A novel phytyltransferase from *Synechocystis* sp. PCC 6803 involved in tocopherol biosynthesis

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Abstract The deduced polypeptide sequence of open reading frame *slr1736* reveals homology to chlorophyll synthase and 1,4-dihydroxy-2-naphthoic acid phytyltransferase in *Synechocystis* sp. strain PCC 6803. In tocopherol and plastoquinone biosynthesis, a condensation reaction mechanistically similar to that of these two enzymes is performed. To analyze the function of this novel prenyltransferase, a deletion mutant of *slr1736* was generated by homologous recombination. The mutant showed a markedly decreased tocopherol content, while plastoquinone levels remained unchanged. Since the aromatic precursor homogentisic acid accumulated in the mutant, the function of the enzyme was proven to be a novel tocopherol phytyltransferase.

Key words: Prenyl/phytyltransferase; Plastoquinone; Vitamin E; Tocopherol; *Synechocystis*

1. Introduction

Predominantly delivered by the ingestion of vegetable oils, vitamin E is an essential component in the human diet, playing an important role as a membrane-associated antioxidant scavenger [1]. During the past years, several additional functions of vitamin E as an anti-hypercholesterolemic and immunostimulatory agent in humans have been proposed [2]. Vitamin E comprises a class of eight lipid-soluble components, being subdivided into tocopherols and tocotrienols (Fig. 1). While tocopherols share an isoprenoid side chain derived from phytyl-diphosphate (PP), tocotrienol side chains are derivatives of geranylgeranyl-PP. The α, β, γ and δ members of these subclasses differ in their degree of methylation in the 6-chromanol ring structure.

The pathway of tocopherol biosynthesis is not yet well characterized. Although many activities have been measured in vitro using radiolabelled precursors, the functional characterization of individual enzymes has been severely hindered by difficulties in their purification. This is mainly due to their membrane-bound localization and to their low abundance in vivo. Recently, three of at least six genes involved in the pathway have been cloned and functionally characterized from *Arabidopsis*, i.e. *p*-hydroxyphenylpyruvate dioxygenase, geranylgeranyl-diphosphate reductase and γ-tocopherol methyltransferase [3–5]. Still other genes, encoding a prenyltransferase, a tocopherol cyclase and one more methyltransferase, are unknown.

The prenyltransferase involved in tocopherol biosynthesis is expected to mark a branching point leading to the formation of tocopherols, tocotrienols and plastoquinones (Fig. 1). By analysis of a mutant in *Arabidopsis* (PDS2) impaired in carotenoid, vitamin E and plastoquinone biosyntheses, it has been proposed that this prenyltransferase is multifunctional, accepting geranylgeranyl-PP, phytyl-PP and solanesyl-PP as substrates for plastoquinone, as well as for vitamin E biosynthesis (Fig. 1) [6]. This enzyme is supposed to catalyze the condensation with homogentisic acid by a complex prenylation/phytylation reaction involving decarboxylation of the aromatic moiety. For identification, we took advantage of the fact that the quinone pattern of the cyanobacterium *Synechocystis* sp. PCC 6803 is similar to that of plants, also comprising the two major plastidic classes of plastoquinones and vitamin E. The presence of genes involved in biosynthetic events similar to plastidic isoprenoid pathways in *Synechocystis* offers the opportunity to analyze deletion mutants by their loss of function in tocopherol biosynthesis. Using this approach, we report here on the molecular identification of this prenyltransferase leading to the formation of 2-methyl-6-phytylplastoquinol.

