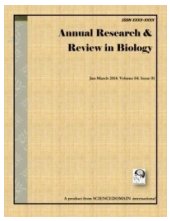

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A Deletion of a Putative Glucosyl Transferase Increases Carotenoids Production in *Synechocystis* sp. PCC 6803

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Author's contribution

The study was designed, analyzed and discussed by the author. The author takes full responsibility for the whole study including data collation, manuscript drafting and editing.

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ABSTRACT

Aim: Deletion the putative glucosyl transferase (Slr1125) in order to investigate its relationship with the unique carotenoid glycoside myxoxanthophyll.
Methodology: In *Synechocystis* sp. PCC 6803, *slr1125* ORF was replaced by streptomycin resistance cassette. The resulted homologous mutant strain was analyzed by high pressure liquid chromatography and electron microscope in order to assess the impact of the gene deletion on myxoxanthophyll and the cell wall ultrastructure of the Δ*slr1125*S strain.
Results: In the Δ*slr1125*S mutant strain, myxoxanthophyll and chlorophyll decreased and photomixotrophic growth was very poor at a low light intensity of 0.5 µE m^{-2} s^{-1}. However, at higher light intensity myxoxanthophyll accumulates in the mutant reach (1120ng/ml/OD_{730}) that is 5-fold higher than that of wild type strain. Photosynthetic pigments increased in the mutant strain and growth improved at a light intensity of 100µE m^{-2} s^{-1}. In addition, a pool of novel carotenoids accumulated part of which are precursor intermediates of myxoxanthophyll biosynthetic pathway. Cell wall dysfunction observed during cell growth in liquid and on plate and verified by ultrastructure

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deformation of the cell wall layers. S-layer disappeared and peptidoglycan was poorly developed.
Conclusion: The putative glucosyl transferase (Slr1125) plays an important role in cell wall biogenesis of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 that is indirectly affecting the carotenoid biosynthetic pathway specially the glycosylated carotenoid myxoxanthophyll. Indeed, these results highlight the notion that cross talk and interconnectedness between cellular processes exist that is critical for metabolic engineering designs.

Keywords: Cyanobacteria*; carotenoid; glucosyltransferase; metabolic engineering.*

1. INTRODUCTION

Carotenoids are emerging anti-obese natural products with strong antioxidant property [1]. They reduce and treat rheumatoid arthritis and other forms of arthritis, heart diseases, diabetes, Alzheimer's disease, irritable bowel syndrome, allergies, asthma, Parkinson's disease, and cancer [2]. Carotenoid play effective role as an anti-diabetic and anti-photo aging compounds [3]. They affect gene expression of osteoblastic bone formation and osteoclastic bone resorption processes [4]. They found to block oxidative DNA damage, lower C-reactive protein as inflammation biomarkers, and boost immunity, decrease triglycerides, raise HDL-cholesterol, improve blood flow [5-12].

Carotenoids are widely distributed in nature but they are essential in photosynthetic bacteria, cyanobacteria, algae and plants. Cyanobacteria are large and diverse group of bacteria and distinguish themselves by their oxygenic, photosynthetic and prokaryotic nature. Cyanobacteria are self-sufficient bio-factories [13] and easy to genetically manipulate their metabolic circuit to modify their metabolites [14].

In cyanobacteria, the occurrence of many different carotenoids has been reported [15-18]. Unique carotenoid glycosides are quite common in cyanobacteria like the methylated fucosylmyxol carotenoids in *Synechocystis* [19-22]. Myxoxanthophyll is found in cyanobacteria, and is named after the synonym for this group of organisms [23]. Myxoxanthophyll is a xanthophyll glycoside found in cyanobacteria [24]. The carotenoid moiety is myxol (1′,2′-dihydro-3′,4′-didehydro-3,1′,2′-trihydroxy-γ-carotene; m/z = 584) [21]. Myxoxanthophyll synthesized in cyanobacteria with a variety of sugars and sugar derivatives. In *Oscillatoria* and *Spirulina* spp., fucosides, chinovosides and methyl fucoside soccur [25,26]. Myxoxanthophyll of the fucose derivatives have been identified in *Nostoc punctiforme* strain PCC 73102 and *Nostoc* sp. strain PCC 7120 [27]. Several other myxol glycosides with fucose derivatives have been found, including myxol 2'-(3-*O*-methyl-α-L-fucoside) in *Oscillatoriabornetii* [25] and the non-methylated myxol2'-α-L-fucoside from *Oscillatoria limnetica* [26]. In *Synechocystis* the sugar moiety of myxoxanthophyll was identified as dimethylated fucose [21], and was found to be essential for myxoxanthophyll biosynthesis [28]. In *Synechocystis* sp. PCC 6803 the normally accumulated carotenoids are myxoxanthophyll (myxol 2'-dimethyl-fucoside; m/z = 758), β-carotene (β,β-carotene; m/z = 536), echinenone (β,β-caroten-4-one; m/z = 550), and zeaxanthin (β,β-carotene-3, 3'-diol; m/z = 568) [21,24].

In this study, an attempt to analyze the role of Slr1125 in the carotenoid biosynthesis pathway, a putative glucosyl transferase was annotated as zeaxanthin glucosyl transferase in Cyano Base. In Δ*slr1125*S mutant, myxoxanthophyll increased and novel carotenoids accumulated.

