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Faculty of Science

Biogenesis of Photosystem II in the Model Cyanobacterium *Synechocystis* sp. PCC 6803

– The Role of Selected Auxiliary Protein Factors and Subcellular Localisation

Ph.D. Thesis

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Annotation

This thesis explores localisations and roles of three auxiliary protein factors involved in the biogenesis of Photosystem II (PSII) in the cyanobacterium *Synechocystis* sp. PCC 6803 and contributes to subcellular localisation of the initial steps of PSII biogenesis and repair-related D1 synthesis. The main results consist in i) identification of a functional interaction of the protein factor Psb27 with a lumenal domain of the Photosystem II subunit CP43, ii) discovery of a novel pigment binding complex formed by the Ycf39 protein and high-light-inducible proteins implicated in photoprotection and delivery of recycled chlorophyll to newly synthesized D1 protein during the PSII reaction centre formation, iii) providing evidence that the early steps of PSII assembly and the repair-related D1 synthesis occur in the thylakoid membrane of *Synechocystis*, and iv) revealing that the cyanobacterial PsbP orthologue, CyanoP, assists in the early phase of PSII biogenesis as an assembly factor facilitating the association of D2 and D1 assembly modules.

Declaration [in Czech]

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I would like to dedicate this work to my wonderful sons, Matěj, Ondřej and Kája, as compensation for all the dinners which they have had to make themselves over recent years, and to my Karel whose original way of life have made me get emancipated.

List of papers and author's contribution

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

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  JKno was responsible for physiological experiments and gel protein separations, contributed to preparation of mutants and participated in data processing and evaluation.
- Knoppová J, Sobotka R, Tichý M, Yu J, Konik P, Halada P, Nixon PJ, Komenda J (2014). Discovery of a Chlorophyll Binding Protein Complex Involved in the Early Steps of Photosystem II Assembly in *Synechocystis*. The Plant Cell 26: 1200-1212. IF 9.34
  JKno carried out all experiments except of HPLC pigment analysis and MS, prepared mutants except of ΔYcf39, participated in data processing and evaluation and contributed to writing the manuscript.
- Selão TT, Zhang L, Knoppová J, Komenda J, Norling B (2016). Photosystem II Assembly Steps Take Place in the Thylakoid Membrane of the Cyanobacterium *Synechocystis* sp. PCC6803. Plant and Cell Physiology 57: 95-104. IF 4.93
  JKno carried out experiments concerning the localisation of early PSII complexes and their components, processed and evaluated the results of these experiments and contributed to writing the manuscript.
- Knoppová J, Yu J, Konik P, Nixon PJ, Komenda J. CyanoP is Involved in the Early Steps of Photosystem Two Assembly in the Cyanobacterium *Synechocystis* sp. PCC 6803**.** Submitted to Plant and Cell Physiology
  JKno carried out all experiments except of MS, prepared mutants except of ΔCyanoP and His-D2/ΔD1, participated in data processing and evaluation and contributed equally to writing the manuscript.

Josef Komenda, the corresponding author of papers I, II and IV, and the supervising author of paper III, approves the contribution of Jana Knoppová in these papers as described above.

Prof. RNDr. Josef Komenda, CSc., DSc.

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List of Abbreviations

A. thaliana	*Arabidopsis thaliana*
BN	blue native electrophoresis
CN	clear native electrophoresis
PAGE	polyacrylamide gel electrophoresis
pD1	precursor form of D1 protein
PSI	Photosystem I
PSII	Photosystem II
PM	plasma membrane
RCII	Photosystem II reaction centre assembly complex lacking CP43 and CP47
RC47	Photosystem II reaction centre core lacking CP43
RCC1	Photosystem II reaction centre core monomer
RCC2	Photosystem II reaction centre core dimer
Synechocystis	*Synechocystis* sp. PCC 6803
T. elongatus	*Thermosynechococcus elongatus*
T. vulcanus	*Thermosynechococcus vulcanus*
TM	thylakoid membrane
WT	wild type

1. Introduction

This thesis has been aimed to elucidate locations and putative functions of three cyanobacterial proteins, Psb27, Ycf39 and CyanoP, supposed to participate in the complex process of Photosystem II (PSII) biogenesis. It also includes a study dealing with localization of PSII biogenesis and repair at the level of the main membrane systems of cyanobacteria, i.e. the plasma and thylakoid membranes.

Photosystem II is a component of the photosynthetic machinery embedded in the thylakoid membranes of oxygenic autotrophic organisms, i.e. plants, algae and cyanobacteria (see Figure 1). Powered by light, it mediates the highly energetically demanding reaction of water splitting. This reaction provides the driving force for photosynthetic electron transport and formation of the transmembrane proton gradient used to generate ATP and NADPH for CO_2 fixation into the organic matter, and supplies the atmosphere with oxygen.

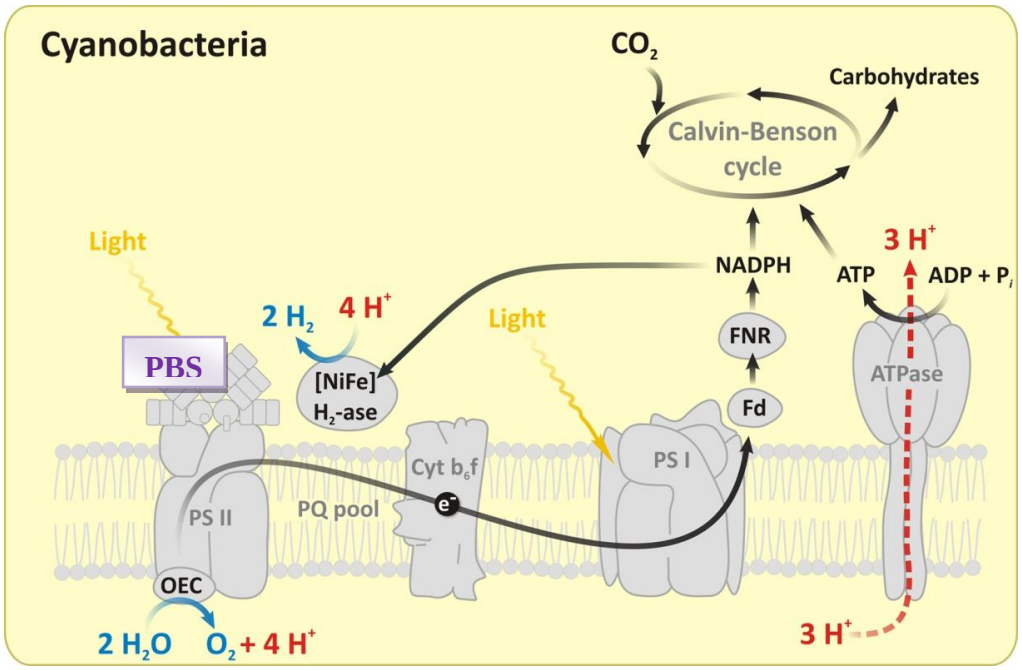

Figure 1 – A schematic representation of the photosynthetic machinery in cyanobacteria. Abbreviations: PQ, plastoquinone; Fd, ferredoxin; FNR, ferredoxin–NADP reductase; ATPase, adenosine triphosphate synthase; PBS, phycobilisome antenna. The figure is modified from Govindjee and Shevela (2011).

PSII consists of at least 20 membrane intrinsic and extrinsic protein subunits binding pigments and other co-factors involved in the electron transport. It is believed to

form a homodimer as a functional form (Barber, 2006). The biogenesis of this multicomponent enzyme requires highly sophisticated coordination of the apoprotein synthesis and membrane incorporation with the synthesis and insertion of chlorophylls and other pigments and cofactors (Nixon et al., 2010). The individual newly synthesized pigment-proteins then need to precisely assemble into the functional unit. In addition to the general enzymatic machineries involved in these processes (protein, pigment and co-factor biosynthesis), the photosystem formation is supported by a range of specific protein factors which bind transiently to PSII intermediates in certain steps of the biogenesis but are not components of the mature complex. These auxiliary proteins appear to serve as stabilising or protecting factors, factors facilitating insertion of pigments and cofactors into apoproteins or mediate the correct association of PSII subunits (Komenda et al., 2012a; Chi et al., 2012; Nickelsen and Rengstl, 2013). They are supposed to participate not only in *de novo* biogenesis, but also in the process of PSII repair following the light-induced damage.

The subcellular localization of PSII biogenesis and repair in cyanobacteria has not been unequivocally elucidated until now. It has been thought for a long time that the initial steps of PSII assembly occur in the plasma membrane and the early assembly complexes are translocated into the thylakoids to complete the biogenesis of the fully active PSII (Zak et al., 2001). However, later findings (see the Overview) raised doubts about this hypothesis and necessitated a revision study which demonstrated the localization of both the entire process of PSII biogenesis and the repair in the thylakoid membranes (Selão et al., 2016, and this thesis). Nevertheless, whether these processes occur in specific compartments within the thylakoids as indicated for instance in chloroplast of *Chlamydomonas* (Uniacke and Zerges, 2007) remains to be elucidated.

The experimental model employed in this thesis is the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) (Kaneko et al., 1996; Tajima et al., 2011). This unicellular cyanobacterium (Figure 2) is an extremely useful model organism serving as a standard of cyanobacteria in various areas of research, such as photosynthesis, stress response and metabolism. The nucleotide sequence of its genome was fully sequenced as early as in 1996 as the first genome of photosynthetic organism. It is naturally transformable, i.e. the cells actively accept foreign DNA without any special pre-treatment, and efficient in homologous gene recombination. Importantly, its ability to grow on glucose as a source of carbon allows us to knock out genes essential for the photoautotrophic growth. A large number of site-directed single and multiple mutants affected in the functioning of

photosynthesis are available or can be easily constructed. Moreover, cyanobacteria as progenitors of chloroplasts share very similar photosynthetic apparatus with their eukaryotic descendants. Thus, the principles of photosynthetic processes elucidated in cyanobacteria are in most cases applicable also to plants and algae.

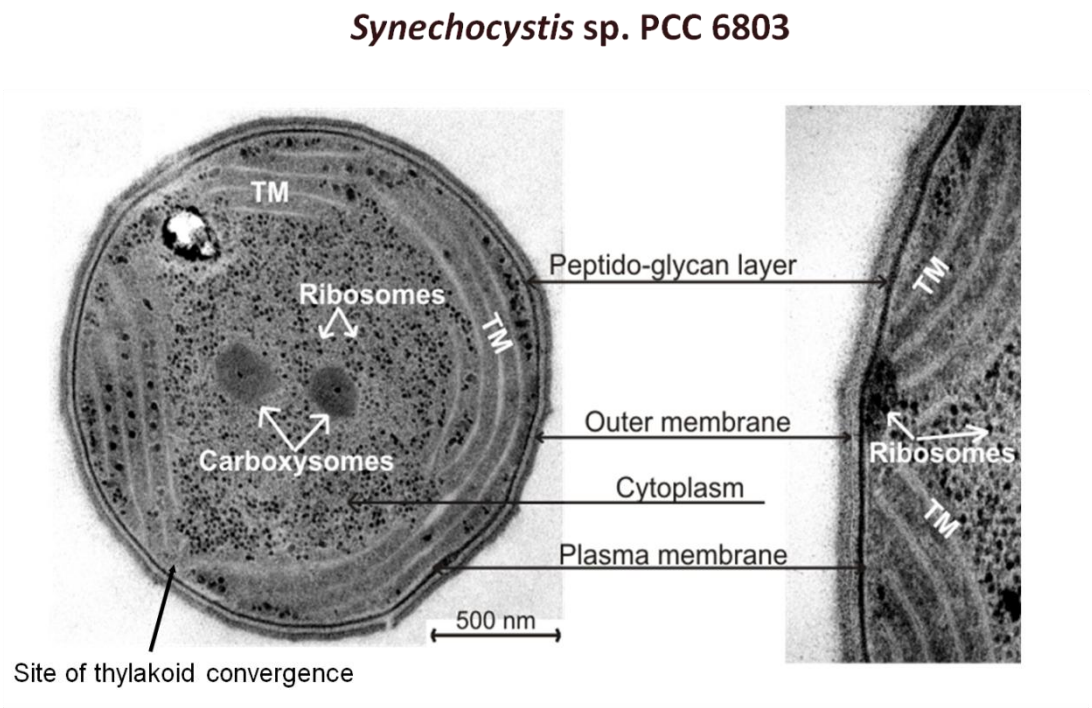

Figure 2 – Wild type cell of *Synechocystis* sp. PCC 6803. Transection electron microscopic image showing details of ultrastructural and membrane organization. TM, thylakoid membranes. Photo by Lenka Bučinská, Institute of Microbiology ASCR and University of South Bohemia.

