Acta Physiol Plant (2016) 38:49
DOI 10.1007/s11738-015-2043-0

ORIGINAL ARTICLE

Cyanobacteria use both *p*-hydroxybenozate and homogentisate as a precursor of plastoquinone head group

Beatrycze Nowicka[1] · **Jerzy Kruk**[1]

Received: 26 May 2015 / Revised: 16 November 2015 / Accepted: 7 December 2015 / Published online: 29 January 2016

Abstract Until recently it was believed that cyanobacterial pathway of plastoquinone biosynthesis is analogical to that of higher plants. In plants, homogentisate is a precursor of the hydrophilic head group of plastoquinone. Recent experiments on *Synechocystis* sp. PCC 6803 have shown that this organism takes advantage of another pathway that resembles ubiquinone biosynthetic pathway of α-, β- and γ-proteobacteria. In the present work, we have analysed the content of plastoquinone, tocopherol and tocopherolquinone in six strains of cyanobacteria and compared the obtained results with search for genes of homologues of enzymes participating in tocopherol and ubiquinone biosynthesis. We have shown that inhibition of homogentisate synthesis lowers tocopherol content but does not affect plastoquinone synthesis in *Synechococcus* sp. PCC 7002. Inhibitors of *p*-hydroxybenzoate and homogentisate prenyltransferases selectively influenced plastoquinone and tocopherol biosynthesis in *Synechocystis* sp. PCC 6803. Radiolabelled ^{14}C-*p*-hydroxybenzoate was incorporated into plastoquinone by three cyanobacteria species investigated. Although, when ^{14}C-homogentisate was added to growth medium, the labelled plastoquinone was found in extracts of the cyanobacteria. *Synechocystis* sp. PCC 6803 grown in the presence of ^{14}C-homogentisate showed also small amounts of the labelled tyrosine, suggesting that cyanobacteria are able to incorporate exogenously added homogentisate into shikimate pathway.

Communicated by E. Schleiff.

✉ Beatrycze Nowicka
beatrycze.nowicka@uj.edu.pl; beatrycze.nowicka@wp.pl

[1] Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

Keywords Cyanobacteria · Homogentisate · *p*-Hydroxybenzoate · Plastoquinone · Plastoquinone biosynthesis

Abbreviations

HBA	*p*-Hydroxybenzoate
HGA	Homogentisate
HPP	4-Hydroxyphenylpyruvate
HPPD	4-Hydroxyphenylpyruvate dioxygenase
HPT	Homogentisate prenyltransferase
HBA PRT	*p*-Hydroxybenzoate prenyltransferase
ML	Medium light
PQ	Plastoquinone
PQH_2	Plastoquinol
TC	Tocopherol cyclase
γ-TMT	γ-Tocopherol methyltransferase
Toc	Tocopherol
α-Toc	α-Tocopherol
γ-Toc	γ-Tocopherol
α-TQ	α-Tocopherolquinone
UQ	Ubiquinone

Introduction

Isoprenoid quinones and chromanols are important groups of amphipathic compounds, both containing isoprenoid side-chain and sharing some common steps in their biosynthetic pathways. Among isoprenoid quinones, plastoquinone (PQ), present in all cyanobacteria, participates both in photosynthetic and respiratory electron transport chains (Nowicka and Kruk 2010). In most species of cyanobacteria, PQ is also a cofactor of phytoene

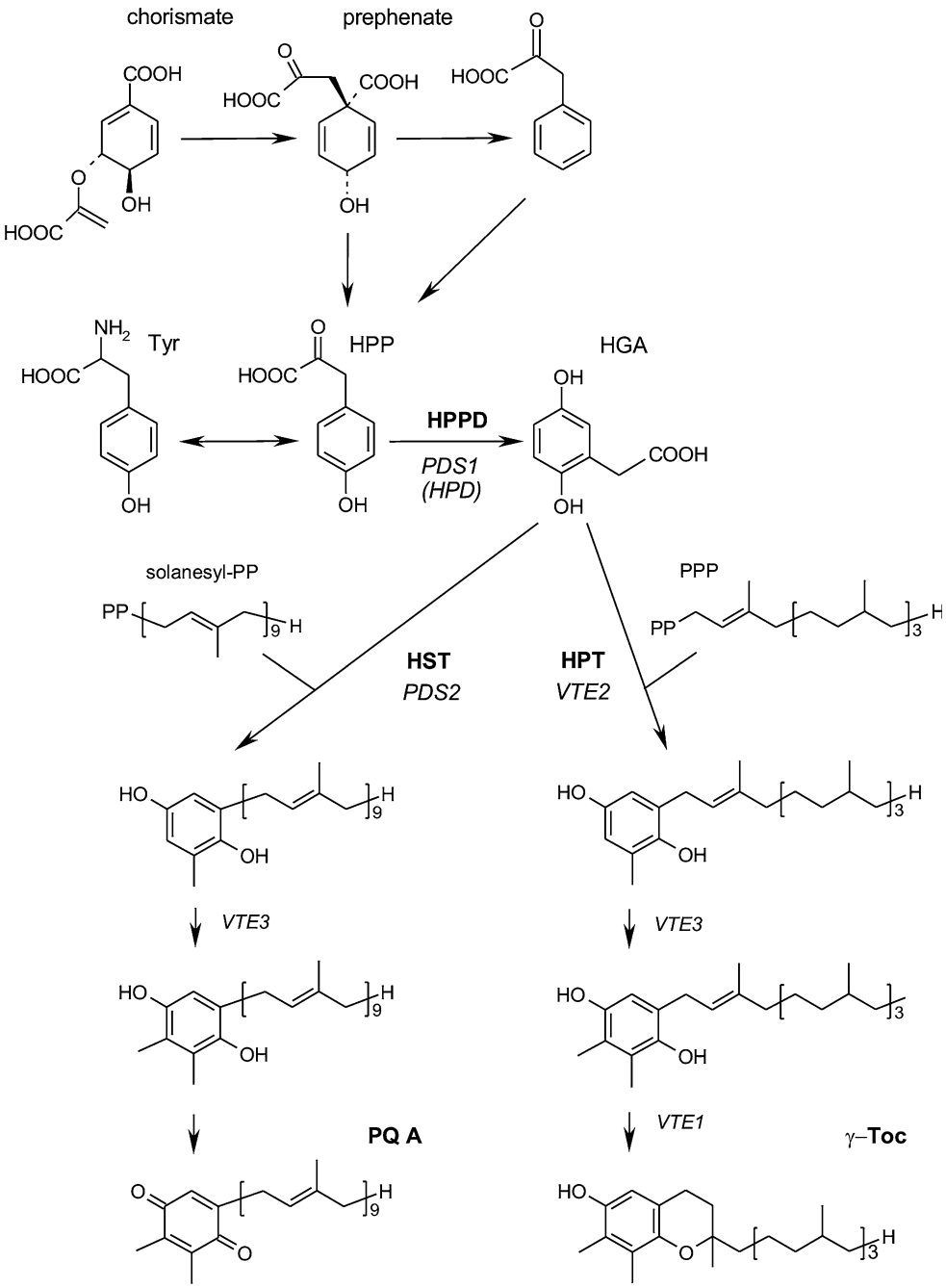

Fig. 1 Plastoquinone and tocopherol biosynthesis in higher plants. *HGA* homogentisate, *HPP* *p*-hydroxyphenylpyruvate, *HPPD* *p*-hydroxyphenylpyruvate dioxygenase, *HPT* homogentisate phytyltransferase, *HST* homogentisate solanesyl transferase, *PPP* phytyl diphosphate, *PQ A* plastoquinone A, *γ-Toc* γ-tocopherol, *Tyr* tyrosine. Names of the genes are in *italics*: *PDS1* (*HPD*) gene of *p*-hydroxyphenylpyruvate dioxygenase, *PDS2* gene of homogentisate solanesyltransferase, *VTE1* gene of tocopherol cyclase, *VTE2* gene of homogentisate phytyltransferase, *VTE3* gene of 2-methyl-6-phytyl benzoquinol methyltransferase

