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Chlorophyll *a* phytylation is required for the stability of photosystems I and II in the cyanobacterium *Synechocystis* sp. PCC 6803

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SUMMARY

In oxygenic phototrophic organisms, the phytyl 'tail' of chlorophyll *a* is formed from a geranylgeranyl residue by the enzyme geranylgeranyl reductase. Additionally, in oxygenic phototrophs, phytyl residues are the tail moieties of tocopherols and phylloquinone. A mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 lacking geranylgeranyl reductase, Δ*chlP*, was compared to strains with specific deficiencies in either tocopherols or phylloquinone to assess the role of chlorophyll *a* phytylatation (versus geranylgeranylation). The tocopherol-less Δ*hpt* strain grows indistinguishably from the wild-type under 'standard' light photoautotrophic conditions, and exhibited only a slightly enhanced rate of photosystem I degradation under strong irradiation. The phylloquinone-less Δ*menA* mutant also grows photoautotrophically, albeit rather slowly and only at low light intensities. Under strong irradiation, Δ*menA* retained its chlorophyll content, indicative of stable photosystems. Δ*chlP* may only be cultured photomixotrophically (due to the instability of both photosystems I and II). The increased accumulation of myxoxanthophyll in Δ*chlP* cells indicates photo-oxidative stress even under moderate illumination. Under high-light conditions, Δ*chlP* exhibited rapid degradation of photosystems I and II. In conclusion, the results demonstrate that chlorophyll *a* phytylation is important for the (photo)stability of photosystems I and II, which, in turn, is necessary for photoautotrophic growth and tolerance of high light in an oxygenic environment.

Keywords: geranylgeranyl reductase, chlorophyll, tocopherol, phylloquinone, photo-oxidative stress, *Synechocystis*.

INTRODUCTION

Chlorophyll (Chl) *a* is the key pigment involved in the primary reactions of oxygenic photosynthesis – the global biological process that provides primary biomass and energy for almost all living beings, and, additionally, supplies oxygen for respiration.

In Chl biosynthesis, the enzyme geranylgeranyl reductase (GGR, also designated ChlP) reduces either geranylgeranyl diphosphate to phytyl diphosphate or a side chain of geranylgeranylated Chl *a* (Chl a_{GG}) to yield (phytylated) Chl *a*. In parallel, Chl synthase (ChlG) esterifies chlorophyllide *a* using either geranylgeranyl diphosphate or phytyl diphosphate, producing Chl a_{GG} or Chl *a*, respectively (Figure S1) (Soll and Schultz, 1981; Keller *et al.*, 1998; Shpilyov *et al.*, 2005; Rüdiger, 2006). Additionally, Chls with di- and tetrahydrogeranylgeranyl 'tails' (Chl a_{DHGG} and Chl a_{THGG}, respectively) may be formed due to incomplete reduction of geranylgeranyl residues by GGR (Table S1) (Maloney *et al.*, 1989; Domanskii *et al.*, 2003).

In oxygenic phototrophs, GGR is also involved in the synthesis of tocopherols (Figure S1) (Keller *et al.*, 1998; Tanaka *et al.*, 1999; Shibata *et al.*, 2004a). Together with tocotrienols – analogs that have an unsaturated isoprenoid side chain – these compounds comprise a group of lipid-soluble antioxidants collectively referred to as tococromanols (or vitamin E), with α–tocopherol being the predominant natural form (Table S1) (reviewed by

Dörmann, 2007; Falk and Munné-Bosch, 2010; DellaPenna and Mène-Saffrané, 2011). The major role assumed for α–tocopherol is prevention of oxidation of membrane lipids triggered by reactive oxygen species. Additionally, α–tocopherol has been suggested to protect photosystem (PS) II from photoinhibition (Trebst, 2003; Krieger-Liszkay and Trebst, 2006; Inoue *et al.*, 2011). Moreover, α–tocopherol is believed to be involved in the regulation of intracellular signaling, macronutrient homeostasis, osmotolerance, seed longevity, seedling and root development, growth rate, etc. (reviewed by Dörmann, 2007; Falk and Munné-Bosch, 2010; DellaPenna and Mène-Saffrané, 2011).

In addition to Chl and tocopherol synthesis, GGR participates in formation of phylloquinone (vitamin K_1) (Figure S1 and Table S1) in oxygenic phototrophs. Phylloquinone functions as secondary electron acceptor at the A_1 site of PSI (Keller *et al.*, 1998; Johnson and Golbeck, 2004; Shibata *et al.*, 2004b; Srinivasan and Golbeck, 2009; Ohashi *et al.*, 2010).

In GRR-deficient plant and cyanobacterial mutants, Chl a_{GG} accumulates (in some cases together with Chl a_{DHGG} and Chl a_{THGG}) instead of phytylated Chl *a*. Chl a_{GG} may be incorporated into photosynthetic pigment–protein complexes and even mediate light-induced electron transport in the mutants (Tanaka *et al.*, 1999; Shibata *et al.*, 2004a,b; Shpilyov *et al.*, 2005). However, Chl a_{GG}, Chl a_{DHGG} and Chl a_{THGG} do not naturally occur in mature chloroplasts or cyanobacterial cells (Tamiaki *et al.*, 2007). Moreover, full replacement of Chls with counterparts that have unsaturated tails abolishes photoautotrophic growth in plants (Shibata *et al.*, 2004a), green algae (Henry *et al.*, 1986) and cyanobacteria (Shpilyov *et al.*, 2005). Altogether, these facts appear to indicate that Chl *a* species with only partially saturated tails may not be able to fulfill all the role (s) of phytylated Chl *a*, and may even have deleterious effects in oxygenic photosynthetic organisms.

However, more information is required for better understanding of the importance of the phytyl tail of Chl *a* and thus the significance of the GGR enzyme for oxygenic photosynthesis – also taking into account that the GGR-catalyzed reaction requires energy and redox equivalents (Schoch and Schäfer, 1978). The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is a suitable organism to study mutations that impair photosynthesis, as glucose-tolerant strain(s) are able to grow heterotrophically using glucose as external energy/carbon source (Ikeuchi and Tabata, 2001). We have previously described a GGR-deficient mutant (Δ*chlP*) of *Synechocystis* (Shpilyov *et al.*, 2005). The goal of the present work was to distinguish the effects of replacement of (phytylated) Chl *a* with Chl a_{GG} from possible effects of α–tocopherol and phylloquinone deficiency in the mutant. Δ*chlP* was compared to the *Synechocystis* Δ*hpt* and Δ*menA* mutants with inactivated homogentisate phytyl transferase (HPT) and 1,4–dihydroxy-2–naphthoate phytyl transferase (DHNA phytyl transferase, MenA), respectively, which specifically control tocopherol and phylloquinone formation (Figures S1 and S2) (Johnson *et al.*, 2000; Collakova and DellaPenna, 2001).

Together with published data, the results demonstrate that, in the Δ*chlP* mutant, deficiency of neither tocopherol nor phylloquinone but instead accumulation of Chl a_{GG} leads to instability of both PSI and PSII. Hence, Chl *a* phytylation appears to be crucial for photoautotrophic growth and prevention of photo-oxidative stress by ensuring the (photo)stability of photosynthetic pigment–protein complexes.

RESULTS

Comparison of Δ*chlP* with the tocopherol-deficient Δ*hpt* mutant

In *Synechocystis*, α–tocopherol accumulates as the only vitamin E species (Collakova and DellaPenna, 2001; Savidge *et al.*, 2002; Shpilyov *et al.*, 2005) (see also Figure S3a). As reported previously, inactivation of GGR in the Δ*chlP* mutant leads to accumulation of α–tocotrienol instead of α–tocopherol (Table S1) (Shpilyov *et al.*, 2005). This provides evidence of the ability of the HPT enzyme to utilize geranylgeranyl diphosphate as a substrate for condensation with homogentisate *in vivo* in *Synechocystis* (Figure S1), corroborating results obtained *in vitro* (Collakova and DellaPenna, 2001). In accordance with the established pathway of vitamin E formation, no tocochromanols were detected in cells of the HPT-deficient Δ*hpt* strain (Figures S1–S3b).

The Δ*chlP* mutant only grows photomixotrophically (Shpilyov *et al.*, 2005). In contrast, Δ*hpt* possesses a phenotype similar to the wild-type (WT) strain when grown photoautotrophically at a light intensity of 40 μmol photons m^{-2} sec^{-1} ('standard' light). This has also been documented for other *Synechocystis* mutants that are impaired in synthesis of vitamin E (Collakova and DellaPenna, 2001; Dähnhardt *et al.*, 2002; Savidge *et al.*, 2002; Sattler *et al.*, 2003; Sakuragi *et al.*, 2006). However, photomixotrophic cultivation at the same light intensity was found to have some adverse effects on the Δ*hpt* strain. The mutant has a tendency to grow slightly more slowly than the WT, although the difference between the doubling times is not dramatic (Table 1). Both Δ*hpt* and Δ*chlP* exhibit reduced Chl *a* and total carotenoid contents. However, the reduction of these pigments in Δ*chlP* is more pronounced than in Δ*hpt* (Figure 1 and Table 1). Furthermore, phycobilisome (PBS) content is reduced in Δ*hpt* but increased in Δ*chlP* (Figure 1).

The 77 K fluorescence emission spectrum of Δ*hpt* cells recorded upon Chl *a* excitation (at 435 nm) is essentially identical to that of the WT (Figure 2). The only difference found is a slight relative decrease in PSI as indicated by

Table 1 Growth rates and pigment contents in the WT, Δ*hpt*, Δ*menA* and Δ*chlP* strains[a]

	WT	Δ*hpt*	Δ*menA*	Δ*chlP*
Photoautotrophic growth, doubling time (h)				
At 40 μmol photons m^{-2} sec^{-1}	11.4 ± 1.0	11.6 ± 1.1	NG	NG
At 20 μmol photons m^{-2} sec^{-1}	18.2 ± 3.1		42.28 ± 5.3	NG
Photomixotrophic growth, doubling time (h)				
At 40 μmol photons m^{-2} sec^{-1}	9.3 ± 0.5	9.8 ± 0.7	NG	9.2 ± 0.4
At 20 μmol photons m^{-2} sec^{-1}	12.1 ± 0.8		32.45 ± 4.1	
Chlorophyll content[b] (μg ml^{-1} $OD_{750}{}^{-1}$)				
At 40 μmol photons m^{-2} sec^{-1}	2.59 ± 0.25	2.31 ± 0.23	NG	1.98 ± 0.20
At 20 μmol photons m^{-2} sec^{-1}	2.56 ± 0.19		2.03 ± 0.12	
Total carotenoid content[b] (μg ml^{-1} $OD_{750}{}^{-1}$)				
At 40 μmol photons m^{-2} sec^{-1}	0.65 ± 0.08	0.60 ± 0.06	NG	0.47 ± 0.04
At 20 μmol photons m^{-2} sec^{-1}	0.63 ± 0.07		0.62 ± 0.06	

NG, no growth.
[a]During growth, aeration was provided with ambient air by stirring. Data are mean values derived from three to five measurements.
[b]Pigments were extracted using 90% v/v methanol/water from cells grown exponentially in the presence of 10 mM glucose.

