 9: 365-367
Sattler SE, Gilliland LU, Magallanes-Lundback M, Pollard M, DellaPenna D (2004b)
Vitamin E is essential for seed longevity, and for preventing lipid peroxidation during
germination. Plant Cell 16: 1419-1432
Sattler SE, Menè-Saffranè L, Farmer EE, Krischke M, Mueller MJ, DellaPenna D
defense markers in Arabidopsis tocopherol-deficient mutants. Plant Cell 18: 3706-3720
Savidge B, Weiss JD, Wong YHH, Lassner MW, Mitsky TA, Shewmaker CK,
Post-Beittenmiller D, Valentin HE (2002) Isolation and characterization of
homogentisate phytlytransferase genes from Synechocystis sp PCC 6803 and
Arabidopsis. Plant Physiology 129: 321-332
Synechocystis sp PCC 6803 involved in tocopherol biosynthesis. Febs Letters 499:
15-20


FIGURE LEGEND

Figure 1. Tocopherol biosynthetic pathways in *C. reinhardtii*. VTE1, tocopherol cyclase; VTE2, homogentisate phytoltransferase; VTE3, MPBQ methyltransferase; VTE4, \( \gamma \)-tocopherol methyltransferase.

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, which 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*; lane 3 and 4, *rbcL-SynVTE2* line 47 and 62; lane 5 and 6, *rbcL-SynVTE2* line 11 and 36.

Figure 3. Tocopherol content and composition in different transgenic strains. Cells were grown photoautotrophically in HS medium under LL to a density of 1-1.5 \( \times 10^6 \) cells/ml. Cells from 4 ml samples were extracted and analyzed for tocopherols by normal-phase HPLC. Tocopherol levels are expressed as the 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).

Figure 4. Tocopherol accumulation kinetics in wild type, WT CrVTE2, *npq1 lor1* and *npq1 lor1 CrVTE2*. Cells were grown photoautotrophically in HS medium under LL for 15 days.
Cells from 2 to 6 ml samples harvested at indicated time points were extracted, and individual tocopherols were separated and quantified by norma-phase HPLC. Tocopherol levels are shown as the means ± SD (n=3). Total tocopherol levels in pmol per 10^6 cells are indicated above the error bar for each strain.

Figure 5. Growth phenotypes of *C. reinhardtii* strains under different light conditions. Photoheterotrophic growth on Tris-Acetate-Phosphate (TAP) agar medium was assayed in air at 50 μmol photons m⁻² s⁻¹ (LL) and at 500 μmol photons m⁻² 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. Photosystem II efficiency (Fv/Fm) of *C. reinhardtii* strains during HL exposure. Cells were grown in 100 ml minimal medium (HS) under continuous 50 μmol photons m⁻² s⁻¹ (LL) to the early exponential phase (1-1.5 × 10^6 cells/ml) before transfer to 500 μmol photons m⁻² s⁻¹ (HL). The chlorophyll fluorescence parameter Fv/Fm representing the maximal photosynthetic activity of PSII in the dark-adapted state was measured as a function of time in HL. WT, wild type.

Figure 7. Growth phenotypes of *C. reinhardtii* strains under other oxidative stress conditions. Serial dilutions of cells were spotted on Tris-Acetate-Phosphate (TAP) agar medium containing the indicated pro-oxidants at concentrations that were sublethal for the wild-type strain. The plates were incubated for 4 days at 50 μmol photons m⁻² s⁻¹ (LL). RB, rose bengal; H₂O₂, hydrogen peroxide; MeV, methyl viologen; MZ, metronidazole; WT, wild type.
Table 1. Pigment content of WT, WT CrVTE2, npq1 lor1, and npq1 lor1 CrVTE2.

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<th>total car</th>
<th>N+Lo</th>
<th>L</th>
<th>V</th>
<th>A</th>
<th>Z</th>
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<th>β-car</th>
<th>α-car</th>
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Pigment measurements were performed before and after transferring LL (50 μmol photons m⁻² sec⁻¹) -grown cells to HL (500 μmol photons m⁻² sec⁻¹) for 48 hours. Data are normalized to cell numbers and presented as the means ± SD (n=3) (pmol/10⁶ cells).

Chl, chlorophyll; Car, carotenoid; N, neoxanthin; Lo, loryoxanthin; L, lutein; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; β-car, β-carotene; α-car, α-carotene.
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Figure 6. Photosystem II efficiency ($F_v/F_m$) of *C. reinhardtii* strains during HL exposure.
Cells were grown in 100 ml minimal medium (HS) under continuous 50 μmol photons m$^{-2}$ s$^{-1}$ (LL) to the early exponential phase ($1-1.5 \times 10^6$ cells/ml) before transfer to 500 μmol photons m$^{-2}$ s$^{-1}$ (HL). The chlorophyll fluorescence parameter $F_v/F_m$ represents the maximal photosynthetic efficiency of PSII in the dark-adapted state measured as a function of time in HL. WT, wild type.
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