Tocopherols Protect *Synechocystis* sp. Strain PCC 6803 from Lipid Peroxidation[1]

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Tocopherols (vitamin E) are lipid-soluble antioxidants synthesized only by photosynthetic eukaryotes and some cyanobacteria, and have been assumed to play important roles in protecting photosynthetic membranes from oxidative stress. To test this hypothesis, tocopherol-deficient mutants of *Synechocystis* sp. strain PCC 6803 (*slr1736* and *slr1737* mutants) were challenged with a series of reactive oxygen species-generating and lipid peroxidation-inducing chemicals in combination with high-light (HL) intensity stress. The tocopherol-deficient mutants and wild type were indistinguishable in their growth responses to HL in the presence and absence of superoxide and singlet oxygen-generating chemicals. However, the mutants showed enhanced sensitivity to linoleic or linolenic acid treatments in combination with HL, consistent with tocopherols playing a crucial role in protecting *Synechocystis* sp. strain PCC 6803 cells from lipid peroxidation. The tocopherol-deficient mutants were also more susceptible to HL treatment in the presence of sublethal levels of norflurazon, an inhibitor of carotenoid synthesis, suggesting carotenoids and tocopherols functionally interact or have complementary or overlapping roles in protecting *Synechocystis* sp. strain PCC 6803 from lipid peroxidation and HL stress.

Oxygenic photosynthetic organisms continuously produce oxygen in the presence of light, and as such cellular damage from various reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•), is a constant threat. Photosynthetic organisms have therefore evolved extensive detoxifying and protective mechanisms, which both limit the production of and potential damage by ROS. Examples include superoxide dismutase (SOD), which reduces O_2^- to H_2O_2; ascorbate peroxidase, which reduces H_2O_2 to H_2O; and nonphotochemical quenching that quenches singlet state chlorophylls (^{1}Chl*) and harmlessly dissipates excessive excitation energy as heat, thereby reducing $^1O_2$ production (Asada, 1999; Muller et al., 2001).

ROS, such as OH•, can trigger a lipid peroxidation chain reaction by abstracting an allylic hydrogen from polyunsaturated fatty acid (PUFA)-containing lipids producing lipid radicals that are converted to lipid peroxyl radicals (LOO•) upon O_2 addition. LOO• can subsequently attack another PUFA generating a second LOO• and propagating a chain reaction of lipid peroxidation that perturbs membrane structure and function (Porter, 1986). Given the susceptibility of PUFAs to ROS damage, it seems counterintuitive that the PUFA-enriched thylakoid membranes would house the photosynthetic machinery, a potential ROS generator. In contrast to the well-studied mechanisms of water-soluble ROS detoxification in photosynthetic organisms (Asada, 1999), the mechanisms preventing or limiting oxidative damage in photosynthetic membranes are less well understood. Several peroxiredoxins have been implicated in reducing lipid hydroperoxides (LOOH) to the less toxic lipid hydroxides (LOH) in both plants and cyanobacteria (Gaber et al., 2001, 2004; Dietz, 2003). In Arabidopsis (*Arabidopsis thaliana*) and *Chlamydomonas reinhardtii*, specific carotenoids have also been shown to play roles in limiting lipid peroxidation, presumably by direct scavenging of free radicals (Havaux and Niyogi, 1999; Baroli et al., 2004). Tocopherols, a second major class of lipid-soluble antioxidants in photosynthetic membranes, are also believed to play important roles in this process (Fryer, 1992; Munne-Bosch and Alegre, 2002). However, there is surprisingly little direct experimental evidence supporting such functions for tocopherols in photosynthetic organisms.

Tocopherols consist of a polar chromanol head group attached to a hydrophobic phytyl tail, both of which are critical to their roles as lipid-soluble antioxidants. Based on studies in artificial and animal cell-derived membranes, tocopherols can efficiently quench $^1O_2$ and scavenge various radicals (Bramley et al., 2000). The chromanol ring of tocopherols can reduce radicals by the donation of a single electron, resulting in the formation of a relatively stable tocopheroxyl radical, which in animals can be recycled back to the corresponding tocopherol by other

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antioxidants such as ascorbate or coenzyme Q (Stoyanovsky et al., 1995; May et al., 1998). Subsequent donation of a second electron from the tocopheroxyl radical forms the nonradical product, tocopherol quinone.

The tocopherol biosynthetic pathway has recently been fully elucidated in *Synechocystis* sp. PCC 6803 (Fig. 1; Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). Homogentisate phytyltransferase (HPT) catalyzes the committed step in tocopherol synthesis by condensing homogentisate (HGA) and phytyl-diphosphate to produce 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ). HGA is produced from hydroxyphenylpyruvate (HPP) by HPP dioxygenase (HPPD). MPBQ is converted to 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by MPBQ methyltransferase. Both MPBQ and DMPBQ are substrates for tocopherol cyclase (TC) to produce δ- and γ-tocopherols, respectively, which are then converted to β- and α-tocopherols by γ-tocopherol methyltransferase. During analysis of the biosynthetic pathway in *Synechocystis* sp. PCC 6803, mutants disrupting each biosynthetic enzyme have been isolated and characterized (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). The TC (*slr1737*) mutant lacks tocopherols entirely but accumulates the quinonol intermediate, DMPBQ, whereas the HPT (*slr1736*) mutant lacks all tocopherols and pathway intermediates (Fig. 1; Collakova and DellaPenna, 2001; Schledz et al., 2001; Sattler et al., 2003).

We have utilized the *slr1736* and *slr1737* mutants to assess the roles that tocopherols play in ROS homeostasis, membrane protection, and how tocopherols are functionally integrated into the antioxidant network. In this study, these mutants were challenged with combinations of chemicals and/or abiotic stresses to induce the formation of different types of ROS, and the ability of the mutants to withstand these stresses was evaluated. The increased sensitivity of tocopherol-deficient mutants to specific treatments indicates that tocopherols play a crucial role in limiting lipid peroxidation in *Synechocystis* sp. PCC 6803 in vivo.

RESULTS

Growth of Tocopherol-Deficient Mutants under High Intensity Light and ROS-Generating Conditions

The previously reported tocopherol-deficient *Synechocystis* sp. PCC 6803 mutants containing gene disruptions in *HPT* (*slr1736*) and *TC* (*slr1737*) were originally isolated and maintained under photomixotrophic conditions, i.e. on Glc-containing media (Collakova and DellaPenna, 2001; Sattler et al., 2003). As described previously (Sakuragi, 2004), we now know that photomixotrophic selection is lethal for both mutant lines due to a Glc-sensitive phenotype that is a consequence of tocopherol deficiency. Thus, the original *slr1736* and *slr1737* mutant lines isolated had varying genotypes and physiologies presumably due to the unintentional selection of additional secondary suppressors of this Glc-sensitive phenotype. When the *aphII*-containing kanamycin-resistance DNA cartridge was reinserted into the *slr1736* and *slr1737* genes of wild-type *Synechocystis* sp. PCC 6803 and mutant selection was performed under photoautotrophic conditions, fully segregated populations were obtained that were genotypically and physiologically homogenous (Sakuragi, 2004). These authentic, photoautotrophically isolated *slr1736*::*aphII* and *slr1737*::*aphII* mutants were used under photoautotrophic conditions for all experiments in this study.

To test the susceptibility of tocopherol-deficient mutants to high-light (HL) intensity stress, the wild type and the *slr1736* and *slr1737* mutants were initially grown at a relatively low-light (LL) intensity for *Synechocystis* sp. PCC 6803 (15 μE m^{-2} s^{-1}), the cells diluted to an appropriate density and transferred to HL (300 μE m^{-2} s^{-1}). As shown in Figure 2A, HL had little impact on growth of the mutant lines in comparison to the wild type, indicating tocopherols are dispensable under the HL stress conditions tested.

To investigate further the susceptibility of tocopherol-deficient mutants to additional oxidative stresses, various ROS-generating and stress-inducing chemicals were applied in combination with HL treatment. Paraquat (methyl viologen) causes generation of O_2^- by transferring electrons from the PSI iron-sulfur clusters to O_2 (Fujii et al., 1990). Treatment with 2 μM paraquat/HL (paraquat in combination with 300 μE m^{-2} s^{-1} HL treatment) slowed the growth of the wild type and the *slr1736* and *slr1737* mutants to the same degree, while 5 μM paraquat/HL completely inhibited growth of all lines (Fig. 2B). Similarly, treatment with a sublethal concentration (3 μM) of Rose Bengal, a $^1O_2$-generating photosensitizer, in HL also inhibited growth of the wild type and the *slr1736* mutant to similar degrees (data not shown). These data suggest that tocopherols do not play an essential role in detoxifying or tolerating the damage of O_2^- and $^1O_2$ in *Synechocystis* sp. PCC 6803, or that other compounds or enzymes can compensate for the lack of tocopherols in this regard.

