Replacement of α-Tocopherol by β-Tocopherol Enhances Resistance to Photooxidative Stress in a Xanthophyll-Deficient Strain of Chlamydomonas reinhardtii

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Tocopherols (vitamin E) comprise a class of lipid-soluble antioxidants synthesized only in plants, algae, and some cyanobacteria. The majority of tocopherols in photosynthetic cells is in the α form, which has the highest vitamin E activity in humans, whereas the β, γ, and δ forms normally account for a small percentage of total tocopherols. The antioxidant activities of these forms of tocopherol differ depending on the experimental system, and their relative activities in vivo are unclear. In a screen for suppressors of the xanthophyll-deficient npq1 lor1 double mutant of Chlamydomonas reinhardtii, we isolated a vte3 mutant lacking α-tocopherol but instead accumulating β-tocopherol. The vte3 mutant contains a mutation in the homolog of a 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase gene found in plants. The vte3 npq1 lor1 triple mutant with β-tocopherol survived better under photooxidative stress than did the npq1 lor1 mutant, but the vte3 mutant on its own did not have an obvious phenotype. Following transfer from low light to high light, the triple mutant showed a higher efficiency of photosystem II, a higher level of cell viability, and a lower level of lipid peroxide, a marker for oxidative stress, than did the npq1 lor1 mutant. After high-light transfer, the level of the photosystem II reaction center protein, D1, was also higher in the vte3 npq1 lor1 mutant, but the rate of D1 photodamage was not significantly different from that of the npq1 lor1 mutant. Taken together, these results suggest that the replacement of α-tocopherol by β-tocopherol in a xanthophyll-deficient strain of Chlamydomonas reinhardtii contributes to better survival under conditions of photooxidative stress.

Tocopherols (vitamin E) are amphipathic molecules (Fig. 1) synthesized exclusively in oxygenic photosynthetic organisms. Because tocopherols are essential nutrients in the human diet, their function and chemistry both in vitro and in animal systems have been studied extensively. Tocopherols have been linked to the prevention of diseases such as cancer, atherosclerosis, and neuronal degeneration (41, 52, 56). They function as potent lipid-soluble antioxidants in both plants and animals (44). There are two main antioxidant functions of tocopherols. One function is the scavenging of harmful radicals, especially lipid peroxyl radicals (42). In this reaction, tocopherols donate an electron from the chromanol head group to a lipid peroxyl radical and stop membrane lipid peroxidation chain reactions. Tocopherol itself becomes a radical and is thought to be regenerated by interacting with ascorbate (48). The second antioxidant function of tocopherol is the quenching of singlet oxygen. In animals, singlet oxygen can be generated, for example, by UV-activated endogenous photosensitizers in the skin (39). In plants, singlet oxygen is generated mostly by triplet chlorophyll in photosystem II (25). Aside from being an antioxidant, tocopherol is also involved in non-antioxidant regulatory functions that affect membrane rigidity, transcription, intracellular signaling, photosynthesis, macronutrient starvation, and carbohydrate metabolism (13, 20, 28, 33, 38, 40, 44).

Tocopherol comes in four different forms: α, β, γ, and δ. All forms are composed of a hydrophilic chromanol head group synthesized from the shikimate pathway and a lipophilic phytyl side chain synthesized from the isoprenoid pathway (Fig. 1). The differences among the four forms are the numbers and positions of the methyl groups on the head group. α-Tocopherol has three methyl groups, β- and γ-tocopherol have two, and δ-tocopherol has one. The tocopherol biosynthetic pathway has been studied for both the model plant Arabidopsis thaliana and a cyanobacterium, Synechocystis sp. strain PCC 6803 (11). The synthesis of tocopherol is initiated by the addition of a phytyl side chain to the homogentisic acid (HGA) head group, creating 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) (Fig. 1). The addition of a different side chain to the HGA head group results in the production of 2-methyl-6-solanesyl-1,4-benzoquinol (MSBQ), an intermediate in plastoquinone biosynthesis. In A. thaliana, the methylation of MPBQ and MSBQ is catalyzed by the same enzyme, MPBQ/MSBQ methyltransferase, generating 2,3-dimethyl-5-phytyl-1,4-benzoquinol (DMPBQ) and plastoquinone, respectively. The cyclization of MPBQ and DMPBQ by tocopherol cyclase produces δ- and γ-tocopherol, which are methylated to form β-tocopherol and α-tocopherol, respectively.