2. MATERIALS AND METHODS

2.1 Strains and Growth Conditions

Synechocystis sp. PCC 6803 was cultivated on a rotary shaker at 30℃ in BG-11 medium [29], buffered with 5 mMN-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid-NaOH (pH 8.2). For growth under photomixotrophic conditions, BG-11 was supplemented with 5 mM glucose. For growth on plates, 1.5% (w/v) Difco agar and 0.3% (w/v) sodium thiosulfate were added. A flux density of 0.5 or 100 µmol of photons m^{-2} s^{-1} from cool-white fluorescent tubes was used for growth in continuous light in liquid medium. For growth in liquid under light-activated heterotrophic growth (LAHG) conditions [30], cells were kept in complete darkness except for one 15-min light period (40µE m^{-2} s^{-1}) every 24h. Growth was monitored by measuring the optical density of the cells at 730nm using a Shimadzu UV-160 spectrophotometer.

2.2 Cloning of slr1125 and Construction of the pΔslr1125S Plasmid

The *Synechocystis* sp. PCC 6803 *slr1125* gene and its flanking regions (Fig. 1) were cloned by polymerase chain reaction (PCR) based on the available *Synechocystis* genomic sequence (CyanoBase:www.kazusa.or.jp/cyano/cyano.htm) [31]. The forward primer was 5′ CTAGAAACGGGAATTCAAGCGGAAT 3′ with an engineered EcoR I site (underlined) and corresponding to base number 85721 – 85745 in CyanoBase; the reverse primer was 5′ GTTTAATAGCATGCTTTGCCAGC 3′ with an engineered Sph I restriction site (underlined) and a sequence corresponding to Cyano Base bases 87845 – 87867 (base changes to introduce restriction sites have been bolded). The PCR-amplified sequence corresponds to *slr1125* with approximately 430-450bp flanking sequence on both sides of the ORF. A PCR product of the expected size (2.1 kb) was purified, restricted with EcoR I and Sph I (using the introduced restriction sites in the primer) and cloned into pUC19 creating p*slr1125*. The *slr1125*gene was deleted by restriction at internal Sty I sites near the beginning and end of the*slr1125* open reading frame and replacing the Sty I fragment (1.2 kb) by a 1.5kb streptomycin resistance cassette. This creates pΔ*slr1125*S, which was used for transformation of *Synechocystis* that was carried out according to Vermaas et al. [32]. Transformants were propagated on BG-11/agar plates supplemented with 5 mM glucose and increasing concentrations of up to 300µg/ml of streptomycin dissolved in sterile water. The segregation states of the transformants were monitored by PCR of transformants DNA using primers recognizing sequences upstream and downstream of the *slr1125*-coding region. *Synechocystis* sp. PCC 6803 genomic DNA used for PCR analysisof mutants was prepared as described in He et al. [33]. In Fig. 1 a schematic representation shows the mechanism of gene deletion used in this work.

2.3 Carotenoids Analysis

Synechocystis sp. PCC 6803 cells in exponential growth phase (optical density at 730 nm ~ 0.5) were harvested by centrifugation. Cell pellets were frozen in liquid nitrogen and freeze-dried. Pigments were extracted from freeze-dried cells by three successive extractions with a 99.9 % methanol/ 0.1 % NH_4OH mixture and extracts were combined and evaporated under a stream of nitrogen until the samples were dry. Dried samples were dissolved in a small volume of the methanol/NH_4OH mixture and immediately subjected to high-performance liquid chromatography (HPLC) on an HP-1100 Chemstation with a Waters Spherisorb S5 OD S2 (4.0 mm x 250 mm) column filled with C-18 reversed phase silica gel, using a linear 18-min gradient of ethyl acetate (0 to 95%) in acetonitrile-water-triethylamine (9:1:0.01, v/v/v) at a flow rate of 1 ml/min. Absorption spectra of the eluted pigments were recorded continuously in the 360-665 nm range with an online photodiode array detector.

2.4 Mass Spectroscopy

To obtain isolated carotenoid fractions suitable for mass spectrometry, pigments were extracted as indicated above but were fractionated by a Waters Spherisorb S10 OD S2 (10 mm x 250 mm) semi-preparative column filled with C_{18} reversed phase silica gel, using a linear 18-min gradient of ethyl acetate (0 to 90%) and methylene chloride (0 to 5%) in acetonitrile-water-triethylamine (9:1:0.01, v/v/v) at a flow rate of 0.8 ml/min. Collected carotenoid fractions were freeze dried. Mass spectra were obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Voyager DE STR Biospectrometry Work Station, Foster City, CA). Before analysis, dried carotenoids were dissolved in 20 µl methylene chloride and 10 µl thereof was mixed with terthiophene (used as a matrix) dissolved in methylene chloride. Ions were generated by a pulsed 337-nm nitrogen laser and were accelerated to 20 kV. All the spectra were obtained in the reflectron mode with delayed extraction of 200 ns and were the result of signal averaging of 200-300 laser shots.