Sensitivity of Tocopherol-Deficient Mutants to Compounds That Enhance Lipid Peroxidation

Tocopherols are known to play a crucial role in protecting animal cells from lipid peroxidation (Ham and Liebler, 1995, 1997) and have been proposed to perform a similar function in photosynthetic organisms (Fryer, 1992; Munne-Bosch and Alegre, 2002). To test this hypothesis, a variety of chemicals were used to induce lipid peroxidation in the wild type and the tocopherol-deficient mutants. PUFAs, which are known to generate LOOH and LOO• by autoxidation

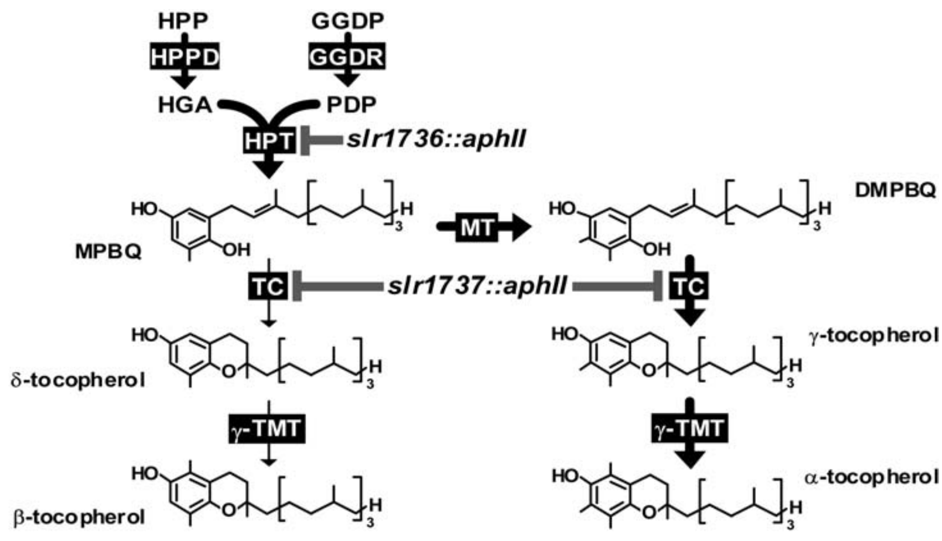

Figure 1. Tocopherol biosynthetic pathway and locations of mutations in *Synechocystis* sp. strain PCC 6803. GGDP, Geranylgeranyl-diphosphate; PDP, phytyl-diphosphate; GGDR, GGDP reductase; MT, MPBQ methyltransferase; γ-TMT, γ-tocopherol methyltransferase; *slr1736*::*aphII* and *slr1737*::*aphII*, disrupted mutants of HPT and TC, respectively. Bold arrows show the primary biosynthetic route in vivo; α-tocopherol is the major tocopherol in *Synechocystis* sp. strain PCC 6803.

reactions in the presence of oxygen (Porter, 1986), have been used to induce lipid peroxidation in yeast (Do et al., 1996) and cyanobacteria (Sakamoto et al., 1998). Linoleic acid ($18{:}2^{\Delta9,12}$) and linolenic acid ($18{:}3^{\Delta9,12,15}$), hereafter referred to as 18:2 and 18:3, respectively, were applied in combination with HL stress to the wild type and the *slr1736* and *slr1737* mutants. In the presence of 10 μM 18:2/HL, growth of the *slr1736* mutant ceased after 20 h, whereas the wild type and the *slr1737* mutant were able to grow as well as untreated controls (Fig. 2C). Treatment with 10 μM 18:3/HL slowed the growth of all strains similarly

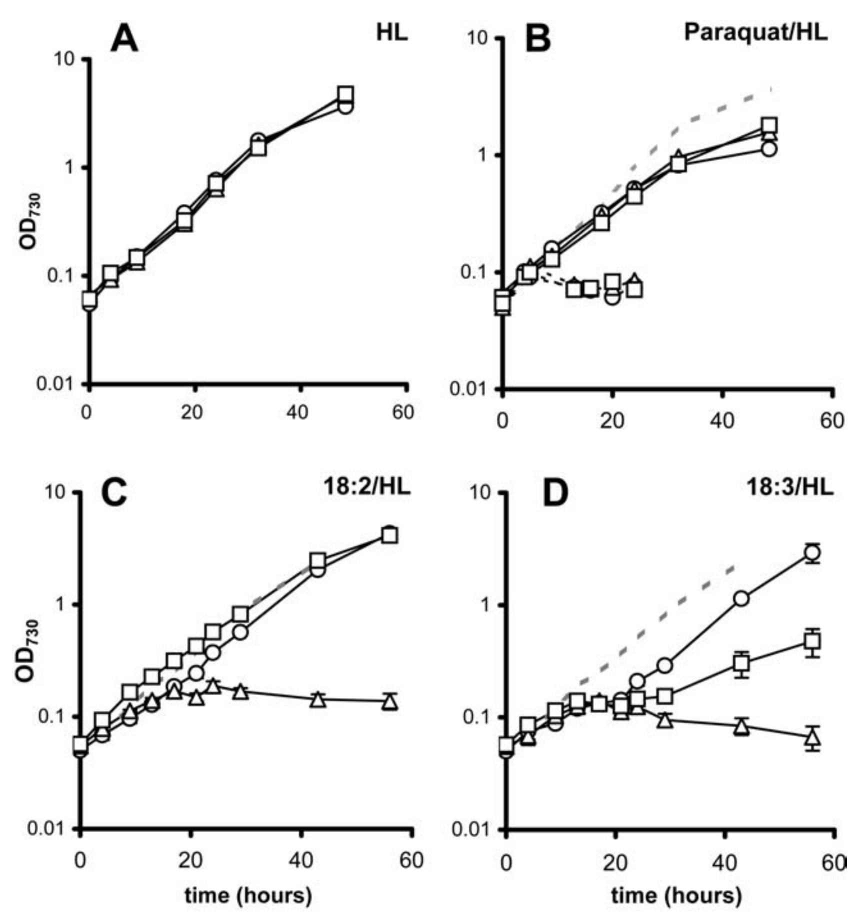

Figure 2. Growth curves of wild type (WT) and the tocopherol-deficient *slr1736* and *slr1737* mutants under different stress and chemical treatments. Wild type (circles), *slr1736* mutant (triangles), and *slr1737* mutants (squares) were grown at 32°C, 1% (v/v) CO_2 in air under A, HL; B, HL with 2 μM (solid lines) and 5 μM (dotted lines) paraquat; C, HL with 10 μM 18:2; and D, HL with 10 μM 18:3. A and B show representative results of at least three independent experiments, while data in C and D are the means ± SD ($n = 4$). SD in C and D are shown only when larger than symbols. In B, C, and D, the HL wild-type growth curve is shown as a gray dotted line with no symbol for reference.

during the initial 20 h of growth. At later time points, the wild-type growth rate fully recovered, the *slr1736* mutant ceased to grow, while the *slr1737* mutant showed an intermediate growth rate (Fig. 2D). These data indicate that the tocopherol-deficient mutants are more susceptible to PUFA treatments than the wild type and that in *Synechocystis* sp. PCC 6803 tocopherols play critical roles in protecting cells from PUFA-induced stress. The intermediate phenotype of the *slr1737* mutant, which lacks tocopherols but accumulates the redox-active pathway intermediate DMPBQ, suggests that DMPBQ can partially compensate for the absence of tocopherols under these conditions.

Only the wild-type and *slr1736* mutant strains were used for subsequent analyses, and the initial OD_{730} for growth experiments was increased from 0.05 OD_{730} to 0.5 OD_{730} in order to obtain sufficient cells for biochemical analyses. Dose-response curves indicated the 10-fold increase in initial cell concentration required a corresponding increase in PUFA treatment levels to impact growth similarly (data not shown). Treatment of 0.5 OD_{730} cultures with 100 μM 18:3/HL slowed the growth of both the wild-type and *slr1736* mutant strains in the initial 20 h, while at later time points the *slr1736* mutant ceased to grow, and growth of the wild type recovered in a fashion similar to that observed in treating 0.05 OD_{730} cultures with 10 μM 18:3/HL (compare Figs. 2D and 3C). The monounsaturated fatty acid, oleic acid ($18:1^{\Delta 9}$), hereafter referred to as 18:1, was used to test whether the toxicity of 18:3 to the *slr1736* mutant was due to the presence of any free fatty acid in the media (a detergent effect) or was specific to PUFAs. Both the wild-type and *slr1736* mutant strains were unaffected by treatment with 100 μM 18:1/HL (data not shown) and were able to grow unaffectedly even in the presence of 500 μM 18:1/HL (Fig. 3E). These data indicate that the differential effects of 18:3 and 18:2 treatments on the growth of the wild-type and *slr1736* mutant strains are due to the polyunsaturation of these fatty acids. To test whether other PUFAs can also cause growth inhibition, eicosatrienoic acid ($20:3^{\Delta 11,14,17}$), hereafter referred to as 20:3, was applied at 100 μM, the same concentration of 18:3 that impacted growth of the *slr1736* mutant. Surprisingly, 100 μM 20:3/HL did not show a toxic effect on either the wild type or the *slr1736* mutant (Fig. 3G). These data suggest that factors in addition to the degree of polyunsaturation determine the toxicity of different PUFAs in the tocopherol-deficient mutants.

tert-Butyl hydroperoxide (*t*-BOOH) is a lipid-soluble hydroperoxide that has been used to induce lipid peroxidation in yeast and animal cells (Masaki et al., 1989; Pereira et al., 2003). Dose-response curves indicated growth of both the wild-type and *slr1736* mutant strains was negatively impacted at 150 μM *t*-BOOH/HL while 200 μM was lethal (data not shown). Growth of the wild-type and the *slr1736* mutant strains in 150 μM *t*-BOOH/HL was initially inhibited, but both recovered rapidly to a similar extent (Fig. 3I), indicating that tocopherols are not essential for acclimation to *t*-BOOH-induced stress.

