The hydroxyphenylpyruvate dioxygenase from *Synechocystis* sp. PCC 6803 is not required for plastoquinone biosynthesis

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Abstract The disruption of the *Synechocystis* open reading frame Δ*slr0090* encoding a gene with high homology to plant genes encoding 4-hydroxyphenylpyruvate dioxygenase results in an impairment of tocopherol biosynthesis without affecting levels of plastoquinone, carotenoids and chlorophyll as well as cell growth and photosynthesis. Our results indicate that in *Synechocystis* in contrast to the situation in higher plants the 4-hydroxyphenylpyruvate dioxygenase is not required for the synthesis of plastoquinone.

Key words: 4-Hydroxyphenylpyruvate dioxygenase; Plastoquinone; Vitamin E; Tocopherols; *Synechocystis*

1. Introduction

In all plants and cyanobacteria plastoquinone serves as an electron carrier between photosystem (PS) II and the cytochrome b_6/f complex. It has been shown that in higher plants an important step of the plastoquinone biosynthesis is the formation of homogentisate from hydroxyphenylpyruvate, catalyzed by the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD). This reaction of the plastoquinone biosynthesis pathway is shared by the synthesis of tocopherols and tocotrienols, which are collectively known as vitamin E [1].

Therefore homogentisate is the common aromatic precursor of plastoquinones and tocopherols. At least in *Synechocystis* the branching point in the formation of both groups of quinones is the prenylation of homogentisate which is catalyzed by specific polyprenyltransferases for the biosynthesis of tocopherols and plastoquinone, respectively [2,3].

The *hpd* gene encoding HPPD has been cloned from different plant species including *Arabidopsis thaliana*, *Daucus carota* and *Hordeum vulgare* [4–6]. The *Arabidopsis* mutant (*pds1*) is deficient in both tocopherols and plastoquinone [7]. Since plastoquinone is a cofactor catalyzing the desaturation of phytoene in carotenoid biosynthesis (NADPH-dependent quinone oxidoreductase), the mutation of the *Arabidopsis* gene also leads to an inhibition of carotenoid synthesis. This phenotype was also found after treatment of higher plants with herbicides (triketones), which are known as competitive inhibitors of the HPPD [8,9].

Since bacteria related to cyanobacteria are thought to be ancestors of plastids, it is likely that HPPD as a key enzyme in tocopherol and plastoquinone biosynthesis, in cyanobacteria has the same function as in higher plants. Indeed, in the case of *Synechocystis* PCC 6803 the composition of quinones including vitamin E derivatives and plastoquinone is similar to higher plant plastids [2]. To investigate whether HPPD has the same function in cyanobacteria as in plants we investigated the impact of a deletion of the *hpd* gene on the contents of tocopherols, plastoquinone, chlorophylls and carotenoids as well as on cell growth and 77 K fluorescence spectra. In this paper, we report on a deletion of the *hpd* gene in *Synechocystis* resulting in a complete loss of tocopherols without effecting the plastoquinone level and the photosynthetic apparatus. This suggests that in *Synechocystis* the *hpd* gene is not required for plastoquinone biosynthesis.

2. Materials and methods

2.1. Plasmid construction, transformation and generation of the deletion mutant Δslr0090

To delete the *hpd* gene (*slr0090*) two PCR products were amplified from 20 ng *Synechocystis* PCC 6803 genomic DNA. The first 415 bp PCR fragment amplified by the primer pair upstream I (5′-GTCTGATTTTAGCGACCTGG-3′) and upstream II (5′-AAGATAGTCGGATCCCATAATTAC-3′) represents a part of the 3′ coding region of *slr0089* and the first 18 bp of *slr0090*, whereas the second 418 bp PCR product amplified with the downstream primer I (5′-GCGGTGGAGAATTCAGAAAAACAG-3′) and downstream primer II (5′-ACTACACCAAGGGGTTCTGG-3′) contains the last 39 bp of *slr0090* and a part of the 5′ region of the *slr0091* gene. PCR was carried out with the proofreading Pfx polymerase (Gibco BRL, Germany).