desaturase. This compound also shows antioxidant properties, especially in the reduced form (Nowicka and Kruk 2010). α-Tocopherol (α-Toc), a well-known antioxidant, occurs in majority of cyanobacteria, although there are species lacking it, e.g., *Synechococcus elongatus* sp. PCC 7942 (Munne-Bosch and Alegre 2002). Among oxidation products of α-Toc, α-tocopherolquinone (α-TQ) can be found in minor amounts in some species of cyanobacteria (Carr and Hallaway 1965).

In higher plants, biosynthetic pathways of PQ and tocopherols (Tocs) are well known. Both pathways share first steps, as both use homogentisate (HGA) as a precursor of hydrophilic head groups of PQ and Toc (Fig. 1) (Mène-Saffrané and DellaPenna 2010). Experiments performed on *Synechocystis* sp. PCC 6803 showed that biosynthetic pathway of α-Toc in cyanobacteria is analogical to that of higher plants (DellaPenna 2005). What is more, synthesis of phylloquinone, a cofactor of photosystem I, is very similar in cyanobacteria and plants (Gross et al. 2006). In experiments carried out by Whistance and Threlfall, where radiolabelled HGA was added to the growth medium of *Synechococcus elongatus*, the presence of labelled PQ in

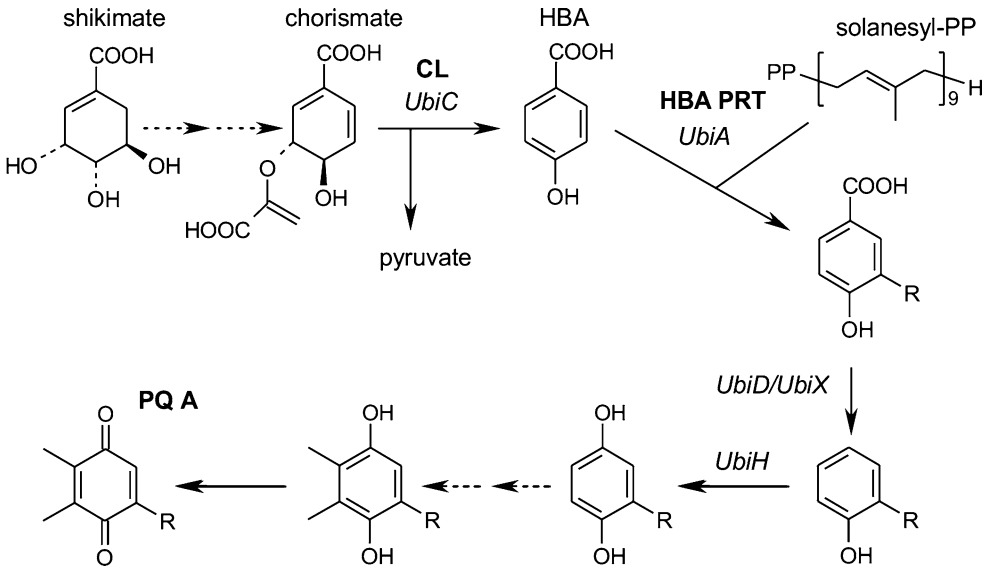

Fig. 2 Plastoquinone biosynthetic pathway in cyanobacteria proposed by (Sakuragi and Bryant 2006). *CL* chorismate lyase, *HBA* *p*-hydroxybenzoate, *HBA PRT* *p*-hydroxybenzoate prenyltransferase, *PQ A* plastoquinone A, *R* solanesyl side-chain. Names of the genes are in *italics*: *UbiA* gene of *p*-hydroxybenzoate prenyltransferase, *UbiC* gene of chorismate lyase, *UbiD* gene of 3-octaprenyl-4-hydroxybenzoate decarboxylase, *UbiH* gene of 2-octaprenyl-6-methoxyphenol hydroxylase, *UbiX* gene of 3-octaprenyl-4-hydroxybenzoate carboxy-lyase

extracts from cyanobacteria was observed (Whistance and Threlfall 1970). Therefore, it was assumed that cyanobacteria synthesize PQ via the same pathway as plants.

First data challenging this hypothesis came from experiments on mutants lacking 4-hydroxyphenylpyruvate dioxygenase (HPPD). *Arabidopsis thaliana pds1* mutant is deficient both in PQ and Tocs, therefore, it is lethal at early stage of seedling development (Norris et al. 1995). The HPPD mutant of *Synechocystis* sp. PCC 6803 lacks only α-Toc, while its PQ level remains unchanged as compared to the wild type; cyanobacterium *Synechococcus elongatus* lacks α-Toc but synthesizes PQ (Henninger et al. 1965; Dasilva and Jensen 1971; Dahnhardt et al. 2002). On the basis of sequence analysis, which has shown that homologues of genes encoding five enzymes participating in ubiquinone-8 (UQ-8) biosynthesis in *E. coli* (*UbiA, UbiD, UbiE, UbiH, UbiX*) are present in 14 cyanobacterial genomes, Sakuragi and Bryant proposed that PQ biosynthetic pathway in cyanobacteria resembles that of UQ in α-, β- and γ-proteobacteria (Fig. 2) (Sakuragi and Bryant 2006).

For elucidation of the PQ biosynthetic pathway in cyanobacteria, experiments on deletion mutants were performed. *Synechocystis* sp. PCC 6803 mutants lacking *UbiA* homologue were not viable (Schultze et al. 2009). As PQ is the only photosynthetic and respiratory quinone electron carrier in cyanobacteria, this compound is indispensable for metabolism of the cell. To overcome this obstacle, Sadre et al. (2012) used a system with heterologous expression of examined cyanobacterial genes in *E. coli* mutant strains lacking ubiquinone. As *E. coli* takes advantage of both UQ-8 and menaquinone-8 in its respiratory electron transfer chain, mutants lacking UQ are able to grow. Expression of cyanobacterial homologue of proteobacterial *p*-hydroxybenzoate prenyltransferase (HBA PRT), encoded by *Slr0926*, in *E. coli* mutants lacking *UbiA*, restored UQ-8 biosynthesis. Moreover, it was shown that ^{14}C-labelled HBA was used for PQ biosynthesis in *Synechocystis* sp. PCC 6803 (Sadre et al. 2012). Sadre et al. also examined properties of *Slr0926* product isolated from transgenic *E. coli* and proved that the transgenic protein indeed was HBA prenyltransferase (Sadre et al. 2012). Using an analogical approach, the same group confirmed that cyanobacterial homologues of *UbiC*, *UbiD* and *UbiX*, encoding chorismate lyase and two 4-hydroxy-3-solanesylbenzoate decarboxylases, respectively, participate in PQ biosynthesis (Pfaff et al. 2014).

