Abstract

A major target of light-induced, irreversible damage in the thylakoid membrane is the photosystem II (PSII) complex, and in particular its D1 reaction center subunit. A repair cycle (Aro et al., 1993) operates to maintain PSII activity by removing and degrading damaged D1 protein from PSII reaction centers and by replacing it with newly synthesized copies of D1. The molecular details of the PSII repair cycle are unclear; however recently the FtsH protease (*slr0228*) was assigned a key role in the degradation of the D1 protein in *Synechocystis* sp. PCC 6803 (Silva et al., 2003).

In *S. cerevisiae* it has been found that unassembled proteins of the inner mitochondrial membrane are removed and degraded by proteases homologous to the FtsH protease in *Synechocystis* sp. PCC 6803 (Steglich et al., 1999), whereas heterooligomeric prohibitin (PHB) complexes operate to protect newly synthesized copies of these proteins from degradation (Nijtmans et al., 2000). Protein complexes containing the FtsH protease and the prohibitin homologues, HflK and HflC, have also been found in *E. coli* (Kihara et al., 1996; Saikawa et. al, 2004).

We have identified five putative prohibitins in *Synechocystis* sp. PCC 6803 that belong to the same protein superfamily, the Band 7 protein family, with four subfamilies: HflK, HflC, stomatins and prohibitins (Tavernarakis et al., 1999). It therefore seems possible that the prohibitins may interact with FtsH (*slr0228*) in *Synechocystis* sp. PCC 6803. Several single and multiple inactivation mutants have been generated by directed mutagenesis. Oxygen evolution measurements and pulse-chase experiments combined with biochemical methods were applied to test a possible involvement of these proteins in the PSII repair cycle and photoinhibition and furthermore to characterize these prohibitin homologues.

Table of Contents

Figures and Tables

Complete list of Figures:

Complete list of Tables:

Abbreviations

APS	Ammonium persulphate
BN PAGE	Blue native polyacrylamide gel electrophoresis
bp	Base pair
BSA	Bovine serum albumine
CTAB	Hexadecyltrimethyl-ammonium bromide
ddH2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
β-DM	N-dodecyl-β-D-maltoside
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EtOH	Ethanol
FeCN	Ferricyanide
IPTG	Isopropyl-D-thiogalactopyranoside
kbp	Kilo-base pair
KCN	Potassium cyanide
kDa	Kilo Dalton
KOAc	Potassium acetate
LB	Luria-Bertani media
M	Molar
NaOAc	Sodium acetate
OD	Optical density
PBS	Phosphate buffered sline
PCR	Polymerase chain reaction
PSI	Photosystem I
PSII	Photosystem II
p.s.i.	Pounds per square inch
RNAi	RNA-mediated interference
RNase	Ribonuclease A
ROS	Reactive oxygen species
r.p.m.	Rounds per minute
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	40 mM Tris-Acetate, 1 mM EDTA pH = 8.0
TE	10 mM Tris/HCl, 1 mM EDTA
TCA	Trichloroacetic acid
TEMED	N,N,N´,N´ tetramethylenediamine
Tween20	Polyoxyethylene sorbitanonelaurate
v/v	volumne per volume
WT	Wild type
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter I: Introduction and Aims

1.1 Introduction

Photoinhibition is the loss of photosystem two (PSII) function under the influence of high light intensities (Ohad et al., 1984). FtsH, a member of the AAA protease family, has been found to be involved in the early events of a repair cycle that operates to counteract this loss (Silva et al., 2003). Interestingly, Band 7 proteins in *E. coli* and *S. cerevisiae* have been found to be associated with the regulation of AAA proteases (Kihara et al., 1996; Steglich et al., 1999). This study focuses on the role of prohibitins, members of the Band 7 protein family (Tavernarakis et al., 1999), in the cyanobacterium *Synechocystis* sp. PCC 6803 and in particular the possible involvement in the PSII repair cycle.

1.1.1 On the relevance of photoinhibition

The intriguing conversion of light energy into biochemical energy, and ultimately into biological material, by photosynthesis seems to hold the answers to the energy needs that mankind will soon be facing and many researchers dream to unravel its secrets. Photoinhibition is a particular and long known aspect of photosynthesis (Ohad et al., 1984). It occurs when more light is available to the photosynthetic apparatus than it can deal with and it ultimately leads to the irreversible damage of the reaction center where the crucial steps of photosynthesis occur. Briefly, photoinhibition causes photosynthetic losses by excess light.

An evergrowing world population inescapably leads to an increased demand of food which can only be met with significant increases in the yields of major crops. In one study it has been calculated that in rice an increase of 50 % is necessary by the year 2030 (Khush and Peng, 1996). Another study states that the world rice production must increase annually by 1 % in order to meet future demands (Rosegrant et al., 1995). However, the crucial part of any strategy to achieve this can only be the increase in the maximum yields of crops (Khush and Peng, 1996). But to prevent environmental degradation, destruction of natural ecosystems and

loss of biodiversity most of this increase must come from greater yields on existing cropland (Cassman, 1999; Tilman et al., 2002). One way out is to increase total crop biomass which is limited by photosynthesis and respiration losses that themselves dependent on various factors (Yoshida, 1981). The influence of photoinhibition on photosynthetic losses has not yet been quantified, but could be significant where plants are exposed to constant high light intensities in arid habitats (Horton, 2000). Thus, a good understanding of photoinhibition might help to increase crop yields by genetically altering the plants.

1.1.2 Photoinhibition

When oxygen-evolving, photosynthetic organisms are exposed to excess light their photochemical efficiency decreases. This phenomenon is generally known as photoinhibition (Ohad et al., 1984; Prasil et al., 1992; Aro et al., 1993).

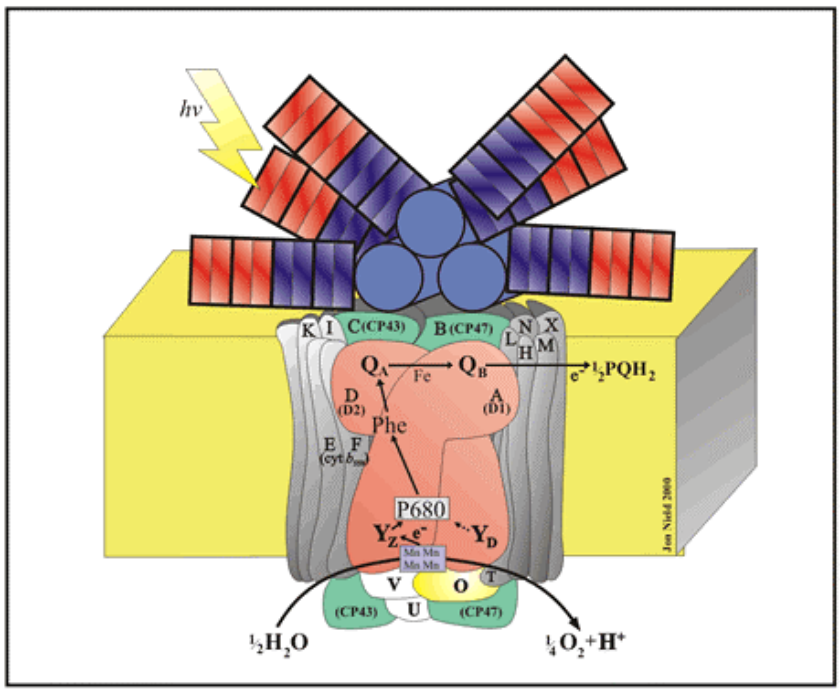

Figure 1.1: A model of PSII in cyanobacteria (kindly provided by Jon Nield). This Figure highlights the structural arrangement of key subunits of the PSII complex from cyanobacteria: the lumenal extrinsic proteins PsbO, PsbU and PsbV; the stromal extrinsic phycobilisome light harvesting antenna; tyrosine residue (Y_Z); the primary electron donor chlorophyll a (P680); pheophytin (Phe); the primary and secondary quinone electron acceptors (Q_A and Q_B,); the Rieske iron-sulphur protein (Fe) and plastoquinone (PQ).

Under prolonged, strong irradiance PSII complexes, which are the thylakoid membrane complexes with a light-driven water-plastoquinone-oxidoreductase activity (see Figure 1.1), are rendered nonfunctional (Prasil et al., 1992; Aro et al. 1993; Ohad et al., 1994). The molecular details of photoinhibtion *in vivo* are still unclear. However two major pathways, referred to as acceptor- and donor-side mechanisms, have been implicated on the basis of *in vitro* studies (Barber and Andersson, 1992) (see Figure 1.2).

Acceptor-side photoinhibition typically occurs under strong illumination at the level of the primary quinone electron acceptor Q_A. When Q_A is being double reduced, it dissociates from its binding site in the D2 protein (Styring et al., 1990; Vass et al., 1992) and the probability of charge recombination between the primary radical pair ($P680^+$ and $Pheo^-$) increases. This leads to an enhancement of P680 triplet states formation (Vass et al., 1992) which are able to react with molecular oxygen to produce toxic, singlet oxygen that ultimately damages PSII. Donor-side photoinhibition takes place when the donation of electrons to PSII occurs more slowly than their removal from the acceptor side. This leads to the formation of long-lived, highly oxidizing radicals such as Tyr Z^+ and $P680^+$ (Blubaugh and Cheniae, 1990; Jegerschöld et al., 1990; Bumann and Oesterhelt, 1995). These species have the capacity to extract electrons from their surroundings and cause irreversible damage to PSII.

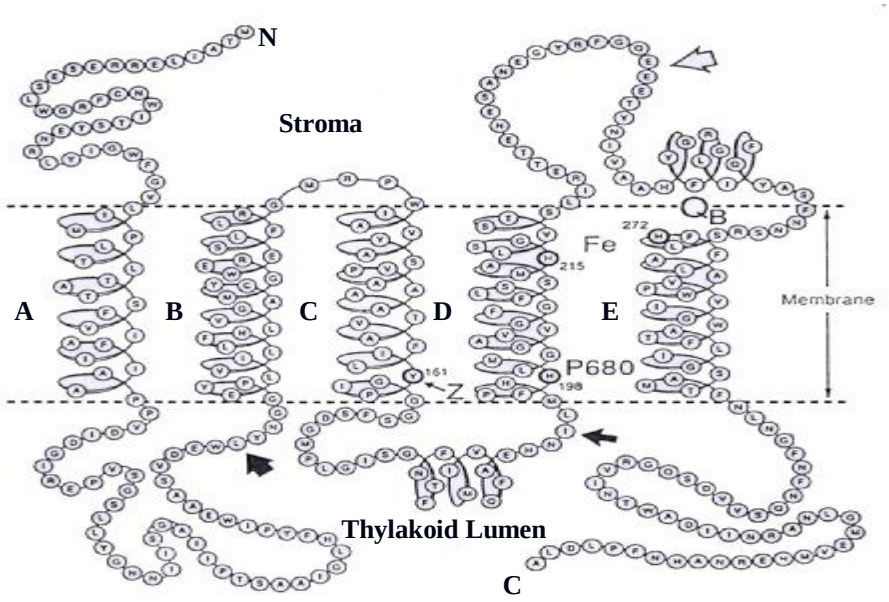

Figure 1.2: Schematic drawing of the D1 protein (Barber and Andersson, 1992). This drawing features the main characteristics of D1. Five transmembrane domains (A-E) span the thylakoid membrane with the N-terminus facing the stromal and the C-terminus the lumenal side. Other important D1 components are labeled: tyrosine residue (Z), primary electron donor chlorophyll a (P680), Rieske iron-sulphur protein (Fe) and plastoquinone B binding pocket (Q_B). Black arrows indicate initial cleavage sites for the donor side photoinhibition. The white arrow marks the cleavage site for acceptor side photoinhibition.

1.1.3 D1 turnover and the PSII repair cycle

Although light is essential for oxygenic, photoautotrophic organisms, excess light can cause significant damage on the cellular level by inducing the formation of reactive oxygen species (ROS) during photosynthetic electron transport (Asada, 1999). A major target of light-induced, irreversible damage is the PSII complex in the thylakoid membrane, and in particular the D1 reaction center subunit (see Figures 1.2 and 1.3). In order to maintain PSII activity the cell needs to remove and degrade damaged D1 protein from the reaction center of PSII and replace it with a newly synthesized copy of the D1 protein. This mechanism is called the PSII repair cycle, which is represented as a hypothetical scheme in Figure 1.3. In its functional state, PSII is prevalent as a dimer which disassembles upon a damaging event into a monomer. Subsequently, the extrinsic proteins PsbO, PsbV and PsbU and the intrinsic CP43 subunits dissociate. Now the D1 protein of the CP47-reaction center (CP47-RC) subcomplex can be replaced in a synchronized manner. Damaged D1 protein is removed and subsequently degraded while existing *psbA* mRNA pools are translated and D1 is contranslationally inserted into the CP47-RC subcomplex. The monomeric state of PSII is reconstituted by C-terminal processing of D1, the ligation of cofactors and the reassembly of the CP43 subunit into PSII. Finally the extrinsic subunits bind and allow PSII to form the dimeric complex.

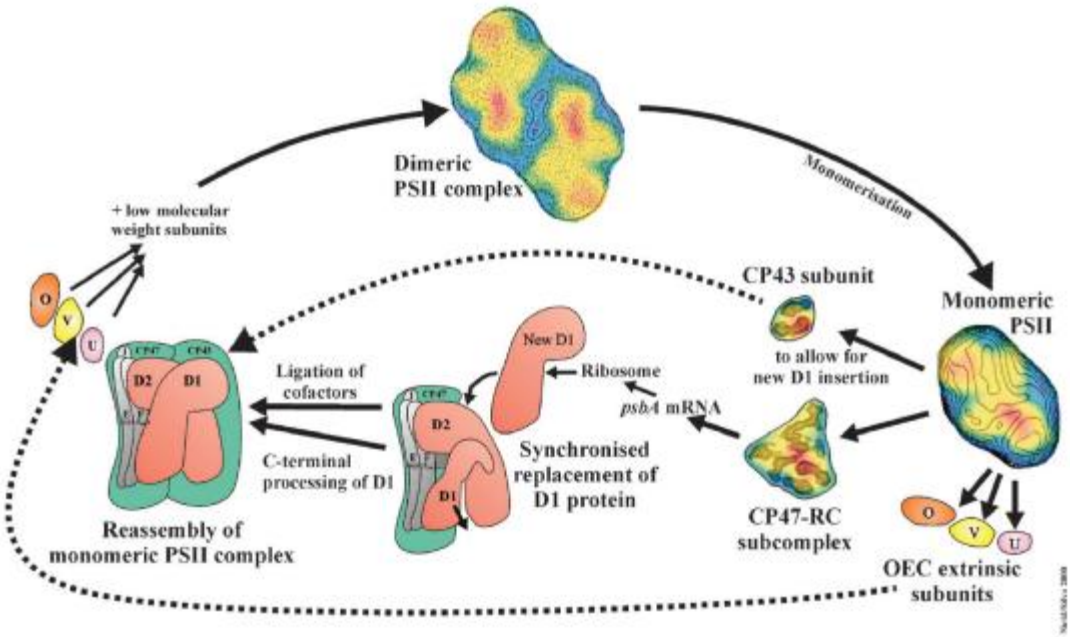

Figure 1.3: Model of the PSII repair cycle in Synechocystis sp. PCC 6803 (kindly provided by Paulo Silva and Jon Nield). The disassembly of dimeric PSII complexes into CP47-reaction center subcomplexes is triggered by a damaging event. The damaged D1 protein is then replaced by a newly synthesized copy and PSII reassembles into its dimeric state.

Even though PSII complexes consist out of more than 25 subunits (Ferreira et al., 2004), the D1 protein represents the major target of photodamage (Ohad et al., 1984; Aro et al., 1993; Adir et al., 2003). A possible reason for this might be that the D1 protein is in close proximity of the oxygen-evolving complex and holds a lot of the cofactors that are involved in the charge recombination reaction (see Figures 1.1 and 1.2).

The molecular mechanisms of the D1 protein degradation are not yet completely understood, however, recently the FtsH protease (*slr0228*) was assigned a key role in this process in *Synechocystis* sp. PCC 6803 (Silva et al., 2003).

1.1.4 The role of FtsH in the PSII repair cycle in cyanobacteria

In our laboratory we are working on the PSII repair cycle, pursuing a particular interest in the synchronized replacement of the D1 protein. Most of the molecular details are still unclear,but in the course of this chapter some of the current models for the removal of the D1 protein from PSII and its degradation will be introduced.

In chloroplasts a variety of *in vitro* studies lead to a model in which two different proteases are proposed to remove and degrade the D1 protein (Adam and Clarke, 2002). The primary cleavage event in the Q_B-binding pocket (see Figure 1.2) by the serine-type DegP2 protease (Haußühl et al., 2001) is proposed to be followed by the degradation of the resulting 10- and a 23-kDa fragments by an FtsH protease (Lindahl et al., 1996 and 2000). This process, involving two proteases, is the current, widespread model for D1 protein degradation.

In *Synechocystis* sp. PCC 6803, however, several lines of evidence lead to a solely FtsH protease catalyzed model for the removal and degradation of the D1 protein (Silva et al., 2003). Even though three potential members of the DegP/HtrA family, termed HtrA (*slr1204*), HhoA (*sll1679*) and HhoB (*sll1427*), have been identified in *Synechocystis* sp. PCC 6803 (Sokolenko et al., 2002), oxygen evolution and pulse-chase assays on a triple mutant, in which these proteases had been insertionally inactivated, did not reveal an impaired PSII repair cycle (personal communication Dr. PJ Nixon). When the same experiments were performed with a mutant in which the FtsH homologue (*slr0228*) had been inactivated, the

opposite was true and the PSII repair cycle was impaired. Another obeservation from pulse-chase labelling studies was that full length D1 protein was stabilized even under the constant high irradiance and that no accumulation of breakdown fragments could be observed. Furthermore growth experiments with this mutant strain under elevated light intensities (~100 $\mu E * m^{-2} * s^{-1}$) revealed a lethal phenotype when *slr0228* was deleted. All these data point in the direction that this particular member of the FtsH family is required to degrade the D1 protein under high light illumination (Silva et al., 2003). The fundamental differences between the two models for D1 protein degradation during photoinhibition in chloroplasts and cyanobacteria, could be rationalised by an early evolutionary diversion after which the DegP2 protease obtained its role in the PSII repair cycle in chloroplasts.

FtsH proteases are members of the AAA protease familiy (ATPase associated with various cellular activities). Based on what is known about FtsH in *E. coli* (Akiyama and Ito, 2003) and AAA proteases in mitochondria (Langer, 2000) a general model for the FtsH catalyzed degradation of the D1 protein in chloroplasts and cyanobacteria has been proposed (Silva et al., 2003; Nixon et al., 2004). In this model damaged D1 protein is translocated in an ATP dependent manner through a central pore of a hexameric FtsH ring that is located in the thylakoid membrane. Upon translocation the D1 protein is degraded at the catalytic Zn^{2+} center. In *E. coli* FtsH mediated proteolysis can occur from either end (Chiba et al. 2002), including newly generated ends from an endoproteolytic cleavage (Shotland et al., 2000), and is highly processive (Akiyama, 2002). Taking into account that the catalytic Zn^{2+} center of the FtsH protease is likely to be located on the stromal side of the membrane (Lindahl et al, 1996), the degradation of the D1 protein would be initiated from stromally exposed regions such as the cleavage site in Q_B-binding pocket (see Figure 1.2), or the N-terminus. In the literature the D-E loop of the D1 protein is described to form an essential structural component of the Q_B pocket (Trebst, 1986) which contains a specific amino acid sequences, the PEST-like sequence and the putative cleavage region (Greenberg et al., 1987). However deletion of the PEST-like sequence modified the Q_B pocket, but did not prevent rapid turnover of the D1 protein (Nixon et al., 1995). One opinion favors general destabilization of PSII and particularly the D1 protein by accumulating photodamage in conjunction with an accessible N-terminus to be the triggering signal for FtsH mediated proteolysis (Nixon et al., 2004). Substrate recognition could be mediated by transmembranes helices or a highly conserved 81-amino-acid sequence of FtsH on the luminal side (Bailey et al, 2002).

1.1.5 Regulation of AAA proteases by Band 7 proteins

More than 550 proteins from all organisms have been grouped into the Band 7 protein superfamily, which can be divided into four distinct protein subfamilies: HflK, HflC, stomatins and prohibitins (Taveranakis et al., 1999). Some studies, that will be discussed in this section, have revealed that some Band 7 proteins seem to exhibit a regulatory function on AAA proteases.

1.1.5.1 FtsH interacts with the HflKC complex in *E. coli*

The FtsH homologue in *E. coli* (HflB) is a plasma membrane-bound protease (Ogura and Wilkinson, 2001) and is required for the proteolytic degradation of, for example, the cytosolic proteins λ cII and heat shock sigma factor σ^{32} (Herman et al, 1993; Ogura and Wilkinson, 2001; Tomoyasu et al. 1995) as well as the integral membrane proteins SecY and the α subunit of the F_1F_0 ATPase (Akiyama et al., 1996; Kihara et al., 1995, 1998). FtsH possesses two N-terminal transmembrane regions and a large, C-terminal cytosolic domain which contains an AAA domain (Krzywda et al., 2002; Niwa et al., 2002) and a Zn^{2+}-metallo protease active-site motif (Akiyama and Ito, 2000, 2001).

When bacteriophage λ infects *E. coli* the λ cII protein plays a key role in the lysis (viral replication and cell lysis) versus lysogeny (chromosomal integration) decision (Herskowitz and Hagen, 1980; Gottesman et al., 1981). This unstable and rapidly degraded protein is a transcriptional activator of the λ cI phage repressor and an integrase protein, which both favour the lysogenic pathway. Hence high levels of the λ cII protein promote lysogeny (Banuett et al., 1986). Stabilization of the λ cII protein was observed in *hflA* (Hoyt et al., 1982; Banuett et al. 1986) and *hflB* mutants (Banuett et al., 1986) leading to a high-frequency lysogenization phenotype (Friedman et al., 1984). Furthermore it was found that a *hflA / hflB* double mutation resulted in an additive effect, suggesting two independent pathways for the observed phenomenon (Banuett et al., 1986). *In vitro* studies revealed that HflB was responsible for the degradation of the λ cII protein, whereas the HflA proteins interacted with HflB to determine its substrate specificity (Kihara et al., 1997). Thus, HflB was defined by the

λ lysogeny phenotype and was later found to be an allele of the *ftsH* gene (Herman et al., 1993). The *hflA* locus on the other hand encodes an operon of three genes (*hflX-hflK-hflC*). HflX is a putative GTP-binding protein, and HflKC are found in the plasma membrane (Zorick and Echols, 1991) forming a multimeric complex (Cheng et al., 1988; Kihara and Ito, 1998) that interacts with HflB (FtsH) (Saikawa et al., 2004) and negatively regulates its activity (Kihara et al., 1996, 1997).

Another well studied proteolytic process catalyzed by the FtsH homologue in *E. coli* is the degradation of the integral membrane protein SecY, which is thought to be a quality control mechanism for the membrane to protect it from the highly toxic accumulation of unassembled protein subunits (Kihara et al., 1995). SecY spans the membrane ten times resulting in six cytoplasmic and five periplasmic domains. Under normal conditions this protein forms a heterotrimeric complex with SecE and SecG (Brundage et al., 1990; Douville et al., 1994) that acts as a protein translocase in ATP dependent protein secretion across the inner membrane (Schatz and Beckwith, 1990; Ito, 1996; Mori and Ito, 2001). In contrast to a stable, assembled SecYEG complex, unassembled SecY subunits are rapidly degraded with a half life of less than 2 min (Matsuyama et al., 1990; Taura et al., 1993). A mutant form of the SecY protein, SecY24 is also readily degraded at 42°C supposedly due to weakened interactions with the SecE subunit (Baba et al., 1994). Both excess SecY and Sec24 proteins were found to be stabilized in a mutant with a compromised FtsH activity (Kihara et al., 1995). Studies in a Δ*hflKC* mutant reported an enhanced degradation of the SecY protein in this genetic background, which suggested a negative regulatory effect of the HflKC complex on FtsH (Kihara et al., 1996).

In mutants in which the interaction between HflKC and FtsH was impaired due to the removal of a periplasmic region of the FtsH protease, the degradation of the λ cII protein became more effective, whereas no effect on the SecY protein degradation could be observed *in vivo* (Akiyama et al., 1998). In another experiment a mutant form of the YccA protein, a membrane-bound substrate of FtsH, selectively interfered with the degradation of other membrane-bound substrates by forming a complex with FtsH/HflKC. Moreover it was shown that the HflKC complex was required for this inhibition by YccA (Kihara et al., 1998). These findings suggest that the degradation pathways for membrane-bound and soluble substrates are in part different from each other and that HflKC is involved in this differentiation.

HflK and HflC proteins are Band 7 proteins that only exist in bacteria (70 HflKs and 66 HflCs; InterPro database). Summarizing the data from *E. coli*: the HflK and HflC homologues are found in the plasma membrane (Zorick and Echols, 1991) and form a large, multimeric complex (Cheng et al., 1988). In *E. coli* the HflKC complex was reported to interact with the FtsH protease and negatively regulated its activity (Kihara et al., 1996).

1.1.5.2 An AAA protease interacts with a prohibitin complex in yeast mitochondria

The inner mitochondrial membrane has a high protein content and the biogenesis of its protein complexes like those of the respiratory chain or the ATP-synthase call for a precise coordination of protein translocases and assembly factors. Not surprisingly a distinct proteolytic quality control system has evolved which selectively removes unassembled polypeptides. Key components of this quality control system are two AAA proteases that expose their catalytic sites on alternative sides of the membrane and a chaperone-like prohibitin complex (see Figure 1.4).

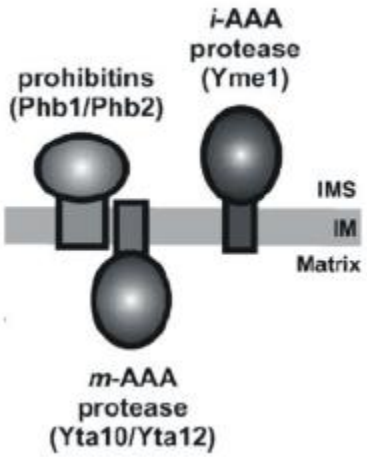

Figure 1.4: Schematic drawing of the proteolytic quality control system in the inner mitochondrial membrane (adapted from Arnold and Langer, 2002). Two AAA proteases are embedded in the inner mitochondrial membrane (IM). The m-AAA protease has its catalytic site on the matrix side, whereas that of the i-AAA protease faces the intermembrane space (IMS). A chaperone-like prohibitin complex is also involved in this quality control system (see text for further explantions).

The involvement of the prohibitin complex in this proteolytic quality control system has lead to an extensive description of prohibitins in yeast mitochondria. Several studies have lead to a better understanding of the function (Steglich et al., 1999; Nijtmans et al., 2000) and the structural organization of the prohibitin complex (Tatsuta et al., 2005).

Two prohibitin homologues, designated Phb1p and Phb2p, have been identified in *S. cerevisiae* (Coates et al., 1997). Both proteins are anchored to the mitochondrial inner membrane via an N-terminal transmembrane segment, expose their C-termini to the intermembrane space and form a high molecular weight complex of about 1.2 MDa (Berger and Yaffe, 1998; Coates et al., 1997). To form this prohibitin complex and for their stability *in vivo*, both subunits exhibit an interdependence and neither of the two proteins could be detected when the other protein had been deleted (Berger and Yaffe, 1998; Steglich et al., 1999).

One function in which prohibitins in *S. cerevisiae* seem to play an important role is the maintenance of mitochondrial morphology and inheritance. In an either *mdm10* or *mdm12* genetic background, where both these mitochondrial outer membrane proteins that are essential for mitochondrial inheritance had been deleted, the additional deletion of either *phb1* or *phb2* had the most severe effect and lead to a lethal phenotype (Berger and Yaffe, 1998).

Despite these interesting phenotypes, the focus of this section will be on the interaction that has been observed between prohibitins and an AAA protease in the inner mitochondrial membrane (Steglich et al., 1999) which exhibits some similarities to the interaction between FtsH and the HflKC complex in the plasma membrane of *E. coli* (Saikawa et al, 2004). The m-AAA and the i-AAA proteases of the inner mitochondrial membrane constitute a quality control mechanism that is responsible for the maturation and turnover of proteins in this particular compartment of the cell (Steglich et al., 1999; Langer, 2000) (see Figures 1.4). It has been shown by co-immunoprecipitation experiments that the m-AAA protease physically interacts with the prohibitin complex, whereas the i-AAA protease does not (Steglich et al., 1999). When the m-AAA protease and prohibitins were deleted in the same mutant, this resulted in a slow growing phenotype (Steglich et al., 1999). In pulse-chase experiments in a genetic background where distinct nuclear encoded subunits from respiratory chain protein complexes (COX4 = subunit 4 of the cytochrome c oxidase; Atp10p = a subunit of the F_0 moiety of the ATPase complex) and the prohibitins had been deleted, the half life times of some of the other subunits of these respiratory complexes were monitored (Cox2p, Cox3p and Atp6p) (Steglich et al., 1999). The deletion of COX4 (Nakai et al., 1994) or Atp10p (Tzagoloff et al., 2004) normally results in the proteolysis of the nonassembled, mitochondrially encoded subunits. It was found that the deletion of either of the prohibitins

lead to an even higher decrease in the half life time of the Cox3p and Atp6p proteins that are degraded by the m-AAA protease (Arlt et al., 1996; Guelin et al, 1996). The half life time of Cox2p, which is degraded by the i-AAA protease (Nakai et al, 1995), did not change significantly. In a different series of pulse-chase and co-immunoprecipitation experiments it was found that the prohibitin complex binds to newly synthesized mitochondrial translation products and stabilizes them. Interestingly, this study also reported a stabilization of the Cox2p subunit of cytochrome oxidase c (Nijtmans et al., 2000). The pulse-chase data also implied that this interaction between the translation products and the prohibitin complex is relatively stable and even after 40 min some labeling of Cox3p could still be detected to be comigrating with the prohibitin complex (Nijtmans et al., 2000). Both observations suggested that the prohibitins at least partly function as a membrane associated chaperone / "holdase" that has a negative regulatory effect on the m-AAA protease activity by binding its potential substrates. A small but significant sequence homology with chaperonins of the GroEL/Hsp60-class supports the chaperone / "holdase" idea (Nijtmans et al., 2000).

In a study by Tatsuta et al (2005) the focus lay on structural aspects of the prohibitin complex and several distinct steps of its biogenesis could be resolved. Both prohibitin subunits are nucleus encoded and targeted to the mitochondria by unconventional non-cleavable targeting sequences at their N-terminal ends. The insertion into the inner mitochondrial membrane of Phb1p and Phb2p is mediated by the Tim23-translocase. Prior to its incorporation into the target membrane, Phb1p binds to Tim13p as determined by crosslinking experiments. Tim13p forms together with Tim8p a soluble, 70 kDa complex in the intermembrane space that functions in the biogenesis of inner and outer membrane proteins (Leuenberger *et al*., 1999; Davis *et al*., 2000; Paschen *et al*., 2000; Curran *et al*., 2002; Hoppins and Nargang, 2004). After integration into the inner mitochondrial membrane the prohibitins assemble into ~120 kDa intermediate complexes and later into large, ring shaped complexes. Their assembly into these larger complexes is dependent on the conserved C terminal coiled coil regions of both subunits. Given the native molecular mass of the assembled complex of 1.0 – 1.4 MDa (without detergent and lipid), the resulting ring complex should be composed of 16 – 20 subunits of both Phb1 (31 kDa) and Phb2 (35 kDa) which are arranged as alternating Phb1p and Phb2p subunits (Back et al, 2002).

1.1.6 Unifying hypothesis and working model

Prohibitins are members of the Band 7 protein family and have been found to be an evolutionary conserved gene family (McClung et al., 1995) with homologues in organisms ranging from procaryotes (Banuett and Herskowitz, 1987) to higher eukaryotes (Nuell et al., 1991). In accordance with a high degree of conservation and a widespread distribution, prohibitins have been functionally linked to many important cellular processes, such as cellular signaling and transcriptional control (Terashima *et al.*, 1994; Montano *et al.*, 1999; Sun *et al.*, 2004), apoptosis (Vander Heiden *et al.*, 2002; Fusaro *et al.*, 2003), cellular senescence (McClung *et al.*, 1992; Coates *et al.*, 1997; Coates *et al.*, 2001; Piper *et al.*, 2002), early development of *Caenorhabditis elegans* (Artal-Sanz *et al.*, 2003) or mitochondrial biogenesis (Berger and Yaffe, 1998; Steglich *et al.*, 1999; Nijtmans *et al.*, 2000; Artal-Sanz *et al.*, 2003).

This report however focuses on the reported interactions between members of the Band 7 protein family and AAA proteases. Summarizing the data that has been presented in the previous sections, it became obvious that in at least two, well-studied cases (*E. coli* and *S cerevisiae* mitochondria) prohibitins or at least prohibitin homologues regulated the function of an AAA protease homologue. Parallels that could be observed were that in both cases the Band 7 proteins formed large protein complexes that physically interacted with the protease. The other common feature in both systems was the negative regulatory effect that the Band 7 proteins exhibited on the protease resulting in an increased stability of unassembled subunits of membrane protein complexes.

After revealing the role of the FtsH protease (also an AAA protease) in D1 turnover and finding a prohibitin gene in *Synechocystis* sp. PCC 6803, an interaction between the two proteins, based upon the presented results from the literature, immediately suggested itself and the following hypothesis and working model (see Figure 1.5) was developed.

Our working model and hypothesis henceforth was that prohibitin complexes protect newly synthesized copies of the D1 protein from FtsH mediated degradation prior to their incorporation into reassembling PSII complexes.

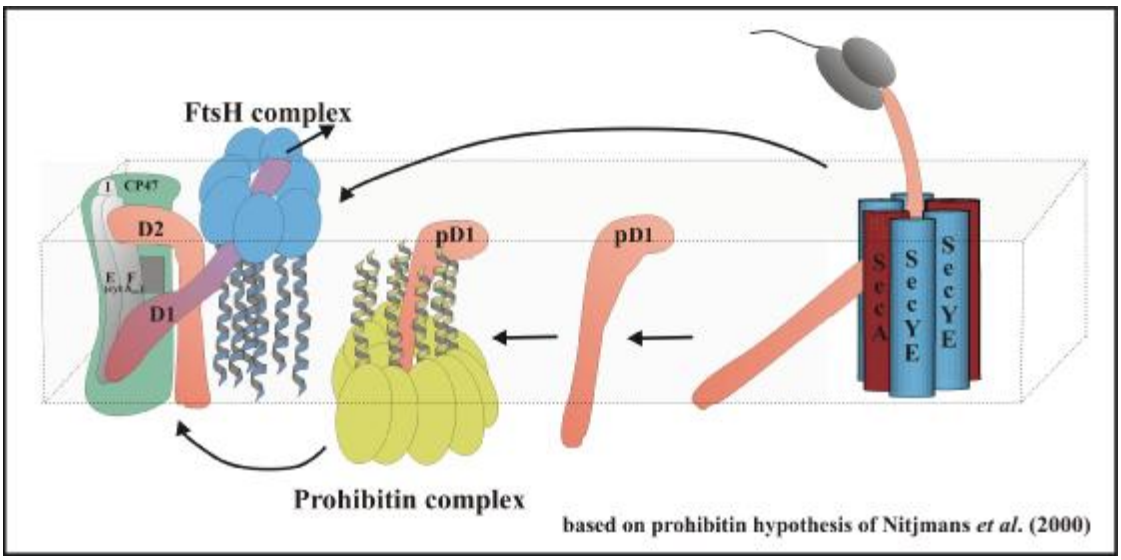

Figure 1.5: Simplified model for the D1 replacement during the PSII repair cycle (Silva et al., 2003). D1 degradation is catalysed by a large FtsH complex. The replacement D1 may be inserted into the reassembling PSII complex cotranslationally via the Sec translocon or post-translationally via a large prohibitin complex. This model is based on the prohibitin hypothesis described in *S.cerevisiae* by Nitjmans *et al.*(2000).

