Functional studies on the Light-harvesting-Like (LiL) Proteins in Cyanobacteria and Cryptophytes

Tania Tibiletti
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Akademisk avhandling

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Abstract
The light-harvesting like (LiL) proteins are a widely spread group of proteins within photosynthetic organisms. They are membrane proteins composed of one to four transmembrane helices and—in homology to the light-harvesting complexes of algae and higher plants—at least one of these transmembrane helices contains the chlorophyll \( a/b \)-binding (CAB) domain. Opposite to the light-harvesting antenna complexes, LiL proteins are stress induced and they have been shown to be involved in protection of the photosynthetic apparatus. The work presented in this thesis is focused on understanding the function of one-helical LiL proteins of the cryptophyte algae \textit{Guillardia theta} and the cyanobacterium \textit{Synechocystis sp.} \textit{PCC 6803}. \textit{G. theta} contains two genes encoding LiL proteins, one is localized in the plastid (hlipP), the other in the nucleomorph (HlipNm). Both genes are expressed in normal growth condition, but they are not induced by high light. Immunostaining indicated that HlipNm is translated, but not light-induced. These proteins therefore seem not to be involved in photoprotective mechanisms of \textit{G. theta}. In the cyanobacterium \textit{Synechocystis sp.} \textit{PCC 6803} four one-helical LiL proteins were identified, they are called Small CAB-like Proteins (SCPs); a fifth LiL (ScpA) is fused with the ferrochelatase (FC), an enzyme involved in the heme synthesis. Our analysis revealed that SCPs are involved in the \textit{de novo} assembly/repair cycle of Photosystem II, stabilizing the chlorophyll pigments at their protein scaffold. The \textit{in vitro} characterization of the recombinant FC showed that ScpA is involved in the product-release of the catalytic domain of the enzyme, thereby regulating substrate availability for chlorophyll- or heme- biosynthesis. Finally, using a transcriptomic and metabolomic approaches, I was able to show that deletion of all SCP genes has profound impact on the cell organization and metabolism. In SCP-depleted cells, production of reactive oxygen species (ROS) is increased, while the amount of Photosystem II per cell volume is decreased, causing a macronutrient-deficient phenotype. Therefore, SCPs are important for stress protection and help to maintain a metabolic equilibrium within the cell.

Keywords
Photosynthesis, cyanobacteria, \textit{Guillardia theta}, photosystem II, chlorophyll-binding proteins, one-helix LiL proteins, photoprotection
Functional studies on the Light-harvesting-Like (LiL) Proteins in Cyanobacteria and Cryptophytes

Tania Tibiletti
To my family
“A person who never made a mistake never tried anything new”
Albert Einstein
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Abstract

The light-harvesting like (LiL) proteins are a widely spread group of proteins within photosynthetic organisms. They are membrane proteins composed of one to four transmembrane helices and – in homology to the light-harvesting complexes of algae and higher plants – at least one of these transmembrane helices contains the chlorophyll a/b-binding (CAB) domain. Opposite to the light-harvesting antenna complexes, LiL proteins are stress induced and they have been shown to be involved in protection of the photosynthetic apparatus. The work presented in this thesis is focused on understanding the function of one-helical LiL proteins of the cryptophyte algae *Guillardia theta* and the cyanobacterium *Synechocystis* sp. PCC 6803. *G. theta* contains two genes encoding LiL proteins, one is localized in the plastid (*hlipP*), the other in the nucleomorph (*HlipNm*). Both genes are expressed in normal growth condition, but they are not induced by high light. Immunostaining indicated that *HlipNm* is translated, but not light-induced. These proteins therefore seem not to be involved in photoprotective mechanisms of *G. theta*. In the cyanobacterium *Synechocystis* sp. PCC 6803 four one-helical LiL proteins were identified, they are called Small CAB-like Proteins (SCPs); a fifth LiL (*ScpA*) is fused with the ferrochelatase (FC), an enzyme involved in the heme synthesis. Our analysis revealed that SCPs are involved in the *de novo* assembly/repair cycle of Photosystem II, stabilizing the chlorophyll pigments at their protein scaffold. The *in vitro* characterization of the recombinant FC showed that *ScpA* is involved in the product-release of the catalytic domain of the enzyme, thereby regulating substrate availability for chlorophyll- or heme- biosynthesis. Finally, using a transcriptomic and metabolomic approaches, I was able to show that deletion of all SCP genes has profound impact on the cell organization and metabolism. In SCP-depleted cells, production of reactive oxygen species (ROS) is increased, while the amount of Photosystem II per cell volume is decreased, causing a macronutrient-deficient phenotype. Therefore, SCPs are important for stress protection and help to maintain a metabolic equilibrium within the cell.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ALA</td>
<td>5-aminolevulinic acid</td>
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<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>CABs</td>
<td>chlorophyll a/b-binding proteins</td>
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<tr>
<td>CAO</td>
<td>chlorophyllide a oxigenase</td>
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<td>Chl</td>
<td>chlorophyll</td>
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<tr>
<td>Chlide</td>
<td>chlorophyllide</td>
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<td>CM</td>
<td>cytoplasmic membrane</td>
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<td>Cyt</td>
<td>cytochrome</td>
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<td>glutamate</td>
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<td>ELIPs</td>
<td>Early Light-Induced Proteins</td>
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<td>FC</td>
<td>ferrochelatase</td>
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<td>FCPs</td>
<td>fucoxantin chlorophyll a/c-binding proteins</td>
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<tr>
<td>Fd</td>
<td>ferredoxin</td>
</tr>
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<td>Fe2+</td>
<td>iron ion</td>
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<tr>
<td>FNR</td>
<td>ferredoxin-NADP reductase</td>
</tr>
<tr>
<td>G3P</td>
<td>glyceraldehyde 3-phosphate</td>
</tr>
<tr>
<td>GluTR</td>
<td>glutamyl-tRNA reductase</td>
</tr>
<tr>
<td>Gya</td>
<td>billion years ago</td>
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<tr>
<td>H</td>
<td>histidine</td>
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<tr>
<td>HL</td>
<td>high light</td>
</tr>
<tr>
<td>HLIP</td>
<td>high light-induced protein</td>
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<tr>
<td>HLR1</td>
<td>high light regulatory 1 sequence</td>
</tr>
<tr>
<td>LAHG</td>
<td>light-activated-heterotrophic-growth</td>
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<td>LCM</td>
<td>linker core-membrane</td>
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<td>LiL</td>
<td>light-harvesting like</td>
</tr>
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<td>LL</td>
<td>low light</td>
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<td>normal light</td>
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<tr>
<td>NPQ</td>
<td>non-photochemical-quenching</td>
</tr>
<tr>
<td>OCP</td>
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</tr>
<tr>
<td>OEC</td>
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</tr>
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<td>protochlorophyllide a</td>
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<td>POR</td>
<td>protochlorophyllide oxidoreductase</td>
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<tr>
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</tr>
<tr>
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<td>qE</td>
<td>energy-dependent quenching</td>
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<td>qI</td>
<td>photoinhibitory quenching</td>
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<td>qT</td>
<td>state transition</td>
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<td>arginine</td>
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<td>RC</td>
<td>reaction center</td>
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<td>reactive oxygen species</td>
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<td>SCP</td>
<td>small CAB-like proteins</td>
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<td>stress-enhanced proteins</td>
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<tr>
<td>α-KG</td>
<td>alpha-ketoglutarate</td>
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1. Introduction

1.1 Introduction to Photosynthesis

1.1.1 Description of the overall photosynthetic process

Photosynthesis is one of the most important processes on Earth and it is one of the few processes that converts low energy carbon molecules (oxidized carbon) in high energy molecules (reduced carbon) using the sun light as energy source (Falkowsky and Raven 2007). The word photosynthesis derives from the Greek words _phos_ meaning light and _syntithenai_ translating into “put together”, and it describes the sum of processes using light to fuel energy into most of the ecosystems (Gest, 2002). The photosynthetic process can be written as an equation:

\[
2H_2A + CO_2 + \text{light} \rightarrow (\text{CH}_2O) + H_2O + 2A \tag{1.1}
\]

The nature of the $H_2A$ molecule can vary. Photosynthetic bacteria, like heliobacteria, acidobacteria, green sulfur bacteria, purple bacteria and filamentous anoxygenic phototrophs (non-green sulfur bacteria in the old nomenclature) fix carbon only in anaerobic conditions and therefore perform anoxygenic photosynthesis. They absorb sunlight with the help of bacteriochlorophylls and use hydrogen, sulfides, sulfur or organic molecules as electron donors.

Oxygenic photosynthetic organisms use water as electron donor. Equation 1.1 in this case can be modified as follows:

\[
2H_2O + CO_2 + \text{light} \rightarrow (\text{CH}_2O) + H_2O + O_2 \tag{1.2}
\]

Cyanobacteria, prochlorophytes, eukaryotic algae and higher plants perform oxygenic photosynthesis (oxygen is a waste product in 1.2) and beside other pigments they use chlorophyll $a$ (chl $a$) as ubiquitous pigment for absorbing light. Equation 1.2 can be split in two reactions;

\[
2H_2O + \text{Light} \rightarrow (\text{Chl } a) \rightarrow 4H^+ + 4e^- + O_2 \tag{1.3}
\]

which is called the “light reaction” of oxygenic photosynthesis. The light reaction of photosynthesis occurs within membrane-inserted protein complexes and has the function to convert light energy into chemical energy (ATP and NADPH) that it will be used during carbon fixation.

The second part of this reaction, the reduction of CO$_2$, can be presented as equation 1.4:
This reaction is light independent and therefore often is referred as “dark reaction” that occurs in the aqueous phase of the photosynthetic cell/organelle and not in membranes. In most of the photosynthetic organisms, the carbon fixation occurs in the Calvin-Benson cycle but other CO₂ fixation pathways exist (Berg et al., 2011).

1.1.2 The origin of photosynthesis

Molecular biomarkers, phylogenic and chemical analyses of ancient rocks provide evidence that anoxygenic photosynthesis evolved first, earliest 3.4 billion years ago (Gya, Figure 2). The ancient photosynthetic organisms were probably using hydrogen as electron donor, but also reduced iron and sulfides should not be excluded. Oxygenic photosynthesis performed by ancient cyanobacteria arose 2.7 Gya, the byproduct, oxygen, accumulated over hundreds of billions of years to give rise to today’s atmosphere (Figure 2). Evidence about the slow increase of oxygen comes from nitrogen-oxygen redox cycle (Godfrey and Falkowsky 2009), the chromium signatures (Frei et al., 2009) and the sulfur fractionation (Canfield et al., 2000, Farquhar et al., 2007). It might be caused by the inability of the ancient cyanobacteria to protect themselves against reactive oxygen species (ROS) and by the presence of buffers preventing oxygen to enter the atmosphere. Ferrous iron, for example, forms with oxygen insoluble compounds, that gave rise to a structure called banded iron formation (BiFs, Holland 2006). BiFs formation

![Figure 1. Important events in the evolution of photosynthesis. Anoxygenic photosynthesis evolved first (at 3.4 Gya). Based on fossil records, the first cyanobacterial form evolved 2.7 Gya. Oxygen accumulated over a period of hundreds of billion years. Based on fossil records the first eukaryotic alga was dated back 1.2 Gya.](#)
stopped when oxygen accumulated in larger amount in the atmosphere (Holland 2006). Fossil records are also used to estimate the occurrence of life. While it is relative certain that the first photosynthetic eukaryote appeared 1.2 Gya ago (Butterfield 2000), the evolution of the first cyanobacterial form is still highly debated. The true identity and the biologic origin of the earliest cyanobacterial records (at 3.5 Gya) have been questioned (Schopf 2006). Nevertheless, globule formation resembling the modern stromatolites has been dated back to 2.72 Gya (Lepot et al., 2008).

1.1.3 The endosymbiotic origin of plastids

It is now widely accepted that eukaryotic photosynthesis was acquired from an ancient bacteria via a process called endosymbiosis (McFadden and VanDorren 2004). An ancient heterotrophic eukaryote engulfed an ancient cyanobacterium and instead of being digested it was retained becoming the specialized organelle where photosynthesis occurs in plants and algae, the chloroplast (Figure 3). The primary endosymbiosis gave rise to three plastid lineages: the Glaucophyta (glaucophyta algae), the Rhodophyta (red algae), and the Viridiplantae (green algae and land plants). Most likely this event occurred just once and not in three different and separated times. The primary endosymbiotic event has been dated back to ca 1.5 Gya (Yoon et al., 2004). However, the amoeba *Pauliniella chromatophora* could be an example of a second primary endosymbiosis, separated from the event that gave origin to plants (Nowack et al., 2008). In the chloroplast, remnants of the cyanobacterial origin are the prokaryotic transcription and translation mechanisms, and the two membranes surrounding the plastid, one originating from the cytoplasmic membrane of the ancestral cyanobacterium and the outer one from the host organism. In addition, the major part of the ancestral cyanobacterial genome was transferred in the nucleus of the host during evolutionary period of time. Secondary and the tertiary endosymbiotic events have led to a high biodiversity amongst algae; an eukaryotic phototroph was incorporated into another eukaryote (Keeling 2010, Archibald 2009). Secondary endosymbiosis gave rise to euglenids and chlorarachniophytes by incorporation of a green alga and to the ancestor of chromalveolates by incorporation of a red alga (Figure 3). From chromalveolates diverged cryptomonads and haptophytes; later, stramenopiles (heterokonts), ciliates, apicomplexans, and dinoflagellates evolved. The haptophytes are covered by calcium carbonate scales, their decomposition formed the fossil hydrocarbon in the past era. The stramenopiles are a variegated group of organisms including huge algae like kelps as well as the tiny diatoms, one of the most important groups of primary producers in the ocean (Field et al., 1998). The classes of
cryptomonads, haptophytes and stramenopiles together form the “old” phylogenetic group of Chromista, since they are closely related (Cavalier-Smith, 1999, Yoon et al., 2004). All the Chromista contain chlorophyll c and have a chloroplast of red algae origin (red line) surrounded by four membranes; the inner two membranes derived from the primary plastid, the third membrane origins from the plasma membranes of the red algae and the outer membrane from the phagosomal host membrane. Cryptophytes maintained the vestigial nucleus of the red alga, termed nucleomorph, which is located in between the second and the third membrane (starting from the inside). Ciliates, apicomplexans and dinoflagellates form a group called the Alveolata. Their common ancestor is believed to have contained a plastid, which it was completely lost in the ciliate branch. Apicomplexans instead still contain a vestigial plastid called apicoplast. Dinoflagellates are marine organisms and most species are non-photosynthetic. Dinoflagellates underwent several tertiary endosymbiotic events with a haptophyte, a cryptomonad, a diatom or a green alga giving raise to many important groups of protists (Archibald and Keeling 2002). The plastid derived after tertiary endosymbiosis contains a unique set of pigments: chl c₂, peridinin, dinoxanthin and diadinoxanthin. A series of secondary endosymbiosis events of green algae gave origin to *Lepidodinium*. After incorporation of a dinoflagellat-type alga evolved *Durinskia* evolved, *Karlodinium* arose after incorporation of a haptophyte-type alga, and *Dinophysis* was generated by incorporation of a cryptophyte-type alga (Figure 3).