The kanamycin resistance gene from pUC4K [10] was first subcloned into the pBluescript SK+ vector and then reisolated by digestion with *Bam*HI and *Eco*RI. It was ligated to the upstream PCR product digested with *Bam*HI and the downstream PCR product digested with *Eco*RI. The kanamycin resistance cassette was included in sense orientation as confirmed by sequencing (Licor, MWG Biotech, Germany). The DNA construct was inserted into the pCR 2.1 TOPO vector (Invitrogen, The Netherlands). The resulting vector was transformed into wild-type *Synechocystis* PCC 6803 [11]. Homologous re-

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Abbreviations: ORF, open reading frame; HPPD, 4-hydroxyphenylpyruvate dioxygenase; *hpd*, gene encoding 4-hydroxyphenylpyruvate dioxygenase

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combination led to an exchange of the *slr0090* gene by the kanamycin resistance gene. Only the first 18 and last 39 bp of *slr0090* were preserved in the mutant. Complete segregation of the mutant was obtained by restreaking cells of single colonies several times on plates containing kanamycin (50 μg/ml).

2.2. Segregation analysis

Complete segregation of the mutant was verified by PCR using the primers *synslr0089* (5′-GGCTTATTAACGGGGATAAAGCCT-3′) and *synslr0091* (5′-GCAACAACAGTTTTAGCAGTATTC-3′), indicated as primers 1 and 2, respectively (Fig. 1A). *Synslr0089* is located 30 bp upstream of the start codon of *slr0090*, whereas *synslr0091* is located 60 bp distant from the *slr0090* stop codon. Using total DNA isolated from wild-type *Synechocystis* as a template, a 1080 bp PCR product was amplified, whereas the PCR product from DNA of fully segregated mutant cells has a size of 1320 bp (Fig. 1B). Complete segregation of the mutant was also confirmed by Southern blot analysis with wild-type and mutant genomic DNA (data not shown).

2.3. Growth of Synechocystis cultures

Throughout this study *Synechocystis* sp. PCC 6803 was grown in BG-11 medium bubbled with air [12] at 28°C under illumination with constant white fluorescent light at an intensity of 50 μE m^{-2} s^{-1}. Growth of cultures initially inoculated with fresh BG-11 medium to an OD_{730} of 0.02 and grown on a shaker at 28°C and with light of 30 μE m^{-2} s^{-1} was monitored by measuring the turbidity spectrophotometrically at 730 nm wavelength (Shimadzu MPS-2000, Kyoto, Japan).

2.4. Analysis of tocopherols

For extraction of lipophilic compounds log-phase cultures of wild-type *Synechocystis* and mutant strain Δ*slr0090* were used. Extraction of tocopherols and plastoquinone was done as described [2]. Before analysis by HPLC the solvent was evaporated under a stream of nitrogen and the dried extract was redissolved in *n*-heptane. For tocopherol as well as for plastoquinone and phylloquinone analysis a volume of 20 μl extract in *n*-heptane derived from 1 mg (dry weight) of cells of the wild-type and mutant Δ*slr0090*, respectively, was subjected to the HPLC system. Chromatograms were analyzed using the Class VP software (Shimadzu, Japan).

Vitamin E derivatives were separated by HPLC using a 510 HPLC pump (Waters, Germany), a SIL-10ADVP autosampler (Shimadzu, Japan), and a LiChrospher® Si60 (5 μm) HPLC column (Merck, Darmstadt, Germany). Tocopherols were detected at an emission wavelength of 328 nm using a RF-10AXL fluorescence detector (Shimadzu, Japan) and an excitation wavelength of 290 nm. The flow rate was 1 ml/min with *n*-heptane/2-propanol (99.5:0.5; v/v) as a solvent.

α-Tocopherol was identified by comparison with external tocopherol standards (Merck, Germany).

2.5. Analysis of plastoquinone and phylloquinone

Analyses of plastoquinone and phylloquinone were performed with the same extracts as used for vitamin E analysis. The HPLC system comprised a 501 HPLC pump (Waters, Germany), a 484 absorbance detector (Waters, Germany) and an SPD-M10AvP photodiode array detector (Shimadzu, Japan). Separation was achieved on a LiChrosorb Si100 (5 μm) column (VDS-Optilab, Germany). The flow rate was also 1 ml/min with the solvent system *n*-heptane/2-propanol (99.95:0.05; v/v). Plastoquinone and phylloquinone were detected by absorbance at 254 nm. Both quinones were identified by comparison of photodiode array spectra with literature values. Additionally, plastoquinone was analyzed using a external decyl-plastoquinone (Sigma, Germany) as well as a plastoquinone-9 standard prepared from tobacco leaves as described by Lichtenthaler [13].