1.2 Aims

The major aim of this project was to test our working model that prohibitins in *Synechocystis* sp. PCC 6803 are indeed involved in the synchronized replacement of the D1 protein, particularly during the PSII repair cycle. Of further interest for the role of prohibitins in this organism were the experiments on the characteristics of the prohibitin complex and the hypothesized interaction with and regulation of the FtsH protease. To achieve these aims, the following objectives were defined:

- Identification of potential prohibitin homologues in *Synechocystis* sp. PCC 6803.

- Identification of prohibitin homologues in plants and test a potential association of these proteins with the chloroplast (presence or function).

- Bioinformatic analysis of identified prohibitin homologues (e.g. phylogenetic trees and protein properties).

- Raising polyclonal antibodies against the prohibitin homologues in *Synechocystis* sp. PCC 6803.

- Generation of single and multiple prohibitin mutants to:
    - test whether the prohibitin homologues are essential for cell viability.
    - reveal potential phenotypes under distinct conditions.
    - test the involvement of prohibitins in the PSII repair cycle.
    - study the characteristics of possible prohibitin complexes.

- Identification and characterization of possible prohibitin complexes *in vivo* with emphasis on the:
    - subcellular localization and prevalent membrane topology.
    - determination of the complex size and stochiometry.
    - structural analysis.
    - identification of interaction partners (e.g. FtsH) and potential substrates.

Chapter II: Materials and Methods

2.1 Bioinformatics

Bioinformatic analysis were mostly performed over the internet and more specific details are included in chapter 3.

protein family databases:

PFAM (Version 16.0; Bateman et al. 2004) (http://www.sanger.ac.uk/Software/Pfam/)

InterPro (Release 8.1; Mulder et al., 2003) (http://www.ebi.ac.uk/interpro/)

PROSITE (Release 18.0; Hulo et al., 2004; Sigrist et al., 2002) (http://us.expasy.org/prosite/)

Sprint (Release 36.0; Attwood et al., 2003) (http://umber.sbs.man.ac.uk/dbbrowser/sprint/)

protein databases:

Cyanobase (http://www.kazusa.or.jp/cyano/cyano.html)

Uniprot (Release 3.5; Bairoch et al., 2005) (http://www.ebi.uniprot.org/index.shtml)

BLAST (Release 2.2.10; Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/BLAST/)

SWISSPROT (Release 45.5) (http://us.expasy.org/sprot/)

protein properties prediction server:

EXPASY (http://us.expasy.org/cgi-bin/protparam)

TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html)

multiple sequence alignment and phylogenetic tree generation:

ClustalW (Higgins et al., 1994; Gonnet et al., 1992) (http://www.ebi.ac.uk/clustalw/)

Treeview (Version 1.6)

protein targeting prediction server:

ChloroP (Version 1.1; Emanuelsson et al., 1999) (http://www.cbs.dtu.dk/services/ChloroP/)

Predotar (Version 1.03) (http://genoplante-info.infobiogen.fr/predotar/predotar.html)

2.2. *E. coli* strains and growth conditions

2.2.1. E. coli strains

The *E. coli* strains that were used for cloning and protein expression in this project are listed in table 2.1.

Table 2.1: *E. coli* strains used in this project.

strain	reference	genotype	used for
DH5α	(Hanahan, 1983)	F‘, endA1, deoR, hsdR17 ($r_K^- m_K^+$), supE44 thi-1, recA1, gyrA96, (Nal^r), relA1 Δ, (lacZYA-argF)$_{U169,}$ (80lcZΔM15)	cloning
BL21-Gold(DE3)pLysS	(Stratagene)	B, F^-, *ompT, hsdS*(r_B– m_B–), *dcm*$^+$, Tet^r, *gal,* λ(DE3), *endA,* Hte [pLysS Cam^r]	expression
Rosetta™	(Novagen)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lacY1* pRARE2[2] (Cm^R)	expression

2.2.2 E. coli growth conditions

E. coli was either grown in liquid Luria-Bertani (LB) media (Sambrook et al., 1989) on a shaker in a temperature controlled room (37 °C) or on solid LB plates in a growth cabinet (37 °C). Plates for routine use were stored at 4 °C and long term stocks were kept at –80 °C in liquid LB medium containing 10 % glycerol (v/v) (Sambrook et al., 1989). Following antibiotics at indicated concentrations were used for selective growth: ampicilin (AMP; 50 µg/ml), carbenicilin (CARB; 50 µg/ml), chloramphenical (CAM; 30 µg/ml), erythromycin (ERM; 200 µg/ml), kanamycin (KAN; 50µg/ml) and spectinomycin (SPEC; 50 µg/ml).

2.3 Cyanobacterial strains and growth conditions

2.3.1 Synechocystis sp. PCC 6803 strain

The glucose tolerant *Synechocystis* sp. Strain PCC 6803 (Williams, 1988) was a gift to this laboratory from Dr. J.K Williams (DuPont, Delaware, USA).

2.3.2 Thermosynechococcus elongatus strain

Aliquots from already grown liquid cultures of *Thermosynechococcus elongatus* (~50 ml) were obtained from Prof. Barber´s group.

2.3.3 Synechocystis sp. PCC 6803 growth conditions

Synechocystis strains were grown in or on BG11 mineral medium (Rippka, 1972) supplemented with 5 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) pH = 8.2 and 5 mM of glucose. Liquid cultures were either grown in an orbital shaker incubator (Gallenkamp, Germany) at 100 rpm at a light intensity of about 20 $\mu E*m^{-2}*s^{-1}$ or on a magnetic stirrer with air bubbling as the source of aeration at a light intensity of about 70 $\mu E*m^{-2}*s^{-1}$ in a temperature-controlled room (29 °C – 33 °C). The air used for the bubbling was pumped through a solution of copper sulphate for humidification. Routine maintenance of the strains was done on BG11 plates (BG11 with 0.3 % sodium thiosulphate, 10 mM TES pH 0 8.2 and 1.5 % agar, added separately after autoclaving) in the presence of 5 mM glucose. For the maintenance of mutant strains following antibiotics were added to the media at the indicated levels: kanamycin (KAN; 50 µg/ml), chloramphenicol (CAM; 10 or 30 µg/ml) and spectinomycin (SPEC; 25 or 50 µg/ml). The stock plates were restreaked every 2-4 weeks. Prior to inoculation of a liquid culture, cells were restreaked onto fresh BG11 plates containing 5 mM glucose and the appropriate antibiotic and used within one week. For long term storage, cells from fresh plates or liquid cultures were suspended in 1 ml of liquid BG11 and 10 % glycerol (v/v), flash frozen and kept at –80°C.

2.3.4 Estimation of cell concentration of liquid Synechocystis sp. PCC 6803 cultures

The optical density of a liquid culture at 730 nm (OD_{730}) was determinded using a Shimadzu spectrophotometer (model UV-1601). An OD_{730} of 0.25 corresponds to approximately 1 x 10^8 cells/ml (Williams, 1988).

2.4 Molecular biology techniques

2.4.1 Standard buffers and solutions

Standard buffers and solutions were prepared according to Sambrook et al (1989). Chemicals and organic solvents were analytical grade reagents and purchased from Sigma and BDH. Bacteriological agar, tryptone and yeast extract were purchased from DIFCO. Exceptions are stated in the methods were applicable. Nanopure water (NanopureII, Barnstead) was routinely used to prepare the solutions. Solutions and other materials for recombinant DNA techniques were sterilized by autoclaving for at least 40 min at 121 °C (130 kPa). Thermolabile solutions were sterilized by filtration through a 0.2 µm sterile filter (Schleicher and Schuell, Germany). Antibiotics were purchased from Sigma. Liquid stocks of antibiotics were kept at –20 °C and added to the medium just before use.

2.4.2 Oligonucleotide primers

All oligonucleotide primers that have been used in this work are listed in Table 2.2 and were purchased from MWG Biotech, Germany. The primers to clone the five prohibitin genes were designbed based on the sequences in CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html).

Table 2.2: Oligonucleotide primers used in this project. Primers 1-8 were used for cloning and general purposes. Primers 9-11 were used to delete the TM domains of the respective prohibitin genes for the expression constructs. Primers 12-24 were used during the course of the HIS-tagging of the prohibtins. Primers 25-26 were used for sequencing.

1	*slr1768*-Fw	5´- GCCCATATGGGTGCTGTTATCTCGGCGATC – 3´ *NdeI*
2	*slr1768*-Rev	5´- GGGGGATCCTTAGGGGTCCGACATGATAATGGG - 3´ *BamHI*
3	*slr1128*-Fw	5´- GCCCATATGGAAGCCTTTTTTCTCTTCTTTCTCGTC – 3´ *NdeI*
4	*slr1128*-Rev	5´- GGGGGATCCCTAAACTGCCCGATGGCGGTCTAC - 3´ *BamHI*
5	*sll0815*-Fw	5´- GCCCATATGGCACGACAAGCTCGCTATCAA – 3´ *NdeI*
6	*sll0815*-Rev	5´- GGGGGATCCTCAACTATCCGATGTTATTTTTTC - 3´ *BamHI*
7	*sll1021*-Fw	5´- GCCCATATGCAAAGTAAATTTTGGTTTGAATTTCTCC – 3´ *NdeI*
8	*sll1021*-Rev	5´- GGGAGATCTCTAAATTTCCTCCGGGGAAAAATT - 3´ *BglII*
9	*slr1768*-TM-Fw	5´- GCCCATATGACTCCCCTTACCTCTGGGGTT – 3´ *NdeI*
10	*slr1128*-TM-Fw	5´- GCCCATATGATTTTGGATCGGGTCGTTTTT– 3´ *NdeI*
11	*sll1021*-TM-Fw	5´- GCCCATATGTTTAAAAAAGAGCAAATGGTGATT– 3´ *NdeI*
12	*slr1106*-HIS-1	5´- CCGCTAGTGATGGTGATGGTGATGGTGATGGTTAGCCAGGTCAGTTAGGTTAAA – 3´
13	*slr1106*-HIS-2	5´- GCTAACCATCACCATCACCATCACCATCACTAGCGGCAGCGGGGAAGTTATAGG – 3´
14	*slr1106*-down	5´- CCAATGGCCCTTACCTGTCCTGGATTA – 3´
15	*slr1768*-HIS-1	5´- GGGTTAGTGATGGTGATGGTGATGGTGATGGGGGTCCGACATGATAATGGGCAA – 3´
16	*slr1768*-HIS-2	5´- GACCCCCATCACCATCACCATCACCATCACTAACCCAACCCTGCGGCGGCATGG – 3´
17	*slr1768*-down	5´- TTAACCCCCATTCCAAACTTTGCA – 3´
18	*slr1128*-HIS-1	5´- TGCCTAGTGATGGTGATGGTGATGGTGATGAACTGCCCGATGGCGGTCTACCTT – 3´
19	*slr1128*-HIS-2	5´- GCAGTTCATCACCATCACCATCACCATCACTAGGCAGTTAGCTTGGGGGCTGGG – 3´
20	*slr1128*-down	5´- AAAAATGTTGTAATGTTGGACATTGGG – 3´
21	*sll0815*-HIS-1	5´- ATTTCAGTGATGGTGATGGTGATGGTGATGACTATCCGATGTTATTTTTTCTGA – 3´
22	*sll0815*-HIS-2	5´- GATAGTCATCACCATCACCATCACCATCACTGAAATTCAGCAAATTAAAATCTG – 3´
23	*sll0815*-down	5´- TTGGCCCTACTACCCACCAACCAATCC – 3´
24	His-Primer	5´- GTGATGGTGATGGTGATGGTGATG – 3´
25	M13-Fw	5´- GTAAAACGACGGCCAGTGA - 3´
26	M13-Rev	5´- GGAAACAGCTATGACCATG - 3´

2.4.3 Vectors

The originating vectors that have been used for cloning and expression in this work are shown in Table 2.3, whereas the generated, recombinant plasmids are shown in Table 2.4. Stocks of recombinant plasmids were maintained in bacterial cultures stored in 10 % (w/v) glycerol at –80 °C or as DNA plasmids in water stored at –20°C.

Table 2.3: Originating plasmids used for cloning and expression in this project.

plasmid	used for	source
pBluescript SK(+) (pBS)	cloning	Stratagene
pGEM-T Easy	cloning	Promega
pET-16b	expression	Novagen

Table 2.4: Generated, recombinant plasmids that were constructed in this project. p = plasmid, AMP = Ampicilin, CARB = Carbenicilin, CAM = Chloramphenical, ERM = erythromycin, KAN = kanamycin, SPEC = spectinomycin.

plasmid	used for	source	gene	selectable marker	restriction sites
pPHB1	Cloning	pGEM-T Easy	*slr1106*	AMP	none
pPHB2	Cloning	pGEM-T Easy	*slr1768*	AMP	none
pPHB3	Cloning	pGEM-T Easy	*slr1128*	AMP	none
pPHB4	Cloning	pGEM-T Easy	*sll0815*	AMP	none
pPHB5	Cloning	pGEM-T Easy	*sll1021*	AMP	none
pPHB1::KAN	Mutagenesis	pPHB1	*slr1106*	AMP, KAN	*Nae*I
pPHB2::CAM	Mutagenesis	pPHB2	*slr1768*	AMP, CAM	*Eco*NI
pPHB3::SPEC	Mutagenesis	pPHB3	*slr1128*	AMP, SPEC	*Msc*I
pPHB4::SPEC	Mutagenesis	pPHB4	*sll0815*	AMP, SPEC	*Hind*III
pPHB5::CAM	Mutagenesis	pPHB5	*sll1021*	AMP, CAM	*Hpa*I
pET16b-phb2	Expression	pET16b	*slr1768*	AMP, CARB	*Bam*HI / *Nde*I
pET16b-phb2-TM	Expression	pET16b	*slr1768*	AMP, CARB	*Bam*HI / *Nde*I
pET16b-phb3	Expression	pET16b	*slr1128*	AMP, CARB	*Bam*HI / *Nde*I
pET16b-phb3-TM	Expression	pET16b	*slr1128*	AMP, CARB	*Bam*HI / *Nde*I
pET16b-phb4	Expression	pET16b	*sll0815*	AMP, CARB	*Bam*HI / *Nde*I

2.4.4 DNA transformation of cells

2.4.4.1 Preparation of competent *E. coli* cells

Competent *E. coli* cells were produced for the transformation of plasmid DNA. It was absolutely crucial to work as sterile as possible and to autoklave any material that is used during the procedure to prevent contamination. A single colony was inoculated into 2.5 ml liquid LB media and grown overnight. On the next day the starter culture was inoculated into 250 ml liquid LB media. The optical density was monitored with a spectrophotometer (UV-1601; Shimadzu Corporation, Europe) until the culture reached an OD_{600} of 0.5. The cells were then pelleted by centrifugation (JA-14; 4,500 x g; 10 min; 4 °C) and resuspended in 100 ml ice-cold TFB1 (100 mM RbCl; 50 mM $MnCl_2$; 30 mM potassium acetate; 10 mM $CaCl_2$; 15 % (v/v) glycerol; pH = 5.8; filter sterile). The cell suspension was kept on ice for 5 min and pelleted again (JA-14; 4,500 x g; 10 min; 4 °C). The resulting cell pellet was resuspended in 10 ml of ice-cold TFB2 (10 mM MOPS, pH = 6.5; 75 mM $CaCl_2$; 10 mM RbCl; 15 % (v/v) glycerol; pH = 6.5; filter sterile) and incubated on ice for 30 min. Subsequently the cells were aliquotted, flash frozen in liquid nitrogen and stored at –80 °C.

2.4.4.2 Transformation of competent *E. coli* cells

Competent *E. coli* cells were thawed on ice. Roughly 1 µg of plasmid DNA was mixed with 100 µl of competent cells and incubated for 20 min on ice. The cells were heat shocked for 45 sec at 42 °C and incubated for 2 min on ice. After adding 800 µl of liquid SOC media (2 % peptone; 0,5 % yeast extract; 0,05 % NaCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose; pH = 7,5) the cells were incubated for 1 hour of vigorous shaking at 37 °C. The cells were plated onto LB plates with a suitable antibiotic selection and incubated at 37 °C overnight.

2.4.4.3 Transformation of *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 cells were transformed with recombinant plasmid DNA according to the protocol described by Williams (1988). A 100 ml culture of recipient

Synechocystis sp. PCC 6803 cells (wild type or mutant) was grown in BG11 medium supplemented with 5 mM glucose and if necessary appropriate antibiotic selection. An aliquot of the culture in exponential growth phase (OD_{730} < 1) was harvested by centrifugation (GS 3.8; 2,000 x g; 15 min; 29 °C) and diluted in a volume of approximately 2 ml to a final OD_{730} of 5.0. 100 µl of the cell resuspension were mixed for each transformation with 1 to 10 µg of recombinant plasmid DNA and incubated for 4 to 6 h under continuous illumination (~ 5 $\mu E*m^{-2}*s^{-1}$) and occasional, gentle agitation. Half of each transformation mix was plated onto a 0,45 µm cellulose nitrate membrane filter (Schleicher and Schuell) that was placed on a BG11 plate (with 5 mM glucose). The filter was kept on the plate for 48 h in order to allow expression of the antibiotic resistance. Subsequently, the filter was transferred to a BG11 plate that contained the suitable antibiotic(s). Resistant colonies appeared after 7 to 10 days and were restreaked onto plates with a rising concentration of antibiotic(s).

2.4.5 Extraction and purification of nucleic acids

2.4.5.1 Mini plasmid preparation from *E. coli*

Mini plasmid DNA preparation from *E. coli* was performed according to the method described in Birnboim and Doly (1979). A single colony was used to inoculate 5 ml of liquid LB media containing the appropriate antibiotic(s) for selection of the cells carrying the target plasmid. The culture was incubated overnight at 37 °C under vigorous shaking. Cells of 1.5 ml culture were harvested by centrifugation in a microfuge for 1 min. The harvested cells were resuspended in 100 µl of ice-cold cell resuspension solution (25 mM Tris-HCl, pH = 8.0; 10 mM EDTA; 50 mM glucose) and incubated for 5 min at room temperature. 200 µl of freshly prepared cell lysis solution (0.2 M NaOH; 1% (w/v) SDS) was added and the mixture was left on ice for further 5 min. Then 150 µl of ice-cold potassium acetate solution (3 M KOAc; 10 % (v/v) glacial acetic acid; pH = 4.8) were added to neutralize the lysate. The sample was properly mixed and left for 5 min on ice to precipitate contaminations. The sediment was spun down in a microfuge and the supernatant transferred to a new tube. The sample was incubated with 0.5 µl of 100 µg/µl DNase-free RNase A (Boehringer, Germany) and incubated for 5 min at room temperature. A volume of 450 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added, the mixture vortexed for 1 min and spun down in a microfuge

for 5 min. The upper aqueous phase was transferred to a fresh tube and 450 µl of chloroform:isoamyl alcohol (24:1) were added, the mixture vortexed for 30 sec and spun down in a microfuge for 2 min. The upper aqueous phase was transferred to a fresh tube and 1 ml of ice-cold 100 % ethanol was added. The sample was incubated for 5 min on dry ice to allow DNA precipitation. The DNA was pelleted in a microfuge for 5 min and the pellet was washed with 70 % (v/v) ethanol. The pellet resulting from the last centrifugation step was dried and resuspended in 100 µl of nuclease-free water. The obtained plasmid DNA was stored at –20 °C.

2.4.5.1. Midi plasmid preparation from *E. coli*

Midi plasmid DNA preparations from *E. coli* were performed using the Qiagen-Plasmid-Midi-Kit according to the suppliers´ instructions (see Qiagen Plasmid Purification Handbook 11/1998; p. 13ff). The dried, final plasmid DNA pellet was resuspended in 500 µl nuclease-free water and stored at –20 °C.

2.4.5.3. Estimation of DNA concentration and quality

The concentration of plasmid DNA midi preparations was determined with a spectrophotometer (UV-1601; Shimadzu Corporation, Europe) at a wavelength of 260 nm (A_{260}). An A_{260} of 1.0 is equivalent to a concentration of approximately 50 µg/ml of double stranded DNA, 33 µg/ml of single stranded DNA or 40 µg/ml RNA (Sambrook et al., 1989). The degree of contamination in the preparations could be estimated by measuring the A_{260}/A_{280} ratio. A value between 1.8-2.0 suggests a clean sample, whereas lower values indicate the presence of contaminants.
The concentration of mini plasmid DNA preparations was visually estimated after agarose gel electrophoresis. Ethidium bromide (EtBr) in the gel (at 1 µg/ml) allows the visualization of DNA with UV illumination. By comparing the intensity of the signal for the DNA with the unknown concentration to that of a marker DNA with a known concentration, it is possible to roughly estimate the concentration of the unknown sample. Contaminating RNA is also stained and runs at the bottom of the gel.

2.4.5.4. Total cellular DNA extraction from *Synechocystis* sp. PCC6803

Genomic DNA preparation of *Synechocystis* sp. PCC 6803 was performed according to a protocol devised by Dexter Chisholm (DuPont, Delaware, USA). A pea-sized glob of cells from a healthy plate was suspended in 400 µl TES (5 mM Tris-HCl, pH = 8.5; 50 mM NaCl; 5 mM EDTA). 100 µl of lysozyme (50 mg/ml stock) was added to the sample and incubated for 15 min at 37 °C. To avoid the cells from settling out during the incubation, the sample was occasionally mixed. 50 µl of 10 % (w/v) sarkosyl (N-dodecanoyl-N-methylglycine sodium salt) and 600 µl of phenol were added and mixed on a rotating wheel for 15 min at room temperature. The sample was spun down in a microfuge (12,000 x g; 5 min) and the aqueous phase was transferred to a fresh tube. Then 5 µl of DNase-free RNase was added (Boehringer, Germany) and the sample incubated for 15 min at 37 °C. After adding 100 µl of 5 M NaCl, 100 µl of CTAB-NaCl solution (700 mM NaCl; 10 % (w/v) cetyl trimethyl ammonium bromide) and 600 µl of chloroform, the sample was mixed again on a rotating wheel for 15 min at room temperature. The sample was centrifuged in a microfuge (12,000 x g; 2 min) and the upper aqueous phase was transferred to a new tube. The DNA was precipitated with 700 µl of isopropanol and pelleted in a microfuge (12,000 x g; 15 min). The obtained pellet was washed with 70 % (v/v) ethanol and pelleted again. Finally the DNA was resuspended in 100 µl TE (10 mM Tris-HCl, pH = 7.5; 1 mM EDTA) or nuclease-free water and stored at –20 °C.

2.4.6 DNA manipulations

2.4.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis allows the separation of DNA fragments according to their sizes. Gels were prepared with agarose (molecular grade agarose; Bioline, London, UK; final [0.5-1.0 % (w/v)]), TAE-buffer (40 mM Tris-acetate; 1 mM EDTA pH = 8.0) and EtBr (final [1 µg/ml]). Samples were loaded in the wells after the addition of 6 x loading buffer (40 % (w/v) sucrose; 0.25 % (w/v) bromophenol blue; final [1x]). The gels were run in TAE-buffer at 50-100 V. DNA could be visualized directly by using a UV transilluminator and a photograph was taken (BioDoc-IT^{TM} System; UVP;CA; USA)(VideoGraphicPrinter UP-890CE; Sony; Japan). To estimate the size of unknown DNA fragments and compare the size of known

DNA fragments, a marker (2-log DNA marker ladder, NEB, Herts) was loaded in one lane of all the agarose gels which contained fragments of the following sizes (in kb): 10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.5, 1.2, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1.

2.4.6.2. DNA purifcation from agarose gels

DNA fragments from agarose gels were purified using a gel extraction kit (QIAGEN Ltd., West Sussex) according to the manufacturers´ instructions.

2.4.6.3. Gene amplification

Synechocystis sp. PCC 6803 genes were amplified using polymerase chain reaction (PCR) with either Techne Touchgene Gradient (FTGrad2D; Techne Ltd., Cambridge, UK) or Techne Techgene (FTGene5D; Techne Ltd., Cambridge, UK). A typical reaction mixture contained 5 µl of 10x reaction buffer (dependent on polymerase; as supplied by Bioline, London), 2 µl 10 mM dNTP mixture (ABgene, Surrey), 1.5 to 4 µl $MgCl_2$ (50mM; Bioline, London), 1 µl of 50 mM forward primer (see table 2.2), 1 µl of 50 mM reverse primer (see table 2.2), 0.5 µl BioTaq or BioXact long (both Bioline, London) and ~ 100 ng template DNA. ddH_2O was added to a final volume of 50 µl and the reaction mixture was overlaid with 50 µl of mineral oil. The PCR program consisted of an initial denaturation step at 94 °C for 3 min and 30 subsequent cycles of 94 °C for 30 sec (denaturation), from 46 to 60 °C for 30 sec (primer annealing), 72 °C for 4 min (primer extension). The final extension step was performed at 72 °C for 10 min. After the PCR reaction had finished the PCR machine would hold a temperature of 4 °C. The reaction mixture and the PCR program were varied when the standard procedure did not lead to optimal amplification.

2.4.6.4. Restriction endonuclease digestion

Restriction endonuclease digestions were performed under suitable conditions as recommended by the manufacturers´ instructions (NEB, Herts). Generally, approximately 1

µg of DNA was digested in 15 µl with 10-20 units of restriction endonuclease in the recommended buffer with acetylated bovine serum albumin (BSA; final [100 µg/ml]) at the optimum temperature in 1.5 hours. The restriction digests were analysed by agarose gel electrophoresis.

2.4.6.5. Ammonium acetate DNA precipitation

Ammonium acetate precipitation is a suitable method for the purification of DNA, especially for PCR products, as the vast amount of dNTP´s (deoxyribonucleoside triphosphates) is not precipitated in the procedure. 1.6 volumes of 7.5 M ammoniumacetate and 8 volumes of 100 % (v/v) ethanol were added to the sample and the mixture was incubated for 15 min at –80 °C. The DNA was pelleted in a microfuge (12,000 x g, 10 min) and the supernatant discarded. The pellet was then washed in 70 % (v/v) ethanol and air dried. The DNA was resuspended in the original volume of nuclease-free water.

2.4.6.5. Removal of 5´ overhangs from DNA

In order to insert a blunt ended DNA fragment into a 5´ overhang restriction site, it was necessary to fill in the 5´ overhangs before the ligation. The Klenow enzyme fragment of *E. coli* DNA polymerase I was utilized for this purpose, because it lacks the 5´ to 3´ exonuclease activity. Each reaction contained 5 µl 10x restriction endonuclease buffer (buffer 2; as supplied by NEB, Herts), 5 µl 10x BSA (1 mg/ml; NEB, Herts), 1 µl dNTP-mix (20 mM; ABgene, Surrey), 1 unit of Klenow polymerase (Promega Corp., Wisconsin), 150 ng of DNA and ddH_2O was added to a final volume of 50 µl. The reaction mixture was incubated for 15 min at room temperature and afterwards heat inactivated for 20 min at 75 °C.

2.4.6.6. DNA ligation

DNA fragments were inserted into various recipient vectors using T4 DNA ligase according to the manufacturers´ instructions (Promega Corp., Wisconsin). Excess insert fragment was

provided and in most cases the insert/vector molar ratio was 4:1. The reaction mixture was incubated over night at 4 °C.

2.5 Microarray analysis

The presented microarray data and the method with which it was obtained was kindly provided by Dr. Iwane Suzuki (National Institute for Basic Biology; Nishigonaka 38; Myodaiji; Okazaki 444-8585; Japan).
Wld type *Synechocystis* sp PCC 6803 (glucose-tolerant strain) was grown in liquid BG-11 medium under continuous light at 70 µE * m^{-2} * sec^{-1} with an aeration of 1% CO_2 / air (v/v) at 34 °C. At an OD_{730} of 0.3 to 0.5 the cells, which had been growing for 12 to 16 h after inoculation into fresh BG-11 medium, were placed for 20 min under various stress conditions: salt stress (0.5 M NaCl), hyperosmotic stress (0.5 M sorbitol), heat stress (shift from 34°C to 44 °C), oxidative stress (0.25 mM H_2O_2), cold stress (shift from 34 °C to 22 °C) and high light stress (shift from 70 µE * m^{-2} * sec^{-1} to 500 µE * m^{-2} * sec^{-1}) before RNA was isolated for the microarray analysis. *Synechocystis* DNA microarrays (Cyano-CHIP) were purchased from TaKaRa Bio Co. Ltd. (Ohtsu, Japan), and the analysis was performed as described previously (Suzuki et al. 2001; Kanesaki et al. 2002). All experiments were performed with CyanoCHIP version 1.6, which included 3074 out of the 3264 genes on the *Synechocystis* chromosome, and results were quantified with the IMAGENE version 5.5 program (BioDiscovery, El Segundo, CA). Changes in the levels of transcripts of individual genes relative to the total level of mRNA were calculated after normalization by reference to the total intensity of signals from all genes with the exception of genes for rRNAs.

2.6 Protein biochemistry

2.6.1 Antibody generation

The prohibitin homologues had to be overexpressed and purified for the generation of polyclonal antibodies from rabbits. After the antigens were produced and purified, they where send to SEQLAB (Germany, Göttingen) for a three months immunization procedure.

2.6.1.1 Small scale protein overexpression

As not every bacterial expression strain seemed to be able to produce a protein from every expression construct, small scale experiments to find the optimal strain / construct combination had to be performed, before the prohibitin homologues could be overexpressed on a large scale. A 20 ml liquid culture of transformed bacteria was grown in LB media at 37 °C and constant shaking until it reached an OD_{600} of ~0.5. The culture was then equally divided and expression was induced in one half by adding IPTG (+) to a final concentration of 1 mM. The other half remained uninduced (-). After further three hours at 37 °C and constant shaking a 1 ml aliquot was taken to determine the final OD_{600} and the rest of the cells were pelleted. The cell pellet was resuspended in such an amount of 50 mM Tris pH = 7.5 that 10 µl corresponded to an OD_{600} of 0.1. To assess whether the desired protein had been produced, 10 µl of the total cell extract were analysed by 1-D SDS PAGE.

2.6.1.2 Large scale protein overexpression

After finding the appropriate expression construct / bacterial strain combination (constructs: pET16b-phb2-TM; pET16b-phb3-TM and pET16b-phb4; strain: *E. coli* BL21-(DE3)-pLysS) the proteins were expressed on a large scale for their subsequent purification. A liquid 1 l culture of transformed BL21-(DE3)-pLysS *E. coli* cells was grown in LB media at 37 °C and constant shaking until it reached an OD_{600} of ~0.5. The protein expression was then induced by adding IPTG (+) to a final concentration of 1 mM. After another three hours at 37 °C and constant shaking the final OD_{600} was determined. Before the cells were pelleted and stored at

–80°C another 1 ml aliquot was taken and processed as described for the small scale expression to test whether protein expression had indeed been induced.

2.6.1.3 Bug buster procedure

The bug buster procedure (Novagen, Nottingham) is a fast method to lyse bacterial cells and test whether the overexpressed protein is soluble or forms inclusion bodies. The procedure was performed according to the manufacturer´s manual (TB245 11/99) and the final products were purified inclusion bodies of the overexpressed prohibitin homologues.

2.6.1.4 Purifcation of overexpressed prohibitins homologues

The inclusion bodies that were obtained from the bug buster procedure were solubilized for one hour on ice in binding buffer (20 mM Tris/HCl pH = 7.9; 0.5M NaCl; 6 M guanidium hydrochloride). Then the sample was filtered through a 0.2 µm filter and centrifuged (JA-14, 16,000 x g; 10 min; 4 °C) to remove large particles. An aliquot of the cleaned sample was removed for 1-D SDS PAGE analysis. The filtered sample was incubated on a torture wheel with 5 ml of the Ni^{2+} charged resin (FastFlow Chelating Sepharose; Amersham Pharmacia; charging of the resin according to the manufacturer´s manual) at 4 °C over night to allow proper binding of the overexpressed proteins. After over night incubation, the resin was washed with 25 ml of several buffers containing an increasing imidazole concentration (20 mM Tris/HCl pH = 7.9; 0.5M NaCl; 6 M guanidium hydrochloride and imidazole concentrations from 5 mM [used twice], 20 mM and 100 mM). The flow throughs were collected and analysed by 1-D SDS PAGE. The bound protein was eluted with elution buffer (20 mM Tris/HCl pH = 7.9; 0.5M NaCl; 6 M guanidium hydrochloride; 300 mM imidazole) and the sample was collected for further processing. The rest of the bound protein was eluted from the resin with buffer containing 500 mM imidazole. The eluted flow through fraction (elution with 300 mM imidazole) was then dialysed over night against 1 x PBS buffer (150 mM NaCl; 7.5 mM Na_2HPO_4; 2.5 mM NaH_2PO_4) (dialysis membrane name: Spectrum, Cambs; Spectra/Por 1 membrane tubing, cut off ~6 to 8 kDa). The dialysate was centrifuged to collect precipitated protein and the supernatant was analysed by 1-D SDS PAGE. The

pellet of this centrifugation step was resuspended in a smaller volume of 1 x PBS buffer. To reduce the volume to fit in an eppendorf tube, the sample had to be centrifuged again and was finally resuspended in 500 µl PBS buffer. The overexpressed protein was solubilized by adding 300 µl 10 % (w/v) SDS leading to a final SDS concentration of around 3.8 %. The amount of sample that was used for the 1-D SDS PAGE analysis was normalized, so 50 µl of the sample corresponded to 25 ml of the original flow through volume. All samples were acetone precipitated and resuspended in 10 µl of 50 mM Tris / HCl pH=7.5.

2.6.1.5 Acetone precipitation of proteins

The protein sample was precipitated by adding four times the sample volume of ice-cold acetone (precooled at –20 °C). The mixture is vortexed and incubated for 60 min at -20 °C. After incubation the sample was centrifuged in a microfuge (12,000 x g; 10 min). The supernatant was decanted thoroughly and the protein pellet is resuspended in desired buffer.