α-Tocopherol is the most studied form of tocopherol, because it has the highest vitamin E activity due to its preferential retention by the tocopherol transfer protein in animals (21). α-Tocopherol is also the predominant form in cells of photosynthetic organisms, except in seeds, which contain mostly the
To increase vitamin E content, the tocopherol composition of soybean seeds has been altered by the overexpression of *A. thaliana* MPBQ/MSBQ methyltransferase together with \( \gamma \)-tocopherol methyltransferase, resulting in the accumulation of \( \gamma \)- instead of \( \beta \)-tocopherol (54). Increasing evidence has led to a better understanding of other forms of tocopherols in biological systems. For instance, it was previously shown that \( \gamma \)-tocopherol is a better antioxidant than \( \beta \)-tocopherol against reactive nitrogen species in liposomes (8). Transgenic tobacco accumulating \( \beta \)-tocopherol showed a higher tolerance toward sorbitol and methyl viologen but was more sensitive to salt stress than the wild-type strain, which accumulates \( \gamma \)-tocopherol (1). The relative antioxidant activity of tocopherols has been shown to be \( \alpha > \beta > \gamma > \delta \) according to data from liposome studies (15). On the other hand, in vitro studies examining the antioxidant activity in fats and oils showed the relative antioxidant activity to be \( \delta > \beta > \gamma > \alpha \) (23). Additionally, the relative physical quenching of singlet oxygen by tocopherols is \( \alpha \geq \beta > \gamma > \delta \), whereas the relative chemical quenching is \( \alpha > \gamma > \delta > \beta \) (22). Due to its low chemical reactivity with singlet oxygen, it was previously suggested that \( \beta \)-tocopherol might be a suitable form under conditions where harmful oxidation products may not be readily eliminated (22).

We isolated a *Chlamydomonas reinhardtii* mutant that accumulates \( \beta \)-tocopherol instead of \( \alpha \)-tocopherol. In *A. thaliana*, defects in MPBQ/MSBQ methyltransferase cause a similar tocopherol phenotype (7). However, because this enzyme is also responsible for the synthesis of plastoquinone in *A. thaliana*, comparison of the role of \( \beta \)- and \( \alpha \)-tocopherol in vivo has been difficult due to the plastoquinone deficiency (7, 32). In *Synechocystis* sp. strain PCC 6803, a mutant of MPBQ methyltransferase (sll0418) showed no effect on the plastoquinone level (4), but both the tocopherol content and composition were only slightly affected, with only a 35% reduction in the amount of total tocopherol, the majority of which remained in the \( \gamma \)-form (40). Interestingly, our *C. reinhardtii* mutant completely lacks \( \alpha \)-tocopherol and synthesizes normal levels of plastoquinone. This mutant provides us with an in vivo system to investigate the role of \( \beta \)-tocopherol compared to \( \alpha \)-tocopherol in survival under oxidative stress conditions without affecting photosynthesis.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Wild-type *Chlamydomonas reinhardtii* strain 4A*+* (137c background) was used in this work (12). The npq1 lor1 mutant was previously described (35). All strains were maintained on Tris-acetate-phosphate...
plates (17) at 10 μmol photons m−2 s−1 before being transferred to appropriate conditions for experiments. For all high-performance liquid chromatography (HPLC) analyses and physiological characterizations, cells were grown photoautotrophically in 100 ml liquid high-salt minimal medium (17) to a density of 1 × 10^8 to 2 × 10^8 cells/ml in continuous low light (50 μmol photons m−2 s−1) and, when indicated, shifted to high light (500 μmol photons m−2 s−1). Cell viability was measured by plating a known number of cells and counting the number of colonies after 10 days in low light.

**HPLC analysis.** Tochopherols were extracted from 2 to 4 ml of cell culture by vortexing the cell pellet in 200 μl acetonitrile at maximum speed for 30 s. Normal-phase HPLC was used to identify the forms of tochopherols that accumulated. The acetone extract was evaporated under N2 gas, and the residue was reconstituted in 200 μl hexane. The hexane extract was filtered through a 2-μm nylon filter, and 25 μl of the extract was subjected to normal-phase HPLC on a 4.6- by 250-mm Luna 5-μm silica column (Phenomenex, Torrance, CA) as described previously (46). Reverse-phase HPLC was used routinely to quantify the amounts of chlorophyll a and chlorophyll in all strains. The acetone extract was filtered and subjected to HPLC on a 4.6- by 250 mm Spherisorb S5 ODS1 cartridge column (Waters, Milford, MA) at 30°C as previously described (5). Tocopherol was detected by fluorescence at 325 nm (295-nm excitation). Tocopherol and chlorophyll a concentration were calculated from standard curves generated from known concentrations of standards.

