Psb31, a novel photosystem II associated protein in *Synechocystis* sp. PCC 6803

Thesis

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Abbreviations

BSA	Albumine bovine serum
cDNA	Complementary deoxyribose nucleic acid
Chl	Chlorophyll
DCBQ -	2,6 dichloro-p-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DUF477	Domain of unknown function number 477
DM	Dodecyl maltoside
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTP	Desoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetate
HCl	Hydrochloric acid
HL	High light
HEPES	N-2-Hydroxyethylpiperazin-N`-2-ethanolsulfonic acid
IPTG	Isopropylthio-β-D-galactoside
LED	Light-emitting diode
mRNA	Messenger ribonucleic acid
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PSI	Photosystem I
PSII	Photosystem II
RNA	Ribonucleic acid
RC	Reaction center
SD	Standard derivation
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
Tris	Tris-(hydroxymethyl)-aminomethane
WT	Wild type

Measuring Units

Å	Angström
bp	Base pairs
°C	Degree celsius
h	Hour
K	Degree kelvin
kb	Kilo base pairs
kDa	Kilodalton
l	Litre
M	Molar concentration
min	Minute
nm	Nanometer
$\mu E/m^2s$	Lightintensity in microeinstein per square meter per second
rpm	Rotations per minute
sec	Second
µmol	Micromole
w/v	Weight per volume

I Introduction

1.1 Cyanobacteria

Cyanobacteria are a large and diverse group of photosynthetic prokaryotes also re ferred to as blue-green algae. Cyanobacteria are resilient and tough and can be found in a wide variety of environments including fresh water, marine, terrestrial environments and extremes like hot springs and rock surfaces in Antarctica and deserts. As photoautotrophic organisms, cyanobacteria utilize light as energy to reduce inorganic CO_2 into energy rich carbohydrates. Fossilized cyanobacteria have been dated as far back as 2.8 billion years and they are thought to be responsible for the transformation of the earths atmosphere from reducing to oxidizing by producing molecular oxygen. Most cyanobacteria contain thylakoids, an extensive internal membrane system containing the components of photosynthesis and respiration. The mechanism of cyanobacterial photosynthesis is remarkably similar to the one in higher plants. In fact, the semiautonomous eukaryotic chloroplast is likely the result of an endosymbiosis of a cyanobacterium by a non-photosynthetic protoeukaryotic cell. Higher plants chloroplasts still contain coding DNA that is organized and regulated in a bacterial manner. Over time a significant number of chloroplast genes were transferred to the host nucleus so the chloroplast no longer contains enough information to be completely free of the nucleus. Many chloroplast proteins are therefore synthesized in the plant cell cytoplasm and transported into the plastid. This requires a highly sophisticated interaction between the plant cell organelles. In contrast, cyanobacteria have a relative simple genetic structure compared to higher plants and are therefore ideal to study oxygenic photosynthesis.

Cyanobacteria have attracted attention these days with their potential to serve as food stock or alternative food fuel sources. The best known example of commercially used cyanobacteria is *Arthrospira platensis,* widely known as Spirulina. Its potential as food stock was described as early as 1983 by Orio Ciferri and it is widely available as food supplement in health stores. Some species of cyanobacteria have the potential to produce hydrogen and others have high lipid content. It has been estimated that cyanobacteria could generate 100 times more biodiesel per biomass compared to any plant system (Rittmann, 2008). There is clearly a need for more detailed studies of cyanobacterial physiology, energy transduction pathways and metabolisms to demonstrate their utility for bioenergy production.

1.2 *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 is an important model organism for photosynthesis research (**figure 1**). Its photosynthetic structures and reactions are similar to the processes in higher plants. There are glucose tolerant strains allowing heterotrophic growth and thus the study of photosynthetic knock-out mutants. Genetic manipulation of *Synechocystis* sp. PCC 6803 is possible due to its ability to uptake and incorporate DNA via highly efficient double homologous recombination (Williams, 1988). Coupled with its doubling time of 6-10 hours under optimal photoautothrophic conditions, homozygous mutants can be generated very fast in *Synechocystis* 6803.

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 was the third prokaryote and first photosynthetic organism whose genome was completely sequenced (Kaneko et al., 1996). Today there have been more than 40 cyanobacterial genomes sequenced. Their size ranges from the most primitive *Prochlorococcus* strains with less than 2 Mb to the nitrogen fixing *Nostoc punctiforme* PCC 73102 with a genome of 9 Mb. *Synechocystis* 6803 has a genome of 3.9 Mb and approximately 3600 genes.

Figure 1 - *Synechocystis* sp. PCC 6803: *Synechocystis* can be grown in liquid BG11 media or on Agar containing plates.

1.3 Photosynthesis

Photosynthesis is a series of enzyme catalyzed reactions in which light energy is converted into chemical energy by living organisms. It is performed by many different organisms, namely plants, algae and certain bacteria. The best known form of photosynthesis is oxygenic photosynthesis where water is oxidized to molecular oxygen. It is considered the most important biochemical pathway as most organisms depend on the oxygen and reduced carbon produced. Photosynthesis occurs in two phases, the light dependent and the light independent dark reactions or Calvin-Benson Cycle. The first phase of photosynthesis utilizes light energy to oxidize water and frees molecular oxygen, protons and electrons.

The electrons are transferred through the photosynthetic electron transfer chain to donate a reducing equivalent to NADP and form the cellular reductant NADPH.

ATP and NADPH are used in the Calvin-Benson-Cycle to fix CO_2 by chemical reduction and to make precursors of carbohydrates.

A simplified equation of photosynthesis:

$$6\ CO_2 + 12\ H_2O + \text{photons} \rightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$$

carbon dioxide + water + light energy → glucose + oxygen + water

While cyanobacteria perform oxygenic photosynthesis, some bacteria perform anoxygenic photosynthesis and use light energy to extract electrons from molecules other than water such as H_2S and therefore do not generate molecular oxygen. Anoxygenic phototrophs are represented in four bacterial phyla - purple bacteria, green sulfur bacteria, green gliding bacteria, and gram positive bacteria. Contrary to cyanobacteria these organisms have only one reaction center, either a type I or a type II and they are thought to have evolved before oxygenic photosynthetic organisms (Blankenship, 2002). For the purpose of this thesis, I will only refer to oxygenic photosynthesis performed by cyanobacteria.

The light reactions require a series of specialized multi-subunit pigment-protein complexes which form the photosynthetic electron transport chain. The photosystem II (PSII) complex is responsible for water oxidation during oxygenic photosynthesis generating electrons used to reduce the mobile electron carrier, plastoquinone.

Protons liberated into the thylakoid lumen during water oxidation form an electrochemical gradient over the thylakoid membrane which utilized by the ATP synthase. The cytochrome b_6f complex transfers electrons from the membrane soluble reduced plastoquinone to the soluble copper protein plastocyanin. Additional protons are translocated into the lumen during this process.

The photosystem I (PSI) uses light energy to transfer the electrons from thylakoid lumen located plastocyanin to the stromal (cytoplasmic in cyanobacteria) ferredoxin. With the help of ferredoxin $NADP^+$ reductase the electron is transferred to $NADP^+$ to generate the reductant NADPH.

1.4 Photosystem II

Photosystem II is a large multisubunit structure in thylakoid membranes that catalyzes light-driven charge separation accompanied by water splitting during oxidative photosynthesis (**figure 2**). The cyanobacterial PSII complex acts as a dimer where each monomer contains at least 20 protein subunits and 77 cofactors including chlorophylls, pheophytins, carotenoids, lipids, plastquinones, manganese, calcium, chloride and both non-heme and heme iron (Loll et al., 2005).

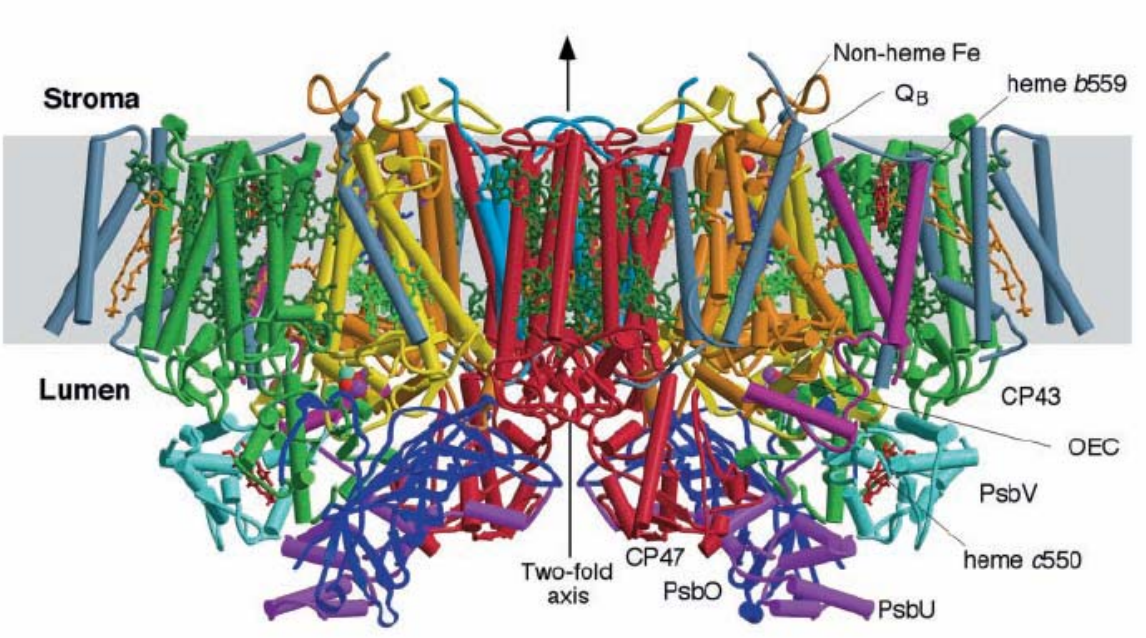


Figure 2 Architecture of the PSII dimer: Isolated from *Thermosynechococcus elongates* at a resolution of 3.5 Å adapted from (Ferreira et al., 2004).

Over the last couple of years a series of x-ray crystallography has revealed intricate details of the PSII architecture. The current structural model provides a 3.0 Å resolution of the *Thermosynechococcus elongatus* and is reviewed by Frank Müh (2008) and Jim Barber (2008).

The D1 and D2 proteins form a heterodimer at the PSII core which in turn binds all the necessary cofactors needed to transfer electrons from water to plastoquinone.

The CP43 and CP47 proteins bind the majority of the 35 chlorophyll molecules, 13 and 16 respectively and they form the proximal antenna around the core. All four proteins contain multiple transmembrane helices. The crystal structure also contains a number of single membrane-spanning proteins including cytochrome b_{559} bound by PsbE and PsbF, PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbT and PsbZ as well as three extrinsic proteins PsbO, PsbU and PsbV.

The picture illustrated by the crystal structure is not complete. For example, cyanobacterial PSII containing more than 30 proteins including a number novel polypeptides has been isolated using affinity tagged CP47 (Kashino et al., 2002). Several of the additional proteins have subsequently shown to be part of PSII, in particular the lumenal PsbP and PsbQ (Thornton et al., 2004), Psb27 (Nowaczyk et al., 2006; Roose and Pakrasi, 2008) as well as Psb29 (Keren et al., 2005b). One more protein identified as part of this highly active oxygen evolving PSII preparation is Sll1390, or Psb31.

1.5 PSII reaction

PSII facilitates the light driven electron transfer from water to plastoquinone. The oxidation of water frees molecular oxygen as a byproduct. The absorption of light by the antenna leads to the excitation of the P680 chlorophyll, which initiates a charge separation.

The electron is channeled via the cofactors pheophytin and plastoquinone Q_A to a second plastoquinone Q_B. Accumulation of two electrons on the Q_B molecule releases the molecule as plastoquinol from PSII and transfers the electrons to the cytochrome b_6f complex. P680 is replenished via a redox active tyrosine residue on D1 by electrons originating from the water oxidation complex thus resetting the PSII center.

The water oxidating center is comprised around a $Mn_4Ca_1Cl_x$ complex which is held in position and shielded form the environment by the lumenal extrinsic proteins (PsbO, PsbP, PsbQ, PsbU, PsbV) and ligands of CP43 and D1. This provides a proper redox environment. The Manganese atoms of the complex cycle through several oxidation states (S-states) from Mn^{2+} to Mn^{4+} or possibly Mn^{5+}. The Manganese cluster in the highly oxidized state is able to pull electrons from water leaving molecular oxygen behind. The high energy involved in the PSII function damages the complex requiring a continuous repair cycle.

1.6 Accessory PSII proteins

Photosynthesis is a complex reaction that requires constant monitoring and adapting to changing conditions. The membrane complexes, especially PSII are dynamic systems in a constant cycle of assembly and degradation. The PSII reaction generates reactive oxygen species like singlet oxygen as byproducts. These molecules damage the reaction center as well as the surrounding environment. Singlet oxygen generated by PSII is efficiently quenched by β-carotene (Telfer et al., 1994b) and PSII crystal structure shows close to a dozen β-carotene molecules as part of a PSII dimer. Due to the high energy of the photosynthetic reactions, D1 in the core of PSII is continuously photo-damaged and needs to be replaced. This PSII repair process entails multiple steps such as partial disassembly to remove the damaged D1 protein followed by reassembly of the manganese cluster and binding of the extrinsic proteins PsbO, PsbU, PsbV, and PsbQ in

cyanobacteria. The PSII lifecycle in *Synechocystis* 6803 is thought to be involving both, thylakoid membrane and plasma membrane with the lumenal subunits binding when the complex is transferred to the plasma membrane (Keren et al., 2005a). However, it is unknown how the core center is transferred between the membranes.

A number of intermediate assembling steps have been identified and an increasing number of proteins have been shown to be acting as auxiliary factors and regulators.

These include CtpA (Anbudurai et al., 1994), PratA (Klinkert et al., 2004), Ycf48 (Komenda et al., 2008), Psb27 (Roose and Pakrasi, 2008), Psb29 (Keren et al., 2005b), Lpa1 (Peng et al., 2006), Lpa2 (Ma et al., 2007) and Slr2013 (Kufryk and Vermaas, 2003). Most of the factors are required transiently and are not part of the active PSII complex. CtpA for example is directly involved in maturation of preD1 protein prior the assembling of the Manganese cluster (Roose and Pakrasi, 2004) and PratA is a periplasmic protein required for preD1 processing, further supporting the theory that the initial steps of PSII assembly is located at the plasma membrane (Klinkert et al., 2004).

Psb27 has been shown to interact with PSII pre-complexes that are incapable of evolving oxygen (Roose and Pakrasi, 2004; Nowaczyk et al., 2006) and facilitates assembly of the Manganese cluster (Roose and Pakrasi, 2008).

Lpa2 is assisting the CP43 incorporation into PSII (Ma et al., 2007) and Ycf48 is involved in assembly of PSII and selective replacement of D1 in the repair cycle.

In a photosynthetic cell there are always a variety of different PSII complexes present in stages of assembly, degradation and repair. The heterogeneous population of PSII molecules makes it difficult to ascertain the true function of each individual component of the isolated complexes. Therefore, careful analysis of individual and multiple mutants in combination with biochemical assays will facilitate a more detailed understanding of PSII assembly and repair pathways.

1.7 Sll1390 – Psb31

Sll1390, named Psb31 throughout this thesis, was first identified in a highly active PSII preparation of *Synechocystis* sp. PCC 6803 (Kashino et al., 2002). This study identified a number of novel proteins by mass spectrometry and C-terminal sequencing. Several of the new proteins could be subsequently assigned to function in PSII, namely Psb29 (Keren et al., 2005b) and PsbQ, a lumenal PSII protein previously thought to be present in eukaryotic PSII complexes only (Thornton et al., 2004).

At the same time a homologue to Sll1390 has been identified in proteomic studies of the *Arabidopsis thaliana* thylakoid membrane (Peltier et al., 2002) and thylakoid lumen (Schubert et al., 2002).

The *Arabidopsis* protein was also found in thylakoid-associated polysome nascent chain complexes. It has been studied recently by Sirpiö et al. (2007) and was named TLP18.3 due to its localization in the thylakoid lumen and its apparent size on a SDS-PAGE gel. It was found in both stroma and grana thylakoids but not stably associated with any membrane protein complexes. The authors state that plants missing the TLP18.3 protein showed a higher susceptibility of PSII to high light stress and an increased amount of PSII monomers compared to wild type (WT) plants. Two steps in the repair process of photosystem II were impaired in the mutant; the turnover of damaged D1 and the dimerization of PSII. The lack of TLP18.3 did not lead to a severe collapse of PSII complexes suggesting a redundancy in these repair steps. A visible growth defect was only observed under fluctuating light conditions, the plants do not seem to be able to adapt to hourly changing light intensities.

II Material and Methods

2.1 Materials

2.1.1 Chemicals

Basic chemicals were obtained from:

- Sigma-Aldrich, Saint Louis, MO
- Fisher, Pittsburgh PA
- USB, Cleveland, OH
- Malinckrodt, Phillipsburg, NJ

2.1.2 Enzymes and kits

• Dnase RQ1	Promega, Madison, WI
• Fideltaq proof reading polymerase	USB, Cleveland, OH
• *KlenTaq* DNA polymerase	DNA Polymerase Tech, St.Louis, MO
• Rapid alkaline phosphatase	Roche, Nutley, NJ
• Rapid T4 ligase	Roche, Nutley, NJ
• Restriction endonucleases	Fermentas, Glen Burnie, MD
	New England Biolabs, Ipswich, MA
	Promega, Madison, WI
• Ribonuclease	Sigma, St.Louis, MO
• SuperscriptII reverse transciptase	Invitrogen, Carlsberg, CA
• T4 DNA ligase	Fermentas, Glen Burnie, MD
• T4 polymerase	Fermentas, Glen Burnie, MD

- BugBuster protein extraction reagent — Novagen, San Diego, CA
- Gen elute plasmid miniprep kit — Sigma-Aldrich, St.Louis, MO
- pET41 protein expression vector system — Novagen, San Diego, CA
- QIAfilter plasmid midi kit — Qiagen, Valencia, CA
- Wizard SV gel and PCR clean-up system — Promega, Madison, WI

2.1.3 Markers for electrophoresis

- 1kB DNA ladder — Invitrogen, Carlsberg, CA
- prestained broad range protein standard — Bio-Rad, Hercules, CA

2.1.4 General buffers and media

Resuspension buffer (RB):	50 mM MES - NaOH pH 6.0, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 25% glycerol
TBS buffer:	150 mM $NaCl_2$, 10 mM Tris-HCl pH 7.5
50x TAE buffer:	2M Tris base, 1M glacial acetic acid, 50mM EDTA (pH8.0)
BG11 medium	as described by (Rippka, 1988)
LB medium:	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl (pH 7.0); for solid medium 1 % (w/v) agar was added

2.1.5 Oligonucleotides

- Random Hexamer primer — Invitrogen, Carlsberg, CA
- dNTP set — Invitrogen, Carlsberg, CA
- M13 Forward primer — Invitrogen, Carlsberg, CA
- M13 Reverse primer — Invitrogen, Carlsberg, CA

1	Psb31F up	5'-ACG AAG CTT AAT AGC CGT TGT TCC GCT CA-3'
2	Psb31R up	5'- TCA TGG ATC CTT GAT TGC TCT GGC TGG CTT -3'
3	Psb31F down	5'- TCA TGG ATC CAG CGG AAG AAA CCG ACG ATA -3'
4	psb31R down	5'- GCA GGA ATT CTG CAC GGC AGT ACC AAA GTT -3'
5	1390oepF	5`-GCT CGA GCA TAT GTC CCC TTA TGA CCT GCC AAT TTT GT-3`
6	1390oepR	5`ATG ATT AGT ATT CTC GAG GGT GGC ACT GGT ATC GTC GG -3`
7	1390 HX	5`- GAT AGT CTC GAG CCG TCC CGG TAA GCC CAC ATA -3`
8	1390-HE3	5'- GCC AGA GAA TTC TAT GAC CTG CCA ATT TTG TC-3'
9	1390-HP2	5`- GCG AGA CTG CAG A TAA CAA TTA GTG GTG GTG- 3`
10	1390-dHP2	5`- GCG CTG CAG ACA TAT TCC GCT GGT AAT AAT AA –3`
11	1390-dHH2	5`- GTG AAG CTT GGC TGA GGC AAT CTA GAC AA –3`
12	RT-PCR 1390 F	5'- TGG TGC TAG ATA CCC TCA CCA AGC A -3'
13	RT-PCR 1390 R	5'- GGG TTT CCC GCA GTA AAC TAT CCA -3'
14	RT PCR 1866 F	5' ATA TTG CCG AAA CTT TCC CTG CCG -3'
15	RT PCR 1866 R	5'- CCC TTG GCG CAG AAT TTG GAA GAT -3'
16	RT PCR RNAseP F	5'- CAA ACT TGC TGG GTA AC -3'
17	RT PCR RNAseP R	5'- ACC AAA TTC CTC AAG CG –3'

Table 1 - Oligonucleotides: Primers used to clone the psb31 gene (1-4), generate the overexpression plasmid (5-6), the psb31-HIS construct (7- 11) and RT-PCR experiments (12-17). Primer were designed with the Integrated DNA Technology (IDT) Primerquest software and synthesized at IDT (Coralville, IA)

2.2 Methods

2.2.1 Bioinformatic analysis

The protein and nucleotide sequences for the *Synechocystis* 6803 Psb31 was obtained from Cyanobase (http://bacteria.kazusa.or.jp/cyanobase) and used to blast against the integrated microbial database at JGI (http://img.jgi.doe.gov) and the NCBI BLASTP database (http://blast.ncbi.nlm.nih.gov).