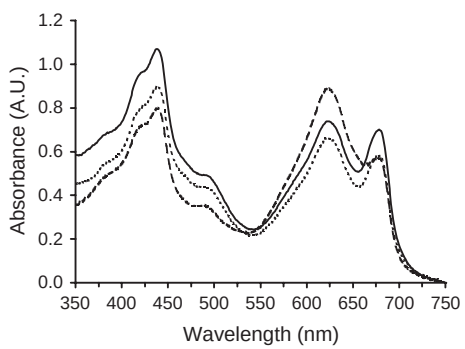

Figure 1. Room-temperature absorption spectra of WT (solid line), Δ*hpt* (dotted line) and Δ*chlP* (dashed line) cells grown photomixotrophically at 40 μmol photons m^{-2} sec^{-1}. Spectra were normalized on a per cell basis at 750 nm.

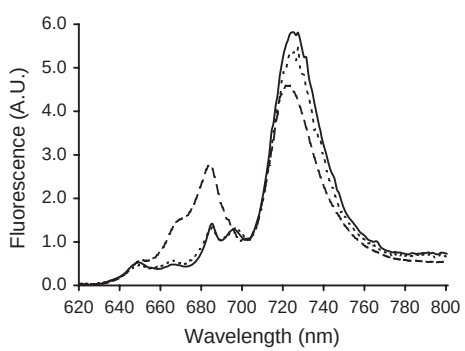

Figure 2. 77 K fluorescence emission spectra of WT (solid line), Δ*hpt* (dotted line) and Δ*chlP* (dashed line) cells grown photomixotrophically at 40 μmol photons m^{-2} sec^{-1}. Excitation was at 435 nm.

the decreased 725 nm peak (PSI fluorescence) and an unaffected 695 nm maximum (PSII). Thus, the reduction of the cellular Chl *a* level in Δ*hpt* is due to a decreased PSI content. In contrast to Δ*hpt*, the PSI emission peak in the fluorescence spectrum of Δ*chlP* is considerably decreased. Moreover, the PSI emission maximum is blue-shifted by 2 nm. A fluorescence band peaking at 684 nm is increased in the mutant (Figure 2). These spectral properties suggest that the structures of both PSI and PSII may be somewhat perturbed in the Δ*chlP* mutant. This was not observed for Δ*hpt*.

Although it grows well at 40 μmol photons m^{-2} sec^{-1}, Δ*chlP* cannot grow under light of approximately 100 μmol photons m^{-2} sec^{-1} and higher, intensities that are permissive for the WT (Shpilyov *et al.*, 2005). To clarify whether α–tocopherol deficiency is responsible for light sensitivity in Δ*chlP*, we compared this mutant to Δ*hpt* under a high light intensity of approximately 500 μmol photons m^{-2} sec^{-1} together with external glucose supply (see Experimental procedures). Following transfer to increased illumination, growth ceased immediately in all strains (Figure 3a). *Synechocystis* sp. PCC 6803 was previously reported to be able to grow photoautotrophically at such a light intensity (Steiger *et al.*, 1999; He *et al.*, 2001). Hence, the observed growth arrest – particularly in the WT – was probably a result of glucose supply. For cyanobacteria, glucose is known to donate additional electrons to the photosynthetic electron transport chain (ETC) by reduction of plastoquinone–9 via respiratory dehydrogenases (Vermaas, 2001; Wang *et al.*, 2002; Egorova *et al.*, 2006). Under excess light conditions, glucose probably causes over-reduction of the plastoquinone pool and thereby the whole ETC, thus provoking strong photo-oxidative stress and abolishing cell division (Krieger-Liszkay, 2005; Ledford and Niyogi, 2005; Telfer, 2005). Note that glucose improves growth in glucose-tolerant *Synechocystis* strains at light intensities up to approximately 150–200 μmol photons m^{-2} sec^{-1} (Ikeuchi and Tabata, 2001; Johnson *et al.*, 2001; Wang *et al.*, 2002; Sakuragi, 2004; Shpilyov *et al.*, 2005) (see also Table 1),

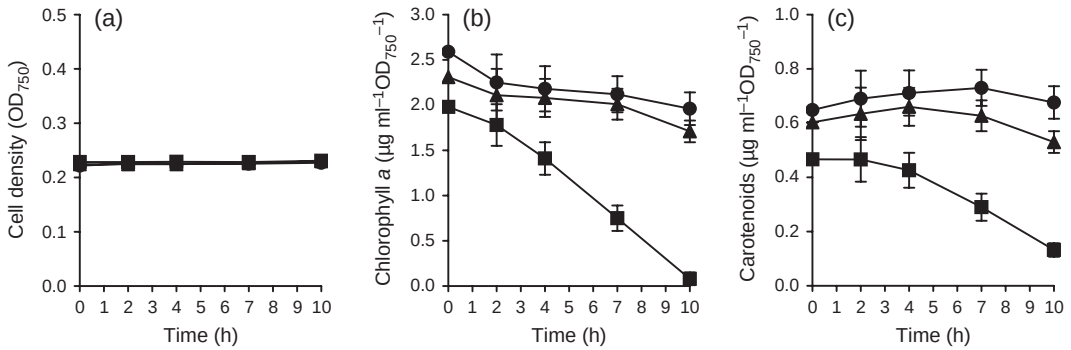

Figure 3. Cell densities (a), Chl *a* (b) and total carotenoid (c) contents in WT (circles), Δ*hpt* (triangles) and Δ*chlP* cultures (squares) during incubation at 500 μmol photons m^{-2} sec^{-1} in the presence of glucose. The 0 h time point corresponds to cells grown photomixotrophically at 40 μmol photons m^{-2} sec^{-1}.

but has an inhibitory effect under stronger irradiation (this study).

When subjected to high light intensity, all strains showed pigment degradation. Both WT and Δ*hpt* exhibited similarly slow Chl degradation kinetics, as assessed by spectrophotometry and HPLC (Figures 3b and 4, respectively) and whole-cell absorption spectra (Figure 6a,b). After 10 h of strong light, WT and Δ*hpt* still retained relatively high Chl levels, i.e. approximately 76 and 74% of the initial values, respectively (Figure 3b). Additionally, WT and Δ*hpt* showed a transient increase in carotenoid contents during the first 4–7 h. Thereafter, total carotenoids returned to close to the initial levels, approximately 104 and 88% for WT and Δ*hpt*, respectively (Figure 3c). The transient accumulation of carotenoids in the WT and Δ*hpt* strains indicates that they were both able to combat photo-oxidative stress (at least for some time) by up-regulation of carotenoid synthesis, in accordance with published data (Steiger *et al.*, 1999; Maeda *et al.*, 2005). However, after 4–7 h, all pigments started to degrade. Degradation proceeded slightly faster in Δ*hpt* than in the WT (Figures 3b,c and 4, Figure 6). The latter observation implies a somewhat increased light sensitivity for the Δ*hpt* strain that lacks vitamin E.

However, the Δ*chlP* mutant displayed a rather different pigment profile (beyond replacement of Chl *a* with Chl a_{GG} as reported previously; Shpilyov *et al.*, 2005) as well as different degradation kinetics. Δ*chlP* eventually lost nearly 100% of its Chl over the course of the experiment (Figures 3b and 4, Figure 6d). Additionally, in contrast to Δ*hpt* and WT, Δ*chlP* exhibited no increase in total carotenoid content, and the carotenoid level significantly decreased (to approximately 28%) in Δ*chlP* cells during high-light treatment (Figure 3c). A distinctive exception appears to be myxoxanthophyll. Increased levels of this carotenoid (exceeding those in WT and Δ*hpt*) were found in Δ*chlP* cells even under standard light conditions, and remained relatively high until 4 h of light stress, when significant decay of all other pigments had already occurred (Figure 4). Myxoxanthophyll (myxol 2′–dimethyl-fucoside in *Synechocystis* sp. PCC 6803) is assumed to be a photo-protective carotenoid that is specific to cyanobacteria (Takaichi *et al.*, 2001). An elevated level of myxoxanthophyll is considered to be symptomatic of photo-oxidative stress because it is commonly observed under photo-inhibitory conditions, e.g. high light, UV irradiation and low temperature (Ehling-Schulz *et al.*, 1997; Steiger *et al.*, 1999; Miśkiewicz *et al.*, 2000; Takaichi *et al.*, 2001; Maeda *et al.*, 2005; Schäfer *et al.*, 2005) (see also Figure 4 for WT and Δ*hpt*). Thus, the increased amount of myxoxanthophyll in Δ*chlP* – especially under standard light conditions – indicates that the mutant is already stressed by moderate light.

The impact of excess light on both photosystems was also assessed using a series of 77 K fluorescence emission spectra recorded upon Chl *a* excitation (Figure 5). WT and Δ*hpt* display essentially identical spectral patterns, revealing a slight decrease in both PSI and PSII contents during incubation under high light intensity. Again, Δ*hpt* appears to be somewhat more affected by high light than the WT, at least with respect to the PSI content (Figure 5a,b). However, the decrease in PSI fluorescence in the Δ*chlP* spectra is much more dramatic (Figure 5c). Moreover, inversion of the 667/684 nm peak ratio with a sharp decrease in emission at 684 nm is also observed. These data indicate that both PSI and PSII underwent rapid degradation in Δ*chlP*. Interestingly, the decrease in emission at 684 nm, indicative of PSII decay, occurred after 4 h, which correlates with the kinetics of myxoxanthophyll decrease in the mutant during high-light exposure (Figures 4 and 5). This may indicate that, under photo-inhibitory conditions, myxoxanthophyll may specifically contribute to protection of PSII in cyanobacteria.

