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OXYGEN PHOTOREDUCTION IN CYANOBACTERIA

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ABBREVIATIONS

ARTO	alternative oxidase
ATP	adenosine triphosphate
BG11/BG11o	growth medium for cyanobacteria with/without combined N
BN-PAGE	blue native polyacrylamide gel electrophoresis
C_i	inorganic carbon
C_3	metabolic pathway of carbon fixation with 3-PGA primary product
C2	plant-type photorespiration cycle
cDNA	complementary DNA
CET	cyclic electron transfer
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Chl	Chlorophyll *a*
CCM	carbon concentrating mechanism
cis-asRNA	antisense RNA encoded by the gene overlapping the target gene
Cm^R	chloramphenicol resistance cassette
Cox	cytochrome *c* oxidase
Cyd	quinol oxidase
Cyt	cytochrome
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCP	dichlorophenol
DCPIP	2,6-dichlorophenol indophenol
DIGE	differential gel electrophoresis
DMBQ	2,5-dimethyl-*p*-benzoquinone
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
F_0	the minimal level of fluorescence in the dark
F_m	the maximum fluorescence in the dark
F_m'	the maximum level of fluorescence under the light
F_s	the level of steady-state fluorescence under the light
FAD	flavin adenine dinucleotide
FD	ferredoxin
FDP, Flv	flavodiiron protein

FL	fluctuating light
FLD	flavodoxin
FMN	flavin mononucleotide
FNR(S/L)	ferredoxin:$NADP^+$ oxidoreductase (short/long form)
Fox^-	incapable of N_2 fixation in the presence of O_2
GA	glycolaldehyde
HEP	polysaccharide layer of heterocyst-specific envelope
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGL	glycolipid layer of heterocyst-specific envelope
HL	high light phase of fluctuating light
HPLC	high-performance liquid chromatography
IAC	iodoacetamide
Km^R	kanamycin resistance cassette
LED	light-emitting diode
LET	linear electron transfer
LL	low light phase of fluctuating light
MIMS	membrane inlet mass spectrometry
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NDH	NADH dehydrogenase
nif	N_2 fixation-related genes
Nm^R	neomycin resistance cassette
OCP	orange carotenoid protein
OD_{750}	optical density at 750 nm
OEC	oxygen evolving complex
OPPP	oxidative pentose phosphate pathway
$P680/P680^+$	red/ox primary electron donor of Photosystem II
$P700/P700^+$	red/ox primary electron donor of Photosystem I
P_m	the maximum level of oxidizable P700
P	the level of the P700 signal under the light

P_m'	the maximum level of oxidizable P700 under the light
PAR	photosynthetically active radiation
2-PG	2-phosphoglycolate
3-PGA	3-phosphoglycerate
PBS	phycobilisomes
PC	plastocyanin
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
PETC	photosynthetic electron transfer chain
pH	negative logarithm of the proton concentration
PS	photosystem
PQH2/PQ	plastoquinol/plastoquinon
RNA	ribonucleic acid
ROS	reactive oxygen species
RTO	respiratory terminal oxidase
RT-qPCR	real-time quantitative reverse transcription PCR
Rubisco	ribulose bisphosphate carboxylase/oxygenase
RuBP	ribulose-1,5-bisphosphate
SHAM	salicylhydroxamic acid
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulfate
sp.	species
Sp^R	spectinomycin resistance cassette
TCA	tricarboxylic acid cycle
TES	2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid
TSS	transcription start site
WT	wild type
Y(I)	the yield of Photosystem I
Y(II)	the yield of Photosystem II
Y(NA)	the yield of acceptor side limitation of Photosystem I
YFP	yellow fluorescent protein
Y_Z	tyrosine residue of the D1 protein of Photosystem II.

ABSTRACT

Cyanobacteria are well-known for their role in the global production of O_2 via photosynthetic water oxidation. However, with the use of light energy, cyanobacteria can also reduce O_2. In my thesis work, I have investigated the impact of O_2 photoreduction on protection of the photosynthetic apparatus as well as the N_2-fixing machinery.

Photosynthetic light reactions produce intermediate radicals and reduced electron carriers, which can easily react with O_2 to generate various reactive oxygen species. To avoid prolonged reduction of photosynthetic components, cyanobacteria use "electron valves" that dissipate excess electrons from the photosynthetic electron transfer chain in a harmless way. In *Synechocystis* sp. PCC 6803, flavodiiron proteins Flv1 and Flv3 comprise a powerful electron sink redirecting electrons from the acceptor side of Photosystem I to O_2 and reducing it directly to water. In this work, I demonstrate that upon C_i-depletion Flv1/3 can dissipate up to 60% of the electrons delivered from Photosystem II. O_2 photoreduction by Flv1/3 was shown to be vital for cyanobacteria in natural aquatic environments and deletion of Flv1/3 was lethal for both *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 under fluctuating light conditions. The lethal phenotype observed in the absence of Flv1/3 results from oxidative damage to Photosystem I, which appeared to be a primary target of reactive oxygen species produced upon sudden increases in light intensity. Importantly, cyanobacteria also possess other O_2 photoreduction pathways which can protect the photosynthetic apparatus. This study demonstrates that respiratory terminal oxidases are also capable of initiating O_2 photoreduction in mutant cells lacking the Flv1/3 proteins and grown under fluctuating light. Photoreduction of O_2 by Rubisco was also shown in C_i-depleted cells of the mutants lacking Flv1/3, and thus provided the first evidence for active photorespiratory gas-exchange in cyanobacteria. Nevertheless, and despite the existence of other O_2 photoreduction pathways, the Flv1/3 route appears to be the most robust and rapid system of photoprotection.

Several groups of cyanobacteria are capable of N_2 fixation. Filamentous heterocystous N_2-fixing species, such as *Anabaena* sp. PCC 7120, are able to differentiate specialised cells called heterocysts for this purpose. In contrast to vegetative cells which perform oxygenic photosynthesis, heterocysts maintain a microoxic environment for the proper function of the nitrogenase enzyme, which is extremely sensitive to O_2. The genome of *Anabaena* sp. PCC 7120 harbors two copies of genes encoding Flv1 and Flv3 proteins, designated as "*A*" and "*B*" forms. In this thesis work, I demonstrate that Flv1A and Flv3A are expressed only in the vegetative cells of filaments, whilst Flv1B and Flv3B are localized exclusively in heterocysts. I further revealed that the Flv3B protein is most responsible for the photoreduction of O_2 in heterocysts, and that this reaction plays an important role in protection of the N_2-fixing machinery and thus, the provision of filaments with fixed nitrogen. The function of the Flv1B protein remains to be elucidated; however the involvement of this protein in electron transfer reactions is feasible. Evidence provided in this thesis indicates the presence of a great diversity of O_2 photoreduction reactions in cyanobacterial cells. These reactions appear to be crucial for the photoprotection of both photosynthesis and N_2 fixation processes in an oxygenic environment.

TIIVISTELMÄ

Syanobakteerien tiedetään osallistuvan maailmanlaajuiseen hapentuotantoon fotosynteesissä tapahtuvan veden hajotuksen, eli kemiallisesti veden hapetuksen, kautta. Valoenergian avulla syanobakteerit myös pelkistävät ilmakehän happea, jolloin lopputuotteena syntyy vettä. Väitöskirjatyössäni olen tutkinut hapen valopelkistyksen molekyylimekanismeja ja roolia syanobakteerien fotosynteesi- ja typensidontakoneistojen suojelemisessa.

Fotosynteesin valoreaktioissa syntyy välituotteina monenlaisia radikaaleja ja pelkistyneitä elektroninsiirtäjiä, jotka reagoivat herkästi hapen kanssa synnyttäen erilaisia reaktiivisia happilajeja. Välttääkseen fotosynteesikoneiston pitkittynyttä pelkistymistilaa, syanobakteerit käyttävät ”elektroniventtiilejä”, joiden kautta ne purkavat fotosynteesikoneistoon kertyneet ylimääräiset elektronit vaarattomasti. *Synechocystis* sp. PCC 6803:n flavodiironproteiinit Flv1 ja Flv3 muodostavat tehokkaan elektroninielun ohjaamalla elektroneja valoreaktio I:n pelkistävältä puolelta suoraan hapelle pelkistäen sen vedeksi. Tässä työssä osoitan, että kun syanobakteereja kasvatetaan matalassa hiilidioksidipitoisuudessa (tasapainossa ympäröivän ilman kanssa), Flv1/3 proteiinipari pystyy kuluttamaan jopa 60% valoreaktio II:n veden hajotuksesta johdetuista elektroneista. Flv1/3-välitteisen hapen valopelkistyksen näytettiin olevan elintärkeää syanobakteereille luonnollisessa vesiympäristössä ja Flv1/3 proteiinien poistaminen johti sekä *Synechocystis* sp. PCC 6803:n että *Anabaena* sp. PCC 7120:n kuolemaan vaihtelevan valon olosuhteissa. Flv1/3:n puuttumisen aiheuttaman letaalin ilmiasun osoitettiin johtuvan valoreaktio I:n foto-oksidatiivisesta vaurioitumisesta: äkillinen valointensiteetin nousu johtaa reaktiivisten happilajien muodostumiseen, ja valoreaktio I näytti olevan näiden happilajien pääasiallinen kohde. Flv1/3:n lisäksi syanobakteereilla on myös muita hapen valopelkistämisreittejä, jotka voivat toimia fotosynteesikoneiston suojaamisessa. Tämä tutkimus osoittaa, että hengitysreitin terminaaliset oksidaasit kykenevät myös hapen valopelkistykseen vaihtelevan valon olosuhteissa mutanttisoluissa, joilta puuttuvat Flv1 ja 3 proteiinit. Matalassa hiilidioksidissa näissä mutanttisoluissa voitiin mitata myös Rubisco-välitteistä hapen valopelkistymistä, osoittautuen ensimmäiseksi raportiksi syanobakteerien aktiivisesta fotorespiratorisesta kaasujen vaihdosta. Huolimatta muista hapen valopelkistysreiteistä, Flv1/3 välitteinen reitti näyttäisi olevan kuitenkin vahvin ja nopein fotosynteesikoneiston suojausmekanismi.

Monet syanobakteeriryhmät kykenevät ilmakehän typen sidontaan. Rihmamaiset typpeä sitovat syanobakteerit, kuten *Anabaena* sp. PCC 7120, kykenevät muodostamaan typen sidontaan erilaistuneita soluja: heterokystejä. Toisin kuin yhteyttävissä vegetatiivisissa soluissa, heterokysteissä ylläpidetään lähes hapetonta ympäristöä, joka vaaditaan happiherkän nitrogenaasientsyymin toimintaan. *Anabaena* sp. PCC 7120:n genomissa on Flv1 että Flv3 proteiineja koodaavista geeneistä kaksi kopiota, niin kutsutut ”A” ja ”B” muodot. Väitöskirjatyössäni osoitan, että Flv1A ja Flv3A ilmentyvät vain syanobakteeririhmojen vegetatiivisissa soluissa, kun taas Flv1B ja Flv3B sijaitsevat ainoastaan heterokysteissä. Lisäksi näytän, että Flv3B on pääasiassa vastuussa hapen valopelkistyksestä heterokysteissä ja että tämä reaktio on tärkeä typpeä sitovan koneiston suojaamisessa ja siten takaa rihmojen typen saannin. Flv1B proteiinin tarkka rooli on vielä selvittämättä, mutta on mahdollista, että se osallistuu elektroninsiirtoreaktioihin. Tässä väitöskirjassa esitetty todistusaineisto viittaa siihen, että syanobakteereilla on useita hapen valopelkistysreittejä. Nämä reaktiot ja reitit ovat välttämättömiä sekä fotosynteettisen että typpeä sitovan koneiston suojelemiseksi hapelta, jota fotosynteesi jatkuvasti tuottaa veden hajotusreaktioissaan.

Моей маме

1. INTRODUCTION

1.1 Significance of cyanobacteria

Cyanobacteria comprise a unique group of prokaryotes capable of oxygenic photosynthesis. During photosynthesis, sunlight energy is converted into the energy of chemical bonds using electrons extracted from water and releasing O_2 as a by-product. This process was initiated by cyanobacteria at least 2.5 billion years ago, when the first traces of O_2 in the atmosphere and surface of the ocean can be tracked (Knoll 2008, Bekker et al. 2004, Holland 2006). However, some evidence points to the appearance of cyanobacteria as far as 3.5 billion years ago, in the Archean Eon (Schopf 2006). After this time, and until the end of Proterozoic Eon (0.5 billion years ago), cyanobacteria were the principal primary producers on the Earth.

Nowadays, cyanobacteria contribute to about 30% of global annual CO_2 fixation. However, when considering ancient cyanobacteria as the ancestors of plant chloroplasts (McFadden 2001, Gould et al. 2008), they can be designated as responsible for the majority of primary production on our planet (Bryant 2003). Time-wise, the estimated period for the endosymbiotic event in which cyanobacteria enabled eukaryotic photosynthesis is in the mid-Proterozoic Eon (Yoon et al. 2004). However, cyanobacteria have not only given a rise to the evolution of plants, but have also tremendously contributed to the appearance and heyday of O_2-based heterotrophy. Initially, the Earth's atmosphere did not contain any O_2, but the photosynthetic activity of cyanobacteria gradually led to an increase in O_2 concentration. As a consequence, organisms developed mechanisms of aerobic respiration in order to obtain energy using O_2 as a strong terminal acceptor of electrons; this being a key factor in the appearance of mitochondria and complex eukaryotic life. In addition, the ozone layer, which was created upon O_2 accumulation, provided protection from UV light and allowed the later terrestrial distribution of life (Blankenship 2010). Yet, cyanobacteria did not only determine the development of the biosphere in past. Currently, photosynthesis-based biofuel production is a promising approach for solving the energetic issues of the future (Dismukes et al. 2008, Janssen et al. 2014).

Aside from photosynthesis, another impact of cyanobacteria in global life is based on their N_2-fixing ability. Atmospheric N_2 is mostly inert and cannot be easily used by living organisms. Diazotrophic Bacteria and Archaea are entirely responsible for the conversion of N_2 into biologically available form, and

cyanobacteria account for the bulk of N_2 fixation in oceans (Montoya et al. 2004). It is possible that a "lag-phase" in O_2 accumulation, preceding the great oxygenation event, was caused by a limitation in the photosynthetic activity of cyanobacteria, related to their inability to perform N_2 fixation (Grula 2005). The later evolution of cyanobacterial N_2 fixation, combined with oxygenic photosynthesis, led to the ultimate dominance of cyanobacteria until the end of Proterozoic Eon and further increased the biological capacity of ancient oceans (Anbar and Knoll 2002).

