Characterization of factors for the formation and function of thylakoid membrane biogenesis centers in *Synechocystis* sp. PCC 6803

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SUMMARY

In organisms performing oxygenic photosynthesis, the thylakoid membrane harbors all pigment-protein complexes of the light-dependent photosynthetic reactions, i.e. photosystems I (PSI) and II (PSII), cytochrome b_6f (Cyt b_6f) as well as the ATP synthase. In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), thylakoids are organized in membrane sheets following the cellular periphery. Occasionally, they converge toward the plasma membrane to form so-called biogenesis centers. The PratA-defined membrane (PDM) resides in the biogenesis center and has been described as the site of early PSII assembly. The *de novo* assembly of PSII is a process highly ordered in space and time, which requires the stepwise attachment of several precomplexes facilitated by a number of specific assembly factors. However, the exact function and importance of biogenesis centers, in particular concerning PSII assembly, remained a matter of debate.

The absence of CurT, which represents the cyanobacterial homolog of the grana-shaping CURVATURE THYLAKOID1 protein family from *Arabidopsis thaliana*, resulted in massive alterations of the thylakoid membrane ultrastructure. Accordingly, the thylakoid membrane in *curT*$^-$ ran in circular structures without converging to the plasma membrane, thereby offered the possibility to analyze the consequences of the lack of biogenesis centers for the first time. Hence, *curT*$^-$ displayed a severe increase in doubling time and a decrease in photosynthetic activity. The absence of biogenesis centers gave rise to a reduction in PSII accumulation, assembly and repair whereas PSI and Cyt b_6f were not affected. In the wild-type, CurT was distributed all over the thylakoids, locally accumulating in biogenesis centers. Moreover, CurT formed high-molecular-weight complexes in PDMs and thylakoids, differing in size and isoform composition. Furthermore, a massive relocalization of CurT to the plasma membrane occured during osmotic stress and interestingly, the absence of CurT resulted in an increased sensitivity to osmotic stress in *curT*$^-$. Since *curT*$^-$ was able to accumulate osmotic stress-related compatible solutes in wild-type-like amounts and dynamics, an independent function of CurT via membrane stabilization in osmotic stress tolerance is proposed. Thus, the establishment of thylakoid membrane architecture and the formation of biogenesis centers depend on CurT, which enhances photosynthetic performance and viability of *Synechocystis*.

The protein Slr0151 has previously been assigned as a factor contributing to PSII repair. Here, evidence of an additional involvement of Slr0151 in *de novo* assembly of PSII is provided. In addition, a partial localization of PSII repair to biogenesis centers is discussed.

In conclusion, the data presented in this thesis significantly enhances the current knowledge on biogenesis center formation and their PSII-specific function in *Synechocystis*.

ZUSAMMENFASSUNG

In Organismen, die oxygene Photosynthese betreiben, sind alle Pigment-Protein-Komplexe der Lichtreaktion – die Photosysteme I (PSI) und II (PSII), Cytochrom b_6f (Cyt b_6f) sowie die ATP-Synthase – in der Thylakoidmembran lokalisiert. Eine Eigenschaft des Cyanobakteriums *Synechocystis* sp. PCC 6803 (hiernach *Synechocystis*) ist, dass die Thylakoide parallel zur Zellhülle in übereinanderliegenden Schichten angeordnet sind. Die Thylakoidmembranen konvergieren in gewissen Abständen zur Plasmamembran und bilden an jenen Annäherungspunkten sogenannte Biogenesezentren. Ein Bestandteil der Biogenesezentren ist die PratA-definierte Membran (PDM), der Ort früher Schritte in der PSII-Assemblierung. Die *de novo* Assemblierung von PSII ist streng geordnet hinsichtlich ihrer räumlichen und zeitlichen Abfolge und erfordert die schrittweise Anlagerung mehrerer Vorkomplexe, was durch eine Vielzahl an spezifischen Assemblierungsfaktoren ermöglicht wird. Die genaue Funktion sowie die Bedeutung der Biogenesezentren insbesondere für die PSII-Assemblierung sind jedoch noch immer Gegenstand von Diskussionen.

Die Abwesenheit von CurT, das das cyanobakterielle Homolog der granaformenden CURVATURE THYLAKOID1 Proteinfamilie aus *Arabidopsis thaliana* darstellt, resultierte in schwerwiegenden Veränderungen der Ultrastruktur der Thylakoidmembran. In der *curT*$^-$-Mutante bildete die Thylakoidmembran zirkuläre Strukturen, die nicht in Richtung Plasmamembran konvergierten und somit keine Biogenesezentren aufwiesen. Diese Entdeckung eröffnete erstmalig die Gelegenheit zu untersuchen, wie sich die Abwesenheit von Biogenesezentren auswirkt. Die *curT*$^-$-Mutante wies geringeres Wachstum sowie einen Abfall der Photosyntheseaktivität auf. Im Detail führte die Abwesenheit der Biogenesezentren zu einer Reduktion der Akkumulation, Assemblierungseffizienz sowie Reparatur von PSII, wohingegen Cyt b_6f und PSI nicht beeinflusst wurden. Im Wildtyp war CurT über die gesamte Thylakoidmembran verteilt und akkumulierte lokal an Biogenesezentren. Des Weiteren bildete CurT hochmolekulare Komplexe in PDMs und Thylakoiden, die sich in ihrer Größe sowie Isoformkomposition unterschieden. Darüber hinaus wurde unter osmotischem Stress eine umfangreiche Relokalisierung von CurT in Richtung der Plasmamembran beobachtet. Im Gegensatz zum Wildtyp war *curT*$^-$ anfälliger gegenüber osmotischem Stress. Sogenannte Kompatible Solute stellen in Cyanobakterien die wichtigste Antwort auf osmotischen Stress dar und konnten in *curT*$^-$ in gleichem Maße und gleicher Dynamik wie im Wildtyp akkumulieren. Daher wurde angenommen, dass eine CurT-vermittelte Stressantwort unabhängig von Kompatiblen Soluten sondern per Membranstabilisierung existiert. Zusammenfassend führt die Gegenwart von CurT zur Steigerung der Photosyntheseeffizienz

sowie der Lebensfähigkeit von *Synechocystis*, da es sowohl die gesamte Thylakoidmembran strukturiert sowie die Bildung von Biogenesezentren bedingt.

Dem Protein Slr0151 wurde bislang eine Funktion in der Reparatur von PSII zugeschrieben. Dass Slr0151 darüber hinaus eine Rolle in der *de novo* Assemblierung von PSII spielt, wird in dieser Arbeit dokumentiert. Weiterhin wird eine partielle Lokalisierung der PSII-Reparatur in Biogenesezentren diskutiert.

In ihrer Gesamtheit tragen die in dieser Arbeit dargestellten Daten maßgeblich zu einem besseren Verständnis der Bildung und Funktion von Biogenesezentren in *Synechocystis* bei.

I. INTRODUCTION

I.1 Oxygenic photosynthesis

Oxygenic photosynthesis is a process converting sunlight into chemical energy, which utilizes the photolysis of water to create an electron transport chain and thereby it releases oxygen into the atmosphere. It emerged more than 2.4 billion years ago culminating in a great oxygenation event changing earth's atmosphere, which was the cornerstone for the evolution of life as we know it today (Hohmann-Marriott and Blankenship, 2011; Nelson, 2011). Oxygenic photosynthesis occurs in cyanobacteria, green algae and plants, where it evolved further and specialized (Nelson and Ben-Shem, 2005).

The photosynthetic electron transport chain consists of several thylakoid membrane-embedded pigment-protein complexes, namely the photosystems I (PSI) and II (PSII) as well as the cytochrome b_6f complex (Cyt b_6f). In order to increase the efficiency of harvesting light energy, photosynthetic organisms possess specialized complexes, which are attached to the photosystems. In plants and green algae, these complexes are called light-harvesting complexes (LHCs) and are integrated into the thylakoid membrane, whereas, in contrast, cyanobacteria absorb light energy via soluble phycobilisomes (Mullineaux, 2005; Neilson and Durnford, 2010; Ruban, 2015). For the transport of an electron the initial excitation of one of the pigments in the light-harvesting complexes is required by a photon originating from sunlight (Figure 1). This energy is transferred to the reaction center of PSII by Förster

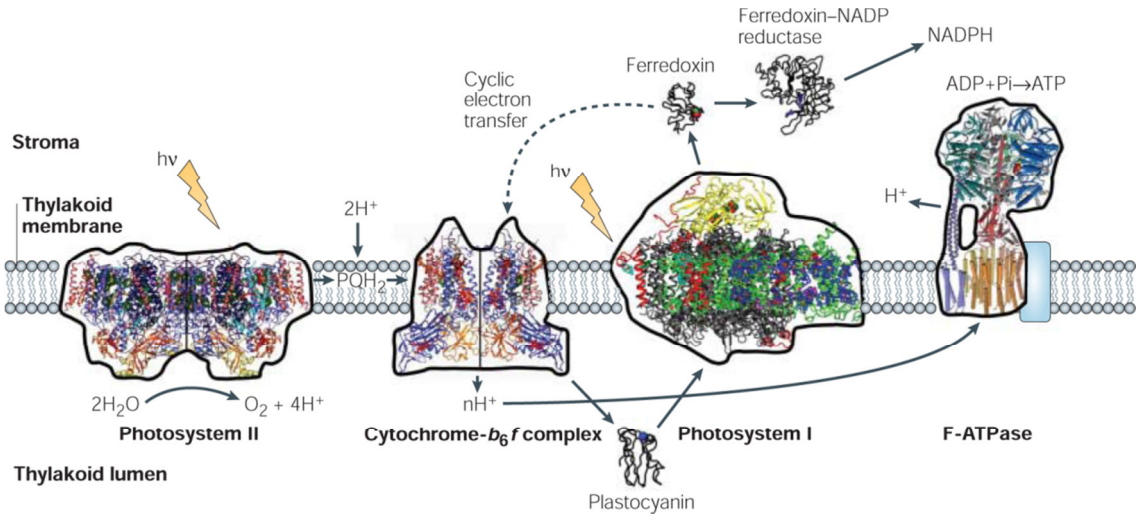

Figure 1: Schematic model of the light reations of photosynthesis. The model presents the path of the electrons throughout the pigment-protein complexes, enzymes as well as electron carriers of the photosynthetic electron transport chain. The profiles and structures of the complexes are depicted with a black line. Solid and dashed gray lines present the linear and cyclic electron transfer, respectively. The yellow lightning indicates the necessity of light energy in PSII and PSI. Adapted from Nelson and Ben-Shem (2004).

resonance energy transfer, where the energy provided by the sunlight excites an electron in one of the two chlorophyll molecules called the special pair of PSII (see below; Saito et al., 2011; Şener et al., 2011; Nelson and Junge, 2015). Thus, the photosynthetic electron transport chain is activated, causing the consecutive transfer of the excited electron from the special pair to a pheophytin to the two plastoquinones Q_A and Q_B (Nelson and Yocum, 2006). The lacking electron in the special pair is immediately filled-up by another electron, which arises from the photolysis of water, a process facilitated by the manganese cluster of the oxygen-evolving complex (OEC; Suga et al., 2015). Thereby the OEC releases molecular oxygen as a byproduct. The transfer of the electron to the special pair is catalyzed via the specific redox-active tyrosine residue Y_Z of the D1 subunit (Shen, 2015). When the quinone Q_B is reduced twice by the consecutive transport of two electrons, it dissociates from PSII and enters the plastoquinone pool circling between PSII and the Cyt b_6f complex (Figure 1). The two electrons from the reduced quinone are transferred to the Cyt b_6f complex, thereby releasing two protons into the thylakoid lumen. One electron passes the complex via cytochrome b_6 to reenter the plastoquinone pool. The other electron follows the linear electron transport chain including its transfer by the Rieske iron-sulfur protein and cytochrome *f*, wherefrom the electron is passed on to PSI via plastocyanin or cytochrome c_6 (Figure 1; Nelson and Ben-Shem, 2004). There, another photon is absorbed by the light-harvesting machinery and the energy is transferred to the special chlorophyll pair of PSI to excite the electron for a second time. By its transport via several chlorophylls, a quinone and three iron-sulfur clusters, the electron traverses PSI and effects the reduction of the soluble electron carrier ferredoxin (Melkozernov et al., 2006; Nelson and Yocum, 2006). Via ferredoxin, the electron is finally delivered to the ferredoxin-$NADP^+$ reductase, which mediates the reduction of $NADP^+$ resulting in the formation of the reducing equivalent NADPH (Figure 1; Mulo, 2011).

Over the course of the electron transport chain, protons are released into the thylakoid lumen, thereby giving rise to a proton gradient. These protons emerge from the oxidation of water at the OEC as well as from the plastoquinone pool. The gradient acts as proton-motive force, which is necessary to power the proton-driven ATP synthase located in the thylakoid membrane (Figure 1; Junge and Nelson, 2015). Together, the synthesized NADPH and ATP provide the energy required for the carbon reactions of photosynthesis. During the carbon reaction, CO_2 is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the Calvin-Benson-Cycle, which leads to the synthesis of sugars for further metabolic processes (Michelet et al., 2013; Buchanan, 2016b, a).

The origin of photosynthesis in algae and plants dates back to an ancient cyanobacterium that lived about 1.5-1.2 billion years ago (Zimorski et al., 2014; Archibald, 2015). This cyanobacterial cell has been incorporated in a eukaryotic host cell, a process called endosymbiosis (Gould et al., 2008; McFadden, 2014; Martin et al., 2015). However, the origin of plastids in all algae and plants dates back to this single primary endosymbiotic event (Schimper, 1883; Mereschkowsky, 1905; Sagan, 1967). Over time, the endosymbiont as well as cyanobacteria adapted, evolved and diversified, but the basic principle of oxygenic photosynthesis is still highly conserved (Cavalier-Smith, 2000; Keeling, 2010; Allen et al., 2011). Some of the most striking acquisitions of plastids are the very sophisticated regulation of organellar gene expression or the development of light-harvesting complexes integrated into the thylakoid membrane, enabling the tight organization of the thylakoid membrane in grana stacks (Mullineaux, 2005; Gould et al., 2008; Nickelsen and Rengstl, 2013; Pribil et al., 2014; Nelson and Junge, 2015). A complex regulation became necessary, since the majority of former endosymbiotic genes has been transferred to the nucleus of the eukaryotic cell during evolution restricting the organellar gene expression to a small number of genes (Gray, 1999; Ku et al., 2015). Therefore, an accurate coordination of gene expression in plastid and nucleus is required including the rise of an efficient and directed protein import machinery (Wobbe et al., 2008; Nickelsen et al., 2014; Sommer and Schleiff, 2014).

In addition to the primary endosymbiosis between cyanobacterium and eukaryotic host, further events involving the uptake of an endosymbiont, which already contained a primary plastid, occurred during evolution. These events are called secondary or tertiary endosymbiosis and led to an increase in plastid diversity and complexity (Gould et al., 2008; Archibald, 2009; Zimorski et al., 2014; section VI.2, Rast et al., 2015).

I.2 *Synechocystis* sp. PCC 6803 – A cyanobacterial model organism

Oxygenic photosynthesis first developed in an ancestor of present-day cyanobacteria (Blankenship, 2010). Of the cyanobacteria known today, *Gloeobacter violaceus* is probably the most ancient representative. *Gloeobacter* is the sole cyanobacterial species that possesses no internal thylakoids (Rippka et al., 1974). However, its plasma membrane presents some specialized domains containing all the complexes required for oxygenic photosynthesis, thereby reflecting a very primordial form of photosynthetic compartmentalization (Rexroth et al., 2011). From an evolutionary point of view, this might be the origin of thylakoid membrane formation (Gupta and Mathews, 2010; Rexroth et al., 2011; Bernát et al., 2012).