The same reverse-phase HPLC system was used to detect plastoquinone by reading the absorbance at 255 nm. Cells harvested from 100 ml of culture were lyophilized, and plastoquinone was extracted from 10 to 15 μg of dry pellet in the same manner as described above for tochopherol extraction except that the extraction was done three times. The supernatants were pooled, evaporated under N2 gas, and redissolved in 200 μl acetonitrile. Pure plastoquinone used to generate a standard curve was a gift from D. Creed (University of Southern Mississippi).

**Chlorophyll fluorescence and lipid peroxidation measurements.** Samples were taken at the indicated time points for chlorophyll a measurement by spectrophotometry (37). Chlorophyll fluorescence was measured using an FMS2 pulse-amplitude-modulation fluorometer (Hansatech, King’s Lynn, United Kingdom) as previously described (6). The number of cells corresponding to 5 μg chlorophyll a were deposited onto a 25-mm-diameter, 12-μm-pore-size nitrocellulose filter (Millipore, Bedford, MA) by filtration and were dark adapted in a moist petri dish for 15 min prior to measurements. Thiobarbituric acid-reactive substances were measured as previously described (5) except that 1 ml of trichloroacetic acid-thiobarbituric acid solution was used per sample.

**Genetic analysis.** Genetic crosses and tetrad analysis were performed according to established methods (17). For dominance testing, the allelic arg7-1 and arg7-8 mutations, which exhibit intragenic complementation, were used to select for stable diploid strains on TAP agar medium without arginine (17). For linkage analysis, the mutant was crossed to polymorphic wild-type strain S1-D2 (for stable diploid strains on TAP agar medium without arginine (17). For linkage analysis, the mutant was crossed to polymorphic wild-type strain S1-D2 (17). All sequence analyses and alignments were performed using the ImageJ program (2).

**RESULTS**

**Isolation and genetic characterization of vte3.** The npq1 lor1 double mutant is unable to synthesize the carotenoids lutein and zeaxanthin, resulting in photobleaching and cell death in high light (6, 35). Several suppressors of the npq1 lor1 double mutant were identified by their ability to survive in high light (5). HPLC analysis showed that one of the suppressors lacked α-tochopherol and accumulated a different form of tochopherol. The mutation responsible for this phenotype was named vte3.

Backcrosses to the npq1 lor1 parent revealed that vte3 was not sufficient for the suppressor phenotype and that a fourth mutation was primarily responsible for the ability to grow in high light. The fourth mutation was eliminated during subsequent backcrossing, and the vte3 mutant in the npq1 lor1 background was then crossed to a wide-type strain five times to isolate isogenic vte3, npq1 lor1, and vte3 npq1 lor1 strains for further characterization.

A normal-phase HPLC column that separates all four forms of tochopherol was used for tochopherol identification and quantitation. Wild-type and npq1 lor1 strains accumulated α-tochopherol with only a small percentage of other tochopherols (Table 1). In contrast, the vte3 and vte3 npq1 lor1 strains completely lacked α-tochopherol but accumulated β-tochopherol with a small amount of δ-tochopherol (Table 1). The total amounts of tochopherol in these strains were similar (Table 1).

Tetrad analysis of the progeny from backcrosses of vte3 strains to the wild type showed a 2:2 segregation of β-tochopherol and α-tochopherol accumulation based on HPLC of over 50 complete tetrads (data not shown), indicating that the tochopherol phenotype of vte3 is due to a single nuclear mutation. Similar to haploid strains, a wild-type diploid strain accumulated α-tochopherol, and a vte3/ vte3 diploid accumulated β-tochopherol (Table 1). A heterozygous vte3/VT3 strain accumulated mostly α-tochopherol, but a significant amount of β-tochopherol was observed (Table 1), indicating that vte3 is semidominant.

