A novel phytoltransferase 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 phytoltransferase 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 phytoltransferase.

Key words: Prenyl/phytoltransferase; 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). Vitamin E is an essential component in the human diet, playing a part of the 3-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. 4-hydroxyphenylpyruvate dioxygenase, geranylgeranyl-diphosphate reductase and 7-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/phytolation 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'-coding 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'-AACCGCTTCGAGGCTTCTTCTTCA-GCGG-3') and upstream II (5'-GAAGCGCGAAAAGGTTGGATAGTGGC-3') was used for delta 5. Amplification of delta 3 was performed with downstream I (5'-GGTTGGGAATTCGTTT-GATTATGTCGAG-3') and downstream II (5'-GCCAAAGGT-ATCCAGAAGTACTTAAAG-3'). Both fragments were cloned sequentially into pKScm2, a pBluescript KS with a cat gene cassette integrated into the EcoRV site, after *XhoI*/*HindIII* (for delta5) and EcoRI/*BamHI* (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-
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 Δslr1736 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 µm/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 achieved by removing 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 PRhys1 (5'-TGGTTTCTTAGTATCCTGCAGA-3') and PRhys2 (5'-CCCTCTAAGTTGCATCTGCGA-3'). PRhys1 corresponds to a sequence about 30 bp upstream of the start codon of open reading frame (ORF) slr1736. PRhys2 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 Δslr1736 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 chloroform, equivalent to 1.5 mg dry weight, and measured against a water reference. 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 sonication. After centrifugation (10 min, 3000 × 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 K₁ (2-methyl-3-phytlyl-1,4-naphthoquinone; Sigma, Germany) was quantified spectrophotometrically (Uvikon, Kontron, Italy) in absolute ethanol at 248 nm, using a molar extinction coefficient (ε248) of 18 900 [8] and 30 nmol was subjected to high performance liquid chromatography (HPLC) (see below) for calibration. Dicyl-plastoquinone (Sigma, Germany) was quantified and expressed as its dicyl derivative at 255 nm prior to and after reduction with NaBH₄ in absolute ethanol (Δε255 = 15 000) [9], 15 nmol was used for calibration. D-α-Tocopherol acetate (Sigma, Germany) was reduced with NaBH₄ and measured in absolute ethanol at 284 nm (E284 = 45; value given by the manufacturer). Tocopherol and tocotrienol standards (Merck, Germany) were quantified as described ([E284] see [10]) and also analyzed in the form of their oxidized quinones after treatment with 200 µM potassium ferricyanide.

For calibration the absorption/emission peaks 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 phyloquinone 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/diethyl ether (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): 8.0×10⁻³×A₅₆₀ [13]; total carotenoids (in µg/ml): 3.77×(A₄₇₀−0.89×A₆₄₉₀) [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 C18 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/1% formic acid/0.3% sodium dithionite/water (60:12:12, v/v) and B: methanol/1% formic acid/0.3% sodium dithionite/water (50:50, v/v). A linear gradient was performed from 100% A to 57% A in 25 min, followed by an isotropic step for 5 min and a linear gradient to 0% A in 25 min.

Separation of vitamin E: 20 µl of wild type or Δslr1736 extracts in chloroform, equivalent to 1.5 mg dry weight, were subjected to C₅₀ reversed HPLC.

Separation of plastoquinones and plastoquinone-9: 200 µl of wild type or Δslr1736 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/acetic acid (40:10:25, v/v). Phyloquinones 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 20000×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 phytlytransferase 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 phytlytransferase: in phylloquinol biosynthesis by 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/phytlytransferase, we generated a null mutant of slr1736 by homol-
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 Δ1736 a 1540-bp fragment could be amplified (Fig. 3A). Since this was the only PCR product amplified from *slr1736* genomic DNA, it was excluded that wild type meropsoids were selected for further analysis. Cultures of Δ1736 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 phytene 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 Δ1736 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 Δ1736 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 Δ1736 mutant (Table 2). The

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**Table 1**

Pigment and quinone concentrations in wild type and Δ1736 mutant *Synechocystis* PCC 6803

<table>
<thead>
<tr>
<th>Extraction of <em>Synechocystis</em> PCC 6803</th>
<th>Chlorophyll (µg/mg)</th>
<th>Carotenoids (µg/mg)</th>
<th>Plastoquinones (nmol/mg)</th>
<th>Phylloquinones (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>16.1 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>0.50 ± 0.01</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Δ1736</td>
<td>16.2 ± 0.4</td>
<td>3.3 ± 0.2</td>
<td>0.56 ± 0.06</td>
<td>0.26 ± 0.02</td>
</tr>
</tbody>
</table>

*Lyophilized bacterial 4000 × g pellet.*
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 Δslr1736 genomic DNA. The amplification of 25 ng of wild type (wt) and mutant (Δslr1736) 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; EcoRI/HindIII digest; 1: water control; 2: wild type PCR product; 3 and 4: Δslr1736 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<sup>R</sup>). The transcriptional orientation of the genes is 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 (Δslr1736) *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, Δslr1736: 49.3 min/255 nm; decyl-PQ as external standard: 13.6 min/259 nm.
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 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 Δ1736 mutant due to a lack of phytyltransferase 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, phyloquinones, 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.

References