Comparison of Δ*chlP* with the phylloquinone-deficient Δ*menA* mutant

In oxygenic phototrophs, GGR is additionally involved in formation of phylloquinone (Figure S1 and Table S1). Thus, Δ*chlP* was also compared to a *Synechocystis* mutant

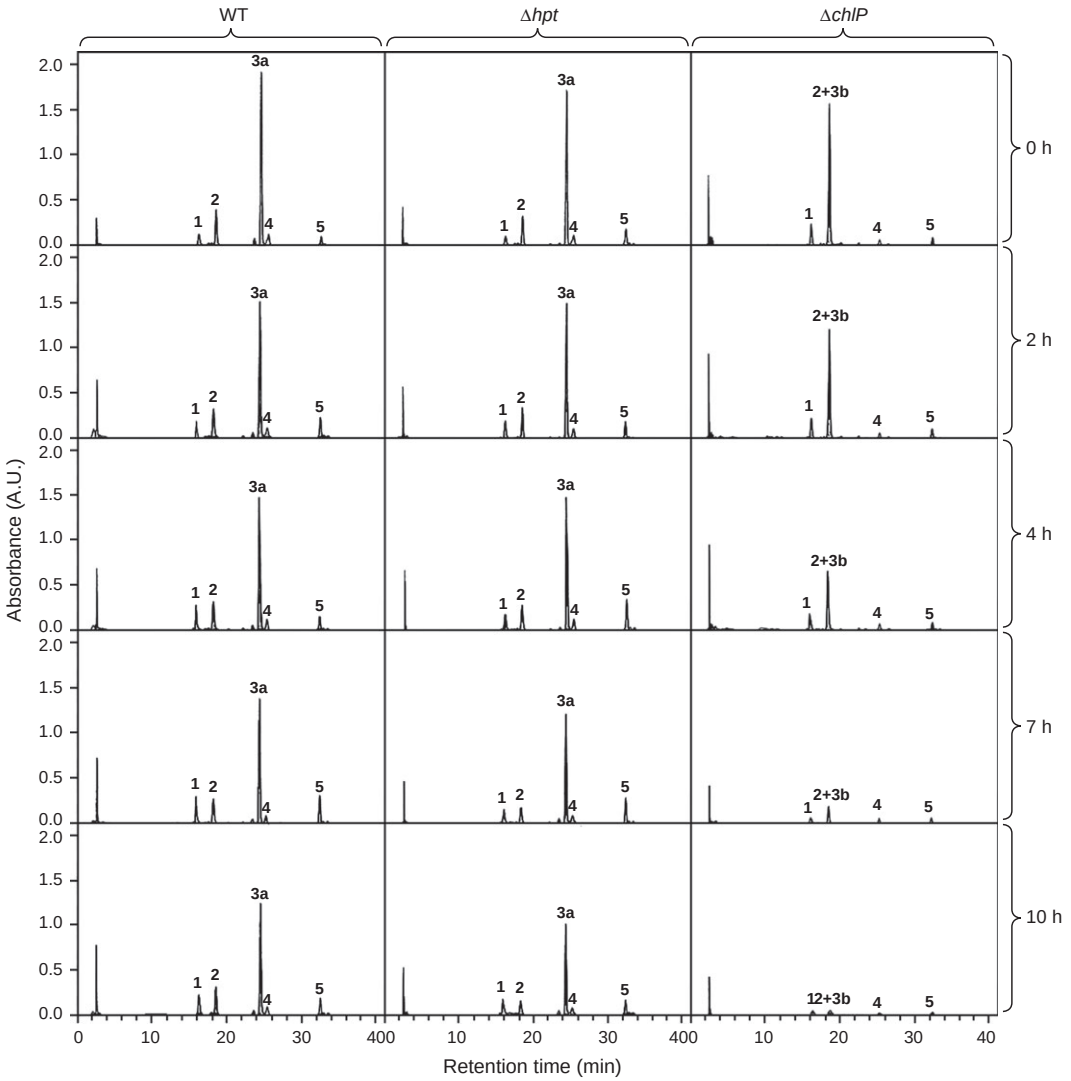

Figure 4. HPLC chromatograms of pigment extracts from WT, Δ*hpt* and Δ*chlP* cells at various time points of incubation at 500 μmol photons m^{-2} sec^{-1} in the presence of glucose. Strains are indicated at the top, and time points (hours) of incubation are indicated on the right. The 0 h time point corresponds to cells grown photomixotrophically at 40 μmol photons m^{-2} sec^{-1}. Peaks: 1, myxoxanthophyll; 2, zeaxanthin; 3a, (phytylated) Chl *a*; 3b, Chl a_{GG}; 4, echinenone; 5, β–carotene.

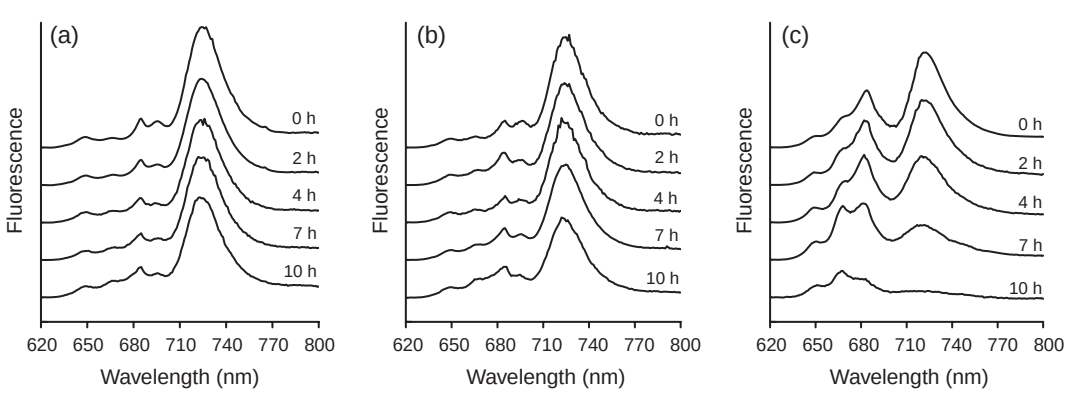

Figure 5. 77 K fluorescence emission spectra of WT (a), Δ*hpt* (b) and Δ*chlP* (c) cells at various time points of incubation at 500 μmol photons m^{-2} sec^{-1} in the presence of glucose. Excitation was at 435 nm. The corresponding time points (hours) of incubation are given on the right. The 0 h time point corresponds to cells grown photomixotrophically at 40 μmol photons m^{-2} sec^{-1}.

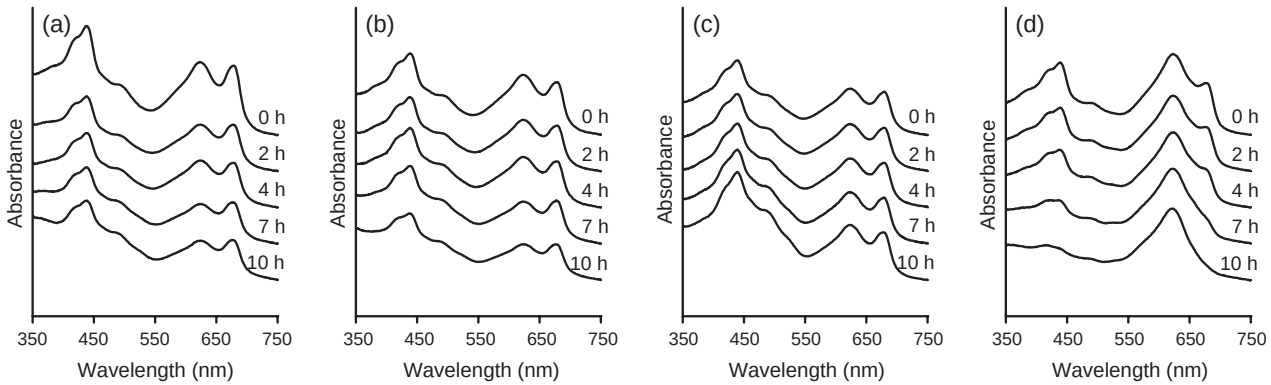

Figure 6. Room-temperature absorption spectra of WT (a), Δ*hpt* (b), Δ*menA* (c) and Δ*chlP* (d) cells at various time points of incubation at 500 μmol photons m^{-2} sec^{-1} in the presence of glucose. The corresponding time points (hours) of incubation are given on the right. The 0 h time point corresponds to cells grown photomixotrophically at 40 μmol photons m^{-2} sec^{-1}, except Δ*menA*, which was grown at 20 μmol photons m^{-2} sec^{-1} before incubation at 500 μmol photons m^{-2} sec^{-1}.

with specifically interrupted phylloquinone synthesis. The latter, designated Δ*menA*, was constructed by genetic knockout of 1,4–dihydroxy-2–naphthoate (DHNA) phytyl transferase (MenA), an enzyme that is specific to the phylloquinone biosynthetic pathway (Figures S1 and S2).

Synechocystis mutants disrupted in MenA activity (*menA*) (Figure S1) and earlier committed steps of phylloquinone biosynthesis (*menB*, *D* and *E* mutants) have been previously described in detail, and appear to be rather similar to each other (reviewed by Johnson and Golbeck, 2004; Srinivasan and Golbeck, 2009). The phenotype of the Δ*menA* strain is identical to the aforementioned mutants, particularly the analogous mutant *menA*, previously described by Johnson *et al.* (2000). Δ*menA* shows a pale olive green coloration due to decreased Chl and PBS contents (Table 1 and Figure S4). Additionally, the mutant cannot grow under illumination exceeding approximately 30–35 μmol photons m^{-2} sec^{-1} (Table 1). Thus, our Δ*menA* mutant shares an obvious light sensitivity consistent with the reported *menA*, *B*, *D* and *E* mutants. Also consistent with previous reports, Δ*menA* exhibits slow but steady growth at reduced light intensity (e.g. 20 μmol photons m^{-2} sec^{-1}), particularly under photoautotrophic conditions (Table 1). As established previously, photoautotrophy is retained due to recruitment of plastoquinone–9 (Table S1) into the A_1 site of PSI instead of phylloquinone (Johnson *et al.*, 2000; Zybailov *et al.*, 2000). Upon incorporation of plastoquinone into PSI, the whole-chain electron transport rate is reduced by approximately 40% (Johnson *et al.*, 2000; own observations). However, the ability of the *men* mutants to grow photoautotrophically suggests two remarkable conclusions for *Synechocystis*. First, phylloquinone is not strictly required to sustain electron transport through PSI and may be substituted for by other quinone (s). Second, even when binding a rather different quinone (e.g. plastoquinone–9; Table S1), PSI remains stable enough to retain photoautotrophy in cells. These conclusions are complemented and extended by numerous *in vitro* and *in vivo* studies (see Discussion). In contrast, both PSI and PSII are unstable in Δ*chlP*, abolishing the capability for photoautotrophic growth in the mutant (Shpilyov *et al.*, 2005).

To assess the resistance of the photosystems towards irradiation in Δ*menA*, the mutant was also compared to the other strains in high-light experiments as described above. Interestingly, despite the light sensitivity of Δ*menA* mentioned above, the mutant retained stable levels of pigments – particularly Chl – under strong illumination as shown in a series of absorption spectra (Figure 6c) and photometric measurements (Figure S5). These data clearly indicate the stability of both PSI and PSII in the Δ*menA* mutant under high-light conditions, in contrast to Δ*chlP*.