2. Materials and methods

2.1. Cloning procedures

A 534-bp fragment (delta5), representing a stretch of the 5′-noncoding region of *slr1736*, and a 534-bp fragment (delta3), representing a part of the 3′-coding region of *slr1736*, were amplified from 25 ng *Synechocystis* PCC 6803 genomic DNA. Proof-reading PCR with Advantage polymerase (Clontech, Germany) was carried out after a denaturation of 1 min at 94°C for 30 cycles (45 s at 94°C, 45 s at 55°C, 2 min at 68°C), followed by an extension at 68°C for 3 min. The primer pair upstream I (5′-AACCGCCTCGAGGGCTTCTCCTACCCGG-3′) and upstream II (5′-GAAGCGCCAAAAAGCTTGGATAGTTGC-3′) was used for delta5. Amplification of delta3 was performed with downstream I (5′-GGTGGGAATTCGTTTGATTATTGGCACG-3′) and downstream II (5′-GCCAAGGGATCCAGCAAGTACTCTAAG-3′). Both fragments were cloned sequentially into pKSCm2, a pBluescript KS with a *cat* gene cassette integrated into the *Eco*RV site, after *Xho*I/*Hin*dIII (for delta5) and *Eco*RI/*Bam*HI (for delta3) digestion. The resulting vector pPrenCm2, bearing a chloramphenicol resistance cassette between delta5 and delta3 in antisense orientation, was checked by sequencing and trans-

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Abbreviations: ORF, open reading frame; TLC, thin layer chromatography; PP, diphosphate; E_m, molar extinction coefficient

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formed into *Synechocystis* PCC 6803. After homologous recombination, it led to a DNA sequence of *slr1736*, where a stretch of 341 bp was deleted, including the putative prenyltransferase encoding domain.

2.2. Transformation and propagation of Synechocystis wild type and Δ1736 mutant

Wild type and mutants of *Synechocystis* PCC 6803 were grown in liquid BG-11 medium supplemented with 5 mM glucose at 30°C at 50 μE/m^{2}s on a rotary shaker. For mutant growth 25 mg/l chloramphenicol was added. Transformation of *Synechocystis* was carried out essentially as described by Williams [7]. Transformed cells were directly plated on BG-11 medium containing 0.3% sodium dithionite, 1.5% agar, 5 mM glucose, 10 mM HEPES–KOH, pH 8.0 and 5 mg/l chloramphenicol. Complete segregation was obtained by restreaking of the cells on plates containing increasing concentrations of chloramphenicol. The final concentration was 50 μg/ml.

2.3. Segregation analysis

The complete segregation was verified by PCR using the primers PRhyp1 (5′-TGGGTTTAGGTACACTGCAAAG-3′) and PRhyp2 (5′-CTTTCTAAGTGTACATCTCGAC-3′). PRhyp1 corresponds to a sequence about 30 bp upstream of the start codon of open reading frame (ORF) *slr1736*. PRhyp2 corresponds to a sequence of the non-coding strand of ORF *slr1736* (bases 794–815). Using chromosomal DNA isolated from wild type *Synechocystis* cells as template, a fragment of 0.85 kb was amplified, whereas with DNA isolated from fully segregated mutant cells a fragment of 1.54 kb was amplified.

2.4. Extraction of lipophilic compounds

Log-phase cultures of wild type *Synechocystis* PCC 6803 and mutant strain Δ*1736* were harvested at 4000×*g*, washed once with water, frozen in liquid nitrogen and then lyophilized overnight. 20 mg of cell dry weight was subjected to the following extraction procedure: after resuspension in 1 volume of water, cells were broken by use of a French press. Three volumes of acetone were added and cells sonicated (Branson Sonifier, USA). Lipid-soluble material was partitioned into the organic phase by adding 1 volume of petroleum ether to the combined acetone extracts followed by a 15-fold excess of water. The aqueous phase was re-extracted twice with petroleum ether, the organic phases were combined and evaporated under a stream of nitrogen. Dried extracts were redissolved in an appropriate volume of chloroform. Alternatively and without notable changes with respect to the product pattern, the dried cells were resuspended in acetone, stored overnight at −20°C and subsequently broken by sonification. After centrifugation (10 min, 5000×*g*) the supernatant was removed and the pellet re-extracted five times with acetone. Combined supernatants were evaporated under a stream of nitrogen and the dried extract redissolved in chloroform.

2.5. Quantification

Oxidized vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone; Sigma, Germany) was quantified spectrophotometrically (Uvikon, Kontron, Italy) in absolute ethanol at 248 nm, using a molar extinction coefficient (E_m) of 18 900 [8] and 30 nmol was subjected to high performance liquid chromatography (HPLC) (see below) for calibration. Decyl-plastoquinone (Sigma, Germany) was quantified spectrophotometrically at 255 nm prior to and after reduction with $NaBH_4$ in absolute ethanol ($\Delta E_m = 15\,000$) [9], 15 nmol was used for calibration. D-α-Tocopherol acetate (Sigma, Germany) was reduced with $NaBH_4$ and measured in absolute ethanol at 284 nm ($E_{cm}^{1\%} = 45$; value given by the manufacturer). Tocopherol and tocotrienol standards (Merck, Germany) were quantified as described ($E_{cm}^{1\%}$ see [10]) and also analyzed in the form of their oxidized quinones after treatment with 200 μM potassium ferricyanide.

