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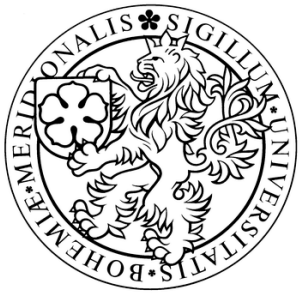

Regulation of the chlorophyll biosynthesis in the cyanobacterium *Synechocystis* sp. PCC 6803

Ph.D. Thesis

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Annotation

The thesis focuses on regulation of the chlorophyll biosynthetic pathway and its coordination with synthesis of chlorophyll-binding proteins in the cyanobacterium *Synechocystis* sp. PCC 6803. One of the aims was to analyze correlation between syntheses of photosystems and chlorophyll in *Synechocystis* cells using radioactive labeling of proteins and chlorophyll by ^{35}S and ^{14}C, respectively. I also investigated the role of enzymes catalyzing protochlorophyllide reduction step in the chlorophyll biosynthesis by analyzing the synthesis and accumulation of photosynthetic proteins in *Synechocystis* mutants lacking one of the enzymes. Further, roles of Ycf54 and Psb27 proteins in stability and assembly of oxidative cyclase and CP43, respectively, are also described.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Jana Kopečná

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The biggest acknowledgements belong to my family and friends for being with me and making my life so great and exciting.

List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Kopečná J**, Komenda J, Bučinská L, Sobotka R (2012) Long-term acclimation of the cyanobacterium *Synechocystis* PCC 6803 to high light is accompanied by an enhanced production of chlorophyll that is preferentially channeled to trimeric PSI. Plant Physiology (DOI:10.1104/pp.112.207274) (IF = 6.535).
Jana Kopečná performed all the experiments except for TEM and participated in writing and reviewing the manuscript.

II. **Kopečná J**, Sobotka R, Komenda J (2012) Inhibition of chlorophyll biosynthesis at the protochlorophyllide reduction step results in the parallel depletion of Photosystem I and Photosystem II in the cyanobacterium *Synechocystis* PCC 6803. Planta (DOI: 10.1007/s00425-012-1761-4) (IF = 3.000).
Jana Kopečná prepared all the mutant strains, performed all the experiments and participated in writing and reviewing the manuscript.

III. Hollingshead S, **Kopečná J**, Jackson PJ, Canniffe DP, Davison PA, Dickman MJ, Sobotka R, Hunter CN (2012) Conserved chloroplast open-reading frame *ycf54* is required for activity of the magnesium-protoporphyrin monomethylester oxidative cyclase in *Synechocystis* PCC 6803. Journal of Biological Chemistry 287: 27823-27833 (IF = 4.773).
Jana Kopečná participated in FLAG-Sll1214 purification, immunodetection analyses and detection of Sll1214 mRNA in the Synechocystis strains by Northern blot analysis.

IV. Komenda J, Knoppová J, **Kopečná J**, Sobotka R, Halada P, Yu J, Nickelsen J, Boehm M, Nixon PJ (2012) The Psb27 assembly factor binds to the CP43 complex of photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Physiology 158, 476-486 (IF = 6.535).
Jana Kopečná participated in CP47-His and CP43-His purifications and analyses of the eluates by a two dimensional - clear native/denaturing electrophoresis.

The research group leaders, who are also corresponding authors in the manuscripts included in this thesis, hereby confirm that Jana Kopečná contributed to the mentioned publications as described above.

Ing. Roman Sobotka, Ph.D. Prof. RNDr. Josef Komenda, CSc.

Contents

INTRODUCTION

I. Introduction

The phototrophic way of life implies the capture of electromagnetic energy, its conversion into chemical energy, and its use for cellular maintenance and growth. The vast majority of phototrophic organisms use chlorophyll-based systems for the conversion of light into chemical energy (Overmann and Garcia-Pichel 2006). They consist of light-harvesting antennae and two reaction centers – photosystem I and II, where the excitation energy is converted into a proton gradient across the membrane. In these systems, chlorophyll (Chl) molecules are essential for capturing and transferring photons to reaction centers and it is also Chl in the form of a "special pair" that serves in reaction centers of both photosystems as the primary electron donor and drives the conversion of light into chemical energy.

In plants, algae and many bacteria, all the tetrapyrroles, including Chl and heme, originate from a common biosynthetic pathway (Masuda and Fujita 2008). As more end products are formed simultaneously and the demand for each strongly depends on a fluctuating environment, the process obviously requires a high degree of metabolic regulation. Indeed, Chl as the key pigment for assembly and function of the photosynthetic apparatus has to be produced in an adequate amount that would not limit biogenesis of photosynthetic membranes. However, demand for Chl-binding proteins varies according to environmental conditions and Chl biosynthesis also has to be controlled to prevent an accumulation of Chl or its intermediates since most of these molecules are strong photosensitizers. When present in excess and not properly protected within the protein complexes that quench the absorbed energy, reactive oxygen species are formed causing oxidative damage of the cell (Foyer et al. 1994). It is important to mention that although the last step of Chl biosynthesis is the moment when the Chl molecule is made, from a regulatory point of view Chl itself is just a toxic intermediate until it is not attached to a protein. And even at this point it is just a start of another pathway – a sophisticated process of assembly of central parts of photosystems from different Chl-proteins. The long journey of Chl, starting at the first step of the branched tetrapyrrole biosynthesis and finished by assembly of functional photosystems, is tightly connected and appears to be under perfect control. However, although the enzymatic steps of the tetrapyrrole pathway are almost completely elucidated at the genetic level (Tanaka and Tanaka 2007), current knowledge of the regulation of tetrapyrrole metabolism and its coordination with protein synthesis or with the process of photosystem assembly is very limited.

Synechocystis ***sp. PCC 6803 as a model organism for studies on the biosynthesis of Chl-protein complexes***

Cyanobacteria, which have been model organisms since the early 70s of the past century (Allen and Smith 1969), are a widespread group of photoautotrophic microorganisms, which originated, evolved, and diversified early in Earth's history (Tamagnini et al. 2002). It is generally accepted that cyanobacteria played a crucial role in the Precambrian phase by contributing oxygen to the atmosphere (Schopf 2000). Even today, cyanobacteria and their close relatives, the prochlorophytes, are responsible for a large portion of the photosynthetic production in aquatic environments and thus play an important role in global CO_2 assimilation and oxygen recycling (Falkowski and Raven 2007). Relevance of this phylum covers from evolutionary studies (Shi and Falkowski 2008) to biotechnological applications, including biofuel production or drug discovery (reviewed in Ruffing 2011).

Among the diverse cyanobacterial strains, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is one of the most frequently used organisms for studies on biogenesis of photosynthetic apparatus including biosynthesis of photosynthetic pigments (Komenda et al. 2012, Xu et al. 2002, 2004). The strain was isolated from a fresh water lake, and was deposited in the Pasteur Culture Collection (PCC) in 1968. Use of *Synechocystis* benefits from the availability of necessary genetic tools and a rich compendium of genomic, biochemical, and physiological data. Perhaps most significantly, *Synechocystis* was the first photosynthetic organism for which the entire genome sequence was determined (Kaneko et al. 1996). Moreover, in contrast to higher plants, gene inactivation, modification and regulatable expression became routine in this organism due to natural transformation and efficient homologous recombination. Another fundamental advantage is the ability to survive and grow in a wide range of conditions including heterotrophy and mixotrophy, where the only energy and carbon source is glucose, or where light is present as well as a combination of two carbon sources - glucose and CO_2, respectively.

In terms of photosynthesis, cyanobacteria, as the progenitors of plant plastids, use the same set of enzymes to produce Chl *a* and very similar photosynthetic apparatus as plants (Vavilin and Vermaas 2002). Thus, it is rational to expect that the regulatory aspects of Chl metabolism in cyanobacteria operate also in chloroplasts and that obtained results are also largely applicable on plants. This is similar to the research on structure and biogenesis of the photosynthesis apparatus in cyanobacteria that was invaluable for understanding energetic processes in the chloroplast (Kanervo et al. 2007). There are only minor differences regarding the set of enzymes needed for Chl *a* synthesis. It should be noted that cyanobacteria generally possess anaerobic oxidases and also so called dark protochlorophyllide oxidoreductase (DPOR, see later); however, their activities appear to be quite limited under aerobic conditions and the respective genes can be readily inactivated in *Synechocystis* (Minamizaki et al. 2008, Yamazaki et al. 2006, He et al. 1998,

Kopečná et al. 2012b). Advantageously, *Synechocystis* has only one gene copy for all the other enzymes (with the exception of Mg protoporphyrin monomethyl ester cyclase), compared to plants that possess multiple gene copies of several enzymes involved in Chl biosynthesis (Tanaka and Tanaka 2007, Masuda 2008).

II. Overview of the chlorophyll biosynthetic pathway

Chl is chemically a tetrapyrrole molecule with a magnesium atom in the center and with one ring esterified with phytol (Fig. 1). The tetrapyrrole biosynthetic pathway is well studied in terms of the enzymes involved. Recent reviews concerning tetrapyrrole biosynthesis in photosynthetic organisms include Bollivar 2006, Tanaka and Tanaka 2007, Masuda 2008, Mochizuki et al. 2010. The biosynthesis of the most important Chl *a* requires usually 15 enzymatic steps that can be divided into three main sections: (1) formation of the initial precursor molecule aminolevulinic acid (ALA), (2) formation of protoporphyrin IX – the last common precursor for both heme and Chl branches (3) the Chl branch.

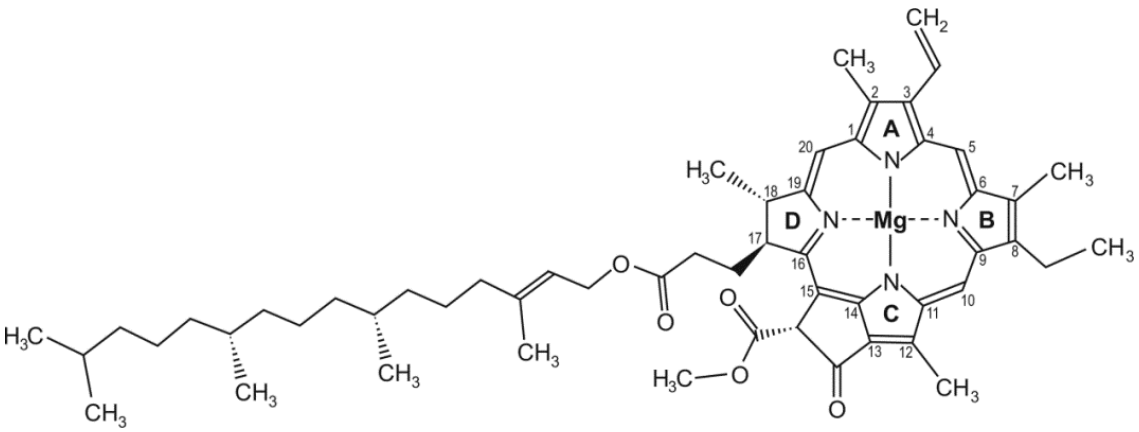

Figure 1. The chemical structure of chlorophyll *a*.

Formation of 5-aminolevulinic acid

In plants, algae, cyanobacteria, sulphur bacteria, and archaea, Chl biosynthesis is initiated from the amino acid, glutamate, which is ligated to $tRNA^{Glu}$ by glutamyl-tRNA synthetase (GluRS) (Beale and Weinstein 1991). Resulting glutamyl-$tRNA^{Glu}$ is also used in the biosynthesis of proteins (Precali and Falkowski 1983). Glutamyl-tRNA reductase (GluTR) then reduces the activated carboxyl group of glutamyl-$tRNA^{Glu}$ to a formyl group to produce glutamate-1-semialdehyde. The reaction is the first committed step of the tetrapyrrole biosynthesis. Subsequently, glutamate-1-semialdehyde aminotransferase (GSA-AT) converts glutamate-1-semialdehyde into ALA, the universal tetrapyrrole precursor in all organisms (Fig. 2). In contrast, animals, fungi, yeast, non-sulphur purple bacteria, and many bacteria that do not contain Chls generate ALA via the Shemin pathway (C_4+C_1 pathway) by condensation of succinyl-coenzyme A with glycine (Beale 2006). The reaction is catalyzed by ALA synthase. Apart from organisms which use either pathway for the synthesis of ALA, there has been a few organisms reported that utilize both pathways, namely *Euglena gracilis* (Mayer and Beale 1992), *Scenedesmus obliquus* (Dreschler-Thielmann et al.

1993) and *Streptomyces nodosus* (Petříček et al. 2006). However, the latter uses the Shemin pathway exclusively for the production of the antibiotic asukamycin and is not incorporated into heme (Petříček et al. 2006). Interestingly, Kořený and coworkers (2011) recently demonstrated that the coral-associated alveolate *Chromera velia*, the closest known photosynthetic relative to Apicomplexa, synthesizes Chl from glycine and succinyl-CoA by ALA synthase, It seems that the Chl biosynthesis in *Chromera velia* starts in mitochondria (Kručinská J, Oborník M, personal communication) making it attractive to speculate that acquisition of the ALA synthase for tetrapyrrole biosynthesis could be an important prerequisite for a switch from autotrophy to parasitic style of life.