2.6.1.6 Immunization procedure

SEQLAB immunization protocol (3 months)

day 0	1. injection (extraction of > 5 ml preimmune serum)
day 21	2. injection
day 35	first bleeding (extraction of 10 – 20 ml serum)
day 49	3. injection
day 63	second bleeding (extraction of 10 – 20 ml serum)
day 77	4. injection
day 98	final bleeding (extraction of > 50 ml serum)

For the immunization procedure two 1 ml aliquots of each antigen (125 µl of resuspended and solubilized protein [~5 mg] from 2.6.1.4 with an additional 875 µl of 1 x PBS; final SDS concentration ~ 0.47 %) were send off to SEQLAB (Germany, Göttingen). The antigens (Phb2-TM; Phb3-TM and Phb4) were injected into two rabbits each (first injection 100 to 500 µg and the following injections 100 µg) and the final bleeding sera were send back after the three month immunization protocol. Preimmun serum, first and second bleeding for testing purposes were sent back shortly after the respective sera had been extracted.

2.6.2 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is performed to separate a complex protein mixture into distinctive bands in a polyacrylamide gel matrix (Laemmli, 1970). The separation is based on size differences of proteins and the use of particular markers allows to determine the molecular weight of particular proteins or protein complexes.

2.6.2.1 1-D sodium dodecyl sulphate gel electrophoresis (SDS PAGE)

1-D SDS PAGE analysis is the most common technique applied in protein biochemistry to assess denatured proteins. A sample for 1-D SDS PAGE analysis is mixed with the same volume of 2 x SDS sample buffer (6% (w/v) SDS; 30 % Sucrose; 20 mM sodium carbonate (Na_2CO_3); 0.09 % bromophenol blue and 0.6 % (v/v) β-mercapto-ethanol) and incubated (a) for 5 min at 95 °C [*E. coli* total cell extracts] (b) 1 h at RT [thylakoid membrane and soluble extracts from *Synechocystis* sp. PCC 6803] or (c) for 5 min at 95 °C [thylakoid membrane and soluble extracts from *Thermosynechococcus elongatus*]. Subsequently, unsolubilized sample is pelleted in a microfuge (12,000 x g; 10 min). Various protein gel systems were available: BioRad Protean II, BioRad protean III and Hoefer vertical electrophoresis unit SE 400. Gels were self cast and their size was dependent on the gel system that was used. Stacking gel (5 % final concentration of polyacrylamide from 40 % Acrylamide / Bisacrylamide = 37.5 / 1 stock solution; 0.125 M Tris / HCl pH = 6.8; 0.01 % (v/v) TEMED; 0.1 % (w/v) APS) routinely had a polyacrylamide concentration of 5 % whereas the polyacrylamide concentration of the separation gel (10 to 12.5 % final concentration of polyacrylamide from 40 % Acrylamide / Bisacrylamide = 37.5 / 1 stock solution; 0.375 M Tris / HCl pH = 8.9; 6 M Urea; 0.01 % (v/v) TEMED; 0.1 % (w/v) APS) was dependent for which region the resoltution was supposed to be optimized. As a guideline 12.5 % gels were used when the focus was on the 30 kDa region and 10 % gel were used when higher molecular weight proteins were of particular interest. To obtain focused bands it proved crucial to adjust the pH of the separation gel solution to 8.9 just before adding the TEMED and APS. The gel was run with a constant voltage of 100 Volts and a Tris / Glycine running buffer (25 mM Tris; 190 mM Glycine; 0.1 % (w/v) SDS; pH = 8.3). Gels were either stained or used for further immuno blotting analysis.

2.6.2.2 1-D Blue Native polyacrylamide gel electrophoresis (BN PAGE)

Blue Native polyacrylamide gel electrophoresis (BN PAGE) is performed to separate protein complexes under "native" conditions according to their molecular weights (Schägger et al 1991 and 1994). The stacking gel (4 % final concentration of polyacrylamide from 30 % Acrylamide / Bisacrylamide = 30 / 0.8 stock solution; 500 mM ε-amino caproic acid; 50 mM Bis-Tris / HCL pH = 7.0; 0.05 % (v/v) TEMED; 0.015 % (w/v) APS) was poured on top of the polymerised separation gel (6 - 12 % final concentration of polyacrylamide from 30 % Acrylamide / Bisacrylamide = 30 / 0.8 stock solution; 500 mM ε-amino caproic acid; 50 mM Bis-Tris / HCL pH = 7.0; 20 % (w/v) glycerine [only in the 12 % gel pouring solution]; 0.05 % (v/v) TEMED; 0.015 % (w/v) APS). The separation gel was a 6 to 12 % linear gradient gel that was poured with a gradient mixer (2117 Multiphor II gradient maker; LKB Bromma) (see Figure 2.1). After pouring the gel, it was covered in wet tissue paper and stored until it was used (maximum of 4 days).

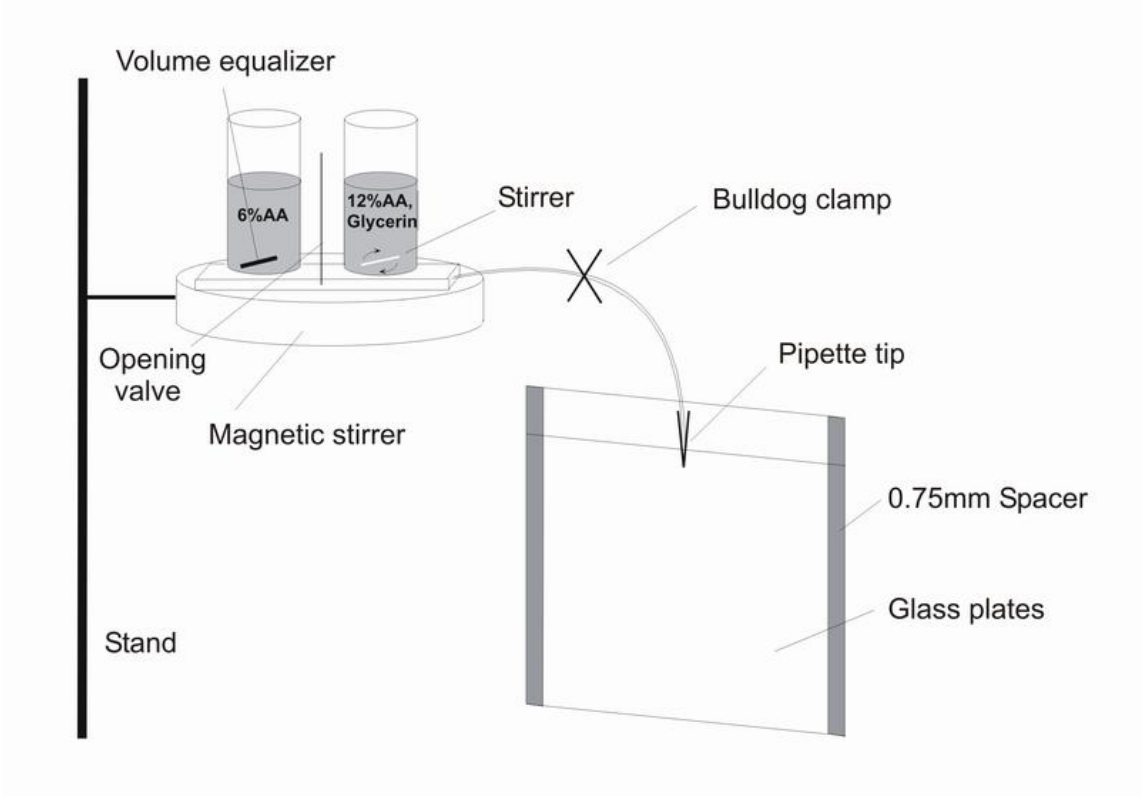

Figure 2.1: Schematic drawing of the gradient gel pouring process (adapted from Bisle, 2000). Before the gel solutions were poured into the gradient maker (heavy solution into the chamber nearest to the exiting opening) it needed to be checked whether the opening valve was closed and the bulldog clamp in place. At the end of the tubing a pipette tip ensured that the gel solution would be directed in between the prepared glass plates. After adding the appropriate amounts of TEMED and APS the opening valve was opened and the proper mixing of the solutions with the magnetic stirrer was controlled. Removing the bulldog clamp initiated the gel pouring process.

1/10 of the sample volume of a 10 % (w/v) DM (N-dodecyl-β-maltoside) was added to the sample, suspended in ACA buffer (750 mM ε-amino caproic acid; 50 mM Bis-Tris / HCL pH = 7.0; 0.5 mM EDTA), to mildly solubilize the protein complexes for 10 minutes on ice. Unsolubilized proteins and other insoluble material was pelleted in a microfuge (12,000 x g, 10 min) and 1/10 of the original sample volume loading buffer (5 % (w/v) Sigma Coomassie Brilliant Blue G; 750 mM ε-amino caproic acid) was added. Now the samples could be applied into the wells of the prepared gel and could be run in the Biorad Protean II system (size of 20 cm x 20 cm x 0.075 cm). The necessary buffers: anode buffer (50 mM Bis-Tris / HCL pH = 7.0), cloured cathode buffer (50 mM Tricine; 10 mM Bis-Tris / HCL pH = 7.0; 0.02 % (w/v) Sigma Coomassie Brilliant Blue G) and colourless cathode buffer (50 mM Tricine; 10 mM Bis-Tris / HCL pH = 7.0) were precooled at 4 °C. The gel was run for 3 to 4 hours at 4 °C and 12 mA per gel (It was possible to run two gels at the same time in the Protean II gel system). After the blue coloured buffer front had reached the middle of the gel, the upper coloured cathode buffer was exchanged for the uncoloured cathode buffer. The gels resulting from this procedure will be referred to as first dimensions.

2.6.2.3 SDS PAGE for the second dimension

The successive application of 1-D BN PAGE and SDS PAGE as a second dimension divided separated protein complexes from the first dimension into their subunits in the second dimension. Separation in both dimensions was according to the molecular weight of proteins and their respective complexes. After the polymerisation of the separation gel (for Ettan DALT six; Amersham Pharmacia; 12.5 % final concentration of polyacrylamide from 30 % Acrylamide / Bisacrylamide = 30 / 0.8 stock solution; 0.375 M Tris / HCl pH = 8.9; 4 M Urea; 0.01 % (v/v) TEMED; 0.1 % (w/v) APS) the gel cassette needed to be sealed at the sides. Thus, 0.5 % (w/v) agarose in running buffer (25 mM Tris; 190 mM Glycine; 0.1 % (w/v) SDS; pH = 8.3) was used to seal the gaps through which the stacking gel solution might have run off otherwise. The stacking gel solution (5 % final concentration of polyacrylamide from 30 % Acrylamide / Bisacrylamide = 30 / 0.8 stock solution; 0.125 M Tris / HCl pH = 6.8; 0.01 % (v/v) TEMED; 0.1 % (w/v) APS) was then poured to a height of approximately 2 cm on top of the separation gel.

In order to disjoint the protein complexes into their respective subunits, excised gel lanes from the first dimension had to be incubated for 20 min at room temperature under gentle agitation in solubilization buffer (2 % (w/v) SDS; 4 M Urea; 66 mM sodium carbonate [Na_2CO_3]; 2 % (v/v) β-mercapto-ethanol). Afterwards the gel slice was slipped in between the glass plates of the prepared separation gel and a piece of whatman paper, soaked in 10 µl of low molecular weight marker (Amersham Pharmacia) or prestained protein marker (NEB, Herts), was slipped on top of the stacking gel as well. The remaining space in between the glass plates was filled with more 0.5 % (w/v) agarose in running buffer. Once the gels were prepared in this manner the lower buffer chamber was filled with running buffer, the gels loaded and then the upper gel chamber could be filled witht running buffer. Finally the gel was run at a constant temperature of approximately 25 °C controlled by a thermostatic circulator (2219 MultiTEMP II; LKB Bromma). At the beginning a current of 30 mA per gel was applied for 20 min which was then reduced to 16 mA per gel for an overnight run. The Ettan DALT six system allows to run six gels simultaneously.

2.6.2.4 Coomassie-Brilliant-Blue-R-250-staining of polyacrylamide gels

The Coomassie staining procedure is a fast but not a very sensitve method for the staining of protein gels. To stain the gel it is incubated in a staining solution (40 % (v/v) EtOH; 10 % (v/v) Acetic acid and 0.2 % (w/v) Coomassie-Brilliant-Blue-R-250) for at least one hour. The first destaining solution (40 % (v/v) EtOH and 10 % (v/v) Acetic acid) destains the background as well as the proteins. Thus, it is advisable to follow the destaining process from time to time. After approximately 15 to 30 min the gel in transferred into the second destaining solution (10 % (v/v) Acetic acid) in which the background stain will be completely removed. After the gels were completely destained, they were either sealed in plastic foil and filed away or dried on whatman paper using a gel dryer (Biorad; Model 583).

2.6.2.5 Silver staining

The silver staining procedure is approximately ten times more sensitive and was performed as outlined in table 2.5. To obtain the best results large containers should be used to ensure

proper mixing and incubation with the respective solutions. Touching of the gels without gloves should in any case be avoided because of the sensitivity of this detection method.

Table 2.5: Silver staining protocol.

step	solution	amount per 200 ml solution	incubation time [min]
fixation	50 % methanol	100 ml	4 h
	12 % acetic acid	24 ml	
	0,05 % formaldehyde; 37% (w/v)	100 µl	
	ddH_2O	76 ml	
wash	50 % methanol	100 ml	3x20
pretreat	0,02 % sodium thiosulfate ($Na_2S_2O_3$; 5 x H_2O)	0,04 g	1
wash	ddH_2O	-	3 x 20 sec
stain	0,2 % silvernitrate	0,4 g	20
	0,075 % formaldehyde; 37 % (w/v)	150 µl	
wash	ddH_2O	-	2 x 20 sec
develop	6 % sodium carbonate (Na_2CO_3)	12 g	by eye
	0,05 % formaldehyde; 37 % (w/v)	100 µl	
	2 % pretreat stock solution	4 ml	
	ddH_2O	196	
wash	ddH_2O	-	2 x 5 sec
stop	50 % methanol	100 ml	10
	12 % acetic acid	24 ml	
wash	50 % methanol	100 ml	> 20

2.6.2.6 Protein markers

The following protein markers were used for PAGE analysis in this work:

- low molecular weight marker for SDS PAGE (Amersham Biosciences)
- high molecular weight marker for SDS PAGE (Amersham Biosciences)
- high molecular weight marker for native electrophoresis (Amersham Biosciences)
- prestained protein marker, broad range (NEB, Herts)

2.6.3 Size exclusion experiment using FPLC

Solubilized proteins and protein complexes from *Synechocystis* sp. PCC 6803 thylakoid membrane extracts were separated by size on a Superdex200 (Amersham Pharmacia) column using the ÄKTA FPLC setup (Amersham Pharmacia). 3 ml of thylakoid membrane extract from a large scale thylakoid membrane preparation were diluted in 20 ml TMK buffer (10mM Tris / HCl pH = 6.8; 10 mM $MgCl_2$; 20 mM KCL). The membranes were pelleted in an ultracentrifuge (Ti70; 103.000 x g; 30 min; 4°C), the supernatant discarded and the pellet resuspended in 300 µl 1 x PBS (150 mM NaCl; 7.5 mM Na_2HPO_4; 2.5 mM NaH_2PO_4) with 0.05 % (w/v) DM (N-dodecyl-β-maltoside). Unsolubilized material was pelleted in a microfuge (12,000 x g; 10 min). Approximately 100 µg were solubilized for 10 min on ice with 1/10 of the sample volume 10 % (w/v) DM. After solubilization the sample was once again centrifuged in an ultracentrifuge (MLA-130; 120,000 x g; 30 min; 4 °C) and the supernatant was loaded on the FPLC column (100 µl could be applied to the loading loop). The run was controlled by the ÄKTA unit (high pressure alarm at 1.25 MPa; flow rate 0.5 ml / min) and fractions of 500 µl were collected and analysed using 1-D SDS PAGE. To assign molecular weigths to the eluted proteins, markers were run using the same conditions and setup. Following markers (high molecular weight marker kit for FPLC; Amersham Pharmacia) were used: Dextran Blue 2000 (eluted after 7.8 ml), Thryoglobulin (669 kDa; eluted after 8.7 ml), Catalase (232 kDa; eluted after), Aldolase (158 kDa; eluted after 12.15 ml), BSA (67 kDa; eluted after 13.98 ml) and Ribonuclease A (13.7 kDa; eluted after 18.06 ml).

2.6.4 Immunoblotting analysis

Unstained polyacrylamide gels from 1-D SDS PAGE, 1-D BN PAGE or 2-D SDS PAGE were used for the immuno blotting analysis. Gels were incubated in transfer buffer (3 mM sodium carbonate [Na_2CO_3]; 10 mM sodium hydrogen carbonate [$NaHCO_3$]; 20 % (v/v) MetOH) and transferred to a nitrocellulose membrane (Trans Blot, Transfer medium nitrocellulose membrane, 0.2 µm; BioRad) using either a semidry setup (2117 Multiphor II, electrophoresis unit; LKB Bromma) (Bjerrum und Schäfer-Nielsen, 1986) or a tank blot method (TransBlot; BioRad) (Burnette, 1981; Towbin et al., 1979). The Protein transfer took place with a constant current of 400 mA for at least 1 h. When a gel was destined for

immunoblotting analysis, prestained protein marker (Prestained protein marker, broad range; NEB, Herts) was loaded in one well of the gel, to allow a visiual confirmation of the transfer. After transferring the proteins, the nitrocellulose membrane was blocked for 1 h against unspecific antibody binding to the membrane with either a BSA containing blocking solution (3 % (w/v) BSA in 1 x PBS with 0.1 % (v/v) Tween 20) or with milk powder (5 % (w/v) milkpowder in 1 x PBS with 0.1 % (v/v) Tween 20). The membrane was then washed three times for 10 min with 1 x PBS with 0.1 % (v/v) Tween 20. The incubation with the primary antibody usually took place over night at 4 °C on a rocking shaker (preferably in small boxes). On the next day the membrane was washed three times for 20 min with 1 x PBS with 0.1 % (v/v) Tween 20 to remove unbound primary antibody.

Table 2.6: Primary antibodies that have been used in this work.

antibody	originating organism	dilution	dilute in	2nd antibody
αPHB1	*Synechocystis* sp. PCC 6803	1:7500	1 x PBS 0.1% Tween 20	αrabbit
αPHB2-1	*Synechocystis* sp. PCC 6803	1:5000	1 x PBS 0.1% Tween 20	αrabbit
αPHB3-1	*Synechocystis* sp. PCC 6803	1:5000	1 x PBS 0.1% Tween 20	αrabbit
αPHB4-1	*Synechocystis* sp. PCC 6803	1:5000	1 x PBS 0.1% Tween 20	αrabbit
αD1 (#304-F)	*Synechocystis* sp. PCC 6803	1:5000	1 x PBS 0.1% Tween 20	αrabbit
αFtsH	*E. coli*	1:5000	1 x PBS 0.1% Tween 20	αrabbit
αSTREP	synthetic peptide	1:1000	1 x PBS 0.1% Tween 20	αmouse
αNdhI (#182)	*Synechocystis* sp. PCC 6803	1:2000	1 x PBS 0.1% Tween 20	αrabbit
αPsbO	*Pisum sativum*	1:5000	1 x PBS 0.1% Tween 20	αrabbit

After the washing procedure, the appropriate secondary antibody (Horseradish peroxidase coupled αmouse and αrabbit secondary antibody, 1:10000 dilution in 1 x PBS with 0.1 % (v/v) Tween 20; Amersham Pharmacia) was applied for 1 h at room temperature to the membrane. The secondary antibody was detected on the membrane by the enhanced chemiluminescence procedure (ECL; Durrant, 1990; Schneppenheim et al., 1991). Therefore the membrane was incubated for 1 min in a 1:1 mixture of ECL reagent A (100 mM Tris / HCl pH = 8.5, 0.4 mM p-coumaric acid [in DMSO], 250 mM luminol) and ECL reagent B (100 mM Tris / HCl pH = 8.5, 100 mM H_2O_2), transferred into an A4 reinforced pocket and exposed to a X-Ray film (SuperRX, X-Ray Film, 100 NIF, 18 x 24 cm; Fuji medical) for 1 sec to 10 minutes. The film was developed according to the manufacturers´ instructions.

2.7 Analysis of *Synechocystis* sp. PCC 6803

2.7.1 Thylakoid extraction from Synechocystis sp. PCC 6803

2.7.1.1 Small scale preparation

Sufficient *Synechocystis* sp. PCC 6803 cells were harvested by centrifugation (3,000 x g; 10 min; 4 °C) to yield ~ 100 µg of chlorophyll a. The resultant pellet was resuspended in a 1.5 ml eppendorf tube with 500 µl of ice cold buffer A (25 mM Tris / HCL pH = 7.5; 1 mM ε-amino caproic acid) and recentrifuged in a microfuge (7,000 x g; 1 min). The supernatant was then discarded and 250 µl of buffer A was added with 200 µl glass beads (150-200 µm; Sigma). The mixture was vortexed for 1 min, placed on ice for 1 min and this process repeated. The supernatant was then transferred to a new tube and the glass beads were washed with approximately 1 ml of buffer A. The supernatants were pooled and centrifuged in a microfuge (7,000 x g; 1 min) to pellet unbroken cells. The supernatant was transferred to a new eppendorf tube and again centrifuged (12,000 x g; 20 min; 4 °C). The blue supernatant was kept separately and the pellet was resuspended in 100 µl of buffer B (25 mM Tris / HCL pH = 7.5; 1 mM ε-amino caproic acid; 1 M sucrose). The chlorophyll a content was determined, the sample flash frozen and stored at –80°C.

2.7.1.2 Large scale preparation

The large scale thylakoid preparation uses a French press to break the cyanobacterial cells. All materials (French press, centrifuges, rotors, centrifuge tubes, buffers, etc) should be precooled and the procedure should be performed in the cold room whenever possible. After the cells have been broken, containers with the sample should be kept in the dark and be covered with aluminum foil to minimize unnecessary damage. Cyanobacterial cells, of a 1 l liquid culture growing in log phase (OD_{730} between 0.5 and 1.0), were harvested by centrifugation (JA-14; 6,500 x g; 15 min; 4°C). The pellet was resuspended in 30 ml buffer A (25 mM Tris / HCL pH = 7.5; 1 mM ε-amino caproic acid) and transfered to a 50 ml Falcon tube. Cells could be flash frozen in liquid nitrogen and stored away at –80°C at this point. The cells were applied to the French press with two passages at 20,000 psi and collected drop wise in a aluminum foil covered Falcon tube on ice. Unbroken cells were pelleted in the benchtop centrifuge (GS

3.8; 2,000 x g; 15 min; 4 °C) and the supernatant containing the thylakoid membranes was ultracentrifuged (Ti70 or Ti55.2; 103,000 x g; 30 min; 4°C). Aliquots of the blue supernatant of this centrifugation step can be kept for the analysis of the soluble fraction and the rest can be discarded. The pellet could be resuspended in either buffer B for storage (25 mM Tris / HCL pH = 7.5, 1 mM ε-amino caproic acid, 1 M sucrose) or in ACA buffer for immediate 1-D BN PAGE (750 mM ε-amino caproic acid; 50 mM Bis-Tris / HCL pH = 7.0; 0.5 mM EDTA). In any case the chlorophyll a content of the sample was determined.

2.7.2 Differential protein extraction

Differential protein extraction is performed to assess whether a protein complex is peripheral or integral and to qulitativley estimate how strongly the protein complex is attached to the respective membrane that is studied. Several thylakoid membrane extracts from wild type *Synechocystis* sp. PCC 6803 cells were isolated on a small scale. Equal amounts of approximately 20 µg were pelleted in a microfuge (12,000 x g; 20 min) and resuspended in 100 µl of various buffers: buffer B (25 mM Tris / HCL pH = 7.5, 1 mM ε-amino caproic acid, 1 M sucrose; stored away right away as a control), extraction buffer (20 mM Tricine pH = 8.0), extraction buffer plus various additives (2 M NaCl, 6.8 M Urea and 0.5 M EDTA, all pH = 8.0 and 100 mM Na_2CO_3, pH = 12.0). 10 µl of the resupended pellets were used for chlorophyll a determination. The samples were incubated for 15 min at room temperature and then treated twice with a freeze (30 min at –80°C) and thaw (20 min at room temperatur) cycle. After this treatment the thylakoid membranes were pelleted in a benchtop ultracentrifuge (TLA 120.1; 100,000 x g; 20 min; 4°C). The supernatant was removed and kept as the soluble fraction whereas the pellet was resuspended in 90 µl buffer B as the pellet fraction (samples could be stored at –80 °C at this stage). Equal amounts of the soluble and the pellet fraction corresponding to 1 µg chlorophyll a were loaded on a gel and analysed by 1-D SDS PAGE followed by immunoblotting analysis.

2.7.3 Photoinhibition

Initially, 100 ml cultures of wild type and mutant *Synechocystis* sp. PCC 6803 cells were grown up in liquid BG-11 and inoculated into 1000 ml liquid BG-11. The larger cultures were grown to an OD_{730} between 0.5 and 0.9 and used for photoinhibition. The chlorophyll a concentration of the cultures was then estimated and sufficient cells centrifuged (GS 3.8; 2,000 x g; 15 min; 30 °C) with gentle resuspension to produce 100-300 ml of cells wih a chlorophyll a concentration of 10-25 µg/ml. Resultant suspensions were placed in a sterile flat bottomed flask and incubated at 30 °C and a light intensity of 100 µE * m^{-2} * s^{-1}. After that the cultures were split, lincomycin added (a protein synthesis inhibitor, final [100 µg/ml] in MetOH) and the light intensity raised to 1200 µE * m^{-2} * s^{-1}. 3 ml samples were taken every hour for six hhours and diluted to 5 µg chlorophyll a / ml for oxygen evolution experiments. Two 1 ml aliquots of the diluted sample were flash frozen for a later chlorophyll a concentration determination.

2.7.4 Oxygen evolution measurements

The activity of photosystem II was assessed by means of oxygen evolution. Cultures with a chlorophyll a concentration of 5 µg / ml were assessed for their oxygen evolution rate by use of an oxygen electrode (DW2/2 unit, Hansatech instruments Ltd., Norfolk). Measurements requird the use of 2,6 dichlorobenzoquinone (DCBQ, Eastman Kodak Co., New York; final [2 mM] in EtOH), a photosystem II Q_A electron acceptor and $K_3Fe(CN)_6$ (final [1 mM]), a DCBQ oxidising agent. Both reagents were mixed with the sample to a total volume of 1 ml and cells were kept in the dark until oxygen evolution had stabilized. After stabilization actinic light illumination commenced at 800 µE * m^{-2} * s^{-1} for ~ 3 min and oxygen evolution was recorded. Oxygen evolution rates were calculated in terms of µmol oxygen * mg chlorophyll a $^{-1}$ * h^{-1} and plotted.

2.7.5 Pulse-chase experiment

The pulse-chase experiment is applied in photosynthesis research to monitor selective D1 turnover under strong illumination. The chlorophyll a concentration of a 100 ml *Synechocystis* sp. PCC 6803 culture that was grown in liquid BG11 was determined (a chlorophyll a concentration between 5 and 6 µg/ml is the optimum, 10 µg/ml is too old). A volume corresponding to 300 µg of chlorophyll a was spun down in two Falcon tubes (GS 3.8; 2,000 x g; 15 min; 30 °C; no break) and the supernatants were disposed. The pellets were resuspended in 1 ml of fresh plain BG11 each by pipetting up and down. The cells were transferred into new 1.5 ml screw cap tubes and washed two more times with BG11 (spun down in the microfuge 12,000 x g, 2 min and resuspended in BG11). After the washing steps the volume in both screw cap eppendorf tubes was adjusted to 1 ml and the pellets were thoroughly resuspended. Then 0.5 ml were transferred into the four prepared pulse-chase vials which already contained 0.5 ml of BG11 leading to four pulse-chase vials with approximately 75 µg of chlorophyll in each vial. The samples were kept on a rocking shaker to allow proper mixture at 30 °C for 30 min at a light intensity of 250 µE * m^{-2} * s^{-1}. After the 30 min incubation time the samples were pooled and 20 µC radioactive L-methionine (Amersham Pharmacia) were added and the samples equally divided into the four vials. The samples were incubated again at 30 °C for 30 min at a light intensity of 250 µE * m^{-2} * s^{-1}. After this initial pulse labeling the radioactive L-methionine was removed by transfering the samples to new screw cap tubes and pelleting the cells (12,000 x g; 2 min). Cells were then resuspended in plain BG11 and the procedure was repeated twice (three times altogether; supernatants containing radioactive waste were disposed in a an appropriate sink). The samples were pooled, 100 µl of 180 mM unlabeled L-methionine were added (final [~4.5 mM]) and then divided into the four vials. The samples were shaken at 30 °C for 5 min at a light intensity of 250 µE * m^{-2} * s^{-1} and after pooling the samples a 1 ml aliquot was removed and shock frozen in a screw cap tube (t = 0 min). Now the light intensity was put to 1000 µE * m^{-2} * s^{-1} and other aliquots were taken in the same manner after 45, 90 and 180 min. The cells were stored at –80 °C until thylakoid membrane extracts were obtained by a small scale preparation which could then be analyed by 1-D SDS PAGE. The gels were dried on whatman paper using a gel dryer (Biorad; Model 583) and exposed to a X-Ray film (SuperRX, X-Ray Film, 100 NIF, 18 x 24 cm; Fuji medical) for 1 to 10 days. The film was developed according to the manufacturers´ instructions.

Chapter III:
Results – Bioinformatic analysis of prohibitins and their homologues

Prohibitins and their homologues have been found in many organisms ranging from procaryotes (Banuett and Herskowitz, 1987) to higher eukaryotes (Nuell et al., 1991) (see Figure 3.1). They belong to a very abundant protein family and have been extensively described in the literature (Nijtmans et al., 2002). But even though they have been linked to various cellular processes (McClung et al., 1995) their function still remains unclear.

To identify the prohibitin homologues in *Synechocystis* sp. PCC 6803 various databases on the internet were searched. Subsequently the identified proteins were analysed and compared to the vast amount of other prohibitin homologues that have previously been found and partly described in the literature (Kihara et al., 1998; Steglich et al., 1999) using bioinformatic tools.

The main question of this chapter is, whether the prohibitin homologues from *Synechocystis* sp. PCC 6803 are closely related to other described prohibitins, in order to see whether it is reasonable to draw parallels between these proteins. Another question that was addressed was if the prohibitin homologues in higher plants had the potential to be targeted to the chloroplast.

3.1 Identification of SPFH / Band 7 domain proteins

Initially, only one prohibitin gene (*slr1106*) had been identified in *Synechocystis* sp. PCC 6803 (CyanoBase database) and the search for more homologues was stimulated by a publication by Tavernarakis et al. (1999). In this work prohibitins were assigned as members of a large protein superfamily that share the SPFH domain as a common motive (named after the initials of the related protein families: **s**tomatins, **p**rohibitins, **f**lotillins and **H**flK/C) and whose members were implicated in regulating targeted protein turnover.

The SPFH domain protein family is also known as the Band 7 protein family. This family was found to be listed in the PFAM database (**p**rotein **fam**ily;

http://www.sanger.ac.uk/Software/Pfam/; PFAM entry: Band_7, accession number: PF01145) and also in the InterPro database (http://www.ebi.ac.uk/interpro/; InterPro entry: Band_7, accession number: IPR001107). The PFAM database lists 569 members for the Band 7 protein family (including the members of the PROSITE PDOC00977 family and proteins with high BLAST scores to known Band 7 proteins, such as HflC and HflK from *E. coli* and human prohibitin), while the family in the InterPro database is composed out of 668 members (see Figure 3.1; criteria for the composition of this database entry is unclear). Both databases refer to the PROSITE database (http://us.expasy.org/prosite/), where the consensus pattern for the core motif of this protein family is described (see table 3.1). A further distinction was made in the InterPro database where four distinct subfamilies were listed to be "children" of the Band 7 protein family: prohibitins (92 members), stomatins (305 members), HflC (63 members) and HflK (69 members). In Figure 3.1 A-C the distribution among various taxonomic groups is shown for the proteins from the three distinct protein families. It was found that there were five Band 7 proteins present in *Synechocystis* sp. PCC 6803 of which one belongs to the prohibitin protein family and another one to the stomatin protein family.

Table 3.1: Consensus pattern for the Band 7 / SPFH domain. All proteins in the Band 7 domain protein superfamily consist of a short N-terminal domain followed by a transmembrane region and a variable size (from 170 to 350 residues) C-terminal domain. As a signature pattern a conserved region located about 110 residues after the transmembrane domain was selected (PROSITE database).

Consensus pattern	R-x(2)-[LIV]-[SAN]-x(6)-[LIV]-D-x(2)-T-x(2)-W-G-[LIVT]-[KRH]-[LIV]-x-[KRA]-[LIV]-E-[LIV]-[KRQ]

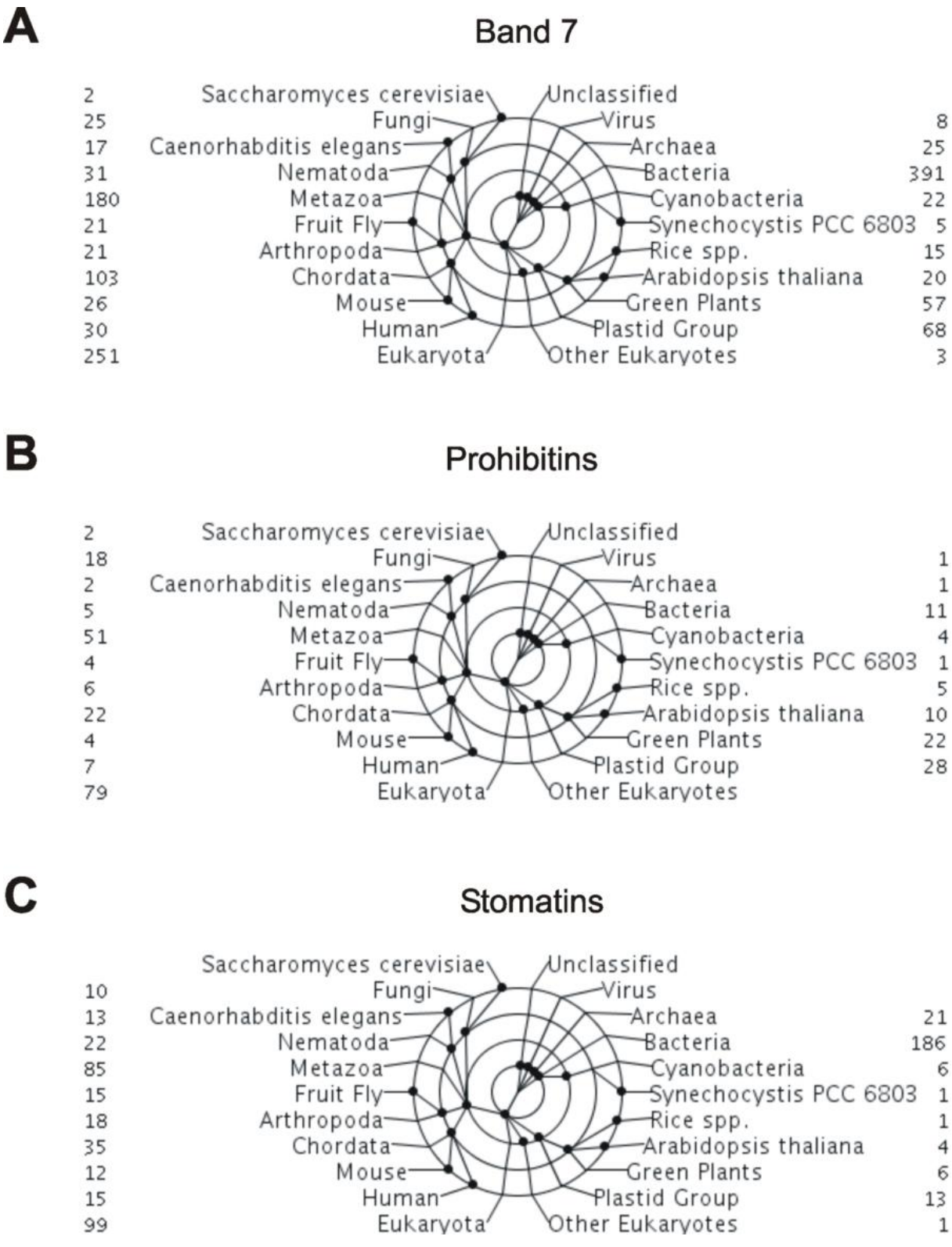

Figure 3.1: Taxonomy plots for the Band 7, prohibitin and stomatin protein families from the InterPro database (Source http://www.ebi.ac.uk/interpro/). Taxonomy plot for the (A) Band 7 (B) prohibitin and (C) stomatin protein family. The taxonomy plot aims to provide a, 'at a glance', view of the taxonomic range of the sequences associated with the InterPro entry and the number of sequences associated with each lineage. The circular display has the taxonomy-tree root as its center. The model organisms selected populate the outer most circle. Nodes of the taxonomy-tree are placed on the inner

circles. Radial lines lead to the description for each node. No significance is attached to the position of the node on a particular inner-circle, other than convenience, though some attempt has been made to group nodes. The nodes themselves are either true taxonomy nodes and have a NCBI taxonomy number or are artificial nodes created for this display; of which there are three: 'Unclassified', 'Other Eukaryota (Non-Metazoa)' and the 'Plastid Group'.