For calibration the absorption/emission peak areas of the quantified standards were analyzed by HPLC. Tocopherols and tocotrienols were detected by use of a fluorescence detector setting excitation and fluorescence emission to 290 nm and 324 nm (40 nm band width), respectively. All other standards were calibrated at their maximal absorbance.

The tocopherol content in the samples was determined by internal standardization adding 350 μg D-α-tocopherol acetate to the lyophilized bacterial pellet prior to extraction.

Plastoquinone and phylloquinone were both quantified by external standardization.

To identify plastoquinones in *Synechocystis*, a plastoquinone-9 standard from spinach leaves was prepared by acetone extraction [11]. It was purified by thin layer chromatography (TLC) (silica gel 60, Merck, Germany) using petroleum ether/diethylether (7:1, v:v) [12].

Pigments, i.e. chlorophyll and carotenoid contents, were determined in 80% (v/v) acetone using the following formulas: total chlorophyll *a* (in μg/ml): $8.02 \times A_{663}$ [13]; total carotenoids (in μg/ml): $3.77 \times (A_{440} - 0.89 \times A_{660})$ [14].

2.6. HPLC analysis

The HPLC system (Waters, Germany) consisted of two 510 HPLC pumps, a 717plus autosampler, a 996 photodiode array detector monitoring UV/Vis spectra, a 474 scanning fluorescence detector and a C_{30}-reversed HPLC column (YMC Europe). Chromatograms were analyzed using the Millenium PDA software package (Waters, Germany). The column was developed at a flow rate of 1 ml/min with the solvent system A: methanol/*tert*-butylmethyl ether/water (60:12:12, v:v) and B: methanol/*tert*-butylmethyl ether (50:50; v:v). A linear gradient was performed from 100% A to 57% A in 25 min, followed by an isocratic step for 5 min and a linear gradient to 0% A in 25 min.

Separation of vitamin E: 20 μl of wild type or Δ*1736* extracts in chloroform, equivalent to 1.5 mg dry weight, were subjected to C_{30}-reversed HPLC.

Separation of phylloquinones and plastoquinones: 200 μl of wild type or Δ*1736* extracts in chloroform, equivalent to 15 mg dry weight, were subfractionated by TLC (silica gel 60, Merck, Germany) with the solvent system petroleum ether/diethyl ether/acetone (40:10:25, v:v). Phylloquinones and plastoquinones were isolated by acetone extraction from the solvent front. After evaporation, the compounds were dissolved in chloroform and an amount equivalent to 2 mg of dry weight was applied to HPLC in 20 μl.

2.7. Detection of ochronotic pigment in cyanobacterial lysates

For analysis of ochronotic pigment, the oxidative polymer of homogentisic acid, 5 ml of log-phase wild type and mutant *Synechocystis* cell culture were harvested and centrifuged for 5 min at 4000×*g*. The bacterial pellets were lysed by the addition of 20 μl 5 N NaOH, vortexed, diluted with 980 μl water and centrifuged for 10 min at 20 000×*g*. Cytosolic supernatants were removed, exposed to air for 10 min and then measured spectrophotometrically in the range of 350–750 nm, using the wild type extract as the reference. A homogentisic acid standard (Sigma, Germany) was treated in the same way and measured against a water reference.