2. Overview

2.1. Photosystem II – Structure and Function

Photosystem II (PSII) is a key component of the machinery responsible for the transformation of solar energy into chemical energy via photosynthetic electron transport and formation of the transmembrane proton gradient providing ATP and reduced coenzymes for CO_2 fixation into the organic molecules. The uniqueness of this complex enzyme consists in catalysing the highly energetically demanding reaction of water splitting which drives the highly efficient photosynthetic charge separation and provides the Earth with the oxygen.

PSII is a multisubunit complex embedded in the thylakoid membranes of plants, algae and cyanobacteria. Its native functional form in both cyanobacteria and plants is a homodimer with total molecular mass of 700 kDa. The first three-dimensional structures of PSII fully active in water oxidation were published by Zouni et al. (2001) and Ferreira et al. (2004) on the basis of X-ray crystallography of the preparations isolated from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (Figure 1). These structures at resolutions of 3.8 and 3.5 Å, respectively, revealed spatial organisation of most of the protein subunits and cofactors involved in excitation energy transfer and electron transport. It also provided direct information regarding the size, shape and location of the manganese/calcium cluster responsible for water oxidation. The following structure of Guskov et al. (2009) at 2.9 Å resolution allowed the unambiguous assignment of all 20 protein subunits (17 intrinsic and 3 extrinsic ones) and complete modelling of 35 chlorophyll-a molecules and 12 carotenoid molecules, 25 integral lipids and 1 chloride ion per monomer. The latest structure at a resolution of 1.9 Å, obtained using PSII crystals from *Thermosynechococcus vulcanus* (Umena et al., 2011), located all the atoms of the Mn_4CaO_5 cluster, together with their ligands. It has been found that five oxygen atoms serve as oxo-bridges linking the five metal atoms, and that four water molecules are bound to the Mn_4CaO_5 cluster; some of them might therefore serve as substrates for dioxygen formation. More than 1,300 water molecules were identified in each photosystem II monomer; some of them formed extensive hydrogen-bonding networks that might serve as channels for protons, water or oxygen molecules.

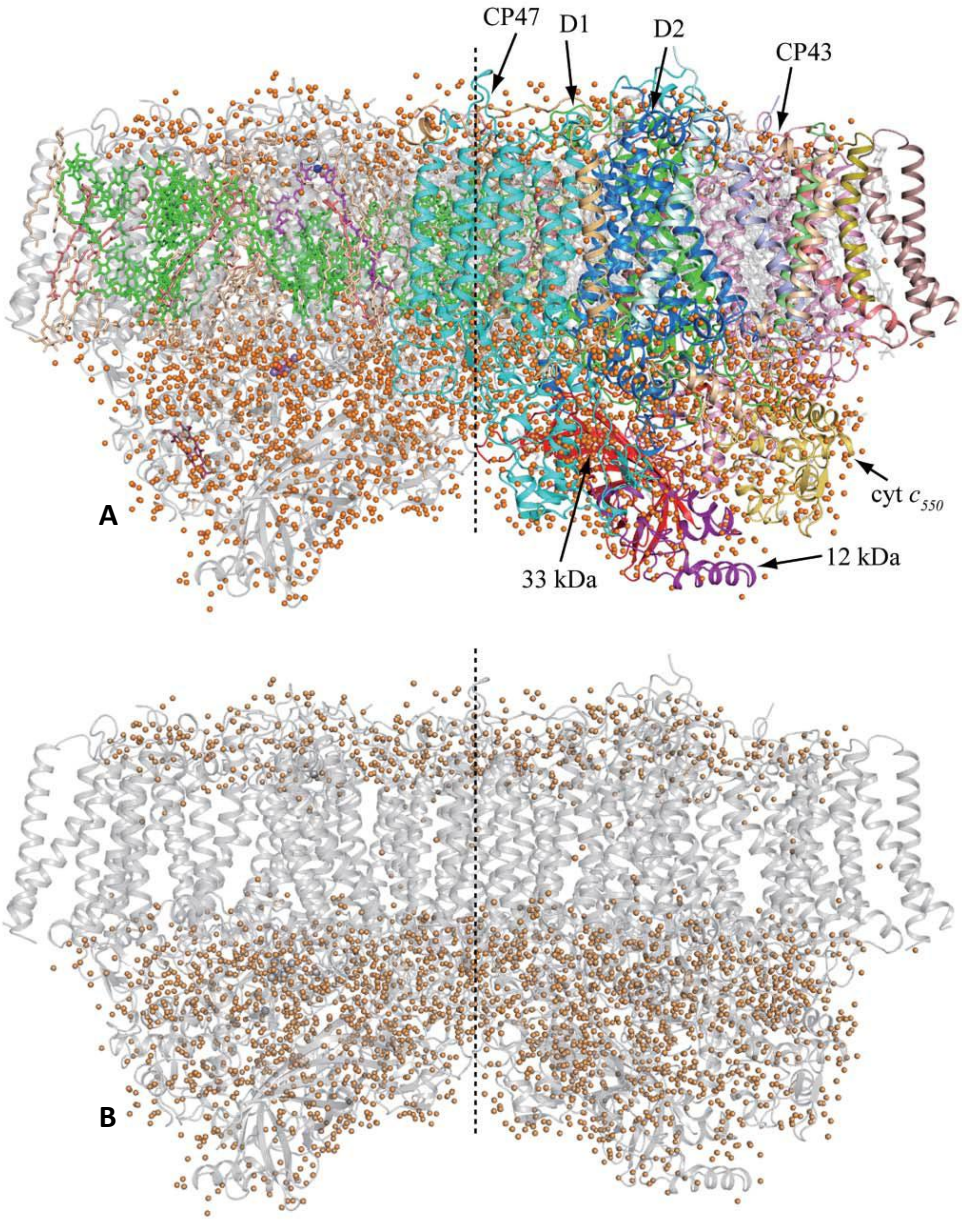

Figure 3 - Overall structure of PSII dimer from *T. vulcanus* at a resolution of 1.9 A˚ from Umena et al. (2011). View from the direction perpendicular to the membrane normal. A, Overall structure. The protein subunits are coloured individually in the right hand monomer and in light grey in the left-hand monomer, and the cofactors are coloured in the left-hand monomer and in light grey in the right-hand monomer. Orange balls represent water molecules. B, Arrangement of water molecules in the PSII dimer. The protein subunits are coloured in light grey and all other cofactors are omitted. The central broken lines are the noncrystallographic two-fold axes relating the two monomers. Designation: 33kDa, PsbO; 12 kDa, PsbV; cyt c550, PsbV.

At the centre of the complex (Figure 3) there are two reaction centre (RCII) subunits called D1 and D2, which bind cofactors involved in the primary charge separation,

including the chlorophylls forming the P_{680} excited state (Barber, 2006). These proteins also carry secondary electron donor (Tyr 161 residue of D1) and primary and secondary electron acceptors (pheophytin and two quinone acceptors Q_A and Q_B). On either side are CP43 and CP47, each binding chlorophyll-a and β-carotene molecules and serving as inner light harvesting antennae. D1 and CP43 coordinate ions of the oxygen evolving Mn_4CaO_5 cluster. On the periphery of these large subunits there are 13 small, mostly single helix subunits (Ferreira et al., 2004; Barber, 2006).

The lumenal part of the complex is shielded by three extrinsic subunits supposed to optimise the binding environment of the oxygen-evolving complex (Roose et al., 2007). The set of extrinsic lumenal subunits differs among oxygenic autotrophs (Bricker et al., 2012). In cyanobacteria, it consists of PsbO, PsbU and PsbV proteins. In plants and green algae, the latter ones had been replaced by structurally different proteins, PsbP and PsbQ. While the orthologues of PsbU and PsbV have not been found in the green lineage of eukaryotes implying that they were probably lost during the evolution (Ifuku et al., 2010), the existence of cyanobacterial orthologues to plant PsbP and PsbQ, called CyanoP and CyanoQ, have been well established (early studies by Kashino et al. (2002), De Las Rivas et al. (2004), Thornton et al. (2004)). However, neither of them has been found even in the latest crystallographic models of the cyanobacterial PSII (Guskov et al., 2009; Umena et al., 2011) and their locations and roles seem to be different from those in green eukaryotes. In the cyanobacterium *Synechocystis* sp. PCC 6803, CyanoQ was shown to be a stoichiometric subunit of PSII complexes highly active in oxygen evolution (Thornton et al., 2004; Roose et al., 2007). Michoux et al. (2014) have demonstrated that CyanoQ of *Thermosynechococcus elongatus* co-purifies with PSII and is actually present in highly pure PSII samples used to generate PSII crystals. Liu et al. (2014) proposed a model in which PsbQ binds to PSII through a close association with PsbO and CP47, thereby stabilizing the PSII dimer and permitting high rates of oxygen evolution. In contrast, the PsbP homologue, CyanoP, is markedly substoichiometric in PSII complexes isolated from *T. elongatus* (Michoux et al., 2014). Recent studies on this protein imply that it plays a role of a PSII-related auxiliary factor rather than a constitutive subunit of the functional PSII (Cormann et al. (2014); Chapter 4.4. - Knoppová et al., submitted).

2.2. Photoinhibition of PSII and Photoprotection

Photosystem II is extremely vulnerable to photoinhibition, the loss of PSII photochemical activity due to light induced damage (Adir et al., 2003; Edelman and Mattoo, 2008; Tyystjärvi, 2013). The rate of damage to PSII is directly proportional to light intensity (Tyystjärvi and Aro, 1996). However, due to efficient recovery mechanisms, net photoinhibition occurs only in strong light when the rate of damage exceeds the rate of repair. In addition to visible light, UV radiation causes photoinhibition (Hakala et al., 2005). Several hypotheses about the mechanism of photoinhibition have been previously suggested, and the role of reactive oxygen species (ROS) in PSII damage, still unclear, is under discussion (Takahashi and Badger, 2011; Tyystjärvi, 2013). In sharp contrast to PSII, the Photosystem I (PSI) centres are very efficiently protected against photodamage, but the subsequent recovery of PSI is extremely slow and the damage, when occurring in rare cases, is practically irreversible (Tikkanen et al., 2014).