The aim of the present experiments was to broaden our knowledge concerning PQ biosynthesis in cyanobacteria and to elucidate discrepancies between results of early and recent research on this topic. As in early research carried out by Whistance and Threlfall (Whistance and Threlfall 1970), radiolabelled HGA was incorporated into PQ, whereas in latest research (Sadre et al. 2012; Pfaff et al. 2014), the pathway analogical to UQ biosynthetic pathway present in proteobacteria, in which *p*-hydroxybenzoate (HBA) serves as a precursor of PQ head group was proved to occur in *Synechocystis* sp. PCC 6803, there is a need to explain these differences. The other interesting question is

if the pathway of PQ biosynthesis found in model cyanobacterium *Synechocystis* sp. PCC 6803 is common in other cyanobacterial species.

The obtained results indicate that the pathway of PQ biosynthesis proposed by Sakuragi and Bryant occurs also in cyanobacteria species other than *Synechocystis* sp. PCC 6803. We also provide data concerning PQ, α-Toc and α-TQ content in selected strains of cyanobacteria. The results of experiments with inhibitors of α-Toc and UQ biosynthesis also confirm the UQ-like pathway of PQ biosynthesis of cyanobacteria. Finally, we show that exogenously applied ^{14}C-HGA indeed can be used for PQ biosynthesis, besides HBA. This effect is probably a result of incorporation of HGA into shikimate pathway.

Materials and methods

Sequence analysis

Sequence analysis was performed using BLAST ver. 2.2.25+ (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) with default settings for protein blast (Altschul et al. 1997, 2005). The search was performed for homologues of ten enzymes participating in α-Toc or UQ biosynthesis in genomes of *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* (PCC 7942 and PCC 6301), *Synechococcus* sp. PCC 7002 and *Thermosynechococcus elongatus* BP-1. The following sequences were used as queries: NP_442493.1 (HPPD from *Synechocystis* sp. PCC 6803), BAA17774.1 (homogentisate phytyltransferase from *Synechocystis* sp. PCC 6803), BAK50662.1 (2-methyl-6-phytyl-1,4-benzoquinol methyltransferase from *Synechocystis* sp. PCC 6803), BAA17775.1 (tocopherol cyclase from *Synechocystis* sp. PCC 6803), BAK51348.1 (γ-tocopherol methyltransferase from *Synechocystis* sp. PCC 6803), BAE78042.1 (HBA PRT from *E. coli*), NP_418285.1 (3-octaprenyl-4-hydroxybenzoate decarboxylase, UbiD from *E. coli*), NP_288885.1 (3-octaprenyl-4-hydroxybenzoate carboxy-lyase, UbiX from *E. coli*), YP_491108.1 (2-octaprenyl-6-methoxyphenol hydroxylase, UbiH from *E. coli*), YP_491609.1 (bifunctional 2-octaprenyl-6-methoxy-1,4-benzoquinone methylase/*S*-adenosylmethionine:2-DMK methyltransferase, UbiE from *E. coli*).

Growth conditions

Cyanobacteria were grown for 6 weeks in sterile liquid media. *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* (PCC 7942 and PCC 6301), *Thermosynechococcus elongatus* BP-1 and *Phormidium laminosum* were grown in BG-11 medium, while *Synechococcus* sp. PCC 7002 was grown in medium A for marine cyanobacteria (Stevens et al. 1973). *Thermosynechococcus elongatus* BP-1 and *Phormidium laminosum* were grown in a shaker at 45 °C, in low light (10 μmol photons/m^{2}s). Other strains were grown at 28 °C, 2–5 μmol photons/m^{2}s.

Prenyllipid extraction and HPLC analysis

Every sample was obtained from 50 ml of the culture. Cultures were centrifuged (10 min × 5500*g*, 4 °C) and lyophilized overnight. The obtained lyophilisates (usually 5–15 mg) were extracted with methanol overnight in a shaker at 4 °C, then re-extracted three times using methanol. Pooled supernatants were evaporated and resuspended in methanol. In the case of α-TQ measurements, for 10 mg of lyophilisate used for the extraction, 300 μl of final extract was obtained. For α-Toc and PQ measurements, final extract was diluted five times. PQ and α-Toc were analysed in following RP-HPLC system: C_{18} column (Nucleosil 100, 250 × 4 mm, 5 μm, Teknokroma, Spain), methanol:hexane (340:20, v/v), flow rate 1.5 ml/min, absorbance detection at 255 nm, fluorescence detection at $\lambda_{ex} = 290$ nm, $\lambda_{em} = 330$ nm. For α-TQH$_2$ determination, acetonitrile:methanol:water (72:8:1, v/v) was used. For α-TQ determination C_{18} column with platinum post column was used, developed in methanol:water (95:5, v/v), at the flow of 1 ml/min and fluorescence detection as described before (Nowicka and Kruk 2012).

Experiments with inhibitors

In the experiment concerning response of *Synechococcus* sp. PCC 7002 to high light, 6-week-old culture was exposed to medium light (ML, 70 μmol photons/m^{2}s) for 120 h. Samples were taken at $t = 0$, 8, 24, 48, 120 h, extracted and analysed as described before. For α-Toc measurements, methanol:water (99:1, v/v) and flow rate 1 ml/min were used.

In the experiment with pyrazolate, *Synechococcus* sp. PCC 7002 was grown for 5 weeks as described previously with or without 10 μM pyrazolate [4-(2,4-dichlorobenzoyl)-1,3-dimethylpyrazol-5-yl toluene-4-sulfonate, Wako Pure Chemicals, Japan]. After 5 weeks, second portion of pyrazolate (10 μl of 50 mM pyrazolate solution for 50 ml of the culture) was added. Then, cultures were exposed to ML. Samples were taken before and after 120 h of ML exposure, extracted and analysed using HPLC.

In experiment with inhibitors of HGA- and HBA-prenyltransferases, haloxydine (3,5-dichloro-2,6-difluoropyridin-4-ol, Wuhan Yitongtai Biological Co. Ltd., China) and 4-nitrobenzoate (Sigma-Aldrich), respectively, were used. *Synechocystis* sp. PCC 6803 was grown in BG-11 medium. Concentration of the inhibitors was 175 μM. For all cultures, the same volume of stock culture was used

as inoculum. After 4 weeks of growth, cyanobacteria were centrifuged (10 min × 5500*g*, 4 °C). Next, the cells were resuspended in 0.5 ml of distilled water and sonicated three times for 13 min, 60 % duty cycle, 35 % of maximal power (Vibra cell, Sonics and Materials Inc.). Between sonication cycles, cultures were frozen in liquid nitrogen and thawed. After sonication, 1.5 ml of chloroform:methanol mixture (2:1, v/v) was added to each sample. Samples were then extracted for 2 h on a vortex in 4 °C and centrifuged (10 min × 9000*g*). Chloroform phases were pooled, evaporated, dissolved in 200 µl of methanol and analysed with HPLC system.