3.1.1 The prohibitin domain

The prohibitin domain protein family is listed separately in the InterPro database (see Figure 3.1; InterPro entry: Prohibitin, accession number: IPR000163) and the consensus motifs are described in the SPRINT database (http://umber.sbs.man.ac.uk/dbbrowser/sprint/; SPRINT entry: Prohibitin, accession number: PR00679). The signature to identify members of the prohibitin family is a 7-element fingerprint which derived from an initial alignment of 7 sequences (BAP37 from *Mus musculus*; prohibitin from rat; prohibitin from human; prohibitin 1 and 2 from yeast [Prohibitin 1 seems to have been entered twice in the initial alignment; second sequence was annotated SCU16737] and prohibitin 2 from *C. elegans*).

The documentation entry in the SPRINT database also includes some remarks on possible functions of prohibitins: Prohibitins negatively regulate proliferation inside the cell and are thereby of considerable interest because of the implications in processes such as development and cancer (Nuell et al., 1991). Moreover, prohibitins are widely expressed in a variety of tissues and inhibit DNA synthesis. It was found that pohibitin may be a suppressor gene and associated with tumor development and/or progression of at least some breast cancers (Sato et al., 1922).

3.1.2 The stomatin domain

The stomatin domain protein family is listed separately in the InterPro database (see Figure 3.1; InterPro entry: Stomatin, accession number: IPR001972) and the consensus motifs are described in the SPRINT database (http://umber.sbs.man.ac.uk/dbbrowser/sprint/; SPRINT entry: Stomatin, accession number: PR00721). Stomatin-like proteins had been identified in various organisms, including *C. elegans* and *Mus musculus*. The 7-element fingerprint that now provides the signature to identify members of this protein family derived from an initial

alignment of 10 sequences (Sto-1, Sto-2, Sto-3, Sto-4, Mec-2 and Unc-1 from *C.elegans*, three stomatins from *Mus musculus* [annotated Ban7_Mouse, MMEMPEPB7 and MMU17297], Stomatin isoform A from human).

The documentation entry in the SPRINT database includes additional information and some remarks on possible functions of stomatins: The eponym of this protein domain family, stomatin, is also known as erythrocyte membrane protein band 7.2b and is a 31 kDa membrane protein. It was named after the rare human disease, haemolytic anaemia hereditary stomatocytosis. The protein contains a single hydrophobic domain, close to the N-terminus, and is phosphorylated (Stewart, 1997). The human gene of stomatin is encoded by seven exons spread over 25 kb of genomic DNA (Schlegel et al., 1997). Stomatin is believed to be involved in regulating monovalent cation transport through lipid membranes. Absence of the protein in hereditary stomatocytosis is believed to be the reason for the leakage of Na^+ and K^+ ions into and from erythrocytes (Stewart, 1997).
A second function of stomatin is to act as a cytoskeletal anchor. One possible example of this is its interaction with some anti-malarial drugs. Current opinion speculates that such drugs bind to high density lipoproteins in serum. The lipoproteins are delivered to erythrocytes, where it is believed they interact with stomatin as a means of transfer to the intracellular parasite, via a pathway used for the uptake of exogenous phospholipid (Foley and Tilley, 1997).

3.2 Band 7 proteins in cyanobacteria

Searching the InterPro database revealed 22 Band 7 proteins in cyanobacteria (see Figure 3.1). These proteins are distributed among eight different species:

- *Anabaena* sp. PCC 7120 (6 Band 7 proteins/ 1 prohibitin / 2 stomatins)
- *Anabaena variabilis* (1/0/1)
- *Prochlorococcus marinus* MIT 9313 (2/0/0)
- *Prochlorococcus marinus* subsp. pastoris CCMP 1378 / MED4 (1/0/0)
- *Prochlorococcus marinus* (1/0/0)
- *Synechococcus elongatus* (2/1/1)
- *Synechococcus* sp. PCC 7942 (2/0/0)
- *Synechococcus* sp. WH8102 (2/0/0)
- *Synechocystis* sp. PCC 6803 (5/1/1)

Another prohibitin and stomatin that were not recognized as Band 7 proteins are present in the cyanobacterium *Gloeobacter violaceus.* Proteins were assigned to the prohibitin [IPR000163] and stomatin [IPR001972] subfamilies according to the InterPro database. The proteins from *Synechococcus elongatus* and *Synechocystis* sp. PCC 6803 (see Figure 3.2) will be analysed and discussed further in the following chapters.

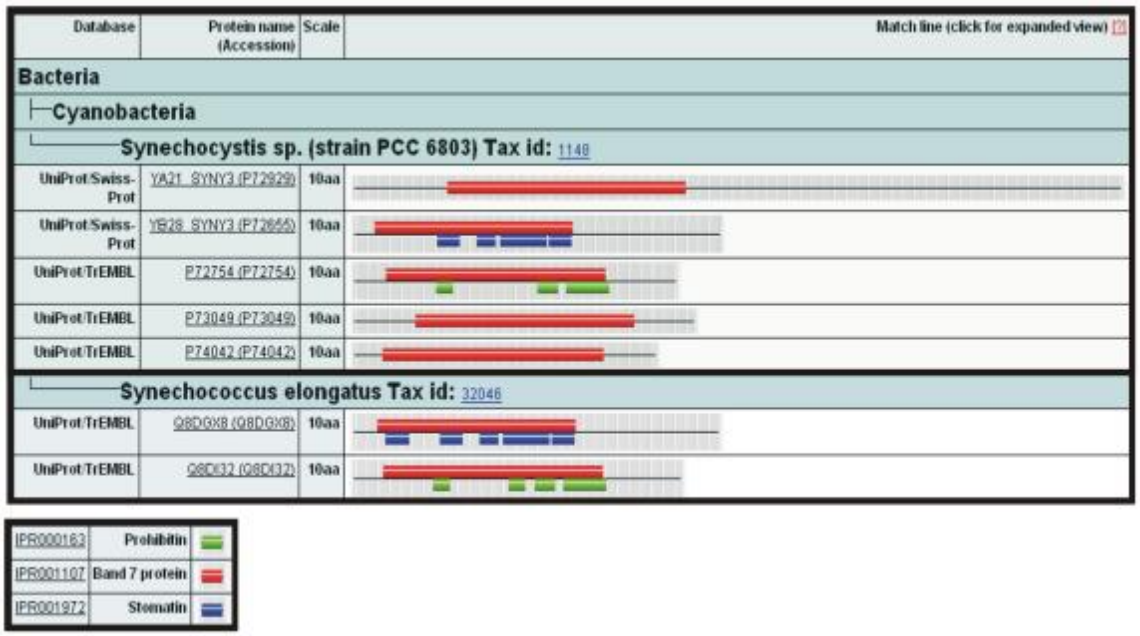

Figure 3.2: Matches for the InterPro entry IPR001107 (Band 7 proteins) from *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* (source: http://www.ebi.ac.uk/interpro/). Five Band 7 proteins were identified in *Synechocystis* sp. PCC 6803, among which one was an assigned prohibitin and one an assigned stomatin. In *Synechococcus elongatus* two Band 7 proteins were identified of which one was an assigned prohibitin and one an assigned stomatin. A key to the nomenclature is given in table 3.2 in the next subchapter. The little grey boxes in the background of the domain plot correspond to 10 amino acids.

3.2.1 Notes on the nomenclature

The vast amount of information that is stored in various databases can sometimes lead to confusion when it comes to the nomenclature of proteins. Researchers or laboratories might follow their own conventions for naming the proteins of their interest. Thus it is of crucial importance to outline the criteria of the nomenclature and maintain it coherently.
In this work all the identified Band 7 proteins in *Synechocystis* sp. PCC 6803 are termed Phb1 to Phb5 and in *Synechococcus elongatus* (equivalent to *Thermosynechococcus elongatus*) TE_Phb1 and TE_Phb2 respectively. This is because the aim of the project was to study the role of prohibitins in *Synechocystis* sp. PCC 6803 and consequently all the prohibitin homologues that were found were termed Phb (for **p**ro**hib**itin). To see which name corresponds to which gene or database accession number refer to table 3.2.

The term prohibitin derives from the originally observed function of these proteins which was the inhibition of DNA synthesis and thus prohibiting progression of the cell cycle (McClung et al., 1989). Later studies by Nijtmans et al. 2000 assigned prohibitins in *S. cerevisiae* as mitochondrial chaperones. Subsequently they suggested to keep the phb abbreviation, because the term prohibitin was already established in the literature, but to adjust the meaning of the abbreviation to “protein that holds badly folded subunits”.

Table 3.2: Nomenclature of Band 7 proteins from *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus*. The Band 7 gene and protein names from *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus* are listed in this table. Information about the proteins can be retrieved from the UniProt database via their accession number. All proteins belong to the Band 7 protein family and it is indicated whether a protein belongs to the prohibitin or stomatin subfamily (InterPro database).

organism	gene	name this work	UniProt accession #	type
***Synechocystis* sp. PCC 6803**	*slr1106*	Phb1	P72754	prohibitin
	slr1768	Phb2	P73049	-
	slr1128	Phb3	P72655	stomatin
	sll0815	Phb4	P74042	-
	sll1021	Phb5	P72929	-
Thermosynechococcus elongatus	*tlr1760*	TE_Phb1	Q8DI32	prohibitin
	tlr2184	TE_Phb2	Q8DGX8	stomatin

3.2.2 Properties of selected Band 7 proteins in cyanobacteria

Certain properties of a protein can be calculated or predicted from its primary sequence e.g. the molecular weight, the isoelectric point or a transmembrane spanning domain (TM). These predictions were carried out for the identified Band 7 proteins in *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus* and listed in table 3.3. All these Band 7 proteins are of approximately the same size of around 30 kDa, except Phb5 which has a molecular weight of about 74 kDa. The isoelectric points for the prohibitin homologues are in a range from 5.1 to 5.6 making them acidic proteins and only Phb4 with an isoelectric point of 8.4 has basic properties. Most of the Band 7 proteins have one N-terminal transmembrane domain, except Phb2 which has two N-terminal TM domains and Phb4 which has none.

Table 3.3: Various protein properties of the Band 7 proteins in *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus*. Several protein properties like molecular weight (MW), isoelectric point (pI) (both http://us.expasy.org/cgi-bin/protparam) and the number of transmembrane (TM) domains (http://www.ch.embnet.org/software/TMPRED_form.html) were calculated.

protein	UniProt accession #	bps	aas	calculated MW	pI	expected TM-domains
Phb1	P72754	849	282	30.57	5.21	1
Phb2	P73049	897	298	32.83	5.58	2
Phb3	P72655	966	321	35.73	5.55	1
Phb4	P74042	795	264	30.37	8.36	-
Phb5	P72929	2022	673	74.42	5.08	1
TE_Phb1	Q8DI32	864	287	31.55	5.32	1
TE_Phb2	Q8DGX8	963	320	35.68	5.55	1

3.2.3 ClustalW alignment of selected Band 7 proteins in cyanobacteria

In order to detect sequence similarities and relatedness among the prohibitin homologues from *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus* a multiple sequence alignment was performed using the ClustalW algorythm (http://www.ebi.ac.uk/clustalw/; Version 1.82). The matrix type was set to Gonnet and the other settings were left at default. To further demonstrate relations among the selected proteins the provided dendrogram from this alignment is included (see Figure 3.3).

ClustalW multiple sequence alignment

```
TE_Phb2         ------------------------------------------------------------
SS_Phb3         ------------------------------------------------------------
TE_Phb1         ----------------------------------------------------MNQRLSS  7
SS_Phb1         ----------------------------------------------------MSKQPSF  7
SS_Phb2         ---------------------------MGAVISAIAALASWTVFITVKNPENPKIPKLL  32
SS_Phb5         MQSKFWFEFLQTLPTLPGDTVPVMAIQASPGETSGELIIAQAPNQTLDNNNSALGGLSPL  60
SS_Phb4         ----------------------------------------------------------MAR  3

TE_Phb2         MGQLLGLLSLLLGFGVWYSA-SAIRVVNQGNMALVERLG-RYNRR---------LGPGFS  49
SS_Phb3         MEAFFLFFLVFFGSAIG----TSVKIVNEKNEYLVERLG-SYNKK---------LTPGLN  46
TE_Phb1         R-LTSAVAAIAIVLLVLL-L-NAVVIINPGQAGVLSILGKAQDTP---------LLEGIH  55
SS_Phb1         DGWQSIVGGLIAALLVLLSF-NSFVVINPGQAGVLSVLGKAQDGA---------LLEGIH  57
SS_Phb2         RPLLFFIALLMSALFVQQSLGRALVVIPAGEVGVIETMGTVDTTP---------LTSGVY  83
SS_Phb5         LFFPVVIIAVIFLILVTIFLYTRFYVIAPNNEALVRTGGVFKKEQMVILHGGCIVIPGFH  120
SS_Phb4         QARYQNLLGLNPRTESRSLIWCRRFSVRPNCLGLLYRKN-CFEKT---------LEPGIY  53

TE_Phb2         LIWPVFERVVFEETIREKVLDIPPQQCITRDN-VTITVDAVVYWR-----IVDMERAYYR  103
SS_Phb3         FTVPILDRVVFKQTTREKVIDIPPQSCITKDN-VAITADAVVYWR-----IIDMEKAYYK  100
TE_Phb1         WKPPFIASVDVYDVT-VQKFEVP-AESATKDL-QDITASFAINFRLDPMAIVDVRRTQGT  112
SS_Phb1         FKPPLVSSVDIYDVT-VQKFEVP-AQSSTKDL-QDLSASFAINFRLDPTEVVTIRRTQGT  114
SS_Phb2         FLNPLSKVVTYSTRLQDIKETVD---TSSKEG-LNFNIDVSLQYRLNPEKAGEVFSSLGS  139
SS_Phb5         EITRVSLREISIDVVRAGNLAVRTQDYMRANMRVTFYVCITPNRNEILTAAARLSKKGQI  180
SS_Phb4         SFWDFRSELELFLIPRSDQFFIVTNQEVLTKDNIPLRFSYIVNYRITDGQKLLTYIDPAQ  113

TE_Phb2         V------ENLKMAMVN-LVQTQIRAEMGKLELDETFT-ARTQVNETLLRDLDIATDPWGV  155
SS_Phb3         V------ENLQSAMVN-LVLTQIRSEIGKLELDQTFT-ARTEINELLLRELDISTDPWGV  152
TE_Phb1         L------ENIVAKIIAPQTQEAFKIAAARRTAEEAIT-KRDELKQDFDHALEERLSKYHI  165
SS_Phb1         L------QNIVAKIIAPQTQESFKIAAARRTVEEAIT-KRSELKEDFDNALNSRLEKYGI  167
SS_Phb2         E------EQQREIIIS-RFRSLIRENTAKYDLSSIYGDKRAEISGVLVQSMKEQLEPLGF  192
SS_Phb5         S------EADIKDALEKRADDAIRAAAKKKKLAELDS-DKLGFADEVLNLIQGDLRKVGL  233
SS_Phb4         MGYIEGLASMIQTLIHPLTQIYWRSAISVINSLELNE-QWEAFIPNIPDELQESAQKFGV  172

TE_Phb2         KVTRVELRDIAPS-----------------QAVQDSMELQMSAERKKRAAI--------  189
SS_Phb3         KVTRVELRDIMPS-----------------KAVLDSMELQMTAERKKRAAI--------  186
TE_Phb1         LVLDTSVVNLDFS-----------------EEFSKAVEDKQIAEQRAQRAV--------  199
SS_Phb1         IVLDTSVVDLAFS-----------------PEFAKAVEEKQIAEQRAQRAV--------  201
SS_Phb2         VVEEALMRNVILP-----------------ENIQKAIQAKVEVEQSNQKKQ--------  226
SS_Phb5         TLNNIAISEIEESDTYDENNFFDAQGVRLRTETIQRSIQQKREVELTTRVAIEQGELEAE  293
SS_Phb4         MIEAMKLRDITFP-----------------KNIQTLFALQLEAKIRGQTDL--------  206

TE_Phb2         ----------------------------LTS---EGEREAAINSARGKAEAQVLAAEAE  217
SS_Phb3         ----------------------------LTS---EGQRDSAINSAQGDAQARVLEAEAK  214
TE_Phb1         ----------------------------YIAQEAAQQAQAEINRAQGKAEAQRLLAETL  230
SS_Phb1         ----------------------------YVAQEAEQQAQADINRAKGKAEAQRLLAETL  232
SS_Phb2         ----------------------------LELISARRDAERKIIEAQGVADSQRILSQSL  257
SS_Phb5         KKSLAIKREQEDANITQQKEIELLKLAQRKELESQEAQQQREIQEAKDKEEAKKERNKIL  353
SS_Phb4         -----------------------------------ENARTAVATARALKNASQLIG---  227

TE_Phb2         QKAAILSAEAEQKVVVLRA------QAERQNQILRAQGTAEAMKIIAAALHEDPKAKEAL  271
SS_Phb3         KKAAILNAEAEQ-----------------QKKVLEAKATAEALSILTEKLSSDNHAREAL  257
TE_Phb1         KAP-------------------------GGQLVLQKEAIEAWREGGAQVP---------  255
SS_Phb1         KAQ-------------------------GGELVLQKEAIEAWREGGAPMP---------  257
SS_Phb2         TDQ-----------------------------IIKLKAIEATQKLAESPN---------  278
SS_Phb5         QEQAVEEERIQKELAIQNSQIASAIALEERNKELKVAQALQKQEAEVAEIQRKKTIEASQ  413
SS_Phb4         ----------------------------DDQNIKFLQYLETLIKIASSGK---------  249

TE_Phb2         QFLLAQSYLDMGRTIGHSDSSKVLFMDPSSIPATIEGVKSLIEQ--------------S  316
SS_Phb3         QFLLAQQYLNMGTTIGSSDSSKVMFLDPRNILSTLEGVRSIVGDGALDEGLEAALNKVDR  317
TE_Phb1         -----QVIVINGQ-EG-LPPFLLNWSSEQSVRERSPKSP--------------------  287
SS_Phb1         -----KVLVMGGEGKGSAVPFMFNLTDLAN-------------------------------  282
SS_Phb2         -----TKVLIMGSGEG---NLPIIMSDP---------------------------------  298
SS_Phb5         LQAKAEIALAEQKTQITEQTAAIAIANKQKERLEAEALRAEAESGVITAQEVEAAERAQK  473
SS_Phb4         -----HTFVVGGSEKITSDS---------------------------------------  264
```

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TE_Phb2         PREV------------------------------------------------------ 320
SS_Phb3         HRAV------------------------------------------------------ 321
TE_Phb1         -------------------------------------------------------------
SS_Phb1         -------------------------------------------------------------
SS_Phb2         -------------------------------------------------------------
SS_Phb5         LAVIVAQQDAQQHRIAEQNVVEIDVFRRRRQAESARQAAELEAESIRTLADANRHKAMAE 533
SS_Phb4         -------------------------------------------------------------

TE_Phb2         -------------------------------------------------------------
SS_Phb3         -------------------------------------------------------------
TE_Phb1         -------------------------------------------------------------
SS_Phb1         -------------------------------------------------------------
SS_Phb2         -------------------------------------------------------------
SS_Phb5         AEGQKAIIEAHNSLSNANRTAELLKTIWPELVTQLPDLIKALAPQPGVLGESRIYSFPGL 593
SS_Phb4         -------------------------------------------------------------

TE_Phb2         -------------------------------------------------------------
SS_Phb3         -------------------------------------------------------------
TE_Phb1         -------------------------------------------------------------
SS_Phb1         -------------------------------------------------------------
SS_Phb2         -------------------------------------------------------------
SS_Phb5         SGSNGNGSNSGDINKLLLSTSGLTLLNGLLNEGKLSTVVDQVKSLLQDPPSVSPPSAAVS 653
SS_Phb4         -------------------------------------------------------------