2.5 Identification of Carotenoids

Carotenoid species were identified by their absorption spectra, retention time and molecular mass [21,34]. Standard samples of lycopene, neurosporene, γ-carotene, and deoxymyxoxanthophyll, zeaxanthin, β-carotene, echinenone and myxoxanthophyll were available for verification. Lycopene and neurosporene were produced by *E. coli* strains harboring the pAC-LYC and pAC-NEUR plasmids, respectively (the plasmids were kindly provided by Dr. F. X. Cunningham Jr.). γ-carotene was purified from *Chlorobium tepidum* (kindly provided by Dr. R. E. Blankenship) and deoxymyxoxanthophyll was obtained from the Δcrt Rmutant strain [35].

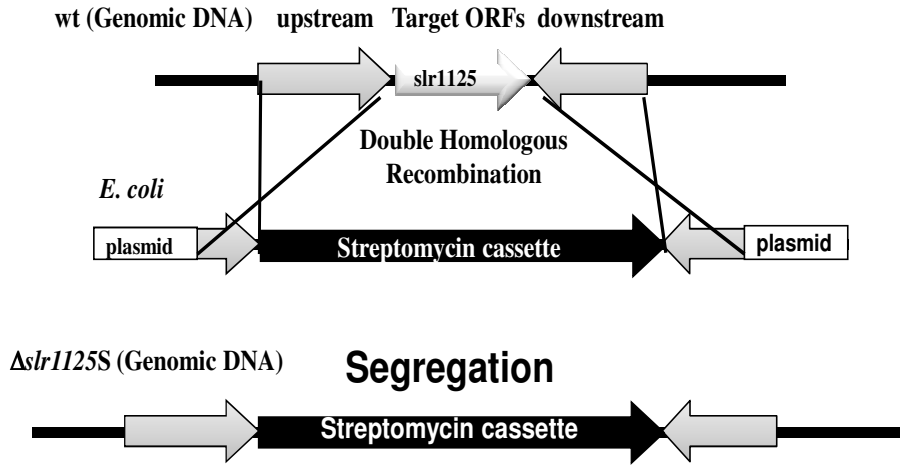


Fig. 1. Adepiction of the plasmid DNA construct containing the streptomycin resistance cassette (shown in black) and flanked by a portion of the genomic DNA (shown in gray) that surrounds the wild type *slr1125* gene (shown in light grey) undergoing double homologous recombination into the genome of *Synechocystis* sp. PCC 6803

3. RESULTS

3.1 Mutants Segregation and Phenotype

Homozygous segregated mutant was confirmed by PCR (Fig. 2). The fully segregated Δ*slr1125*S strain grew slowly on agar plates as well as in liquid culture under light-activated heterotrophic growth (LAHG) and photomixotrophic growth conditions at a light intensity of 0.5 μE m^{-2} s^{-1}. In addition, yellow pigments were excreted when grown at 100 μE m^{-2} s^{-1}. The growth medium of the mutant was shown to contain a mixture of carotenoids that were similar to carotenoids in the living cell. Therefore, this pigment presumably originated from lysed cells, a typical phenotype of cell wall dysfunction. However, photoautotrophically grown cells of the Δ*slr1125*S strain were bleached by exposure to a light intensity above 200 μE m^{-2} s^{-1}.

3.2 Ultrastructure of the ΔSlr1125SMutant Cell Wall

The ultrastructure of the mutant Δ*slr1125*S cell wall reveals a clear reduction in the peptidoglycan layer and absence of the S-layer protein (Fig. 3). Therefore, the cell wall layers seem to connect and anchor each other to form a viable active wall. Indeed, cell wall ultrastructure is consistent with the leakage phenotype observed during the growth of the Δ*slr1125*Smutant.

3.3 Pigment Analysis

3.3.1 At low light

The Δ*slr1125*S strain propagated at low light intensity of 0.5 μE m^{-2} s^{-1} showed a drastic decrease in the chlorophyll and carotenoid levels. Chlorophyll was decreased 10-fold relative to that of wild type on a per-cell basis. Major carotenoids were decreased 4-20 fold (Table 1), with myxoxanthophyll showing the most drastic reduction in the absorption fine structure (%III/II = 20-33 myxoxanthophyll isomer vs. 57 of the native myxoxanthophyll in wild type; Fig. 4). Indeed, Δ*slr1125*S cells still synthesize and accumulate the same carotenoids species that are present in wild type strain (Table 1).

3.3.2 At high light

Remarkable variety of new carotenoids accumulate in the Δ*slr1125*S strain when grown at 100 μE m^{-2} s^{-1} (Fig. 5). The total amount of these novel accumulated carotenoids represents 22.9% of the total amount of carotenoids present in the mutant (Table 1, 2). The absorption spectra of these compounds are shown in Fig. 6, and the maximum absorption wavelengths, absorption fine structures and molecular mass are provided in Table 3.