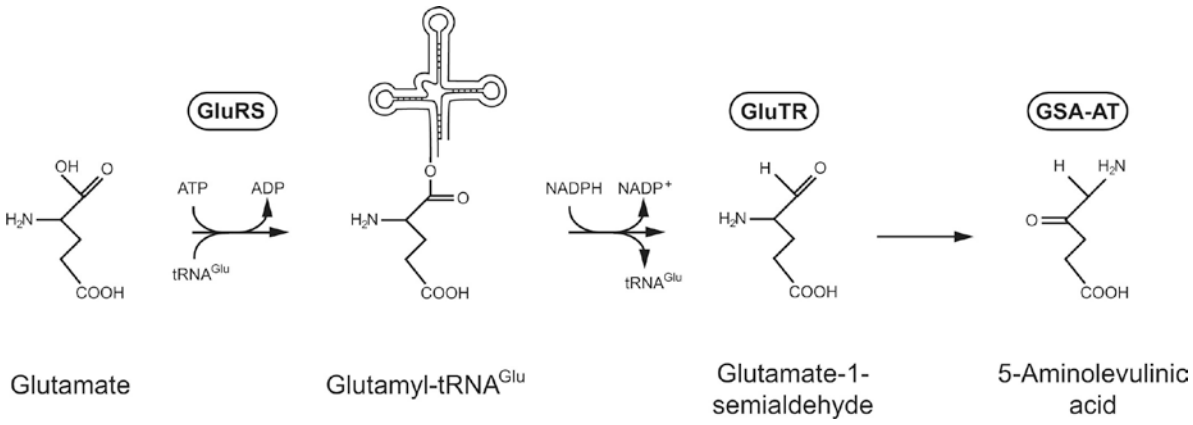

Figure 2. The C_5 biosynthetic pathway of 5-aminolevulinic acid. Abbreviations used: GluRS, glutamyl-tRNA synthetase; GluTR, glutamyl-tRNA reductase; GSA-AT, glutamate-1-semi-aldehyde aminotransferase.

Formation of protoporphyrin IX

Six enzymatic reactions converting ALA into an empty porphyrin ring protoporphyrin IX are common for heme and Chl biosynthesis (Fig. 3), although in different organisms oxidation steps can be catalyzed by structurally unrelated enzymes (reviewed in Vavilin and Vermaas 2002). At the beginning two ALA molecules are asymmetrically condensed by ALA dehydratase to form the pyrrole derivative porphobilinogen. Four molecules of porphobilinogen are then sequentially polymerized into a linear tetrapyrrole, 1-hydroxymethylbillane, by porphobilinogen deaminase. Uroporphyrinogen III, the first closed macrocycle (´porphyrin ring´) in the pathway, is produced by uroporphyrinogen III synthase. The pathway is branched at this point leading to siroheme cofactors of nitrite and sulfite reductases and to vitamin B_{12}. In the following steps of the heme/Chl pathway, the side chains of the macrocycle are modified through different decarboxylation reactions resulting in intermediates coproporphyrinogen III and protoporphyrinogen IX. Subsequently, the ring system is oxidized giving rise to the planar, fully conjugated protoporphyrin IX. This compound serves as a substrate for two different chelatases; either Fe^{2+} is incorporated by ferrochelatase (FeCH) or the Mg^{2+} ion is inserted by magnesium chelatase (MgCH). These

represent the final step of the heme biosynthesis or the first committed step of the separated Chl branch (Fig. 4).

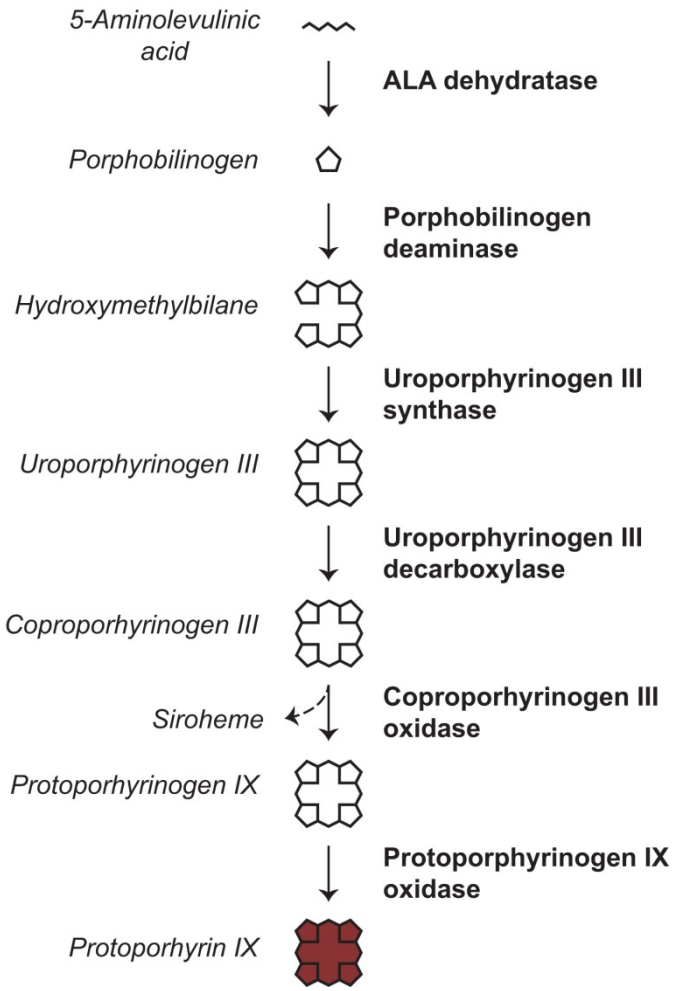

Figure 3. Formation of proto-porphyrin IX from 5-amino-levulinic acid in cyanobacteria. Enzymes are indicated in **bold**; intermediates are indicated in *italics*.

The chlorophyll branch

The Chl branch is unique to photosynthetic organisms (reviewed in Masuda 2008). As mentioned above the Mg chelation is catalyzed by MgCH, a heterogenous complex consisting of three subunits and requiring ATP for catalysis. Resulting Mg protoporphyrin IX is then methylated by Mg protoporphyrin IX methyl transferase (MgPMT) to produce Mg protoporphyrin IX monomethyl ester. In the next reaction, Mg protoporphyrin IX monomethyl ester cyclase (MgPMC) incorporates atomic oxygen into its substrate to form divinyl protochlorophyllide. This oxidative cyclization creates the isocyclic ´fifth´ ring of Chl. The following enzyme divinyl (proto)chlorophyllide reductase (DVR) catalyzes reduction of the 8-vinyl group on the B ring of the tetrapyrrole to an ethyl group using NADPH as the reductant. Although DVR can also use the later

precursor (divinyl)-chlorophyllide (Parham and Rebeiz 1995) as a substrate, in *Synechocystis* most of protochlorophyllide is in monovinyl form (Sobotka R., unpublished data). Protochlorophyllide oxidoreductase then reduces the ring D to form the last Chl precursor chlorophyllide. The Chl *a* molecule is completed by esterification of a propionate group on the D ring of chlorophyllide with phytol-pyrophosphate by teamwork of the Chl synthase and geranylgeranyl reductase.

Nonetheless, there are more chemically distinct Chls known to date in oxygenic photosynthetic organisms. Higher plants, green algae, and a few groups of cyanobacteria (prochlorophytes) possess Chl *b* together with Chl *a*. In the Chl cycle where Chl *b* is formed, chlorophyllide *a* is converted to chlorophyllide *b* by chlorophyllide *a* oxygenase. Moreover, Chl *b* can be reversibly converted to Chl *a* through 7-hydroxymethyl Chl *a*. These reactions are carried out by Chl *b* reductase and 7-hydroxymethyl Chl *a* reductase. Chl *c* is formed in a branched pathway prior to the synthesis of protochlorophyllide in chromophytes (Jeffrey 1980). Also a rare, red shifted form of Chl, Chl *d*, was found in an epiphytic cyanobacterium, *Acaryochloris marina* (Hu et al. 1998). Recently, a new form of Chl, Chl *f*, was extracted from cultures of cyanobacteria and other oxygenic microorganisms that form stromatolites (Chen et al. 2010).

The heme branch and synthesis of linear tetrapyrroles

Heme biosynthesis is completed by FeCH, a single-subunit enzyme that inserts Fe^{2+} into the protoporphyrin IX macrocycle in a reaction that does not require input of energy. The resulting heme B molecule either serves as the prosthetic group for many proteins and enzymes or is further processed to different derivatives in bacteria and plants. Heme oxygenase linearizes protoheme to biliverdinIXα, a precursor for phytochromobilins and various phycobilins (Alvey et al. 2011). In cyanobacteria and red algae, phycobilins accumulate to high amounts in the form of the light-harvesting pigments associated with phycobilisomes.

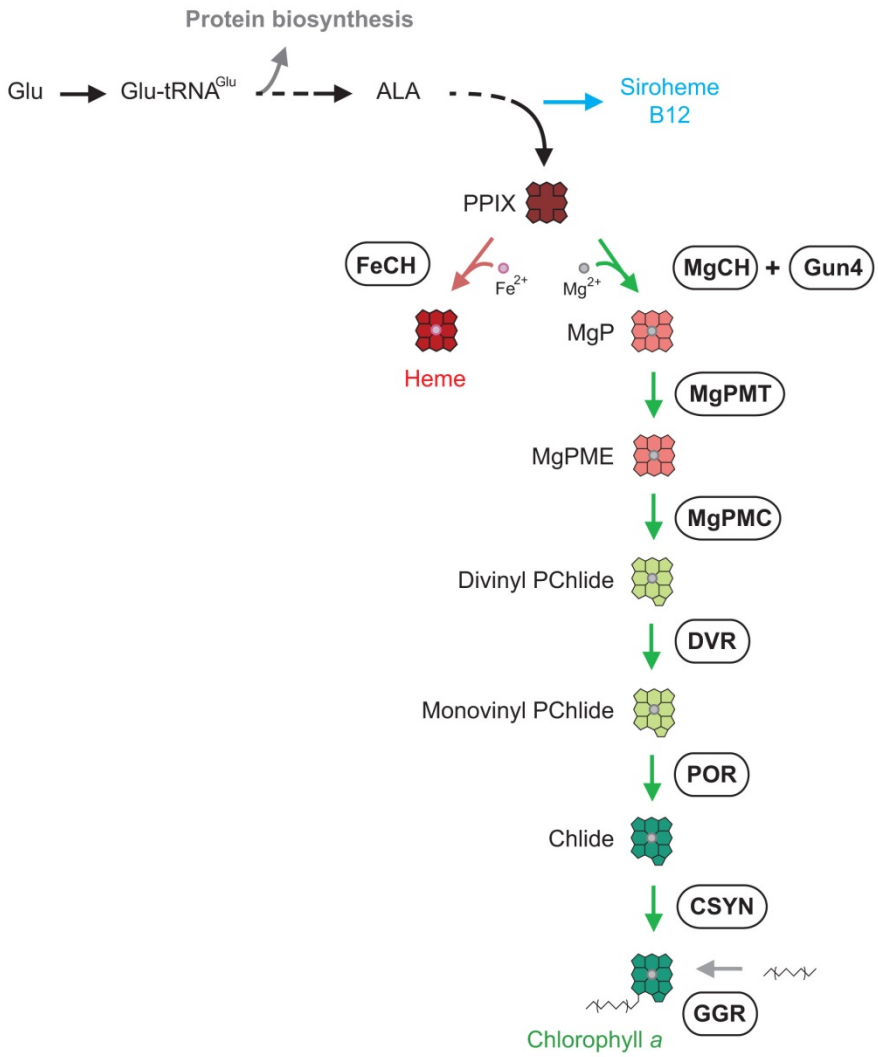

Figure 4. The tetrapyrrole biosynthetic pathway in cyanobacteria. Abbreviations of enzymes (in circles): FeCH, ferrochelatase; MgCH, Mg-chelatase, which consists of three subunits ChlI, ChlH and ChlD and another protein Gun4 is also required for the fully-active Mg-chelatase; MgPMT, Mg protoporphyrin IX methyltransferase; MgPMC, Mg protoporphyrin IX monomethyl ester oxidative cyclase; DVR, 3,8-divinyl chlorophyllide 8-vinyl reductase; POR, protochlorophyllide oxidoreductase; CSYN, chlorophyll synthase; GGR, geranylgeranyl reductase. Abbreviations of Chl intermediates: Glu, glutamate; Glu-tRNA^{Glu}, glutamyl tRNA^{Glu}; ALA, 5-aminolevulinic acid; PPIX, protoporphyrin IX; MgP, Mg protoporphyrin IX; MgPME, Mg protoporphyrin IX monomethyl ester; PChlide, protochlorophyllide *a*; Chlide, monovinyl chlorophyllide *a*. Adapted from Kopečná et al. (2012b).

III. Overview of the regulatory mechanisms

Tight regulation of Chl metabolism is essential for several reasons: (1) Different amounts of tetrapyrrole end products (Chl, heme, siroheme) are needed in various amounts. In single cell organisms like cyanobacteria regulation has to follow rapidly changing growth conditions and, of course, in plants the situation is further complicated by different requirements for tetrapyrroles during development or in various tissues (Castelfranco and Jones 1975). (2) Many tetrapyrroles are readily excited by light and that, when not properly protected, can lead to the formation of highly toxic singlet oxygen species. These radicals, in turn, promote growth retardation, cell damage or death (reviewed in Vavilin and Vermaas 2002). Tetrapyrrole synthesis must therefore be accomplished while preventing the accumulation of pools of intermediates that might endanger the photosynthetic organism. By binding to a variety of different proteins, the level of free tetrapyrroles within a cell is kept to a minimum. Thus it is expected that there is an explicit coordination of tetrapyrrole and cognate apoprotein synthesis. (3) Photosynthetic organisms cope with light fluctuations and possess complex regulatory machinery to optimize utilization of light energy and to protect photosynthetic apparatus against the damage induced by excessive light. In cyanobacteria, this machinery involves the regulation of stoichiometry between both photosystems (PSI/PSII ratio; reviewed in Walters 2005, Muramatsu and Hihara 2012). Interestingly, the PSI/PSII ratio was shown to be controlled via Chl availability (Hihara et al. 1998, Sonoike et al. 2001, Kopečná et al. 2012b), which also highlights the central role of Chl metabolism in cellular signaling network.