During evolution, internal thylakoids arose and cyanobacteria developed into a very divers phylum, which can be found in various environments on earth (Rippka et al., 1979). Unicellular cyanobacteria range from marine genera like *Prochlorococcus* or *Cyanothece* to freshwater genera as *Synechocystis* to rather extreme species, e.g. the thermophilic *Thermosynechococcus elongatus* or the halotolerant *Aphanothece halophytica* (Miller et al., 1976; Rippka et al., 1979; Komárek and Cepák, 1998; Nakamura et al., 2002; Rocap et al., 2003; Scanlan et al., 2009). Some other cyanobacteria can be found in organized multicellular filamentous structures, for example *Spirulina*, *Anabaena* or *Nostoc*, whereas the two latter ones are able to differentiate into vegetative cells and nitrogen-fixing heterocysts (Schirrmeister et al., 2011; Komárek et al., 2014).

The model organism *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is a unicellular, non-nitrogen fixing cyanobacterium. It was isolated from a freshwater lake in California by R. Kunisawa in 1968 (Stanier et al., 1971). *Synechocystis* cells show typically a diameter of ~2 µm and are Gram-negative (Rippka et al., 1979). They consist of an outer membrane followed by the plasma membrane. Between these two membranes resides the periplasmic space, which also contains a layer of peptidoglycan (Figure 2). In *Synechocystis*, the thylakoid membrane is arranged in layers located at the cellular periphery. Occasionally they converge toward the plasma membrane forming so-called biogenesis centers (Figure 2; Liberton et al., 2006; van de Meene et al., 2006; Stengel et al., 2012). It is unclear, whether

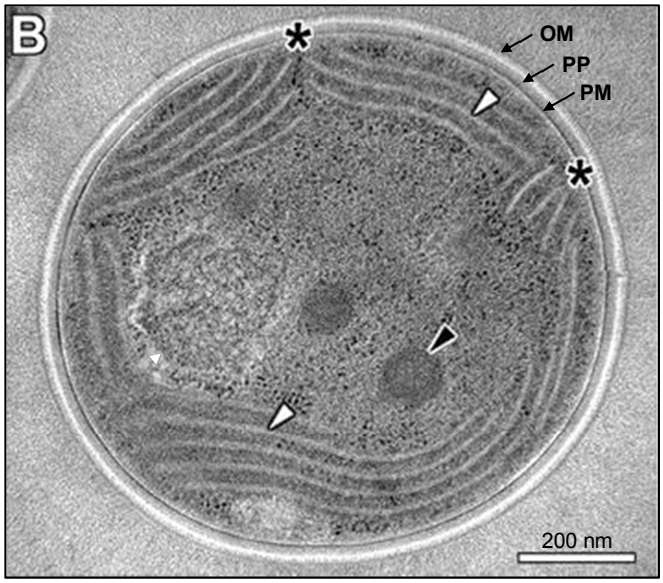

Figure 2: Ultrastructure of a *Synechocystis* wild-type cell. The transmission electron micrograph shows a representative wild-type cell. It displays the outer membrane (OM), the plasma membrane (PM) as well as the periplasmic space (PP) in between. The thylakoid membranes (white arrowhead) are visible as sheets inside the cytoplasm (C). Regions, where the thylakoids converge toward the plasma membrane to form biogenesis centers, are highlighted with black asterisks and a carboxysome is labeled with a black arrowhead. Adapted and modified from van de Meene et al. (2006).

biogenesis centers establish a direct connection between thylakoids and plasma membrane, but nevertheless, several cellular processes e.g. ion homoeostasis or PSII assembly take advantage from the spatial proximity of thylakoid and plasma membrane (see section I.4; Schottkowski et al., 2009a; Stengel et al., 2012; Nickelsen and Rengstl, 2013). Studies on the composition of biogenesis centers revealed a rod-like structure called thylakoid center at the cellular periphery, which attaches the thylakoid membrane to the plasma membrane (Kunkel, 1982). In more detail, thylakoid membranes appear to emerge from or rather converge toward a rod-like structure, which possibly resembles the thylakoid center (van de Meene et al., 2006). It has been hypothesized, that the cyanobacterial homolog of the vesicle-inducing protein in plastids (Vipp1) is involved in thylakoid center formation since *in vitro* assemblies of Vipp1 showed high structural similarities to thylakoid centers (Fuhrmann et al., 2009; Rütgers and Schroda, 2013). Moreover, Vipp1-dependent membrane fusion of liposomes has been observed in *in vitro* studies, indicating the possibility of Vipp1-mediated membrane attachment or even the establishment of direct connections (Hennig et al., 2015; Heidrich et al., 2016). However, *in vivo* proof for the involvement of Vipp1 in biogenesis center formation has not yet been provided. Another recent study suggested a semicircular membrane structure surrounding the thylakoid center, which is called PratA-defined membrane (PDM) since it depends on the presence of the PSII assembly factor PratA (Stengel et al., 2012). This membrane subcompartment harbors the early steps of PSII assembly and remarkably, active PSII as well as PSI are absent from PDMs (Rengstl et al., 2011). Therefore, the heterogeneity of the thylakoid membrane enables a clear differentiation between functional and developing thylakoids in *Synechocystis*. Despite all the efforts on solving the nature of biogenesis centers, their exact ultrastructural organization as well as their multifarious functions have been a matter of intensive debate for several years and still remain elusive (section VI.2, Rast et al., 2015).

Synechocystis was the first photosynthetic organism with a completely sequenced genome (Kaneko et al., 1996). It consists of one main chromosome as well as seven minor plasmids with a total size of about 3.9 Mb encoding 3725 genes (Yang and McFadden, 1993, 1994; Kaneko et al., 1996; Xu and McFadden, 1997; Kaneko et al., 2003). The natural competence for the uptake of DNA in combination with its integration via homologous recombination enables the targeted mutagenesis of genes in *Synechocystis* (Grigorieva and Shestakov, 1982; Barten and Lill, 1995). Together with the possibility to utilize glucose as additional or sole carbon source, *Synechocystis* became an excellent tool in photosynthetic research (Williams, 1988; Vermaas, 1996). Over the years a multitude of mutants has been

generated by targeted knock-out of photosynthetic genes, leading to several fundamental contributions to the understanding of photosynthesis. For example, this includes the effects of inactivation of single or multiple major PSII subunits, namely D1, D2, CP47 and CP43, on each other's accumulation and stability as well as the basic principle of stepwise assembly of PSII or the existence and/or function of diverse assembly factors (Yu and Vermaas, 1990; Komenda et al., 2004; Nickelsen and Rengstl, 2013). Recently, *Synechocystis* also attracted attention for the utilization of the photosynthetically fixed CO_2 in biotechnological applications, e.g. biofuel production (Yu et al., 2013; Branco dos Santos et al., 2014; Gao et al., 2016).

I.3 Structure and composition of photosystem II in cyanobacteria

Over the last few years, several studies provided high-resolution crystal structures of cyanobacterial PSII and thereby gained valuable insight into subunit and cofactor organization in PSII (Ferreira et al., 2004; Guskov et al., 2009; Umena et al., 2011). Cyanobacterial PSII is a pigment-protein complex, which is present as a dimer in its functional state. Two identical PSII monomers form the PSII dimer, which is associated with the light-harvesting complexes of cyanobacteria called phycobilisomes (Arteni et al., 2009; Chang et al., 2015). Based on the most recent structure at 1.9 Å from *Thermosynechococcus vulcanus*, a PSII monomer consists of 20 protein subunits and a multitude of cofactors, including 35 chlorophyll a molecules, 2 pheophytins, 11 β-carotenes, over 20 lipids, 2 plastoquinones, 2 haems, 1 non-haem iron, 4 manganese atoms, 3-4 calcium atoms, 3 Cl^- ions, 1 bicarbonate ion and more than 15 detergents (Umena et al., 2011).

An active PSII monomer is a 350 kDa complex embedded in the thylakoid membrane and consists of the reaction center proteins D1 and D2 which are attached to the inner antenna proteins CP43 and CP47 (Figure 3; Ferreira et al., 2004; Nelson and Yocum, 2006). The D1/D2 reaction center forms the functional heart of PSII. The special pair of chlorophylls as well as the other cofactors directly involved in the electron transport chain, i.e. pheophytin, the quinones Q_A and Q_B, the tyrosine Y_Z as well as most of the ions of the manganese cluster are bound to D1 and D2 (Umena et al., 2011). The manganese cluster of the OEC consists of four manganese ions and a calcium ion which are interconnected by five oxygen atoms (Chernev et al., 2016). In close proximity of the manganese cluster, two chloride ions have been identified, which are most likely involved in stabilizing or regulating the amino acid ligands of the manganese cluster (Rivalta et al., 2011). Notably, one of the amino acid residues serving as ligands in binding the manganese cluster is provided by CP43, whereas the

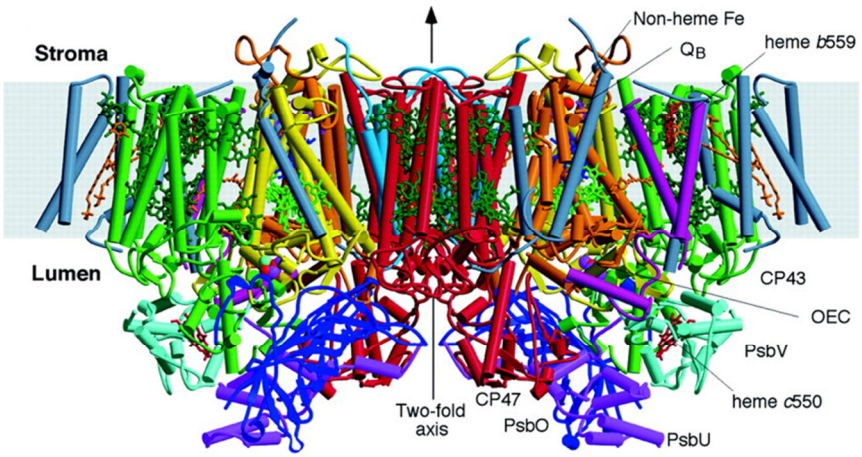

Figure 3: Structural model of a cyanobacterial PSII dimer. The X-ray structure of isolated PSII from *Thermosynechococcus elongatus* illustrates the structures and organization of cyanobacterial PSII subunits in a dimer. Protein subunits are depicted as follows; D1, yellow; D2, orange; CP47, red; CP43, green; Cyt b_{559}, purple; low-molecular-weight subunits, light blue; PsbO, dark blue; PsbU, dark purple; PsbV, cyan. The color-coded pigments include chlorophylls of D1 and D2, light green; chlorophylls of inner antenna proteins, dark green; pheophytins, blue; β-carotenes, orange; hemes, red; nonheme iron, red and Q_A and Q_B, purple. In the OEC, manganese is illustrated in purple, calcium in cyan and oxygen in red. Adapted from Ferreira et al. (2004).

remaining residues originate from D1 (Shen, 2015). Moreover, four water molecules were described to be attached to the manganese cluster with presumably at least one of them serving as substrate for water oxidation (Umena et al., 2011).

The majority of chlorophyll molecules of PSII is bound to the inner antenna proteins CP43 and CP47, indicating their importance in energy transfer to the reaction center proteins during light-harvesting. Furthermore, the α- and β-subunits of the haem-binding cytochrome b_{559} (Cyt b_{559}; PsbE and PsbF, respectively) are attached to D2 close to the Q_B binding site and the PsbI subunit stabilizes the D1 protein (Figure 3). In addition, several low-molecular-weight subunits with a single transmembrane domain are attached to the complex including PsbH, PsbJ, PsbK, PsbL, PsbM, PsbT, PsbX, PsbY, PsbZ and Psb30 (Figure 3; Pagliano et al., 2013). However, the composition of the low-molecular-weight subunits of PSII differs between cyanobacteria and higher plants. Moreover, significant variations are also displayed in the lumenal subunits of PSII protecting the OEC. In cyanobacteria, the extrinsic subunits are made up of PsbO, PsbU and PsbV, while the two latter ones are replaced by PsbP and PsbQ in plants. Interestingly, homologs of the PsbP- and PsbQ-like protein families are also present in cyanobacteria, called CyanoP and CyanoQ. Albeit their absence in the most recent crystal structure, their presence in the active complex is still under debate (Umena et al., 2011; Bricker et al., 2013). Nonetheless, CyanoP has been found to be expressed in substoichiometric amounts compared to PSII (Thornton et al., 2004). Consistent with that finding, several recent studies suggested a function related to PSII assembly for CyanoP,

thereby substantiating its absence from the final complex (Cormann et al., 2014; Jackson and Eaton-Rye, 2015; Knoppová et al., 2016). In contrast, there are contradictory studies regarding the role of CyanoQ. On the one hand, CyanoQ is absent from the most recent PSII crystal structures obtained from *T. elongatus* and *T. vulcanus* (Ferreira et al., 2004; Guskov et al., 2009; Umena et al., 2011). On the other hand, CyanoQ has been found exclusively in biochemically isolated PSII complexes, exhibiting the highest oxygen evolution activity as compared to other PSII isolates lacking CyanoQ in *Synechocystis* (Roose et al., 2007). Moreover, in *T. elongatus* CyanoQ has been detected substoichiometrically compared to PSII, but more than one CyanoQ proteins were present per PSII in quantifications of *Synechocystis* protein extracts (Thornton et al., 2004; Michoux et al., 2014). Another study even identified a PSII complex from *Synechocystis* with four copies of CyanoQ attached to the OEC, but lacking PsbU and PsbV, which most likely reflects a late assembly intermediate (Liu et al., 2015). These observations might indicate species-specific differences in PSII structure at least on the level of the extrinsic lumenal subunits of PSII. In particular, it might argue for differences between cyanobacterial species in binding of CyanoQ to the mature PSII dimer.

I.4 *De novo* assembly of photosystem II in *Synechocystis*

All the subunits and cofactors described above require a coordinated assembly process to form and accumulate active PSII dimers. *De novo* biogenesis of PSII in cyanobacteria is a stepwise process, highly ordered in space and time. The formation of a functional PSII dimer occurs in a defined chronological order of subsequent attachments of precomplexes, which are catalyzed and regulated by a number of specific assembly factors. These factors interact transiently with PSII assembly intermediates during the assembly, thereby determining the efficiency of this process.