PUFA Treatments Increase Peroxides in the Growth Media

Because PUFA treatment has previously been shown to cause accumulation of lipid peroxides in yeast (Do et al., 1996) and the cyanobacterium *Synechococcus* sp. PCC 7002 (Sakamoto et al., 1998), the level of total peroxides in the growth media of wild-type and *slr1736* mutant strains during different treatments were measured using the ferrous oxidation-xylenol orange (FOX) assay (Griffiths et al., 2000; Sattler et al., 2004) and correlated with growth rates. LL, HL, and 18:1/HL treatments did not differentially affect growth of the wild-type and *slr1736* mutant strains (Fig. 3, A and E) and did not increase the peroxide levels of the media above background levels (Fig. 3, B and F). *t*-BOOH/HL, 18:3/HL, and 20:3/HL treatments all resulted in high levels of peroxides in the media but had different impacts on growth. Media peroxide levels in *t*-BOOH/HL-treated wild-type and *slr1736* mutant cells were elevated at 30 min, returned to background levels by 4 h, but were much lower than in the absence of cells at all time points (Fig. 3J). Therefore, it appears that both the wild type and the *slr1736* mutant can rapidly reduce *t*-BOOH, which would explain the limited and similar impact of *t*-BOOH treatment on cell growth of both lines (Fig. 3I).

Media peroxide levels in cells treated with 18:3/HL and 20:3/HL were near background levels at 30 min, increased to their highest levels by 4 or 8 h, and decreased thereafter. In the absence of cells, media peroxide levels increased linearly in treatments with both 18:3/HL and 20:3/HL (Fig. 3, D and H). The media peroxides produced during the 18:3/HL treatment were separated into water and lipid phases, and more than 90% of the total peroxides were found in the lipid phase (data not shown), indicating the peroxides detected in the media are mainly lipid-derived peroxides. The media peroxide levels of *slr1736* mutant cells treated with 18:3/HL and 20:3/HL were always equivalent or higher than the levels in treated wild-type cells. However, despite the apparent correlation of higher medium peroxide levels, especially at early time points, with more severe growth inhibition in *slr1736* mutant cells treated with 18:3/HL, it is clear that media peroxide levels are not the root cause of growth inhibition. Indeed, cells of the wild type and the *slr1736* mutant treated with 20:3/HL had media peroxide profiles and levels similar to 18:3/HL-treated cells (Fig. 3H); however, there was no impact on growth of either genotype by 20:3 treatment (Fig. 3G). This suggests that other processes within the PUFA-treated cells, such as the differential incorporation and/or the oxidation of specific fatty acids in membranes, contribute to the observed growth inhibition of the *slr1736* mutant.

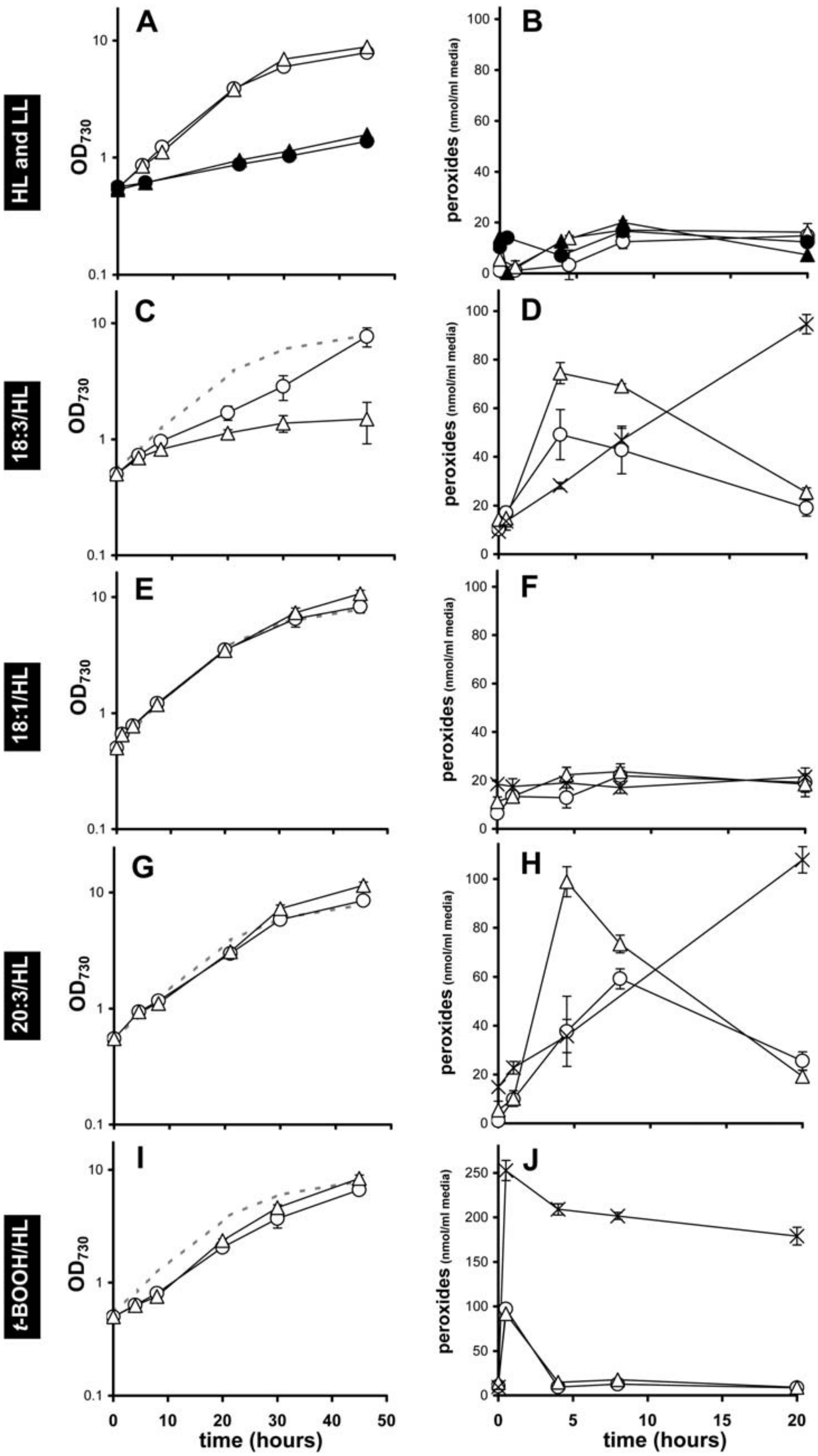

Figure 3. Growth curves and medium peroxide levels of the wild type and the tocopherol-deficient *slr1736* mutant under HL with various chemical treatments. Wild type (circles) and *slr1736* mutant (triangles) were grown at 32°C, 1% (v/v) CO_2 in air under HL. A and B, Control (HL, white symbols; LL, black symbols); C and D, HL with 100 μM 18:3; E and F, HL with 500 μM 18:1; G and H, HL with 100 μM 20:3; I and J, HL with 150 μM *t*-BOOH. A, C, E, G, and I are 45-h growth curves, while B, D, F, H, and J are the respective medium peroxide levels during the first 20 h. Peroxide levels before addition of chemicals are shown at 0 h (B, D, F, H, and J). The *y* axis scale of J is different from B, D, F, and H. The gray dotted lines in C, E, G, and I are the growth curve of HL-treated wild type from A for reference. Crossed marks in D, F, H, and J are media peroxide levels in the absence of cells. The data shown are the means ±SD of cultures grown in triplicate, except the LL media peroxide levels, which are representative of three independent experiments (B). The SD is shown only when it is larger than the symbols except for the LL media peroxide levels in B.