1.2 Taxonomy of cyanobacteria

Cyanobacteria can be found in wide variety of forms and therefore traditional taxonomy is based on the morphology and development of cells and filaments. On the basis of this, five principal sections have been recognized by Rippka et al. (1979): (I) unicellular species which could also aggregate in colonies (order Chroococcales); (II) unicellular species capable of division in multiple planes (order Pleurocapsales); (III) filamentous cyanobacteria without differentiation of cells (order Oscillatoriales); (IV) heterocyst-forming filamentous cyanobacteria; and (V) filamentous species with complex branched trichomes. Phylogenetic approaches later confirmed that orders Nostocales (IV) and Stigonematales (V) are monophyletic groups, but other sections were found to consist of species of different phylogenetic origin (Sanchez-Baracaldo et al. 2005). Nevertheless, both morphological and molecular approaches are consistent in suggesting the evolution of filamentous cyanobacteria from unicellular, and the later appearance of cell differentiation.

Another classification of cyanobacteria is based on the type of Rubisco and carboxysomes and reflects the ecological distribution of different species. The α-cyanobacteria are marine species mostly found in oceans; they have α-carboxysomes with Form-IA Rubisco (Badger et al. 2002, 2006). This group of cyanobacteria contributes to about one half of primary productivity in the ocean (Field 1998). The β-cyanobacteria are ecologically diverse and might be found in a broad range of habitats from fresh water to hot springs; they have β-carboxysomes and Form-IB Rubisco (Badger et al. 2002, 2006). Carboxysomes of both types share a common origin, but have distinguished interior proteins (Kerfeld et al. 2005). However, they have likely appeared via convergent evolution and there is no significant difference in the amount and capacity of Rubisco between α and β-cyanobacteria (Whitehead et al. 2014).

Synechocystis sp. PCC 6803 and *Anabaena* (also called *Nostoc*) sp. PCC 7120 (hereafter *Synechocystis* and *Anabaena,* respectively) are model organisms used in this work. *Synechocystis* is a unicellular, non-N_2-fixing, freshwater cyanobacterium. It belongs morphologically to Section I and ecologically to β-cyanobacteria (Rippka et al. 1979). The *Synechocystis* genome was the third sequenced of prokaryotes and the first of photosynthetic organisms (Kaneko et al. 1996). The amenability of *Synechocystis* to genetic transformation, with exogenous DNA via homologous double recombination, ensured its use as a model organism for research of photosynthesis and CO_2 fixation, stress reactions and plastid evolution (Labarre et al. 1989, Koksharova and Wolk 2002).

Anabaena is a filamentous, heterocystous, N_2-fixing β-cyanobacterium and is well-known for forming blooms in aquatic ecosystems. Under certain conditions, some cells of *Anabaena* filaments terminally differentiate into heterocysts, special cells performing fixation of atmospheric N_2 into ammonium (Flores and Herrero 2010). *Anabaena* belongs to Section IV and has a close phylogenetic relationship to the genera *Nostoc*, *Calothrix*, and *Nodularia*. Available genome sequence and methods developed for genetic transformation facilitated a wide use of *Anabaena* as a model organism for cell differentiation and N_2 fixation (Wolk et al. 1984, Kaneko et al. 2001).

1.3 Photosynthetic machinery of cyanobacteria

Cyanobacteria are gram-negative bacteria and their cell wall includes an outer membrane and plasma membrane with a peptidoglycan layer in between (Hoiczyk and Hansel 2000). Beyond the cell wall, carbohydrate-enriched glycocalyx protects cells from desiccation and predators. The membrane system inside the cells also includes photosynthetic membranes, which are structurally and functionally different from the plasma membrane (Liberton and Pakrasi 2008). Carboxysomes, lipid bodies and polyphosphate bodies can also be visually recognized in the cytosol of cyanobacteria (Nierzwicki-Bauer et al. 1983).

Photosynthetic thylakoid membranes form a continuous intact compartment separated from cytosol, designed as lumen, with similar functions as in plant chloroplasts. Similarly, the cytoplasmic space around thylakoid membranes is functionally related to the stroma of chloroplasts. Therefore, the photosynthesis-related mechanism of ATP production, including translocation of protons from stroma (or cytoplasm) to the lumen, is conserved in both cyanobacteria and plants. However, as prokaryotes, cyanobacteria do not have a developed, compartmentalized cell system. Therefore, in contrast to eukaryotic algae and

plants, in cyanobacteria respiratory and photosynthetic pathways share, at least partially, the thylakoid membranes (Mullineaux 2014b). This increases the complexity of the electron transport network in cyanobacteria and contributes to a fine-tuning of electron flows.

Photosynthetic reactions are in general divided into "light" and "dark" phases. The light-dependent phase is carried out by the major photosynthetic protein complexes embedded in the thylakoid membranes. In the light phase, the transfer of electrons from water-oxidizing Photosystem II (PS II) to Cytochrome b_6f (Cyt b_6f), then to Photosystem I (PS I) and finally to $NADP^+$ through the membrane, establishes a proton gradient across the membrane, resulting in ATP synthesis. Linear electron transfer (LET) involves all above mentioned components but electrons can also be directed to auxiliary electron transfer pathways, including cyclic electron transfer (CET). NADPH and ATP obtained under the light are used in the so-called "dark" phase of photosynthesis for the fixation of CO_2 and production of sugars. This process is known as Calvin-Benson-Bassham cycle (hereafter Calvin cycle).

1.3.1 Components of photosynthetic electron transfer chain (PETC)

Four multi-subunit protein complexes are involved in the light reactions of photosynthesis: PS II, Cyt b_6f complex, PS I, and ATP synthase (for a review, see Battchikova and Aro 2014). The major complexes are linked by lipophilic and soluble electron carriers to NADPH production: (1) plastoquinones (PQ) forming a pool inside the membrane between PS II and Cyt b_6f; (2) plastocyanin (PC) working on the lumenal side of the thylakoid membrane between Cyt b_6f and PS I; (3) cyt c-553 (also known as cyt c_6), an analog of PC in some cases; (4) ferredoxin (FD) and flavodoxin (FLD) accepting electrons from PS I on the cytosolic side of membrane; and (5) ferredoxin:$NADP^+$ oxidoreductase (FNR) catalyzing NADPH production on the reducing side of PS I.

To perform efficient light-harvesting, most cyanobacteria have phycobilisomes (PBS). These PBS are soluble supramolecular complexes (up to 3000 kDa), located on the cytosolic side of the thylakoid membrane. The PBS covalently bind phycobilins, linear tetrapyrrol molecules, which define the specific color of cyanobacteria. Absorbed light energy is transferred along the rods of the PBS towards the core; this occurs according to the absorption and emission ranges of the PBS components. The structure and composition of PBS varies between species. In *Anabaena*, phycoerythrocyanin, situated on the ends of rods (absorbtion peak at 570 nm), transfers energy via phycocyanin, located on the

base of rods (615 nm), to allophycocyanin, forming the core of PBS (651 nm) (Ducret et al. 1996). The structure of the PBS in *Synechocystis* is described in detail in Arteni et al. (2009).

PBS can direct energy to both photosystems, and so-called "state transitions" are a mechanism defining the recipient (Kirilovsky 2014). In contrast to plants, PQ pool in cyanobacteria is under strict homeostatic regulation and, plausibly, it does not determine the state stransitions (Schuurmans et al. 2014). Recently, the interaction of PBS with both photosystems in megacomplex PS II-PBS-PS I was demonstrated (Liu et al. 2013). Therefore, the balance of energy transfer between two photosystems is maintained, not only by the mobility of the PBS (Joshua and Mullineaux 2004), but also by the modulation of energy transfer to the photosystems. The dosage effect might be achieved by a spillover of energy (McConnell et al. 2002), or by local energy quenching mechanisms. Orange carotenoid protein (OCP) participates in non-photochemical quenching by releasing the excess of excitation energy as heat in antenna, thus regulating the energy transfer from PBS to reaction centers (Kirilovsky and Kerfeld 2013).

Linear electron transfer is based on the "Z-scheme" and involves two photosystems and the Cyt b_6f complex (Hill and Bendall 1960). It starts with the Photosystem II, which crystal structure was resolved at 1.9 Å from *Thermosynechococcus vulcanus* (Umena et al. 2011). Photons excite the reaction center of PS II (P680) leading to charge separation, and the electron ejected from P680 is transferred by the chain of PS II-incorporated cofactors: pheophytin and two protein-bound plastoquinones, Q_A and Q_B. $P680^+$ is the strongest biological oxidizing agent, with the negative potential established by oxidized P680 able to split water molecules. The oxygen evolving complex (OEC), composed of Mn_4CaO_5 cluster and situated on the lumenal side of PS II, is a site of water oxidation. The Kok cycle (Kok et al. 1970) postulates that in a four-step reaction, four electrons, originated from two water molecules, are forwarded to $P680^+$ via the tyrosine residue of the D1 protein (Y_Z), four protons are released into lumenal space, and one molecule of O_2 is evolved as a by-product.

PQ, initially located in the Q_B pocket of PS II, receives two electrons upon two turnovers of the reaction center P680, and captures two protons from the cytosolic side of the thylakoid membrane. Thus it is reduced to plastoquinol (PQH_2) and migrates to the PQ pool. PQH_2 is further oxidized in the Q cycle of the Cyt b_6f complex, so that two protons are released into the lumen, one electron is returned to PQ by cyt b_6, and the second electron is transferred via the Rieske

Fe-S protein and cyt *f* to soluble carriers on the lumenal side (Smirnov and Nori 2012). PC or cyt c_6 act further to carry electrons to PS I, reducing the oxidized P700, primary donor of PS I. Electrons ejected upon excitation of P700 are transferred to FD or FLD on the cytosolic side via the chain of PS I-based redox cofactors: chlorophyll A_0, phylloquinone A_1, and three Fe_4-S_4 clusters: F_A, F_B, and F_X. The structure of PS I, at a resolution of 2.5 Å, is available from *Synechococcus elongatus* (Jordan et al. 2001). Finally, electrons from FD are used for the reduction of $NADP^+$ via FNR, and the proton gradient, established by splitting water and oxidation of PQH_2, is used for ATP production by ATP synthase.

1.3.2 Auxiliary electron transfer pathways

Besides LET, cyclic electron transfer around PS I might take place if the demand on ATP increases related to that of NADPH (Fig.1). In this case, electrons from the acceptor side of PS I (from reduced FD and/or NADPH) are returned back into the PQ pool, or might be donated to the Cyt b_6f complex and, therefore, the proton gradient is increased without the production of NADPH. In plants, two major routes of CET are known: the Antimycine A-sensitive pathway possibly involving PROTON GRADIENT REGULATION5 (PGR5) and PGR5-LIKE PHOTOSYNTHESIS PHENOTYPE1 (PGRL1) proteins, and the Antimycine A-insensitive pathway mediated by chloroplast NADH dehydrogenase-like (NDH) complex (Shikanai 2014). In cyanobacteria, the NDH-1 complex-mediated CET route reducing the PQ pool is the most traditionally accepted pathway, and both FD and NADPH are suggested as possible electron donors (Mi et al. 1995, Battchikova et al. 2011, Ifuku et al. 2011). The alternative CET pathway proposed in *Synechocystis* might involve the PGR5-like protein (Ssr2016) possibly acting as a FD:PQ oxidoreductase (Yeremenko et al. 2005). In addition, the gene encoding FNR in cyanobacteria produces both short and long forms of the enzyme (FNRS and FNRL, respectively) (Thomas et al. 2006). FNRL reduces $NADP^+$, whilst FNRS oxidizes NADPH and, conceivably, returns electrons to the PQ pool or Cyt b_6f and thus might contribute to CET (van Thor et al. 2000).

The respiratory electron transfer chain also has a direct impact on photosynthetic activity (Fig. 1). Besides NDH-1, succinate dehydrogenase (SDH) reduces the PQ pool by oxidation of the tricarboxylic acid cycle (TCA) intermediate succinate to fumarate (Cooley et al. 2000, Cooley and Vermaas 2001). Both complexes translocate protons from the cytosol into the lumen and, therefore, participate in membrane energization and ATP production (Peschek et al. 2004). In

Synechococcus elongatus PCC 7942, NDH-1 and SDH react to the redox status of the PQ pool under light, and are redistributed within the thylakoid membrane to balance the electron flow (Liu et al. 2012). NDH-2 is an alternative NADPH oxidizing enzyme found in cyanobacteria, which consists of three different proteins and cannot translocate protons across the membrane. However, NDH-2 might be involved in the regulation of the $NADP^+$/NADPH ratio (Howitt et al. 1999).

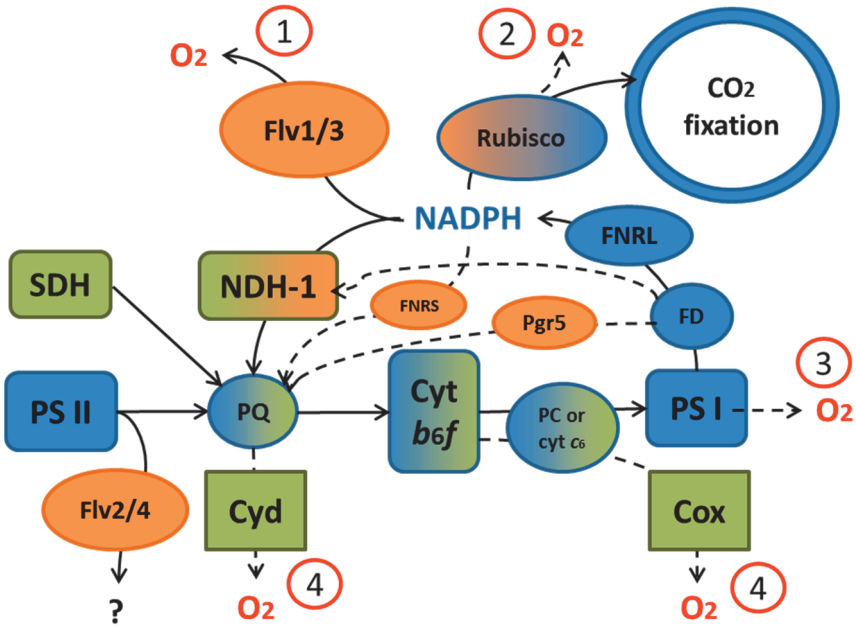

Figure 1. Electron transfer routes in thylakoid membranes of cyanobacteria. The components of linear photosynthetic electron transfer chain are marked with blue, the respiratory electron transfer chain components with green, auxiliary electron transfer pathways components are marked with orange. Shared components are marked with a gradient of two colors according to which pathways they belong to. Dashed lines indicate putative electron transfer routes. Red circles indicate possible ways of O_2 photoreduction: (1) flavodiiron proteins Flv1/3; (2) photorespiration; (3) "true" Mehler reaction; (4) respiratory terminal oxidases.