The biogenesis of PSII starts with the co-translational integration of the precursor of the D1 protein (pD1) into the membrane via the SecYEG translocon assisted by the YidC insertase (Spence et al., 2004; Ossenbühl et al., 2006; Gathmann et al., 2008; Sachelaru et al., 2013; Chidgey et al., 2014; Plöchinger et al., 2016). The pD1 insertion machinery is closely linked with the final steps of chlorophyll synthesis, since a complex consisting of Ycf39, two chlorophyll-binding one-helix proteins HliC and HliD, which are representatives of the high-light inducible protein (Hlip) family, as well as the chlorophyll synthase (ChlG) interacts with pD1 upon its membrane integration (Figure 4; Chidgey et al., 2014; Knoppová et al., 2014). ChlG catalyzes the transformation from chlorophyllide to chlorophyll before HliD mediates the immediate chlorophyll transfer and incorporation into pD1. This interaction depends on

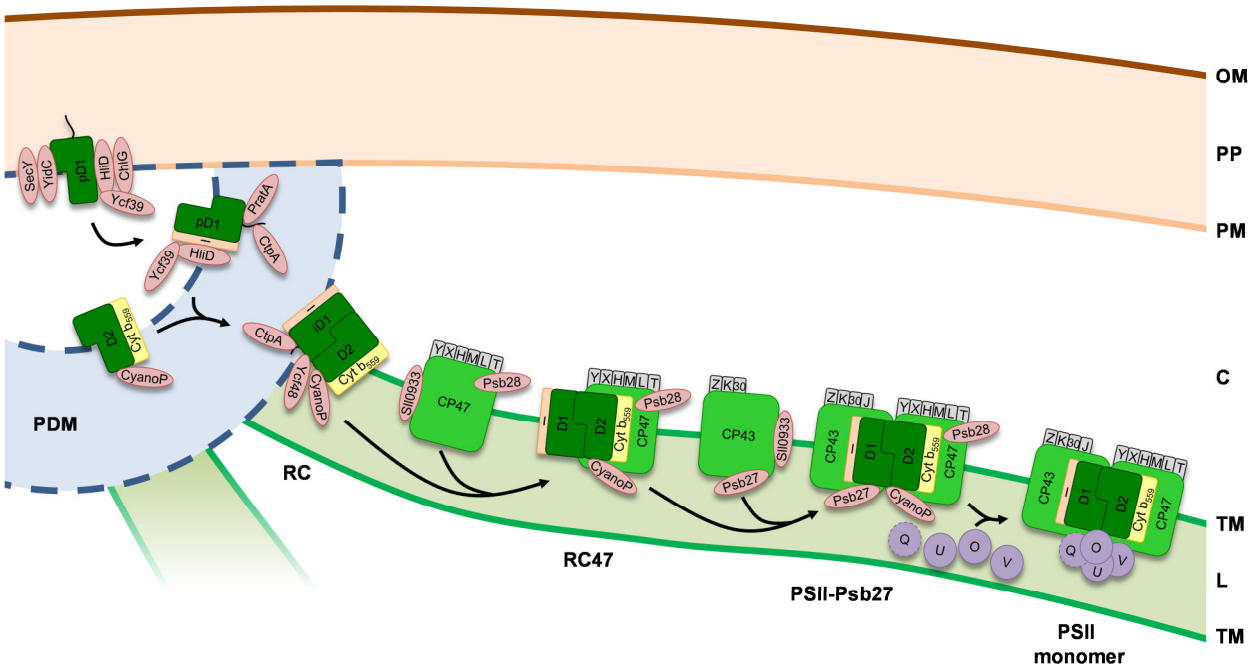

Figure 4: Working model of the spatiotemporal assembly of PSII in *Synechocystis*. The step-wise assembly of PSII is shown from pD1 insertion through precomplex attachment up to the functional PSII monomer. Subsequently, two PSII monomers dimerize and a phycobilisome is attached. Early steps of PSII biogenesis take place in the PDMs and subsequently the process continues in the thylakoid membrane. Since the exact ultrastructure of biogenesis centers still remains elusive, PDMs are illustrated as dashed blue lines. The model is derived from yeast two-hybrid interaction studies, mass spectrometry, further biochemical analysis and functional considerations. Please see the text for more detailed description. The major chlorophyll-binding subunits D1, D2, CP47 and CP43 are depicted in green, Cyt b_{559} in yellow, PsbI in orange, other intrinsic low-molecular-weight subunits in gray, extrinsic subunits stabilizing the OEC in purple and assembly factors in red. A dashed line defines CyanoQ since its presence in the functional PSII complex is still under debate. pD1, precursor of D1; Cyt b_{559}, cytochrome b_{559} (composed of PsbE and PsbF); RC, reaction center complex lacking CP47 and CP43 modules; RC47, reaction center complex lacking CP43 module; PSII-Psb27, PSII monomer lacking the extrinsic lumenal subunits; C, cytoplasm; L, lumen; OM, Outer membrane; PDM, PratA-defined membrane; PM, plasma membrane; PP, periplasm; TM, thylakoid membrane. Adapted and modified according to Figure 1 in section VI.1 (Heinz et al., 2016a).

the presence of Ycf39, which thereby enables the co-translational delivery of chlorophyll to the newly synthesized, growing pD1 protein (Chidgey et al., 2014; Knoppová et al., 2014). Consecutively, the inserted, folded and cofactor-loaded pD1 binds to the low-molecular-weight subunit PsbI and together they form the pD1-PsbI precomplex (Dobáková et al., 2007).

The question concerning the site of pD1 insertion has been the subject of many controversial studies over the years. Early investigations suggested an insertion into the plasma membrane and the subsequent transport to the thylakoids, but more recent work indicated an important role of thylakoid membrane biogenesis centers, in particular the PDM membrane subcompartment (Smith et al., 1992; Zak et al., 2001; Schottkowski et al., 2009a; Rengstl et al., 2011). This subfraction connects plasma and thylakoid membrane and harbors

the early steps of PSII assembly (Figure 4). The early PSII assembly factor PratA serves as marker for this region, since it has been located to biogenesis centers via immunogold-labeling (Stengel et al., 2012). Membrane fractionation via two-step sucrose density gradient centrifugation demonstrated a co-migration of PratA and pD1 alongside other early assembly factors in this fraction (Schottkowski et al., 2009a; Rengstl et al., 2011). Intriguingly, another protein involved in chlorophyll synthesis, namely the light-dependent protochlorophyllide oxidoreductase (POR), as well as its interaction partner Pitt (POR-interacting TPR protein) were biochemically detected in PDMs, emphasizing the close spatial relationship between chlorophyll synthesis and early PSII assembly (Schottkowski et al., 2009b).

However, the presumably most important feature of PDMs is constituting the site of pD1 preloading with at least some of the manganese ions, which are required for the formation of the OEC (Stengel et al., 2012). The manganese is delivered via PratA, mediated through its direct interaction with the C-terminus of pD1 (Figure 4; Klinkert et al., 2004; Schottkowski et al., 2009a). The accumulation of pD1 strongly depends on the presence of the assembly factor Ycf48, which interacts and thereby stabilizes pD1 during this stage of PSII biogenesis (Komenda et al., 2008). In *Synechocystis*, the C-terminal extension of pD1 is comprised of 16 amino acids. The cleavage of the extension, which is facilitated by the C-terminal processing protease (CtpA), occurs in a two-step manner, with the first processing step resulting in the intermediate form of D1 (iD1; Anbudurai et al., 1994; Komenda et al., 2007). Subsequently, the D2 precomplex including the reaction center protein D2 and Cyt b_{559} is attached, thereby forming the RC complex (Figure 4; Komenda et al., 2004). The assembly factor CyanoP has recently been described to be involved in early steps of PSII assembly, since it possesses a high affinity to the lumenally exposed D2 C-terminus and is already associated to the D2 precomplex (Figure 4; Cormann et al., 2014; Knoppová et al., 2016). From there it most likely accompanies the growing PSII complex until the extrinsic subunits protecting the OEC are attached. Formation of the RC complex most likely depends on an interaction of the D2-associated CyanoP and Ycf48 bound to the D1 module (Knoppová et al., 2016).

Subsequent to RC formation in the PDMs the complex migrates into the thylakoid membrane, where the remaining steps of PSII biogenesis take place (Nickelsen and Rengstl, 2013; section VI.2, Rast et al., 2015; section VI.1, Heinz et al., 2016a). The formation of the RC47 assembly intermediate necessitates the attachment of the CP47 module to the RC complex. This precomplex consists of the inner antenna protein CP47 as well as the low-molecular-weight subunits PsbH, PsbL, PsbM, PsbT, PsbX and PsbY (Figure 4; Boehm et al.,

2011; Boehm et al., 2012a). During formation of the RC47 complex, the second CtpA-mediated cleavage step transforming iD1 into the mature D1 protein takes place resulting in the completely processed protein as the major form of D1 in RC47 (Komenda et al., 2007). Moreover, the assembly factors Psb28 and Sll0933 are both involved in the stabilization of the CP47 precomplex, whereas Psb28 can also be found in the RC47 intermediate complex (Figure 4; Dobáková et al., 2009; Rengstl et al., 2011). In addition, Sll0933 has been shown to directly interact with CP47 as well as Ycf48, thereby suggesting a role as mediator of CP47-attachment to the RC complex (Rengstl et al., 2013). Furthermore, three members of the Hlip protein family, namely HliA, HliB and HliC, have been described to interact with CP47 to photoprotect it prior and after its attachment to the RC complex (Promnares et al., 2006; Boehm et al., 2012a; Yao et al., 2012; Staleva et al., 2015; Komenda and Sobotka, 2016). Thereafter, RC47 interacts with the CP43 module adding the second inner antenna protein CP43 and the low-molecular-weight subunits PsbK, PsbZ and Psb30 to the complex (Figure 4; Boehm et al., 2011). The CP43 precomplex is stabilized by the lumenal assembly factor Psb27, whose presence defines the resulting PSII-Psb27 complex, which only lacks the external subunits protecting the OEC (Komenda et al., 2012). In addition to Psb27, Sll0933 has been shown to interact also with CP43 (Rengstl et al., 2013). It appears likely, Sll0933 facilitates again the attachment of the precomplex to the assembly intermediate. However, this process also depends on the assembly factor Sll0606, indicated by the loss of PSII monomers or dimers in its absence (Zhang et al., 2010). Thus, with the attachment of the CP43 module all the membrane-integrated subunits of PSII are assembled including the low-molecular-weight subunit PsbJ, which has neither been detected in the CP47 nor the CP43 module (Boehm et al., 2012a). In absence of PsbJ, the PSII-Psb27 assembly intermediate accumulated, thereby indicating the importance of PsbJ for PSII biogenesis, but mechanism and time of its attachment to the complex remains elusive (Nowaczyk et al., 2012).

According to the latest crystal structure, all ligands required for the binding of the manganese cluster of the OEC are present during this stage of PSII biogenesis (Umena et al., 2011; Shen, 2015). Therefore, the light-driven assembly of the OEC called photoactivation occurs in this state. It is triggered by the photooxidation of one manganese ion resulting in a defined series of manganese rearrangements as well as the incorporation of the calcium ion culminating in the active manganese cluster of PSII (Becker et al., 2011). During photoactivation Psb27 most likely prohibits an early attachment of the extrinsic subunits by occupation of the PsbV docking site (Mamedov et al., 2007; Cormann et al., 2016). A similar role has been implicated for CyanoP, since it binds to the lumenal side of PSII and therewith

blocks the site of PsbO binding (Cormann et al., 2014). Due to their close proximity to the manganese cluster during photoactivation, a potential role for Psb27 and CyanoP in facilitating this process has been hypothesized although proof for this assumption is still lacking (Roose and Pakrasi, 2008; Cormann et al., 2014). Thus, with the detachment of Psb27 and CyanoP, the extrinsic subunits stabilizing the manganese cluster, namely PsbO, PsbU, PsbV and possibly CyanoQ, are able to bind to the complex forming a functional PSII monomer (Figure 4; Roose and Pakrasi, 2008).

The transition from PSII monomer to dimer is promoted by the interplay of PsbI and PsbM (Kawakami et al., 2011). Subsequently, with the attachment of the phycobilisome, the biogenesis of the active PSII dimer is completed.

I.5 Repair of photodamaged PSII in *Synechocystis*

In Cyanobacteria as well as in chloroplasts, D1 is the target of a constant turnover due to its high susceptibility to photodamage. Therefore, the permanently damaged D1 protein is fast and selectively replaced, which requires the partial disassembly of PSII. Consecutively, a newly synthesized D1 protein is inserted into the complex. Under high-light intensities, *Synechocystis* D1 has a half-life time of ~20 minutes and its replacement is indispensable to prevent the cell from permanent damage (Tyystjärvi et al., 1994).

Photosynthesis as well as other metabolic processes continuously produce highly toxic reactive oxygen species (ROS) as byproduct (Apel and Hirt, 2004). Unfortunately, the production of ROS strongly increases under high-light conditions due to limitations in the photosynthetic capacity of PSII. Besides several other mechanisms, ROS formation under high-light conditions has been observed on the electron acceptor and donor sides of PSII caused by ineffective electron transport, e.g. when the light-harvesting machinery of PSII absorbs more energy than PSII can utilize. However, on the acceptor side, ROS are produced due to an overreduction of the plastoquinone pool, whereas on the donor side of PSII, the incomplete oxidation of water favors the formation of ROS (Pospíšil, 2009). Under high-light intensities, the high accumulation of ROS radicals often gives rise to undesired redox states of the special pair of chlorophylls in PSII. In turn, the special pair's increased reactivity damages nearby residues resulting in irreversible inactivation of the D1 protein (Ohad et al., 1984; Yu and Vermaas, 1990). If the damage to PSII exceeds its repair, the term photoinhibition is used and the viability of the organism is seriously endangered. Since damaged PSII is no longer able to facilitate the regular redox reactions required for electron transport, the production of further ROS species is implied. Thus, the detoxification of ROS and the reutilization of PSII

by repairing the damaged complex is necessary to keep up the cellular metabolism (Nixon et al., 2010).

Similar to the *de novo* assembly of PSII, the replacement of D1 demands for auxiliary proteins acting as specific repair factors facilitating the steps of the repair cycle. However, several of these factors involved in PSII repair have been identified and interestingly, some of these factors are already known from their function in PSII assembly (Nickelsen and Rengstl, 2013). To make photodamaged D1 accessible for degradation, the extrinsic subunits as well as the CP43 module has to be detached from the PSII monomer (Figure 5). As a consequence, PSII resides in a state similar to the RC47 complex from *de novo* assembly (Mabbitt et al., 2014). Without CP43 bound to the complex, the D1 protein is exposed and can be targeted by

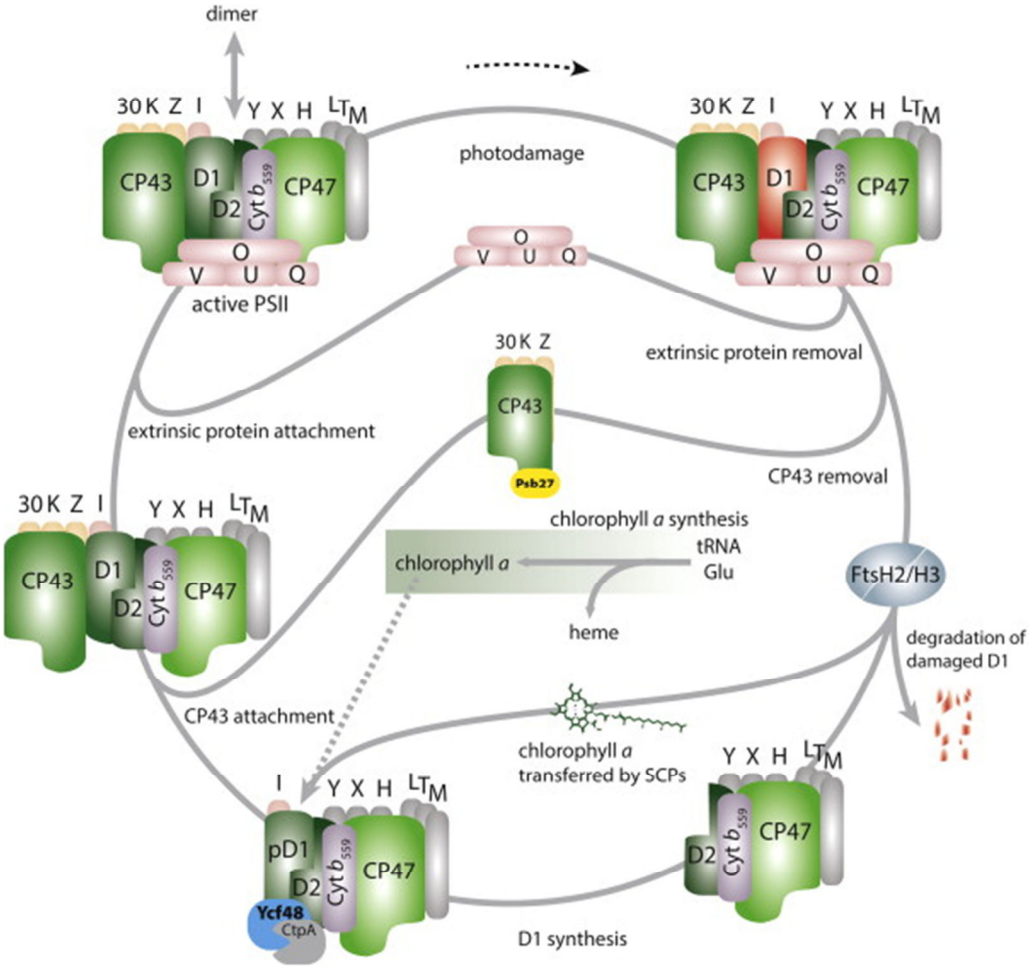

Figure 5: Working model of the repair cycle of PSII. This recent working model graphically summarizes the current knowledge on PSII repair. It displays the steps required to replace a photodamaged D1 protein including disassembly and reassembly of the PSII complex. SCPs (small Cab-like proteins) is another term for the high-light inducible proteins (Hlips). Please see the text for more detailed description. Concerning the major PSII subunits, D1 and D2 are depicted in dark green, the inner antenna proteins in light green and photodamaged D1 in red. The other subunits as well as repair factors of PSII are illustrated as follows: Cyt b_{559}, purple; PsbI, rose; low-molecular-weight subunits attached to CP47, grey; low-molecular-weight subunits attached to CP43, orange; external subunits protecting the OEC, pink; FtsH2/FtsH3 complex, blue-gray; Psb27, yellow; Ycf48, blue; CtpA, dark gray. Adapted and modified according to Mabbitt et al. (2014).