Incorporation of 18:3 and 20:3 Fatty Acids into Membrane Lipids

The possibility that the toxicity of 18:3/HL may be associated with more efficient uptake/incorporation of 18:3 into membranes in comparison to 20:3 was examined by analyzing the esterified fatty acid composition of membrane lipids after 4 h of 18:3/HL and 20:3/HL treatments. 18:3/HL and 20:3/HL treatments both resulted in increased levels of esterified 18:3 and 20:3, respectively, in both the wild type and the *slr1736* mutant relative to HL controls, though the increase from the 18:3/HL treatment was about 3-fold greater than that of the 20:3/HL treatment (Fig. 4). Some incorporated 18:3 also appeared to be further desaturated to stearidonic acid ($18{:}4^{\Delta6,9,12,15}$) or elongated to 20:3. As a consequence of the increased incorporation of 18:3 relative to 20:3, the total membrane PUFA content in cells of both the wild-type and *slr1736* mutant strains was increased significantly by 18:3/HL treatment but only slightly by 20:3/HL treatment relative to HL controls (Fig. 4). These results suggest that differential lethality of 18:3/HL and 20:3/HL treatments in the *slr1736* mutant are associated with the more efficient uptake/incorporation of 18:3 relative to 20:3. Because of carry over of exogenously applied free PUFAs in washed cell pellets, we were unable to assess the relative free PUFA pool sizes of 18:3- and 20:3-treated wild-type and *slr1736* mutant cells.

Attempts were made to assess the cellular levels of lipid peroxidation by-products, LOOH and LOH, in 18:3/HL-treated cells of the wild-type and *slr1736* mutant strains using the FOX assay (Griffiths et al., 2000) and HPLC analysis (Sattler et al., 2004), respectively, but the results were inconclusive. LOOH and LOH levels in washed cell pellets did increase severalfold in response to 18:3 and 20:3 treatments, but these increases were highly variable and in all cases paralleled the LOOH and LOH levels detected in the media. Therefore, as with analysis of cellular-free PUFA levels, it appears that the high background level of LOOH and LOH in the media of PUFA-treated cells precludes distinguishing and quantifying lipid peroxidation products that were specifically generated in cells or cell membranes.

Changes in Carotenoids, Chlorophyll *a*, and Tocopherols during HL and 18:3/HL Treatments

The effect of HL and 18:3/HL treatments on photosynthetic pigment composition (carotenoids and chlorophyll *a*) and tocopherols was analyzed by HPLC. In the absence of any treatment (LL-grown cells), the total carotenoid and chlorophyll contents of the wild-type and *slr1736* mutant strains were identical (Fig. 5, A and C, at 0 h). Individual carotenoid levels were also nearly identical with the exception of myxoxanthophyll and zeaxanthin, which were slightly lower and higher, respectively, in the *slr1736* mutant in comparison to the wild type (Fig. 6, A and C, at 0 h). The total carotenoid content of HL-treated wild-type cells was unchanged during the first 20 h (Fig. 5A), but there was a significant increase in myxoxanthophyll and a corresponding decrease in zeaxanthin and echinenone levels (Fig. 6, A, C, and E). By 45 h, the total carotenoid content of HL-treated wild type had increased 20%,

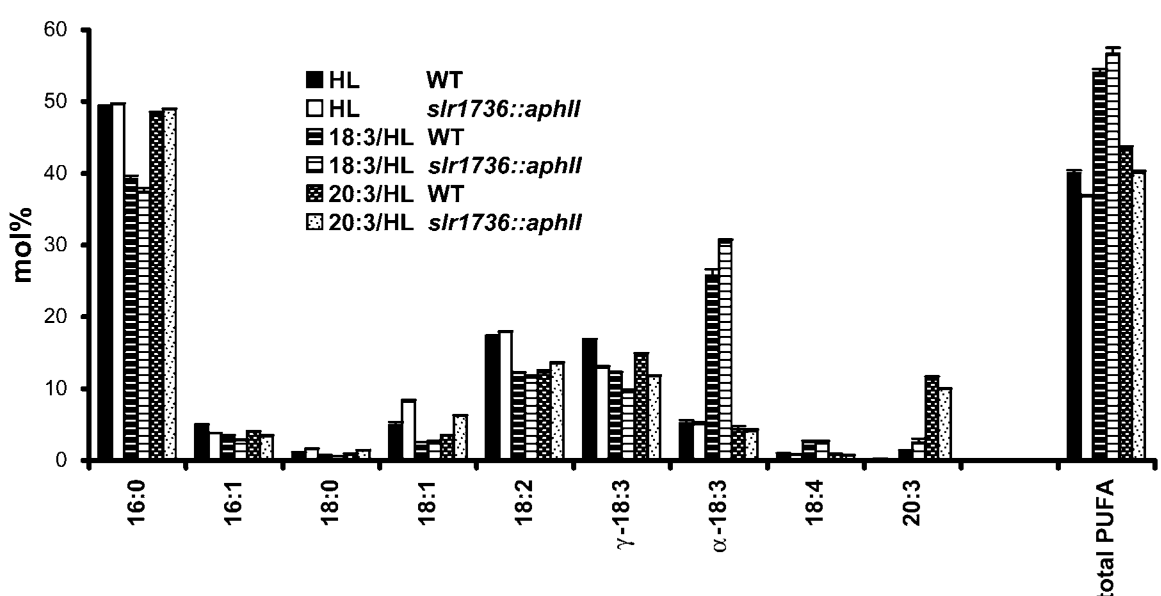

Figure 4. Fatty acid composition of total membrane lipid extracts from the wild type (WT) and the tocopherol-deficient *slr1736* mutant after 4 h of PUFA treatment. The molar percentage of each fatty acid species esterified to membrane lipids is indicated. Total PUFA levels were calculated as sums of 18:2, γ-linolenic acid (γ-18:3), α-linolenic acid (α-18:3), stearidonic acid (18:4), and 20:3 in each genotype. Data shown are the means ± SD ($n = 4$). SD is shown only when it is larger than symbols.

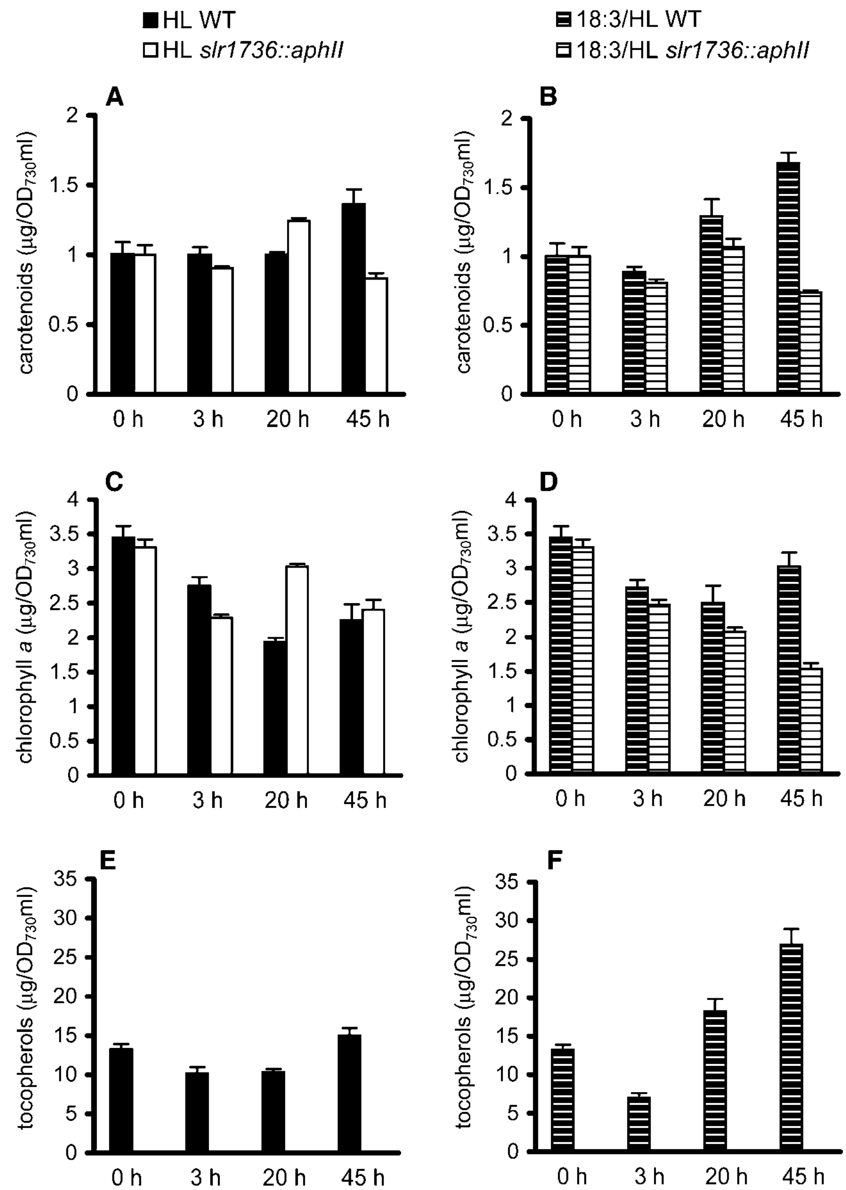

Figure 5. Total carotenoid, chlorophyll, and tocopherol contents in the wild type (WT) and the tocopherol-deficient *slr1736* mutant during HL and 18:3/HL treatments. Total carotenoid (A and B), chlorophyll *a* (C and D), and tocopherol (E and F) levels were measured at 0, 3, 20, and 45 h of HL (A, C, and E) and 100 μM 18:3/HL (B, D, and F) treatments at 32°C, 1% (v/v) CO_2 in air. Tocopherol was not detected in the *slr1736* mutant in any experiments. Data shown are the means ± SD (n = 3).