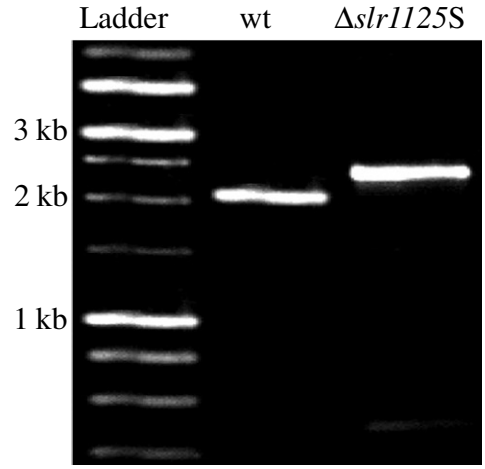


Fig. 2. DNA Agarose gel electrophoresis showing fully segregated mutant with a fragment of 2.3 kb compared to wild type (2.1 kb) of *slr1125* gene loci amplified with the same primers

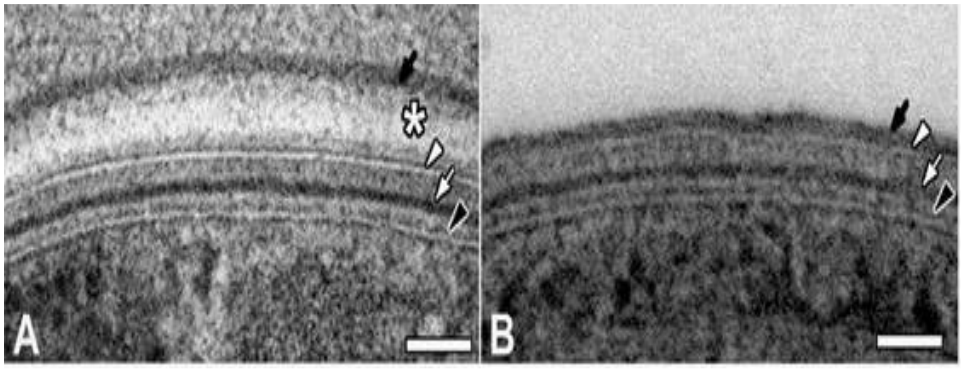


Fig. 3. The wall layers of wild type cells (A) consisted of the lipopolysaccharides (black arrow), S-layer (asterisk), outer membrane (white arrowhead), and peptidoglycan layer (white arrow). The cytoplasmic membrane (black arrowhead) is pointed out. The outer membrane (white arrowhead), peptidoglycan layer (white arrow) and cytoplasmic membrane (black arrowhead) of Δ*slr1125*S cells are shown (B). Scale bars in A-B = 50 nm

3.4 Carotenoids Accumulated in Δ*slr1125*S Mutant

In the Δ*slr1125*S mutant, Peak 1 exists in low quantity that makes it hard to identify. Peaks, 2 and 3, are unique to the mutant and accumulated over 8% of the total novel carotenoids. Peak 2 is a lycopene-type chromophore according to its absorption spectrum, and its retention time suggests that the chromophore has been modified in a way similar to myxoxanthophyll. According to its absorbance spectrum, the chromophore accumulated in peak 3 resembles neurosporene with nine conjugated double bonds (λ_{max} = 281-347-415-437-465); the molecule presumably is hydroxylated and glycosylated similar to myxoxanthophyll (m/z = 762), its presumable chemical structure is in Fig. 7. The reduction in the absorption fine structure (%III/II) from 82 for neurosporene to 54 suggests one or more cis-bonds in the molecule. At least three different carotenoids were eluted between zeaxanthin and chlorophyll (peaks 4, 5 and 6 in Fig. 5). These carotenoids are expected to be more hydrophobic than myxoxanthophyll or zeaxanthin, and may lack one of the polar groups. Peak 5 appears is deoxymyxoxanthophyll, based on its absorption spectrum and mass value (m/z= 742) and confirmed by comparing it to standard isolated from Δ*crtR* mutant [35], chemical structure presented in Fig. 7. The carotenoid eluting in

peak 6 has an absorption spectrum similar to γ-carotene (peak 10). The reduced retention time of the compound in peak 6 vs. that of γ-carotene suggests a deoxymyxoxanthophyll-type modification: hydroxylation and glycosylation was confirmed by the molecular mass (744 m/z). As the molecular mass is 2 mass units more than that of deoxymyxoxanthophyll (peak 5), the compound of peak 6 appears to have one less conjugated double bond, in agreement with its absorbance spectrum. For this reason, peak 6 is a 3′,4′-dihydro-deoxymyxoxanthophyll (1',2'-dihydro-γ-carotene 2'-(2,4-di-*O*-methyl-α-L-fucoside; Fig. 7). The third carotenoid eluting between zeaxanthin and chlorophyll was present in very small amounts (peak 4), but is interesting in that its absorbance spectrum suggests the presence of 12 conjugated double bonds (λ_{max} = 307-355-461-489-519 nm) (Fig. 6). Due to its low abundance, no mass determination could be performed for this carotenoid.

Carotenoids in peaks 7-11 that are found in extracts from the Δ*slr1125*S strain have a longer elution time than echinenone but shorter than β-carotene, suggestive of rather hydrophobic compounds. Based on comparison with a lycopene standard, peak 7 was identified as lycopene (λ_{max} = 297-449-475-505 nm, %III/II = 65), without any side modification (m/z = 536). The amount of lycopene accumulating at a light intensity of 100 μE m^{-2} s^{-1} was 160 ng/LOD_{730} cells (Table 2). Of the non-standard carotenoids accumulating in the Δ*slr1125*S strain but absent in the wild type. The carotenoid in peak 8 is similar to that found in peak 4 apart from a spectral shift suggestive of 11 (λ_{max} = 503 nm) (in peak 8) instead of 12 (λ_{max} = 519 nm) (in peak 4) conjugated double bounds. Peak 9 was assigned to a neurosporene isomer. Peak 10 was identified to be γ-carotene based on comparison with a standard and confirmed by its spectroscopic and mass analysis (λ_{max} = 287-441-465-494 nm, %III/II = 33 and m/z = 536; Table 3).