Cultures with radiolabelled precursors

Radiolabelled precursors, i.e., ^{14}C-HBA and ^{14}C-HGA, were synthesized from ^{14}C-Tyr (Hartmann Analytic, Germany, 250 µCi in 2.5 ml, 485 mCi/mmol) as described in Threllfall and Whistance (1971) and Ashby and Edwards (1990). For experiments with labelled precursors, *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002 were used. In the case of *Synechococcus* sp. PCC 7002, sterilized solutions of radiolabelled HGA and HBA, mixed with the respective unlabelled compounds, were added to 6-week-old cultures. Then, the cultures were exposed to ML for 120 h. Samples (25 ml of each culture) were taken after 48 and 120 h. The whole experiment was repeated and samples were taken after 5, 10, and 24 h. In the case of *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, radiolabelled compounds were added to 12-day-old cultures. Samples (50 ml of each culture) were taken after 1 and 3 weeks of growth in the presence of labelled compounds. For incorporation experiments, 2.71 ml of the ^{14}C-HGA stock solution (0.92 mM, ≈5 µCi/ml) or the corresponding amount of ^{14}C-HBA was added to 50 ml of cell culture (final precursor conc. 50 µM).

Samples were centrifuged (15 min × 5500*g*, 4 °C), the pellet was suspended in 600 µl of distilled water, sonicated (2 × 15 min at 70 % duty cycle, Sonicator 4710, Cole Parmer). Between sonication cycles, samples were frozen in liquid nitrogen and thawed. For *Synechococcus elongatus* PCC 7942, one sonication for 6 min was used. Next, samples were extracted with chloroform:methanol (2:1, v/v) for 2 h on a vortex in 4 °C. Extracts were centrifuged (10 min × 9000*g*), then chloroform phases were collected, evaporated, dissolved in 100 µl of chloroform and spotted on TLC plate with silica gel (20 × 20 cm, thickness 0.2 mm, DC-Alufolien, Kieselgel F_{254}, Merck). The plates were developed in chloroform stabilized with amylene (Chempur, Poland), exposed to Kodak phosphor screen for 72 h and scanned with phosphorimager (BioRad Molecular Imager FX). Cultivation with labelled precursors, extraction and TLC separation was repeated twice for each strain.

Alternatively, 150 µl of ^{14}C-HGA solution was added to 100 ml of 7-week-old culture of *Synechocystis* sp. PCC 6803. Fifty ml of the culture was collected after 17 and 25 h, and extracted as described in the experiment with haloxydine and 4-nitrobenzoate. Hydrophilic phases were collected and concentrated by evaporation. Then, samples were spotted on TLC plate with silica gel. The plates were developed in toluene:acetic acid:water (25:25:1, v/v), then exposed to Kodak phosphor screen for a week and scanned with phosphorimager.

For an experiment with ^{14}C-Tyr, the labelled precursor was added to 5-week-old cultures of *Synechococcus elongatus* PCC 6301 and PCC 7942. Samples (75 ml of each culture) were taken after 72 h and extracted as described above. Chloroform phases of the obtained extracts were evaporated, resuspended in a small volume of chloroform and separated using TLC as described above, with chloroform as a mobile phase.

Results

Comparison of genes encoding enzymes of plant α-Toc/PQ biosynthetic pathway and UQ-like pathway with prenyllipid content in selected species of cyanobacteria

As *Synechococcus elongatus* (formerly known as *Anacystis nidulans*) was used in early experiments by Whistance and Threlfall (1970), whereas *Synechocystis* sp. PCC 6803 was used for experiments concerning α-Toc and PQ biosynthesis more recently (Dahnhardt et al. 2002; Sadre et al. 2012; Pfaff et al. 2014), these species were used also in our experiments. Two other species chosen were: *Thermosynechococcus elongatus*, in which there are no close homologues of genes encoding enzymes of plant α-Toc and PQ biosynthetic pathway and *Synechococcus* sp. PCC 7002, in which these homologues are present.

Analysis of prenyllipids and bioinformatic analysis of genes encoding enzymes required for PQ and UQ biosynthesis in selected strains of cyanobacteria were performed. The obtained results enabled to correlate the presence of homologues of enzymes engaged in prenyllipid biosynthetic pathways with the occurrence of the prenyllipids analysed.

The results of sequence analysis are shown in Table 1, whereas prenyllipid content is presented in Table 2. It is evident that strains lacking homologues of HPPD, homogentisate phytyltransferase and tocopherol cyclase, are deficient also in α-Toc and α-TQ. On the other hand, the genes required for the postulated PQ biosynthetic pathway in cyanobacteria are found in all strains. Unfortunately, for *Phormidium laminosum*, only sequences of

Table 1 Presence of genes for homologues of enzymes participating in α-tocopherol biosynthesis and selected enzymes of ubiquinone biosynthetic pathway of *E. coli* in the examined cyanobacterial strains

Species/strain	Enzymes of α-Toc biosynthetic pathway				
	slr0090 (HPPD)	slr1736 (HPT)	sll0418 (MPBQ MT)	slr1737 (TC)	slr0089 (γ-TMT)
Synechococcus elongatus PCC 6301	–	–	BAD79117.1	–	BAD79117.1
Identities/positives			74/84 %		36/52 %
Synechococcus elongatus PCC 7942	–	–	ABB56628.1	–	ABB56628.1
Identities/positives			74/84 %		36/52 %
Synechococcus sp. PCC 7002	WP_012308304.1	WP_041443778.1	WP_012308332.1	WP_012306299.1	WP_012307402.1
Identities/positives	33/49 %	59/72 %	78/88 %	47/63 %	57/73 %
Synechocystis sp. PCC 6803 Genes used as a query	WP_010873791.1	WP_010872404.1	WP_010873108.1	WP_010872405.1	WP_014407146.1
Thermosynechococcus elongatus BP-1	–	–	NP_682516.1	–	NP_682516.1
Identities/positives			76/88 %		37/51 %
Species/strain	Enzymes of UQ biosynthetic pathway in *E. coli*				
	HBA PRT (UbiA)	UbiD	UbiE	UbiH	UbiX
Synechococcus elongatus PCC 6301	BAD80321.1	BAD79712.1	BAD80228.1	BAD80228.1	BAD80375.1
Identities/positives	32/53 %	48/64 %	34/53 %	31/50 %	42/58 %
Synechococcus elongatus PCC 7942	ABB57994.1	ABB58618.1	ABB58085.1	ABB58085.1	ABB57940.1
Identities/positives	33/53 %	48/64 %	34/53 %	31/50 %	42/58 %
Synechococcus sp. PCC 7002	WP_012307014.1	WP_012306736.1	WP_012307559.1	WP_012307559.1	WP_012307025.1
Identities/positives	36/57 %	46/63 %	32/53 %	33/52 %	39/57 %
Synechocystis sp. PCC 6803	WP_010874074.1	WP_010871507.1	WP_010871480.1	WP_010871480.1	WP_010871388.1
Identities/positives	34/54 %	45/62 %	31/51 %	32/47 %	42/59 %
Thermosynechococcus elongatus BP-1	NP_681192.1	NP_683015.1	NP_683079.1	NP_683079.1	NP_681822.1
Identities/positives	34/56 %	45/62 %	40/57 %	32/46 %	43/60 %