proteases. An involvement in D1 degradation has been discussed for two families of proteases, namely FtsH and Deg, which consist of four and three copies in *Synechocystis*, respectively (Nixon et al., 2005). Earlier, Deg proteases have been hypothesized to be the key players in D1 degradation, but more recent studies provided growing evidence, that the proteases of the FtsH family are the ones actually degrading D1 (Huesgen et al., 2009; Nixon et al., 2010). FtsH proteases form high molecular mass complexes and FtsH2 is directly involved in early cleavage of photodamaged D1 as well as degradation of the resulting fragments (Silva et al., 2003; Yoshioka-Nishimura and Yamamoto, 2014). In the absence of FtsH2, which appears to be the most important protease in the D1 repair cycling, the D1 turnover is strongly reduced (Komenda et al., 2006). Nevertheless, a minor level of degradation can still be observed, implicating other factors contributing to D1 degradation, possibly due to partial functional replacement by other FtsH or Deg proteases (Komenda et al., 2010). Furthermore, a hetero-oligomeric complex consisting of FtsH2 and FtsH3 has been described as the major complex degrading D1 during PSII repair (Boehm et al., 2012b).

Subsequent to the extraction of the impaired D1, a newly synthesized copy of pD1 is inserted co-translationally into the complex via the same machinery as in *de novo* assembly. Similar to PSII *de novo* biogenesis, the newly synthesized protein is most likely stabilized by Ycf48, since it has been shown to promote pD1 accumulation during PSII repair (Komenda et al., 2008). The insertion of pD1 gives rise to a complex that is again similar to the RC47 assembly intermediate (Figure 5; Nickelsen and Rengstl, 2013). Subsequently, CP43 is attached to the complex and the inserted pD1 is processed by CtpA forming a complex that resembles the PSII-Psb27 complex (Figure 5; Mabbitt et al., 2014; Theis and Schroda, 2016). Also the availability of the CP43 module depends on factors with identical functions during *de novo* assembly, namely Sll0933 and Psb27. In particular, Psb27 has been described as important factor in PSII repair, where it most presumably stabilizes the dissociated CP43 module. Furthermore, Psb27 and Sll0933 are presumably involved in the attachment of CP43 to the RC47-like complex (Nowaczyk et al., 2006; Grasse et al., 2011; Komenda et al., 2012; Rengstl et al., 2013). Remarkably, an increased accumulation of the PSII-Psb27 complex has been observed under high-light, underlining the importance of the assembly/repair intermediate during stress conditions (Nowaczyk et al., 2006). As in PSII assembly, an involvement of CyanoP in the assembly of the extrinsic subunits has been proposed (Cormann et al., 2014). For the sake of completeness, the factors Slr1768 and Psb32 have been described as important factors for PSII repair, but their exact function remains elusive (Bryan et al., 2011; Wegener et al., 2011).

It is noteworthy, that chlorophyll does not turn over in a comparable rate as D1 under high-light conditions (Vavilin et al., 2005). This strongly suggests the idea of reutilization of chlorophyll (Vavilin and Vermaas, 2007). Indeed, members of the Hlip protein family have been described to be involved in chlorophyll recycling during PSII repair and an increase in chlorophyll turnover has been observed in their absence. Therefore, it has been proposed, that Hlip proteins bind chlorophyll released from PSII during the replacement of D1 (Figure 5; Vavilin et al., 2007). This process is an important feature of PSII repair, since free chlorophyll tends to ROS production in the light (Apel and Hirt, 2004; Pospíšil, 2012). In addition, the Ycf39 protein is involved in the reutilization of chlorophyll and presumably acts as a mediator of Hlip-dependent transport of chlorophyll molecules to newly synthesized pD1 in repair and/or *de novo* assembly (Knoppová et al., 2014).

Recently, the protein Slr0151 has been described as a factor involved in PSII repair (Yang et al., 2014). Slr0151 is an intrinsic membrane protein and possesses two tetratricopeptide repeat (TPR) domains. TPR domains have been shown to form two antiparallel α-helices, which facilitate protein-protein interactions (Blatch and Lässle, 1999; Bohne et al., 2016). Moreover, TPR proteins can be found in all organisms and are involved in various processes including PSI and PSII assembly. For example, TPR motifs are present in Ycf3 and Ycf37, which are related to PSI, as well as in the above mentioned PratA facilitating manganese preloading of pD1 and the Pitt protein enhancing chlorophyll synthesis (Boudreau et al., 1997; Wilde et al., 2001; Klinkert et al., 2004; Schottkowski et al., 2009b). Yeast two-hybrid analysis demonstrated an interaction of Slr0151 with CP43 and D1. In addition, the *slr0151*$^{-}$ mutant was slightly reduced in growth rate and PSII activity under normal light conditions (Wegener et al., 2008; Yang et al., 2014). Interestingly, this phenotype was even more pronounced when high-light intensities were applied. Furthermore in high-light, the absence of Slr0151 led to a slower repair rate of the D1 protein as well as to a decrease in disassembly of photodamaged PSII. Since PSII activity in *slr0151*$^{-}$ is already affected under normal conditions, a dual function of Slr0151 in PSII repair as well as assembly appears plausible, as seen for e.g. Psb27 or Ycf48.

Since the complexes formed during the PSII repair cycle are similar to complexes known from the later stages of PSII *de novo* assembly, repair was assigned to be also located in the thylakoid membrane (Nickelsen and Rengstl, 2013). Notably, recent localization studies of GFP-tagged FtsH2 and FtsH3 proteases are inconsistent with that hypothesis, since they argue for the localization to specialized repair zones. Strinkingly, some of them are overlapping with regions of reduced chlorophyll autofluorescence, which most likely

represent biogenesis centers (Komenda et al., 2006; Sacharz et al., 2015). Thereby the data indicates, that the compartment harboring PSII repair as well as the possible separation of PSII repair from *de novo* assembly need further evaluation.

I.6 The CURVATURE THYLAKOID1 protein family

Thylakoid membranes in chloroplasts of higher plants form a sophisticated interconnected network of tightly packed grana stacks and non-appressed stroma lamellae in between. Furthermore, a functional separation between grana and stroma lamellae has been observed, in particular concerning the distribution of the photosynthetic complexes they harbor. Thus, grana stacks contain active PSII supercomplexes, whereas PSI and the ATP synthase are restricted to the stroma lamellae and the Cyt b_6f complex is present in both compartments (Pribil et al., 2014). The strict separation into the two membrane subdomains requires defined margins as well as the adjustability of the system to response to changing conditions, e.g. PSII repair in high-light or state transitions (Mullineaux, 2005; Kirchhoff, 2013).

Recently, a new protein family called CURVATURE THYLAKOID1 (CURT1) comprised of the four members CURT1A-D has been described in *Arabidopsis thaliana*. Homologs of CURT1 proteins are present in plants, algae and cyanobacteria and it acts as key player in defining thylakoid membrane ultrastructure (Armbruster et al., 2013). In the absence of all four CURT1 proteins in a *curt1abcd* quadruple mutant, grana stacking was almost completely lacking resulting in structures resembling loose layers of stroma lamellae or very flat grana stacks (Armbruster et al., 2013). In contrast, plants overexpressing CURT1A exhibited the very opposite phenotype, i.e. an increase in membrane layers per granum as well as a decrease in diameter. Accordingly, it can be concluded the level of CURT1 protein defines the level of grana stacking (Armbruster et al., 2013). In addition, a regulation of the level of grana stacking via phosphorylation of CURT1 proteins has been suggested. Moreover, immunogold-labeling experiments showed that CURT1 proteins are localized almost exclusively to the margins of grana stacks, thus the region where thylakoid membranes are highly curved (Armbruster et al., 2013). Furthermore, CURT1 proteins are able to form homo and hetero oligomers and intriguingly, they have been shown to tubulate liposomes resembling the lipid composition of the thylakoid membrane. Therefore, the CURT1 protein family has been assigned to be a key player in shaping the thylakoid membrane architecture by directly inducing curvature to grana margins (Armbruster et al., 2013). However, the strong alterations in thylakoid membrane ultrastructure in *curt1abcd* had only a minor effect

on photosynthesis. This is expressed in wild-type-like accumulation and basic activity of photosynthetic complexes, but mutants lacking CURT1 proteins revealed slight reductions in e.g. cyclic electron transport and nonphotochemical quenching. Notably, replacement of the cyanobacterial homolog of the CURT1 family with *Arabidopsis* CURT1A led to slight changes in *Synechocystis* thylakoid membrane ultrastructure indicating only partial complementation (Armbruster et al., 2013).

Furthermore, CURT1 proteins contain sequence similarities to the CAAD domain (cyanobacterial aminoacyl-tRNA synthetases appended domain) found in cyanobacteria, which can be fused to some special aminoacyl-tRNA synthetases (aaRSs). These specific CAAD-fused enzymes have been described as exclusively thylakoid membrane localized with a putative role in protein sorting (Olmedo-Verd et al., 2011; Luque and Ochoa de Alda, 2014). However, besides the structural similarities no functional connection between CURT1 proteins and aaRSs has been established so far.

II. AIMS OF THIS WORK

Various factors are involved in forming and defining the cyanobacterial thylakoid membrane as well as the bioenergetic processes it harbors. Although many outstanding studies over the last years shed light on a number of components as well as their specific function, plenty remained elusive or the subject of speculations and theories. One of the topics of interest are cyanobacterial biogenesis centers, which have been controversially discussed for years concerning their formation and function. However, these regions are formed in close proximity to the cellular periphery and present a special ultrastructural element of the thylakoid membrane in *Synechocystis*. Whereas chloroplasts of higher plants display a complex system of thylakoid membrane architecture consisting of stroma lamellae and grana stacks, cyanobacteria possess a rather simple thylakoid system. Nevertheless, a cyanobacterial homolog of the grana-shaping CURT1 protein family exists in *Synechocystis* despite the absence of grana-like membrane stacking (Armbruster et al., 2013). Therefore, CurT as a novel factor that shapes the cyanobacterial thylakoid membrane ultrastructure is analyzed, particularly focusing on the implications for PSII assembly and repair in its absence (see section III.1). Strikingly, CurT presented not only a function in defining thylakoid membrane ultrastructure, but also displayed a role in osmotic stress response via its relocalization to the plasma membrane. Therefore, one additional chapter of this thesis investigates CurT's role in the molecular mechanism of the cyanobacterial response to osmotic stress (see section III.2).

Several PSII assembly factors have been assigned an additional role during the repair of PSII indicating an overlapping mode of action between these processes. Thus, the function of the TPR protein Slr0151, previously assigned as repair factor, was investigated in view of its localization as well as its potential role during PSII assembly (see section III.3; Yang et al., 2014).

Thus, the data presented in this thesis aims to contribute to the current knowledge and understanding of thylakoid membrane biogenesis and maintenance in *Synechocystis* by focusing on novel factors as well as their functions. In particular, this thesis intends to add a significant piece to the sophisticated puzzle of the formation and function of cyanobacterial thylakoid membrane biogenesis centers.

III. RESULTS

The following section consists of three studies, which are all published in international peer-reviewed journals. The main conclusions as well as the contributions of the author to the articles are summarized in the beginning of each chapter.

III.1 Thylakoid membrane architecture in *Synechocystis* depends on CurT, a homolog of the granal CURVATURE THYLAKOID1 proteins

Heinz, S., Rast, A., Shao, L., Gutu, A., Gügel, I.L., Heyno, E., Labs, M., Rengstl, B., Viola, S., Nowaczyk, M.M., Leister, D., and Nickelsen, J. (2016b). Plant Cell 28: 2238-2260

The research presented in this study focusses on the functional characterization of the cyanobacterial homolog of the CURT1 protein family from *Arabidopsis*. Similar to its higher plant homologs, CurT is a membrane protein that revealed an *in vitro* membrane tubulating activity and its knock-out mutant presented a severely altered thylakoid membrane ultrastructure. Thus, the absence of CurT led to circular membrane structures resulting in the complete absence of biogenesis centers. Their importance for PSII was underlined by a decline in PSII assembly, accumulation and repair in *curT*$^-$. In addition, photosynthetic performance reflected by PSII-dependent oxygen evolution was also reduced to ~50%. Furthermore, photoautotrophic and photoheterotrophic growth rates as well as pigment contents were decreased in *curT*$^-$. Two-step sucrose density centrifugation demonstrated a mislocalization of CP47 and pD1 in the absence of CurT. Importantly, the relative electron transport rate indicated an intact electron transport downstream of PSII, which is consistent with unchanged protein levels of Cyt b_6f and PSI subunits. Therefore, the analysis of the *curT*$^-$ mutant lacking biogenesis centers strongly suggests a PSII-specific function of these biogenic regions.

CurT has been found to be distributed over the complete thylakoid membrane including PDMs, but live-cell imaging depicted local concentration of CurT at regions with a reduced chlorophyll autofluorescence, which represent biogenesis centers. In fact, the CurT-CFP signal revealed a network-like signal of CurT around the cellular periphery. Moreover, CurT presented differences in complexation as well as post-translational modifications of its isoforms present in PDMs and thylakoids. Immunogold-labeling analysis indicated a membrane-bending activity via CurT's asymmetric intercalation into the convex and the concave side of thylakoid membrane sheets.

Also, *curT*$^-$ showed an increased accumulation of the stress related protein IsiA (iron stress-induced protein A) under normal conditions and furthermore, the mutant was highly susceptible to salt and osmotic stress, represented by a strong reduction in growth rates. In combination with a relocalization of CurT to the plasma membrane under these conditions, it indicates an involvement of CurT in osmotic stress tolerance in *Synechocystis*.