TE_Phb2         --------------------
SS_Phb3         --------------------
TE_Phb1         --------------------
SS_Phb1         --------------------
SS_Phb2         --------------------
SS_Phb5         EDDWPDLAPPTETNFSPEEI 673
SS_Phb4         --------------------
```

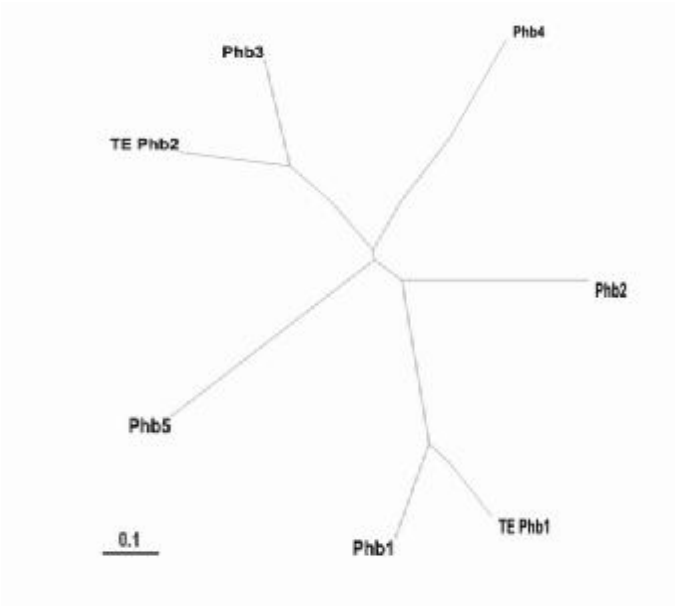

Figure 3.3: Unrooted phylogenetic tree for the Band 7 proteins in *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus*. This unrooted phylogenetic tree derived from the ClustalW alignment performed on the Band 7 proteins from *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus*.

The results from the ClustalW multiple sequence alignment show that the assigned prohibitin and stomatin homologues from both cyanobacterial species group together, whereas the other Band 7 proteins seem not to display any significant sequence similarity or relatedness towards these proteins or among each other. This finding is in accordance with the data found in the various protein domain databases.

3.3 Band 7 proteins in other organisms

In this section the Band 7 proteins from organisms in which these proteins have previously been studied, as well as Band 7 proteins from higher plants (see table 3.4) will briefly be described. Members of the Band 7 protein family have been identified in *S. cerevisiae* and *E. coli* where they have been extensively studied and where some light has been shed upon their function (see 1.1.5). The Band 7 proteins from higher plants are of special interest, because if these homologues could be found to be targeted to the chloroplast an evolutionary link towards cyanobacteria could be established.

Table 3.4: Band 7 proteins from various organisms. The number of proteins belonging to the Band 7, prohibitin or stomatin protein family according to the InterPro database are listed for *S. cerevisiae*, *E. coli*, *A. thaliana*, *Zea mais* and *N. tabacum*.

	all	***S. cerevisiae***	***E. coli***	***A. thaliana***	***Zea mais***	***N. tabacum***
Band 7	668	2	6	20	9	1
prohibitin	92	2	0	10	4	1
stomatin	305	0	2	4	1	0

3.3.1 Band 7 proteins in *S. cerevisae*

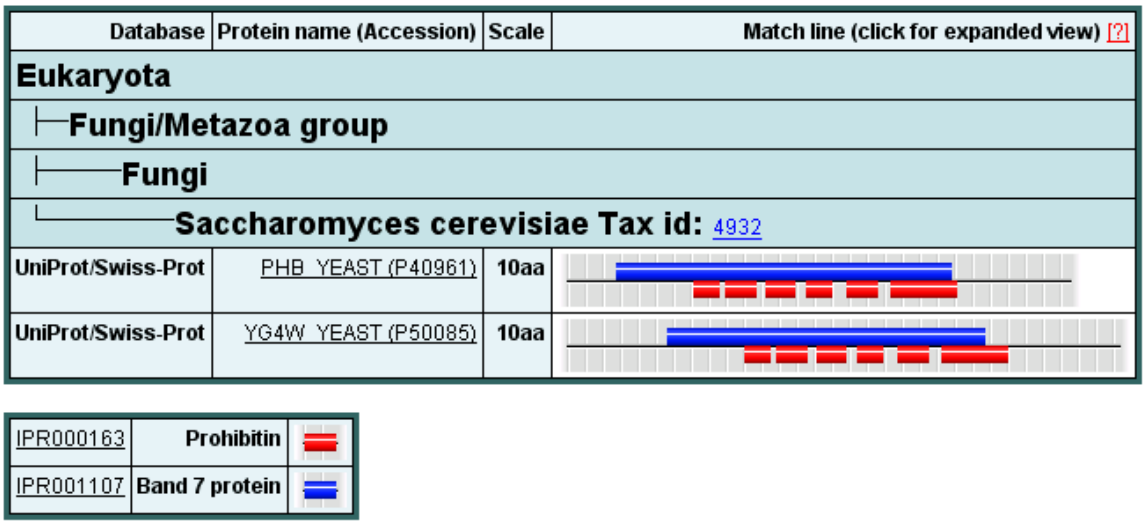

Figure 3.4: Matches for the InterPro entry IPR001107 (Band 7 proteins) in *S. cerevisae* (source: http://www.ebi.ac.uk/interpro/). Two Band 7 proteins were identified in *S. cerevisiae* of which both were assigned prohibitins (P40961 = SC_Phb1 and P50085 = SC_Phb2). The little grey boxes in the background of the domain plot correspond to 10 amino acids.

An InterPro database search revealed that both members of the Band 7 family in *S. cerevisiae* are prohibitins (SC_Phb1 and SC_Phb2; see table 3.4 and Figure 3.4). In fact most of what is known about prohibitins has been found in studies using this organism (see 1.1.5.2). A ClustalW analysis that has been performed with these proteins lead to a score of 48 indicating a medium degree of relatedness among both proteins (data not shown).

3.3.2 Band 7 proteins in *E. coli*

In *E. coli* the Band 7 protein family has six members and seemed to be more diverse than in *S. cerevisae* or in higher plants (see Figure 3.5). This is because *E. coli* or bacteria in general possess two more, closely related, Band 7 protein subfamilies termed HflC and HflK (members and eponyms of the SPFH domain family). No prohibitin was found in any of the Band 7 proteins of this organism, but instead two proteins HflK (P25662) and YbbK (P77367) contained stomatin domains. A HflC domain was found in the HflC protein (P25661) and HflK domains were detected in HflK (P25662) and YhdA (Q9F507). The two other Band 7 proteins are YqiK (P77306) and a putative YqiK (Q93D69).

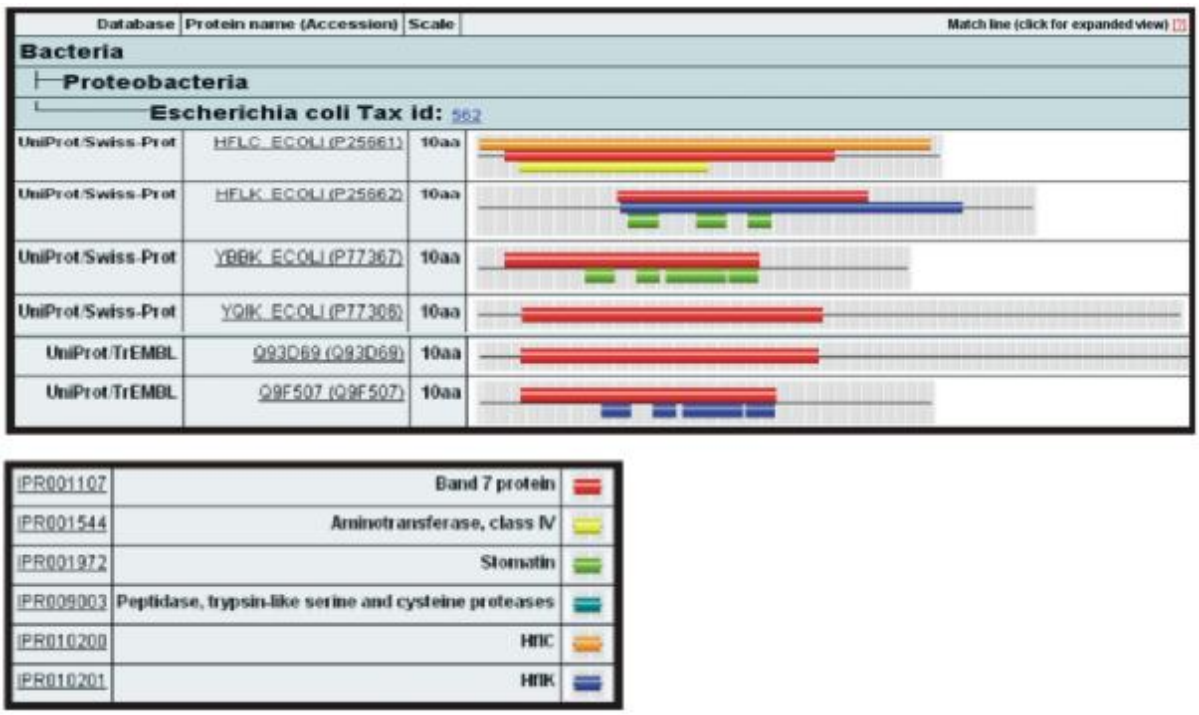

Figure 3.5: Matches for the InterPro entry IPR001107 (Band 7 proteins) in *E. coli* (source: http://www.ebi.ac.uk/interpro/). Six Band 7 proteins were identified in *E. coli*, among which two contain a stomatin domain, two a HflK domain and one a HflC domain. The little grey boxes in the background of the domain plot correspond to 10 amino acids.

3.3.3 Band 7 proteins in higher plants

Band 7 proteins from higher plants are of particular interest, because of the evolutionary link that could be drawn to cyanobateria if these proteins were targeted to the chloroplast. Indeed many Band 7 proteins were found in higher plants (see table 3.4) and *A. thaliana* alone possesses 20 Band 7 homologues (ten prohibitins and four stomatins). In *Zea mais* nine Band 7 proteins were found of which four are prohibitins and one is a stomatin. One Band 7 protein was identified in *N. tabacum* and is a member of the prohibitin protein family. Some details, particularly about the prohibitins in higher plants, are listed in table 3.5.

Table 3.5: Prohibitins in higher plants. Prohibitins from *A. thaliana* (AT), *Zea mais* (ZM) and *N. tabacum* (NT) are listed with UniProt accession #, protein name and gene name with synonym. A reference is given were applicable.

origin	UniProt accession #	protein names	gene name and synonyms	reference
AT	O04331	Prohibitin 3	Atphb3 At5g40770	(Millar et al., 2001)
AT	O49460	Prohibitin 1	Atphb1 At4g28510 F20O9.200	(Snedden and Fromm, 1997) (Millar et al., 2001)
AT	Q84WL7	At3g27280		(Kleffmann et al. 2004)
AT	Q8LA39	Prohibitin-like protein		-
AT	Q8LBC7	putative Prohibitin		-
AT	Q9FFH5	Prohibitin	At5g44140	-
AT	Q9LK25	Prohibitin	BAB02123	(Millar et al., 2001)
AT	Q9LY99	Prohibitin-like protein	At5g14300 F18O22_90	-
AT	Q9SIL6	putative Prohibitin	At2g20530	(Millar et al., 2001)
AT	Q9ZNT7	Prohibitin 2	Atphb2 At1g03860 F21M11.21	(Kleffmann et al. 2004) (Millar et al., 2001)
ZM	Q9M586	Prohibitin	PHB4	(Nadimpalli et al., 2000)
ZM	Q9M587	Prohibitin	PHB3	(Nadimpalli et al., 2000)
ZM	Q9M588	Prohibitin	PHB2	(Nadimpalli et al., 2000)
ZM	Q9M589	Prohibitin	PHB1	(Nadimpalli et al., 2000)
NT	O04361	Prohibitin	T03843	(Snedden and Fromm, 1997) (Baginsky et al. 2004)

3.4 Targeting of Band 7 proteins in higher plants

This section deals with the question whether Band 7 proteins in higher plants are predicted to be targeted to the choloroplast by using different targeting prediction softwares. The analysis was performed for all identified Band 7 proteins in higher plants (see table 3.4) although the particular focus was on the prohibitins of which some had already been studied elsewhere (see table 3.5; Snedden and Fromm, 1997; Kleffmann et al. 2004; Nadimpalli et al., 2000; Baginsky et al. 2004).
A first indication that prohibitin homologues from higher plants might be associated with the chloroplast was given by Kleffmann et al. (2004) who analysed the *A. thaliana* chloroplast proteome. They detected two prohibitin homologues Q84WL7 and Q9ZNT7 (Atphb2) in the envelope fraction of their preparation. For this report only bioinformatic means were available to address the question and the two commonly used prediction servers ChloroP and Predotar analysed the proteins from *A. thaliana*, *Zea mais* and *N. tabacum*.

The analysis of the Band 7 proteins of higher plants revealed that most of the proteins were not predicted to be targeted to the chloroplast. However, a few proteins were predicted to possess a transit peptide. At this point it has to be said that the predictions from both programs did not necessarily agree. In *A. thaliana* for example none of the prohibitin homologues was predicted to be targeted to the chloroplast by the Predotar server, although ChloroP predicted a transit peptide for one of the proteins (O04331; AT_Phb3). However this protein was not detected in the proteomics approach by Kleffmann et al. (2004). All of the four stomatin homologues in *A. thaliana* on the other hand were predicted to have a transit peptide by ChloroP. Predotar only made a prediction for two of the four stomatins assigning them to be possibly mitochondrial (Q93VP9 and Q9T082).

In *Zea mais* one of the four prohibitin homologues (Q9M587; PHB3) was predicted to be possibly targeted to the chloroplast by both prediction servers. ChloroP also assigned the stomatin from *Zea mais* to be targeted to the chloroplast, whereas Predotar assigned it to be mitochondrial. No predictions were made for the other Band 7 proteins from *Zea mais*.

The prohibitin homologue from *N. tabacum* was predicted to have a transit peptide by ChloroP whereas Predotar did not predict the presence of such a sequence.

Table 3.6: Targeting predictions for Band 7 proteins in higher plants. Proteins (UniProt accession #) have been analysed using ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) and Predotar (http://genoplante-info.infobiogen.fr/predotar/predotar.html). The length of the protein, presence of a transit peptide (**Y**ES or **N**O), the score for the transit peptide to be there and the length of the transit peptide are given for the ChoroP analysis. For the Predotar analysis only the predicted compartment is indicated ignoring to list the calculated scores.

protein type	UniProt accession #	ChloroP transit peptide				Predotar prediction
		length of protein	transit peptide Y/N	score	length of transit peptide	
AT_Phb	O04331	277	Y	0.521	51	none
AT_Phb	O49460	288	N	0.440	-	none
AT_Phb	Q84WL7	279	N	0.488	-	none
AT_Phb	Q8LA39	288	N	0.440	-	none
AT_Phb	Q8LBC7	279	N	0.492	-	none
AT_Phb	Q9FFH5	278	N	0.453	-	none
AT_Phb	Q9LK25	279	N	0.488	-	none
AT_Phb	Q9LY99	249	N	0.469	-	ER
AT_Phb	Q9SIL6	286	N	0.441	-	none
AT_Phb	Q9ZNT7	286	N	0.473	-	none
AT_Sto	Q8LDI0	401	Y	0.563	34	none
AT_Sto	Q93VP9	411	Y	0.573	45	possibly mitochondrial
AT_Sto	Q9LVW0	401	Y	0.563	34	none
AT_Sto	Q9T082	515	Y	0.573	45	possibly mitochondrial
AT	Q84TE3	316	N	0.434	-	none
AT	Q9CAR7	286	N	0.435	-	none
AT	Q9FHM7	292	N	0.431	-	none
AT	Q9FM19	286	N	0.438	-	none
AT	Q9SRH6	285	N	0.448	-	none
AT	Q9ZQ87	356	N	0.440	-	none
ZM_Phb1	Q9M586	289	N	0.453	-	none
ZM_Phb2	Q9M587	282	Y	0.514	25	possibly plastid
ZM_Phb3	Q9M588	284	N	0.482	-	none
ZM_Phb4	Q9M589	289	N	0.472	-	none
ZM_Sto	Q9M585	394	Y	0.528	25	mitochondrial
ZM_Hir1	Q9M584	284	N	0.435	-	none
ZM_Hir2	Q9M583	284	N	0.440	-	none
ZM_Hir3	Q9M582	287	N	0.440	-	none
ZM_Hdr10	Q9FS40	284	N	0.435	-	none
NT_Phbb	O04361	279	Y	0.501	24	none
AT	D1 protein	352	N	0.432	-	no result
AT	psbO	332	Y	0.570	29	plastid

3.5 Phylogenetic analysis of Band 7 proteins

Phylogenetic analysis were performed to investigate possible relationships among Band 7 proteins. As various Band 7 proteins have been extensively studied and described in the literature, it seemed important to test how related the Band 7 proteins from *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus* were to these known homologues. Thus, two dendrograms were drawn of which one was based on a publication by Nadimpalli et al. (2000) in which the Band 7 proteins from *Zea mais* were studied and classified (see Figure 3.6). The second dendrogram was drawn of the Band 7 proteins that have been mentioned in this report (see table 3.4 and Figure 3.7).

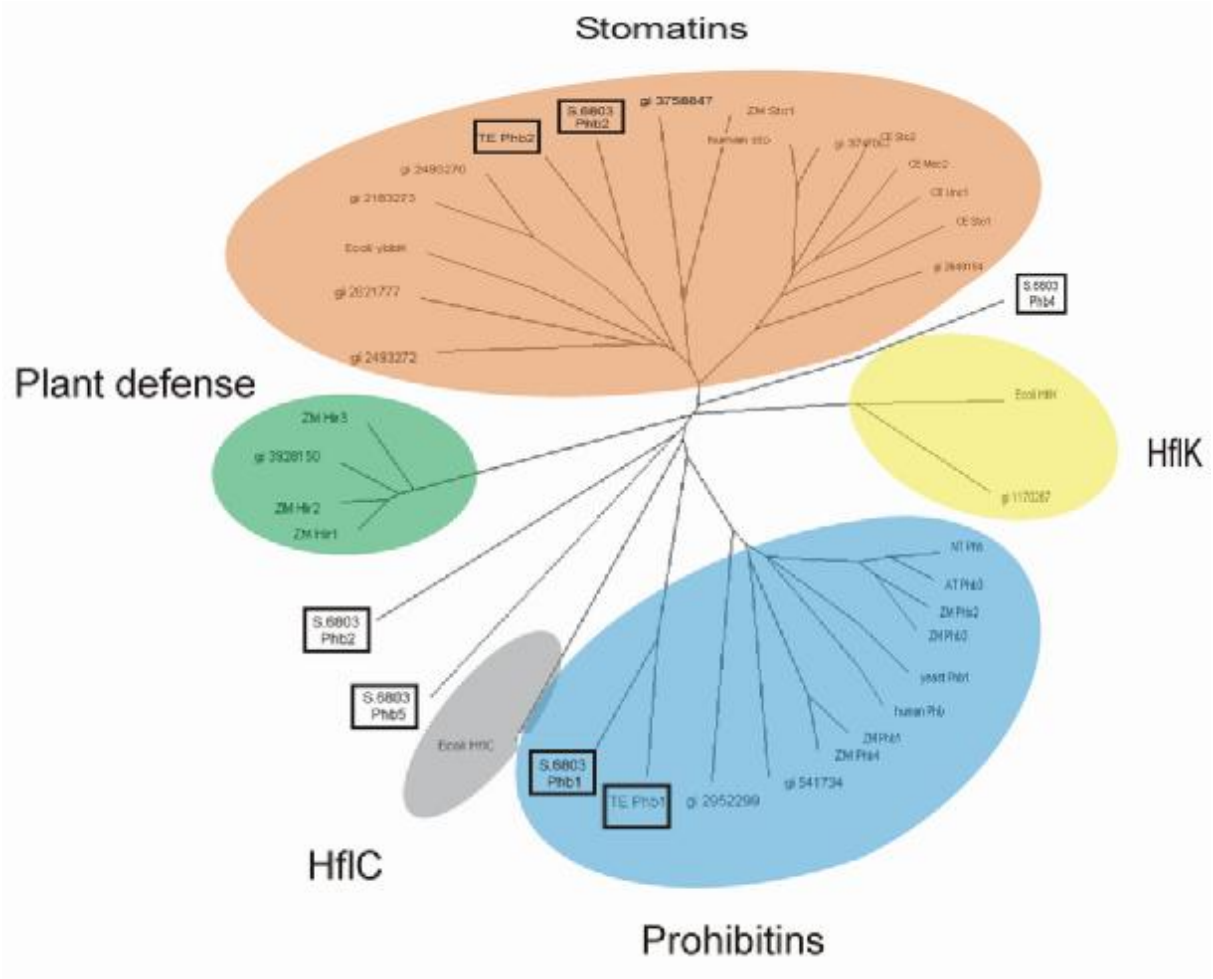

Figure 3.6: An unrooted dendrogram showing relationships among selected Band 7 proteins. The selection of proteins that was used for this analysis was based on Nadimpalli et al. (2000), only the Band 7 proteins from *Synechocystis* sp. PCC 6803 and *Thermosynechococcus elongatus* were added and one protein (gb:I38527) excluded. The dendrogram was generated from a ClustalW multiple sequence alignment (http://www.ebi.ac.uk/clustalw/; Version 1.82; Gonnet matrix, rest default). The partitioning of families within the Band 7 superfamily can clearly be observed: stomatin (red), HflK (yellow), prohibitin (blue), HflC (grey) and plant defense proteins (green). The proteins of interest from cyanobacteria have marked with a slim, surrounding box.

The dendrogram by Nadimpalli et al. (2000) was based on the analysis of Band 7 proteins from *Zea mais* and showed the classic separation into the four distinct protein families that are listed in the InterPro database: stomatin, prohibitin, HflC and HflK. The fifth protein subfamily present in the dendrogram, associated with plant defense, did not appear separately in any of the searched protein domain databases. This dendrogram provided an ideal starting ground for the analysis of the prohibitin homologues from *Synechocystis* spec. PCC 6803 and *Thermosynechococcus elongatus* (see Figure 3.6).
Including the cyanobacterial Band 7 proteins in the analysis partly grouped into the existing families and allowed an estimation of how related these proteins really are. The first observation was that the assigned prohibitins (Phb1 and TE_Phb1) and stomatins (Phb3 and TE_Phb2) were correctly placed into their respective families, although the branching point for the procaryotic proteins from the eukaryotic prohibitins and stomatins was rather early, suggesting only a distant relationship. The other three prohibitin homologues from *Synechocystis* sp. PCC 6803 did not group with either of the existing protein families. As the set of sequences that was used for this analysis included various animal proteins, it seemed appropriate to repeat the analysis with more Band 7 proteins from plants.

The second dendrogram includes the Band 7 proteins mentioned in table 3.4, which are partly described and higher plant Band 7 protein homologues (see Figure 3.7). Again the classic protein family partitioning, including the fifth plant defense protein family, became obvious and the cyanobacterial prohibitins and stomatins were grouped correctly.
Looking at the protein families individually, revealed that the procaryotic prohibitins grouped among each other and branched off early from their eukaryotic homologues suggesting only a distant relationship. In case of the prohibitins it was even more intriguing that the prohibitins of eukaryotic origin separated into two branches (each branch consisting of several prohibitins from each *A. thaliana* and *Zea mais*). Particularly, because the two prohibitins in *S. cerevisiae* were found to form large, interdependent protein complexes (Nijtmans et al, 2002).
Interestingly, the procaryotic stomatin homologues seemed to branch off rather early from their eukaryotic homologues as well and the cyanobacterial Phb3 and TE_Phb2 showed some degree of relationship with the ybbK and YhdA proteins from *E. coli*. Another distant relationship was observed between Phb5 and the YqiK proteins of *E. coli*. Strikingly none of the cyanobacterial proteins was found to share a relationship towards either HflC or HflK from *E. coli*.

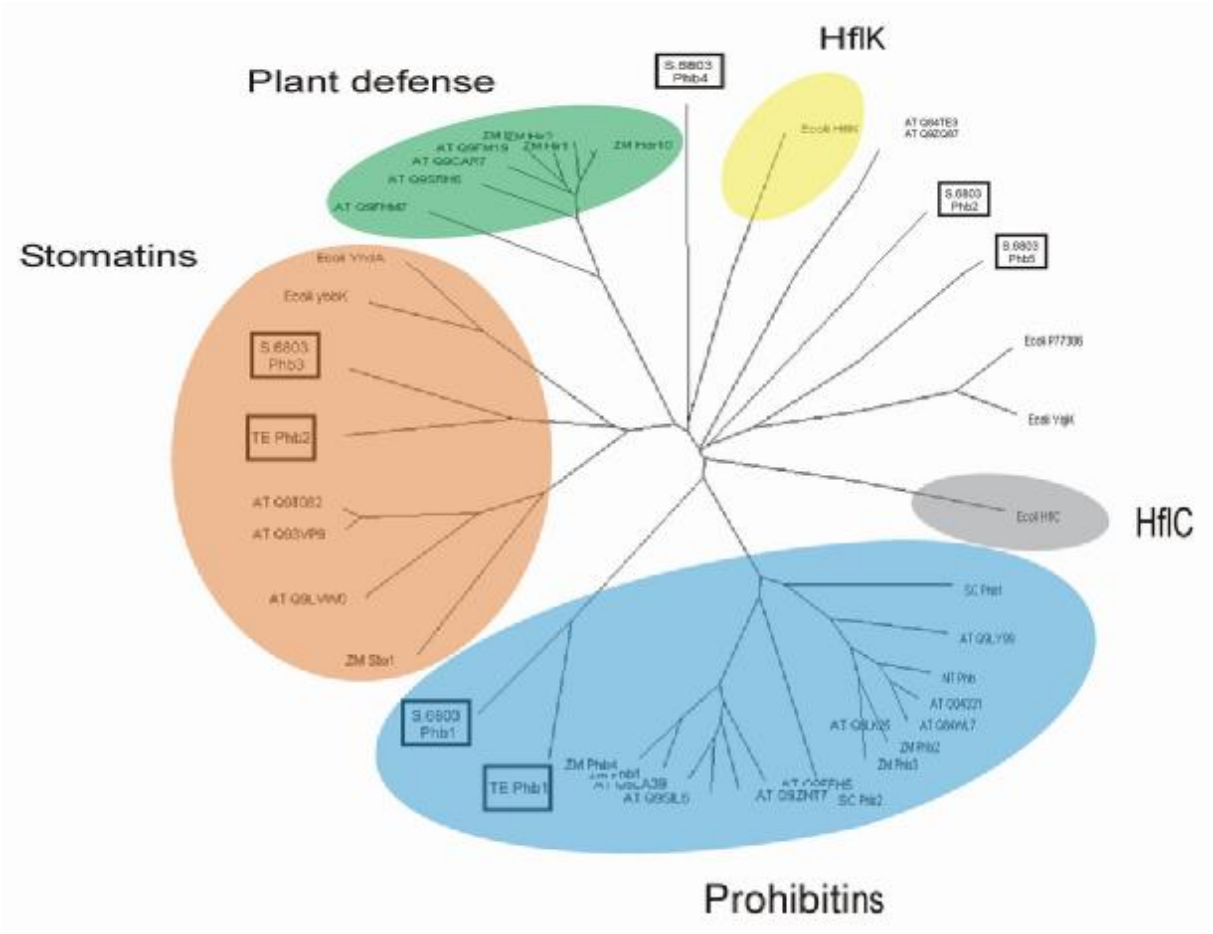

Figure 3.7: An unrooted dendrogram showing relationships among cyanobacterial Band 7 proteins and Band 7 proteins that have been described or are found in higher plants. Partly described and higher plant Band 7 protein homologues were used for this analysis. The dendrogram was generated from a ClustalW multiple sequence alignment (http://www.ebi.ac.uk/clustalw/; Version 1.82; Gonnet matrix, rest default). The partitioning of families within the Band 7 superfamily can clearly be observed: stomatin (red), HflK (yellow), prohibitin (blue), HflC (grey) and plant defense proteins (green). The proteins of interest from cyanobacteria have marked with a slim, surrounding box.

3.6 Conclusions and Discussion

3.6.1 Relationships among Band 7 proteins

We have identified five Band 7 proteins in *Synechocystic* sp. PCC 6803 and two in *Thermosynechococcus elongatus* (see 3.2). In each organism one of the Band 7 proteins was an assigned prohibitin and one a stomatin. The other three proteins in *Synechocystis* sp. PCC 6803 have not been recognized as a member of any other particular subfamily. To compare

the cyanobacterial Band 7 proteins among each other, we performed a multiple sequence alignment using CLUSTALW (see 3.2.3). This alignment suggested only a limited relationship among the analysed proteins (see Figure 3.3). Only the two prohibitins and the two stomatins from both organisms seemed to exhibit some similarity. All in all, based on their primary sequence, the identified, cyanobacterial Band 7 proteins can only be considered to be distantly related and make up a diverse protein family.

To put the Band 7 proteins from *Synechocystic* sp. PCC 6803 and *Thermosynechococcus elongatus* into a broader context, we reproduced a dendrogram from Nadimpalli et al. 2000 and included our proteins of interest in the dataset (see Figure 3.6). This analysis proved to place the cyanobacterial prohibitins and stomatins in the correct protein subfamilies, but revealed that the other three Band 7 proteins from *Synechocystic* sp. PCC 6803 cannot be grouped into a related protein family. The second dendrogram that we generated included more prohibitin and stomatin homologues from higher plants and also all the Band 7 proteins from *E. coli* and *S. cerevisiae* of which some homologues are well-described (see Figure 3.7). This dendrogram pointed out that within the prohibitin and stomatin subfamily there is a clear distinction between procaryotic and eukaryotic proteins which branched quite early. The other three Band 7 proteins from *Synechocystic* sp. PCC 6803 could still not be assigned into another protein family.

Summarizing, these results suggested that to draw parallels concerning the function of the prohibitin homologues in *Synechocystic* sp. PCC 6803 or *Thermosynechococcus elongatus* with *E. coli* and especially with the eukaryotic *S. cerevisiae* is highly speculative.

3.6.2 Targeting of the Band 7 proteins in higher plants

After having identified Band 7 proteins in higher plants (see table 3.4), we analysed the potential of these proteins to be targeted to the chloroplast (see 3.4). If this could be shown, then a link between the Band 7 proteins and photosynthesis would appear more plausible. Two main approaches can be considered in order to determine whether a protein is targeted to a particular compartment of the cell. One way is to analyse the primary amino acid sequence of the protein in question with a targeting prediction software and the other is to identify the

compartment(s) in which the protein resides experimentally. In this study we only pursued the bioinformatic approach, whereas some experimental data was retrieved from the literature.

Commenting on the bioinformatic approach, it has to be emphasized that the obtained results are only predictions from mathematical calculations and certain algoryhthms. Even though the software programs (ChloroP and Predotar) returned the correct predictions for the controls that we included in the analysis, in most cases both programs were not even in agreement with each other. Only the ZM_Phb2 protein from *Zea mais* was predicted to be possibly targeted to the chloroplast by both programs. In all the other cases the Predotar server never assigned a protein to be targeted to the chloroplast, where ChloroP predicted a transit peptide. Although there is not much evidence that Band 7 proteins, especially prohibitins, in higher plants are targeted to the chloroplast, neither was the opposite proven by these bioinformatic means.

As the prediction programs did not return satisfactory results for the targeting of Band 7 protein, we screened the literature for experimental evidence where to these proteins and especially the prohibitin homologues are targeted.
Several proteomics approaches identified prohibitin homologues in different organelles of *A. thaliana*. Five of the ten prohibitin homologues in this organism (O04331, O49460, Q9LK25, Q9SIL6, **Q9ZNT7**; UniProt accession numbers) were found in the mitochondria (Millar et al., 2001). Two of the ten prohibitin homologues (Q84WL7, **Q9ZNT7**; UniProt accession numbers) were found in the envelope fraction of *A. thaliana* chloroplasts (Kleffmann et al., 2004). This also suggests that even experimental data is not always unambiguous, as one of the prohibitin homologues Q9ZNT7 is found in both organelles.
The best evidence for a prohibtin that is targeted to the chloroplast has been found in *N. tabacum*. Here a proteomics approach in which undifferentiated heterotrophic plastids (tobacco bright yellow-2 cell culture plastids) were used, the one prohibitin homologue in this organism (O04361; UniProt accession numbers) could be identified (Baginsky et al., 2004). The protein was found in the fraction that corresponded to integral membrane proteins.

Considering both, bioinformatic and experimental data, the possibility that prohibitin homologues are targeted to the chloroplast and do have a function that is involved in photosynthesis, can still not be ruled out.

Chapter IV: Results - Generation of Polyclonal Prohibitin Antibodies

The working model for the prohibitin homologues in *Synechocystis* sp. PCC 6803 (see Figure 1.5) proposes a multimeric complex, that shields a newly synthesized copy of the D1 protein from degradation by the FtsH protease prior to its incorporation into the reassembling PSII complex.

As it is our aim to identify and characterize possible prohibitin complexes and their interaction partners, we generated polyclonal antibodies against the prohibitins in *Synechocystis* sp. PCC 6803. Once generated and purified, the antibodies will be used in immunoblotting analysis following 1-D- and 2-D-PAGE (polyacrylamide gel electrophoresis), affinity purification and for immunoprecipitation experiments. Previously, one polyclonal antibody against Phb1 has been produced in the lab. Thus, only four antibodies remained to be produced.