Tyystjärvi (2013) put forward a most recent working hypothesis linking the up-to-date knowledge about the mechanism of PSII photoinhibition. In this model, photodamage of the oxo-metal (Mn_4CaO_5) cluster is regarded as a primary event. Thus, photoinhibition begins with inactivation of the cluster caused by excitation of Mn ions leading to their release from the structure. This inhibits electron transfer from the oxygen-evolving complex to the oxidized primary electron donor, P_{680}^+. The long lived P_{680}^+ alone can cause an oxidative damage to the D1 protein and the entire Mn-inactivated complex. Alternatively, P_{680}^+ can recombine with Q_A^- producing the triplet state of P_{680} ($^3P_{680}$), which is known to react with O_2 to form the highly reactive singlet oxygen ($^1O_2$) causing further damage to the Mn-inactivated complex and other PSII units. The singlet oxygen may also be generated in PSII antennae, as well as in PSI, via chlorophyll photosensitisation, i.e. a spontaneous spin change of any excited antenna chlorophyll molecule to the triplet state. Triplet formation is particularly efficient if a chlorophyll molecule is energetically uncoupled from the photosystems and other chlorophylls.

The above described mechanism is strongly supported by the data on the action spectra of photoinhibition which differs from the absorption spectra of chlorophylls and carotenoids and resembles those of model manganese compounds and manganese containing proteins having their maxima in the UV range (Hakala et al., 2005). In agreement with these findings it was also demonstrated that photodamage to PSII under incident sunlight is primarily associated with UV

wavelengths and secondarily with yellow light wavelengths, and less associated with light absorbed by photosynthetic pigments (Takahashi et al., 2010).

Cyanobacterial cells and chloroplasts produce also other ROS, superoxide anion radical ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). While the singlet oxygen is produced mainly in PSII, the main site of superoxide and hydrogen peroxide formation is PSI. When the quantity of generated electrons exceeds the capacity of the Calvin cycle to absorb them, the excess electrons reduce oxygen to produce the $^{\cdot}O_2^-$, which is converted to H_2O_2 by the action of superoxide dismutase.

H_2O_2, $^{\cdot}O_2^-$ or $^1O_2$ can cause direct damage to PSII, however, the separate monitoring of photodamage and repair in cyanobacterial cells revealed that ROS act primarily by inhibiting the repair of PSII on the level of protein translation essential for the repair process (Murata et al., 2007; Nishiyama and Murata, 2014). It is the oxidation of the elongation factor G that was suggested to inhibit the *de novo* synthesis of proteins, namely the D1 (Kojima et al., 2007). It is also thought that the main sites of $^{\cdot}O_2^-$ and H_2O_2 production are so distant from PSII that their enzymatic scavenging, employing peroxiredoxin and catalase in cyanobacteria and ascorbate peroxidase in plants, is believed to provide efficient protection *in vivo* (Takahashi and Badger, 2011; Tyystjärvi, 2013).

When combined with illumination, other kinds of abiotic stress, such as CO_2 depletion, moderate heat stress, low temperature and high concentrations of salt, can enhance photoinhibition. Nishiyama and Murata (2014) summarize the recent studies on photoinhibition and emphasize that abiotic stresses act primarily by inhibiting the repair of PSII rather than accelerating photodamage itself. They suggest that various kinds of stress might suppress CO_2 fixation and, thereby, stimulate generation of ROS inhibiting PSII repair. In addition, singlet oxygen may also be formed under heat, heavy metal and mechanical injury stresses by less known mechanisms different from the chlorophyll photosensitisation (Pospíšil and Prasad, 2014).

Photosynthetic organisms developed a broad variety of mechanisms protecting them against the excess of light. As reviewed by Takahashi and Badger (2011), these mechanisms are associated with avoiding light absorption by the manganese cluster and successfully consuming or dissipating the light energy absorbed by photosynthetic pigments.

Plants can dissipate excessive light energy absorbed by the light-harvesting complexes (LHC) of PSII as harmless heat energy (thermal energy dissipation, qE).

The mechanism responsible for qE is associated with the conversion of violaxanthin to zeaxanthin, via antheraxanthin, by the violaxanthin de-epoxidase (VDE) and the protonation of the PSII protein subunit PsbS in plants (Takahashi and Badger, 2011). In cyanobacteria, thermal energy dissipation is related to the functioning of the orange carotenoid protein (OCP) which interacts with the core of phycobilisome (PBS) outer antenna under strong blue-green light and mediates thermal energy dissipation at the level of the PBS antenna and thereby reduces the energy arriving at reaction centres (Kirilovsky et al., 2014).

A putative mechanism of photoprotection based on thermal dissipation mediated by the cyanobacterial ancestors of plant antennae, high-light-inducible proteins (Hlips), has been described recently in the cyanobacterium *Synechocystis*. Staleva et al. (2015) brought direct evidence that the Hlips bind both chlorophyll-a and β-carotene and are able to dissipate energy via direct energy transfer from an excited Chl-*a* Qy state to the β-carotene S1 state. The interaction of Hlips with assembly modules and intermediates of Photosystem II (Promnares et al., 2006; Chidgey et al., 2014; Knoppová et al., 2014) implies that they might provide photoprotection during the process of PSII biogenesis (Chidgey et al., 2014; Knoppová et al., 2014; Staleva et al., 2015; Komenda and Sobotka, 2016) .

2.3. PSII Repair

The high photosensitivity leading to frequent photoinactivation of Photosystem II is compensated by an efficient repair mechanism allowing continuous restoration of PSII activity. Despite uncertainty about the precise mechanism of photoinhibition, it is generally accepted that the main target of photodamage is the D1 subunit which shows an extraordinary rapid turnover compared to other PSII core subunits. It has been demonstrated that the repair of PSII is based on selective elimination of the damaged D1 protein and its replacement with a newly synthesized functional copy in so called PSII repair cycle (Nixon et al., 2005; Edelman and Mattoo, 2008; Nixon et al., 2010). This process consists of a partial PSII disassembly, involving detachment of the lumenal extrinsic subunits and the D1 shielding CP43 antenna, allowing access of a protease complex degrading the damaged D1 protein. After the insertion and maturation of a new D1, the complex is reassembled and reactivated (Nixon et al., 2010; Boehm et al., 2012a; Komenda et al., 2012a).

In both cyanobacteria and chloroplasts, the damaged D1 is specifically degraded by FtsH proteases (Nixon et al., 2005; Kato et al., 2009; Kato and Sakamoto, 2009),

membrane-bound zinc metalloproteinases and members of the AAA (ATPases associated with a variety of cellular activities) class of proteins (Tomoyasu et al., 1993). In *Synechocystis*, a thylakoid membrane bound FtsH2/FtsH3 heterocomplex has been identified to be responsible for the specific D1 turnover (Boehm et al., 2012a).

The molecular mechanism of the recognition of D1 protein for its enzymatic degradation has not been fully described to date. However, it has been revealed that the important prerequisite for the D1 to be recognized for degradation is the accessibility of this protein, either it is damaged or not (Krynická et al., 2015).

2.4. Biogenesis of PSII - Principles

PSII biogenesis occurs by step-wise assembling of smaller sub-complexes or modules as demonstrated in *Synechocystis* using mutants specifically blocked in a particular assembly step (rev. Nixon et al., 2010, Komenda et al., 2012a). Each module consists of one large pigment-binding protein (D1, D2, CP47, or CP43) accompanied by low molecular mass PSII subunits and specific protein factors assisting in the assembly (so called auxiliary or assembly factors, see following chapter).

The key protein subunit of PSII is the D1 protein. It binds majority of the co-factors involved in PSII electron transfer including those of the primary charge separation and water oxidation. In *Synechocystis*, D1 is inserted into the membrane as a precursor (pD1) containing a C-terminal extension, which is matured in a two-step process by a specific C-terminal processing protease CtpA (Inagaki et al., 2001; Komenda et al., 2007). The insertion of *Synechocystis* pD1 was shown to require the YidC (SynOxa) membrane insertase (Spence et al., 2004; Ossenbuhl et al., 2006). During or shortly following the insertion, pD1 associates with PsbI (Dobáková et al., 2007) and together with specific auxiliary proteins forms the D1 module. This module then associates with the D2 module, consisting of (at least) the D2 protein and subunits PsbE and PsbF of cytochrome (cyt) b_{559}, forming the PSII reaction centre assembly complex, RCII (Komenda et al. 2004; Komenda et al. 2008) – see Figure 4. At this phase of PSII assembly pD1 is cleaved at the residue A352, yielding a D1 processing intermediate iD1 (Komenda et al., 2007). Subsequently, CP47 module, containing also PsbH, PsbL and PsbT subunits, is attached to form the assembly complex termed RC47 (Boehm et al., 2011; Boehm et al., 2012b). This promotes the cleaving-off of the remaining eight amino acids of the extension to

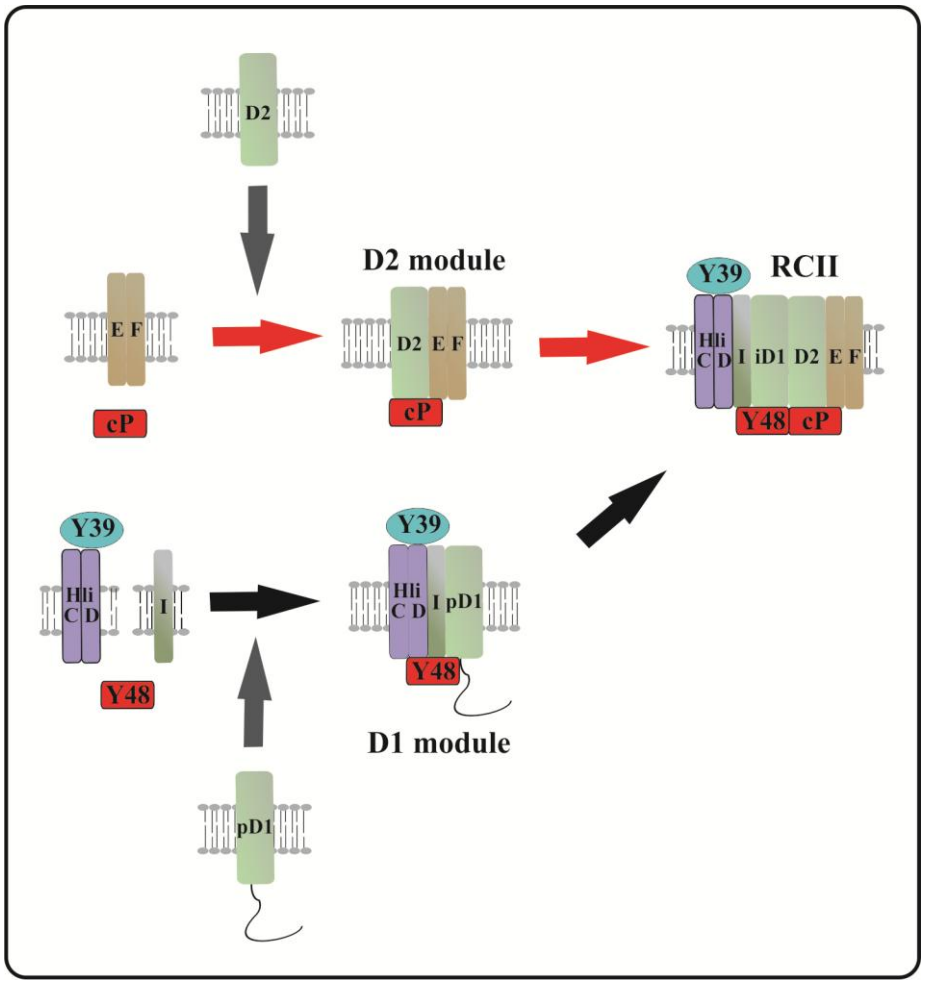

Figure 4 – Initial steps of Photosystem II assembly in *Synechocystis* sp. PCC 6803 leading to the formation of the reaction centre assembly complex, RCII. The auxiliary protein factors participating in this phase are included. The Figure was modified and adapted from Nixon et al. (2010). Designation is as follows: pD1, precursor form of D1; Y48, Ycf48; Y39, Ycf39; HliCD, High-light-inducible proteins C and D; cP, CyanoP.

yield the mature D1 protein. The attachment of the CP43 module binding also PsbK, PsbZ and Psb30 subunits plus the auxiliary factor Psb27 (Boehm et al., 2011; Komenda et al., 2012b) allows formation of the reaction centre core monomer, RCC1. Fully functional PSII is then completed by the light-driven assembly of the oxygen-evolving complex coordinated with the attachment of the lumenal extrinsic proteins, and the PSII dimerization (Komenda et al., 2008; Nixon et al., 2010, Komenda et al., 2012a) - see Figure 5. PSII is supposed to be assembled in essentially the same order in cyanobacteria and chloroplasts which reflects the conservation of the subunits involved and the need for careful coordination of the process.