3. Results

3.1. A null mutant of slr1736 in Synechocystis

As an alternative to identify the tocopherol phytyltransferase by standard biochemical methods, we used the amino acid sequences of two functionally characterized prenyltransferases to search the *Synechocystis* genomic database (CyanoBase). The gene products of *slr0056* (ChlG) and *slr1518* (MenA) were expected to reveal some homology to the unidentified phytyltransferase: in phylloquinol biosynthesis by MenA a similar phytyltransferase reaction between an aromatic head group and phytyl-PP has been proposed (Fig. 1) [15]. A prenylation/phytylation is also catalyzed by chlorophyll synthase (ChlG). Enzymatic in vitro assays demonstrated that phytyl-PP as well as geranylgeranyl-PP were accepted as its substrate [16]. The search revealed an ORF, *slr1736*, sharing 33.3% similarity (19.2% identity) to ChlG and 31.3% similarity (13.8% identity) to MenA in alignments performed with the deduced protein sequence. Fig. 2 shows an alignment of *slr1736* with MenA, UbiA, and ChlG, as performed using the CLUSTALW algorithm. In the protein sequence of *slr1736* a region homologous to polyprenyltransferase domain II can be observed.

To identify the function of this unknown prenyl/phytyltransferase, we generated a null mutant of *slr1736* by homol-

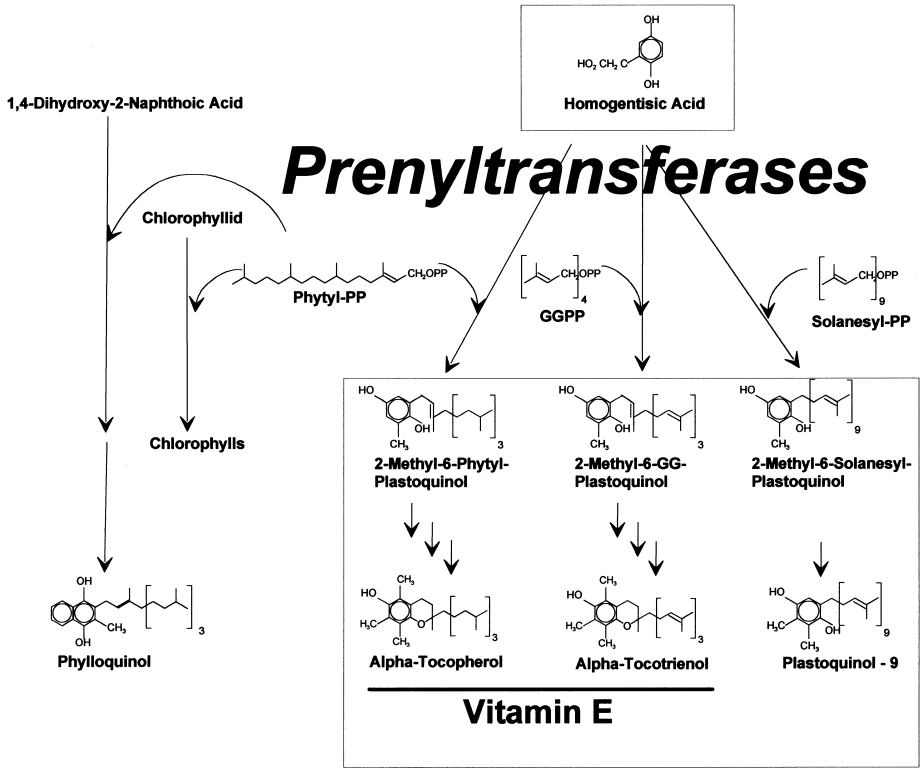

Fig. 1. Similar prenyl/phytyltransferase reactions in biosynthetic pathways of plants. In vitamin E and plastoquinol biosynthesis the same aromatic head group homogentisic acid and different diphosphorylated isoprenoids are required as substrates.

ogous recombination in *Synechocystis*. A 5′-terminal deletion sequence of *slr1736* removing the putative prenyltransferase domain (Fig. 2) was integrated into pPrenCm2. After transformation with this vector, homoplasmic mutants were selected by their chloramphenicol resistance. The homologous integration of the chloramphenicol cassette flanked by 5′-terminal and 3′-terminal sequences of *slr1736* into the genome of the cyanobacterium was checked by PCR (Fig. 3B). Wild type *Synechocystis* revealed a 850-bp PCR product, in mutant strain Δ*1736* a 1540-bp fragment could be amplified (Fig. 3A). Since this was the only PCR product amplified from Δ*slr1736* genomic DNA, it was excluded that wild type meroploids were selected for further analysis. Cultures of Δ*1736* grew normally and were subjected to extraction and HPLC analysis.