In addition to these main pathways, a number of auxiliary electron pathways serve as "electron valves" and remove electrons under certain conditions to prevent over-reduction of the PETC and the formation of reactive oxygen species (ROS) (Fig. 1). For this purpose, in *Synechocystis*, the Flv1 and Flv3 proteins work on the reducing side of PS I (details in 1.4.2 and 1.5.2), Flv2 and Flv4 release excess electrons from the acceptor side of PS II (details in 1.5.2), and some respiratory

terminal oxidases (RTOs) might help to oxidize the PETC (1.4.3). Also, bidirectional hydrogenase might briefly serve to release electrons from the acceptor side of PS I upon the beginning of illumination, after long periods of darkness in anoxic conditions (Appel et al. 2000, Gutekunst et al. 2014).

1.3.3 Carbon concentration and CO_2 fixation

The ultimate aim of photosynthetic reactions is the conversion of C_i in the form of organic sugar molecules. For this purpose, cytosolic enzymes of the Calvin cycle utilize NADPH and ATP, produced during photosynthetic electron transfer reactions, to perform CO_2 fixation and following regeneration of trioses to pentoses. The first reaction of the Calvin cycle incorporates CO_2 into ribulose-1,5-bisphosphate (RuBP) and is catalyzed by *r*ib*u*lose *bis*phosphate *c*arboxylase/*o*xygenase (Rubisco), the most abundant enzyme in the world. Rubisco of plants and cyanobacteria is built of large and small subunits, RbcL and RbcS, to form a L_8S_8 molecule (Whitney et al. 2011). Unlike plants, cyanobacteria pack their Rubisco into special micro-compartments, carboxysomes, and have developed a carbon concentrating mechanism (CCM) to increase the amount of CO_2 available for fixation.

The CCM consists of multiple active transport systems enabling the accumulation of C_i in the form of HCO_3^- inside the cells at several orders of magnitude more than in the extracellular environment (Price et al. 2008). A comprehensive overview of the selection of carbon acquisition pumps in different species is available in the work of Kaplan et al. (2008). Carboxysomes are icosahedral structures formed by hexagonal-shape proteins connected into sheets and pentagonal-shape proteins situated on the vertexes of the adjacent sheets (Kerfeld et al. 2005). This protein envelope provides a physiological barrier with the possibility of selective transport achieved by incorporation of specific channels into the protein shell. It is expected that RuBP is extensively transported into carboxysomes, and 3-phosphoglycerate (3-PGA), resulting from the carboxylation reaction, is exported. Besides Rubisco, carboxysomes are expected to contain carbonic anhydrase which converts HCO_3^-, concentrated by CCM in the cytoplasm, into CO_2 inside carboxysomes. This is thought to occur in close proximity to Rubisco active centers (Cameron et al. 2014).

CO_2 fixing activity is usually delayed during dark-to-light transition of the cells, implying the existence of mechanisms regulating the activity of Calvin cycle enzymes. Unlike plant homologues, cyanobacterial glyceraldehyde-3P-dehydrogenase (Gap), and phosphoribulokinase are not redox-regulated by

ferredoxin/thioredoxin system (Tamoi et al. 1998). The double function enzyme, fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase, is unique for cyanobacteria and does not have a typical single-function plant enzyme structure. However, in *Synechococcus* sp. PCC 7942 it was suggested that, during dark-to-light transitions, Gap and phosphoribulokinase are regulated by the ratio of NADPH/NADH via complex formation with a small protein, CP12 (Tamoi et al. 2005).

1.4 O_2 photoreduction

The beginning of oxygenic photosynthesis and subsequent oxygenation of the atmosphere initiated the first large extinction of species in the Earth's history, since most of strict anaerobic species were not able to cope with new conditions (Knoll 2008). However, cyanobacteria themselves should have survived the dramatic increase of O_2 concentration inside the cells; therefore there are several photosynthesis-related O_2 reduction systems in cyanobacteria. These systems were initially the mechanisms for maintenance of microoxic conditions inside the cells in order to avoid interaction of O_2 with the reduced components of PETC. However, in the course of evolution, O_2 photoreduction pathways engaged the use of O_2 as an acceptor of photosynthetic electrons and therefore as a "sink" for the dissipation of excess light energy absorbed by light-harvesting systems (Fig.1).

1.4.1 Photorespiratory pathways

The active center of Rubisco does not effectively distinguish between O_2 and CO_2, so both carboxylation and oxygenation reactions are possible. The oxygenation reaction leads to reduction of O_2 and formation of toxic 2-phosphoglycolate (2-PG). Photosynthetic organisms utilize multiple approaches to favor CO_2 fixing activity of Rubisco and to neutralize harmful consequences of oxygenation reaction (Moroney et al. 2013). Photorespiration is an essential set of reactions employed to metabolize harmful 2-PG.

Although cyanobacterial Rubisco has a low affinity to CO_2 (yet high catalytic rate) compared to C_3 plants, the presence of the CCM and carboxysomes should nearly eliminate the possibility of reaction with O_2 (Savir et al. 2010). However, cyanobacteria have genes encoding enzymes for three hypothetical pathways of photorespiration: the plant-like C2 cycle, the bacterial glycerate pathway and, in some species, the complete decarboxylation of glyoxylate (Eisenhut et al. 2007, 2008). The photorespiratory glycolate pathway, or C2 cycle, converting two 2-

PG molecules into 3-PGA and CO_2, has been studied extensively in plants (Foyer et al. 2009), but was found to originate from cyanobacteria (Hagemann et al. 2013).

Synechocystis possesses the genes encoding all three pathways, but *Anabaena* lacks the genes required for complete decarboxylation of glyoxylate (Eisenhut et al. 2006, 2008). A functional redundancy of photorespiratory pathways might explain the fact that mutant cells of *Synechocystis*, deficient in one of the proteins of glycine decarboxylase complex (GcvT), and therefore in the C2 cycle, did not differ from WT cells (Hagemann et al. 2005). Nevertheless, double mutants, lacking both the C2 cycle and glycerate pathway, and a triple mutant, deficient in all three photorespiratory pathways, had a high-CO_2-requiring phenotype (Eisenhut et al. 2006, 2008). Therefore, the oxygenation reaction of Rubisco should naturally occur in cyanobacteria, especially in low CO_2 conditions, and the activity of photorespiratory pathways is essential to detoxify 2-PG. Despite this suggestion, the actual rate of photorespiratory gas exchange in cyanobacteria has not been monitored previously, but has been demonstrated for the first time in this work.

1.4.2 Cyanobacterial "Mehler-like" reaction

It was demonstrated by Badger et al. (2000) that light-induced O_2 uptake in cyanobacteria is not coupled to, neither limited by ATP consumption. Therefore, the authors concluded that this O_2 is consumed not by Rubisco, but by the Mehler reaction. The Mehler reaction refers to the photoreduction of O_2 to hydrogen peroxide on the reducing side of PS I, first shown in spinach chloroplasts by Mehler (1951). Later studies revealed that in this reaction O_2 probably interacts with reduced Fe-S clusters of PS I and the primary product of Mehler reaction is superoxide (Asada 1994). Consequent events involve the activity of several stromal and thylakoid enzymes which dismutate superoxide to peroxide and eventually to water, therefore completing a water-water cycle (Asada 1999). Importantly, plants developed such effective systems for scavenging of superoxide and peroxide produced in the Mehler reaction, that there is no excess ROS accumulation in intact chloroplasts under standard growth conditions (Asada 2006).

Cyanobacteria appeared to have a unique water-water cycle, different from plants (Helman et al. 2003, 2005). In 2002, Vicente et al. demonstrated that recombinant flavodiiron protein (FDP) Flv3 from *Synechocystis* might *in vitro* reduce O_2 directly to water in the presence of NADPH or NADH without production of ROS.

Furthermore, while in WT cells O_2 consumption was strongly stimulated upon the dark-to-light transition, the mutants of *Synechocystis* deficient in FDPs Flv1 and Flv3 did not exhibit light-induced O_2 uptake (Helman et al. 2003). A strong acceptor side limitation of PS I during first 30 s after the beginning of illumination was also observed in the mutants (Helman et al. 2003). Altogether, the data suggest that Flv1 and Flv3 obtain electrons downstream of PS I, most likely from NADPH to reduce O_2 directly to water under the light. This reaction is further referred to as the Mehler-like reaction, or cyanobacterial Mehler reaction in our studies. More details on the diversity and function of FDPs will be given in Chapter 1.5.

The important cooperation of the photorespiratory C2 cycle and Mehler-like reaction in *Synechocystis* during high light acclimation was revealed by Hackenberg et al. (2009). Importantly, the double mutant lacking both Flv3 and GcvT could not be completely segregated and sometimes could not survive a shift from dim light and low CO_2 to high light and high CO_2. Therefore, the presence of at least one of these proteins is essential for the cells and, likely, Flv-mediated O_2 uptake and photorespiratory O_2 uptake are both important protective mechanism for dissipation of energy during excessive pressure on the photosynthetic apparatus. However, the double mutant deficient in Flv1 and GcvT could be segregated and had a growth rate similar to the WT (Hackenberg et al. 2009).

Concerning the "true" Mehler reaction, there has not been clear evidence for its presence in cyanobacteria. However, it has been demonstrated that cyanobacterial blooms could be fought by an addition of H_2O_2 directly into lakes (Matthijs et al. 2012). Since the "true" Mehler reaction requires effective elimination of ROS, increased sensitivity of cyanobacteria to peroxide implies no sufficient activity of peroxidase and, likely, no condition for the function of the "true" Mehler reaction.

1.4.3 O_2 photoreduction by respiratory terminal oxidases

The types and amount of respiratory terminal oxidases (RTOs) varies between species: *Synechocystis* has three RTOs while *Anabaena* contains five (Schmetterer, 1994). RTOs of *Synechocystis* represent three types of cyanobacterial RTOs (Pils et al. 1997, Pils and Schmetterer, 2001): (1) a typical aa_3-type cytochrome *c* oxidase encoded by *coxBAC* locus (hereafter Cox); (2) alternative oxidase (hereafter ARTO), a homologue to cytochrome *c* oxidase, encoded by the *ctaCDEII* genes; and (3) quinol oxidase, a homologue of *Escherichia coli* cytochrome *bd* ubiquinol

oxidase encoded by the *cydA* and *cydB* genes (hereafter Cyd). Different approaches involving combinations of RTO mutants with various inhibitors led to the conclusion that all three RTOs are active and that ARTO is localized in the plasma membrane, while Cox and Cyd are instead restricted to thylakoid membranes, and therefore can have a direct impact into photosynthetic electron transfer (Berry et al. 2002).

Despite the evidence that Cyd is more active in cells of *Synechocystis* grown at high light intensity, the overall activity of RTOs under the light is still under question (Berry et al. 2002, Helman et al. 2005). However, a recent study demonstrated that a double mutant lacking both Cox and Cyd could not survive 12 h dark/12 h high light square-cycles (Lea-Smith et al. 2013). Also, pulsing light with 5 min on /5 min off cycles of light at the intensity of 1,500 μmol photons m^{-2} s^{-1} caused higher PS II photoinhibition in the double mutant Δ*cox*/*cyd*, compared to the WT (Lea-Smith et al. 2013). Therefore, Cox and Cyd serve as an "electron valve" for the photosynthetic electron transfer chain, at least during abrupt dark-to-high light transitions.

1.5 Flavodiiron proteins (FDPs)

Flavodiiron proteins comprise a protein family taking an origin from Bacteria and Archaea (Wasserfallen et al. 1998), but can also be found in some eukaryotic species of Protozoa and lower plants (Zhang et al. 2009, Peltier et al. 2010). These proteins are important in maintaining microoxic conditions in anaerobic organisms and in protection from nitrosative stress, but, as was demonstrated in cyanobacteria, might have more diverse functions. The common core of FDPs includes two conserved domains: the C-terminal metallo-*β*-lactamase module harboring the non-heme Fe-Fe active center, which is the apparent site of O_2/NO reduction; and the N-terminal flavodoxin-like module, which binds FMN as a cofactor. The bi-domain structure is already operative; it comprises Class A and represents the majority of found FDPs (Vicente et al. 2008). Typically, Class A FDPs require one or two redox partners to complete an electron transfer from NADH to O_2 and/or NO (Gomes et al. 1997, Seedorf et al. 2004).

Other variants of the FDP structure include a common core fused with different N-terminus extensions. These additional modules reflect the composition of redox partners required for the function of Class A FDPs, and the resulting proteins have combined properties of several electron carriers. Class C FDPs of cyanobacteria and other photosynthetic eukaryotes have a unique structure

which, besides the core domain, includes a flavin reductase domain which binds FAD as a cofactor and serves as NAD(P)H:flavin oxydoreductase. As a result, Class C FDPs may perform direct transfer of electrons from NAD(P)H to O_2/NO.

1.5.1 Diversity of flavodiiron proteins in (facultative) anaerobic species

FDPs have mostly been studied in anaerobic Bacteria and Archaea, as well as some eukaryotic parasites. First, the O_2 reducing activity of flavodiiron protein Dg_ROO was shown in anaerobic sulphate-reducing bacteria *Desulfovibrio gigas,* where it is required for the elimination of toxic O_2 during short-term periods of O_2 exposure (Chen et al. 1993). The crystal structure of Dg_ROO demonstrated that the protein forms a homodimer of 43-kDa monomers (Frazão et al. 2000). The monomers are arranged in a head-to-tail manner so that the Fe-Fe center of one protein is situated closely to the FMN cofactor of the flavodoxin-like domain of another protein, thus making electron transport possible. Later, the crystal structures of other flavodiiron proteins confirmed that FDPs from (facultative) anaerobic species are typically arranged in homodimers and homotetramers (Silaghi-Dumitrescu et al. 2005, Seedorf et al. 2007, Petoukhov et al. 2008).

A search of homologous sequences among available genomes revealed the wide distribution of FDPs in prokaryotes: from thermophilic methanogens to mesophilic organisms, including *E. coli* and *Synechocystis* (Wasserfallen et al. 1998). Flavorubredoxin from *E. coli*, has been demonstrated to reduce NO, likely, in order to suppress the immune response of the host (Gardner et al. 2002). Some FDPs might reduce both O_2 and NO without preference; however, in some cases the choice of substrate is strict (Vicente et al. 2007, Seedorf et al. 2007).