For calculation of the plastoquinone content, oxidized plastoquinone-9 purified from tobacco leaves was used as a standard. For spectrophotometric measurements in *n*-heptane at 254 nm wavelength, a molar extinction coefficient (E_m) of 17.94 mM^{-1} cm^{-1} was used [14].

2.6. Analysis of carotenoids and chlorophylls

Concentrations of chlorophylls were measured in 100% methanol (v/v), whereas total carotenoids were measured in 80% acetone (v/v) using the following formulae: total chlorophyll *a* (in μg/ml): $(A_{666.5}-A_{750})/0.0809$ [15] and total carotenoids (in μg/ml): $3.77\times(A_{440}-0.89\times A_{660})$ [16].

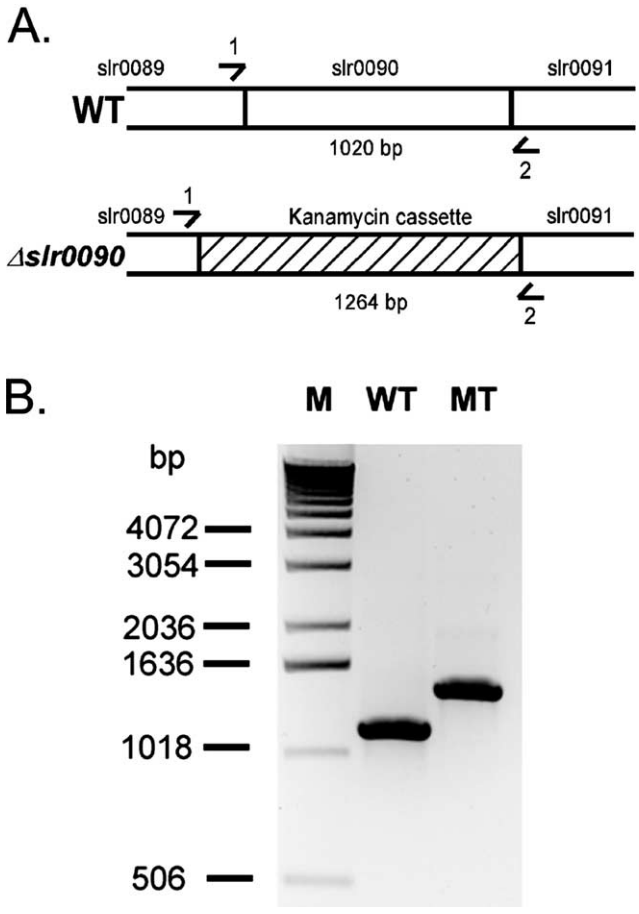

Fig. 1. PCR analysis of wild-type and Δ*slr0090* genomic DNA confirming the segregation of the mutant. A: Physical map of wild-type gene and the mutated *hpd* gene. The kanamycin resistance cassette in the Δ*slr0090* mutant is indicated by hatching. The primer pair used for PCR analysis, *synslr0089* (1) and *synslr0091* (2), bind to the neighboring genes of *slr0090*, *slr0089* and *slr0091*, respectively. B: PCR products electrophoretically separated on a 1% (w/v) agarose gel. M: Smart Ladder (Eurogentec); WT: wild-type PCR product (1080 bp); MT: Δ*slr0090* PCR product (1320 bp).

2.7. Analysis of 77 K fluorescence emission spectra

77 K fluorescence spectra were recorded using an Aminco Bowman Series 2 fluorescence spectrometer with the cuvette replaced by a dewar containing liquid nitrogen. A shortpass filter (580 nm) was inserted to eliminate interferences of higher order. Cell suspensions were set to an OD_{680} of 0.9. This corresponds to 2.5 μg chlorophyll/ml. For measurements these suspensions were filled into glass capillaries of 5 mm diameter, quickly frozen in liquid nitrogen and inserted into the dewar.

3. Results

3.1. Preparation of deletion mutant of slr0090 in Synechocystis

In the genomic database of *Synechocystis*, CyanoBase [17], the gene *slr0090* has been identified as a 4-hydroxyphenylpyruvate dioxygenase gene (*hpd*). The predicted amino acid sequence derived from this gene shows indeed highest similarity to HPPD from other organisms. It has significant identities with the HPPD enzymes from *A. thaliana* (34%), *H. vulgare* (34%), *D. carota* (33%) and *Streptomyces* (38%). The *Synechocystis slr0090* open reading frame represents the fourth gene in a putative operon comprising 10 genes.