Given all these factors, it is apparent that the regulation of Chl metabolism is complex and includes several levels of control, which allow balancing of the total metabolic flow as well as the distribution of all tetrapyrroles in response to external and endogenous factors. While long term acclimatory changes involve predominantly regulation at the transcriptional level, on a shorter timescale, the synthesis of tetrapyrroles is apparently controlled posttranslationally (Nogaj et al 2005, Jensen 2004, Huber and Hardin 2004). A dominant regulatory response simply reflects the presence of light, which is necessary for protochlorophyllide (PChlide) reduction catalyzed by light-dependent POR. Another prompt regulatory event occurs at the ALA formation step. It was shown in plants that ALA synthesis arrests within minutes after transition from light to dark and recovers immediately upon re-illumination (Richter et al. 2010). While transcriptional control in the tetrapyrrole biosynthetic pathway was studied extensively, the elucidation of posttranslational control of the pathway has just begun.

In the following chapters of my thesis I focus on the proposed key control points in the pathway primarily from the posttranslational point of view. Particularly, it covers the control over the total metabolic flow through the pathway, distribution of protoporphyrin IX into heme or Chl at the main branching point, and the role of light-driven PChlide reduction step.

IV. Regulation of ALA formation

Considering the biological roles of particular tetrapyrroles, the activity of each branch in the pathway reflects the cellular demand for each end product. The cell is apparently able to adjust the overall metabolic flow through the pathway according to the actual demand for each tetrapyrrole without accumulating pools of phototoxic precursors. In the current model, the rate of ALA synthesis is thought to be the rate-limiting point that determines the total flux through the tetrapyrrole pathway (reviewed in Masuda and Fujita 2008, Czarnecki and Grimm 2012). This conclusion was established in early experiments demonstrating that the lag phase in Chl accumulation in the light could be abolished by the addition of ALA (reviewed in Beale and Weinstein 1991).

In animals, yeast and non-photosynthetic bacteria, which do not synthesize Chl, ALA formation is regulated by feedback from heme (Wang et al. 1999, Andrew et al. 1990). Subsequently, it was also shown in photosynthetic organisms, that heme inhibits the synthesis of ALA from glutamate *in vitro* (Castelfranco and Zheng 1991, Weinstein and Beal 1985), whereas other intermediates including Mg protoporphyrin IX and protochlorophyllide had much less effect (Weinstein and Beal 1985). An inhibitory effect at the step of ALA formation is in agreement with the phenotype of the *yellow-green-2* tomato mutant, which lacks heme oxygenase. This mutation suppresses PChlide accumulation in etiolated seedlings and this effect has been ascribed to enhanced levels of heme in the organism (Cornah et al. 2003; Terry and Kendrick 1999). The heme feedback loop very probably also operates in cyanobacteria, as in the *Synechocystis* strain with reduced activity of the FeCH, ALA synthesis was found to be significantly increased (Sobotka et al. 2008).

The site of downregulation by heme appears to be at GluTR, which expression and activity is controlled by a wide range of regulatory signals – cytokinin (Masuda et al. 1995), the circadian clock (Papenbrock et al. 1999), plastid signals (McCormac et al. 2001) and light, through the action of the phytochrome and cryptochrome families of photoreceptors (McCormac et al. 2001, 2002). The GluTR enzyme holds a strategic position, since it catalyzes the first committed step of the tetrapyrrole biosynthetic pathway, the conversion of glutamyl-$tRNA^{Glu}$ to glutamate-1-semialdehyde. Compelling evidence exists that GluTR and GSA-AT form a complex, which should ensure an efficient synthesis of ALA and minimize the loss of glutamate-1-semialdehyde, a highly unstable compound (Moser et al. 2001). The existence of such a complex has already confirmed in *Escherichia coli* and *Chlamydomonas reinhardtii* (Lüer et al. 2005, Nogaj and Beale 2005). The important finding is that the level of GluTR/GSA-AT itself in *Chlamydomona reinhardtii* had no direct effect on the rate of Chl synthesis (Nogaj and Beale 2005). It supports the idea that the control of the GluTR activity is mainly achieved via feedback or other kinds of allosteric regulations, rather than just by adjusting the enzyme concentration per cell. Direct interaction

between heme and GluTR is also supported by an analysis of recombinant GluTRs. This enzyme from green sulphur bacteria *Chlorobium vibrioforme* and barley were purified from *Escherichia coli* with bound heme (Srivastava and Beale 2005, Pontoppidan and Kannangara 1994). Finally, exogenous as well as cellular levels of heme inhibited activity of both enzymes by acting on the N-terminal 30 amino acids of the enzyme (Vothknecht et al. 1998, de Armas-Ricard et al. 2011).

Although this data emphasizes the importance of heme as an inhibitor of ALA synthesis, they do not exclude the possibility that other effectors of feedback control of ALA synthesis may exist together with heme regulation (Yaronskaya et al. 2003, Cornah et al. 2003). To identify such regulators, mutants with a defect in the control of Chl biosynthesis have been screened. One such mutant was the *fluorescent* (*flu*) mutant of *Arabidopsis thaliana* (Meskauskiene et al. 2001). Etiolated *flu* seedlings over-accumulate PChlide; however, no obvious changes were detected at the heme level. Based on these data it was proposed that the FLU protein operates independently from heme control. Consistently with the key role of GluTR in integrating regulatory signals, FLU interacts with GluTR (but not GSA-AT) in yeast two-hybrid assays (Meskauskiene and Apel 2002). Interaction between these two proteins requires the C-terminus of GluTR and a tetratricopeptide repeat domain of FLU (Meskauskiene and Apel 2002, Goslings et al. 2004). As inhibition of the GluTR by heme requires 30 N-terminal amino acids of the enzyme, heme and FLU seem to act independently on the same target (Goslings et al. 2004). While the inhibitory activity of heme is though to originate in Fe-branch, the FLU has been proposed to play a similar and complementary role for the Chl-branch of the tetrapyrrole pathway (Meskauskiene et al. 2001, Kauss et al. 2012). Recently, a protein complex consisting of FLU, MgPMC, and POR was identified by co-immunoprecipitation (Kauss et al. 2012). It implies that FLU indeed mediates feedback control from Chl branch rather than just modulates the sensitivity of the heme dependent regulation. A hypothetical mechanism of FLU action has been proposed. GluTR would interact with the FLU-POR-MgPMC complex only when PChlide was also bound to this complex and ALA synthesis would be thus inhibited (Kauss et al. 2012). However, the exact role of PChlide in modulation FLU activity needs to be elucidated.

BtpA forms a complex with the GluTR enzyme

There are no doubts that the GluTR enzyme is a critical target for the regulatory network, however, the model with ‘free’ heme directly interacting and inhibiting the GluTR activity appears to be over-simplified. It should be noted that accumulation of ‘free’ heme in the cell is toxic. Particularly, the most dangerous is iron released from oxidized heme ring that generates reactive oxygen and nitrogen species via Fenton reactions (Gutteridge 1986, Bian et al. 2003). Indeed, in *Chlamydomonas reinhardtii*, the inhibition of the GluTR by heme depends on the presence of an unidentified soluble protein (Strivastava et al. 2005). One possibility is that in the heme-mediated

regulation the GluTR is located in the vicinity of, or directly interacts with FeCH. It would enable fast transmission of heme to GluTR keeping heme safely bound to proteins and avoiding its uncontrolled release into the cell space.

As a part of my PhD project I focused on the identification of GluTR interacting partners in *Synechocystis*. First, we have prepared a strain expressing the GluTR fused with 3xFLAG-tag on the N-terminus. The FLAG-GluTR was purified by batch binding to anti-FLAG-M2-agarose (Sigma) under native conditions. Buffer used for the preparation of cell fractions as well as for protein purification contained 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 20% glycerol, and 0.04% dodecyl-β-maltoside. For details about the construction of the FLAG-tagged *Synechocystis* mutant, preparation of soluble and membrane fractions as well as the purification procedure see Hollingshead et al. (2012).

Immunodetection with an anti-FLAG antibody revealed that the tagged GluTR enzyme is located in both soluble and membrane fractions and from both fractions the GluTR was successfully purified (Fig. 5A) (Kopečná J., unpublished data). Proteins co-purified with the FLAG-GluTR from the soluble fraction were analyzed in details. Elution was separated by SDS-PAGE, stained by Coomassie blue and visible bands identified using mass spectroscopy (Fig. 5B, Table 1). This approach revealed several putative interacting partners of the GluTR enzyme: pyruvate kinase 1, BtpA, Slr1098 and ArgJ. Except for the FLAG-GluTR itself used as bait, we also identified another GluTR band with a smaller mass, which suggests the formation of a GluTR oligomer in the *Synechocystis* cell. It is consistent with the crystal structure of the recombinant GluTR from the Archaea *Methanopyrus kandleri*, which is organized as a homodimer (Moser et al. 1999). Pyruvate kinase is considered to be one of the key enzymes of the glycolytic pathway in cyanobacteria; it catalyzes phosphorylation of ADP at the expense of phosphoenolpyruvate, producing ATP and pyruvate (Knowles et al. 2001), and thereby, can possibly provide ATP for the synthesis of GluTR substrate, glutamyl-$tRNA^{Glu}$. The Slr1098 is an uncharacterized protein with a high similarity to HEAT domain-containing proteins (Kopečná J., unpublished data). The HEAT (huntingtin-elongation-A subunit-TOR) motif has a repetitive rod-like structure (helix-turn-helix-turn), similar to tetratricopeptide, and appears to function as a protein-protein interaction surface (Hausrath and Goriely 2006). Many HEAT repeat-containing proteins are involved in intracellular transport or translation (Groves et al. 1999); however, it is difficult to hypothesize what role Slr1098 plays in relation to GluTR. Another protein co-purified with the FLAG-GluTR is the ArgJ, a bifunctional protein involved in the biosynthesis of arginine. Considering the interaction of ArgJ with GluTR, it is useful to mention that arginine can be stored as a nitrogen reserve in cyanophycine granules and later metabolized to glutamate (Liberton et al. 2006, Quintero et al. 2000). This offers speculation that a physical connection of the initial steps of the tetrapyrrole pathway with arginine biosynthesis could ensure fast mobilization of nitrogen reserves and its conversion into glutamate and thus maintain tetrapyrrole biosynthesis under a nitrogen shortage.

Interestingly, one of the co-purified proteins was identified to be BtpA, a protein already suggested to play a role in biogenesis of PSI in *Synechocystis* (Bartsevich and Pakrasi 1997; Zak et al. 1999). In the following work, Zak and Pakrasi (2000) described the *btpA* deletion mutant that had dramatically reduced the steady-state levels of most of the PSI proteins without noticeable reduction in PSII activity. To verify this phenotype, another *btpA* mutant was constructed by Martin Tichý using the identical construct and the non-motile glucose tolerant *Synechocystis* wild type as a genetic background. Intriguingly, this strain was strongly deficient in Chl and practically devoid of both photosystems as well as phycobilisomes. In addition, this mutant is quite unstable and frequently forms 'green' colonies presumably possessing suppressor mutations (Tichý M., personal communication). Close relatives of the BtpA protein have also been found in non-photosynthetic organisms such as the bacterium *Escherichia coli*, or the nematode *Caenorhabditis elegans* implying that this protein family is involved in more general process(es) than is the biogenesis of PSI. Taking into account the lack of all tetrapyrroles in the 'true' *btpA* mutant our findings indicate that the BtpA protein is involved in the initial steps of the tetrapyrrole pathway.

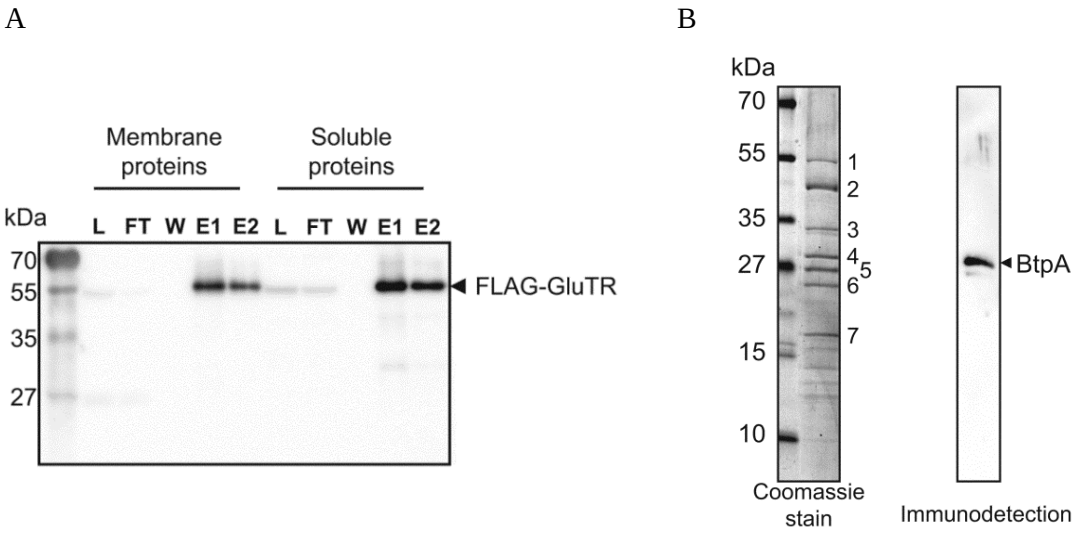

Figure 5. Purification of the FLAG-GluTR from *Synechocystis* cells. (A) Purification steps from membrane and soluble protein fractions were separated by 10% SDS-PAGE and transferred to a PVDF membrane. Immunodetection was performed using a polyclonal antibody raised against the FLAG peptide (Sigma). Abbreviations: L, fractions loaded on the FLAG-affinity resin (Anti-FLAG-M2-Agarose, Sigma); FT, flow through fraction; W, wash fraction; E1, the first elution; E2, the second elution. (B) The elution of FLAG-GluTR purified from the soluble fraction and separated by 12-20% SDS-PAGE. Numbered protein bands were identified by mass spectroscopy: 1 - pyruvate kinase 1, GluTR; 2 - GluTR; 3 - tryptophanyl tRNA synthetase; 4 - BtpA; 5 – BtpA, Slr1098; 6 - ArgJ; 7 - ArgJ. BtpA protein was also identified using a specific antibody kindly provided by Prof. Himadri B. Pakrasi. Tryptophanyl tRNA synthetase was present also in the control wild-type sample (not shown), and was regarded as false positive.