Purple bacteria might perform anoxygenic photosynthesis, and *Rhodobacter capsulatus* also has a nitrogenase enzyme that is able to fix atmospheric N_2 in anaerobic conditions. The genome of *R. capsulatus* contains a gene encoding flavoprotein FprA, which was found inside the *nif* (*ni*trogen *f*ixing) gene cluster and has been demonstrated to be important for nitrogenase activity. The role of FprA is not clear, but it was suggested to function as ferredoxin:O_2 oxidoreductase in order to maintain anaerobic conditions inside the cells (Jouanneau et al. 2000).

Among eukaryotic anaerobic species, FDPs were found in some protozoan pathogens. Human parasites *Trichomonas vaginalis* and *Giardia intestinalis* engage flavodiiron proteins to reduce toxic O_2. FDPs from both species are strictly

selective towards O_2 and have common structural features which likely determine the O_2-specificity (Vicente et al. 2009, Smutna et al. 2009, Goncalves et al. 2014).

1.5.2 Flavodiiron proteins in *Synechocystis*

The *flv* genes have been conserved throughout the evolution of photosynthetic organisms. Cyanobacteria, green algae and lower plants, such as mosses and lycophytes, have genes encoding FDPs in their genomes (Zhang et al. 2009, Peltier et al. 2010). The genome of *Synechocystis* contains four genes encoding FDPs: *sll1521* (*flv1*), *sll0219* (*flv2*), *sll0550* (*flv3*), and *sll0217* (*flv4*) (Helman et al. 2003). Phylogenetically, these genes, along with all other genes coding for cyanobacterial-type FDPs, can be distributed into two distinct clusters. Cluster FlvA includes *flv1* and *flv2*, while cluster FlvB contains *flv3* and *flv4*. This strict sequence-based distribution is supported on both structural and functional levels. In contrast to FDPs from anaerobic bacteria, cyanobacterial-type FDPs were suggested to be arranged in heterodimers, so that two proteins from different clusters form an active unit (Zhang et al. 2009, 2012). In the case of Flv2 and Flv4, this fact has been confirmed by protein modeling and by immunodetection of the heterodimer in native conditions (Zhang et al. 2012). One possible explanation for the heterodimeric arrangement of cyanobacterial FDPs is an absence of canonical ligands in the Fe-Fe center of FlvA cluster-proteins (Goncalvez et al. 2011a, 2011b). This modification might make FDPs of cluster FlvA dependent on the presence of protein from cluster FlvB, at least in electron transfer reactions to O_2.

Various gene expression studies have demonstrated the involvement of Flv2 and Flv4 in photoprotection. Transcript abundances of *flv2* and *flv4* were increased in response to low CO_2 and high light treatment (Wang et al. 2004, Eisenhut et al. 2007, Hihara et al. 2001, Muramatsu and Hihara 2012, Zhang et al. 2009). The latest studies have revealed that the Flv2/Flv4 heterodimer (hereafter Flv2/4) releases excess electrons from the acceptor side of PS II, preferably from Q_B pocket, in order to avoid the over-reduction of the PQ pool and photoinhibition of PS II (Zhang et al. 2009, 2012, Bersanini et al. 2014). However, the exact interaction partners for Flv2/4 are yet to be elucidated. Together with a gene encoding the small membrane protein Sll0218, *flv2* and *flv4* form an operon (*flv4-2*), which is controlled by the low CO_2-inducible transcription factor NdhR and is also under tight regulation of *cis*-asRNA (Eisenhut et al. 2012). The operon is conserved in most β-cyanobacteria, but not in α-cyanobacteria or lower plants.

Flv1 and Flv3 function in the Mehler-like reaction, and the corresponding gene expression is responsive to various environmental conditions. The amount of *flv3* mRNA has been found to increase in response to low CO_2 and high light treatments (Wang et al. 2004, Eisenhut et al. 2007, Hihara et al. 2001, Zhang et al. 2009) and the shift of cells from high CO_2 to low CO_2 also induced significant increase of Flv3 protein amount (Zhang et al. 2009). In addition, Flv3 has been demonstrated to interact with FD 9, one of the minor ferredoxins of *Synechocystis*, known to be expressed under high light and high temperature stress (Singh et al. 2010, Cassier-Chauvat and Chauvat 2014).The transcript abundance of the *flv1* gene was shown to be quite low and lacked a response to high light and low CO_2 treatments (Zhang et al. 2009). However, the amount of *flv1* transcripts increased after H_2O_2 and heat treatments, and in response to nitrosative stress (Houot et al. 2007, Rowland et al. 2010, Gonsalves et al. 2011a). Despite the fact that the function of Flv1 and Flv3 has been previously reported, the physiological significance of this pathway and interplay with other routes of O_2 photoreduction has become an object of research in present work.

1.5.3 Cyanobacterial-type FDPs in other organisms

Phylogenetic analysis has revealed the presence of FDPs in photosynthetic eukaryotes including the unicellular green alga *Chlamydomonas reinhardtii*, moss *Physcomitrella patens* and lycophyte *Selaginella moellendorffii* (Zhang et al. 2009, Peltier et al. 2010). All three organisms contain two genes encoding FDPs in their genomes and, though they are not functionally characterized yet, these genes are likely homologues of *flv1* and *flv3* in *Synechocystis*. This is supported by an observation that the function of *flv4-2* may have been substituted by the appearance of a high light-tolerant copy of PsbD1 protein upon evolution (Bersanini et al. 2014). The possible involvement of FLVA and FLVB proteins in the regulation of electron transport and their importance in low carbon and high light acclimation was discussed recently in *C. reinhardtii* (Dang et al. 2014).

Although the majority of unicellular β-cyanobacteria have four genes encoding FDPs, filamentous N_2-fixing heterocyst-forming β-cyanobacteria usually possess two more *flv* genes. Phylogenetic analysis clusters the "extra" *flv* genes of *Anabaena* and *Nostoc punctiforme* together with *flv1* and *flv3* from *Synechocystis*; so *flv1* and *flv3* are duplicated in filamentous cyanobacteria. Microarray and RNA-sequencing analysis revealed that one copy of *flv1* and one copy of *flv3* in *Anabaena* are arranged in operon *all0178-all0177*, which is positively regulated after combined N step-down, and there is more mRNA of these genes in heterocyst-

enriched cell fractions (Ehira et al. 2003, Flaherty et al. 2011, Mitshke et al. 2011). On the protein level, these two FDPs were also found to be more abundant in heterocyst-enriched cell fractions (Ow et al. 2008). Furthermore, the NtcA-regulated promotor sequence has recently been found upstream of *all0178*, strongly suggesting the relationship of this operon to nitrogen metabolism (Picossi et al. 2014). In this work, I studied the reasons for duplication of *flv1* and *flv3* in genomes of filamentous heterocystous cyanobacteria and the functions of the "extra" *flv* genes.

1.6 Filamentous heterocystous cyanobacteria

Filamentous heterocyst-forming cyanobacteria comprise a unique group, remarkably different from unicellular cyanobacteria. They are multicellular and, moreover, they have different types of cells in one filament. Conceivably, cell differentiation appeared as a strategy to adapt to changing environmental conditions upon the course of evolution. Species of order Nostocales, including *Anabaena*, can differentiate vegetative cells into three types of cells: N_2-fixing heterocysts, motile hormogonia and dormant akinetes. Heterocysts are terminally differentiated, while akinetes and hormogonia may turn back into vegetative cells and resume cell division in favorable conditions (Kaplan-Levy et al. 2010, Meeks et al. 2002). Unlike akinetes and hormogonia, after differentiation, heterocysts stay connected to filaments and perform their function in communication with vegetative cells. Vegetative cells fix CO_2 and heterocysts fix N_2.

While growing in an environment rich in combined nitrogen (nitrate or ammonia) filaments of *Anabaena* are comprised of vegetative cells only. Functionally, vegetative cells match cells of *Synechocystis*, with a similar structure of membrane system and photosynthetic apparatus (Cordona et al. 2009, Watanabe et al. 2011). Combined N step-down provokes differentiation of certain vegetative cells of filaments into heterocysts. This involves a differential transcriptional program in these cells and the development of highly complex mechanisms of cell communication. Importantly, ca. 15 to 25% of the *Anabaena* DNA sense strand is transcribed only in heterocysts (Lynn et al. 1986). The differential program triggers rebuilding of cell components and metabolic pathways and takes approximately 24 h under laboratory conditions.

1.6.1 Heterocyst pattern formation

The process of heterocyst development, as a simple model system for cell differentiation, became the subject of numerous studies and reviews (Haselkorn

1998, Golden and Yoon 2003, Kumar et al. 2010, Flores and Herrero 2010, Muro-Pastor and Hess 2012). The exchange of metabolites and signal molecules between cells of the filament is one of the most important prerequisites for heterocyst differentiation. It has been suggested that the cells of filaments are connected by continuous periplasm and encapsulated by an outer membrane (Fig. 2). However, the exchange via periplasm is not accessible for all metabolites (Flores et al. 2006, Zhang et al. 2008). Septal junctions (or microplasmadesmata), directly connecting the cytoplasm of neighboring cells, also provide the possibility for exchange of substances between cells (Mullineaux et al. 2008) (Fig. 2). These structures appeared to be protein channels and formed by SepJ (FraG) and FraC/FraD protein complexes (Flores et al. 2007, Merino-Puerto et al. 2011). The proteins localized in intercellular septa are essential for the complete differentiation of heterocysts and diazotrophic growth of *Anabaena* by contribution to intercellular molecular exchange (Nayar et al. 2007, Merino-Puerto et al. 2010).

NtcA, the global nitrogen regulator, and HetR, the differentiation-specific regulator, are pivotal for heterocyst differentiation (Herrero et al. 2013). PII proteins, known as regulators of nitrogen metabolism in cyanobacteria and some diazotrophic heterotrophs, are not involved in heterocyst development (Zhang et al. 2007, Huergo et al. 2012).

Differentiation starts within a cluster of several cells, and in a few hours a cluster will be resolved into a single cell (Zhao and Wolk 2008). This developmental program is initiated through sensing of an increased amount of 2-oxoglutarate, an intermediate of TCA, which is used as a primary carbon skeleton for the incorporation of ammonium and, therefore, is an important link between C and N metabolism (Muro-Pastor et al. 2001). A step-down in combined N increases the amount of *ntcA* mRNA and 2-oxoglutarate facilitates the binding of NtcA to DNA (Zhao et al. 2010). NtcA binds to the TGTA-(N_8)-TACA consensus binding site, centered at about -41.5 nucleotides upstream of transcription start sites (TSSs) (Herrero et al. 2004). The estimated amount of TSSs up-regulated after combined N step-down under the control of NtcA is at least 158 (Mitshke et al. 2011). But recent data suggest that the total NtcA regulon is extended to over 2000 genes with functions far beyond N assimilation (Picossi et al. 2014).

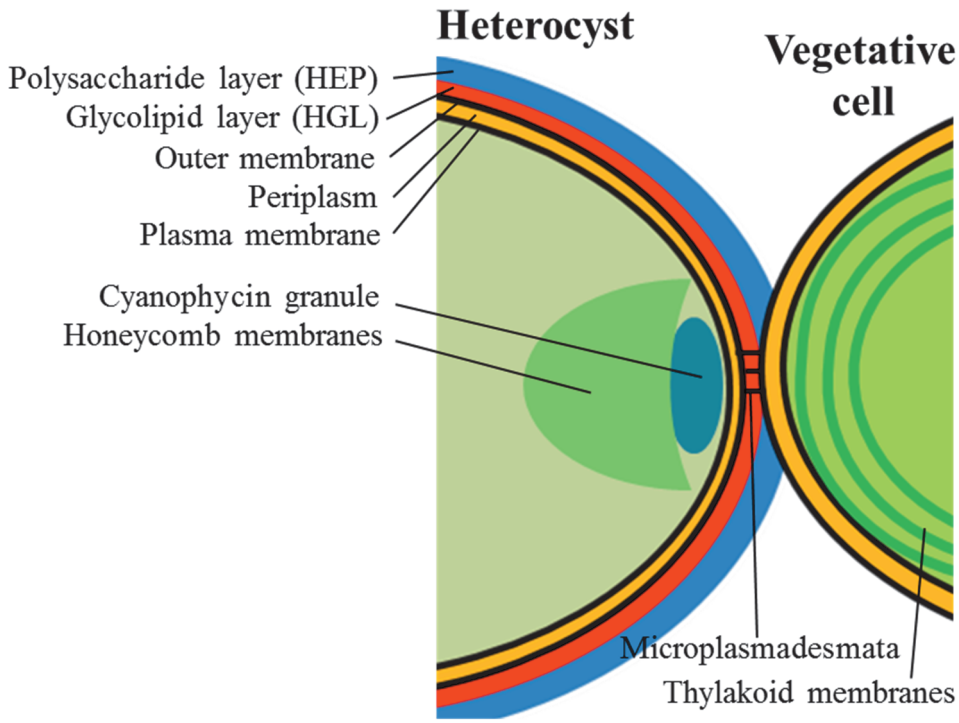

Figure 2. Schematic representation of parts of a heterocyst and adjacent vegetative cell (modified from Flores and Herrero 2010).

NtcA initiates the transcription of HetR, which, in turn, transiently increases the transcription of NtcA (Frias et al. 1994, Muro-Pastor et al. 2002). HetR is a serine type protease with autoprotease activity, which binds DNA in the form of a homodimer (Zhou et al. 1998, Huang et al. 2004). It has been suggested that the 5′TCCGGA palindrome close to the −35 position upstream of TSS is a site of HetR binding, and it is conserved in the promotor regions of many heterocyst-specific genes (Mitshke et al. 2011). HetR is responsible for the morphogenesis of heterocysts and for pattern formation, since the Δ*het*R mutant fails to differentiate heterocysts, but the strain overexpressing HetR has an increased amount of heterocysts (Buikema and Haselkorn 1991, Buikema and Haselkorn 2001). An increase of Ca^{2+} concentration, detected in differentiating cells, has been suggested to promote protease activity of HetR. NrrA, HetF, PatA and other proteins are directly or indirectly involved in the positive regulation of HetR (Shi et al. 2006, Ehira and Ohmori 2006, Risser and Callahan 2008).

Heterocyst pattern formation also requires negative regulation in order to prevent differentiation of neighboring cells. PatS is an inhibitory pentapeptide RGSGR, which can interfere with the DNA binding activity of HetR and, therefore,

prevent differentiation (Huang et al. 2004). The *patS* gene is strongly up-regulated in differentiating heterocysts and encodes PatS together with a cleavable twin-arginine signal peptide targeting PatS to the periplasm (Mariscal et al. 2007). During transport, the signal peptide is removed and the residual pentapeptide diffuses into nearby vegetative cells via the periplasm, creating an inhibitory gradient (Corrales-Guerrero et al. 2013). By this mechanism, developing heterocysts prevent neighboring vegetative cells from differentiation, explaining the similar intervals of vegetative cells between heterocysts (Haselkorn 1998, Yoon and Golden 2001). HetN also contains an inhibitory peptide sequence and has the same function as PatS, but it is synthetized by mature heterocysts (Callahan et al. 2001). During the growth of filaments, and after the appearance of a sufficient amount of vegetative cells between two heterocysts, a new heterocyst will appear in the middle of the vegetative cells' interval.

