

**Ph.D. Thesis**

**CAROTENOIDS ASSIST IN ASSEMBLY AND FUNCTIONS OF  
PHOTOSYNTHETIC COMPLEXES IN CYANOBACTERIA**

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## TABLE OF CONTENTS

0.0 PROLOG.....	5
1.0 INTRODUCTION .....	6
1.1 Photosynthesis.....	6
1.1.1 Light Harvesting Antenna System.....	7
1.1.2 Major photosynthetic complexes in thylakoid membrane.....	8
1.1.2.1 Photosystem II .....	8
1.1.2.2 Cytochrome <i>b<sub>6</sub>f</i> .....	11
1.1.2.3 Photosystem I .....	11
1.2 Carotenoids .....	13
1.2.1 Chemical structure and some physiological properties of carotenoids.....	13
1.2.2 Functional roles of carotenoids in photosynthesis.....	15
1.2.2.1 Light harvesting .....	15
1.2.2.2 Photoprotection.....	16
1.2.2.3 Membrane structure organization .....	17
1.3 Model Organism: Cyanobacterium <i>Synechocystis</i> sp. PCC 6803 .....	18
1.4 Biosynthetic pathway of carotenoids in <i>Synechocystis</i> .....	18
1.5 Localization of carotenoids in photosynthetic complexes .....	21
1.5.1 Localization of $\beta$ -carotene molecules in photosystem II.....	21
1.5.2 Localization of a $\beta$ -carotene molecule in cytochrome <i>b<sub>6</sub>f</i> .....	23
1.5.3 Localization of $\beta$ -carotene molecules in photosystem I .....	23
1.6 Phosphatidylglycerol in photosynthesis.....	24
1.7 Earlier studies on carotenoid mutants in cyanobacteria.....	25
2.0 AIMS.....	28
3.0 MATERIALS AND METHODS.....	29
3.1 Construction of <i>Synechocystis</i> $\Delta$ <i>crtH/B</i> mutant.....	29
3.2 Organisms and growth conditions .....	29
3.3 Chlorophyll content analysis.....	30
3.4 Carotenoid content analysis .....	30
3.5 Absorption spectroscopy.....	31
3.6 Low-temperature (77 K) fluorescence spectroscopy .....	31
3.7 RNA isolation and Northern blot analysis .....	31
3.8 Radioactive labeling of the cells .....	31
3.9 Thylakoid membrane isolation .....	32
3.10 Protein analyses .....	32
3.11 Oxygen-evolving activity measurements.....	33

3.12 Chl <i>a</i> fluorescence efficiency (Fv/Fm) and the maximum changes in P700 signal (Pm-Po), and oxidation-reduction measurements of P700 kinetics .....	34
4.0 RESULTS .....	35
4.1 Generation of carotenoid-less $\Delta crtH/B$ strain in <i>Synechocystis</i> .....	35
4.2 Physiological consequences of the carotenoid deficiency .....	36
4.2.1 Growth rate and light sensitivity .....	36
4.3 Structural consequences of carotenoid deficiency .....	40
4.3.1 Level of the large protein subunits of photosynthetic complexes .....	40
4.3.2 Assembly of the photosynthetic complexes and their large protein subunits.....	41
4.3.3 Stabilization of the small protein subunits binding to the large protein subunits in photosystem II .....	43
4.3.4 Assembly of the functioning photosystem II core complex .....	45
4.3.5 Level of genes encoding PSII large protein subunits .....	47
4.4 Functional consequences of carotenoid deficiency.....	48
4.4.1 Oxygen-evolving activity of the cells.....	48
4.4.2 Maximal photosystem II efficiency in dark adapted state (Fv/Fm).....	48
4.4.3 Maximal redox changes in P700 signal in dark adapted state (Pm-Po) .....	49
4.4.4 Oxidation-reduction kinetics of P700.....	51
4.5 Phosphatidylglycerol (PG) depletion induces an increase in myxoxanthophyll and echinenone biosynthetic activities in <i>Synechocystis</i> PCC 6803 cells.....	52
5.0 DISCUSSION .....	57
5.1 Structural and functional consequences of the carotenoid deficiency in the <i>Synechocystis</i> .....	57
5.2 Phosphatidylglycerol (PG) depletion induces an increase in myxoxanthophyll and echinenone biosynthetic activities in <i>Synechocystis</i> PCC 6803 cells.....	65
6.0 CONCLUSIONS.....	68
AKNOWLEDGEMENTS.....	69
REFERENCES .....	70
ÖSSZEFOGLALÓ.....	80
SUMMARY.....	85
PUBLICATIONS.....	90

## ABREVIATIONS

Abs <sub>max</sub>	Absorbance maximum
Chl	Chlorophyll
CP43 and CP47	Photosystem II antenna chlorophyll-protein subunits
Cyt <i>b<sub>6</sub>f</i>	Cytochrome <i>b<sub>6</sub>f</i> complex
D1 and D2	Photosystem II core complex protein subunits
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
Fo	Fluorescence yield of open PSII RCs in dark-adapted state
Fm	Maximum fluorescence yield of closed PSII RCs in dark-adapted state
Fv	Maximum fluorescence change in dark-adapted state (Fm-Fo)
iD1	Intermediate form of D1 protein subunit after removal of part of the C-terminal extension
HPLC	High performance liquid chromatography
LAHG	Light-activated heterotrophic growth
NPQ	Non-photochemical quenching
OD	Optical density
OEC	Oxygen-evolving complex
PBS	Phycobilisome
pD1	Precursor form of D1 protein subunit with complete C-terminal extension
PG	Phosphatidylglycerol
<i>pgsA</i>	The mutant deficient in phosphatidylglycerol (PG) synthesis
Pm	Maximum P700 signal in dark-adapted state
Po	Minimum P700 signal in dark-adapted state
PsaA and PsaB	Photosystem I core complex protein subunits
P680	Primary electron donor of PSII reaction center
P700	Primary electron donor of PSI reaction center
RC47	Intermediate PSII complex lacking inner antennae CP43 protein subunit
RCC(1) and RCC(2)	Monomeric and dimeric PSII complexes, respectively
PSI(1) and PSI(3)	Monomeric and trimeric PSI complexes, respectively

RC	Reaction center
RCa	Intermediate PSII complex containing D1 (or iD1), D2 and Cyt <i>b</i> -559 but lacking CP47 and CP43
ROS	Reactive oxygen species
2D-BN/SDS-PAGE	Two-dimensional blue native/sodium dodecyl sulfate polyacrylamide gel electrophoresis
$\Delta crtH$	The mutant deficient in <i>cis</i> - to <i>trans</i> - carotene isomerase encoded by <i>crtH</i> gene in <i>Synechocystis</i>
$\Delta crtH/B$	The mutant deficient in the phytoene synthase encoded by <i>crtB</i> gene

## **0.0 PROLOG**

Cyanobacteria are a fascinating and versatile group of bacteria of immense biological importance. In earth history cyanobacteria as nitrogen and carbon-fixing organisms have a special position and they play a significant role in Earth's nitrogen and carbon cycles. Cyanobacteria are able to perform nitrogen and carbon fixation, which are often competing metabolic processes in the same cell. Moreover, their ancestors are the first oxygen-evolving organisms, which were responsible for the oxygen in the atmosphere due to their photosynthetic activity, which was invented 2.8 billion years ago. Cyanobacteria even nowadays contribute up to 30% of the yearly oxygen production on Earth.

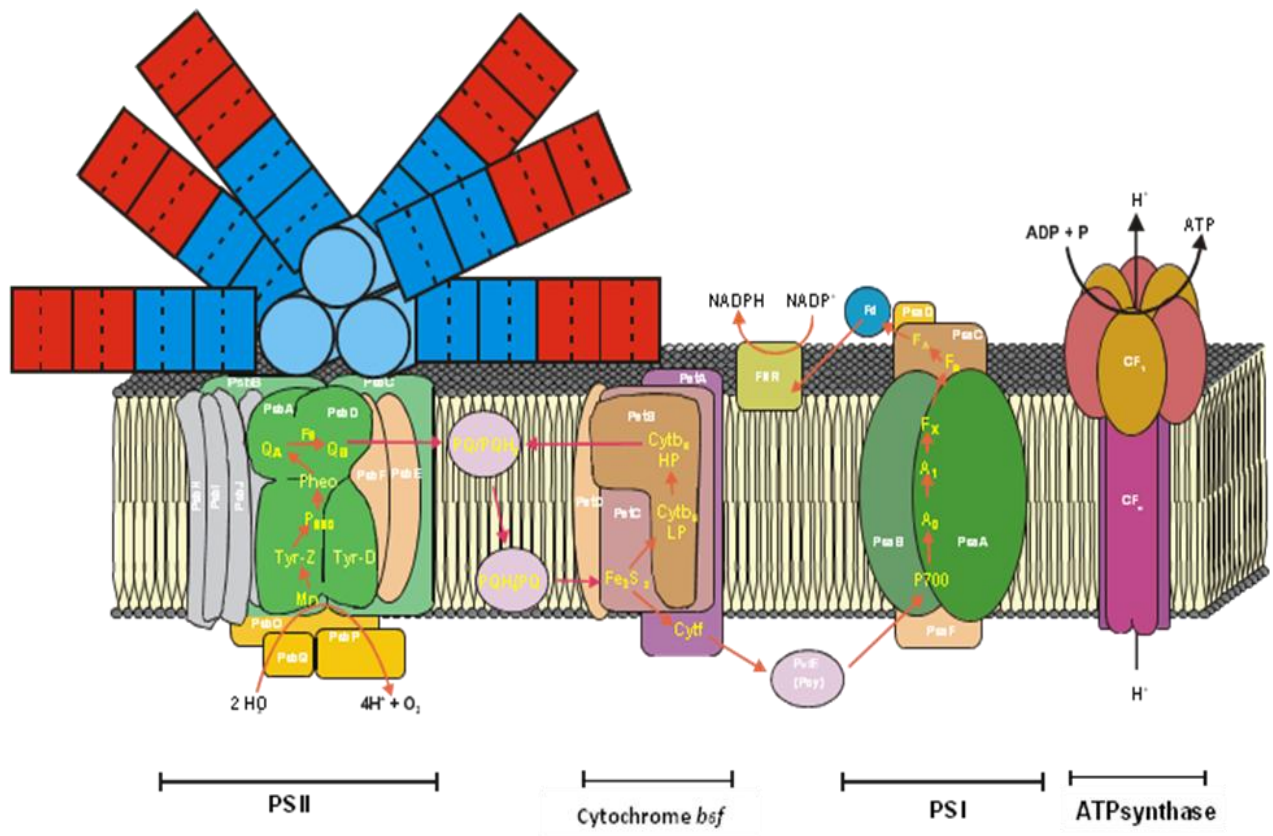
Carotenoids are ubiquitous compounds playing a role in a range of special processes. Thus, they are regarded as essential compounds for life mainly due to their various roles in photosynthesis. During the last couple of year interest in these pigments get into the focus of plant biology. Recently, various genes involved in carotenoid biosynthesis of several bacterial organisms have been indentified, which offers new opportunities for the genetic engineering of carotenoid synthesis in vivo.

Complete genome sequences and the transformability of several cyanobacterial strains allow us to generate various mutants and study the importance of carotenoids in photosynthetic processes. We aimed to generate a carotenoid deficient mutant in order to understand the structural and functional roles of carotenoids in photosynthesis.

## 1.0 INTRODUCTION

### 1.1 Photosynthesis

Photosynthesis is a process by which the energy of sunlight is converted into chemical energy in higher plants, algae and cyanobacteria. Photosystem II (PSII), photosystem I (PSI), cytochrome *b<sub>6</sub>f*, (Cyt *b<sub>6</sub>f*), and ATP-synthase are the four major protein complexes embedded in the thylakoid membranes. Both PSI and PSII reaction centers (RCs), which carry out light-driven electron transfer, are surrounded by light-harvesting antenna systems, which consist of protein-pigment complexes. Both photosystems are coupled functionally by a plastoquinone pool, the Cyt *b<sub>6</sub>f* complex, and plastocyanin or cytochrome *c<sub>6</sub>* (Fig. 1).



**Figure 1: Major photosynthetic complexes of thylakoid membrane of cyanobacteria**

Major complexes (Photosystem II, cytochrome *b<sub>6</sub>f*, photosystem I and ATPsynthase) and light driven electron transfer chain across the membrane. The figure was adapted from Dr. Donald A. Bryant (1994) and modified by Dr. László Kovács.

### 1.1.1 Light Harvesting Antenna System

Photosynthetic organisms have additional peripheral light harvesting antenna complexes containing different pigments, which are specifically associated with proteins in a unique structure of photosynthetic apparatus in order to overcome the light limited conditions by extending the spectral range of absorbance. The large protein antenna complexes of higher plants contain chlorophyll *a* (Chl *a*), chlorophyll *b* and xanthophyll pigments. Some marine organisms, such as diatoms and brown algae utilize xanthophylls, Chl *a*, and chlorophyll *c* within a single light harvesting antenna complex. Red algae and cyanobacteria have phycobilisomes. Although the various peripheral light-harvesting antennas differ significantly among the photosynthetic organisms, their common functions are to absorb light energy and to control dissipation of excess excitation energy under light stress, and to regulate energy flow between the two photosystems.

In cyanobacteria, there are specific light harvesting antenna complexes, phycobilisomes (PBSs), which consist of phycobiliproteins (Fig. 1). In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) there are two blue-pigmented phycobiliproteins: phycocyanin (Abs<sub>max</sub> 620 nm) and allophycocyanin (Abs<sub>max</sub> 650 nm). Some cyanobacteria also have phycoerythrin, a red-pigmented phycobiliprotein (Abs<sub>max</sub> 560 nm). The PBSs contain core and rod substructures. Phycoerythrin and phycocyanin form the rods, which efficiently absorb and transfer excitation energy to the PBS core, which consist predominantly of allophycocyanin (Liu et al. 2005). The PBS core also contains terminal emitters (Abs<sub>max</sub> 683 nm), which tunnel light energy from the PBSs to the RCs. The exact interaction between the PBS and the photosystems is still under debate. It is known that a PBS can also be decoupled from the Photosystems, leading to long-lived excitation (Bittersmann and Vermaas 1991). PBS excitation energy distribution between the two photosystems is regulated, in order to optimize photosynthetic performance (Mullineaux 2008). PBSs are also subject to carotene-triggered dissipation of absorbed energy as heat. The phenomenon is known as non-photochemical quenching (NPQ) in cyanobacteria (Rakhimberdieva et al. 2010)



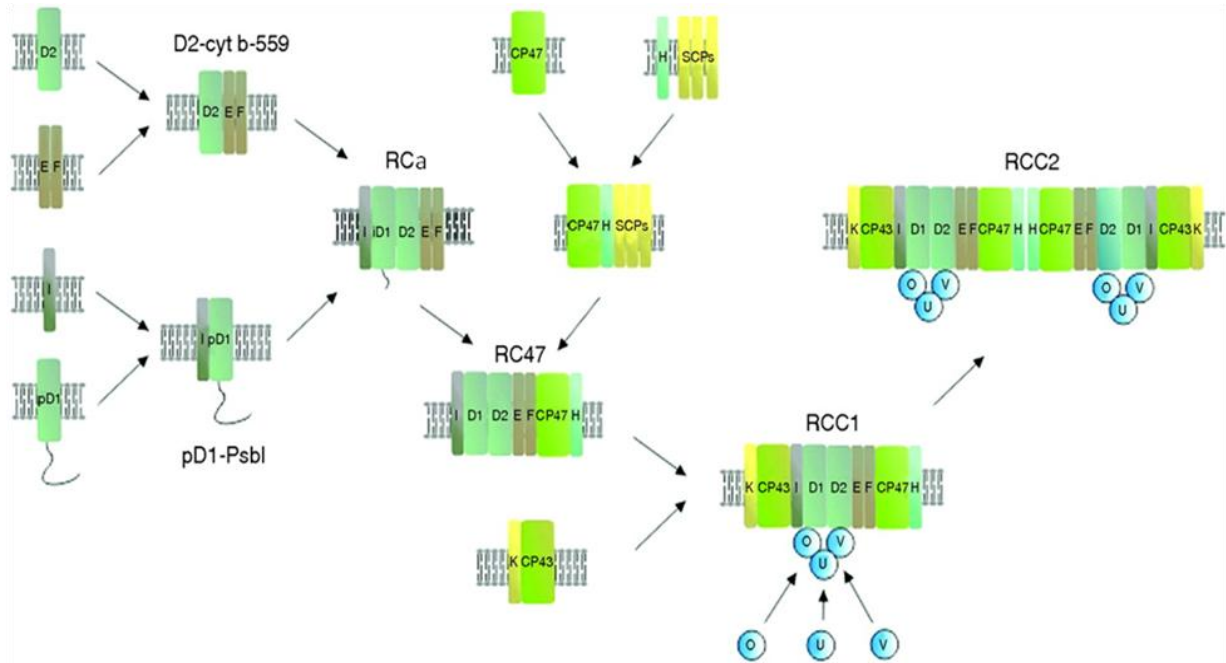
## 1.1.2 Major photosynthetic complexes in thylakoid membrane

### 1.1.2.1 Photosystem II

The PSII complex has been conserved among cyanobacteria, algae and higher plants. The X-ray crystallographic structure of the monomeric PSII complex from *Thermosynechococcus elongatus* (*T. elongatus*) consists of 17 intrinsic and 3 extrinsic protein subunits, 35 Chl *a*, 11  $\beta$ -carotene, 2 haem, and one non-haem iron molecules, two calcium ions, at least 25 integral lipids, 1-2 chloride ion(s), 3 plastoquinones and  $\text{CaMn}_4$  cluster (Ferreira et al. 2004, Loll et al. 2005, Guskov et al. 2009, Guskov et al. 2010).

The membrane intrinsic part of monomeric PSII consists of the core complex protein subunits D1 and D2, each containing 5 transmembrane  $\alpha$ -helices which bind Chl, pheophytin and plastoquinone co-factors (Rappaport and Diner 2008). In addition, there are intrinsic antenna protein subunits CP47 and CP43, each containing 6 transmembrane  $\alpha$ -helices, which bind 26 Chl *a* and 9  $\beta$ -carotene molecules (Loll et al. 2005). These four large protein subunits are surrounded by 13 low-mass small protein subunits on the periphery of the PSII. Cytochrome b-559 (Cyt b-559) consists of two transmembrane  $\alpha$ -helices; PsbE and PsbF represents two of those small protein subunits. Cyt b-559 is able to accumulate in the membrane in the absence of D1 and D2 (Komenda et al. 2004). It seems to act as a nucleation factor to initiate PSII assembly: the formation of the first Cyt b-559-D2 subcomplex (Fig. 2) (Komenda et al. 2004, Komenda et al. 2008). After the integration of D1 and PsbI, (Dobakova et al. 2007), an intermediate PSII complex, RCa is formed (Fig. 2) (Komenda et al. 2008). In the absence of CP47, CP43 is unable to attach stably to the subcomplex (Komenda et al. 2008), although in the absence of CP43, CP47 is able to attach to the subcomplex and forms the next intermediate PSII complex, RC47 (Fig 2) (Komenda et al. 2004). RC47 is unable to oxidize water but is still able to drive oxidation of redox-active tyrosine, TyrZ, in D1. The subsequent attachment of CP43 allows the formation of monomeric PSII complex (RCC(1)) (Fig. 2), which is the starting point for the light-driven assembly of the  $\text{CaMn}_4$  cluster together with the attachment of the extrinsic protein subunits PsbO, PsbV and PsbU. They are located at the luminal side of the core complex assembly and contribute to the stability of the  $\text{CaMn}_4$  cluster (Fig. 2) (Roose et al. 2007, Enami et al. 2008). Finally two monomeric PSII complexes form a dimeric PSII complex (RCC(2)) (Fig. 2). Both monomeric and dimeric PSII complexes can be formed in whole cells and isolated

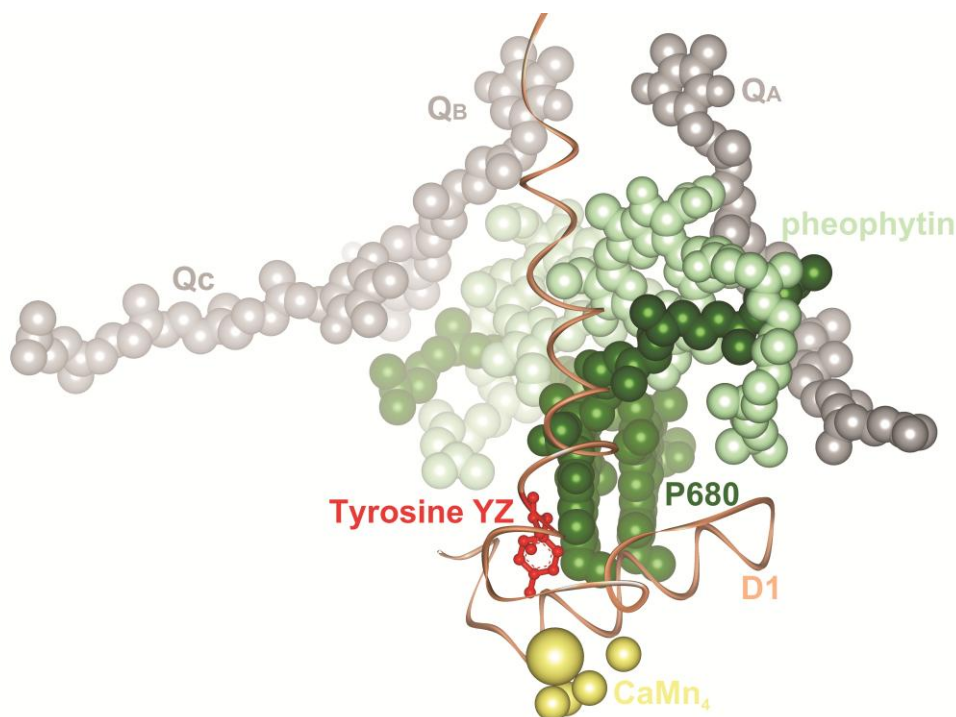
thylakoid membranes of *Synechocystis* and *T. elongatus* with dimeric form being the more active form (Nowaczyk et al. 2006).



**Figure 2: Assembly of PSII complex in *Synechocystis*.**

The small protein subunits PsbE, PsbF, PsbH, PsbI and PsbK and the extrinsic protein subunits PsbO, PsbU and PsbV are designated by the upper case letter. Cytochrome b-559 (cyt b-559) is composed of a heterodimer of PsbE and PsbF. Types of PSII complexes: RCa, intermediate PSII complex containing either mature D1, intermediate D1 (iD1), or precursor D1 (pD1) but lacking CP43 and CP47; RC47, intermediate PSII complex lacking CP43; RCC(1), monomeric PSII complex; RCC(2), dimeric PSII complex. For clarity, assembly of the cofactors and many of the small protein subunits is not included. The figure was adapted from Nixon et al. (2010).

During biogenesis of PSII, D1, which is synthesized in most organisms as a precursor protein, pD1, with a C-terminal extension (16 amino acid extension), must be cleaved to allow the assembly of a functional  $\text{CaMn}_4$  cluster. During C-terminal extension processing, the intermediate of D1, designated iD1 can be found (8 amino acid extension), (Komenda et al. 2007). The iD1 is found mainly in PSII complexes in vivo (Komenda et al. 2004). C-terminal extension is not required for assembly of the oxygen-evolving complex (OEC) of PSII (Satoh and Yamamoto 2007). However, mutants either lacking the extension or containing modified C-terminal extension are in general much more susceptible to photoinhibition (Ivleva et al. 2000, Kuvikova et al. 2005).



**Figure 3: Electron transport chain in PSII RC**

Major participants: P680, (dark green); pheophytin, (light green);  $Q_A$ ,  $Q_B$ , and  $Q_C$  (grey);  $CaMn_4$  cluster (yellow) and tyrosine Z (red). The figure was created by the software Discovery Studio Visualizer (DSV) (<http://accelrys.com/products/discovery-studio/visualization>) and the PDB file 3BZ1 (Guskov et al. 2009).

Light energy captured by the light harvesting antenna complexes is transferred to PSII RC. In the RC, light-induced charge separation takes place leading to the oxidation of P680 ( $P680^+$ ), which is a heterodimeric Chl *a* pair. The electron released from excited P680 ( $P680^*$ ) travels along the redox-active cofactors of the electron transfer chain. Pheophytin is the first electron acceptor. Then two plastoquinone molecules  $Q_A$  and  $Q_B$  receive electrons from pheophytin. The tightly bound  $Q_A$  acts as electron transmitter and the mobile  $Q_B$  is the substrate of the quinone reductase part of PSII complex (Fig. 3). An additional plastoquinone molecule ( $Q_C$ ) with unresolved function has been located next to the  $Q_B$  binding site. After two charge separation events, the doubly reduced  $Q_B$  ( $Q_B^{2-}$ ) binds two protons from the stromal side of the membrane yielding a fully reduced plastohydroquinone,  $PQH_2$ .

$P680^+$  has an exceptionally strong oxidizing power and is able to abstract electrons via redox active tyrosine Z from the OEC, a heteronuclear  $CaMn_4$  cluster located at the luminal side of PSII (Fig. 3). After the accumulation of four positive charges, water is oxidized to molecular oxygen. The oxidation of two water molecules in PSII results in the

release of four protons in the lumen. This contributes to the formation of the electrochemical gradient that drives ATP synthesis.

#### **1.1.2.2 Cytochrome *b<sub>6</sub>f***

The Cyt *b<sub>6</sub>f* complex, which is another large membrane-bound protein complex, lies between PSII and PSI complexes in the thylakoid membrane. In general the native dimeric form consist of eight protein subunits and several cofactors legated to the proteins (Kurusu et al. 2003). Four of these are considered the large protein subunits and include Cyt *f*, the Rieske iron-sulfur protein, Cyt *b<sub>6</sub>*, and subunit IV.