mostly due to an increase in myxoxanthophyll content (Fig. 6A). When the wild type was subjected to 18:3/HL treatment, the total carotenoid content decreased slightly at 3 h (Fig. 5A) due to small but significant decreases in myxoxanthophyll and zeaxanthin (Fig. 6, B and D). Total carotenoid levels then increased at 20 h and were 67% higher by 45 h (Fig. 5B) due to a large increase in myxoxanthophyll levels and smaller increases in zeaxanthin and β-carotene (Fig. 6, B, D, and H). These data indicate that carotenoid synthesis in the wild type is up-regulated in response to both HL and 18:3/HL treatments.

The total carotenoid level of *slr1736* mutant cells treated with HL and 18:3/HL were similar to the wild type for the initial 3 h of treatment and transiently increased at 20 h before decreasing to approximately 80% of the initial control level by 45 h (Fig. 5, A and B). The decrease in total carotenoid levels in the *slr1736* mutant during HL treatment was due almost entirely to a precipitous drop in myxoxanthophyll levels by 45 h (Fig. 6A). This drop also occurred in the 18:3/HL-treated *slr1736* mutant along with a severe decrease in zeaxanthin levels (Fig. 6, B and D). This reduction in individual and total carotenoid levels in HL- and 18:3/HL-treated *slr1736* mutant cells sharply contrasts with the wild type and suggests that, in the absence of tocopherols, specific carotenoids in the *slr1736* mutant cells undergo more rapid turnover/degradation than in wild-type cells.

The chlorophyll *a* contents of the wild-type and *slr1736* mutant cells during HL treatment were very similar with the exception of 20 h, where the *slr1736* mutant showed a transient increase (Fig. 5C). This similarity in chlorophyll content is consistent with the

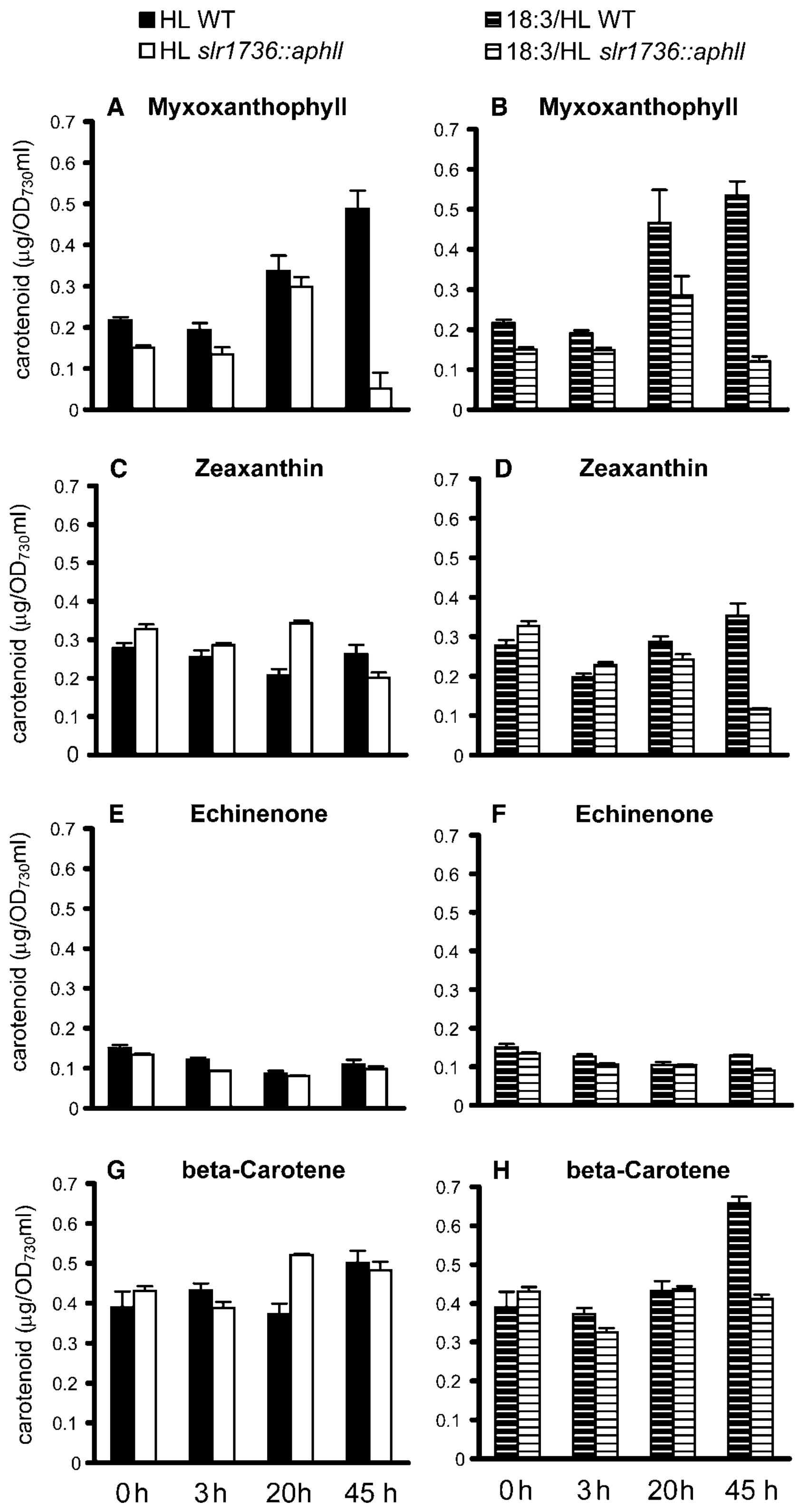

Figure 6. Levels of individual carotenoids in the wild type (WT) and the tocopherol-deficient *slr1736* mutant during HL and 18:3/HL treatments. Myxoxanthophyll (A and B), zeaxanthin (C and D), echinenone (E and F), and β-carotene (G and H) were measured at 0, 3, 20, and 45 h of HL (A, C, E, and G) and 100 μM 18:3/HL (B, D, F, and H) treatments at 32°C, 1% (v/v) CO_2 in air. Data shown are the means ± SD (n = 3).

growth of the wild type and the *slr1736* mutant being indistinguishable in HL (Fig. 3A). When the wild type was subjected to 18:3/HL treatment, chlorophyll levels initially decreased before recovering by 45 h, in parallel with the increase in total carotenoids (Fig. 5, B and D). By contrast, the chlorophyll content of 18:3/HL-treated *slr1736* mutant cells continuously decreased at all time points to 46% of the initial value by 45 h (Fig. 5D), suggesting that impaired growth of the *slr1736* mutant (Fig. 3C) was coincident with the loss of photosynthetic capacity as reflected by the lower chlorophyll content.

The total tocopherol content was also measured in wild-type and *slr1736* mutant cells subjected to HL and 18:3/HL treatment (Fig. 5, E and F). No tocopherols were detected in the *slr1736* mutant cells at any time point or treatment, consistent with the nature of the mutation. The tocopherol content of HL-treated wild type was reduced approximately 20% at 3 and 20 h before recovering by 45 h. When wild-type cells were subjected to 18:3/HL treatment, a more severe reduction in tocopherols was observed after 3 h followed by a sharp increase at 20 and 45 h to twice the initial level. This initial decrease followed by accelerated accumulation of tocopherols during 18:3/HL treatment of the wild type suggests tocopherols play a key role in the response of *Synechocystis* sp. PCC 6803 to 18:3-induced oxidative stress.