**vte3 contains a mutation in a plant-type MPBQ methyltransferase homolog.** The accumulation of β-tochopherol in vte3 suggested a defect in MPBQ methyltransferase. Previously reported studies showed that divergent genes encode this enzyme in plants and cyanobacteria (7). C. reinhardtii is the only known organism so far that contains orthologs of both the
planted- and cyanobacteria-type (vte3) and cyanobacteria-type (sll0418) MPBQ methyltransferase genes. Before the *C. reinhardtii* genome draft sequence was completed, the two orthologs of MPBQ methyltransferase were found by a similarity search of the EST Database (47). The sequences of the expressed sequence tags were used to design primers to amplify and sequence full-length cDNAs of both genes from wild-type *C. reinhardtii*. The deduced protein sequences were used to generate alignments with *A. thaliana* and *Synechocystis* sp. strain PCC 6803 MPBQ methyltransferases (Fig. 2). Linkage analysis was performed to determine if either of the two orthologs might be affected in the vte3 mutant. A PCR-based marker was developed for each gene, and over 50 progeny from a cross between the vte3 mutant and a polymorphic wild-type strain were scored for the cosegregation of the markers with the β-tocopherol accumulation phenotype. Unexpectedly, both markers cosegregated with the β-tocopherol accumulation phenotype in all of the progeny (Fig. 3), indicating that the phenotype was closely linked to both orthologs. The two genes were subsequently found to be 144 kb apart on linkage group XV (Fig. 3C). Sequencing cDNAs of both genes from vte3 revealed that the plant-type ortholog carried an A-to-C point mutation in the sixth exon of the gene. This mutation translates into a change of a conserved threonine residue at position 137 to a proline residue (Fig. 2). The cyano- bacteria-type ortholog was unaffected.

To confirm that the mutation in the plant-type VTE3 gene was responsible for the phenotype observed, a wild-type copy of the gene was transformed into the mutant. The expression of VTE3 cDNA under the control of the *PSAD* promoter (14) complemented the vte3 mutation, as shown by the restoration of the wild-type tocopherol phenotype albeit with a higher total tocopherol level (Table 1). The higher tocopherol level could be due to the expression vector used, or VTE3 might be a limiting enzyme for α-tocopherol production. No transformants with a construct carrying the cyanobacteria-type gene were complemented. Altogether, these results show that the accumulation of α-tocopherol in vte3 is caused by a mutation in the plant-type MPBQ methyltransferase gene, VTE3.

**Analysis of plastoquinone in vte3**. Plant-type MPBQ methyltransferase mutants that lack α-tocopherol and/or accumulate β-tocopherol have been isolated in other model organisms such as *A. thaliana* and maize (7, 9, 32). Because this enzyme catalyzes both the conversion of MPBQ to DMPBQ in the tocopherol biosynthetic pathway and the conversion of MSBQ to plastoquinone, an electron carrier in the photosynthetic membrane, the mutants also exhibit a decrease or absence of plastoquinone, which leads to a seedling-lethal phenotype. In contrast, the *Chlamydomonas* vte3 mutant did not show any obvious differences in photoautotrophic growth compared to that of the wild type (Fig. 4), implying that plastoquinone must be present in the mutant. Plastoquinone measurement by HPLC confirmed that the vte3 mutation of *C. reinhardtii* does not affect plastoquinone synthesis (Table 1).

**Phenotypes of vte3 under conditions of photooxidative stress.** To investigate the antioxidant function of β-tocopherol in vivo, we examined the growth of vte3 strains under oxidative stress conditions. The vte3 and complemented vte3 strains showed no growth phenotype relative to the wild type in continuous low light or high light or following a shift from low light to high light (Fig. 4). Both the npq1 lori and vte3 npq1 lori strains were able to grow in low light but underwent photooxidative bleaching in high light. Even though both strains completely bleached in high light, only the vte3 npq1 lori strain survived and resumed growth when the plate was shifted back to low light (Fig. 4). Even when left longer in low light, the npq1 lori strain never recovered to the same extent as the vte3 npq1 lori strain (data not shown).

We further explored the underlying basis for the difference in growth phenotypes between the npq1 lori and vte3 npq1 lori strains. One proposed function of tocopherol is to protect the photosynthetic membrane from photooxidative damage, which can be monitored by photosynthesis parameters and the amount of lipid peroxide produced. $F_{v}/F_{m}$ values (a measurement of photosystem II efficiency), the lipid peroxidation level, and cell viability were determined, along with chlorophyll $a$ and tocopherol contents, by using cultures that had been exposed to high-light stress. The wild-type and vte3 strains did not exhibit differences in any of the parameters measured except for the tocopherol content at 48 h. In contrast, the vte3 npq1 lori strain showed significant differences in most of the parameters compared to the npq1 lori strain. All strains exhibited similar $F_{v}/F_{m}$ values before high-light transfer, and they showed an initial decrease in $F_{v}/F_{m}$ values during the first 6 h (Fig. 5A). Subsequently, the $F_{v}/F_{m}$ value for the vte3 npq1 lori strain remained relatively constant, whereas the $F_{v}/F_{m}$ value