4. DISCUSSION

4.1 Bioinformatics of the slr1125 Open Reading Frame

Slr1125 of *Synechocystis* sp. PCC 6803 was annotated as a zeaxanthin glucosyl transferase in CyanoBase even though no glycosylated zeaxanthin has been observed in *Synechocystis* to date. The assignment presumably originated from a 31% identity between Slr1125 (403 AA) and the known bacterial zeaxanthin glucosyl transferase (CrtX) from *Pantoea ananatis* (431 AA) [37]. Four conserved glycosyltransferase domains are present in Slr1125 and CrtX. The first domain (residues 1-70) in Slr1125 is similar to glycosyltransferases that catalyze glycosylation of polyketide antibiotics by a methylated rhamnose [38]. The second domain (residues 80-270) is specifically conserved in CrtX proteins but not commonly present in other glycosyltransferases. The third and fourth domains (residues 270-350 and 350-402) are similar to the conserved domains of the glycosyltransferases that are involved in peptidoglycan biosynthesis. Interestingly, in *Crocosphaera watsonii* WH 8501 (previously known as *Synechocystis* sp. WH 8501) Slr1125 apparently has been split up into two ORFs (ZP_00177831 and ZP_00174102). The first ORF is expected to encode a small 137 aa protein that is annotated as a protease-like polypeptide. However, Blast analysis of this protein shows a clear similarity with the N-terminal region of members of the glycosyl transferase family that includes Slr1125. The second ORF encodes a 238 aa protein that is annotated as UDP-glucuronosyl transferase. This protein is similar to the C-terminal part of Slr1125 with 57% identity. Therefore, Slr1125 presumably is a product of gene fusion, which explains that Slr1125 appears to have functionally heterogeneous domains.

Highly conserved homologous of *slr1125* are very common in prokaryotes, over 700 sequences that are belongs to 93 species possess similar ORFs to the *slr1125*. Surprisingly, the agreed annotation of all these orthologs indicate that this ORF encode transglycosylase (MGTs) that uses lipid II precursor (undecaprenyl–pyrophosphate-N-acetylglucosaminyl-N-acetylmuramoyl-penta-peptide) to synthesize the glycan chain of the bacterial wall peptidoglycan. In Fig. 8 a strong identity is shown with the sequence alignment of Slr1125 protein and MGTs from *Nitrosococcus* sp. Bioinformatics data and leakage phenotype of the mutant is indicative that Slr1125 necessary to the cell wall biosynthesis.

Table 1. Pigments analysis of *Synechocystis* sp. PCC 6803 strains. Wild type and Δ*slr1125*S grown under 0.5 µE m^{-2} s^{-1} light intensity (^{LL}) and 100 µE m^{-2} s^{-1} light intensity (^{HL}). Values are averages from nine determinations performed on three different cultures

	Pigments content (µg/ml/10^8 cells)				
Pigments	**Chlorophyll**	**Myxoxanthophyll**	**Zeaxanthin**	**Echinenone**	**β-Carotene**
Wild type	4.1±0.20	0.26±0.050	0.44±0.040	0.21±0.030	0.47±0.050
Δ*slr1125*S^{LL}	0.2±0.02	0.02±0.003	0.10±0.004	0.03±0.005	0.04±0.005
Δ*slr1125*S^{HL}	5.6±0.02	1.12±0.003	0.35±0.004	0.22±0.005	0.38±0.005

Table 2. Novel carotenoids recorded in the methanol extract of Δ*slr1125*S grown at 100 µE m^{-2} s^{-1}. S: synechoxanthin, M: myxoxanthophyll, Z: zeaxanthin E: echinenone, β: β-carotene, the numbering is according to the peaks recorded in Fig. 5

Peak	**Carotenoid species**	**Relative amount ng/ml/10^8 cells**	
		Δ*slr1125*S mutant	**wild type**
S	Synechoxanthin	115	125
M	Myxoxanthophyll	1120	260
Z	Zeaxanthin	350	440
E	Echinenone	220	210
β	B-carotene	380	470
1	Unknown 1	12	NR
2	Lycopene glycoside	20	NR
3	Neurosporene glycoside	34	NR
4	Unknown 2	31	NR
5	Deoxymyxoxanthophyll	110	NR
6	γ-carotene-glycoside	50	NR
7	Lycopene	160	NR
8	Unknown 3	37	NR
9	Unknown 4	85	NR
10	γ-carotene	110	NR
T	Normal carotenoids	2185	1505
T	New carotenoids	649	NR
T	All carotenoids	2834	1505