3.2. Analysis of pigment and quinone composition in the Δ1736 mutant

Plastoquinones, phylloquinones, chlorophylls and carotenoids are related with respect to their biosynthesis, all of them being involved in photosynthesis. It was found necessary to monitor all of these compounds taking into account that first, the novel enzyme could be multifunctional and second, the deletion may lead to the upregulation of the other prenyl lipids. No significant changes were observed in either phylloquinone or chlorophyll concentration in the mutant as compared to the wild type (Table 1). Since the desaturation of phytoene in carotenoid biosynthesis is dependent on a NADPH-dependent quinone oxidoreductase [6,17], carotenoid levels were also analyzed. There was no significant quantitative and qualitative change in this respect (Table 1).

Plastoquinone-9 separated at 49.3 min in wild type and Δ*1736* mutant extracts (Fig. 4A, 1). Their retention times and absorption spectra (Fig. 4B) were identical to a plastoquinone-9 standard purified from spinach leaves. Concentrations of plastoquinone-9 in extracts, externally standardized with decyl-plastoquinone (Fig. 4B), remained unchanged in the Δ*1736* mutant compared to the wild type at 0.50 nmol/mg dry weight (Table 1), indicating that *slr1736* gene product is not involved in plastoquinone biosynthesis.

3.3. Deletion of slr1736 leads to a decrease of tocopherols and accumulation of homogentisic acid

HPLC analysis revealed a four-fold decrease in the tocopherol content from 443 ng/mg dry weight in the wild type to 113 ng/mg dry weight in the Δ*1736* mutant (Table 2). The

Table 1
Pigment and quinone concentrations in wild type and Δ*1736* mutant *Synechocystis* PCC 6803

Extraction of *Synechocystis* PCC 6803	Concentrations per dry weight[a]			
	chlorophyll (μg/mg)	carotenoids (μg/mg)	plastoquinones (nmol/mg)	phylloquinones (nmol/mg)
Wild type	16.1 ± 0.3	2.6 ± 0.3	0.50 ± 0.01	0.23 ± 0.03
Δ*1736*	16.2 ± 0.4	3.3 ± 0.2	0.56 ± 0.06	0.26 ± 0.02

[a]Lyophilized bacterial 4000×*g* pellet.

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                1                            30
1736   (1)  -------------------------MATIQA
MenA   (1)  ----------------MTESSPLAPSTAPAT
ChlG   (1)  MSDTQNTGQNQAKARQLLGMKGAAPGESSI
UbiA   (1)  -------------MVAQTPSSPPLWLTIIYL

                31                           60
1736   (7)  FWRFSRPHTIIGTTLSVWAVYLLTILGDGN
MenA  (16)  RKLWLAAIKPPMYTVAVVPITVGSAVAYG-
ChlG  (31)  WKIRLQLMKPITWIPLIWGVVCGAASSGGY
UbiA  (19)  LRWHKPAGRLILMIPALWAVCLAAQG----

                61                           90
1736  (37)  SVNSPASLDLVFGAWLACLLGNVYIVGLNQ
MenA  (45)  -LTGQWHGDVFTIFLLSAIAIIAWINLSND
ChlG  (61)  IWSVEDFLKALTCMLLSGPLMTGYTQTLND
UbiA  (45)  ----LPPLPLLGTIALGTLATSGLGCVVND

                91                          120
1736  (67)  LWDVDIDRINKPN----LPLANGDFSIAQG
MenA  (74)  VFDSDTGIDVRKAHSVVNLTGNRNLVFLIS
ChlG  (91)  FYDRDIDAINEPY----RPIPSGAISVPQV
UbiA  (71)  LWDRDIDPQVERTK--QRPLAARALSVQVG

                121                         150
1736  (93)  RWIVGLCGVASLAIAWGLGLWLGLTVGISL
MenA(104)  NFFLLAGVLGLMSMSWRAQDWTVLELIGVA
ChlG(117)  VTQILILLVAGIGVAYGLDVWAQHDFPIMM
UbiA  (99)  IGVALVALLCAAGLAFYLTPLSFWLCVAAV