DISCUSSION

Effects of tocopherol deficiency

In *Synechocystis*, α–tocopherol has been proposed to have specific (probably regulatory) homeostatic roles beyond its antioxidant activity. A *Synechocystis* HPT-deficient mutant (Figure S1) was reported to be extremely glucose-sensitive (Sakuragi *et al.*, 2006). However, our Δ*hpt* strain is only slightly compromised under photomixotrophic conditions. The discrepancy between the data is resolved if the pH of the growth medium is considered. The previously described mutant (*slr1736*) was inhibited by added glucose at pH approximately 7.2 and lower, whereas at higher pH (e.g. 7.6 and 8.0), the mutant grew similarly to the WT (Sakuragi *et al.*, 2006). In our experiments, the cultures were started at an initial pH approximately 8.0, not buffered and aerated with ambient air. The pH rose gradually to approximately 9.0 during growth until the stationary phase was reached. Good photomixotrophic growth was also observed by other researchers for an analogous mutant under a similar aeration regime with no buffer

added (Sakuragi, 2004). The initial pH of their medium was also 8.0; hence, the growth conditions were similar to ours (Sakuragi, 2004). However, if the medium was bubbled with 3% CO_2-enriched air and not buffered, the pH eventually decreased to approximately 7.0. Consequently, growth was inhibited, with concomitant strong bleaching of the culture (Sakuragi, 2004). However, if under the same conditions, the pH was kept at the initial value (8.0) using HEPES buffer, the mutant displayed persistent growth and phenotype (Sakuragi, 2004; Sakuragi *et al.*, 2006). Thus, the cultivation conditions used in our study were permissive for the Δ*hpt* strain. Under photoautotrophic conditions, pH does not influence the *Synechocystis* strains that lack α–tocopherol, e.g., *slr1736, slr0089 and slr0090* (Sakuragi, 2004; Sakuragi *et al.*, 2006).

Nevertheless, adverse effects of α–tocopherol deficiency, such as reduced PBS and PSI contents, were observed in the Δ*hpt* strain grown photomixotrophically, even at high pH (Figures 1 and 2, and Table 1). These observations are consistent with results obtained by other researchers: in the presence of glucose at pH 8.0, the HPT-deficient mutant used by Sakuragi (2004) showed a pale green–yellow coloration, as did our Δ*hpt* strain, due to slight de-pigmentation, particularly decreased PBS content (Figure 1). Thus, the present study corroborates previous data indicating that α–tocopherol may play specific homeostatic roles in *Synechocystis* (Sakuragi, 2004; Sakuragi *et al.*, 2006). Consequently, although Δ*chlP* accumulates α–tocotrienol (Shpilyov *et al.*, 2005), which also has a high antioxidant potency (Yoshida *et al.*, 2003), loss of α–tocopherol was hypothesized to be critical in the mutant. Thus, one may expect the phenotypes of Δ*chlP* and Δ*hpt* to be very similar, given that both strains lack α–tocopherol.

However, the comparative analyses revealed specific features for the Δ*chlP* mutant. The most striking trait is loss of photoautotrophic growth. Glucose appears to provide an additional energy/carbon source to maintain steady-state levels (through high rates of re-synthesis) of PSI and PSII in Δ*chlP* (Shpilyov *et al.*, 2005). In contrast, the stability of PSI/II in Δ*hpt* is comparable to that of the WT, as deduced from its robust photoautotrophic growth. Additionally, the 77 K fluorescence emission spectra imply that both PSI and PSII in Δ*chlP* are structurally perturbed, which is not observed in Δ*hpt* (Figure 2).

The slightly increased light sensitivity of Δ*hpt* (Figures 3–6) cannot be interpreted unambiguously. A possible reason is the absence of α–tocopherol as an antioxidant. Alternatively, this and other specific traits of the strain, e.g. somewhat different PBS, PSI and carotenoid contents (Figures 1–4 and Table 1), may be a side effect of glucose in a tocopherol-less background. Clarification of this issue requires further investigations beyond the scope of the present study.

The present study revealed extremely fast degradation of PSI and PSII induced by strong light in Δ*chlP* (Figures 3, 5 and 6). Evidently, α–tocopherol deficiency is not the determining factor in this regard, as, under the same conditions, Δ*hpt* displayed only a slightly increased PSI instability in comparison to the WT (Figure 5). Furthermore, the abundant myxoxanthophyll in Δ*chlP* cells grown under standard light (Figure 4) indicates that the mutant experiences photo-oxidative stress, even under moderate illumination. This observation is corroborated by the more rapid degradation of PSI/II in the mutant under light of 40 μmol photons m^{-2} sec^{-1} than in darkness (Shpilyov *et al.*, 2005). Under stronger irradiation (e.g. 500 μmol photons m^{-2} sec^{-1}), photo-oxidative stress is further aggravated in the mutant, leading to very fast destruction of PSI and PSII. This type of enhanced light sensitivity was not observed in Δ*hpt* and the other *Synechocystis* strains that lack vitamin E (Dähnhardt *et al.*, 2002; Savidge *et al.*, 2002; Sattler *et al.*, 2003; Sakuragi, 2004; Sakuragi *et al.*, 2006).

Thus, the present data indicate that both PSI and PSII are structurally altered in Δ*chlP*, unstable and very vulnerable to photodegradation, and these traits are not due to α–tocopherol deficiency in the mutant.

Effects of phylloquinone deficiency

The function of phylloquinone (vitamin K_1) (Table S1) in the A_1 site of PSI has been extensively explored for more than two decades. Approaches such as reconstitution of the A_1 site with foreign quinones *in vitro* as well as *in vivo* were very informative. These studies revealed that diverse benzo-, naphtho- and anthraquinones – and even so-called 'quinonoids' – bind to the A_1 site and function as efficient redox co-factors (Table S1) (reviewed by Ikegami *et al.*, 2000; Itoh *et al.*, 2001; Johnson and Golbeck, 2004; Srinivasan and Golbeck, 2009). Even the tail-less phylloquinone analog menadione (vitamin K_3) (Table S1) may occupy the A_1 site in the correct orientation and sustain $A_0 \rightarrow F_X$ electron transfer *in vitro* (Iwaki and Itoh, 1989, 1994; Kumazaki *et al.*, 1994; reviewed by Ikegami *et al.*, 2000; Itoh *et al.*, 2001). Thus, the 'head' group of a quinone (phylloquinone in most oxygenic phototrophs; see below also) is the main factor determining its binding, coordination and redox activity in PSI. However, the 'tail' is assumed to improve the quinone binding affinity through hydrophobic interaction with proteins (Iwaki and Itoh, 1989, 1994; Kumazaki *et al.*, 1994; reviewed by Ikegami *et al.*, 2000; Itoh *et al.*, 2001), but the chemical nature of the tail appears not to be critical, as different long-chain isoprenoids, or even non-branched alkyl substituents, may also bind efficiently to the A_1 site (Biggins, 1990; Srinivasan and Golbeck, 2009). For the phylloquinone-deficient *Synechocystis* mutants (*menA*, *B*, *D* and *E*), it has been shown *in vivo* that plastoquinone–9 (Table S1) functions at the A_1 site but with diminished efficiency due to the more oxidizing redox

potential (Johnson *et al.*, 2000; Semenov *et al.*, 2000; Zybailov *et al.*, 2000; reviewed by Johnson and Golbeck, 2004; Srinivasan and Golbeck, 2009). Additionally, incorporation of plastoquinone into PSI leads to a decrease in PSI content (Johnson *et al.*, 2000). Together, both effects result in a reduction of the whole ETC capacity by approximately 40% (Johnson *et al.*, 2000; own observations). However, the capability for photoautotrophic growth is not abolished in these mutants (Johnson and Golbeck, 2004; Srinivasan and Golbeck, 2009; own observations). Altogether, the available data demonstrate that PSI possesses a considerable capacity to accommodate quinones of various structure and size at the A_1 site (Table S1).

The incorporation of phylloquinone into PSI requires neither enzymatic activity nor *de novo* protein synthesis, and is not accompanied by disassembly/re-assembly of PSI *in vitro* (reviewed by Ikegami *et al.*, 2000; Itoh *et al.*, 2001) and *in vivo* (Johnson *et al.*, 2001). Moreover, PSI containing a variant quinone, which may be considerably different in size and/or binding affinity, or even with an empty quinone-binding pocket, remains relatively stable *in vitro* (reviewed by Ikegami *et al.*, 2000; Itoh *et al.*, 2001) as well as *in vivo* (Johnson *et al.*, 2000, 2001). The available data indicate that – in contrast to the role of Chl *a* in biogenesis of PSI (and PSII) (Eichacker *et al.*, 1992, 1996; Adamska *et al.*, 2001) – phylloquinone does not determine assembly and stabilization of PSI, at least not in cyanobacteria such as *Synechocystis* (Johnson and Golbeck, 2004; Srinivasan and Golbeck, 2009).

So far, the nature of the quinone in PSI of the Δ*chlP* mutant remains to be established. However, several lines of evidence suggest that this may be the geranylgeranylated phylloquinone analog menaquinone–4 (vitamin $K_{2(20)}$) (Figure S1 and Table S1). First, there is no GGR activity in the mutant (Shpilyov *et al.*, 2005). Hence, formation of (phytylated) phylloquinone is impossible (Figure S1). Second, if synthesis of the tailed vitamin K is interrupted at the MenA stage (Figure S1) or earlier, only plastoquinone–9 occupies the A_1 site in the respective *Synechocystis* mutants (Johnson and Golbeck, 2004; Srinivasan and Golbeck, 2009). Third, the Δ*chlP* mutant possesses a fully functional ETC (Shpilyov *et al.*, 2005) indicative of a highly active PSI, which appears to exclude the presence of plastoquinone–9 at the A_1 site. Therefore, PSI in Δ*chlP* appears to contain a quinone with a redox capacity comparable to that of phylloquinone, but not phylloquinone itself.

According to the phylloquinone biosynthetic pathway, menaquinone–4 is indeed such a candidate (Figure S1). It possesses the same head group as phylloquinone and differs from phylloquinone only by three additional double bonds in the tail moiety, which appear not to be critical (see above). Indeed, *in vitro* reconstitution studies established menaquinone–4 to be a fully functional analog of phylloquinone, i.e. displaying comparable binding and electron-transfer properties at the A_1 site (Iwaki and Itoh, 1989; Biggins, 1990; Iwaki and Itoh, 1991, 1994; Kumazaki *et al.*, 1994; reviewed by Ikegami *et al.*, 2000; Itoh *et al.*, 2001). Furthermore, menaquinone–4 synthesis and efficient operation in PSI have been demonstrated using isolated spinach chloroplasts (Kaiping *et al.*, 1984) and GGR-deficient rice mutants (Shibata *et al.*, 2004b). Finally, menaquinone–4 was found to function at the A_1 site in several oxygenic phototrophs, which, however, use phytylated Chls (Table S1).