The phylogenetic tree was constructed under the parsimony criterion using the software PAUP* V4b10 (Swofford, 2002) and was midpoint rooted. SignalP and TMHMM V2.0 were used to predict signal peptide and transmembrane helices respectively (http://www.cbs.dtu.dk/services/).

Microarray data for *Cyanothece* sp. ATCC 51142 and *Synechocystis* sp. PCC 6803 were provided by Dr. Jana Stöckel (Stockel et al., 2008).

2.2.2 Strains

The Δ*psb31* deletion mutant was generated by PCR amplifying two fragments (nucleotides 1821694-1821255 and 1821003-1820558 of the *Synechocystis* 6803 genome at http://bacteria.kazusa.or.jp) of the *psb31* locus omitting a 252 bp part of the open reading frame. The fragments were cloned into pUC118 where they flanked a chloramphenicol resistance cassette. The construct was confirmed by sequencing at the PNACL-center at Washington University in Saint Louis and then transformed into *Synechocystis* 6803 and selected on BG11 media containing 10 µg/µl chloramphenicol.

Primers were designed by using Oligo Designer software (Integrated DNA Technology (IDT), Coralville, IA) and were synthesized by IDT (**table 1**).

Unique restriction sites were added to the primers allowing directional cloning of the PCR fragments.

The original Δ*sll1390*-Sp strain was constructed by Hiroshi Ohkawa. In this case the whole *psb31* gene was cloned into *E.coli* and a spectinomycin resistance cassette replaced base pairs 137 to 217 of the open reading frame. The resulting plasmid was used to transform wild type (WT) *Synechocystis* 6803. Mutants were selected on BG11 supplemented with 10 µg/µl spectinomycin and segregation confirmed by PCR.

The HT47GM strain containing a C-terminal HIS tag on CP47 and a gentamycin selection marker was obtained from Dr. Johnna Roose.

The Δ*psb31*-HT47GM double mutant was generated by transforming the Δ*psb31* plasmid into the HT47GM strain. Complete segregation was confirmed by PCR and RT-PCR analysis of the *psb31* locus.

The CP47 tagged double mutants Δ*psb27*-HT3 was generated in the lab by Dr. Johnna Roose and the, Δ*psbV*-HT3 and the Δ*psbQ*-HT3 strain have been described before (Wegener et al., 2008)

The Δ*psbQ* strains (Thornton et al., 2004) as well as the Δ*psbO* (Chandler et al., 2003) and the Δ*psbU* strains (Inoue-Kashino et al., 2005) have been described before and the Δ*psbV* strain was generated by Dr. Victor Bartsevich.

The double Mutants O1390, Q1390, U1390 and V1390 were generated by transforming the Δ*psb31* construct into a single mutant background. Complete segregation was confirmed by PCR analysis of the *psb31* locus.

2.2.3 Culture conditions

Synechocystis 6803 was grown in BG11 media (Rippka, 1988). Strains were maintained on BG11 plates at 45 μE/m^2s in presence of the required antibiotic with the concentrations of 10 μg/ml for chloramphenicol, spectinomycin and kanamycin or 5 μg/ml for gentamycin. Exceptions were the Manganese sensitive Δ*psbV* derivated strains which were grown on Manganese free BG11, supplemented with 1μM Manganese.

Liquid cultures were grown in 50 ml volume shaking in 250 ml Erlenmeyer flasks. For large scale biochemical assays the strains were grown 3 l batches in the presence of 5 mM glucose and the required antibiotic.

Growth assays were done in 12 or 6 well microtiter plates. Aliquots were taken frequently to monitor OD_{730} on a μQuant plate reader (Bio-Tek Instruments, Winooski, VT). For starvation conditions cells were re-suspended in EDTA-wash buffer (20 mM HEPES pH 7.8, 10 mM EDTA) incubated for 10 minutes, spun down and washed twice in BG11 lacking the particular component before starting the assay.

2.2.4 DNA isolation

Plasmid DNA was isolated from *E.Coli* with the Gen Elute Plasmid Miniprep Kit (Sigma, St.Louis, MO) or the QIAfilter Plasmid Midi Kit (Qiagen, Valencia, CA).

Genomic DNA from *Synechocystis* 6803 was isolated by spinning down 25 ml of a log phase culture and resuspend the pellet in 1 ml STET buffer (8% (w/v) sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-HCl (pH 8.0)). The suspension was frozen for 30 min at –80°C and then directly transferred to 70°C and incubated for 30 min. Subsequently, the tube was incubated over night at 37°C with lysozyme. The next morning 2% SDS was added and after further 30 min at 37°C DNA was extracted with a Phenol-Chloroform extraction.

DNA was precipitated with 0.7 V isopropanol and 300 mM sodium-acetate (pH 7.0). The DNA pellet was washed in 70% ethanol, resuspended in water and quantified with a NanoDrop spectrophotometer (Ocean Optics, Dunedin, FL).

2.2.5 PCR

Analyses using the polymerase chain reaction (PCR) were carried out as described by Sambrook and Russell (Sambrook, 2002) using an Eppendorf Thermocycler. (Eppendorf, Hamburg GER).

2.2.6 Reverse transcriptase - PCR

RT-PCRs with gene specific primers were performed to determine weather the modification of the *psb31* gene had an effect on the expression of the downstream gene *sll1866*. RNA was isolated with the RNAwiz kit (Ambion, Austin, TX), treated with DNAse RQ1 (Promega, Madison WI) to remove DNA contamination. Random hexamer primers (Invitrogen, Carlsbad, CA) were used to generate cDNA with Superscript II reverse transcriptase (Invitrogen).

2.2.7 Mixed culture experiments

The mixed culture experiments were performed essentially as described by Natasha Ivleva (2002). WT and mutant were grown in a 50 ml liquid culture of BG11 till they reached mid-exponential phase.

Mixed cultures containing an equal number of WT and mutant cells were started at OD_{730} of 0.05 on the plate reader (Bio-Tek Instruments, Winooski, VT) and grown under 5 (LL), 45 (GL) or 150 (HL) µmol photons/m^2s. A sample was taken to extract DNA every 72 h and the culture was diluted back to OD_{730} of 0.05. Each mixed culture was sub-cultured five times. PCR was used to analyse the locus of the psb31 gene in each isolated DNA sample. PCR products were separated on 1% agarose gels and visualised using the Kodak 1D Image Analysis software (Rochester, NY).

2.2.8 Gel electrophoresis

DNA was separated on 1% Agarose TAE buffered gels containing Ethidiumbromide.

Proteins were separated on SDS-PAGE (Laemmli, 1970) in presence of 6 M urea on 16% acrylamide minigels or 20%-25% gradient gels. Protein gels were stained with Coomassie or silver stained or blotted onto nitrocellulose membranes with a Semi-Dry blotter (Biorad, Hercules, CA).

2.2.9 Antibody generation

The polyclonal aPsb31 antibody was generated by cloning the psb31 gene without transit sequence and C-terminal transmembrane sequence into the pET41b expression system (Novagen, San Diego, CA) and purifying the protein via a HIS-tag. Antibodies against the purified truncated protein were raised in rabbits (Cocalico Biologicals, Reamstown, CA).

The polyclonal aPsbV antibody was raised in rabbits against the surface exposed PsbV peptide N-CGLADLAGAEPRRDN-C (Sigma Genosys, The Woodlands, TX).

To confirm the specificity of the antibodies, samples of WT and deletion mutants were run on SDS-PAGE, blotted onto nitrocellulose, probed with 1:1500 diluted antibodies and detected with a Fuji imager (IR-LAS-1000 PRO V3.1, Fuji, Japan).

2.2.10 Immunoprobing

Nitrocellulose membranes were blocked with 3% BSA in TBS buffer, probed with various antibodies of an appropriate dilution and chemiluminescence (Immobilon Western substrate, Millipore, Billerica, MA) was detected with a Fuji Imager (IR-LAS-1000 PRO V3.1 Fuji, Japan).

Membranes could be stripped by incubating for 30 min at 60°C with stripping buffer (100 mM β-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.1) and re-probed several times.

2.2.11 Purification of total membranes and PSII complexes

Cultures were harvested, briefly frozen at –80°C, thawed on ice and re-suspended in resuspension buffer (RB: 50 mM MES - NaOH pH 6.0, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 25% glycerol). ½ volume of glass beads were added and cells were broken 10x 1 minute on a vortexer. The supernatant was briefly spun to remove unbroken cells and debris. The total membranes were pelleted by centrifugation (Sorval SS34, 17.000 rpm 20 min) and resuspended in RB.

PSII was purified from CP47 HIS-tagged strains as described in 2002 by Yasuhiro Kashino et al. and resuspended in RB.

2.2.12 Two phase partitioning

The fractionation of total membranes into hydrophobic and hydrophilic fractions was essentially done as described by Terry Bricker (1984) with a concentration of 100 µg Chl/ml. Triton X-114 was purchased from Sigma (St.Louis, MO).

Plasma membranes and thylakoid membranes were separated with a two-phase dextran PEG polymer system as described by Nir Keren (2005a).

2.2.13 Separation of membrane complexes

Complexes were isolated based on a protocol from Vera Göhre (2006) by solubilizing membranes for 30 min at 4°C with 0.9 % ß-dodecyl maltoside and separating the soluble material onto a continuous sucrose gradient (0,1 – 2 M) in 20 mM Tricine pH 7,5 and 0,05% ß-dodecyl maltoside by centrifugation for 17 h at 160.000 xg. The whole gradient was aliquoted and equal protein amounts were run on a 16% SDS-PAGE gel, blotted onto nitrocellulose and immunoprobed against PSII and PSI associated proteins.

2.2.14 Determination of protein concentration

Protein concentrations were determined on a microtiter plate reader (Bio-Tek Instruments, Winooski, VT) with a BCA-assay kit (Pierce, Rockford, IL, USA).

2.2.15 Determination of pigment concentrations

Pigments were extracted from log phase cells. One ml culture was spun down, the supernatant all discarded but 10 µl in which the pellet was resuspended. Chlorophyll concentration was determined by Methanol extraction (Porra, 1989). Carotenoid concentration was determined likewise (Lichtenthaler, 1987).

Phycobiliprotein concentration was calculated after Bennett and Bogorad (1973). The methanol extracted pellet was re-suspended in water and the cells were broken with glass beads. The supernatant was collected and absorbance was measured on a DW2000 spectrophotometer (SLM-Aminco, Urbana, IL).

2.2.16 UV-visible absorption spectroscopy

Absorption spectra between 400 nm and 750 nm were recorded on a DW2000 spectrophotometer (SLM-Aminco, Urbana, IL) and normalized to the chlorophyll absorption peak at 678 nm.

2.2.17 77 K fluorescence spectroscopy

Cells at the same OD_{730} were frozen with liquid nitrogen and low temperature fluorescence emission spectra were taken on a FluoroMax II fluorometer (Jobin Ivon, France) using two different excitation wavelengths: 420 nm which excites chlorophyll and 600 nm which excites phycocyanin. Because the absolute amplitudes of low-temperature spectra are unreliable, spectra were routinely normalized to the PSII specific peak at 685 nm for the 420 nm excitation and the phycocyanin peak at 646 nm for the 600 nm excitation.

2.2.18 Atomic absorption spectroscopy

Manganese concentrations in isolated PSII samples were measured using an AA600 atomic absorption spectrometer (PerkinElmer, Waltham, MA). Samples were diluted to 3 µg/ml chlorophyll.

2.2.19 Oxygen evolution and light saturation curves

Log phase cultures of WT and the Δ*psb31* mutant were re-suspended in fresh BG11 to a chlorophyll concentration of 5-7 µg/ml and incubated in a 30°C water bath for one hour. Oxygen evolution was measured on a Clark-type electrode (Yellow Springs Instruments) in triplicate for each light intensity. Light intensities were adjusted between 400 and 8250 µE/m^2s using neutral density filters.

Electron acceptors (1 mM Ferricyanide and 0.5 mM DCBQ) were added to measure PSII specific activity in whole cell samples. The activity of isolated PSII was determined by diluting CP47 tagged samples to a chlorophyll concentration of 3 µg/ml in pre-warmed RB buffer and measuring in triplicate. Whole chain oxygen evolution was measured from whole cell samples in the absence of before mentioned electron acceptors.

2.2.20 Fluorescence kinetics

Q_A reoxidation was measured at room temperature using a Double-Modulation Fluorometer (FL200, Photon System Incorporated, Czech Republic) with the FluorWin software (ver3.6.3.3). A three ml aliquot of log phase cultures was dark incubated for one minute prior measurement. 10 µM DCMU was added afterwards and the measurement was repeated.

For the fluorescence induction measurement, log phase cultures of the same OD_{730} were dark incubated for 3 minutes and then illuminated with actinic light while the chlorophyll fluorescence was monitored on a FL200 (Photon System Incorporated, Czech Republic) with FluorWin software (ver3.6.3.3). 10 µM DCMU was added and a second induction curve was measured after one further minute of dark incubation.

2.2.21 Photoinhibition and recovery

Two day old cultures of WT and Δ*psb31* were re-suspended in fresh BG11 to an chlorophyll concentration of 3 µg/ml. Cultures were incubated in a 30°C water bath for one hour at 5 µmol photons/m^2*s light intensity with a LED light source (Photon System Incorporated, Czech Republic) and bubbled with air.

To induce photoinhibition, samples were taken (t_0), 20 µg/ml of the protein synthesis inhibitor lincomycin (Sigma; St.Louis, MO) was added to prevent *de novo* synthesis of proteins and the light intensity was subsequently increased to 200 µE/m^2s (equivalent to ~1000 photons/m^2s white incandescent light). Variable fluorescence yield (F_v/F_m) was measured every 15 minutes with a Double-Modulation Fluorometer (FL200; Photon System Incorporated, Czech Republic). When the F_m over F_0 ratio dropped below 50%, the cultures were spun down 3 min at 6,000 rpm and washed twice with BG11 to remove the lincomycin. Subsequently it was re-suspended in a pre-wash volume of warmed BG11 and incubated at 10 µmol photons/m^2s. The recovery of the PSII was monitored by fluorescence measurements.

2.2.22 Blue light experiments

For the incubation under blue light the Δ*psb31* mutant and WT were grown in a 50 ml liquid culture till mid-exponential phase. Cells were spun down and re-suspended to 2x 50 ml with an OD_{730} of 0.035 on the DW2000 spectrophotometer (SLM-Aminco, Urbana, IL). Cultures were incubated bubbling in a 30°C water bath and exposed to 5 µ µmol photons/m^2s light from a LED light source (Photon System Incorporated, Czech Republic). After 90 min at 5 µE/m^2s red and blue light the t_0 sample was taken. The red light (620.5 – 645 nm) was turned off and blue light (and blue (440 – 460 nm) intensity was increased to 30 µE/m^2s. After 24 h the blue light sample was taken.

77 K spectra with an excitation of 420 nm were taken for both time points on a low temperature spectrophotometer (FluoroMax II, Jobin Yvon, France). Spectra were normalized to the PSII specific chlorophyll peak at 694 nm.

2.2.23 Pull down experiments with truncated Psb31 expressed in *E.coli*

The central part of the *psb31* gene (Nucleotides 1821491-1820973 in the *Synechocystis* 6803 genome on Cyanobase http://bacteria.kazusa.or.jp), coding for the whole Psb31 protein except the N-terminal signal peptide and the C-terminal transmembrane helix, was cloned as a C-terminal HIS-tag translational fusion into the pET41 expression system (Invitrogen). The Truncated Psb31 was over-expressed in the *E.coli* BL21 strain (Invitrogen) after induction with IPTG. Soluble proteins were isolated with BugBuster reagent (Novagen).

The over-expressed truncated Psb31 protein was purified following the QIAexpressionist protocol (http://www1.qiagen.com/HB/QIAexpressionist_EN) by Nickel affinity chromatography using Ni-Agarose (Qiagen) and concentrated by centrifugation through a Millipore Amicon Ultra 3,000MWCO filter. This was followed by a second purification step using a Superdex 200 10/300 GL size exclusion column and a further concentration with Millipore Amicon Ultra 10,000MWCO filter. As a control, a pET41-NrtA expression vector was obtained from Nicole Koropatkin (Tom Smith Lab, Donald Danforth Plant Science Center, Saint Louis, MO). NrtA-HIS, a membrane bound nitrate transporter, was purified using the same protocol.

Synechocystis 6803 protein extracts were isolated as described in the PSII preparation protocol (Kashino et al., 2002). A three liter culture of wild-type *Synechocystis* 6803 was re-suspended in 40 ml RB-buffer and broken with glass beads.

Larger debris was removed by a brief spin and membranes were collected by centrifugation for 30 min at 20.000*x*g on a Sorval Biofuge, leaving the soluble proteins in the supernatant.

Membranes were re-suspended in RB-buffer and solubilized by adding 0.8% Dodecylmaltoside and stirring in the dark on ice for 30 minutes. Non-solublized membranes were removed by centrifugation.

The pull downs were performed by first binding the target protein onto a Ni-Agarose column followed by addition of *Synechocystis* 6803 proteins diluted 1:10 in binding buffer (40 mM Immidazole, 500 mM $NaCl_2$, 20 mM Sodiumphosphate pH 7.4). Bound proteins were eluted with elution buffer (500 mM Immidazole, 500 mM $NaCl_2$ and 20 mM Sodiumphosphate pH 7.4) after ample washing.

2.2.24 Purification of the Psb31 HIS-tagged protein from *Synechocystis*

The C-terminal of the *psb31* gene was modified by adding a histidine tag before a stop codon. Nucleotides 1821491-1820892 of the *Synechocystis* 6803 genome (http://bacteria.kazusa.or.jp) were amplified via PCR and cloning into the pET41b vector in front of the HIS-tag. From there the fragment was amplified including the HIS tag and cloned into pUC118. The *E.coli* construct comprising of the modified *psb31* sequence, a chloramphenicol resistance marker directly behind the stopcodon and a 600 bp long downstream sequence (nucleotides 1820888-1820138) was transformed into WT *Synechocystis* 6803 via double homologues recombination. Clones were selected for chloramphenicol resistance. Segregation was confirmed by PCR of the *psb31* locus and the expression of the HIS-tagged Psb31 protein was confirmed by Western analysis of membrane extracts.

The purification of Psb31-HIS followed the protocol for the isolation of HIS-tagged PSII Yahuhiro Kashino (2002) using a ÄKTA-FPLC System (GE-Healthcare, Chalfont St. Giles, UK) with following modifications:

The solubilization with 0.8% dodecyl maltoside (DM) was done twice to increase the yield. Solubilized membranes were loaded onto a Qiagen Ni-Agarose column equilibrated with wash buffer (RB buffer +0.04% DM). Elution of the tagged protein was achieved by running a continuous Histidine gradient (0-50 mM). Protein absorption was monitored at 280 nm. Peak fractions were pooled and concentrated by centrifugation in presence of polyethylene glycol.

III Results

3.1. Bioinformatic analysis

The Psb31 protein is conserved in the majority of photosynthetic organisms, with homologues found in cyanobacteria, eukaryotic algae (*Chlamydomonas*), moss (*Physcomitrella*) and higher plants (*Arabidopsis*). This gene is not present in non photosynthetic organisms indicating that its function is linked to photosynthetic mechanisms. A homologue could be identified in 34 out of 47 sequenced cyanobacteria.

The closest cyanobacterial homologue to the *Synechocystis* sp. PCC 6803 protein is found in *Cyanothece* sp. ATCC 51142, with 81% similarity and 62% identity (**figure 3**). In contrast, there is a higher divergence compared to the eukaryotic proteins: for example, the *Arabidopsis* homologue TLP18.3 has a 49% similarity and 29% identity. A similar level of conservation is seen in *Prochlorococcus* sp. MIT 9313. As reviewed by Frédéric Partensky in 1999, *Prochlorococcus* strains are the smallest cyanobacteria known. They have a strongly reduced genome and no phycobilisomes. Most of them are lacking the *psb31* gene as does *Gloeobacter violaceous*, the most early branching cyanobacteria (Nelissen et al., 1995). *Gloeobacter violaceous* does not even have thylakoid membranes (Rippka et. al., 1974). These two groups of cyanobacteria make up majority of organisms missing a *psb31* gene and they also lack a number of other extrinsic PSII subunits (Thornton et al., 2005), so the absence of a *psb31* homologue is not surprising as their photosynthetic apparatus is different compared with the rest. A third group of cyanobacteria missing the *psb31* gene are the thermophilic *Synechococcus* strains, again a group that branches early from the other cyanobacteria.

A phylogenetic tree (**figure 4**) with representatives of the classes of organisms that include a *psb31* homologue gene shows a clustering similar to 16S RNA trees, a broadly used tool to characterize phylogenetic relationships (Woese et al., 1990).