                151                         180
1736(123)  IIGT--------AYSVPPVRLKRFSLLAALC
MenA(134)  IFLG-------YTYQGPPFRLGYLGLGELIC
ChlG(147)  VLTLGGAFVAYIYSAPPLKLKQNGWLGNYA
UbiA(129)  PVIVAYPGAKRVFPVPQLVLS---IAWGFA

                181                         210
1736(146)  ILTVRGIVVNLGLFLFFRIGLGYPPTLITP
MenA(158)  LITFG--------PLAIAAAYYSQSQSFSWN
ChlG(177)  LGAS--------YIALPWWAGHALFGTLNPT
UbiA(156)  VLIS-------------WSA---VTGDLTDA

                211                         240
1736(176)  IWVLTLFILVFTVAIAIFKDVPDMEGDRQF
MenA(181)  LLTPSVFVGISTAIILFCSHFHQVEDDLAA
ChlG(200)  IMVLTLIYSLAGLGIAVVNDFKSVEGDRQL
UbiA(171)  TWVLWGATVFWTLGFDTVYAMADREDDRRI

                241                         270
1736(206)  KIQTLTLQIGKQNVFRGTLILLTGCYLAMA
MenA(211)  GKKSPIVRLGTKLGSQVLTLSVVSLYLITA
ChlG(230)  GLKSLPVMFG-----IGTAAWICVIMIDVF
UbiA(201)  GVNSSALFFG-------QYVGEAVGIFFALT

                271                         300
1736(236)  IWGLWAAMPLNTAFLIVSHLCLLALLWWRS
MenA(241)  IGVLCHQAPWQTLLIIASLPWAVQLIRHVG
ChlG(255)  QAGIAGYLIYVHQQLYATIVLLLLIPQITF
UbiA(225)  IGCLFYLGMILMLNPLYWLSLAIAIVGWVI

                301                         330
1736(266)  RDVHLESKTEIASFYQFIWKLFFLEYLLYP
 MenA(271)  QYHDQPE--QVSNCKFIAVNLHFFSGMLMA
ChlG(285)  QDMYFLRNPLENDVKYQASAQPFLVFGMLA
UbiA(255)  QYIQLSAPTPEPKLYGQIFGQNVIIGFVLL

                331         343
1736(296)  LALWLPNFSNTIF
MenA(299)  AGYGWAGLG----
ChlG(315)  TGLALGHAGI---
UbiA(285)  AGMLLGWL-----
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Fig. 2. Amino acid sequence alignment of prenyl/phytyltransferases in *Synechocystis* sp. strain PCC 6803. Comparison of chlorophyll synthase (ChlG; *slr0056*), 1,4-dihydroxy-2-naphthoic acid phytyltransferase (MenA; *slr1518*) and putative 4-hydroxybenzoate-octaprenyltransferase (UbiA; *slr0926*) with the hypothetical phytyltransferase of *slr1736*. The alignment was generated using the CLUSTALW algorithm of AlignX in the Vector NTI program (InforMax, USA). Regions of homology are shaded in gray, those of identity in black. Gaps in the alignment are indicated by dashes. The potential prenyltransferase domain is underlined.