In this thesis the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) and the cryptophyte *Guillardia theta* have been studied. *Synechocystis* 6803 is a fresh water cyanobacterium widely used as model organism to study photosynthesis, as it is naturally transformable and it integrates foreign DNA by homologous recombination. In addition, it can grow mixotrophically and heterotrophically and the genome is fully sequenced (Kaneko et al., 1996). The cryptomonad *Guillardia theta* (hereafter *G. theta*) is the first cryptophyte whose genomes have been sequenced (including the nucleomorph genome, Douglas et al., 2001).
**Figure 2.** Schematic view of plastid evolution. Endosymbiotic events are boxed in grey; the different plastid lineages are colored as follow: the green algal lineage is colored in green, the red algal lineage in red and lines that lost their plastids are given in grey. At the bottom of the figure the primary endosymbiotic event is shown that gave raise to glaucophytes, red algae and green algae. At the lower right, secondary endosymbiotic events (with a green alga) are given leading to the origin of euglenids. On the lower left, the event is shown when a red alga was taken by an ancient heterotroph, generating the ancestor of chromalveolates. From the chromalveolate ancestor, haptophytes and cryptomonads diverged. Chlorarachniophytes and Paulinella regained a plastid after their symbiosis with a green alga and a cyanobacterium, respectively. At the top left a schematic view is given, where stramenopiles diverged from alveolates. Plastids were lost in ciliates and apicomplexa. At the top right a tertiary endosymbiotic event is shown of dinoflagellates with haptophytes, diatoms, cryptomonads, and a green alga (a series of secondary endosymbiotic events).
1.1.4 The general organization of the photosynthetic membrane system

The photosynthetic apparatus has a general conformation that is conserved in all photosynthetic organisms. It is always constituted by an antenna system collecting and transferring the light energy to a membrane-integral proteinaceous structure called reaction center (RC), where charge separation and stabilization takes place. RCs have the same structural organization in photosynthetic organisms, and thus are believed to have evolved just once. Light-induced charge separation requires a specific structure of proteins and cofactors. On the contrary, light harvesting can be obtained in many different ways and several antenna systems evolved in photosynthetic organisms.

In heliobacteria and green sulfur bacteria, the only photosystem is an integral component of the cytoplasmic membrane and large pigment-protein complexes located at the inside of the membranes work as antenna. These pigment-protein complexes are called chlorosomes (Figure 3A, Oostergetel et al., 2010). In purple and filamentous phototrophic bacteria, the cytoplasmic membrane exhibits profound invaginations that can assume different conformations (tubular, lamellar or vesicular forms are known). Together with the RC, the antenna is an integral-membrane system (Figure 3B, Law and Cogdell 2008).

In cyanobacteria, RCs are inserted in specialized membranes named thylakoids located parallel in several layers to the plasma membrane (Figure

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**Figure 3.** Schematic representation of membrane systems containing the photosynthetic apparatus in prokaryotic cells and chloroplasts of plants. (A) Anoxygenic bacteria (heliobacteria and green sulfur bacteria) with chlorosomes (round green shaped) that are attached to the cytoplasmic membranes (CM), OM, outer cell membrane. (B) Anoxygenic bacteria with intracytoplasmic membranes (ICMs) that contain the photosynthetic membranes (red dots); OM, outer cell membrane, (C) Cyanobacterial cell; OM, outer cell membrane, CM, cytoplasmic membranes and thylakoid membranes are indicated in green. (D) Chloroplast from higher plants; OChM, outer chloroplast membrane, IchM, inner chloroplast membrane, thylakoid membranes are indicated in green and the luminal space is colored in yellow.
Components of the photosynthetic electron transfer and the complexes of the respiratory chain are localized in the thylakoid membranes (Cooley and Vermaas 2001, Schultze et al., 2009). In plants, photosynthesis takes place in specialized organelles, the chloroplasts. Many differences exist between the chloroplasts of higher plants and those of algae with respect to size, shape and number per cell; nevertheless, the general structure is similar. Depending on the organism, chloroplasts are surrounded by two to four plastid envelope membranes. Within the colorless matrix called stroma a complex membrane structure is imbedded, known as thylakoid membranes. Thylakoids contain pigment-binding protein complexes of the photosynthetic light reaction and therefore have a green color when isolated. While higher plants contain a membrane-integrated antenna system, and sometimes also extrinsic antennas can be found in different algal families. In higher plants thylakoids are organized in stacked areas called grana and unstacked regions, stroma lamellae, connecting the grana. Enclosed between the lipid bilayers of the thylakoid membrane is the aqueous lumen (Figure 3D).

1.1.5 The photosynthetic electron transfer in oxygenic photosynthesis

Embedded in the thylakoid membranes, oxygenic phototrophs have two RCs, Photosystem II (PSII) and Photosystem I (PSI) both working in series and linked by a third protein complex, the cytochrome (cyt) b$_6$f and a series of carriers. The forth component of the photosynthetic electron transport is the ATP synthase (Figure 6, Blankenship 2002, Falkowsky and Raven 2007). PSII and PSI are structurally similar to the RC of purple and filamentous phototrophic bacteria and the RC in heliobacteria and green sulfur bacteria, respectively; therefore, a single common ancestor for the origin of RCs is hypothesized (Hohmann-Marriot and Blankenship 2011). Cyt b$_6$f complex has similarities with the cytochrome bc$_1$ found in Archea, Bacteria and in mitochondria of eukaryotic organisms (Kallas 2012).

At PSII, one photon is absorbed by a strong reductant, called P680. P680 is a special pair of chlorophyll that releases one electron at the time. The electron is captured by a pheophytin (Pheo) molecule that subsequently reduces the primary acceptor QA, a quinone molecule bound to protein. In QA the charge separation is stable and the probability of a back-reaction is very low. Two electrons are needed to fully reduce the second acceptor QB, a plastoquinone molecules, that can diffuse in the lipid bilayer of the membrane until it reaches cyt b$_6$f. The electron transfer is coupled with the transport of two protons over the thylakoid membrane from the stromal side (cytoplasmatic side in cyanobacteria) to the thylakoid lumen. Additional two protons are shuttled into the thylakoid lumen via the cyt b$_6$f complex. The electron hole
in P680⁺ is filled by oxidizing a tyrosine (Tyr), which is reduced in turn by manganese (Mn) atoms of the Oxygen Evolving Complex (OEC). Splitting water into oxygen at the same time releases four protons in the thylakoid lumen. Electrons from the reduced Qb are transported further, first to cyt b₆, then to cyt f and then to a soluble, mobile protein, depending on the organism either plastocyanin (a copper containing protein) or cyt c₆ (a heme protein). Within PSI the electrons reduce the special dimer of chl α, P700, when a second photon leads to a second charge separation. The electron then is carried to A₀, a chlorophyll monomer, A₁, a phylloquinone, Fₓ, an iron sulfur cluster (Fe-Sₓ), Fe-Sₐ/Fe-Sₘ, two iron-containing proteins, and then to Fd, a molecule of ferredoxin. The last step of the photosynthetic electron transfer is the reduction of NADP⁺ to NADPH by ferredoxin-NADP reductase (FNR) that receives two electrons from ferredoxin. In the absence of iron flavodoxin, a flavin protein is produced instead of ferredoxin. Ferredoxin can also directly be used in the reduction of nitrate and sulfate. The production of reducing power in the form of NADPH is associated with ATP formation by a protein complex called ATP synthase. The ATP synthase uses both the difference in pH and the electrical potential across the membranes to produce ATP.

**Figure 4.** The photosynthetic electron transport chain in oxygenic phototrophs. In PSII-RC, a photon (hv) is absorbed by the special pair of pigment and causes release of an electron. Pheophytin (Pheo) is the first acceptor, the Q₁ and Q₂ are primary and secondary quinone electron acceptors, respectively. Electrons are transferred through mobile plastoquinones (PQ) to cyt b₆f, composed of two cyt b (cyt b₆ and cyt b₃-high and low potential), Rieske cluster (FeSₓ), and cyt f. Plastocyanin (PC) or cyt c₅ transfer electrons to the P₇00 RC of PSI. The primary acceptors in PSI are A₀, a chl, and A₁, a quinone species. Fe-Sₓ, Fe-Sₐ, and Fe-Sₘ are iron sulfur clusters associated to PSI that transfer electrons to NADPH via ferredoxin (Fd) and a ferredoxin-NADP oxidoreductase (FNR). The oxygen-evolving complex (OEC) of PSII donates electrons via a conserved tyrosine (Tyr) to P680. The produced electrochemical gradient is utilized by the ATP synthase to generate ATP.
1.1.6 Structure of PSII and PSI

Crystallographic X-ray structures of PSI have been determined for both cyanobacteria and higher plants while only the cyanobacterial structure of PSII has been solved so far (Ferreira et al., 2004, Loll et al., 2005, Gusakov et al., 2009, Jordan et al., 2001, Amunts et al., 2007). Photosystem I and II from cyanobacteria and plants are structurally similar, and the major differences exist between the smaller subunits that surround the central core. In the following section I will focus on cyanobacterial PSII and PSI, being important for the main subject of my doctoral thesis.

Recently, a high-resolution crystal structure of PSII from the cyanobacterium *Thermosynechococcus vulcanus* has been reported (Umena et al., 2011). PSII is a dimer (Folea et al., 2008), each monomer is composed of 17 intrinsic and three extrinsic sub-units and a number of cofactors: 35 chlorophylls, two pheophytins, 11 β-carotenes, more than 20 lipids, two plastoquinones, two heme irons, one non-heme iron, four manganese atoms, three or four calcium atoms and three Cl− ions per monomer have been identified (Umena et al., 2011). The reaction center of PSII is a hetero-dimer composed of the D1 and D2 subunits (Figure 5, cyan and yellow respectively). Each of these proteins is composed of five transmembrane helices harboring chlorophyll, pheophytin and plastoquinone involved in the charge separation. Additional RC proteins are the low molecular mass proteins Cyt $b_{559}$ and PsbI. The RC is surrounded by CP43 and CP47 (Figure 5, pink and green respectively). Each of them is composed of six transmembrane helices binding most of the chlorophyll in PSII as well as β-carotene. Three PSII subunits are extrinsic, PsbO, PsbU and PsbV; they are located at the luminal site of PSII and stabilize the OEC. On the periphery of this complex, there are 13 low molecular mass protein subunits (Shi et al., 2012), with one or at most two transmembrane helices (Figure 6, colored in grey). The function of many of these proteins is under investigation; some of them seem to be involved in the binding of cofactors (Müh et al., 2008).

Lipids in the PSII structure seem to be important for the correct assembly and function of PSII. Lipids might give flexibility to the PSII structure to facilitate exchange of damaged proteins (i.e. D1). They also could be important for the assembly of the complex by improving the mobility of PSII subunits and the recognition between subunits. Moreover, lipids participate in the creation of a hydrophobic environment of the Q$_B$ pocket (Loll et al., 2005). Carotenoids are located around the core antenna consisting of CP47 and CP43 and within D1 and D2 subunits. They are involved both in energy transfer to chl and in the quenching of triplet state of the chl.

Cyanobacterial PSI complex is composed by 12 protein subunits and 127 cofactors: 96 chls, 22 carotenoids, three iron-sulfur clusters, two phylloquinones and four lipids (Jordan et al., 2001). The two largest intrinsic
subunits, PsaA and PsaB, both consist of 11 transmembrane helices (Figure 5B). The C-terminal five helices are the reaction center core and they bear the cofactors involved in charge separation (chl $a$, phyloquinone and FeS center). The other six N-terminal helices function as antenna and they bind most of the chl $a$ molecules of PSI. Three subunits are extrinsic (PsaC, PsaD and PsaE) and they are located at the cytoplasmic site. The other seven protein subunits are intrinsic and are involved in the complex stabilization and in some cases in chl binding.

Figure 5. (A) Subunits and pigments organization of the homo-dimeric PSII complex isolated from _Thermosynechococcus vulcanus_ viewed from the cytoplasmic site of the membrane. The major subunits are colored: D1 in cyan, D2 in yellow, CP43 in pink, CP47 in green and small subunits in grey. The luminal subunits PsbO, PsbV and PsbU are not shown for clarity. Green, chl $a$; orange, carotenoids; yellow, lipids. The figure was created with the software Jmol and the PDB file 3ARC (Umena et al., 2011). (B) Subunit and pigment organization of the PSI complex from _Synechococcus elongatus_ viewed from the membrane plan. PsaA is in red, PsaB in yellow and small subunits in grey. The figure was created with the software Jmol and the PDB file 1JB0 (Jordan et al., 2001).