1.6.2 Prerequisites for N_2 fixation in heterocysts

Dynamic changes in cell morphology, gene expression, and metabolomics during heterocyst development have been studied in *Anabaena* and other heterocystous cyanobacteria. The deposition of a specific heterocyst envelope beyond the outer membrane is one of the first events during differentiation which requires the activity of regulatory proteins and enzymes for the synthesis and export of the produced material (Nicolaisen et al. 2009). The heterocyst-specific envelope is composed of the external layer (polysaccharide layer, HEP) and internal layer (glycolipid layer, HGL) (Fig. 2). Mutant studies have demonstrated that both HEP and HGL are essential for nitrogenase activity in oxic, but not in microoxic, conditions. It has therefore been suggested that these layers prevent O_2 diffusion into the cells (Wolk et al. 1988, Ernst et al. 1992). The phenotype of mutants "incapable of N_2 *f*ixation in the presence of *o*xygen" was designated as Fox^- and was found to be valid for many mutants deficient in heterocyst-specific genes (Ernst et al. 1992, Huang et al. 2005, Lecho-Yossef et al. 2011, Fan et al. 2005). Genes responsible for the formation of HEP are up-regulated in the early/medium phase of heterocyst differentiation, 6-12 h after combined N step-down, while a strong up-regulation of genes required for the deposition of HGL was observed 12-21 h after combined N step-down (Moslavac et al. 2007, Nicolaisen et al. 2009, Flaherty et al. 2011). After the deposition of HEP, reorganization of the membrane system in heterocysts proceeds.

Besides thylakoid membranes, heterocysts contain special "honeycomb" membranes situated in the polar regions of heterocyst, near to junctions with

vegetative cells (Lang and Fay, 1971) (Fig. 2). These membranes host components of a highly active respiratory chain and also PS I (Wolk et al. 1994, Kumazaki et al. 2013). Due to the structural alterations of heterocysts, their contact area with vegetative cells is extremely reduced. Nevertheless, this surface is a major pathway for O_2 to penetrate heterocysts (Walsby 2007). Therefore, the respiratory complexes of honeycomb membranes provide immediate protection against O_2 diffusing via the septum. Two RTOs, Cox2 and Cox3, were found to be expressed exclusively in heterocysts, and the presence of at least one of them is essential for N_2 fixation and diazotrophic growth (Jones and Haselkorn 2002, Valladares et al. 2003, 2007). Cox2 is a typical cytochrome *c* aa_3-type oxidase, and Cox3 is likely an ARTO and might be a quinol oxidase (Valladares et al. 2007). These oxidases are expressed in both developing and mature heterocysts and, importantly, participate in the structural assembly of honeycomb membranes (Valladares et al. 2007).

Concerning photosynthetic components, heterocysts do not retain enzymes of CO_2 fixation, but still contain photosynthetic complexes. PS I is an unambiguously essential partner of nitrogenase, and therefore the amount and oligomeric status of PS I does not undergo significant changes during heterocyst development, *e.g.* in *Anabaena variabilis* (Kumazaki et al. 2013). PS I of *Anabaena* 7120 was shown to exist as a tetramer in contrast to *Synechocystis* and many other cyanobacteria which have trimeric PS I (Watanabe, 2011). Tetrameric PS I in *Anabaena* 7120 also has its own antennae system resembling a rod of PBS, and therefore PS I in heterocysts can retain its own light-harvesting system despite the degradation of conventional PBS (Watanabe et al. 2014).

The fast and synchronous degradation of PS II and allophycocyanin during heterocyst differentiation in *Anabaena variabilis* is physiologically critical to suppress energy transfer to PS II, and thus O_2 evolution (Kumazaki et al. 2013). However, some studies demonstrate that some amount of PS II is still present in mature heterocysts (Ferimazova et al. 2013). Separated subunits of PS II were identified in isolated heterocysts of different species (Thiel 1990, Baier et al. 2004), but also PS II monomers were shown to be present in heterocysts of *Nostoc punctiforme* (Cardona et al. 2009). Importantly, residual PS II of heterocysts was not able to transfer electrons from water to the artificial electron acceptor DCPIP, but could reduce DCPIP in the presence of DCP, an exogenously added electron donor (Cardona et al. 2009).

ATP synthase and Cyt b_6f, as essential components of both photosynthetic and respiratory electron transfer chains, are retained in heterocysts (Ow et al. 2008, 2009). Regarding the NDH-1 complex, NDH-1L would be expected to be active in heterocysts, since these cells rely on respiration and a heterotrophic supply of energy production (Wolk et al. 1994). However, only trace levels of NDH-1L were found in heterocysts of *Nostoc punctiforme*, but NDH-1M was abundant in the cell-wall fraction (Cardona et al. 2009). Despite the assignment of NDH-1M to CO_2 concentration in *Synechocystis* (Battchikova et al. 2011), the functions of different NDH-1 complexes have not been investigated in *Anabaena*. Only one study has attempted to knock-out the NdhK subunit, common for all NDH-1 complexes, but the mutant clones were not viable (Howitt et al. 1996). Therefore, NDH-1M might substitute NDH-1L in heterocysts. In addition, various studies have revealed increased amounts of enzymes of TCA and of the carbon catabolic pathways, the oxidative pentose phosphate pathway (OPPP) and glycolysis, in heterocyst-enriched cell fractions (Stensjö et al. 2007, Ow et al. 2008, 2009).

1.6.3 N_2 fixation

Since heterocysts cannot obtain electrons from water splitting, they rely on electrons and reducing power provided by vegetative cells. By tracking molecules transported between heterocysts and vegetative cells, it was revealed that heterocysts import sucrose, glutamate and alanine (Jüttner 1983, Cumino et al. 2007, Pernil et al. 2010). Glutamate serves for incorporation of ammonium, and resulting glutamine is transported back to vegetative cells (Thomas et al. 1977, Martín-Figueroa et al. 2000). Fixed N might be stored in heterocysts in polar granules of cyanophycin (Fig. 2), an aminoacid polymer composed of an aspartate-based backbone with side groups of arginine (Gupta and Carr 1981, Sherman et al. 2000). On demand, cyanophycin is hydrolyzed and transported into vegetative cells as β-aspartyl-arginine (Burnat et al. 2014).

Electrons extracted from sucrose in glycolysis and OPPP are preserved in NADH and NADPH. The NDH-1M complex (and possibly FNRS) oxidizes the reducing equivalents and feed electrons into the PQ pool, where they might be directed either to RTOs or PS I. Importantly, only short form of FNR is present in heterocysts (Valladares et al. 1999, Omairi-Nasser et al. 2014). Respiratory activity reduces dangerous O_2 and creates a proton gradient to synthetize ATP. Light-driven PS I is thought to perform active CET to generate more ATP, but exact components forming CET units are not known in heterocysts (Böthe et al. 2010). However, heterocyst-specific FD, FdxH, receives electrons from PS I and

is possibly involved in CET. FdxH is also considered to be a donor of electrons for nitrogenase, although it is not essential for N_2 fixation (Böhme and Haselkorn 1988, Masepohl et al. 1997), and might also be reduced directly by NADPH or pyruvate in darkness (Neuer and Böthe 1985, Böhme and Haselkorn, 1988). Nevertheless, without light, supply of nitrogenase with electrons becomes limited very quickly, upon the depletion of carbon stores, and therefore, N_2 fixation is (indirectly) a light-dependent process (Neuer and Böthe 1985, Valladares et al. 2007).

Molybdenum-containing nitrogenase is a well-conserved enzyme in all N_2-fixing organisms, performing the following reaction:

$$N_2+16ATP+8H^++8e^-\rightarrow 2NH_3+H_2+16ADP+16P_i$$

The enzyme consists of two components: dinitrogenase (FeMo protein comprised of NifD and NifK) and dinitrogenase reductase (Fe protein, NifH); and its synthesis places a high demand on Fe (Rubio and Ludden 2008, Böthe et al. 2010). In heterocyst-forming cyanobacteria, components of Mo-nitrogenase and its maturation machinery are usually encoded by genes of the *nif* operon expressed exclusively in heterocysts during late differentiation. In *Anabaena*, before nitrogenase can be expressed, a specific enzyme XisA excises an 11-kb DNA element within the *nifD* gene and performes the gene rearrangement to allow nitrogenase transcript formation to proceed (Golden et al. 1991).

Some filamentous strains may contain genes encoding additional Mo-nitrogenases. The *nifH2* gene cluster of *Anabaena variabilis,* encoding an "alternative" Mo-nitrogenase, is expressed in vegetative cells in anaerobic conditions (Thiel and Pratte 2001). In some cases, only "alternative" copies of particular nitrogenase genes are present. In *Anabaena* 7120, *nifH1* is situated inside the *nifHDK* operon and *nifH2,* a copy of *nifH1*, is situated independently. The function of *nifH2* is not clear. However, based on analogy to the *nifH2* gene cluster of *Nostoc punctiforme*, which is surrounded by *nifE2* and *nifN2* genes involved in the biosynthesis of the FeMo cofactor of nitrogenase, it might be also assigned to the nitrogenase maturation machinery (Meeks 2005, Rangaraj and Ludden 2002). Additional nitrogenases with other metal cofactors, such as vanadium, might also be present. *Anabaena variabilis*, for example, also possesses a Va-containing nitrogenase encoded by the *vnfDGK* genes (Thiel 1993).

Some heterotrophic diazotrophs develop an O_2-sensing system preventing the transcription of *nif* if O_2 is present inside the cells (Martinez-Argudo et al. 2005).

In contrast, cyanobacterial expression of *nif* is mainly subjected to developmental control (Elhai and Wolk 1990, Wolk et al. 1994). Even the Δ*cox2/cox3* double mutant, despite an apparent increase of O_2 inside heterocysts and a lack of honeycomb membranes, expresses *nif* at 18-24 h after a combined N step-down, thus behaving in a similar manner to the WT (Valladares et al. 2007, Flaherty et al. 2011). An unknown post-translational modification of NifH has been proposed to serve as a fast mechanism for the regulation of the pool of active nitrogenase and, therefore, for the protection of the enzyme from O_2 damage (Gallon et al. 2000, Huergo et al. 2012). However, rhythmical activity of the *hesAB* genes, possibly involved in the regulation of nitrogenase synthesis, was demonstrated to peak at subjective dawn, indicating the *de novo* synthesis of the enzyme each day (Kushige et al. 2013). This may be explained by the inactivation of nitrogenase each night, due to a depletion of carbon stores and a gradual increase of O_2 inside heterocysts (Walsby 2007).

1.6.4 Maintenance of microoxic conditions

Uptake hydrogenase, another heterocyst-specific enzyme, is encoded by the *hupSL* operon and recovers electrons from H_2 that is produced as a by-product of N_2 fixation by nitrogenase (Tamagnini et al. 2007). It is suggested that electrons from H_2 are returned in the electron transfer chain of heterocysts and are further used for the reduction of O_2 via RTOs or PS I (Böthe et al. 2010). However, it was demonstrated that uptake hydrogenase not only protects nitrogenase by removing excess O_2 in heterocysts, but also indirectly protects photosynthetic apparatus from photoinhibition in vegetative cells (Kosourov et al. 2014). Uptake hydrogenase itself is also sensitive to O_2, being irreversibly damaged by even trace amounts. In *Anabaena* 7119, isolated uptake hydrogenase was shown to be activated by reduced thioredoxin and, therefore, it was suggested to operate in a light-dependent manner (Papen et al. 1986).

Other heterocyst-specific systems for O_2 and ROS reduction were found to be important for nitrogenase activity and the diazotrophic growth of *Anabaena* and other heterocystous cyanobacteria. Lactate oxidase encoded by the *lox* gene is another enzyme possibly reducing O_2, while rubrerythrin, RbrA, functions as an FNR-dependent peroxidase (Zhao et al. 2007b, Hackenberg et al. 2013). Mn-superoxide dismutase, SodB, also has an impact on the protection of nitrogenase, and ferritin-like Dps proteins likely protect the DNA of heterocysts from H_2O_2-mediated oxidative stress (Zhao et al. 2007a, Ekman et al. 2014).

The reduction of O_2 is an essential prerequisite for nitrogenase activity, especially under the light. Light-driven photosynthesis promotes diffusion of O_2 from vegetative cells into heterocysts and, therefore, the O_2 uptake of isolated heterocysts is significantly higher under the light compared to that in darkness (Murry et al. 1981, Milligan et al. 2007). Even though the reduction of O_2 in heterocysts under the light, likely, includes the activity of terminal oxidases, the actual light-induced component has not been known thus far (Valladares et al. 2007). In the present work, the nature of light-induced O_2 uptake by heterocysts and its significance for nitrogenase activity and the diazotrophic growth of *Anabaena* was investigated.

2. AIMS OF THE STUDY

Cyanobacteria are capable of inhabiting highly variable environments and are therefore equipped with effective and flexible acclimation mechanisms. In my thesis, I examined the roles and functional mechanisms of O_2 photoreduction in protection of the photosynthetic apparatus under various environmental conditions using WT and several mutants of non-N_2-fixing *Synechocystis* sp. PCC 6803 and N_2-fixing *Anabaena* sp. PCC 7120. In the latter species, particular emphasis was put on O_2 photoreduction in protection of the N_2-fixing machinery in specific heterocyst cells. The workflow included the following aspects of cyanobacterial electron transfer pathways to molecular oxygen:

1. Evaluation and analysis of the multiple systems carrying out photosynthesis-related O_2 photoreduction.
2. Characterisation of a specific O_2 photoreduction pathway, mediated by Flv1 and Flv3 flavodiiron proteins, in protection of the photosynthetic apparatus in vegetative cells under naturally occurring fluctuating light conditions.
3. Discovery and significance of a heterocyst-specific and FDP-mediated O_2 photoreduction pathway in protection of the N_2-fixing machinery.

3. METHODOLOGY

3.1 Cyanobacterial strains and growth conditions

Table 1. Cyanobacterial strains used in this work. Construction of the mutants is described in detail in provided references.