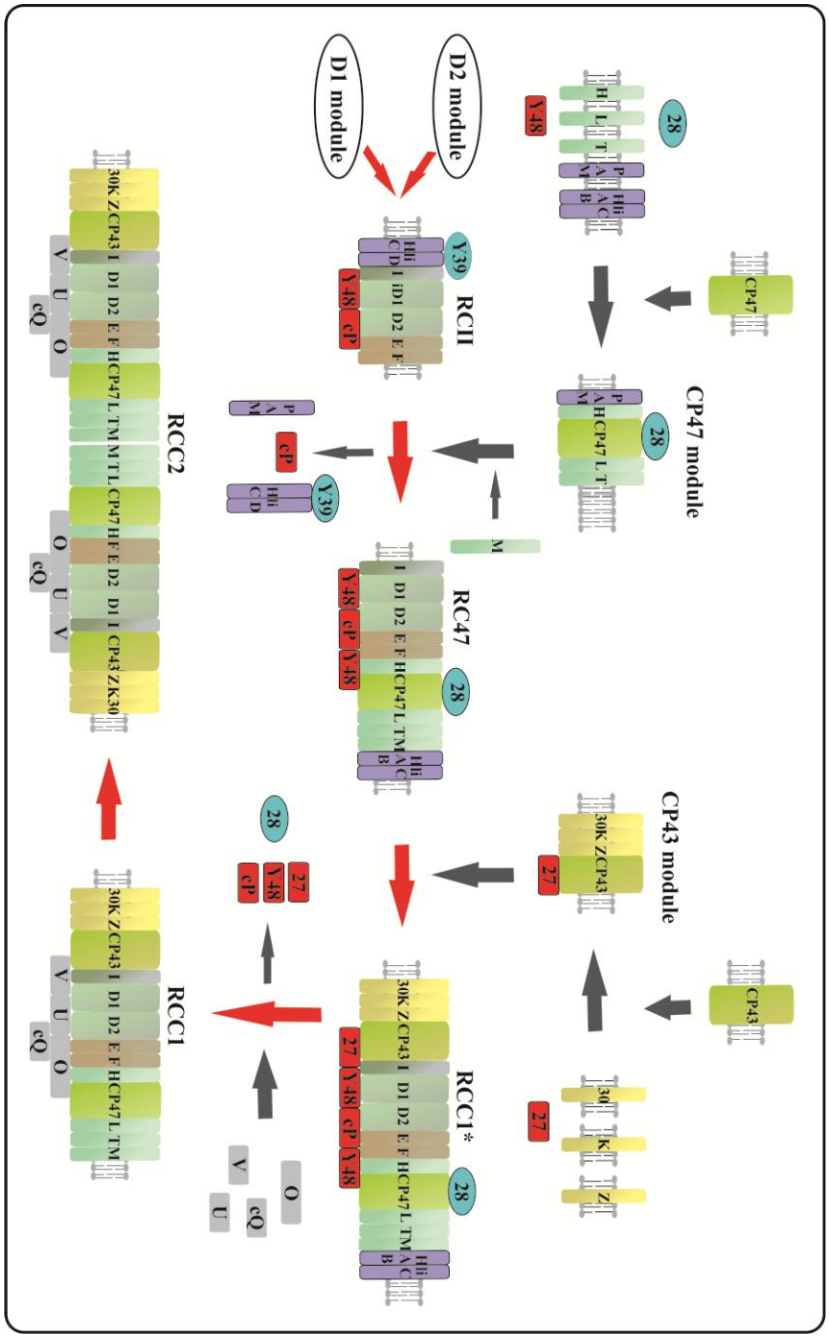

Figure 5 – The assembly of Photosystem II in *Synechocystis* sp. PCC 6803 as described in the text. The auxiliary protein factors and low molecular mass subunits are included. For details of the RCII formation see Figure 4. The Figure was modified and adapted from Nixon et al. (2010). Designation of complexes and auxiliary factors is as follows: RC47, reaction centre core lacking CP43 subunit; RCC1* and RCC1, a non-active and active reaction centre core monomer, resp.; RCC2, reaction centre core dimer; Y48, Ycf48; Y39, Ycf39; HliCD and HliABC, High-light-inducible proteins C and D, and A, B and C, resp.; cP, CyanoP; PAM, PAM68; cQ, CyanoQ; 27, Psb27; 28, Psb28.

The assembly of PSII is closely coordinated with the insertion of pigments and other cofactors. It has been demonstrated that isolated CP47 and CP43 modules as well as the reaction centre sub-complex (RCII) are preloaded with chlorophyll and β-carotene pigments before they are incorporated into the PSII (Boehm et al., 2011; Komenda et al., 2012b; Knoppová et al., 2014). It was also shown that RC47 is unable to oxidize water but is still able to drive oxidation of Yz, a redox-active tyrosine in D1, which is the immediate oxidant of the manganese cluster (Rogner et al., 1991).

The enzymes involved in chlorophyll biosynthesis are suspected to form a multienzyme complex *in vivo*, allowing effective synthesis of chlorophyll and avoiding release of potentially phototoxic precursors (Komenda et al., 2012a). In consistence with this idea, the protochlorophyllide oxidoreductase (POR) was shown to form a complex with chlorophyll synthase (Domanskii et al., 2003), and it was co-purified with Mg-protoporphyrin methylester cyclase (Sobotka, 2014). Chidgey et al. (2014) demonstrated that the chlorophyll synthase catalyzing the final step of chlorophyll biosynthesis can form larger assemblies involving components of the translation-translocon machinery (ribosomal subunits, YidC insertase) and the Ycf39-Hlip complex participating in early steps of PSII biogenesis. It is supposed that this close physical contact between the chlorophyll biosynthesis pathway and PSII assembly machinery, including the co-translational insertion of the proteins, could optimise the insertion of chlorophyll molecules into apoproteins without any chlorophyll-carrier proteins and eliminate unwanted release of potentially dangerous chlorophylls.

2.5. Auxiliary Protein Factors Involved in PSII Biogenesis

2.5.1. Overview

Owing to its highly complex nature, the precise assembly of functional PSII requires the involvement of various auxiliary protein factors which are supposed to facilitate individual assembly steps but are not components of the functional complex (Nixon et al., 2010; Chi et al., 2012; Komenda et al., 2012a; Nickelsen and Rengstl, 2013). They are generally not essential for the photoautotrophic growth, but they help to optimize the biogenesis process and, thereby, to cope with changing environmental conditions.

In *Synechocystis*, most of these factors were identified as components of specific PSII assembly intermediates which accumulate in mutants arrested in particular assembly steps due to the absence of a following "building" module (Komenda et al., 2004; Komenda et al., 2008; Dobáková et al., 2009; Knoppová et al., 2014). According to our recent knowledge, each assembly complex appears to be accompanied by specific auxiliary factor(s) detaching either during the subsequent assembly step or before the finishing of the functional photosystem (see Figure 4 and 5).

Several protein factors have been observed to interact with early PSII intermediates in *Synechocystis*, including Ycf48 (Slr2034), PratA (Slr2048), Ycf39 (Slr0399), and CyanoP (Sll1418). The Ycf48 protein, located in the thylakoid lumen (Hynds et al., 2000), has been implicated in stabilizing the newly synthesized D1 and the reaction centre assembly complex, RCII (Komenda et al., 2008). It has been recently detected also in higher PSII intermediates (Komenda, unpublished results) indicating that it might participate also in later phases of the assembly. The knocking-out of this protein in *Synechocystis* is not fatal but it results in a significantly reduced photoautotrophic growth. Interestingly, the phenotype of the *Arabidopsis ycf48* knock-out is much more pronounced, the mutants could only survive on sucrose-containing medium, caused by their inability to accumulate PSII complexes (Meurer et al., 1998).

The PratA, characterised as a periplasmic Mn^{2+}- binding protein, has not been directly detected in any PSII assembly intermediate, however, it was co-localized in a specific membrane sub-fraction called "PratA defined membranes" (PDM) with substantial amounts of D1 precursor (Schottkowski et al., 2009; Rengstl et al., 2011; Stengel et al., 2012). The PDM is hypothesised to be located at the interface between the plasma and thylakoid membranes, and it is proposed to be the site of

initial steps of PSII biogenesis where the early PSII intermediates are preloaded with manganese (Stengel et al., 2012; Nickelsen and Rengstl, 2013; Rast et al., 2015).

Characterisation of the Ycf39 and CyanoP, including their localisation and putative functions within the photosystem, has been the subject of this thesis. The reviews are in Chapters 2.6.2. and 2.6.3., respectively, and the results are included in Chapter 4 (Knoppová et al., 2014; Knoppová et al., submitted).

Other PSII-related auxiliary factors described so far in cyanobacteria, Psb28 (Sll1398), Psb27 (Slr1645) and PAM68 (Sll0933), are implicated in later steps of the assembly. As shown by Dobáková et al. (2009), Psb28 transiently associates with the RC47 assembly complex at the cytoplasmic side of the thylakoid membrane. Psb27 (see below for review) binds to the lumenal side of the non-active PSII core complexes and is supposed to block premature binding of the extrinsic subunits of the oxygen evolving complex (Nowaczyk et al., 2006). More recent data pointing to the association of Psb27 with the CP43 antenna are included in Chapter 4 of this thesis (Komenda et al., 2012b). The PAM68 (Sll0933) factor potentially interacts with a number of PSII subunits and assembly factors, and it is supposed to be involved in the process of RC47 and PSII core formation (Armbruster et al., 2010; Rengstl et al., 2013).

Most of the PSII assembly factors are common for both cyanobacteria and chloroplasts, presumably indicating a conserved role. There are, however, also others which are specific for plants and algae having no clear cyanobacterial homologues. These probably emerged after the evolution of the chloroplast and therefore reflect possible differences in the PSII biogenesis between chloroplasts and prokaryotes (Nixon et al., 2010; Chi et al., 2012; Nickelsen and Rengstl, 2013).