1.1.7 The assembly and repair of PSII

PSII has a more oxidized redox potential compared to PSI; therefore, in case of excess of light, PSII is damaged first. If PSII is not repaired, a phenomenon called photoinhibition occurs (Vass 2011, Allahverdiyeva and Aro 2012), causing a decrease of photosynthesis and thus a decrease of growth. To keep PSII homeostasis, assembly and repair mechanisms maintain to certain level the amount of functional PSII in the thylakoids. _Synechocystis_ 6803 is widely used for studying these processes. The assembly of PSII is a stepwise process, where sub-complexes are initially formed and later fused to form the active complex. Involved in the assembly are a number of accessory protein factors, which only transiently belong to PSII and therefore are absent in the X-ray structure (reviewed by Nixon et
Briefly, cytochrome $b_{559}$ (cyt $b_{559}$) functions as nucleation factor initiating PSII assembly. D2, together with PsbE and PsbF (Figure 6A1), attaches to cyt $b_{559}$ forming the D2/cyt $b_{559}$ subcomplex. D1 precursor (pD1), together with PsbI (Figure 6A2) attaches stably to this D2/cyt $b_{559}$ subcomplex, forming a PSII RC-like complex (Figure 6A3). In presence of CP43, a CP47-PsbH/PsbL/PsbT complex (Figure 6A4) binds to the PSII RC-like complex, forming the so-called RC47 complex (Figure 6A5). The subsequent attachment of CP43-PsbZ/PsbK/Psb30 subcomplex (Figure 6A6) builds the monomeric PSII core complex (Figure 6A7), which induces the assembly of the OEC (Figure 6A8). To assemble the OEC, the C-terminal extension of the D1 subunit must be cleaved off by a specific protease, CtpA. The final step is the assembly of PSII dimers. This repair mechanism involves the partial disassembly of PSII, the synthesis and the incorporation of newly synthetized polypeptides and the reassembly of the complex (Nixon et al., 2005). The reaction center protein D1 is most sensible towards photoinhibition, and it has the highest turnover rate of all the PS proteins (Yao et al., 2012 a,b). Disassembly of PSII begins with the

![Figure 6](image_url)

**Figure 6.** Model for the (A) assembly and (B) repair of PSII. (1) The D2 subunit forms a pre-complex with PsbE, PsbF and subunits of cytochrome $b_{559}$, the D2/cyt $b_{559}$ complex. (2) The pD1-PsbI pre-complex is formed by the association of the D1 precursor and PsbI. (3) This D2/cyt $b_{559}$ complex binds to the pD1-PsbI pre-complex, forming the RC complex. (4) PsbH, PsbL, and PsbT bind to CP47 and then attach to the RC complex forming the RC47 (5). Monomeric PSII (6) is formed by binding a CP43-complex, containing beside CP43 the subunits PsbK, PsbZ, and Psb30 (7). (8) The final step prior the PSII dimerization (not shown) is the assembly of the OEC. When PSII is damaged (9), D1 subunit has to be replaced. PSII is disassembled (10) and the damaged D1 (D1*) is degraded by FtsH (and Deg) proteases (10). New D1 subunits are synthetized (11) and incorporated. Stress-induced small CAB-like proteins (SCPs) seem to be involved in the repair as it will be described later.
monomerization of PSII, the detachment of the extrinsic proteins and CP43. The damaged D1 subunit is degraded by proteases members (Figure 6B10) and a new D1 subunit is synthetized and incorporated (Figure 6B11). D1 replacement seems to be facilitated by the belt of lipids present separating D1 and CP43. The PSII is reassembled (Figure 6B12), allowing to the OEC to re-attach. Recently, several accessory proteins have been identified to play an important function in the assembly and repair of PSII. Some of them share homology between cyanobacteria and eukaryotic photosynthetic organisms, others are found only in prokaryotes or eukaryotes, respectively (reviewed by Nixon et al., 2010, Shi et al., 2012). Ycf48 and PAM68 are assembly factors conserved in A. thaliana and Synechocystis 6803. Ycf48 stabilizes the unassembled pD1 and aids PSII formation (Komenda et al., 2008). PAM48 promotes early steps in PSII biogenesis interacting with numerous PSII subunits and assembly factors (Armbruster et al., 2010). Psb27 is small lipoprotein that has recently been identified to be associated with unassembled CP43 in Synechocystis 6803 (Komenda et al., 2012b). Some data also suggest Psb27 to bind to PSI (Cormann et al., 2009, Komenda et al., 2012b). It is proposed that Psb27 has a role in the biogenesis of CP43 during PSII repair, regulating the assembly of the OEC. Psb28 has been localized attached to the RC47, probably at the cytoplasmic site (Dobakova et al., 2009). Deletion of psb28 does not affect the functional properties of PSII, but the amount of CP47 and of both PsaA and PsaB subunits of PSI are decreased in the mutant. The Psb28 deletion mutant shows impairment in the chl biosynthetic pathway. Therefore, it has been proposed that Psb28 might regulate chl availability during PSI and PSII biogenesis.

1.1.8 The biosynthetic pathways of tetrapyroles
Chlorophylls (or bacteriochlorophylls in anoxygenic photosynthesis) are ubiquitous participants of photosynthesis. The chl molecule is characterized by a tetrapyrole ring structure that centrally binds a magnesium (Mg$^{2+}$) ion. In addition, a long hydrophobic side chain, known as phytol chain, is attached to the tetrapyrole ring structure of most of the chls (chl c is an exception) and renders them extremely unpolar. The various chls existing in nature mainly differs for their substituents around the ring, modulating their absorption spectra and their interaction with the protein scaffold. The central Mg$^{2+}$ atom of the tetrapyrole ring enables the most stable interaction with the protein. Mg$^{2+}$ has an extra ligand that in most of the case is histidine (H); in other cases the binding might occur through glutamate (E), glutamine (Q), asparagine (N), tryptophan (W), serine (S) and also water (Jordan et al., 2001, Murray et al., 2006, Mühl et al., 2008, Loll et al., 2005). However, oxygen groups in the tetrapyrole ring and the phytol chains of chl
molecules can form extra H-bonding with different groups of the amino acid chains of proteins.

Cyanobacteria, algae and plants synthesize chl, heme and linear tetrapyrroles (phycobilins) via a common branched pathway (Tanaka et al., 2011, Tanaka and Tanaka 2007, Vavilin and Vermaas 2002, Masuda and Fujita 2008, Czarnecki and Grimm 2011, Tripathy and Pattayak 2012). The common precursor is 5-aminolevulinic acid (ALA) synthesized from glutamate via glutamyl-tRNA in a 3-step reaction that involves three different enzymes. ALA formation is one of the most important control points regulating the flux through the chl/heme pathway. A wide range of regulatory signals modulate the glutamyl-tRNA reductase (GluTR), the second enzyme in the pathway and the first enzyme of tetrapyrrole biosynthesis as the substrate, Glu-tRNAGlu, is also used for protein synthesis. Heme feedback inhibition on GluTR is observed in many organisms (Vothknecht et al., 1998, Javor and Febre 1992, Scrivastava et al., 2005). In plants, FLU, a nuclear-encoded chloroplast protein, represses GluTR in the presence of protochlorophyllide (Pchlide) or other chl intermediates, binding to the C-terminal part of GluTR (Goslings et al., 2004). The enzymatic reaction converting ALA to protoporphyrin IX (PPIX) is common to all photosynthetic organisms, although the orthologues are structurally different. PPIX is the common substrate for both chl and heme biosynthesis depending on its cofactor binding, either Fe$^{2+}$ or Mg$^{2+}$. In the heme branch, ferrochelatase incorporates Fe$^{2+}$ into the porphyrin ring, while Mg-chelatase inserts Mg$^{2+}$ forming Mg-protoporphyrin IX (Mg-PPIX), a precursor of chl. Mg-chelatase is an ATP-dependent enzyme formed by three subunits, CHLI, CHLD and CHLH. Deletion of a single subunit lowers the chl content in the mutant in higher plants. Mg-chelatase activity is positively regulated by a porphyrin-binding protein, GUN4, which has been found to interact with the CHLH subunit of the Mg-chelatase (Peter and Grimm, 2009, Sobotka et al., 2008b). A gun4 gene was also identified in Synechocystis 6803 (Wilde et al., 2004). Disruption of gun4 either in Arabidopsis thaliana (hereafter A. thaliana) or Synechocystis 6803 decreased the cellular chl (Wilde et al., 2004, Peter and Grimm 2009). GUN4 is also involved in anchoring Mg-chelatase to the membrane (Sobotka et al., 2008b), which may allow a rapid exchange of tetrapyrrole intermediates with other enzymes within chl biosynthesis. In plants, Gun4 might bind chl-intermediates to avoid damages, regulating the pathway of chl synthesis (Peter and Grimm 2009). In the Mg-branch, Mg-PPIX is methylated forming the Mg-protoporphyrin methyl ester (Mg-Proto IX ME) that is sequentially modified by Mg-protoporphyrin IX monomethylester cyclase (MgPVC) forming divinyl-protoporphyllide a (DV-Pchlide a). DV-Pchlide a can be converted to monovinyl-protoporphyllide a (MV-Pchlide a) by divinyl reductase (DVR). Protochlorophyllide oxidoreductase (POR)
catalyzes the conversion of Pchlide to chlorophyllide (Chlide). All photosynthetic organisms, with the exception of angiosperms, contain two forms of POR, a light-dependent and a light-independent protochlorophyllide vinyl reductase (LPOR and DPOR, respectively). The last step in the chlorophyll synthesis is the esterification of MV-chlide with phytyl pyrophosphate by chlorophyll synthase. In plants, the enzyme chlorophyllide a oxygenase (CAO) converts chlide a to chlide b, that will be used by chl synthase to produce chl b.

Chlorophyll synthesis and synthesis of chl-binding apoproteins have to be coordinated to avoid the production of free pigments, which might promote the production of reactive oxygen species (ROS). Chlorophyll

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**Figure 7.** The biosynthetic pathway of tetrapyrroles in photosynthetic organisms. Glutamyl-tRNA is converted to ALA by a 3-steps process. The second enzyme, GluTR is an important control point in the pathway. After several modifications ALA is converted to PPIX, the common substrate used by either Fe-chelatase or Mg-chelatase. Fe-chelatase inserts Fe$^{2+}$ into the porphyrin ring of PPIX within the heme pathway, while Mg-chelatase inserts Mg$^{2+}$ within the chl pathway. Regulation of Mg-chelatase is an important step within the tetrapyrrole pathway (it is regulated by GUN4, Mg$^{2+}$, ATP and thioredoxin). Mg-PPIX formed by Fe-chelatase, is converted to Pchlide in two enzymatic reactions. PPIX ME is the intermediate of these two reactions. Pchlide is converted to Chlide a by POR, an enzyme that exists in a light-dependent (LPOR) or light-independent (DPOR) form. In organisms containing chl b CAO converts Chlide a to Chlide b. Both are phytylated by GGR (geranylgeranyl reductase), using phytyl pyrophosphate of the non-mevalonate pathway (MEP pathway).
always should be in close proximity to quenchers like carotenoids, and therefore be bound to carotenoid-containing proteins. The binding of chl to protein involves that either all pigment-binding PSII sub-complexes have to be in proximity to the enzymes of the chl biosynthesis and/or that specific (car-binding) carrier proteins are involved to transport chl to the apoprotein. In *Synechocystis* 6803, it has been proposed that the assembly and repair of PSII occurs in specific regions of the membrane where thylakoids and cytoplasmic membranes converge. Biochemical fractionation studies and immunoblotting identified D1, D2, cyt $b_{559}$ and PsbO in cytoplasmic membranes and thylakoids (Zak et al., 2001). In addition, enzymes involved in chl biosynthesis (POR), proteins involved in assembly of PSII (Prat, Pitt) and pD1 have been identified in the biogenesis area (Schottowskii et al., 2009 a,b, Armbruster et al., 2010). Nevertheless, the purity of these membrane fractionations has been questioned. Therefore, a second proposal suggests that areas within the thylakoids are sites of assembly/repair of PSII (Komenda et al., 2008). This proposal relies on the recent finding that cyanobacterial membranes present high heterogeneity (Srivastava et al., 2006, Nevo et al., 2007, Vermaas et al., 2008).

### 1.1.9 Rapid mechanisms of light dissipation

In addition to the repair mechanism of PSII, photosynthetic organisms have developed other mechanisms to dissipate the excess of absorbed light. Rapid processes involve changes in the antenna i.e. non-photochemical-quenching (NPQ; Niyogi, 1999, Kirillovsky and Karfeld, 2012), which includes state transition ($q_T$) (van Thor et al., 1998, William and Allen, 1987), energy-dependent quenching ($q_E$) and photoinhibitory quenching ($q_I$).

State transition is a process to acclimate to the light quality; it aims to equilibrate the amount of energy absorbed by the antenna complexes between the two PSs, preventing over-reduction of the quinone pool. It occurs in plants, algae and cyanobacteria. In higher plants and the green alga *Chlamydomonas reinhardtii* phosphorylated LHCII migrates from PSII towards PSI, while dephosphorylation induces migration back to PSII. This phosphorylation/deshphorilation cycle is performed by membrane-embedded kinases and phosphatases sensible to the redox state of PQ. Cyanobacterial phycobilisomes (PBSs) can move on the surface of the thylakoid membranes toward PSI in response to high light (Joshua and Mullineaux 2004). However, red algal PBSs seem to be immobile, as PSI of red algae contains antennae of the LHC superfamily.

Energy-dependent quenching of NPQ in plants and green algae is induced under high light condition, when high electron transfer activity increases the accumulation of protons in the thylakoid lumen. At low pH, the xanthophyll cycle, the protonation of PsbS (see 1.2.3.4.1, Li et al., 2004, Bonente et al.,
and conformational changes in the LHC antenna (Ruban et al., 2007) are activated to efficiently dissipate the surplus of energy. In the xanthophyll cycle, the carotenoid violaxanthin bound to LHC, is converted to zeaxanthin by the enzyme violaxanthin de-epoxidase that becomes active at low pH. Possibly, light energy is transferred from chl to zeaxanthin and dissipated by heat. In cyanobacteria NPQ is not induced by lowered pH in the thylakoid lumen during high light exposure. Instead, strong blue-green light activates a soluble carotenoid binding protein called orange carotenoid protein (OCP), which interacts with the PBSs (Wilson et al., 2006). OCP is a soluble protein that binds the ketocarotenoid 3'-hydroxyechinenone. Absorption of blue-green light induces conformational changes in the carotenoid as well as in the protein and dark- or dim light-form of OCP (OCPo) is converted into the metastable red form OCPr. OCPr interacts with PBSs to dissipate light as heat and decreases the light arriving to the PSs. Recently, a second protein, the fluorescence recovery protein, has been identified, which accelerates the conversion of OCPr back to OCPo (Boulay et al., 2010).
1.2. The light-harvesting antenna in oxygenic photosynthesis

Light-harvesting antennas are a large group of pigmented protein complexes that have the function to absorb and transfer light energy to the photosynthetic reaction centers. As mentioned in the previous paragraph, they also are important for light protection and energy dissipation. Among the oxygenic photosynthetic organisms, cyanobacteria and some algae contain phycobilisomes and/or the IsiA-Pcb Chl-proteins as major light harvesting antennas; while green algae and higher plants have evolved intrinsic antenna consisting of the light-harvesting complex (LHC) superfamily.