To investigate the function of the *slr0090* gene in *Synecho-*

Table 1
Cell growth, pigment and quinone concentrations in cells of wild-type and Δ*slr0090* mutant *Synechocystis* PCC 6803 ($n = 5$)

Parameter	Cell growth, doubling time (h)	Chlorophyll (μg/mg)	Carotenoids (μg/mg)	Plastoquinones (nmol/mg)
Wild-type	23.5 ± 2.3	8.9 ± 0.89	0.48 ± 0.05	0.15 ± 0.04
Δ*slr0090*	23.8 ± 2.5	8.04 ± 0.24	0.51 ± 0.04	0.14 ± 0.06

Cultures were grown for 5 days at 28°C under illumination with constant white fluorescent light at an intensity of 50 μE m^{-2} s^{-1}. Doubling time of the cells was determined every 24 h.

cystis, we generated a deletion mutant by homologous recombination. The transformation vector contained a kanamycin resistance cassette flanked by 5′- and 3′-terminal sequences of the two neighboring genes of *slr0090*, *slr0089* and *slr0091*, respectively. Except the first 18 and the last 39 bp, the complete *slr0090* gene was deleted and replaced by the kanamycin resistance cassette (Fig. 1A).

Accomplishment of the mutation of the *slr0090* gene by homologous integration of the kanamycin cassette into the genome of the cyanobacterium was investigated by PCR (Fig. 1B) and Southern blot analysis (data not shown). While in case of the *Synechocystis* wild-type a 1080 bp fragment was obtained by PCR, with the same primers a 1320 bp fragment was amplified in case of the mutant strain Δ*slr0090* (Fig. 1B). After six times restreaking the cells on plates containing kanamycin the only PCR product amplified from Δ*slr0090* genomic DNA had the expected size of 1320 bp showing that the segregation was complete.

3.2. Analysis of pigment content and the organization of the photosynthetic apparatus

In plants HPPD catalyzes the first step in the synthesis of both plastoquinones and tocopherols. Mutation of the *hpd* gene in *Arabidopsis* leads to an albino phenotype completely lacking tocopherols, plastoquinone, chlorophyll and carotenoids [7]. In contrast to the *Arabidopsis* mutant, cultures of the *Synechocystis* mutant Δ*slr0090* are green and are able to grow photoautotrophically. Analysis of chlorophyll and carotenoid concentrations did not reveal significant differences between wild-type and Δ*slr0090* mutant (Table 1). HPLC analysis of the carotenoid composition revealed that the relative proportions of echinenone, β-carotene, zeaxanthin and myxoxantophyll are similar in *Synechocystis* wild-type and Δ*slr0090* cells (data not shown). Furthermore the doubling times of both strains were similar. To test whether wild-type and mutant strain show differences in the organization of the photosynthetic apparatus, we analyzed chlorophyll fluorescence at 77 K. It was obvious that the ratio of the PSI peak to the PSII peak is similar in cells of the wild-type (ratio 4.9) and the mutant (ratio 5.1) (Fig. 2).

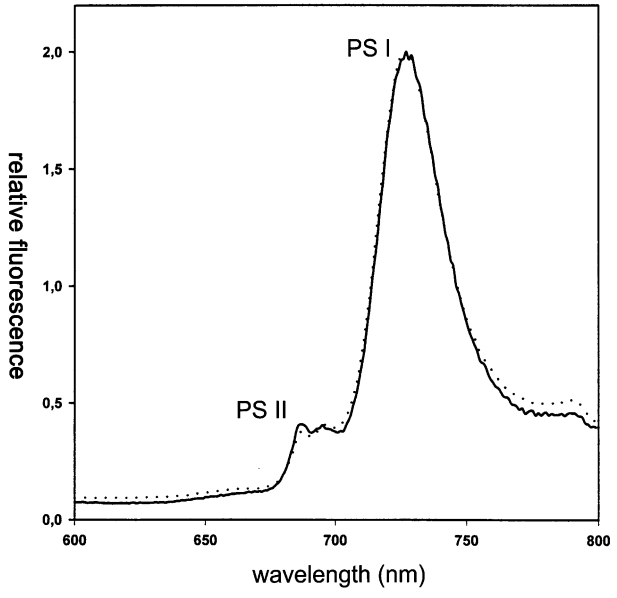

Fig. 2. 77 K fluorescence spectra of wild-type (solid line) and mutant cells (dotted line). The excitation wavelength was set at 435 nm. The corrected spectra are normalized to the PSI peak at 730 nm. The ratio of the PSI peak to the PSII peak is 4.9 in wild-type and 5.1 in mutant cells, respectively.