A role in PSII biogenesis in cyanobacteria has been ascribed also to a family of high-light-inducible proteins (Hlips), called also small cab-like domain proteins (Scps). They are one-helix relatives of the light harvesting chlorophyll a/b-binding proteins of plants and algae, containing domains implicated in chlorophyll and carotenoid binding (Dolganov et al., 1995; Funk and Vermaas, 1999). Several studies provided indirect evidence for binding of pigments to Hlips/Scps (Xu et al., 2002, 2004; Storm et al., 2008) and the experimental evidence has been recently given by Knoppová et al. (2014) and Staleva et al. (2015). Although the Hlips typically accumulate in cells under high light stress, they have been found to associate with various PSII intermediates also under normal non-stressing conditions. They have been suggested to protect the assembly complexes against the photodamage via a quenching mechanism based on the transfer of chlorophyll excitation to the β-

carotene bound to the Hlips (Knoppová et al., 2014; Staleva et al., 2015; Komenda and Sobotka, 2016). The Hlips are also supposed to participate in chlorophyll delivery to newly synthesized chlorophyll-proteins of PSII (Chidgey et al., 2014; Knoppová et al., 2014).

2.5.2. Psb27

Psb27, an approx. 11-kDa protein encoded by the *slr1645* gene in *Synechocystis*, was identified by Kashino et al. (2002) in His-tagged PSII preparations as one of the additional PSII-related proteins absent in the crystal structure of PSII (Zouni et al., 2001; Umena et al., 2011). Psb27 is targeted to the thylakoid lumen and possesses a lipidation site on the N-terminus. Its attachment to the thylakoid membrane via a lipid moiety was confirmed by Nowaczyk et al. (2006) for *Thermosynechococcus elongatus* and by Ujihara et al. (2008) for *Synechocystis*.

PSII complexes purified from a CtpA-less mutant of *Synechocystis* specifically bound the Psb27 and lacked the extrinsic subunits PsbO, PsbV and PsbU and manganese (Roose and Pakrasi, 2004). Similarly, *T. elongatus* Psb27 was localized with a non-active PSII monomer lacking the manganese cluster and the other extrinsic subunits (Nowaczyk et al., 2006). The Psb27-PSII association was therefore suggested to block premature binding of the extrinsic subunits to the arising complex. However, Liu et al. (2011a) found that PsbO, but not PsbV, is still able to bind to monomeric PSII purified via His-tagged Psb27 in *Synechocystis*. Dimeric PSII-Psb27 complexes inactive in oxygen evolution were isolated from *T. elongatus* grown at low temperature (Grasse et al., 2011). Similarly, in *Synechocystis* Psb27 was found to associate not only with monomeric but also with dimeric PSII and even larger complexes, possibly PSII-PSI supercomplexes (Komenda et al., 2012b).

Using independent approaches, Liu et al. (2011b) and Komenda et al. (2012b) gave evidence of a direct interaction between the Psb27 and the lumenal domain of CP43 subunit of PSII in *Synechocystis*. This indicates that the function of Psb27 is mediated through its specific interactions with the CP43. The results of Komenda et al. (2012b) are included in Chapter 4.1. of this thesis.

The solution structure of the soluble domain of Psb27 from *Synechocystis* was initially determined by nuclear magnetic resonance (NMR) spectroscopy (Cormann et al., 2009; Mabbitt et al., 2009). Later, the crystal structure with a resolution of 1.6 Å was obtained by Michoux et al. (2012) from *T. elongatus*. All these structures

revealed the presence of a four-helix bundle in the protein. Based on the structure of Mabbitt et al. (2009) Psb27 was docked *in silico* onto the *T. elongatus* monomeric PSII lacking PsbO, PsbU and PsbV beneath CP43 and D1 in a position usually occupied by PsbO (Fagerlund and Eaton-Rye, 2011). The docking experiments of Michoux et al. (2012) suggested a possible binding site for Psb27 close to the position of PsbV in mature PSII, thus interacting hypothetically with CP43 and D1. In this model Psb27 does not occupy the binding site for PsbO which is consistent with the findings of Liu et al. (2011a) mentioned above. Although the precise location of Psb27 is still unsure, both the models are in agreement with the biochemical data of Liu et al. (2011b) and Komenda et al. (2012b) suggesting the interaction between the Psb27 and CP43.

Physiological studies on mutants of *Synechocystis* deficient in Psb27 demonstrated that the protein is not essential for the photoautotrophic growth. However, competitive growth between wild type and mutant cells indicated that Psb27 provides a selective advantage. Moreover, recovery from photodamage was slowed in the mutant cells (Roose and Pakrasi, 2008). The same authors also demonstrated abolished photoactivation of PSII in the mutant after the chemically induced disassembly of the manganese cluster implying a role for Psb27 in the assembly of the oxygen-evolving complex. The physiological role of the PSII-Psb27 pool existing in the membranes is still unclear. However, it might act as a reservoir of preassembled complexes that can be rapidly photoactivated to replace damaged photosystems under conditions when the rate of PSII damage suddenly exceeds the rate of repair (Komenda et al., 2012b).

Two Psb27 homologues were found in *Arabidopsis thaliana*, Psb27:Psb27-H1 (At1g03600) and Psb27-H2 (At1g05385), and both were identified in the thylakoid lumen (Fagerlund and Eaton-Rye, 2011). The first one has been proposed to participate in PSII repair (Chen et al., 2006) and the second one, called also LPA19, seems to be required for efficient maturation of the D1 precursor (Wei et al., 2010).

2.5.3. Ycf39

The *Synechocystis* open reading frame *slr0399* encodes a protein with a calculated molecular weight of app. 36-kDa. The protein shows high similarity to hypothetical eukaryotic proteins termed Ycf39, encoded in the cyanelle genome of *Cyanophora paradoxa*, chloroplast genome of some non-green algae, and nuclear genome of *Arabidopsis thaliana*. The Ycf39s are characterized by a conserved NAD(P)H-binding

motif of unclear function localized near the N-terminus. They are classified as atypical short-chain dehydrogenases /reductases SDRs-5a, with a cofactor binding motif GXXGXXG (see NCBI for the details).

Cyanobacterial Ycf39 was described by Ermakova-Gerdes and Vermaas (1999) in a study on heterotrophic D2 mutants impaired in Q_A function when they found an autotrophic pseudorevertant with the site of secondary mutation located in the ORF *slr0399*. Deletion of the *slr0399* restored the photoautotrophy in the mutant, but it had no significant effect in the wild type. The authors speculated that the Ycf39 is a chaperon-like protein involved in (but not crucial for) the Q_A insertion into the PSII reaction centre. This idea is, however, difficult to accept. If this protein was a quinone chaperone, its absence would hardly improve the incorporation of the quinone into the Q_A binding pocket. It seems more likely that the Ycf39 protein binds to the reaction centre in the vicinity of the Q_A binding site and that its modification or absence may result in the improved access/binding of the quinone to its binding site altered by the mutation.

The *slr0399* was further mentioned in bioinformatic studies among cold inducible genes, probably regulated by the cold sensor Hik33, as revealed using the DNA microarray technique (Suzuki et al., 2001; Mikami et al., 2002). It is also one of the open reading frames up-regulated in response to inorganic carbon limitation (Wang et al., 2004).

In *Synechocystis* there are two other homologues of Ycf39: *sll1218* and *slr0317*. They both contain the N-terminal NAD(P)H-binding motif and are characterized as atypical SDRs. The putative function of either of these hypothetical proteins has not been studied so far. The attempts to delete the *sll1218* ORF failed implying that the gene product is essential (Sobotka, unpublished).

Protein analysis of Photosystem II precursors of *Synechocystis* mutant lacking the CP47 antenna (PsbB) surprisingly revealed the presence of Ycf39 (Slr0399) in the PSII reaction centre assembly complex (RCII) accumulating in this mutant. A detailed study on the localisation and function of this protein published by Knoppová et al. (2014) is included in this thesis (see Chapter 4.2.). Briefly, Ycf39 has been found to form a previously undiscovered pigment-protein complex with specific members of Hlip family of proteins binding chlorophyll-a and β-carotene (Chapter 2.5.1). This complex binds to the D1 assembly module and becomes a component of the RCII. The data of Knoppová et al. (2014) and the results simultaneously obtained by Chidgey et al. (2014) and Staleva et al. (2015) imply that the Ycf39-Hlip complex is involved in the delivery of chlorophyll to newly synthesized D1 and that it may

protect both the D1 and the RCII from light damage via dissipating energy of Chl excited states by energy transfer to the β-carotenes of the Hlips.

The Ycf39 seems to play a role in PSII biogenesis also in chloroplasts as the Ycf39 homolog HCF244 (High Chlorophyll Fluorescence 244) of *Arabidopsis* has been found to be needed for biosynthesis of the D1 protein and for normal accumulation of PSII (Link et al., 2012).

2.5.4. CyanoP

CyanoP, a product of *sll1418* gene in *Synechocystis* with a predicted molecular weight of 21 kDa, is a distant homologue of eukaryotic PsbP family of proteins located in the thylakoid lumen. The presence of a *psbP* homologue in the genome of cyanobacteria was predicted by De Las Rivas et al. (2004) and the first experimental evidence for the protein expression was given by Thornton et al. (2004). Despite the low sequence similarity between the CyanoP and other members of the PsbP family, they all share a common structural fold, and it is believed that the cyanobacterial homologue represents the basal form of the family. The structural differences derived from both crystal and NMR structures and sequence analyses are supposed to reflect the phylogenetic differentiation within the family and distinct roles which the various members play in cyanobacteria and chloroplasts (Sato, 2010; Michoux et al., 2010; Bricker et al., 2013). However, an interesting difference of the CyanoP which is not shared by the eukaryotic homologues is the presence of an N-terminal lipobox for type II signal peptidase cleavage and a cysteine residue for lipidation, implying that the protein is attached to the thylakoid membrane via a lipidated N-terminus (Thornton et al., 2004; Ishikawa et al., 2005; Fagerlund and Eaton-Rye, 2011).

The most important member of the PsbP family in plants and green algae is the "authentic" PsbP which is one the three extrinsic subunits of PSII shielding the oxygen evolving cluster (Sato, 2010; Bricker et al., 2013). Although non-essential, it has been repeatedly demonstrated to optimize oxygen evolution at physiological concentrations of calcium and chloride, probably via conformational changes required for Ca^{2+} and Cl^{-} retention around the catalytic manganese cluster (Bricker et al., 2013). The most recent crystal structure of the PsbP from spinach by Cao et al. (2015) implies that the protein could be involved in the delivery of manganese ions to the Mn_4CaO_5 cluster through a conformational change of a flexible loop which has been newly revealed to coordinate manganese. However, as mentioned

in Chapter 2.1., the composition of the set of PSII extrinsic subunits has changed during the evolution from cyanobacteria to green plants, and the cyanobacterial homologue plays another, apparently less prominent role (Ifuku et al., 2010; Bricker et al., 2012; Ifuku, 2015).