### Table 1. HPLC analysis of tocopherols and plastoquinone

| Strain | Total tocopherol | α-Tocopherol | β-Tocopherol | γ-Tocopherol | δ-Tocopherol | Mean plastoquinone level (nmol/g [dry weight]) ± SD
|--------|----------------|-------------|-------------|-------------|-------------|-----------------------------------|
| Wild type | 10.1 ± 1.7 | 8.2 ± 1.4 (81) | 0.4 ± 0.1 (4) | 1.0 ± 0.5 (10) | 0.5 ± 0.1 (5) | 294 ± 24 |
| vte3 mutant | 14.0 ± 1.9 | 0 (0) | 13.7 ± 1.8 (98) | 0 (0) | 0.3 ± 0.1 (2) | 318 ± 32 |
| Complemented vte3 mutant | 27.0 ± 5.5 | 25 ± 5.4 (92) | 0.2 ± 0.0 (1) | 1.7 ± 0.1 (6) | 0.1 ± 0.0 (1) | NA |
| npq1 lori mutant | 10.0 ± 0.2 | 9.0 ± 0.2 (90) | 0.3 ± 0.1 (4) | 0.5 ± 0.1 (4) | 0.2 ± 0.1 (2) | NA |
| vte3 npq1 lori mutant | 10.1 ± 1.0 | 0 (0) | 9.9 ± 1.0 (98) | 0 (0) | 0.2 ± 0.1 (2) | NA |
| VTE3/VTE3 diploid | 29.6 ± 4.3 | 24.3 ± 4.1 (82) | 2.7 ± 1.7 (9) | 2.0 ± 1.3 (7) | 0.6 ± 0.3 (2) | NA |
| vte3/vte3 diploid | 12.3 ± 3.4 | 0 (0) | 11.7 ± 3.1 (95) | 0 (0) | 0.6 ± 0.6 (5) | NA |
| VTE3/vte3 diploid | 36.5 ± 11.3 | 25.5 ± 5.8 (70) | 7.3 ± 4.5 (20) | 2.1 ± 1.5 (6) | 1.6 ± 1.2 (4) | NA |

$a$ The data are means ± SD (n = 3 to 6).

$b$ The percentage of each tocopherol in the total pool is given in parentheses.

$^c$ NA, not applicable.
for the npq1 lor1 mutant decreased further, reaching a minimum at 24 h. A loss of chlorophyll a was observed for both the double and the triple mutants upon high-light exposure. Nevertheless, the triple mutant was able to retain a significantly higher chlorophyll a content, especially at later time points (Fig. 5B). The vte3 npq1 lor1 strain also exhibited slightly but significantly higher cell viability than the npq1 lor1 strain (Fig. 5C). Thiobarbituric acid-reactive substances were used to measure the degree of lipid peroxidation of all strains under stress. The wild-type and vte3 strains exhibited a low and constant level of lipid peroxidation (Fig. 5D). The npq1 lor1 double mutant showed a level of lipid peroxidation that was similar to that of the vte3 npq1 lor1 triple mutant at up to 24 h in high light. At 48 h, however, the lipid peroxidation level continued to increase in the double mutant but stayed constant in the triple mutant. The tocopherol content in the wild-type and vte3 strains stayed relatively constant for the first 24 h. At 48 h, however, the levels of tocopherol increased in both strains, with the wild type having a significantly higher tocopherol level. A difference in tocopherol levels between the double and the triple mutants was observed at as early as 6 h until the end of the experiment. The α-tocopherol level in the npq1 lor1 strain followed the same trend as that of chlorophyll a, staying at a constant level for the first 12 h but exhibiting a dramatic decrease at 24 and 48 h. In contrast, the β-tocopherol level in the vte3 npq1 lor1 strain decreased in the first 12 h before remaining relatively constant. Because tocopherol has been shown to protect the D1 reaction center protein from degradation by singlet oxygen generated in photosystem II during oxidative stress (26, 53), we investigated D1 protection by different forms of tocopherol in the npq1 lor1 and vte3 npq1 lor1 strains. Cultures exposed to high light were used for immunoblotting and quantitation of total D1 (Fig. 6A and B). The double mutant immediately exhibited a steady decrease in levels of total D1 protein after high-light transfer (Fig. 6B). In contrast, the triple mutant
showed a delay in the decrease in the D1 level in the first 6 h. After 6 h, the level of D1 in the triple mutant also decreased steadily, as in the double mutant. After 48 h in high light, the steady-state level of D1 was significantly lower in the double mutant than in the triple mutant. To assay D1 photodamage and degradation, a similar experiment was performed with lincomycin added to inhibit the synthesis of new D1 after degradation (Fig. 6C and D). The initial rates of D1 photodamage and degradation were not significantly different between the two strains (Fig. 6D). However, 4 h after lincomycin was added, the amount of D1 was significantly higher in the npq1 lor1 strain than in the vte3 npq1 lor1 strain.