The unicellular cyanobacteria most closely related to *Synechocystis* sp. PCC 6803 cluster together, as do the filamentous strains *Anabaena variabilis* and *Nostoc punctiforme*. *Prochlorococcus* sp. MIT 9313 and the *Synechococcus elongatus* strains are more distant from the other cyanobacteria as are the eukaryotic organisms. This shows that the *psb31* gene was conserved over evolution suggesting its importance.

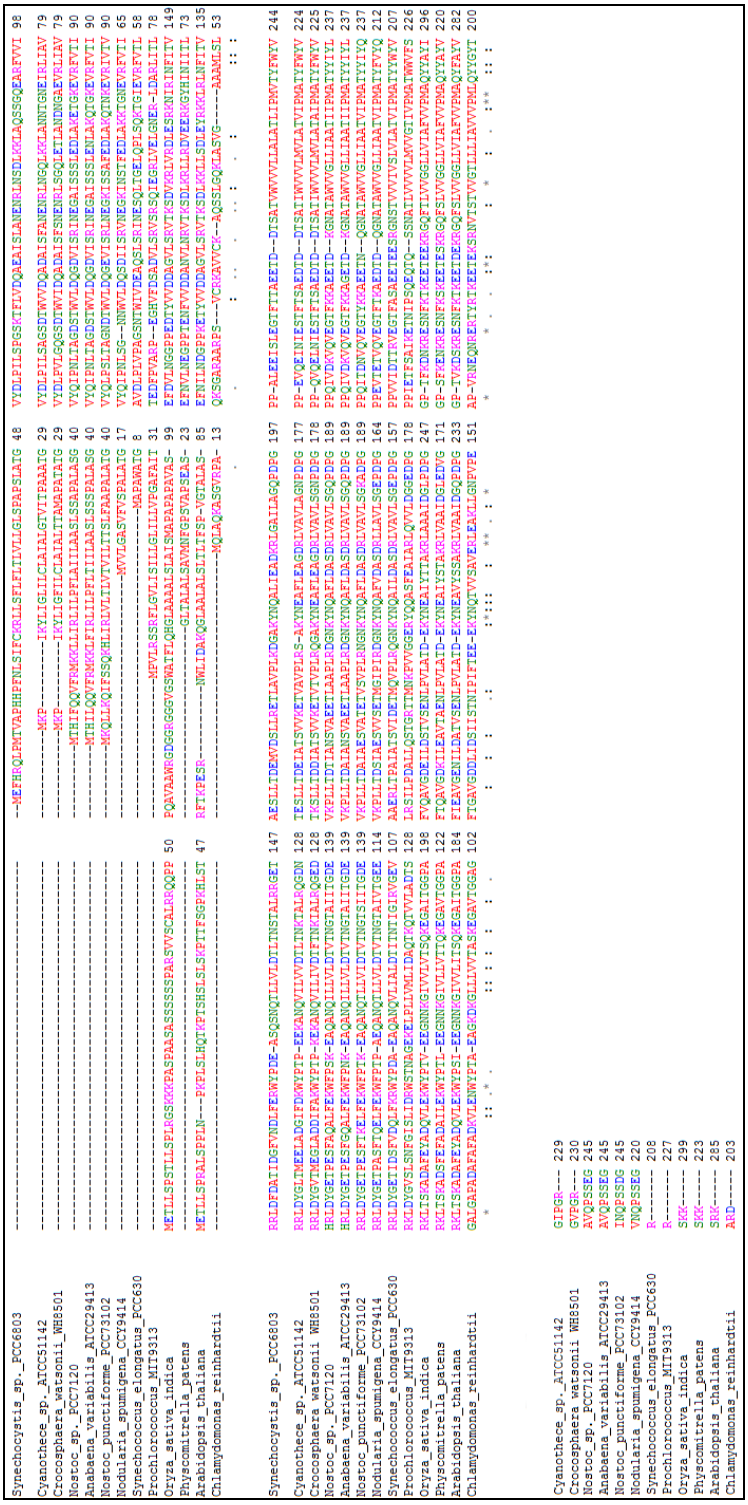


Figure 3 - Alignment of the *Synechocystis* Psb31 protein to its homologues in various photosynthetic organisms: Color coding represents the various classes of amino acids. Note the longer transit peptide in the two plants represented, *Arabidopsis* and *Oryza sativa*, needed for import into the chloroplast thylakoid membrane.

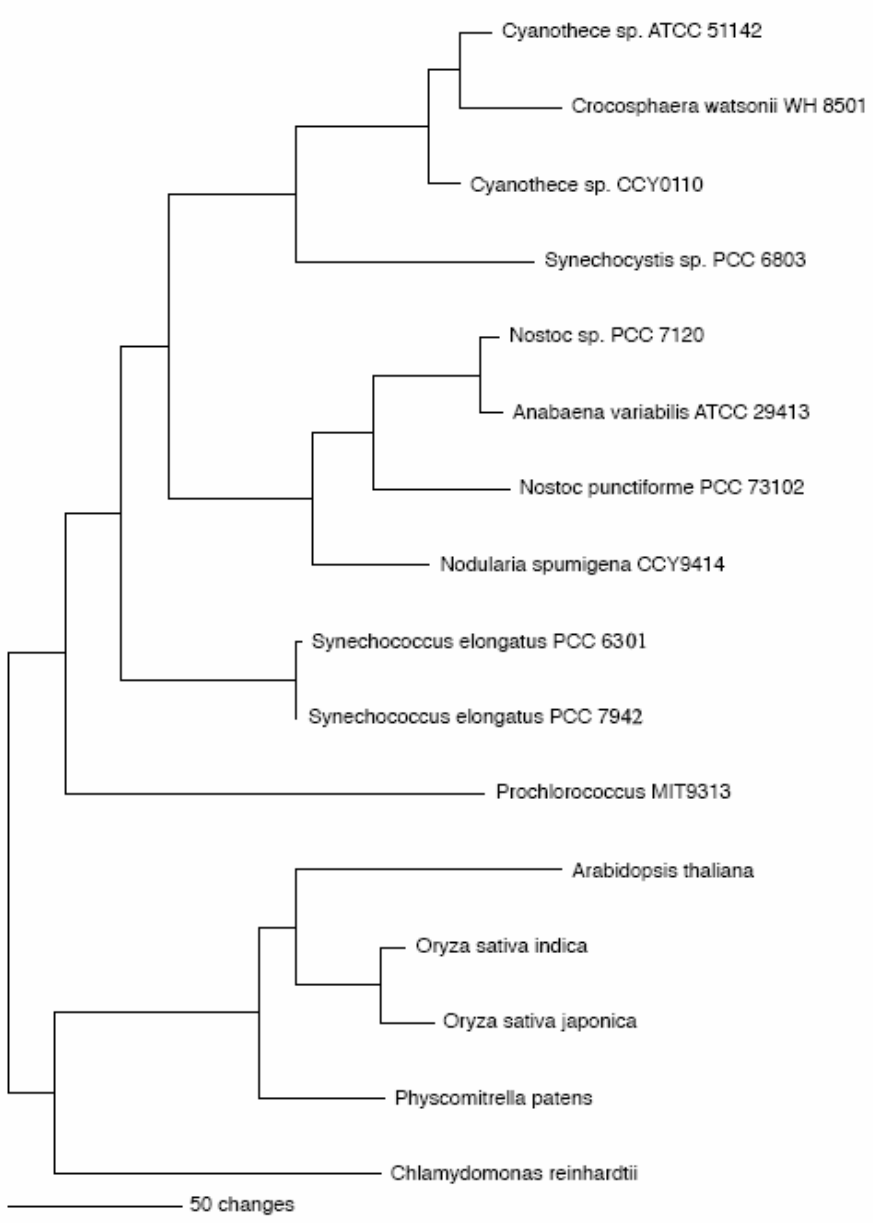


Figure 3 – Phylogenetic tree: The phylogenetic tree of the Psb31 protein was constructed under the parsimony criterion using the software PAUP* 4b10 and was midpoint rooted.

Computational analysis predicted a signal peptide with typical endopeptidase I typical SLA-TG recognition site to direct Psb31 to the thylakoid lumen (**figure 5A**). There are also two transmembrane helices predicted, one in the signal peptide and one at the C-terminus (**figure 5B**).

Blasting the protein sequence shows the presence of a domain of unknown function (DUF477) that is present in both eukarya and eubacteria. This domain has similarities to *β*-propellersheets of the methanol dehydrogenase type. These analyses lead to the presented schematics of the Psb31 protein (**figure 5C).**

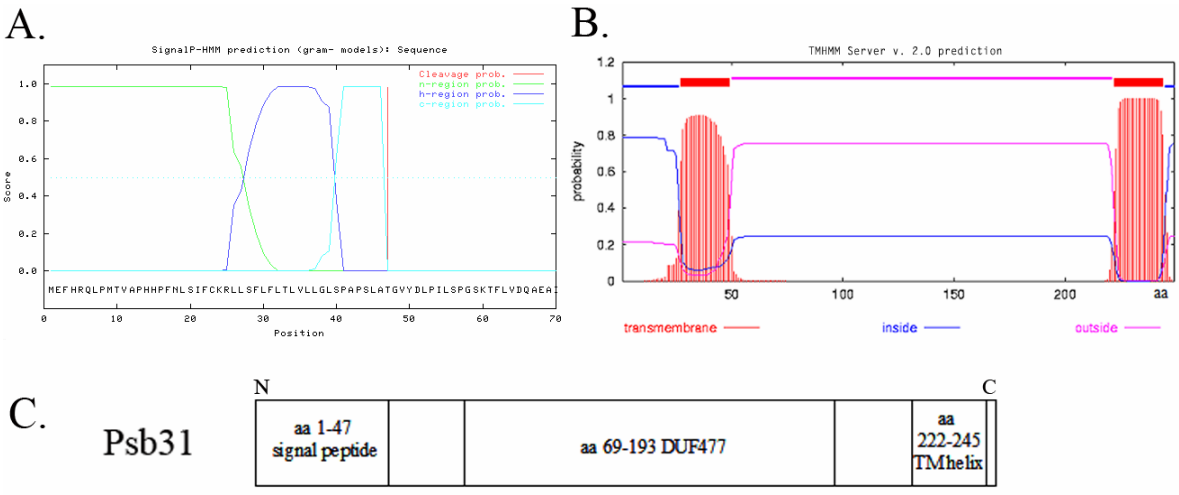


Figure 4 - Computational analysis of the Psb31 protein: (A) Prediction of a SLA-TG transit peptide with SignalP (probability 0.978). **(B)** Prediction of transmembrane helices with TMHMM. V2.0. **(C)** Schematic model of the Psb31 protein with its distinctive domains.

There is no conservation in the *psb31* gene cluster. Its locus is very diverse over different organisms. In *Synechocystis* sp. PCC 6803 the gene is flanked by two hypothetical genes both coding for the YrdC class of hypothetical proteins. These proteins are predicted to be involved in rRNA maturation. *sll1390* (*psb31*) is located 186 bp downstream of *ssl2717* and 52 bp upstream of *sll1866* (**figure 6)**

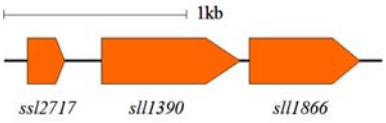


Figure 5 - Scaled locus of the *psb31* (*sll1390*) gene: The gene is 186 bp downstream of *sll2717* and 52 bp upstream of the *sll1866* gene. Both flanking genes code for hypothetical proteins of the YrcD class.

Additional results could be obtained from DNA mircoarray analysis of the closely related cyanobacterium *Cyanothece* sp. ATCC 51142 under alternating 12h light/dark cycles (Stöckel et al., 2008). *Cyanothece* sp. ATCC 51142 is a unicellular diazotrophic cyanobacterium that performs photosynthesis during the day and nitrogen fixation during the night. This temporal separation protects the oxygen sensitive nitrogenase enzyme from oxygen generated during photosynthesis. The microarrays revealed, that the expression of the two core PSII genes, *psbA1* and *psbD1* as well as all PSI genes (*psaA-psaF*) peak later during the light period, whereas *nifE*, which encodes the nitrogenase molybdenum-iron cofactor biosynthesis protein, shows highest expression in the early dark phase. Notably, the expression of the *psb31* homologue (*cce_4116*) mirrors the expression of other PSII genes (*psbB, psbC, psbE, psbO, psbV*) with a maximum in the early light phase (**figure 7)**. This temporal co-expression pattern suggests a possible interaction with PSII.

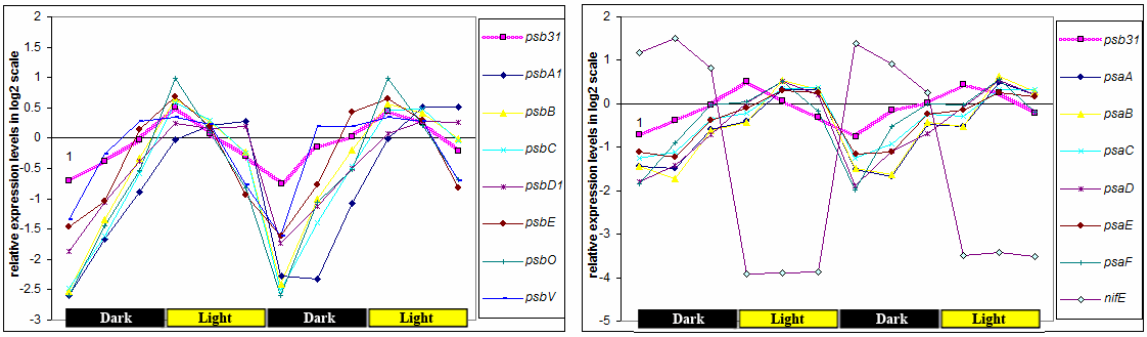


Figure 6 - Diurnal gene expression: Expression profiles of the *psb31* homologue in *Cyanothece* sp. ATCC 51142, a closely related cyanobacterium that shows strong diurnal rhythms in gene expression. RNA was isolated every 4 hours over a 12h dark/light cycles. The expression of the *psb31* homologue (thick line) is aligned with the expression of PSII genes (**left**) and PSI genes (**right**). Expression of the *nifE* gene shows the opposite pattern, with peak expression early in the dark period

3.2 Localization of Psb31

We could raise a polyclonal antibody against the hydrophilic part of the Psb31 protein. A truncated version of the Psb31 gene without the N-terminal signal peptide and the C-terminal transmembrane region was expressed in *E.coli* and purified due to a C-terminal HIS-tag. Expression of the right protein was confirmed my mass-spectroscopy and the protein was used to generate polyclonal antibodies in rabbits. The antibody was tested against PSII preparations of *Synechocystis* 6803 WT and a Δ*psb31* deletion line and specificity could be confirmed (**figure 8A and 8B**). There are only limited cross reactions to a protein of roughly the size of D2. The polyclonal Psb31 specific antibody was tested against membranes isolated from various photosynthetic organisms used in our lab (**figure 8C**). No specific signal could be detected in either *Cyanothece* sp. ATCC 51142, a closely related cyanobacterium, or the higher plants *Arabidopsis thaliana* and *Spinacia oleracea*.

The generated antibody is highly specific for the *Synechocystis* sp. PCC 6803 Psb31 protein and can be used to characterize the location and abundance of the protein.

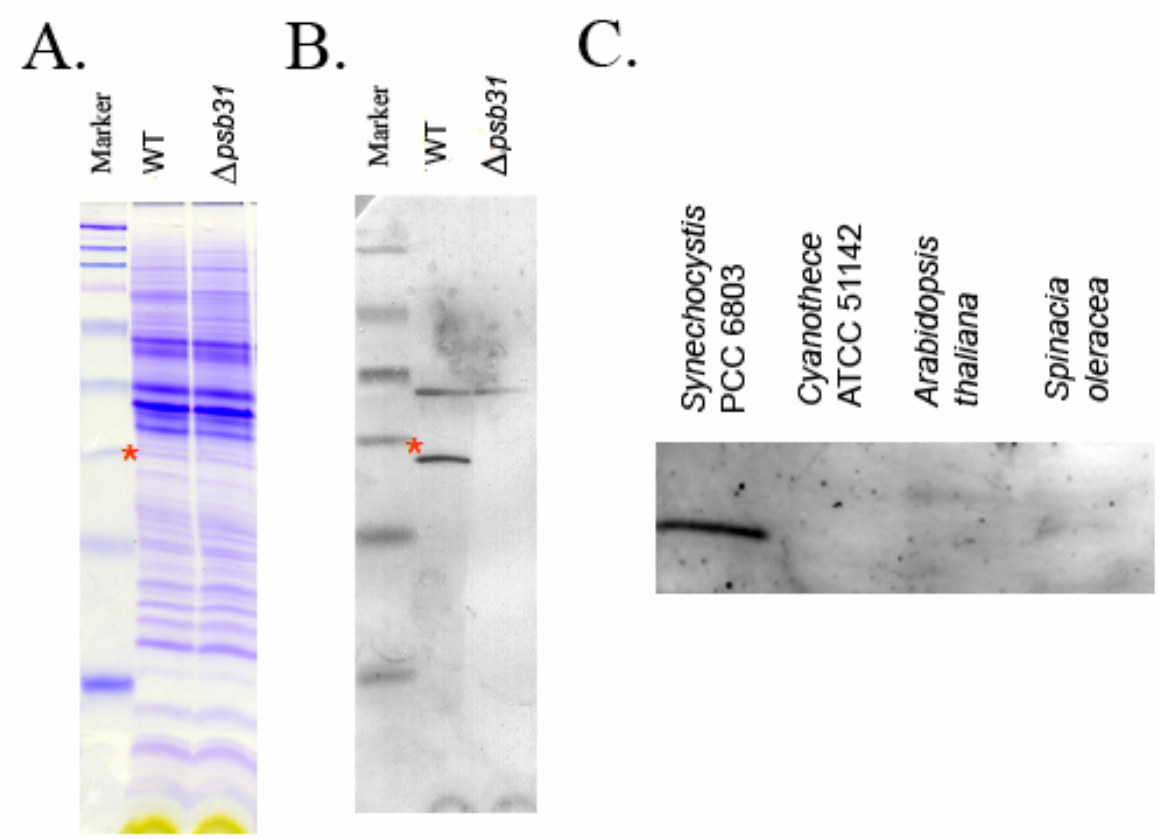


Figure 7 - Psb31 specific antibody: A truncated version of the *Synechocystis* Psb31 protein was over-expressed in *E.coli* and purified via an added HIS-tag. Polyclonal antibodies were raised in rabbits. Purified PSII of WT and mutant (3 µg chlorophyll) was loaded onto a gradient SDS-PAGE and Coomassie stained (**A**.) or blotted and probed with the antibody (**B**.)**.** Psb31 is marked with a red *. Membrane extracts of various organisms probed against the polyclonal *Synechocystis* Psb31 specific antibody. 3 µg chlorophyll were loaded in each lane of a 16% SDS-PAGE gel.

In order to localize the Psb31 protein, WT *Synechocystis* 6803 cells were broken and total membranes isolated. These membranes were separated with Triton X-114, a well established method to separate hydrophilic and hydrophobic proteins (Bricker and Sherman, 1984). This allows to determine the hydrophobicity of the Psb31 protein and therefore if it is a membrane associated protein. The protein could be only detected in the hydrophobic fraction as expected from the prediction programs. As control the fractions were also probed against the hydrophobic D2 protein and the hydrophilic PsbV (**figure 9**). This demonstrates that Psb31 is a membrane protein.

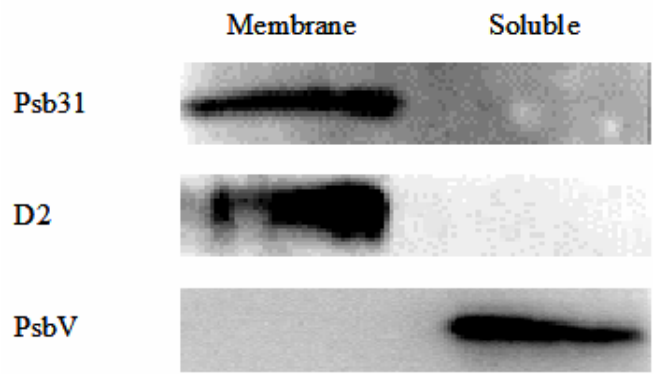


Figure 8 - Triton X-114 phase partitioning: Cells were broken with glass beads and separated 3 times with a 1% Triton X-114 solution. Membrane proteins (hydrophobic) stay in the Triton phase and soluble proteins (hydrophilic) in the buffer. The same amount of proteins (30 µg) was separated by SDS-PAGE, blotted and probed against Psb31, D2 and PsbV.

Total membranes also underwent a second different separation process, namely a phase partitioning with a two phase PEG – Dextran polymer system to separate plasma membranes and thylakoid membranes (Keren et al., 2005a). CP47 could only be identified in the thylakoid membranes and the sodium-dependent bicarbonate transporter (SbtA) predominantly in the plasma membrane. Psb31 is mainly found in the thylakoid membrane but a significant amount is present in the plasma membrane (**figure 10**).