Irrespective of the quinone species in PSI in the Δ*chlP* mutant, this cannot be the reason for PSI instability, as the quinone is not a factor influencing PSI assembly and stabilization, as mentioned above. 77 K fluorescence emission spectra of an analogous *menA* mutant indicated only depletion in PSI content, with no sign of structural alterations (Johnson *et al.*, 2000). Moreover, the instability of PSII in Δ*chlP* is certainly not related to the quinone present in PSI, as the biogenesis and function of PSII are independent of those of PSI in cyanobacteria, as deduced from data obtained using phylloquinone-deficient (Johnson *et al.*, 2000) and PSI-lacking *Synechocystis* mutants (Shen *et al.*, 1993; Wu and Vermaas, 1995).

Hence, as in the case of tocopherol, impaired phylloquinone synthesis also cannot be the origin of the instability and high vulnerability of PSI and especially PSII to photodegradation in the Δ*chlP* mutant.

Requirement for chlorophyll phytylation

Active ETCs in GGR-deficient mutants of oxygenic phototrophs (Shibata *et al.*, 2004a,b; Shpilyov *et al.*, 2005) indicate that geranylgeranylated electron transfer co-factors, i.e. Chl a_{GG}, pheophytin a_{GG} and menaquinone–4 (see above), are functional. However, when assembled with Chl a_{GG}, PSI and PSII complexes become unstable and tend to degrade spontaneously (i.e. in complete darkness; Shpilyov *et al.*, 2005). The geranylgeranyl residue is more rigid than a phytyl residue due to three additional double bonds. This increased rigidity probably perturbs the association of $Chls_{GG}$ with apoproteins, possibly also disturbing the interaction of protein subunits with each other. Reaction centre preparations from a GGR-deficient mutant of the purple bacterium *Rhodobacter capsulatus* synthesizing bacteriochlorophyll (BChl) a_{GG} were also found to be much less stable than similar preparations from WT cells (Bollivar *et al.*, 1994). Thus, phytylation of (B)Chls appears to be generally important for the stability of pigment–protein complexes among chlorophototrophs, with the exception of one purple bacterium, *Rhodospirillum rubrum* (see below), and heliobacteria. The latter accumulate BChl *g* with a fully unsaturated C_{15} isoprenoid (farnesyl) tail (Madigan, 2006). It should be noted that heliobacteria only grow photoheterotrophically or even heterotrophically (Madigan, 2006).

From an evolutionary perspective, (B)Chl tail saturation has apparently become more critical for oxygenic photosynthesis. For example, GGR-deficient mutants of anoxygenic photosynthetic bacteria are still capable of photoautotrophic growth, although less efficiently (Bollivar *et al.*, 1994; Addlesee and Hunter, 1999; Harada *et al.*, 2008a). Moreover, depending on growth stage and conditions, these organisms naturally accumulate various amounts (6–30%) of BChls with unsaturated tail moieties (Bollivar *et al.*, 1994; Addlesee and Hunter, 1999; Mizoguchi *et al.*, 2006; Harada *et al.*, 2008b). The purple bacterium *Rhodospirillum rubrum* even accumulates only BChl a_{GG} – although bacteriopheophytin *a* in its reaction centre is phytylated due to the activity of a special bacteriopheophytin a_{GG} reductase (Addlesee and Hunter, 2002). In striking contrast, Chls with only partially saturated tails never occur in the mature photosynthetic apparatus of oxygenic phototrophs (Tamiaki *et al.*, 2007), and GGR deficiency in these organisms is lethal (Henry *et al.*, 1986; Shibata *et al.*, 2004a; Shpilyov *et al.*, 2005).

Remarkably, similar light sensitivity as in the cyanobacterial Δ*chlP* mutant was observed in GGR-deficient plants, e.g. tobacco (Tanaka *et al.*, 1999) and rice (Shibata *et al.*, 2004a,b). The likely explanation is that perturbed binding and spatial orientation of Chl a_{GG} in the pigment–protein complexes impedes interactions and thus efficient excitation energy transfer among Chls and possibly also to carotenoids. In turn, this may lead to the build-up of triplet excited states of Chls (^{3}Chl*), leading to the generation of destructive reactive oxygen species (particularly $^1O_2$) upon illumination. This is apparently the reason for aggravated photo-oxidative stress and the high vulnerability of pigment–protein complexes to light-induced degradation in GGR-deficient mutants of oxygenic phototrophic organisms. Thus, beyond stabilizing photosynthetic pigment–protein complexes by Chl phytylation, the second important role of the GGR enzyme in oxygenic phototrophs appears to be to ensure the optimal coordination of Chl molecules in these complexes that is necessary for efficient utilization of light energy and thus protection from photo-oxidative stress in an oxygenic environment.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

Mutants were derived from the same glucose-tolerant non-motile *Synechocystis* sp. PCC 6803 strain (collection of the Genetics Department, Lomonosov Moscow State University, Russia) used as the wild-type (WT). The Δ*chlP* mutant was generated by disruption of the *ggr* (*chlP*) gene encoding the GGR (ChlP) enzyme using a kanamycin resistance (Km^r) cassette as described previously (Shpilyov *et al.*, 2005). For construction of the Δ*hpt* and Δ*menA* mutants, the Km^r cassette was inserted inside the *hpt* and *menA* genes encoding the HPT and MenA enzymes, respectively. The biosynthetic pathways, Δ*hpt* and Δ*menA* genetic maps and mutant construction protocols are shown in Figures S1 and S2.

Cyanobacteria were cultivated in liquid BG–11 medium (Rippka *et al.*, 1979) or solidified BG-11 containing 1% agar (Difco, www.bd.com) at 34°C under continuous illumination provided with white fluorescent lamps and aeration with ambient air by magnetic stirring (for liquid cultures). The WT and Δ*hpt* strains were propagated under a light intensity of 40 μmol photons m^{-2} sec^{-1} ('standard' light) with no glucose supply (photoautotrophic conditions). Dim light of approximately 2–4 μmol photons m^{-2} sec^{-1} was used together with 10 mM glucose (photomixotrophic conditions) for propagation of the Δ*chlP* and Δ*menA* mutants. Cyanobacteria were maintained on agar plates at room temperature in dim light in the presence of glucose in the case of Δ*chlP* and Δ*menA*, and without glucose in case of WT and Δ*hpt*. Kanamycin (40 μg ml^{-1}) was supplied to propagate and maintain the mutants.

In the comparative studies, the strains were preliminary adapted to photomixotrophic conditions (unless indicated otherwise) through two re-inoculations. Cells were taken from plates used to maintain the strains and grown in liquid medium to mid-log phase in the presence of 10 mM glucose under aeration with ambient air and illumination of 40 μmol photons m^{-2} sec^{-1} for WT, Δ*hpt* and Δ*chlP* and 20 μmol photons m^{-2} sec^{-1} for Δ*menA* (and WT for comparison with Δ*menA*). Thereafter, cells were re-inoculated into fresh BG–11 medium under the same conditions, and, when they had reached mid-log phase, were subjected to the experiments/measurements. In the high-light experiments, cells were diluted with fresh 10 mM glucose-containing BG–11 medium to an absorbance at 750 nm (OD_{750}) of approximately 0.25, and exposed to illumination of approximately 500 μmol photons m^{-2} sec^{-1} under the same aeration and temperature conditions. Cell growth was monitored by measurements of OD_{750}.

Biochemical and biophysical analyses

Contents of Chls, total carotenoids and PBS were estimated from the whole-cell absorption spectra recorded at room temperature. Furthermore, Chls and total carotenoids were quantified spectrophotometrically in cell extracts obtained using 90% v/v methanol/water. Individual pigments and tocochromanols were assayed by HPLC as described previously (Shpilyov *et al.*, 2005). PSI/II levels were assessed by 77 K fluorescence emission spectroscopy. The spectra were recorded with 435 nm excitation for cell samples of OD_{750} = 1, i.e. Chl concentrations of 2.59, 1.98 and 2.31 μg ml^{-1}, respectively, in the WT, Δ*chlP* and Δ*hpt* liquid cultures growing exponentially under photomixotrophic standard light conditions. Cell samples of equal volumes and densities were analyzed during the high-light experiment. The measurement/estimation procedures and equipment used in the present work are the same as described previously (Shpilyov *et al.*, 2005).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Biochemical network of coordinated chlorophyll *a*, α-tocopherol and phylloquinone syntheses in oxygenic phototrophic organisms.

Figure S2. Mutant construction.

Figure S3. HPLC chromatograms demonstrating vitamin E contents in WT (a) and *Δhpt* (b) cells. Peak in the chromatogram (a) corresponds to α-tocopherol.

Figure S4. Room-temperature absorption spectra of WT (black line) and *ΔmenA* (red line) cells grown photomixotrophically at 20 μmol photons m^{-2} sec^{-1}. Spectra were normalized on a per cell basis at 750 nm.

Figure S5. Cell densities (a), Chl *a* (b) and total carotenoid (c) contents in WT, *Δhpt*, *ΔmenA* and *ΔchlP* cultures during incubation at 500 μmol photons m^{-2} sec^{-1} in the presence of glucose.

Table S1. Chlorophylls, tocochromanols, quinones

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SUPPORTING INFORMATION

Figure S1. Biochemical network of coordinated chlorophyll *a*, α-tocopherol and phylloquinone syntheses in oxygenic phototrophic organisms. Dashed lines and question marks correspond to the hypothetical pathways and enzymes, respectively. π carbon bonds eliminated by geranylgeranyl reductase (GGR) are marked in red in a yellow background.

Abbreviations: GGR, geranylgeranyl reductase; ChlG, chlorophyll synthase; CHL, chlorophyllase; PPH, pheophytinase; PK[b], phytol kinase, PPK[c], phytyl phosphate kinase; HPT, homogentisate phytyl transferase; HGGT[e], homogentisate geranylgeranyl transferase; MenA, 1,4-Dihydroxy-2-naphthoate phytyl transferase (DHNA phytyl transferase); P, phytol; GG, geranylgeraniol; PP, phytyl phosphate; GGP, geranylgeranyl phosphate; PDP, phytyl diphosphate; GGDP, geranylgeranyl diphosphate; Chlide *a*, chlorophyllide *a*; Chl *a*, (phytylated) chlorophyll *a*; Chl a_{GG}, geranylgeranylated chlorophyll *a*; Phe *a*, (phytylated) pheophytin *a*; Phe a_{GG}, geranylgeranylated pheophytin *a*; HGA, homogentisic acid; DHNA, 1,4-Dihydroxy-2-naphthoic acid; α-Toc-3 and β-, γ-, δ-Toc-3 are α-tocotrienol and β-, γ-, δ-tocotrienols, respectively; α-Toc and β-, γ-, δ-Toc are α-tocopherol and β-, γ-, δ-tocopherols, respectively; MQ-4, menaquinone-4; PhQ, phylloquinone.