My contribution to the research presented in this article include the generation and characterization of the *curT*$^-$ mutant in regard to estimation of growth rates, chlorophyll level as well as cell number. In addition, I determined several of the *curT*$^-$ protein levels, performed membrane sublocalization analysis by two-step sucrose density gradient centrifugation for the wild-type control, ^{35}S pulse-labeling experiments as well as the identification of CurT's isoforms in PDMs and thylakoids. I addressed the turnover of D1 in *curT*$^-$ under high-light intensities and the detailed statistical analysis of the immunogold-labeling data was also performed by myself. Moreover, I investigated CurT's stress phenotype including growth experiments, protein level determinations and the biochemical analysis of alterations in membrane localization in presence of salt. I also contributed to the bioinformatical analysis and many of the control experiments described in the supplemental material (see appendix, section VI.4). The paper was written by J. Nickelsen, A. Rast and me, afterwards it was revised by all co-authors.

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III.2 CurT contributes to osmotic stress tolerance in *Synechocystis* independent of compatible solutes

Heinz, S., Pade, N., Hagemann, M. and Nickelsen, J. (2016c). Endocyt. Cell Res. 27: 52-55

As described in section III.1, the analysis of the *curT*$^-$ mutant revealed the very intriguing involvement of CurT in tolerance of salt and osmotic stress. As most important response to these stress conditions, cyanobacteria activate the synthesis of compounds called compatible solutes, which prevent water efflux by increasing the internal osmolality of the cells. To gain further insights in CurT's function during stress situation, the ability to accumulate compatible solutes as well as the dynamic of the process was investigated in *curT*$^-$. The wild-type-like induction of compatible solute synthesis in *curT*$^-$ reasoned for a function of CurT independent of compatible solutes. Furthermore, it argues for CurT as a so far unknown but important player in stress response in *Synechocystis*.

I contributed to this paper by performing the salt treatments for wild-type and *curT*$^-$ and the isolation of compatible solutes. The gas-chromatographic analysis was performed by N. Pade. The manuscript was written by me and revised by J. Nickelsen and M. Hagemann.

III.3 The role of Slr0151, a tetratricopeptide repeat protein from *Synechocystis* sp. PCC 6803, during Photosystem II assembly and repair

Rast, A., Rengstl, B., **Heinz, S.**, Klingl, A., and Nickelsen, J. (2016). Front. Plant Sci. 7: 605

This study focusses on the function of the TPR protein Slr0151, which has previously been described as PSII repair factor (Yang et al., 2014). Since PSII activity and growth have been shown to already be impaired under normal light conditions, the research aimed on the characterization of a mutant lacking Slr0151 in matters of PSII assembly.

The *slr0151*$^{-}$ mutant revealed an increase in the protein levels of the inner antenna protein CP43 and the chlorophyll synthesis-related factor Pitt. Moreover, the protein level of Slr0151 was altered in several mutants deficient in a variety of PSII assembly factors. Ultrastructural analysis displayed an enlargement in thylakoid lumen in *slr0151*$^{-}$ indicating an additional structural effect in the absence of Slr0151. In addition, it has been biochemically detected in PDMs as well as in the thylakoids and interestingly, a minor fraction was found in the plasma membrane. Immunofluorescence microscopy confirmed a distribution to the entire thylakoids and additionally demonstrated local concentrations under normal and high-light intensities. Remarkably, these concentrations were often found in regions with reduced chlorophyll autofluorescence representing biogenesis centers. In its entirety, the data implicates a PSII-related involvement of Slr0151 not only during repair, but also under normal light conditions, thus the biogenesis of PSII.

I contributed to this publication by membrane fractionation experiments. I performed sucrose density gradient centrifugation for the *Synechocystis* wild-type including the Western analysis of the obtained fractions. A. Rast and J. Nickelsen wrote the article and I revised the manuscript.

https://doi.org/10.3389/fpls.2016.00605

IV. DISCUSSION

IV.1 Formation of biogenesis centers and thylakoid membrane architecture depend on CurT

The thylakoid membrane architecture in *Synechocystis* has been the subject of several studies, including the structure of biogenesis centers (Liberton et al., 2006; van de Meene et al., 2006; Stengel et al., 2012; van de Meene et al., 2012). However, less information has been gathered about factors required for thylakoid membranes to form biogenesis centers. Several hypotheses concerning the formation of biogenesis centers have been suggested over the years, many of them involving the Vipp1 protein. Despite several attempts, complete segregation of a Vipp1 knock-out mutant has never been achieved in *Synechocystis* indicating that Vipp1 is essential for the survival of the cell. Nevertheless, a knock-down mutant of Vipp1 in *Synechocystis* exhibited a completely disturbed ultrastructure, in particular, thylakoids were reduced to membrane-like structures lacking photosynthetic activity (Westphal et al., 2001). This rather extreme phenotype in combination with the partial knock-out severely impedes conclusions on specific functions of Vipp1 in thylakoid membrane biogenesis.

Intense analysis of the cyanobacterial CURT1 homolog CurT confirmed its anticipated function in shaping thylakoid membrane ultrastructure in *Synechocystis*. In contrast to the Vipp1 knock-down mutant, a wild-type-like number of thylakoid layers were observed in the *curT*$^-$ ultrastructure. Strikingly, the thylakoids formed circular structures instead of ordered layers at the cellular periphery and are devoid of any sites converging to the plasma membrane (Figure 2 in section III.1, Heinz et al., 2016b). As demonstrated by two fluorescence-based localization approaches, CurT is distributed over the complete thylakoid membrane, but specifically accumulates at biogenesis centers (Figures 8 and 9 in section III.1, Heinz et al., 2016b). Consistent with the *curT*$^-$ mutant phenotype lacking convergence sites, the observed localization pattern and CurT-induced tubulation of thylakoid-like liposomes indicated CurT's significance in defining the structure of biogenesis centers (Figures 1D, 2, 8 and 9 in section III.1, Heinz et al., 2016b). Furthermore, CurT was shown to be distributed over the complete thylakoid membrane and the PDMs by immunogold-labeling and density gradient centrifugation (Figures 7 and 10 in section III.1, Heinz et al., 2016b). Strikingly, an increased ratio of CurT-related immunogold signals on the convex side of thylakoid sheets compared to the concave side was significantly pronounced in regions of the thylakoid membrane displaying the highest level of curvature, i.e. where they converge towards the

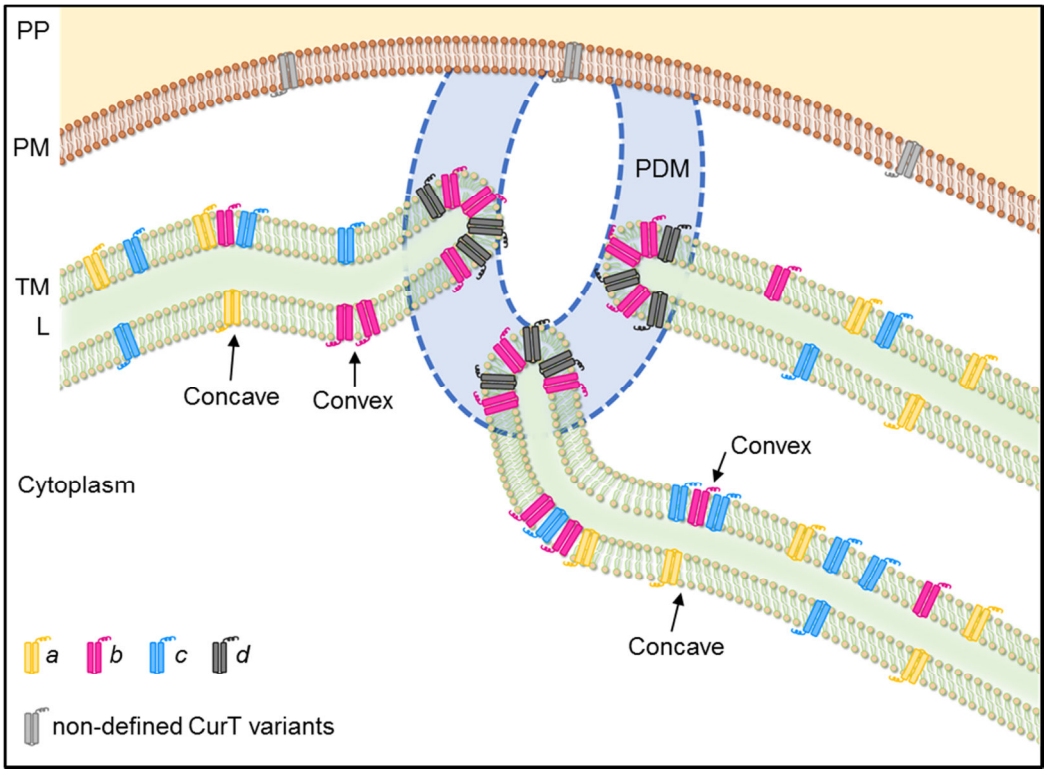

Figure 6: Working model of CurT distribution and function in shaping biogenesis centers in *Synechocystis*. The model shows the distribution of CurT in thylakoids and PDMs based on the localization studies described in Heinz et al. (2016b). Different CurT isoforms *a*, *b*, *c* and *d* identified via isoelectric focusing are displayed in yellow, pink, cyan and black, respectively. Since the identity of the CurT isoform present in the plasma membrane remains unknown, it is displayed in gray. The dashed blue lines of the PDMs indicate, that the exact structure of these regions remains elusive. L, lumen; PDM, PratA-defined membrane; PM, plasma membrane; PP, periplasm; TM, thylakoid membrane. Modified and adapted from Figure 13 in section III.1 (Heinz et al., 2016b).

plasma membrane (Figure 6). Thereby local differences in the degree of CurT-dependent membrane bending are mediated by the ratio of asymmetric intercalation into the thylakoid bilayer (section III.1, Heinz et al., 2016b). Differences in CurT function in PDMs and TM was suggested by the identification of four CurT-isoforms causing the formation of different high-molecular-weight complexes (Figure 11 in section III.1, Heinz et al., 2016b). This process might be regulated by post-translational modifications of CurT, since isoelectric focusing revealed at least four isoforms of CurT and at least one phosphorylation site has been detected for CurT in a phosphoproteomic study (Spät et al., 2015). Consequently, PDMs and thylakoids contain differentially modified CurT isoforms, presumably regulating its localization and/or activity (Figure 6). Altogether, CurT guides the thylakoid membrane towards convergence zones next to the plasma membrane, where thylakoid membrane ultrastructure culminates in the CurT-dependent formation of biogenesis centers.

Moreover, several other factors have been described, which are contributing to thylakoid membrane ultrastructure but nevertheless CurT represents the only one known in

biogenesis center formation. In wild-type cells, PDMs have been described as a semicircular structure which is present in regions, where thylakoids converge toward the plasma membrane to form biogenesis centers (Stengel et al., 2012). In more detail, thylakoids appear to originate from these structures, but it remains unclear, whether the semicircular PDM-structure is directly connected to the plasma membrane. An intermediate ultrastructural phenotype has been described in a mutant lacking PratA. In *pratA*$^-$, the semicircular structures of PDMs are absent, but thylakoid sheets do still converge toward the plasma membrane (Stengel et al., 2012). Thereby the dependence of the semicircular structure on the presence of PratA is demonstrated.

A very recent study investigated the effects of hydrocarbons on membrane bending in *Synechocystis* and *Synechococcus* sp. PCC 7002, since these compounds are embedded into the thylakoid membrane. A mutant unable to synthesize hydrocarbons (C15-C19 alkanes or alkenes) exhibited a slight reduction in membrane curvature (Lea-Smith et al., 2016). The proposed model includes an aggregation of hydrocarbons in the middle of the bilayer, thereby inducing curvature due to disruption of one of the lipid layers. However, the phenotype described in the absence of hydrocarbon synthesis displays only a minor effect on the level of thylakoid membrane curvature. Biogenesis centers were still formed and membranes were still curved, just to a slightly lesser extent (Lea-Smith et al., 2016). Therefore, hydrocarbons are presumably not shaping the thylakoid membrane in a way as significant as CurT does, but they appear to at least enhance the flexibility of the thylakoid membrane, which is a very important mechanism in adaptation to changing light conditions (Stingaciu et al., 2016). It might be interesting to see, if future work can establish a connection between CurT and the accumulation of hydrocarbons in terms of membrane bending.

Reconsidering the function of Vipp1, there are several arguments challenging the involvement of Vipp1 in defining thylakoid membrane ultrastructure. Despite massive changes in thylakoid membrane organization, neither the Vipp1 protein level nor its subcellular localization is altered in *curT*$^-$ (Figures 3 and 7 in section III.1, Heinz et al., 2016b). Overall, Vipp1 presents a wild-type like behavior, not affected by the ultrastructural changes in *curT*$^-$ and apparently not involved in responding to them. Therefore, the involvement of Vipp1 in shaping biogenesis centers seems to be unlikely, since the absence of biogenesis centers in *curT*$^-$ has evidently no effect on Vipp1. Of course, an indirect effect of Vipp1 in biogenesis center formation cannot be excluded, but it appears not to be the main player in this process as suggested earlier (section III.1, Heinz et al., 2016b). In fact, two recent studies imply another function of Vipp1. An increase in Vipp1 localization to the

thylakoids including spot-like accumulations has been observed for a GFP-tagged Vipp1 fusion protein under high-light conditions (Bryan et al., 2014). In combination with a study arguing for an *in vitro* membrane fusion activity of Vipp1 under specific conditions, Vipp1 function seems to be dedicated to the maintenance of the thylakoid membrane, since it probably enhances membrane organization or stability under stress conditions (Hennig et al., 2015; McDonald et al., 2015; Heidrich et al., 2016). Moreover, in *A. thaliana* and *Synechococcus* sp. PCC 7002, Vipp1 has been described as factor required for the biogenesis of PSII and PSI, respectively (Zhang et al., 2014; Walter et al., 2015). Thus, further research is required to understand and confirm the variety of actions Vipp1 has been assigned to in various organisms (Zhang and Sakamoto, 2015; Heidrich et al., 2016).

Notably, a study on thylakoid membrane ultrastructure in the unicellular green alga *Chlamydomonas reinhardtii* revealed multiple invaginations of the inner envelope of the chloroplast, which occasionally result in connections to the thylakoid membrane (Engel et al., 2015). Thereby the intermembrane space of the chloroplast's envelope and the thylakoid lumen are directly connected. It has to be mentioned, that these connections are very rare structures and they are likely to be very dynamic. Presumably, they function in the transport of lipids, proteins or other factors that have to be imported from the cytosol (Engel et al., 2015). Most importantly, this finding raises the question whether cyanobacterial thylakoid membrane and plasma membrane exhibit similar connections. In this regard, a study by van de Meene et al. (2012) on *Synechocystis* wild-type cells investigated the thylakoid membrane ultrastructure under low-light (0.5 µmol photons m^{-2} s^{-1}). Besides structural changes in the size of the thylakoid lumen, the authors observed fusions between thylakoids and the plasma membrane in biogenesis centers as a result of the applied low-light conditions (van de Meene et al., 2012). Therefore, it can be hypothesized, that these connections might also arise under normal light conditions, most likely as very transient and dynamic structures. In particular, the studies by Engel et al. (2015) and van de Meene et al. (2012) demand for closer inspections of biogenesis centers to determine whether direct connections to the plasma membrane develop under normal growth conditions or not.

Moreover, the data presented by Rast et al. (2016) in section III.3 identified the PSII-related protein Slr0151 as a new factor in the organization of thylakoid membrane ultrastructure. In case of a mutant lacking Slr0151, an increase of the thylakoid lumen was observed, indicating a less tight arrangement of the photosynthetic membranes (Figure 2 in section III.3, Rast et al., 2016). Although the data argues for a function in thylakoid

membrane organization, however, the exact mode of action remains elusive (for further discussion see section IV.2.2).