Another GluTR-binding protein (GluTRBP) was recently identified in the chloroplasts of *Arabidopsis thaliana;* particularly, as a part of a 300-kD protein complex located in thylakoid membranes (Czarnecki et al. 2011). Although the protein does not modulate activity of ALA synthesis, the *Arabidopsis* GluTRBP knockout is lethal in, whereas mutants expressing reduced levels of GluTRBP contain less heme (Czarnecki et al. 2011). It indicates that the formation of a membrane-bound complex containing GluTR is an essential prerequisite for the functioning of tetrapyrrole pathway. Our purification of the FLAG-tagged GluTR from the membrane protein fraction is in progress and could possibly reveal regulatory factors similar to *Arabidopsis* GluTRBP.

Table 1. Proteins identified by LC-MS/MS and database searching in soluble FLAG-GluTR elution fraction. The tryptic peptides identified by database searching are shown with their neighboring residues in the sequence in brackets. Peptides originated from the FLAG-GluTR protein used as bait are not shown.

Protein	Uniprot identifier gene name	Mass	Coverage	Peptides
		Da	*%*	
Pyruvate kinase 1	Q55863	53,071	55	(R)AEASDVANAILDGTDAVMLSGESAVGQYPVK(S) (K)IVATIGPASSSVEVIR(Q) (R)SVEQEMDTPITLLQDLQGPK(I) (R)HPGAVGIDYPHLATEAK(V) (K)GDQLLIMAGIPTK(I) (R)LNFSHGSYEDHATMVR(L) (R)ILLDDGLLEMK(V) (K)GVNLPGLVLTLPSMTTK(D) (K)IEKPQAIDNLEEIVAVSNGIMVAR(G) (R)GHPDLPVIAK(I) (K)VSLVPVEIGDR(H) (K)VSLVPVEIGDRHPGAVGIDYPHLATEAK(V) (R)KGVNLPGLVLTLPSMTTK(D) (R)GDLGVEVNPEK(V) (R)QMVDAGMNVAR(L) (K)YIVTFTTSGFTSLLASNQRPSVPVIAFTPSEK(V) (R)IGQLPGGEK(Q) (K)SVQMLR(K) (K)FMRPLSHR(T)
BtpA	P72966	30,023	85	(R)AEQEATALAAGGVDGIIVENFFDAPFPK(Q) (R)VSAFIEAMAEGLK(S) (R)VNVLTGVMATDQGLIEGNAHELLR(Y) (R)GLADGIILSGWATGSPPNLEDLELATNAAK(G) (R)LQNLVVAPVGINVLR(N)

				(R)ELSSDVAILADVLVK(H) (R)VDPAVVSAMTLIVDR(L) (R)HGNINEAIDPIR(V) (R)WGGNLTAVIER(A) (K)QRVDPAVVSAMTLIVDR(L) (-)MDLFQTFQTHNPVIGVVHLLPLPTSAR(W) (K)GTPVFIGSGADEDNIGQLIQAANGVIVASSLK(R) (K)HARPLGTPNLTTAVTDTIER(G) (D)PAVVSAMTLIVDR(L) (A)PVGINVLR(N) (I)LADVLVK(H)
Slr1098	P72742	27,657	54	(K)LAYEDVIATIPPLIEGLR(R) (R)LWSASSLPNIAK(L) (R)NTDPTIVEPLIQSLQTDFNGYVR(K) (R)GLQMIEDLEMDGLI(-) (R)ALAPLIHALK(T) (R)VSAAYALGR(N) (K)TDISAVR(L) (R)NQDDLLLEDSWVDPLDQLDDDVAPPPPDPDEM LVLLTSR(E) (K)SALLR(L) (I)PPLIEGLR(R)
ArgJ	P74122	43,292	48	(M)ADWQVIEGNITAPK(G) (K)TFNQVTVDGDTSTNDSLFALANGESR(T) (K)LGNYVLMDQGQPLEFDRPGASNYLK(Q) (R)TAAITEMGPNAEK(L) (K)FDQNNLLIK(L) (K)AAGITAGLKPSGSPDLSLIYSETEAIAAGVFTTSQ VR(A) (R)AILVNSGQANAGTGK(Q) (K)TIALETEIDGRPVR(M) (I)MTTDLVPK(T) (R)AIMTTDLVPK(T) (R)TIAGSSLVK(S) (I)ALETEIDGRPVR(M) (M)TTDLVPK(T) (K)AIARDGEGATCLMEIQVTGAPDDQSAR(A)

V. Regulation of the branching point leading to heme and chlorophyll biosynthesis

The distribution of protoporphyrin IX into either FeCH to produce heme, or MgCH, which inserts Mg^{2+} for Chl biosynthesis, is thought to be another key regulatory point (for recent reviews, see Eckhardt et al. 2004, Masuda 2008, Masuda and Fujita 2008, Czarnecki et al. 2012). How is the distribution of protoporphyrin IX driven in the cell remains to be elucidated, although the roles of both chelatases have become evident (Papenbrock et al. 2000, 2001, Sobotka et al. 2005). Expression levels and/or activities of these chelatases are controlled by light, diurnal and circadian rhythmicity (Matsumoto et al. 2004, Papenbrock et al. 1999). In higher plants, the activities of both chelatases oscillate with almost inverse amplitude when plants are grown in cycles of 12 h of light/12 h of dark. It was suggested that the coordination of expression and enzyme activities allowed, in the light phase, an extensive flow of substrates into the Chl branch and, after the transition from light to dark, a channeling into the heme and heme-derived tetrapyrroles (Papenbrock et al. 1999). This oscillation, however, cannot explain fine tuning of tetrapyrrole pathway on a short time scale. Moreover, unlike plants, which use Chl-containing antenna complexes for light harvesting, the major light-harvesting pigments of cyanobacteria are phycobilines produced by oxidation of heme. In cyanobacteria, protoporphyrin IX has to be directed into the iron branch to a much larger extent than in plants and also the regulation mechanisms have to reflect demand for new phycobilinoproteins.

Regulation signals originating from the haem branch

FeCH is a single membrane-associated and ATP-dependent enzyme requiring only Fe^{2+} and protoporphyrin IX as substrates to produce heme. The regulatory role of this enzyme is quite obvious (Sobotka et al. 2005, Yaronskaya et al. 2003). Theoretically, the FeCH activity may regulate direction of protoporphyrin IX down to both branches as well as the total flux through the pathway via increased production of heme, which then exerts feedback control by inhibiting synthesis of ALA at the beginning of the pathway (Beck and Grimm 2006, Srivastava and Beale 2005).

A striking feature of cyanobacterial and algal FeCHs as well as FeCH isoform II from plants is C-terminal Chl *a/b*-binding (CAB) domain that shares a high degree of similarity to the first and third helices of the plant light-harvesting complex II. Particularly highly conserved is the Chl-binding motif (Dolganov et al. 1995). It was demonstrated the CAB domain of *Synechocystis* FeCH promotes oligomerization of this enzyme *in vivo* but it is not essential for the FeCH activity (Sobotka et al. 2011). Nonetheless, an expression of truncated FeCH lacking CAB domain caused aberrant accumulation of PSI under high light accompanied by high levels of the Chl precursor chlorophyllide (Sobotka et al. 2011). Thus, the CAB domain appears to serve mainly a regulatory

function, possibly in directing of Chl into Chl-binding PSI subunits PsaA/B. This conserved CAB domain is connected to the FeCH catalytic core by a more variable region II, a short hydrophobic proline-rich sequence (Sobotka et al. 2011). In contrast to the deletion of CAB domain only, the deletion of both of these FeCH features in the *Synechocystis* strain ΔH324 dramatically reduced the stability and activity of FeCH (Sobotka et al 2005). The ΔH324 strain accumulated very high amounts of protoporphyrin IX that was even exported into the medium; however, only a limited amount of overproduced protoporphyrin IX was metabolized by the Chl branch (Sobotka et al. 2008). This shows that the distribution of substrate between Fe and Mg chelatases is an important regulatory step in the tetrapyrrole trafficking. Moreover, the ΔH324 mutant also accumulated PChlide but no chlorophyllide, suggesting that there might be an additional regulatory or feedback mechanism that operates within the Chl branch similar to that regulated by the FLU protein in higher plants (Sobotka et al. 2008, Meskauskiene et al. 2001).

Regulation signals originating from the magnesium branch

MgCH, which competes with FeCH for protoporphyrin IX, is composed of three subunits conserved from prokaryotes to plants that are commonly referred to as ChlH, ChlD, and ChlI (Jensen et al. 1996). The catalytic insertion of Mg^{2+} into protoporphyrin IX is an ATP dependent complex mechanism that contrasts to the 'cheap and simple' activity of FeCH (Castelfranco et al. 1994, Bollivar 2010). An initial activation step includes ChlI and ChlD that are related to AAA+ ATPases and form two associating hexameric rings. The ChI-ChlD complex is proposed to interact with the protoporphyrin IX binding ChlH and drives the ATP-dependent metallation of protoporphyrin IX (Lundquist et al 2010). According to results of *in vitro* studies the ChlH subunit subsequently transfers Mg protoporphyrin IX to MgPMT (Hinchigeri et al. 1997, Shepherd et al. 2005). It has been speculated that the ChlD:ChlI rings of MgCH may undergo conformational changes as part of the catalytic mechanism similar to ATP synthase having three different active sites at different stages in the catalytic process (Bollivar 2010).

The ChlH subunit is a big protein (~120 KDa) occupying probably a strategic position in cellular signaling network. It was demonstrated that the ChlH functions as a repressor of sigma factor SigE in cyanobacteria (Osanai et al. 2009). In the proposed model, ChlH transduces light signals by Mg^{2+}, not by protoporphyrin IX or Mg protoporphyrin IX, and regulates SigE at the posttranslational level (Osanai et al. 2009). A bit controversial is the role of ChlH as a receptor of the plant hormone abscisic acid. According to results of Shen et al. (2006) the ChlH in plants mediates abscisic acid signaling as a positive regulator of seed germination, post-germination growth, and stomatal movement. However, these results need to be clarified (Müller et al. 2009).

Using antisense technology an intensive cross-talk between MgCH and other enzymes was revealed in plants. Tobacco with down-regulated *chlH* transcripts and MgCH activity, lower levels

of transcripts encoding GluTR and ALA dehydratase were found (Papenbrock et al. 2000a). Subsequently, the decreased ALA-synthesizing capacity led to lower levels of Chl and heme in the transgenic plants. Interestingly, the activities of GluTR and ALA dehydratase were adjusted to the same level as MgCH activity, indicating a feedback-controlled mechanism balancing the activity of early steps to later steps in the pathway (Papenbrock et al. 2000a). In congruence with these data, *chlI* antisence tobacco plants also showed reduced biosynthesis of ALA and had lower heme/Chl contents than control plants (Papenbrock et al. 2000b). Furthermore, Hedtke and coworkers (2007) showed that RNAi silencing of GluTR and GSA-AT did not affect transcript levels of the other tetrapyrrole biosynthesis genes but enzyme activities of MgCH and FeCH were lower in parallel to the loss of Chl/heme content. This is another of much evidence that the flux through the tetrapyrrole biosynthesis is controlled on a posttranslational basis.

There are several lines of evidence that the MgCH and particularly its ChlH subunit, interacts with following enzyme MgPMT. Addition of ChlH accelerates the activity of MgPMT (Hinchigeri et al. 1997, Johnson and Schmidt-Dannert 2008) and a direct interaction between these two proteins was proven by yeast two-hybrid assays (Alawady et al. 2005). In addition, using an *in vitro* assay it was demonstrated that the ChlH affects MgPMT kinetics by enhancing the accumulation and decay of the reaction intermediate and thus increasing the rate of product evolution (Shepherd et al. 2005). Alawady and Grimm (2005) also showed that the low MgPMT activity was correlated with reduced MgCH activity and lowered ALA synthesis, but with enhanced FeCH activity and *vice versa*. High MgPMT activity lead to exactly inverse activity profiles, indicating a direct influence of MgPMT in combination with MgCH on the metabolic flux of ALA and the distribution of protoporphyrin IX into the branched pathway.

Although three subunits of MgCH are sufficient for catalysis, activity of this enzyme is strongly enhanced by so called Gun4 protein. This protein was identified by screening of *Arabidopsis thaliana* mutants that cannot transduce signals from plastid to nucleus. Several *gun* (genome uncoupled) mutants have been identified (Susek et al. 1993) and, interestingly, one of the genes, *gun5*, encodes ChlH subunit of MgCH, and another one, *gun4*, encodes a protein interacting with the ChlH (Mochizuki et al. 2001, Larkin et al. 2003). Gun4 is able to bind porphyrins and was demonstrated to strongly enhance the MgCH activity *in vitro* (Larkin et al. 2003; Davison and Hunter 2011). Moreover this protein was shown to interact with ChlH *in vivo* in plants (Larkin et al 2003) and with the same subunit in cyanobacteria (Sobotka et al. 2008b). The *Synechocystis* mutant lacking Gun4 retains no more than 25% of Chl and accumulates high quantity of protoporphyrin IX but only traces of Mg-protoporphyrin IX (Sobotka et al. 2008b). Although it is not clear yet, the Gun4 activates ChlH subunit to perform the chelation probably via the porphyrin binding (Walker and Willows 1997, Karger et al. 2001). Since physical interaction between ChlH and MgPMT has been demonstrated (Alawady et al. 2005), it is attractive to speculate that the Gun4 is also involved in the transfer of Mg protoporphyrin IX from MgCH to MgPMT. Indeed, Gun4 was found to bind

Mg protoporphyrin IX with significantly higher affinity than other porphyrins, such as protoporphyrin IX or Mg protoporphyrin IX methyl ester (Adhikari et al. 2009). Interestingly, the thylakoid-bound Gun4 was found as a part of a >500 kDa complex also containing the ChlH and it was suggested that Gun4 helps to channel porphyrins into Chl biosynthesis by binding to ChlH on chloroplast membranes (Larkin et al. 2003, Sobotka et al. 2008b, Adhikari et al. 2009). However, Gun4 probably uses a mechanism that depends on binding both porphyrins and activated ChlH to associate with chloroplast membranes; the ChlH association with chloroplast membranes seems to be influenced rather by MgCH activity than Gun4 activity (Adhikari et al. 2011). Moreover, accumulation and membrane binding of ChlH and Gun4 is differently regulated than that of ChlI and, even more strikingly, ChlD, thus they both can play more roles than just Mg^{2+} chelation (Kopečná et al. 2012b).