HBA PRT 4-hydroxybenzoate prenyltransferase, *HPPD* 4-hydroxyphenylpyruvate dioxygenase, *HPT* homogentisate phytyltransferase, *MPBQ MT* 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase, *TC* tocopherol cyclase, *γ-TMT* γ-tocopherol methyltransferase, *α-Toc* α-tocopherol, *UbiD* 3-octaprenyl-4-hydroxybenzoate decarboxylase, *UbiE* bifunctional 2-octaprenyl-6-methoxy-1,4-benzoquinone methylase/*S*-adenosylmethionine:2-DMK methyltransferase, *UbiH* 2-octaprenyl-6-methoxyphenol hydroxylase, *UbiX* 3-octaprenyl-4-hydroxybenzoate carboxy-lyase, *UQ* ubiquinone

Table 2 Composition of isoprenoid lipids in the examined cyanobacterial strains

Species/strain	PQ_{tot} (μmol/g DW)	α-Toc (μmol/g DW)	α-TQ (nmol/g DW)
Phormidium laminosum	0.65 ± 0.05	0.369 ± 0.023	3.9 ± 0.3
Synechococcus elongatus			
PCC 6301	0.61 ± 0.08	–	–
PCC 7942	0.59 ± 0.03	–	–
Synechococcus sp. PCC 7002	0.57 ± 0.05	0.012 ± 0.001	–[a]
Synechocystis sp. PCC 6803	0.70 ± 0.05	0.198 ± 0.004	12.1 ± 3.4
Thermosynechococcus elongatus BP-1	3.63 ± 0.56	–	–

The procedure used, resulted in oxidation of quinols to quinones, so only total content of both of these forms was determined

DW dry weight, PQ_{tot} total plastoquinone, *α-Toc* α-tocopherol, *α-TQ* α-tocopherolquinone

The data are mean ± SE, $n = 3$–10

[a] Results of other experiments have shown that in high-light stressed cyanobacteria, trace amounts of α-TQ were observed

selected genes are known presently. Although, the prenyllipid content of that cyanobacterium was included because it is interesting as *P. laminosum*, similar to *T. elongatus,* is a thermophilic species (see "Discussion" section).

Interestingly, PQ content was similar in all the strains, except for *T. elongatus* BP-1, where it was about six times higher (Table 2). α-Toc and α-TQ were present only in some species. The level of α-Toc varied among the investigated species. The highest content of α-Toc was found in thermophilic *P. laminosum* (Table 2). In *Synechococcus* sp. PCC 7002, high variations in the content of α-Toc were observed. In most of the cultures (9), its level was low, but there were some cultures (3) where only trace amounts of α-Toc were detected, whereas in three other cultures, high level of α-Toc was found (up to 0.463 μmol/g DW). Very low content of α-TQ was observed only in species having α-Toc (Table 2).

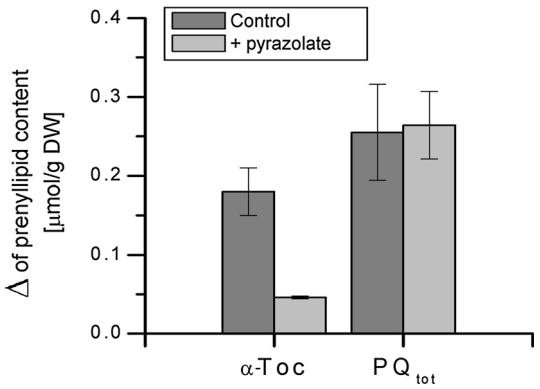

Fig. 3 α-Tocopherol and plastoquinone content in *Synechococcus* sp. PCC 7002: control and culture with pyrazolate, during 120 h of exposure to medium light (70 μmols photons/m^2s, ML) expressed as a difference between prenyllipid content after and before ML treatment. α-Toc, α-tocopherol; PQ_{tot}, total plastoquinone. The data are mean ± SE

Experiments with selective inhibitors of enzymes participating in plant α-Toc/PQ biosynthetic pathway and UQ biosynthetic pathway in proteobacteria

Pyrazolate is an inhibitor of HPPD, a key enzyme participating in α-Toc and PQ biosynthesis in plants. Preliminary experiments, where inhibitor was added during inoculation of the cultures, which were then grown for 4 weeks in low light, showed that there were no differences in prenyllipid content as compared to the control culture (data not shown). Either inhibitor was degraded or, as inhibition was only partial, when growth was slow, α-Toc accumulated over time. However, in other preliminary experiments, we have discovered that *Synechococcus* sp. PCC 7002 was able to acclimate to light of medium intensity (ML, 70 μmol photons/m^2s), which was accompanied by an increase in α-Toc level during 120 h of ML treatment (data not shown). During such fast response, the effect of pyrazolate should be visible. Therefore, the impact of pyrazolate on ML-induced α-Toc increase in *Synechococcus* sp. PCC 7002 was investigated. The inhibitor was added to 5-week-old culture of *Synechococcus* sp. PCC 7002 and then the culture was exposed to ML. The increase in α-Toc content in response to ML treatment was partially inhibited in the presence of pyrazolate, whereas there was no difference in the case of the change of PQ content when compared to the control (Fig. 3).

Impact of inhibitors of prenyltransferases participating in quinone biosynthesis on the content of α-Toc and PQ was also examined in model cyanobacterium *Synechocystis*

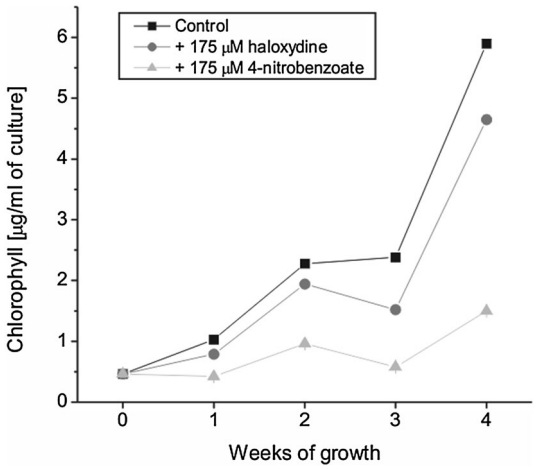

Fig. 4 Chlorophyll a content in cultures of *Synechocystis* sp. PCC 6803 grown on the medium with inhibitors of plant α-Toc/PQ biosynthetic pathway (haloxydine) and UQ biosynthesis (4-nitrobenzoate)

sp. PCC 6803. Haloxydine is an inhibitor of HGA prenyltransferases, participating in PQ and α-Toc biosynthesis in higher plants, whereas 4-nitrobenzoate is an inhibitor of HBA prenyltransferase engaged in UQ biosynthesis (Sadre et al. 2010; Quinzii et al. 2012). Addition of 4-nitrobenzoate significantly slowed down the culture growth, monitored as chlorophyll content in the liquid culture (Fig. 4). In cultures with haloxydine, content of α-Toc was lower than in the control, whereas PQ level remained unchanged. In the culture with 4-nitrobenzoate, PQ content was lower than in the control, whereas α-Toc level increased (Table 3).