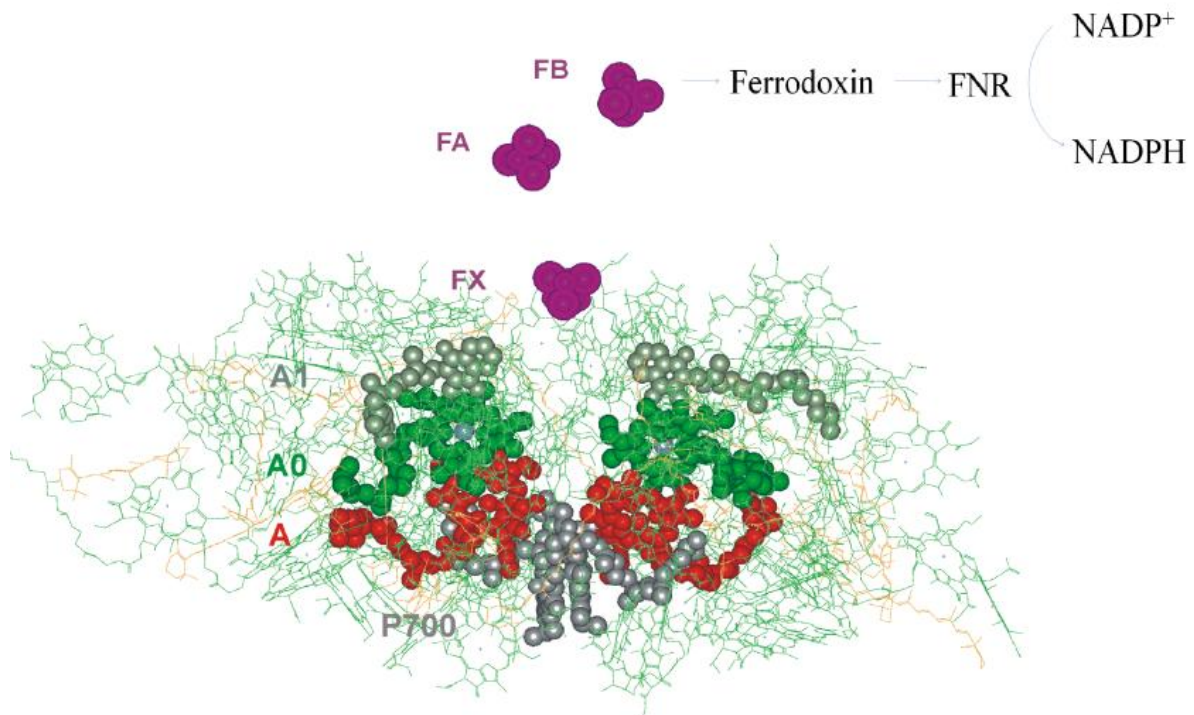
Electrons are transferred through mobile plastoquinone (PQH<sub>2</sub>) molecules from PSII to the Cyt *b<sub>6</sub>f* complex. Cyt *b<sub>6</sub>f* donates the electrons to one of the lumenal electron carrier proteins, plastocyanin, (or in some cases to Cyt *c<sub>6</sub>*), which carries the electron, to PSI. Further, the reoxidation of two PQH<sub>2</sub> by Cyt *b<sub>6</sub>f* also results in the release of an additional four protons in the lumen, thereby contributing to the formation of the electrochemical gradient that drives ATP synthesis.

#### **1.1.2.3 Photosystem I**

X-ray crystal structure of the monomeric PSI complex from *T. elongatus* exhibits 12 protein subunits which are bound to 127 cofactors performing light capturing and electron transfer (Jordan et al. 2001, Fromme et al. 2001, Grotjohann and Fromme 2005, El-Mohsnawy et al. 2010). These cofactors include 96 Chl *a* and 22 carotenoids, two phylloquinones, three iron-sulfur cluster and four lipid molecules. The central part of the core complex is formed by the heterodimer of the two large protein subunits PsaA and PsaB, comprising 22 transmembrane helices, located in the center of the monomeric PSI complex. They coordinate most of the Chl *a*, carotenoids and the redox cofactors of electron transport (Amunts and Nelson 2009). PSI exists in vivo in monomeric and trimeric forms. The trimeric PSI is the most abundant in the thylakoid membrane of *T. elongatus* and the formation of trimeric form is essential for the growth of the cells at low light intensity (Grotjohann and Fromme 2005). Trimeric PSI form also plays an important role in the assembly and structural integrity of PSI (Grotjohann and Fromme 2005). Interaction of antenna Chl *a* of the monomeric forms within the trimeric form leads to a larger amount of long-wavelength Chl *a*, resulting in a higher photochemical activity of the trimeric form under red or far-red illumination. The dynamic equilibrium between monomeric and trimeric

forms in the thylakoid membrane may indicate a transient monomeric population in the course of biogenesis and could also be the basis for short-term adaptation of the cell to changing environmental conditions (El-Mohsnawy et al. 2010).

PSI catalyzes the light driven electron transfer from plastocyanin, which is located on the luminal side of the thylakoid membrane, to ferredoxin, which is located on the stromal side of the membrane. In some cyanobacteria and green algae, Cyt *c*<sub>6</sub> can replace plastocyanin as an electron donor to PSI. Excitation energy captured by antenna pigments is funneled to P700, a heterodimer of Chl *a* pair in PSI RC where the charge separation takes place. The electron is transferred from P700 stepwise to A and A<sub>0</sub> (Chl *a* molecules), A<sub>1</sub> (a phylloquinone molecule) and from there subsequently to the iron-sulfur clusters, FX, FA and FB. The latter mediates electron transfer to ferredoxin, which act as soluble electron carrier. Ferredoxin transfers the electron to the ferredoxin NADP<sup>+</sup>-reductase enzyme (FNR), which then finally reduces NADP<sup>+</sup> to NADPH (Fig. 4). To complete the cycle, P700<sup>+</sup> is re-reduced.



**Figure 4: Electron transport chain in PSI RC.**

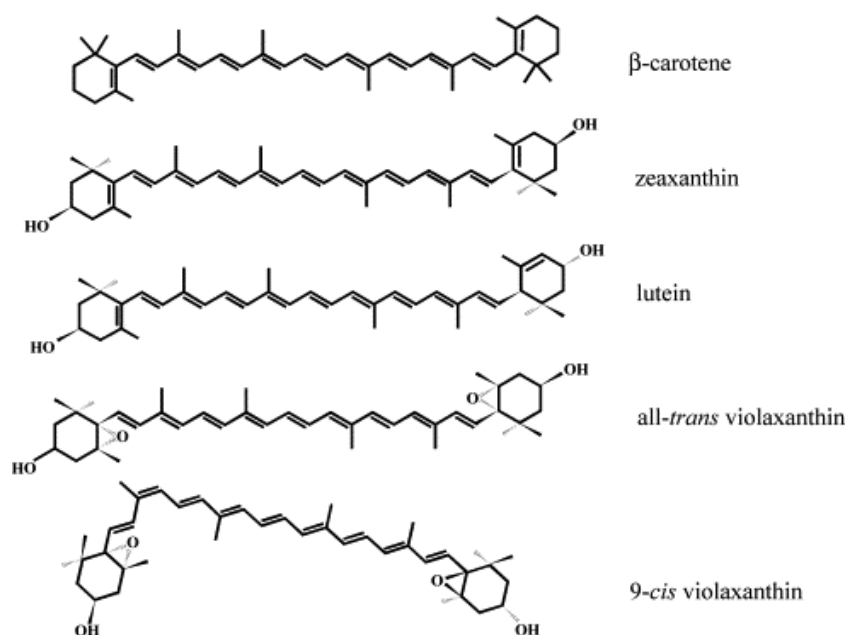
Major participants: P700, grey; A and A<sub>0</sub>, red and green, respectively; A<sub>1</sub>, pale green; FX, FA and FB (magenta). The figure was created by the software DSV and the PDB file 1JB0 (Jordan et al. 2001).

## **1.2 Carotenoids**

Carotenoids represent the most fascinating, abundant and widely distributed classes of natural pigments. From photosynthetic organisms to anoxygenic bacteria through cyanobacteria, algae and higher plants (Frank and Cogdell 1996), as well as numerous non-photosynthetic bacteria and fungi (Goodwin 1980) produce carotenoid pigments. Until now, more than 700 naturally-occurring carotenoid pigments have been identified (Britton et al. 2004).

### **1.2.1 Chemical structure and some physiological properties of carotenoids**

Their structures are consistent with the assumption that they are produced by joining together isoprene units, usually in a "head to tail" fashion. Isoprene is the common name for 2-methyl-1,3-butadiene. Most naturally occurring carotenoid pigments are tetraterpens (eight isoprene units) (Fig. 5). A very important property of carotenoids, both from spectroscopic and structural points of view, is the presence of double bonds in conjugated system which forms the chromophore responsible for the molecule color. A conjugated double bond system of a polyene longer than nine is responsible for the pigment properties of carotenoids (Fig. 5) (Gruszecki and Strzalka 2005). The conjugated double bond system constitutes a rigid, rod-like skeleton of carotenoid molecules. This feature seems to play a key role in the stabilization function of carotenoids, both with respect to lipid membranes and proteins.



**Figure 5: Chemical formulas of serial carotenoid pigments**

All-*trans*-  $\beta$ -carotene, zeaxanthin, lutein, violaxanthin and 9-*cis*- violaxanthin

Carotenoids are classified in two subgroups, carotenes and xanthophylls. Carotenes are pure hydrocarbons with cyclic ionone rings at one or at two sides.  $\beta$ -carotene which is responsible for the orange color in plants and animals is the most known carotene (Fig. 5). In several cases, the cyclic ionone rings at one or at both sides are complemented with oxygen functional groups such as hydroxy, keto or epoxy (Fig. 5). These oxygenated carotene derivatives are called xanthophylls. Since carotenes are hydrocarbons without any polar group, they are fat-soluble and extremely hydrophobic, non-polar molecules. In contrast to xanthophylls, which contain polar groups, carotenes are hardly soluble in water. Thus, carotenes are expected to be restricted to hydrophobic region in the cell, such as the inner core of membranes, except in association with a protein, which allows them to access the aqueous phase. Obviously, polar functional groups alter the polarity of carotenoids and affect their interactions with other molecules (Britton 1995a).

In general, carotenoids may exist as different stereoscopic isomers. Each double bond can exist in *trans*- or *cis*- configuration (Goodwin 1980). In nature carotenoids exist primarily in the thermodynamically more stable all-*trans*- configuration rather than in *cis*-configuration. It has been well-documented that polar carotenoids, in the all-*trans*-configuration, are linear, rigid molecules and they are effective modulators of the fluidity of

natural and model lipid membranes (Gruszecki and Strzalka 2005). The *cis*-isomers, however, are not simple linear molecules. Their overall shape differs substantially from that of the all-*trans* form, so their ability to fit into subcellular structures is greatly altered. The tendency of *cis*-isomers to crystallize or aggregate is usually much less; therefore *cis*-isomers may be more readily solubilized, absorbed, and transported than their all-*trans*- counterparts (Widomska et al. 2009). Minor *cis*-isomers can be regarded as products of chemical, thermal and photo isomerization (Molnar and Szabolcs 1993, Masamoto et al. 2004).

## **1.2.2 Functional roles of carotenoids in photosynthesis**

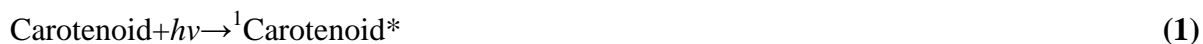
### **1.2.2.1 Light harvesting**

In photosynthesis, carotenoids act as accessory antenna pigments by absorbing light energy in the blue-green region of the solar spectrum (approximately between 400-550 nm). Absorption of UV radiation by a carotenoid molecule excites it from a vibrational level in the electronic ground state ( $S_0$ ) to vibrational levels in the excited singlet state ( $^1\text{Car}^*$ ) (all electrons in the molecule are spin-paired), (1). Classically, carotenoid photophysics has been interpreted in terms of two low-lying excited singlet states, called  $S_2$  and  $S_1$  (Frank and Cogdell 1996). Due to the selection rules, the one photon allowed transition from the ground state goes to  $S_2$ . Then  $S_2$  internally is converted to  $S_1$  by dissipating the excess energy as heat (Kandori et al. 1994). Then the decay from  $S_1$  to  $S_0$  occurs. Carotenoids can donate energy from their both lowest singlet excited states  $S_1$  and  $S_2$  to the neighboring Chl and excite it to the level of the singlet excited state ( $^1\text{Chl}^*$ ), the so called singlet-singlet energy transfer (2). The overall efficiency of this energy transfer varies from organism to organism. Singlet-singlet energy transfer which depends on the antenna complex involved, is either from  $S_2$  to the Chl  $Q_x$  singlet excited state or from  $S_1$  to the Chl  $Q_y$  singlet excited state (Ritz et al. 2000); in some complexes, both pathways are active (Macpherson et al. 2001).

Carotenoids orient nearby Chl in precise configurations that are designed for optimal singlet energy capture and efficient migration of excitation energy with minimal energy losses (van Amerongen and van Grondelle 2001). The Chl subsequently transfers the excitation energy to the RC where it is used to induce a charge separation (3).

The above mechanisms involved in light-harvesting are briefly shown as follows:





*Singlet–singlet energy transfer:*



*Then,*



### 1.2.2.2 Photoprotection

Under excess light conditions carotenoids quench singlet excited state Chl (Horton et al. 1996). The effect of excess light on a photosynthetic organism can be extremely harmful. In fact, the absorption of excess light required for maximum CO<sub>2</sub> fixation will lead to the formation of a rather long lifetime triplet excited state Chl (<sup>3</sup>Chl\*) (one set of electron spins is unpaired) (1) which can react with molecular oxygen and can produce excited singlet oxygen (<sup>1</sup>O<sub>2</sub>\*) (5), a very reactive oxygen species (ROS) formed mainly in PSII RC (Knox and Dodge 1985). The photooxidative damage may result in oxidation of lipids (Havaux et al. 1996), proteins and pigments (Formaggio et al. 2001), which can lead to photoinhibition of the photosynthetic machinery and to photobleaching. At this point, carotenoids can act in two ways: either by quenching the triplet excited state of Chl (2) (Schodel et al. 1999) or by directly scavenging the singlet excited state oxygen, which can induce interaction of triplet excited state of Chl with molecular oxygen (3), (Farmilo and Wilkinson 1973). This capacity relies on the photoprotection property of the carotenoid triplet state (<sup>3</sup>Carotenoid\*), whose energy is sufficiently low to effectively quench triplet state Chl and singlet state oxygen (Polivka and Sundstrom 2004).

In PSII RC, β-carotenes are not in close contact with P680, hence they cannot quench triplet excited state of P680 (<sup>3</sup>P680\*) by triplet-triplet transfer instead scavenge the singlet excited state oxygen, which is formed by the interaction of the triplet excited state of P680 with molecular oxygen. In both cases triplet excited carotenoids are formed and then relax to their ground state by heat dissipation (4). For an efficient triplet-triplet transfer between Chl

and carotenoid both molecules must be positioned in close contact with each other (Cogdell and Frank 1987), which is maintained by the proteins, via coordinating pigments.

The above-mentioned mechanisms involved in carotenoid protection are briefly summarized as follows:



*Triplet-triplet energy transfer:*



*Or,*



*Then,*



*Otherwise,*



### 1.2.2.3 Membrane structure organization

Several recent studies indicate that protection of biomembranes against oxidative damage can be also rendered via modification of the physical properties of the lipid phase of the membranes. The hydrophobic core of biomembranes composed of polyunsaturated fatty acids is a potential target of ROS which may directly lead to membrane degradation. The protection against ROS is one of the main biological functions of carotenoids. A direct effect of carotenoid pigments particularly on structural and dynamic properties of lipid membrane is a significant decrease in the lipid-membrane susceptibility to oxidative degradation (Cogdell and Frank 1987, Gruszecki and Strzalka 2005). The effect of carotenoids on the modulation of physical properties of lipid membranes is the result of the carotenoid-lipid interactions therefore highly depends on the physiological properties of the lipids composing the membrane and embedded carotenoids. The presence of hydroxyl groups which are anchored at the opposite sides of lipid bilayers in the carotenoid molecule seems to significantly affect

the membrane properties by modulating membrane fluidity and forming penetration barrier of small molecules, including oxygen (Berglund et al. 1999).

### **1.3 Model Organism: *Cyanobacterium Synechocystis* sp. PCC 6803**

Cyanobacteria are remarkably resistant organisms that inhabited almost all environments where light is available, ranging from freshwater, marine and terrestrial environments to extreme environments such as hot springs, deserts and Antarctica.

They are regarded as the origin of plant chloroplasts originated by endosymbiosis due to their ability to perform the oxygen-evolving photosynthesis, a characteristic of photosynthetic eukaryotes. Cyanobacteria were formerly named as blue-green algae based on the provisions of the Botanical Code, but they are in fact prokaryotes, lacking internal organelles, and having cell wall (Rippka et al. 1979).

Cyanobacteria are amenable to genetic manipulation. Some strains are naturally transformable. The transformability of cyanobacteria is determinant in the development of the molecular biology of these organisms. Genomes of the most popular cyanobacteria have been analyzed extensively and complete genome sequencing data for various strains are now available. Their unique photosynthetic abilities combined with their usefulness as experimental model organisms have made cyanobacteria popular photosynthetic subjects for genetic and physiological studies.

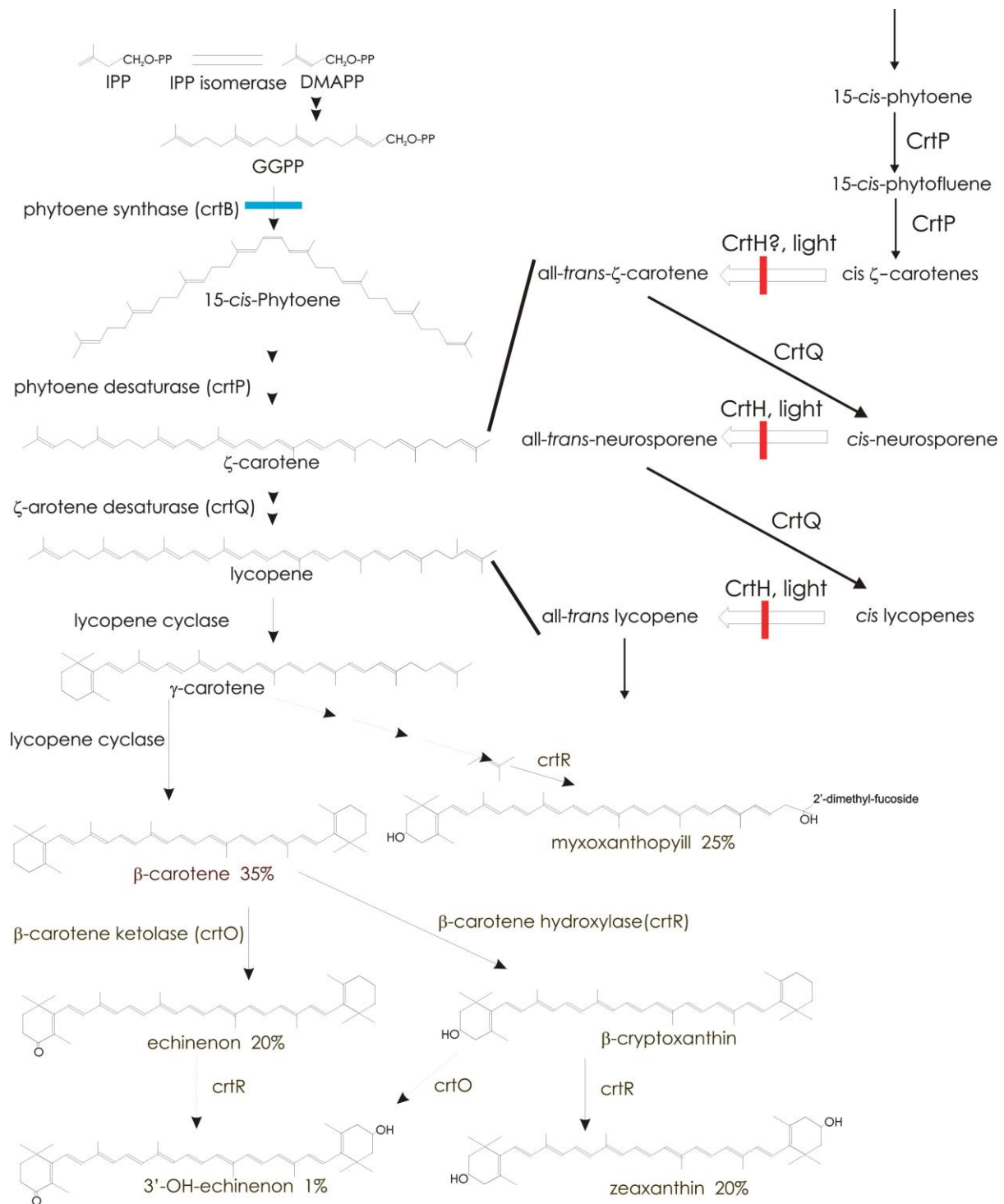
*Synechocystis* is one of the most highly studied cyanobacteria which can grow both in photoautotrophic and heterotrophic conditions in the absence of light. Complete genomic sequences have been obtained for the *Synechocystis* by Dr. Satoshi Tabata and coworkers (Nakamura et al. 1996). It is transformable at high efficiency and integrates DNA by homologous double recombination. The photosynthetic apparatus is very similar to that found in higher plants.

### **1.4 Biosynthetic pathway of carotenoids in *Synechocystis***

Farnesyl pyrophosphate ( $C_{15}$ ) (FPP) is synthesized from three isopentenyl pyrophosphates (IPPs) by IPP isomerase. Then one IPP ( $C_5$ -isoprenoid units) is added to farnesyl pyrophosphate by geranylgeranyl pyrophosphate synthase (CrtE) to yield geranylgeranyl pyrophosphate ( $C_{20}$ ) (GGPP). During a condensation of the two  $C_{20}$  compounds, the first carotene of 15-*cis*-phytoene ( $C_{40}$ ) is formed by phytoene synthase (CrtB). Geranylgeranyl pyrophosphate synthase (CrtE) and phytoene synthase (CrtB)

(Martinez-Ferez et al. 1994) are the first enzymes of carotenoid biosynthesis in *Synechocystis* (Fig. 6). The subsequent conversion of phytoene to  $\beta$ -carotene requires desaturases and cyclases steps. Phytoene desaturase (CrtP), (Martinez-Ferez and Vioque 1992) converts 15-*cis*-phytoene to *cis*- $\zeta$ -carotenes via 15-*cis*-phytofluene,  $\zeta$ -carotene desaturase (CrtQ), (Bautista et al. 2005a) converts all-*trans*- $\zeta$ -carotenes to *cis*-lycopene via *cis*-neurosporene) (Fig. 6). *Cis*-to-*trans* carotene isomerase (CrtH) or light (Masamoto et al. 2001) converts *cis*-carotenes to all-*trans*-lycopene (Fig. 6).

All *trans*-lycopenes are cyclized to  $\beta$ -carotene via  $\gamma$ -carotene. There are two possible branching points in the synthesis of  $\beta$ -carotene and its derivatives, and myxoxanthophyll. One is at lycopene, where two lycopene cyclases may be present: lycopene  $\beta$ -cyclase, which produces  $\beta$ -carotene, and lycopene monocyclase, which produces only  $\gamma$ -carotene for myxoxanthophyll. The other possible branching point is at  $\gamma$ -carotene: lycopene cyclase produces  $\beta$ -carotene via  $\gamma$ -carotene, which is also used for myxoxanthophyll synthesis. In any case, the branching points are still unknown (Takaichi and Mochimaru 2007).  $\beta$ -carotene is further modified by the enzymes such as  $\beta$ -carotene ketolase (CrtO) (Fernandez Gonzalez et al. 1997) and  $\beta$ -carotene hydroxylase (CrtR) (Lagarde and Vermaas 1999). CrtO catalyzes  $\beta$ -carotene to echinenone (Fernandez Gonzalez et al. 1997). CrtR catalyzes  $\beta$ -carotene to zeaxanthin via  $\beta$ -cryptoxanthin, and echinenone to 3-hydroxyechinenone and deoxymyxol 2-dimethyl-fucoside to myxoxanthophyll (Masamoto et al. 1998, Lagarde and Vermaas 1999, Takaichi et al. 2001).



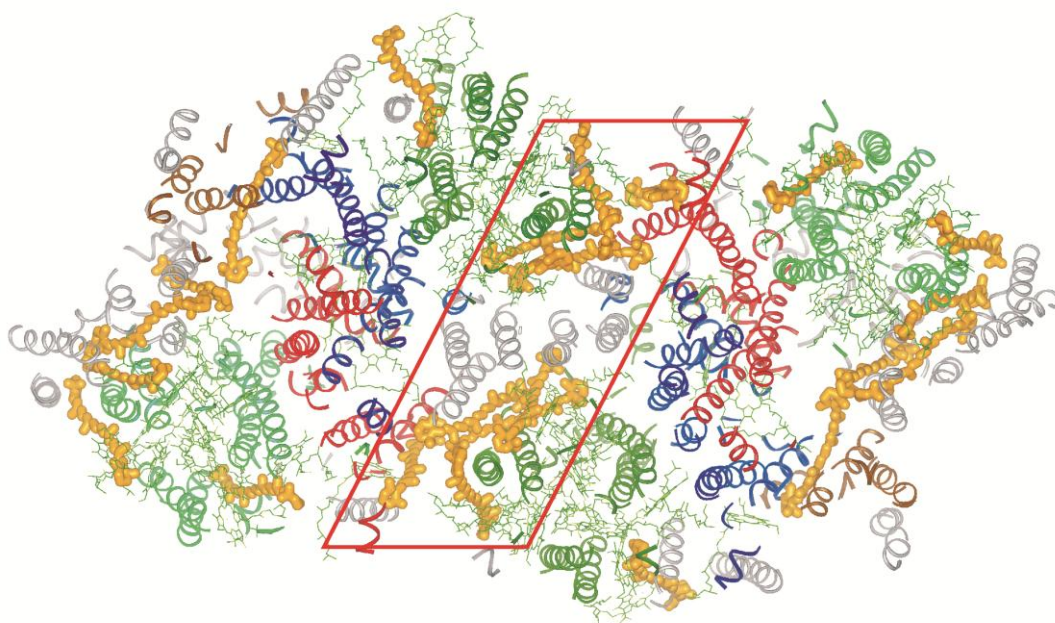
**Figure 6: The pathway of carotenoid biosynthesis in *Synechocystis***

General carotenoid biosynthesis pathway together with accumulated end-products: β-carotene, zeaxanthin, echinenone, 3'-hydroxyechinenone and myxoxanthophyll. Blue line indicates the inactivation of *crtB* gene. On the right side: A putative carotenogenesis pathway from 15-*cis*-phytoene to all-*trans*-lycopene. Red line indicates the inactivation of *crtH* gene.