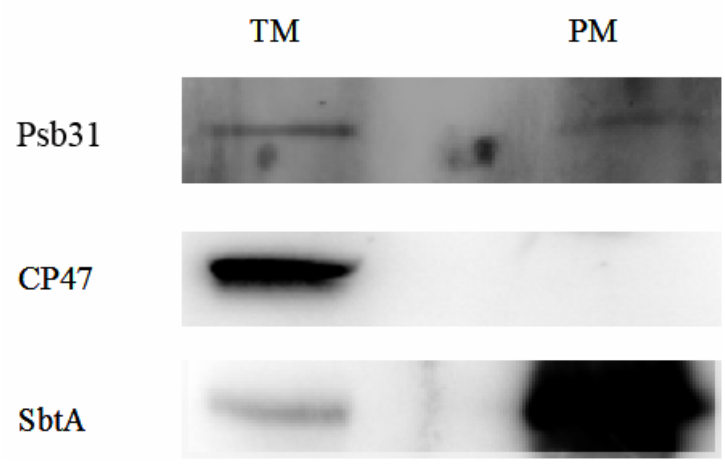


Figure 9 – Separation thylakoid membrane (TM) and plasma membrane (PM): Membrane preparations were further separated by a PEG/ Dextran two phase system. 30 µg protein were separated by SDS-PAGE and immunoprobed against Psb31, CP47 and the Sodium-dependent bicarbonate transporter (SbtA).

Photosynthetic complexes in thylakoid membranes were further separated by sucrose gradient centrifugation. Proteins tightly bound to PSII or PSI, migrate with the heavy complexes whereas loosely attached proteins sediment on their own. Psb31 was not found associated with either D2, a PSII core protein, or the PSI core PsaA/B, but it migrated as a free protein as did PsbV (**figure 11**).

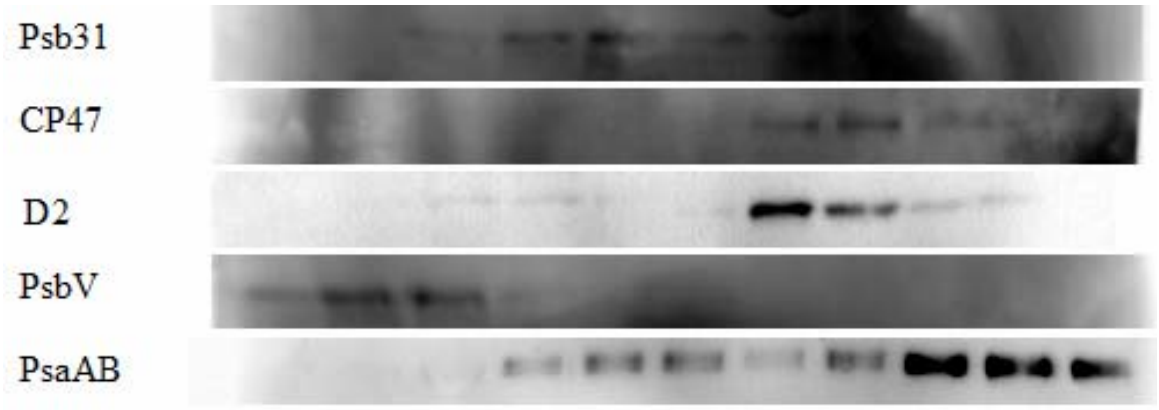


Figure 10 - Sucrose density gradient: Photosynthetic complexes were separated on a sucrose density gradient (10%-30% m/v). The gradient was fractionated and an equal volume of every fraction was separated by SDS-PAGE, starting with the low density on the right, blotted and immunoprobed against Psb31, D2, CP47, PsaA/B and PsbV.

A dilution series of the pure truncated Psb31 protein was used to generate a standard curve to ascertain the amount of Psb31 in PSII complexes. In PSII complexes isolated via a CP47-HIS tag, there is roughly 400 pMol Psb31 / µg Chl present (**figure 12**). This relates to approximately Psb31 protein for 70 CP47 molecules in the PSII preparation. The PSII preparation contains more than 10x more Psb31/Chl than a membrane extract. To calculate the ratios of Psb31/CP47 we used the assumption of 41 Chl / PSII complex as determined by Yasuhio Kashino (2002).

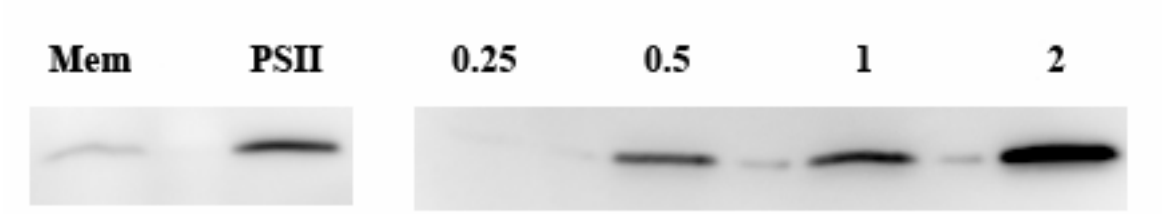


Figure 11 - Quantification of Psb31: 3 µg Chl containing membranes and PSII were probed for Psb31 and quantification was performed by generating a standard curve with 0 - 2 pMol of the pure truncated Psb31. The known ratio of Chl/PSII allows to calculate the relative abundance of Psb31 in the PSII preparation.

3.3 Lumenal Subunits

The Psb31 specific antibody makes it possible to examine the protein levels in various photosynthetic mutants.

There is less Psb31 in PSII preparations of Δ*psbV* compared to WT (**figure 13**). The same goes for the lumenal PSII subunit PsbO.

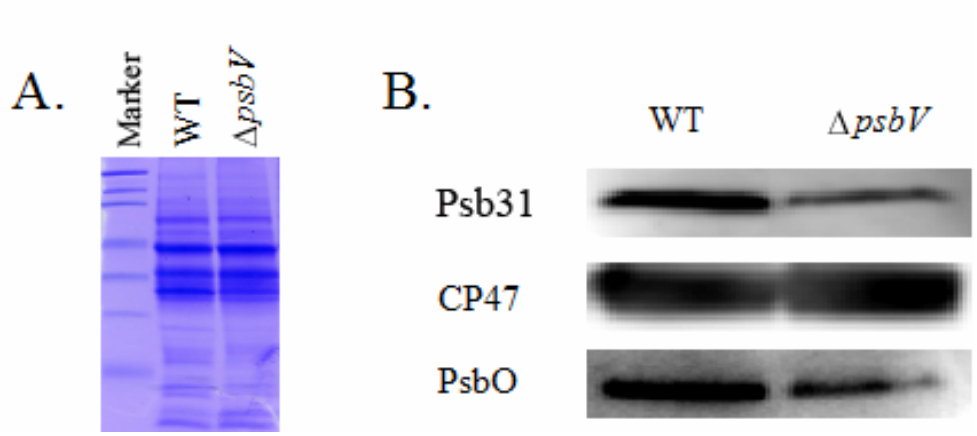


Figure 12 - PSII prep of WT and Δ*psbV*: 3 µg Chl containing PSII isolated from CP47-HIS-tagged WT and Δ*psbV* were separated on SDS-PAGE, stained with Coomassie (**A**) or blotted onto nitrocellulose and probed against Psb31, D2 and PsbO (**B**).

In membrane preparations isolated from lumenal PSII mutants there is much more Psb31 in the Δ*psbQ* mutant compared to a WT preparation (**figure 14**). Psb31 is also more abundant in the Δ*psbU* mutant. But this does not reflect the levels in PSII preparations where Psb31 is similarly abundant in Δ*psbQ* and WT (**figure 15**).

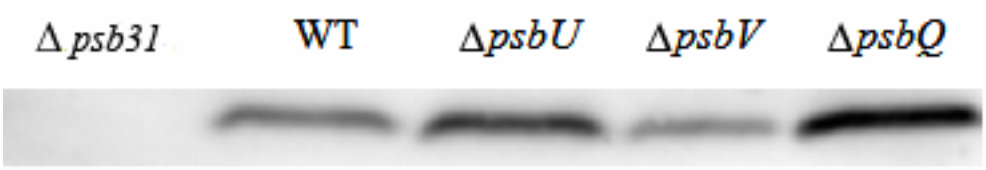


Figure 13 - Psb31 levels in membranes: 3 ug chlorophyll containing membranes of different PSII deletion mutants were separated by SDS-PAGE, blotted and probed against Psb31.

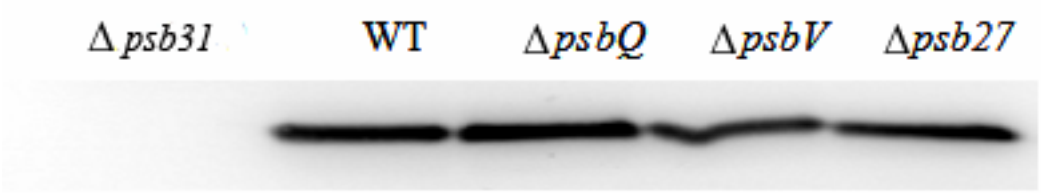


Figure 14 - Psb31 levels in PSII preparations: 3 ug Chlorophyll containing PSII samples of different PSII deletion mutants in the CP47GM background were separated by SDS-PAGE, blotted and probed against Psb31.

3.4 Construction of the Δ*psb31* deletion mutant

To study the function of the Psb31 protein two Δ*psb31* deletion lines were constructed (**figure 16**).

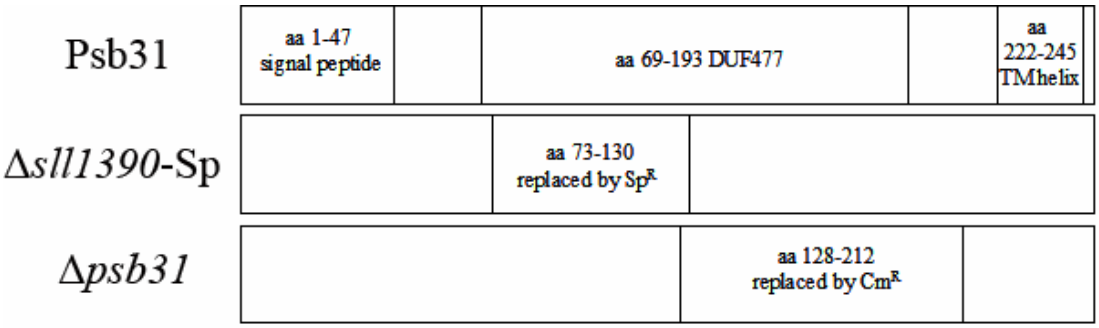


Figure 15 – Schematic layout of the two Psb31 deletion mutants: Parts of the DUF477 domain were replaced by antibiotic resistance cassette, creating deletion mutants.

A first knock out mutant (Δ*sll1390*-Sp) has been previously constructed in the Pakrasi lab by Hiroshi Ohkawa but was not been studied in detail. A problem with this strain occurred when attempting competition experiments. The antibiotic resistance cassette jumped out of the *psb31* locus making it impossible to perform these experiments. Subsequent Western analysis showed that the Psb31 protein was still not expressed, even when the antibiotic cassette was decoupled from the locus. That was expected considering the deletion of 80 bp of the open reading frame. Simply retransforming WT with the plasmid did not solve the problem as the cassette would start jumping again. This forced the construction of a completely new Δ*psb31* construct with a different antibiotic resistance (chloramphenicol) and a different insertion site. All experiments were done or repeated with the new Δ*psb31* construct if not stated otherwise.

The new Δ*psb31* deletion mutant was constructed by replacing 252 bp of the coding region of the *psb31* gene with a 850 bp chloramphenicol resistance cassette. Segregation was confirmed by PCR with gene specific primers. No WT specific signal (1177 bp) is observed in the knock out line (**figure 17A**). Instead the mutant PCR product is about 1776 bp as expected.

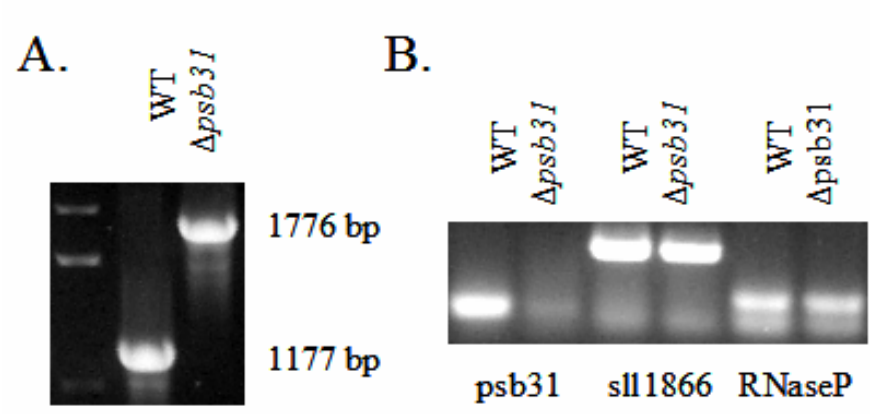


Figure 16 - Segregation analysis (A) and RT-PCR (B) of the Δ*psb31* strain: PCR was performed with genomic DNA of WT and Δ*psb31* using gene specific primers. The PCR product is 1177 bp in WT and in the Δ*psb31* mutant 1776 bp. RT-PCR was performed after generating cDNA with random hexamer primers. For the RT-PCR, specific primers for *psb31*, *sll1866* and *RNAseP* were used.

There are only 50 bp between *psb31* and the next gene downstream *sll1866*. To determine whether the disruption of the *psb31* locus prevents the expression of the downstream gene *sll1866* RT-PCR with *sll1866* specific primers was performed. The downstream hypothetical gene *sll1866* is still expressed. *psb31* specific primers showed that there is no transcript of the gene present in the mutant. *RNaseP* served as positive control (**figure 17B)**. This experiment shows that any phenotype observed is specific for the absence of Psb31 and not the downstream gene.

3.5 Physiological analysis of the Δ*psb31* mutant

Growth assays

Deletion mutant and WT were grown under a variety of conditions (**figures 18A and B**). Neither different light intensities of 10, 45 or 150 µE/m^{2}s, nor modification of the micronutrient levels did alter the capability of photoautothrophic growth. The latter experiments were performed with the 2nd Δ*psb31* deletion strain, Δ*sll1390*-Sp.

The mutant did also not show growth defects under photomixotrophic (in presence of glucose as external carbon source) and photoheterotrophic (glucose + DCMU) growth conditions.

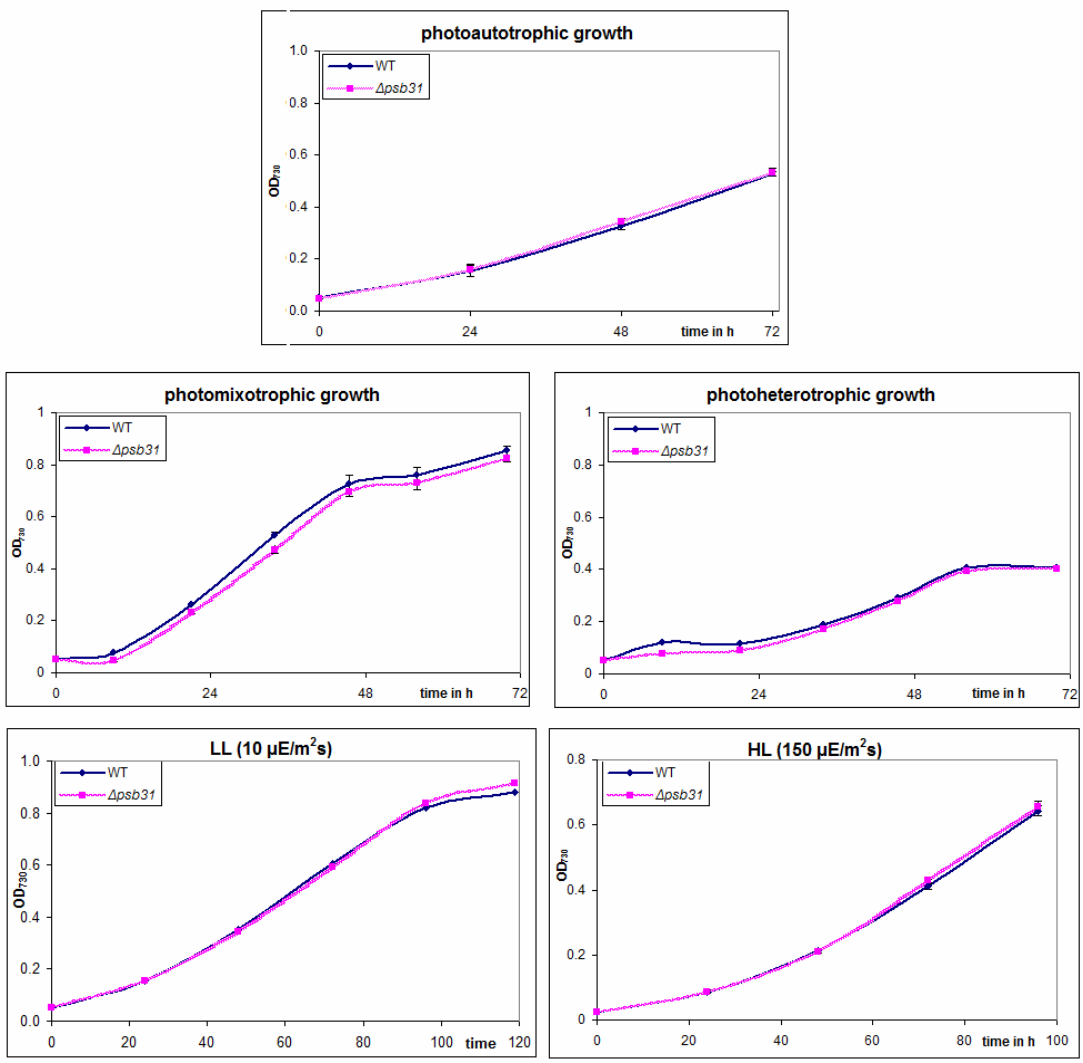


Figure 17A - Phototrophic growth curves: WT and Δ*psb31* cultures grown photoautotrophic under 45 µE/m^{2}s (**top panel**); photomixothropic (supplemented with 5 mM glucose) and photoheterotrophic (supplemented with 5 mM glucose and 10 µM DCMU) (**middle panels**) and photoautrhophic under low light (LL – 10 µE/m^{2}s) and high light (150 µE/m^{2}s) (**lower panels**).

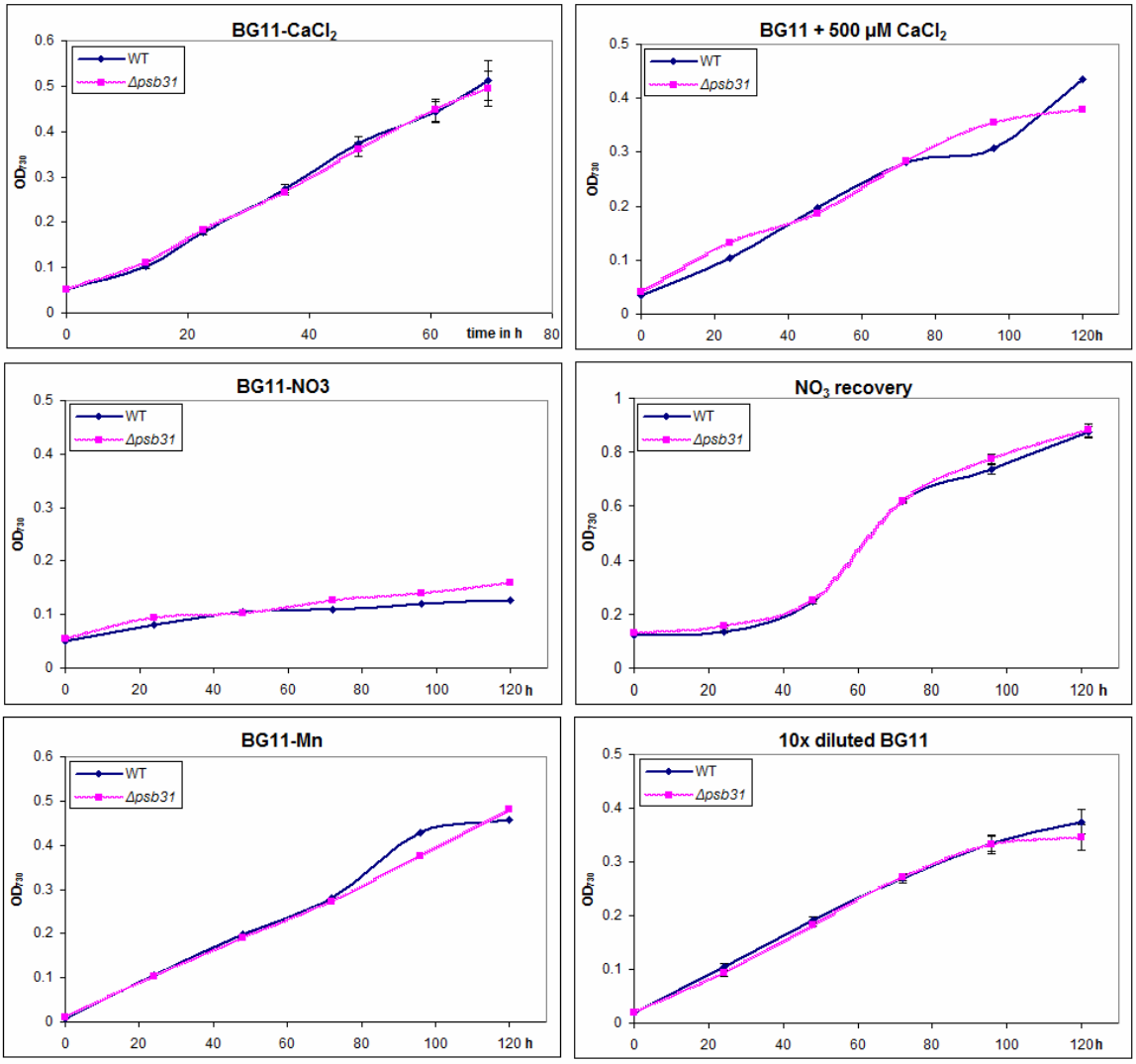


Figure 18B - Growth curves in modified media: Nitrogen starvation and recovery **(top panels),** $CaCl_2$ starvation and over supply of $CaCl_2$Starvation **(middle panels),** starvation of Manganese and general limitation of micronutrients in 10x diluted BG11 **(lower panels).**

Oxygen evolution

The photosynthetic capabilities of the mutant were tested by measuring oxygen evolution rates. The molecular oxygen evolution rate of the PSII reaction is not altered in whole cells or isolated PSII complexes in the presence of excess electron acceptors (**figure 19**).