VI. Reduction of protochlorophyllide

Regarding regulation of the tetrapyrrole biosynthesis as well as the biogenesis of photosynthetic apparatus, the reduction of the C_{17}-C_{18} double bond of the protochlorophyllide has to be one of the key regulatory points just due to the fact that energy for this enzymatic step is provided by light (Fig. 6) (reviewed in Masuda 2008). Although, in contrast to higher plants, cyanobacteria and algae possess a second, light independent POR enzyme (DPOR), but it cannot replace the light dependent POR (LPOR, Kopečná et al. 2012a, see below) making light indispensable for production of a sufficient amount of Chl. Taking into account fluctuating light intensity in the environment it would make sense that PChlide, the substrate of POR enzyme, operates as a signal resembling to some extend heme signaling discussed above. When a higher plant is shifted to dark conditions, Chl biosynthesis stops at the step of PChlide, which starts to accumulate. It appears that once a threshold level of PChlide is reached, ALA synthesis is shut off. However, upon illumination, PChlide is converted to chlorophyllide and the block in ALA synthesis is released (Reinbothe and Reinbothe 1996). As already discussed in context with the FLU protein such feedback control of ALA synthesis has been attributed to a coordinated heme and PChlide signaling (reviewed in Czarnecki and Grimm 2012).

Light 'sensitivity' thus makes LPOR an ideal sensor of an actual light intensity, which the cell exposed and this enzyme has also a potential to modulate other parts of the tetrapyrrole biosynthesis. In addition, chlorophyllide, the product of the LPOR enzyme and the direct precursor of Chl, was shown to be an intermediate of the constant process of Chl recycling, which will be described later. However, the situation in cyanobacteria, algae and gymnosperms is more complicated due to presence of the second light-independent POR. To clarify roles of both enzymes was another object of my PhD project. In the course of evolution two genetically and biochemically different strategies of PChlide reduction have developed (reviewed in Reinbothe et al. 2010). The LPOR is specific for oxygenic phototrophs, belonging to a family of short-chain alcohol dehydrogenases that covers a variety of NAD(P)(H)-dependent enzymes, and is very similar in all organisms performing this reaction (He et al. 1998). A structural model for the ternary complex PChlide-NADPH-LPOR and processing of this complex is relatively well characterized (Fig. 7A; Townley et al. 2001, Heyes et al. 2006, 2008). It is strictly dependent on light (Fujita and Bauer 2003), since the reaction is driven by the direct absorption of two photons by PChlide (Heyes et al. 2003). The first photon activates the enzyme, whereas the second photon induces catalysis (Sytina et al 2008). The energy is used to transfer the hydride from NADPH to the PChlide molecule (Schoefs and Franck 2003). The reactions as well as the binding and releasing of the substrate and product of the reaction correlate with changes in the conformation of the LPOR structure (Sytina et al. 2008, Heyes et al. 2007, 2008). Despite the recent progress in the kinetic analysis of LPOR using *in vitro* systems (Heyes and Hunter 2005, Sytina et al. 2008, 2012), how

this enzyme harvests light or how is structurally organized in the cell remains unknown (Reinbothe et al. 2010).

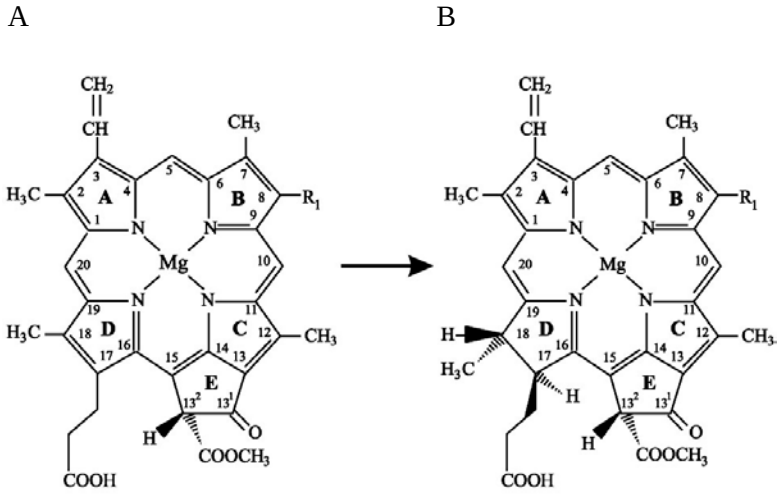

Figure 6. Structures of (A) protochlorophyllide and (B) chlorophyllide molecules. R1 is $CH_2 - CH_3$ for the monovinyl pigment form and $CH = CH_2$ for the divinyl form (adapted from Belyaeva and Litvin 2007).

Since angiosperms employ LPOR as the sole PChlide reduction system, their seedlings become etiolated in the dark. In such etiolated plants, LPOR accumulates in a highly stable complex with PChlide and NADPH and becomes a backbone of membrane structures of prolamellar bodies. This complex is responsible for a regular, paracrystalline structure of prolamellar bodies (Blomqvist et al. 2006). Upon illumination, LPOR photoreduces PChlide to chlorophyllide and prolamellar bodies transform into the grana and stroma of thylakoids (Adam et al. 2011). The assembly of the photosynthetic apparatus within the thylakoid membranes leads to the visible greening of the seedling and etioplasts differentiate into chloroplasts (Rudowska et al. 2012).

Prolamellar body-like structures were once observed in *Chlamydomonas reinhardtii*; however their appearance was not understood since growth in the dark, by itself, is insufficient to induce the formation of this structure (Friedberg et al. 1971). Recently, similar prolamellar body-like structures formed by co-accumulation of LPOR and PChlide in the dark were reported for the cyanobacterium *Leptolyngbya boryana* (Fujita Y., abstract on the conference 14th International Symposium on Phototrophic Prokaryotes, Porto 2012).

The second enzyme, DPOR is present in anoxygenic and most oxygenic phototrophs with the exception of flowering angiosperm plants. This enzyme was first studied in purple bacteria in which products of three genes *bchB*, *bchL* and *bchN* were recognized to be essential for synthesis of bacteriochlorophyll in the dark (Fig. 7B, C) (Yang and Bauer 1990). Since then, homologues of these genes were identified in cyanobacteria, algae and gymnosperms and named *chlB*, *chlL* and *chlN* (Wu and Vermaas 1995). Amino acid sequences of the DPOR subunits show significant

similarities to those of *nifH*, *nifD* and *nifK*, which encode subunits of nitrogenase catalysing the reduction of dinitrogen to ammonia. DPOR from *Rhodobacter capsulatus* consists of two separable components, L-protein (a BchL dimer) and NB-protein (a BchN-BchB heterotetramer), which are homologous to nitrogenase Fe protein and MoFe protein, respectively (Rees et al 2005, Nomata 2005). The L-protein accepts electrons from a ferredoxin and transfers them to NB-protein in an ATP-dependent manner (Nomata et al. 2006). The [4Fe-4S] cluster and the MgATP binding sites of L-protein are highly conserved. In contrast, the charge distribution in the probable docking site of the L-protein with the NB-protein is distinct from that of the Fe protein. Moreover, C-terminal domain of BchB is well conserved in the known BchB/ChlB proteins but is missing in NifK of nitrogenase. This domain probably plays an important role in PChlide reduction (Muraki et al. 2010). The reaction mechanism of DPOR inferred from structural data is fundamentally different from that of LPOR (Fig. 7). Firstly, an electron is transferred from the [4Fe-4S] cluster of L-protein to the NB-cluster. Subsequently, a second electron transfer from the L-protein to PChlide via NB-cluster completes the reaction and a chlorophyllide molecule is released from the enzyme (Reinbothe et al. 2010).

The reduction of PChlide is thought to be a rate-limiting step in the Chl branch (Fujita 1996), and as the rate of Chl protein synthesis depends strictly on Chl availability (Kim et al. 1994), a shortage but also a surplus in total POR activity could have fatal consequences on the cell viability. Indeed, in organisms possessing both LPOR and DPOR, the enzymes are expected to cooperate together to produce an optimal flow of chlorophyllide under various growth conditions. One of the most significant parameters is light intensity. DPOR is the sole operative reductase in the dark as LPOR requires light energy for its activity. Although both LPOR and DPOR contribute to Chl synthesis in the cells growing in the light, the extent of contribution by LPOR increases with increasing light intensity and LPOR is indispensable in high light conditions (Ford et al. 1983, Fujita et al. 1998, Kopečná et al. 2012a). However, in contrast to *Leptolyngbya boryana*, we showed that the *Synechocystis* LPOR-less strain has strongly reduced Chl levels also under low light, which means that the DPOR enzyme in *Synechocystis* could not compensate for the loss of LPOR even under very limited light irradiation (Kopečná et al. 2012a). Given that the Δ*chlL* (DPOR-less) strain did not exhibit any decrease in Chl except when grown in dark, these observations imply that the LPOR is responsible for the synthesis of the majority of Chl, even under low light. An explanation could be given by differences between both organisms. DPOR is highly oxygen sensitive and was demonstrated to be almost completely inhibited by oxygen levels >3 % (Yamazaki et al. 2006). In contrast to *Synechocystis*, *Leptolyngbya boryana* is capable of fixing nitrogen under anaerobic conditions when the activity of DPOR is expected to be increased and this activity may be partly maintained even under dim light. Nevertheless, the relationship between LPOR and DPOR enzymes remains unclear. In terms of evolution, the creation of LPOR is explained as a compensation for an older, oxygen-sensitive DPOR enzyme, during the transition

from the anaerobic Archaean Earth´s atmosphere to an aerobic atmosphere (Reinbothe et al. 1996). This however does not say nothing why an atypical, light-powered mechanisms, was chosen just for this step of Chl biosynthesis. The benefit from ability to sense light directly with no need for a signaling cascade from photoreceptors might be the reason.

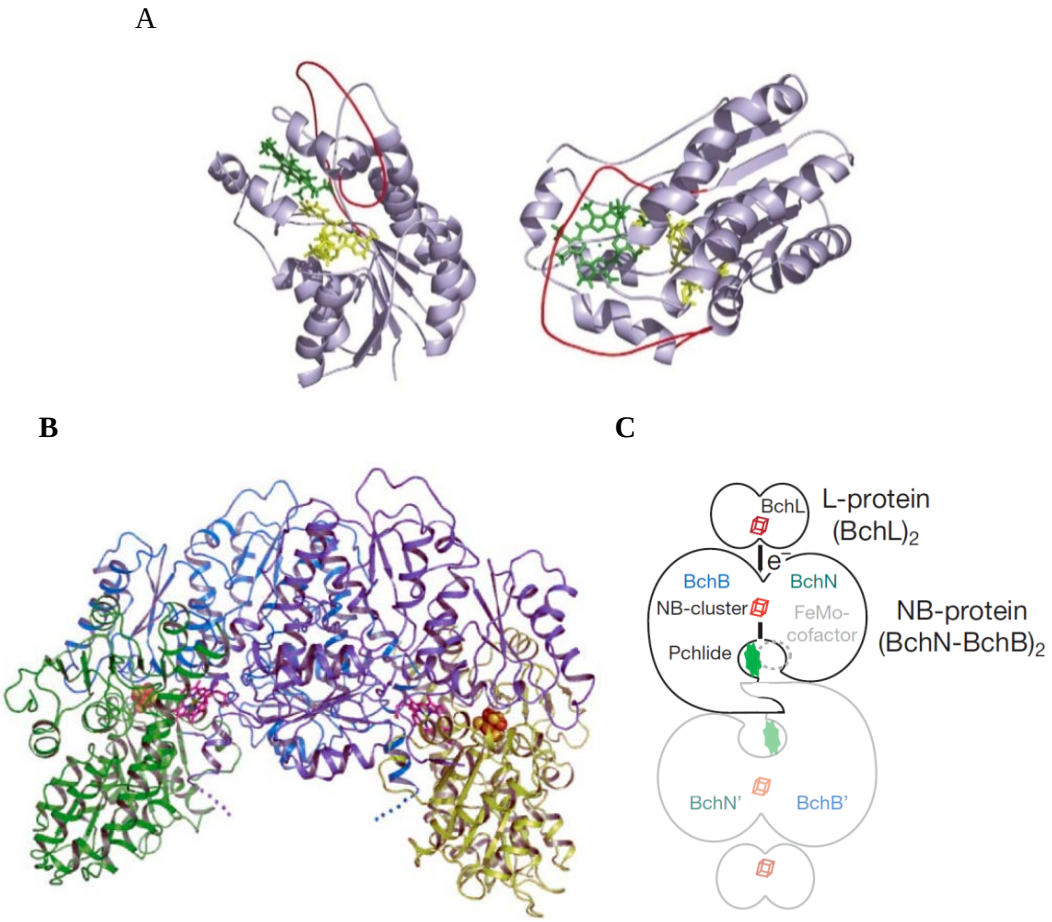

Figure 7. Structural models of LPOR and DPOR enzymes. (A) Two views of a model of *Synechocystis* LPOR calculated according to homology with other short-chain dehydrogenases. The template structure contains NADPH (yellow) and PChlide (green). The 33-residue insertion (red) is unique to LPOR and is thought to be involved in PChlide binding (adapted from Heyes and Hunter 2005). (B) Crystal structure of NB protein. The [4Fe–4S] clusters (NB-clusters) are shown in a space-filling model and the PChlide molecules are shown in a stick model. The BchN and BchB subunits in one dimer are respectively coloured green and blue, and the identical subunits related by non-crystallographic two-fold symmetry are respectively coloured light green and light blue (adapted from Muraki et al. 2010). (C) A conceptual model of DPOR complex with architecture similar to that of the nitrogenase complex. Two symmetric functional units (BchN–BchB) of NB-protein interact through some amino-acid residues of BchB subunits (adapted from Muraki et al. 2010).