## 1.5 Localization of carotenoids in photosynthetic complexes

### 1.5.1 Localization of $\beta$ -carotene molecules in photosystem II

Twelve carotenoids were found in monomeric PSII complex of *T. elongates* by X-ray crystallographic analysis (Guskov et al. 2009). All carotenoid molecules were identified as  $\beta$ -carotene in all-*trans*-configuration (Loll et al. 2005, Guskov et al. 2009). Recently, the absorption spectroscopy of redissolved crystals confirmed 11  $\beta$ -carotene molecules (Guskov et al. 2010), the missing one  $\beta$ -carotene in the structure of the monomeric PSII is located actually at the monomer-monomer interface in dimeric PSII and therefore may detach due to its exposed localization in the monomer (Guskov et al. 2010). The best characterized  $\beta$ -carotene molecule is in the monomeric PSII which is close D2 and Cyt *b*-559 (Guskov et al. 2010). The almost identical structure of monomeric and dimeric PSII is also reflected by the location of the tetrapyrrole cofactors and the non-heme iron. In agreement with this finding, the presence of 11  $\beta$ -carotene at positions expected from dimeric PSII could also be confirmed in monomeric form (Guskov et al. 2010). As five of all  $\beta$ -carotene molecules in the dimeric PSII are located at the monomer-monomer interface it is remarkable that only one of these five molecules is missing in the monomeric form (Fig. 7). This  $\beta$ -carotene is found in a bridging position across the monomer-monomer interface (Fig. 7), (Guskov et al. 2010).

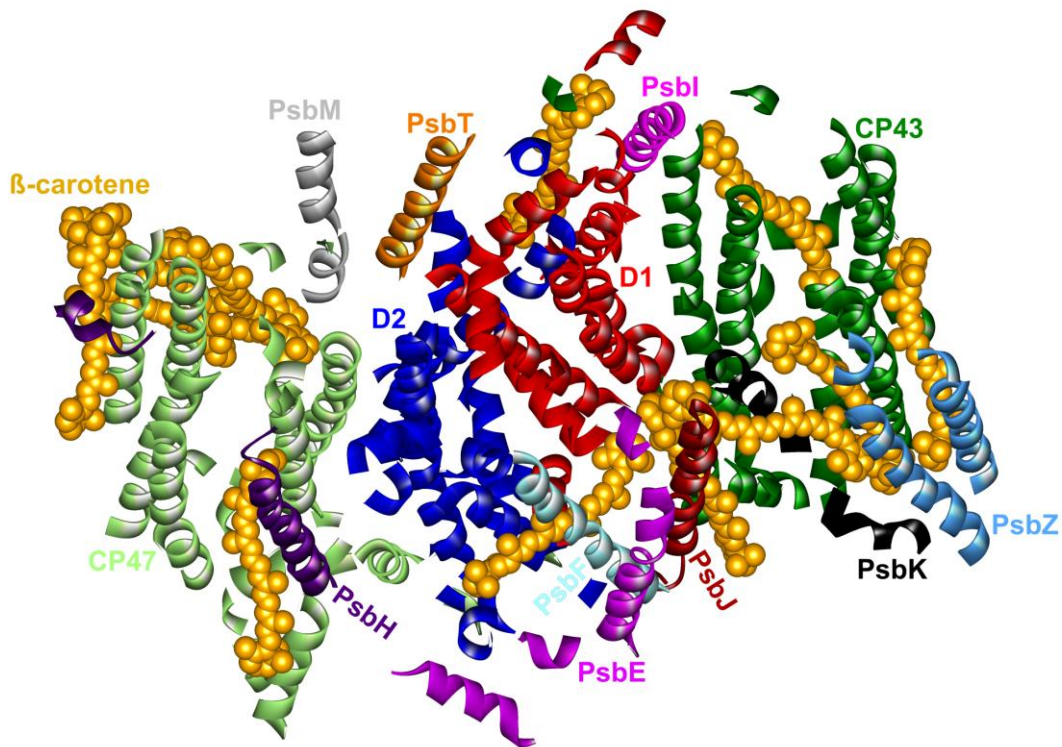


**Figure 7: Stromal side view of  $\beta$ -carotene molecule localization on the monomer-monomer interface**

Red frame emphasizes 5  $\beta$ -carotene molecules (orange) located on the monomer-monomer interface. The figure was created by the software DSV and the PDB files 3BZ1 and 3BZ2 (Guskov et al. 2009)

Only two  $\beta$ -carotenes are structurally related to the D1 and D2 protein subunits, five are associated with CP47 and four with CP43 (Fig. 8). Most of the  $\beta$ -carotene molecules associated with CP47 are clustered at the monomer-monomer interface between CP47 of one monomer and D1 and PsbT of the other monomer. A number of  $\beta$ -carotene molecules at the outside of the dimeric PSII core complex is nestled between transmembrane helices of the large and small membrane intrinsic protein subunits, so that PsbF, PsbH, PsbJ, PsbK and PsbZ are apparently involved in carotenoid binding (Fig. 8) (Muh et al. 2008).

Three Chl *a* in CP47 and two Chl *a* in CP43 are in close contact with carotenoids. Thus, an efficient quenching of excited triplet states become possible (Muh et al. 2008). Five carotenoid molecules are in close contact with each other and are possibly coupled for fast electron and/or exciton transfer (Guskov 2009).



**Figure 8: Stromal side view of  $\beta$ -carotene and membrane-intrinsic protein subunits localization in the monomeric PSII complex.**

Orange,  $\beta$ -carotene; red, D1; navy, D2; light green CP47; dark green, CP43; purple, PsbH; light blue, PsbF; magenta, PsbE, orange, PsbT; dark red, PsbJ; black, PsbK; dark blue, PsbZ; violet, PsbI; grey, PsbM. The figure was created by the software DSV and the PDB file 3BZ1 (Guskov et al. 2009).

### 1.5.2 Localization of a $\beta$ -carotene molecule in cytochrome *b<sub>6</sub>f*

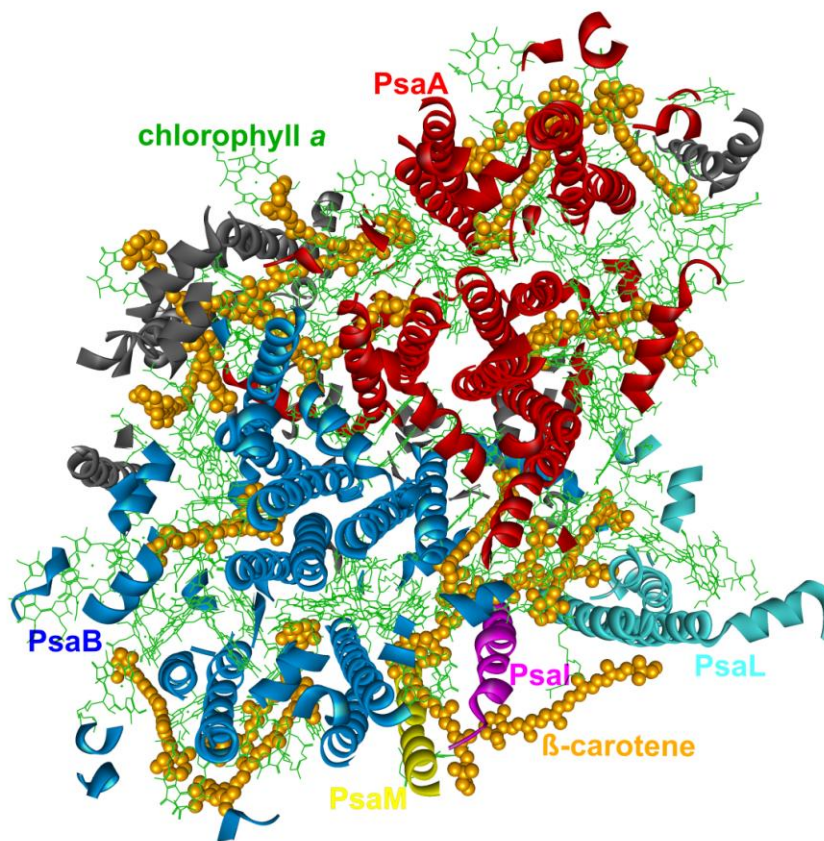
The crystallographic structure of the Cyt *b<sub>6</sub>f* complex from the filamentous cyanobacterium *Mastigocladus laminosus*, *Nostoc* sp. PCC 7120 exhibited stoichiometrically bound Chl *a* and  $\beta$ -carotene at a ratio of approximately one molecule of each per Cyt *f* (Baniulis et al. 2009). A 9-*cis*- $\beta$ -carotene molecule which is in agreement with Raman spectra results is inserted near the center of the transmembrane region between the PetL and PetM helices, in contact with the PetB, PetE, and PetG helices (Kurusu et al. 2003). It is oriented obliquely to the membrane plane and Chl *a*. It is too far from Chl *a* for effective quenching of the excited triplet state Chl *a* (Cramer et al. 2006).

### 1.5.3 Localization of $\beta$ -carotene molecules in photosystem I

Twenty two carotenoids have been identified in the structure of monomeric PSI from *T. elongatus* (Jordan et al. 2001, Fromme et al. 2001, Grotjohann and Fromme 2005) (Fig. 9). They were modelled as  $\beta$ -carotene (Jordan et al. 2001) which has been previously shown as the dominant carotenoid in PSI (Palsson et al. 1998). Seventeen carotenoid molecules showed the all-*trans* configuration, 2 are 9-*cis*, and 3 contain one of 9, 9'-*cis*, 9, 13'-*cis* and 13-*cis* bonds. The 'fit-into-space' consideration would be the easiest explanation for the incorporation of the *cis*-carotenoids. Other options may include the possibilities that there may be a different efficiency for quenching for the *cis*- and *trans*-carotenoids, due to the higher energy level for the *cis*- compared to the *trans*- carotenoids (Grotjohann and Fromme 2005).

According to crystallographic structure of *T. elongatus* (Jordan et al. 2001, Grotjohann and Fromme 2005), six carotenoid clusters are present in PSI. Four of six clusters are associated with PsaA and PsaB, and contain three, three, two and two carotenoids, respectively. Cluster five contains six carotenoid molecules and interacts with PsaA, PsaB, PsaF and PsaJ and is located peripherally, on the outside of the complex. Cluster six contains also six carotenoids and is near a trimeric axis and participates in hydrophobic contacts with PsaA, PsaB, PsaI, PsaL and PsaM.





**Figure 9: Luminal side view of the  $\beta$ -carotene molecules localization in the monomeric PSI complex**

Orange,  $\beta$ -carotene; red, PsaA; blue, PsaB; light blue, PsaL; magenta, PsaI, yellow, PsaM and dark green, chlorophyll *a* pigments. The figure was created by the software DSV and the PDB file 1JB0 (Jordan et al. 2001).

The majority of the carotenoids are deeply inserted into the hydrophobic region of membrane, and only a few of the head groups located closer to the stromal or luminal side. In PSI, all carotenoids are in direct vicinity to Chl *a* head groups and they are in van der Waals contacts with them. More than 60 of the 96 Chl *a* are in close contact with carotenoids, thereby facilitating efficient triplet quenching and energy transfer (Fromme et al. 2001).

### 1.6 Phosphatidylglycerol in photosynthesis

. Lipid-protein and lipid-pigment interactions play an important role in the regulation of pigment-protein complexes embedded in thylakoid membrane (Gruszecki and Strzalka 2005). Thylakoid membrane of cyanobacteria possesses a characteristic membrane lipid composition that consists of glycolipids and phospholipid. In cyanobacteria and in the thylakoid membrane of higher plants, phosphatidylglycerol (PG) is the only phospholipid.

The recent X-ray crystallographic structure of *T. elongatus* showed that there are PG molecules in PSII complex localized between CP43 and D1 (Guskov et al. 2009) as well as in PSI (Jordan et al. 2001).

### 1.7 Earlier studies on carotenoid mutants in cyanobacteria

The major carotenoids in *Synechocystis* are  $\beta$ -carotene; its hydroxyl derivative, zeaxanthin; its keto derivative, echinenone and 3'-hydroxyechinenone and the carotenoid glycoside, myxoxanthophyll (Britton et al. 2004, Takaichi and Mochimaru 2007) (Fig. 6). In the  $\Delta crtO$  mutant, absence of echinenone affected photosynthetic electron transport to only a small extent (Fernandez Gonzalez et al. 1997). In contrast,  $\Delta crtR$  lacking both zeaxanthin synthesis and myxoxanthophyll accumulation resulted in strong photoinhibition of overall photosynthetic electron transport as well as the PSII activity under high light treatment (Schäfer et al. 2005). However,  $\Delta crtR$  showed that these two missing carotenoids are not required for the assembly of RCs (Steiger et al. 1999). In the double mutant  $\Delta crtRO$  (Schäfer et al. 2005) the effect was additive. The oxygen-evolving activity and maximum quantum yield of PSII dramatically dropped under high-light treatment. Moreover, the remaining  $\beta$ -carotene and modified myxoxanthophyll are not able to protect the photosynthetic machinery.  $\Delta crtH$ , *cis*- to *trans*-isomerase mutant (Masamoto et al. 2001), under light-activated heterotrophic growth (LAHG) conditions (described in materials and methods), produced primarily *cis*-lycopenes and small amounts of all-*trans* carotenes, which are intermediate carotenes for all-*trans* lycopene synthesis, but no xanthophylls. Under light conditions, however, this mutant produced all carotenoids which are typical for wild-type strain (Masamoto et al. 2001).  $\Delta crtH$  can accumulate  $\beta$ -carotene under light conditions. However, the mutant cells grown under LAHG condition did not contain detectable levels of either  $\beta$ -carotene or D1 protein subunit and there were no oxygen-evolving activity of PSII observed.  $\beta$ -carotene and D1 protein subunit appeared and a high level of PSII activity was detected after the cells were transferred to continuous light condition. The PSI activity of  $\Delta crtH$  cells were almost the same as those of WT cells, both before and after transfer to continuous light. These results suggest that  $\beta$ -carotene is required for the assembly of PSII but not for that of PSI (Masamoto et al. 2004). Moreover, genetic manipulation of the carotenoid synthesis suggested that PSII assembly requires the presence of a carotenoid with at least one  $\beta$ -ionylidene ring, which may play a structural role in early stages of assembly (Bautista et al. 2005b).

Cyanobacteria are well protected against stress-mediated photooxidation. They possess a well-balanced antioxidative system to cope with photoinhibition. Due to their antioxidative properties, carotenoids are essential for the protection of the photosynthetic apparatus against different stress conditions. The substitutions of the  $\beta$ -carotene molecule determine antioxidative properties (Britton 1995a). In general, hydroxy carotenoids efficiently scavenge peroxy radicals (Woodall et al. 1997b) whereas keto derivatives are more efficient in quenching of reactive singlet oxygen (Woodall et al. 1997a) and possess efficient stability against peroxy radicals and photooxidation (Steiger et al. 1999). In *Synechocystis*, genetically modified cells containing over-expressed zeaxanthin were more efficient in photoprotection than the WT (Schäfer et al. 2005). Similar results obtained on *Synechococcus* PCC 7942 also support the efficient antioxidant activity of zeaxanthin, since UV-B stress increased the level of zeaxanthin (Gotz et al. 1999). Under high-light treatment of *T. elongatus*, the level of zeaxanthin was the highest and level of myxol glycoside greatly increased while there were only minor alterations in the level of  $\beta$ -carotene (Kilian et al. 2007). Investigations on the photoprotective function of myxoxanthophyll in the cyanobacterium *Plectonema boryanum* cells exposed to UV radiation revealed a reduced efficiency of energy transfer to PSII RCs and an elevated level of myxoxanthophyll (Ivanov et al. 2000). Another stress factor that can generate elevated levels of carotenoids is low temperature stress. Low temperature stress increased the myxoxanthophyll content of *Cylindrospermopsis raciborskii* cells (Várkonyi et al. 2002). Carotenoids are known to affect the structure of photosynthetic membranes (Gruszecki and Strzalka 2005). Stress-induced carotenoid accumulation was observed in both thylakoid and cytoplasmic membranes (Masamoto and Furukawa 1997, Masamoto et al. 1999).

Other photoprotective mechanism involves down-regulation of photosynthesis by increasing thermal dissipation of the energy absorbed by the PSII antenna. While this process has been well studied in plants, the equivalent process in cyanobacteria was only recently discovered (Kerfeld 2004, Wilson et al. 2006). The orange carotenoid protein (OCP) binding 3'-hydroxyechinenone as well as echinenone (Punginelli et al. 2009) is the key inducer of this photoprotective mechanism. Light causes structural changes within both carotenoids and the proteins, leading to the conversion of an orange inactive form into a red active form. The activated red form induces an increase of energy dissipation leading to a decrease in the fluorescence of the PBSs, the cyanobacterial antenna, and thus of the energy arriving to the RCs. The OCP, which senses light and triggers photoprotection, is a unique example of a

photoactive protein containing a carotenoid as the photoresponsive chromophore in cyanobacteria (Wilson et al. 2010).

Studies on *Synechocystis* cells demonstrated that PG plays an important role in oxygen evolution (Hagio et al. 2000), in the formation of dimeric PSII (Sakurai et al. 2003), and in the formation of trimeric PSI (Domonkos et al. 2004). PG depletion resulted in a decrease of the Chl content and a suppression of electron transport from  $Q_A$  to  $Q_B$ , which may lead to over-reduction of  $Q_A$  and to the generation of  $Q_A^{2-}$  (Gombos et al. 2002, Laczkó-Dobos et al. 2008) indicating the general slowdown of the photosynthetic processes. PG plays an essential role against low temperature stress (Murata et al. 1982, Murata 1983, Moon et al. 1995) and high light stress (Vass et al. 1992).

## 2.0 AIMS

Changes in global environment might lead to stress conditions in photosynthetic organisms and limit the efficiency of photosynthesis. Understanding photosynthesis and its protective mechanisms might lead to the development of plant, algae and cyanobacteria with improved growth characteristics even under stress conditions. Cyanobacteria, our model organisms, are regarded as an origin of the plant chloroplast; therefore they are excellent to study higher plant photosynthesis. Carotenoids are very important protective agents in photosynthesis due to their anti-oxidative properties against photooxidative damage. Their structural and functional features in protection mechanisms are not completely elucidated yet. Complete genome sequences and the transformability of several cyanobacterial strains allow us to generate various mutants and study the importance of carotenoids in photosynthetic processes. Therefore, the aims of my thesis are:

- I. To generate the first viable oxygenic photosynthetic prokaryotic in the cyanobacterium *Synechocystis* sp. PCC 6803, which is completely deficient in carotenoid synthesis in order to study, for the first time, *in vivo*:
  - i. The structural consequences of carotenoid deficiency on the assembly of the photosynthetic complexes
  - ii. The functional consequences of carotenoid deficiency on the activity of the photosynthetic reaction centers
- II. Studying how carotenoids respond to stress conditions other than light stress; to this end PG depletion was induced in the *Synechocystis* sp. PCC 6803 *pgsA* strain.

### 3.0 MATERIALS AND METHODS

#### 3.1 Construction of *Synechocystis* $\Delta crtH/B$ mutant

In order to generate the carotenoid deficient mutant, partial carotenoid deficient  $\Delta crtH$  cells were used as a host strain due to their adaptation to partial carotenoid deficiency and LAHG conditions (check in 3.2) that could increase the probability of transformation. Restriction enzymes *Bgl*III and *Not*I were used to cut the cosmid clone cs0798 (<http://genome.kazusa.or.jp/cyanobase/Synechocystis/map/Chr/orf16>) provided by S. Tabata. A 7.7 kb *Bgl*III fragment containing the phytoene synthase encoding *crtB* gene of *Synechocystis* was cloned into the *Bam*HI site of pMPMA2 (Mayer 1995). A part of the *crtB* gene coding region was removed by *Apa*I-*Hind*III digestion and replaced with an omega cassette. Transformants were selected under LAHG conditions on BG11 agar plates supplemented with glucose and increasing amount of spectinomycin by several restreaking of single colonies. Complete segregation of  $\Delta crtH/B$  cells was confirmed by PCR using the primers *crtBup* (5'-CGGTGCCCAACTTTTACCTA-3'), and *crtBdown* (5'-TCACCTAAGGGGAAACATCG-3').

#### 3.2 Organisms, growth conditions and light treatment

The mutant strains used in this study were derived from the glucose-tolerant strain of *Synechocystis*  $\Delta crtH$ , and  $\Delta crtH/B$  strains were grown at 30 °C in BG11 medium (Allen 1968) supplemented with 5 mM HEPES buffer (pH 7.5) and 10 mM glucose under light-activated heterotrophic growth (LAHG) conditions (Anderson and McIntosh 1991). In LAHG, the cells grown in the dark were supplied by white fluorescent lights for 10 min once a day at an intensity of 15  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . For low-light treatment the cells were treated at 35  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  only for protein analysis) and for high-light treatment the cells were treated at 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Forty  $\mu\text{g ml}^{-1}$  kanamycin and 40  $\mu\text{g ml}^{-1}$  spectinomycin, and 20  $\mu\text{g ml}^{-1}$  kanamycin were added to the medium of  $\Delta crtH/B$  and  $\Delta crtH$  cells, respectively. Cultures were aerated on a gyratory shaker at 100 rpm.

*Synechocystis* *pgsA* mutant cells were grown photoautotrophically in BG11 medium (Allen 1968) supplemented with 5 mM HEPES-NaOH (pH 7.5), 20  $\mu\text{g ml}^{-1}$  kanamycin and 20  $\mu\text{M}$  dioleoyl-PG (18:1/18:1 PG) (P-9664, Sigma, St Louis, MO, USA) at 30°C under continuous illumination at a light intensity of 30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . PG depletion was

achieved by washing the cells twice with PG-free medium and keeping them afterwards in PG-free medium. Cultures were aerated on a gyratory shaker at 100 rpm.

### 3.3 Chlorophyll content analysis

*Chlorophyll content analysis for HPLC measurements* – Chl concentration was measured by absorbance at 665 nm, using a 90% methanol extract (Lichtenthaler 1987).

*Chlorophyll content analysis for protein measurements* – Sedimented cells or membranes were extracted with 100% methanol and the Chl content of the extract was calculated from the absorbances at 666 nm and 720 nm (Welburn and Lichtenthaler 1984).

### 3.4 Carotenoid content analysis

*Synechocystis* cells and subcellular fractions isolated from them were extracted with acetone:methanol (7:2 v/v) and centrifuged for 3 min at 4°C in a Sigma K-18 centrifuge (20,000×g). The supernatant fluid was evaporated under nitrogen gas and the extracted pigments were dissolved in HPLC-grade ethanol.

Pigments were separated on a Prostar HPLC system (Varian, Miami, FL, USA), equipped with a photodiode array spectrophotometric detector Tidas I (World Precision Instruments, Sarasota, FL, USA) and a Nucleosil 100 C18 reversed phase column, 5 µm particle size (Technokroma, Barcelona, Spain), using the solvent system described by Lagarde and coworkers (Lagarde et al. 2000). Samples (100 µl) were filtered through a stainless steel filter ( $\phi = 0.22 \mu\text{m}$ ) and loaded on the column equilibrated with Solvent A (acetonitril:water:triethylamine, 9:1:0.01 by vol.). The column was eluted with Solvent B (ethyl acetate 100%), by a three-step gradient (0-40% B for 10 min, 40-60% B for 10 min, 60-100% B for 3 min) followed by an isocratic hold (2 min) at 100% B. During separation, a constant flow rate of  $1.5 \text{ ml min}^{-1}$  was ensured. The absorption spectra of the eluates (380–800 nm) were recorded every 0.2 s.

Carotenoid derivatives were identified on the basis of both their absorption spectra and their retention times. The relative content of each pigment was estimated by a comparison of peak areas on chromatograms recorded at 440 nm. The concentrations of carotenoid species and chlorophyll were calculated from Beer–Lambert's law using their specific extinction coefficients at 440 nm (Mantoura and Llewellyn 1983) and at 665 nm (Lichtenthaler 1987) (Only the echinenone was calculated at 460 nm (Britton 1995b)). Values are means  $\pm$  SD from at least three individual experiments.

### 3.5 Absorption spectroscopy

Absorption spectra of cell suspensions were recorded with a UV-3000 (Shimadzu, Japan) spectrophotometer. The cell densities were adjusted to an identical optical density at 730 nm. The absorption spectra of cell suspensions were scanned in the visible region from 400 nm to 800 nm. All these absorption spectra were taken at room temperature and were not corrected for spectral sensitivity.

### 3.6 Low-temperature (77 K) fluorescence spectroscopy

Low temperature steady-state fluorescence emission spectra (600 to 800 nm) were recorded at 77 K using an Aminco Bowman Series 2 luminescence spectrometer (Spectronic Unicam, USA). The cells were excited at 435 nm. Spectra were corrected for the sensitivity of the photomultiplier and normalized to the maximum of PSI emission around 725 nm.

### 3.7 RNA isolation and Northern blot analysis

Total RNA from *Synechocystis* WT and  $\Delta crtH/B$  cells grown under LAHG conditions was isolated using the hot phenol method (Mohamed and Jansson 1989), with the following modification: the first phenol extraction was done in a boiling water bath for 3 min. Northern blot analysis was performed as described by Kis and coworkers (Kis et al. 1998). DNA probes were generated by PCR using *Synechocystis* genomic DNA and gene-specific primers as shown: *psbA* gene; F:5'-GACATCGACGGTATCCGTGAG-3', R:5'-ACAGCAGGAGCGGTCAAAG-3'. *psbDII* gene; F:5'-TGTCCTCGACGATTGGCTAAAG-3', R:5'-AAACCGACGATACCCACAGAAC-3'. *psbDIC* gene; F:5'-CTTGGTGGTCGGGAAATG-3', R:5'-GTGAAGGCTTGGGATTGG-3'. *psbB* gene; F:5'-TGCCCACATCGTTCTATC-3', R: 5'-TGCGGAATACACCATCAG-3'. All membranes were probed also with the *rnpB* as loading and transfer control.

### 3.8 Radioactive labeling of the cells

For radioactive labeling, cells containing 75  $\mu\text{g}$  of Chl were resuspended in 250  $\mu\text{l}$  of BG11 in a microcentrifuge tube, shaken at 50  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  for 15 min and then a mixture of [ $^{35}\text{S}$ ]Met and [ $^{35}\text{S}$ ]Cys (Trans-label, MP Biochemicals, Irvine, USA) was added (final specific activity 400  $\mu\text{Ci ml}^{-1}$ ). The suspension was exposed to 50  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  white light for 15 min (pulse), then chloramphenicol (1  $\text{mg ml}^{-1}$  final concentration) and a



mixture of cold Met and Cys (5 mM final concentration) were added and incubation continued for an additional 15 min (pulse-chase). Afterwards the cells were frozen in liquid nitrogen and used for isolation of thylakoid membranes.