In conclusion, the identification of CurT as factor shaping the thylakoids and defining biogenesis centers greatly enhances the knowledge on thylakoid membrane organization. Future research has to focus on revealing its more detailed function as well as other factors contributing to the ultrastructure. Furthermore, the current understanding of biogenesis center function would greatly benefit from the identification of their precise ultrastructure.

IV.2 PSII-specific role of biogenesis centers

Earlier investigations of the subcellular distribution of photosynthetic complexes in *Synechocystis* indicated a separation between fully functional and developing thylakoids. Especially the early steps of PSII biogenesis have been referred to as biogenesis center related (Nickelsen and Rengstl, 2013; section VI.1, Heinz et al., 2016a). That includes the PratA-mediated delivery of manganese to pD1, which depends on the presence of PDMs, as well as the formation of the RC complex (Rengstl et al., 2011; Stengel et al., 2012). Several early PSII assembly factors and subunits are localized to PDMs including PratA, Ycf48 or YidC (Schottkowski et al., 2009a; Rengstl et al., 2011). In contrast, the biochemical localization of the PSI subunit PsaA as well as its assembly factor Ycf37 is restricted to the thylakoid membrane. Moreover, PsaA and Ycf37 are not affected in their localization in *pratA*$^{-}$, where PDMs are absent and especially pD1 as well as other PSII assembly factors are relocated (Rengstl et al., 2011).

Strikingly, the analysis of the *Synechocystis* mutant lacking CurT offered the opportunity to study the effects of the absence of biogenesis centers including secondary effects for the first time (section III.1, Heinz et al., 2016b). The absence of CurT resulted in a decrease of PSII accumulation and activity as well as its biogenesis and repair, whereas the protein levels of PsaA and Cytf remained unchanged (Figures 3, 4 and 5 in section III.1, Heinz et al., 2016b). In addition, the relative linear electron transport downstream of PSII was not affected in *curT*$^{-}$ indicating fully functional Cyt b_6f and PSI (Figure 4 in section III.1, Heinz et al., 2016b). Thus, the phenotype clearly confirms earlier hypotheses stating the specific PSII-related function of biogenesis centers (discussed in sections IV.2.1 and IV.2.2). As additional evidence, a bioinformatical coexpression analysis of *curT* identified five PSII subunits within the first ten hits, i.e. *psbE*, *psbO*, *psbF*, *psbL* and *psb30*, which exhibit a similar expression pattern (Supplemental Table 4 in appendix, section VI.4, Heinz et al., 2016b).

Compared to the other photosynthetic complexes, PSII is the target of a very high turnover due to the photodamage to D1 resulting in constant repair as well as *de novo* assembly (section I.5). Therefore, none of the other thylakoid-embedded complexes demand for such a frequent synthesis. This fact might favor the requirement of a biogenic compartment specialized in these PSII-related mechanisms by concentrating factors necessary for their efficient function. Moreover, it separates functional from developing PSII complexes giving rise to thylakoid membrane heterogeneity, thereby ensuring the highest efficiency of the thylakoid membrane (section VI.2, Rast et al., 2015).

Nevertheless, there is one shared feature of photosystem biogenesis i.e. the requirement of pigment synthesis, which in the case of chlorophyll depends on biogenesis centers. The complex consisting of Ycf39, HliD and HliC is involved in co-translational insertion of chlorophyll into pD1 (Knoppová et al., 2014). Therefore, the complex interacts with ChlG, which catalyzes the final step of chlorophyll synthesis (Chidgey et al., 2014). Moreover, since the insertion of pD1 takes place in biogenesis centers, the Ycf39-mediated delivery of chlorophyll is thought to occur in these regions as well (Komenda and Sobotka, 2016). In accordance with this hypothesis, the localization of POR, which facilitates the second to last reaction of chlorophyll synthesis, as well as of its stabilizing interaction partner Pitt are affected in the absence of PDMs in *pratA*$^{-}$ (Schottkowski et al., 2009b). Furthermore, PDMs show an accumulation of the late chlorophyll precursor chlorophyllide a (Rengstl et al., 2011). Moreover, very recent proteomic analysis of PDM and thylakoid fractions in our group identified two subunits of the magnesium chelatase subunits of chlorophyll synthesis, i.e. ChlD and ChlH to relocalize from PDMs to the thylakoid membrane in absence of PratA. This is in accordance with the above-mentioned PDM-dependence of several other enzymes catalyzing chlorophyll synthesis, namely ChlG, POR and Pitt. Nevertheless, POR and Pitt localization is not restricted to PDM fractions. Thereby it is indicated, that despite the interconnections of PSII assembly and chlorophyll synthesis, there has to be an additional pool besides biogenesis centers containing the enzymes synthesizing chlorophyll (Rengstl et al., 2011). Presumably, this might be the site of chlorophyll synthesis for PSI biogenesis and/or PSII repair. In the absence of biogenesis centers in *curT*$^{-}$, chlorophyll synthesis is disturbed, which is reflected by the significantly altered protein levels of POR and Pitt as well as the decrease of chlorophyll content by ~20% (Figure 3 and Table 1 in section III.1, Heinz et al., 2016b). On the other hand, mutants lacking either the PSII subunit PsbO or the assembly/repair factors CtpA and Psb28 are all decreased in chlorophyll content and interestingly, also in PSI level (Dobáková et al., 2009; Komenda et al., 2010). Since the

proteins play crucial roles in the repair of PSII, which is affected in these mutants, the authors hypothesized a limitation of chlorophyll depending on the rate of PSII repair. Thereby an indirect effect influencing the availability of chlorophyll for PSI is suggested (Komenda et al., 2010). These points argue for a sophisticated regulation of chlorophyll synthesis as well as a partial localization to biogenesis centers, where it is synchronized with PSII assembly and/or repair. In addition, similar to cyanobacterial biogenesis centers, a recent study from *Arabidopsis* reported the grana margins, which are defined by CURT1 proteins, to be the major site of chlorophyll synthesis (Wang et al., 2016). However, this indicates an evolutionary conservation of the CURT1 proteins in constituting the site of chlorophyll synthesis as an indirect effect of their membrane bending activity.

IV.2.1 Biogenesis centers are required for efficient PSII assembly

The PDMs of cyanobacterial biogenesis centers have been hypothesized to harbor early stages of PSII assembly (Rengstl et al., 2011; Stengel et al., 2012). In the absence of this biogenic subcompartment in *curT*$^-$, PSII is severely affected in assembly, accumulation, sublocalization and activity (section III.1, Heinz et al., 2016b). Protein levels of PSII subunits were reduced to less than 50%, which is consistent with a similar decrease of oxygen evolution activity (Figure 3 and Table 1 in section III.1, Heinz et al., 2016b). The reduction in growth rate represented by the doubling time in *curT*$^-$ was even more pronounced under photoautotrophic conditions, indicating the importance of biogenesis-center-dependent photosynthetic efficiency for the viability of the cells (Table 1 in section III.1, Heinz et al., 2016b). In addition, the protein levels of several assembly factors were significantly altered in *curT*$^-$ including PratA, YidC and Sll0933 (Figure 3 in section III.1, Heinz et al., 2016b). Strikingly, these proteins have been described to form an interdependent network of assembly factors in the PDMs together with POR and Pitt of the chlorophyll synthesis pathway (Rengstl et al., 2011).

Moreover, almost no PSII dimers are present in the absence of CurT, resulting in an increased accumulation of PSII monomers and even earlier assembly intermediates as compared to the wild-type (Figure 5 in section III.1, Heinz et al., 2016b). A general slowdown of PSII assembly in *curT*$^-$ was shown by ^{35}S pulse-labeling, especially by the high level of early RC intermediate complexes, which are supposed to form in biogenesis centers (section VI.1, Heinz et al., 2016a; Figure 5 in section III.1, Heinz et al., 2016b). Therefore, the data argues for an impaired formation of the RC47 assembly intermediate by the attachment of the CP47 module. This interaction is supposed to take place in the thylakoid membrane but still in

close proximity of biogenesis centers (Nickelsen and Rengstl, 2013). In favor of this hypothesis, the sublocalization of CP47 by sucrose density centrifugation is changed as well as the protein level of the assembly factor Sll0933, which is one of the mediators of this interaction (Figures 3 and 7 in section III.1, Heinz et al., 2016b). Thus, both points suggest a decrease in CP47 attachment efficiency. The reduced formation or accumulation of PSII dimers mentioned above is a very striking observation, since PDMs have been thought to harbor only the early steps of PSII assembly with the subsequent steps occurring in the thylakoid membrane. Thus, the reduction is most likely not caused by the absence of biogenesis centers but due to secondary effects of the altered thylakoid membrane ultrastructure and/or the altered pigment contents of *curT*$^-$ (section III.1, Heinz et al., 2016b).

Moreover, the biochemical localization studies revealed a change in pD1 sublocalization. In the wild-type, pD1 co-migrates with PratA in the PDMs, where the preloading with manganese occurs. In *curT*$^-$, no biogenesis centers are formed, raising the question of how and where PDM-like structures are constituted in the mutant. Intriguingly, fractions defined by the presence of PratA can still be obtained in *curT*$^-$ by sucrose density gradient centrifugation, but they are devoid of pD1 (Figure 7 in section III.1, Heinz et al., 2016b). Without the presence of PratA and pD1 in the same subcompartment, manganese preloading is most likely impaired, especially since PDMs have been hypothesized to act as high-throughput manganese import machinery (Stengel et al., 2012; section VI.2, Rast et al., 2015). If and to what extent the compromised function of PratA-mediated delivery of manganese can be compensated by other manganese uptake machineries or binding pathways, e.g. involving the MntABC transporter or the periplasmic manganese binding protein MncA, remains elusive (Bartsevich and Pakrasi, 1995; Bartsevich and Pakrasi, 1996; Tottey et al., 2008; Zorina et al., 2016).

As stated before, the TPR protein Slr0151 has previously been shown to be involved in the repair of photodamaged PSII (Yang et al., 2014). Nevertheless, a PSII-related function was already implied by a slight decrease in PSII activity under normal growth conditions, suggesting an additional role in *de novo* assembly of PSII (Wegener et al., 2008; Yang et al., 2014). Thus, the data presented by Rast et al. (2016) in section III.3 provided further evidence for this hypothesis. In particular, the analysis of Slr0151 protein level in several PSII-related mutants under standard growth conditions exhibited altered accumulations, thereby confirming the connection of Slr0151 to PSII (Figure 1 in section III.3, Rast et al., 2016). In a *sll0933*$^-$ mutant, Slr0151 was strongly increased and moreover, disturbed in its membrane sublocalization (Figure 1 in section III.3, Rast et al., 2016). This is of particular interest,

especially since Sll0933 mediates the attachment of the CP43 precomplex to the RC47 intermediate in the wild-type (Armbruster et al., 2010; Rengstl et al., 2013). However, this assembly step involves the interplay of CP43 and D1, two proteins Slr0151 has been shown to directly interact with (Yang et al., 2014). Therefore, Slr0151 presumably is involved in facilitating this inner antenna attachment and might have a functional relationship with Sll0933 in PSII biogenesis. Accordingly, CP43 displayed an increased accumulation in *slr0151*$^{-}$, whereas other PSII subunits were expressed in a wild-type-like manner (Figure 1 in section III.3, Rast et al., 2016). Thus, a high level of unassembled CP43 is suggested, due to the lack of Slr0151-mediated attachment.

Localization of Slr0151 via membrane fractionation revealed its presence in the thylakoid membrane and in the PDMs as well as minor amounts in the plasma membrane (Figures 3 and 4 in section III.3, Rast et al., 2016). Changes of Slr0151 in sublocalization and in accumulation in *pratA*$^{-}$, which lacks PDMs, indicated a connection to these biogenic regions (Figure 4 in section III.3, Rast et al., 2016). Likewise, a similar phenotype has been observed in the *curT*$^{-}$ mutant. In the absence of biogenesis centers in *curT*$^{-}$, Slr0151 accumulation is also reduced and its sublocalization is slightly altered as well (Figures 3 and 7 in section III.1, Heinz et al., 2016b). Thereby a connection of Slr0151 to biogenesis centers is further substantiated. Notably, CP47 was the only protein that depicted a pattern diverging from the wild-type in membrane fractionation experiments in *slr0151*$^{-}$ (Figure 4 in section III.3, Rast et al., 2016). This observation suggests a bottleneck in PSII assembly, presumably due to inefficient turnover of RC47 to later assembly intermediates. This is also reflected by the increase in RC47 complexes, which has been previously described in *slr0151*$^{-}$ (Yang et al., 2014). Concluding, Slr0151 is not just involved in the repair cycle of PSII, it also enhances PSII *de novo* assembly, possibly including a functional overlap with Sll0933.

Over the years, the function of biogenesis centers has been questioned by controversial studies using different fractionation techniques to yield fractions of *Synechocystis* membranes. For example, the localization of PratA has recently been referred to as solely plasma membrane associated, in contrast to several studies assigning it as marker protein defining the PDM subcompartment between plasma and thylakoid membrane (Schottkowski et al., 2009a; Rengstl et al., 2011; Selão et al., 2016). Moreover, exclusive plasma membrane localization has been proposed for CtpA, despite its involvement in PSII repair, which has long been accepted to take place in the thylakoid membrane (Zak et al., 2001; Nixon et al., 2010). Nevertheless, CtpA was reconsidered to be located to the thylakoid membrane in a recent approach applying a mutant in which PSII biogenesis is blocked at the RC assembly

intermediate (Selão et al., 2016). These quite contradictory results can be explained, if the diverging techniques are compared to each other in more detail. The commonly used two-phase partitioning of cyanobacterial membranes according to Norling et al. (1998) as well as earlier approaches based on density centrifugation resulted in a simple separation of plasma and thylakoid membrane (Murata et al., 1981; Smith et al., 1992; Zak et al., 2001). In contrast, consecutive two-step sucrose density gradient centrifugation according to Schottkowski et al. (2009a) enables to separate even the PDM subcompartment from functional thylakoid membranes. It is worth mentioning, that the first gradient is also able to simply distinguish plasma and thylakoid membrane, but it necessarily requires the second linear gradient to isolate PDMs (Schottkowski et al., 2009a; section VI.1, Heinz et al., 2016a). Thus, the separation suggests a more delicate differentiation of membranes compared to two-phase partitioning. Therefore, the differential localization patterns obtained by the two techniques might possibly arise from a differential association of PDMs with the various fractions during the isolation process (discussed in Selão et al., 2016). The latest approaches applying the one or other technique agree, that PSII assembly is supposed to not occur in the plasma membrane and both implicate the involvement of specialized regions, i.e. biogenesis centers (Heinz et al., 2016b; Selão et al., 2016).

The identification of structural similarities in CurT and the CAAD domain potentially represents a more direct link between CurT-dependent biogenesis center formation and protein synthesis (Luque and Ochoa de Alda, 2014). CAAD domains have been shown to be fused to some specific aminoacyl-tRNA synthetases (aaRSs) and have been hypothesized to form clusters of aaRSs resulting in specific gene translation foci (Olmedo-Verd et al., 2011). It remains elusive, whether CurT might act in a similar way by creating such regions via protein-protein interactions, maybe even in the PDMs of biogenesis centers. To further investigate this potential function, the identification of CurT-interaction partners will be essential for future experiments.