[a]Analogous pathway, *i.e.*, reduction of geranylgeranylated bacteriopheophytin *a* to (phytylated) bacteriopheophytin *a*, exists in *Rhodospirillum rubrum* and is catalyzed by a variant of geranylgeranyl reductase named geranylgeranyl-bacteriopheophytin (GG-Bphe) reductase (Addlesee and Hunter, 2002).

[b]The abbreviation is applied here. Originally, the enzyme was designated VTE5 in *Arabidopsis thaliana* due to a decreased vitamin E (α-tocopherol) content observed in the respective mutant (Valentin *et al*., 2006). Alternative name in *Synechocystis* is Slr1652 according to the respective ORF *slr1652* (Vavilin and Vermaas, 2007).

[c]The abbreviation is applied here. The enzyme/gene has not been identified yet in oxygenic phototrophs. However, its activity has been shown, in particular in *Arabidopsis thaliana* (Ischebeck *et al*., 2006).

[d]Cyanobacterial HPT, *e.g*., from *Synechocystis* (Collakova and DellaPenna, 2001; Shpilyov *et al*., 2005). HPTs from dicot plants cannot perform this reaction.

[e]The pathway/enzyme is specific for monocot plants (Yang *et al*., 2011).

[f]The pathway naturally exists in the cyanobacteria *Synechococcus* sp. PCC 7002 (Sakuragi, 2004; Sakuragi *et al*., 2005) and *Gleobacter violaceus* (Mimuro *et al*., 2005), red algae *Cyanidium caldarium* (Yoshida, E. *et al*., 2003) and a marine diatom *Chaetoceros gracilis* (Ikeda *et al*., 2008). Additionally, the pathway was revealed as possible in rice (*Oryza sativa*) (Shibata *et al*., 2004a,b), and most likely can operate in the cyanobacterium *Synechocystis* sp. PCC 6803 (current work).

Figure S2. Mutant construction.

Construction of Δhpt (a). Construction of the *Δhpt* mutant strain was carried out by inactivation of the *hpt* gene (ORF *slr1736*; CyanoBase ID: slr1736) coding for the HPT enzyme (Figure S1) (Collakova and DellaPenna, 2001; Savidge *et al*., 2002; Sakuragi *et al*., 2006). A 920-bp DNA fragment including a part of the *slr1736* locus was amplified by PCR from the *Synechocystis* WT genome with the 5'-ggtcctgggcaatgatggcgggg-3' and 5'-gccgcccataagccccagattgc-3' primers (F and R arrows, respectively, in Figure S2a). The

amplified fragment was cloned into the pGEM-T Easy vector (Promega, Mannheim). In the resultant construct, the 379-bp *Hpa*I-segment containing the promoter region and a part of the *hpt* coding sequence was replaced with a 1252-bp fragment containing kanamycin-resistance (Km^r) cassette derived from the pUC4K plasmid (Pharmacia) by digestion with *Hinc*II restrictase. This final plasmid was cut with *Pvu*II to obtain a linear DNA segment containing a part of the *hpt* locus carrying Km^r insertion. Such a linearized DNA thereafter was used to transform *Synechocystis* WT cells to Km resistance indicative of insertion of the Km^r cassette into chromosomal *slr1736* locus *via* a double-crossover event (Figure S2a). Transformants were selected on plates with the BG-11 medium (Rippka *et al*., 1979) solidified with 1% agar (Difco) and supplemented with 20 μg ml^{-1} of Km under photoautotrophic conditions at light intensity of 40 μmol photons m^{-2} sec^{-1}. Four transformed colonies were picked for segregation. The segregation was carried out under photoautotrophic conditions at 40 μmol photons m^{-2} sec^{-1} by re-streaking on plates with increasing concentrations of Km (with step 20 μg ml^{-1}). Homozygous state of the transformants was verified by PCR (data not shown). All four transformants appeared to have a wild-type phenotype under photoautotrophic conditions as judged from the growth rates and absorption spectra. One of the transformants was chosen for further studies and designated *Δhpt* mutant. Lack of the HPT function (Figure S1) in the mutant cells was revealed by the HPLC analysis (Figure S3b). Conditions of propagation, maintainence and experiments applied to the *Δhpt* mutant are described in the 'Experimental procedures' section of the article.

Construction of ΔmenA (b). Construction of the *ΔmenA* mutant strain was carried out by insertion of a Km^r cassette inside the *menA* gene (ORF *slr1518*; CyanoBase ID: slr1518) coding for the MenA enzyme (Figure S1) (Johnson *et al*., 2000). A 789-bp DNA

segment including a part of the *slr1518* locus was amplified by PCR from the *Synechocystis* WT genome with the 5'-agcctgatgacacaaaacgccga-3' and 5'-ggaagcggaagcccaggtagttt-3' primers (F and R arrows, respectively, in Figure S2b) and cloned into the pGEM-T Easy vector. The same 1252-bp *Hin*cII-fragment from pUC4K containing Km^r cassette was inserted into the *Mls*I restriction site inside the cloned *slr1518* locus. The linear fragment, including a part of the gene disrupted with Km^r cassette, was derived from the resultant plasmid by *Pvu*II-digestion and used to transform *Synechocystis* WT cells to Km resistance indicative of incorporation of the Km^r cassette into the chromosomal *menA* gene (Figure S2b). Transformants were selected on plates with the BG-11 medium solidified with 1% agar and supplemented with 20 μg ml^{-1} of Km under photoautotrophic conditions at light intensity of 40 μmol photons m^{-2} sec^{-1}. Four transformed colonies were picked for segregation. The segregation was carried out in dim light of ~2–4 μmol photons m^{-2} sec^{-1} in the presence of 10 mM glucose by re-streaking on plates with increasing concentrations of Km (with step 20 μg ml^{-1}). Homozygous state of the transformants was verified by PCR (data not shown). All four transformants appeared to show de-pigmentation resulting in 'olive'-like coloration. Absorption spectra revealed lowered levels of phycobilisomes and chlorophyll (Figure S4). All transfomants could grow photomixotrophically and photoautotrophically but in light not exceeding ~30–35 μmol photons m^{-2} sec^{-1}. One of the transformants, designated *ΔmenA* mutant, was taken for studies.

Conditions of propagation, maintainence and experiments applied to the *ΔmenA* mutant are described in the 'Experimental procedures' section of the article.

E. coli strain JM109 along with LB medium (supplied with X-gal and IPTG for white-blue screening of the cloned PCR products) (Sambrook and Rassell, 2001) were used to engineer plasmid constructs.

Figure S3. HPLC chromatograms demonstrating vitamin E contents in WT (a) and *Δhpt* (b) cells. Peak in the chromatogram (a) corresponds to α-tocopherol.

Figure S4. Room-temperature absorption spectra of WT (black line) and *ΔmenA* (red line) cells grown photomixotrophically at 20 μmol photons m^{-2} sec^{-1}. Spectra were normalized on a per cell basis at 750 nm.

Figure S5. Cell densities (a), Chl *a* (b) and total carotenoid (c) contents in WT, *Δhpt*, *ΔmenA* and *ΔchlP* cultures during incubation at 500 μmol photons m^{-2} sec^{-1} in the presence of glucose. The 0-h time point corresponds to the cells grown photomixotrophically at 40 μmol photons m^{-2} sec^{-1}, except *ΔmenA*, which was grown at 20 μmol photons m^{-2} sec^{-1} prior subjection to high light.

Table S1 Chlorophylls, tocochromanols, quinones

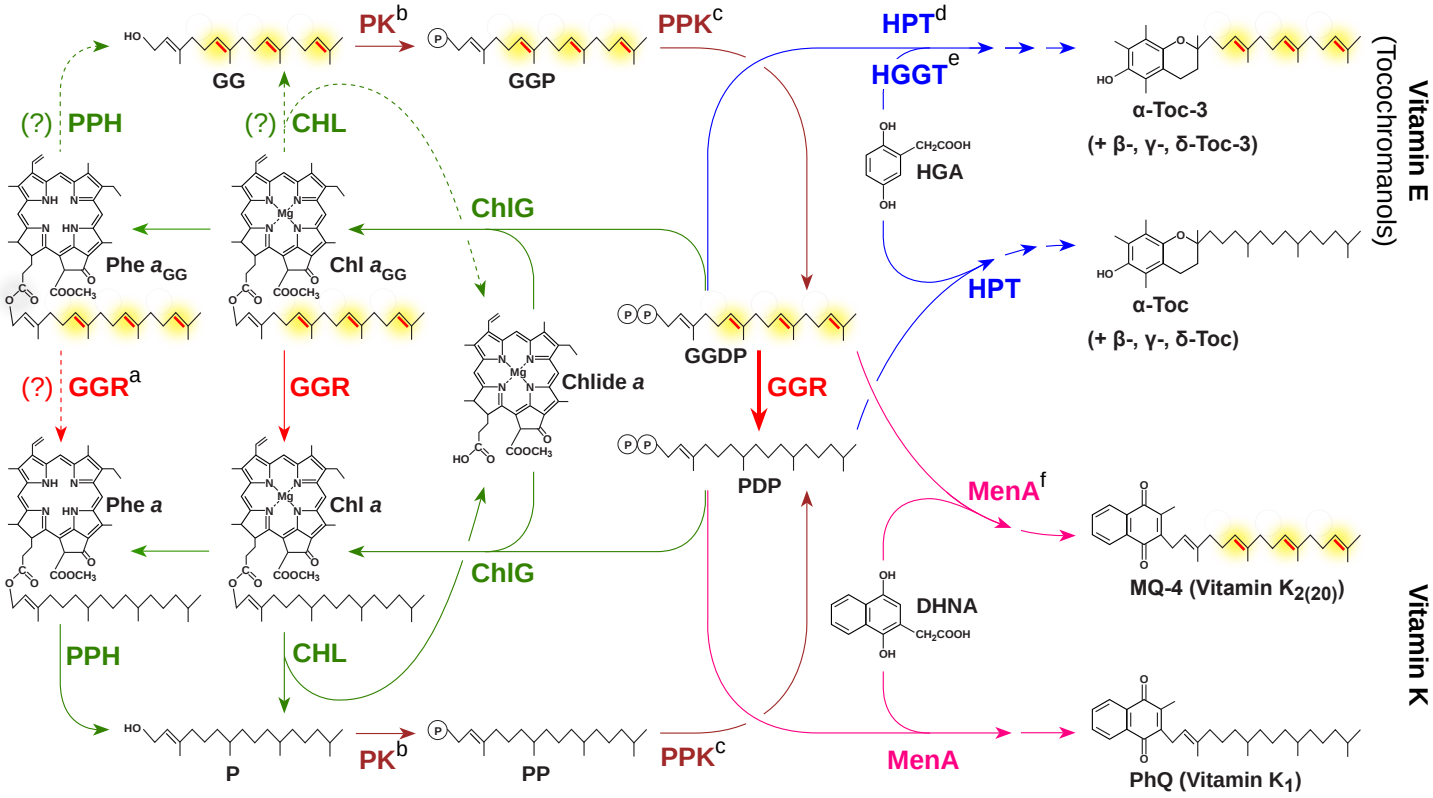

GG
PK^b
GGP
PPK^c
HPT^d
HGGT^e
HGA
α-Toc-3
(+ β-, γ-, δ-Toc-3)
Vitamin E
(Tocochromanols)
(?) PPH
(?) CHL
ChlG
Phe a_GG
Chl a_GG
HPT
α-Toc
(+ β-, γ-, δ-Toc)
(?) GGR^a
GGR
Chlide a
GGDP
GGR
Phe a
Chl a
PDP
MenA^f
DHNA
MQ-4 (Vitamin K_2(20))
Vitamin K
ChlG
PPH
CHL
P
PK^b
PP
PPK^c
MenA
PhQ (Vitamin K_1)