Table 3 The effect of inhibitors of plant α-Toc/PQ biosynthetic pathway (haloxydine) and UQ biosynthesis (4-nitrobenzoate) on α-tocopherol and plastoquinone content in *Synechocystis* sp. PCC 6803 cultures

	Prenyllipid content in 4-week-old cultures (mol/100 mols of Chl)		
	Control	+175 μM haloxydine	+175 μM 4-nitrobenzoate
PQ_{tot}	1.77 ± 0.03	1.90 ± 0.11	0.25 ± 0.03
α-Toc	0.27 ± 0.02	0	0.61 ± 0.01

The data are mean ± SE

PQ_{tot} total plastoquinone, *α-Toc* α-tocopherol

Experiments with radiolabelled precursors known to be the intermediates of PQ biosynthesis in plants and UQ biosynthesis in proteobacteria

Addition of radiolabelled precursors to the cultures resulted in incorporation of both ^{14}C-HBA and ^{14}C-HGA predominantly into PQ (Figs. 5, 6). In 6-week-old culture of *Synechococcus* sp. PCC 7002 exposed to ML for 120 h after addition of precursors, detectable amounts of labelled PQ were found after 24 h. There was also another product with high R_f for all samples observed, except for those with

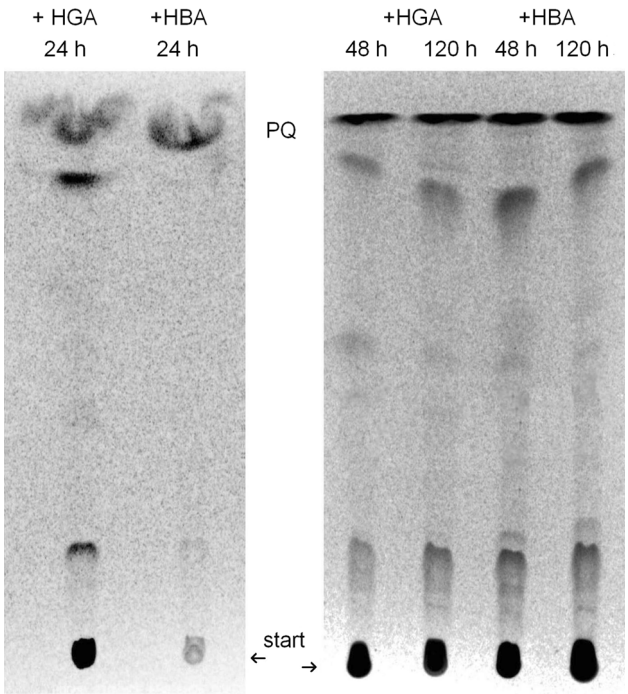

Fig. 5 Autoradiogram of TLC plates of extracts from *Synechococcus* sp. PCC 7002 exposed to ML. In the first experiment, samples were collected after 48 and 120 h. In the second experiment, samples were collected after 24 h. In the third, after 5 and 10 h, although labelled compounds were present only in trace, barely detectable amounts (data not shown). TLC plates were developed in chloroform. In this mobile phase, HGA and HBA do not move from the start point. *HBA* *p*-hydroxybenzoate, *HGA* homogentisate, *PQ* plastoquinone

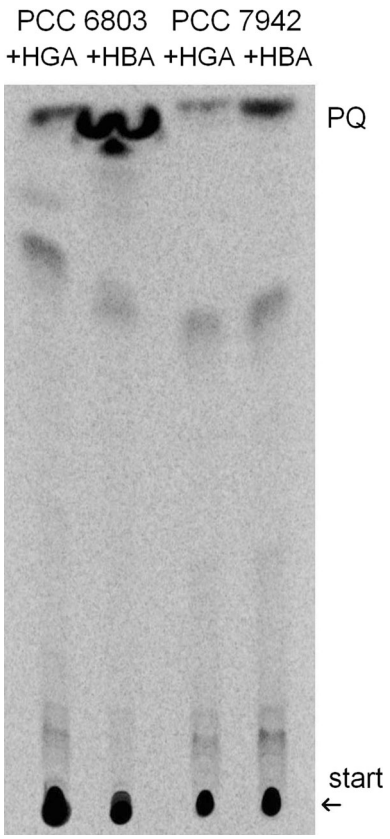

Fig. 6 Autoradiogram of TLC plate of extracts from *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 grown in low light for 1 week after addition of the labelled precursors to 12-day-old culture. TLC plates were developed in chloroform. *HBA* *p*-hydroxybenzoate, *HGA* homogentisate, *PQ* plastoquinone

^{14}C-HBA collected after 24 h. In the case of *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, ^{14}C-HBA seemed to be incorporated more efficiently than ^{14}C-HGA and 3 weeks of growth with precursors turned out to be too long; the labelled compounds were degraded (data not shown).

Analysis of hydrophilic compounds in the extract of *Synechocystis* sp. PCC 6803, 17 and 25 h after addition of ^{14}C-labelled HGA, showed the presence of labelled Tyr (Fig. 7). The amount of labelled compounds increased with time.

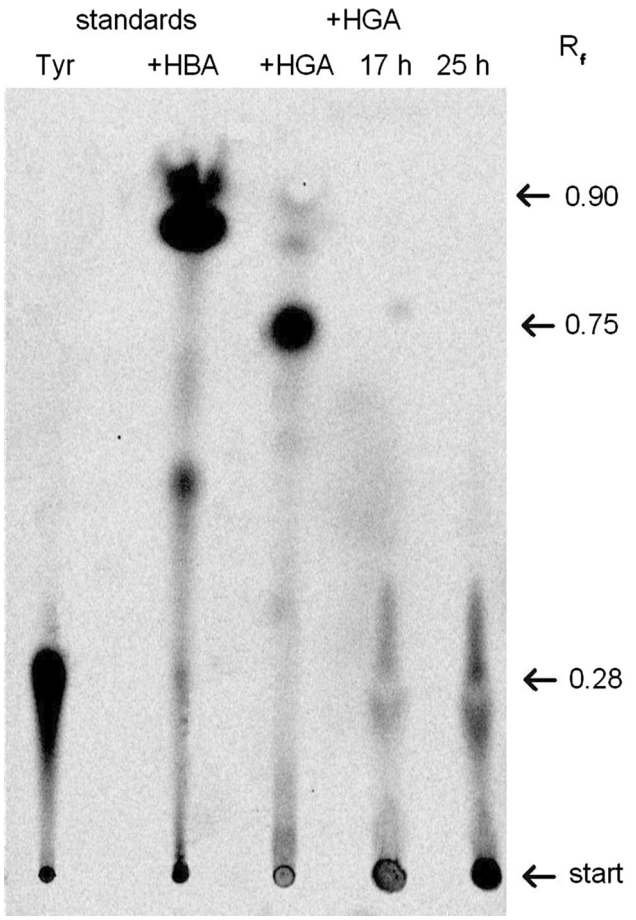

Fig. 7 Autoradiogram of TLC plate of extracts from *Synechocystis* sp. PCC 6803 after 17 and 25 h since the addition of ^{14}C-HGA. TLC plates were developed in toluene:acetic acid:water (25:25:1, v/v). *HBA* *p*-hydroxybenzoate, *HGA* homogentisate, *Tyr* tyrosine

Fig. 8 Autoradiogram of TLC plate of extracts from *Synechococcus elongatus* PCC 6301 and PCC 7942 after 72 h since the addition of ^{14}C-Tyr. TLC plates were developed in chloroform. *PQ* plastoquinone

In the last experiment, ^{14}C-Tyr was added to the cultures of two strains of *Synechococcus elongatus,* a cyanobacterium that does not have genes encoding HPPD homologues. Therefore, it is not able to convert Tyr into HGA via well known degradation pathway of Tyr, where HPPD performs the most important irreversible step (see Fig. 1). The incorporation of labelled aromatic ring into PQ has been observed, although less evident than in the case of ^{14}C-HBA and ^{14}C-HGA (Fig. 8).