Norflurazon/HL Treatment

The experiments described above (Fig. 5A) further suggested a possible functional interaction between carotenoids and tocopherols in *Synechocystis* sp. PCC 6803. To assess any potential interaction, carotenoid synthesis was inhibited with norflurazon (NF), a herbicide that specifically inhibits phytoene desaturase (Breitenbach et al., 2001; He et al., 2001). Dose-response experiments indicated that growth of the *slr1736* mutant was much more sensitive to inhibition of carotenoid synthesis at levels as low as 5 μM NF/HL (Fig. 7A). During treatment with 25 μM NF/HL, the wild type grew more slowly than HL treatment alone but was still viable, while growth of the *slr1736* mutant was completely abolished after 30 h (Fig. 7B). Under LL conditions, treatments with 25 μM or 100 μM NF did not affect the growth of either wild type or the *slr1736* mutant relative to untreated cells (data not shown). These results indicate that, when *Synechocystis* sp. PCC 6803 cells are subjected to HL stress, the simultaneous inhibition of both carotenoid and tocopherol synthesis is more deleterious than inhibition of either pathway alone.

Pigment analyses during NF/HL treatment revealed that total carotenoid levels decreased much faster in the *slr1736* mutant cells compared to wild-type cells (Fig. 8A). While both the wild type and the *slr1736* mutant reached a lower steady-state carotenoid level by 20 h of NF/HL treatment, the steady-state carotenoid level in the *slr1736* mutant cells was less than half that of wild-type cells. Chlorophyll levels were similar in the *slr1736* mutant and the wild type up to 30 h (Fig. 8B), but by 45 h the *slr1736* mutant had lost almost all carotenoids and chlorophyll, while wild-type cells maintained constant levels of both. Because carotenoid synthesis is presumably inhibited to the same degree by 25 μM NF treatment in the wild type and the *slr1736* mutant, these results suggest that carotenoids were degraded more rapidly during the NF/HL treatment in the absence of tocopherols; this loss of carotenoids in turn led to bleaching and eventual death of the *slr1736* mutant cells. When individual carotenoids were analyzed during NF/HL treatment, all were found to decrease in both the wild type and the *slr1736* mutant, but myxoxanthophyll and β-carotene decreased to lower levels in the *slr1736* mutant than in wild type (Fig. 9). The combined results of NF/HL

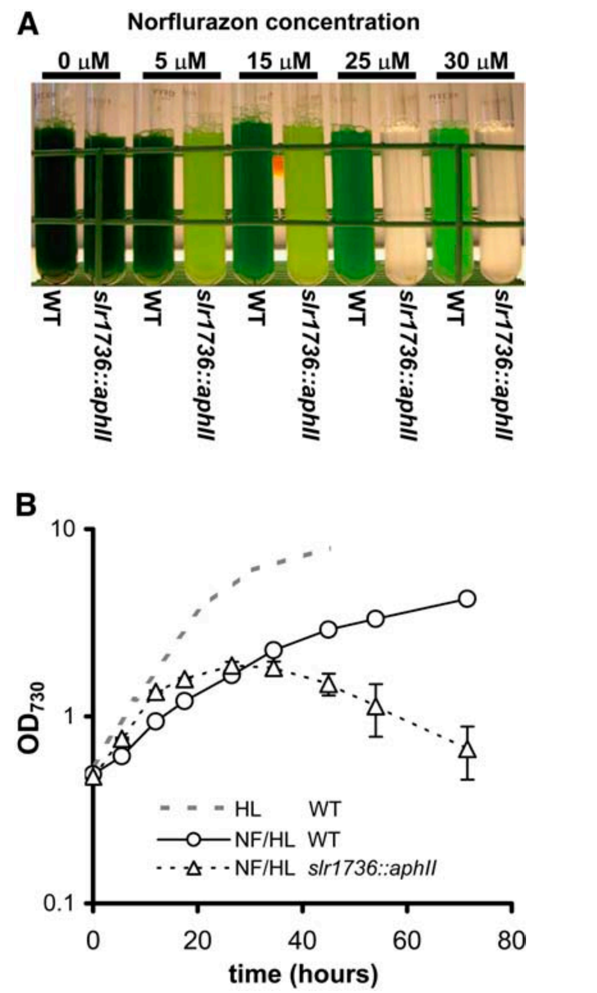

Figure 7. Growth of the wild type (WT) and the tocopherol-deficient *slr1736* mutant in the presence of NF in HL. A, Wild type and the *slr1736* mutant were grown at the indicated concentration of NF for 90 h under HL at 32°C, 1% (v/v) CO_2 in air. B, Growth curves of wild type (circles) and the *slr1736* mutant (triangles) during 25 μM NF/HL treatment. Data shown are the means ± SD (n = 4). SD is shown only when it is larger than the symbols. The growth curve for the wild type under HL was shown as a gray dotted line.

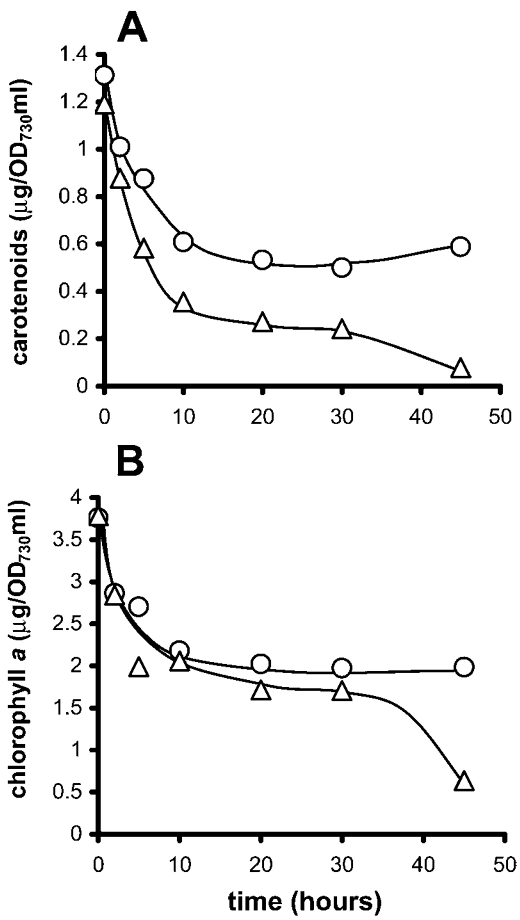

Figure 8. Changes in total carotenoids and chlorophyll *a* contents in the wild type and the tocopherol-deficient *slr1736* mutant during NF/HL treatment. Total carotenoids (A) and chlorophyll *a* levels (B) of the wild type (circles) and the *slr1736* mutant (triangles) were measured during 25 μM NF/HL treatment at 32°C, 1% (v/v) CO_2 in air. These data are representative of two independent experiments.

treatment on growth and photosynthetic pigments demonstrate that tocopherols and carotenoids play important and complementary roles in protecting *Synechocystis* sp. PCC 6803 cells from HL stress.

DISCUSSION

In contrast to the well-established roles of tocopherols in animals (Brigelius-Flohe and Traber, 1999; Ricciarelli et al., 2002), assessing tocopherol functions in photosynthetic organisms has only recently become experimentally approachable as a result of the complete molecular dissection of the biosynthetic pathway and isolation of mutants in cyanobacteria and plants (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Porfirova et al., 2002; Shintani et al., 2002; Cheng et al., 2003; Sattler et al., 2003). The evolutionary conservation of tocopherol synthesis in oxygenic phototrophs, the localization of tocopherols in photosynthetic membranes, and the increased tocopherol accumulation in response to a variety of stresses suggest a key role for tocopherols in photosynthetic organisms during stress (Munne-Bosch and Alegre, 2002; Collakova and DellaPenna, 2003). However, such lines of evidence are circumstantial, and this hypothesis has not yet been rigorously tested.

Light is required for photosynthesis, but light intensity in excess of that required for photosynthesis can also create ROS resulting in oxidative damage to the photosystems. Somewhat surprisingly, HL treatment did not differentially affect the growth (Figs. 2A and 3A), membrane lipid fatty acid composition (Fig. 4), or chlorophyll *a* content (Fig. 5C) of the tocopherol-deficient mutants and the wild type. The only observed differences were total carotenoid levels, which, unlike the wild type, did not remain elevated in the HL-treated cells of the *slr1736* mutant, primarily due to a severe drop in myxoxanthophyll levels at 45 h (Figs. 5A and 6A). The results are consistent with those of another tocopherol-deficient mutant in *Synechocystis* sp. PCC 6803 (*slr0090*::*aphII*, disrupted mutant in the HPPD enzyme), which when grown at 500 μE m^{-2} s^{-1} was also indistinguishable from the wild type (Dahnhardt et al., 2002). These combined data indicate that tocopherols are not essential for tolerating/acclimating to moderate (<500 μE m^{-2} s^{-1}) HL conditions in *Synechocystis* sp. PCC 6803.