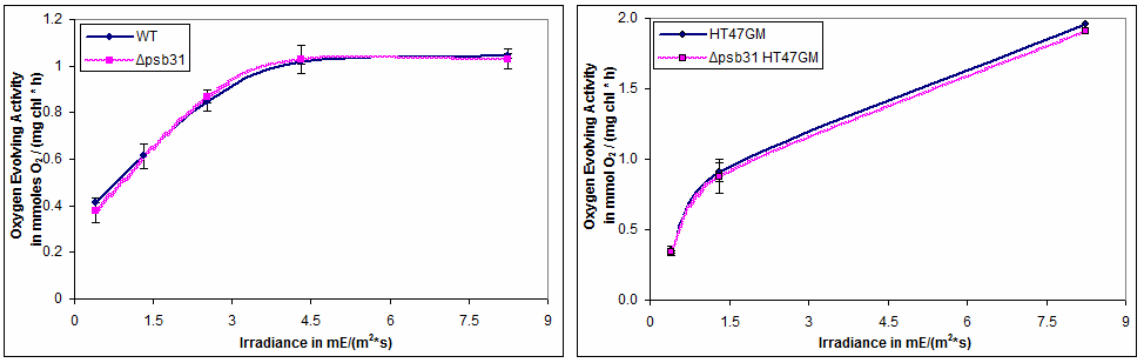


Figure 18 - Oxygen evolution of whole cells (left) and PSII preparations (right): Log phase cultures were diluted to 6 µg/ml Chlorophyll and molecular oxygen evolution was measured at various light intensities in the presence of the electron acceptors DCBQ and Ferricyanide. PSII samples were diluted to 3 µg/ml Chlorophyll and also measured in presence of the electron acceptors. Error bars represent SD of triplicate measurements.

Supportive data for the unchanged oxygen evolving capacity of isolated PSII could be obtained by determination of the manganese content by Atomic Absorption Spectroscopy. The molar ratio between manganese and PSII was determined on the assumption of 41 Chlorophyll molecules per PSII reaction center (Kashino et al., 2002). The manganese per PSII ratio was found to be 3.50 ± 0.18 for WT-HT47GM and 3.45 ± 0.14 for Δ*psb31*-HT47GM. This corresponds to the Manganese levels found previously in CP47 HIS-tagged PSII preparations by Yasuhiro Kashino (2002) and Johnna Roose (2007a). It shows that the oxygen evolving complex is assembled to the same extent in both WT and mutant. A fully assembled PSII contains 4 manganese atoms and the lower numbers indicate that not all PSII isolated are fully assembled. Most likely degradation did occur during the purification process.

In absence of excess electron acceptors, the electrons generated by PSII have to be channeled down the photosynthetic electron transport chain via the cytochrome b_6f complex and PSI.

Under these conditions the oxygen evolution rate is also similar comparing WT and mutant. This indicates that the electron transport downstream of PSII is not affected by a deletion of the *psb31* gene (**table 2**).

Irradiance in mE/(m^2*s)	**Oxygen evolving activity in mmol O_2 / (mg chl *h)**	
	WT	**Δ*psb31***
800	216 ± 6	233 ± 26
8250	266 ± 16	256 ± 20

Table 2 Whole chain oxygen evolution of WT and deletion mutant: Samples were diluted to 6 µg Chlorophyll and measured on a Clark-type oxygen (n=3).

Fluorescence Kinetics

Analysis of fluorescence kinetics is an established method for characterizing key aspects of photosynthetic electron transport and was thoroughly reviewed in 2004 by Govindjee. In particular, chlorophyll fluorescence can be used to determine various parameters of the PSII reaction center. The rates of reoxidation of the primary PSII electron acceptor, Q_A, exhibit no significant difference between WT and the Δ*psb31* strain (**figure 20**). In the absence of DCMU, Q_A reoxidation takes place through a mixture of forward electron transfer to plastoquinones and backward electron transfer through charge recombination (van Best and Duysens, 1975). In the presence of DCMU, forward electron transfer reactions are blocked, and the reoxidation is caused solely through back-reactions. These normal rates of Q_A- reoxidation indicate that both the donor and acceptor sides of PSII are intact.

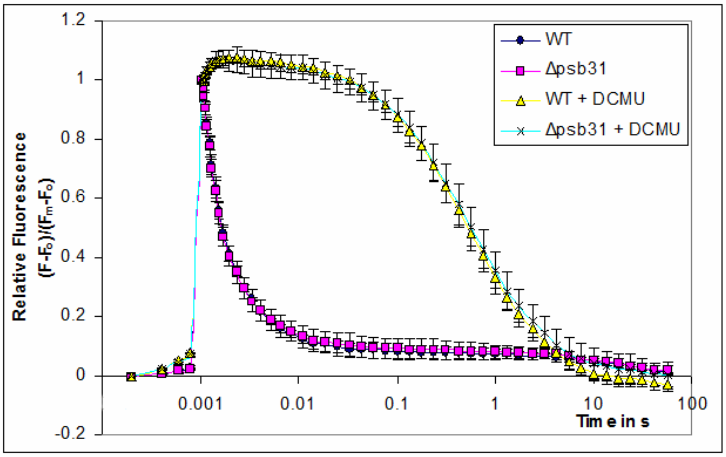


Figure 19 - Q_A reoxidation kinetics: Q_A reoxidation was measured in the presence and absence of DCMU. Error bars represent the standard error of the mean (n = 3).

Chlorophyll fluorescence induction kinetics can be measured in order to monitor the electron acceptor side reactions downstream of the Q_A site, the PSII associated electron acceptor pool heterogeneity and pool size (Govindjee, 2004). The presence of the inhibitor DCMU further allows one to investigate effects on the donor side. The fast *transient of Chlorophyll a Fluorescence (OJIP)* is not altered in the Δ*psb31* deletion mutant compared to WT, indicating that electron transfer through PSII is not affected by the Psb31 protein.

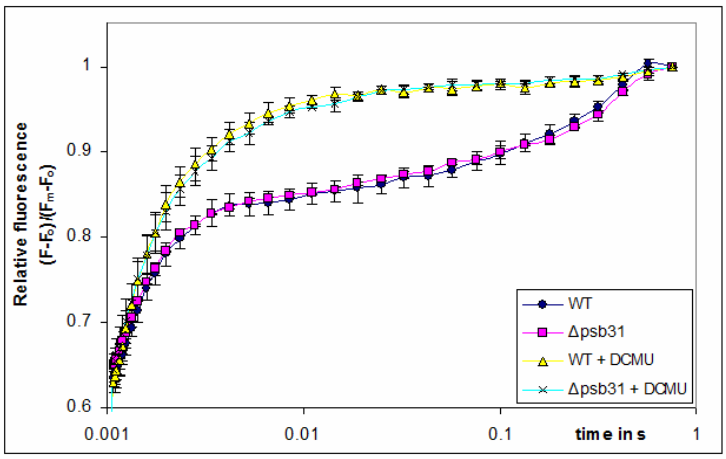


Figure 20 - Fluorescence induction: Fluorescence induction of log phase samples was measured in presence and absence of DCMU. Error bars represent the standard error of the mean (n = 3**).**

77 K Spectroscopy

77 K fluorescence emission spectra were taken of whole cells and normalized for better comparability (**figure 22**). Excitation at 420 nm or 600 nm does not yield any differences. The chlorophyll specific excitation at 420 nm indicates that the chlorophyll stoichiometry between the two photosystems is not altered. The excitation at 600 nm allows statements about the connectivity of the phycobilisomes to the photosystems. In the Δ*psb31* mutant, both PSII with the phycobilisome linker pigment (at 685 nm) and PSI (at 720 nm) seem to bind similar amounts of phycobilisomes compared to WT.

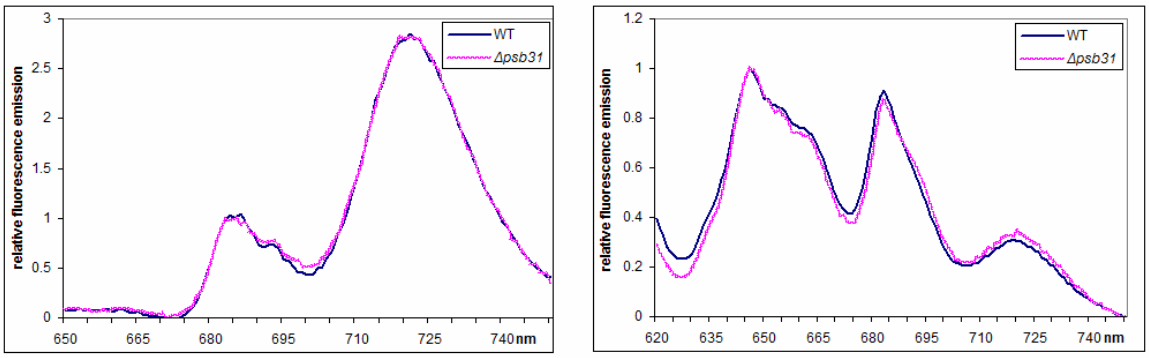


Figure 21 - 77 K fluorescence emission spectra: Samples were measured at 77 K with excitation wavelengths of 420 ± 5 nm (left) and 600 nm (right). Spectra were normalized to the phycocyanin peak at 646 nm or to the PSII specific peak at 685 nm respectively.

Pigment composition

Cyanobacteria produce a variety of pigments. Phycobiliproteins, in particular the blue-colored phycocyanin and the red-colored phycoerythrin are the major light-harvesting pigments used to drive photosynthesis. Chlorophylls are in general directly bound to the two cores of the photosystems and facilitate the transfer from the antenna to the core complexes. A third group of pigments are the carotenoids with mainly photo-protective functions. To determine the total amounts of the various pigments they were extracted and quantified from mid-log phase cultures of similar OD_{730} (**table 3**).

The pigment levels in the Δ*psb31* mutant are comparable to those in WT under standard conditions. This finding is supported by the absorption spectra obtained from whole cells which are virtually identical (**figure 23**).

Pigment	WT	Δ*psb31*
Chlorophyll	32.7 ± 0.3	32.6 ± 1.4
Carotenoids	13.0 ± 0.3	12.9 ± 0.8
Phycocyanin	132 ± 3	143 ± 6
Phycoerythrin	187 ± 3	193 ± 3

Table 3 - Pigment composition of WT and Δ*psb31*: Chlorophylls and carotenoids were extracted with methanol and the watersoluble phycobiliproteins were extracted from methanol extracted cells by breaking them with glass beads. Absorbance ratios were used to quantify the amounts of pigments. Values in fg/cell ± the standard derivation (n=3).

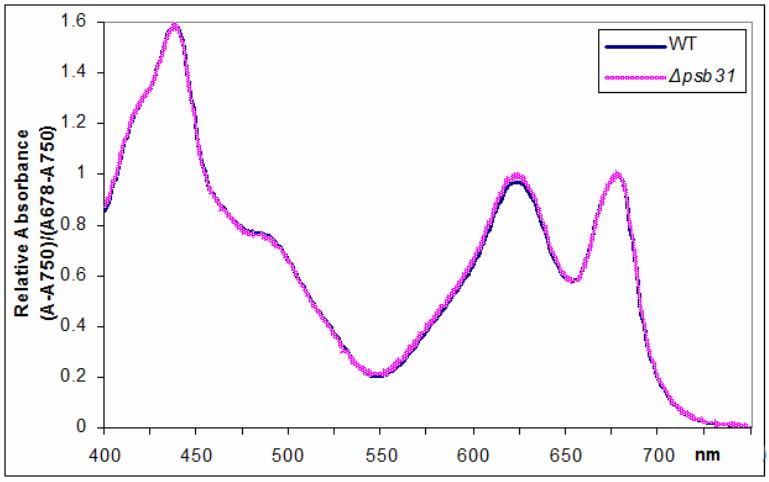


Figure 22 - Room temperature absorption spectra: Whole cell absorption spectra from 400 to 750 nm were taken of log phase cultures with a DW2000 spectrophotometer. This spectra is compiled from three independent experiments, averaged and normalized to the chlorophyll peak at 678 nm.

Protein composition

Membrane extracts of WT and Δ*psb31* were probed for photosynthetic proteins and subunits of both, PSI and PSII, seem to be similarly abundant in both strains (**figure 24**). The composition of isolated PSII complexes was determined by Coomassie stain and also shows no difference (**figure 8**).

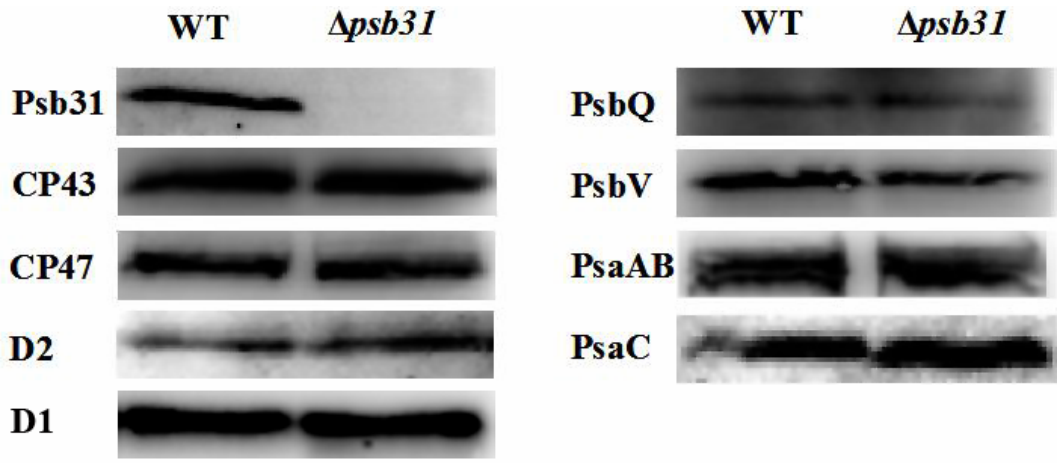


Figure 23 - Protein composition of membrane extracts: Membranes were isolated from WT and Δ*psb31*, 3 µg chlorophyll containing samples separated with SDS-PAGE, blotted onto nitrocellulose and probed against various PSII and PSI proteins.

3.6 Mixed culture experiments

There was no significant growth difference observed between WT and mutant under various light intensities (**figure 18A**). Mixed culture experiments can often increase the resolution of minor defects. For this purpose the same number of WT and mutant cells were mixed and grown together in one culture. DNA was extracted on several time points to determine the relative abundance of each strain. If the absence of Psb31 reduces the fitness of *Synechocystis* 6803, than the mutant should be out-competed. During growth under lower light intensities of 5 (LL) and 45 (GL) µmol photons/m^2s there was no change in the WT/mutant ratio.

However, under high light of 150 (HL) µmol photons/m^2s the signal for the mutant locus disappears after several rounds of sub-culturing (**figure 25**). Therefore Psb31 provides significant benefits to cells grown under high light stress but is not essential under lower light intensities.

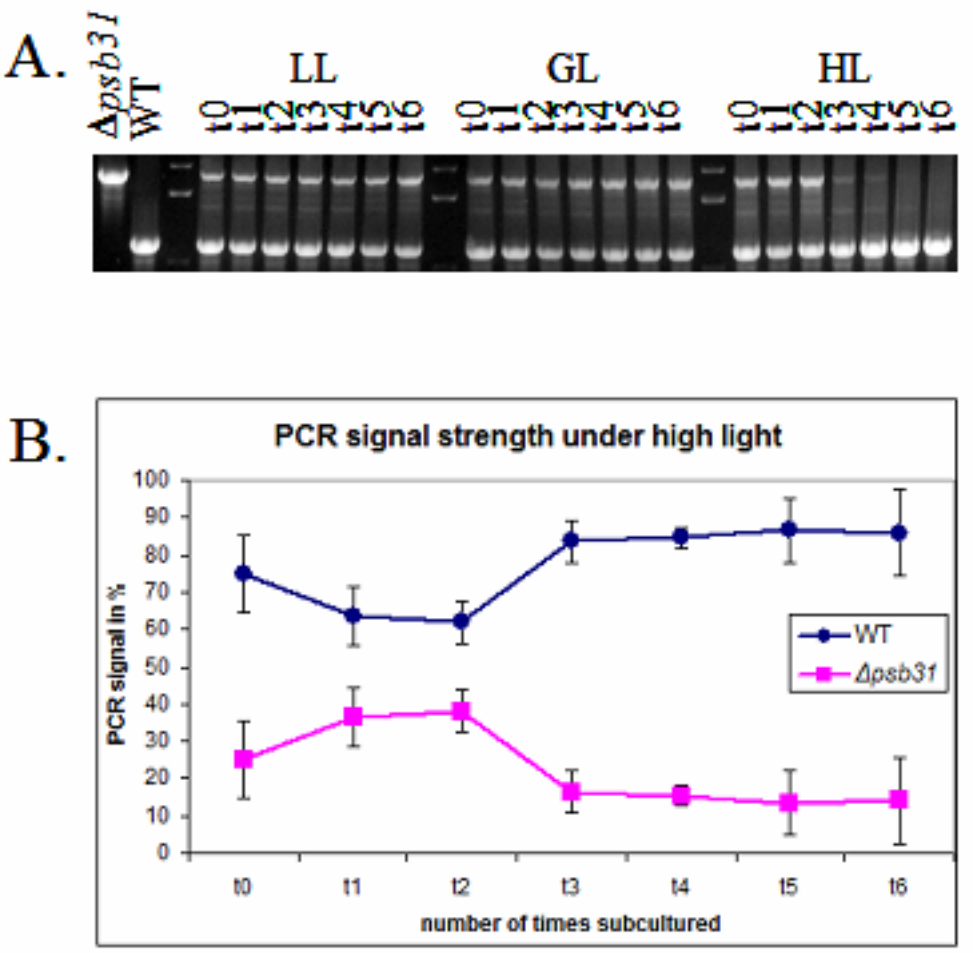


Figure 24 - Competition experiments:

A. Equal amounts of WT and Δ*psb31* were mixed in a shaking culture and incubated under low light (**LL** 5 µE/m^2s), growth light (**GL** 45 µE/m^2s) and high light (**HL** 150 µE/m^2s). Samples were taken every three days and the cultures were diluted back to the starting OD. DNA was isolated and amplified via PCR with gene specific primers. The lower band of 1177 bp is the WT and the upper band of 1776 bp represents the mutant. On the far left are cultures of WT and Δ*psb31*.

B. The signals on the gel were quantified with ImageJ, plotted and normalized so that the respective signals add up to 100% for each time point. The graph represents the average of three independent experiments with the error bars showing the SD.

3.7 Oxidative stress

To further increase the HL stress, separate cultures were grown at 220 µmol photons/m^2s in the presence or absence of Rose Bengal which induces singlet oxygen stress. No growth difference could be observed in absence of Rose Bengal. However, in presence of 7.5 µM Rose Bengal the Δ*psb31* mutant bleached completely and recovered only partially after 72 hours whereas WT, although some bleaching occurs, is still able to grow (**figure 26**).