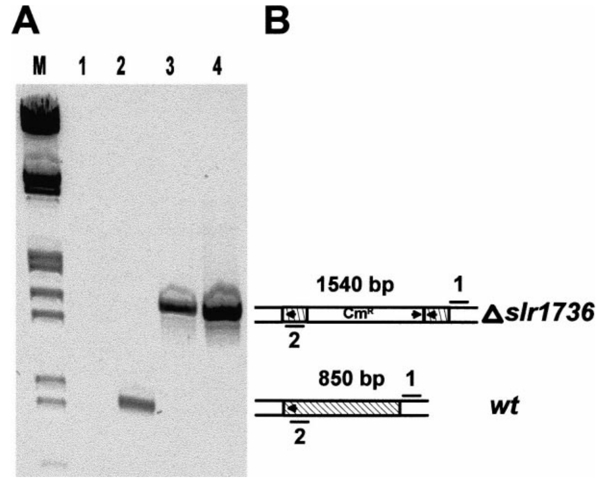

Fig. 3. Segregation analysis of wild type and Δ*slr1736* genomic DNA. The amplification of 25 ng of wild type (wt) and mutant (Δ*1736*) genomic DNA by the primer pair PRhyp1 and PRhyp2 in 50 μl PCR assay yields a single product, i.e. a 850-bp and a 1540-bp product respectively. A: Separation of PCR products on a 1% (w/v) TBE agarose gel. M: λDNA; *Eco*RI/*Hin*dIII digest; 1: water control; 2: wild type PCR product; 3 and 4: Δ*slr1736* PCR product. The amount of chromosomal DNA used for PCR was 0 ng (lane 1), 20 ng (lanes 2 and 3), 60 ng (lane 4). B: Physical map of the PCR products amplified with primer pair PRhyp1 (1) and PRhyp2 (2). In fully segregated mutant cells, a fragment of 1540 bp was amplified. The increase in size was due to a replacement of 320 bp of ORF *slr1736* (shaded) by the 1.1-kb fragment carrying the chloramphenicol resistance gene (Cm^R). The transcriptional orientation of the genes are indicated by arrowheads.

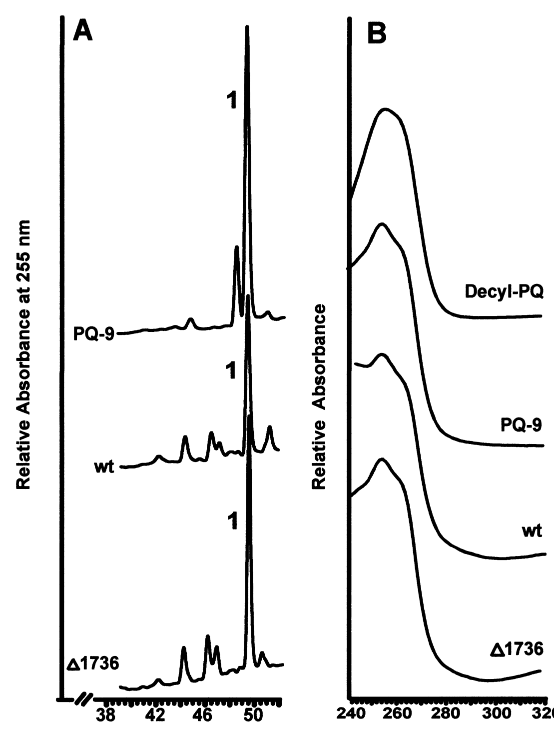

Fig. 4. HPLC separation of plastoquinones. A: Prominent plastoquinone-9 (1) was detected at 49.3 min in a plastoquinone extract from spinach leaves (PQ-9) and in wild type (wt) and mutant (Δ*1736*) *Synechocystis* extracts. The spectra in B were taken by use of a photodiode array detector at the respective retention time and revealed the respective absorption maximum. PQ-9, wt, Δ*1736*: 49.3 min/255 nm; decyl-PQ as external standard: 13.6 min/259 nm.

Table 2
Vitamin E concentrations in wild type and Δ*1736* mutant *Synechocystis* PCC 6803

Extraction of *Synechocystis* PCC 6803	Vitamin E concentrations in ng/mg dry weight[a]				
	α-tocopherol	β/γ-tocopherol	δ-tocopherol	α, β, δ-tocotrienols	Σ vitamin E
Wild type	251 ± 15	22 ± 0,3	170 ± 9	n.d.	443
Δ*1736*	37 ± 10	4 ± 0,4	72 ± 16	n.d.	113

n.d., not detectable.
[a]Lyophilized bacterial 4000×*g* pellet.

qualitative analysis (for a representative HPLC trace, see Fig. 5) showed that this decrease was not co-linear with all tocopherol species but declined in the order of α-tocopherol, β/γ-tocopherol, δ-tocopherol. No α-, β- and δ-tocotrienols were detected in extracts of either wild type or mutant cells. It cannot be judged whether γ-tocotrienol is a constituent in the tocotrienol complement since it co-eluted with an additional hitherto unidentified and spectrally unrelated compound that was regularly observed in the wild type and which declined in the mutant (Fig. 5, peak 5).