**DISCUSSION**

In *A. thaliana*, the enzyme MPBQ/MSBQ methyltransferase catalyzes both the conversion of MPBQ to DMPBQ, a precursor of γ- and α-tocopherols, and the conversion of MSBQ to plastoquinone. A mutation in the gene encoding this enzyme therefore affects the synthesis of both molecules. Plastoquinone-deficient mutants of maize and *A. thaliana* exhibit severe growth defects and seedling lethality (7, 9, 32). They are also α-tocopherol deficient. For cyanobacteria, the situation is different. First, the cyanobacterial enzyme is very diverged from the plant version, with less than 20% amino acid identity between VTE3 from *A. thaliana* and the sll0418 gene product from *Synechocystis* sp. strain PCC 6803 (7). Second, *Synechocystis* sp. strain PCC 6803 sll0418 mutants did not completely lack α-tocopherol or plastoquinone. Instead, the tocopherol content decreased to 35% of the wild-type level, and its composition was only slightly affected, with a small increase in β- and δ-tocopherol levels (40). The plastoquinone level appeared to be unaffected (4). These phenotypes suggest that *Synechocystis* must possess additional methyltransferases that have activity toward MPBQ and MSBQ. There is also evidence supporting the existence of a second plastoquinone biosynthetic pathway in *Synechocystis* sp. strain PCC 6803 (10).

*C. reinhardtii* is the only known organism so far that carries orthologs of both the plant and cyanobacterial MPBQ methyltransferase genes (7). A previously reported study showed that the *C. reinhardtii* cyanobacterium-type enzyme can use both MPBQ and MSBQ as substrates in vitro (7). Therefore, it seemed likely that this enzyme has a role in both tocopherol and plastoquinone syntheses in this organism. For our study, however, the *C. reinhardtii* plant-type VTE3 enzyme appears to be the only enzyme functioning as an MPBQ methyltransferase in tocopherol synthesis in vivo, because the vte3 mutant does not accumulate any trace amount of α-tocopherol. The fact that the level of plastoquinone is unaffected in this mutant

**FIG. 3.** Molecular genetic analysis of vte3. (A and B) Cosegregation analysis of vte3 and the ortholog of *A. thaliana* VTE3 (A) and the ortholog of the *Synechocystis* sp. strain PCC 6803 sll0418 gene product (B). The vte3 mutant was crossed to a polymorphic wild-type strain (WT) to obtain haploid progeny. A single-nucleotide polymorphism was scored for each gene. The form of tocopherol accumulated in each strain is indicated by α or β. 1a and 2d are progeny of two complete tetrads. A total of 45 progeny were tested. (C) Relative positions of both orthologs on linkage group XV. The orthologs are located 144 kb apart on linkage group XV. EST894011B10, IDA2, DHC7, and ZSP2 are chromosomal markers. VTE3, *A. thaliana* ortholog; sll0418, *Synechocystis* sp. strain PCC 6803 gene product ortholog.