VII. Coordinated synthesis of chlorophyll and chlorophyll-binding proteins

Role of chlorophyll availability in the synthesis and assembly of photosynthetic complexes

Synthesis of Chl-binding proteins must be tightly synchronized with supply of Chl molecules. Theoretically, if the synthesis of Chl exceeds available binding sites in translated apoproteins, a pool of Chl not bound to proteins would form in the cell generating destructive radical oxygen species (Sinha et al. 2012). On the other hand, a shortage in Chl will limit synthesis of new subunits of photosystems, which can have fatal consequence, particularly under stress conditions (Tanaka and Tanaka 2007, 2011). Mechanisms coordinating Chl and protein biosyntheses remain to be unraveled, however, it has been repeatedly suggested that Chl availability is one of factors that control the accumulation of individual Chl-proteins (Hihara et al. 1998, Müller and Eichacker 1999). It appears that the insertion of Chl molecules into a growing polypeptide is a prerequisite for the correct folding and finishing of Chl-binding protein synthesis. Neither chlorophyllide nor phytyl diphosphate but only Chl can stabilize translated apoproteins, which was proven for subunits of both photosystems (Kim et al. 1994, Eichacker et al. 1996, 2001). In higher plants, a similar mechanism was demonstrated for Chl *b* that is necessary for stabilizing the conformation of major light-harvesting complexes that are otherwise recognized and degraded by specific proteases (Yang et al. 1998, Horn et al. 2007).

The role of Chl in accumulation and assembly of photosystems *in vivo* has been also confirmed using mutants of *Synechocystis*, in which the Chl pathway was either downregulated or completely blocked by various mutations (He and Vermaas 1998, He et la. 1998, Sobotka et al. 2008a,b and 2011, Hihara et al. 1998). The *Synechocystis* strains Δ*por* as well as Δ*chlL*, which cannot synthetize Chl when grown in the dark, exhibited a strong decrease in the content of both PSI and PSII complexes (Kopečná et al. 2012a). In this respect, the phenotype is similar to the Δ*gun4* mutant, which was affected in the function of Mg-chelatase (Sobotka et al. 2008a). Interestingly, both Δ*por* and Δ*gun4* mutants accumulate PSII core complexes (RCa and RC*) that immediately precede the incorporation of CP47 during the process of PSII assembly (Kopečná et al. 2012a). In addition, the second PSII antenna CP43 was also synthesized and accumulated in both Δ*por* and Δ*gun4* mutants to a higher level than CP47. These results suggest that either CP47 has the lowest affinity to Chl in comparison to D1, D2 and CP43, or these three PSII Chl-proteins can use Chl molecules more ‘easily’ than CP47 (Kim et al. 1994, Kopečná et al. 2012a).

The regulatory role of Chl in accumulation of photosynthetic complexes is best characterized in adjustment of the PSI/PSII ratio, a parameter required for maintaining a high quantum efficiency of photosynthesis during varying light conditions (Chow et al. 1990, Melis et al. 1996). Cyanobacteria acclimate to high-light conditions by adjusting photosystem stoichiometry through a selective decrease of PSI abundance in thylakoid membranes. A physiological significance of this

process was demonstrated on the *Synechocystis* mutant *pmgA* that lost the ability to decrease the PSI content during acclimation to high light and its growth was severely inhibited under prolonged high light cultivation (Hihara et al. 1998, Sonoike et al. 2001). As PSI complexes bind the majority of Chl in cyanobacterial cells it is accepted that the mechanism controlling PSI level/synthesis is tightly associated with the Chl biosynthetic pathway. The downregulation of PSI was reported to be determined primarily by the limited availability of Chl, suggesting that the accumulation of Chl-binding PSI subunits is more sensitive to deficiency in *de novo* Chl biosynthesis than subunits of PSII (Kada et al. 2003, Muramatsu et al., 2009). The proposed mechanism is based on an assumption that a surplus of PsaA/B proteins is continuously translated but finally degraded due to a shortage in Chl (Muramatsu et al. 2009). However, in contrary to this model, we found that inhibition of the PChlide reduction step in the Δ*por* mutant results in the parallel decline of PSII and PSI content (Kopečná et al. 2012a). It clearly demonstrates that the PSI/PSII ratio in *Synechocystis* cannot be controlled by a simple general upregulation/downregulation of the Chl pathway, as it would lead to parallel changes in the content of both photosystems. According to our recent publications (Kopečná et al. 2012a,b) we expect that such regulation has to involve a selective delivery of Chl for synthesis of each individual Chl-protein and an important role in maintaining of PSII synthesis is played by recycled Chl.

Chlorophyll recycling and distribution into chlorophyll-binding proteins

Since the lifetime of Chl molecules is much longer than that of proteins, each Chl molecule has to be used several times before it is degraded (Vavilin et al. 2005, Vavilin and Vermaas 2007, Yao et al. 2012a). Thus, there is an important question as to how the new and recycled Chl is distributed into individual Chl-proteins. To clarify this issue we employed radiolabeling of Chl by ^{14}C and proteins by ^{35}S in *Synechocystis* cells to follow the flow of *de novo* Chl correlated with synthesis of Chl-proteins. Interestingly, *de novo* Chl was found to be predominantly directed to the PSI trimer, whereas only a small part of labeled Chl was detected in PSII, even though the PSII subunits were much more intensively synthetized than the core subunits of PSI. This data implies that PSII subunits are mostly synthesized using the recycled Chl molecules previously released during Chl-protein degradation (Kopečná et al. 2012b). The exact mechanism of the Chl recycling process is not known, nevertheless, it seems to include a dissociation of the phytol chain and the chlorophyllide ring (Vavilin and Vermaas 2007) upon/during degradation of Chl-proteins. Chlorophyllide is than probably delivered back to Chl-synthase to be re-used (Vavilin et al. 2005, Vavilin and Vermaas 2007). Despite the apparent importance of the Chl recycling it appears that there always needs to be a certain input of newly synthesized Chl even into PSII complexes replacing damaged Chl molecules and also allows the cell to continue in proliferation (Kopečná et al. 2012b).

Chl recycling is thought to be closely connected to the process of PSII repair. PSII is prone to various types of light-induced irreversible damage, which unless repaired leads to an inhibition of PSII activity and hence to a reduction in oxygenic photosynthesis and growth (reviewed by Adir et al. 2003). In order to maintain PSII homeostasis, a PSII repair cycle operates to replace damaged protein subunits, mainly D1, by a newly synthesized copy (reviewed by Nixon et al. 2010). It was suggested that during assembly and/or repair of PSII, Chl is bound transiently by specific Chl carrier proteins within the membrane before being delivered to its final destination (Wu and Vermaas, 1995). The cyanobacterial small membrane proteins called high-light induced proteins (HLIPs), which are also termed small-CAB-like proteins (SCPs), owing to the presence of a conserved Chl *a*/*b* binding (CAB) motif, have been speculated to play such a role (Dolganov et al. 1995, Funk and Vermaas 1999, Xu et al. 2004). When the *Synechocystis* strain lacking all HLIPs proteins is grown at high irradiance, the half-life of Chl is much shorter and formation of singlet oxygen is enhanced (Vavilin et al. 2007, Sinha et al. 2012). The role of these proteins thus might be to scavenge Chl/chlorophyllide molecules released during PSII damage and recycle them during PSII repair/*de novo* assembly. Indeed, HLIPs were shown to be associated with PSII and to be particularly abundant in the RC47 subcomplex involved in both PSII assembly and repair (Promnares et al. 2006, Yao et al. 2007, Kufryk et al. 2008). On the contrary, Wang and coworkers (2008) reported that His-tagged HLIPs expressed under the control of their native promoters are associated with the PSI complex. They further found that the quadruple *hli* mutant loses >90% of PSI trimers after a 24-h incubation under high-light and concluded that HLIPs are involved in stabilizing PSI trimers during high light acclimation. This issue needs to be resolved in the future since it appears that the presence of His-tags at the C-terminus is responsible for an aberrant association of HLIPs with large supercomplexes containing PSII (Komenda J, personal communication). In chloroplasts, related proteins such as one helix proteins (OHPs), early-light induced proteins (ELIPs), or stress-enhanced proteins (SEPs) might play a similar role in the process of Chl recycling (Engelken et al. 2010). Interestingly, HLIPs and ELIPs were also proposed to modulate early steps of Chl biosynthesis, possibly the rate of ALA formation to synchronize *de novo* synthesis of Chl with its recycling (Yao et al. 2012a; Hernandez-Prieto et al. 2011, Tzvetkova-Chevolleau et al. 2007). Furthermore, cyanobacterial as well as plastidic FeCH enzymes are fused at the C-terminus with an HLIP protein forming the so-called CAB domain. A deletion of this domain in *Synechocystis* has no effect on the FeCH activity or stability but the resulting mutant accumulates significantly higher Chl level under high light (Sobotka et al. 2011). In the light of these findings, it would be interesting to investigate the direct impact of the deletion of one or more HLIP proteins on the distribution of the newly synthesized Chl into photosynthetic complexes.

It is still not completely clear whether Chl once bound to a target protein remains permanently attached until the protein is degraded or is able to move from one protein to another. Early work

showed that the expression of a plant light-harvesting complex II in the *Synechocystis* strain engineered to produce Chl *b* helped to significantly increase the accumulation of Chl *b* in the PSII complex of (Xu et al., 2001). It indicates that such a Chl-chaperone function might operate *in vivo*. In another report the same authors proposed that the pre-existing Chl molecules in a periphery of PSI can be, in some circumstances, released and redistributed for PSII biosynthesis (Xu et al. 2004). The *Synechocystis* mutant lacking the Psb28 protein, a PSII assembly factor that binds to CP47, exhibits a defect in Chl biosynthesis at MgPMC step (Dobáková et al. 2009). Interestingly, this defect specifically limited synthesis of the PSII subunit CP47 and core PSI subunits PsaA/B; the synthesis of D2 and CP43 was not affected and the turnover of the D1 subunit was even accelerated (Dobáková et al. 2009). It is tempting to speculate that Chl can be interchanged between PsaA/B and CP47, perhaps as an alternative route for the Chl distribution to PSI and/or to CP47 (PSII). The CP47 is the subunit of PSII, of which its synthesis is the most sensitive for Chl deficiency (Kim et al. 1994, Kopečná et al. 2012a) and the reason might be the competition with PsaA/B. Indeed, these PSI proteins are the main sink for newly made Chl, at least in *Synechocystis* (Kopečná et al. 2012b). Plenty of Chl molecules are needed per one translated PsaA/B protein and if supply of Chl is impaired, the PsaA/B could drain off Chl from the CP47 synthetized nearby.

Is there a protein factory in the cell producing Chl-binding subunits?

Organization of Chl biosynthesis in the cell, its specific cellular location and the connection to protein synthesis are questions that remain to be unraveled. Nonetheless, it has been proven that at least the CP47 and CP43 PSII subunits contain both Chl and carotenoid molecules before these proteins are assembled into larger pre-complexes of PSII (Boehm et al. 2011). In contrast to Chl, an attachment of carotenoids is not critical for stability of synthetized Chl-binding proteins. In the β-carotene-less mutant of *Synechocystis* the synthesis of CP47 and CP43 is impaired, however, these two proteins accumulate to some extend and, moreover, the absence of β-carotene has only little effect on the synthesis of PsaA, PsaB, D1 and D2 (Sozer et al. 2010). Given the critical need for Chl during Chl-protein synthesis it sounds sensible that the terminal steps of Chl pathway are placed in the cellular or chloroplast compartment where Chl-proteins are translated and then assembled into photosystems.

The first steps in *de novo* PSII assembly seem to take place at or very close to the cytoplasmic membrane and not the thylakoids of *Synechocystis* (Zak et al. 2001, Keren et al. 2005), later steps of the assembly then occur in the thylakoid membranes (Zak et al. 2001, Rengstl et al. 2011). It was thus suggested that discrete regions of the plasma membrane harbor sites at which assembly of PSII is initiated (Srivastava et al. 2006). Recently, a specific membrane fraction containing pre-matured D1 and PSII assembly factors PratA and Ycf48 has been isolated from *Synechocystis* (Schottkowski et al. 2009a, Rengstl et al. 2011). This membrane subfraction might represent a transfer and/or connection region between plasma and thylakoid membrane, possibly as a part of

'thylakoid centers'. Thylakoid centers were observed in the periphery of cyanobacterial cells (Kunkel et al. 1982). These structures are thought to be essential in establishing, maintaining, and biogenesis of thylakoid membranes due to their intimate thylakoid membrane association and apparent continuity with the cytoplasmic membrane and periplasmic space (van de Meene et al. 2006). Moreover, recently identified a tetratricopeptide repeat protein of *Synechocystis*, termed Pitt, has also been identified in this PratA-defined membrane fraction (Schottkowski et al. 2009b). The Pitt protein seems to be involved the spatial organization of early PSII assembly since maturation of D1 is affected in its absence. Pitt also interacts with LPOR and a lack of this protein causes a reduction in light-dependent chlorophyll synthesis (Schottkowski et al. 2009b, Rengstl et al. 2011). Accordingly, LPOR, geranylgeranyl reductase and FeCH enzymes were found to be located in thylakoid membranes but also in a minor fraction of plasma membrane and authors of this work suggest that Chl biosynthesis occurs in a specific sub-compartment of thylakoid membrane in a close proximity to plasma membrane (Pisareva et al. 2011).