To show unequivocally that the biosynthetic block introduced by the mutation was indeed due to a lack of phytyltransferase activity, we investigated mutant and wild type cells for the presence of the non-prenyl acceptor, homogentisic acid, which was expected to accumulate. We analyzed the presence of homogentisic acid by treating wild type and mutant cells with 100 mM NaOH. Alkali and oxygen lead to the oxidation of homogentisic acid and to the formation of ochronotic pigment [18] which can be recognized by its red-brownish color. Mutant cell supernatants turned immediately brown when exposed to air, whereas wild type supernatants remained colorless. The accumulation of ochronotic pigment in these supernatants was further analyzed by comparing the spectrum of mutant with wild type supernatant. The spectrum obtained

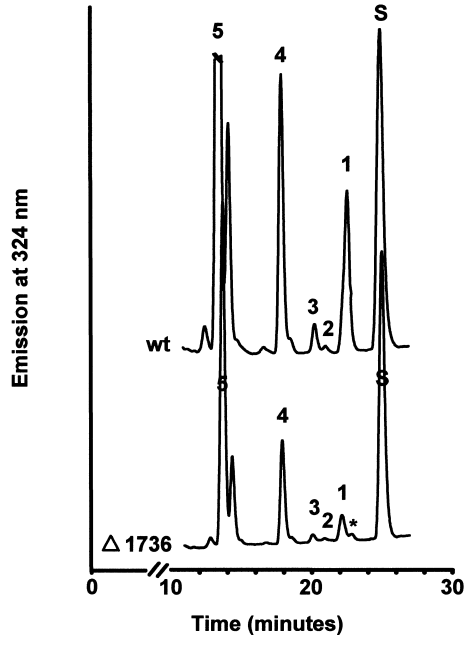

Fig. 5. HPLC separation of tocopherols in wild type and Δ*1736* organic extracts. Peak S: D-α-tocopherol acetate as internal standard; peak 1: α-tocopherol, in Δ*1736* additional shoulder generated by oxidized form (α-tocopheryl quinone; *); peak 2: β-tocopherol; peak 3: γ-tocopherol; peak 4: δ-tocopherol; peak 5: unknown compound.

from mutant cells was very similar to the one obtained with the oxidation product of a homogentisic acid standard (not shown). It can therefore be safely concluded that homogentisic acid accumulates in the Δ*1736* mutant due to a lack of prenyltransferase activity. Thus, the *slr1736* gene product encodes a phytyltransferase involved in tocopherol biosynthesis.

4. Discussion

As we have shown, the deletion of the ORF *slr1736* in *Synechocystis* sp. strain PCC 6803 leads to a mutant phenotype exhibiting markedly reduced levels of tocopherols. As a second consequence of this mutation there is also a strong accumulation of homogentisic acid, analyzed in the form of its polymeric oxidation product, ochronotic pigment. This indicates that the metabolism of this prenyl acceptor molecule is severely hindered by the absence of the *slr1736* gene product. Since the mutant showed unchanged levels of plastoquinones, phylloquinones, chlorophylls and carotenoids, we conclude that *slr1736* encodes a phytyltransferase acting specifically in the tocopherol biosynthetic pathway. No other intermediates in the biosynthesis of tocopherols were detectable in the mutant. Thus, the identity of *slr1736* with any other gene involved in tocopherol formation can be excluded.

There are conflicting results with respect to the formerly proposed presence of a multifunctional prenyltransferase serving both plastoquinone and tocopherol/tocotrienol biosynthesis. This suggestion stems from investigations of an *Arabidopsis* PDS2 mutant being defective in plastoquinone as well as tocopherol biosynthesis (the simultaneous lack of colored carotenoids in this mutant being a consequence of plastoquinone absence [6]). On the other hand, there are biochemical data pointing to the presence of specific prenyltransferases for either pathway, the observation being that in tocopherol synthesis solely phytyl-PP condenses with homogentisate while geranylgeranyl-PP is inactive [19,20].

We propose the presence of two different enzymes in *Synechocystis*, a tocopherol phytyltransferase as identified here and a yet unidentified plastoquinol/tocotrienol prenyltransferase. The measured residual level of tocopherols in our deletion mutant may be the result of low selectiveness for phytyl-PP and homogentisate of this additional prenyltransferase involved in plastoquinol biosynthesis. This problem is currently being investigated.

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