4.1 Cloning of the prohibitin expression constructs

The prohibitin genes (*phb2*, *phb3*, *phb4* and *phb5*) were amplified from genomic *Synechocystis* sp. PCC 6803 wild type (WT) DNA using PCR and specific primers. The fragments were then cloned into the pET16b expression vector (Novagen) (see Figure 4.1) that adds a N-terminal His tag to the expressed protein which can be used for the purification procedure.

In the course of the project it proved impossible to overexpress the *phb2, phb3* and *phb5* genes as full length proteins in the bacterial strains that were available. We decided to generate expression constructs with truncated versions of these proteins (Phb2-TM, Phb3-TM and Phb5-TM) in which the coding sequences for the transmembrane (TM) domains had been deleted. Even after deletion of its TM domain coding sequence Phb5 could still not be expressed.

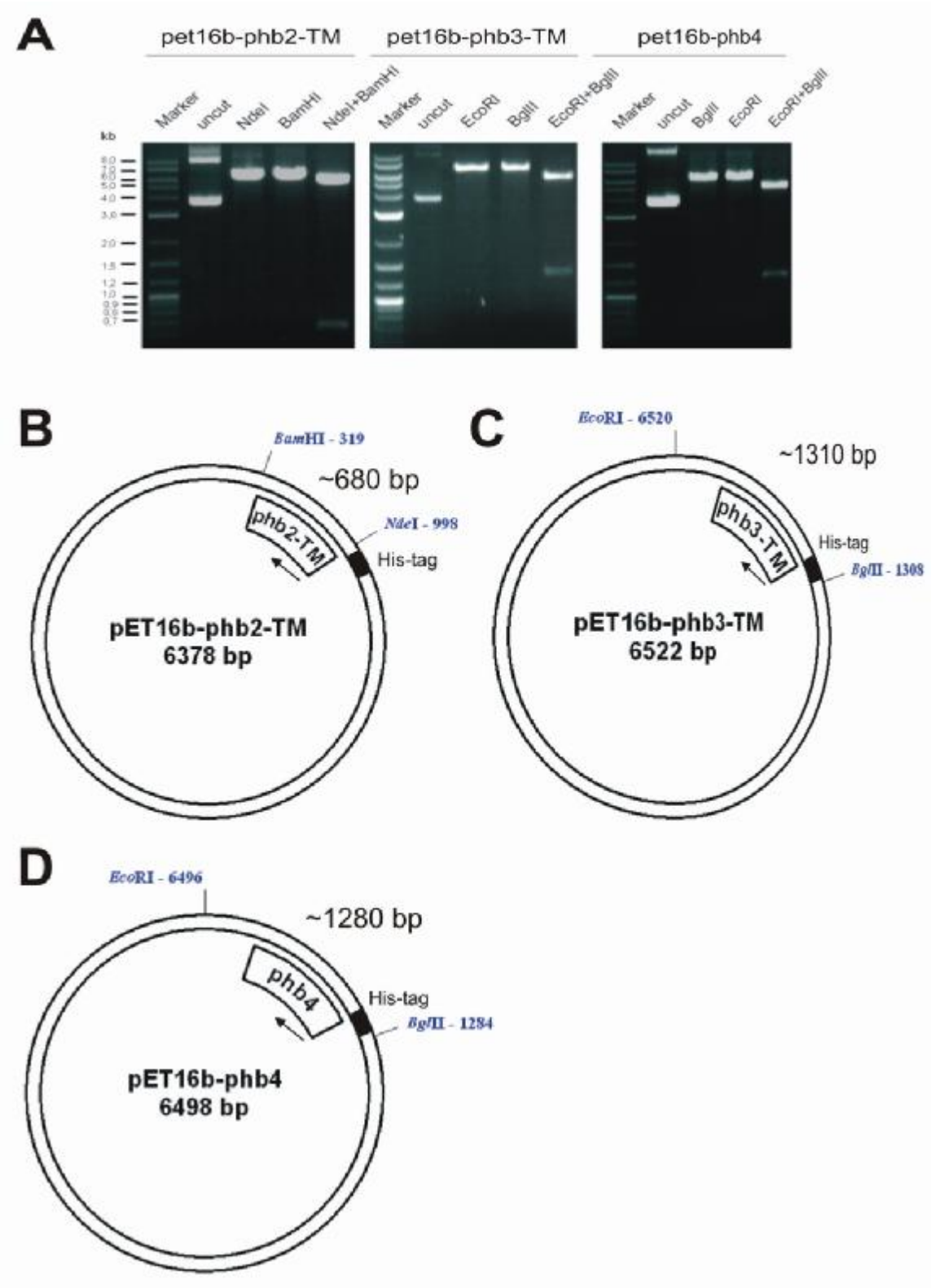

Figure 4.1: Prohibitin expression constructs. (A) Diagnostic digests of the expression constructs pET16b-phb2-TM, pET16b-phb3-TM and pET16b-phb4. (B-D) Schematic drawings of the expression constructs pET16b-phb2-TM (A), pET16b-phb3-TM (B) and pET16b-phb4 (C). The restriction enzymes that have been used for the diagnostic digest (A) and the sizes of the excised fragments are indicated. The direction of transcription is indicated by the little arrow and the position of the introduced His-tag is marked as a black box.

4.2 Expression of recombinant prohibitins

Initially, protein expression was tested on a small scale. The pET16b-phb constructs (see table 4.1) were transformed into various bacterial strains (see table 4.2) and their expression was induced. Whole cell extracts were analysed using SDS PAGE (see Figures 4.2 and 4.3). Once an appropriate construct / strain combination was identified, the proteins were produced on a larger scale for their purification.

The *phb4* gene could readily be expressed and yielded a full length product of ~ 30 kDa in the BL21-(DE3)-pLysS strain. Both the *phb2* and *phb3* gene could only be expressed in this strain as truncated versions of ~ 25 kDa and ~ 30 kDa respectively, after their transmembrane domain coding sequences had been deleted (see Figure 4.2). In the BL21-(DE3)-rosetta strain the Phb2-TM and Phb3-TM proteins were expressed constitutively (see Figure 4.3). Ultimately, the BL21-(DE3)-pLysS strain was used to produce the Phb2-TM, Phb3-TM and Phb4 proteins on a large scale.

Table 4.1: Expression constructs from this study. The constructs that were ultimately used are indicated in bold.

Expression Constructs	Protein
pET16b-phb2	Phb2
pET25b-phb2	Phb2
pET16b-phb2-TM	**Phb2-TM**
pET16b-phb3	Phb3
pET16b-phb3-TM	**Phb3-TM**
pET16b-phb4	**Phb4**
pET16b-phb5	Phb5
pET16b-phb5-TM	Phb5-TM

Table 4.2: Bacterial expression strains from this study. The strain that were ultimately used is indicated in bold.

Bacterial Expression Strains
BL21
BL21-(DE3)-pLysS
BL21-(DE3)-Codon Plus
BL21-(DE3)-Rosetta

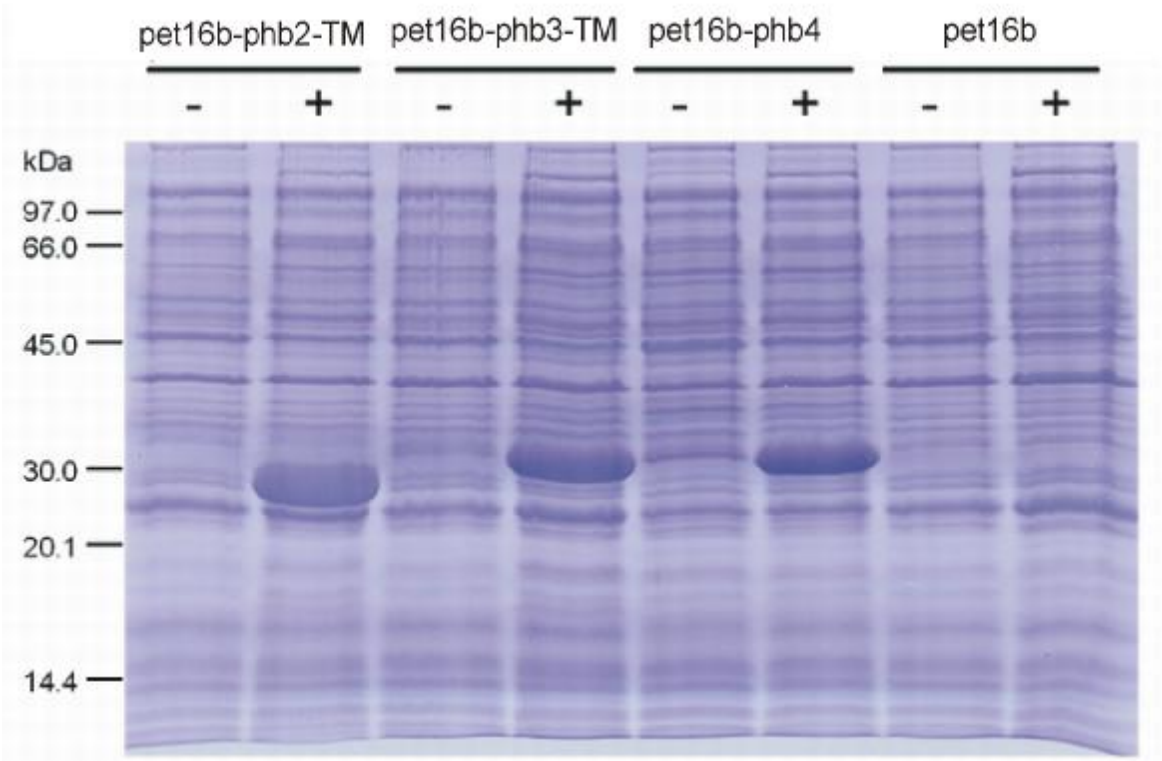

Figure 4.2: Expression of Phb2-TM, Phb3-TM and Phb4 in BL21-(DE3)-pLysS. Protein expression from the various pET16b-phb constructs was assessed after 3 h in the absence (-) and presence (+) of IPTG. Whole cell extracts of an amount of cells corresponding to an OD_{600} of 0.1 were loaded in each lane of a 12 % SDS gel.

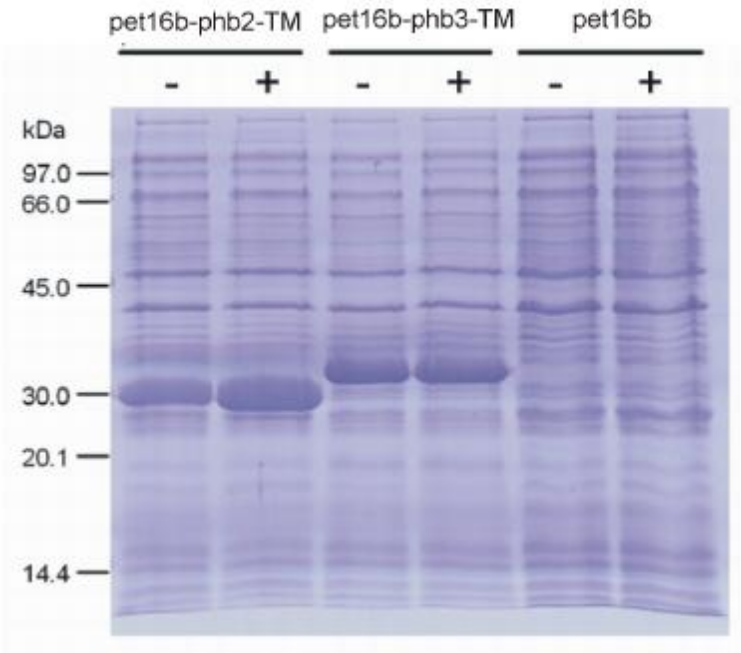

Figure 4.3: Expression of Phb2-TM and Phb3-TM in BL21-(DE3)-rosetta. Protein expression from the various pET16b-phb constructs was assessed after 3 h in the absence (-) and presence (+) of IPTG. Whole cell extracts of an amount of cells corresponding to an OD_{600} of 0.1 were loaded in each lane of a 12 % SDS gel.

4.3 Purification of overexpressed prohibitins

To obtain sufficient protein for the immunization procedure, the cloned expression constructs (see Figure 4.1) were transformed into BL21-(DE3)-pLysS and overexpressed on a large scale. After IPTG induction and 3 h of incubation the cells were harvested and lysed using the bug buster procedure (Novagen). The bug buster procedure is a fast method to assess overexpressed proteins and allows to test whether the protein is soluble or present in inclusion bodies. Quantitative purification of the prohibitins was performed using a Ni^{2+} charged resin in a batch method.

At first it was tested whether the overexpressed prohibitins were soluble or prevalent as inclusion bodies. As it became apparent that the prohibitins formed inclusion bodies (data not shown), these were then purified. Representative of all prohibitins, the purification for Phb3-TM is shown in Figure 4.4. Aliquots were taken at various timepoints during the procedure and analysed using SDS PAGE.

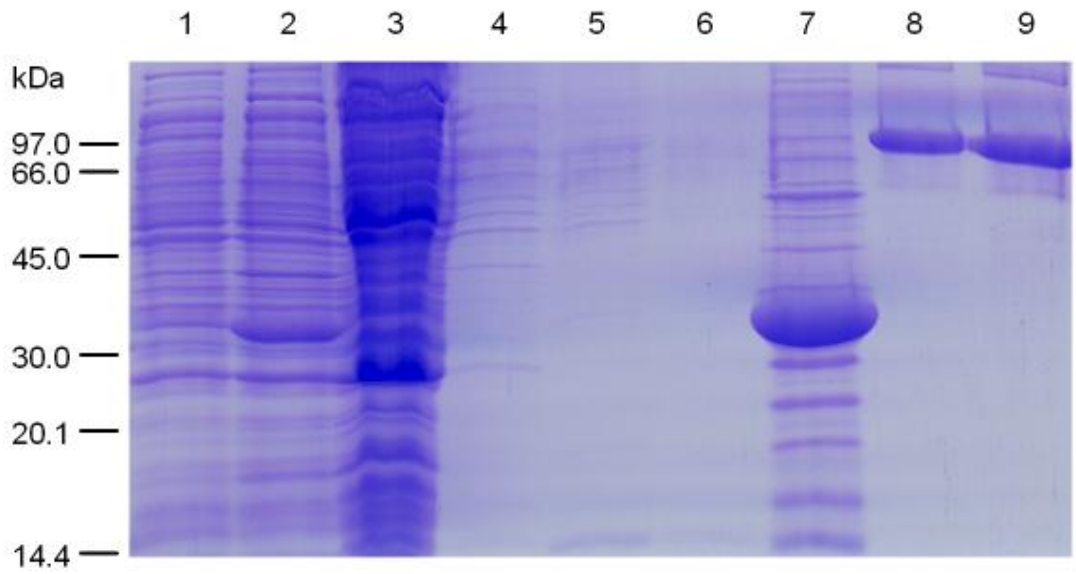

Figure 4.4: Bug buster procedure on BL21-(DE3)-pLysS cells expressing Phb3-TM. (1 and 2) crude cell extracts of induced cells @ 0 h and @ 3 h (both samples correspond to an OD_{600} of ~0.1). The amounts of sample loaded in lanes 3-7 were not normalized (10µl of every fraction was loaded on the gel, disregarding the initial volume of the individual sample). Lysed cells were centrifuged and the supernatant (3) was taken off. The pellet was resuspended, centrifuged again and the supernatant (4) was taken off. The pellet was then washed twice and the supernatants (5) and (6) were taken off. Lane (7) is the resuspended pellet before it was centrifuged again to yield the supernatant (6) and the inclusion body pellet. (8 and 9) 5 and 10 µg BSA were loaded as an internal standard on the gel to estimate the amounts of protein present.

The specific appearance of a ~30 kDa band in the IPTG induced cells after the 3 h incubation accounts for the expression of Phb3-TM (compare Figure 4.4, lane 1 and 2). After lysing the cells the sample was centrifuged and the pellet washed several times. Aliquots for SDS PAGE from the supernatants were taken (see Figure 4.4, lane 3-6) and a lot of protein could be washed away, whereas Phb3-TM was enriched in the fraction (see Figure 4.4, lane 7). The resulting inclusion body pellet was stored away for further purification at –80°C.

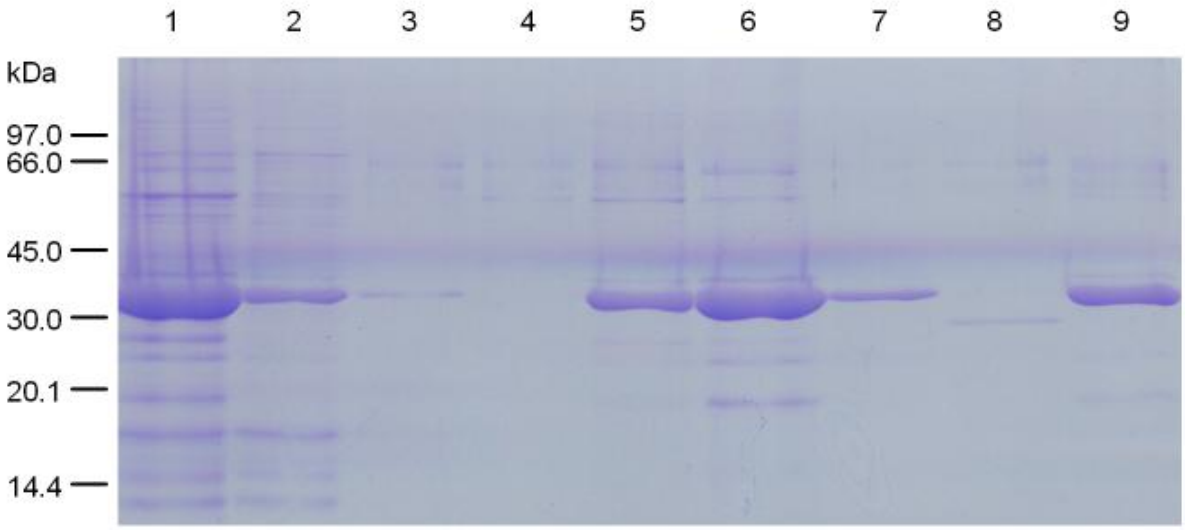

Figure 4.5: Affinity chromatography of His-tagged Phb3-TM using a Ni^{2+} charged resin. All samples (except lane 8 where 50 µl were used directly) had been normalized to 50 µl of sample per 25 ml of original flow through. All samples were acetone precipitated and resuspended in 10 µl of 50 mM Tris / HCl pH=7.5. (1) Solubilized inclusion body pellet. (2) Flow through after over night binding of the sample to the resin. (3, 4 and 5) Washing steps with increasing imidazole concentrations (5, 20 and 100 mM)(6) Phb3-TM fraction that was eluted with 300 mM imidazole. (9) Eluents that came off the resin with 500 mM imidazole. The eluted Phb3-TM fraction (6) was dialysed over night against PBS buffer. The dialysate was centrifuged to collect precipitated Phb3-TM and the supernatant was analysed on the gel (7). The pellet was resuspended in a smaller volume and concentrated further (supernatant of this centrifugation step analysed in lane 8).

The next step in the purification procedure was to solubilize the obtained inclusion body pellet and affinity purify the overexpressed proteins using a Ni^{2+} charged resin in a batch method (see Figure 4.5). The inclusion body pellet was successfully solubilized in binding buffer (see Figure 4.5 lane 1) and could be applied for binding to the resin. After overnight incubation there was still some overexpressed protein left in the sample (see Figure 4.5 lane 2). This could be due to the slowness of the binding process or the binding capacity of the resin had been reached. Nevertheless sufficient protein had bound to the resin and was washed

with various buffers containing increasing concentrations of imidazole. The washing steps with binding buffer and 20 mM imidazole washed away some contaminants (see Figure 4.5 lane 3 and 4). At an imidazole concentration of 100 mM more proteins, including Phb3-TM, were washed from the resin (see Figure 4.5 lane 5). The Phb3-TM fraction that was eluted with 300 mM imidazole still contained contaminating proteins, but was significantly purer than the starting material (compare Figure 4.5 lane 1 and 6). A purer fraction, which contained less Phb3-TM, was obtained by eluting with 500 mM imidazole (see Figure 4.5 lane 9). This fraction was not processed further.
After dialysis precipitated Phb3-TM was pelleted and some Phb3-TM was found in the supernatant (see Figure 4.5 lane 7). This effect was even more obvious for the soluble Phb4 protein during its purification. This could either be due to contamination from the pellet or to Phb3-TM respectively Phb4 that was soluble in the supernantant. This finding might lead to further experiments e.g. size exclusion experiments or single particle analysis. No Phb3-TM was observed in the supernatant of the concentrating step (see Figure 4.5 lane 8).

Ultimately, the precipitated and pelleted overexpressed prohibitins were resuspended in PBS buffer (phosphate buffered saline) and solubilized by adding 10% SDS. The degree of purity and an estimation of the obtained amounts was determined by SDS PAGE (see Figure 4.6). Two aliquots of each Phb2-TM, Phb3-TM and Phb4 containing ~5 mg/ml of protein (final SDS concentration ~0.47%) were send to SEQLAB (Göttingen, Germany) for a 3 months immunization protocol in rabbits.

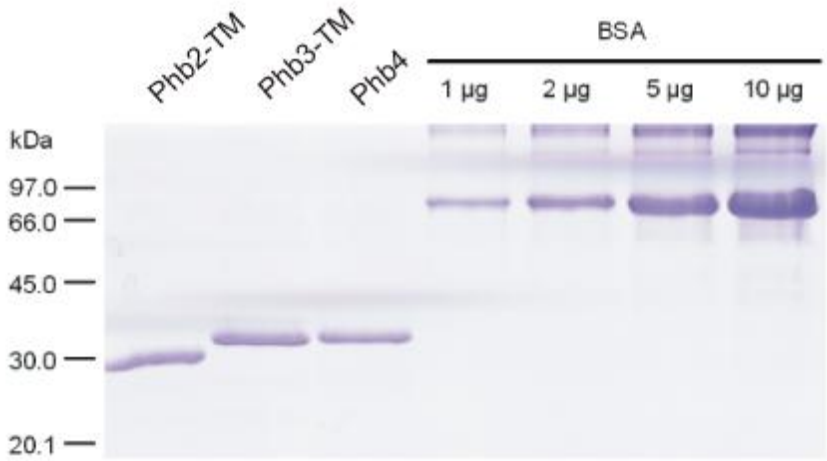

Figure 4.6: Purified overexpressed prohibitins. The purified proteins were diluted and analysed by SDS PAGE. Phb2-TM (diluted 1:20, expected size ~25 kD). Phb3-TM (diluted 1:20, expected size ~30 kD). Phb4 (diluted 1:20, expected size ~30 kD). Internal BSA standards (1µg, 2 µg, 5µg and 10µg) were loaded on the gel to estimate the amounts of protein present.

4.4 Testing of the polyclonal antisera

After completion of the three months immunization protocol, two antisera per prohibitin were sent back by SEQLAB. Initially, the antisera were tested on the overexpressed and purified prohibitins, which had served as the antigens and in another experiment soluble and thylakoid extracts from wild type and mutant *Synechocystis* spec. PCC 6803 cells were probed.

4.4.1 Testing the polyclonal antisera on the antigens

To test whether antibodies against the antigens had been raised during the immunization, a immunoblotting analysis was performed on the overexpressed prohibitins (Phb2-TM, Phb3-TM and Phb4) (see Figure 4.7). The other question that was addressed in this experiment was whether the antisera were specific for their respective antigens.
The results of the immunoblotting analysis clearly demonstrated that the tested antisera contained antibodies that were capable of specifically recognizing their respective antigens (see Figure 4.7 A-C). Immunoblotting analysis with preimmune serums showed no cross reactions with the used antigens (data not shown). Even though the antibodies were polyclonal and raised against homologous proteins, the antisera were specific for their respective antigen and showed no cross reaction with the other prohibitins.

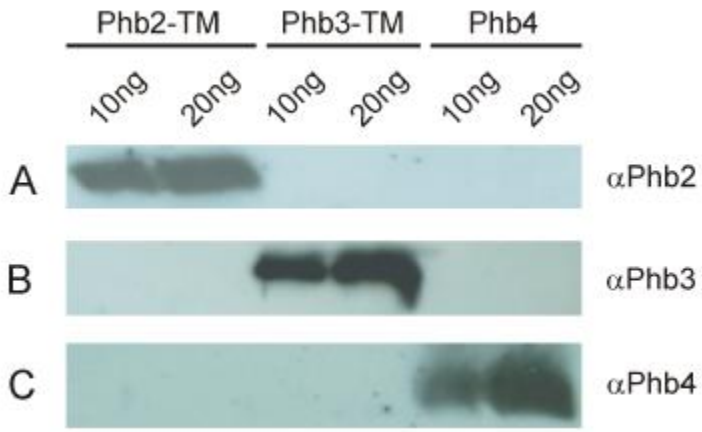

Figure 4.7: Immunoblotting analysis with polyclonal antisera on purified overexpressed prohibitins. 10 and 20 µg of each overexpressed prohibitin (Phb2-TM, Phb3-TM and Phb4) were loaded on a SDS gel and probed in a immunoblotting analysis with αPhb2-1 (A), αPhb3-1 (B) and αPhb4-1 (C) antibodies.

4.4.2 Testing the polyclonal antisera on soluble and thylakoid extracts from *Synechocystis* spec. PCC 6803

After establishing that the antibodies recognized the antigens they were raised against, it needed to be tested whether they would also give a specific signal when being probed on soluble or thylakoid extracts from *Synechocystis* spec. PCC 6803 cells. Therefore immunoblotting analysis were carried out where soluble and thylakoid extracts from wild type and mutant cells specifically lacking the antigen were probed with preimmune serum and the antiserum to be tested. Although two batches of antisera were obtained for each antigen, only one immunoblotting analysis will be shown here.

4.4.2.1 The αPhb2-1 antibody

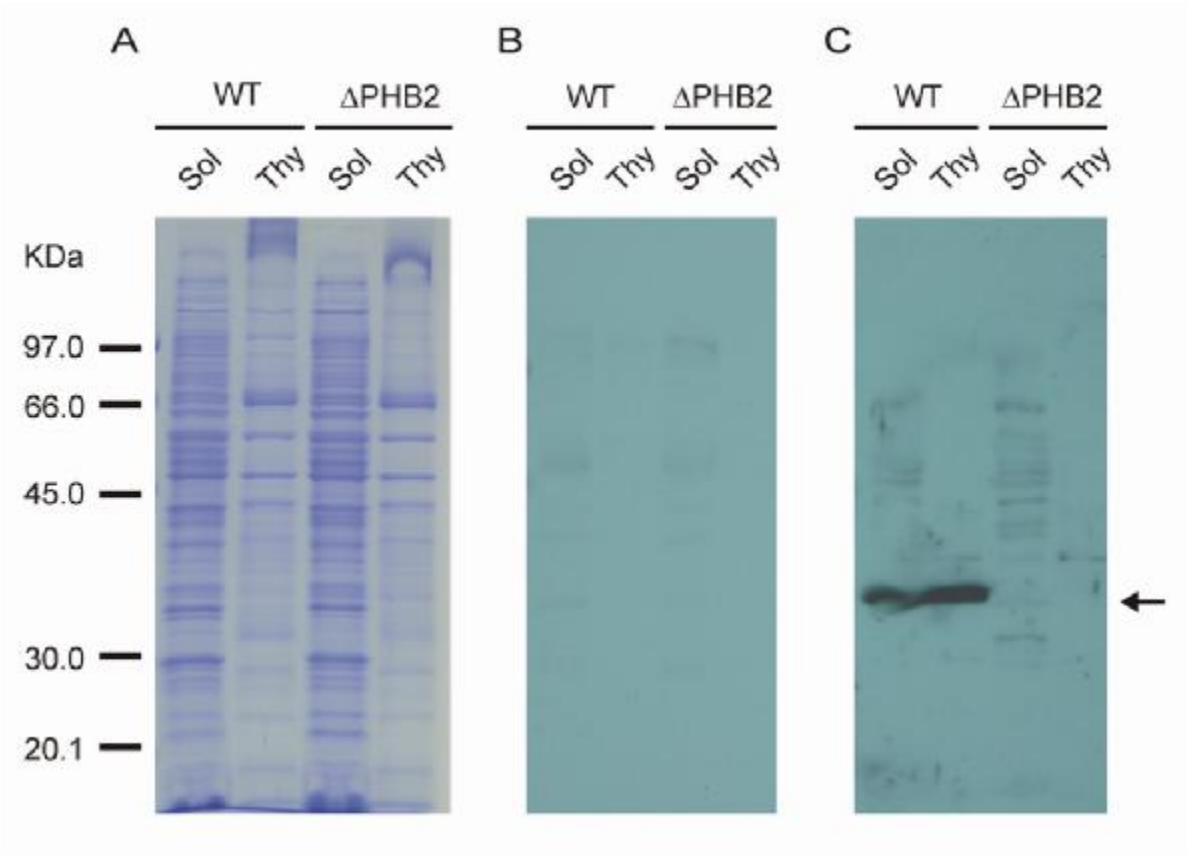

Figure 4.8: Immunoblotting analysis to test the polyclonal antisera directed against Phb2. Thylakoid and soluble extracts were obtained from wild type and mutant (specifically lacking Phb2) cells. The loaded amount of thylakoid extracts corresponded to 1 µg chlorophyll a, whereas the amount of soluble extracts was non-quantified. The extracts were separated on a SDS gel and Coomassie stained (A) or used for the immunoblotting analysis with either preimmune serum (B) or antiserum against Phb2-1 (C).

The immunoblotting analysis for the αPhb2-1 antibody showed that it specifically recognized Phb2 at a size of ~ 32 kDa in the soluble and thylakoid extracts obtained from wild type cells (see Figure 4.8 C arrow). The signal that was observed in the soluble fraction is most likely due to contaminating thylakoid membranes in this fraction. A specific signal for Phb2 could not be observed in the lanes where soluble and thylakoid extracts from the mutant cells lacking Phb2 had been loaded. This proved that the signal in the wild type lanes were indeed due to the presence of Phb2. Furthermore no signal that could correspond to Phb2 was generated when the membrane was incubated with preimmune serum (see Figure 4.8 B), indicating that the antibodies responsible for the signal were produced after the immunization. Furthermore this antibody recognizes Phb2 rather specifically without many cross reactions with other proteins, so that this antibody would not necessarily have to be purified.

4.4.2.2 The αPhb3-1 antibody

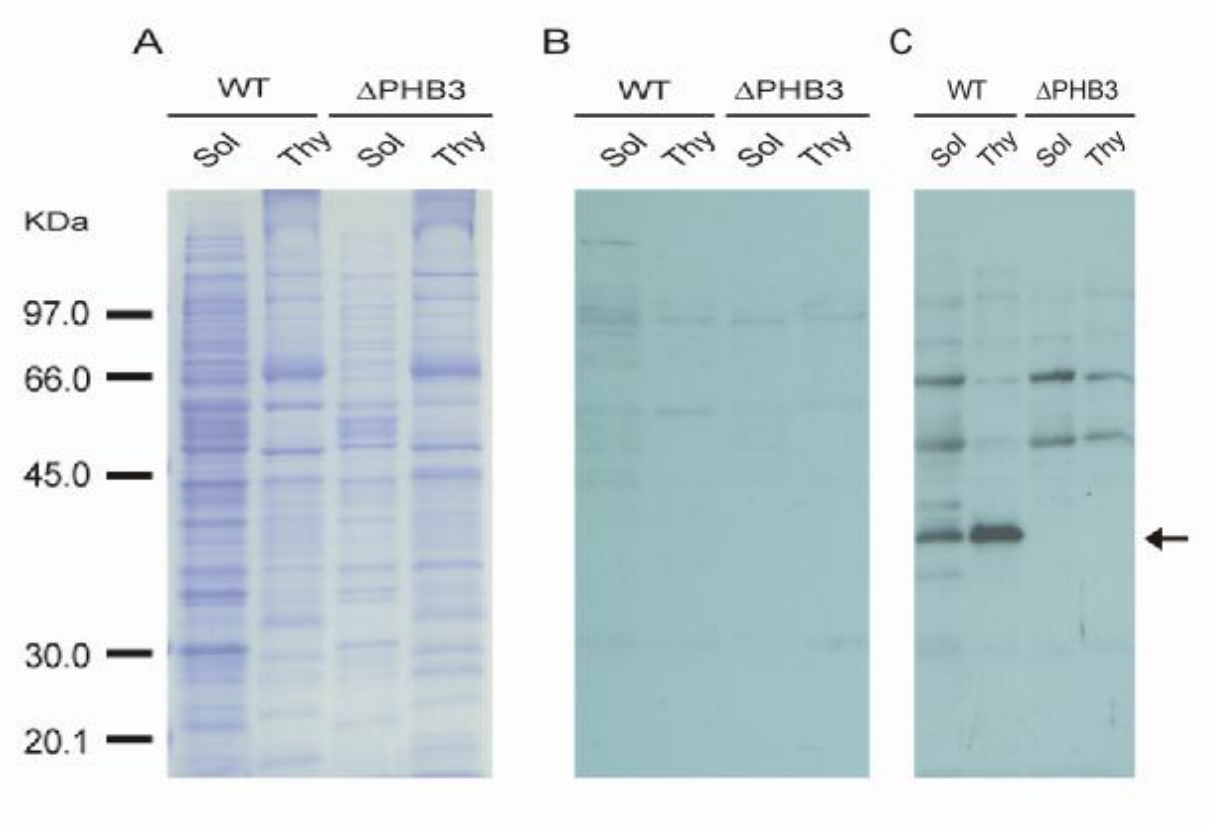

Figure 4.9: Immunoblotting analysis to test the polyclonal antisera directed against Phb3. Thylakoid and soluble extracts were obtained from wild type and mutant (specifically lacking Phb3) cells. The loaded amount of thylakoid extracts corresponded to 1 µg chlorophyll a, whereas the amount of soluble extracts was non-quantified. The extracts were separated on a SDS gel and Coomassie stained (A) or used for the immunoblotting analysis with either preimmune serum (B) or antiserum against Phb3-1 (C).

The immunoblotting analysis for the αPhb3-1 antibody showed that it recognized Phb3 at a size of about 36 kDa in the soluble and thylakoid extracts obtained from wild type cells (see Figure 4.9 C arrow). The signal that was observed in the soluble fraction is most likely due to contaminating thylakoid membranes in this fraction. A specific signal for Phb3 could not be observed in the lanes where soluble and thylakoid extracts from the mutant cells lacking Phb3 had been loaded. This proved that the signal in the wild type lanes were indeed due to the presence of Phb3. Furthermore no signal that could correspond to Phb3 was generated when the membrane was incubated with preimmune serum (see Figure 4.9 B), indicating that the antibodies responsible for the signal were produced after the immunization. This antiserum interacts with various other proteins that are present in both extracts. Thus, this antibody would have to be purified before it could be used for other applications.

4.4.2.3 The αPhb4-1 antibody

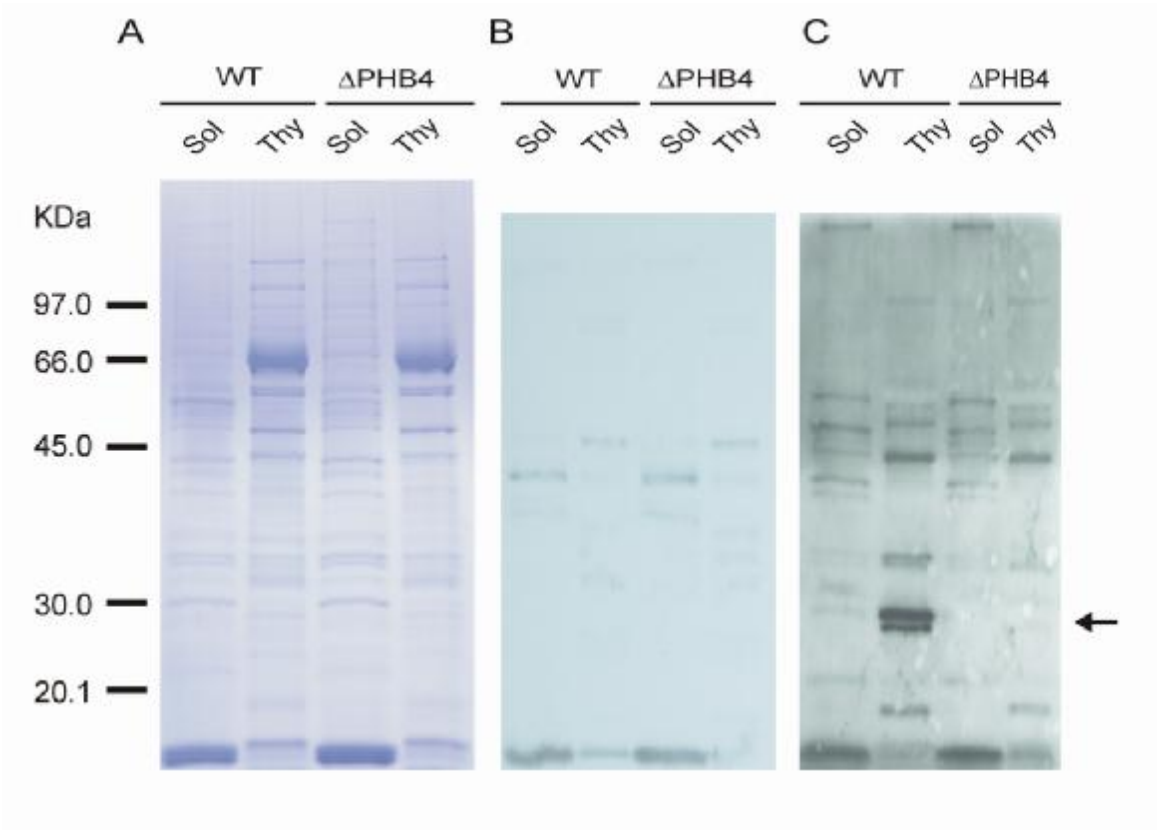

Figure 4.10: Immunoblotting analysis to test the polyclonal antisera directed against Phb4. Thylakoid and soluble extracts were obtained from wild type and mutant (specifically lacking Phb4) cells. The loaded amount of thylakoid extracts corresponded to 1 µg chlorophyll a, whereas the amount of soluble extracts was non-quantified. The extracts were separated on a SDS gel and Coomassie stained (A) or used for the immunoblotting analysis with either preimmune serum (B) or antiserum against Phb4-1 (C).

The immunoblotting analysis for the αPhb4-1 antibody showed that it recognized Phb4 at a size of about 30 kDa in the soluble and thylakoid extracts obtained from wild type cells (see Figure 4.10 C arrow). The signal that was observed in the soluble fraction is most likely due to contaminating thylakoid membranes in this fraction. A specific signal for Phb4 could not be observed in the lanes where soluble and thylakoid extracts from the mutant cells lacking Phb4 had been loaded. This proved that the signal in the wild type lanes were indeed due to the presence of Phb4. Furthermore no signal that could correspond to Phb4 was generated when the membrane was incubated with preimmune serum (see Figure 4.10 B), indicating that the antibodies responsible for the signal were produced after the immunization. This antiserum interacts with various other proteins that are present in both extracts. Thus, this antibody would have to be purified before it could be used for other applications.

4.5 Conclusions

4.5.1 Generation of polyclonal antibodies

We generated three polyclonal antibody sera from rabbit during a three months immunization protocol. Immunoblotting experiments have shown that the antisera were specific for their respective antigens, the prohibitin homologues Phb2, Phb3 and Phb4 from *Synechocystis* sp. PCC 6803. Nevertheless the antibodies αPhb3-1 and αPhb4-1 also exhibited a considerable amount of non-specific cross reactions, so that these antibodies will have to be purified for future experiments. Especially the planned immunoprecipitation and affinity purification experiments will require high quality antibody sera.

Chapter V:
Results - Generation and Characterization of Prohibitin Mutants

Mutants are important tools in biological studies. In particular knock out mutants are widely used to test whether a certain gene product is involved in a particular process. After knocking out a specific gene, any changes in the phenotype compared to the WT situation suggest a link between the affected gene and the observed process. In this study various mutants have been generated where prohibitins have been insertionally inactivated (see table 5.1). These mutants have been and will be studied under various environmental stress conditions to elucidate the role of prohibitins in *Synechocystis sp.* PCC 6803.

Another use for directed mutagenesis is the tagging of a target gene. In these mutants the target gene is altered in a way that the respective protein is tagged (in this study: His tagged) e.g. to facilitate the purification of this protein. Generation of His-tagged prohibitin mutants is still ongoing, but the progress that has been achieved so far is shown in this report.

Table 5.1: Generated *Synechocystis sp.* PCC 6803 knock out mutants. The mutants listed in the table below have been generated in this study and complete segregation has been confirmed. All mutants have been generated in the glucose tolerant Dexter wild type strain. KAN (kanamycin); CAM (chloramphenicol); SPEC (spectinomycin).

#	segregated mutants	knocked out genes
1	ΔPHB1-KAN	*slr1106*
2	ΔPHB2-CAM	*slr1768*
3	ΔPHB1-KAN + ΔPHB2-CAM	*slr1106 + slr1768*
4	ΔPHB3-SPEC	*slr1128*
5	ΔPHB3-CAM	*slr1128*
6	ΔPHB1-KAN + ΔPHB2-CAM + ΔPHB3-SPEC	*slr1106 + slr1768 + slr1128*
7	ΔPHB4-SPEC	*sll0815*
8	ΔPHB3-CAM + ΔPHB4-SPEC	*slr1128 + sll0815*
9	ΔPHB5-CAM	*sll1021*

5.1 The DPHB1 + DPHB2 + DPHB3 *triple mutant*

The ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant has been generated in this study and should show the most obvious phenotype of the mutants that have been generated so far.

5.1.1 Generation of the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant

Knock out mutants in *Synechocystis* sp. PCC 6803 are routinely generated by transforming the cells with a plasmid that contains the target gene disrupted by an antibiotic resistance cassette. Under selective pressure the antibiotic resistance cassette integrates into the genome via homologous recombination and disrupts the target gene. In case of the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant the genes were successively knocked out.

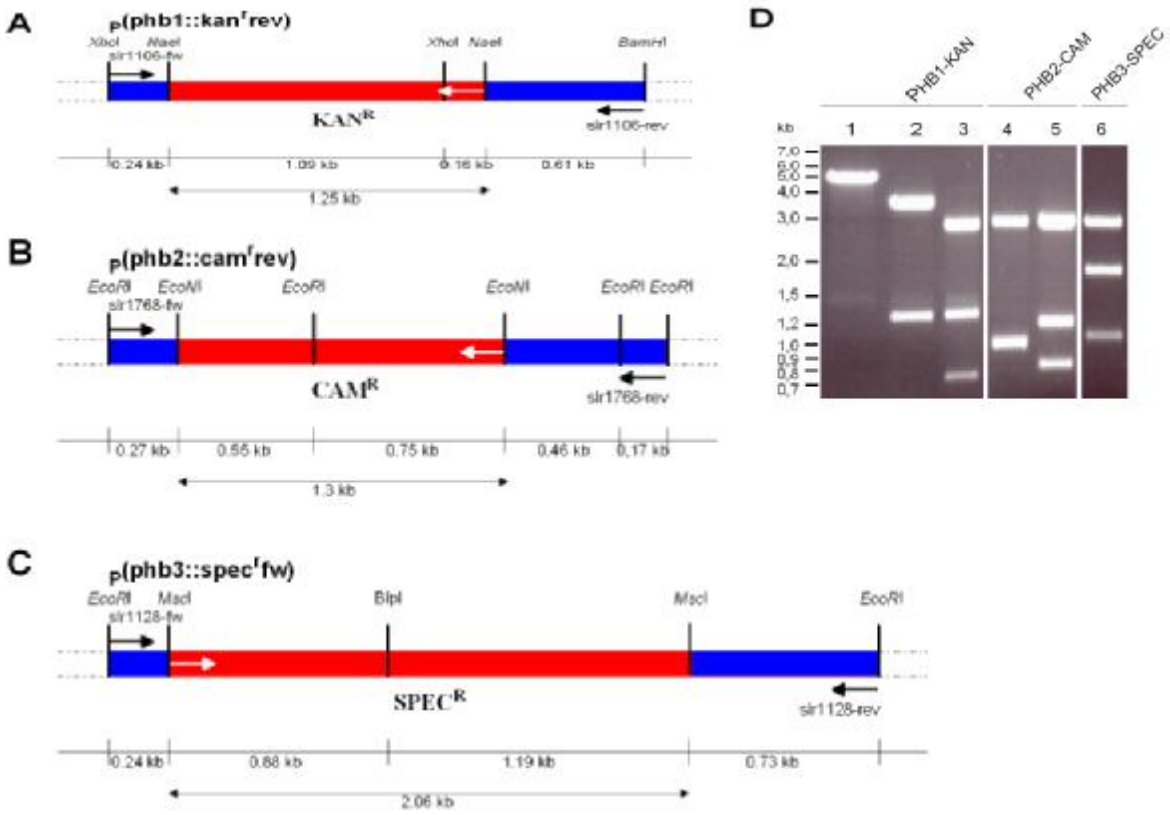

Figure 5.1: DNA constructs for the generation of the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant. (A-C) Schematic drawings of the DNA constructs used for the transformation of *Synechocystis* sp. PCC 6803 to generate the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant. (A) the *phb1* gene (*slr1106*; 849 bp) has been disrupted with a kanamycin resistance cassette (KAN^R) that was inserted into the *Nae*I site at position 236. (B) The *phb2* gene (*slr1768*; 897 bp) has been disrupted with a chloramphenicol resistance cassette (CAM^R) that was inserted into the *Eco*NI site at position 265. (C) The *phb3* gene (*slr1128*; 966 bp) has been disrupted with a spectinomycin resistance cassette ($SPEC^R$) inserted into the *Msc*I site at position 238. The orientations of the resistance cassettes with direction of transcription are indicated (white arrow). (D) Diagnostic digests of constructs shown in (A-C). Lane 1-3 digests of PHB1-KAN in pBluescript (~5080 bp) with *BamH*I (lane1; linearized plasmid), *Xho*I (lane 2; fragments of ~1.3 kb and ~3.8 kb) and *BamH*I + *Xho*I (lane3; fragments of ~ 3 kb, ~1.3 kb and ~770 bp). The PHB2-CAM construct was available with two orientations of the CAM^R cassette. Lane 4+5 digests of both PHB2-CAM constructs with *Eco*RI (lane 4; PHB2-CAM-A; fragments of ~1020 bp, ~1010 bp and ~170 bp) (lane 5; PHB2-CAM-B; fragments of ~1210 bp, ~820 bp and ~70bp). The PHB1-KAN and PHB2-CAM constructs had kindly been provided by Dr. Peter Nixon. Lane 6 digest of PHB3-SPEC with *Eco*RI and *Blp*I (fragments of ~1920 bp and ~1120 bp).