3.3. Analyses of tocopherols, plastoquinone and phylloquinone

The impact of the mutation on plastoquinone content was analyzed by HPLC. In contrast to the *Arabidopsis hpd* mutant, the *Synechocystis* mutant Δ*slr0090* has about the same plastoquinone content (0.14 nmol plastoquinone/mg dry weight) as the wild-type (0.15 nmol/mg dry weight) (Fig. 3, Table 1). Likewise the phylloquinone content was similar in

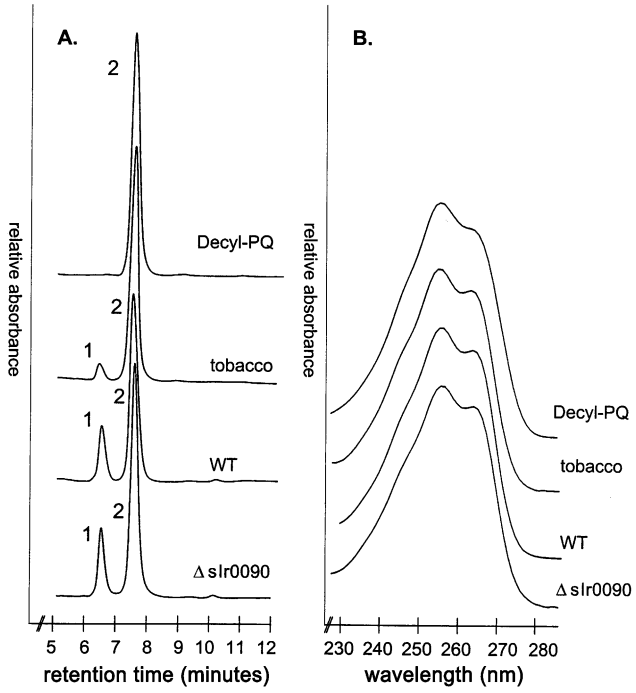

Fig. 3. HPLC analysis and absorption spectra of plastoquinone from *Synechocystis* wild-type and Δ*slr0090* mutant cell extracts. A: Isocratic HPLC separation of quinones. Plastoquinone was detected at a retention time of 7.5 min (2) in *N. tabacum* L. (plastoquinone-9) and *Synechocystis* extracts of both wild-type (WT) and Δ*slr0090* mutant extracts. Decyl-plastoquinone (decyl-PQ; Sigma) was used as a standard. The peak at a retention time of 6.4 min (1) represents phylloquinone as identified by photodiode array spectra (data not shown). B: Photodiode array spectra at a retention time of 7.5 min revealed the respective absorption maximum of plastoquinone at 255 nm wavelength.

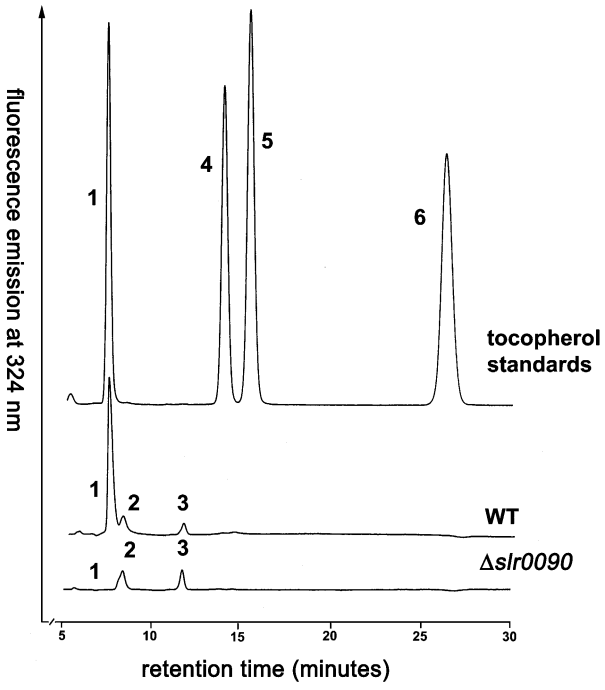

Fig. 4. HPLC diagram of tocopherol standards and vitamin E derivatives in extracts from wild-type (WT) and Δ*slr0090* cells. Peak: 1: α-tocopherol; 2 and 3: unknown compounds; 4: β-tocopherol; 5: γ-tocopherol and 6: δ-tocopherol.

cells of the wild-type and the mutant, respectively (Fig. 3A). Phylloquinone eluted from the column after 6.4 min whereas plastoquinone-9 eluted after 7.5 min in wild-type and Δ*slr0090* mutant extracts (Fig. 3A). The retention time and absorption spectra in mutant and wild-type extracts were identical to plastoquinone-9 purified from tobacco leaves and to a decylplastoquinone standard (Fig. 3B).