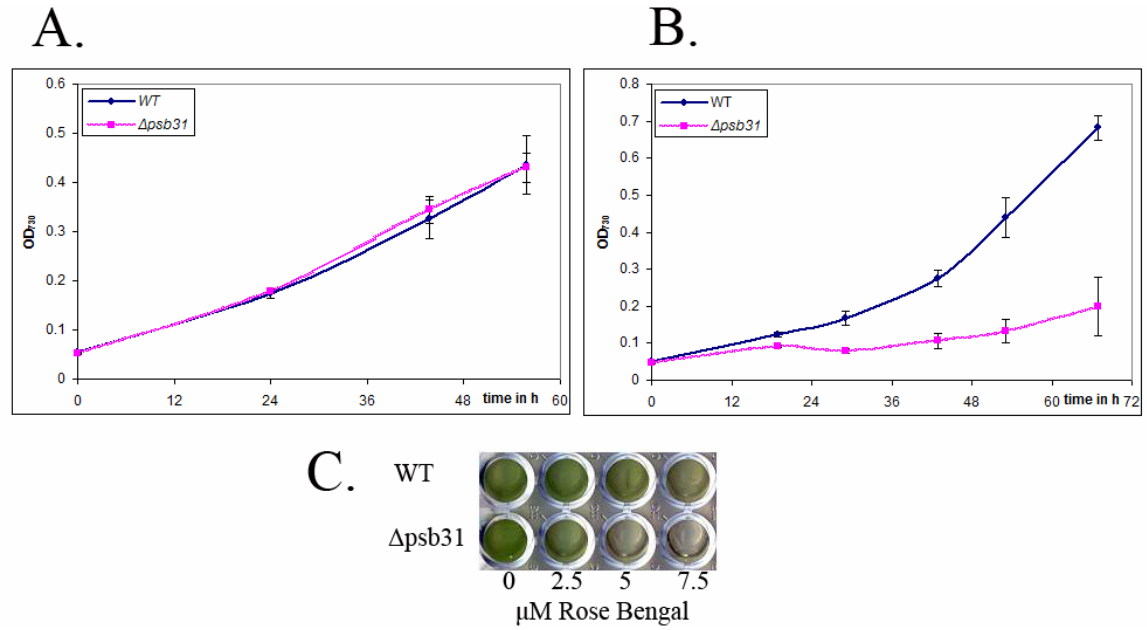


Figure 25 - Growth in presence of Rose Bengal: Growth under 220 µE/m2s was monitored by OD_{730} measurements. Errorbars represent SD of n=3. (**A**) Growth curve without Rose Bengal. (**B**) Growth in presence of 7.5 µM Rose Bengal.(**C**) Cultures 24 h after addition of Rose Bengal.

The effect of Rose Bengal to PSII can be monitored by chlorophyll fluorescence spectroscopy. After adding Rose Bengal, the variable fluorescence $F\text{-}F_0$ drops drastically for both WT and mutant. This indicates that the electron transfer through PSII is disturbed. The initial drop is similar but while the WT retains some fluorescence the mutants drops further. WT recovers after 24h whereas the almost no active PSII is left in the mutant (**figure 27**).

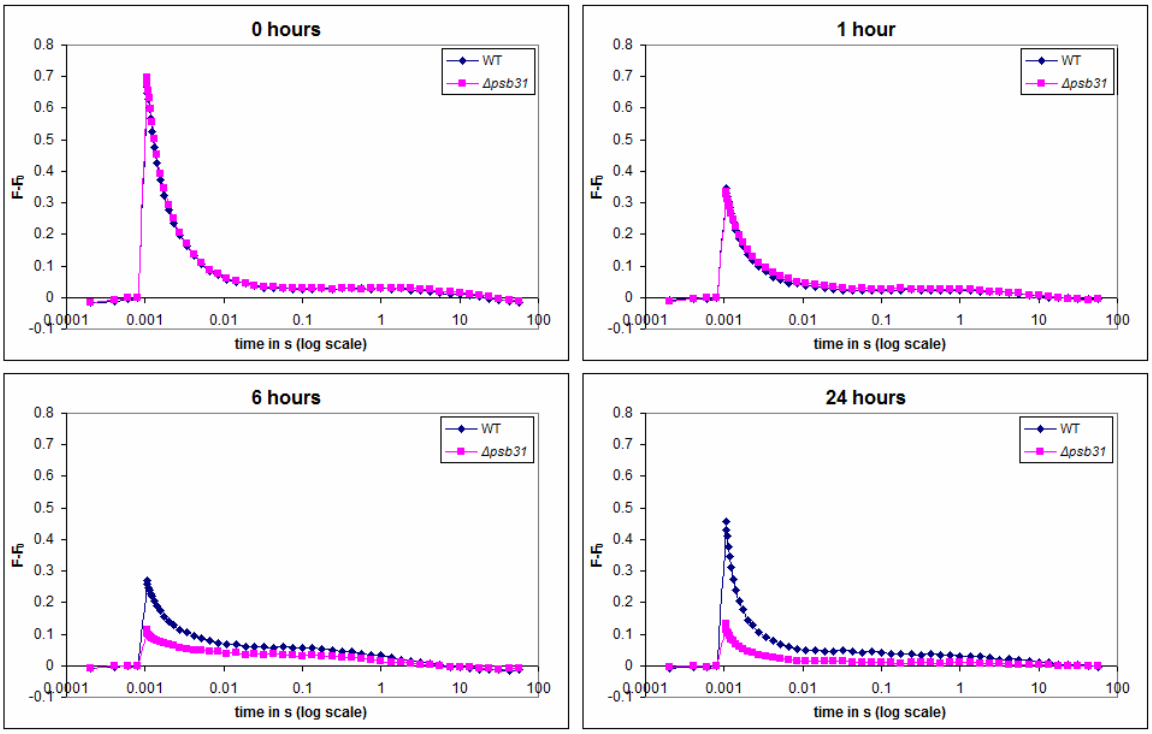


Figure 26 - Q_A reoxidation kinetics after Rose Bengal treatment: The kinetics were measured before adding 7.5 µM Rose Bengal and after 1, 6 and 24 hours of incubation at 220 µE/m^{2}s. Graphs are normalized to F-F_0.

When exposed to hydrogen peroxide, a different reactive oxygen species, the deletion mutant and WT show the same sensitivity (**figure 28**). In presence of 2 mM H_2O_2 both WT and Δ*psb31* mutant can grow with similar growth rates whereas both are killed with 2.5 mM H_2O_2.

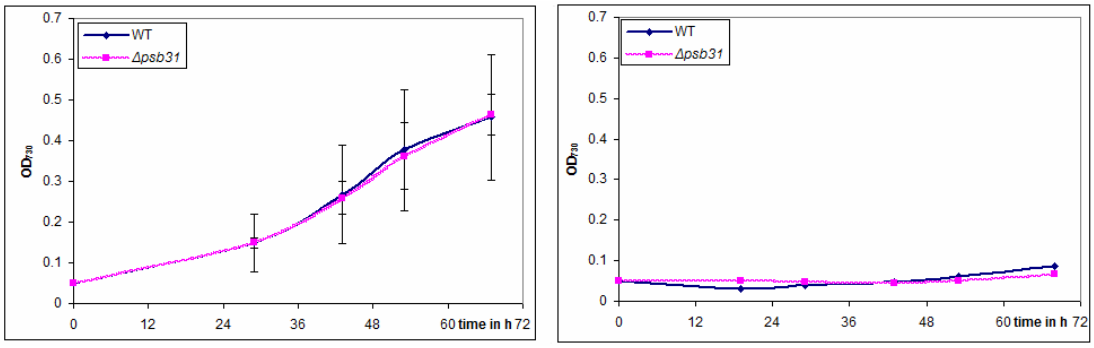


Figure 27 - Growth curve in presence of H_2O_2: Growth under 220 µE/m2s in presence of 2 mM (left) and 2.5 mM H_2O_2 (right) was monitored by OD_{730} measurements. Error bars represent SD of n=3.

These findings indicate that Psb31 is necessary to overcome oxidative stress, in particular singlet oxygen stress generated by Rose Bengal.

3.8 Photoinhibition and recovery

To determine if the increased sensitivity to oxidative stress is due to a direct effect on PSII, photoinhibition under high light (200 µE/m^{2}s red and blue LED light – equivalent to 1000 µE/m^{2}s white light) and recovery under low light were monitored by fluorescence measurements (**figure 29**). The rates of photoinhibition and subsequent recovery are comparable for WT and Δ*psb31* knockout mutant, showing that the high light sensitivity is not directly linked to defects on the PSII reaction center.

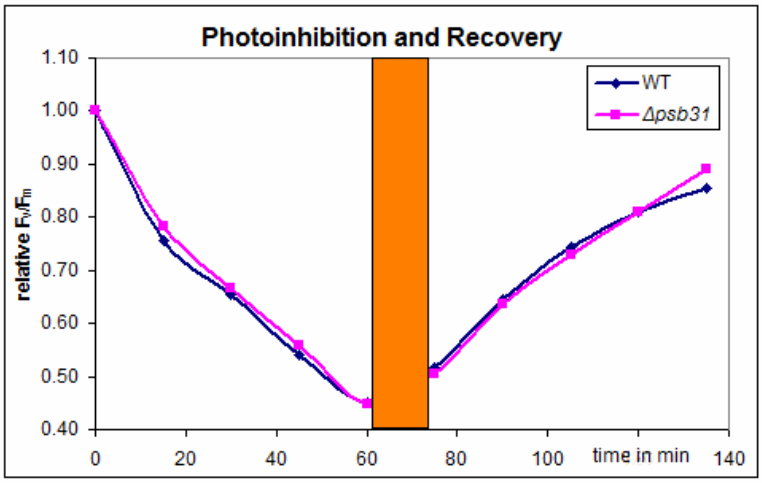


Figure 28 - Photoinhibition and recovery: WT and Δpsb31 cells were incubated for 1 h under high light (200µE/m^{2}s red and blue LED) in the presence of the protein synthesis inhibitor lincomycin. Cells were then washed twice with fresh medium to remove the lincomycin (duration marked in orange) and then incubated at a lower, non-inhibiting light intensity. Variable fluorescence yield (F_v/F_m) was measured throughout the experiment.

3.9 Psb31 expression under HL

To determine if the Psb31 protein is present in a higher abundance under higher light intensities, a WT culture was grown under HL (150 μE/m^{2}s) and GL (45 μE/m^{2}s) intensities and membranes were isolated. Protein gels loaded on the same chlorophyll or protein basis were run, blotted and probed against the D2 protein and Psb31. Under HL the chlorophyll to protein ratio goes down as cells try to limit the absorption of excess photons to prevent photo damage. On protein basis there is no difference in total Psb31 protein detected between HL and GL showing that there is no greater abundance under HL (**figure 30**).

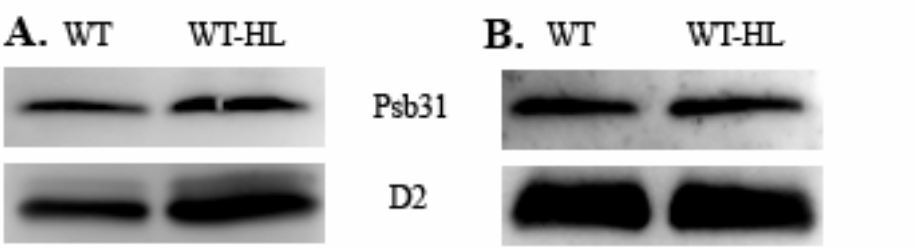


Figure 29 - Protein levels under high light: Membranes isolated from cultures grown under regular light (45 μE/m^{2}s) and high light (150 μM/m^{2}s) were loaded on chlorophyll (**A**.) or protein (**B**.) basis, blotted onto nitrocellulose and probed against Psb31 and D2.

3.10 Blue light treatment

The effect of light quality on the Δ*psb31* deletion was investigated by exposing mutant and WT cells to blue light, predominately exciting PSI. Prior exposing to blue light both show a similar 77 K emission spectra when exciting chlorophyll at 420 nm. The fluorescence emission ratio of PSI to PSII is 6:1. After 24 h under blue light the ratios between the PSI and PSII specific signals drops for both, but more drastically for the Δ*psb31* mutant. The lower PSI/PSII ratio indicates higher amount of PSII compared to the WT. On protein level, blue light leads to an increase of PSII (**figure 31**).

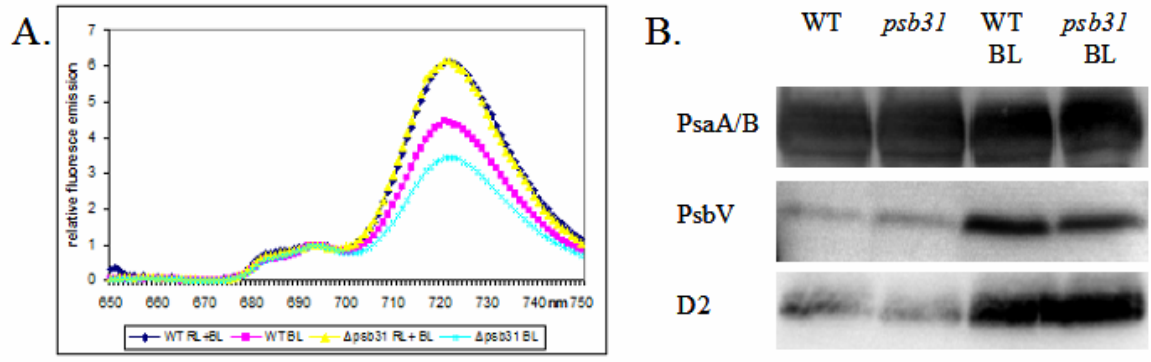


Figure 30 - Blue light treatment: Cells were exposed for 24 h to a blue LED light source (450 nm, PSI Czech Republic). (**A**.) 77 K spectra were taken before and after the treatment with an excitation of 420 nm and normalized to the PSII peak at 694 nm. The graph represents the average of three independent experiments. (**B.**) Membranes were isolated and probed against various proteins.

3.11 Pull down assays with *E.coli* synthesized truncated Psb31

The truncated Psb31 expressed with a C-terminal HIS-tag in *E.coli,* which was originally used to generate the Psb31 specific antibody, was used in pull down assays in order to identify binding partners of Psb31. The fragment contains the whole Psb31 protein except for the C-terminal transmembrane region and the N-terminal signal peptide, so it is not to be confused with the full length *psb31*-HIS construct used for expression in *Synechocystis* 6803.

The pull downs of the *E.coli* expressed truncated Psb31 with solubilized *Synechocystis* 6803 membranes yielded an orange colored eluate (**figure 32**). Absorption spectra of the eluate taken with a DW2000 spectrophotometer revealed a triple peak at 457, 481 and 520 nm which is typical for carotenoids (**figure 33**). Pure *β*-carotene, dissolved in DMSO was used as a reference.

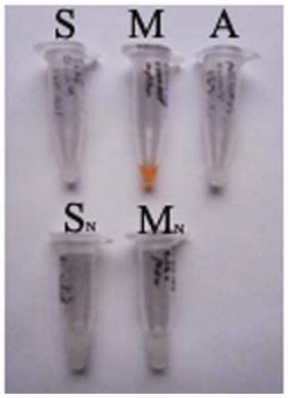
The orange pigment could neither be captured by running solubilized *Synechocystis* 6803 membranes directly over a Nickel-Agarose column without Psb31 as

bait, nor when run over a column prepared with a different HIS-tagged bait, NrtA. Pull down of soluble *Synechocystis* 6803 proteins also did not capture the carotenoid. This shows that the interaction is specific for the truncated Psb31 and not due to interaction with the HIS-tag or the column matrix.

There is less Psb31 in Δ*psbV* PSII isolations (**figure 13**). To address the question weather Psb31 and PsbV bind each other, Western analysis of the pull downs was performed with a PsbV specific antibody. For that purpose, a new polyclonal antibody against a surface peptide was raised in rabbits at Sigma-Genosys (**figure 34**).

No PsbV could be detected in the pull downs. Neither could any other protein binding partner be identified by silver staining for either soluble *Synechocystis* 6803 proteins or solubilized membranes. This still does not rule out a binding partner as the amount present could be below the detection limit.

Name	Bait	*Synechocystis* extract
A	Truncated Psb31	---
S	Truncated Psb31	Soluble proteins
M	Truncated Psb31	Solubilized membranes
S_N	NrtA	Soluble proteins
M_N	NrtA	Solubilized membranes



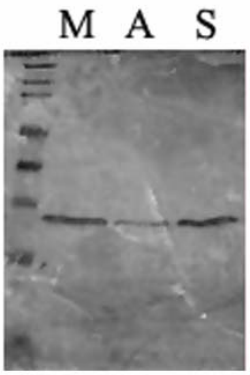


Figure 31 – Pull down with truncated Psb31 protein expressed in *E.coli*: The center of the figure shows the collected eluates of different pull down experiments as described on the left. On the right is a silver stained SDS gel of the truncated Psb31 pull downs.

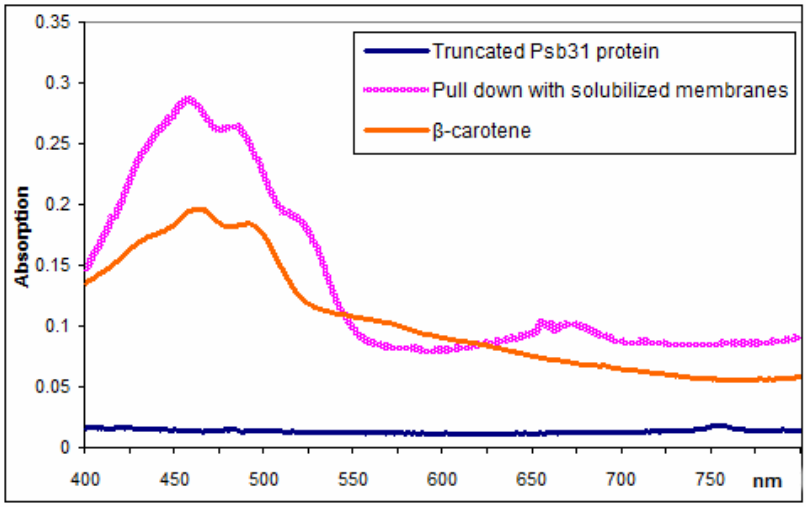


Figure 32 – Pull down absorption spectra: Spectra of the purified truncated Psb31 protein and the pull down of solubilized membranes, both in elution buffer. As control serves pure β-carotene dissolved in DMSO.

3.12 PsbV antibody

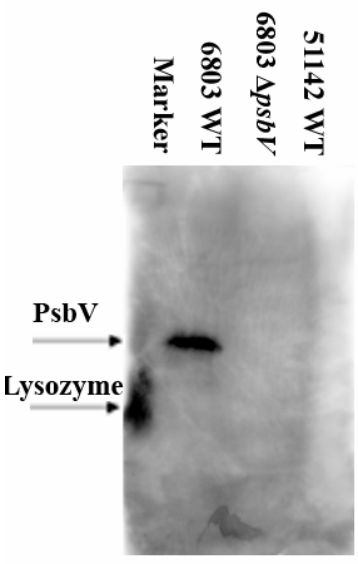


Figure 33 – Specificity of the polyclonal antibody against PsbV: The antibody was probed against membranes isolated from *Synechocystis* 6803 WT and Δ*psbV* as well as *Cyanothece* 51124 WT. Note the cross reaction against the 20 kDa marker protein, lysozyme.

The polyclonal antibody against PsbV was tested against membranes isolated from *Synechocystis* 6803 (**figure 34**). We could detect PsbV previously only by heme stain. The antibody detects the same protein but an additional advantage is an unexpected cross reaction of the antibody with lysozyme, the 20kDa protein of the ladder we use in our lab. This cross reaction can be used as a positive control for studies using the PsbV specific antibody. Like the Psb31 specific antibody, the PsbV specific antibody was used to probe against *Cyanothece* sp. ATCC51142, and once again the antibody is specific for *Synechocystis* sp. PCC 6803 only. Only 10 out of the 14 amino acids in the peptide used to generate the antibody are conserved between the two cyanobacteria which explains the specificity. No eukaryotic samples were probed with the antibody as PsbV is only present in cyanobacteria.

3.13 Purification of HIS-tagged Psb31

In order to identify binding partners of Psb31 a polyhistine tag was fused to the C-terminus of the protein. The expression of the fusion protein was confirmed by Western analysis of membrane extracts showing a slightly bigger protein compared to WT Psb31. The Psb31-HIS protein was isolated using a FPLC system. The well established method for purification of an active PSII preparation by Yasuhiro Kashino *et al.* (2002) was adopted. Absorption was monitored at 280 nm and the elution with a histidine gradient yielded a specific peak (**figure 35**) which was concentrated and further investigated.