The *phb1 (slr1106)*, *phb2 (slr1768)* and *phb3 (slr1128)* genes were amplified via PCR from WT genomic DNA and cloned into the pGEMT easy vector (Promega). The respective antibiotic resistance cassettes were inserted into the cloned genes and the resulting constructs (see Figure 5.1) were transformed sucessively into *Synechocystis* sp. PCC 6803 cells. The transformants were grown on selective plates with rising levels of antibiotics in order to enforce segregation, as *Synechocystis* sp. PCC 6803 possesses multiple copies of its genome. The progress of segregation was monitored by PCR. In the end a segregated and viable ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant was generated (see Figure 5.2).

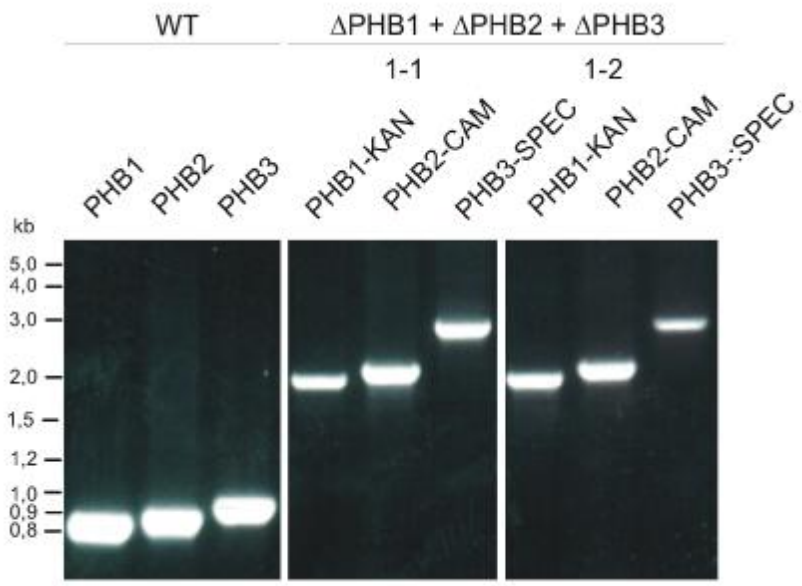

Figure 5.2: Confirmation of the segregation of the D**PHB1 +** D**PHB2 +** D**PHB3 triple mutant.** Wild type prohibitin genes (*phb1*, *phb2* and *phb3*) were amplified via PCR using specific primers from *Synechocystis* sp. PCC 6803 wild type DNA. The disrupted prohibitin genes (phb1:KAN, phb2:CAM and phb3:SPEC) were amplified via PCR using specific primers from ΔPHB1 + ΔPHB2 + ΔPHB3-triple mutant DNA.

5.2 Stress conditions

In order to assess the significance of the prohibitin homologues in *Synechocystis* sp. PCC 6803, it would be important to identify a distinctive phenotype. That is a varying behaviour from wild type cells under particular environmental conditions. Thus, the generated mutants will be exposed to various environmental stress situations.

In our working model, prohibitins are thought to protect newly synthesized D1 protein from degradation by the FtsH protease prior to its incorporation into reassembling PSII complexes. Thus, the prohibitin mutants need to be exposed to conditions where PSII complexes are damaged and their phenotypes can be assessed. The major environmental factor that causes damage to PSII complexes is excessive light.

Other potentially interesting stress conditions for the prohibitin mutants include growth at lower or higher temperature, oxidative or salt stress and variation of the ambient CO_2 level.

5.2.1 Microarray data for wild type *Synechocystis* sp. PCC 6803

Global gene expression under various stress conditions can be assessed using microarray data, in order to get a first notion on which environmental factors could be significant to reveal the role of prohibitins. Microarray data for wild type *Synechocystis* sp. PCC 6803 (see table 5.2) was kindly provided by Dr. Iwane Suzuki.

The induction ratios (IR) for prohibitins did not seem to vary significantly under the observed conditions. Only peroxide and cold stress affected the gene expression of some prohibitin genes in a minor way. Under peroxide stress *phb1* and *phb3* were slighty upregulated (IR = 2.89 and IR = 3.13) whereas *phb4* was slightly downregulated (IR = 0.37). Cold stress downregulated the expression of *phb3* (IR = 0.43) and *phb5* (IR = 0.46) whereas *phb4* was upregulated (IR = 4.48). High light stress only caused changes in the expression of the *phb5* gene which was downregulated slightly (IR = 0.45).

Table 5.2: Microarray data from wild type *Synechocystis* sp. PCC 6803 cells. Changes in induction ratios for global gene expression were tested under various stress conditions (cells treated for 20 min): salt (0.5 M NaCl), hyperosmotic (0.5 M sorbitol), heat (34 °C to 44 °C), oxidative (0.25 mM H_2O_2), cold (34 °C to 22 °C), high light (70 µE * m^{-2} * sec^{-1} to 500 µE * m^{-2} * sec^{-1}). This table shows the induction ratios for prohibitins, FtsH and the D1 proteins. Upregulated genes (induction ratios >2.0) are marked red and downregulated genes (induction ratios <0.5) are marked blue.

		Stress Condition						
Gene	**Name**	**Salt**	**Hyperosmolarity**	**Heat**	**Peroxide**	**Cold**	**High light**	**Low CO_2**
slr1106	phb1	0.94	1.63	0.63	2.89	1.39	0.77	1.13
slr1768	phb2	0.53	0.92	1.08	1.37	0.55	0.88	1.12
slr1128	phb3	0.51	1.63	1.18	3.13	0.43	0.72	1.23
sll0815	phb4	0.96	1.72	0.80	0.37	4.48	1.30	0.86
sll1021	phb5	0.62	0.56	1.03	0.81	0.46	0.45	1.52
slr0228	ftsH	1.75	1.95	2.09	6.93	2.60	2.06	1.56
slr1181	psbA1	1.13	1.54	2.32	1.50	1.26	1.29	1.32
slr1311	psbA2	1.80	1.48	2.85	2.05	1.44	1.67	1.43
sll1867	psbA3	1.78	1.51	1.42	2.66	1.77	1.37	1.93

5.2.2 Growth of the DPHB1 + DPHB2 + DPHB3 triple mutant under high light

Even though the microarray data (see table 5.2) provided no evidence for an elevated expression of prohibitins under high light conditions, we tested whether the generated ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant would reveal a phenotype when grown under high light.

Wild type *Synechocystis* sp. PCC 6803, the ΔPHB1 + ΔPHB2 + ΔPHB3 triple and the 0228::CAM mutant (positive control) were grown under low and high light conditions (BG11 plates; 5 mM glucose and 5 or 100 µE * m^{-2} * s^{-1}) (see Figure 5.3). Under high light conditions the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant was still viable and had the same phenotype as the wild type cells. The 0228::CAM mutant in which slr0228 (FtsH) has been inactivated is light sensitive and did not survive the treatment of continuous high light exposure. As the FtsH protease is inactivated in this mutant, it lacks the ability to remove damaged D1 protein which consequently accumulates under these photoinhibitory conditions. In this preliminary experiment it appeared that the inactivation of the three prohibitin genes *phb1*, *phb2* and *phb3*, did not have the expected dramatic effect on cell viability. Nevertheless other

experiments were performed on the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant to investigate the role of prohibitins in the PSII repair cycle in more detail.

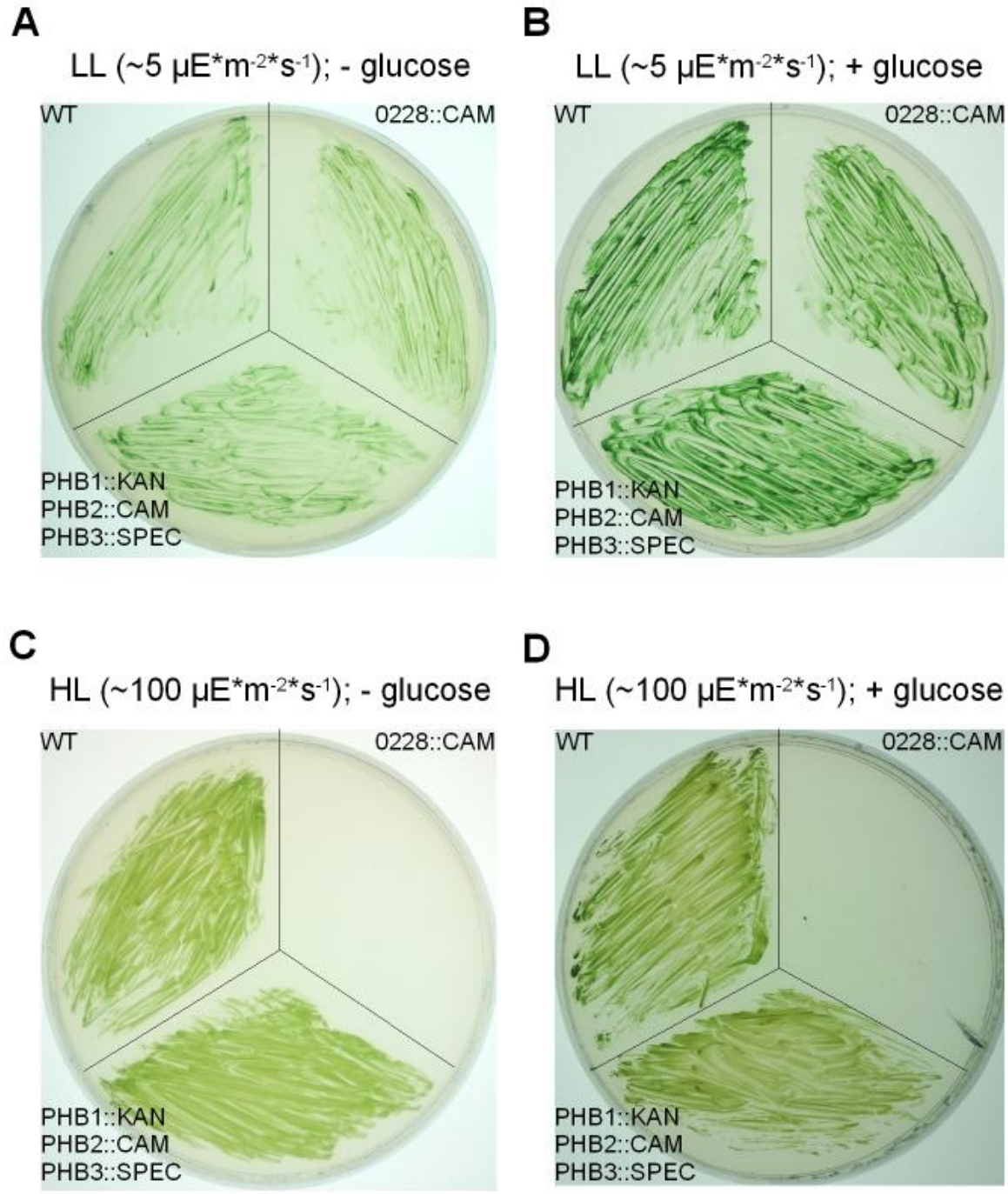

Figure 5.3: Growth of the D**PHB1** + D**PHB2** + D**PHB3 triple mutant under low and high light conditions.** (A-D) Dexter wild type cells (DWT), a glucose tolerant *Synechocystis* sp. PCC 6803 strain, Δ0228 the high light sensitive FtsH knock out mutant and the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant have been restreaked on (A) BG11 plates, low light conditions (LL, ~5 µE * m^{-2} * s^{-1}) (B) BG11 with 5 mM glucose plates, low light conditions (LL, ~5 µE * m^{-2} * s^{-1}) (C) BG11 plates, high light conditions (HL, ~100 µE * m^{-2} * s^{-1}) (D) BG11 with 5 mM glucose plates, high light conditions (HL, ~100 µE * m^{-2} * s^{-1}). All mutants are derivatives from the Dexter wild type strain.

5.3 Photoinhibition experiment

A photoinhibition experiment, where oxygen evolution is monitored after exposure to high light illumination, was performed to determine whether the PSII repair cycle is functional in the triple mutant. The results of the photoinhibition experiment that was conducted for wild type and the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant are shown in Figure 5.4.

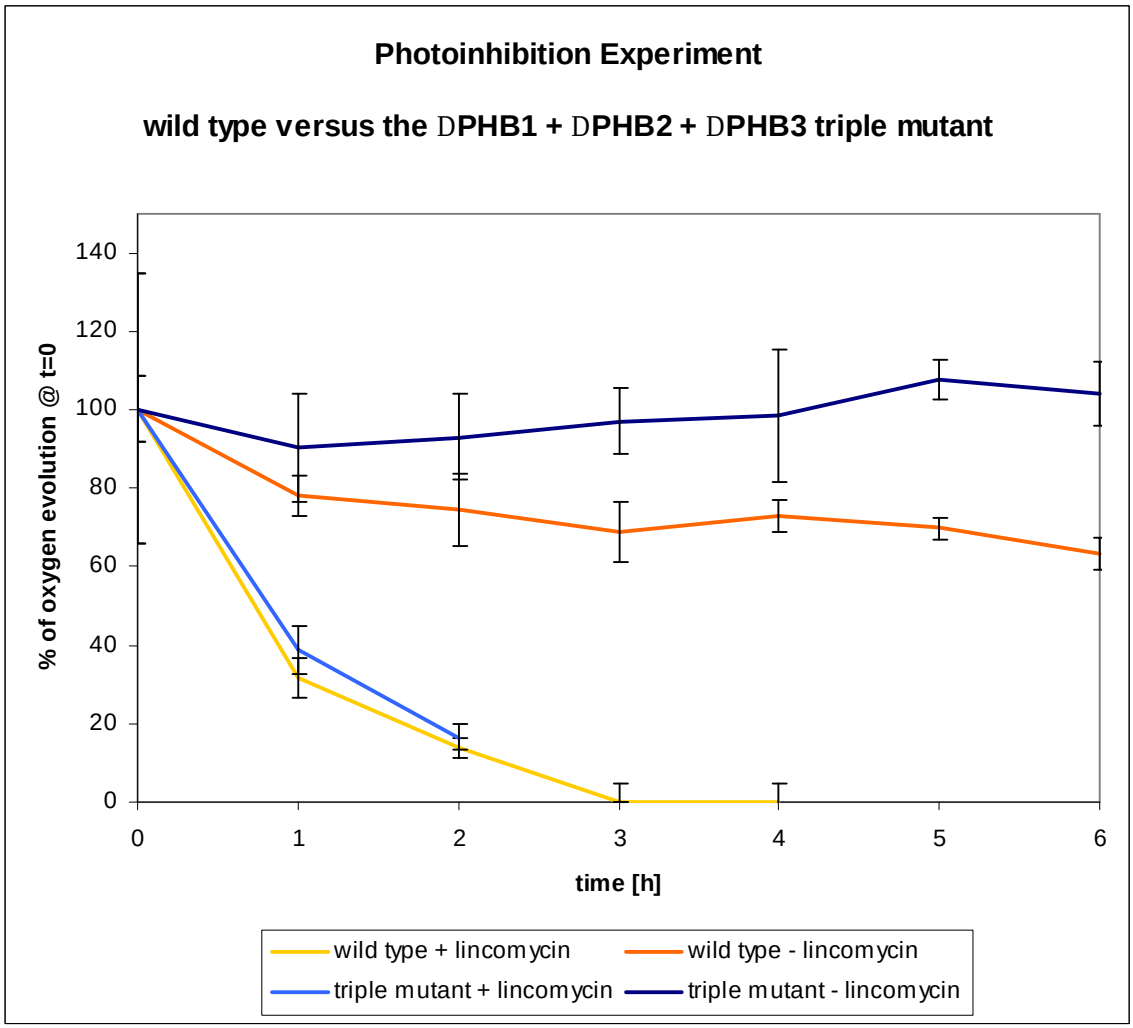

Figure 5.4: Plot of normalized oxygen evolution rates during the photoinhibition experiment with wild type and ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant cells. The photoinhibition experiment was performed at 1200 µE * m^{-2} * s^{-1} for six hours. Oxygen evolution was measured in the presence of 2,6 dichlorobenzoquinone (DCBQ) and $K_3Fe(CN)$ as an indication of PSII activity. Cultures were incubated with or without lincomycin (a protein synthesis inhibitor). Relative oxygen evoltion is plotted with the oxygen evolution at t=0 as 100%.

The oxygen evolution values in the graph were normalized to the values at t=0 as 100 % for both wild type and mutant cells. Cells that were not lincomycin treated could maintain their

rate of oxygen evolution during the entire experiment after an initial drop (20% for the wild type and 10% for the triple mutant). The oxygen evolution of the samples that have been incubated with lincomycin dropped dramatically to 0 % within the first three hours of constant strong illumination (photoinhibiting conditions). The rate of decrease was similar for both wild type and mutant cells. All in all the behaviour of the triple mutant did not differ significantly from that of the wild type cells. Thus the PSII repair cycle does not seem to be impaired in the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant as oxygen evolution maintains stable after inflicting damage to the PSII complexes.

5.4 Pulse-chase experiment

To investigate the role of prohibitins in the PSII repair cycle, selective D1 protein turnover was monitored in both wild type and mutant cells, using the pulse-chase approach. In this experiment ^{35}S methionine labelled cells are exposed under high light conditions (~1000 µE * m^{-2} * s^{-1}) which leads to the damage of PSII complexes. The selective degradation of the D1 protein can be monitored in the chase period. In conjunction with the photoinhibition experiment (see 5.3) this assay has the potential to reveal if prohibitins are involved in the PSII repair cycle.

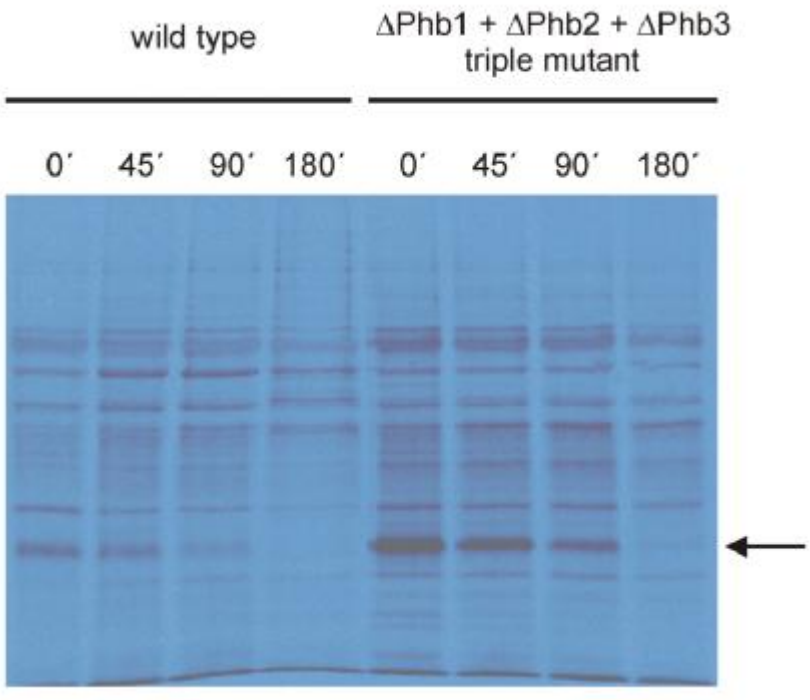

Figure 5.5: Pulse-chase experi-ment of wild type and the D**PHB1** + D**PHB2** + D**PHB3 triple mutant.** Pulse-chase experiments were performed for wild type and triple mutant cells. Samples were taken at the indicated time points and thylakoid membranes were isolated. A 12 % SDS gel was run and each lane contains a sample corresponding to approximately 1 µg of chlorophyll. The gel was dried and exposed for 1 days to a radiography film. The band resulting from the D1 protein is marked with an arrow (←).

The results of the pulse-chase experiments are represented in Figure 5.5. It was observed that the rate of D1 protein (see arrow) turnover is similar for both wild type and mutant cells. Although initial labelling (@ t=0) was more efficient for the triple mutant, the labelling of the D1 protein was completely diminished after 180 min of strong illumination.

5.5 His-tagging of prohibitins

In order to be able to purify possible prohibitin complexes, four of the prohibitins will be His-tagged (Phb1, Phb2, Phb3 and Phb4) by transforming *Synechocystis* sp. PCC 6803 cells with recombinant DNA constructs, that have been generated using overlap extension PCR. This part of the project is ongoing, but recent results are included in this report.

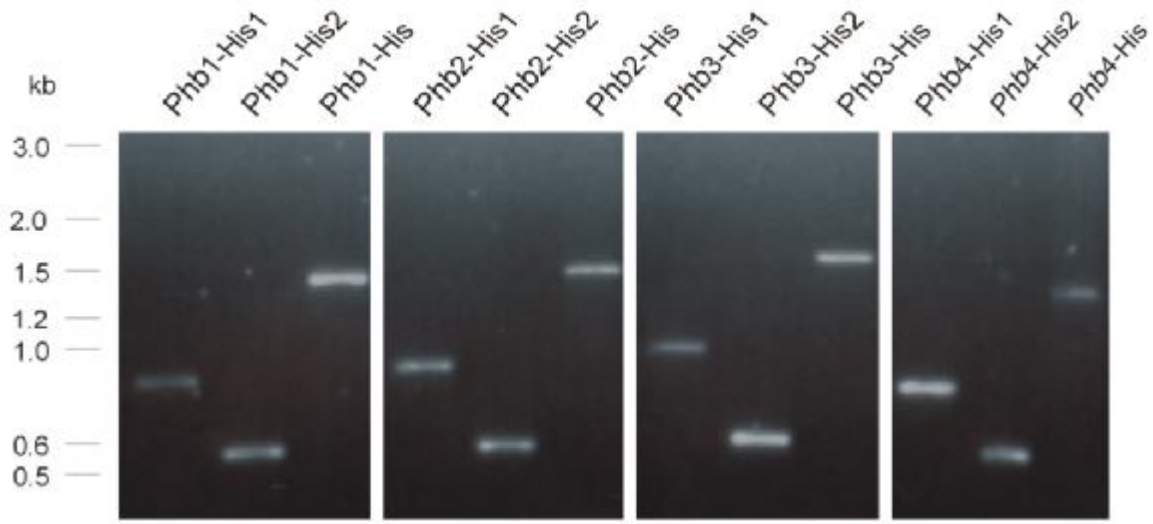

Figure 5.6: Phb-His PCR fragments. The Phb-His fragments have been generated by PCR using primer overlap extension. The His-tag will be at the C-Terminus of the prohibitins. Phb-His1 corresponds to the amplified prohibitin gene with the His-tag coding sequence extension. Phb-His2 is the respective, amplified downstream region of each prohibitin. Phb-His is the resulting PCR fragment after combining Phb-His1 and Phb-His2.

So far four plasmids have been cloned that contain the coding sequence of the prohibitin, the coding sequence of the His-tag and the downstream region of each prohibitin respectively. The fragments that were cloned into pGEMT easy were obtained by overlap extension PCR (see Figure 5.6) and a diagnostic *Eco*RI digest of these plasmids is shown in Figure 5.7. After inserting a selective marker these constructs can be transformed into *Synechocystis* sp. PCC 6803.

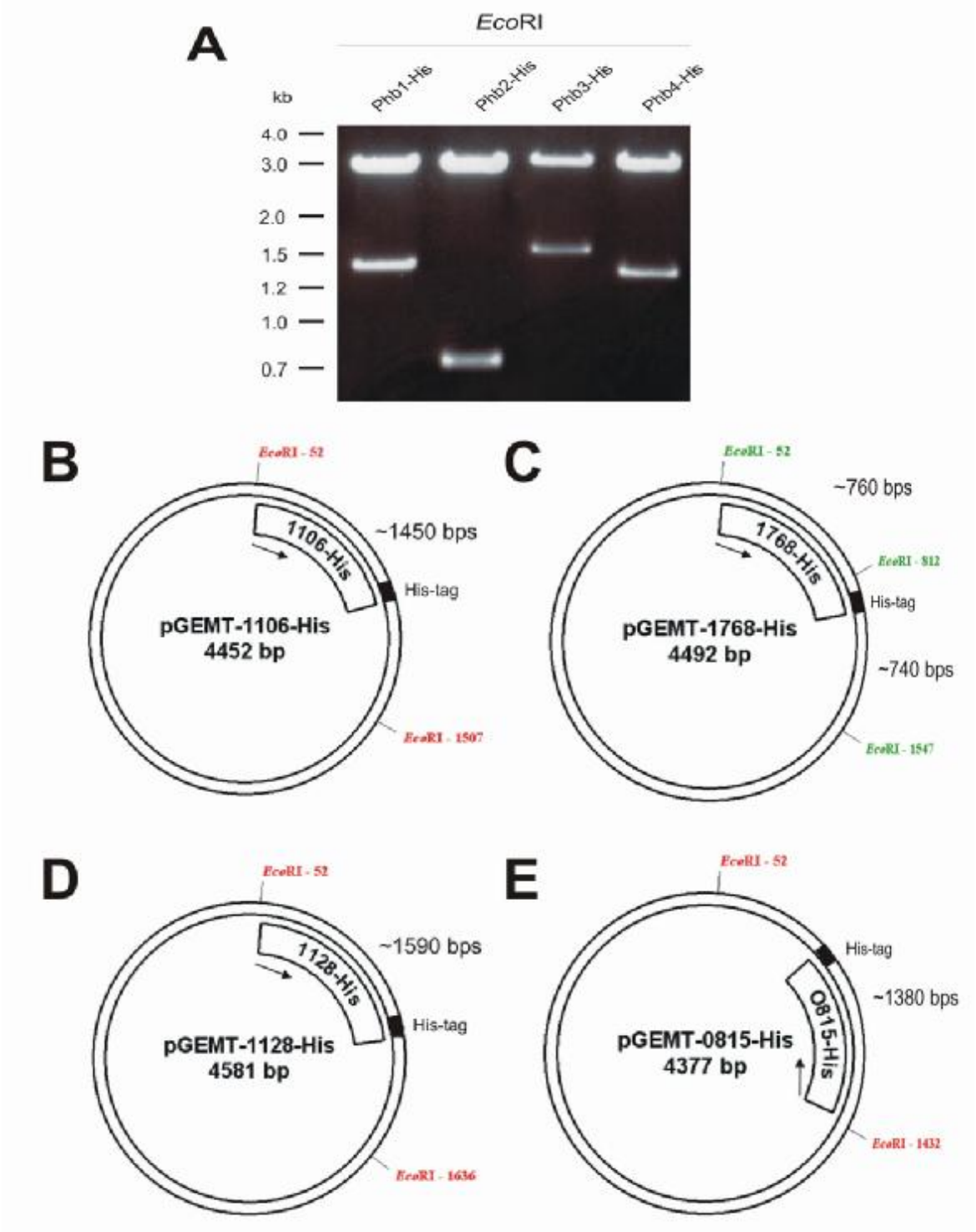

Figure 5.7: Diagnostic digests and schematic drawings of pGEMTeasy vectors containing the Phb-His fragments. (A) Diagnostic digest: *Eco*RI cuts two times in the multiple cloning site of the pGEMTeasy vector and releases the ligated fragment. The 3 kb band corresponds to the linearized pGEMTeasy vector. Phb1-His ~1450 bps, Phb2-His ~1470 bps (has an internal *Eco*RI site around 700 bps), Phb3-His ~1590 bps and Phb4-His ~1380 bps. (B-D) Schematic drawings of the His-tagged Phb fragments that were cloned into pGEMTeasy (B) pGEMT-1106-His, (C) pGEMT-1768-His (D) pGEMT-1128-His and (E) pGEMT-0815-His. The restriction sites for *Eco*RI and the sizes of the excised fragments are indicated. The direction of transcription is indicated by the little arrow and the position of the introduced His-tag is marked as a black box.

5.6 Conclusions and Discussion

We have generated single mutants of all the identified prohibitin homologues in *Synechocystis* sp. PCC 6803 (see table 5.1) and found that the prohibitin genes are not required for cell viability. We also generated a ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant (see Figure 6.2) and showed by photoinhibition (see 5.3) and pulse-chase experiments (see 5.4) that the prohibitin homologues Phb1, Phb2 and Phb3 do not seem to be involved in the PSII repair cycle and in D1 protein turnover respectively. None of the generated mutants showed an obvious phenotype under normal growth conditions or under the influence of elevated light. The generation of His-tagged prohibitin mutants for the purification of the respective protein complexes is ongoing.

5.6.1 Phenotypes of prohibitin mutants in other organisms

Prohibitin homologues have been found in many organisms ranging from procaryotes (Banuett and Herskowitz, 1987) to higher eukaryotes (Nuell et al., 1991). These proteins attracted particular interest, because they were found to be expressed at decreased levels in mammalian tumor cells and had the ability to negatively regulate cell proliferation (McClung et al., 1989; Nuell et al., 1991). These observations lead to further experiments in various organisms in which the respective prohibitin homologues had been inactivated (Eveleth et al., 1986; Coates et al., 1997; Artal-Sanz et al., 2002).

Mutations in the prohibitin gene had the most severe effect in *Drosophila*, where the phenotype was found to be lethal during the larval development (Eveleth et al., 1986). In another study the effect of prohibitin depletion by RNA-mediated interference (RNAi) at different developmental stages of the nematode *C. elegans* was monitored. During embryogenesis the depletion of the prohibitins resulted in developmental arrest, whereas a later depletion during postembryonic development lead to somatic and germline effects (Artal-Sanz et al., 2002). In *S. cerevisiae* however the observed phenotype after the disruption of the prohibitin genes was less severe and resulted in a shortened replicative life span due to premature aging (Coates et al, 1997). A more dramatic effect could be observed when the

deletion of prohibitins in yeast was paired with defects in the mitochondrial inheritance machinery (Berger and Yaffe, 1998) or of the mitochondrial phosphatidylethanolamine biosynthetic machinery (Birner et al., 2003) which then also turned out to be lethal. A slow growing phenotype was observed when the prohibitins were knocked out together with the mitochondrial m-AAA protease (Steglich et al, 1999).

These observations seem to suggest that prohibitins are essential for one or more steps in the differentiation of multicellular organisms. The lack of a clear growth phenotype of the probibitin mutants in yeast might reflect a redundancy in assembly factors, or could imply that prohibitin mutations may in general have a stronger phenotype in organisms or tissues with a greater dependence on mitochondrial energy generation. In any case it needs to be emphasized that so far only eukaryotic prohibitins have been mutated, which we found to be only distantly related to the prohibitin homologues that are found in procaryotes and consequently in cyanobacteria (see Figure 3.7).

5.6.2 Searching for a function of prohibitin mutants in *Synechocystis* sp. PCC 6803

The single and multiple prohibitin homologue mutants that we generated in *Synechocystis* sp. PCC 6803 did not exhibit an obvious phenotype under the normal growth conditions. The possible involvement in the PSII repair cycle and in D1 degradation was assessed by photoinhibition and pulse-chase experiments respectively performed with the ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant. Both experiments clearly demonstrated that the mutant did not exhibit a phenotype that differed significantly from the situation in the wild type cells. Nevertheless redundancy, i.e. overlap in function, could be responsible for the obtained results and the experiments will have to repeated with a mutant in which at least the *phb4* gene and preferably also the *phb5* gene has been inactivated.

So far the efforts to find an obvious phenotype in the *Synechocystis* sp. PCC 6803 prohibitin mutants have been limited for various reasons. In the future the search for effects under environmental stress conditions in these mutants will be pursuit more intensively. Microarray data however already revealed that the expression of prohibitin homolgues is rather stable

during some environmental stresses (see table 5.2) which implies that a severe phenotype resulting from an inactivation of a prohibitin homologue cannot necessarily be expected. In any case it seems important to inactivate as many prohibitin homologues in *Synechocystis* sp. PCC 6803 as possible in order to overcome a possible redundancy. More promising even seems to be the approach to inactivate other genes, like FtsH or a PSII subunit, in the same mutant as the prohibitin homologues. Judging from the observations that have been made in the eukaryotic backgrounds (Berger and Yaffe, 1998; Birner et al., 2003; Steglich et al, 1999), this should increase the possibility of revealing a phenotype.

Since the attempts to show an involvement of the prohibitin homologues in *Synechocystis* sp. PCC 6803 in the PSII repair cycle or photosynthesis in general have been unsuccessful so far, new approaches will have to be pursuit. Localization experiments which will identify the compartment(s) or membrane(s) in which the prohibitin homologues reside, will help to elucidate the role of prohibitins. Some of the proteins (Phb1, Phb2, Phb3 and Phb5) have transmembrane domains (see table 3.3) and a proteomics approach has identified these proteins in the plasma membrane of *Synechocystis* sp. PCC 6803 (Huang et al., 2002). This finding could be of significance for the possible role of prohibitin homologues as “holdases” or assembly factor, because some of the initial steps of biogenesis of the cyanobacterial photosystems occur in the plasma membrane (Zak et al., 2001). Immunoprecipitation experiments and the purification of the prohibitin homologue complexes however might lead to the identification of some of the interaction partners of prohibitins and thereby give a better understanding of the function that the prohibitins fulfil in *Synechocystis* sp. PCC 6803.

Chapter VI: Results – Characterization of Prohibitins *in vivo*

This chapter presents the results from experiments that have been performed to characterize the prohibitin homologues *in vivo*, that is either in thylakoid membranes extracts of *Synechocystis* sp. PCC 6803 or *Thermosynechococcus elongatus*. We intended to confirm the inactivation of the prohibitin homologues in the generated mutants, that has been shown on the genetic level in chapter IV, on the protein level. Moreover, the first results from immuno-blotting analysis following Blue Native (BN) PAGE and fast protein liquid chromatography (FPLC) will be discussed. A differential protein extraction experiment concludes this chapter.

6.1 Immunoblotting analysis following 1-D PAGE of thylakoid membrane extracts from prohibitin mutants

After the results from the PCR analysis confirmed that mutants were completely segregated and the prohibitins inactivated, thylakoid membrane extracts were isolated from each of the mutants and used for 1-D PAGE. In a first step SDS PAGE was used to test for the absence of the prohibitins on the protein level. Later 1-D BN PAGE was applied to analyse possible prohibitin complexes. Thylakoid membrane extracts from wild type, all the single mutants and the triple mutant (ΔPHB1, ΔPHB2, ΔPHB3, ΔPHB4, ΔPHB5 and ΔPHB1 + ΔPHB2 + ΔPHB3) were analysed in these experiments.

6.1.1 Immunoblotting analysis following 1-D SDS PAGE

This immunoblotting analysis was performed to confirm the PCR results for the respective mutants on the protein level. Moreover the protein band pattern was observed for any obvious changes resulting from the inactivation of the prohibitin homologues.
Thylakoid membrane extracts from wild type and mutant cells were isolated, separated on a 12.5 % SDS gel (Coomassie stain; see Figure 6.1 A) and immunoblotting analysis were performed using antibodies against Phb1, Phb2, Phb3, Phb4 and D1 (see Figure 6.1 B to F). In the thylakoid extract from wild type cells all proteins (Phb1, Phb2, Phb3, Phb4 and D1)

could be detected. In the mutants the proteins that were insertionally inactivated were indeed not present. The immunoblotting analysis with the D1 antibody was performed as a control to show that thylakoid membrane proteins had been loaded and properly transferred to the filter. Interestingly, some changes in the pattern of the protein bands could be observed on the Coomassie stained gel. The most obvious change was an additional protein with an apparent molecular weight of about 35 kDa in the ΔPHB3 and in the triple mutant (see Figure 6.1 *).