By HPLC analysis of vitamin E only α-tocopherol (186 ng/mg dry weight) was detectable (detection limit of HPLC system is 0.4 ng α-tocopherol). α-Tocopherol elutes from the column after 7.2 min (Fig. 4). This result is in accordance with the data reported by Collakova and DellaPenna [3].

In contrast to the wild-type, in cell extracts of Δ*slr0090* deletion mutant α-tocopherol is undetectable (Fig. 4). Wild-type as well as Δ*slr0090* mutant cell extracts contain two further compounds which do not co-elute with any known tocopherol or tocotrienol derivative.

4. Discussion

In this study we have shown that deletion of the *Synechocystis* open reading frame *slr0090* results in a complete loss of tocopherol biosynthesis without affecting the synthesis of plastoquinone. Moreover, mutant cells are not impaired in their doubling rates and in the content of photosynthesis pigments. In addition the organization of the photosynthetic apparatus does not differ between wild-type and Δ*slr0090* mutant cells as analyzed by 77 K chlorophyll fluorescence spectra. These results are in conflict with the functional characterization of the *hpd* gene mutant *pds1* of *Arabidopsis* [7]. In this case mutation of the *hpd* gene and loss of HPPD activity resulted in albino plants lacking plastoquinone as well as vitamin E. Similar results were obtained by inhibiting the HPPD activity with competitive inhibitors of the enzyme [8,9]. Treatment of *Synechocystis* wild-type cells with the same competitive inhibitors of the HPPD enzyme had no effect on plastoquinone, whereas tocopherol biosynthesis was impaired (data not shown). These results are in accordance with the phenotype of the *Synechocystis hpd* deletion mutant indicating that in contrast to higher plants plastoquinone synthesis in *Synechocystis* is not strictly dependent on the activity of HPPD. Based on these results an alternative or additional pathway for plastoquinone synthesis in *Synechocystis* has to be claimed.

A molecule with a very related chemical structure and function to plastoquinone is ubiquinone which is well known as a mobile electron transfer cofactor in the mitochondrial electron transfer chain in eukaryotes. *Synechocystis* does not contain ubiquinone, but uses plastoquinone both for respiration and for photosynthesis. Nevertheless, the genes specific for enzymes of the ubiquinone pathway are still present. These include *slr0926* for a UbiA prenyltransferase that uses hydroxybenzoic acid as a head group for prenylation, *slr1099* for a UbiX carboxylase which removes the carboxyl group from the UbiA product and *slr1300* putatively encoding a UbiH-like protein which could easily transfer another hydroxyl group to the aromatic ring in *para* position [17]. To synthesize plastoquinone two further methylations are necessary. One of these methyltransferases could be encoded by the gene *sll0418*. Further mutations have to be done in order to investigate whether these genes are involved in a alternative or additional pathway for plastoquinone synthesis in *Synechocystis*.

While in *Synechocystis* the deletion of the *hpd* gene has no impact on the contents of pigments, photosynthesis and growth, tocopherol biosynthesis is completely impaired. Furthermore, no differences between wild-type and *hpd* mutant were detected in cultures grown at high light intensity (500 μE m^{-2} s^{-1}) instead of low light intensity (50 μE m^{-2} s^{-1}) (data not shown). A light intensity 500 of μE m^{-2} s^{-1} is indeed sufficient to impose a light stress on cells of *Synechocystis* [18]. This result is surprising considering that tocopherols in higher plants and most other photosynthetic organisms may have an important function as lipophilic antioxidants similar as vitamin E in human diet [19,20]. By scavenging membrane-associated free radicals tocopherols are supposed to play a pivotal role for photosynthesis [21]. Differences in the photochemical reactions between the wild-type and a mutant of *Scenedesmus obliquus* deficient in vitamin E support this view [22]. Nevertheless, some cyanobacteria, e.g. *Synechococcus*, are capable of performing photosynthesis without tocopherol biosynthesis [23,24]. It is likely that in cyanobacteria other compounds may serve as substitutes for tocopherols.

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