**FIG. 4.** Growth phenotype of *C. reinhardtii* mutants under different light conditions. Serial dilutions of cells were spotted onto minimal agar medium and grown under conditions of low light for 1 week before being shifted to the indicated conditions. Cells were incubated for 72 h in low light (LL) (left) and high light (HL) (middle). For light shift conditions (right), cells were treated in the same way as they were for high light except that they were later transferred back to low light for 5 days. Low light, 50 μmol photons m⁻² s⁻¹; high light, 500 μmol photons m⁻² s⁻¹. WT, wild type.
FIG. 5. Chlorophyll fluorescence, chlorophyll a content, cell viability, lipid peroxidation, and tocopherol content upon high-light (HL) exposure of _C. reinhardtii_ strains. (A) The chlorophyll fluorescence parameter $F'_F_\infty$, representing the maximal photosynthetic efficiency of photosystem II in the dark-adapted state. (B) Chlorophyll (Chl) a content per cell. (C) Cell viability expressed as CFU/ml of culture relative to the initial value (rel. to initial). (D) Lipid peroxidation quantified as malondialdehyde (MDA) equivalents per cell. (E) Tocopherol content per cell. Data shown are means ± standard deviations ($n = 4$ to 7). Error bars are shown where they are larger than symbols. The differences between the _npq1 lor1_ and _vte3 npq1 lor1_ strains were statistically significant (Student’s $t$ test) (* indicates a $P$ value of <0.05, and ** indicates a $P$ value of <0.01). The difference in tocopherol contents between the wild-type and _vte3_ strains at 48 h was statistically significant (Student’s $t$ test) (+ indicates a $P$ value of <0.05). Open circles, _npq1 lor1_ mutant; filled circles, _vte3 npq1 lor1_ mutant; open triangles, wild type; filled triangles, _vte3_ mutant. (Table 1) indicates that a methyltransferase other than VTE3 functions in plastoquinone synthesis or that there is a functional redundancy of methyltransferases in this step of plastoquinone synthesis. One obvious candidate for MSBQ methyltransferase activity is the cyanobacterium-type enzyme. In the _Chlamydomonas vte3_ background, we generated RNA interference lines targeting the gene encoding the cyanobacterium-type enzyme, but they showed no reduction of the plastoquinone level (data not shown). Three scenarios are then possible. First, it is possible that neither VTE3 nor the cyanobacterium-type enzyme is involved in plastoquinone synthesis. Second, both VTE3 and the cyanobacterium-type enzyme might be involved, but this particular _vte3_ allele might not affect plastoquinone synthesis. Last, either VTE3 or the cyanobacterium-type enzyme (or both) are normally used for plastoquinone synthesis; however, one or more other methyltransferases can function in plastoquinone synthesis if the other enzyme(s) is absent. Resolution of this issue will require further investigation.

It is not surprising that the _vte3_ mutation on its own shows no growth phenotype even under conditions of high light. Results from previous studies of other organisms support this observation. _Synechocystis_ sp. strain PCC 6803 and _A. thaliana_ tocopherol mutants lacking all tocopherols or having a reduced level of α-tocopherol also showed no growth phenotype under conditions of high-light treatment (19, 29, 40). Only under extreme conditions, such as a combination of very high light and very low temperature or high light with linoleic or linolenic acid treatment, did the phenotypes emerge. Because tocopherol-deficient mutants showed no phenotype, it was previously suggested that other mechanisms such as the presence of other antioxidants can compensate for the loss of tocopherol (19, 36).

The most plausible mechanism of compensation for a tocopherol deficiency is the antioxidant activity of carotenoids. Both tocopherols and carotenoids have been shown to be essential for preventing lipid peroxidation and protecting photosystem II from singlet-oxygen damage (29, 43). Several studies have demonstrated interactions or overlapping roles of these two groups of lipid-soluble antioxidants. For example, α-tocopherol and zeaxanthin were previously shown to have a synergistic effect on protection against lipid peroxidation in liposomes (55). The _A. thaliana npq1_ mutant, lacking zeaxanthin and antheraxanthin, is able to grow in high light, but it accumulates larger amounts of α-tocopherol during photoacclimation, suggesting that tocopherol can compensate for the missing carotenoid (18). Conversely, the _A. thaliana vte1_ mutant, which lacks tocopherol, accumulates larger amounts of zeaxanthin (19). _Synechocystis_ sp. strain PCC 6803 tocopherol mutants, which showed no phenotype in high light, were sensitive to a combination of high light and treatment with a carotenoid synthesis inhibitor, norflurazon (29). The previously characterized _C. reinhardtii npq1 lor1_ mutant, which lacks the carotenoids lutein and zeaxanthin and is sensitive to photooxidative stress (6, 35), is therefore an ideal background in which to study _vte3_. The isolation of _vte3_ in a screen for suppressors of _npq1 lor1_ and the demonstration of _vte3_ phenotypes in this background provide further evidence of the relationship between tocopherol and carotenoids.

When exposed to high light, the _npq1 lor1_ mutant exhibits
severe photobleaching and a loss of cell viability, photosystem II function, and the D1 protein, along with increased levels of lipid peroxidation (6). The substitution of α-tocopherol with β-tocopherol in the vte3 npq1 lor1 strain resulted in a partial rescue of these phenotypes (Fig. 4 to 6). The maintenance of photosystem II activity seems to be very sensitive to the form of tocopherol present, as the differences in $F_v/F_m$ values between the npq1 lor1 and vte3 npq1 lor1 strains appeared as early as 6 h after high-light transfer. The $F_v/F_m$ value remained lower in the double mutant than in the triple mutant at all time points, indicating higher photosystem II activity in the triple mutant.