1.2.1 The phycobilisomes

Phycobilisomes are evolutionarily old light harvesting systems found in some oxygenic phototrophs. Today they still are the most important antennae of cyanobacteria, glaucophytes and red algae, but they have been lost in green algae and plants. Phycobiliproteins are soluble proteins that bind phycobilins, open chain tetrapyrroles (Adir 2005, MacColl 1998). There are four major types of phycobiliproteins: allophycocyanin (APC), phycocyanin (PC), phycoerythrocyanin (PEC) and phycoerythrin (PE). Each pigment-protein consists of a hetero-dimeric protein complex of an α- and β-subunit that covalently binds the chromophore. Stacks of this basic structure form the phycobilisome (PBS). In cyanobacteria, the PBS is constituted of a core of APC, from which several rods made of PC, PEC and/or PE protrude radially towards the outside (Figure 8). PC is localized in proximity to the APC core, PEC and PE (when present) are localized at a distal position in the rod. This organization of the pigments allows directed funneling of the light energy from PE to APC and further to the reaction center. A linker protein

![Figure 8. Schematic model of the cyanobacterial antenna system. The phycobilisome is composed of a central core of allophycocyanin (APC) and radial structures (rods) of phycocyanin (PC) and phycoerythrin (PE). The composition of the pigments in the rods can vary. The linker protein (Lcm), ApcF and ApcD anchor and transfer light to the photosystem II of 23 aa.](image)
(ApcE or LCM – linker core-membrane) stabilizes the structure of the PBS and together with ApcD and ApcF subunits transfers the energy mainly to PSII (Figure 8, Dong et al., 2009, Ashby and Mullineaux, 1999). Cryptophytes also contain phycobiliproteins, however α and β subunits do not assemble in fully organized phycobilisomes. Heterodimers of α and β subunits are tightly packed into the luminal side of the thylakoid membranes and contribute to the light absorption (Spear-Bernstein and Miller 1989).

1.2.2 Chl a/b (Pcb) antenna

In most PBS-containing cyanobacteria, IsiA is a stress-related protein that is induced mostly during iron starvation (Geiss et al., 2001, Ivanov et al., 2007), but also during oxidative stress (Singh et al., 2005) and high light treatment (Havaux et al., 2005). Under these conditions, IsiA forms a ring of 18 subunits surrounding PSI, each subunit binds around 13 chlorophyll a molecules (Boekema et al., 2001). The light-harvesting capacity of PSI is increased to avoid damage on PSII. *Synechocystis* 6803 exposed to severe iron limitation was found to accumulate aggregates of IsiA in the membrane, dissipating light via carotenoids (Yeremenko et al., 2004, Ihalainen et al., 2005, van der Weij-de Wit et al., 2007).

Green oxyphotobacteria, formerly called *Prochlorophytes*, are a group of cyanobacteria that contain chl a and chl b. During evolution, they lost PBSs and acquired membrane-intrinsic chl a/b (Pcb) binding antenna proteins, which are related to IsiA and CP43 (Murray et al., 2006). The number of pcb genes varies depending on the species, strain and ecotype. The Pcb antenna forms either a ring structure around PSI (Bibby et al., 2001, Bibby et al., 2003), similar to the ring that IsiA form in PBS containing cyanobacteria, or an arc structure flanking the PSII dimer (Bibby et al., 2003).

*Acaryochloris* is a chl-d binding cyanobacterium that is grouped within the *Prochlorophytes*, because its chl-d-binding antenna is similar to the chl a/b binding antenna of green oxyphotobacteria. Interestingly, *Acaryochloris* contains two pcb genes, one of them is expressed under iron limitation similar to IsiA of PBS containing cyanobacteria, the other one is a constantly expressed as antenna system (Bumba et al., 2005, Chen et al., 2005). Therefore, the IsiA/Pcb family members seem to be involved in the acclimation to light and Fe-limitation by balancing the delivery of light energy to the PSs.

However, these proteins have been completely lost during the primary endosymbiosis, no traces have been found, neither in the red nor in the green lineage.
1.2.3 The light harvesting complex (LHC) superfamily

The acquisition of photosynthesis in eukaryotes was associated with a change in light harvesting strategies; membrane integral chlorophyll binding complexes, the LHCs, progressively substituted phycobilisomes. The LHC proteins form a large family of proteins that share a homologous domain, the chlorophyll-binding motif (CAB-domain), which is part of a trans-membrane helix. All LHC proteins have a similar structural organization, being composed of three trans-membrane helices.

Phylogenetic analyses show that the LHC superfamily consists of several families that include the chlorophyll a/b-binding proteins (CABs), the fucoxantin chlorophyll a/c-binding proteins (FCPs), the chlorophyll a-containing LHCs of some rhodophytes and cryptomonads (LhcaR), the LHCSR/LI818 group and the LHCZ (Dittami et al., 2010, Koziol et al., 2007, Neilson and Durnford 2010). Relatives of this superfamily are the light-harvesting-like (LiL) proteins, which also contain the conserved CAB domain, but are not involved in light harvesting (Jansson 1999). They have a different structural organization, being composed of one to four transmembrane helices and they will be described later. Despite their more distant relationship to the LHC superfamily, Alboresi et al. (2008) showed that all the LI818 sequences identified are clear orthologs of the LiL proteins. Nevertheless, LI818 proteins are considered more closely related to LHC than the LiL (Elrad and Grossman 2004). Changes in the phylogenic trees of the LHC superfamily can be expected when more sequenced genome data will be available.

1.2.3.1 The chlorophyll a/b-binding proteins (CABs)

The light harvesting antenna system of green plants is composed by LHC protein associated with PSI (LHCI or Lhca) and PSII (LHCII or Lhcb, reviewed by Dekker and Boekema 2005). The PSII core is surrounded by an inner, minor, LHC system and by a more peripheral LHC antenna, which forms trimeric structures and is the main light harvesting antenna. Together they form the PSII-LHCII supercomplex. The minor antenna complexes are Lhcb4, 5 and 6, and historically these proteins are termed according their molecular mass CP29, CP26 and CP24. They are involved in thermal dissipation of light energy and in the organization of the thylakoid membrane. The major LHCII trimers can biochemically be separated into different populations depending on their bonding strength to PSII. They are important for state transition. PSI and LHCI form the PSI-LHCI supercomplex, which binds to one site of the PSI core. In higher plants LHCI form heterodimer while in green algae monomers interact with the core.
1.2.3.1.1 Crystal structure of the Lhcb4 (CP29) in plants

All LHC polypeptides of higher plants have high sequence similarity resulting in a conserved structural organization: as shown in Figure 9 they all consist of three transmembrane helices, of which the first and third helix share high homology, additional to one amphipathic helix (Liu et al., 2004, Strandfuss et al., 2005, Pan et al., 2011). The crystal structure of LHCII is available at 2.5 Å (Liu et al., 2004 and Strandfuss et al., 2005) and the one of Lhcb4 at 2.8 Å (Pan et al., 2011). The monomers consist of three transmembrane helices called A, B and C connected by stroma- and lumen-exposed loops and two amphipathic helices, named D and E, which are exposed to the luminal surface (Figure 9). In Lhcb4 9 chl $a$, 4 chl $b$ three xanthophylls, and one lipid molecule (glyceraldehyde 3-phosphate, G3P) are coordinated by each monomer. The 28 amino acid long helices A and C display high similarity: they contain the characteristic LHC motif/CAB domain (ExxxxRxAM), in which the glutamate (E) of one helix and the arginine (R) of the second helix interlock the two helices by a symmetrical pair of salt bridges. The two luteins located at both sides of the two helices A and C and the six chl $a$ closest to them build the central structural motif of Lhcb4, which is conserved within the family. Helix B is 20 amino acids long and also participates in chl binding, as well as the lumen exposed α-helix at the C-terminus of the protein. In Lhcb4, seven chl are coordinated through amino acid side chains of histidine (H), glutamic acid (E) and glutamine (Q), four via oxygen atoms of water molecules and one is bound to G3P via the phosphate group. LHC requires carotenoids for proper function; they are involved in light-harvesting, have a structural role and a photoprotective function. In Lhcb4, three carotenoids have been identified: two luteins and one neoxantin.

1.2.3.2 The fucoxanthin chlorophyll a/c-binding proteins (FCPs)

FCP proteins have high level of similarity to LHCII in plants with an average of 30 % of identity compared to Lhcb1 of A. thaliana (Ballottari et al., 2012). Their three membrane spanning helices bind chl $a$, chl $c$ and carotenoids like fucoxanthin, diadinoxanthin and diatoxanthin (Lepetit et al., 2010, Bailleul et al., 2010). Diadinoxanthin and diatoxanthin seem to be involved in a xanthophyll cycle similar to the violaxanthin/zeaxanthin in plants (Lavaud et al., 2002). One main difference between CABs and FCPs is the chlorophyll/carotenoid ratio bound to the proteins. FCPs bind equal amount of both pigment types (4:4, Papagiannakis et al., 2005), while CABs in plants bind much more chlorophyll (14:4, Liu et al., 2004). Little is known about the macro-organization of the FCP complexes, but in diatoms they seems form trimers or higher oligomeric complexes (Beer et al., 2006, Lepetit et al., 2007).
1.2.3.3 The LHCSR group

This group contains most of the stress-induced LHC proteins and they are mainly induced by high light (Richard et al., 2000, Elrad and Grossman, 2004, Eppard et al., 2000, Oeltjen et al., 2002, Zhu and Green 2010). LHCSR proteins are found in diatoms, brown algae, haptophytes and in green green algae and mosses, but seem to be absent in higher plants (Neilson and Durnford 2010). A structural model based on their amino acid sequences suggests that LHCSR proteins consist of three trans-membrane helices and they contain several chl-binding residues. The LHCSR proteins are well characterized in C. reinhardtii and Physcomitrella patens. In C. reinhardtii there are three genes encoding LHCSR proteins, but only LhcSR3 is involved in NPQ (Peers et al., 2009). The LhcSR3 from C. reinhardtii has been reconstituted in vitro with pigments (Bonente et al., 2011). The recently characterized LhcX1 of the diatom Phaeodactylum tricornutum also seems to be involved in NPQ, although it is also expressed under non-stressful light regimes (Bailleul et al., 2010). The same feature is reported for other members of the LHCSR clade in Thalassiosira pseudonana (Zhu and Green 2010). These data suggest similarity to the LiL proteins.

1.2.3.4 The LiL family

The light-harvesting like (LiL) proteins are a group of proteins that share similarity with the LHC superfamily. Like LHC proteins, they contain the CAB domain in (at least one of their) transmembrane helices (Jansson 1999). The function of many LiL proteins is still not known; nevertheless, they are usually up-regulated in response to different stresses, opposite to the LHCs.
Their expression pattern suggests that they are involved somehow in photoprotection, rather than light harvesting. Evolutionary LiL proteins are older than LHC proteins and they might have been their ancestors (Jansson 2005). LiL proteins in higher plants contain one to four membrane-spanning helices, while the cyanobacterial and algal LiL proteins consist of one to two helices. Traditionally these proteins are grouped based on the number of their predicted transmembrane helices.

1.2.3.4.1 PsbS protein

PsbS is the only LiL protein with four predicted trans-membrane helices (Funk, 2001). Similar to the LHC proteins, its first and third helix share high homology to each other, while the second and fourth helix are less conserved. PsbS is localized within PSII in land plants (Funk et al., 1994) and might have been one of the evolutionary progenitors of LHCs (Engelken et al., 2010). Whether PsbS protein binds chl has been long debated. PsbS was isolated with chl attached to it (Funk et al., 1994, Funk et al., 1995b); however, pigments were weakly bound compared to Lhcb1 (Funk et al., 1995b) and the protein was stable even without pigments attached to it (Funk et al., 1995a). Furthermore, Dominici et al. (2002) were not able to reconstitute in vitro PsbS with pigments. PsbS fulfills the important role to initiate the faster component of the NPQ process. Photosynthetic acidification of the lumen triggers the thermal dissipation through protonation of two lumen-exposed glutamate rests of PsbS. The exact mechanism of PsbS in NPQ is not clear: the pH acidification might activate two xanthophyll-binding sites on PsbS, which are directly involved in the quenching by interaction with the LHCII-PSII (Li et al., 2004); alternatively the acidification induces a conformational change of PsbS that activates other quenching sites on the antenna system (Bonente et al., 2008a). Interestingly, the PsbS gene has been identified in green algae, but it seems not to be expressed (Bonente et al., 2008b).