Another component of putative biosynthetic centers may be the Psb27 protein. In *Synechocystis* this protein binds CP43 in a specific PSII complexes lacking oxygen evolving complex (Nowaczyk et al. 2006, Komenda et al. 2012b). Strikingly, the Psb27 interacts also with PSI in megadalton supercomplexes containing both PSI and PSII. Deletion of the *psb27* gene did not have drastic effects on PSII assembly and repair but did compromise short-term acclimation to high light (Komenda et al. 2012b). What is even more interesting, Psb27-enriched PSII complexes purified by copper affinity chromatography was eluted with enzymes of Chl biosynthesis, particularly with MgPMC and LPOR (Komenda J., Sobotka R., unpublished data). There is a possibility that the core of the proposed biosynthetic center is formed by a supercomplex of monomeric PSI and inactive Psb27-PSII offering a scaffold for Chl biosynthetic pathway.

The concept of biosynthetic centers anticipates that terminal enzymes of Chl biosynthesis are located at the membrane and channel Chl or chlorophyllide directly to a nascent polypeptide. Such a scenario would ensure a quick and safe passing of potentially dangerous Chl molecules into apoproteins and also facilitate coordination between Chl and Chl-protein syntheses. However, the high reactivity of Chl intermediates has to be considered as well. It would be reasonable to assume that enzymes involved in Chl biosynthesis form complexes for efficient substrate channeling. In fact, a megacomplex of enzymes, "Chl biosynthetic center", was proposed more than four decades ago based on physiological and biochemical experiments (Shlyk 1971). Such a multienzyme complex would reduce the pool of phototoxic intermediates to minimum, protect unstable intermediates by maintaining them in the complex, and allow an efficient regulation of the whole pathway. Pieces of evidence that some enzymes of the tetrapyrrole pathway physically interact already exist and further cumulate. For instance, GluTR, the first enzyme of the tetrapyrrole pathway, forms a complex with the following enzyme GSA-AT (Fig. 2) (Luer et al. 2005, Nogaj and Beale 2005). Also, an interaction between GluTR and GluRS, which is provides glutamyl-

$tRNA^{Glu}$ for tetrapyrrole and protein syntheses, has been suggested in *Chlamydomonas reinhardtii* and more recently in *Mycobacterium tuberculosis* (Jahn 1992, Paravisi et al. 2009). As described earlier we have identified several other proteins interacting with GluTR and we expect that large assemblies containing enzymes of tetrapyrrole are rather a rule than an exception (J. Kopečná, unpublished data). Although there is no data yet about a complex formation of the next five enzymes following GSA-AT (Fig. 3) it is expected they physically interact in order to channel highly unstable intermediates. Moving down along the pathway the next enzyme is protoporphyrinogen oxidase, the last common enzyme for both heme and Chl branches. In the cyanobacterium *Thermosynechococcus elongatus* protoporphyrinogen oxidase was identified in a complex with FeCH (Masoumi et al. 2008). The question of delivery of protoporphyrin IX to MgCH is still open. Perhaps it is assisted by Gun4, which interacts with the ChlH subunit of MgCH (Larkin et al. 2003; Sobotka et al. 2008a). Furthermore, physical interactions between MgCH and MgPMT have been reported (Shepherd et al. 2005). LPOR was found to bind MgPMC together with FLU protein in higher plants (Kauss et al. 2012). Finally, alternate data suggests the possibility of substrate channeling between LPOR and CSYN (Domanskii et al. 2003) and between CSYN and GGR (Rüdiger et al. 2005).

In order to identify putative protein partners of MgPMC in cyanobacteria, we used the same strategy as for the GluTR enzyme, tagging the N-termini of the enzyme with 3xFLAG and subsequent purification of the protein from *Synechocystis* cell extracts (Hollingshead et al. 2012). This experiment identified the Ycf54 protein as a possible partner for MgPMC. A reciprocal pulldown assay using FLAG-Ycf54 showed that MgPMC was trapped as prey. We suggested that the Ycf54 is an auxiliary factor essential for the assembly of MgPMC complex or facilitates formation of a catalytic complex between cyclase and preceding and/or following enzymes that would be required for MgPMC activity. Interestingly, the structure of Ycf54 exhibits a similarity with the structure of the Psb28 protein (Hollingshead et al. 2012). Deletion of the gene encoding Psb28 also affects PChlide synthesis in *Synechocystis* (Dobáková et al. 2009), although not to the extent observed in the *ycf54* mutant. Given that the Psb28 binds CP47 during the process of PSII assembly (Dobáková et al. 2009); this protein may be a suitable candidate for a factor harmonizing Chl biosynthesis and PSII biogenesis. Given the strong phenotype of the *ycf54* mutant, this strain seems to be promising for further understanding interactions between enzymes of the Chl pathway and, from my point of view inevitably, for a connection between Chl and protein synthesis.

List of abbreviations

ALA	Aminolevulinic acid
CAB domain	Chl *a/b*-binding domain
Chl	Chlorophyll
ELIP	early-light induced protein
FeCH	Ferrochelatase
DPOR	Light-independent protochlorophyllide oxidoreductase
DVR	Divinyl (proto)chlorophyllide reductase
GluRS	Glutamyl-tRNA synthetase
GluTR	Glutamyl-tRNA reductase
GluTRBP	GluTR-binding protein
GSA-AT	Glutamate-1-semialdehyde aminotransferase
HEAT	Huntingtin-elongation-A subunit-TOR
HLIP	High-light induced protein
LPOR	Light-dependent protochlorophyllide oxidoreductase
MgCH	Magnesium chelatase
MgPMC	Mg protoporphyrin IX monomethyl ester cyclase
MgPMT	Mg protoporphyrin IX methyl transferase
OHP	One helix protein
Pchlide	Protochlorophyllide
PSI	Photosystem I
PSII	Photosystem II
SCP	Small-CAB-like protein
SEP	Stress-enhanced protein

SUMMARY

Tetrapyrroles are probably one of the most ancient prosthetic groups in all organisms and built into proteins they play critical roles in a broad spectrum of fundamental reactions like photosynthesis. Indeed, without the heme, chlorophylls or bilins life as we know it would not exist. However, these life-giving molecules, when not properly protected within the protein complexes, like to form free radicals causing oxidative damage of cellular structures. Cells thus handle with tetrapyrroles very carefully, which is especially true for photosynthetic organisms accumulating high concentration of chlorophyll (Chl) to utilize light energy. All tetrapyrroles are produced by a biosynthetic pathway starting with the amino acid glutamate or, alternatively, glycine. The pathway is later branched to several different end products, these must be safely incorporated into proteins and these proteins bearing tetrapyrroles are usually further assembled into larger protein complexes. Indeed, all described steps are tightly connected and under perfect control.

This thesis explores how the synthesis and assembly of photosynthetic complexes is coordinated with Chl biosynthesis using the cyanobacterium *Synechocystis* PCC 6803 (hereafter *Synechocystis*) as a model organism. Although the enzymatic steps of Chl biosynthesis are almost completely elucidated at the genetic level (Tanaka and Tanaka 2007), current knowledge of the regulation of Chl metabolism and its synchronization with Chl-protein synthesis is very limited. The first publication listed provides new insight into distribution of newly synthesized Chl into photosystems under different light regimes. Using radioactive labeling by ^{35}S and ^{14}C combined with the native electrophoresis we showed that higher light intensities stimulate synthesis of Chl as well as all Chl-binding subunits of photosystems. Strikingly, the majority of newly synthesized chlorophyll was localized in the trimeric photosystem (PS) I, whereas Chl molecules in PSII were labeled only weakly. We concluded that mostly 'recycled' Chl molecules are used for assembly and/or repair of PSII.

In the second publication, we are discussing the relationship between light-dependent (LPOR) and light-independent (DPOR) protochlorophyllide oxidoreductases concerning the possibly differential effect of these enzymes on the synthesis and accumulation of individual Chl proteins. *Synechocystis* strains lacking either LPOR or DPOR allowed us to compare consequences of Chl deficiency caused by inactivation of these enzymes. In both cases, a decrease in the cellular content of Chl led to a parallel disappearance of both photosystems. These data have important relevance to the regulation of photosystem stoichiometry in thylakoid membrane. In fact, we excluded the PSI/PSII ratio in *Synechocystis* in controlled simply by upregulation/downregulation of the Chl pathway, a model proposed in a recent review (Muramatsu and Hihara 2012). We rather expect that such regulation has to involve a selective delivery of *de novo* synthesized and reused chlorophyll according to actual demand.

The third publication elucidates role of the Ycf54 protein in Chl biosynthesis. We have identified Ycf54 in a tight complex with Mg protoporphyrin IX monomethylester oxidative cyclase, an enzyme catalyzing oxidative cyclization of the 'fifth' Chl ring. Ycf54 is apparently required for the enzyme activity since its inactivation dramatically decrease Chl level as well as the level of the cyclase enzyme. In addition, other enzymes of Chl biosynthesis, the light-dependent protochlorophyllide oxidoreductase and Mg protoporphyrin IX methyl transferase, are significantly impaired. Finally, the fourth paper concerns possible roles of the Psb27 protein, an auxiliary protein that is found in PSII complexes lacking a functional Mn cluster. The Psb27 makes a complex with the PSII subunit CP43 and probably is also involved in the biogenesis of this subunit. However, deletion of the *psb27* gene does not impair the PSII assembly or repair, but affects acclimation of *Synechocystis* cells to high light. Interestingly, we also demonstrated an interaction of Psb27 with PSI in a large supercomplex containing also PSII.

RESULTS

I. Long-term acclimation of the cyanobacterium *Synechocystis* PCC 6803 to high light is accompanied by an enhanced production of chlorophyll that is preferentially channeled to trimeric PSI
Kopečná J, Komenda J, Bučinská L, Sobotka R
Plant Physiology, DOI:10.1104/pp.112.207274

II. Inhibition of chlorophyll biosynthesis at the protochlorophyllide reduction step results in the parallel depletion of Photosystem I and Photosystem II in the cyanobacterium *Synechocystis* PCC 6803
Kopečná J, Sobotka R, Komenda J
Planta, DOI: 10.1007/s00425-012-1761-4

III. Conserved chloroplast open-reading frame *ycf54* is required for activity of the magnesium-protoporphyrin monomethylester oxidative cyclase in *Synechocystis* PCC 6803
Hollingshead S, Kopečná J, Jackson PJ, Canniffe DP, Davison PA, Dickman MJ, Sobotka R, Hunter CN
Journal of Biological Chemistry, 287: 27823-27833

IV. The Psb27 assembly factor binds to the CP43 complex of photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803
Komenda J, Knoppová J, Kopečná J, Sobotka R, Halada P, Yu J, Nickelsen J, Boehm M, Nixon PJ
Plant Physiology, 158: 476-486

I.

Long-term acclimation of the cyanobacterium *Synechocystis* PCC 6803 to high light is accompanied by an enhanced production of chlorophyll that is preferentially channeled to trimeric PSI.

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ABSTRACT

Cyanobacteria acclimate to high-light conditions by adjusting photosystem stoichiometry through a decrease of photosystem I (PSI) abundance in thylakoid membranes. As PSI complexes bind the majority of chlorophyll (Chl) in cyanobacterial cells it is accepted that the mechanism controlling PSI level/synthesis is tightly associated with the Chl biosynthetic pathway. However, how Chl is distributed to photosystems under different light conditions remains unknown. Using radioactive labeling by ^{35}S and by ^{14}C combined with native/2D electrophoresis we assessed the synthesis and accumulation of photosynthetic complexes in parallel with the synthesis of Chl in *Synechocystis* cells acclimated to different light intensities. Although cells acclimated to higher irradiances (150 and 300 μE $.m^{-2}.s^{-1}$) exhibited markedly reduced PSI content when compared to cells grown at lower irradiances (10 and 40 μE $.m^{-2}.s^{-1}$) they grew much faster and synthesized significantly more Chl, as well as both photosystems. Interestingly, even under high irradiance almost all labeled *de novo* Chl was localized in the trimeric PSI, whereas only a weak Chl labeling in PSII was accompanied by the intensive ^{35}S protein labeling, which was much stronger than in PSI. These results suggest that PSII subunits are mostly synthesized using 'recycled' Chl molecules previously released during PSII repair driven protein degradation. In contrast, most of 'fresh' Chl is utilized for synthesis of PSI complexes likely to maintain a constant level of PSI during cell proliferation.

Na vysoké světlo se sinice aklimatizují přizpůsobením stechiometrie fotosystémů a to snížením množství fotosystému I (PSI) v tylakoidních membránách. Protože komplexy PSI váží většinu chlorofylu v buňkách sinic, předpokládá se, že mechanismus kontrolující množství a syntézu PSI je úzce spojen s biosyntézou chlorofylu. Nicméně, způsob distribuce chlorofylu do fotosystémů při různých světelných podmínkách není doposud znám. Pomocí radioaktivního značení ^{35}S a ^{14}C, kombinovaného s nativní/2D elektroforézou, jsme stanovili syntézu a akumulaci fotosyntetických komplexů společně se syntézou chlorofylu v buňkách *Synechocystis* přizpůsobené na různé světelné podmínky. I když buňky aklimatizované na vyšší světlo (150 a 300 μE $.m^{-2}.s^{-1}$) vykazují

výrazně zredukované množství PSI ve srovnání s buňkami aklimatizovanými na nižší světlo (10 a 40 μE $.m^{-2}.s^{-1}$), rostou mnohem rychleji a syntetizují výrazně více chlorofylu i obou fotosystémů. Zajímavé je, že většina radioaktivně značeného chlorofylu je i na vysokém světle lokalizována v trimeru PSI, zatímco značení chlorofylu v komplexech PSII bylo velmi slabé ve srovnání se značením proteinů, které bylo na rozdíl od PSI velmi intenzivní. Tyto výsledky naznačují, že podjednotky PSII se syntetizují zejména za použití recyklovaného chlorofylu, který byl uvolněn během oprav PSII a degradace proteinů. Naopak většina „čerstvého" chlorofylu je využita pro syntézu komplexů PSI, pravděpodobně kvůli zachování konstantního množství PSI během buněčného dělení.