** NR = Not recorded, T = total*

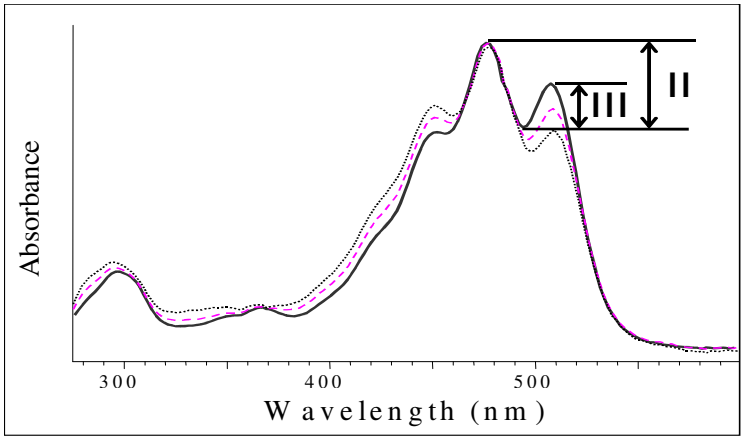


Fig. 4. The absorption spectra of myxoxanthophyll and myxoxanthophyll isomers (myxoxanthophyll peaks in wild type and mutant; Fig. 5). The fine absorption structure of the native myxoxanthophyll present in wild type (—; %III/II = 58) and the carotenoid glycoside (myxoxanthophyll isomers) present in the Δ*slr1125*S mutant (- - -; %III/II = 33 and ……; %III/II = 20). Where, %III/II is the ratio of the longest-wavelength absorption peak (III) divided by that of the middle absorption peak (II) multiplied by 100 and the minimum between these two peaks is the baseline [36]

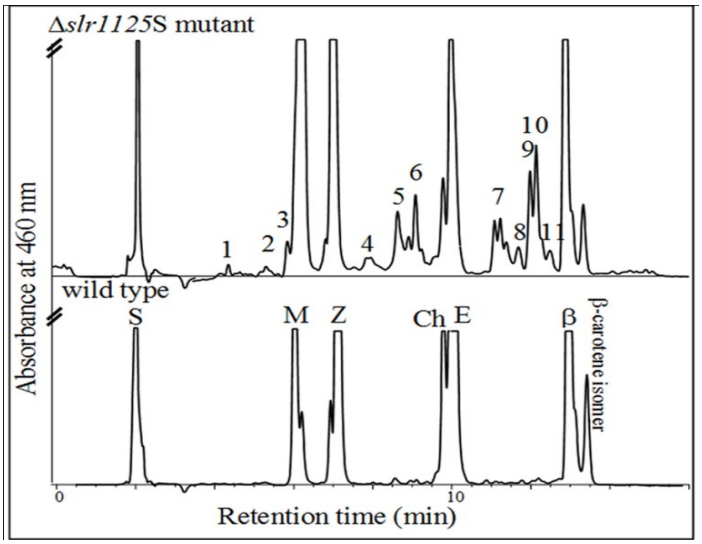


Fig. 5. HPLC analysis of pigments extract from the Δ*slr1125*S mutant and wild type. The methanol extracts of pigments from the Δ*slr1125*S mutant and wild type were analyzed using cultures grown at a light intensity of 100 µE m^{-2} s^{-1}. Detection was at 460 nm. β: β-carotene, E: echinenone, Ch: chlorophyll, Z: zeaxanthin, M: myxoxanthophyll and S: synechoxanthin.Wavelength maxima and the absorption fine structure of the peaks (1 – 11) observed in the mutant are listed in Table 3

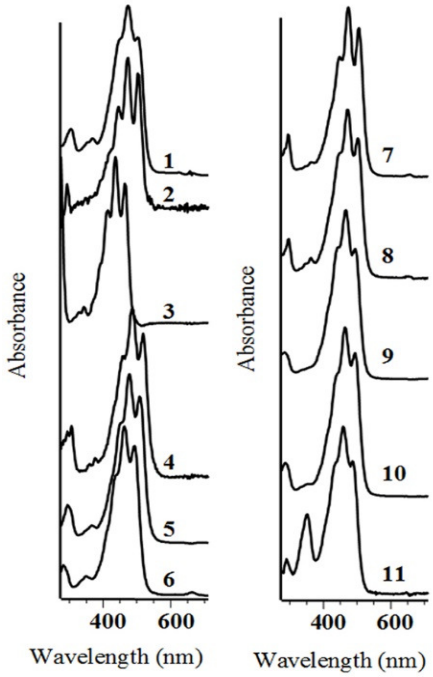
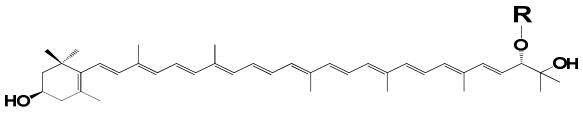


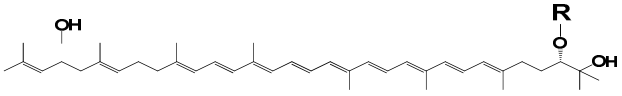
Fig. 6. Photodiode array absorption spectra of novel carotenoids present in (peaks 1 – 11) of the Δ*slr1125*S mutant grown at a light intensity of 100 µE m^{-2} s^{-1} (Fig. 5). Numbers near absorption spectra correspond to the number peaks in Fig. 5. Wavelength maxima and the absorption fine structure of these spectra are listed in Table 3