Fig. S1

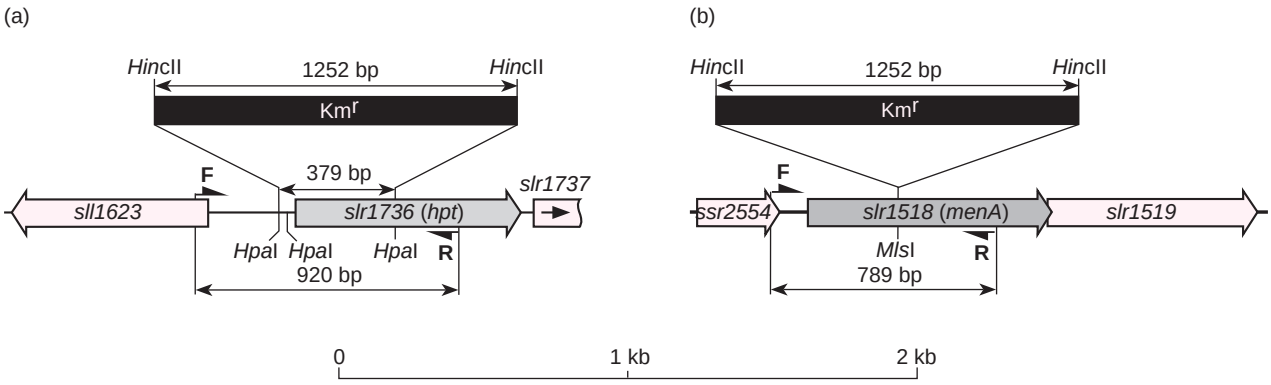

(a)
HincII
1252 bp
HincII
Km^r
379 bp
F
slr1737
sll1623
slr1736 (hpt)
HpaI
HpaI
HpaI
R
920 bp
(b)
HincII
1252 bp
HincII
Km^r
F
ssr2554
slr1518 (menA)
slr1519
MlsI
R
789 bp
0
1 kb
2 kb

Fig. S2

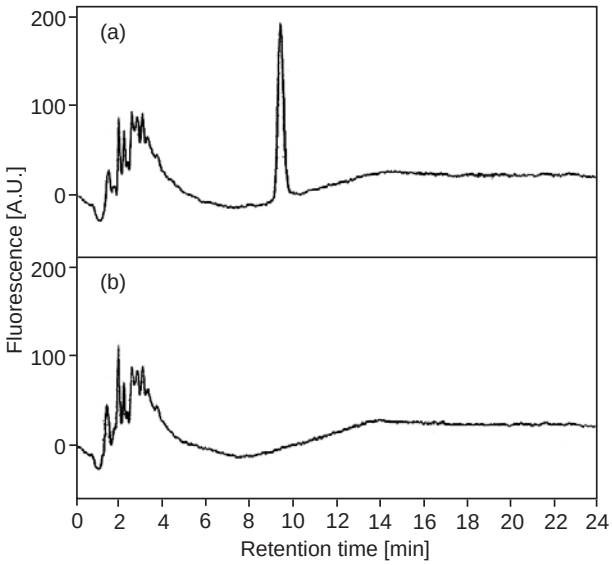

(a)
(b)
200
100
0
200
100
0
Fluorescence [A.U.]
0 2 4 6 8 10 12 14 16 18 20 22 24
Retention time [min]

Fig. S3

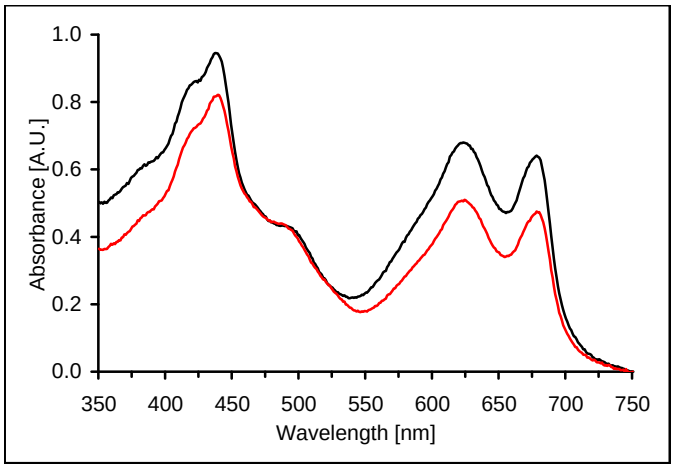

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance [A.U.]
350
400
450
500
550
600
650
700
750
Wavelength [nm]

Fig. S4

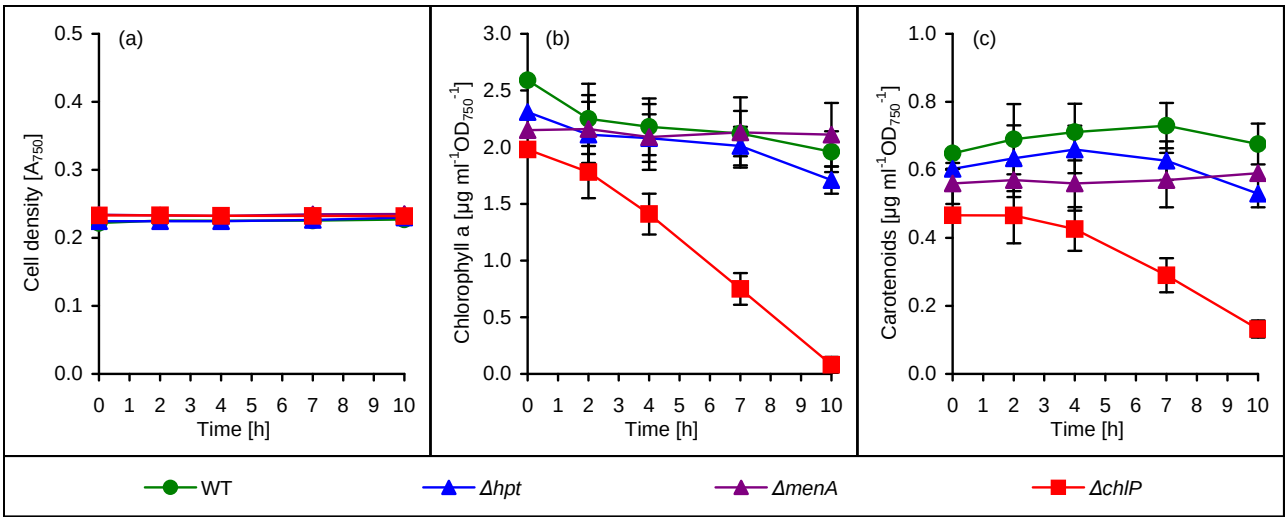
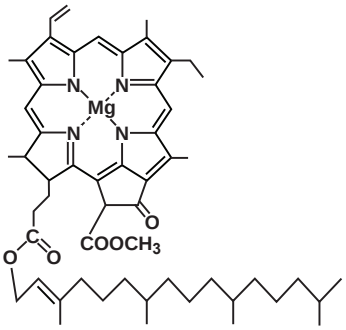

(a)
Cell density [A750]
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10
Time [h]
(b)
Chlorophyll a [µg ml-1OD750-1]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10
Time [h]
(c)
Carotenoids [µg ml-1OD750-1]
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7 8 9 10
Time [h]
WT
Δhpt
ΔmenA
ΔchlP

Fig. S5

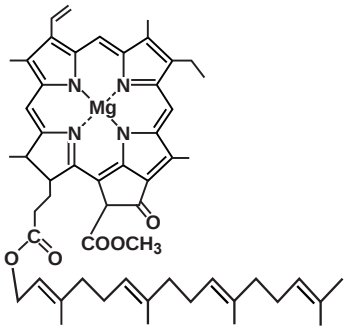
Table S1. Chlorophylls, tocochromanols, quinones

Chlorophyll *a* species

Chl *a,* chlorophyll *a*; Chlide *a*, chlorophyllide *a*



(Phytylated) Chl *a*

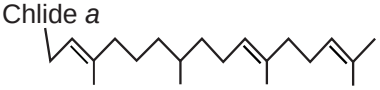


Geranylgeranylated Chl *a*

Chlide *a*

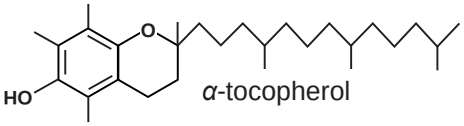
Tetrahydrogeranylgeranylated Chl *a*

Chlide *a*

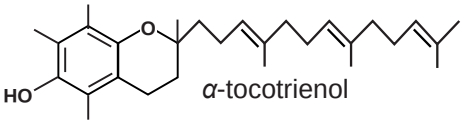


Dihydrogeranylgeranylated Chl *a*

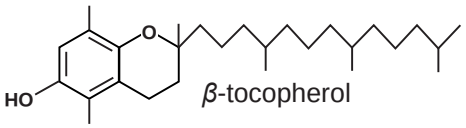
Tocochromanols (vitamin E)