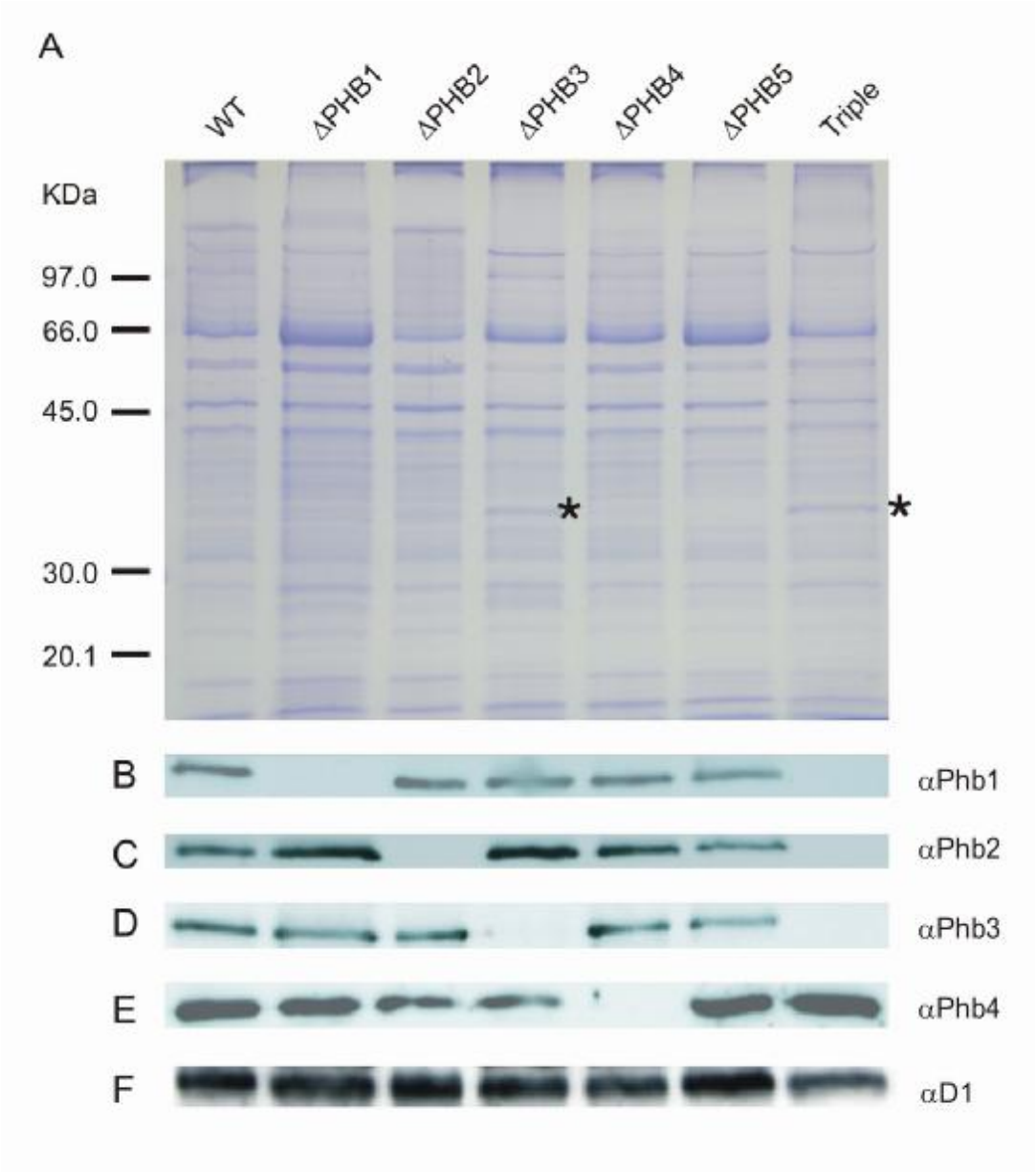

Figure 6.1: Immunoblotting analysis following 1-D SDS PAGE of thylakoid extracts from wild type and mutant cells. Thylakoid membrane extracts from wild type and the prohibitin mutants corresponding to 1 µg of chlorophyll a were loaded into each lane of a 12.5 % SDS gel. (A) Coomassie stained gel where the newly appearing band is marked (*). Immunoblotting analysis using the indicated antisera were performed and the region corresponding to the respective antigen are shown in (B) αPhb1 (C) αPhb2-1 (D) αPhb3-1 (E) αPhb4-1 and (F) αD1.

6.1.2 Immunoblotting analysis following 1-D BN PAGE

1-D BN PAGE is one technique to study the compostion and dynamics of protein complexes. As prohibitins have been reported to form large multimeric complexes, we wanted to test if these are present in *Synechocystis* sp. PCC 6803 and whether the inactivation of a particular prohibitin would affect other possible prohibitin complexes.

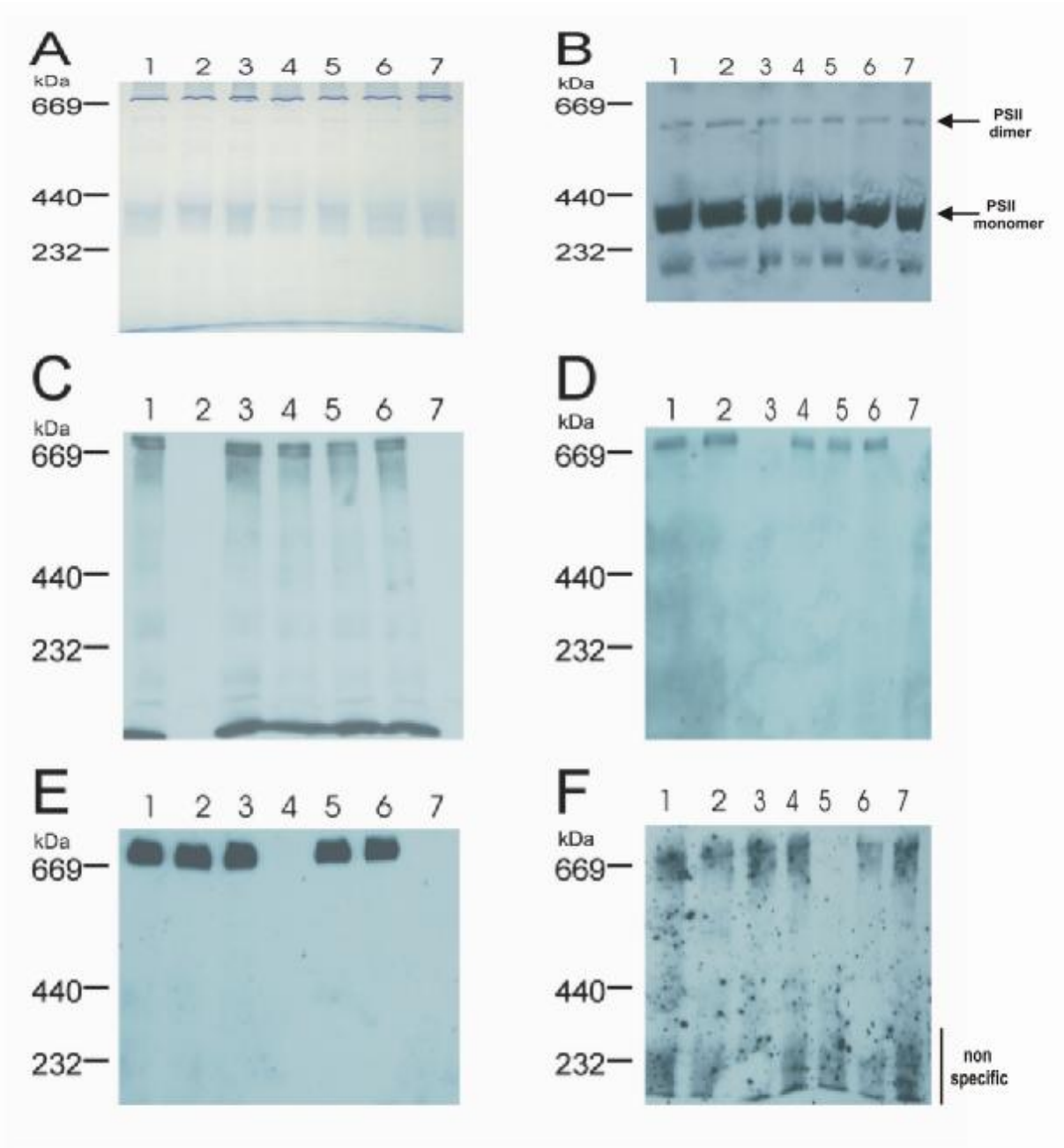

Figure 6.2: Immunoblotting analysis following 1-D BN PAGE of thylakoid extracts from wild type and mutant cells. Thylakoid membrane extracts from wild type and the prohibitin mutants corresponding to 1 µg of chlorophyll a were loaded into each lane of a 6 to 12 % BN gradient gel. (A) Coomassie stained gel. Immunoblotting analysis using (B) αD1 (C) αPhb1 (D) αPhb2 (E) αPhb3 and (F) αPhb4 were performed.

Thylakoid membrane extracts from wild type (Figure 6.2 A to F lane 1) and mutant cells (Figure 6.2 A to F; ΔPHB1 [lane 2], ΔPHB2 [lane 3], ΔPHB3 [lane 4], ΔPHB4 [lane 5], ΔPHB5 [lane 6] and ΔPHB1 + ΔPHB2 + ΔPHB3 [lane 7])were isolated, separated on a 6 to 12 % BN gel and immunoblotting analysis were performed using different antibodies against D1, Phb1, Phb2, Phb3 and Phb4 (see Figure 6.2 B to F).

The immunoblotting analysis of the D1 protein (Figure 6.1 B) showed that samples were loaded in every lane and that dimeric (~600 kDa) and monomeric (~300 kDa) photosystem II were equally present in the wild type and in every mutant thylakoid extract.

Using the αPhb1 antibody (Figure 6.1 C) no signals were observed in the lane corresponding to the ΔPHB1 and ΔPHB1 + ΔPHB2 + ΔPHB3 triple mutant, confirming the results obtained in 5.1.1. In all the other lanes however several signals corresponding to molecular masses ranging from about 1 MDa to less than 100 kDa could be detected, suggesting the presence of several distinct Phb1 protein complexes. Remarkably the inactivation of the other prohibitin homologues did not seem to affect the Phb1 complexes in these mutants which suggested that the Phb1 complexes are homooligomeric or a composition with another protein. Interestingly substantial amount of possibly monomeric Phb1 were also detected at the bottom of the gel.

The immunoblotting analysis with the αPhb2 (Figure 6.1 D) and αPhb3 (Figure 6.1 E) antibodies revealed that these two proteins also from large multimeric complexes (>1 MDa) which did not seem to depend on the presence of another prohibitin homologue. No monomeric Phb2 or Phb3 was detected.

The αPhb4 antibody that was used in this analysis exhibited several cross reactions which were not specific to the Phb4 protein. Nevertheless, the absence of a signal in the high moleclar range of > 1 MDa in the lane for the ΔPHB4 mutant suggested that this protein also forms a large multimeric protein complex that is associated with the thylakoid membrane. No monomeric Phb4 was detected.

6.2 Westernblotting analysis following FPLC

FPLC (fast protein liquid chromatography) is an alternative protein separation technique, which was used in conjunction with immunoblotting analysis to study possible prohibitin homologue complexes.

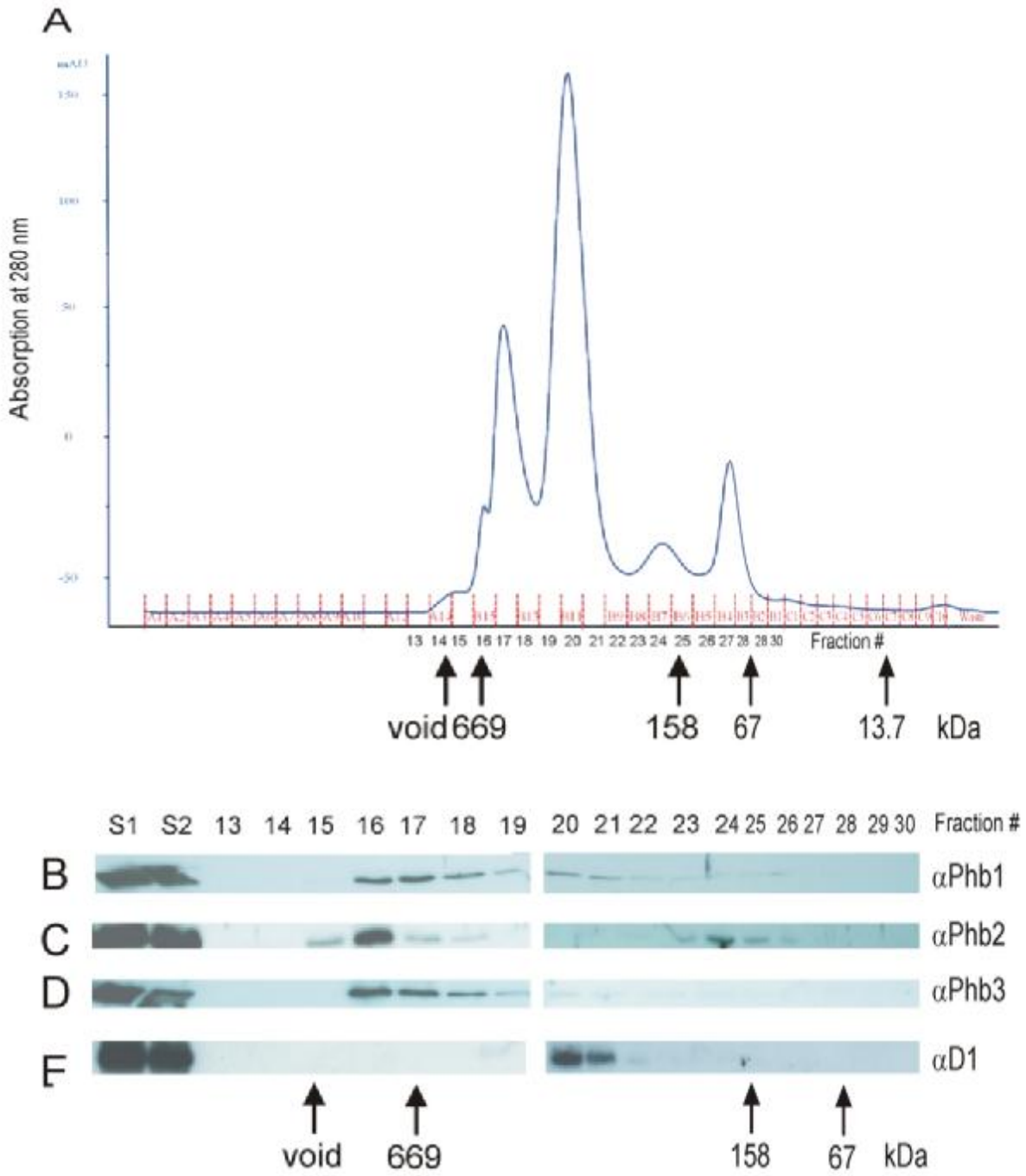

Figure 6.3: Immunoblotting analysis following FPLC of thylakoid membrane extract from *Synechocystis* sp. PCC 6803. (A) Absorption spectrum @ 280 nm that was recorded on the eluate during the FPLC run. Fractions of 500 µl were collected and analysed by SDS PAGE. (B-E) 10 µl aliquots of solubilized thylakoid extract before (S1) and after (S2) ultracentrifugation as well as 10 µl aliquots of the collected fractions were run on a 10 % SDS gel and analysed using different antibodies: (B) αPhb1 (C) αPhb2 (D) αPhb3 and (F) αD1.

Wild type thylakoid membrane extract (see Figure 6.3 lanes S1 and S2) was applied to a Superdex200 column, fractions were collected and analysed using 1-D SDS PAGE. Subsequently, immunoblotting analysis were performed with various antibodies against Phb1, Phb2, Phb3 and D1. To estimate the molecular weight markers were run separately and their elution time recorded.

The protein absorption spectrum of the eluate from the thylakoid membrane extract (see Figure 6.3 A) showed four peaks of around 600 kDa, 300 kDa, 180 kDa and 100 kDa. The two major peaks of 600 kDa and 300 kDa corresponded to among others dimeric and monomeric photosystem II respectively which was in agreement with the signals oberved in the immunoblotting analysis for the αD1 antibody. Here the signal for the monomer (fractions 20 and 21) was rather strong, whereas the signal for the dimer (fraction 17) was very weak. Using the αPhb1 and αPhb3 antibody (Figure 6.3 B and D) showed tailing signals that constantly became weaker over the fractions 16 to 22. Fraction 16 corresponded to high molecular masses of above 670 kDa and fraction 22 contained proteins and their complexes with a molecular weight of roughly above 250 kDa. This suggested large Phb1 and Phb3 complexes that seemed to degrade rather easily.
Phb2 on the other hand led to a strong signal in fraction 16 and signals of lesser intensity in the fractions 15, 17 and 18 suggesting a more stable protein complex. Moreover other signals for the Phb2 protein were observed at lower molecular weight in the fractions 23 to 26 with the most intense signal in fraction 24 (~180 kDa), implying the presence of a smaller second Phb2 protein complex or a distinct breakdown product of the large protein complex.

6.3 Immunoanalysis following 2-D PAGE of thylakoid membrane extracts from Thermosynechococcus elongatus

Two dimensional PAGE is a powerful technique to study protein complexes, especially their dynamics during assembly and disassembly. Thus, in order to establish this technique and test whether it is suitable for the cyanobacterial Band 7 protein complexes, thylakoid extracts from *Thermosynechococcus elongatus* were isolated and analysed. Being a thermophilic organism, protein complexes from *Thermosynechococcus elongatus* are generally more stable and were therefore used preferentially in this experiment. Specific antibodies against D1,

Phb1 and FtsH were used in the subsequent immunoblotting analysis to test for an interaction between TE_Phb1 and FtsH. Such an interaction has been reported in *E. coli* where a complex between the FtsH homologue and the Band 7 proteins HflK and HflC had been observed (Saikawa et al., 2004).

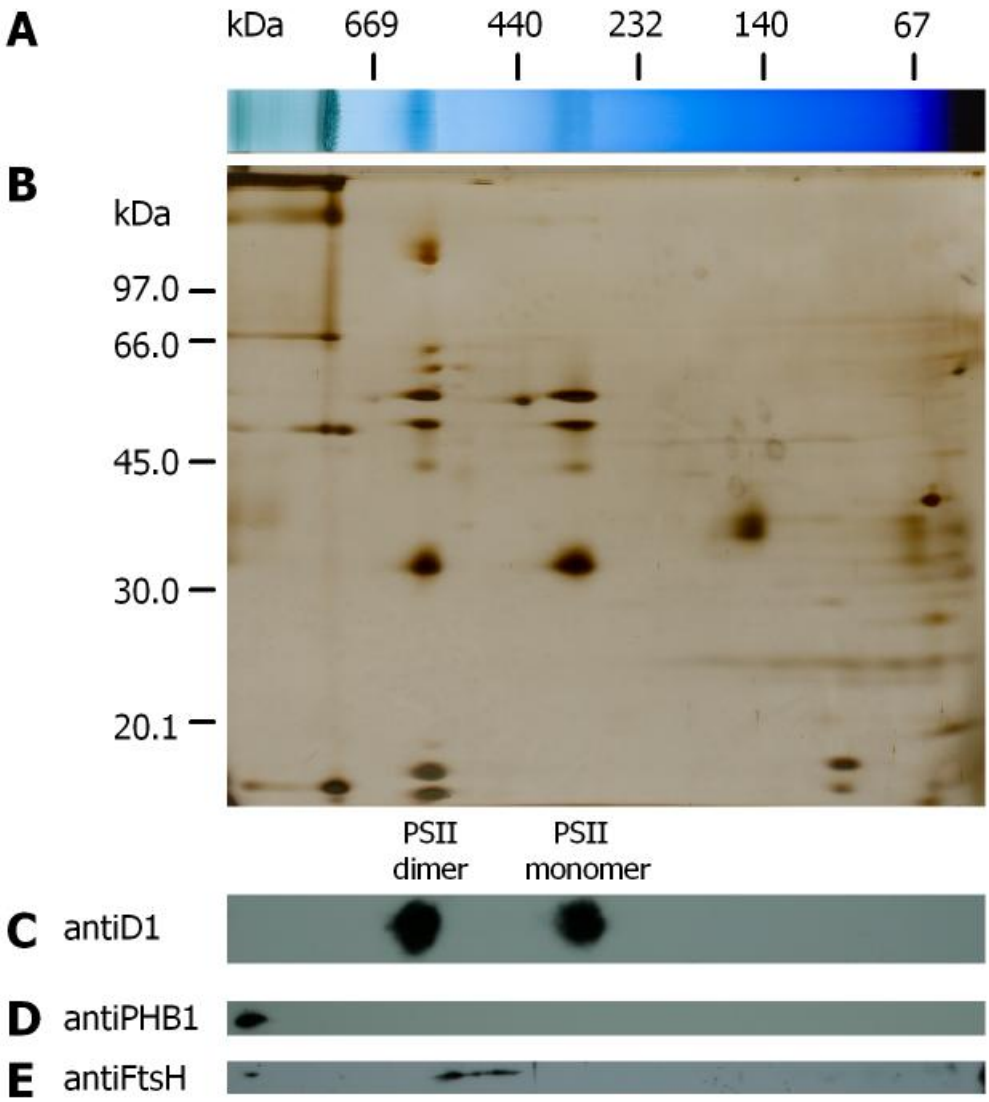

Figure 6.4: Immunoblotting analysis following 2-D PAGE of thylakoid membrane eatract from *Thermosynechococcus elongatus*. (A) Thylakoid membrane extract from *Thermosynechococcus elongatus* corresponding to 10 µg of chlorophyll a were loaded into each lane of a 6 to 12 % BN gradient gel. (B) One lane was incubated for 30 min in solubilization buffer, transfered and run on the 12 % second dimension SDS gel that was silver stained. (C-E) Immunoblotting analysis were performed on the second dimension gel using various antibodies (C) αD1 (from *Synechocystis* sp. PCC 6803) (D) αPhb1 (from *Synechocystis* sp. PCC 6803) and (E) αFtsH (from *E. coli*).

Figure 5.4 shows the two dimensional PAGE analysis of a thylakoid membrane extract from *Thermosynechococcus elongatus* with a subsequent immunoblotting analysis. The protein

complexes were separated into several distinct bands on the 1-D BN gel (see Figure 6.4 A) and large protein supercomplexes have migrated into the low percentage (6 %) upper region of the gel. Above 669 kDa one large, green band could be seen which consited out of trimeric photosystem I. The next lower bands with apparent molecular masses of about 600 and 300 kDa represented dimeric and monomeric photosystem II respectively, which is in agreement with the immunoblotting analysis (see Figure 6.4 C).
In the second dimension 12 % silver stained polyacrylamide gel the separation of the protein complexes (see Figure 6.4 A) into their subunits could be seen (see Figure 6.4 B). Immunoblotting analysis with the various antibodies αD1 (C) αPhb1 (D) and αFtsH (E) were performed and only the regions where the antibodies generated specific signals are shown (see Figure 6.4 C to E). As already mentioned the αD1 antibody produced two signals which corresponded to dimeric and monomeric photosystem II. The TE_Phb1 protein was found in the region that corresponded to the high molecular weight in the first dimension, suggesting it to have been in a complex of a molecular weight of above 1 MDa. In this analysis no monomeric TE_Phb1 could be detected at the bottom of the gel. Two signals were observed for the αFtsH antibody, of which one was in the same high molecular weight region as the signal for TE_Phb1. This could imply the existance of an interaction between the TE_Phb1 and the FtsH homologue as it has been observed between the FtsH homologue and HflKC in *E. coli*. The other signal generated by the αFtsH antibody was in the molecular weight region of around 450 kDa which would correspond to an homohexameric FtsH complex. The FtsH monomer is usually detected with an apparent molecular weight of around 75 kDa.

6.4 Differential protein extraction from Synechocystis sp. PCC 6803 thylakoid membrane extracts

A differential protein extraction on *Synechocystis* sp. PCC 6803 thylakoid membrane extracts (see Figure 6.5) was performed to test the interaction between prohibitin complexes and the thylakoid membrane. This was of particular interest for the Phb4 homologue which was not predicted to contain a transmembrane domain and which had in previous experiments been found to be only loosely attached to the thylakoid membrane (data not shown). Unfortunately the immunoblotting analysis for this particular protein contained too many unspecific interactions, so that the data was not of sufficient quality to be presented in this report (compare 4.4.2.3).

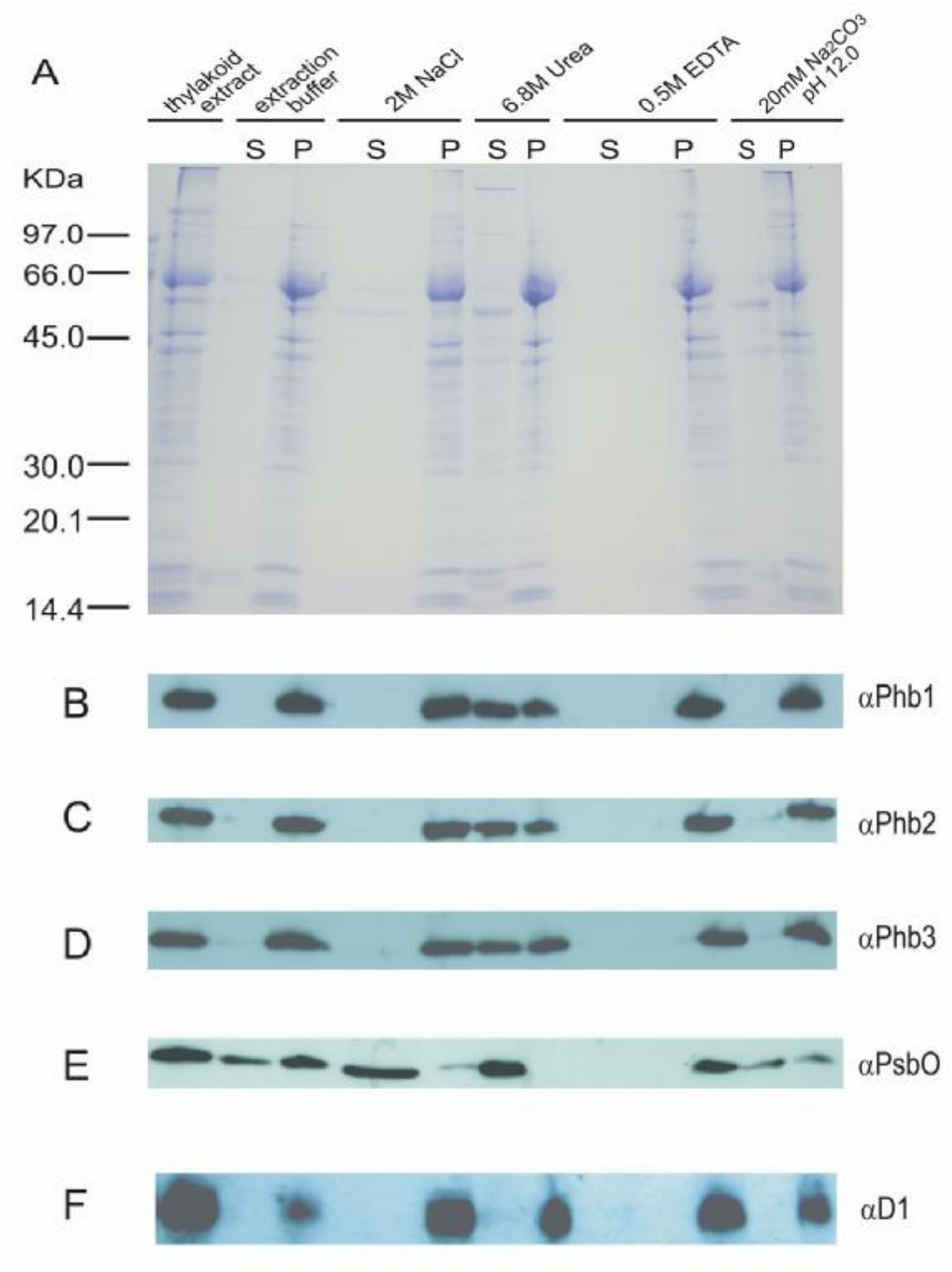

Figure 6.5: Immunoblotting analysis following differential protein extraction of thylakoid membrane extracts from *Synechocystis* sp. PCC 6803. Thylakoid membrane extracts were isolated and washed in various buffers: extraction buffer (EB), 2M NaCl in EB, 6.8M Urea in EB, 0.5 M EDTA in EB and 20 mM Na_2CO_3 in EB pH = 12.0. After two consecutive freeze / thaw cycles the membranes were ultracentrifuged and separated into a soluble (S) and a pellet (P) fraction. Original thylakoid extract and aliquots corresponding to an amount of 1 µg of chlorophyll a were loaded into each lane. The gels were Coomassie stained (A) or used for a further immunoblotting analysis (B to F) with various antibodies: (B) αPhb1, (C) αPhb2, (D) αPhb3, (E) αPsbO and (F) αD1.

Thylakoid membrane extracts from *Synechocystis* sp. PCC 6803 wild type cells were isolated and treated with extraction buffer containing different additives. After the buffer treatment the membranes were pelleted in an ultracentrifuge and the soluble and the pelleted fractions were analysed by 1-D SDS PAGE and immunoblotting analysis. The following antibodies were applied: αPhb1 (see Figure 6.5 B), αPhb2 (see Figure 6.5 C), αPhb3 (see Figure 6.5 D), αPsbO (see Figure 6.5 E) and αD1 (see Figure 6.5 F). The antibodies against PsbO (an extrinsic photosystem II protein of the oxygen evolving complex; should be readily extracted) and against D1 (an integral membrane protein of the photosystem II reaction center; should not be affected be the buffer treatment) were used as controls.
The immunoblotting analysis of the thylakoid membrane extract showed that all proteins were present in the samples before the buffer treatment.
The treatment with extraction buffer did not have any significant effects on the prohibitins which could all still be found in the pellet fraction. The D1 protein was also only present in this fraction. The PsbO protein on the other hand could already be extracted under these conditions and was partly found in the soluble fraction (an estimated 30 %).
Extraction buffer including 2M NaCl did neither extract the prohibitins nor the D1 protein, whereas almost all of the PsbO protein (an estimated 95 %) was found in the soluble fraction.
Addition of 6.8 M Urea to the extraction buffer created denaturing conditions that were harsh enough to extract some of the prohibitin complexes from the thylakoid membrane (an estimated 40 to 50 %) which were detected in the soluble fraction. Under these conditions all of the PsbO and still none of the D1 protein was extracted and found in the soluble fraction.
The addition of 0.5 M EDTA even seemed to stabilize the PsbO protein and none of the proteins were found in the soluble fractions.
When 20 mM Na_2CO_3 were added to the extraction buffer at an pH of 12 the prohibitins could not be extracted from the thylakoid membrane whereas most of the PsbO protein was found in the soluble fraction (an estimated 70 %). The D1 protein remained in the pellet fraction.

6.5 Conclusions and Discussion

We analysed the protein compostion of thylakoid extracts that were isolated from wild type and mutant *Synechocystis* sp. PCC 6803 cells by denaturing and native PAGE. Immuno-blotting analysis using specific antibodies against the prohibitin homologues after 1-D SDS

PAGE showed, that these proteins were indeed inactivated in the mutants (see Figure 6.1). The appearance of additional bands on a Coomassie stained SDS PAGE gel was observed in some cases. High molecular weight Phb1, Phb2, Phb3 and Phb4 complexes were identified by 1-D BN PAGE (see Figure 6.2) and FPLC (see Figure 6.3) followed by immunoblotting analysis. Differential protein extraction revealed that the prohibitin homologues Phb1, Phb2 and Phb3 are integral membrane proteins (see Figure 6.5). Interestingly, we observed signals in an immunoblotting analysis after 2-D PAGE of thylakoid membrane extracts from the cyanobacterium *Thermosynechococcus elongatus* that implied a possible interaction between the TE_Phb1 and FtsH homologue in this organism (see Figure 6.4).

6.5.1 A hint of a phenotype for prohibitin mutants?

So far no function could be assigned to any of the prohibitin homologues in *Synechocystis* sp. PCC 6803 from observing the mutant strains in which these proteins had been inactivated. However, when we analysed the thylakoid membrane extracts from wild type and mutant cells, we observed slight changes in the banding patterns between the wild type and the mutants thylakoid extracts in which the Phb3 protein (stomatin-like protein) had been inactivated. The intensity of a band with a molecular weight corresponding to about 35 kDa increased significantly on the Coomassie stained gel in the lane where the thylakoid extract from the *phb3* and triple mutant were analysed. This band was reproducible and will be subjected to analysis by mass spectrometry. The SDS PAGE analysis will be repeated and we will try to identify more bands that differ in the wild type and the mutant thylakoid extracts.

6.5.2 Prohibitin homologue complexes in Synechocystis sp. PCC 6803

The main focus of future experiments will be dedicated to the characterization of the identified prohibitin homologue complexes. Recently, a study on the prohibitins in *S. cerevisae* revealed insights into the nature and biogenesis of the PHB complex in yeast (see 1.1.5.2). We also identified large, multimeric protein complexes for Phb1, Phb2, Phb3 and Phb4 by 1-D BN PAGE (see Figure 6.2) and FPLC (see Figure 6.3). After these experiments

there is evidence that the prohibitin homologues form homomultimeric complexes, because all the identified complexes can form even when the other four prohibitin homologues have been inactivated. However, it is still possible that these complexes contain a subunit other than a prohibitin homologue or that an overlap of function is observed where one prohibitin replaces the inactivated homologue in the complex. In any case the question will be addressed again, once the complexes have been purified. Other experiments that will be performed after the prohibitin homologue complexes have been purified to reveal further details the complexes will include single particle analysis. Just recently could a 3D model for the PHB complex in *S. cerevisiae* be proposed (Tatsuta et al., 2005). The PHB complex in yeast is a large, heteromeric complex of about 1.2 MDa and forms a ring-like structure. Tatsuta et al (2005) also reveal several distinct steps in the biogenesis of this complex. They observed a stepwise assembly and idetified 120 kDa intermediates. A stepwise assembly is also likely to be be found for the prohibitin homologue complexes in *Synechocystis* sp. PCC 6803. Analysing the immonoblotting analsys for the Phb1 protein, we already observed a smear like signal that stretched over the entire lane. This signal is a signal specific to Phb1, as it could not be found in the *phb1* mutant lane. Thus, this smear either represents distinct assembly stages of the Phb1 complex or is due to degradation that appeared during sample preparation. Both the Phb2 and Phb3 complexes did not exhibit this smear and appeared as single, intense signals at the top of the gel. The Phb1, Phb2 and Phb3 complexes have been proven to be membrane associated by differential protein extraction. Only under harsh buffer conditions (for example 6.8 M Urea) could these proteins be extracted from the membrane. Gentler treatments washed the control protein PsbO off the membranes, but did not extract the prohibitin homologues.

In a thylakoid membrane extract from *Thermosynechococcus elongatus* we observed a possible interaction between the TE_Phb1 and FtsH homologue. The antibodies that were used in this immunoblotting analysis were from *Synechocystis* sp. PCC 6803 (αPhb1) and *E. coli* (αFtsH) respectively. Sequence alignments of the antigens and TE_Phb1 and FtsH from *Thermosynechococcus elongatus* displayed sufficient sequence similarity to be confident about assigning the obtained signals to TE_Phb1 and FtsH. Immunoprecipitation experiments will have to be performed to gather more evidence for the interaction of these two proteins and hopefully FtsH will be found to co-purify with any of the prohibitin homologue complexes.

Chapter VII: Future work

7. Future Work

After the progress that has been achieved so far, the following experiments need to be performed within the next 22 months in order to reach the objectives that have been outlined in section 1.2. The plan has been visualized in a GANNT-chart.

To purify the polyclonal antibody sera is of the highest priority, because they will be used in most of the biochemical approaches and need to be of an adequate quality. Only then can further experiments to characterize the prohibitin homologue complexes be conducted.

The purification of the prohibitin homologue complexes will be attempted by two approaches. One way will be to couple the purified antisera to a resin or by immunoprecipitation and the second approach will be to His-tag the prohibitin homologues. To obtain purified prohibitin homologue complexes is of particular importance, because once purified the material can be used for single particle analysis and for the identification of interaction partners. Dr. Jon Nield is an expert in the field of single particle analysis and structure modeling and has signalled his interest to cooperate on this issue. Secondly, 1-D SDS PAGE and mass spectrometry could identify copurified interaction partners of the prohibitin homologues.

Two phase separation localization studies will be performed to identify the membrane in which prohibitins are present and differential protein extraction experiments will be repeated.

Further mutants will be generated, including the His-tagged and prohibitin quadruple mutants as well as mutants in which *slr0228* (FtsH) will be inactivated. These mutants and the ones that have already been generated will be analysed for an obvious phenotype under various stress conditions. Photoinhibition and pulse-chase experiments will be performed on the quadruple mutants to test for an impaired PSII repair cycle and an overlap in function among the prohibitin homologues. Mutants will also be analysed by 2-D SDS PAGE and mass spectrometry to study the biogenesis and dynamics of the prohibitin homologue complexes.

A longer vacation is planned for spring next year and several short breaks will be taken every once in a while. The thesis will be written between October / December 2006 so that the final PhD viva can be held early in the year 2007.

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