Table 3. Spectroscopic, chromatographic and mass spectrometry data of the normal carotenoids and newly accumulated peaks in Δ*slr1125*S strain of *Synechocystis* sp.PCC 6803.The retention time (R.T.), wavelength (nm), absorption fine structure (%III/II), molecular mass (m/z), and number of double conjugated bond (C.D.B.) for carotenoids are provided. S: synechoxanthin, M: myxoxanthophyll, Z: zeaxanthin E: echinenone, β: β-carotene, the numbering is according to the peaks recorded in Fig. 5. (----) indicates that no data is available

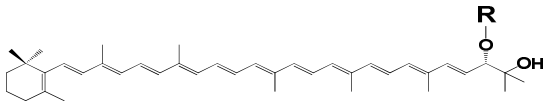
Peak	Spectroscopic and mass data of carotenoids				
No.	R.T. (min)	Wavelength (nm)	III/II (%)	Mass (m/z)	C.D.B.*(no.)
S	2.02	448-471-501	11%	588	9
1	4.02	305-369-451-475-503	12%	584	11
2	5.26	447-473-505	75%	----	11
2	5.81	281-347-415-437-465	54%	762	9
M	6.20	453-477-509	58%	758	11
Z	7.20	430-450-479	23%	568	9
4	7.82	307-355-461-489-519	52%	----	12
5	8.62	295-371-455-477-509	50%	742	11
6	9.08	285-351-441-463-493	45%	744	10
E	10.10	455	----	550	9
7	11.08	297-449-475-505	65%	536	11
8	11.36	295-363-449-473-503	40%	536	11
9	11.81	441-471	12%	538	9
10	12.12	287-441-465-494	33%	536	10
11	13.31	347-429-449-475	12%	536	9
β	12.00	427-450-478	18%	536	9



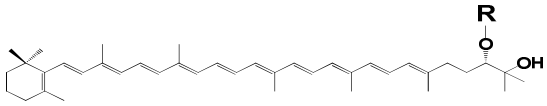
Myxoxanthophyll (Peak M in Fig. 5): (3R,2'S)-myxol 2'-(2,4-di-O-methyl-α-L-fucoside) [m/z= 758]



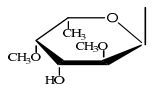
Neurosporene glycoside (Peak 3; Fig. 5): (1',2',?-trihydroneurosporene 2'-(2,4-di-O-methyl-α-L-fucoside) [m/z= 762]



Deoxymyxoxanthophyll (Peak 5; Fig. 5): (Deoxymyxol 2'-(2,4-di-O-methyl-α-L-fucoside) [m/z= 742]



γ-carotene glycoside (Peak 6; Fig. 5): 1',2'-dihydro-γ-carotene 2'-(2,4-di-O-methyl-α-L-fucoside) [m/z= 744]



R = **Dimethyl fucose**

Fig. 7. Chemical structure of selected carotenoids species accumulated in the Δ*slr1125*S mutant and compared to the known chemical structure of myxoxanthophyll identified from *Synechocystis* sp. PCC 6803 [17]

```
slr1125          ----------------MLPLGKELQQRGHTVTMFGVLDAQAKTLAAGLNFQAIATTEFP
Nitrosococcus    MTHFGLLCPALTGHLNTLLPLGQALQKRGHRVTLVGLLDAEPPTRAAGLEFRPIGETERP
                                 :****: **:*** **:.*:***:. * ****:*:.*. ** *

slr1125          LGAQAEFMAELGKLSGIKALQYTVAKITQKAAAFFEEAPGVMAKAGVEVLLVDQVSQEGG
Nitrosococcus    AGAIAELTAQASTLSGRAALRYAVAVFQQDAALLLREAPAVIKAAGIDALLVDQFSRGGG
                  ** **: *: ..***  **:*:** : *.** ::.***.*:  **::.*****.*: **

slr1125          TIGDRLGIPFISICSAVVLNREPTIPPYATPWPYDPSWLGQLRNRLGYGLLNRATKPITA
Nitrosococcus    TVADFLNLPFITLCSGVVLNREPSIPPFNTSWNYHPAWWAQLRNRLGYGLLSRVTQPLTE
                 *:.* *.:***::**.*******:***: *.* *.*:* .***********.*.*:*:*

slr1125          LINDYRQRWNLPAQSSPNDRYSPLAQISQQPAAFEFPRECLPSRFHFTGPFHSNVGRDIA
Nitrosococcus    VVAEYRRTWNLPPLSHPNEAYSQLAQISQQPAELEFPRQQLPPWFHFTGPYHSSGSREPV
                 :: :**: ****. * **: ** ********* :****: **. ******:**. .*: .

slr1125          DFPWEQLTDQPIIYASLGTIQNQLMSTFKIIAEACMDLDAQLIISLGG-AKLESMPALPG
Nitrosococcus    PFPWEKLTGQPLIYASMGTVLGRFKGVFQQIAAACEGLDAQLVISLGGSVPPEALPALPG
                  ****:**.**:****:**: .:: ..*: ** ** .*****:***** .  *::*****

slr1125          NPLVVNYAPQLELLQRTALTITHAGLNTTLECLNNAVPMVAIPIANDQPGVAARIAWAGV
Nitrosococcus    SPLVVGYAPQLELLQRAALTITHAGMNTTLESLSNGVPLVAIPIANDQPGVAARVAWTGA
                 .****.**********:********:*****.*.*.**:***************:**:*.

slr1125          GEFIPLSKLNTNNLRAALEKVLTEDSYKRNTLQLQQAIKTAGGLTKAADIIEQVTAEAMG
Nitrosococcus    GVRVPLKRLRVPRLRQAIAQVLTHDSYRKHALRFQAAIQRAGGVDQAVEIILQAVSTGKP
                 *  :**.:*.. .** *: :***.***::::*::* **: ***: :*.:** *..: .

slr1125          ------------
Nitrosococcus    VLRETNAPGMGG
```