1.2.3.4.2 Three-Helices ELIPs

The early light-induced proteins (ELIPs) are LiL proteins with three trans-membrane helices; similar to LHC proteins the first and third helix are highly homologous to each other. ELIPs are found in Viridiplantae, but they are absent in red algae, diatoms and cyanobacteria (Neilson and Durnford 2010). ELIPs accumulate transiently during different stress conditions (high light -HL-, cold, drought, heat) that would cause photoinhibition (Heddad et al., 2012). In A. thaliana, there are two ELIP genes coding for two polypeptides, Elip1 and Elip2, which contain 81% sequence similarity. They are differentially expressed during light-stress, greening and senescence.
(Heddad et al., 2006). It is believed that they have a photoprotective role during high light stress either by transiently binding the free chl, thus preventing photo-oxidation, and/or by dissipating excess energy to protect PSII (Hutin et al., 2003, Montané and Kloppstech 2000). However, a double mutant deficient of ELIPs, elip1/elip2, in A. thaliana does not display a photosensitive phenotype (but it contains a reduced amount of chl), suggesting the presence of compensatory processes (Casazza et al., 2005, Rossini et al., 2006). In contrast, the analysis of an overexpressor mutant of Elip2 indicates that ELIPs might reduce photoinhibition by interfering with the chl biosynthesis pathway (Tzvetkova-Chevolleau et al., 2007). In HL conditions Elip1 and Elip2 associated with monomeric and trimeric LHCII, prolonged exposure to stress increases the amount of ELIPs in trimeric LHCII compared to monomeric (Heddad et al., 2006). Biochemical isolations using sucrose gradients identified Elip1 and Elip2 in different LHCII subpopulations (Heddad et al., 2006). In pea, ELIPs were localized in the non-appressed regions of thylakoids membranes in the vicinity of PSII (Adamska and Kloppstech 1991). In HL-stressed pea leaves, ELIPs were co-purified with chl.a and lutein, but strong evidence for their pigment-binding is still lacking (Adamska et al., 1999). In the green algae Dunaliella, an ELIP homolog Cbr, was identified. It seems to form a complex with the minor LHCII proteins (Levy et al., 1992, Levy et al., 1993), which are enriched in zeaxanthin.

1.2.3.4.3 Two-Helices SEPs

The two-helical LiL proteins (SEPs, stress-enhanced proteins) are difficult to classify using phylogeny. Neilson and Dunford (2010) found them to be restricted to chlorophyll a/b containing organisms. The two SEPs they identified, one in diatoms and in one brown alga, were quite distinct and not related to the ones present in green algae and plants. Engelken et al. (2010), believe that SEPs are ubiquitous distributed among photosynthetic eukaryotes, even though there is currently no evidence that they form a monophyletic group. A. thaliana contains six two-helical proteins termed Sep1, Sep2, Sep3-1 (Li3:1), Sep3-2 (Li3:2), Sep4 and Sep5. The transcripts of Sep1 and Sep2 were present in plants exposed to low light condition, and their level increased after HL illumination (Heddad and Adamska 2000). Li3 transcript, instead, remains unchanged upon transferring plants to high light (Jansson 1999). Li3 was found to bind chl a, protochlorophyll a and carotenoids in barley seedling during de-etiolation (Resinger et al., 2008). Recently, both isoforms of Li3 present in A. thaliana were found to stabilize geranylgeranyl reductase (GGR), the enzyme synthesizing phytyl-pyrophosphate, which is required for chl and tocopherol biosynthesis (Tanaka et al., 2010).
1.2.3.4.4 One-Helix, OHP/HLIP/SCPs

Two classes of one-helix Proteins (oHPs) can be distinguished: the OHP1/HLIP/SCP-type of prokaryotic evolutionary origin, present in cyanophages, cyanobacteria and photosynthetic eukaryotes (Dolganov et al., 1995, Funk and Vermaas 1999, Jansson et al., 2000), and the OHP2-type restricted to eukaryotic organisms (Andersson et al., 2003). One-helical LiL proteins have been so far detected in all organisms performing oxygenic photosynthesis. Genes coding for some one-helix LiL proteins are located in the plastid genomes (Neilson and Durnford 2010). Nuclear-encoded one-helix LiLs were identified in red algae, green algae, land plants, glaucophytes and in organism with secondary plastids (Neilson and Durnford 2010). In the cryptophyte G. theta a one-helix LiL protein was found to be nucleomorph-encoded (Douglas et al., 2001, Neilson and Durnford 2010) and a second one has been identified in the chloroplast.

In A. thaliana there are two genes that codify one-helix proteins, Ohp1 and Ohp2. Ohp2 transcript was found in low light (LL) plants, but transcription and translation was enhanced in HL exposed plants (Andersson et al., 2003). OHP2 was found to be specifically associated to PSI during HL conditions, but not during other stresses (Andersson et al., 2003).

1.2.3.5 Evolution of the LHC

HLIPs/SCPs/OHPs have been identified in all oxygenic photosynthetic organisms, the cyanobacterial SCPs therefore are considered to be the evolutionary ancestors of the LiL and LHC protein families. It has been proposed that LHCs and LiLs evolved from SCP-like genes that after duplication and fusion encoded proteins with three membrane-spanning helices. Multiple theories describe how this process might have occurred (Green and Pichersky 1994, Green and Kühlbirad 1995, Heddad and Adamska 2002, Montané and Kloppestech 2000). According to the model of Green and Pichersky (1994, Figure 10A) two HLIP-SCP-like genes fused during the evolution, resulting in the generation of a two helical ancestor. After duplication, an ancestral PsbS protein with four membrane-spanning helices evolved. Finally, loss of the forth helix of this PsbS-ancestor gave rise to the three helical proteins similar to LiL and LHC proteins.

Based on a recent phylogenetic analysis a new model of the evolution of this superfamily has been proposed (Figure 10B, Engelken et al., 2010). Similar to the first model, also this model suggests that the cyanobacterial HLIP/SCP formed a central group of SEPs after gene duplication. However, because LHCs of the red and green lineage differ to the ones in glaucophytes, the authors suggest the first LHC protein to occur after formation of the glaucophytes, but before the green and red algae diverged evolutionary. In each lineage, the LHC ancestor evolved into different antenna proteins. As
ELIPs are found only in green algae and plants, it seems unlikely that they were precursors of LHC. Instead they most likely evolved independently from a different group of SEPs. Another group of SEPs might be the ancestor of PsbS, after internal gene duplication events. This hypothesis implies the loss of SEPs in several taxa like haptophytes and cryptomonas (Engelken et al., 2010). New data derived from more genome sequences will clarify the role of SEPs during evolution.

The LHCSR protein family is spread in chromalveolates, this family therefore might have originated early during evolution; via secondary endosymbiosis it was passed on to the chl a/c-containing organisms. LHCSR proteins are not present in red algae, they might have been lost. However, lateral transfer of the LHCSR-encoding gene cannot be ruled out (Moustafa et al., 2009, Dittami et al., 2010).

Figure 10. Proposed model for the evolution of the LHC protein superfamily. (A) Proposed model by Green and Pichersky (1994). A one-helix HLIP-SCP protein acquired a second helix resulting in a two-helix progenitor. An internal duplication gave then origin to the four helix PSBS ancestor. Loss of the fourth helix led to the three-helix ELIPs and LHC ancestor. (B) Newly proposed model by Engelken et al. (2010). In this model the one-helix HLIP-SCP protein gave origin to a central SEPs group, from which independent evolution of PsbS, LHCs and ELIPs occurred. (*) internal gene duplication.
1.2.4 Ferrochelatase

Ferrochelatase (FC) is the enzyme inserting Fe$^{2+}$ into the porphyrin ring of PPIX during heme biosynthesis. Two different FCs exist in plants, one enzyme is located in mitochondria (type I), while the other in plastids (type II). Interestingly, only the plastid-imported paralog contains a C-terminal extension with a chlorophyll-binding motif (CAB-domain). The CAB-domain and the catalytic core of the FC are connected by a linker region that is variable in length and sequence among different organisms (Sobotka et al., 2011). Type II FC is the only FC of the green alga C. reinhardtii and cyanobacteria. In Synechocystis 6803 the hemH gene is 1161 bp long and codifies for a protein of 387 amino acids (Figure 11). The N-terminus of the protein (324 amino acids long) resembles the catalytic domain of the FC enzyme, whereas the C-terminus contains the LiL part (30 amino acids long, ScpA) connected by a linker region of 23 amino acids. Insertion of a kanamycin cassette at the end of the linker region (at the amino acid 332 or 347) generated deletion mutants without any particular phenotype (Funk and Vermaas 1999, Sobotka et al., 2011). Insertion of a stop codon at the end of the catalytic domain (at amino acid 324) decreased the activity of the FC with severe consequences on the tetrapyrrole biosynthesis (Sobotka et al., 2008). This knock-out mutant accumulates large amounts of PPIX (that is released into the medium) and is not able to grow in HL. These results suggest that the C-terminus of the FC is important for the tetrapyrrole biosynthesis and for both the stabilization and the function of the FC in Synechocystis 6803. The CAB-domain might regulate the dimerization and the complex formation of the enzyme or, eventually, the organization of a super-complex for better delivery of PPIX to the catalytic core. It has also been suggested that the CAB-domain could sense the excess of free chl in membranes, thereby increasing FC activity. This feedback would decrease the flux of PPIX through chl branch, avoiding the accumulation of photoactive toxic intermediates of chl when not needed (Funk and Vermaas 1999).

![Figure 11](image.png)

**Figure 11.** Schematic representation of the ferrochelatase. The N-terminus of the Fe-chelatase of Synechocystis 6803 is the catalytic core of the enzyme (1-324 aa). The C-terminus is called ScpA and is composed of 30 aa. It contains the CAB-motif. C- and N-terminus are connected by a linker region of 23 aa.
1.2.5 HLIP/SCPs in viral genomes

The presence of photosynthetic genes, including HLIP genes, in genomes of several cyanophages has been reported (Mann et al., 2003, Lindell et al., 2004). HLIPs are believed to be important to maintain photosynthetic activity in the host during phage infection (Lindell et al., 2004, 2005).

1.2.6 Other proteins containing the CAB-motif

The CAB-motif has been found in proteins that are not known to be directly connected to light-harvesting and photoprotection. In Synechococcus, a protein with two trans-membrane helices has been identified that appears to be a fusion between one HLIP and a second trans-membrane helix (Kilian et al., 2007). In plants, a trans-membrane helix containing the CAB-motif was identified at the C-terminus of a Rieske iron-sulfur protein (Neilson and Durnford 2010).
1.3 The small-CAB like proteins in *Synechocystis* sp. PCC 6803

As described in the previous chapter, cyanobacterial HLIPs or SCPs are considered to be the progenitor of LHC proteins in photosynthetic eukaryotes. This hypothesis is widely accepted, although the exact evolutionary pathway is still under debate. HLIP/SCPs proteins seem to be present in all cyanobacterial genomes sequenced so far; moreover, many similar *HLI/SCP* genes can be present in the same organism. The cyanobacterium *Prochlorococcus marinus* strain MED4, which is adapted to high light intensity at the surface of open water contains 24 *HLI* genes in its genome (Bhaya et al., 2002). In *Synechocystis* 6803, there are five *scp* genes; four of them, *scpBCDE* (Funk and Vermaas 1999), corresponding to *hliC, hliA, hliB* and *hliD* (He et al., 2001), codify for small one helical proteins of around 6 kDa, while *scpA* encodes the C-terminal extension of the ferrochelatase (see 1.2.4, Figure 12). The sequences of ScpC and ScpD have high similarity (87.1%), whereas ScpB and ScpE are less related (44.7% similarity). ScpBCDE are stress induced proteins.

**Figure 12.** Sequence alignment of SCPs from *Synechocystis* 6803 and the TMH3 of Lhcb1 from *Pisum sativum*. Conserved residues are indicated by a star (*), the TMH is underlined. The alignment was generated using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalW2) and the prediction of the secondary structure was performed using PSIPRED protein prediction server (bioinf.cs.ucl.ac.uk/psipred/).

SCP genes are monocistronic, with the exception of *scpD* (Kufryk et al., 2008), which is co-transcribed with another gene *slr1544* in several cyanobacteria including *Synechocystis* 6803 (Kufryk et al., 2008). Slr1544 has also been named LiLa (light harvesting-like protein A) as two residues of the chl-binding domain are conserved in its predicted transmembranal helix. Despite this fact LiLa has low sequence similarity with the other LiL proteins.

1.3.1 Gene expression and regulation of SCPs

HliA of *Synechococcus* is controlled by the sensor kinase NblS (van Waasbergen et al., 2002). In the same way, SCP genes of *Synechocystis* 6803 are regulated by the sensor kinase DspA (also known as Hik33), which is homologous to NblS (Kappell et al., 2006). NblS/DspA functions as sensor of the photosynthetic redox state (Salem and van Waasbergen 2004). A high light regulatory 1 (HLR1) sequence was identified upstream of the SCP genes.
in *Synechocystis* 6803 and other cyanobacteria, indicating that this regulation is important and widespread in this group (Kappell et al., 2006). Numerous gene expression data of *Synechocystis* 6803 have been accumulated during the past years, investigating different environmental and genetic backgrounds. If corrected, they can be integrated and used for correlation cluster analysis (Singh et al., 2010). Cluster analysis of SCP gene expression using the database created by Hernandez-Prieto and Futschik (2012) shows that scpB, scpC and scpD have similar expression patterns during different experimental conditions (Figure 13). They cluster with non-photosynthetic genes and genes with unknown function. In agreement with the regulation via a histidine kinase system, SCP genes are induced in stress conditions that affect the lipid membrane. ScpB, scpC and scpD are up-regulated after addition of DBMIB to the media at low light and down-regulated or unaffected by DCMU addition (Hihara et al., 2003). Desiccation stress, sorbitol- (Paithoorangsarid et al., 2004), salt – (Shoumskaya et al., 2005, Allakverdiev et al., 2002), hydrogen peroxide treatment (Houot et al., 2005), PSI light (Singh et al., 2009), low temperature (Prakash et al., 2010) and CO₂ limitation as well as high CO₂ (Wang et al., 2004) up-regulate the SCP genes. During light-dark cycle growth or in presence of cadmium (Cd, Houot et al., 2007) transcription of scpB, scpC and scpD is reduced. In HL condition, SCP genes are mostly up-regulated, even though the various genes respond with different expression pattern (Muramatsu and Hihara 2011, Hihara et al., 2001, Singh et al., 2008). Interestingly, HL induction of the SCP genes in the presence of glucose is repressed (Tu et al., 2004). SceE has a similar expression pattern to scpB, scpC and scpD, but its transcript accumulates in lower amount (Figure 13). Although lilA is co-transcribed with scpD, some microarray analyses report different expression of these two genes (Figure 13). LilA seems to be stronger expressed after hydrogen peroxide addition, desiccation, UV light and HL compared to scpD (Houot et al., 2007, Singh et al., 2008).