### 3.9 Thylakoid membrane isolation

*Isolation of thylakoid membranes of  $\Delta crtH/B$  cells* -Thylakoid membranes were prepared by breaking the cells using glass beads according to Komenda et al. (2005) with the following modifications: the cells were washed, broken and resuspended in 25 mM MES/NaOH, pH 6.5, containing 10 mM  $\text{CaCl}_2$ , 10 mM  $\text{MgCl}_2$  and 25% glycerol. Glass beads were subsequently removed by filtering and thylakoid membranes were obtained by differential centrifugation

*Isolation of thylakoid and cytoplasmic membranes of  $pgsA$  cells*- Thylakoid and cytoplasmic membranes were isolated (Murata and Omata 1988), with some modifications. The 0.2% lysozyme-treated (37°C, 2 h) and pelleted cells were disrupted with 0.1 mm glass beads in a Bead Beater homogenizer (Biospec Products, Bartlesville, OK, USA) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor. The disrupted cells were treated with 0.1% DNase for 15 min, and the unbroken cells were removed by centrifugation (10 min, 7,000×g). The membrane vesiculi were ultracentrifuged in a discontinuous sucrose density gradient (130,000×g, 16 h, 4°C). After flotation centrifugation, the cytoplasmic membranes formed a yellow band in the 30% sucrose layer, and the thylakoid membranes formed a green band at the interface between the 39 and 50% sucrose layers.

Cytosolic fractions were isolated from PG-supplemented and PG-depleted cells that were suspended in a buffer containing 50 mM MES (pH 6.5), 2 mM  $\epsilon$ -amino-caproic acid, 5 mM EDTA, 1 mM PMSF and 1 mM benzamidine. Cells were disrupted with 0.1 mm glass beads in a Bead Beater homogenizer. Intact cells, cell walls and all membranes were removed by ultracentrifugation (45 min, 145,000× g, 4°C). Pigments were extracted from lyophilized supernatants.

### 3.10 Protein analyses

*2D-BN/SDS-PAGE* - For the analysis of protein complexes, the isolated membranes were solubilized with dodecyl- $\beta$ -D-maltoside (DM/Chl = 40:1 w/w) and analyzed by blue-native electrophoresis at 4 °C in a 5 to 14% polyacrylamide gel according to Schagger and

coworkers (Schagger et al. 1994). Samples with 6 µg Chl content were loaded onto the gel. The protein composition of the complexes was assessed by a 2<sup>nd</sup> electrophoresis in a denaturing 12 to 20% linear gradient polyacrylamide gel containing 7 M urea (Komenda et al. 2002). The lanes from the native gel were excised along their entire length, incubated for 30 min in 25 mM Tris/HCl, pH 7.5 containing 1% SDS (w/v) and placed on top of the denaturing gel. Proteins separated in the gel were stained by Coomassie Blue.

*Western blot analysis of PSI and PSII large protein subunits* – Samples containing 1 µg Chl were loaded onto the denaturing gel (described above) and the separated proteins were transferred onto a PVDF membrane. Membranes were incubated with specific primary antibodies and then with a secondary antibody-horseradish peroxidase conjugate (Sigma, St. Louis, USA). The primary antibodies used in this study were raised in rabbits against: (i) residues 58 to 86 of the spinach D1 polypeptide, (ii) the last 12 residues of the D2 polypeptide from *Synechocystis*, (iii) residues 380 to 394 of barley CP47; (iv) the whole isolated CP43 from *Synechocystis*; and (v) the last 14 residues of the PsbI protein from *Synechocystis*.

For autoradiography, the gel or the membrane with labeled proteins was visualized on X-ray films exposed at room temperature for 2 to 3 days or on Phosphorimager plates (GE Healthcare, Vienna, Austria) overnight. Quantitation of bands was done using ImageQuant 5.2 software (GE Healthcare, Vienna, Austria).

*Western blot analysis of Cyt b<sub>6</sub>f large protein subunits*– SDS-PAGE was performed according to the standard procedure (Schagger and von Jagow 1987), using 12% gels. In each lane a sample containing 3 µg Chl was loaded. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Protran BA 85; Schleicher & Schuell, Keene, NH) according to Towbin et al. (Towbin et al. 1979). Blots were probed with rabbit polyclonal antibodies raised against Cyt b<sub>6</sub>f protein subunits of *Synechocystis* (anti-Cyt b<sub>6</sub> and anti-Cyt f). Blots were developed by using goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase according to the standard nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining protocol (Ausubel et al. 1995).

### **3.11 Oxygen-evolving activity measurements**

Oxygen-evolving activity in whole cells was measured with a Clark-type oxygen electrode (Hansatech Instruments, Kings Lynn, U.K.) as described by Gombos and coworkers (Gombos et al. 2002). PSII oxygen-evolving activity was measured from H<sub>2</sub>O to

parabenzoquinone (artificial acceptor), at a concentration of 500  $\mu\text{M}$ . The cells were washed with BG11 medium and re-suspended in fresh BG11 medium for the measurement of oxygen evolution. An incandescent lamp equipped with a red optical filter was the light source. This arrangement was used for all the oxygen evolution measurements at a saturating light intensity of 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The Chl concentration of the cells was adjusted to 5  $\mu\text{g ml}^{-1}$ .

### **3.12 Chlorophyll *a* fluorescence measurements ( $F_v/F_m$ ) and the changes in P700 signal ( $P_m-P_o$ ), and oxidation-reduction measurements of P700 kinetics**

Redox changes of P700 and Chl *a* fluorescence were measured by a Dual-PAM-100 Measuring System (Heinz Walz GmbH, Germany) equipped with DUAL-E Measuring Head (difference of intensities of 830 nm and 875 nm) with P700 Near Infra Red Emitter (720 nm) and DUAL-DR (red) Measuring Head (620 nm). The same amount of sample (equivalent to 20  $\mu\text{g}$  Chl concentration) was filtered onto a Whatman GF/C glass-fiber disc. Three independent repetitions were made for each type of measurement.

*Maximum PSII yield, ( $F_v/F_m$ )* was determined after 20 min dark-adaptation to allow relaxation of the photosynthetic electron transport and determination of the fluorescence yield of open PSII RCs ( $F_o$ ). Maximal fluorescence yield of close PSII RCs in dark adaptate state ( $F_m$ ) is detected during a 2000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  saturation pulse. Using these parameters, the following ratios were calculated.  $F_v$  ( $F_m-F_o$ ), variable fluorescence, indicates the maximum fluorescence change in dark-adapted cells.  $F_v/F_m=(F_m-F_o)/F_m$  is a sensitive indicator of maximum photosynthetic efficiency of PSII in the dark-adapted state.

*Maximum changes of P700 signals ( $P_m-P_o$ )* were determined after 20 min dark adaptation that allows reduction of P700 ( $P_o$ ). Then, P700 was oxidized by far red light pre-illumination for 10 seconds and then maximum signal level was induced by 20000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  actinic red light in the presence of far red light. Maximum changes of P700 signal levels between P700 fully reduced ( $P_o$ ) and P700 fully oxidized ( $P_m$ ) were recorded on a millisecond time scale.

*Oxidation-Reduction Kinetics of P700* was determined by 53  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  continuous actinic red light illumination after 20 min dark adaptation at room temperature. P700 absorbance changes were recorded on a millisecond time scale. Linear electron transport was inhibited by the addition of 100  $\mu\text{M}$  3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

#### 4.1 Generation of carotenoid-less $\Delta crtH/B$ strain in *Synechocystis*

**A**

*crtBup* → *HindIII* *ApaI* *crtB* ← *crtBdown*

slr1150 slr1253 *crtP* slr1147 slr1256 slr1257 slr1258

T4 term Omega T4 term

**B**

**M** **W** **ΔcrtH/B**

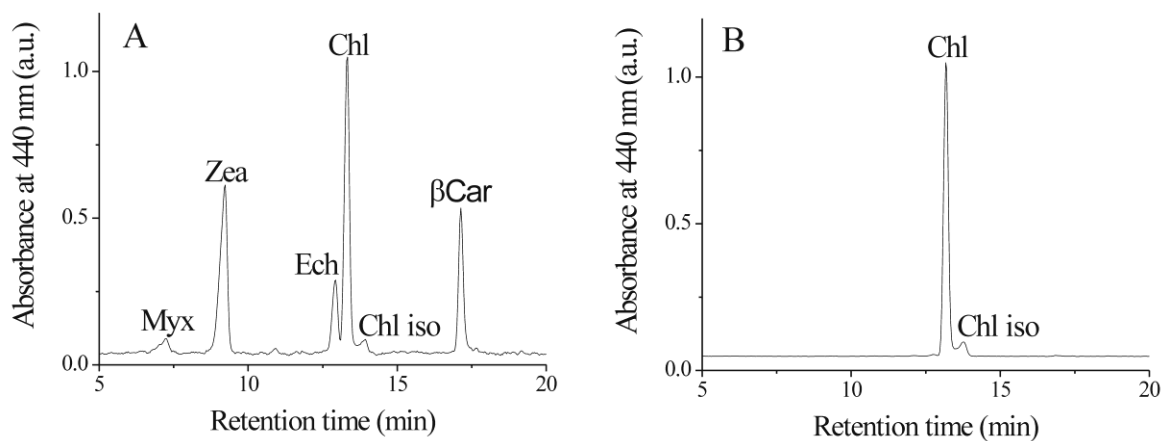
4kb  
3kb  
2 kb  
1.6kb  
1kb

3.3kb  
1.4kb

(A) Physical map of the *Synechocystis* genome fragment containing *crtB*. In the inactivated strain the omega cassette replaces a 140-bp-long *ApaI-HindIII* fragment. Black arrows indicate position of PCR primers used for checking the complete segregation. (B) PCR analysis of WT and  $\Delta crtH/B$  transformants. Sizes of the fragments containing WT and mutant allele are indicated on the right and size marker (M) on the left.

Transformation was performed by insertion of an omega cassette that provides spectinomycin resistance (Fig. 10A) as a selection marker. Complete segregation of the  $\Delta crtH/B$  mutant (Fig. 10B) could be carried out only under light-activated heterotrophic growth (LAHG) conditions. In LAHG conditions cells are grown in the presence of 5-10 mM glucose in the dark with brief, 5-10 min daily illumination (Anderson and McIntosh 1991).

To prove the absence of carotenoids in the mutant, elution profiles of the extracted pigments of WT and  $\Delta crtH/B$  cells grown under LAHG conditions were compared by high-performance liquid chromatography (HPLC) (Fig. 11). The peaks corresponding to carotenoid and Chl *a* were identified according to both their absorption spectra and retention times (Fig. 11A). WT cells contained myxoxanthophyll, zeaxanthin, echinenone,  $\beta$ -carotene and Chl *a*, while in cells of  $\Delta crtH/B$  only Chl *a* was found, neither carotenoids nor any of their intermediates were detectable (Fig. 11B). The same results were obtained with cells illuminated for 24 hours (data not shown).



**Figure 11: Pigment analysis by of WT (A) and  $\Delta crtH/B$  (B) cells by high-performance liquid chromatography**

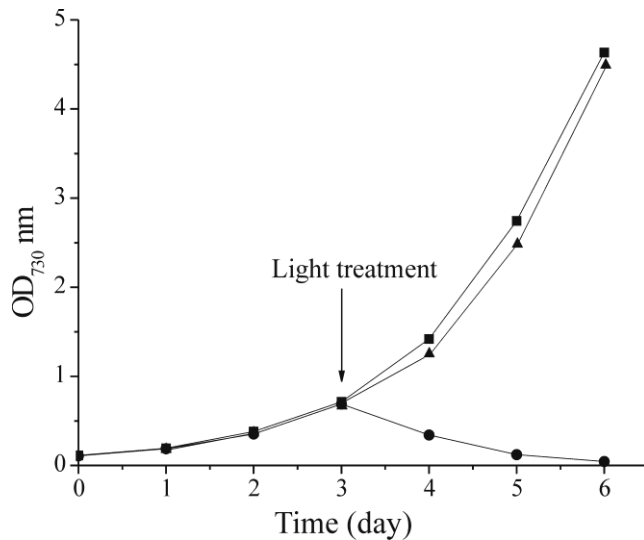
The cells were grown under LAHG conditions. The HPLC chromatograms were recorded at 440 nm. Carotenoid derivatives were identified on the basis of both their absorption spectra and their retention times. Abbreviations:  $\beta$ -Car,  $\beta$ -carotene; Myx, myxoxanthophyll; Zea, zeaxanthin; Ech, echinenone; Chl, chlorophyll and Chl iso, chlorophyll isomers.

## 4.2 Physiological consequences of the carotenoid deficiency

### 4.2.1 Growth rate and light sensitivity

The growth rate of  $\Delta crtH$  and  $\Delta crtH/B$  cells was similar to that of WT cells under LAHG conditions. In 3 days the cell density of all strains gradually increased from 0.2 to 0.8-

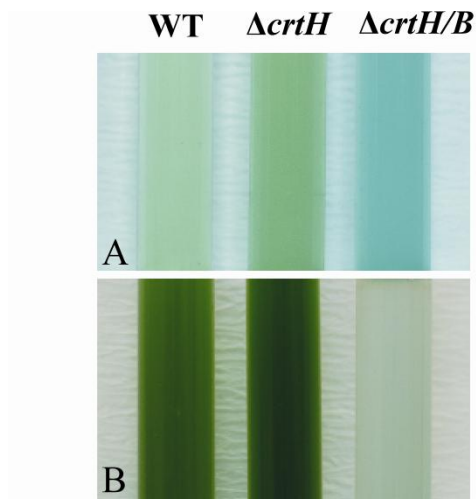
0.9 (optical density recorded at 730 nm,  $OD_{730}$ ) under LAHG conditions (Fig. 12). Following 3 days of culturing the cells were exposed to a light intensity of  $35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . WT and  $\Delta crtH$  cells adapted to light and kept growing. Under LAHG and photomixotrophic growth conditions  $\Delta crtH$  and WT cells gave similar growth profiles indicating that disruption of the *crtH* gene had no serious effect on growth (Masamoto et al. 2001). In contrast, the  $\Delta crtH/B$  cells stopped growing and the cells bleached and died.



**Figure 12: Growth curve of *Synechocystis* WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells**

WT (squares),  $\Delta crtH$  (triangles) and  $\Delta crtH/B$  (circles) cells grown at  $30^\circ\text{C}$  under LAHG conditions for 3 days and then illuminated at  $35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Data derived from a representative experiment are shown.

WT and  $\Delta crtH$  cells have typical green coloration both under LAHG and light conditions (Fig. 13A, 13B). However,  $\Delta crtH/B$  cells grown under LAHG conditions had bluish color, indicating the presence of phycobiliproteins and suppression of Chls (Fig. 13A). Following a short-term light treatment at  $35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  the  $\Delta crtH/B$  cells were photo-bleached (Fig. 13B). These results suggested that the carotenoid-less mutant is extremely light sensitive.

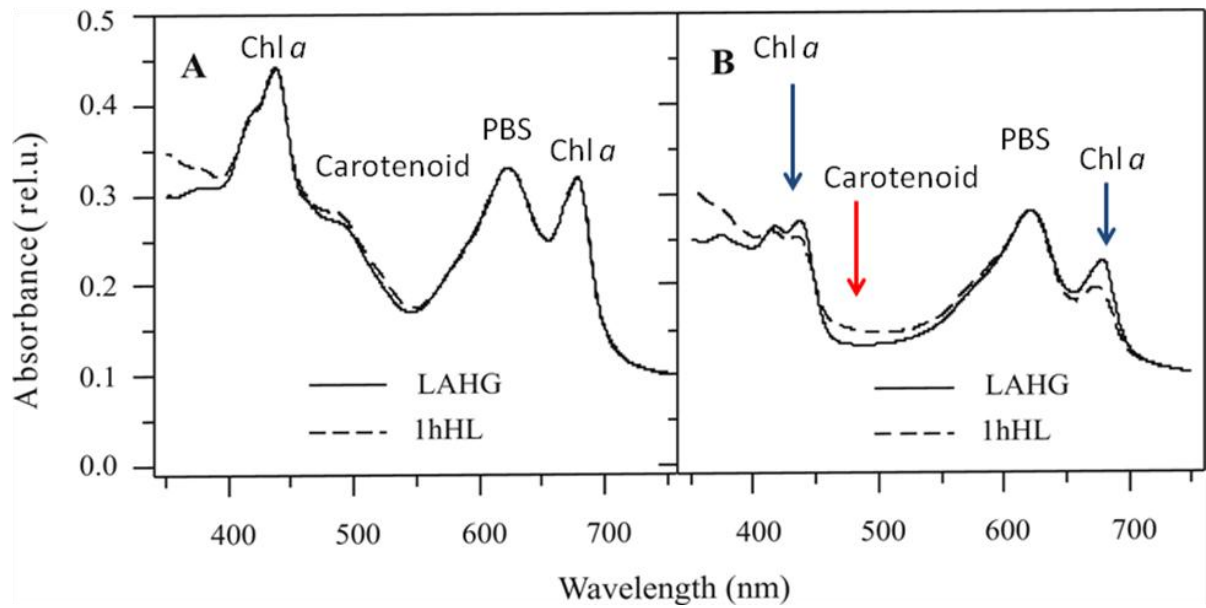


**Figure 13: Effect of light on the pigmentation of WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells.**

**(A):** *Synechocystis* WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells grown at 30 °C at the end of 3<sup>rd</sup> day under LAHG conditions. **(B):** *Synechocystis* WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells after a 48 hour light treatment at 35  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ .

#### 4.2.2 Spectroscopic properties

Absorption spectra of *Synechocystis* WT cells taken at room temperature showed four distinct regions. The absorption range between 435 and 450 nm corresponds to Sorret region of Chl *a*. The absorption ranges of 400 to 550 nm, 550 to 650 nm and 650 to 700 nm belong to visible regions of carotenoids, phycobiliproteins and Chl *a*, respectively (Fig. 14). In the absorption spectra of the  $\Delta crtH/B$  cells there was a sharp decrease of absorption in the 450 to 550 nm region corresponding to carotenoids, confirming the complete absence of carotenoids in the cells (Fig. 14, red arrow). Furthermore,  $\Delta crtH/B$  cells exhibited a decrease in absorption with a shoulder at around 435 and 680 nm corresponding to Chl *a* (Fig. 14, navy-blue arrows). One-hour high light treatment at 500  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  had no effect on the Chl *a* absorption profile of the WT cells (Fig. 14A dashed line). However, Chl *a* content of  $\Delta crtH/B$  cells significantly decreased after one hour high light treatment (Fig. 14B solid line at around 680 nm) compared to that of the cells grown under LAHG conditions (Fig. 14B dashed line at around 680).



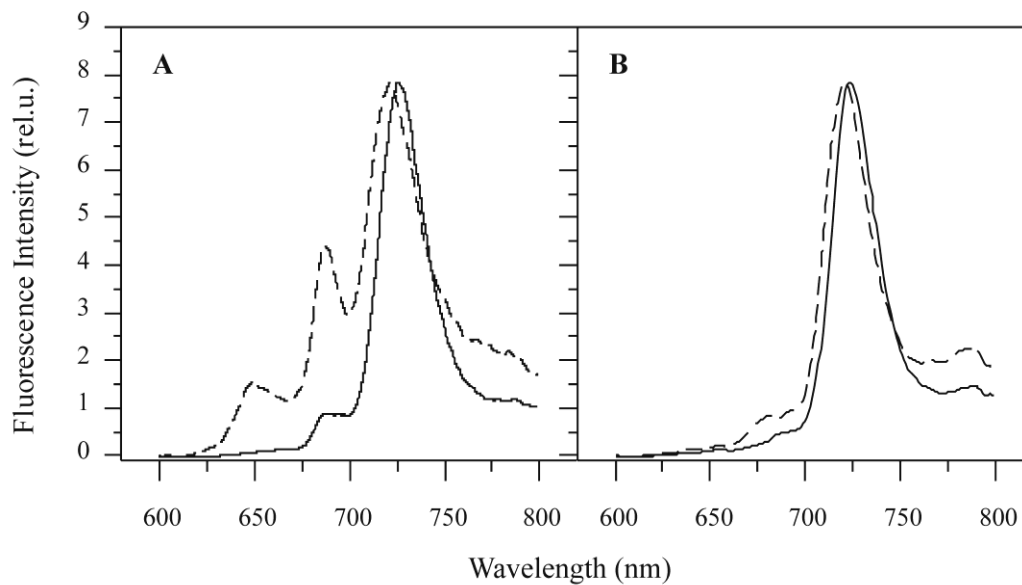
**Figure 14: Absorption spectra of WT and  $\Delta crtH/B$  cells**

(A) WT and (B)  $\Delta crtH/B$  cells grown under LAHG conditions (LAHG, solid line) and after one hour illumination at  $500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  white light (1hHL, dashed line). The spectra were taken at an identical absorbance of 750 nm. Blue and red arrows indicate the changes in absorption bands of Chl *a* and carotenoid, respectively.

Low temperature Chl fluorescence emission spectra of WT and  $\Delta crtH/B$  cells ( $\text{OD}_{730} \sim 0.8$ ) were recorded. Chl *a* was excited at 435 nm. The emission spectrum of WT cells showed a minor band at around 685 nm that might correspond to PSII and a major band at 725 nm, which corresponds to PSI-associated Chl *a* (Fig. 15A). The fluorescence spectrum of  $\Delta crtH/B$  cells was distinctly different from that of WT cells. It showed three bands at 650, at approximately 685 and at 723 nm (Fig. 15A). The latter is the highest peak corresponding to PSI-associated Chl *a* and it is shifted by 2-3 nm to the blue region comparing with that of WT, most probably as a consequence of missing carotenoids. The peak at 650 nm is emitted by phycocyanin (MacColl and Guard-Friar 1987) and the peak at around 685 nm is emitted either by PSII-associated Chl *a* or by APC-B and APC- $\text{L}_{\text{CM}}$  (Rakhimberdieva et al. 2010), terminal emitters of PBSs. These two increased fluorescence emission peaks suggested that the linear electron transfer between PBS and PSI could not function efficiently. During thylakoid isolation process, PBSs are easily detached from the membrane. To distinguish between PBS-specific and Chl-specific fluorescence properties, emission spectra of isolated and well washed thylakoid membranes from WT and  $\Delta crtH/B$  cells were measured (Fig. 15B). Majority of the large fluorescence emission peaks at 650 and at around 685 nm was suppressed in the spectra of the thylakoid membranes of  $\Delta crtH/B$  cells, indicating that these



bands were related to PBS terminal emitters emitting at 683 nm at 77 K (Rakhimberdieva et al. 2010).



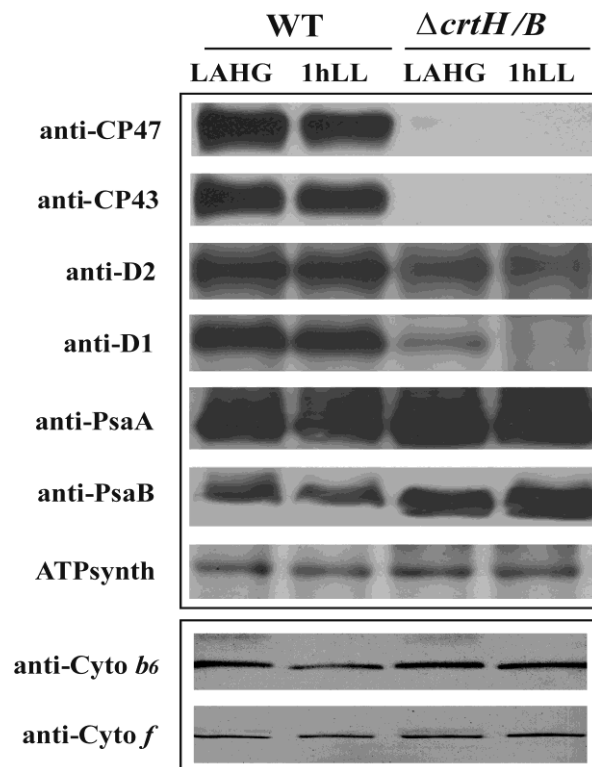
**Figure 15: Low-temperature (77 K) fluorescence emission spectra of WT and  $\Delta crtH/B$  cells**

(A) Whole cells and isolated thylakoid membranes (B) of WT (solid lines) and  $\Delta crtH/B$  (dashed lines) cells cultured under LAHG conditions. The excitation wavelength was 430 nm. The spectra were recorded at 77 K, corrected for the sensitivity of the photomultiplier and normalized to long wavelength maxima.

### 4.3 Structural consequences of carotenoid deficiency

#### 4.3.1 Level of the large protein subunits of photosynthetic complexes

The accumulation of PSII as well as PSI and Cyt *b<sub>6</sub>f* protein in carotenoid deficient  $\Delta crtH/B$  cells grown in LAHG conditions were investigated by semi-quantitative western blot analysis (Fig.16). The level of PSI antenna protein subunits, PsaA and PsaB and Cyt *b<sub>6</sub>f* protein subunits, Cyt *b<sub>6</sub>* and Cyt *f* of the  $\Delta crtH/B$  cells were similar to that of WT (Fig. 16). On the contrary, the levels of PSII core complex protein subunits D1 and D2, in  $\Delta crtH/B$  were much lower in comparison with those in the WT. Antenna protein subunit CP47 was hardly detectable and CP43 was completely absent (Fig. 16). The effect of low light on the accumulation of PSII, Cyt *b<sub>6</sub>f* and PSI protein subunits was also investigated in WT and  $\Delta crtH/B$  cells. After an exposure to 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for an hour, a further decrease in the amount of PSII protein subunits, D1, D2 and CP47 was detected while the level of PSI and Cyt *b<sub>6</sub>f* protein subunits did not change significantly in the carotenoid deficient mutant (Fig. 16) (Lupinkova and Komenda 2004).