Photosystem II constantly undergoes a damage-and-repair cycle, even under normal light conditions, which does not cause oxidative stress (24). This cycle involves the proteolytic degradation of the damaged D1 protein, followed by the synthesis and assembly of new D1 along with other proteins into photosystem II (3, 31). Under oxidative stress conditions such as high light, the rate of repair cannot keep up with the rate of damage, leading to a process called photoinhibition (50). Several previously reported studies have shown that various stresses, including oxidative stress, inhibit photosystem II repair but do not accelerate the rate of photosystem II damage (34, 49–51). When the npq1 lor1 and vte3 npq1 lor1 strains were exposed to high light in the presence of lincomycin, there was no significant difference in the initial rate of D1 degradation (Fig. 6D). Four hours after lincomycin was added, the vte3 npq1 lor1 strain showed a significantly higher level of D1 degradation than did the npq1 lor1 strain. However, the steady-state level of D1 after high-light exposure in the absence of lincomycin was higher in the vte3 npq1 lor1 mutant (Fig. 6B). Thus, in order to have a higher steady-state level of D1, the accumulation of β-tocopherol in the triple mutant must somehow allow the triple mutant to maintain a higher rate of new D1 synthesis. Although D1 degradation in the presence of lincomycin seemed to be higher in the vte3 npq1 lor1 strain at 4 h than in the npq1 lor1 strain, we cannot conclude that β-tocopherol is a less efficient singlet-oxygen quencher than α-tocopherol because there might be other factors that affect D1 degradation. Moreover, we tested the growth of these two strains in the presence of rose bengal, a singlet-oxygen generator, and found no difference in their sensitivities (data not shown).

Differences in photosystem II inactivation were followed by differences in the loss of chlorophyll, cell viability, lipid peroxidation, and tocopherol (Fig. 5). Interestingly, the loss of tocopherol generally did not follow the same trend as other parameters. The steady-state level of β-tocopherol in the vte3 npq1 lor1 strain decreased initially and then remained constant, whereas the α-tocopherol pool in the npq1 lor1 strain was relatively constant initially and decreased only after 12 h as the cells were bleaching. Assuming that the rate of tocopherol synthesis does not differ between the two strains, the initial decline in β-tocopherol levels in the vte3 npq1 lor1 strain would
indicate a higher rate of turnover of tocopherol. Even though the level of \( \beta \)-tocopherol in the \( \text{vte} 3 \) \( npq1 \) \( lor1 \) strain was much lower than that of \( \alpha \)-tocopherol in the \( npq1 \) \( lor1 \) strain at 12 h, the triple mutant was better able to withstand photodiss oxidative stress than the double mutant. When the wild-type and \( \text{vte} 3 \) strains were compared, the level of \( \beta \)-tocopherol was lower in the \( \text{vte} 3 \) strain at 48 h, but there was no difference in any of the parameters measured even though the difference in the tocopherol level was clear.

There are several possibilities that might explain the better survival of the \( \text{vte} 3 \) \( npq1 \) \( lor1 \) strain than the \( npq1 \) \( lor1 \) strain in high light. Damaged photosystem II reaction centers might generate free chlorophyll molecules that photosensitize singlet oxygen, leading to further oxidative damage and cell death. It is possible that \( \beta \)-tocopherol is a better quencher of singlet oxygen or scavenger of lipid peroxyl radicals than \( \alpha \)-tocopherol in vivo. A previous study reported that \( \beta \)-tocopherol physically quenches singlet oxygen in vitro with the same efficiency as that of \( \alpha \)-tocopherol but exhibits very little chemical reactivity (22). This might allow \( \beta \)-tocopherol to avoid direct oxidation by singlet oxygen, thereby maintaining its capacity for physical quenching. This could be particularly important in the \( npq1 \) \( lor1 \) background, which shows an increased generation of singlet oxygen in high light (6, 27). Alternatively, the two forms of tocopherol might activate different gene expressions in response to oxidative stress. We cannot exclude the possibility that it is the absence of \( \beta \)-tocopherol, rather than the presence of \( \alpha \)-tocopherol, that contributed to the better survival of the triple mutant. Future experiments will address these possibilities and explore the specific molecular mechanisms involved. Nevertheless, it is clear from our results that the accumulation of \( \beta \)-tocopherol instead of \( \alpha \)-tocopherol in a xanthophyll-deficient mutant confers greater resistance to photooxidative stress in vivo.

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