1.3.2 Protein expression and regulation

SCPs have been found to be expressed after cell exposure to high light, cold stress or nutrient limitation (He et al., 2001). During high light stress, ScpCD accumulate faster and in larger amount compared to ScpB and ScpE (He et al., 2001). Both proteins can be detected in the cell within one hour of high light stress and they accumulate up to 6 hours, then their amount gradually decreases. ScpB accumulates at a slower rate (after 3 hours), but it is stable for longer than 6 hours in high light. ScpE is expressed at lowest amount, its optimal abundance can be detected after 6-9 hours of high light exposure, and then it declines rapidly (He at al., 2001). If cells are transferred back to normal light conditions after short exposure to HL stress, ScpBCDE
expression declines even faster; all SCPs are degraded within 3 hours of LL.
SCPs were found to be constitutively expressed in *Synechocystis* 6803 mutants depleted of PSI (PSI-less) and in different mutants lacking PSI and PSII (PSI-less/PSII-less, Funk and Vermaas 1999).

Single and multiple SCP deletion mutants have been constructed to study their function. Single SCP deletion mutants show no phenotype different to wild type (WT), neither when grown in normal light (NL) nor HL. However, in a competition experiment in HL grown, a scpC mutant is defeated against WT. A scpBCDE quadruple deletion mutant is unable to grow in HL condition (He et al., 2001, Havaux et al., 2003). It shows an altered pigmentation compared to WT, exhibiting reduced amount of chl - even in low light – and increased carotenoid amount, in particular mixoxanthophyll and zeaxanthin (Havaux et al., 2003). The quadruple mutant exhibits enhanced photochemical activities (seen in fluorescence, photo-acoustic and O2 evolution measurements) leading to the hypothesis of SCPs being involved in photoprotection via the dissipation of light energy (Havaux et al., 2003), which is not correlated to an increase in the light-harvesting antenna size (Xu et al., 2002, Havaux et al., 2003, Xu et al., 2004). Therefore, light dissipation of the SCPs can either be performed by direct interaction with the PS or by an indirect mechanism, e.g. regulation of chl biosynthesis (or both). Recently, ScpC and ScpD have been localized with PSII complexes, mainly in the monomeric PSII and the smaller reaction center core complex (RC47), which is depleted of CP43 (Promnares et al., 2006, Yao et al., 2007). The finding of these two LiL proteins within PSII indicates a similar function, which also is supported by their high sequence similarity and expression pattern. The closest neighbor of ScpD is CP47, connected with PsbH (Promnares et al., 2006, Yao et al., 2007). PsbH seems to be important for the association of ScpCD; in its absence, ScpCD are not attached to CP47 (Promnares et al., 2006). SCPs also have been reported to associate with PSI (Wang et al. 2008) however, the PSII-localization of these LiL proteins is supported by mass spectrometry data, immunoblotting and immunogold labeling.

Also ScpB has been detected within purified PSII, but not ScpE (Yao et al., 2007). Immunoblot localization detects ScpE exclusively in the thylakoid membranes, not associated to any complexes (Yao et al., 2007). Tagging ScpB with strep-tagII also co-purifies ScpE in a green band containing PSII protein together with other proteins (Kufryk et al., 2008). Due to their lower sequence similarity and different expression pattern compared to ScpCD, ScpB and ScpE are proposed to have a different function in the dissipation of light energy. It is proposed that ScpB and ScpE regulate the tetrapyrrole biosynthesis depending on pigment availability (Xu et al., 2002). In *Synechocystis* 6803 with PSI-less/chlL background (this mutant is not able to synthesize chl in the dark), deletion of ScpB and ScpE causes a reduction
of chl, phycobilisomes, and, in the case of the PSI-less/chlL-/scpE-less mutant, also protoheme during light-activated-heterotrophic-growth (LAHG, Xu et al., 2002). The chl synthesis pathway is proposed to be inactivated at ALA formation. In fact, supplement of ALA in darkness increases the level of Mg-Proto IX and Mg-Proto IX ME in PSI-less/chlL-/scpE-less, whereas Pchlide accumulates in PSI-less/chlL-/scpB-less. No differences in the amount of tetrapyrrole intermediates are found in the control strain (Xu et al., 2002). Deletion of all SCP genes in a PSI-less/PSII-less mutant background reduces the amount of chl per cell 30-fold, but in the presence of either ScpB or ScpE the chl level only decreased 6- or 20-fold, respectively (Xu et al., 2004). The kinetics of chlorophyll degradation upon SCP-deletion is faster in the absence of SCPs and the degradation rate is proportional to the number of inactivated SCPs (in the PSI-less background) (Xu et al., 2004, Vavilin et al., 2007a). Upon removal of the SCPs, the rate of chl synthesis is slower compared to the control strain (Xu et al., 2004, Vavilin et al., 2007a). Based on the results obtained on single scp-deletion mutants, ScpB seems to be most important for chlorophyll stability. Interestingly, the authors observed that in general chl molecules turn over much slower than their protein scaffolds, implying that most of the chlorophyll molecules have to be reused/recycled during PSII assembly/disassembly (Vavilin et al., 2007a). In this chl-cycle, the pigment is degraded to chlorophyllide and phytol, both are re-used after chlorophyll de-esterification (Vavilin et al., 2007a,b). It seems that SCPs have no influence on chlorophyllide recycling, although ScpE deletion appears to inhibit the chlorophyllide conversion to chl (Vavilin et al., 2007a). Therefore, SCPs seem to inhibit the chl degradation associated to PSII by temporarily binding chl, and thereby preventing it from degradation.

Using in vitro reconstitution experiments, it was shown that SCPSs have the capacity to interact with a pigment-mix extracted from Synechocystis 6803 (Storm et al., 2008). In similarity to the cross-linked structure of the first and third helices in LHCII two SCPS might form a homo- or hetero-dimer (Storm et al., 2008).
Figure 13. Microarray expression data of scpB, scpC, scpD, scpE, lilA and hemH using the public database CyanoEXpress (cyanoexpress.sysbiolab.eu). RNA was analyzed of cells exposed to different environmental condition. Green: increase of expression, red: decrease of expression, black: no changes in expression, grey: data not available.
2 Aims of the thesis

One-helical proteins of the LiL family are widespread within photosynthetic organisms, but their function is still elusive. The general aim of my thesis was to increase the understanding of their function in the cryptophyte alga Guillardia theta and the cyanobacterium Synechocystis sp. PCC 6803.

Below I specify the aim of my work resulting in manuscripts/publications.

Paper I

Cryptophytes like Guillardia theta originated after secondary endosymbiosis. They still contain a vestigial nucleus of the engulfed red alga, called nucleomorph. At the beginning of my work, it was neither known if nucleomorph-encoded proteins were expressed in G. theta, nor if (or how) G. theta performs photoprotection. Two LiL genes are encoded in the genomes of the alga, one is plastid-located, the other one in the nucleomorph. The aim of my work was to investigate the function of the two LIL proteins and their possible role in photoprotection.

Paper II and III

Five LiL genes were detected in the cyanobacterium Synechocystis sp. PCC 6803 encoding small CAB-like protein (SCPs). In analogy to the plant LiL proteins, they were assumed to be involved in a general mechanism of photoprotection. Three SCPs had been found to be associated with Photosystem II. The aim of my studies was to further investigate the function and mechanism of photoprotection of the SCPs. In order to do that I used two different approaches:

- I closely investigated how the structural organization of photosystem II is changed in cells depleted of the SCPs using fluorescence techniques and focusing on their possible role in photosystem II assembly/repair mechanisms (Paper II).

- I combined high-throughput profiling strategies, transcriptomics, proteomics and metabolomics, to investigate the effect of SCP-deletion on the cellular organization (Paper III).

Paper IV

In Synechocystis 6803 ScpA is fused to a ferrochelatase, an enzyme involved in the heme biosynthetic pathway. The aim of this project was to understand if ScpA has a regulatory function on the activity of the ferrochelatase and if possibly pigment binding to ScpA might influence this regulation.
3 Results and discussion

Photosynthetic organisms are exposed to various environmental factors such as light, temperature, inorganic carbon and nutrients. They respond to environmental changes depending on the intensity and length of the perturbation. Organisms can either not change their living strategy performing homeostatic responses (Montechiaro et al., 2006, Montechiaro and Giordano 2006) or they can acclimate by changing the cell organization. These responses are directed to reestablish the energetic balance. Therefore, primarily the composition and organization of the photosynthetic apparatus is modulated, but other cellular processes can also be affected (Walters 2005, Eberhard et al., 2008, Pfannschmidt and Yang 2012). If acclimation strategies do not work properly, ROS are generated, which can damage the macromolecules within the cell.

The large families of LHCs, LHCSR and LiL proteins are involved in acclimation responses. The antenna proteins, CABs and FCPs, mainly function in light harvesting, but also are involved in light adaptation and photoprotection, while the LiL family and the LHCSR clade are mainly involved in photoprotection; LiL family and the LHCSR clade are induced by high light intensities, opposite to LHC (Heddad et al., 2012, Heddad and Adamska 2002). Photoprotective mechanisms involve both rapid and slow changes in the photosynthetic apparatus, ranging from seconds to hours (Eberhard et al., 2008). General mechanisms of photoprotection are conserved in photosynthesis, but the effectors and the regulation may differ between organisms. Conformational changes within the antenna modify the interaction between chl and carotenoid that dissipate the excess of light by heat. This mechanism is called qE and it occurs rapidly in response to the light stress. LHCs operate using this modality with the support of some LiL proteins that function creating new quenching sites, as PsbS in plants and possibly LhcSR3 in C. reinardtii (Bonente et al., 2008a, Bonente et al., 2011). An efficient PSII assembly/repair system is another mechanism important for photoprotection that involves many different proteins (Nixon et al., 2010); between them LiL protein members, as SCPs as it will be described later. In Viridiplantae, ELIPs appear to have a similar function of SCPs (Tzvetkova-Chevolleau et al., 2007), but their mechanism is still unclear. This photoprotective mechanism (qI) occurs on longer time scale it seems to involve the regulation of the chl biosynthesis pathway. Therefore, members of LiL family proteins are indubitably important for protection and interestingly although their similarity, LiL members are able to support and modulate different photoprotective mechanisms in different manner. For many LiL the function is unknown or uncertain, therefore further studies are needed in order to clarify their photoprotective mechanisms.
In my work I was interested to investigate the function of the one helical LiL proteins of the cryptophyte alga *Guillardia theta* and the cyanobacterium *Synechocystis* sp. PCC 6803.

### 3.1 HLIPs in G. theta and their role in photoprotection

As described earlier, *G. theta* has undergone two endosymbiotic events during evolution. It contains two small LiL proteins; one is encoded in the plastid (*hlipP*) and is of cyanobacterial origin, the other is encoded in the remaining of the red algal nucleus, the nucleomorph (*HlipNm*). We hypothesized that the two HLIPs were involved in NPQ. First we could observe that *G. theta* indeed performs NPQ (Paper I). As *G. theta* possesses FCPs antenna complexes, we tested the existence of a xanthophyll cycle, one of the main players in NPQ in plants and algae. However, none of the xanthophyll carotenoids present in plants, green algae and diatoms were identified by pigment analysis. The nuclear genome of *G. theta* contains neither *PsbS* nor *LhcSR*, genes encoding proteins involved in NPQ in higher plants, green algae and diatoms. So *HlipP* and *HlipNm* could indeed perform this function. However, even though both genes were transcribed, both *hlipP* and *HlipNm* were not induced by HL (Figure 14, Paper I), opposite to members of the LiL family and LHCSR clade. In contrary, the stronger light stress was applied to the cells, the faster transcription declined. A peptide-directed antibody against *HlipNm* visualized that protein expression of *HlipNm* protein was constant and independent of the light exposure (Figure 15, Paper I). It is worth to mention that at the time of the work, it was not known if nucleomorph-encoded genes are transcribed or translated.

At the moment, there is no evidence that the two HLIPs of *G. theta* are involved in photoprotection. However, in the diatom *P. tricornutum*, *Lhcx1* (related to LHCSR of green algae) is not high light responsive, but knock-
down mutants generated using RNAi had a significantly reduced NPQ capacity (Bailleul et al., 2010). It has been proposed that, constitutive expression of LhcX1 might be an advantage in the highly dynamic environment of the water column. Various orthologues of LhcX1 have been detected and not all of them are light responsive (Nymark et al., 2009, Bailleul et al., 2010, Zhu and Green, 2010). As a member of phytoplankton and being distantly related to diatoms (chl c, chloroplast with four membranes), it is possible that G. theta’s light adaptation is different from the one in green algae (Lepetit et al., 2012). Cryptophytes contain phycobiliproteins situated at the luminal site of the thylakoid membrane. These seem to be involved in light absorption, but it is unknown whether or not they are involved in NPQ, in similarity to cyanobacteria.

Figure 15. Protein expression of HlipNm in cells exposed to high light stress. HlipNm with molecular mass of 13 kDa was immunodecorated using a peptide-directed antibody recognizing the N-terminus of the protein. Total cell extract was isolated from G. theta cells grown on 12 h day/12 h night cycle under NL (30 µmol photons m⁻² s⁻¹) or after exposure to 500 µmol photons m⁻² s⁻¹ (upper panel) or 1000 µmol photons m⁻² s⁻¹ (lower panel). Harvest was started (time point 0, lane 1) at the end of the night, just before the light was turned on. Thirty micrograms of protein were loaded per lane.

3.2 Cyanobacterial SCPs are not involved in NPQ

Contrary to the HLIPs of G. theta, SCPs in Synechocystis 6803 have been studied extensively during the past decade. They are induced under different stress conditions and are believed to function in stress protection (He et al., 2001, Havaux et al., 2003). In my work, I compared a PSI-less mutant, in which the SCPs are constitutive expressed, with a PSI-less/ScpABCDE-mutant to investigate their function.