Strain	Mutated genes	Phenotype	Paper	Reference
Synechocystis				
WT sp. PCC 6803			I, II	
Δ*flv1*	*sll1521*::Cm^R	FL-sensitive	I, II	Helman et al. 2003
Δ*flv3*	*sll0550*::Sp^R	FL-sensitive	I, II	Helman et al. 2003
Δ*flv1/3*	*sll1521*::Cm^R *sll0550*::Sp^R	FL-sensitive	I, II	Paper I
Δ*pgr5*	*ssr2016*::Km^R		II	Paper II
Anabaena				
WT sp. PCC 7120			II, III, IV	
Δ*flv1A*	*all3891*::Nm^R	FL-sensitive	II	Paper II
Δ*flv3A*	*all3895*::Nm^R	FL-sensitive	II	Paper II
Δ*flv1B*	*all0177*::Nm^R		IV	Paper IV
Δ*flv3B*	*all0178*::Nm^R	Fox^-	IV	Paper IV
Δ*flv1B/3B*	*all0177*::Sp^R *all0178*::Nm^R	Fox^-	IV	Paper IV
Flv1A-YFP	*all3891*::YFP		III	Paper III
Flv3A-YFP	*all3895*::YFP		III	Paper III
Flv1B-YFP	*all0177*::YFP		III	Paper III
Flv3B-YFP	*all0178*::YFP		III	Paper III

Strains of *Synechocystis* (Table 1) were grown in BG11 medium (Rippka et al. 1979) containing 17 mM $NaNO_3$ and supplemented with 20 mM TES-KOH (pH 8.2). *Anabaena* WT and mutants (Table 1) were grown in either BG11, or BG11o (without combined N) medium. Standard growth conditions comprised cool white fluorescent light (L 30W/865, Osram) at the intensity of about 50 µmol photons m^{-2} s^{-1} PAR and temperature of 30 °C. Cells were routinely cultivated in Erlenmeyer flasks under gentle agitation or, for creating microoxic conditions, bubbling using a mixture of 99.96% N_2 and 0.04% CO_2. In some experiments,

cells were subjected to treatments of high light (220 μmol photons m^{-2} s^{-1}), dim light (10 μmol photons m^{-2} s^{-1}), or darkness. Different experimental conditions also included various levels of CO_2 supply: 3% (high CO_2); 0.03% (air level, low CO_2); or C_i starvation (when cells were incubated under the light in air-tight Eppendorf tubes in BG11 medium without $NaHCO_3$ for 4 h). Fluctuating light (FL) experiments were performed in growth chambers (AlgaeTron AG 130-ECO, PSI) equipped with LED white light, where the background light intensity of either 20 or 50 μmol photons m^{-2} s^{-1} was interrupted by 30 s pulses of 500 μmol photons m^{-2} s^{-1} light every 5 min (FL 20/500 and FL 50/500, respectively). Cells were harvested at the logarithmic growth phase (OD_{750}=1.0) and subjected to biophysical measurements, RNA, protein, or other analysis. The concentration of Chlorophyll *a* (hereafter Chl) in samples was determined in 90% methanol by absorbance measurement at 665 nm and calculated with an extinction coefficient of 78.74 L g^{-1} cm^{-1} (Meeks and Castenholz 1971).

3.2 Biophysical analysis

3.2.1 O_2 exchange by Clark-type electrode

Steady-state O_2 consumption and evolution rates were measured with a Clark-type O_2 electrode (DW1, Hansatech) in darkness and at a saturating light intensity of 400 μmol photons m^{-2} s^{-1}, respectively. To measure PS II activity and net photosynthesis, cells at the Chl concentration of 5 μg mL^{-1} were supplemented with 2 mM DMBQ (2,5-dimethyl-p-benzoquinone) and 10 mM $NaHCO_3$, respectively. To inhibit dark respiration in isolated heterocysts, 1 mM KCN, 10 mM salicylhydroxamic acid (SHAM), or 10 μM Antimycin A (final concentrations) were added to cell suspensions adjusted to the Chl concentration of 15 μg mL^{-1}.

3.2.2 Membrane Inlet Mass Spectrometry (MIMS)

Online measurements of $^{16}O_2$ (mass 32), $^{18}O_2$ (mass 36) and CO_2 (mass 44) exchange were performed on cell suspensions in a measuring chamber directly connected, via teflon membrane, to a mass spectrometer (Prima-B, Thermo Fisher Scientific). The spectrometer adjusted the magnet current to the mass peaks corresponding to different gases and estimated their abundances. Since photosynthetic cells produce $^{16}O_2$ from water, the heavy isotope $^{18}O_2$ (99% isotope content, Euriso-Top) was used to differentially monitor the O_2 uptake by cells. $^{18}O_2$ was injected into the cell suspension just before vessel closure. The initial conditions in the experimental vessel required equilibrium between the two

O_2 isotopes, and therefore, isotope ratio as a function of time could be used to estimate O_2 uptake and production flow rates. For *Synechocystis*, N_2 (78.09% in air) was used as a reference gas for the calculation of gas abundances, whilst Ar (1% in air) was used for *Anabaena*.

A temperature of 30°C was maintained in the chamber, and cell suspensions at the Chl concentration of 15 μg mL^{-1} were continuously stirred with a magnetic stirrer. To create different C_i conditions, we either supplied the cell suspensions with 5 mM $NaHCO_3$, or did not provide any source of C_i for the cells. The saturating light intensity of 500 μmol photons m^{-2} s^{-1} was provided by LED-powered fiber optic illuminator (PerkinElmer Life Sciences), when required. Fluctuating light conditions were mimicked during the measurements by changing actinic light intensity in the experimental cell following the FL 20/500 regime described above. The final concentrations of inhibitors used in MIMS analysis were 1 mM KCN, 8 mM iodoacetamide (IAC) and 10 mM glycolaldehyde (GA).

3.2.3 Flash-fluorescence analysis

The single flash-induced Chl fluorescence increase and following decay were measured by a fluorometer FL 3500 (PSI Instruments). For the measurements, cells at the Chl concentration of 5 μg mL^{-1} were dark adapted for 5 min before the application of 10-μs saturation flash either in the absence, or in the presence, of 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). Obtained data were normalized on a scale from 0 to 1 to compare the kinetics.

3.2.4 Yields of photosystems

PS I and PS II activities were monitored using a pulse amplitude modulated fluorometer Dual-PAM-100 (Walz), directly on cell suspensions at the Chl concentration of 10-20 μg mL^{-1}. Measurements were performed at 30°C, and actinic red light (635 nm) was used to illuminate the cells. To mimic FL, actinic light intensity between 58 and 530 μmol photons m^{-2} s^{-1} was applied. Saturating pulses of red light (5000 μmol photons m^{-2} s^{-1}, 300 ms) were applied during the measurements to transiently close all PS I and PS II centers and to probe photosynthetic parameters. Also, built-up "light curves" analysis was used to estimate the performance of photosystems upon increasing light intensities. In this case, cells were subjected to 30-s periods of illumination by a series of actinic light intensities, starting from the lowest intensity and gradually increasing. At the conclusion of each illumination period, a saturating pulse was applied to monitor photosynthetic parameters.

During FL measurements, cells were pre-incubated in darkness for 10 min and F_0, the dark minimal level of fluorescence, was determined after switching on the measuring light. Next, a saturating pulse was applied to determine F_m, the maximum fluorescence in the dark. Further, upon the illumination of samples, F_s, the steady-state level of fluorescence, and F_m' values, the maximal levels of fluorescence under the light, were monitored. The yield of PS II, Y(II), was calculated as $(F_m'-F_s)/F_m'$ for each saturating pulse applied. The maximal photochemical efficiency of PS II in darkness was more precisely estimated in the presence of 10 μM DCMU as $(F_m-F_0)/F_m$.

The maximum level of oxidizable P700 (P_m) was calculated by the application of a saturating pulse after pre-illumination of cells using far-red light (75 watts m^{-2}) for 10 s. Further, upon illumination with actinic light, the P level of the P700 signal was monitored and P_m' values were collected by the application of saturating pulses. The Y(NA), non-photochemical quantum yield of PS I, demonstrating the level of PS I acceptor side limitation, was calculated as $(P_m-P_m')/P_m$. The yield of PS I, Y(I), was calculated as $(P_m'-P)/P_m$.

3.3 Transcript analysis

Total RNA was isolated from cells collected from 10 mL of culture at the logarithmic growth phase using the "hot phenol" method with TRIsure reagent (Bioline). In the case of *Synechocystis*, cells were incubated with phenol reagent for 10 min at 60°C, whereas *Anabaena* cells were treated for 5 min at 95°C. RNA was cleaned of residual DNA by a treatment with 1 unit of deoxyribonuclease (Turbo DNase, Ambion). The concentration and purity of RNA samples was measured using a NanoDrop spectrophotometer (Thermo Scientific).

RNA of *Anabaena* WT and the Δ*flv1B*, Δ*flv3B* and Δ*flv1B/3B* mutants was subjected to strand-specific transcriptome sequencing by Illumina HiSeq2000 (BGI Tech Solutions, Co., Ltd). The obtained data contained the sum of sequenced 100-nt RNA fragments aligned with the genome sequence of *Anabaena*. Using this data, the transcript amounts for each gene were calculated as reads per kilobase of coding sequence model per million mapped reads in the sample) and could be directly compared between samples (Mortazavi et al. 2008).

To perform real-time quantitative reverse transcription PCR (hereafter RT-qPCR) analysis, 1 μg of total RNA and random hexamer primers (Promega) were used to synthetize cDNA using SuperScript III Reverse Transcriptase (Invitrogen). The 5-fold diluted cDNA was mixed with iQ SYBR Green

Supermix (BioRad) and specific primers to monitor the PCR reaction with the iQ5 Detection System (BioRad). For comparison, we used reference genes with a constitutive transcription: *rnpB* for *Synechocystis* (Wang et al. 2004) and *rpoA* for *Anabaena* (Mella-Herrera et al. 2001). Quantification cycle for PCR reactions was determined by iQ5 Optical System software 2.0. Samples lacking reverse transcriptase during cDNA synthesis were used as a negative control.

3.4 Isolation of heterocysts

Heterocysts were isolated from 400-mL cultures of *Anabaena* grown in N_2-fixing conditions. Filaments were harvested, resuspended in 25 mL of lysis buffer (50 mM Hepes-NaOH pH 7.2, 10 mM NaCl, 10 mM EDTA, 0,4 M sucrose) and treated with lysozyme (1 mg mL^{-1} final concentration) for 1 h at 37°C. Afterward, cell suspensions, kept in an ice-cold water bath, were subjected to sonication at 40W for 1 min by Sonicator Labsonic U (B. Braun Biotech). A series of low-speed centrifugations at +4°C (1000x-250x g) was used to sediment heterocysts separately from vegetative cells. For activity measurements, heterocysts were resuspended in 50 mM Hepes-NaOH (pH 7.5) supplemented with 400 mM of sucrose.

3.5 Analysis of proteins and aminoacids

3.5.1 Protein isolation, SDS-PAGE, BN-PAGE and immunodetection

Cells from 30-50 mL of culture at OD_{750}=0.5-1.0 were collected and resuspended in 50 mM Hepes-NaOH (pH 7.5) buffer containing 30 mM $CaCl_2$, 800 mM sorbitol, and 1 mM ε-amino-n-caproic acid. The total protein was obtained by vortexing the cells in the presence of glass beads (6 times for 1 min with 1-min brakes in ice in between), following by centrifugation at 3000x g and collection of the supernatant fraction (total protein). When required, soluble proteins were separated from the membrane fraction by centrifugation at 18000x g for 20 min. Determination of protein concentration was performed using the Lowry protein assay (BioRad). To analyse samples in native conditions, proteins were separated using 6–13% gradient blue native PAGE (BN-PAGE). Otherwise, samples were solubilized in Laemmli buffer supplemented with 5% β-mercaptoethanol and 6 M urea at 4°C overnight and separated by 12% SDS-PAGE containing 6 M urea. Proteins were transferred from gels to a polyvinylidene fluoride membrane (Immobilon-P, Millipore) and were probed using protein-specific antibodies. For "oxyblot" analysis, membranes were analysed by OxyBlot Protein Oxidation Detection Kit (Millipore).

3.5.2 DIGE and MS/MS

For differential gel electrophoresis (DIGE), total protein was extracted from 400-mL cultures of *Anabaena* grown in N_2-fixing conditions or from isolated heterocysts. Cells were resuspended in 5 mL of 8 M urea and 2 M thiourea and broken using three shots of a 40 kg in^{-2} by TS2 0.75 W Cell Disruptor (one shot machine, Constant Systems Ltd). Samples were then vortexed vigorously, following the addition of 4% CHAPS, and then the supernatant containing total protein was separated by centrifugation at 3000x g. A 2-D Clean-Up Kit (GE Healthcare) was used to obtain the purified protein extract, which was resuspended in 30 mM Tris-HCl (pH 8.8) buffer containing 8 M urea, 2 M thiourea, and 4% CHAPS. The labelling of proteins was performed with CyDye DIGE Fluor minimal dyes (GE Healthcare): WT sample labelled with Cy3, mutant sample with Cy5, and the combined sample of WT and mutant proteins with Cy2. All three samples where pulled together and the proteins were focused in 18-cm strips with immobilized pH gradient (nonlinear gradient, pH 3-11, GE Healhcare) by Ettan IPGphor isoelectric focusing system (GE Healthcare). Afterward, proteins were reduced using 2% dithiothreitol and then alkylated using 2.5% IAC in the presence of buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 10% SDS. 10% SDS-PAGE was used to separate proteins by mass. Images of the fluorescence resulting from resolved, labelled proteins were taken using a Geliance 1000 imaging system (Perkin Elmer) and were analysed using ProFINDER2D software (Perkin Elmer).

Proteins in gels were visualized by silver staining (Blum, 1987) and the spots of interest were excised and digested by Trypsin Gold (Promega). Peptides were then extracted from gel pieces by washing with formic acid and acetonitrile. The protein digests were dried and dissolved in 12 μL of 2% formic acid to be subjected to mass spectrometry analysis. Tandem mass spectrometry (MS/MS) was performed by API QSTAR (Applied Biosystems) complemented with a nano electrospray source (Protana), an in-line nano-HPLC system and an auto-sampler (Ultimate, Switchos and Famos, LC Packing). Analysis of the data was performed with Analyst QS software and the proteins were identified using Mascot. Genome sequence database for *Anabaena* was obtained from Cyanobase (www.kazusa.or.jp/cyanobase).

3.5.3 Analysis of aminoacids

The extraction of free amino acids was performed from frozen cells (collected from 2 mL of culture) with 80% ethanol at 65°C for 3 h. Supernatants were

separated by centrifugation at 13000x g, dried and resuspended in 8 mM Na_2HPO_4 (pH 6.8) with the addition of 2.5% tetrahydrofurane. The content of glycine and serine was determined using a Class-Vp-HPLC system (Shimadzu) (Geigenberger et al. 1996).