**Figure 16: Western blot analysis of large protein subunits of photosynthetic complexes in WT and  $\Delta crtH/B$  cells**

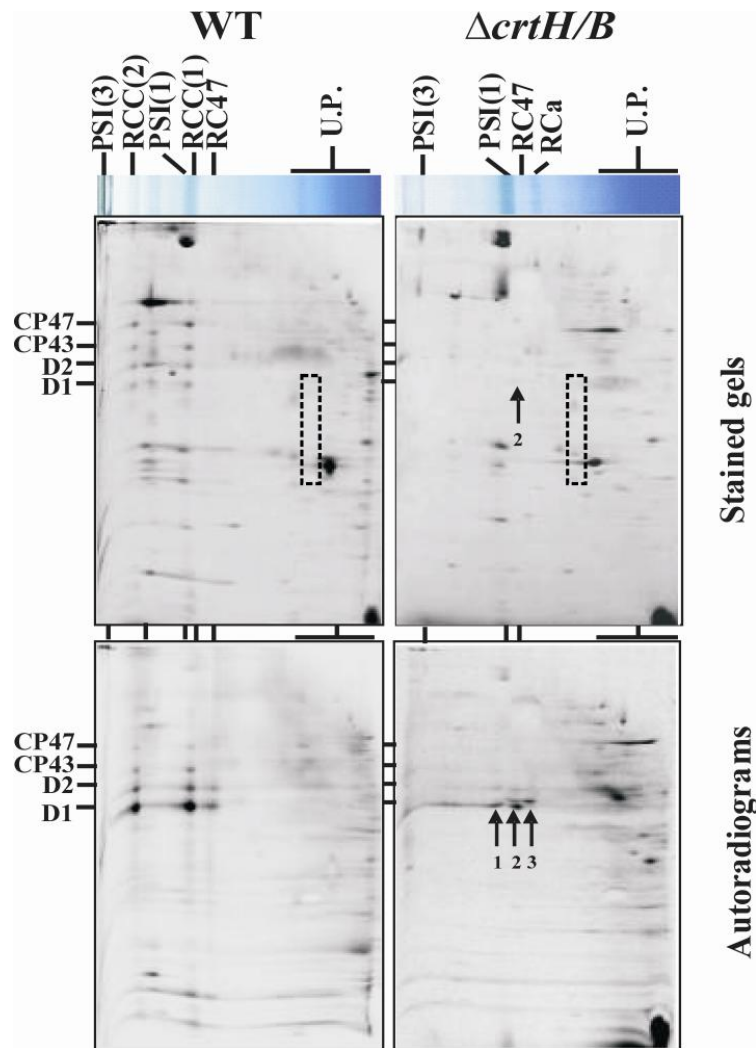
Cells were grown under LAHG conditions followed by 1 hour illumination at a light intensity of  $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . One  $\mu\text{g}$  of Chl per lane was loaded onto the gel. Blots were incubated with specific antibodies and protein subunits of PSII and PSI were detected by peroxidase-conjugated secondary antibodies while Cyt *b*<sub>6</sub>*f* protein subunits by alkaline phosphatase-conjugated secondary antibodies. Stained bands of ATP synthase protein subunits are shown to document equal protein loading.

#### 4.3.2 Assembly of the photosynthetic complexes and their large protein subunits

Accumulation of PSII subunit proteins were severely affected by the absence of carotenoids. Whereas, on the basis of the reason, which might be attributed either to the lack of the synthesis of protein subunits or to the lack of assembly..These effects were characterized in  $\Delta crtH/B$  cells by 2D gel electrophoresis. The first dimension was a blue native PAGE and denaturing PAGE was used as the second dimension (2D BN/SDS-PAGE). Protein levels were studied by autoradiography and immunoblotting. These techniques have recently been used for the characterization of the protein content of various *Synechocystis* mutants (Dobakova et al. 2007, Sobotka et al. 2008, Laczkó-Dobos et al. 2008). The protein complexes detected in WT cells grown under LAHG conditions were similar to those present

in WT cells cultivated under photoheterotrophic growth conditions (Dobakova et al. 2007). Most of the PSI complexes existed in a trimeric form in WT cells and contribution of monomeric form did not exceed 30%. In contrast to the WT cells, the monomeric PSI form was predominant in cells of  $\Delta crtH/B$  and it had a faster mobility (Komenda et al. 2004). About 90% of PSII protein subunits of WT cells accumulated in monomeric and dimeric forms and only a small amount of these protein subunits, less than 10%, was present in RC47, intermediate PSII complex lacking CP43 (Komenda et al. 2004). In the carotenoid-less  $\Delta crtH/B$  cells both monomeric and dimeric PSII complexes were absent and only a negligible amount of RC47 was detected by Coomassie staining. Cyt *b<sub>6</sub>f* complexes were found exclusively in the form of monomeric complexes in WT. The level of the monomeric form of the Cyt *b<sub>6</sub>f* complex in the  $\Delta crtH/B$  was similar to that in the WT (Fig. 17, blue-native PAGE and Coomassie staining).

The labeling patterns of WT and  $\Delta crtH/B$  large protein subunits were shown on the autoradiogram of 2D gel electrophoresis (Fig. 17). The D1 was the most intensively labeled protein subunit in both strains. In WT cells, the D1 bands were present in both monomeric and dimeric PSII complexes and a small amount in RC47 while the majority of the D1 bands accumulated in mainly RC47 in the  $\Delta crtH/B$ , and also in monomeric PSII complex and in RCa (Fig 17., 3 arrows). The RCa is an intermediate PSII complex lacking both inner antenna CP47 and CP43. It contains processed D1 as well as iD1, an incompletely processed precursor of D1 (Komenda et al. 2004). In contrast to typical labeling patterns of WT protein subunits, the majority of the  $\Delta crtH/B$  protein subunits were detected on the unassembled part (U.P), (Fig. 17). In addition, the overall labeling of the  $\Delta crtH/B$  proteins was 5-10 times less intensive than that of the WT proteins, Thus, in order to see the protein labeling of  $\Delta crtH/B$ , the gel was exposed for five times longer period than that of WT.



**Figure 17: 2D-BN/SDS-PAGE of WT and  $\Delta crtH/B$  cells**

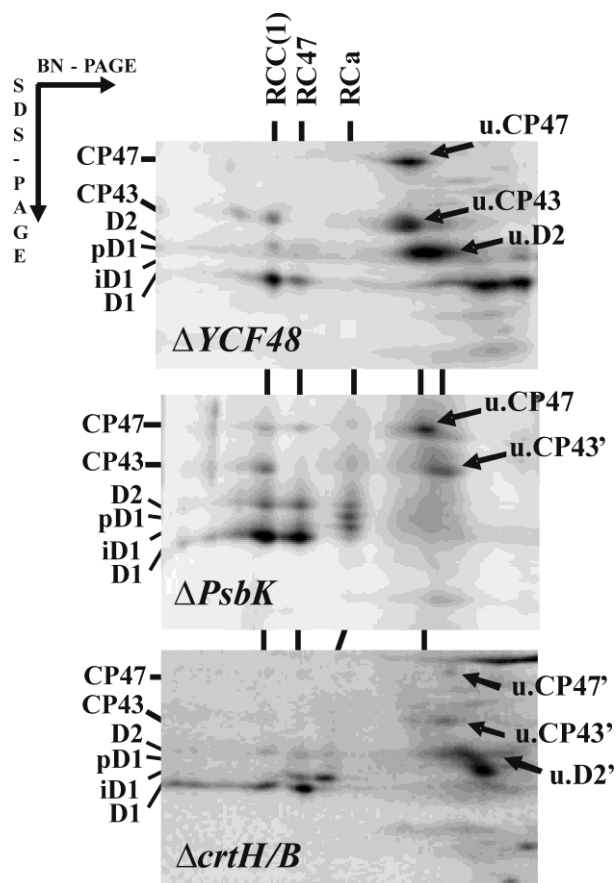
Cells were grown under LAHG conditions and radioactively labeled with [ $^{35}\text{S}$ ] Met and [ $^{35}\text{S}$ ] Cys. The gels were stained by Coomassie Blue and exposed to Phosphorimager plates. Designation of complexes: PSI(3) and PSI(1), trimeric and monomeric PSI complexes, respectively; and RCC(2) and RCC(1), dimeric and monomeric PSII complexes, respectively. RC47 is an intermediate PSII complex lacking CP43, RCa is an intermediate PSII complex lacking both CP43 and CP47. U.P. indicates unassembled part. Boxes define positions of the large protein subunits of the monomeric Cyt *b<sub>6</sub>f* complex. Arrow 1, 2 and 3 designates D1 protein in RCC(1), RC47 and RCa complexes, respectively in the  $\Delta crtH/B$ . Each sample contained 6  $\mu\text{g}$  of Chl.

#### 4.3.3 Stabilization of the small protein subunits binding to the large protein subunits in photosystem II

There are 13 low-mass small protein subunits with less than 10 kDa in each monomer of PSII complex from *T. elongatus* (Guskov et al. 2009). It has been shown that small protein subunits can bind to the large protein subunits and many of them have been shown to have

stabilization function in the PSII assembly (Promnares et al. 2006, Komenda et al. 2007, Dobakova et al. 2007, Muh et al. 2008). Analysis of the PSII complex demonstrated that all large protein subunits may exist in two forms with slower and faster electrophoretic mobilities (Komenda et al. 2004). The faster bands contain only the large protein subunits while the slower bands usually contain both large protein subunits together with binding small protein subunits. For instance, the slowly migrating native pD1 contains not only pD1 but also PsbI small protein subunit (Dobakova et al. 2007), or the slower migrating CP47 band contains not only CP47 but also PsbH small protein subunit, as well as Hlips or Scps (Promnares et al. 2006). Hlips (High Light Induced Proteins) or Scps (Small Cap-like Proteins), are single helix proteins with sequence similar to regions of the plant Chl *a/b* binding proteins (CAB family proteins), and they are mostly induced under stress conditions such as high irradiance or low temperature (Funk and Vermaas 1999, He et al. 2001).

On the autoradiogram of 2D gel electrophoresis of  $\Delta crtH/B$ , small amount of unassembled CP43, CP47, D2 and pD1, the D1 precursor, were detected (Fig. 17- autoradiogram U.P. region and shown more detailed in Fig.19). To evaluate a possible effect of carotenoid deficiency on the binding of small protein subunits to large protein subunits in PSII, we compared the electrophoretic mobilities of unassembled forms of large protein subunits such as CP47, CP43 and D2 in the blue-native gel of the  $\Delta crtH/B$  with using two control strains;  $\Delta YCF48$  and  $\Delta PsbK$ . The  $\Delta YCF48$ , which is an early PSII assembly mutant, accumulates large amounts of unassembled CP47, CP43 and D2 due to the low availability of D1 caused by the absence of the assembly factor YCF48 (Komenda et al. 2008). In the blue-native gel the majority of these unassembled large protein subunits migrated as the slower band (Fig. 18, designated without inverted comma - u.CP47, u.CP43 and u.D2). This indicated the presence of small subunit proteins bound to large unassembled large protein subunits in  $\Delta YCF48$ . On the other hand, the unassembled band of CP43 migrated as the faster band in the blue-native gel of  $\Delta PsbK$  strain (Ikeuchi et al. 1991) (Fig. 18, designated with inverted comma, u.CP43') indicating that the slower band of unassembled CP43 of  $\Delta YCF48$  contained at least PsbK small protein subunit (Fig.18). Interestingly, the faster band of unassembled CP43 also prevailed in  $\Delta crtH/B$  (Fig. 18), as well as that of the majority D2 and CP47 (Fig. 18, designated u.D2' and u.CP47', respectively). These indicated the absence of small protein subunits bound to large unassembled protein subunits in PSII complex of  $\Delta crtH/B$  in the absence of carotenoids.



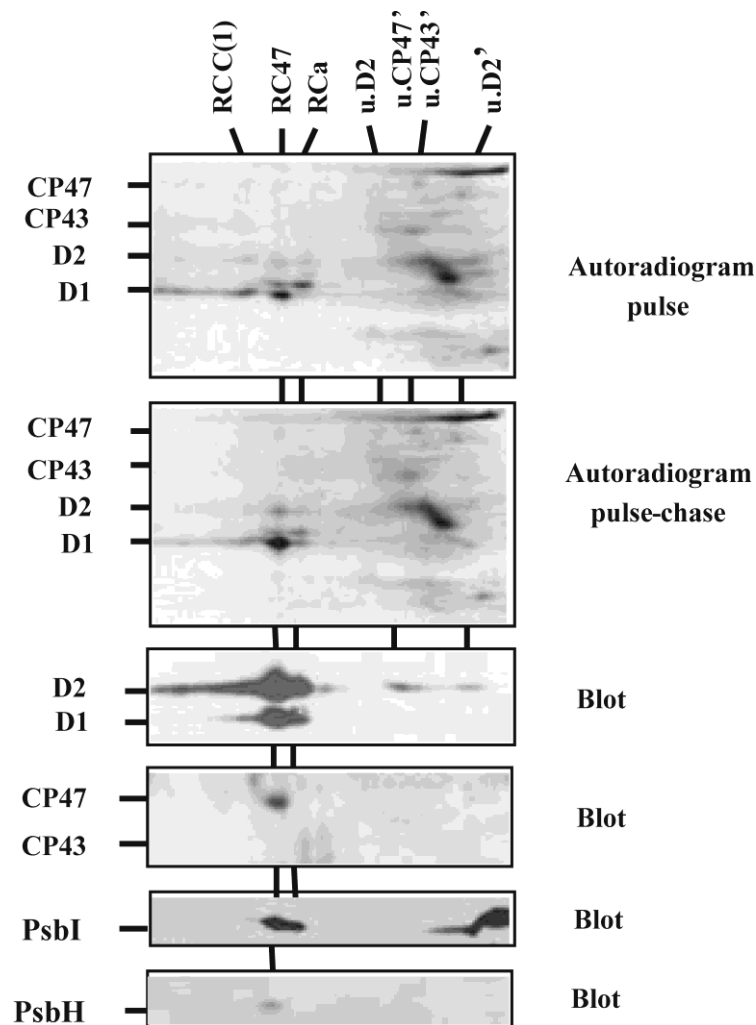
**Figure 18: Autoradiogram of pulse-labeled membrane proteins of  $\Delta YCF48$ ,  $\Delta PsbK$  and  $\Delta crtH/B$  mutants separated by 2D-BN/SDS-PAGE**

Six  $\mu\text{g}$  of Chl were loaded onto each gel. After separation the gels were stained, dried and exposed on PhosphorImager plates. Designation of the PSII complexes is the same as in Fig. 17. Moreover, pD1 indicates unprocessed D1 precursor and iD1 indicates incompletely processed D1 intermediate. The apostrophe designates unassembled proteins with unusually fast mobility in the BN gel most probably lacking bound small subunits.

#### 4.3.4 Assembly of the functional PSII core complex

Taking into account the effect of carotenoid depletion on the migration of particular antenna protein subunits CP43 and CP47, we were interested in whether the absence of carotenoids also affected the assembly of PSII core complex protein subunits D1 and D2. Therefore, we chased the labeled proteins in the presence of chloramphenicol, a protein synthesis inhibitor, and followed their incorporation into complexes by 2D analysis (Fig. 19). Most of the unassembled D2 and pD1 (Fig. 17-pulse) disappeared with a concomitant increase in the labeling of D2 and D1 in RC47. This showed that they were efficiently inserted into the intermediate PSII complexes (Fig. 19-pulse-chase). This reflected partly the transformation of RCa into RC47, and partly the maturation of iD1 into D1, in the remaining

RCa. The amount of weakly labeled protein subunits in monomeric PSII form also significantly decreased during the chase experiment. However, we could not detect any labeled protein subunits in the region of putative dimeric PSII form. This indicated that monomeric form was unstable and was rapidly converted into RC47, which was the main accumulated intermediate PSII complex as identified by Coomassie staining (Fig. 17) and immunoblotting (Fig. 19). Recently it was suggested that PsbI, a small protein subunit of PSII, stabilizes binding of CP43 into the PSII complex (Dobakova et al. 2007). According to X-ray crystallographic measurements, PsbI small protein subunit is bound to D1 in the vicinity of a  $\beta$ -carotene molecule (Loll et al. 2005b, Guskov et al. 2009). Immunodetection using specific antibodies confirmed the presence of PsbI in RC47 (Fig. 19-blot). Since, no increase in the level of unassembled CP43 was observed during the chase experiment we believe that the absence of  $\beta$ -carotene was the reason why CP43 is quickly detached from monomeric PSII complex and probably degraded. In  $\Delta crtH/B$ , RC47 contained another small subunit protein PsbH (Fig. 19-blot), which stabilizes CP47 and facilitates its binding to the D1-D2 heterodimer (Komenda et al. 2002, Komenda et al. 2005).



**Figure 19: Autoradiogram and Western blot analysis of protein subunits of  $\Delta crtH/B$  cells separated by 2D-BN/SDS-PAGE**

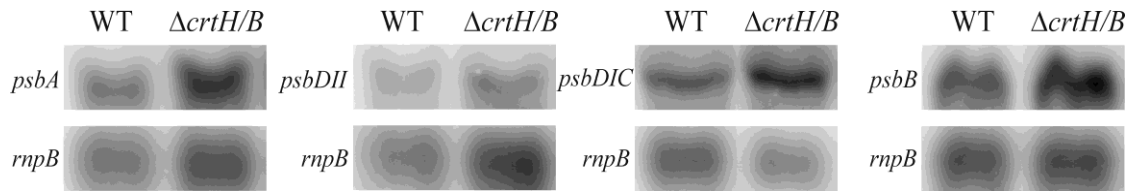
Cells of the  $\Delta crtH/B$  grown under LAHG conditions were labeled with [ $^{35}$ S] Met and [ $^{35}$ S] Cys (pulse experiment), then cold Met and cold Cys together with chloramphenicol were added and incubation continued (pulse-chase experiment) as described in Materials and Methods. Blot only for the pulse experiment is shown as it was identical to that of the pulse-chase experiment. Designation of the PSII complexes is the same as in Fig. 17. The apostrophe designates unassembled proteins with unusually fast mobility in the BN gel. Each loaded sample contained 3  $\mu$ g of Chl.

#### 4.3.5 Level of genes encoding PSII large protein subunits

Carotenoid deficiency seriously affected the accumulation of the major PSII proteins. However, it was not clear whether transcriptional levels of genes encoding these proteins were affected. To this end Northern blot analysis of WT and  $\Delta crtH/B$  cells grown under LAHG conditions was used. Steady-state levels of *psbA*, *psbB*, *psbDII* and *psbDIC* gene transcripts encoding D1, CP47, D2 and D2 together with CP43 protein, respectively, were



detected by gene specific probes. This analysis indicated that the transcription efficiencies of these genes were similar in WT and the  $\Delta crtH/B$  cells (Fig. 20).



**Figure 20: Northern hybridization analyses of genes encoding large protein subunits of PSII**

*psbA*, *psbDII*, *psbDIC* and *psbB* genes in WT and  $\Delta crtH/B$  grown under LAHG conditions. Ten  $\mu\text{g}$  of total RNA was loaded per lane. All membranes were probed also with the *rnpB* (RNase P RNA gene) as a loading and transfer control.

#### 4.4 Functional consequences of carotenoid deficiency

##### 4.4.1 Oxygen-evolving activity of the cells

Oxygen-evolving activity from  $\text{H}_2\text{O}$  to 1,4-parabenzquinone, an artificial electron acceptor of PSII, was  $220 \mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$  in WT cells grown under LAHG conditions. When the cells were transferred to the light at  $35 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  light intensity, the activity increased from 220 to 260 and to  $480 \mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$  in 3 and 48 hour, respectively.  $\Delta crtH$  cells showed no photosynthetic oxygen-evolving activity from  $\text{H}_2\text{O}$  to 1,4-parabenzquinone, however they had active PSI related oxygen uptake (Masamoto et al. 2004) under LAHG conditions. After 48 hours of illumination, the oxygen evolving activity of  $\Delta crtH$  cells detected at  $390 \mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$  which was similar to that of the WT cells indicating recovery of  $\Delta crtH$  cells upon illumination. In contrast,  $\Delta crtH/B$  cells showed no PSII activity measured from  $\text{H}_2\text{O}$  to  $\text{CO}_2$  and from  $\text{H}_2\text{O}$  to 1,4-parabenzquinone either under LAHG conditions or after 3 or 48 hour of illumination.

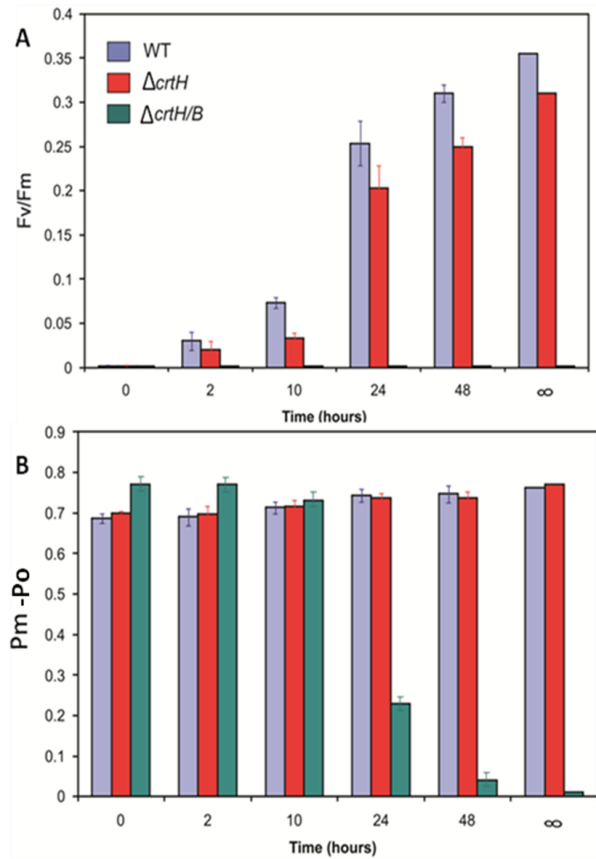
##### 4.4.2 Maximum photosystem II efficiency in dark-adapted state ( $F_v/F_m$ )

Measurements of Chl *a* fluorescence on *Synechocystis* WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells grown under LAHG conditions were performed following illumination at a light intensity of  $35 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  for 0, 2, 10, 24 and 48 hours. Cell cultures were dark adapted for 20 min before each measurement to allow the determination of fluorescence intensity when all PSII RCs are open ( $F_o$ ), i.e. they are capable of transferring electrons from the water

oxidizing complex to the  $Q_A$  electron acceptor before a saturation pulse applied to determine the maximum fluorescence intensity of closed PSII RCs ( $F_m$ ). Based on these two parameters, the  $F_v/F_m$  parameter was calculated that is a sensitive indicator of photosynthetic efficiency in dark-adapted cells. WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells grown under LAHG conditions showed no variable fluorescence ( $F_v$ ) (Fig. 21A). PSII-recovery in both WT and  $\Delta crtH$  cells started within 2 hours of light treatment. The initial rapid increase in  $F_v/F_m$  values of WT and  $\Delta crtH$  accounted within 24 hours which was nearly 70 % of those in light grown forms.  $F_v/F_m$  values in WT and  $\Delta crtH$  cells grown in the light (at  $35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  light intensity) were about 0.35 and 0.3, respectively (Fig. 21A,  $\infty$ ). In *Synechocystis*  $F_v/F_m$  values are rather low ( $\sim 0.3\text{--}0.4$ ) (Ruengjitchatchawalya et al. 2005, Wilson et al. 2006) compared to those of higher plants. In contrast to the WT and  $\Delta crtH$  cells, in  $\Delta crtH/B$  cells no PSII activity was induced by illumination (Fig 21A).

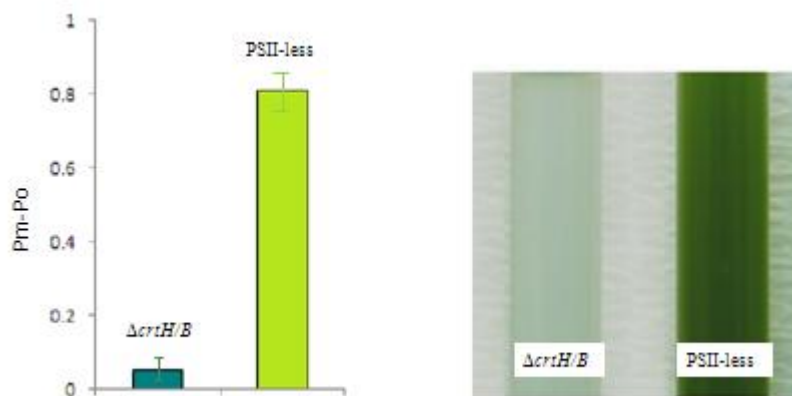
#### 4.4.3 The maximum redox changes in P700 signals in dark-adapted state (Pm-Po)

Measurement of the redox changes in P700 were performed in *Synechocystis* WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells grown under LAHG conditions after light treatment for 0, 2, 10, 24 and 48 hours at  $35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Cells were dark-adapted for 20 min before each measurement for maintaining the P700 in the reduced form (all RCs are open) to determine the minimum P700 signal level ( $P_o$ ). The changes in the level of active PSI centers were determined by applying a far-red pre-illumination for 10 seconds and the maximum P700 signal level ( $P_m$ ) was induced by applying saturating light. The measurements demonstrated the presence of active P700 centers in carotenoid-less  $\Delta crtH/B$  under LAHG conditions. Exposure of cells for 24 hours to light significantly decreased PSI activity and almost no signal was detected after 48 hours of illumination (Fig. 21B). In Figure 21, it was demonstrated that in  $\Delta crtH/B$  cells there was neither PSII activity (Fig. 21A) nor PSI activity at the end of the light treatment (Fig 21B, 48 hours). Nevertheless, PSII-less mutant (psbDI, psbDII and psbC genes encoding D2 and CP43 proteins, respectively were blocked) having carotenoid but lacking PSII activity showed a constant PSI activity and typical green color even when the cells were grown in the light (Figure 22). These results indicate that carotenoid deficiency was the reason for the photoinhibition of PSI in  $\Delta crtH/B$ .



**Figure 21: Photosynthetic activity of WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells**

(A) Maximum PSII efficiency (Fv/Fm) and (B) Maximum changes in P700 signals (Pm-Po), were determined for WT,  $\Delta crtH$  and  $\Delta crtH/B$  under LAHG conditions after exposure to 35  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light for 0, 2, 10, 24 and 48 hours for both measurements. The data from three independent measurements were averaged. Blue; WT, red;  $\Delta crtH$ , dark green;  $\Delta crtH/B$ , 0; cells grown under LAHG conditions;  $\infty$ ; cells grown under continuous light.