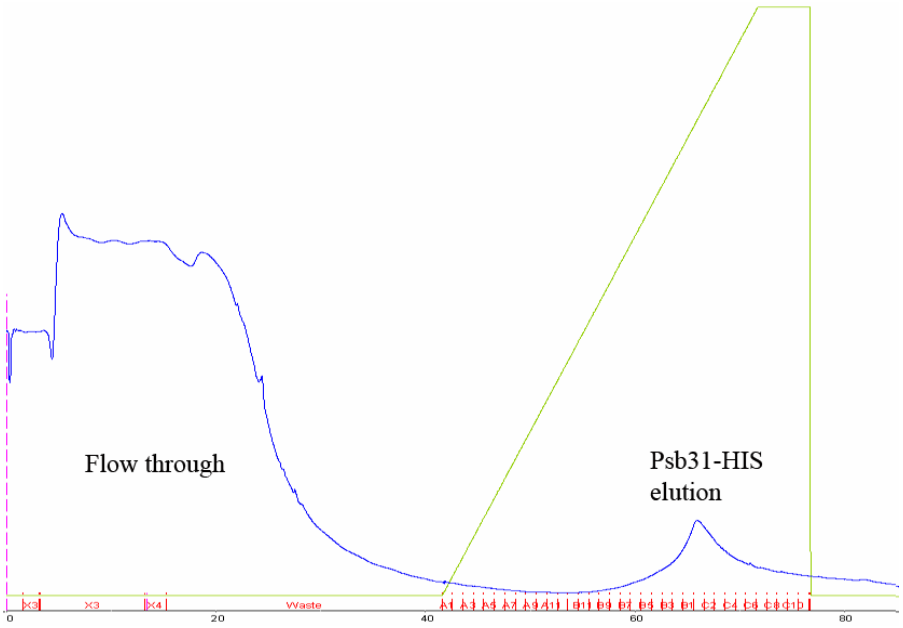


Figure 34 - Chromatogram of the Psb31-HIS preparation: Psb31-HIS was purified from solubilized membranes by FPLC. The protein was eluted from the Ni-Agarose column using a continuous histidine gradient. The absorption was monitored at 280 nm and the elution peak is clearly recognizable. Fractions were pooled and concentrated.

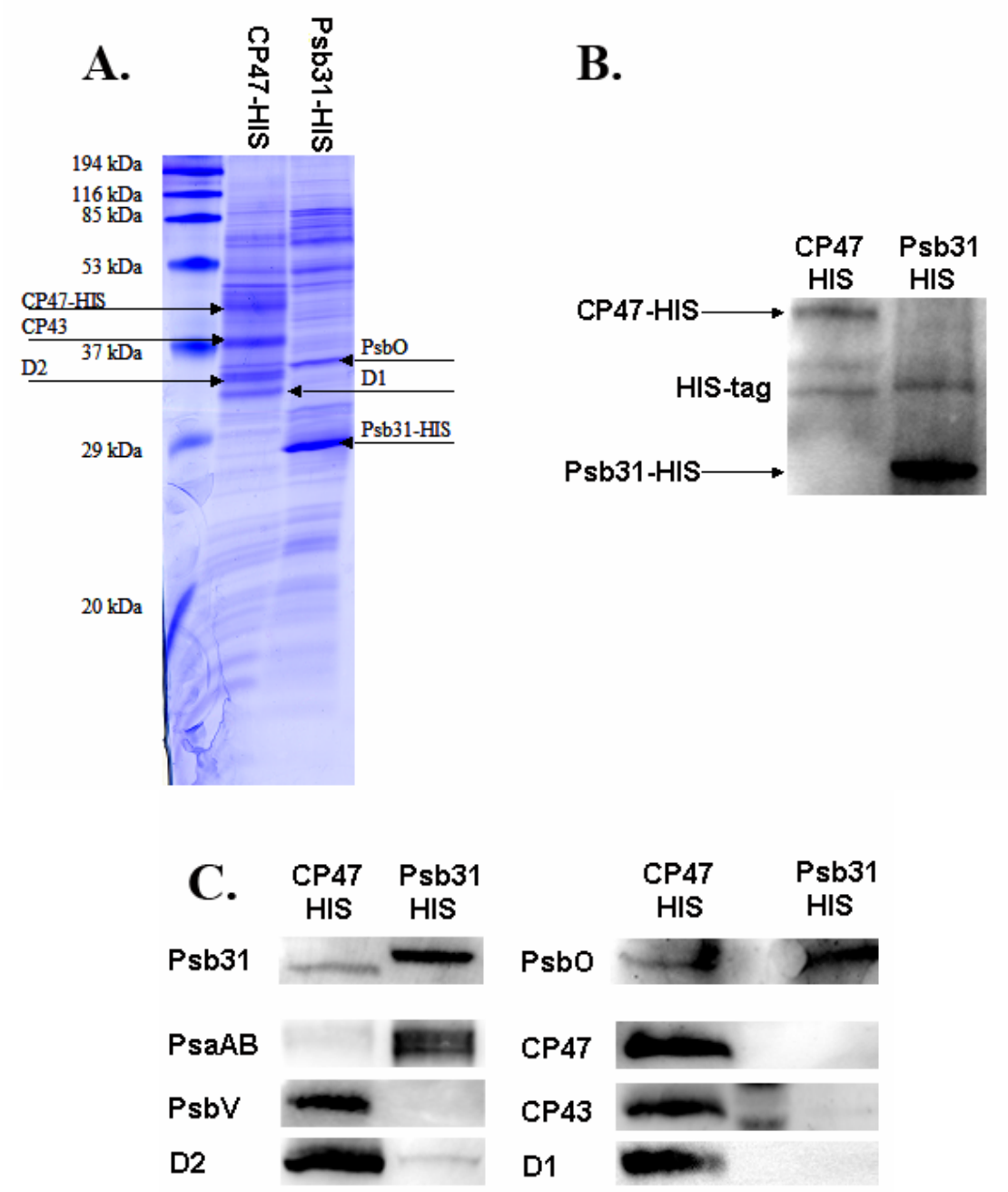


Figure 35 - Polypeptide composition of HIS tagged complexes: SDS-PAGE analysis of polypeptides from HT47GM PSII and Psb31-HIS preparations. 20 µg protein samples were loaded for both preparations.

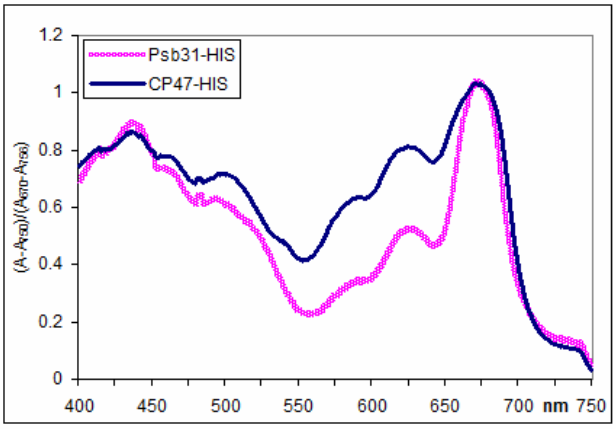
(**A**) Coomassie stained gel including the marker sizes and the identities of the most prominent proteins by arrow arrows.

(**B**) Both complexes probed for the HIS-tag.

(**C**) Both complexes probed against a variety of photosynthesis proteins.

The polypeptide composition of the complexes isolated by affinity chromatography from HT47GM and Psb31-HIS cultures are shown in **figure 36**. Proteins were identified based on their migration pattern in an SDS-PAGE system described by Yasuhiro Kashino *et al.* (2002) and by immunoblot detection. There are dramatic differences in the two preparations. The Psb31-HIS is a little larger than the WT protein due to the affinity tag and this histidine tag could be detected with a HIS specific monoclonal antibody.

In contrast to PsbQ-HIS (Roose et al., 2007a) or CP47-HIS (Kashino et al., 2002), the Psb31-HIS protein can not be used to purify PSII complexes. Notably the core PSII proteins could not be identified on Coomassie stained gels and only traces of D2, D1 and CP47 could be detected by immunoblot analysis. CP47 and PsbV could not be identified as part of the Psb31-HIS purification. Instead there is, compared to a CP47-HIS preparation, a higher amount of the PSI PsaAB protein detectable, although most PsaAB was found in the flow through fraction and did not bind to the column (data not shown). However, while most of the PSII proteins are not present or only present in very low abundance in the Psb31-HIS preparation, one PSII protein could be identified in high quantity. This is the lumenal manganese stabilizing protein, PsbO. These data indicate hat Psb31 does bind primarily to PsbO.



	CP47-HIS	Psb31-HIS
Chlorophyll in ng/µl	1020	30
Protein in µg/µl	17.2	1.2
Ratio µg protein / µg chl	16.8	40

Figure 36 - Absorption spectra of the Psb31-HIS purifications: Absorption spectra of the CP47-tagged PSII and the Psb31-HIS preparation were normalized to the Chlorophyll peak at 670 nm (**left**). The Chlorophyll / protein ratio was determined for both preparations (**right**).

The Psb31-HIS purification retained some chlorophyll, but far less than a PSII preparation (**figure 37**). Both preparations show high absorption in the carotenoid area between 400 and 550 nm. The CP-47 tagged preparation has a higher absorption between 550 and 650 nm corresponding to some retained phycobiliproteins. 77 K fluorescence spectroscopy of the Psb31-HIS complex revealed that no phycobilisomes are present and most of the chlorophyll shows a 77 K emission of 695 nm rather than 720 nm (**figure 38A**). This indicates that the chlorophyll bound to the Psb31-HIS purification is mainly associated to PSII. The small peak at 720 nm is corresponding to a small amount of PSI contamination. This explains the PsaAB protein seen in the immunoblots (**figure 38B**).

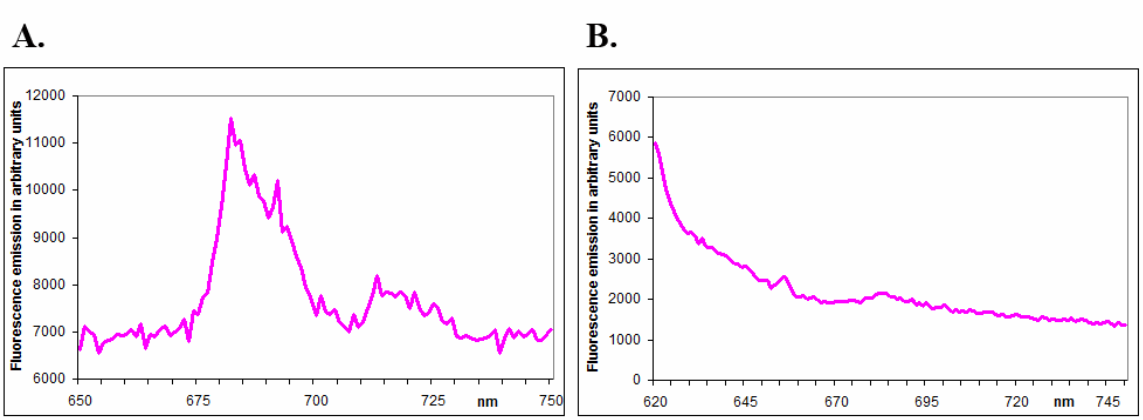


Figure 37 - 77K spectra of the Psb31-HIS complex: 77 K fluorescence emission spectra after excitation of chlorophyll (420 nm – **A**) and phycobilisomes (600 nm – **B**).

3.14 Psb31 Double mutants

Western analysis revealed that Psb31 levels are altered in mutants missing certain lumenal PSII subunits. To further investigate the relationship between Psb31 and the lumenal proteins, various double mutants were created by transforming the Δ*psb31* construct into single PSII gene deletion lines, namely Δ*psbO*, Δ*psbQ*, Δ*psbU*, Δ*psbV*. Segregation was confirmed by PCR of the *psb31* locus (**figure 39**).

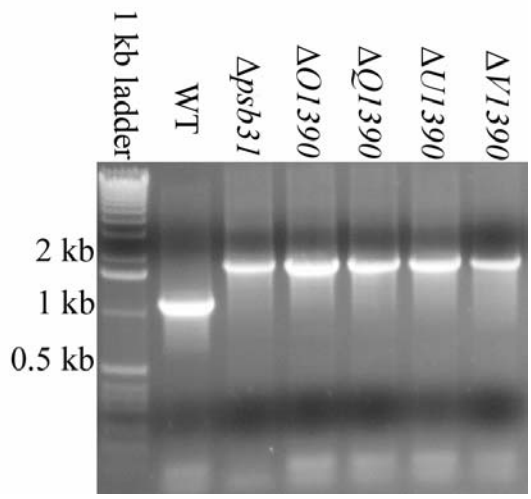


Figure 38 - Segregation of the double mutants: PCR analysis of the *psb31* locus in the Δ*psb31* double mutants to confirm complete segregation. WT (1177 bp) and Δ*psb31* (1776 bp) genomic DNA served as control.

The double mutants were assayed in their capacity to grow photoautotrophically (**figure 40**) and their oxygen evolution rates (**table 4**). The growth rates under regular growth light and under HL are similar. A deletion of *psbV* leads to increased sensitivity to manganese, so the Δ*psbV* mutants were assayed in low manganese media (1 µM instead of 9 µM in regular BG11 media).

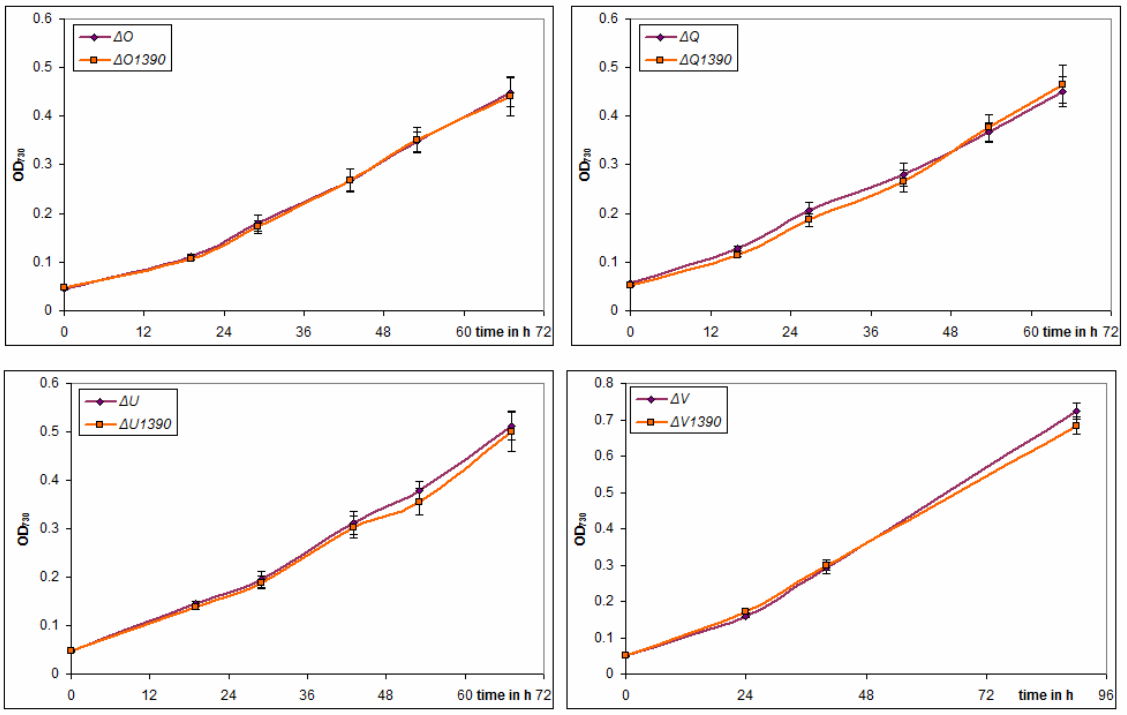


Figure 39 - Growth curves of the Δ*psb31* double mutants: Growth curves of the deletion mutants of lumenal PSII subunits Δ*psbO*, Δ*psbQ*, Δ*psbU* and Δ*psbV* compared to the Δ*psb31* double mutants Δ*O1390*, Δ*Q1390*, Δ*U1390* and Δ*V1390*. Grown under 45 µE/m^{2}s in BG11 except Δ*psbV* and Δ*V1390* (the Δ*psb31* – Δ*psbV* double mutant) which was grown in low manganese BG11 (1 µM). Errorbars represent SD of n=3.

Oxygen evolution capacities were measured on a Cark-type electrode under 2 different light intensities, and again the double mutants are not impaired compared to the single mutants, although a deletion of the lumenal subunits decreases the oxygen evolution rates compared to WT as described previously by Thornton *et al.* (2004) and Inoue-Kashino *et al.* (2005). This is obvious in particular for the Δ*psbO* and Δ*psbV* mutants whose oxygen evolution is strongly impaired under HL due to photodamage of the PSII machinery.

	Δ*psbO*	Δ*O1390*	Δ*psbQ*	Δ*Q1390*	Δ*psbU*	Δ*U1390*	Δ*psbV*	Δ*V1390*
800	130±15	148±17	256±36	226±19	177±23	176±9	185±46	181±10
8250	232±79	248±10	533±21	548±65	525±6	507±14	87±10	93±14

Table 4 - Oxygen evolution of lumenal PSII deletion mutants and Δ*psb31* double mutants: Log phase cultures were diluted to 6 µg/ml Chlorophyll and molecular oxygen evolution was measured at 800 and 8250 µE/m^{2}s light intensity in the presence of the electron acceptors DCBQ and Ferricyanide. Errorbars represent SD of triplicate measurements.

A deletion of the *psb31* gene does not seem to decrease the fitness compared to the single mutants Δ*psbO*, Δ*psbQ*, Δ*psbU* and Δ*psbV*.

IV Discussion

The aim this work is to investigate possible functions of the Psb31 protein in *Synechocystis* sp. PCC 6803. This protein that was originally found as part of a highly active PSII complex (Kashino et al., 2002). This protein was one of 5 novel proteins identified in the study. Two of these have subsequently been characterized as PSII specific proteins with PSII associated activity, namely Psb29 (Keren et al., 2005b) and PsbQ, a lumenal PSII protein previously thought to be present in eukaryotic PSII complexes only (Thornton et al., 2004). A homologue of Psb31 has been recently characterized in *Arabidopsis thaliana* to be involved in the turnover of the PSII core protein D1 and PSII dimerization (Sirpiö et al., 2007).

The *psb31* gene is found only in the genome of photosynthetic organisms. A homologue can be found in most cyanobacteria and all examined photosynthetic eukaryotes. Cyanobacteria lacking a copy of this gene are early branching species which have some key differences in composition and regulation of PSII compared to the majority of the photosynthetic organisms: for example, *Gloeobacter violaceus* lacks thylakoid membranes (Rippka, 1974) and certain lumenal PSII subunits (Thornton et al., 2005). Most *Prochlorococcus* strains and the thermophilic *Syneochcoccus* strains are also missing the *psb31* gene. The tight conservation of the gene shows that Psb31 function is conserved in photosynthetic organisms.

Further supportive evidence for a role of *psb31* in photosynthesis comes from microarray transcriptome analysis. The *psb31* homologue (*cce_4116*) in *Cyanothece* sp. ATCC 51142, a closely related cyanobacterium with robust diurnal patterns, shows co-expression with photosynthetic genes (**figure 7**).

The expression is maximal during the early light phase similar to the majority of the PSII genes. Interestingly, the expression of the core PSII genes *psaA1* and *psaD1* peaks later during the light phase.

There are multiple copies of those two genes in the *Cyanothece* 51142 genome but only one copy of the other PSII genes (Welsh et al., 2008). Multiple gene copies allow cyanobacteria to adapt to different environmental conditions. For example, the *psbA1* gene expression in *Synechocystis* sp. PCC 6803 is induced under low oxygen whereas *psbA2* is expressed under regular growth conditions (Summerfield et al., 2008).

PSI genes reach their maximal expression in *Cyanothece* 51142 hours after the PSII genes. The reason for this shift is unknown but one possibility is that the organism begins to shut down PSII activity earlier to lower the internal oxygen concentration in order to prepare the cell for the dark phase. Expression patterns do not necessarily mirror protein levels as translation and protein stability can also be regulated.

The study of Jana Stöckel *et al.* revealed that only ~30% of the genes in *Cyanothece* 51142 did oscillate during a 12h light / dark period and that these genes are primarily involved in core metabolic processes such as photosynthesis, respiration, energy metabolism, nitrogen fixation and amino acid biosynthesis (Stöckel et al., 2008). Nitrogen fixation takes place predominately during the dark as shown by the expression of *nifE*, which encodes a nitrogenase molybdenum-iron cofactor biosynthesis protein. In contrast to this pattern of expression during the dark period, the cycling pattern of the *psb31* homologue strongly suggests a function of Psb31 associated with photosynthesis, in particular to PSII (**figure 7**).

To study the function of the Psb31 protein, I constructed a *psb31* deletion strain by replacing 255 bp of the coding region of the *psb31* gene with a 850 bp chloramphenicol resistance gene cassette. Physiological characterization of the mutant under normal growth conditions showed no alteration in growth (**figure 18**), oxygen evolution (**figure 19**), fluorescence properties (**figure 20**) or pigment composition (**figure 21**) when compared to the *Synechocystis* 6803 WT. This implies that the Psb31 protein performs a function under non-standard conditions or that there is a redundancy with other proteins.

I generated a polyclonal antibody against the *Synechocystis* 6803 Psb31 protein that shows only limited cross-reactions to proteins far larger than the Psb31 protein (**figure 8**). In *Cyanothece* 51142, the Psb31 homologue is of the same size and shares 78% similarity and 58% identity with the truncated Psb31 protein used to generate the polyclonal antibody. Nonetheless, the antibody could not detect the homologue in *Cyanothece* 51142 membrane extracts. Neither could the antibody be used to visualize the eukaryotic Psb31 homologue TLP18.3 in *Arabidopsis thaliana*, which appears to be a 25 kDa protein (Sirpiö et al., 2007), or the spinach homologue. This shows that the antibody is highly specific for *Synechocystis* sp. PCC 6803.