3.6 Nitrogenase activity

Activity of nitrogenase was measured by an acetylene reduction assay (Dilworth, 1966). Filaments of *Anabaena* grown in N_2-fixing conditions were harvested and then resuspended in fresh BG11o to the Chl concentration of 5 μg mL^{-1}. Vials (20 mL) were filled with 5 mL of cell suspension and sparged with the gas mixture of 20.95% O_2, 0.039% CO_2, and 79.01% Ar (AGA Ab). Then, vials were sealed gas-tight and 10% of the gas phase was replaced with acetylene. After incubation of vials for 24 h in growth conditions, 10-μL samples from the headspace were subjected to ethylene determination by gas chromatographer (Perkin Elmer Autosystem).

3.7 Microscopy

Filaments of mutant *Anabaena* strains expressing modified Flv proteins fused with Yellow fluorescent protein (YFP) were inspected using a laser scanning microscope Zeiss LSM510 META with a multi-spectral analyser. An argon ion laser (488 nm) was used to excite YFP and the fluorescence across 510–550 nm was collected. Fluorescence at 680–720 nm was collected to visualize Chl autofluorescence. Bright field microscopy of the filaments was performed with a Zeiss Axiovert 200M microscope.

4. RESULTS

4.1 O_2 photoreduction in *Synechocystis* under low CO_2 conditions

It has previously been demonstrated that Flv1 and Flv3-mediated O_2 uptake is responsible for O_2 photoreduction in high CO_2-grown cells of *Synechocystis* (Helman et al. 2003). In this work the contribution of the Flv-related "Mehler-like" reaction into light-driven O_2 consumption under different carbon supply regimes was further studied. Furthermore, the inactivation of *flv1* and/or *flv3* has allowed detecting the distinct light-induced O_2 uptake which was previously masked or prevented by FDP activity.

4.1.1 Flv1/3 heterodimer is a major electron sink in low CO_2 conditions

The MIMS technique with $^{18}O_2$ isotope was used to investigate in detail the capacity of FDPs as an electron sink in different conditions. The activity of Flv1 and Flv3 in low CO_2-acclimated cells was the primary focus in Paper I. Illumination of dark-adapted *Synechocystis* WT cells with strong white light significantly stimulated O_2 uptake from 10.4 to 38.2 μmol O_2 [mg Chl]$^{-1}$ h^{-1}, thus demonstrating a light-induced O_2 uptake of 27.8 μmol O_2 [mg Chl]$^{-1}$ h^{-1}. Next, MIMS analysis was performed in the presence of different inhibitors. The addition of KCN eliminated the dark O_2 uptake and instead stimulated light-induced O_2 uptake. IAC and GA, inhibitors of Rubisco and other Calvin cycle enzymes and thus inhibitors of photorespiratory O_2 uptake, also strongly stimulated light-induced O_2 uptake (Paper I).

In the Δ*flv1*, Δ*flv3* and the Δ*flv1/3* mutant cells light-induced O_2 uptake was completely absent (Paper I), indicating that O_2 photoreduction in low CO_2-grown WT cells is carried out by Flv1 and Flv3 proteins. The rate of O_2 photoreduction in the WT was ca. 20% of the gross O_2 evolution rate, as measured by MIMS during dark-to-high light transition, implying that about 20% of electrons originating from water splitting were redirected to Flv1 and Flv3 in these conditions (Paper I).

To investigate if Flv1 and Flv3 function as a heterodimer *in vivo*, BN-PAGE with soluble protein fractions extracted from WT and Δ*flv1* cells was used to separate proteins and immunodetect Flv3 with a specific antibody. In both protein samples, a major band of about 140 kDa was recognized, indicating that Flv3 is capable of forming a homodimer, at least in the absence of Flv1 (Paper I). However, similar behavior of the Δ*flv1* and Δ*flv3* mutants during MIMS experiments and the decreased amount of Flv3 in Δ*flv1* led to the conclusion that

Flv1 and Flv3 function as a heterodimer (hereafter Flv1/3), at least in the "Mehler-like" reaction (Paper I).

4.1.2 C_i deprivation increases electron flow to O_2

CO_2 fixation, as the most physiologically important reaction, consumes the majority of cellular reducing equivalents in the form of NADPH. Flv1/3 also obtains electrons from the reducing side of PS I, likely from NADPH. In order to investigate the fate of electrons where the main terminal acceptor is lacking, MIMS analysis was performed on C_i-deprived cells of the WT, Δ*flv1*, Δ*flv3,* and Δ*flv1/3* (Paper I). C_i-deprivation in WT cells stimulated light-induced O_2 uptake to an even higher level than in low CO_2 conditions (60.0 μmol O_2 [mg Chl]$^{-1}$ h^{-1}). This O_2 consumption could not be inhibited, neither by KCN nor by IAC, suggesting that it originates from Flv1/3. A comparison of rates of gross O_2 evolution and O_2 photoreduction indicated that, in case of unavailability of C_i, up to 60% of electrons extracted from water were redirected to O_2 by Flv1/3 (Paper I).

Interestingly, C_i-deprived Δ*flv1*, Δ*flv3* and Δ*flv1/3* also demonstrated high rates of O_2 photoreduction (about 45-55 μmol O_2 [mg Chl]$^{-1}$ h^{-1}) which was approximately equal to ca. 60% of their gross O_2 evolution rates (Paper I). This O_2 uptake was not affected by KCN, but could be significantly inhibited by IAC. However, some O_2 uptake (about 20% of gross O_2 evolution) was still present even after application of the inhibitor. To further confirm that O_2 photoreduction belonged to photorespiration, the photorespiratory pathways involved in the elimination of 2-PG were studied (Paper I). Indeed, cells of the Δ*flv1/3* mutant accumulated more serine and glycine, intermediates of photorespiration, after 36 h of C_i-deprivation compared to the WT. Transcripts of the genes encoding aminomethyltransferase (GcvT) and acetolactate synthase (IlvB) enzymes, representing the plant-like C2 cycle and bacterial-type glycerate photorespiratory pathway respectively, were increased 2-3-fold in Δ*flv1/3* compared to WT levels.

Thus, the major part of the light-induced O_2 uptake in Δ*flv1/3* was carried out by photorespiration (Paper I). Conceivably, the inability to forward electrons neither to CO_2 fixation nor to O_2 via Flv1/3 created a condition favoring the oxygenation of Rubisco. The residual part of O_2 uptake in Δ*flv1/3*, not inhibited by IAC, could possibly be carried out by the "true" Mehler reaction. The contribution of photorespiration to the light-induced O_2 uptake in C_i-deprived WT cells cannot

be completely excluded, since the addition of IAC increased the rate of O_2 photoreduction in the WT (Paper I).

4.2 Flv1 and Flv3 are crucial for survival of cyanobacteria in natural environments

Flv1/3 appeared to have a high capacity of light-induced O_2 consumption to harmlessly dissipate vast amounts of electrons when required. This feature is especially important in natural aquatic environments, where waves create a lens effect and cause sudden changes of light intensity ranging from very dim to very strong conditions. The significance of the Flv-mediated "Mehler-like" reaction was further studied in detail in the photoprotection of cyanobacteria under fluctuating light (Paper II). Both *Synechocystis* and *Anabaena* were used as model organisms to demonstrate that FDPs are essential for different cyanobacteria.

4.2.1 Flv1(A) and Flv3(A) enable growth of *Synechocystis* and *Anabaena* under fluctuating light

WT and the Δ*flv1*, Δ*flv3,* and Δ*flv1/3* mutants of *Synechocystis*; and WT and the Δ*flv1A* and Δ*flv3A* mutants of *Anabaena* were used in Paper II. Flv1A and Flv3A are expressed in vegetative cells of *Anabaena* and likely function as O_2 photoreduction analogues to Flv1 and Flv3 from *Synechocystis* (see Paper III, chapter 4.3.1). The cells were subjected to two different light regimes: FL 20/500 and FL 50/500. First, the growth capacity of the cells was addressed under FL in liquid cultures starting from OD_{750}=0.1.

FL 20/500 appeared to be a more severe condition for the cells, since after 8 days none of the studied *flv* mutants showed an increase in OD_{750}, whereas WT *Synechocysis* and WT *Anabaena* demonstrated stable growth comparable with the growth observed under continuous light (Paper II). However, if after 4 days under FL 20/500, cells of *flv* mutants and WT *Synechocystis* were transferred to normal growth light conditions (50 μmol photons m^{-2} s^{-1}), mutant cells could restore the growth and reach the OD_{750} of WT cells. Longer periods of treatment (8 days) under FL 20/500 resulted in the death of *flv* mutant cells whereby, even after the transfer to normal growth light, cells could not recover.

The FL 50/500 condition did not kill the cells, but induced a significant decrease of growth in *flv* mutants of *Synechocystis* compared to the WT. The observed effect of FL was not caused by the high light itself, since the growth of the *Synechocystis flv* mutants under constant light of 500 μmol photons m^{-2} s^{-1} did not differ from the WT (Paper II).

4.2.2 Flv1/3 protects Photosystem I under fluctuating light

In paper II, the photosynthetic properties of the WT and *flv* mutants under FL were further analyzed using the Δ*flv1/3* mutant of *Synechocystis*. First, MIMS analysis of gas exchange was performed on cells acclimated to FL 20/500 for 3 days while mimicking FL conditions during the measurement. Both CO_2 consumption and O_2 evolution kinetics of the WT demonstrated that cells were able to increase the photosynthetic performance under sudden increases of light intensity. During the high light (HL) phases, WT cells showed intensive bursts of CO_2 uptake and 10-fold higher rates of gross O_2 evolution compared to the low light (LL) phases. O_2 photoreduction also increased drastically upon low light-high light (LL-HL) transitions (2.4 to 50.4 μmol O_2 [mg Chl]$^{-1}$ h^{-1}); however, the net O_2 evolution was still higher under HL compared to LL (Paper II).

During the LL phases, the Δ*flv1/3* mutant already demonstrated approximately three times lower rates of gross O_2 evolution, compared to the WT. During the LL-HL transitions, the mutant exhibited no bursts of CO_2 uptake and only a moderate increase in gross O_2 evolution. Conversely, the mutant cells demonstrated significant O_2 photoreduction rates of 8.4 (LL) and 16.0 (HL) μmol O_2 [mg Chl]$^{-1}$ h^{-1}. Therefore, the net photosynthesis in the mutant was only just above zero during the LL phases and was three times lower during the HL phases, compared to the WT (Paper II).

The activity of both photosystems in the WT and Δ*flv1/3* under fluctuating light was further checked in Paper II. First the status of the donor and acceptor sides of PS II was addressed by Flash Fluorescence analysis. The kinetics of the dark-relaxation of flash induced fluorescence yield in the presence and absence of DCMU were similar in the WT and Δ*flv1/3*, implying neither significant structural nor functional damage of PS II in the mutant. The value of the maximum quantum yield of PS II (F_m-F_o/F_m) was lower in the Δ*flv1/3* mutant compared to the WT (0.35 and 0.43 respectively) (Paper II). However, this difference did not fully explain the drastic decrease of photosynthetic activity observed in the mutant cells.

Next, the performance of both photosystems under FL cycles was simultaneously monitored by mimicking FL 50/500 using an actinic light in Dual-PAM (Paper II). The P_m value, which indicates the maximum oxidizable amount of PS I centers, of the WT was 0.33, while in Δ*flv1/3* it was only 0.06. In the WT, upon the transition to HL, the reduction of PETC caused an increase in the F_s level and a significant drop in Y(I) and Y(II), which represent the effective yields of

PS I and PS II, respectively. However, LL-HL transitions did not provoke an acceptor side limitation of PS I, Y(NA). P700 was strongly oxidized by HL, but some portion was demonstrated to remain available for oxidation when probed by saturating pulses.

Under the LL phases, the Δ*flv1/3* mutant already had a lower Y(II) than the WT, and, upon the transition to HL, all PS II centers closed. Importantly, during the LL phases P700 stayed reduced and even periodic HL phases could not oxidize P700 in the mutant strain. Some level of PS I acceptor side limitation was already detected in the mutant during the LL phases, and upon the LL-HL transitions cells demonstrated very strong limitation of electron transfer beyond PS I. However, the addition of methyl viologen, an artificial acceptor of electrons from PS I, allowed some portion of P700 to become oxidizable and opened some PS II centers during the HL phases. Therefore, the presence of Flv1/3 ensures an absence of the limitation of electron transport beyond PS I and prevents the over-reduction of PETC (Paper II).

Analysis of the dynamic changes in protein content of the WT and Δ*flv1/3* mutant under FL further demonstrated that PS I was the primary target of oxidative damage in the absence of Flv1/3 (Paper II). While the amount of PsaB (PS I core protein) in WT cells increased after 7 days under FL, PsaB content in the mutant decreased drastically after only 3 days of FL treatment. Moreover, after 7 days under FL, PsaB was almost absent in the mutant cells. In contrast, the amount of PsbA (PS II core protein) in the Δ*flv1/3* cells only started to decrease after 7 days of FL treatment. Although the amount of RbcL (Rubisco large subunit) in the mutant already decreased after 1 day of the FL treatment, it was still about 50% of the WT level after 7 days. The "oxyblot" analysis, detecting the carbonylation of proteins caused by oxidative agents (*e.g.* ROS), revealed increased amounts of carbonylated proteins in the mutant compared to the WT after only 1 day of FL treatment. Therefore, the immediate damage of PS I and consequent damage of other proteins upon the shift of the Δ*flv1/3* cells to fluctuating light was conceivably related to the production of ROS, caused by over-reduction of PETC (Paper II).

4.2.3 Respiratory terminal oxidases can support O_2 photoreduction

As reported in Paper II, WT *Synechocystis* cells demonstrated an increase in O_2 photoreduction during LL-HL transitions. The separate additions of both KCN and IAC further increased the rates of light-induced O_2 uptake during both LL and HL phases. Therefore, under fluctuating light, the photoreduction of O_2 in

the WT was carried out mostly by Flv1/3. Importantly, upon the increase of light intensity during HL phases, electron flow via Flv1/3 to O_2 was highly stimulated (Paper II).

It was interesting that Δ*flv1/3* cells also demonstrated O_2 photoreduction under FL, and that the addition of IAC further stimulated this O_2 uptake (Paper II). However, the addition of KCN could inhibit almost all light-induced O_2 uptake during the LL phases, and a major portion (about 60%) of O_2 uptake during the HL phases. Thus, O_2 photoreduction in the Δ*flv1/3* mutant under FL conditions was carried out by KCN-sensitive RTO(s). It is highly possible that Cox and/or Cyd, RTOs which are known to share the thylakoid membrane with PETC, were responsible for this light-induced O_2 uptake. In addition, the residual O_2 uptake in the mutant, which could not be inhibited by KCN, might be related to the "true" Mehler reaction (Paper II).