II.

Inhibition of chlorophyll biosynthesis at the protochlorophyllide reduction step results in the parallel depletion of Photosystem I and Photosystem II in the cyanobacterium *Synechocystis* PCC 6803

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ABSTRACT

In most oxygenic phototrophs, including cyanobacteria, two independent enzymes catalyze the reduction of protochlorophyllide to chlorophyllide, which is the penultimate step in chlorophyll (Chl) biosynthesis. One is light-dependent NADPH:protochlorophyllide oxidoreductase (LPOR) and the second type is dark-operative protochlorophyllide oxidoreductase (DPOR). To clarify the roles of both enzymes, we assessed synthesis and accumulation of Chl-binding proteins in mutants of cyanobacterium *Synechocystis* PCC 6803 that either completely lack LPOR or possess low levels of the active enzyme due to its ectopic regulatable expression. The LPOR-less mutant grew photoautotrophically in moderate light and contained a maximum of 20 % of the wild-type (WT) Chl level. Both Photosystem II (PSII) and Photosystem I (PSI) were reduced to the same degree. Accumulation of PSII was mostly limited by the synthesis of antennae CP43 and especially CP47 as indicated by the accumulation of reaction center assembly complexes. The phenotype of the LPOR-less mutant was comparable to the strain lacking DPOR that also contained <25 % of the wild-type level of PSII and PSI when cultivated under light-activated heterotrophic growth conditions. However, in the latter case, we detected no reaction center assembly complexes, indicating that synthesis was almost completely inhibited for all Chl-proteins, including the D1 and D2 proteins.

Většina oxygenních fototrofů vč. sinic má dva nezávislé enzymy katalyzující redukci protochlorofylidu na chlorofylid, což je předposlední reakce biosyntézy chlorofylu. Prvním z nich je světelně závislá NADPH:protochlorofylid oxidoreduktáza (LPOR), druhým je pak světelně nezávislá protochlorofylid oxidoreduktáza (DPOR). Abychom objasnili roli těchto dvou enzymů, stanovili jsme syntézu a akumulaci proteinů vážících chlorofyl v mutantech sinice *Synechocystis* PCC 6803, kteří buď zcela postrádají jeden z enzymů nebo jej exprimují jen na velmi nízké úrovni. Kmen bez LPOR rostl fotoautotrofně jen za mírného osvětlení a množství chlorofylu odpovídalo

20% chlorofylu obsaženého v buňkách kontrolního kmenu. Oba fotosystémy byly zredukovány ve srovnatelném rozsahu. Akumulace fotosystému II (PSII) byla limitována převážně syntézou svých anténních podjednotek CP43 a především CP47. Fenotypy obou kmenů, jak bez LPOR tak bez DPOR enzymu, byly srovnatelné. Kmen bez LPOR enzymu rovněž obsahoval méně než 25% fotosystémů. Nicméně, v kmeni bez DPOR enzymu jsme nedetekovali žádná reakční centra, což naznačuje, že byla inhibována syntéza všech chlorofyl-vážících proteinů, včetně podjednotek D1 a D2.

III.

Conserved chloroplast open-reading frame *ycf54* is required for activity of the magnesium-protoporphyrin monomethylester oxidative cyclase in *Synechocystis* PCC 6803

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ABSTRACT

The cyclase step in chlorophyll (Chl) biosynthesis has not been characterized biochemically, although there are some plausible candidates for cyclase subunits. Two of these, Sll1214 and Sll1874 from the cyanobacterium *Synechocystis* 6803, were FLAG-tagged *in vivo* and used as bait in separate pulldown experiments. Mass spectrometry identified Ycf54 as an interaction partner in each case, and this interaction was confirmed by a reciprocal pulldown using FLAG-tagged Ycf54 as bait. Inactivation of the *ycf54* gene (*slr1780*) in *Synechocystis* 6803 resulted in a strain that exhibited significantly reduced Chl levels. A detailed analysis of Chl precursors in the *ycf54* mutant revealed accumulation of very high levels of Mg-protoporphyrin IX methyl ester and only traces of protochlorophyllide, the product of the cyclase, were detected. Western blotting demonstrated that levels of the cyclase component Sll1214 and the Chl biosynthesis enzymes Mg-protoporphyrin IX methyltransferase and protochlorophyllide reductase are significantly impaired in the *ycf54* mutant. Ycf54 is, therefore, essential for the activity and stability of the oxidative cyclase. We discuss a possible role of Ycf54 as an auxiliary factor essential for the assembly of a cyclase complex or even a large multienzyme catalytic center.

Syntéza pátého kruhu během biosyntézy chlorofylu není doposud biochemicky charakterizována, ačkoliv již bylo objeveno několik možných kandidátů na podjednotky cyklázy.. Dva takové proteiny, Sll1214 a Sll1874 ze sinice *Synechocystis* 6803, jsme označili proteinovou kotvou 3xFLAG a použili pro purifikační experimenty. Hmotnostní spektroskopie odhalila proteinYcf54 jako možného navázaného partnera těchto proteinů. Tato interakce byla dále potvrzena vzájemnou kopurifikací za použití FLAG-Ycf54. Inaktivací genu *ycf54* (*slr1780*) v *Synechocystis* 6803 jsme získali kmen s výrazně sníženým množstvím chlorofylu. Detailní analýza prekurzorů chlorofylu v tomto kmeni odhalila vysokou akumulaci Mg protoporfyrinu IX metyl esteru a jen nepatrné stopy po protochlorofylidu. Imunodetekční analýza ukázala, že množství enzymů Sll1214, Mg

protoporfyrin metyltransferázy a protochlorofylid reduktázy, je výrazně sníženo. Protein Ycf54 je tudíž nezbytný pro aktivitu a stabilitu oxidativní cyklázy. Diskutujeme také možnou roli proteinu Ycf54 jako doplňkového faktoru nutného pro správné složení či aktivitu cyklázového komplexu nebo dokonce většího katalytického komplexu složeného z více enzymů.

IV.

The Psb27 assembly factor binds to the CP43 complex of photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803

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ABSTRACT

We have investigated the location of the Psb27 protein and its role in photosystem (PS) II biogenesis in the cyanobacterium *Synechocystis* sp. PCC 6803. Native gel electrophoresis revealed that Psb27 was present mainly in monomeric PSII core complexes but also in smaller amounts in dimeric PSII core complexes, in large PSII supercomplexes, and in the unassembled protein fraction. We conclude from analysis of assembly mutants and isolated histidine-tagged PSII subcomplexes that Psb27 associates with the "unassembled" CP43 complex, as well as with larger complexes containing CP43, possibly in the vicinity of the large lumenal loop connecting transmembrane helices 5 and 6 of CP43. A functional role for Psb27 in the biogenesis of CP43 is supported by the decreased accumulation and enhanced fragmentation of unassembled CP43 after inactivation of the *psb27* gene in a mutant lacking CP47. Unexpectedly, in strains unable to assemble PSII, a small amount of Psb27 comigrated with monomeric and trimeric PSI complexes upon native gel electrophoresis, and Psb27 could be copurified with histidine-tagged PSI isolated from the wild type. Yeast two-hybrid assays suggested an interaction of Psb27 with the PsaB protein of PSI. Pulldown experiments also supported an interaction between CP43 and PSI. Deletion of psb27 did not have drastic effects on PSII assembly and repair but did compromise short-term acclimation to high light. The tentative interaction of Psb27 and CP43 with PSI raises the possibility that PSI might play a previously unrecognized role in the biogenesis/repair of PSII.

Zkoumali jsme lokalizaci proteinu Psb27 a jeho roli při vzniku fotosystému II (PSII) v sinici *Synechocystis* sp. PCC 6803. Nativní gelová elektroforéza odhalila, že se Psb27 vyskytuje v jádrovém komplexu monomeru PSII a v menším množství také v dimeru PSII, velkých PSII superkomplexech a ve frakci volných proteinů. Z analýzy skládání subkomplexů PSII usuzujeme, že Psb27 je asociován s volným CP43 komplexem a také s dalšími komplexy, které obsahují CP43. Psb27 je pravděpodobně navázáno na CP43 v lumenární smyčce spojující trans-membránové

spirály 5 a 6. Inaktivace genu *psb27* vedla ke zvýšené fragmentaci volného CP43, což naznačuje, že se Psb27 účastní vzniku CP43. V kmeni, který nebyl schopen složit funkční PSII, byl Psb27 objeven v komplexu s PSI. Yeast two-hybrid experiment a kopurifikační analýzy potvrdily interakci mezi Psb27 a podjednotkou PSI PsaB. Delece genu *psb27* neměla výrazný vliv na skládání a opravu PSII, ovšem kmen byl výrazně limitován při přechodu na vysoké světlo. Možná interakce Psb27 a CP43 s PSI naznačuje, že tyto proteiny mohou hrát jistou úlohu při vzniku či opravě PSI.

CONCLUSIONS

This PhD thesis has contributed to elucidation of important aspects of chlorophyll biosynthesis regulation and coordination with the synthesis of the chlorophyll-binding proteins in the cyanobacterium *Synechocystis* sp. PCC 6803. It is composed of two first author publications and two co-author publications. The main conclusions are as follows:

- Chlorophyll biosynthesis is upregulated under higher light conditions to maintain a stable chlorophyll level per cell due to faster growth rate of the cells and shorter life time of chlorophyll molecules.
- The majority of the *de novo* synthesized chlorophyll is directed into the trimeric photosystem I, whereas photosystem II subunits are probably mostly synthesized using 'recycled' chlorophyll molecules.
- Although the synthesis of both photosystems is upregulated with increasing light intensity, photosystem I content in cells acclimated to higher irradiances is markedly reduced in comparison to cells grown at lower irradiances, while photosystem II content remains relatively stable. A detail model of this regulatory event is provided.
- Inhibition of chlorophyll biosynthesis at the step of protochlorophyllide reduction results in the comparable depletion of both photosystems, suggesting that the ratio between photosystems is not controlled only by a simple up-/down-regulation of the chlorophyll pathway.
- The *Synechocystis* mutant lacking light-dependent protochlorophyllide oxidoreductase enzyme (LPOR) has strongly reduced chlorophyll levels under low light conditions. It demonstrates that the second enzyme catalyzing the same step (DPOR) could not compensate for the loss of LPOR and that the LPOR is responsible for the synthesis of the majority of chlorophyll even under low light.
- Ycf54 protein was identified in a complex of the Mg-protoporphyrin methyl ester oxidative cyclase and even a decrease in level of the Ycf54 in *Synechocystis* strongly affected the activity of the cyclase enzyme. The Ycf54 probably plays a critical role in the assembly of a cyclase complex or even a large multienzyme catalytic center.
- Psb27 protein was shown to physically interact with the photosystem II subunit CP43 and stabilizes this protein against proteolytic degradation. Deletion of *psb27* in *Synechocystis* does not affect photosystem II assembly and repair but compromises short-term acclimation to high light. The Psb27 is associated with photosystem II but also with supercomplexes containing both photosystems.

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Publications:

Kopečná J, Komenda J, Bučinská L, Sobotka R (2012) Long-term acclimation of the cyanobacterium *Synechocystis* PCC 6803 to high light is accompanied by an enhanced production of chlorophyll that is preferentially channeled to trimeric PSI. *Plant Physiology* DOI:10.1104/pp.112.207274

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Awarded research grants:

2010 **Kopečná J**, *Regulation of the chlorophyll and heme biosynthetic pathways: Role of the glutamyl-tRNA reductase*. Research grant of the German Academic Exchange Service (DAAD), Bonn, Germany

2010 **Kopečná J,** *Chlorophyll biosynthesis under control - regulatable expression of the LPOR enzyme in the cyanobacterium* Synechocystis *PCC 6803*. Grant agency of the University of South Bohemia, České Budějovice, Czech Republic

2009 **Kopečná J**, *Regulation of the chlorophyll and heme biosynthetic pathways: the role of glutamyl-tRNA reductase*. Grant agency of the University of South Bohemia, České Budějovice, Czech Republic

2006 **Kopečná J**, *In vitro activity of the* Trypanosoma brucei *mitochondrial RNA-binding proteins MRP1 and MRP2*. Student grant agency of the Faculty of Biological Sciences, University of South Bohemia, České Budějovice, Czech Republic

2002 **Kopečná J**, *Excystation structures and phylogeny of selected coccidian species.* Student grant agency of the Faculty of Biological Sciences, University of South Bohemia, České Budějovice, Czech Republic

Participation in conferences:

Kopečná J (oral presentation) *Chlorophyll biosynthesis is synchronized with the synthesis of photosystem II core subunits in* Synechocystis *PCC 6803*. 10th International Conference on Tetrapyrrole Photoreceptors of Photosynthetic Organisms, Berlin, Germany, 24-28 Jul 2011

Kopečná J, Komenda J, Sobotka R (poster presentation) *Chlorophyll biosynthesis under control – the regulatable expression of the LPOR enzyme in* Synechocystis *PCC 6803*. 25th Congress of the Czechoslovakian microbiological association, Stará Lesná, Slovakia, 15-18 Sep 2010

Kopečná J, Komenda J, Sobotka R (poster presentation) *Chlorophyll synthesis under control - regulatable expression of the POR enzyme in* Synechocystis *PCC 6803*. Methods in Plant Sciences, Czech Republic, 11-14 Oct 2009

Kopečná J, Komenda J, Sobotka R (poster presentation) *The regulatable expression of the light-dependent protochlorophyllide oxidoreductase in* Synechocystis *PCC 6803*. 9th International Conference on Tetrapyrrole Photoreceptors in Photosynthetic Organisms, Asilomar, California, USA, 31 Jul - 4 Aug 2009

Kopečná J, Tichý M, Sobotka R (poster presentation) *The purification of His-tagged Scp proteins from Synechocystis sp. PCC 6803 using Ni-NTA chromatography*. 7th European Workshop on the Molecular Biology of Cyanobacteria, České Budějovice, Czech Republic, 31 Aug - 4 Sep 2008