The antibody was used to investigate the sub-cellular localization of the Psb31 protein. The protein is predicted to be a thylakoid protein with a C-terminal transmembrane helix (**figure 5**). Phase separation with Triton-X114 into a hydrophobic and a hydrophilic phase clearly showed that that Psb31 is indeed a hydrophobic protein like the PSII reaction center D2 protein and unlike the soluble PsbV protein. (**figure 9**).

Further separation of total membranes into plasma- and thylakoid membranes showed that Psb31 is predominantly located in the thylakoid membranes, but there are

also significant amounts of Psb31 in the plasma membrane (**figure 10**). The PSII core protein CP47 could only be identified in the thylakoid membrane showing that the plasma membrane fraction is free of thylakoid contamination. Furthermore, our own unpublished proteomic data of plasma and thylakoid membrane isolations support that Psb31 is partly localized in the plasma membrane in *Synechocystis* 6803. It has been proposed in *Synechocystis* that PSII reaction center cores are assembled in the plasma membrane and that they are photochemically competent (Zak et al., 2001; Keren et al., 2005a). But these cores are missing the lumenal PSII donor side cofactors and CP47 as well as CP43 which are incorporated only after the transfer of such PSII core complexes to the thylakoid membrane.

Such a stepwise assembling process protects cyanobacterial cells from potentially harmful consequences of performing water oxidation in a partially assembled PSII complex before it reaches its final destination in the thylakoid membrane. The PSII reaction is a high energy reaction as PSII needs to be able to oxidize water. A malfunction of the PSII reaction center generates reactive oxygen species capable of damaging PSII and the surrounding cell. A number of cofactors have been identified which are required during the life cycle of PSII. The periplasmic PratA protein, for example is required for preD1 processing (Klinkert et al., 2004) whereas the Psb27 protein facilitates the assembly of the Manganese cluster (Roose and Pakrasi, 2008) and Lpa2 is required for incorpararion of CP43. (Ma et al., 2007).

One lumenal subunit, PsbO, has also been identified in both plasma membrane and thylakoid membrane (Zak et al., 2001). However, TLP18.3, the *Arabidopsis* homologue of Psb31, has only been identified as part of thylakoid membrane preparations (Peltier et al., 2002; Schubert et al., 2002; Sirpiö et al., 2007). That does not rule out a partial localization in the inner or outer chloroplast membranes, but, in any

case, the dual membrane system of complex assembly does not seem to apply to plant chloroplasts. The presence of Psb31 in both plasma membrane and thylakoid membrane suggests that the protein might be involved in the trafficking of PSII components during the PSII lifecycle.

The fact that only a minority of CP47-HIS tagged PSII complexes contain Psb31 (ratio approximately 70:1) indicates that the protein is not part of the majority PSII complexes but rather bound transiently to a sub-fraction during the PSII life cycle, as it is the case with the PsbP protein. PsbP is bound to only 3% of CP47-HIS tagged PSII complexes (Thornton et al., 2004). The assembly factor Psb27 does also bind in sub-stoichiometric ratio to PSII and has been shown to interact with PSII pre-complexes that are incapable of evolving oxygen (Roose and Pakrasi, 2004; Nowaczyk et al., 2006). This suggests that a sub-stoichiometric amount of Psb31 is sufficient to provide its function in PSII while it is binding to a specific sub-population of PSII complexes.

The Psb31 levels are higher in total membranes of the Δ*psbQ* and Δ*psbU* strains (**figure 14**). However, the PSII complex isolated from Δ*psbQ* do not show a higher Psb31 level (**figure 15**). The altered Psb31 levels in deletion mutants which are missing lumenal subunits of PSII, indicate that the protein is interacting with proteins of the lumenal side of PSII. The high abundance of PsbO in the Psb31-HIS preparation (**figure 36**) makes it probable that Psb31 binds primarily to PsbO. Both Psb31 and PsbO are partially located in the plasma membrane. So it can be hypothesized that their co-localization is linked to their interaction. Maybe the Psb31-PsbO interaction is most important in the plasma membrane of cyanobacteria. If the PsbO protein is not incorporated into PSII until it gets to the plasma membrane, then perhaps Psb31 serves as a shuttle for this protein. Or Psb31 holds onto the PsbO protein in the thylakoid lumen during PSII turnover.

But deleting the *psb31* gene in the Δ*psbO*, Δ*psbQ*, Δ*psbU* or Δ*psbV* background does not further impair the photoautotrophic growth (**figure 40**) or oxygen evolution (**table 4**) of the single mutant under the conditions tested. This implies that the interaction with the lumenal subunits might not be the main function of Psb31 or that the interaction is only crucial under certain, not identified conditions.

There is less Psb31 in PSII isolated from a Δ*psbV* strain compared to WT (**figure 13**). This has been confirmed by a proteomic study of PSII isolated from the Δ*psvV* strain (Wegener et al., 2008). As reviewed by Roose *et al*. (2007b), PsbV is a low potential *c*-type cytochrome found only in cyanobacteria and red algae. It stabilizes the oxygen evolving complex and might act as an electron acceptor to remove excess electrons under conditions where photosynthesis is not maximal.

The same PSII preparation also contains a lower amount of PsbO. It has been shown that the absence of PsbV decreases the binding of PsbO to the PSII core, and a double mutant of Δ*psbO* and Δ*psbV* is no longer capable of photoautotrophic growth (Shen et al., 1995). The DUF477 domain of Psb31 has similarities to *β*-propellersheets of the methanol dehydrogenase type. These enzymes oxidize methanol and transfer the electrons to *c*-type cytochromes in methanogenic bacteria and were reviewed by Christopher Anthony (2004). Since PsbV is a *c*-type cytochrome, there is a possibility for an interaction in scavenging electrons away from the PSII reaction center. But PsbV could not be detected in pull downs with the truncated Psb31 protein expressed in *E.coli* or in the Psb31-HIS preparation. However, I could find PsbO in abundance co-purifying with Psb31-HIS (**figure 36**) , so the lower Psb31 levels in the Δ*psbV* PSII preparation are probably due to the lower PsbO levels rather than to an interaction with PsbV.

The Psb31-HIS preparation bound a significant amount of the PSI core protein PsaAB. Chlorophyll is also present in the preparation although on a much lower Chl/protein ratio compared to a PSII preparation (**figure 37**). 77 K spectroscopy reveals that the majority of the chlorophyll present in the preparation is associated with PSII rather than PSI (**figure 38**). So the high abundance of PsaAB compared to the chlorophyll binding PSII proteins CP43 and CP47 is surprising. The PsaAB antibody is very sensitive and most PsaAB did not bind to the column but was washed out with the flow through, so the presence of PsaAB might be an artifact.

Under blue light, which excites preferentially PSI, the ratio between the chlorophyll fluorescence associated with PSI and PSII drops as more PSII is generated to balance the activity of both photosystems. This process is called chromatic adaptation. The ratio drops more in the Δ*pb31* mutant than in WT. This indicates, that there is even more PSII compared to PSI in the mutant (**figure 31**). Surprisingly, on a protein level both WT and mutant seem to increase the total PSII protein levels in membrane extracts to a similar extend. A possible explanation is that, while the total protein levels are similar, the assembled PSII numbers might differ resulting in the different ratios.

Psb31 is not essential under standard growth conditions in *Synechocystis* sp. PCC 6803. In the mutant missing the *psb31* gene, growth rates and oxygen evolution under various light intensities are similar to WT. But forcing WT and mutant to compete directly with each other under high light (HL150 μE/m^2s) leads to the mutant being out-competed by the WT (**figure 25**). This is caused by only a minor defect, because cultures grown independently under this light intensity or an even higher one (220 μE/m^2s) do not show any growth deficiencies. This indicates that Psb31 is not necessary for growth under normal conditions, but under HL stress it performs a protective function. The total levels of Psb31 are not altered when grown under high light of 150 μE/m^2s indicating that

the protein is not induced under HL (**figure 30**). This corresponds with microarray data where the *psb31* gene was not found to be strongly regulated under HL conditions (Singh et al., 2008).

More photons are absorbed by the peripheral antenna complexes under HL. This leads to an increased activity of the photosynthetic reaction centers. This in turn increases the byproduct generation of reactive oxygen species which leads to oxidative stress. The decreased fitness of the Δ*psb*31 deletion mutant is caused specifically by an increased sensitivity to singlet oxygen stress (**figure 26**) rather then oxidative stress generated by hydrogen peroxide (**figure 28**). Singlet oxygen was induced by the photosensitizer Rose Bengal of which the Δ*psb31* mutant can not tolerate the same levels as the WT. Q_A reoxidation kinetics show that the electron transport in PSII is disrupted after the addition of Rose Bengal (**figure 27**). The initial drop in $F\text{-}F_0$ can be attributed to the sudden HL stress, but usually cultures adapt within 6 hours and regain a higher chlorophyll fluorescence. In presence of Rose Bengal the PSII activity continuous to drop for the Δ*psb31* mutant and only the WT is able to recover within 24 hours. By that time the photosensitizer is degraded but the mutant still shows no PSII activity, moreover the culture is completely bleached and barely re-greens over the next 2-3 days meaning that most Δ*psb31* cells are death. This shows that the Psb31 protein is essential in *Synechocystis* 6803 to overcome singled oxygen stress.

Singlet oxygen is mainly generated *in vivo* by energy transfer from excited pigments in the light-harvesting complexes (Zolla and Rinalducci, 2002), from excited Fe-S centers in PSI (Chung, 1995), and it is also generated from nonfunctional PSII that has been photo-damaged (Anderson, 2001). Hydrogen peroxide is generated mainly by the enzymatic or spontaneous reduction of superoxide, which is produced most abundantly on the acceptor side of PSI (Asada, 1996).

It has been shown in *Synechocystis* 6803 that both reactive oxygen species, singlet oxygen generated by the photosensitzer Rose Bengal (Nishiyama et al., 2004) and hydrogen peroxide (Nishiyama et al., 2001) act primarily by inhibiting the repair of photodamaged PSII *in vivo*. This happens in particular by inhibiting the synthesis of the PSII core protein D1 in the elongation step of the translation. The different sensitivity to the two reactive oxygen species of the Δ*psb31* mutant indicates that there are differences in the damage control mechanisms towards these two reactive oxygen species.

Sirpiö *et al.* described the *Arabidopsis* homologue of Psb31 and linked its function to the turnover of the D1 protein and PSII dimerization (Sirpiö et al., 2007). However, I found photoinhibition and recovery are not affected in the Δ*psb31* deletion mutant in *Synechocystis* 6803 (**figure 29**).

Singlet oxygen has been shown to be generated by illuminated PSII complexes isolated from *Pisum sativum* (Telfer et al., 1994a) and is efficiently quenched by *β*-carotene (Telfer et al., 1994b). There have been 11 *β*-carotenes identified in the 3.0 Å PSII dimmer crystal structure (Loll et al., 2005) and the removal of one carotenoid in close proximity to the D1 protein drastically increased the yield of singlet oxygen (Telfer et al., 1994b). Psb31 does not have any sequence similarity to the other described carotenoid binding proteins. The best characterized is the orange carotenoid protein (OCP) which has been shown to quench singlet oxygen in *Arthrospira maxima* (Kerfeld et al., 2003). A higher susceptibility for singlet oxygen might be due to defects in this detoxification mechanism, but the carotenoid levels are not altered in the Δ*psb31* strain. However, in pull down experiments with the truncated Psb31 protein synthesized in *E.coli,* it did bind a carotenoid pigment (**figure 32-33**). The shift in the absorption compared to *β*-carotene can be explained by the environmental conditions as both, the differences in the buffer composition and the presence of carotene binding partners, do

affect the absorption spectra. Of course, the captured carotenoid might also be a different carotenoid altogether.

No carotenoids were captured in control experiments with a different HIS-tagged bait, the nitrate reductase transporter subunit NrtA, or by running the extracts directly over a Ni-Agarose column (**figure 32**). This shows that the interaction is specific for the truncated Psb31 protein and not due to interaction with the HIS-tag or the column matrix. Carotenoids were also present in the *Synechocystis* 6803 1390-HIS preparation as detected in absorption spectra (**figure 37**), but they were not seen visually due to the abundance of chlorophyll.

The DUF477 domain of unknown function in Psb31 has similarities to *β*-propellersheets of the methanol dehydrogenase type. In methylotrophic bacteria methanol dehydrogenases oxidize methanol and transfer the electrons to *c*-type cytochromes as reviewed by Christopher Anthony (2004). If Psb31 retained some properties of *β*-propellersheets, a function of the protein in the singlet oxygen detoxifying mechanism can be hypothesized. Psb31 could be involved in detoxifying singlet oxygen by assisting its quenching by carotenoids. Reactive oxygen species might also be generated by partial assembled PSII because, if the proper redox environment is not present, the chance of a wrong electron transfer is higher. To prevent this kind of malfunction, partial assembled PSII complexes have to be kept inactive. It is possible that the Psb31 protein does bind to a pre-complex which has just bound PsbO but not the rest of the oxygen evolving complex in order to prevent the accidental generation of reactive oxygen species.

The investigation of the Sll1390 protein lead me to the conclusion that it is indeed a protein involved with PSII and can therefore be named Psb31.

V-1 Summary

The aim of this thesis is to investigate possible functions of the Psb31 protein in the cyanobacterium *Synechocystis* sp. PCC 6803. This protein was originally identified by Yasuhiro Kashino *et al*. (2002) as part of an active PSII preparation and is present only in prokaryotic and eukaryotic photosynthetic organisms. The *Arabidopsis* homologue has been show to be involved in D1 turnover and PSII dimerization. The expression of the psb31 homologue in *Cyanothece* sp. ATCC 51142 is co-regulated with photosynthetic genes, in particular PSII genes.

A deletion of the *psb31* gene does not impair photoautothrophic growth, but in competition experiments the mutant is out-competed under high light stress. This phenotype is minor as when grown separately, the growth rates and physical properties of Δ*psb31* and WT are similar. It could be shown that the decreased fitness is caused by a higher sensitivity to reactive oxygen species, in particular to singlet oxygen rather than to hydrogen peroxide.

Singlet oxygen is generated as byproduct of the PSII reaction and is thought to inhibit D1 protein synthesis by interfering with the elongation step of translation, but I found that photoinhibition and recovery are not affected in the Δ*psb31* mutant. However singlet oxygen is efficiently quenched by carotenoids and I found that a truncated Psb31 protein expressed in *E.coli* binds carotenoids.

A polyclonal antibody against Psb31 was generated and I could show that the protein is a membrane protein located mainly in thylakoid membrane but also present in the plasma membrane. It is part of highly active PSII preparations, but only in sub-stoichiometric amounts. Under HL the total amount of Psb31 is not altered.

In mutants where lumenal subunits of PSII are missing, the Psb31 levels are altered. In particular, there is more Psb31 present in membranes of Δ*psbQ* and Δ*psbU* strains.

In PSII preparations of Δ*psbV* strain, I found lower Psb31 levels compared to WT PSII, but in the Δ*psbV* mutant, the PsbO levels are reduced as well and I could co-purify PsbO with a HIS-tagged Psb31 preparation.

These findings indicate that Psb31 interacts with the lumenal side of PSII, in particular with the PsbO protein. Its function might be to transiently bind and prevent the generation of oxidative stress generated by singled oxygen. It supports quenching by carotenoids in pre-assembled PSII complexes. I suggest there is redundancy in the functions of Psb31, since the deletion mutant does not generate a stronger phenotype.

V-2 Zusammenfassung

Diese Arbeit beschäftigt sich mit der erstmaligen Untersuchung des Psb31 Protein in dem Cyanobakterium *Synechocystis* sp. PCC 6803. Dieses Protein wurde 2002 als Bestandteil einer aktiven PSII Präparation identifiziert (Kashino et al., 2002) und ist ausschließlich in photosynthetisch aktiven Organismen konserviert. Die Expression des Genes in dem Cyanobakterium *Cyanothece* sp. ATCC 51142 ist zyklisch und koregulliert mit PSII Genen.

Ein Ausschalten des *psb31* Genes führt nicht zu einer Beeinträchtigung des photoautotrophen Wachstums, jedoch wird die Mutante in Konkurrenzexperimenten unter hohen Lichtintensitäten vom Wildtyp verdrängt. Der Selektionsnachteil ist jedoch gering, da getrennt wachsende Kulturen von WT und Δ*psb31* die gleiche Wachstumsrate aufweisen.

Ich konnte zeigen, dass der Selektionsnachteil auf eine erhöhte Sensitivität für reaktive Sauerstoffmoleküle zurückzuführen ist. Dabei ist die Sensitivität auf Stress durch Singuletsauerstoff und nicht Wasserstoffoperoxid zurückzuführen.

Es wird angenommen, dass Singuletsauerstoff vor allem die Neusynthese des D1 Protein inhibiert, jedoch konnte ich zeigen, dass in der Δ*psb31* Mutante Photoinhibierung und Erholung nicht beinträchtig sind. Dies lässt auf einen weiteren Wirkmechanismus schließen. Singuletsauerstoff wird *in vivo* durch Carotenoide abgefangen, und ich konnte mit einem verkürzten Psb31 Protein aus Extrakten des Cyanobakteriums Carotenoide isolieren.

Durch die Herstellung eines polyklonalen Antikörpers war ich in der Lage, die intrazelluläre Lokalisierung des Psb31 Proteins in der Thylakoidmembran festzustellen. Jedoch ist das Protein zu einem geringeren Anteil auch in der Plasmamembran nachzuweisen. Das Protein ist Bestandteil einer nur kleinen Fraktion der PSII Komplexe, was auf eine vorübergehende Bindung an den Komplex weist.

Die Menge des Psb31 Proteins ist in Mutanten, in denen eine lumenale Untereinheit des PSII Komplexes ausgeschaltet wurde, verändert. Insbesondere in Δ*psbQ* and Δ*psbU* Membranen ist mehr Psb31 vorhanden. PSII Präpärationen der Δ*psbV* Mutante haben Anteilsmäßig weniger Psb31 aufzuweisen, jedoch ist in dieser Mutante auch weniger PsbO gebunden. Das PSII Protein PsbO konnte als Bestandteil einer Psb31-HIS-tag Aufreinigung nachgewiesen werden. Daher ist anzunehmen, dass PsbO ein Interaktionspartner von Psb31 ist.

Meine Untersuchungen lassen auf eine vorübergehende Interaktion von Psb31 mit den lumenalen PSII Proteinen, insbesondere mit PsbO schließen. Eine mögliche Funktion des Psb31 besteht in darin, dass es an teilweise assemblierte Komplexe bindet und dazu beträgt, dass diese keinen reaktiven Sauerstoff freisetzen. Psb31 kann das Löschen dieser Moleküle durch gebundene Carotenoide fördern. Diese Funktion ist für die Zelle unter regulären Bedingungen nicht überlebenswichtig, denn ein Ausschalten des Genes hat nur einen begrenzten Einfluss auf die Fitness von *Synechocystis* 6803.

VI Literature

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Publications

Stöckel, J., Bennewitz, S., Hein, P., and Oelmüller, R. (2006) The Evolutionarily Conserved Tetratrico Peptide Repeat Protein Pale Yellow Green7 Required for Photosystem I Accumulation in Arabidopsis and Copurifies with the Complex. *Plant Physiol* 141(3): 870–878.

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Bennewitz, S., Pakrasi, H.B. (*In preparation*) Psb31 (Sll1390), a photosystem II protein, provides high light protection in *Synechocystis* sp. PCC 6803.

Poster presentations

03/04 17th Molekularbiologie der Pflanzen Meeting, Dabringhausen, GER
Stöckel J, Hein P, Bennewitz S and Oelmüller R
"Charakterisierung zweier kernkodierter regulatorischer Proteine der Photosystem I-Biogenese"

10/05 31st Midwest/Southeast Photosynthesis Meeting, Turkey Run, IN
Roose J, Sarkar A, Bennewitz S, Pakrasi HB and Bhattacharyya-Pakrasi M
"Manganese and photosynthesis in cyanobacteria"

06/07 9th Cyanobacterial Workshop, Lake Delavan, WI
Bennewitz S, Roose J, Stöckel J, Oelmüller R and Pakrasi HB
"Investigations on Psb30 (Sll1390), a PSII associated polypeptide of unknown function in *Synechocystis sp.* PCC 6803"

10/07 33rd Midwest Photosynthesis Meeting, Turkey Run, IN
Roose J, Bennewitz S, Bhattacharyya-Pakrasi M and Pakrasi HB
"Manganse homeostasis in the Photosystems II *ΔpsbV* mutant"

10/08 34th Midwest Midwest Photosynthesis Meeting, Turkey Run, IN
Bennewitz S, Oelmüller R and Pakrasi HB
"Psb31 (Sll1390), a photosystem II protein, provides high light protection in *Synechocystis* sp. PCC 6803"

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Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Es wurde weder die Hilfe eines Promotionsberaters noch die Hilfe Dritter, nicht in dieser Arbeit erwähnten Personen, in Anspruch genommen. Diese Arbeit wurde weder in dieser noch in ähnlicher Form bei einer anderen Hochschule als Dissertation oder Prüfungsarbeit eingereicht.

Jena, den 6 Januar 2009

Stefan Bennewitz