One could argue that the similar responses of HL-treated cells of the wild type and the tocopherol mutants are because the light intensity used (300 μE m^{-2} s^{-1}) was not sufficiently high to require tocopherol function(s), as treatment of *C. reinhardtii* at 1,500 μE m^{-2} s^{-1} with a herbicide that inhibits HPPD enzyme activity reduced tocopherol levels to 20% of controls and induced concomitant degradation of the D1 protein (Trebst et al., 2002). However, 300 μE m^{-2} s^{-1} is three times the level needed to saturate photosynthesis in *Synechocystis* sp. PCC 6803, and this condition has previously been shown to up-regulate both high light-responsive and oxidative stress-related genes (e.g. HL-inducible proteins, SOD, and glutathione peroxidase; Hihara et al., 2001; Huang et al., 2002). Another plausible explanation is that other components of the antioxidant network may mitigate ROS damage or compensate for the lack of tocopherols in mutants under the conditions tested. Indeed, like most photosynthetic organisms, *Synechocystis* sp. PCC 6803 contains multiple layers of ROS defenses, including carotenoids, peroxiredoxins, SOD, and catalase-peroxidase (Kaneko et al., 1996), some or all of which may mitigate any damage caused by the lack of tocopherols under the HL conditions tested in this study. Future studies utilizing light intensities approaching full sunlight (2,000 μE m^{-2} s^{-1}) may provide additional insights into tocopherol functions in photosynthetic organisms.

To test the hypothesis that tocopherols play a critical role in tolerance to specific types of ROS

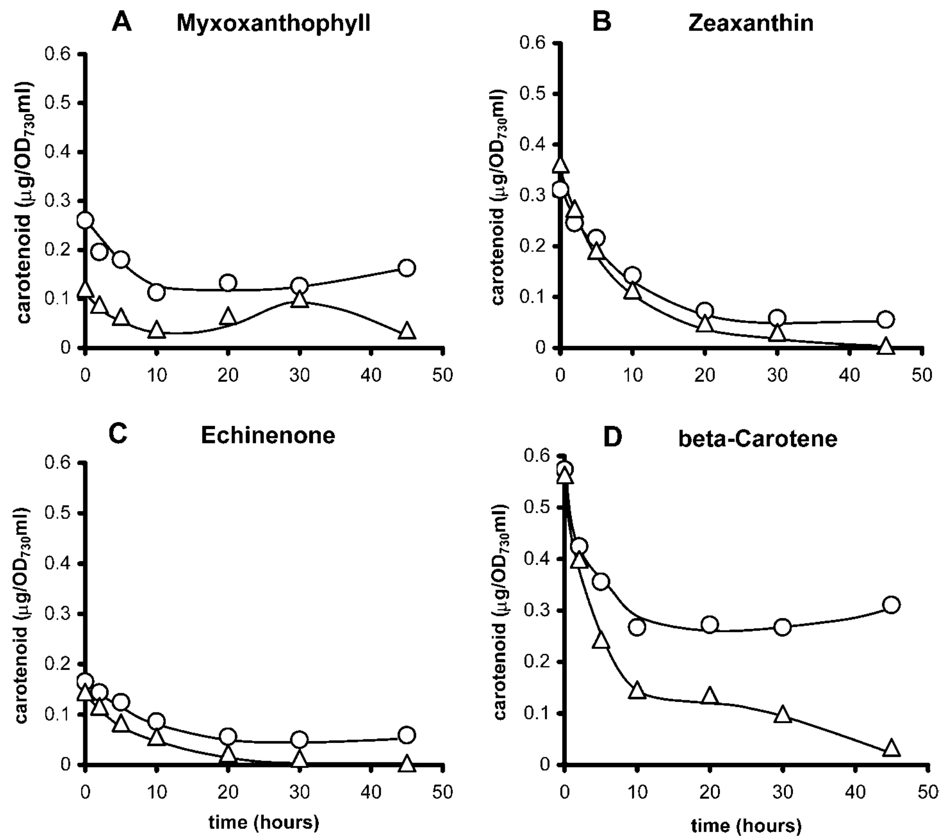

Figure 9. Changes in individual carotenoids in the wild type and the tocopherol-deficient *slr1736* mutant during NF/HL treatment. Myxoxanthophyll (A), zeaxanthin (B), echinenone (C), and β-carotene (D) were measured during 25 μM NF/HL treatment at 32°C, 1% (v/v) CO_2 in air. These data are representative of two independent experiments.

or ROS-induced damage, the tocopherol-deficient mutants and wild type were subjected to chemical treatments in combination with HL stress to generate different types of ROS. The wild type and the tocopherol-deficient *slr1736* mutant did not show differential sensitivity to treatment with the $^1O_2$-generating compound Rose Bengal (data not shown). Similarly, paraquat, an O_2^- generator, did not cause differential effects on the growth of the wild type and the tocopherol-deficient mutants (Fig. 2B). A *Synechococcus* sp. PCC 7942 mutant deficient in SOD showed enhanced sensitivity to paraquat at 100 μE m^{-2} s^{-1}, demonstrating that SOD is essential for O_2^- detoxification at moderate light levels (Thomas et al., 1998). These combined data suggest that tocopherols are not crucial for O_2^- or $^1O_2$ detoxification/tolerance in *Synechocystis* sp. PCC 6803 under the conditions tested.

Tocopherols have long been assumed to protect the membranes of oxygenic phototrophs from oxidative stress. To assess this proposed function, the tocopherol-deficient mutants and wild type were subjected to treatments known to induce lipid peroxidation. *t*-BOOH is an alkyl peroxide routinely used to induce lipid peroxidation in other systems (Masaki et al., 1989; Pereira et al., 2003). Surprisingly, *t*-BOOH did not differentially impact the *slr1736* mutant and wild type (Fig. 3I), suggesting tocopherols might not be essential in protecting *Synechocystis* sp. PCC 6803 cells from lipid peroxidation. As the role of tocopherols as lipid peroxidation chain reaction terminators is well established in vitro and in animal systems (Brigelius-Flohe and Traber, 1999; Wang and Quinn, 2000), this would be most unexpected. The similar and extremely rapid turnover of *t*-BOOH in the media of the wild type and the *slr1736* mutant (Fig. 3J) instead suggests that components other than tocopherols function very efficiently in both genotypes to rapidly reduce *t*-BOOH levels in vivo. Five peroxiredoxins have been characterized in *Synechocystis* sp. PCC 6803, and four of them, Sll0755, Slr1171, Slr1992, and Sll1621, have been shown to reduce *t*-BOOH efficiently in vitro when expressed in *Escherichia coli* (Yamamoto et al., 1999; Gaber et al., 2001; Hosoya-Matsuda et al., 2005). The expression of *slr1171* and *slr1992* is also induced in response to HL (Huang et al., 2002), and it is likely that these peroxiredoxins confer the similar resistance of the wild type and the *slr1736* mutant to *t*-BOOH treatment.

Unlike *t*-BOOH, the tocopherol-deficient mutants did show enhanced sensitivity to treatments with specific PUFAs. Treatment with 18:3 caused more severe growth inhibition than 18:2, while 18:1 was nontoxic (Figs. 2, C and D, and 3, C and E). These results indicate that the extent of toxicity for 18-carbon fatty acids depends on the degree of polyunsaturation. The results of growth curves and lipid peroxide analyses of the growth media further suggested that oxidation of 18:2 and 18:3 in the medium might be associated with their toxicity. However, in comparing the results from 18:3 and 20:3 treatments, which cause similar levels of lipid peroxides in the medium but have opposite effects on growth (Fig. 3, C, D, G, and H), it is clear that lipid peroxide levels in the medium per se are not the primary cause of 18:3 toxicity. The enhanced uptake/incorporation of 18:3 fatty acids into cell membranes relative to 20:3 (Fig. 4) implies that the 18:3 treatment results in more severe lipid peroxidation inside the cell. This could occur due to elevated levels of free or esterified PUFAs in membranes, either of which could initiate or participate in enhanced autocatalytic lipid peroxidation in the mutants. Unfortunately, PUFA treatments resulted in such high background levels of free PUFAs and lipid peroxides in media and cell pellets that it was not possible to reproducibly quantify the levels of free PUFAs and esterified or nonesterified lipid peroxidation byproducts in PUFA-treated cells. As a consequence, we were unable to directly determine whether nonenzymatic or enzyme-mediated lipid oxidation (e.g. lipoxygenases) was enhanced in membranes of tocopherol-deficient mutants. Despite these analytical limitations, our results are consistent with the hypothesis that tocopherols are critical in protecting *Synechocystis* sp. PCC 6803 from lipid peroxidation.

If tocopherols are crucial for protecting *Synechocystis* sp. PCC 6803 from lipid peroxidation, why is the *slr1737* mutant less sensitive to PUFA/HL treatment than the s*lr1736* mutant (Fig. 2), when both are deficient in tocopherols? The Arabidopsis *vte1* and *vte2* mutants (equivalent to the *slr1737* and *slr1736* mutants, respectively) both had reduced seed longevity, but only *vte2* exhibited early seedling developmental defects and a greater than 100-fold increase in lipid peroxidation during germination (Sattler et al., 2004). The attenuated phenotype of *vte1* relative to *vte2* is consistent with the attenuated phenotype of PUFA-treated *slr1737* relative to *slr1736* mutants (Fig. 2) and suggests that the quinol intermediate DMPBQ that accumulates in the *vte1* and *slr1737*, but not in the *vte2* and *slr1736* mutants, functionally compensates for the absence of tocopherols in many regards, most likely by acting as an alternative lipid-soluble antioxidant. In this regard, it is interesting to note that the Arabidopsis *vte1* mutant accumulates slightly but significantly increased levels of glutathione and ascorbate even in the absence of stress (Kanwischer et al., 2005). Whether these water-soluble antioxidants may also play a role in the attenuated phenotype of the *vte1* is as yet unclear.