Besides the "authentic" PsbP, the plant (*Arabidopsis*) family contains two PsbP-like proteins (PPL1/2), and seven PsbP homologues containing a PsbP domain (PPD1-7) (Sato, 2010). They share the same basal structure but lack loops specific for the authentic PsbP (Sato, 2010; Bricker et al., 2013), one of which has been proposed to play a structural and functional role within the oxygen evolving complex (Cao et al., 2015). Some members have clear roles outside PSII including PPL2 which is a subunit of the chloroplast NADH dehydrogenase-like complex (Ishihara et al., 2007; Ifuku et al., 2011) and PPD1 which is required for assembly of PSI in plants (Liu et al., 2012). On the other hand, the PPL1, seems to play a PSII-related role, although there is still no evidence for its direct association with the complex and the abundance of the protein is much lower than that of PsbP. However, the PPL1 *Arabidopsis* mutant is more susceptible to high light photoinhibition and exhibits slower recovery from photodamage than wild type plants (Ishihara et al., 2007). Ifuku et al. (2010) demonstrated that the PPL1 is co-expressed with genes for ribosomal subunits, immunophilins, and several genes for PSII subunits, indicating a possible involvement in stress response regulation of PSII. Interestingly, CyanoP is structurally most related to the PPLs sharing the simplest structure within the family: both PPLs and CyanoP lack short insertions into the loop regions and have a shorter N-terminus (Sato, 2010; Michoux et al., 2010; Bricker et al., 2013).

Analyses of the CyanoP mutants from *Synechocystis* did not reveal any significant phenotype pointing to a clear function of the protein within the PSII (Thornton et al., 2004; Ishikawa et al., 2005; Aoi et al., 2014), although competitive growth experiments showed a selective advantage for the presence of CyanoP even under normal conditions, suggesting its functional significance (Sveshnikov et al., 2007). Conclusions of the cited authors are rather controversial. According to Thornton et al. (2004) it might be a transiently binding regulatory/assembly factor. However, Ishikawa et al. (2005), Sveshnikov et al. (2007) and Aoi et al. (2014) consider it to be a constitutive component of the complex contributing to optimise its functionality.

The *in silico* models attempting to locate the CyanoP within the PSII crystal structures positioned CyanoP on the lumenal face of the D2 protein away from the other extrinsic proteins (Michoux et al., 2010; Bricker et al., 2012). Cormann et al. (2014) performed an *in vitro* interaction analysis between the CyanoP and various

lumenal PSII domains employing the surface plasmon resonance spectroscopy. The results indicated possible interactions between CyanoP and the C-terminal tail of D2 as well as the lumenal loop linking the first two transmembrane helices of D1. These regions are located in the centre of the complex and are normally occupied by the PsbO subunit in the PSII crystal structure (Umena et al., 2011). In addition, *in vitro* reconstitution experiments showed selective binding of CyanoP to the inactive PSII monomer lacking the constitutive extrinsic subunits PsbO, PsbV, and PsbU, while the active PSII dimer bound only trace amounts of CyanoP. Based on these results the authors concluded that the extrinsic subunits and especially PsbO block the binding site of CyanoP, and that CyanoP plays a role in the dynamic PSII lifecycle rather than being a structural subunit of the mature complex.

The approach of our laboratory to determine the function of CyanoP within Photosystem II is based particularly on the interaction analysis of the genetically tagged CyanoP from various PSII mutants blocked in different steps of PSII assembly. It has been demonstrated that this protein binds to PSII in early stage of its biogenesis and that it probably facilitates the attachment of the D2 and D1 assembly modules leading to the RCII formation (Chapter 4.4.). We also show, however, that it accompanies the complex on most of the biogenesis pathway, which is in agreement with its identification within the purified PSII (Thornton et al., 2004; Ishikawa et al., 2005) or with the selective binding of CyanoP to the inactive PSII (Cormann et al., 2014).

2.6. Subcellular Localization of PSII Biogenesis and Repair

2.6.1. Thylakoid or Plasma Membrane?

For a long period it has been believed that the early steps of PSII biogenesis occur in the plasma membrane in *Synechocystis*. This hypothesis was formulated by Zak et al. (2001) analyzing highly pure plasma and thylakoid membranes (PM and TM, resp.) prepared by two-dimensional purification employing separation in sucrose gradient followed by two-phase partitioning as developed by Norling et al. (1998). The components of PSII reaction centre assembly complex (RCII), i.e. D1, D2 and cytochrome b_{559} subunits, were found in both PM and TM, while the D1 maturation protease CtpA was exclusively localized in the plasma membrane and the inner antennae CP43 and CP47 were detected only in the thylakoid membrane fraction. Based on these findings it was concluded that the RCII formation including the CtpA-mediated D1 processing occurs in the plasma membrane and the RCII is then

translocated to the thylakoid membranes, either via hypothetical PM-TM interconnections or by vesicular transport, to complete the biogenesis of the functional PSII.

However, later studies of other laboratories employing the same purification method brought results which were not consistent with this idea. First, contrary to the exclusive presence of the maturation protease in the PM demonstrated by Zak et al. (2001), the precursor form of D1 protein were detected in the thylakoid membranes (Jansen et al., 2002). Later on, the important early assembly factor Ycf48 binding to D1 and facilitating the RCII formation was found predominantly in the TM (Komenda et al., 2008). In addition, Pisareva et al. (2011) reported the TM localization for the YidC (SynOxa), an insertase which is essential for membrane integration of the precursor D1 (Ossenbuhl et al., 2006). Moreover, the FtsH2 component of the FtsH2/FtsH3 protease heterocomplex responsible for the damaged D1 degradation during the PSII repair was detected in the thylakoid membranes using two independent methods, the immunodetection of the enzyme in separated membranes and the green fluorescent protein (GFP)-FtsH2 imaging by confocal fluorescent microscopy (Komenda et al., 2006). During PSII repair, the damaged PSII is partially disassembled to allow access of FtsHs to the damaged D1 protein. After the degradation, the newly synthesized protein is co-translationally inserted into the PSII (Zhang et al., 2001) and simultaneously processed. In the strain lacking CP43 the D1 replacement is especially fast and no unprocessed forms of this protein can be detected even using radioactive labelling (Komenda et al., 2006). The high efficiency of the maturation process implies that the CtpA must be in close proximity to the D1-degrading FtsH proteases, i.e. in the thylakoids.

Also the translocation of the PSII assembly complexes between the membranes suggested by Zak et al. (2001) appears rather controversial as there is still no strong evidence for any kind of the transport of membrane material between the plasma and thylakoid membranes in cyanobacteria, whether via vesicles derived from the plasma membrane or by lateral fusion between the membrane systems (Rast et al., 2015).

The mounting evidence against the plasma membrane location of PSII biogenesis and repair necessitated a revision, which has been done by Selão et al. (2016). This study has been included in this thesis - see Chapter 4.3. Briefly summarised, based on (1) localisation of the initial steps of PSII assembly employing a *Synechocystis* CP47-knock out strain, (2) tracking of D1 synthesis and processing at photoinhibitory light using radioactive pulse-chase labelling, and (3) detection of

the FLAG-tagged CtpA protease, our data allowed to conclude that the early steps of PSII assembly including D1 maturation as well as the repair-related D1 synthesis occur in the thylakoid and not in the plasma membrane.

2.6.2. Communication between the Plasma and Thylakoid Membranes – an Evergreen Enigma

Despite our current belief that the biogenesis of PSII takes place in the thylakoids, there are observations indicating a need of communication between the plasma and thylakoid membranes in cyanobacteria.

This concerns first of all the trafficking of the digalactosyldiacylglycerol (DGDG), one of the major lipid components of both the PM and TM, which is present also in the crystallised PSII. As the cyanobacterial DGDG synthase, DgdA (Awai et al., 2007), seems to occur exclusively within the plasma membrane of *Synechocystis* (Selão et al., 2014), there should exist a transport route for the DGDG delivery into the thylakoids. In theory, there are three ways of lipid trafficking between the membranes: (1) by lateral fusion of the membranes (2) via vesicle transport and (3) by soluble lipid carriers (Rast et al., 2015).

The lateral fusion allowing at least temporary physical connection between the two membrane systems in cyanobacteria is still doubtful. In *Synechocystis* (Figure 2), the thylakoids characteristically converge close to the plasma membrane (Liberton et al., 2006; van de Meene et al., 2006) and these sites of convergence are suspected to be also the sites of PM-TM interconnections (Pisareva et al., 2011; Rast et al., 2015). In reality this was observed in low light grown cells characterized by inflated lumen which allowed the visualization of what appeared to be connections between the thylakoid membrane system and the plasma membrane (van de Meene et al., 2012). These connections were found only in 7% of cells observed in this study which might be considered rather insignificant. However, as the authors argue, they may exist in most cells during cell expansion when thylakoid membranes are actively growing, but have been difficult to detect because of the usually small size and transient nature of the connections. Nevertheless, this is just one finding of this kind which does not allow ultimate conclusions.

The vesicular transport in cyanobacteria is also enigmatic as no membrane vesicles have been ever observed in cyanobacterial cells. It also appears that cyanobacteria do not share homologues of vesicle-related factors found in chloroplasts. The

chloroplast vesicular transport system is most likely of eukaryotic origin, e.g. cytosolic, and it is supposed to be transferred to the chloroplasts during the evolution (Westphal et al., 2003). Employing the bioinformatic approach, homologies between the vesicle-associated proteins from yeasts and cyanobacterial gene products have been indicated recently (Keller and Schneider, 2013), however, the function of these proteins has not been determined yet.

The transport of lipids to assembling membranes or membrane complexes via a soluble transporter has also not been described up to date. Speculations exists, however, that this function might be subscribed to VIPP1 factor which plays an important but still unclear role in biogenesis of thylakoid membranes and maintenance of photosynthetic complexes in plants, algae and cyanobacteria (Gao and Xu, 2009; Nordhues et al., 2012; Zhang and Sakamoto, 2015). The VIPP1 is a typical amphipathic protein, most likely of eubacterial origin, with a characteristic lipid binding region, well suited for membrane binding (Zhang and Sakamoto, 2015). It was originally suggested to participate in vesicle-mediated thylakoid membrane formation (Kroll et al., 2001; Westphal et al., 2001). Recent studies demonstrated a more direct effect of the VIPP1 in maintaining of Photosystem II and I activity in *Synechocystis* (Gao and Xu, 2009) and also its role in the biogenesis of thylakoid membrane core complexes in *Chlamydomonas* (Nordhues et al., 2012). The latter authors hypothesised that its role lies most likely in supplying structural lipids to the assembling complexes. As the VIPP1 of *Synechocystis* has been immunochemically located in both PM and TM fractions by Selão et al. (2016), and the GFP-tagged VIPP1 imaging showed its fast relocation from the cytoplasm to the vicinity of the plasma and thylakoid membranes after the transfer from low to high light (Bryan et al., 2014), the idea of VIPP1 as a mediator of substance transport between the PM and TM cannot be excluded.

The identification of the PSII-related periplasmic factor PratA (Schottkowski et al., 2009; Stengel et al., 2012) also brought an argument supporting the communication between the two membrane systems. This Mn^{2+} - binding protein was co-localised with significant levels of precursor D1 within a specific membrane sub-fraction which has been hypothesised to form specialised compartments at the interface of the plasma and thylakoid membranes, so called biogenesis centres, where the early PSII intermediates might be preloaded with manganese (Stengel et al., 2012; Nickelsen and Rengstl, 2013; Rast et al., 2015). However, an unequivocal confirmation of this hypothesis would require a convincing visualisation of the PratA-pD1 co-localisation at the PM-TM interface which is very difficult due to the small size of *Synechocystis* cells.