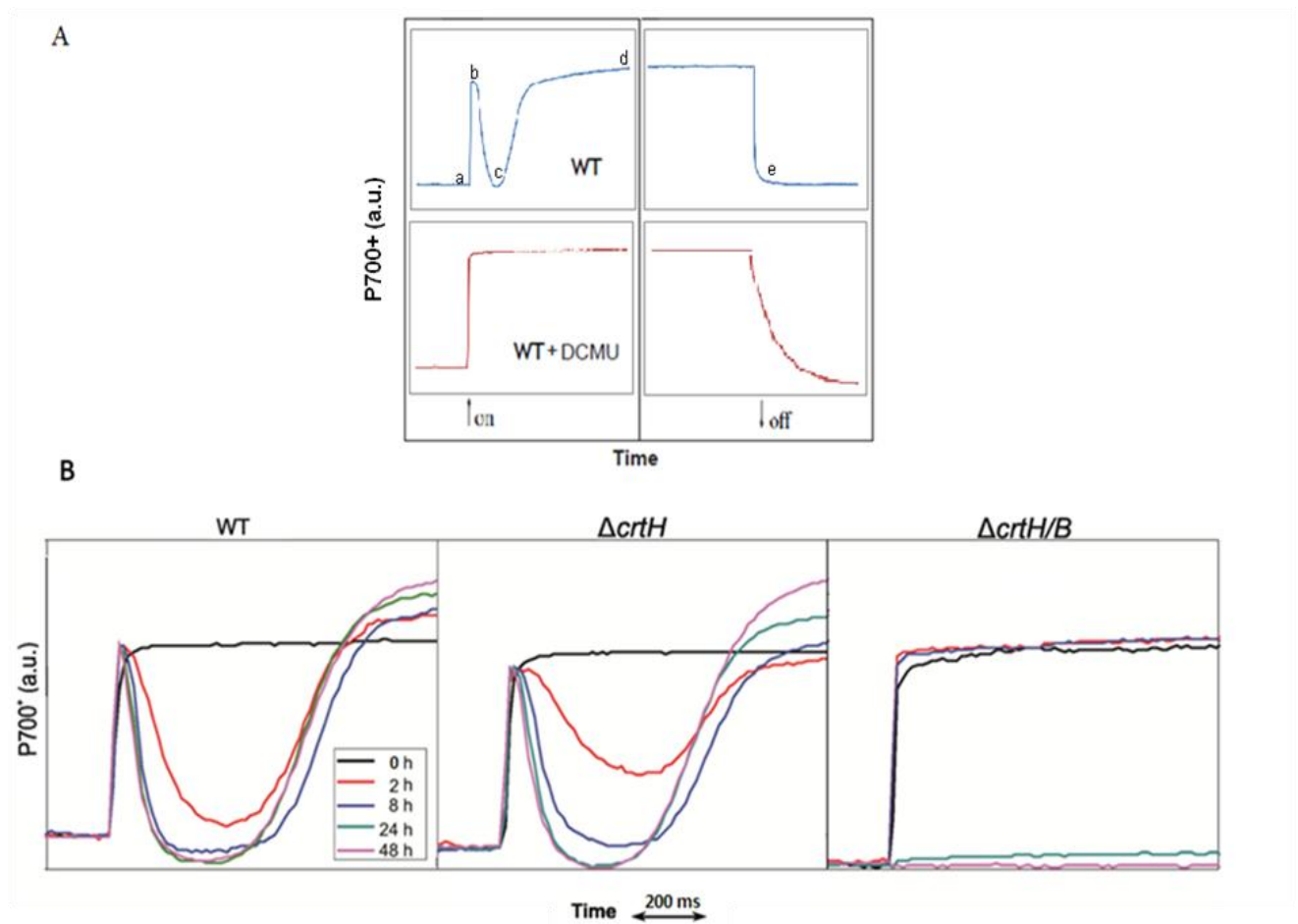
**Figure 22: (A) The maximum changes in P700 signals for  $\Delta crtH/B$  and PSII-less mutants. (B) The effect of light on pigmentation of  $\Delta crtH/B$  and PSII-less mutants**

$\Delta crtH/B$  cells grown under LAHG conditions were collected after 48 hours at 35  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light treatment and PSII-less cells grown in continuous light of 35  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Delete the rest of the sentence. The data from three independent measurements were averaged.

#### 4.4.4 Oxidation-reduction kinetics of P700

Oxidation-reduction of P700 displays complex kinetics before it reaches the final steady-state level, as observed in several species of algae (Maxwell and Biggins 1977) and cyanobacteria (Ruengjitchatchawalya et al. 2005, Sas et al. 2006). In light grown WT cells, the main features of this oxidation kinetics can be described by a rapid initial decrease in the absorption (a) due to a fast photooxidation of P700 (b), followed by an almost complete, transient re-reduction (c), and then a second, slow oxidation to the final steady-state level (d). The transient reduction is caused by the electron flow from the reduced intersystem electron transport components, such as the plastoquinone (PQ) pool, the Cyt *b<sub>6</sub>f* complex, the plastocyanin, and the Cyt *c<sub>553</sub>*. The slow oxidation of P700 reflects the depletion of this electron pool as a consequence of the rate-limiting step of PQH<sub>2</sub> oxidation at the Cyt *b<sub>6</sub>f* complex. The dark reduction of P700<sup>+</sup> (e) by intersystem electron transfer components after illumination follows first-order kinetics, in accordance with a single rate-limiting step between the two photosystems (Hartwig 2001). Figure 23A shows the P700 kinetics of WT cells in the absence and presence of 100  $\mu$ M DCMU. DCMU blocks Q<sub>A</sub> to Q<sub>B</sub> electron transfer and inhibits the transient reduction of P700 by preventing linear electron flow towards PSI.

The oxidation-reduction kinetics of P700 was measured for dark-adapted WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells grown under LAHG conditions and following 0, 2, 10, 24 and 48 hours of light treatment at 35  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>. The cells were dark-adapted for 20 min and then measured. In WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells grown under LAHG conditions the extent of absorbance changes (Fig 23B, black lines) were similar to that of WT cells grown in light in the presence of DCMU (Fig 23A) indicating that there is no transient reduction of P700 by electrons originating from PSII in any of the strains under LAHG conditions. The oxidation kinetics of P700 in the dark-adapted  $\Delta crtH$  is similar to that of the WT. In both strains, transient reduction can be observed after 2 h of illumination. The transient reduction of P700 in  $\Delta crtH$  compared to that in WT was slightly delayed and its initial rate was slightly lower. Its final steady-state level, though, was almost the same after 48 h illumination. The oxidation of P700 in the  $\Delta crtH/B$  cells follows remarkably different kinetics. A transient reduction pattern was not observed in  $\Delta crtH/B$  cells after illumination. These data strongly suggest that, in contrast to PSII-recovery in WT and  $\Delta crtH$  cells exposed to light, in  $\Delta crtH/B$  the rate-limiting step was found at the donor side of P700 indicating absence of functional PSII (Fig. 23B).

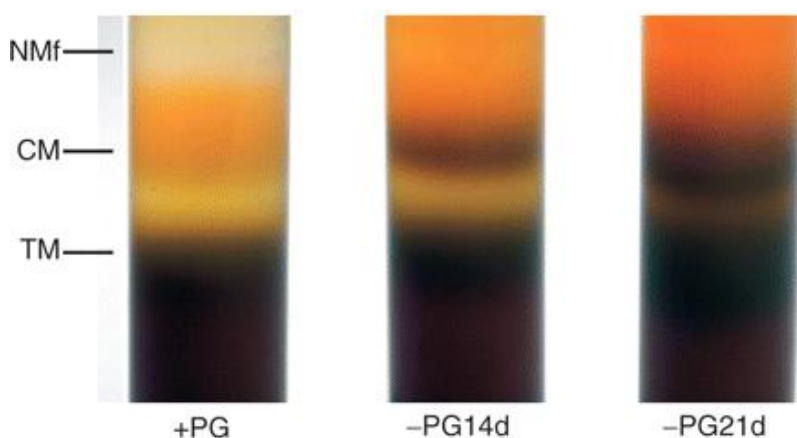


**Figure 23: Oxidation-reduction kinetics of P700**

(A) Typical oxidation-reduction kinetics in WT and WT+100 $\mu$ M DCMU. a, b, c, d and e indicate the consecutive steps of oxidation-reduction kinetics. (B) Transient reduction kinetics of WT,  $\Delta crtH$  and  $\Delta crtH/B$  cells. The cells grown under LAHG conditions and after exposed to 50  $\mu$ mol photons  $m^{-2}s^{-1}$  light for 2, 10 24 and 48 hours for both measurements. The data from 3 independent measurements were averaged.

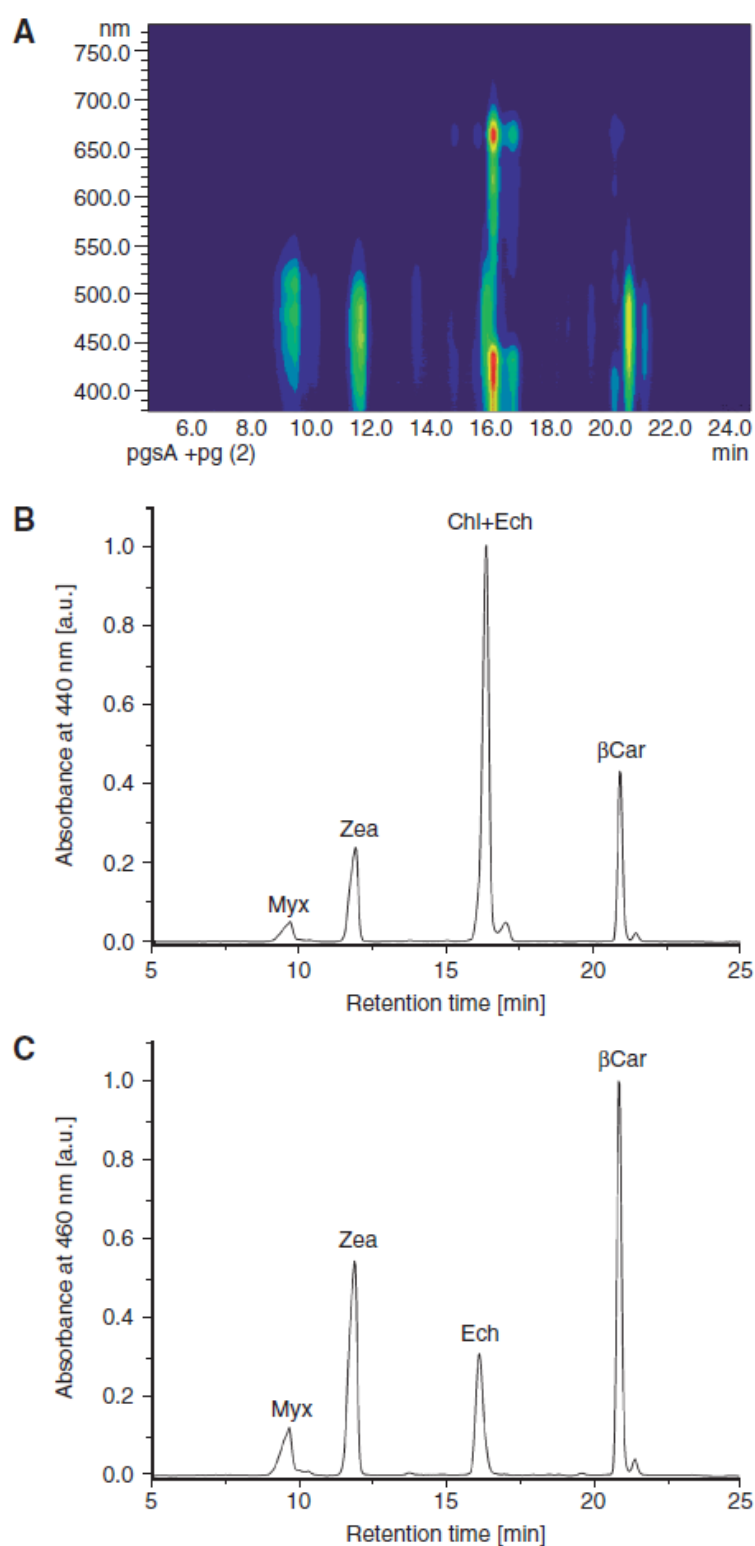
#### 4.5 Phosphatidylglycerol (PG) depletion induces an increase in myxoxanthophyll and echinenone biosynthetic activities in *Synechocystis* PCC 6803 cells

In order to study the role of carotenoids in protection against other physiological stress we used a phosphatidylglycerol (PG)-deficient *Synechocystis* *pgsA* mutant (Hagio et al. 2000). We measured the changes in carotenoid content and composition during longer term PG depletion (Laczko-Dobos et al. 2008, Domonkos et al. 2009, Bogos et al. 2010). In order to analyze and localize the carotenoid accumulation in the PG-deficient mutant we isolated thylakoid and cytoplasmic membrane fractions either from PG-supplemented or from PG-depleted cells. The separation of cytoplasmic and thylakoid membrane layers by ultracentrifugation on a discontinuous sucrose density gradient revealed that the PG-depleted cells (–PG14day and –PG21day) contained higher amount of carotenoids than the PG-supplemented (+PG) cells, especially in the upper part of the gradient which contained the water-soluble non-membrane fraction of the cells (Fig. 24).

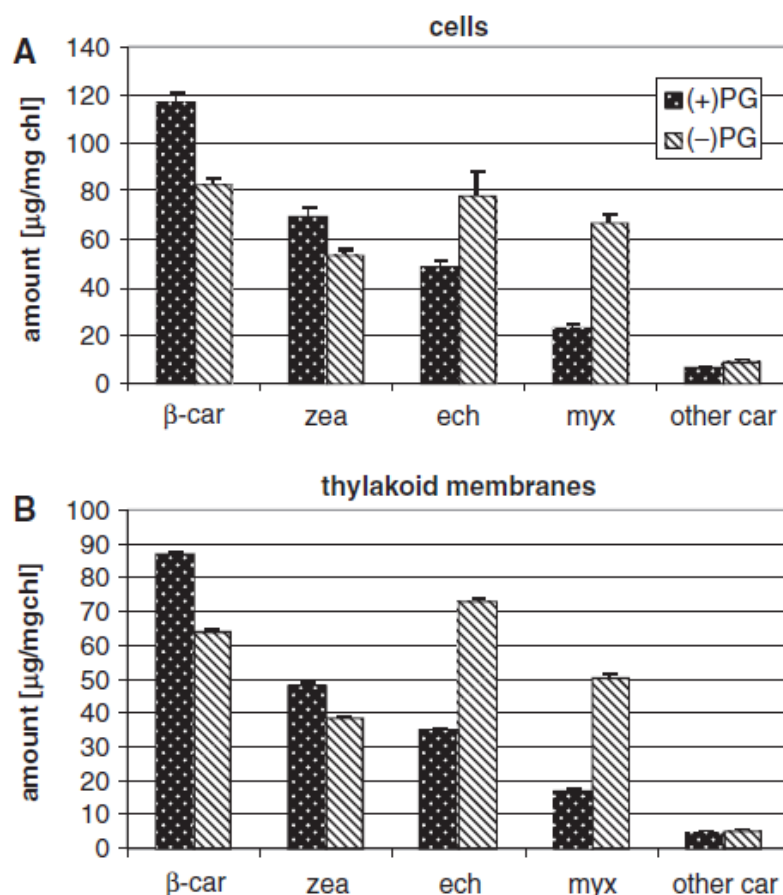


**Figure 24: Accumulation of carotenoids induced by PG depletion**

Isolation of thylakoid and cytoplasmic membranes from *Synechocystis* *pgsA* cells by sucrose gradient centrifugation. The loaded samples were normalized on the wet weight of the pelleted cells. +PG, PG-supplemented cells; –PG14d and –PG21d, PG-depleted cells on 14<sup>th</sup> and 21<sup>st</sup> days of growth, respectively; CM, cytoplasmic membrane; TM, thylakoid membrane; NMf, non-membrane fraction.



**Figure 25: HPLC analysis of the pigment composition of *Synechocystis pgsA* cells**  
 (A) 3D image of the chromatogram, (B) chromatogram recorded at 440 nm, (C) chromatogram recorded at 460 nm. myx, myxoxanthophyll; zeaxanthin; ech, echinenone;  $\beta$ car,  $\beta$ -carotene; Chl, chlorophyll.



**Figure 26: Changes in carotenoid content upon PG depletion**

Amounts of carotenoid species relative to the Chl content (A) of whole cells and (B) of thylakoid membranes in PG-supplemented and PG-depleted *pgsA* cells. +PG, cells grown in the presence of PG; -PG, cells grown in the absence of PG for 21 days; β-car, β-carotene; zea, zeaxanthin; ech, echinenone; myx, myxoxanthophyll; other car, unidentified carotene derivatives. Error bars represent  $\pm$ SD ( $n = 3$ ).

We analyzed the carotenoid species extracted from *pgsA* cells and from isolated membrane fractions by HPLC (Fig 25). In *pgsA* cells, myxoxanthophyll, zeaxanthin, echinenone and β-carotene were identified on the basis of their absorption spectra and their retention times (Fig 25). Since the difference in retention times between Chl *a* and echinenone was too small, the echinenone content was calculated from the absorbance at the maximum (460 nm) of its spectrum. The total amount of carotenoid species was calculated from the HPLC chromatogram on Chl basis in PG-supplemented and PG-depleted *pgsA* cells as well as in thylakoid membranes (Fig. 26). The amounts of β-carotene and zeaxanthin decreased in the PG-depleted samples compared to those of the PG-supplemented samples. The amount of myxoxanthophyll increased approximately 3-fold in all PG-depleted samples, and the amount of echinenone was twice as high in the PG-depleted thylakoid membranes

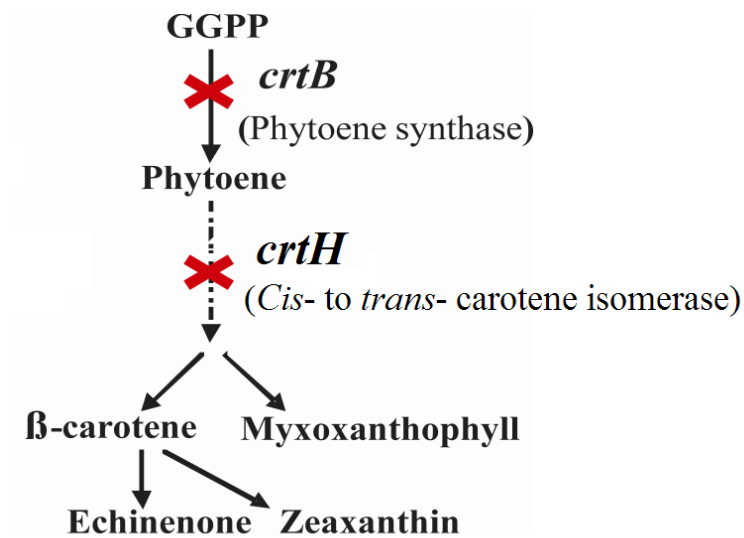


and to a lesser extent in whole cells than in PG-supplemented cells. Relative amount of the individual carotenoid species was also estimated from their peak areas from the HPLC chromatograms. The proportion of myxoxanthophyll and echinenone in the carotenoids of PG-depleted whole cells, isolated membranes and cytosol proved to be higher than in the corresponding PG-supplemented controls (data not shown).

## 5.0 DISCUSSION

### 5.1 Structural and functional consequences of the carotenoid deficiency studied by the *Synechocystis* $\Delta crtH/B$ cells

The structural and functional consequences of the complete lack of carotenoids were studied in a carotenoid-less mutant of the oxygenic photosynthetic organism, *Synechocystis*. The mutant was generated in  $\Delta crtH$  mutant cells by inactivation of the *crtB* gene encoding phytoene synthase, which is responsible for the first committed reaction toward carotenoid biosynthesis (Fig. 27).



**Figure 27: Inactivation of the *crtB* gene, encoding phytoene synthase, in  $\Delta crtH$  mutant in the carotenoid biosynthetic pathway in *Synechocystis***

$\Delta crtH$  was used as a host strain for its better transformability compared to WT. Under photoautotrophic growth conditions carotenoid composition and growth rate of the  $\Delta crtH$  mutant cells were very similar to those of the WT (Breitenbach et al. 1998, Masamoto et al. 2001, Masamoto et al. 2004). Therefore,  $\Delta crtH$  was more suitable substitute than WT in construction of the carotenoid-less mutant  $\Delta crtH/B$ . Under LAHG conditions complete segregation of the  $\Delta crtH/B$  mutant occurred and HPLC analysis demonstrated that the mutant cells contained no carotenoid derivatives (Fig. 11). The bluish color of the mutant cells indicated that phycobiliprotein synthesis and accumulation was not significantly suppressed,

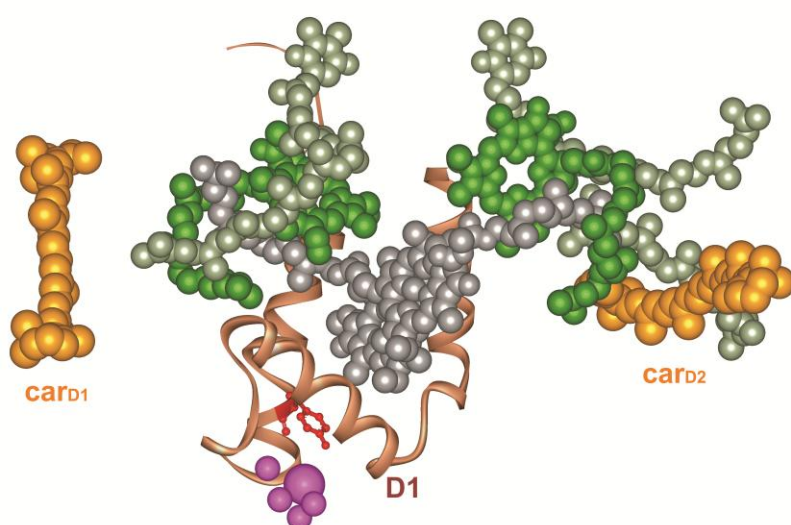
in contrast to chlorophyll synthesis and accumulation (Fig. 12). When the cells were exposed to light, the  $\Delta crtH/B$  cells gradually bleached and died (Fig. 12). This light susceptibility suggested that carotenoids are essential for the cells in the photoprotection mechanism.

As it was mentioned above, pigment availability is a major factor in the formation and function of photosynthetic complexes. In the monomeric PSII complex there are 12 carotenoid pigments identified in *T. elongatus* as  $\beta$ -carotene in all-*trans* configuration (Guskov 2009). Previously it has been suggested that  $\beta$ -carotene is vital for synthesis of D1 protein in the green alga, *Chlamydomonas reinhardtii* (Trebst and Depka 1997) as well as in the cyanobacterium, *Synechocystis* (Masamoto et al. 2004). Moreover, genetic manipulation of the carotenoid synthesis suggested that PSII assembly requires the presence of a carotenoid with at least one  $\beta$ -ionylidene ring, which may play a structural role in early stages of assembly (Bautista et al. 2005b). On the other hand, it is interesting to note that carotenoid-less mutants of purple bacteria with type-2 RCs have normal photosynthesis. These RCs assemble and function properly, although their stability decrease compared to those of the WT (Ouchane et al. 1997). Therefore, in these phototrophic bacteria carotenoids are not essential for structure and function of type-2 RCs. Since photosynthetic complexes are evolutionarily well conserved among cyanobacteria and higher plants, our results demonstrate the general importance of carotenoids in the assembly of photosynthetic complexes and photoprotection of photosynthetic machinery against changing environmental conditions.

In the carotenoid-less  $\Delta crtH/B$  mutant, amount of the large protein subunits of PSII was strongly suppressed as detected by western blotting (Fig. 16). D1 and D2 core complex protein subunits were present at lower level than those of the WT under LAHG conditions. After the cells were exposed to light, even lower level of D1 and D2 protein subunits was detected indicating photooxidation of those proteins in the absence of carotenoids. CP47 and especially CP43 Chl-binding antenna protein subunits were most affected. This is in agreement with the latest X-ray crystallographic model of PSII structure (Guskov et al. 2009, Guskov et al. 2010) which shows that most of the  $\beta$ -carotene molecules are located in the vicinity of the transmembrane  $\alpha$ -helices of CP47 and CP43.

For  $\Delta crtH/B$ , 2D gel analyses of protein subunits in combination with their radioactive labeling provided clues for the explanation of why no active PSII complexes were assembled. The results show that dimeric PSII complex is not formed and monomeric PSII complex could only be detected by radioactive labeling (Fig. 17, 19). Its amount significantly decreased after chase experiment together with chloramphenicol treatment, which is a protein synthesis inhibitor, suggesting that monomeric PSII complex could actually be formed,

however, it was very unstable (Fig 19, chase). RC47 appeared as the most dominant complex, which is an intermediate of PSII complex lacking CP43 that is easily detectable by 2D gel electrophoresis (Fig 17). The increase in the level of RC47 after chase experiments can be a response to the decrease in the level of monomeric PSII form by detachment of CP43. Concomitantly an increase in the level of RCa was also observed, which is also intermediate PSII lacking both CP47 and CP43. It is known that PsbI and PsbH small protein subunits stabilize CP43 and CP47 in PSII complex, respectively (Komenda et al. 2002, Komenda et al. 2005, Dobakova et al. 2007). They were detected in RC47 by immunoblotting following 2D gel separation (Fig. 19). These findings confirmed that not the absence of these two small protein subunits but the absence of  $\beta$ -carotene resulted in instability of CP47 and especially CP43 in the PSII complex of  $\Delta crtH/B$  mutant. These results suggest the importance of carotenoids for PSII biogenesis. However, presence of RC47 and RCa in carotenoid-less cells indicated that at least PSII core complex consisting of D1-D2 heterodimer can be formed and the 2  $\beta$ -carotenes (Guskov et al. 2009) in the vicinity of the D1-D2 heterodimer are not primarily responsible for the PSII core complex assembly (Fig. 28).



**Figure 28: Two  $\beta$ -carotene molecules located in the PSII core complex.**

Two  $\beta$ -carotene molecules named as car<sub>D1</sub> and car<sub>D2</sub> according to Guskov et al., 2009. The figure was created with the software DSV and by the PDB file 3BZ1 (Guskov et al. 2009).

D1 protein was the most intensively labeled protein in RC47, as well as in monomeric PSII complex and RCa. An intense radioactive labeling of pulse-chased D1 protein pointed to a rapid functional D1 turnover in the  $\Delta crtH/B$  and it was in line with the selective

replacement of the D1 protein during PSII repair. However, other newly synthesized PSII proteins such as D2, CP43 and CP47 were detected mainly in the unassembled proteins (U.P.) region and could only transiently be detected by the autoradiogram.