IV.2.2 PSII repair in Synechocystis *and its association to biogenesis centers*

In addition to their function in PSII assembly, growing evidence has been provided, that biogenesis centers might be involved in the repair cycle of PSII as well. The major players in D1 degradation during PSII repair are the proteases FtsH2 and FtsH3 (Komenda et al., 2006; Komenda et al., 2010; Boehm et al., 2012b). Under high-light conditions, the GFP-tagged FtsH2 protease has been localized in spots close to the cellular periphery, suggesting the existence of specialized repair zones in the thylakoid membrane (Sacharz et al., 2015).

Moreover, these regions were partially overlapping with regions of low chlorophyll autofluorescence representing biogenesis centers. Interestingly, a similar pattern has been observed following the signals of immunofluorescence-labeled Slr0151 in cells grown under both high-light or standard conditions. Since the localization of Slr0151 under conditions favoring either high levels of repair or *de novo* assembly remains unchanged, an involvement of these regions in both processes are very likely (Figure 5 in section III.3, Rast et al., 2016). Moreover, following the signals of fluorescence-labeled Slr1051 and the chlorophyll autofluorescence in line-plots, the data might even indicate a slight increase of Slr0151-spots in biogenesis centers under high-light conditions, but this needs to be further evaluated in future statistical experiments.

From a more functional point of view in PSII repair, Slr0151 is presumably involved in the detachment of the CP43 module, what is implied by the slower decrease of PSII monomers after high-light exposure in *slr0151*$^{-}$ in combination with its direct interaction with D1 and CP43 in yeast two-hybrid analysis (Yang et al., 2014). Moreover, Slr0151 might also facilitate the reattachment of the CP43 module subsequent to the insertion of the newly synthesized pD1 protein (Yang et al., 2014). As Slr0151, a number of other factors has been described in PSII assembly as well as repair, including CtpA, Psb27 and Ycf48 (Nickelsen and Rengstl, 2013). Thus, two of them have been analyzed by membrane fractionation experiments, i.e. Ycf48 and Slr0151, and they show a very similar distribution ranging from PDM into thylakoid membrane fractions (Figure 7 in section III.1, Heinz et al., 2016b; Figure 4 in section III.3, Rast et al., 2016). Therefore, the authors in Rast et al. (2016) argue for a characteristic pattern of factors involved in both PSII repair and *de novo* assembly (see section III.3).

In higher plants' chloroplasts, grana stacks have been shown to be very flexible in their ultrastructural organization, thereby adapting to environmental conditions. However, thylakoid lumens increase and grana are less tightly stacked under conditions promoting the repair of PSII, i.e. high-light stress (Kirchhoff, 2013). In consequence, damaged PSII migrates to the stroma lamellae, enabling proteases of the FtsH and Deg family to access and degrade photodamaged D1 (Yoshioka-Nishimura, 2016). Since also cyanobacterial thylakoid membranes have been shown to be a very dynamic compartment, a similar mechanism appears reasonable (Stingaciu et al., 2016). Therefore, the ultrastructural consequences of the absence of Slr0151 are of particular interest, i.e. the enlarged thylakoid lumen as well as the less tightly packed thylakoid sheets (Figure 2 in section III.3, Rast et al., 2016). Since its involvement in PSII repair has been established, analysis of *slr0151*$^{-}$ stimulates the thought of

a repair dependent response in membrane ultrastructure, possibly regulated by Slr0151. Furthermore, a relationship between Slr0151 and organization of thylakoid membrane ultrastructure is supported by its reduced accumulation and slightly changed sublocalization in *curT*$^-$ (Figures 3 and 7 in section III.1, Heinz et al., 2016b). However, co-immunoprecipitation experiments of Slr0151 and CurT were not able to establish that link under the tested conditions.

Moreover, D1 degradation was impaired in the *curT*$^-$ mutant lacking biogenesis centers. This is expressed by the relative reduction of the D1 protein level under high-light conditions, which was less pronounced in *curT*$^-$ than in wild-type cells (Figure 4 in section III.1, Heinz et al., 2016b). It appears unlikely, that D1 is less susceptible to photodamage in *curT*$^-$. Especially, since the relative electron transport rates, which indicate the maximal light-dependent photosynthetic capacity, reached their limits at similar light intensities in wild-type and *curT*$^-$ (Figure 4 in section III.1, Heinz et al., 2016b). Thus, the data indicates a defect in D1 degradation, probably due to a mislocalization of FtsH proteases, which are supposed to be partially located to biogenesis centers (Sacharz et al., 2015).

Furthermore, photoprotection of PSII is an important cellular process, especially under increasing light intensities. In *Synechocystis*, the flavodiiron proteins play a major role in protection from light stress (Bersanini et al., 2014; Chukhutsina et al., 2015). Interestingly, recent data indicates an involvement of the protein Sll0218, which is encoded in the same operon as Flv2 and Flv4 proteins, especially in the stabilization of PSII during repair and coupling of phycobilisomes (section VI.3, Bersanini et al., 2016). It is noteworthy, that Sll0218 has been detected via biochemical and fluorescence-based approaches in the PDMs, thereby increasing the evidence of a relationship between PSII repair and biogenesis centers (Figure 6 in section VI.3, Bersanini et al., 2016). Moreover, the proteins of the Hlip family have been shown to provide photoprotection during assembly and repair of PSII and also are likely to be at least partially present in biogenesis centers due to their involvement in chlorophyll delivery to pD1 (Knoppová et al., 2014; Staleva et al., 2015; Komenda and Sobotka, 2016).

Accordingly, an involvement of biogenesis centers in the PSII repair cycle has been suggested by the data discussed above. It clearly implies that the function of biogenesis centers is not restricted to *de novo* assembly of PSII. Many factors appear to be required for both processes, such as Psb27, Ycf48, CtpA as well as Slr0151. In terms of cellular functionality, it appears reasonable to locate overlapping processes like PSII repair and assembly at least partial to the same subcompartment. This might be of special interest, since

biogenesis centers have been hypothesized as a high-throughput machinery in manganese uptake required for the OEC during assembly as well as repair of PSII (Stengel et al., 2012; section VI.2, Rast et al., 2015). To confirm this hypothesis, the localization of specific steps in the PSII repair cycle remains to be clarified in future research. However, the analysis of the *curT*$^-$ mutant illustrated the importance of biogenesis centers for the fitness of *Synechocystis* cells, in particular their function in PSII assembly as well as repair (section III.1, Heinz et al., 2016b).

IV.3 How conserved is the function of the CURT1 protein family?

Thylakoid membrane architecture in *Synechocystis* is shaped by CurT, since it directs them toward the plasma membrane, where biogenesis centers are formed (ection III.1, Heinz et al., 2016b). Thereby it establishes thylakoid membrane subcompartments separating functional, active thylakoids from developing ones (section IV.1). A similar scenario can be found in plants' chloroplasts, where the CURT1 protein family is also responsible for defining the thylakoid membrane architecture, thus, separating the thylakoids in functional diverse grana stacks and stroma lamellae (Armbruster et al., 2013). Therefore, it appears that the CURT1 family has a common function. Cyanobacterial and plastidic CURT1 proteins have been shown to tubulate liposomes resembling the lipid composition of the thylakoid membrane *in vitro* by inducing membrane curvature (Armbruster et al., 2013; Figure 1 in section III.1, Heinz et al., 2016b). In regard of localization of CURT1 proteins, *Arabidopsis* CURT1 is exclusively localized in grana margins, whereas cyanobacterial CurT can be found all over the thylakoid membrane but locally accumulates in biogenesis centers, i.e. regions of the highest curvature. Interestingly, CURT1 proteins from *Arabidopsis* as well as *Synechocystis* CurT can be phosphorylated, indicating a conserved feature in their regulation (Pribil et al., 2014; Spät et al., 2015; section III.1, Heinz et al., 2016b). Both organisms exhibit dramatic effects on thylakoid membrane architecture upon loss of CURT1 proteins, nevertheless, the consequences on photosynthetic performance and viability of the organism diverge. The *Arabidopsis curt1abcd* mutant displayed only minor reductions in electron transport and nonphotochemical quenching, but the overall photosynthetic activity or growth of the mutant plants was not affected (Armbruster et al., 2013). In clear contrast, the loss of CurT in *Synechocystis* resulted in much more drastic phenotypic consequences, i.e. a reduction in growth as well as in PSII accumulation, assembly and repair capacity (discussed in section IV.2; Figures 3, 4 and 5 in section III.1, Heinz et al., 2016b).

However, attempts to replace the *Synechocystis* CurT with CURT1A from *Arabidopsis* were only able to achieve partial complementation. This was expressed by a reduced growth rate, as it has been shown for a *curT*$^-$ knockout mutant (Armbruster et al., 2013; Table 1 in section III.1, Heinz et al., 2016b). Moreover, in ultrastructural analysis the thylakoid membrane exhibited a slightly altered shape, phycobilisomes appeared to be detached and the thylakoid lumen was reduced (Armbruster et al., 2013). However, similar effects, but to a lower extent, have been observed in a *Synechocystis* strain expressing CURT1 proteins from both organisms at the same time. Notably, the protein level of CurT was reduced in presence of CURT1A expression (Armbruster et al., 2013). Unlike for *Arabidopsis* CURT1 proteins, it was not possible to create a *Synechocystis* strain which is stably overexpressing CurT to study the effects of an overaccumulation. It appears, as if an overaccumulation of CurT is repressed in cyanobacteria, most likely representing a protective mechanism of the cell (Armbruster et al., 2013; section III.1, Heinz et al., 2016b). In Armbruster et al. (2013), the authors hypothesize that a possible explanation for this might be the presence and the requirement of the phycobilisomes, which act as sterical antagonism to increased membrane curvature. Moreover, a putative increase of thylakoid convergence zones and biogenesis centers by CurT overexpression might severely disturb the intracellular balance between active and developing thylakoids. This might result in a massive impairment of photosynthesis, since also the effects of CurT knockout in *Synechocystis* are more severe than of its homologs in *Arabidopsis* (section III.1, Heinz et al., 2016b). Therefore, the necessity for a repression of CurT overaccumulation in *Synechocystis* might be justified. Nevertheless, it might be interesting to see the effects on thylakoid membrane ultrastructure in an *Arabidopsis* line expressing CurT in the *curt1abcd* mutant background.

In conclusion, CURT1 proteins have a similar function in inducing membrane curvature to thylakoid membranes, but to our current understanding, the impact of this action differs in its significance between cyanobacteria and higher plants. Moreover, plant CURT1 is not able to functionally replace its homolog from cyanobacteria, indicating an evolutionary divergence in their mode of function. This is also demonstrated by the induced degree of curvature resulting in CURT1-dependent formation of the sophisticated thylakoid network in chloroplasts.

To add another level of complexity to CURT1 protein function, the cyanobacterium *Anabaena* sp. PCC 7120 encodes two homologs of the CURT1 family (Mitschke et al., 2011; Armbruster et al., 2013). Moreover, *Anabaena* cells organize in multicellular filamentous structures and are able to form heterocysts for nitrogen fixation. Thereby they distinguish

spatially between photosynthetic and nitrogen-fixing cells resulting in massive changes to thylakoid membrane ultrastructure (Magnuson and Cardona, 2016). Strikingly, one of the *Anabaena* homologs of the CURT1 family appears to be specifically expressed under nitrogen-fixing conditions, i.e. in heterocysts (Mitschke et al., 2011). Thus, the question of CURT1 function in comparison of nonphotosynthetic and photosynthetic cells is of particular interest and will be addressed in future research.

IV.4 Evolutionary conservation of thylakoid membrane heterogeneity

As discussed above, CurT establishes the differentiation of the cyanobacterial thylakoid membrane by dividing them in active and developping thylakoids (discussed in sections IV.1 and IV.2). The formation of subcompartments within the thylakoid membrane is a common theme in oxygenic photosynthesis throughout cyanobacteria, green algae and plants. In chloroplasts of green algae and plants, thylakoid membrane architecture is also regulated by CURT1 proteins, resulting in the development of tightly appressed grana stacks and interconnecting stroma lamellae in between. However, the processes, which are divided by CURT1-mediated subcompartmentalization, differ between organisms. Despite the equal distribution of Cyt b_6f complexes in chloroplasts, the other photosynthetic complexes exhibit a spatial separation in the thylakoid membrane. Active PSII and its LHCs are restricted to grana stacks and PSI as well as the ATP synthase reside in the stroma lamellae (Pribil et al., 2014). A shared feature between higher plants and cyanobacteria appears to be the requirement of a CURT1-dependent compartment for chlorophyll synthesis, i.e. the grana margins and biogenesis centers, respectively (discussed in section IV.2; Rengstl et al., 2011; section III.1, Heinz et al., 2016b; Wang et al., 2016).

In higher plants, photosynthesis adapts to changing environmental conditions by partially breaking the thylakoid membrane heterogeneity, e.g. under changing light conditions (Pribil et al., 2014). A sophisticated network of phosphorylation is involved in regulating these processes (Pesaresi et al., 2011; Rochaix et al., 2012). Therefore, the level of membrane stacking is coordinated by phosphorylation of CURT1 proteins resulting in increased thylakoid membrane flexibility (Armbruster et al., 2013; Pribil et al., 2014). Furthermore, variations in light or a number of other factors can trigger so-called state transitions, during which the membrane-embedded LHCs are phosphorylated. Thereby the migration of LHCs between PSII in grana stacks and PSI in stroma lamellae is regulated (Minagawa, 2013). Moreover, it is known, that the repair of PSII occurs in the stroma lamellae, which requires the movement of photodamaged complexes from tightly stacked grana cores into non-

appressed regions (Järvi et al., 2015). Thus, flexible thylakoid membrane architecture is required to adapt quickly to environmental changes. These processes greatly benefit from distinct membrane subcompartmentalization coordinated by CURT1 proteins to precisely target the appropriate molecular responses. Concerning the site of PSII assembly in higher plants' chloroplasts, there is some evidence proposing the stroma lamellae as regions harboring early steps of PSII assembly (Yamamoto et al., 1981; Danielsson et al., 2006). However, not many details on the site of PSII biogenesis in plants are available and further research is clearly required to shed light on this process.

Similar to the PSII-specific role of cyanobacterial biogenesis centers, the unicellular green alga *C. reinhardtii* exhibits a region specialized in early PSII assembly. Localization studies revealed the existence of so-called translation zones (T-zones) close to the Rubisco-accumulating pyrenoid (Nickelsen and Zerges, 2013). In *Chlamydomonas*, T-zones represent the site of protein insertion in PSII *de novo* assembly, since mRNAs of PSII subunits as well as translational factors and PSII assembly intermediates accumulate in these regions (Uniacke and Zerges, 2007, 2009; Bohne et al., 2013). Therefore, the current model suggests the stepwise assembly of PSII in T-zones from where the complexes migrate into the functional thylakoids, interestingly a situation closely resembling the proposed mode of action of cyanobacterial biogenesis centers (Schottkowski et al., 2012; Nickelsen and Zerges, 2013). Moreover, the direct membrane connections observed between inner chloroplast envelope and thylakoid membranes have been found in close proximity to T-zones, thereby suggesting a possible mechanism for the uptake of cofactors, e.g. manganese (discussed in section IV.1, Engel et al., 2015). In addition, localization of the *psbA* mRNA under high-light revealed the absence of PSII repair from T-zones in *Chlamydomonas*, indicating the spatial separation of these two processes by on-site replacement of photodamaged D1 all over the thylakoids (Uniacke and Zerges, 2007; section VI.2, Rast et al., 2015).

However, the formation of functional domains in the thylakoids is a conserved feature through evolution but apparently it differs in the level of complexity. Moreover, the processes as well as the significance of their separation vary between cyanobacteria, green algae and higher plants.