Discussion

In the present experiments, evidence was provided that cyanobacterial strains other than *Synechocystis* sp. PCC 6803 also take advantage of PQ biosynthetic pathway that is different than that of α-Toc, but similar to UQ biosynthesis in proteobacteria. There is a correlation between the presence of HPPD, HPT and TC homologues in strains of cyanobacteria and the presence of α-Toc and α-TQ. Increased levels of prenyllipids (α-Toc in *P. laminosum*, PQ in *T. elongatus*) were observed in thermophilic cyanobacteria, which might result from adaptation to the higher temperature of growth, as the increase in α-Toc level during heat stress was observed in plants (Bergmüller et al. 2003). It is known that α-Toc influences properties of the cell membranes. It was shown in experiments on liposomes that α-Toc present in model membranes in certain proportion to other lipids increase the stability of the membrane (Hincha 2008). The absence of HPPD homologues in *Synechococcus elongatus* PCC 7942 and PCC 6301, as well as in *Thermosynechococcus elongatus* BP-1, has no impact on PQ synthesis in these strains.

Inhibition of HPPD synthesis in *Synechococcus* sp. PCC 7002 had no effect on the increase in PQ content in response to medium light treatment, whereas the increase in α-Toc level was lowered by about 70 % in the presence of pyrazolate. This effect is analogical to the results obtained by Dahnhardt et al.

in the experiment with *Synechococcus* sp. PCC 6803, where mutant with disrupted open reading frame *Δslr0090*, encoding homologue of plant HPPD, had the same PQ level as the wild type, but was lacking α-Toc (Dahnhardt et al. 2002). Results obtained for haloxydine and 4-nitrobenzoate clearly support hypothesis that cyanobacteria use UQ-like pathway for PQ biosynthesis, whereas plant pathway is not branched and used only for α-Toc biosynthesis. Inhibition of the prenylation of HBA led to specific inhibition of PQ biosynthesis, whereas inhibition of the prenylation of HGA led to specific inhibition of α-Toc biosynthesis. The increased α-Toc content in 4-nitrobenzoate treated culture might be an effect of accumulation of shikimate pathway intermediates resulting from inhibition of PQ biosynthesis.

We observed incorporation of labelled HBA into PQ not only in the case of *Synechocystis* sp. PCC 6803, but also for *Synechococcus elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002.

Incorporation of ^{14}C-HGA into PQ was observed in all strains under study. This was also described by Whistance and Threlfall, who observed that the labelled HGA was incorporated into PQ in *Synechococcus elongatus* (Whistance and Threlfall 1970). Interestingly, the ability to utilize exogenous HGA was found not only for strains able to synthesize this compound (*Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002) but also for strains lacking HPPD homologue, therefore unable to synthesize HGA (*Synechococcus elongatus* PCC 7942).

During degradation pathways of aromatic compounds, such as HGA, Tyr or Phe, their benzene ring is cleaved, resulting in the formation of carboxylic acids. In the shikimate pathway, where HGA, HBA, Tyr, Phe and other aromatic compounds are formed, substrates for aromatic ring synthesis are sugars (Caspi et al. 2014). If the labelled ring of HGA is cleaved, the probability that degradation products would be introduced into shikimate pathway with high selectivity and efficiency, enabling observation of strong signal due to labelled PQ, is very low. Therefore, it can be concluded that aromatic ring of the applied HGA is not cleaved. Instead, it is introduced into anabolic pathways leading to PQ biosynthesis.

The question is, if HGA can be prenylated by *UbiA* homologue present in cyanobacteria or if it is converted into other compounds. Sadre and coworkers were not able to observe activity of HGA solanesyltransferase in preparations of recombinant Slr0926, therefore they postulated that HBA prenyltransferase responsible for PQ biosynthesis is specific for HBA (Sadre et al. 2012). In experiments with haloxydine and 4-nitrobenzoate, we showed that these compounds, known to selectively inhibit prenylation of HGA and HBA, respectively, specifically influence both α-Toc and PQ synthesis. Together, these results suggest that HGA is not prenylated by HBA prenyltransferase of cyanobacteria, but rather converted into other compounds.

Next question is, if a product of HGA conversion can be prenylated in a way that it bypasses prenylation of HBA. Pfaff et al. showed that *Synechocystis* sp. PCC 6803 knock-out mutant of chorismate lyase is barely viable and contains about 30 times less PQ than the wild type (Pfaff et al. 2014). In the experiment with ^{14}C-labelled HGA we observed strong signal from the labelled PQ. Therefore, it can be concluded that *Synechocystis* sp. PCC 6803 does not show any effective alternative PQ biosynthetic pathways, where compounds other than HBA are prenylated to PQ. This means that HGA is converted into a compound that is introduced into the shikimate pathway before the synthesis of HBA.

The presence of labelled Tyr in extracts of *Synechocystis* sp. PCC 6803 seems to favour this explanation. One can suspect that if HGA is a product of Tyr degradation, there could be a way to reverse this reaction. The conversion of Tyr into *p*-hydroxyphenylpyruvate (HPP) by tyrosine aminotransferase is reversible. However, the second reaction, conversion of HPP into HGA, is a multistep exergonic reaction involving oxidative decarboxylation, phenyl ring hydroxylation and side-chain rearrangement (Borowski et al. 2004). It is therefore irreversible (Rundgren 1983) and simple conversion of HGA into Tyr is rather impossible. However, as Tyr and HBA are both products of the shikimate pathway, sharing common intermediates (see Figs. 1, 2), it seems possible that if a product of HGA conversion is introduced into shikimate pathway, it would result in Tyr production. It is also possible that intermediates of shikimate pathway and of PQ biosynthesis would be metabolized efficiently, while small amounts of Tyr could accumulate.

The hypothetical scheme of HGA conversion is shown in Fig. 9. The proposed intermediates were selected to ensure the least number of unknown steps required to introduce HGA into the shikimate pathway. However, the scheme should be treated only as a proposal to be revised in further research.

In the prokaryotic pathway of HBA synthesis, explored for *E. coli*, this compound is synthesized via the shikimate pathway. The examined eukaryotes, *S. cerevisiae* and mammals can convert Tyr into HBA (Nowicka and Kruk, 2010). In *Synechococcus elongatus* strains, devoid of an enzyme converting Tyr into HGA, the addition of ^{14}C-labelled Tyr to the medium resulted in synthesis of the labelled PQ. It indicates that *S. elongatus* strains have a pathway converting Tyr into HBA, although this question needs further research.