The available data suggest SCPs to be important for long-term acclimation. Although the SCP genes are induced within minutes (He et al., 2001), in high light stress the proteins begin to appear earliest after one hour and maximal expression is reached upon 6 hours. Havaux et al. (2003) observed that SCP peptides are present only when the cells began to replicate during high light
treatment. Therefore, these data as well as other biochemical measurements suggest the SCPs not to be antenna proteins (Xu et al., 2002, Paper II) and they are not involved in non-radiative dissipation processes (Paper II), although they are associated with PSII (Promnares et al., 2006, Yao et al., 2007). We wanted to investigate further this aspect measuring NPQ using the pulse amplitude modulated (PAM) fluorimeter (Figure 16). As described earlier, the main player in NPQ in cyanobacteria is OCP, a photoactive protein able to bind a carotenoid molecule (reviewed by Kirilovsky and Karfeld 2012). Dim blue light induces conformational change in the OCP₀ dark form converting it in the OCPᵣ active form that is able to bind PBSs thereby reducing light transferred mainly to PSII. We observed the basal fluorescence (F₀) in the PSI-less/ScpABCDE⁻ mutant to be higher than in the PSI-less mutant, indicating disconnected PBSs (Figure 16). Upon exposure light-adapted cells to a dim blue-blue light first and then to a strong-blue light, higher fluorescence quenching could be observed in the SCP-deleted mutant. The higher NPQ in PSI-less/ScpABCDE⁻ is performed by the larger amount of PBS and OCP per PSII in this mutant as shown in Figure 17. When bands were quantified using D1 band as reference ca 60% increase of OCP and the α- subunit of APC was detected in PSI-less/ScpABCDE⁻ compared to PSI-less. We also tested if IsiA, which also is able to protect PSII under stress conditions (Ihalainen et al., 2005, Yeremenko et al., 2004), was expressed under the conditions we used; neither in the control mutant nor in the SCP-depleted mutant (Figure 17). We concluded that SCPs do not have the same function as IsiA and OCP.

Figure 16. Non-photochemical quenching of the (A) PSI-less strain and the (B) PSI-less/ScpABCDE⁻ mutant using PAM chlorophyll fluorescence. Dark-adapted cells with the same chlorophyll concentration were illuminated first with dim blue light for 50 s (LB), then with strong blue-green light for 100 s (DB) and then allowed to recover in non-actinic measuring light (dark bar).
3.3 SCPs (ScpC, ScpD and possibly ScpB) are involved in the stabilization of chlorophyll-binding proteins during PSII de novo assembly/repair

As SCPs are not antenna protein involved in light dissipation (they are not involved in qE), we investigate the possibility of their involvement in the photoinhibitory quenching (qI), supposing that SCPs could be implicated in the assembly/repair mechanism of PSII (Nixon et al., 2010). In addition, we used an inhibitor of chl synthesis (gabaculin) to investigate further into the SCPs’ role of stabilization of chl molecules during PSII assembly/repair (Vavilin et al., 2007a). As expected PSII activity (measured as O2 evolution, Figure 18) was lost during photoinhibition, the process was similar in the PSI-less control strain and the PSI-less/ScpABCDE- mutant. In case no protein inhibitor (lincomycin) was used, PSII activity decreased 10% more in the PSI-less/ScpABCDE- mutant compared to the control. When chl

![Figure 17](image_url)

**Figure 17.** Immunoblot using total cell extract of the PSI-less and the PSI-less/ScpABCDE- mutants in the presence and absence of gabaculine (5 μM). Antibodies directed against the Photosystem II proteins D1 and PsbH as well as against ScpC and ScpD, ScpE, allophycocyanin (APC), OCP and IsiA were used. In the right upper panel ratios of band intensities APC/D1 and OCP/D1 are shown. Quantification of band intensities is presented in percentage. The immunoblots were quantified using Image J (http://rsbweb.nih.gov/ij/).
synthesis was inhibited by gabaculine the activity of PSII decreased ca 20% more in the control strain, whereas it remained unchanged in the SCP-depleted mutant strain. The photoinhibitory treatment that accelerates loss of PSII activity in presence of gabaculine is related to an impaired assembly process of PSII. In the presence of chl and/or SCPs recovery was almost complete, however, recovery was retarded if chl, SCPs (SCP-depleted mutant) or both were absent. These results indicate that chl is required for PSII repair and that SCPs are involved in the stabilization of chlorophyll during PSII de novo assembly/repair. Our results were confirmed by the data of Yao et al. (2012).

SCPs ensure an efficient assembly/repair PSII by reducing the effect of the acceptor-site photoinhibition, which is induced via single oxygen formation (Vass 2011). Single oxygen is known to produce extensive damage to protein complexes. Damaged proteins release chl molecules that still absorb light, but cannot transfer the energy to another pigment, and thus act as strong photosensitizer. SCPs were proposed to stabilize chl molecules by direct binding through the CAB-domain (Xu et al., 2002, 2004, Storm et al., 2008, Sinha et al., 2012) in proximity to a quenching carotenoid molecule. Based on their sequence similarity to the first and the third helix of the LHC proteins, SCPs were proposed to be able to bind carotenoids (Xu et al., 2004). Moreover, circular dicroism of in vitro reconstituted SCPs shows a signal that can be attributed to the interaction of protein and carotenoids (Storm et al., 2008). However, evidences that SCPs bind chl in vivo are still lacking.

**Figure 18.** Dependence of protein- and chlorophyll-synthesis on photodamage and repair of PSII in the (A) PSI-less and the (B) PSI-less/ScpABCDE strain. PSII activity was quantified by the initial amplitude of the flash-induced chlorophyll fluorescence signal. Photoinhibitory treatment was performed in the presence (circles) or absence (squares) of gabaculine (5 μM), or in the presence (closed symbols) and absence (empty symbols) of 300 μg/mL lincomycin. Chlorophyll concentration was adjusted after gabaculine incubation (for 17 h) prior to measurement. High light (200 μmol photons m⁻² s⁻¹) and recovery light (4 μmol photons m⁻² s⁻¹) were applied for 90 min.
3.4 ScpC, ScpD and periodically ScpB stabilize chl molecules in sub-complexes of PSII

ScpC, ScpD and periodically ScpB have been localized in the RC47 complex and in PSII monomers in proximity to CP47 and PsbH (Promnares et al., 2006, Yao et al., 2007, Paper III). SCPs could also bind the complex CP47-PsbH (Boehm et al., 2011). Is has been proposed that CP47 might not only be important as antenna subunit of PSII, but also function as chl storage and hub delivery of chl molecules in the cell (Sobotka et al., 2008b, Dobakova et al., 2009, Boehm et al., 2011). An active exchange of chl molecules between chl-binding proteins has been proposed for both plants and cyanobacteria (Tzinas and Argyroundi-Akyounoglou 1987; Kada et al., 2003). CP47 senses the availability of chl, reduced chl amount leads to lower expression of CP47 (Sobotka et al., 2005, Sobotka et al., 2008b). PsbH in its turn stabilizes CP47, allowing the sub-complex CP47-PsbH to bind to the RC pre-complex D1-D2/cyt b559 (Komenda et al., 2005). CP47 and PsbH have the lowest turnover rate of all PSII subunits, in agreement with their possible role of chl storage proteins (Yao et al., 2012 a,b). Deletion of psbH leads to impaired electron transfer between QA and QB without decreasing the rate of oxygen evolution (Mayes et a., 1993); this phenotype is similar to the one observed in the SCP-deletion mutant (Paper II, Vavilin et al., 2007a). In the psbH-deletion mutant the phenotype was attributed to modified bicarbonate binding at the acceptor site of PSII (Komenda et al., 2002). The psbH mutant is more sensitive to photoinhibition due to inefficient PSII repair and to high oxidative damage (Komenda et al., 2002). Recently, it has been shown that PsbH is in contact with a chl molecule of CP47, having a probable function in chl-protein stabilization (Müh et al., 2008), and it is also essential for the interaction of SCPs with CP47; in case PsbH is removed, SCPs do not co-migrate with PSII sub-complexes in Blue Native-PAGE (BN-PAGE) but they are found not associated with any complex (Promnares et al., 2006). Psb28 was also co-purified with His-ScpD (Yao et al., 2007). Psb28 is mainly attached to RC47 sub-complex, in proximity to PsbH (Dobakova et al., 2009). A psb28 knock-out mutant contains decreased amount of RC47 and it accumulates chl intermediates, similar to the SCP-deletion mutant.

These data indicate that the stabilization of CP47 and PSII sub-complexes (as RC47 and psbH-CP47) might be important for a correct equilibrium between PSII assembly/repair and chl synthesis. Stable CP47 and PSII sub-complexes might serve to re-utilize chl and therefore decrease the rate of chl degradation by chl re-utilization, which decreases the need of chl synthesis in the cell. In the absence of SCPs, PSII sub-complexes containing CP47 and PsbH are destabilized and therefore transcription of most of the genes encoding enzymes of the chlorophyll biosynthesis pathway is enhanced.
(Paper III) and chl intermediates accumulate (Xu et al., 2004). Moreover phytol accumulated 7-fold more in the SCP-deletion mutant compared to the control strain (Paper III) indicating both, higher chl degradation and lower chl re-utilization. Free phytol is highly toxic for proteins and membranes due to its detergent-like properties and therefore it might interfere with the PSII assembly/repair process in the SCP-deletion mutant.

BN-PAGE in Paper III shows a decreased amount of RC47 in PSI-less/ScpABCDE compared to PSI-less (in similarity to Yao et al., 2012a), supporting this hypothesis. However, in our BN-PAGE we could not identify smaller complexes containing CP47 (as CP47-PsbH) neither in PSI-less or PSI-less/ScpABCDE due probably to their low amount. Low temperature (77K) emission spectra show a decrease of the PSII peak at 695 nm in PSI-less/ScpABCDE compared to PSI-less when 435 nm excitation light was used. This peak has been attributed to CP47 fluorescence emission (Vermass et al., 1986, Shen and Vermaas 1994). On the contrary, any change of the

![Figure 19. Low temperature (77K) emission fluorescence spectra of whole cells with an (A) excitation at 435 nm, or (B) excitation at 580 nm. Spectra were normalized to the highest fluorescence peak. On the x-axes, nm are not shown for clarity and the wavelength of the fluorescence peaks is indicated; 650 nm peak is attributed to PC, while 660 nm emission peak is related to APC. (C) Immunoblot analysis of total protein extracts isolated from the same number of cells of the PSI-less and PSI-less/ScpABCDE strain, respectively.](image)
peak at 685 nm attributed to CP43 and LCM of PBS was observed (Figure 19A). Similar results were obtained using the 580 nm excitation wavelength, light that is mainly absorbed by PSBs (Figure 19B). These results support a depletion of CP47 pool in PSI-less/ScpABCDE- (Sobotka et al., 2008b). As the peak at 685 nm is also due to the fluorescence of the LCM of PBS, I also checked the amount of CP47 using immunoblot analysis shown in Figure 19C. While I could detect D1 in both PSI-less and PSI-less/ScpABCDE-, the band corresponding to CP47 was detectable only in PSI-less when the protein extract from the same number of cells was used. Interestingly, this result also indicate that sub-complexes of PSII containing CP47 but not processed D1 (as CP47-PsbH) are present in lower amount compared to RC47 and PSII that contain both processed D1 subunit and CP47 in the PSI-less/ScpABCDE- mutant. This further suggests the importance of SCPs in chl stabilization in CP47. ScpC, ScpD and possibly ScpB might stabilize chl at the protein scaffold by increasing the chl-recycling, and similar to CP47 and PsbH, they sense the amount of chl (Figure 17). Although, their mechanism is still unclear they might form hydrophobic bridges between PSII subunits that help the transferring and re-cycling of chl molecules avoiding the damaging of other molecules. Nevertheless, this hypothesis needs to be experimentally verified.

### 3.5 SCPs regulate the chl biosynthesis

By regulating chl-recycling, SCPs might regulate the chl biosynthesis. Contrary to ScpC and ScpD, the amount of ScpE is not decreasing upon the addition of gabcucin; its function might be different during the acclimation process (Figure 17). ScpE and ScpB have been proposed to regulate chl biosynthesis depending on chl availability at the step of ALA formation (Xu et al., 2002, 2004). This regulation might occur at the enzyme GluTR, the first committed enzyme in the tetrapyrrole biosynthesis branch; its substrate, Glu-tRNAGlu, also is used for protein synthesis.

Alpha-ketoglutarate (α-KG) is a metabolite of the tricarboxylic acid (TCA) cycle that provides the carbon skeleton for nitrogen fixation. Nitrogen is incorporated in carbon skeletons through the action of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT, Figure 20C). Using a glutamate molecule as sustrate, GS incorporates ammonium (NH4+) forming glutamine, that is then used by GOGAT that catalyzes the transfer of one of the amino groups to α-KG. The net product of the GS/GOGAT cycle is a glutamate molecule that is exported and used for protein and chl synthesis (Figure 20C). In *Synechocystis* 6803, α-KG is an important signal molecule involved in the coordination of the carbon (C) and nitrogen (N) metabolism (Muro-Pastor et al., 2001). Addition of α-KG to the SCP-deletion mutant increased the amount of chl (50% increase of chl per cell, Figure 20A), PBSs
and proteins per cell, while the control strain showed only an increase in protein amount (Figure 20B, Paper III). This indicates that both PSI-less and PSI-less/ScpABCDE- use the added KT to produce glutamate and sequentially Glu-tRNA\textsubscript{Glu}, the substrate of GluTR; at this branchpoint Glu-tRNA\textsubscript{Glu} is used only for protein synthesis in PSI-less, while in the SCP-deleted mutant, it is utilized in both the ALA pathway and protein synthesis. This result is in agreement with Xu et al. (2002); among the SCPs, ScpB and/or ScpE are more favorable to inhibit the chl biosynthesis at the ALA step. Most likely this mechanism is indirect; ScpE and ScpB are small membrane-integral proteins, it seems therefore unlikely that they should regulate a soluble enzyme like GluTR. Other, unknown, proteins might be

\[ \begin{align*}
&\text{PSI-less} \\
&\text{PSI-less + α-KG} \\
&\text{PSI-less/ScpABCDE-} \\
&\text{PSI-less/ScpABCDE- + α-KG}
\end{align*} \]

\( \text{nm} \)

**Figure 20.** Effect of α-KT addition on PSI-less control and the PSI-less/ScpABCDE- strain of *Synechocystis* 6803. A. Absorption spectra of total pigments extracted from the same number of cells. B. Coomassie-stained gel of total proteins extracted from the same number of cells. C. Schematic representation of the hypothetical SCP function. The addition of α-KT leads to an increase of proteins, chl and PBSs per cell in the PSI-less/ScpABCDE- strain while only protein amount increases in PSI-less cells, SCPs therefore should inhibit chl synthesis at GluTR.
involved in this feedback inhibition, similar to the process performed by FLU in plants (Meskauskiene et al., 2001, Gosling et al., 2004). GluTR also is known to be feedback-regulated by heme, and our microarray analyses indicate higher transcript amount of ho1 (encoding heme oxygenase) upon SCP-deletion compared to the control mutant. In plant mutants with impaired activity in HO, GluTR is inhibited (Terry and Kendrick 1999). An increase of FC activity led to a decrease in the metabolic flow through the tetrapyrrole pathway in Synechocystis 6803, suggesting a heme feedback (Sobotka et al., 2005). However, rates of ALA formation are not always correlated to the amount of the heme pool suggesting that other factors might be involved in ALA formation (Sobotka et al., 2008a). Recently, FLU was found to co-precipitate with POR and GGR (Kauss et al., 2012), proving that the chl-branch controls the initial step in plants tetrapyrrole synthesis.