3. Summary

The elucidation of the principle of Photosystem II (PSII) biogenesis as a step-wise modular assembly (rev. Nixon et al., 2010) also revealed that this process includes various protein factors which transiently interact with specific PSII intermediates but are not components of the mature complex. They are, therefore, supposed not to affect the functionality of the photosystem but to facilitate particular steps of the assembly. These proteins are usually dispensable for the photoautotrophic growth and the phenotype related to their absence often appears only when the cells are exposed to various stresses. This underlines their role as auxiliary factors which are not essential individually but contribute to optimise the cell growth together with other supporting and protecting mechanisms.

This thesis has been aimed to elucidate localizations and potential roles of three auxiliary proteins, Psb27, Ycf39 and CyanoP, in the cyanobacterium *Synechocystis* sp. PCC 6803. Additionally, it also explores the subcellular localization of the initial steps of PSII biogenesis and repair-related D1 synthesis at the level of the main membrane systems, i.e. plasma and thylakoid membranes, in *Synechocystis*.

The already published data are presented as Parts I - III of the Results (Komenda et al. (2012b), Knoppová et al. (2014) and Selão et al. (2016), respectively). The data concerning CyanoP protein has been recently submitted for publication and the manuscript is included as Part IV.

The study of Komenda et al. (2012b) reveals that the Psb27 protein, previously identified as a factor binding to the lumenal side of the non-active PSII core complexes (Nowaczyk et al., 2006), interacts with the lumenal domain of the CP43 subunit of PSII. Moreover, it has been demonstrated that Psb27 binds to the unassembled CP43 module and as a component of this sub-complex enters the arising PSII core. The evidence is based on several independent experiments showing that (1) Psb27 co-migrates with CP43 in two-dimensional native/SDS gels in both wild type and various PSII mutants, (2) Psb27 specifically co-purifies with the unassembled His-tagged CP43 module, together with the small CP43-interacting PSII subunits, (3) the level of Psb27 correlates with the amount of unassembled CP43 in various PSII mutants and (4) the unassembled CP43 is destabilized in the absence of Psb27. Additionally, the sequence analysis of the CP43 fragments found in Psb27-deficient mutants allowed determining of the putative interaction site in the vicinity of the long lumenal loop of the CP43 between the fifth and sixth transmembrane

helixes. This is consistent with the results of Liu et al. (2011b) who detected a cross-link in this region between the CP43-Asp-321 and Psb27-Lys-63. The absence of Psb27 does not noticeably affect either the assembly or repair. However, it deteriorates the short-time acclimation of the cells to high light. We propose that a plausible physiological role for the non-active PSII core complexes containing Psb27 might be to act as a pool providing preassembled complexes that can be rapidly photoactivated to replace damaged PSII under sudden exposition to photoinhibitory conditions.

Knoppová et al. (2014) identified a novel pigment-binding complex consisting of a predicted PSII assembly factor Ycf39 and two members of the high-light-inducible family of proteins, HliC and HliD. Pull-down experiments employing FLAG-tagged Ycf39 from various PSII mutants combined with the analyses of purified complexes using two-dimensional CN/SDS electrophoresis showed that the Ycf39-Hlip complex is a component of the unassembled D1 module and the D1-D2 reaction centre assembly complex, RCII. While the FLAG-Ycf39 itself was shown to be colourless, separation of the pure FLAG-Ycf39-Hlip complex and the analysis of its absorption spectrum provided the strongest evidence yet for chlorophyll-a and β-carotene binding to Hlips. The FLAG-Ycf39 preparations from the RCII-less mutants also revealed the interaction of the Ycf39 with chlorophyll synthase, the final enzyme of chlorophyll biosynthesis. Our experimental data further suggest that the Ycf39-Hlip is involved in the delivery of recycled chlorophyll to newly synthesized D1 protein during the formation of the RCII. It has been also proposed that the interaction with the Ycf39-Hlip might protect newly synthesized D1 and RCII from light damage. This hypothesis has been recently supported by direct evidence of the energy transfer from chlorophyll excited states to the S1 state of the Hlip-associated β-carotene in the isolated FLAG-Ycf39-Hlip complex (Staleva et al., 2015).

The study of Selão et al. (2016) has been aimed to revise the previous controversial data concerning the localisation of PSII assembly and repair in either plasma (PM) or thylakoid membrane (TM), as reviewed in Chapter 2.6.1. In this study, we employed the CP47-knock out strain of S*ynechocystis* (ΔCP47) accumulating the reaction centre assembly complexes (RCII) due to the block in the subsequent assembly step. This approach allowed plausible detection of the RCII components, including the related assembly factors and the YidC (SynOxa) insertase, in the thylakoid membrane fraction. Moreover, radioactive pulse-chase labelling of the wild type cells under photoinhibitory irradiance stimulating the repair-related D1 synthesis clearly demonstrated that the newly synthesized D1 precursor (pD1) is inserted and processed within the thylakoid membranes. Additionally, the FLAG-tagged version

of the CtpA protease responsible for D1 maturation, identified using a specific commercial antibody against the FLAG-epitope, was also clearly located in TM. These results provide strong evidence that the early steps of PSII assembly as well as the repair-related D1 synthesis occur in the thylakoid membranes, and not in the plasma membrane. Thus, these processes do not require translocation of the related proteins and/or complexes between the two membrane systems as suggested earlier by Zak et al. (2001).

The manuscript included in the Part IV of the Results explores the involvement of CyanoP, an ancestral orthologue of the eukaryotic PsbP subunit of PSII, in the biogenesis of the cyanobacterial Photosystem II. Using the genetic tagging we show here, that CyanoP interacts with the purified PSII reaction centre assembly complex, RCII. Moreover, specific co-purification of D2 and cytb559 subunits with the CyanoP-FLAG and *vice versa* co-purification of CyanoP with the His-tagged D2-cytb559 from the mutant lacking D1 subunit indicate that this protein binds as an auxiliary factor to the D2 assembly module before it associates with the D1 module to form the RCII. It is also demonstrated that the strain lacking CyanoP significantly accumulates newly synthesized unassembled D1 protein compared to the wild type implying retarded D1-D2 association in the mutant. Based on these findings we suggest that the CyanoP assists in the early phase of PSII biogenesis as an assembly factor facilitating the association of D2 and D1 assembly modules. A small amount of PSII core complexes has been also co-purified with tagged CyanoP implying that the protein may accompany PSII on most of its assembly pathway. However, the importance of this interaction remains to be elucidated.

4. Results

4.1. Part I.

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., and Nixon P.J.

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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.

Abstrakt

Zkoumali jsme lokalizaci proteinu Psb27 a jeho úlohu v biogenezi fotosystému II (PSII) u sinice *Synechocystis* sp. PCC 6803. Nativní gelová elektroforéza ukázala, že se Psb27 vyskytuje v monomeru jádra PSII a v menším množství také v jeho dimeru, velkých superkomplexech PSII a ve frakci volných proteinů. Z analýzy mutantů s narušeným skládáním PSII a analýzy subkomplexů PSII izolovaných pomocí histidinového tagu usuzujeme, že se Psb27 váže s volným komplexem CP43 a také s dalšími komplexy, které CP43 obsahují. Místem vazby je pravděpodobně lumenální smyčka spojující 5. a 6. trans-membránovou spirálu proteinu CP43. Snížená akumulace a zvýšená fragmentace volného CP43 po inaktivaci genu pro protein Psb27 u mutanta postrádajícího podjednotku CP47 podporuje názor, že Psb27 hraje úlohu v životním cyklu komplexu CP43. Ve kmeni, který nebyl schopen složit funkční PSII, byl Psb27 překvapivě detekován v komplexu s PSI. Kromě toho byl také kopurifikován s histidin-tagovaným PSI v divokém kmeni. Interakční analýza pomocí eseje ‚yeast two-hybrid' naznačila interakci mezi Psb27 a podjednotkou PSI PsaB. Purifikace potvrdila také interakci mezi CP43 a PSI. Delece genu *psb27* neměla výrazný vliv na skládání a opravu PSII, ale narušila krátkodobou aklimaci buněk při přechodu na vysoké světlo. Naznačená interakce Psb27 a CP43 s PSI poukazuje na možnost, že PSI hraje dosud nerozpoznanou úlohu při vzniku či opravě PSII.

4.2. Part II.

Discovery of a Chlorophyll Binding Protein Complex Involved in the Early Steps of Photosystem II Assembly in *Synechocystis*

Knoppová J., Sobotka R., Tichý M., Yu J., Konik P., Halada P., Nixon P.J., and Komenda J.

The Plant Cell 26: 1200-1212, 2014

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Abstract

Efficient assembly and repair of the oxygen-evolving photosystem II (PSII) complex is vital for maintaining photosynthetic activity in plants, algae, and cyanobacteria. How chlorophyll is delivered to PSII during assembly and how vulnerable assembly complexes are protected from photodamage are unknown. Here, we identify a chlorophyll and β-carotene binding protein complex in the cyanobacterium *Synechocystis* PCC 6803 important for formation of the D1/D2 reaction center assembly complex. It is composed of putative short-chain dehydrogenase/reductase Ycf39, encoded by the *slr0399* gene, and two members of the high-light-inducible protein (Hlip) family, HliC and HliD, which are small membrane proteins related to the light-harvesting chlorophyll binding complexes found in plants. Perturbed chlorophyll recycling in an Ycf39-null mutant and copurification of chlorophyll synthase and unassembled D1 with the Ycf39-Hlip complex indicate a role in the delivery of chlorophyll to newly synthesized D1. Sequence similarities suggest the presence of a related complex in chloroplasts.

Abstrakt

Výkonné skládání a oprava komplexu fotosystému II (PSII) vyvíjejícího kyslík je životně důležitá pro udržování fotosyntetické aktivity v rostlinách, řasách a sinicích. Nevíme však, jak je do PSII dodáván chlorofyl a jak jsou citlivé prekomplexy vznikající při postupném skládání PSII chráněny proti poškození světlem. V tomto článku identifikujeme proteinový komplex vázající chlorofyl a β-karoten, důležitý pro tvorbu prekomplexu reakčního centra obsahujícího podjednotky D1 a D2. Tento komplex je tvořen proteinem Ycf39, který je hypotetickou dehydrogenázou/reduktázou kódovanou genem *slr0399*, a dvěma členy rodiny proteinů indukovaných vysokým světlem, HliC a HliD, což jsou malé transmembránové proteiny příbuzné světlosběrným komplexům rostlin. Narušená recyklace chlorofylu u mutanta postrádajícího Ycf39 a kopurifikace chlorofyl-syntázy a volného D1 s komplexem tvořeným Ycf39 a Hli proteiny (Ycf39-Hlip) naznačují úlohu tohoto komplexu v přísunu chlorofylu nově syntetizovanému proteinu D1. Na základě podobnosti sekvencí můžeme očekávat přítomnost příbuzného komplexu i v chloroplastech.

4.3. Part III.

Photosystem II Assembly Steps Take Place in the Thylakoid Membrane of the Cyanobacterium *Synechocystis* sp. PCC 6803

Selão T.T., Zhang L., Knoppová J., Komenda J., Norling B.