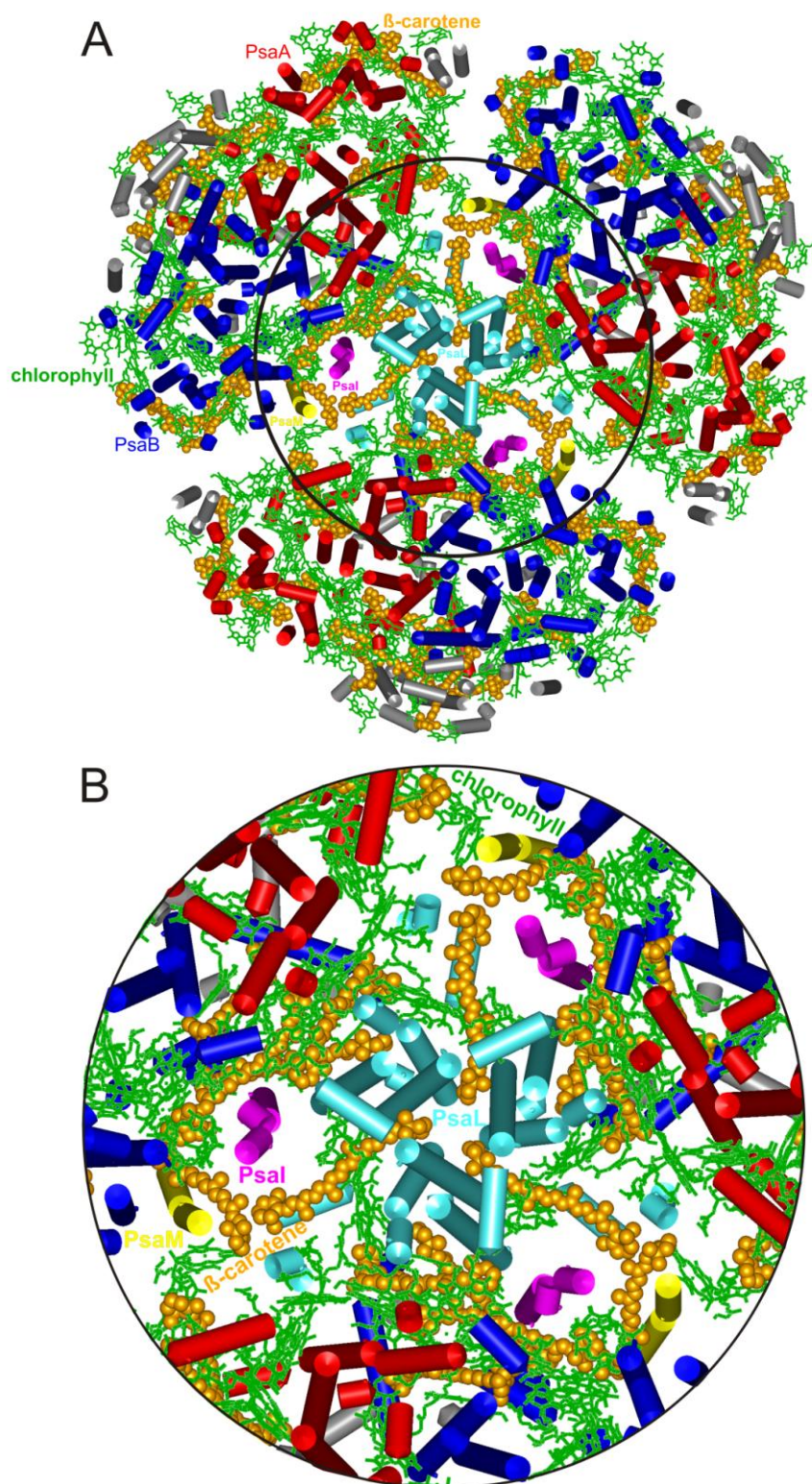
Recent X-ray structural models of cyanobacterial PSII (Ferreira et al. 2004, Loll et al. 2005, Guskov et al. 2009) have demonstrated the presence of  $\beta$ -carotenes at the interface between initial transmembrane helices of PSII large protein subunits and small protein subunits outside the heterodimer D1-D2. This applies to D2-Cyt *b*<sub>559</sub> pair and to CP47-PsbM/PsbT pair and especially to CP43-PsbK/PsbZ pair. It has been shown previously that there are two parallel bands of unassembled PSII large protein subunits on the BN PAGE which are slower and faster electrophoretic mobility protein bands (Dobakova et al. 2007, Komenda et al. 2008). The mobility differences between the bands rise from the absence/presence of PSII small protein subunits.. Additional PSII small protein subunits seem to stabilize the PSII large protein subunits in the complex (Komenda et al. 2005, Dobakova et al. 2007, Komenda et al. 2008). In  $\Delta crtH/B$ , bands were found in faster electrophoretic mobility of radioactively labeled unassembled CP47, CP43 and D2 large protein subunits. These findings indicate that carotenoid deficiency negatively affected the stability of binding small protein subunits to large protein subunits in PSII (Fig. 18). Data from Northern blot analyses indicated that the transcription of genes encoding PSII large protein subunits is not affected by elimination of carotenoids under LAHG conditions (Fig. 20).

The elimination of carotenoids suppressed the synthesis of PSII proteins in LAHG conditions and the synthesis of the overall membrane proteins during light treatment. This pointed to a general detrimental effect of the absence of carotenoids on the protein subunits. The regulation mechanism behind this suppression has not been elucidated yet. However, it has been shown that oxidative stress related to the action of ROS inhibits the elongation step of D1 translation via oxidation of the elongation factor G (Kojima et al. 2007). We assume that there may be a similar reason for inhibited translation in the  $\Delta crtH/B$  mutant, since lack of carotenoids severely decreased the ability of cells to scavenge ROS and to prevent the inactivation of translation factors.

Cyt *b<sub>6</sub>f* complexes possess specific carotenoid-binding sites, suggesting an important structural role for these pigment molecules (Wenk et al. 2005). Despite this expectation,  $\Delta crtH/B$  cells showed a growth rate similar to that of the WT under LAHG conditions and 2D protein analysis revealed similar accumulation of monomeric Cyt *b<sub>6</sub>f* complex in both WT and  $\Delta crtH/B$  cells (Fig. 17). This finding indicates that carotenoids are not essential for the assembly of functional Cyt *b<sub>6</sub>f* complexes.

PSI complex can be assembled in the absence of carotenoids.. Interestingly, PSI in the  $\Delta crtH/B$  was mostly present in the monomeric form while the prevailing form in WT cells is the trimeric form. Blue native gel experiments revealed that the trimer/monomer ratio of PSI drastically decreased in the mutant. On the other hand, we cannot exclude that trimeric form exist in the mutant cells and they are destabilized and decomposed during gel analysis. We suggest that carotenoids have an important stabilization function in PSI trimerization domain. This is in accordance with the fact that trimerization domain is rich in  $\beta$ -carotene (Fig. 29) (Grotjohann and Fromme 2005).

The striking difference between the antenna system in PSI and PSII is the lack of the central antenna domain in PSII. Whereas the PSI central antenna domain contains 96 Chl *a* and 22 carotenoid molecules carrying the electron transport chain in PSI RC. In contrast, the PSII RC contains only the D1-D2 heterodimer, 2  $\beta$ -carotene and 6 Chl *a* molecules (Fig. 28 and Fig. 30). The absence of the central antenna domain in PSII may be the response to its ability for water splitting as an unlimited electron source, which leads to the process of D1 turnover. This process may have hindered the fusion of the RC domain and the antenna domain in PSII,(Fig. 30). Definitely, among photosynthetic membrane complexes in  $\Delta crtH/B$  mutant, PSII was the most severely affected complex by the absence of carotenoids. Indeed, PSII-dependent oxygen-evolving activity of carotenoid-less  $\Delta crtH/B$  cells measured by using an artificial electron acceptor 1,4-parabenzquinone was undetectable indicating the absence of functional PSII complexes. Additionally, the absence of variable Chl *a* fluorescence and the absence of transient reduction in P700 kinetics confirmed that in  $\Delta crtH/B$  cells, no active PSII centers are present (Fig 21A, 23).



**Figure 29: Luminal side view of trimeric PSI complex**

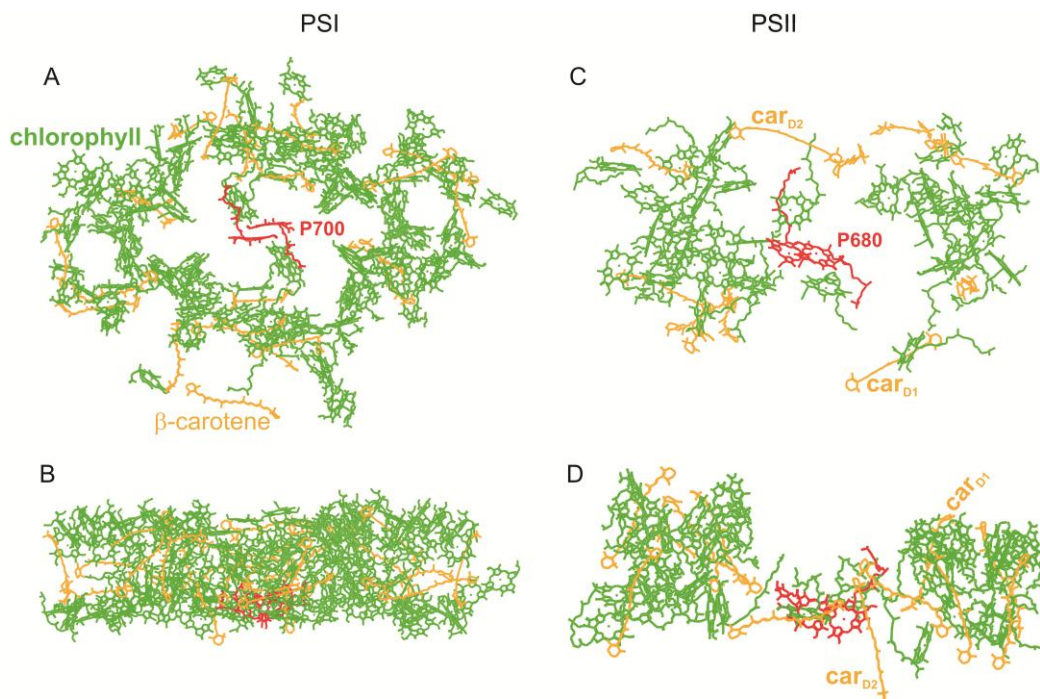
(A) Trimerization domain, rich in  $\beta$ -carotene, shown in black circle and (B) in magnified view. Yellow, carotenoid, green, chlorophyll, light blue, PsaL; violet, PsaI and yellow PsaM. The figure was created with the software DSV and the PDB file 1JB0 (Jordan et al. 2001).



PSII is known to experience radiation damages even at very low illumination intensities. The main ROS produced in the PSII RC is the very reactive singlet oxygen. This is due to the generation of singlet oxygen during charge recombination between  $P680^+$ /Pheo $^-$  and to the fact that carotenoid molecules in PSII RC are located too far from P680 to be able to efficiently quench the triplet state Chl (Telfer 2002) (Fig. 28). On the other hand, if the carotenoids were closer, they would probably be immediately oxidized by  $P680^+$  because of its strong oxidizing potential (Telfer 2002). For this reason PSII RC is poorly protected from the action of singlet oxygen and an efficient mechanism of PSII repair has evolved in order to maintain the photochemical capacity. When light is in excess, the D1 protein is rapidly degraded and resynthesized to reestablish a fully active PSII RC (Baena-Gonzalez and Aro 2002). A similar mechanism is not known for PSI. After radiation damages its recovery requires several days (Kudoh and Sonoike 2002). Furthermore, the repair from photoinhibition does not require the turnover of a single polypeptide, as in the case of D1, but involves degradation and resynthesis of the whole PSI complex. Although the mechanisms and localization are different, the D1 protein might also be considered as a safety fuse: in fact, once D1 is degraded, not only charge separation but also singlet oxygen production are stopped thus protecting the remaining PSII core complex from oxidation damage and photobleaching (Bergantino et al. 2003). Thus, it appears that in both complexes some sacrificial proteins exist functioning like a safety fuse for the rest of the structure. In the case of PSI large antenna Chl-protein domain while in the case of PSII is the D1 protein of the RC to play this role. It is worth mentioning that  $P700^+$  is not such a strong oxidizer as  $P680^+$ , and no massive charge recombination has been reported in PSI. This is probably one major reason explaining why different photoprotection strategies evolved in PSI and PSII. It is interesting to point out that, despite the differences, in both cases the antenna systems are fundamental in photoprotection. Even under high light conditions, large antenna system works very efficiently as the excited triplet state Chl *a* cannot be detected in intact PSI (Grotjohann and Fromme 2005). In addition, PSI photoprotection is mainly mediated by ROS scavenging enzymes (e.g., superoxide dismutase and ascorbate peroxidase) instead of carotenoid scavenging. These enzymes efficiently detoxify reactive species such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^-$ ), hydroxyl radicals (OH) produced at the reducing side of PSI (Asada 1999). Therefore, PSI is generally believed to be less sensitive to light stress and its carotenoid related photoprotection mechanisms were less investigated. Norflurazon (inhibitor of phytoene synthase)-treated *Scenedesmus obliquus* cells lacking carotenoids still contain some PSI and charge separation can be detected (Romer et al. 1995). Samples in which all



carotenoids are extracted with organic solvents lack quinones but are still able to perform electron transfer from P700 to A0 (Ikegami and Katoh 1991). In carotenoid-less  $\Delta crtH/B$ , PSI charge separation and cyclic electron transport could be efficiently performed in LAHG conditions (Fig.21B). However, level of active PSI centers significantly decreased after cells were exposed to low light illumination. Earlier studies suggested that PSI starts to become activated only when as a consequence of unassembled subunits of PSII chlorophyll liberation and degradation (Trebst A and Depka B. 1997). To understand the reason of PSI photoinhibition in carotenoid-less and PSII-less  $\Delta crtH/B$ , we used PSII-less mutant strain which contains carotenoids (psbDI, psbDII and psbC genes encoding D2 and CP43 proteins, respectively were blocked) as a control. Nevertheless, PSII-less mutant showed a significant PSI activity similar to WT even if the cells were grown in constant light (Fig. 22). These results suggested that not the inefficient linear electron transport between PSII and PSI but the carotenoid deficiency was the reason of the photoinhibition of PSI.



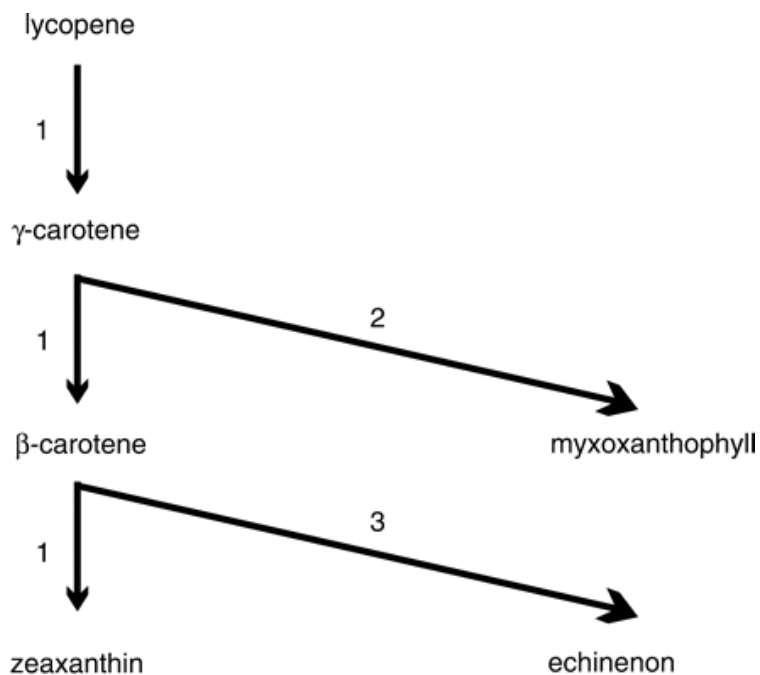
**Figure 30: Arrangement of antenna pigments in the PSI and PSII complexes of *T. elongatus*.**

Luminal side view (A, C), side view along the membrane plane (B, D). Green, chlorophyll; orange,  $\beta$ -carotene; green, Chl *a*, red, P700 and P680 centers.  $car_{D1}$  and  $car_{D2}$ ;  $\beta$ -carotene molecules in PSII RC named by Guskov 2009. The figure was created with the software DSV and the PDB files 3BZ1 (Guskov et al. 2009) and 1JB0 (Jordan et al. 2001).

## 5.2 Phosphatidylglycerol (PG) depletion induces an increase in myxoxanthophyll and echinenone biosynthetic activities in *Synechocystis* PCC 6803 cells

Investigation of pigment-lipid interaction in a membrane environment is important to understand the primary mechanism of antioxidant activity and bioavailability of carotenoids. Our study has demonstrated the importance of pigment-lipid interactions to drive efficient photosynthesis. Phosphatidylglycerol (PG) is the only phospholipid found in photosynthetic reaction centers. PG depletion resulted in a remarkable suppression of photosynthetic oxygen-evolving activity and impaired PSII activity by probably generation of ROS as shown earlier for *pgsA* (Hagio et al. 2000) and *PALcdsA* (Laczkó-Dobos et al. 2008) mutants. Here we studied how carotenoids respond to stress conditions induced by PG depletion in the PG-deficient mutant of *Synechocystis*, *pgsA*.

In *Synechocystis*, carotenoids are synthesized via a branched pathway initiating from common precursors (Fig 6). Lycopene is a central molecule that is present in the synthetic pathway of all carotenoids and their intermediates. Our results suggest PG depletion significantly increased the synthesis of two major carotenoids, myxoxanthophyll and echinenone and on the other hand, the suppression of  $\beta$ -carotene and zeaxanthin synthesis was observed. This differential effect of PG starvation could be explained by differences between the metabolic pathways leading to the biosynthesis of the different carotenoids (Lagarde et al. 2000). Synthesis towards myxoxanthophyll and a group of  $\beta$ -carotenes, echinenone and zeaxanthin, is branched at  $\gamma$ -carotene (Fig. 31). The second branching point is at  $\beta$ -carotene, where the synthesis of echinenone and that of zeaxanthin diverge (Fig 30). Our results suggest that these steps are regulated differently. The increase in the relative content of echinenone can be explained by its higher stability compared with those of other carotenoid species in *Synechocystis* (Steiger et al. 1999), whereas its absolute increase in the cytoplasmic membrane can be due to enhanced biosynthesis. However, the higher amount of myxoxanthophyll, a less stable carotenoid species comparing to zeaxanthin and echinenone, seems to indicate enhanced biosynthesis of this compound.



**Figure 31: Flow sheet of carotenoid biosynthesis in *Synechocystis*.**

Arrow 1 indicates non-regulated or down-regulated catalytic processes, and arrows 2, 3 indicate up-regulated catalytic processes in carotenoid biosynthesis.

The two up-regulated carotenes, myxoxanthophyll and echinenone, are special protective agents. In *Synechocystis*, myxoxanthophyll, which was preferentially synthesized, under high light stress provided the strongest protection and echinenone was the most stable carotenoid under photo-oxidative conditions (Steiger et al. 1999). Investigations on the photoprotective function of myxoxanthophyll in cyanobacterium *Plectonema boryanum* cells under UV radiation stress revealed a reduced efficiency of energy transfer to PSII RCs and an elevated level of myxoxanthophyll (Ivanov et al. 2000). Low temperature stress increased the myxoxanthophyll content of *Cylindrospermopsis raciborskii* cells (Várkonyi et al. 2002). Myxoxanthophyll is also required for the formation of normal cell wall structure and it stabilizes thylakoid membranes (Mohamed et al. 2005). Induction of myxoxanthophyll biosynthesis by high light exposure in *Synechocystis* has been demonstrated by Mohamed and Vermaas (2004). However, very little is known about the biological function of both myxoxanthophyll and echinenone.

Increased myxoxanthophyll and echinenone content of the PG-depleted cells was detected in the membranes; nevertheless, a significant amount of carotenoids was also found in the cytosol. The accumulation of carotenoids was demonstrated in the upper non-membrane layer of the sucrose gradient (Fig. 24). Accordingly, we found increased

myxoxanthophyll and echinenone contents in the cytosol of PG-depleted cells. Since carotenoids are not water-soluble molecules, they should be bound to proteins in the cytosol. In cyanobacteria, orange carotenoid-binding proteins were found to maintain the carotenoids in the aqueous phase (Kerfeld 2004). Carotenoids occur in all parts of the cyanobacterial cell. Earlier studies suggested that carotenoid-binding proteins were present in cytoplasmic membranes (Bullerjahn and Sherman 1986). Carotenoids were also localized to outer membranes (Jurgens and Weckesser 1985). The carotenoids in the outer membrane were found to be oriented (Jurgens and Mantele 1991).

The increased amount of myxoxanthophyll pointed to its protection role against slowdown of photosynthetic processes generated by PG depletion. High amounts of echinenone and myxoxanthophyll, whose synthesis were up-regulated by PG depletion (Fig. 31, arrow 2 and 3), seem to protect the structure and function of the photosynthetic apparatus of cyanobacterial cells. On the other hand, the synthesis of  $\beta$ -carotene and zeaxanthin was non-regulated or down-regulated in the PG-depleted cells (Fig. 31, arrow 1). Synthesis towards myxoxanthophyll and a group of  $\beta$ -carotene, echinenone and zeaxanthin, is branched at  $\gamma$ -carotene. The second branching point is at  $\beta$ -carotene, where the synthesis of echinenone and that of zeaxanthin diverge. These findings suggested that these steps are regulated differently by PG-depleted stress conditions.

## 6.0 CONCLUSIONS

I. We have successfully generated a viable carotenoid-less mutant in an oxygenic prokaryotic species, *Synechocystis*.  $\Delta crtH/B$  cells were extremely light sensitive and could grow only under LAHG conditions, demonstrating the protective role of carotenoids against light stress.

Carotenoid-less mutant of *Synechocystis*,  $\Delta crtH/B$ , allowed us study the structural and functional consequences of the complete carotenoid deficiency on photosynthesis here for the first time, *in vivo*.

i. The structural consequences of the carotenoid deficiency on the assembly of photosynthetic complexes were studied in  $\Delta crtH/B$ :

- Dimeric and monomeric PSII complex could not be formed. Instead, intermediate PSII complexes lacking CP43 and lacking CP43/CP47 were found.
- D1 was the most intensively labeled protein in  $\Delta crtH/B$  indicating D1 turnover was active.
- Small protein subunits binding to the unassembled PSII large protein subunits were not stable without carotenoids.
- PSI and Cyt *b<sub>6</sub>f* complexes could be assembled, however trimeric/monomeric form of PSI ratio significantly decreased.

ii. The functional consequences of the carotenoid deficiency on the activity of photosynthetic reaction centers were studied in  $\Delta crtH/B$  cells:

- There were no active PSII centers.
- PSI centers were active. however they could not maintain their activity in the absence of carotenoids even under low light.

II. Phosphatidylglycerol (PG) depletion leads to a significant increase in the level of both echinenone and myxoxanthophyll suggesting their protection functions under stress conditions. Here we show that the increase of carotenoid content is a response to different stress conditions, not only to high light stress, however, the level of specific carotenoids induced by stress conditions against oxidative damage regulated differently.

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## ÖSSZEFOGLALÓ

### **Karotinoidok részvétele a cianobaktériumok fotoszintetikus komplexek összeszerelődésében és működésében**

A fotoszintézis alapvető biofizikai és biokémiai folyamat, amelynek során a napfény energiája az élethez nélkülözhetetlen kémiai energiává alakul át. A cianobaktériumok a legősibb ismert oxigéntermelő, fotoszintetizáló, prokarióta szervezetek, melyek az endoszimbiózis elmélete szerint a növényi kloroplasztisz őseinek tekinthetők. A cianobaktériumok még napjainkban is Földünk éves oxigéntermelésének közel 30%-át biztosítják.

A cianobaktériumok igen ellenálló, a környezeti változásokhoz jól alkalmazkodó szervezetek, melyek hatékonyan védekeznek a reaktív oxigéngyökök (ROS) fotoszintetikus aktivitást csökkentő, fotoinhibíciót eredményező fotooxidatív károsító hatásai ellen is. A karotinoidok működése nélkülözhetetlen a védekezéshez. A fénykárosodástól azáltal védik a sejtet, hogy képesek kioltani a gerjesztett szinglet és triplet állapotú klorofillt, valamint befogni a szinglet oxigént, amely rendkívül káros hatású ROS (Knox és Dodge 1985). Nélkülözhetetlen védőszerepük mellett a karotinoidok járulékos fénybegyűjtő pigmentként is működnek (Frank és Cogdell 1996) és részt vesznek a membránok felépítésében is (Cogdell és Frank 1987, Gruszecki és Strzalka 2005).

A *crtB* gén által kódolt fitoén-szintáz enzim katalizálja a karotinoidok bioszintézisének első lépését, a fitoén kialakítását. A fitoénből további enzimek közreműködésével különböző karotin- és xantofillmolekulák képződnek.

Az egyik legintenzívebben tanulmányozott cianobaktérium, a *Synechocystis* sp. PCC 6803 jellemző karotinoidjai a következők:  $\beta$ -karotin, hidroxiszármazéka a zeaxantin, ketoszármazéka az echinenon és a 3'-hidroxiechinenon, valamint a mixoxantofill, amely egy karotinoid glikozid (Britton és Mtsai 2004, Takaichi és Mochimaru 2007). Az újabb röntgenkristallográfiás analízisek feltárták, hogy a cianobakteriális fotoszintetikus komplexekben a  $\beta$ -karotin az egyetlen karotinoidfajta, de az összes többi karotinoid is megtalálható a cianobaktériumok tilakoidmembránjában, a citoplazmamembránban és a citoszolban. Különböző stressz körülmények hatására mennyiségük megnő a sejtben. A  $\beta$ -karotin-molekulán levő szubsztituensek kémiai természete meghatározza a molekula antioxidatív tulajdonságát, szerepét a fénykárosodás elleni védelemben (Britton 1995a). A hidroxikarotinoidok általában jók a peroxidgyökök inaktíválásában (Woodall és Mtsai

1997b), míg a ketoszármazékok hatékonyabbak a reaktív szinglet oxigén kioltásában (Woodall és mtsai 1997a), és legstabilabbnak bizonyultak a peroxidgyökök és a fotooxidáció károsításával szemben (Steiger és mtsai 1999).