ScpB seems to be the most important among SCPS (Vavilin et al., 2007a). ScpB has been coisolated with ScpC and ScpD within PSII and the ScpB mutant shows an impaired chl biosynthesis, as described. ScpB alone or together with ScpC and ScpD might connect the process of PSII assembly/repair with chl biosynthesis. It is unknown if SCPS can interact directly with one or more enzymes of the chl biosynthesis. Microarray data in Paper III show an accumulation of transcripts that encode for subunits of POR enzyme (chlB, chlN and chlL), the ChlH subunits of the Mg-chelatase and the anaerobic cyclase BchE, indicating a possible target of SCPS in the chl biosynthesis pathway (Figure 7 and 21). However, SCPS could interact with the chl biosynthesis pathway via Psb28. Psb28 was co-purified with His-ScpD and it seems to interact with the aerobic cyclase (sll1214), that converts Mg-PPIX ME to Pchide (Dobakova et al., 2009). It has been proposed that Psb28 might regulate chl availability during PSI and PSII biogenesis (Dobakova et al., 2009). Nevertheless, ScpB, ScpD and/or ScpC might interfere with the FC activity interacting with ScpA (see 2.6). Sobotka and co-workers (2011) showed that full-length FC of Synechocystis 6803 is able to bind the tagged C-terminal segment of the FC (ScpA) when used as bait. Due to the high sequence similarity, also ScpBCDE could bind to the C-terminal ScpA of the FC. Nevertheless, so far none of ScpBCDE has been co-purified with any enzymes of the chl biosynthetic pathway or the FC in Synechocystis 6803.

2.6 The CAB-domain at the C-terminus of the FC regulates the enzyme activity

Like most photosynthetic organisms, Synechocystis 6803 contains a type II ferrochelatase C-terminal CAB-motif embedded within its hydrophobic tail. In analogy to the other SCPS, this stretch has been called ScpA (Funk and Vermaas 1999). The ferrochelatase enzyme (FC) functions as first enzyme in
the heme and phycobilins-synthesis branch, it competes with the Mg-chelatase for the same substrate PPIX, to insert Fe^{2+} ion, while Mg^{2+} insertion leads to the synthesis of chlorophyll. The branching point between chl- and -heme synthesis therefore has to be tightly regulated to avoid the accumulation of phototoxic precursors. However, this regulation step is not well understood.

We hypothesized that the CAB-domain of the FC could have a regulatory function, sensing an increase of chl availability in the cell, thereby increasing the FC activity (Paper IV). To test this hypothesis, we overexpressed and purified recombinant *Synechocystis* 6803 full-length FC as well as truncated FC depleted of the CAB-domain (truncation was made at aa 347, see Figure 11), and we monitored the production of zinc-protoporphyrinIX (Zn-PPIX) in different conditions over time. First, we observed that both, the full-length FC and the truncated FC, were active in our *in vitro* assay (even though they displayed differences in their kinetics). However, we did not observe any changes in the FC activity in presence of pigments, suggesting that either our *in vitro* system is not suitable for detecting this interaction or such regulation does not occur. It is reasonable to think that the micelle system formed by the detergents used in our assay has different characteristics compared to the lipid bilayer of membranes. It was observed earlier that FC activity measured in native membranes differs from the purified enzyme (Sobotka et al., 2011). We also observed that different detergents affect the activity of both forms of the enzyme (Paper IV). The use of liposomes, artificial vesicles composed of a lipid bilayer, might represent a more suitable system to study the effect of chl on enzyme activity. However, based on our results it seems more reasonable that the CAB-domain regulates the FC activity by a different mechanism, such as modulating the interaction with other proteins (another FC monomer, other SCPs) and/or changing the affinity/delivery of the substrate/product to the catalytic domain (Sobotka et al., 2008, Sobotka et al., 2011). We showed that the turnover number (k\text{cat}) of the truncated FC is 5.5-fold higher than the one of the full-length enzyme, indicating a faster release of the product (Zn-PPIX) from the enzyme, consequently the affinity for PPIX (K_M) is lower in the truncated enzyme (Paper IV). These results support *in vivo* measurements of PPIX and heme pools (Sobotka et al., 2011); in a ΔH347FC mutant, the PPIX is 20% lower compared to the WT while the heme concentration increased, suggesting a higher activity of FC depleted of the CAB-domain. A slightly increase of chl and 2.5-fold higher amount of chlide was also observed in a ΔH347FC mutant, suggesting that the CAB-domain of FC can modulate the PPIX channeling through the chl and heme pathway. Our results point to a regulatory function of the CAB-domain (ScpA) inducing a slower release of Zn-PPXI, thereby influencing the PPIX utilization. Although the biological relevance of this mechanism *in vivo* has to be investigated, it is interesting to
observe that in the cyanobacterium *Thermosynechococcus elongatus* FC was found associated to the protoporphyrinogen IX oxidase (PPO), the enzyme that catalyze the formation of PPIX (Masoumi et al., 2008). It would be interesting to investigate if this complex is also formed in *Synechocystis* 6803 and whether or not it can further modulate the function of the FC (full-length and truncated) and/or the substrate channeling between the heme and the chl branches.

2.7 The effect of SCPs deletion on chronic stressed cell

The important function of the SCPs on the stabilization of PSII sub-complexes is seen in the SCP-deletion mutant, where the total amount of functional PSII is considerably lower, affecting the energy balance and the cell metabolism (Paper III). Electron microscopy images showed a drastic change in cellular organization of *Synechocystis* 6803, visible by a rough surface and few thylakoid membranes in comparison with the control strain (Paper III). Deletion of the *SCP* genes also affected the carbon- and nitrogen-balance and thereby the carbohydrate accumulation at the expenses of the N-rich pools (proteins and chlorophyll), as seen by transcriptomic and metabolomics analysis. The lower amount of PSII in the *SCP*-deletion mutant leads to reduced amount of electrons in the electron transport chain, a decreased proton motive force across the membrane and, consequently, to a lower amount of ATP production. In addition, the impair process of *de novo* synthesis/repair if SCPs are absent increases the ROS damaged proteins as seen in Paper III. Therefore, we concluded that SCPs are important for the adaptation to environmental stresses, especially during the generation of reactive oxygen species.

2.8 Conclusions

To conclude my doctoral thesis, I would like to give an overview of the induction and function for the SCPs based on my own data and data of other groups (Figure 21). Overeduction of the PQ pool induces transcription of the *SCP* genes via the Hik33 kinase pathway (Kappel et al., 2006). The response regulator RpaB binds to the HLR1 sequence at the gene promoter (Kappel and Waasbergen 2007). *SCP* induction seems to be coordinated for *scpB*, *scpC* and *scpD* (Figure 13). *SCP* translation probably occurs in ribosomes associated with thylakoid membranes. We showed that SCPs are involved in the assembly/repair processes of PSII, therefore, I would expect to localize them mainly in the repair/biogenesis area of photosynthetic complexes (Schottowski et al., 2009). Although this is a reasonable assumption for the function that we propose, immunolabelling *in vivo* has not been performed yet for any of the SCPs. At protein level, ScpC and ScpD accumulate first and associate with the PSII-subcomplex; ScpB has its maximum peak of
expression retarded in comparison to ScpCD, and therefore it is likely that it binds PSII later as well (He et al., 2001). ScpB binding to PSII is less clear compared to ScpCD. In presence of ScpCD and/or ScpB, the chl-binding of PSII is stabilized during the repair. As chl is efficiently re-utilized in the presence of SCPs, the cell will not synthetize new chlorophyll and therefore down-regulates or inhibits enzymes involved in chl biosynthesis. ScpB alone or together with ScpCD might be responsible for this, as hypothesized earlier. It is interesting to note that scpB contains non-coding RNA at its 3’ terminus (Georg et al., 2009). Therefore, a more complex regulation of scpB expression might be possible. Further modulation on the chl biosynthesis is carried out by ScpE, which can be induced by ScpB (Kufryk et al., 2007). ScpE expression starts later and in lower amounts (He et al., 2001) and it could be involved in the fine-tuning of the chl biosynthesis.

Figure 21. Schematic representation of a possible mechanism of gene induction and function of SCPs in *Synechocystis* 6803. A source of stress is sensed through an overreduction of the plastoquinone pool (PQH). The histidine kinase Hik33 activates RpaB, the response regulator binding to promoters with a HLR1 sequence (yellow box), like the scp genes. They are induced within minutes, ScpC and ScpD are translated first, they form homo- or heterdimers and bind to PSII, stabilizing its chl binding. ScpB is translated later, this delay might be regulated by the non-coding RNA. ScpB and later ScpE decrease the flux of chl biosynthesis. Dashed arrows point to possible regulative steps. PQH: Plastoquinones, PEP: Phosphoenolpyruvate, OPP: Oxidative pentose phosphate, ALA: Aminolevulinic acid, PPIX: protoporphyrin IX, Mg-PPIX: Mg-protoporphyrin IX, Mg-PPIX ME: Mg-protoporphyrin methyl ester, PChide: protochlorophyllide, Chide: chlorophyllide, TCA tricarboxylic acid, GS: glutamine synthase, GOGAT: glutamate synthase, POR: protochlorophyllide oxidoreductase, BchE: anaerobic cyclase. Thylakoid membranes in green, cytoplasmic membrane in black.
4 Concluding remarks

This thesis provides new insights into the function of the one-helical LiL proteins in the cryptophyte alga *Guillardia theta* and the cyanobacterium *Synechocystis* 6803. We were able to show that the two single-helix LiL genes (*hlipP* and *HlipNm*) in *G. theta* are expressed at normal growth conditions, but their expression is decreased by high light (Paper I). *HlipNm* is translated, but not light-inducible. We concluded that the two single-helix LiL proteins of *G. theta* are not needed in photoprotection. The cyanobacterial SCPs are not involved in OCP-mediated NPQ in *Synechocystis* 6803 (Paper II). SCPs stabilize the chlorophyll-binding proteins of the Photosystem II sub-complex during PSII repair (Paper II). Inhibition of chl biosynthesis reduces the amount of ScpC and ScpD, as well as the amount of other PSII subunits, but the amount of ScpE is not altered. Therefore, ScpE seems to fulfill a different function compared to ScpC and ScpD (Paper II). Deletion of all SCPs in a PSI-less background has profound impact on the cell organization and metabolism (Paper III). The impair of de novo assembly/repair of PSII increase the ROS production in *scp*-deletion mutant (Paper III). The strong decrease of PSII number per cell volume in the *SCP*-deletion mutant leads to decreased proton motor force across the membranes, leading to a chronic macronutrient deficiency seen by transcriptomic and metabolomic analysis (Paper III). Finally, I characterized the recombinant *Synechocystis* 6803 ferrochelatase *in vitro* and I could show that ScpA is involved in the product-release to the catalytic domain of the enzyme, thereby regulating substrate availability for chlorophyll- or heme- biosynthesis (Paper IV).
5 Future perspectives

SCPs stabilize chl during the repair of PSII (Vavilin et al., 2007a, Paper II, Yao et al., 2012), however, their working mechanism is still unknown. It has been proposed that SCPs can bind chl via their conserved CAB-domain. Although chl may interact with SCPs in vitro (Storm et al., 2008), in vivo evidence is still lacking. Studying a mutant with amino acid changes (point mutagenesis) in the CAB-domain might provide some understanding regarding the function of these LiL proteins. Mutagenesis studies on LHCs revealed that arginine (R) and glutamate (E) of the CAB-domain are involved in chl binding and important for proper folding of the protein (Bassi et al., 1999). Modifications of R and G in the SCPs should lead to instable protein, prohibiting a cross-linked structure in analogy to LHCII. Instead, the central asparagine (N) or histidine (H) of the CAB-domain should be the target for the point mutation. However, one should note that point mutagenesis of H might change the SCP protein structure due their small size.

ScpD and ScpC consist of 70 aa, while ScpB and ScpE are shorter, 47 aa and 57 aa, respectively (Figure 12). The longer N-terminals of ScpD and ScpC have been shown to be localized at the stromal side of the thylakoid membrane (Promnares et al., 2006). They contain three arginine (R) residues each, that might be involved in the stabilization of the PSII acceptor side, as proposed for PsbH (Komenda et al., 2002). It would be interesting to further investigate the functional role of these N-terminals of ScpD and ScpC.

While I could show that SCPs stabilize chl-binding in the RC47 sub-complex and in PSII monomers, it is still not known how many SCPs are bound per PSII. Quantitative analyses as well as qualitative pigment determination of these complexes in the presence of absence of SCPs might give important information. In addition, the chl binding properties of these complexes should be studied using fluorescence at cryogenic temperature (Boehm et al., 2011).

The functional relevance of regulatory RNAs in Synechocystis 6803 becomes more obvious (Eisenhut et al., 2012, Sakurai et al., 2012). Regulatory RNA was also identified at the 3′-terminus of scpB (Georg et al., 2009). It would be interesting to investigate the role of this regulatory RNA on ScpB expression and function.
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