Our results indicate that cyanobacteria have still unknown pathways of conversion of aromatic compounds. These pathways enable both to recycle aromatic

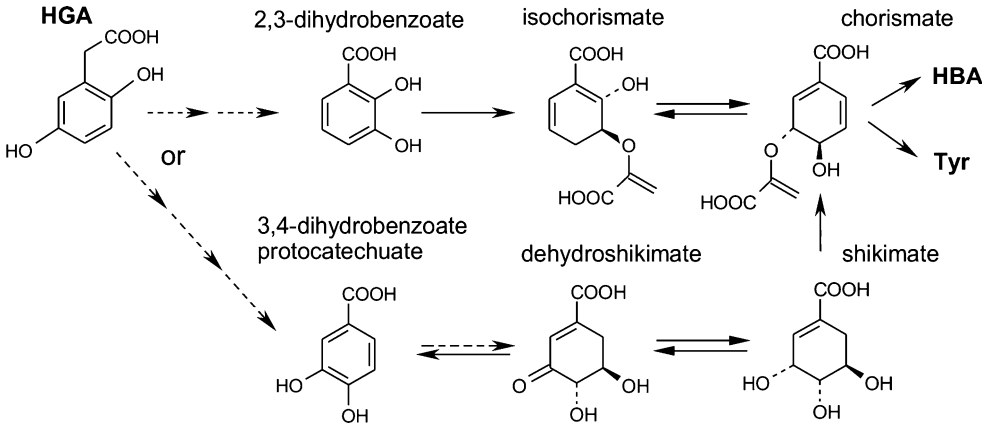

Fig. 9 Hypothetical scheme of homogentisate conversion into *p*-hydroxybenzoate. *Solid lines* are reactions known to occur in nature. *Dashed lines* are hypothetical reactions. *HBA* *p*-hydroxybenzoate, *HGA* homogentisate

compounds produced in the cell, as well as to take advantage of exogenous compounds.

Author contribution statement J. Kruk is responsible for overall design of the experiment. B. Nowicka optimized the methods used and carried out all experiments and analyses. She has also written the preliminary version of the manuscript. Authors carried out synthesis of radiolabelled homogentisate and *p*-hydroxybenzoate together. They also cooperated in developing and improving of the manuscript.

Compliance with ethical standards

Financial support This work was supported by a grant from the National Science Center Poland 2013/11/D/NZ1/00303. Scholarship funded by Society-Environment-Technology project co-financed by the European Union, which enabled employment of B. Nowicka on a postdoc position is also acknowledged.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, Yu YK (2005) Protein database searches using compositionally adjusted substitution matrices. FEBS J 272:5101–5109

Ashby MN, Edwards PA (1990) Elucidation of the deficiency in two yeast coenzyme Q mutants: characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase. J Biol Chem 265:13157–13164

Bergmüller E, Porfirova S, Dörmann P (2003) Characterization of an *Arabidopsis* mutant deficient in γ-tocopherol methyltransferase. Plant Mol Biol 52:1181–1190

Borowski T, Bassan A, Siegbahn PEM (2004) 4-Hydroxyphenylpyruvate dioxygenase: a hybrid density functional study of the catalytic reaction mechanism. Biochemistry 43:12331–12342

Carr NG, Hallaway M (1965) Presence of α-tocopherolquinone in blue-green algae. Biochem J 97:c9

Caspi R, Altman T, Billington R, Dreher K, Foerster H, Fulcher CA, Holland TA, Keseler IM, Kothari A, Kubo A, Krummenacker M, Latendresse M, Mueller LA, Ong Q, Paley S, Subhraveti P, Weaver DS, Weerasinghe D, Zhang P, Karp PD (2014) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res 42(D1):D459–D471

Dahnhardt D, Falk J, Appel J, van der Kooij TAW, Schulz-Friedrich R, Krupinska K (2002) The hydroxyphenylpyruvate dioxygenase from *Synechocystis* sp. PCC 6803 is not required for plastoquinone biosynthesis. FEBS Lett 523:177–181

Dasilva EJ, Jensen A (1971) Content of α-tocopherol in some blue-green algae. Biochim Biophys Acta 239:345–347

DellaPenna D (2005) A decade of progress in understanding vitamin E synthesis in plants. J Plant Physiol 162:729–737

Gross J, Cho WK, Lezhneva L, Falk J, Krupinska K, Shinozaki K, Seki M, Herrmann RG, Meurer J (2006) A plant locus essential for phylloquinone (vitamin K_1) biosynthesis originated from a fusion of four eubacterial genes. J Biol Chem 281:17189–17196

Henninger MD, Bhagavan HN, Crane FL (1965) Comparative studies of plastoquinones. I. Evidence for three quinones in the blue-green alga *Anacystis nidulans*. Arch Biochem Biophys 110:69–74

Hincha DK (2008) Effects of α-tocopherol (vitamin E) on the stability and lipid dynamics of model membranes mimicking the lipid composition of plant chloroplast membranes. FEBS Lett 582:3687–3692

Mène-Saffrané L, DellaPenna D (2010) Biosynthesis, regulation and functions of tocochromanols in plants. Plant Physiol Biochem 48:301–309

Munne-Bosch S, Alegre L (2002) The function of tocopherols and tocotrienols in plants. Crit Rev Plant Sci 21:31–57

Norris SR, Barrette TR, DellaPenna D (1995) Genetic dissection of carotenoid synthesis in Arabidopsis defines plastoquinone as an essential component of phytoene desaturation. Plant Cell 7:2139–2149

Nowicka B, Kruk J (2010) Occurrence, biosynthesis and function of isoprenoid quinones. Biochim Biophys Acta 1797:1587–1605

Nowicka B, Kruk J (2012) Plastoquinol is more active than α-tocopherol in singlet oxygen scavenging during high light stress of *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1817:389–394

Pfaff C, Glinedemann N, Gruber J, Frentzen M, Sadre R (2014) Chorismate pyruvate-lyase and 4-hydroxy-3-solanesylbenzoate decarboxylase are required for plastoquinone biosynthesis in the cyanobacterium sp. PCC6803. J Biol Chem 289:2675–2686

Quinzii CM, Tadesse S, Naini A, Hirano M (2012) Effects of inhibiting CoQ_{10} biosynthesis with 4-nitrobenzoate in human fibroblasts. PLoS One 7:e30606

Rundgren M (1983) Some kinetic properties of 4-hydroxyphenylpyruvate dioxygenase from *Pseudomonas* sp. strain P.J. 874. Eur J Biochem 133:657–663

Sadre R, Frentzen M, Saeed M, Hawkes T (2010) Catalytic reactions of the homogentisate prenyl transferase involved in plastoquinone-9 biosynthesis. J Biol Chem 285:18191–18198

Sadre R, Pfaff C, Buchkremer S (2012) Plastoquinone-9 biosynthesis in cyanobacteria differs from that in plants and involves a novel 4-hydroxybenzoate solanesyltransferase. Biochem J 442:621–629

Sakuragi Y, Bryant D (2006) Genetic manipulation of quinone biosynthesis in cyanobacteria. In: Golbeck JH (ed) Photosystem I: the light-driven plastocyanin: ferredoxin oxidoreductase. Springer, The Netherlands, pp 205–222

Schultze M, Forberich B, Rexroth S, Dyczmons NG, Roegner M, Appel J (2009) Localization of cytochrome b_6f complexes implies an incomplete respiratory chain in cytoplasmic membranes of the cyanobacterium *Synechocystis* sp. PCC 6803. Biochim Biophys Acta 1787:1479–1485

Stevens SE Jr, Patterson COP, Myers J (1973) The production of hydrogen peroxide by blue-green algae: a survey. J Phycol 9:427–430

Threllfall DR, Whistance GR (1971) Biosynthesis of tocopherols and biogenetically related compounds. Methods Enzymol 18:369–396

Whistance GR, Threlfall DR (1970) Biosynthesis of phytoquinones. Homogentisic acid: a precursor of plastoquinones, tocopherols and alpha-tocopherolquinone in higher plants, green algae and blue-green algae. Biochem J 117:593–600