Fig. 8. Amino acid sequence alignment between Slr1125 protein and glycosyltransferase of *Nitrosococcus* sp. (Accession: YP_003528194) that belongs to the MGT family protein. The sequence alignment shows over 77 % identical (*) and highly similar (:) amino acid residues between the two proteins

4.2 Ultrastructure of the ΔSlr1125S Mutant Cell Wall

The ultrastructure of the mutant Δ*slr1125*S cell wall support the bioinformatics predictions that Slr1125 is involved in peptidoglycan biosynthesis revealed by a clear reduction in the peptidoglycan layer and subsequent loss of the S-layer protein (Fig. 3). The absence of the surface layer is due to the presence of a well-conserved protein domain in the S-layer protein [39,40] that encored to the peptidoglycan layer [41] and/or to secondary wall polymers [42]. Indeed, the loss of S-layer is presumably an indirect effect of *slr1125* deletion. Therefore, cell wall layers seem to connect and anchor each other to form a viable active wall layers [43-45]. The reduced cell wall in the mutant is going to save cellular resources that might become available for other biosynthetic pathways in the cell (e.g. carotenoids).

4.3 Carotenoid in *Synechocystis sp.* PCC 6803

In Δ*slr1125*S cells grown at 100 μE m^{-2} s^{-1}, a wide range of novel carotenoids was found. As most of these carotenoids are glycosylated and/or hydroxylated, they appear to have gone through at least part of the myxoxanthophyll biosynthesis process. As at higher light intensity

the excess of glycosylated carotenoids increases. This observation provides evidence that the Δ*slr1125*S cells are likely possess the native carotenoid glycosyl transferase that has been identified as cruG (*sll1004*; Cyano Base) [46]. However, this plethora of glycosylated carotenoids and accumulation of large amount of myxoxanthophyll in the Δ*slr1125*S cells compared to wild type cells raises the question about the relationship between Slr1125 and carotenoid biosynthesis. An apparent explanation is that Slr1125 encode glycosyl transferase that uses sugar moiety that is probably competing with fucose biosynthesis [28]. This implies that fucose biosynthesis may be a rate limiting step in the myxoxanthophyll biosynthesis.

The observation that high light intensity increases the growth rate of the mutant as well as non-specific catalysis of carotenoids in the myxoxanthophyll biosynthesis pathway was made also in the Δ*sll1213*Z mutant of *Synechocystis* sp. PCC 6803 that lacks GDP-fucose synthetase [28]. Light might have two effects: (1) light can induce isomerization of carotenoids [47,48] that may enhance non-specific substrate recognition by carotenogensis enzymes and thereby increase non-specific catalysis; (2) the redox state of component in the photosynthetic electron transport chain affect carotenoid biosynthesis-related gene expression, as is the case in the green alga *Haematococcus pluvialis* [49]. Induction of early carotenoid biosynthesis genes will enhance the level of intermediates in the pathway, enhancing side reactions.

The effect of Slr1125 protein in the carotenoid/chlorophyll biosynthesis pathway explains in part why it is required for optimal growth under photoautotrophic growth conditions [50]. This work delivers interesting evidence that Slr1125 protein affect the photosynthetic ability through the regulation of pigment biosynthesis in this cyanobacterium. The observation that different amount of carotenoid intermediates are accumulated indicating that terminal carotenoids biosynthesis genes are tightly regulated and some enzymes in the pathway are rate limiting steps.

To date, the Δ*slr1125*S strain appears to have the largest spread of carotenoids in *Synechocystis* sp. PCC 6803. Part of these compounds may be biosynthesis intermediates, whereas others may be unusual secondary metabolites formed due to relatively low specificity of the carotenoid biosynthesis enzymes. The deletion and/or a reduction of a glycosylation reaction is a promising scenario to metabolically engineer glycosylated metabolites. Controlling the metabolic flux is evident by employing synthetic biology tool-box as in *E. coli* and yeast [51-54]. Indeed, understanding the cross talk and interconnectedness between cellular processes is critical for metabolic engineering to enrich bioactive library and commercially produce high-value chemicals.

5. CONCLUSION

The deletion of the putative glucosyl transferase (Slr1125) in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 increases myxoxanthophyll and induces the formation of unusual carotenoid species that are distinctive to this strain. The bioinformatics, physiological, and ultrastructure analyses are indicative that Slr1125 is involved in cell wall formation and indirectly affect S-layer edifice. Indeed, understanding the crosstalk between competing biosynthetic pathways delivers an attractive approach that is critical for metabolic engineering to enrich bioactive library and commercially produce high-value chemicals.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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