IV.5 CurT acts as an important player in osmotic stress resistance

Besides its function in shaping thylakoid membrane ultrastructure, CurT exhibited a function in tolerance of stress conditions. This feature was indicated by an increase in 77K fluorescence at 685 nm in *curT*$^{-}$, which is usually contributed by the PSII inner antenna

protein CP43 (Shen and Vermaas, 1994; Figure 6 in section III.1, Heinz et al., 2016b). However, this increase is also characteristic for cells accumulating the CP43 homolog IsiA, which was detected in significantly high levels in *curT*$^-$ (Figure 6 in section III.1, Heinz et al., 2016b). High expression of *isiA* has been described in cells suffering from various stress conditions, including oxidative stress, iron limitation, high-light, heat and salt stress (Laudenbach and Straus, 1988; Havaux et al., 2005; Wilson et al., 2007). Due to the absence of PSII-related biogenesis centers, a high-light sensitivity could be anticipated, but treatment with increased light intensities did not result in an additional effect on the *curT*$^-$ growth phenotype (Figure 5 in appendix, section VI.4; section III.1, Heinz et al., 2016b). Since a previous proteomic approach suggested CurT induction in the plasma membrane under salt stress, the investigation of this stress phenotype depicted a high sensitivity to salt and osmotic stress in the absence of CurT (Huang et al., 2006; Figure 1 in section III.2, Heinz et al., 2016c; Figure 12 in section III.1, Heinz et al., 2016b). Under osmotic stress conditions, *Synechocystis* cells increase the internal osmolality by the synthesis of compounds called compatible solutes to prevent the efflux of water (Pade and Hagemann, 2015). The activation of compatible solute synthesis represents the major stress response in cyanobacteria (Brown, 1976; Reed and Stewart, 1985). Therefore, cells are highly susceptible to salt and osmotic stress in absence of these compounds (Marin et al., 1998). Strikingly, *curT*$^-$ was still able to accumulate compatible solutes in wild-type-like amounts and dynamics (Figure 2 in section III.2, Heinz et al., 2016c). Moreover, the salt- and maltose-dependent presence of CurT in the plasma membrane was confirmed by live-cell imaging as well as biochemical analysis, indicating not just a slight shift to the plasma membrane but a massive relocalization (Figure 12 in section III.1, Heinz et al., 2016b). Taken together, CurT apparently contributes to osmotic stress tolerance independent of compatible solutes, introducing a new feature of CurT function besides shaping the thylakoid membrane.

Considering a previous study by Marin et al. (2006), *Synechocystis* wild-type cells suffered from strong deformation, when they were exposed to very high osmolyte concentrations. They have been shown to exhibit a kidney-like shape, indicating intense forces affecting their general spherical structure. Therefore, the relocalization of CurT under stress conditions might result in a more stable plasma membrane, especially since CurT is already involved in shaping and stabilizing another membrane compartment, i.e. the thylakoids (discussed in section IV.1; section III.1, Heinz et al., 2016b). Consequently, the absence of CurT is likely to result in a lower tolerance of deformation due to reduced membrane flexibility and/or stability, thereby giving rise to disruptions of the cell envelope.

To relocate CurT under osmotic stress, cells have to shuffle the protein from the thylakoids into the plasma membrane. Connections between these membrane systems could possibly also be established by CurT, since it is already localized in biogenesis centers close to the plasma membrane. It appears reasonable, that this response of CurT during osmotic stress is most likely regulated by its post-translational modifications, which result in at least four isoforms (discussed in section IV.1; Figure 11 in section III.1, Heinz et al., 2016b). However, connections like this have not yet been observed under normal light conditions, therefore, the exact mechanism of CurT's movement to the plasma membrane remains elusive.

Moreover, a disturbance in the ion and/or water homoeostasis caused by the ultrastructural changes in *curT*$^-$ might be contributing to the osmotic stress phenotype (section III.2, Heinz et al., 2016c). In agreement with this, ultrastructural changes have been detected in *Synechocystis* cells after the exposure to osmotic stress for 15 minutes (Sinetova et al., 2015). Thylakoids lumen were extremely enlarged, but restored their normal ultrastructure within an hour. Interestingly, this ultrastructural adaptation to salt stress was slowed down and not reversible in a mutant lacking the aquaporin AqpZ. Notably, *aqpZ* is coexpressed with *curT* according to the *Synechocystis* coexpression database CyanoEXpress 2.3, thereby suggesting a potential functional correlation (Hernández-Prieto and Futschik, 2012; Hernández-Prieto et al., 2016). Thus, combined action of AqpZ and CurT might possibly enhance membrane flexibility during salt-induced thylakoid enlargement, but this might be addressed in future experiments.

IV.6 Conclusion and future perspectives

Despite the profound understanding of photosynthesis, our knowledge of thylakoid membrane biogenesis including the assembly of photosynthetic complexes is still very limited. However, the data presented in this thesis adds its piece to the understanding of these processes. Especially the function of cyanobacterial CurT in shaping thylakoid membrane ultrastructure and mediating biogenesis center formation provided valuable new information on thylakoid biogenesis. Moreover, their effect on PSII assembly substantiated the PSII-specific function of biogenesis centers, which has been a matter of debate for a long time. In addition, further analysis of the factor Slr0151, previously assigned as PSII repair factor, identified a function in PSII assembly as well. In conclusion, this thesis notably contributed to the current knowledge on photosynthesis and thylakoid formation in cyanobacteria, but compared to the overall picture, there is still a long way ahead to complete understanding of thylakoid membrane biogenesis and all the factors involved.

Therefore, the major goal of future research subsequent to this thesis has to be the identification of the detailed structure of thylakoid membrane biogenesis centers. Despite the introduction of CurT as factor determining these regions, ultrastructural analysis is required to understand the exact functions of the PDM subcompartment. Furthermore, the relationship of PratA and CurT is of special interest as both proteins are involved in defining and/or shaping biogenic PDMs in biogenesis center. In particular, the localization of the proteins in absence of the other might shed light on biogenesis center formation as well as on the process of manganese delivery to pD1. Moreover, the construction of a double mutant lacking CurT and hydrocarbon accumulation has already been initiated and its analysis will start soon in regard to a possibly combined function in determining thylakoid membrane ultrastructure. The identification of proteins interacting with CurT or regulating its function and isoforms is also a very promising field for future work. Another approach might focus on the identification of PSII assembly intermediates by 2D-PAGE analysis of fractions obtained by membrane fractionation experiments. It will also be exciting to further investigate the dynamics of CurT's plasma membrane relocalization during osmotic stress. Maybe even a connection between osmotic stress responses of CurT and water and/or ion regulation can be established.

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VI. APPENDIX

The following four sections contain two review articles (sections VI.1 and VI.2), an accepted manuscript (section VI.3) as well as a set of supplemental data substantiating the study described in Heinz et al. (2016b) in section III.1 (section VI.4). All of them have been peer-reviewed for publication in international journals. The main findings and contributions are summarized at the beginning of each section.

VI.1 Analysis of photosystem II biogenesis in cyanobacteria

Heinz, S., Liauw, P., Nickelsen, J., and Nowaczyk, M.M. (2016a). Biochim. Biophys. Acta 1857: 274-287

The assembly of cyanobacterial PSII is an intensively studied research field, focused on by many research groups all over the world. This review article briefly summarizes the current knowledge on PSII composition as well as its step-wise assembly process. Moreover, it focusses on the technical aspects of PSII assembly research in more detail by introducing several techniques commonly used for its progress. These techniques include mutant analysis, membrane fractionation, 2D-PAGE, isolation of PSII assembly intermediates, chemical cross-linking, mass spectrometry as well as surface plasmon resonance spectrometry.

I contributed to this publication by extensive literature research and the subsequent writing process. Thus, I wrote the sections on the state-of-the-art understanding of the spatiotemporal assembly of cyanobacterial PSII and elucidated several of the frequently-used techniques, as mutant analysis, membrane fractionation and 2D-PAGE analysis. The remaining chapters of the manuscript were written by P. Liauw and M. Nowaczyk. J. Nickelsen coordinated the writing process and revised the manuscript. P. Liauw and I contributed equally to this article.

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VI.2 Biogenesis of thylakoid membranes

Rast, A., **Heinz, S.**, Nickelsen, J. (2015). Biochim. Biophys. Acta 1847:821-30

Despite the detailed information available on the function and structure of thylakoids, the biogenesis of thylakoid membranes is a biological process of great importance, which is still far from being understood in its basic principles. This review article summarizes the current knowledge of thylakoid formation in plants, green algae and cyanobacteria. Thereby it discusses the main players responsible for shaping the thylakoids, i.e. lipids, pigment-protein complexes and other cofactors. Moreover, the sites of thylakoid membrane biogenesis are compared between the different organisms.

J. Nickelsen and A. Rast wrote the article and I revised the manuscript.

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VI.3 Dissecting the photoprotective mechanism encoded by the *flv4-2* operon: A distinct contribution of Sll0218 in photosystem II stabilization

Bersanini, L., Allahverdiyeva, Y., Battchikova, N., **Heinz, S.**, Lespinasse, M., Ruohisto, E., Mustila, H., Nickelsen, J., Vass, I., Aro, E.-M. (2016). Plant Cell Environ. Accepted

Efficient photoprotection is important to protect PSII from excessive photodamage. An important role in this process has earlier been described for the flavodiiron proteins Flv2 and Flv4. Notably, the genes for the two proteins are encoded in one operon together with the open reading frame *sll0218* of unknown function. Here, the role of Sll0218 has been analyzed. In absence of Sll0218, early PSII assembly complexes accumulated and chlorophyll was reduced in normal and high-light. The *sll0218*$^-$ mutant exhibited a reduction in D1 protein level, but almost wild-type-like PSII activity argued for only a minor effect of Sll0218 knockout under normal conditions. Nevertheless, *sll0218*$^-$ was highly susceptible to photoinhibition and furthermore, 77K measurements indicated uncoupling of phycobilisomes in *sll0218*$^-$. Interestingly, Sll0218 was localized to the PDM fractions in membrane fractionation experiments, which was confirmed by an YFP-based approach. In conclusion, Sll0218 is required for optimal light harvesting and is involved in stabilizing PSII during assembly and repair.

I performed membrane fractionation experiments via consecutive sucrose density centrifugation and was involved in revising the manuscript.

http://dx.doi.org/10.1111/pce.12872

VI.4 Supplemental data - Thylakoid membrane architecture in *Synechocystis* depends on CurT, a homolog of the granal CURVATURE THYLAKOID1 proteins

Heinz, S., Rast, A., Shao, L., Gutu, A., Gügel, I.L., Heyno, E., Labs, M., Rengstl, B., Viola, S., Nowaczyk, M.M., Leister, D., and Nickelsen, J. (2016b). Plant Cell 28: 2238-2260

The information presented in this chapter supplements the research described in section III.1 and provides several controls justifying the original data. It includes the construction of the *curT*$^{-}$ mutant, growth and pigment analysis as well as the ultrastructure of a mutant lacking all three *psbA* genes, which is still forming biogenesis centers at the cellular periphery. It also demonstrates the wild-type-like function of the tagged version of CurT expressed in the *curT-CFP* strain. In addition, immunogold-labeling controls were able to substantiate the significance of CurT's asymmetrical intercalation into the thylakoid membrane.

I contributed by performing the controls, which demonstrated the complete segregation of the mutant as well as the expression analysis via RT-PCR. Moreover, I carried out the more detailed growth analysis of the mutant in presence or absence of glucose as well as under high-light conditions. Furthermore, my experiments included the protein accumulation and sublocalization by membrane fractionation analyzing the *curT-CFP* strain. I extensively evaluated the immunogold-labeling micrographs, which served as controls, including the statistical analysis. The figures and tables as well as their explanation and methods provided in this supplemental data file were arranged by me with assistance by A. Rast. J. Nickelsen approved and revised the data file.

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LIST OF ABBREVIATIONS

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
^{35}S	radioactive sulfur isotope
aaRS	aminoacyl-tRNA synthetase
ATP	adenosine triphosphate
C-terminus	carboxyl-terminus
CAAD	cyanobacterial aminoacyl-tRNA synthetases appended domain
CFP	cyan fluorescent protein
ChlG	chlorophyll synthase
CO_2	carbon dioxide
CtpA	C-terminal processing protease
CURT1	CURVATURE THYLAKOID1
Cyt b_{559}	cytochrome b_{559}
Cyt b_6f	cytochrome b_6f complex
DNA	deoxyribonucleic acid
GFP	green fluorescent protein
Hlip	high-light inducible protein, family members HliA, HliB, HliC and HliD
iD1	intermediate form of D1
IsiA	iron stress-induced protein A
LHC	light-harvesting complex
Mb	mega base pairs
MncA	Mn^{2+}-cupin A
MntABC	manganese transport system consisting of the proteins MntA, MntB and MntC
mRNA	messenger RNA
$NADP^+$	oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
pD1	precursor of D1
PDM	PratA-defined membrane
Pitt	POR-interacting TPR protein
POR	light-dependent protochlorophyllide oxidoreductase
PratA	processing-associated TPR protein
PSI	photosystem I
PSII	photosystem II
PSII-Psb27	PSII monomer lacking the extrinsic lumenal subunits containing Psb27

Q_A, Q_B	plastoquinone A, plastoquinone B
RC	reaction center complex lacking CP47 and CP43 modules
RC47	reaction center complex lacking CP43 module
RNA	ribonucleic acid
ROS	reactive oxygen species
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SecYEG	Sec translocon consisting of the proteins SecY, SecE and SecG
T-zone	translation zone
TPR	tetratricopeptide repeat
tRNA	transfer RNA
Vipp1	vesicle-inducing protein in plastids 1
YFP	yellow fluorescent protein
Y_Z	specific redox-active tyrosine residue of the D1 subunit of PSII

LIST OF PUBLICATIONS

Bersanini, L., Allahverdiyeva, Y., Battchikova, N., **Heinz, S.**, Lespinasse, M., Ruohisto, E., Mustila, H., Nickelsen, J., Vass, I., Aro, E.-M. (2016). Dissecting the photoprotective mechanism encoded by the *flv4-2* operon: A distinct contribution of Sll0218 in photosystem II stabilization. Plant Cell Environ. Accepted.

Heinz, S., Pade, N., Hagemann, M. and Nickelsen, J. (2016c). CurT contributes to osmotic stress tolerance in *Synechocystis* independent of compatible solutes. J. Endocyt. Cell Res. 27: 52-55.

Heinz, S., Rast, A., Shao, L., Gutu, A., Gügel, I.L., Heyno, E., Labs, M., Rengstl, B., Viola, S., Nowaczyk, M.M., Leister, D., and Nickelsen, J. (2016b). Thylakoid membrane architecture in *Synechocystis* depends on CurT, a homolog of the granal CURVATURE THYLAKOID1 proteins. Plant Cell 28: 2238-2260.

Rast, A., Rengstl, B., **Heinz, S.**, Klingl, A., and Nickelsen, J. (2016). The role of Slr0151, a tetratricopeptide repeat protein from *Synechocystis* sp. PCC 6803, during Photosystem II assembly and repair. Front. Plant Sci. 7: 605.

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„We have no right to express an opinion until we know all of the answers“

KurT Cobain

“Ist das noch Punkrock?”

Farin Urlaub

EIDESSTATTLICHE ERKLÄRUNG/ERKLÄRUNG

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, 29. November 2016

Steffen Heinz

Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist. Des Weiteren habe ich mich **nicht** anderweitig einer Doktorprüfung ohne Erfolg unterzogen.

München, 29. November 2016

Steffen Heinz