Carotenoids are the second major group of lipid-soluble antioxidants in photosynthetic membranes and have been shown to play important roles in protecting plant and green algae during photooxidative stress (Havaux and Niyogi, 1999; Baroli et al., 2003, 2004). However, with the exception of their structural roles in photosystems, little work has been done to assess other physiological roles of carotenoids in *Synechocystis* sp. PCC 6803. Prior studies have shown that two carotenoid biosynthetic genes (*slr1254* and *slr0940*) are up-regulated during HL stress in wild-type *Synechocystis* sp. PCC 6803 (Huang et al., 2002), which is consistent with the observed increase in the levels of total and specific carotenoid (myxoxanthophyll being the most prominent) in the wild type in response to HL (Figs. 5A and 6A). By contrast, the *slr1736* mutant did not show corresponding increases in total or specific carotenoids during HL and 18:3/HL treatments. These data indirectly but strongly suggest that carotenoids, most likely myxoxanthophyll, are involved in the adaptation/tolerance of *Synechocystis* sp. PCC 6803 to HL stress and functionally interact with or complement tocopherols.

To assess further the role of carotenoids in adapting to HL stress and any functional interactions between tocopherols and carotenoids, carotenoid synthesis was partially inhibited in the wild type and the tocopherol-deficient *slr1736* mutant by treatment with NF/HL. Phytoene desaturase (Slr1254), one of two carotenoid biosynthetic enzymes induced in response to HL (Huang et al., 2002), is the enzymatic target of NF (Breitenbach et al., 2001). Treatment with 25 μM NF in HL slowed the growth of the wild type but had a much more severe impact on growth of the tocopherol-deficient *slr1736* mutant (Fig. 7B). NF-treated *slr1736* mutant cells also had a steady-state level of carotenoids half that of the wild type (Fig. 8A), mainly due to lower levels of myxoxanthophyll and β-carotene (Fig. 9). Assuming NF inhibits carotenoid synthesis to a similar degree in both mutant and wild type, the higher steady-state level of total carotenoids and better growth rate of the wild type during NF treatment are due to the presence of tocopherols. These data clearly demonstrate that carotenoids are a key component compensating for the absence of tocopherols during HL stress in the mutant cells. Introduction of other mutations that affect the levels of individual carotenoid species (Fernandez-Gonzalez et al., 1997; Lagarde and Vermaas, 1997; Mohamed and Vermaas, 2004) into the tocopherol-deficient mutant background will further clarify the role(s) of individual carotenoids in the adaptation/tolerance of *Synechocystis* sp. PCC 6803 to HL stress in the absence of tocopherols.

In summary, the enhanced sensitivity of tocopherol-deficient mutants of *Synechocystis* sp. PCC 6803 to specific PUFAs provides physiological and biochemical evidence that tocopherols are crucial in protecting oxygenic phototrophs from lipid peroxidation in vivo. These data are consistent with a recent study of tocopherol-deficient mutants of Arabidopsis, which have reduced seed longevity and early seedling developmental defects due to greatly increased lipid peroxidation during germination in the absence of tocopherols (Sattler et al., 2004). From the combined studies in these two model photosynthetic organisms, it can be concluded that a primary function of tocopherols in both eukaryotic and prokaryotic oxygenic photosynthetic organisms is to protect cells from lipid peroxidation. Simultaneous inhibition of carotenoid and tocopherol biosynthesis in *Synechocystis* sp. PCC 6803 clearly demonstrated the two classes of lipid-soluble antioxidants functionally interact or have complementary roles during HL stress. The overlapping functionality of tocopherols and carotenoids in *Synechocystis* sp. PCC 6803 may explain why tocopherols appear to be dispensable during moderate HL stress (up to 500 μE m^{-2} s^{-1}; Figs. 2A and 3A; Dahnhardt et al., 2002). However, under extreme and specific stress conditions, such as during PUFA-induced lipid peroxidation in HL, the absence of tocopherols cannot be fully compensated by carotenoids, and both lipid-soluble antioxidants are required for survival of *Synechocystis* sp. PCC 6803.

MATERIALS AND METHODS

Chemicals

Oleic acid (18:1$^{\Delta 9}$), linoleic acid (18:2$^{\Delta 9,12}$), linolenic acid (18:3$^{\Delta 9,12,15}$), eicosatrienoic acid (20:3$^{\Delta 11,14,17}$), *t*-BOOH, paraquat, Rose Bengal, butylated hydroxytoluene (BHT), and xylenol orange [*o*-cresolsulfonephthalein-3, 3′-*bis*(methylimino-diacetic acid)sodium salt] were purchased from Sigma (St. Louis). Ferrous ammonium sulfate hexahydrate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_20$] was from Aldrich (Milwaukee, WI). NF was from Chem Service (West Chester, PA).

Growth Conditions and Chemical Treatments

The construction, photoautotrophic selection, and molecular and physiological characterization of authentic *slr1736* and *slr1737* mutants of *Synechocystis* sp. strain PCC 6803 are described in detail elsewhere (Sakuragi, 2005). Wild-type and mutant strains of *Synechocystis* sp. strain PCC 6803 were grown photoautotrophically in liquid B-HEPES medium, which is BG-11 (Williams, 1988) supplemented with 4.6 mM of HEPES, pH 8.0, and 18 mg L^{-1} ferric ammonium citrate. Growth was at 32°C with 1% (v/v) CO_2 in air under constant illumination from cool-white fluorescent lamps at 15 μE m^{-2} s^{-1} (LL) or 300 μE m^{-2} s^{-1} (HL). Light intensity was measured by a LI-250 light meter (LI-COR, Lincoln, NE). Cell growth was monitored by the optical density at 730 nm (OD_{730}). For growth and treatments in HL, exponentially growing LL cultures (OD_{730} = 0.7–1.0) were diluted to 0.05 or 0.5 of OD_{730} with fresh B-HEPES medium and transferred to the HL condition described above.

Peroxide Analysis

The peroxide contents in media and cell pellets were measured using the FOX assay (Griffiths et al., 2000). Aliquots of cultures (500 μL–1 mL) were collected at different time points and centrifuged at 15,000*g* for 5 min. The supernatants (60 μL) were mixed with 540 μL of FOX reagent [90% (v/v) methanol, 4 mM BHT, 25 mM sulfuric acid, 250 μM $Fe(NH_4)_2(SO_4)_2$, 100 μM xylenol orange] and incubated for 20 min in darkness, and the A_{560} was measured. The peroxide content was calculated based on a standard curve created by known concentrations of hydrogen peroxide (J.T. Baker, Phillipsburg, NJ).

Lipid Composition Analysis

Cells were collected by centrifugation at 3,500*g* for 15 min and lipid extracts were prepared as described previously (Hara and Radin, 1978). Esterified fatty acids were selectively methyl-esterified by KOH-catalyzed transesterification as described (Ichihara et al., 1996). Fatty acid methyl esters were quantified by gas-liquid chromatography using pentadecanoic acid (Sigma) as an internal standard (Rossak et al., 1997).

Carotenoid, Chlorophyll *a*, and Tocopherol Analyses

The amount of cells equivalent to 10 mL of OD_{730} = 1.0 culture were collected by centrifugation at 8,000*g* for 5 min and washed twice with 25 mM HEPES buffer, pH 7.0. Carotenoids and tocopherols were extracted in 500 μL of methanol with 1 mg mL^{-1} BHT at 4°C. After centrifugation and filtration, 100 μL was subjected to HPLC (Agilent 1100 series; Wilmington, DE) on a Spherisorb ODS-2 5 μm, 250- × 4.6-mm reverse phase column (Column Engineering, Ontario, CA) using a 30-min gradient of isopropanol (0–10 min, 0%; 10–20 min, 0%–80%; 20–25 min, 80%; 25–30 min, 80%–0%) in methanol at a flow rate of 0.75 mL min^{-1}. Photodiode array detection was used to identify each carotenoid species and chlorophyll *a* by their characteristic absorption spectra and their retention times relative to standards. Individual carotenoids and chlorophyll *a* were quantified against a standard equation derived by injection of known amounts of each purified compound. Tocopherols were detected by fluorescence using 290 nm excitation and 325 nm emission and quantified against standard curves generated by commercially available tocopherols (Acros Organics, Hanover Park, IL).

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