Röntgenkrisztallográfiás analízissel (Guskov és mtsai 2009) 12 karotinoidot azonosítottak *Thermosynechococcus elongatus* PSII komplexében monomerenként. A PSII a fotooxidatív stresszel szemben a leginkább érzékeny fotoszintetikus komplex. Korábbi tanulmányok arra engednek következtetni, hogy a  $\beta$ -karotin elengedhetetlenül szükséges a D1 fehérje felhalmozódásához *Chlamydomonas reinhardtii* zöld algában (Trebst és Depka 1997) és *Synechocystis* PCC 6803 cianobaktériumban is (Masamoto és mtsai 2004). Tehát a karotinoidok jelenléte fontos a működőképes PSII felépüléséhez.

A *T. elongatus* PSI komplexek szerkezetében 22 karotinoidmolekulát azonosítottak monomerenként (Jordan és mtsai 2001, Fromme és mtsai 2001, Grotjohann és Fromme 2005). A PSI általában kevésbé érzékeny a fénystresszre, és a karotinoidok részvétele a fénykárosodás elleni védelemben a PSI esetén kevésbé ismert. A fitoén-szintáz gátló norflurazonnal történt kezelés hatására karotinoidmentessé vált *Chlamydomonas reinhardtii* sejtek még tartalmaztak PSI komplexeket, melyekben töltésszeparációt is megfigyeltek (Romer és mtsai 1995). A tilakoidmembránokból szerves oldószerrel kivonták a karotinoidokat és a kinonokat, ezután is még megtörtént az elektronszállítás a P700 és az A0 között (Ikegami és Katoh 1991).

A fotoszintézis folyamatai a PSI- és PSII-reakciócentrumokban játszódnak le, melyek a tilakoidmembránba ágyazott pigment-fehérje komplexek. A lipid-fehérje és lipid-pigment kölcsönhatások fontos szerepet játszanak a fotoszintézis szabályozásában. A cianobaktériumok tilakoidmembránja jellegzetes lipidösszetételű, mely glikolipideket és egy foszfolipidet tartalmaz. A cianobaktériumokban és a magasabbrendű növények tilakoidmembránjában egyetlen foszfolipid fordul elő, a foszfatidil-glicerin (PG). A *T. elongatus* fotoszintetikus komplexeinek röntgenkrisztallográfiás szerkezetvizsgálata PG-molekulákat mutatott ki a PSII komplex CP43 és D1 fehérjéi között (Guskov és mtsai 2009), valamint a PSI szerkezetében is (Jordan és mtsai 2001). A PG eltávolítása a sejtekből a klorofilltartalom csökkenését és a fotoszintetikus folyamatok lassulását okozta. A  $Q_A$  és  $Q_B$  közti elektrontranszport gátlása volt megfigyelhető, ami a  $Q_A$  túlredukálódásához és  $Q_A^{-2}$  képződéséhez vezet (Gombos és mtsai 2002, Laczko-Dobos és mtsai 2008). A PG esszenciális az alacsony hőmérsékleti stressz (Murata és mtsai 1982, Murata 1983, Moon és mtsai 1995) és a fénystressz elleni védekezésben (Vass és mtsai 1992).

A globális környezet változása egyre gyakrabban idéz elő olyan stressz körülményeket, melyek a fotoszintetizáló szervezetekre hatva csökkentik a fotoszintézis hatékonyságát. A stressz körülmények között a fotoszintézisben működő védekező mechanizmusok megismerésének igénye a különböző körülmények között eltérő fejlődési sajátosságokkal rendelkező növények, algák és cianobaktériumok kutatásához vezetett. A cianobaktériumok a növényi kloroplasztisz őseinek tekinthetők, így kiváló modellszervezetek a magasabbrendű növények fotoszintézisének tanulmányozásához. Az antioxidáns tulajdonságokkal bíró karotinoidok fontos szerepet játszanak a stressz körülmények elleni harcban, de a védekezésben elengedhetetlen szerkezeti és funkcionális sajátosságaik csak részben felderítettek. Ezért munkám során a következő célokat tűztam ki:

I. Felderíteni, hogy *in vivo*, a fotoszintetikus apparátusban a karotinoidok hiánya milyen (i) szerkezeti változásokat okoz, és ez milyen (ii) funkcionális következményekkel jár. Ennek érdekében az első olyan oxigéntermelő, fotoszintetikus prokarióta (*Synechocystis* sp. PCC 6803 cianobaktérium) mutánszt létrehozni, melyben a karotinoidok szintézise teljes mértékben gátolt.

II. Ezenkívül a karotinoidok védő szerepét tanulmányozni olyan stressz körülmények között, melyet a PG hiánya váltott ki a *Synechocystis* sp. PCC 6803 *pgsA* mutánsban.

A fitoén-szintáz enzimet kódoló *crtB* gént inaktiváltuk a részlegesen karotinoidhiányos  $\Delta crtH$  mutánsban. Így létrejött egy teljes mértékben karotinoidmentes kettős mutáns, a  $\Delta crtH/B$ , amely csak sötétben, glükóztartalmú tápoldatban volt fenntartható. HPLC analízissel igazoltuk, hogy a  $\Delta crtH/B$  sejtek semmilyen karotinoidszármazékot nem tartalmaznak. A  $\Delta crtH/B$  sejt kultúra kékes színe arra utalt, hogy a sejtekben a fikobilinok a domináns pigmentek. A karotinoidok hiánya nem érintette szignifikánsan a fikobiliproteinek szintézisét és felhalmozódását, a klorofill szintézisét és akkumulációját viszont gátolta. Fénykezelés hatására a mutáns sejtek fokozatosan kifehéredtek, elvesztették pigmentációjukat és elpusztultak, igazolva, hogy a karotinoidok nélkülözhetetlen elemei a fotoprotekciónak.

Western blot analízissel kimutattuk, hogy a karotinoidmentes  $\Delta crtH/B$  mutánsban a PSII nagy fehérjealegységeinek szintje igen alacsony, míg a PSI és a citokróm- $b_6f$  fehérjeinek mennyisége nem csökkent. LAHG körülmények között a D1 és D2 fehérjealegységek szintje sokkal alacsonyabb volt, mint a vad típusban. Miután a sejteket megvilágítottuk, a D1 és D2 fehérjék mennyisége tovább csökkent, jelezve a karotinoidok hiányában bekövetkező fotooxidációt. A CDP47 és különösen a CP43 klorofillkötő antennaalegységek szintje volt a

leginkább érintett. Ez a megfigyelés összhangban van a PSII legutóbbi röntgenkristallográfiás szerkezeti modelljével, mely szerint a  $\beta$ -karotin molekulák többsége a CP47 és CP43 alegységek transzmembrán  $\alpha$ -héliceinek közelében helyezkedik el. A  $\Delta crtH/B$  sejtek fehérje alegységeinek 2D-gélanalízise radioaktív „pulse-chase” jelöléssel kombinálva megadta a kulcsot annak magyarázatához, hogy miért nem épülnek fel aktív PSII komplexek a mutánsban. Az eredmények megmutatták, hogy PSII-dimerek nem képesek kialakulni  $\beta$ -karotin hiányában, sőt még a nagyon instabil PSII-monomerek is csak radioaktív jelöléssel voltak detektálhatóak. A CP47 és különösen a CP43 alegységek nem kötődtek stabilan a PSII komplexben, ezért csak egy intermediér PSII komplex, az RC47 (melyből a CP43 hiányzik) volt képes felhalmozódni gélelektroforézissel detektálható mennyiségben. A  $\Delta crtH/B$  sejtek tilakoidjában a D1 fehérje volt a radioaktívan leginkább jelölődött fehérje, ami jelzi, hogy a D1 fehérje „steady state turnover” karotinoidok hiányában is fennmaradt. A radioaktívan jelölődött D1 fehérje leginkább az RC47 komplexben volt detektálható, valamint a monomer PSII-ben és az RCa-ban, amely egy olyan intermediér PSII komplex, ahonnan a CP43 és a CP47 is hiányzik. Miután a „chase” kísérletet kloramfenikolkezeléssel, azaz a fehérjeszintézis gátlásával kombináltuk, a PSII-monomerek eltűntek a gélből, ami a komplex instabilitására és fényérzékenységre utal. Sőt  $\beta$ -karotin hiányában azoknak a kis fehérje alegységeknek a kapcsolódása sem stabil, amelyek a nagy PSII-alegységekhez kötődnek a komplexek összeszerelődése során. A PSII komplexszel ellentétben, a PSI és a citokróm- $b_6f$  komplexek felépülnek, bár a trimer/monomer PSI aránya szignifikánsan csökkent karotinoidok hiányában.

Kétségtelenül a PSII volt a karotinoidok hiányában leginkább érintett fotoszintetikus membránkomplex a  $\Delta crtH/B$  mutánsban. A karotinoidhiányos mutáns sejtek valóban nem tartalmaztak működőképes PSII komplexeket, amit a PSII-függő oxigénfejlesztő aktivitás méréseivel igazoltunk. Továbbá a PSII klorofill-a fluoreszcencia mérései és a P700 tranziens redukciókinetikájának mérései megerősítették, hogy a  $\Delta crtH/B$  sejtekben nem alakultak ki aktív PSII-reakciócentrumok. Ezzel szemben a PSI-hez köthető töltésszeparáció és ciklikus elektrontranszport működik a LAHG-körülmények között nevelt  $\Delta crtH/B$  sejtekben is. Azonban, miután a sejteket megvilágítottuk, az aktív PSI-reakciócentrumok mennyisége szignifikánsan csökkent. Azért, hogy megértsük a PSI fotoinhibíciójának okát a karotinoidmentes és aktív PSII-mentes  $\Delta crtH/B$  sejtekben, méréseket végeztünk egy másik PSII-hiányos mutáns törzsben, melyben a D2 és a CP43 alegységek szintézise gátolt. A karotinoidokat is tartalmazó PSII-hiányos mutáns konstans PSI-aktivitást mutatott, még akkor is, ha a sejteket folyamatos fényen neveltük. Az eredmények arra utalnak, hogy nem a

kevésbé hatékony lineáris elektrontranszport, hanem a karotinoidok hiánya vezetett a PSI fotoinhibíciójához.

A *Synechocystis* sp. PCC 6803 *pgsA* sejtekben a foszfatidil-glicerin kiürítése csökkentette a PSII aktivitását, valószínűleg a ROS fokozott termelődése következtében. Ezért megmértük a sejtek karotinoidtartalmának és -összetételének változását a PG kiürítésének ideje alatt. A karotinoidok analízise és a felhalmozódás helyének meghatározása céljából tilakoid- és citoplazmamembrán-frakciókat izoláltunk mind a PG-vel kiegészített tápoldatban nevelt, mind a PG-hiányos sejtekből. A citoplazma- és tilakoidmembránokat lépcsős cukorgradiens ultracentrifugálással elválasztva szemmel látható volt, hogy a PG-hiányos sejtek több karotinoidot tartalmaztak, mint a PG-vel kiegészített tápoldatban nevelt sejtek, különösen a gradiens felső rétegében, ahol a sejtek vízőldékony, membránt nem tartalmazó frakciója gyűlt össze. HPLC-vel határoztuk meg a *pgsA* sejtekből és az izolált membránfrakciókból extrahált karotinoidokat. Abszorpciós spektrumuk és retenciós idejük alapján mixoxantofil, zeaxantin, echinenon és  $\beta$ -karotin karotinoidokat azonosítottunk. A PG-vel kiegészített tápoldatban nevelt mintákhoz képest a PG-hiányos mintákban csökkent a  $\beta$ -karotin és a zeaxantin mennyisége. A mixoxantofil mennyisége kb. háromszorosára nőtt a PG-hiányos mintákban, míg az echinenon mennyisége kétszer nagyobb volt a PG-hiányos tilakoidmembránban és kisebb mértékben nőtt az egész sejtben, mint a PG-vel kiegészített mintákban. Az egyes karotinoidfajták arányát is kiszámítottuk a HPLC-kromatogramokból a csúcsok területe alapján. A mixoxantofil és echinenon karotinoidok aránya magasabb volt a PG-hiányos sejtekben, izolált membránokban és a citoszolban is mint a PG-vel kiegészített mintákban. Ezen eredmények alapján arra következtethetünk, hogy a PG-kiürülés következtében fellépő fotooxidatív károsodás egyes specifikus karotinoidok szintjének növekedését indukálta. Ezt a specifikus növekedést valószínűleg a karotinoidok bioszintézisének a stressz hatására megváltozott regulációja okozta.

Vizsgálataink alapján levonhatjuk azt a következtetést, hogy a karotinoidok nélkülözhetetlen résztvevői a működőképes fotoszintetikus komplexek felépülésének, különös tekintettel a PSII-re. Karotinoidok hiányában a  $\Delta crtH/B$  sejtek rendkívül fényérzékenyek, még alacsony intenzitású fénykezelés is erős fotoinhibíciót okoz. Másrészt kimutattuk, hogy a karotinoidok védő szerepe nem csak fénystressz esetén, de másfajta stressz körülmények között is nélkülözhetetlen. A PG-kiürülés okozta fotooxidatív károsodás specifikus karotinoidok mennyiségének a megnövekedését eredményezte nemcsak a fotoszintetikus membránokban, de a citoplazmamembránban és a citoszolban is.

## SUMMARY

Photosynthesis is a vital process by which the energy of sunlight is converted into chemical energy needed for life. Cyanobacteria are the oldest known oxygenic photosynthetic prokaryotic organisms regarded as ancestors of plant chloroplast according to the endosymbiosis theory. Cyanobacteria contribute up to 30% of the yearly oxygen production on Earth.

Cyanobacteria are remarkably resistant organisms to changes in environmental conditions and very well protected against photooxidative damage generated by reactive oxygen species (ROS) whose attacks might result in the decline of the photosynthetic activity (photoinhibition). Carotenoids are indispensable components in the protection. Their photoprotective role is related to their ability to quench excited singlet-state and triplet-state chlorophyll, and scavenge the singlet-state oxygen, that are highly destructive ROS (Knox and Dodge 1985). Besides this very essential protective role, carotenoids act as accessory light harvesting pigments (Frank and Cogdell 1996) and regulate the membrane organization (Cogdell and Frank 1987, Gruszecki and Strzalka 2005).

Phytoene synthase encoded by the *crtB* gene, which is the first committed step in carotenoid biosynthesis pathway, produces phytoene which is converted to various carotenes and xanthophylls. In *Synechocystis* sp. PCC 6803, one of the most studied cyanobacteria, the major carotenoids are as follows;  $\beta$ -carotene; its hydroxyl derivative, zeaxanthin; its keto derivative, echinenone and 3'-hydroxyechinenone and the carotenoid glycoside, myxoxanthophyll (Britton et al. 2004, Takaichi and Mochimaru 2007). Recent X-ray structural analysis revealed that  $\beta$ -carotene is the only carotenoid identified in the photosynthetic complexes of cyanobacteria. Besides  $\beta$ -carotene, all other carotenoids are found in the thylakoid membrane, cytoplasmic membrane and cytosol in cyanobacteria. The increase in their level is detected under various stress conditions. The substitutions of the  $\beta$ -carotene molecule determine the antioxidative properties against photooxidative stress (Britton 1995a). In general, hydroxy carotenoids are good in inactivating peroxy radicals (Woodall et al. 1997b) whereas keto derivatives are more efficient in quenching of reactive singlet oxygen (Woodall et al. 1997a) and possess the best stability against peroxy radicals and photooxidation (Steiger et al. 1999).

Twelve carotenoids were found in monomeric PSII complex of *Thermosynechococcus elongatus* (*T. elongatus*) by X-ray crystallography (Guskov et al. 2009). PSII is believed to be

the most sensitive photosynthetic complex to photooxidative stress which is likely due to the absence of central antenna domain in PSII. Earlier studies suggested that  $\beta$ -carotene is vital for accumulation of D1 in the green alga *Chlamydomonas reinhardtii* (Trebst and Depka 1997) as well as in *Synechocystis* PCC 6803 (Masamoto et al. 2004). Therefore, carotenoid availability seems to be a major factor in the assembly of functional PSII.

Twenty two carotenoids have been identified in the structure of monomeric PSI from *T. elongatus* (Jordan et al. 2001, Fromme et al. 2001, Grotjohann and Fromme 2005). PSI is generally less sensitive to light and its carotenoid-triggered photoprotection is less known. Norflurazon (inhibitor of phytoene synthase)-treated *Scenedesmus obliquus* cells lacking carotenoids still contain some PSI and charge separation can be detected (Romer et al. 1995). Samples in which all carotenoids are extracted with organic solvents lack the quinones but are still able to perform electron transfer from P700 to A0 (Ikegami and Katoh 1991).

Photosynthetic processes occur in PSI and PSII RC composed of pigment-protein complexes embedded in the thylakoid membrane. Lipid-protein and lipid-pigment interactions play an important role in the regulation of photosynthesis. Thylakoid membranes of cyanobacteria possess a characteristic membrane lipid composition that consists of glycolipids and phospholipids. In cyanobacteria and in the thylakoid membrane of higher plants, phosphatidylglycerol (PG) is the only phospholipid. The recent X-ray crystallographic structure of *T. elongatus* showed that there are PG molecules in the PSII complex localized between CP43 and D1 (Guskov et al. 2009) as well as in PSI (Jordan et al. 2001). PG depletion results in a decrease of the Chl content of cells and in a general slowdown of photosynthetic processes. A suppression of electron transport from  $Q_A$  to  $Q_B$  was detected, which may lead to over-reduction of  $Q_A$  and to the generation of  $Q_A^{2-}$  (Gombos et al. 2002, Laczkó-Dobos et al. 2008). PG plays an essential against low-temperature stress (Murata et al. 1982, Murata 1983, Moon et al. 1995) and high light stress (Vass et al. 1992).

Changes in global environment might lead to stress conditions in photosynthetic organisms and limit the efficiency of photosynthesis. Understanding photosynthesis and its protective mechanisms might lead to the development of plant, algae and cyanobacteria with improved growth characteristics even under stress conditions. Cyanobacteria, our model organisms, are regarded as an origin of the plant chloroplast; therefore they are excellent to study higher plant photosynthesis. Carotenoids are very important protective agents in photosynthesis due to their anti-oxidative properties against photooxidative damage. Their structural and functional features in protection mechanisms are not completely elucidated yet. Complete genome sequences and the transformability of several cyanobacterial strains allow

us to generate various mutants and study the importance of carotenoids in photosynthetic processes. Therefore, the aims of my thesis are:

I. Generation of a viable oxygenic photosynthetic prokaryotic mutant in cyanobacterium *Synechocystis* that is completely deficient in carotenoid synthesis. The aim of creating this mutant is to study (i) the structural and (ii) the functional consequences of carotenoid deficiency on photosynthesis for the first time, *in vivo*.

II. Studying how carotenoids respond to stress conditions other than light stress; to this end PG depletion was induced in the *Synechocystis* sp. PCC 6803 *pgsA* strain.

The *crtB* gene encoding phytoene synthase was inactivated in the partially carotenoid-less mutant  $\Delta crtH$ . Thus, a carotenoid-less double mutant,  $\Delta crtH/B$ , was produced.  $\Delta crtH/B$  cells are extremely light sensitive and only grown in light activated heterotrophic growth LAHG conditions (in the presence of 5-10mM glucose and in the dark with an exposure of 5  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  white light for 10 min/day). HPLC analysis demonstrated that the  $\Delta crtH/B$  cells contained no carotenoid derivatives. The bluish color of the  $\Delta crtH/B$  cells indicated that phycobiliproteins were the most dominant pigments and their synthesis and accumulation was not significantly suppressed. On the other hand, chlorophyll synthesis and accumulation was suppressed by carotenoid deficiency. The mutant cells gradually bleached and died in the light indicating that carotenoids are indispensable components in photoprotection mechanism against light stress.

In the carotenoid-less  $\Delta crtH/B$  mutant levels of the large protein subunits of PSII were strongly suppressed, however, not those of PSI and Cyt *b<sub>6</sub>f* as detected by western blotting. D1 and D2 core-complex protein subunits were present at lower level than those of the WT under LAHG conditions. After the cells were exposed to light, even lower level of D1 and D2 protein subunits could be detected indicating photooxidation of those proteins in the absence of carotenoids. The level of CP47 and especially CP43 Chl-binding antenna protein subunits were the most affected. This is in agreement with the latest X-ray crystallographic model of PSII structure, which shows that most of the  $\beta$ -carotene molecules are located in the vicinity of the transmembrane  $\alpha$ -helices of CP47 and CP43. 2D gel analyses of protein subunits of  $\Delta crtH/B$  in combination with their radioactive pulse-chase labeling provided clues how to explain why no active PSII complexes were assembled. The results show that, dimeric PSII complex could not be formed in the absence of  $\beta$ -carotene, even though very unstable monomeric PSII complex could be detected only by radioactive labeling. CP47 and especially CP43 protein subunits were not stable in the PSII complex. Therefore, only the intermediate PSII complex, RC47 lacking CP43 was able to accumulate



to the level detectable by gel electrophoresis. The D1 protein subunit was the most labeled protein in  $\Delta crtH/B$  indicating that the steady state D1 turnover was functioning in the carotenoid deficient mutant. D1 labeling could be detected mostly in RC47 as well as in monomeric PSII complex and RCa, intermediate PSII complex lacking both CP43 and CP47 protein subunits. After chase experiment together with chloramphenicol treatment which is a protein synthesis inhibitor, monomeric PSII complex disappeared indicating instability and light susceptibility of the complex. Moreover, small protein subunits binding to the unassembled PSII large protein subunits were not stable without  $\beta$ -carotene. In contrast to PSII, PSI and Cyt *b<sub>6</sub>f* could be assembled, however the ratio of trimeric/monomeric PSI complexes was significantly decreased in the carotenoid deficiency.

Definitely, among photosynthetic membrane complexes in the  $\Delta crtH/B$  mutant, PSII was the most severely affected complex by the absence of carotenoids. Indeed, PSII-dependent oxygen-evolving activity was not detected in the carotenoid-less  $\Delta crtH/B$  cells under all conditions demonstrating the absence of functional PSII complexes. Moreover, measurements of PSII Chl *a* fluorescence and transient reduction kinetics of P700 confirmed that in  $\Delta crtH/B$  cells no active PSII centers are formed. In contrast, PSI charge separation and cyclic electron transport could be efficiently performed in  $\Delta crtH/B$  cells grown under LAHG conditions. However, changes in the level of active PSI centers significantly decreased after cells were exposed to low light illumination. To understand the reason of PSI photoinhibition in carotenoid-less and active PSII-less  $\Delta crtH/B$ , we made use of PSII-less mutant strain (psbDI, psbDII and psbC genes encoding D2 and CP43 proteins, respectively were blocked). Nevertheless, PSII-less mutants, containing carotenoids showed a constant PSI activity even if the cells were grown in the constant light. These results suggested that not the inefficient linear electron transport between PSII and PSI but the carotenoid deficiency was the reason for the photoinhibition of PSI.

Phosphatidylglycerol (PG) depletion suppressed the PSII activity in *Synechocystis* PCC6803 *pgsA* mutant cells most likely caused by elevated ROS production. Therefore, the changes in carotenoid content and composition during longer-term PG depletion were measured. In order to analyze and localize the carotenoid accumulation in PG-deficient mutant we isolated thylakoid and cytoplasmic membrane fractions either from PG-supplemented or from PG-depleted cells. The separation of cytoplasmic and thylakoid membrane layers by ultracentrifugation on a stepwise sucrose density gradient revealed that the PG-depleted cells (–PG14day and –PG21day) contained higher amount of carotenoids

than the PG-supplemented (+PG) cells, especially in the upper part of the gradient, which contained a water-soluble non-membrane fraction of the cells. We analyzed the carotenoid species extracted from *pgsA* cells and from isolated membrane fractions by HPLC. In *pgsA* cells, myxoxanthophyll, zeaxanthin, echinenone and  $\beta$ -carotene were identified on the basis of their absorption spectra and their retention times. The amounts of  $\beta$ -carotene and zeaxanthin decreased in the PG-depleted samples compared to those of the PG-supplemented samples. The amount of myxoxanthophyll increased approximately 3-fold in all PG-depleted samples, and the amount of echinenone was twice as high in the PG-depleted thylakoid membranes and to a lesser extent in whole cells than in PG-supplemented cells. Relative amount of the individual carotenoid species was also estimated from their peak areas from the HPLC chromatograms. The proportion of myxoxanthophyll and echinenone in the carotenoids of PG-depleted whole cells, isolated membranes and cytosol proved to be higher than in the corresponding PG-supplemented controls. These results suggested that photooxidative damage mediated by PG depletion induced the level of specific carotenoids. This is most likely the result of differently regulated biosynthetic steps of carotenoids under stress conditions.

We can conclude that carotenoids are indispensable components for the assembly and the function of photosynthetic complexes especially that of PSII shown for the first time, *in vivo*. In the absence of carotenoids cells were extremely light sensitive and exposure to the low light resulted in strong photoinhibition. On the other hand we demonstrate that carotenoid accumulation is a response to different stress conditions other than high light stress as well, however, the increase in the level of specific carotenoids induced by stress conditions are regulated differently.

## PUBLICATIONS

### Source of the publication of the thesis

Domonkos I, Malec P, Laczkó-Dobos H, **Sozer O**, Klodawska K, Wada H, Strzalka K, Gombos Z. Phosphatidylglycerol depletion induces an increase in myxoxanthophyll biosynthetic activity in *Synechocystis* PCC6803 cells **Plant Cell Physiol.** 2009; 50(2):374-82

**Sozer O**, Komenda J, Ughy B, Domonkos I, Laczkó-Dobos H, Malec P, Gombos Z, Kis M. Involvement of carotenoids in the synthesis and assembly of protein subunits of photosynthetic reaction centers of *Synechocystis* sp. PCC 6803 **Plant Cell Physiol.** 2010; 51(5):823-35

**Sozer O**, Kovács L, Kis M, Gombos Z

Involvement of carotenoids in the activity of photosynthetic reaction centers of *Synechocystis* sp. PCC 6803 (**In preparation**)

### Other publications

**Sozer O**, Kis M, Gombos Z, Ughy B

Proteins, glycerolipids and carotenoids in the functional photosystem II architecture **Frontiers in Bioscience** 2011; 16: 619-643

Laczkó-Dobos H, Todinova S.J, **Sozer O**, Komenda J, Kis M, Sallai A, Dobrikova A.G, Ughy B, Debreczeny M, Gombos Z, Apostolova E.L, Domonkos I. Identification of thylakoid membrane thermal transitions in *Synechocystis* sp. PCC6803 photosynthetic mutants (**accepted**)