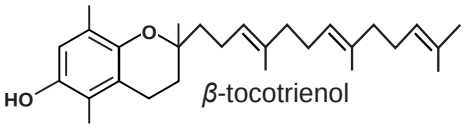
α-tocopherol



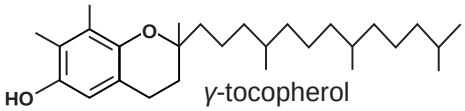
α-tocotrienol



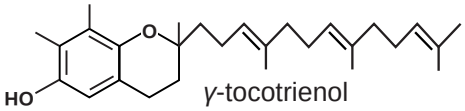
β-tocopherol



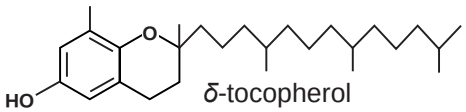
β-tocotrienol



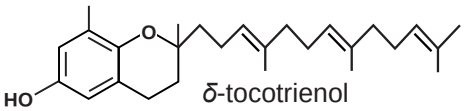
γ-tocopherol



γ-tocotrienol



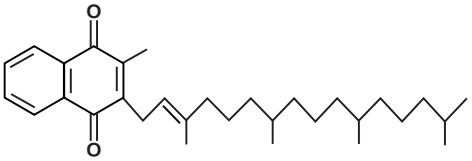
δ-tocopherol



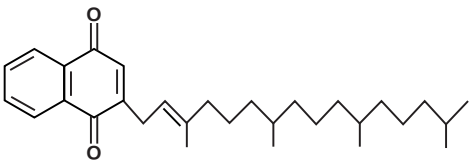
δ-tocotrienol

Quinones, which can operate at the A_1 site of PSI as electron-transfer cofactors

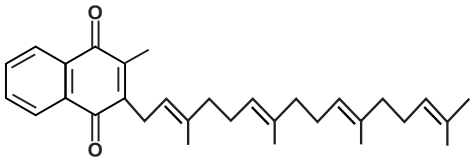
Naphthoquinones



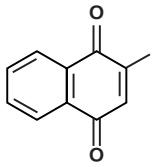
Phylloquinone; vitamin K_1; 2-Methyl-3-phytyl-1,4-naphthoquinone. Naturally occurring as an electron carrier at the A_1 site in PSI of oxygenic phototrophs (reviewed by Srinivasan and Golbeck, 2009; Ohashi *et al*., 2010).



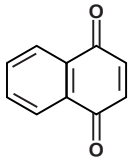
Demethylphylloquinone; 2-Phytyl-1,4-naphthoquinone. *In vivo* studies (Sakuragi, 2004; Sakuragi *et al*., 2002).



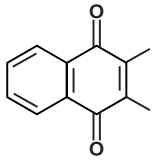
Menaquinone-4; vitamin $K_{2(20)}$;
2-Methyl-3-(isoprenyl)$_4$-1,4-naphthoquinone;
2-Methyl-3-geranylgeranyl-1,4-naphthoquinone.
The quinone naturally functions in PSI of the cyanobacteria *Synechococcus* sp. PCC 7002 (Sakuragi, 2004; Sakuragi *et al*., 2005) and *Gleobacter violaceus* (Mimuro *et al*., 2005), red algae *Cyanidium caldarium* (Yoshida, E. *et al*., 2003) and a marine diatom *Chaetoceros gracilis* (Ikeda *et al*., 2008). Additionally, menaquinone-4 was found to effectively operate in PSI in GGR-defective rice (*Oryza sativa*) mutant (Shibata *et al*., 2004a,b), and most likely can operate in the cyanobacterium *Synechocystis* sp. PCC 6803 (current work). In *in vitro* reconstitution studies, menaquinone-4 acted as a full functional analogue of phylloquinone in PSI (Iwaki and Itoh, 1989; Biggins, 1990; Iwaki and Itoh, 1991; Iwaki and Itoh, 1994; Kumazaki *et al*., 1994).



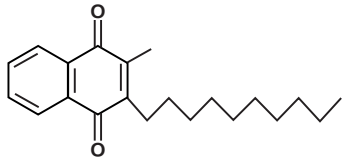
Menadione; vitamin K_3; 2-Methyl-1,4-naphthoquinone. *In vitro* studies (Iwaki and Itoh, 1989; Iwaki and Itoh, 1994).



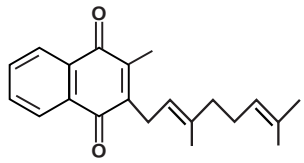
α-Naphthoquinone; 1,4-Naphthoquinone. *In vitro* studies (Iwaki and Itoh, 1989; Iwaki and Itoh, 1991; Sieckman *et al*., 1991; Iwaki and Itoh, 1994).



2,3-Dimethyl-1,4-naphthoquinone. *In vitro* studies (Iwaki and Itoh, 1991; Iwaki and Itoh, 1994).

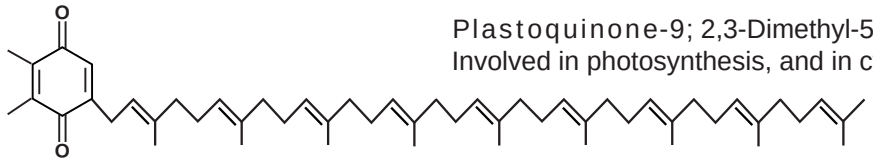


2-Methyl-3-decyl-1,4-naphthoquinone. *In vitro* studies (Biggins, 1990).

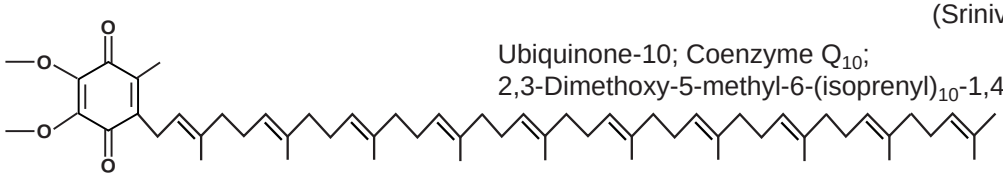


2-Methyl-3-(isoprenyl)$_2$-1,4-naphthoquinone. *In vitro* studies (Biggins, 1990).

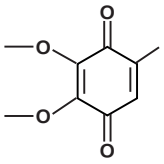
Benzoquinones



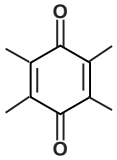
Plastoquinone-9; 2,3-Dimethyl-5-(isoprenyl)$_9$-1,4-benzoquinone.
Involved in photosynthesis, and in cyanobacteria also in respiration. Acts as an electron carrier at the A_1 site of PSI in the phylloquinone-deficient mutants, *menA, B, D, E* (Srinivasan and Golbeck, 2009).



Ubiquinone-10; Coenzyme Q_{10};
2,3-Dimethoxy-5-methyl-6-(isoprenyl)$_{10}$-1,4-benzoquinone.
Involved in respiration. Was tested in PSI insteed of phylloquinone *in vitro* (Iwaki and Itoh, 1989).

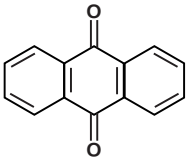


Ubiquinone-0; Coenzyme Q_0;
2,3-Dimethoxy-5-methyl-1,4-benzoquinone. *In vitro* studies (Iwaki and Itoh, 1989).

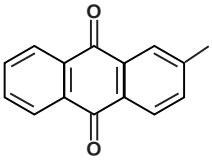


Duroquinone; Tetramethyl-1,4-benzoquinone. *In vitro* studies (Iwaki and Itoh, 1989; Sieckman *et al*., 1991).

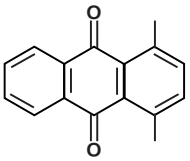
Anthraquinones



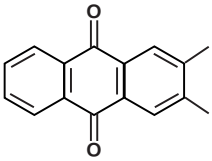
Anthradione; 9,10-Anthraquinone. *In vitro* studies (Iwaki and Itoh, 1989; Iwaki and Itoh, 1994; Pushkar *et al*., 2005); *in vivo* studies (Romberger *et al*., 2005).



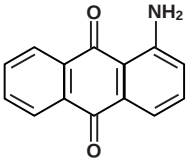
Techtoquinone; 2-Methyl-9,10-anthraquinone. *In vitro* studies (Iwaki and Itoh, 1989; Iwaki and Itoh, 1994).



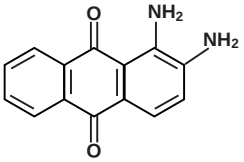
1,4-Dimethyl-9,10-anthraquinone. *In vitro* studies (Iwaki and Itoh, 1991).



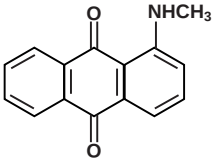
2,3-Dimethyl-9,10-anthraquinone. *In vivo* studies (Romberger *et al*., 2005).



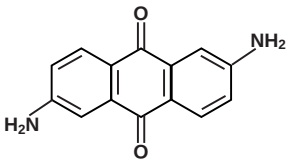
Diazo Fast Red AL; 1-Amino-9,10-anthraquinone. *In vitro* studies (Iwaki and Itoh, 1989; Iwaki and Itoh, 1994).



1,2-Daa; 1,2-Diamino-9,10-anthraquinone. *In vitro* studies (Iwaki and Itoh, 1994).

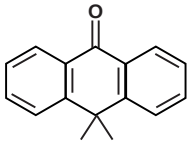


Methane quinone; Disperse red 9; 1-(Methylamino)anthraquinone; 1-(Methylamino)-9,10-anthraquinone. *In vivo* studies (Romberger *et al*., 2005).

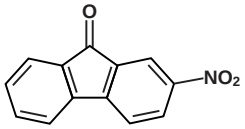


2,6-Diamino-9,10-anthraquinone. *In vitro* studies (Iwaki and Itoh, 1989; Iwaki and Itoh, 1994); *in vivo* studies (Romberger *et al*., 2005).

‘Quinonoids‘



10,10-Dimethylanthrone. *In vitro* studies (Itoh and Iwaki, 1991).



2-Nitrofluorenone; 2-Nitro-9-fluorenone. *In vitro* studies (Itoh and Iwaki, 1991).

***NB*:** Only several quinones, which are able to function at the A_1 site of PSI in place of phylloquinone, are presented in the table. They are different from each other with respect to binding affinity and electron-transfer capacity as well. Among other quinones, menaquinone-4 appears to be a full analogue of phylloquinone. However, interestingly, several anthraquinones [9,10-Anthraquinone; 2,3-Dimethyl-9,10-anthraquinone; 1-(Methylamino)-9,10-anthraquinone (Methane quinone) and 2,6-Diamino-9,10-anthraquinone], if added to cultures of the phylloquinone-deficient *menA, B Synechocystis* sp. PCC 6803 mutants, appear to displace plastoquinone-9 from PSI and fully restore the WT phenotype in the mutants, *e.g.*, the photoautotrophic growth rate and high-light tolerance (Romberger *et al*., 2005).

In general, wider range of quinones can efficiently bind to the A_1 site of PSI; however, many of them are unable to sustain electron transport from A_0 to F_X due to unsuitable redox potential. For details about quinones presented here and other quinones, see the respective references. Additionally, *in vitro* and *in vivo* studies of reconstitution of the A_1 site with different quinones are summarized in reviews of Ikegami *et al*. (2000), Itoh *et al*. (2001), Johnson and Golbeck (2004) and Srinivasan and Golbeck (2009).

(Table S1, Part 4)

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