Plant and Cell Physiology 57: 95-104, 2016

DOI 10.1093/pcp/pcv178

Abstract

Thylakoid biogenesis is an intricate process requiring accurate and timely assembly of proteins, pigments and other cofactors into functional, photosynthetically competent membranes. PSII assembly is studied in particular as its core protein, D1, is very susceptible to photodamage and has a high turnover rate, particularly in high light. PSII assembly is a modular process, with assembly steps proceeding in a specific order. Using aqueous two-phase partitioning to separate plasma membranes (PM) and thylakoid membranes (TM), we studied the subcellular localization of the early assembly steps for PSII biogenesis in a *Synechocystis* sp. PCC 6803 cyanobacterium strain lacking the CP47 antenna. This strain accumulates the early D1–D2 assembly complex which was localized in TM along with associated PSII assembly factors. We also followed insertion and processing of the D1 precursor (pD1) by radioactive pulse–chase labelling. D1 is inserted into the membrane with a C-terminal extension which requires cleavage by a specific protease, the C-terminal processing protease (CtpA), to allow subsequent assembly of the oxygen-evolving complex. pD1 insertion as well as its conversion to mature D1 under various light conditions was seen only in the TM. Epitope-tagged CtpA was also localized in the same membrane, providing further support for the thylakoid location of pD1 processing. However, Vipp1 and PratA, two proteins suggested to be part of the so-called 'thylakoid centers', were found to associate with the PM. Together, these results suggest that early PSII assembly steps occur in TM or specific areas derived from them, with interaction with PM needed for efficient PSII and thylakoid biogenesis.

Abstrakt

Biogeneze tylakoidů je složitý proces, vyžadující přesné a načasované skládání proteinů, pigmentů a dalších kofaktorů do funkčních, fotosynteticky aktivních membrán. Zvláštní pozornost je věnována skládání fotosystému II (PSII), jehož centrální protein D1 je velmi citlivý k poškození světlem a má vysoký obrat, zvláště za vysokého světla. Skládání PSII je modulární proces, jehož jednotlivé kroky následují ve specifickém pořádku. Využitím separace ve dvoufázovém systému pro oddělení plazmatické (PM) a tylakoidní (TM) membrány jsme studovali subcelulární lokalizaci časných kroků skládání PSII u kmene sinice *Synechocystis* sp. PCC 6803 s chybějícím proteinem CP47. Tento kmen akumuluje časný komplex tvořený proteiny D1 a D2, který byl lokalizován v TM spolu s příslušnými pomocnými faktory, které se skládání účastní. Sledovali jsme také inzerci a maturaci prekursoru D1 (pD1) pomocí radioaktivního značení metodou ‚pulse-chase'. Protein D1 je zabudován do membrány s C-terminální extenzí vyžadující štěpení specifickou proteázou, C-terminální proteázou CtpA, aby mohlo posléze dojít k sestavení komplexu vyvíjejícího kyslík. Zabudování pD1 a jeho přeměna na maturovaný protein byly za různých světelných podmínek pozorovány pouze v TM. Proteáza CtpA tagovaná epitopem byla rovněž lokalizována v TM, což poskytuje další důkaz pro lokalizaci maturace D1 v tomto typu membrány. Na druhé straně, proteiny Vipp1 a PratA, o kterých se předpokládá, že jsou součástí tzv. tylakoidních center, byly nalezeny v PM. Tyto výsledky společně naznačují, že časné kroky skládání PSII probíhají v tylakoidech nebo určitých kompartmentech od nich odvozených, ale interakce s plazmatickou membránou je pravděpodobně potřebná jak pro výkonnou biogenezi PSII, tak i tylakoidů.

4.4. Part IV.

CyanoP Is Involved in the Early Steps of Photosystem Two Assembly in the Cyanobacterium *Synechocystis* sp. PCC 6803

Knoppová J., Yu J., Konik P., Nixon P.J., Komenda J.

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Abstract

Although the Photosystem II (PSII) complex is highly conserved in cyanobacteria and chloroplasts, the PsbU and PsbV subunits stabilizing the oxygen-evolving Mn_4CaO_5 cluster in cyanobacteria are absent in chloroplasts and have been replaced by the PsbP and PsbQ subunits. There is, however, a distant cyanobacterial homologue of PsbP, termed CyanoP, of unknown function. Here we show that CyanoP plays a role in the early stages of PSII biogenesis in *Synechocystis* sp. PCC 6803. CyanoP is present in the PSII reaction centre assembly complex (RCII) lacking both the CP47 and CP43 modules and binds to the smaller D2 module. A small amount of larger PSII core complexes co-purifying with FLAG-tagged CyanoP indicates that CyanoP can accompany PSII on most of its assembly pathway. A role in biogenesis is supported by the accumulation of unassembled D1 precursor and impaired formation of RCII in a mutant lacking CyanoP. Interestingly, the pull-down preparations of CyanoP-FLAG from a strain lacking CP47 also contained PsbO indicating engagement of this protein with PSII at a much earlier stage in assembly than previously assumed.

Abstrakt

Ačkoliv je fotosystém II (PSII) fylogeneticky vysoce konzervovaný, podjednotky PsbU a PsbV, které u sinic stabilizující komplex vyvíjející kyslík, nejsou přítomné v chloroplastu, kde byly nahrazeny podjednotkami PsbP a PsbQ. U sinic však byl nalezen vzdálený homolog proteinu PsbP, nazvaný CyanoP, o neznámé funkci. Tato studie ukazuje, že u sinice *Synechocystis* sp. PCC 6803 hraje CyanoP úlohu v časných stádiích biogeneze PSII. Protein CyanoP je přítomen v subkomplexu reakčního centra PSII, tzv. RCII, postrádajícím navázané moduly CP43 a CP47, a váže se také k menšímu modulu D2. Malé množství subkomplexů PSII o vyšší molekulové hmotnosti kopurifikovaných s FLAG-tagovaným CyanoP naznačuje, že tento protein pravděpodobně doprovází PSII téměř celou dráhou jeho biogeneze. Úlohu CyanoP v biogenezi dokládá také akumulace volného prekursoru proteinu D1 a narušená tvorba RCII u mutanta postrádajícího CyanoP. Je zajímavé, že preparát purifikovaného FLAG-CyanoP z kmene s chybějící podjednotkou CP47 obsahuje také PsbO, což naznačuje dosud nepředpokládané angažmá této lumenální podjednotky v biogenezi PSII.

5. Conclusions

This thesis contributes to elucidation of the localization and function of three auxiliary protein factors, Psb27, Ycf39 and CyanoP, involved in the biogenesis of Photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803. Additionally, it includes a study examining localization of the initial steps of PSII biogenesis and repair-related D1 synthesis at the level of the main membrane systems, i.e. plasma and thylakoid membranes, in this cyanobacterium. The thesis is based on two co-author and one first-author publications, and one first-author manuscript. The main results are as follows:

- Psb27 protein factor interacts with a lumenal domain of the Photosystem II subunit CP43 and stabilizes it against degradation. The absence of Psb27 does not noticeably affect the assembly or repair, but it aggravates short-term acclimation of the cells to high light.

- Ycf39 protein forms a complex with two members of high-light-inducible family of proteins, HliC and HliD. The pigment analysis of pure Ycf39-Hlip complex allowed to provide the strongest direct evidence yet for the chlorophyll-a and β-carotene binding to the Hlips.

- The Ycf39-Hlip complex binds to the unassembled D1 protein and the reaction centre assembly complex, RCII. Moreover, it also interacts with chlorophyll-synthase (ChlG), the final enzyme of the chlorophyll biosynthesis pathway. The latter association was confirmed also by Chidgey et al. (2014). Our experimental data suggest that the Ycf39-Hlip is involved in the delivery of recycled chlorophyll to newly synthesized D1 protein during the formation of the RCII and that the interaction of the Ycf39-Hlip might protect newly synthesized D1 and RCII from light damage.

- The evidence has been provided that the early steps of PSII assembly as well as the repair-related D1 synthesis occur in the thylakoid membrane of *Synechocystis*. This revises a previous controversial finding of Zak et al. (2001) localizing these processes in the plasma membrane.

- CyanoP, the cyanobacterial homologue of the PsbP extrinsic subunit of plant Photosystem II, assists in the early phase of PSII biogenesis as an assembly

factor facilitating the association of D2 and D1 assembly modules. Although it appears that it may accompany PSII on most of its assembly pathway, the importance of this interaction is still unclear. CyanoP is considered to be the ancestral form of the PsbP family present in all groups of oxygenic autotrophs. The functional diversification of members of this family presumably reflects the ability of the basal structural fold to accommodate changes in primary structure (Sato, 2010; Bricker et al, 2013). Thus, it seems plausible that PsbP evolved from CyanoP to bind more tightly to the PSII core and at the same time to form new interactions with plant specific components of PSII.

This thesis has brought a number of interesting questions which would deserve attention in future work. The study of Knoppová et al. (2014) revealed a functional link between the Ycf39 (Slr0399) factor and the Hlips. However, the role of this protein itself is still unknown. As mentioned above, the Ycf39 is characterized as a putative dehydrogenase/reductase possessing a conserved NAD(P)H-binding motif at the N-terminus. Confirmation of the functionality of this motif and elucidation of a potential enzymatic activity of the Ycf39 would provide additional information about the function of this protein. A feasible approach might be to construct a mutant modified in the NAD(P)H-domain and to follow its phenotype and characteristics related to the proposed function of the Ycf39 in chlorophyll metabolism or delivery. Moreover, there are two other Ycf39 homologues of unknown functions in *Synechocystis*, encoded by the genes *sll1218* and *slr0317*. At least one of them, Sll1218, seems to be essential for the cell survival as our preliminary attempts to knock out its coding sequence were unsuccessful. Identification of the role of this Ycf39 homologue would give us a hint of a general functioning of the Ycf39 proteins and possibly also of their specific role in the early phase of PSII biogenesis. The construction and characterization of a mutant with regulatable expression of *Sll1218* allowing gradual depletion of the Sll1218 protein is a possible strategy how to determine its function.

The preparation of the reaction centre assembly complex, RCII, isolated from the CP47-less mutant using the FLAG-tagged CyanoP surprisingly contained PsbO, a constitutive subunit of the oxygen evolving complex of mature PSII. This finding which implies a putative engagement of the PsbO in early PSII biogenesis should be confirmed by additional experiments. A suitable approach might be the employment of the genetically tagged PsbO transformed into PSII mutants blocked in various steps of the assembly. Analysis of tag-specific pull-downs should

afterwards reveal the interactions between the PsbO and various PSII intermediates.

Although it has been demonstrated that the biogenesis of PSII occurs in the thylakoid membranes of *Synechocystis*, there are indications that the thylakoids are compositionally and functionally heterogeneous (Agarval et al., 2010; Mullineaux, 2014) and that both the initial steps of PSII assembly and PSII repair occur in specific zones within the TM (Stengel et al., 2012; Nickelsen and Rengstl, 2013; Sacharz et al., 2015). The sub-localisation of the various steps of PSII biogenesis requires specific and reliable *in situ* visualisation methods, which, however, collides with the very small size of the *Synechocystis* cells. It has been confirmed that the immunogold labelling in this cyanobacterium does not provide unequivocal results (Bučinská and Sobotka, unpublished), however, the visualization combining fluorescence labelling of specific proteins and confocal microscopy appears to be more promising (Krynická et al., 2014; Sacharz et al., 2015; Kaňa, unpublished results). In order to localize the initial phases of PSII biogenesis within the thylakoids it would be useful to construct and employ fluorescence labelled derivatives of assembly factors like Ycf39, which associate exclusively with early PSII assembly intermediates (Knoppová et al., 2014). In addition, the confocal microscopy imaging of appropriate specific mRNAs after fluorescent *in situ* hybridization could be used to localize the sites of PSII assembly and repair in cyanobacteria. This approach has been successfully used in *Chlamydomonas* (Uniacke and Zerges, 2007) to visualize the membrane zones related to PSII biogenesis.

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