4.3 O_2 photoreduction in *Anabaena*

Anabaena can be considered a more complex organism than *Synechocystis* since it has a truly multicellular structure containing specialized cells with different functions. Thus, the fact that genome of *Anabaena* contains six genes encoding FDPs compared to four in *Synechocystis* suggested that the "extra" *flv* genes might be related to the specific metabolic activities of *Anabaena*, such as N_2 fixation. In papers III and IV, multiple approaches, targeted to both whole filaments and isolated heterocysts, were used to investigate the relationship between Flv proteins and O_2 photoreduction in *Anabaena*.

4.3.1 Expression of *flv* genes in *Anabaena*

Based on phylogenetic analysis of *flv* genes performed by Zhang et al. (2009) the six *flv* genes of *Anabaena* were designated as: *flv1A* (*all3891*); *flv1B* (*all0177*); *flv2* (*all4444*); *flv3A* (*all3895*); *flv3B* (*all0178*); and *flv4* (*all4446*) (Paper III). The *flv2* and *flv4* genes are arranged in an operon, *flv4-sll4445-flv2*, closely resembling the *flv4-sll0218-flv2* operon of *Synechocystis* (Zhang at el. 2012). According to phylogenetic analysis, other *flv* genes, represent duplicates of *flv1* and *flv3* of *Synechocystis* and share a high degree of identity between each other (Paper III).

Transcript analysis of *flv* genes was performed for WT *Anabaena* in non-N_2-fixing conditions, before and after the shift of cells from high CO_2 to low CO_2, and under different light intensities (Paper III). In high CO_2 conditions, the transcripts of *flv2* and *flv4* were found in the lowest amount compared to other *flv* genes, while the transcripts of *flv1A* and *flv3A* were found in the highest

amounts. Two days after the shift from high to low CO_2, *flv1A* and *flv3A* responded with 3-5 fold increases in transcript amounts, whereas the amounts of *flv2* and *flv4* transcripts increased 400-fold. The *flv1B* and *flv3B* genes demonstrated very low transcript levels and this was independent of the CO_2 regime applied.

To study the response of *flv* transcripts to light treatment, high CO_2-grown WT cells were shifted for 1 h treatments under dim light, high light, or darkness (Paper III). The high light treatment resulted in a 2-3-fold increase of transcript amounts of *flv1A* and *flv3A* genes, while in dim light and in darkness, transcript amounts of these genes decreased compared to the control values under growth light. In contrast, transcript amounts of *flv1B* and *flv3B* were not affected by the light treatment applied.

Since transcripts of *flv1B* and *flv3B* were previously found to be more abundant in N_2-fixing filaments of *Anabaena* (Ehira et al. 2003), the transcription of *flv* genes during the first 24 h after a combined N step-down was studied in detail (Paper III). For this purpose, cells grown in nitrate-containing medium were transferred into the combined N-free medium (BG11o). This shift was intended to cause nitrogen starvation and to initiate the process of heterocyst development, required for the fixation of atmospheric N_2. As a control for nitrogen starvation, we used expression of the *nifH* gene, coding for nitrogenase reductase, which begins to be expressed only in mature heterocysts. The transcript amount of *nifH* rose drastically after 6 h of combined N step-down and stayed constantly high until the last studied time-point, at 24 h. Importantly, *flv1B* and *flv3B* demonstrated similar behavior, with increased transcript abundance following the course of *nifH* transcription. Whilst, after 24 h of nitrogen starvation, transcript amounts of *flv1A* and *flv3A* were halved compared to initial levels (Paper III).

It was clear that, despite the close phylogenetic relationship between pairs of the *flv1* and *flv3* genes, "*A*" and "*B*" *flv* genes of *Anabaena* function in different processes. Therefore, mutant strains where each of Flv1 and Flv3 proteins was co-translated with the small fluorescent protein YFP were constructed, and the latter could be easily visualized in living cells by fluorescent microscopy to localize the expression of Flv proteins (Paper III). Visualization of YFP fluorescence from mutant filaments grown on solid BG11o medium for 5 days revealed that Flv1A and Flv3A proteins are located exclusively in vegetative cells of filaments, while the expression of Flv1B and Flv3B was restricted to heterocysts.

4.3.2 O_2 photoreduction in heterocysts

Single and double mutants lacking functional Flv1B and/or Flv3B proteins were constructed to study the role of these proteins in O_2 photoreduction (Paper IV). Since Flv1B and Flv3B proteins were found to be heterocyst-specific, isolated heterocysts from N_2-fixing filaments of the WT and mutants were subjected to O_2 consumption analysis using an O_2 electrode. As confirmed by Dual-PAM fluorescence analysis, heterocysts in our preparations lacked active PS II centers and, therefore, we could directly measure O_2 uptake. Heterocysts of the WT and Δ*flv1B* demonstrated rates of light-induced O_2 uptake of 2.2 and 1.8 μmol [mg Chl]$^{-1}$ h^{-1}, respectively. In contrast, heterocysts of both mutants lacking Flv3B, whilst demonstrating twice the dark O_2 uptake rate of the WT, did not exhibit any increase of O_2 consumption upon a dark-to-light transition. Thus, they completely lacked light-induced O_2 uptake (Paper IV).

Indeed, the light-induced O_2 uptake of WT heterocysts had different origin than respiratory O_2 uptake. The addition of KCN significantly decreased the O_2 uptake of heterocysts in the dark, but stimulated light-induced O_2 uptake. Moreover, the addition of KCN together with SHAM induced an even greater effect, whereby it almost eliminated dark O_2 uptake, but up-regulated O_2 photoreduction 3-fold (Paper IV). It is conceivable that O_2 photoreduction in the heterocysts of *Anabaena* is carried out by flavodiiron protein Flv3B to maintain, in cooperation with RTOs, microoxic conditions.

An inability to perform O_2 photoreduction resulted in drastic changes in the metabolism and function of heterocysts (Paper IV). Firstly, as measured by the acetylene assay, the nitrogenase enzyme of the Δ*flv3B* mutant demonstrated only half the capacity of the WT nitrogenase. Moreover, as demonstrated by immunoblotting with NifH antibodies and DIGE protein analysis for NifHDK proteins, the mutant had an approximately 50% reduced amount of nitrogenase. Overall, the comparative DIGE protein analysis of heterocyst-enriched fractions of the WT and Δ*flv3B* suggested that the mutant heterocysts had decreased amount of uptake hydrogenase, increased synthesis of heterocyst-specific cell wall components, decreased Chl synthesis, more active glycolysis and less active OPPP, and a modified redox status inside the cells, compared to WT heterocysts (Paper IV).

A comparative transcript analysis revealed significant up-regulation of genes encoding proteins conceivably involved in the reduction of H_2O_2 and O_2 in the heterocysts of the Δ*flv3B* strain (Paper IV). Among these proteins were CoxA3,

subunit of heterocyst-specific RTO Cox3, lactate oxidase, rubrerythrine, and catalase. The transcript abundance levels of *nifH2* and the heterocyst-specific ferredoxin, *fdxH*, were found in higher amounts in Δ*flv3B* compared to the WT.

Neither protein content nor transcript amounts of most of the studied genes were significantly changed in the heterocysts of Δ*flv1B*. However, the transcript level of *nifH1* was lower than in the WT, and the amount of *nifH2* transcripts was increased, resembling the situation in the Δ*flv3B* mutant. Nitrogenase activity of the Δ*flv1B* filaments, determined in acetylene assays, also demonstrated an approximately 50% decrease compared to the WT, even though the amounts of NifHDK proteins were not affected in the mutant (Paper IV).

4.3.3 Importance of heterocyst-specific O_2 photoreduction for the whole filaments

The deletion of Flv3B resulted in a decreased amount and activity of nitrogenase and, therefore, drastically influenced the supply of whole filaments with fixed N (Paper IV). Filaments of Δ*flv3B* were short and pale-green in N_2-fixing conditions, while WT and Δ*flv1B* formed long green filaments. Chl autofluorescence analysis demonstrated that in the Δ*flv3B* mutant, Chl is distributed unequally and the overall signal of Chl autofluorescence is much lower than in the WT. Indeed, the ratio of total Chl amount per OD_{750} was twice lower in the N_2-fixing Δ*flv3B* filaments compared to the WT, as was the ratio of total protein content per OD_{750}.

The growth of the Δ*flv3B* and Δ*flv1B/3B* filaments, but not of Δ*flv1B* filaments, was severely compromised in N_2-fixing conditions. The growth rate constant of mutants lacking Flv3B was twice lower than the WT value. Importantly, when the WT and Δ*flv3B* were grown in microoxic, N_2-fixing conditions, with continuous bubbling of the cultures with N_2 and CO_2, the growth rate constant of the mutant recovered to WT value (Paper IV). Immunoblotting analysis was in agreement with the growth observations, indicating that the amounts of NifH were similar in both the mutant and the WT under microoxic, N_2-fixing conditions.

Whole N_2-fixing filaments of the WT and mutants were subjected to MIMS gas exchange analysis (Paper IV). Both mutants lacking Flv3B demonstrated ca. 20-25% lower rates of gross O_2 evolution and CO_2 uptake compared to the WT and Δ*flv1B*. Regarding O_2 uptake, the dark rate of O_2 consumption in both mutants lacking Flv3B was 150% of the WT rate. However, under the light, the mutants consumed 50% less O_2 compared to the WT, thus demonstrating a strong

decrease of light-induced O_2 uptake. The Δ*flv1B* mutant had similar dark O_2 consumption rates to the WT, but only a 20% lower light-induced O_2 uptake.

The DIGE analysis of Δ*flv3B* demonstrated consistent reduction of proteins related to light harvesting, the Calvin cycle, Chl synthesis, starch synthesis and other biosynthesis pathways. In contrast, the amounts of enzymes catalyzing reactions of glycolysis were increased in the Δ*flv3B* mutant compared to the WT. This was supported by increased amounts of transaldolase and phosphoketolase, which return carbon molecules from OPPP and pentosephosphates metabolism into glycolysis (Paper IV).

The expression of genes encoding the major subunits of both photosystems and other photosynthesis- and general metabolism-related proteins was also addressed (Paper IV). In line with protein data, in the N_2-fixing filaments of Δ*flv3B*, significant decreases in photosynthesis-related genes *psaB1*, *psbA1*, *flv3A*, *rbcL*, *apcA* (PBS core), and *pecA* were found, as compared to transcript levels of these genes in the WT. However, the transcript level of *flv4* was increased in the Δ*flv3B* mutant, compared to the WT. The amount of *coxA1* transcripts was not affected in the Δ*flv3B* mutant, despite the increased dark O_2 uptake by the mutant filaments.

5. DISCUSSION

5.1 FDPs provide ultimate protection of photosynthesis in cyanobacteria

Organisms performing oxygenic photosynthesis constantly experience the negative consequences of photodamage, which originates from the energetic imbalance occurring when absorbed light exceeds the capacity of photosynthesis for CO_2 fixation, and leads to the over-reduction of PETC. The direct interaction of reduced electron carriers, incorporated into photosystems, with O_2 results in the production of ROS and consequently, oxidative damage of the photosynthetic apparatus. Therefore, in the photosynthetic oxygenic environment, it is extremely important to prevent the over-reduction of photosynthetic components by dissipating excess electrons in a harmless way. Importantly, cyanobacteria can utilize O_2 as an electron acceptor for this purpose, by performing the light-induced reduction of O_2 in a controlled manner which avoids the production of ROS.

The main players of this process in cyanobacteria are Flv1 and Flv3 proteins, which catalyze O_2 reduction directly to water during illumination. The ability of the Flv1 and Flv3 proteins to couple O_2 reduction with the oxidation of NADPH, produced on the reducing side of PS I, provides cyanobacteria with the following benefits:

1) The avoidance of acceptor side limitation of PS I by reoxidizing $NADP^+$ and, thus, releasing the over-reduction of PETC and regulating linear electron transport (Paper I, II).

2) The supression of the "true" Mehler reaction (Paper I, II). This is important in protecting cyanobacteria against ROS, particularly H_2O_2, to which they are highly sensitive (Matthijs et al. 2012).

3) The supression of photorespiration by decreasing amounts of O_2 in cytoplasm during C_i-deprivation (Paper I). Although photorespiration is essential for cyanobacterial cells in particular conditions (Eisenhut et al. 2006, 2008), this cycle reoxidizes $NADP^+$ at the expense of ATP and fixed carbon.

4) The regulation of NADPH/ATP balance by provision of cells with extra ATP. To cope with high light stress, all photosynthetic organisms require increased production of ATP (Asada et al. 1998). In cyanobacteria, besides cyclic electron

transport, Flv1/3 serves as an ATP generator by directing electrons from PETC to O_2.

5) The presence of an efficient electron valve able to operate on a very fast timescale and respond to fluctuating light intensities (Paper II). Cyanobacterial cells easily acclimate to different environmental stresses, particularly to high light intensities, by modulating photosynthetic parameters and up-regulating photoprotective mechanisms on a slow timescale (Mullineaux 2014a). However, Flv1/3-mediated photoprotection is crucial during very fast changes of light intensity (at frequencies higher than 1 Hz).

An important feature of the function of Flv1/3 is the very high and fast capacity for light-induced O_2 reduction, making the heterodimer extremely important for the survival of cyanobacteria under natural, highly fluctuating, light conditions. However, it is conceivable that the use of NADPH to conclude the water-water cycle reduces the resourses of cyanobacteria for growth. Therefore, higher plants have evolved more sophisticated mechanism for the prompt regulation of electron transfer. In plants, the balance of photosynthetic electron flow under FL is largely regulated by PGR5. PGR5 is probably not as important in CET as it is in the regulation of linear electron flow via the Cyt *b*$_6$*f* complex (Suorsa et al. 2012). Interestingly, the product of the homologues *pgr5* gene in *Synechocystis* was proposed to participate in cyclic electron transport (Yeremenko, 2005). However, as demonstrated in Paper II, this gene was not important for the survival of *Synechocystis* cells under FL.

Flv1/3 provides immediate protection for PS I, which, despite being considered the more robust of the photosystems, was revealed as the primary target of photodamage in the Δ*flv1/3* mutant under FL (Paper II). Taking into account the importance of Flv2/4 for the protection of PS II from photoinhibition (Zhang et al. 2012, Bersanini et al. 2014), the flavodiiron proteins are key players in the protection of cyanobacterial photosynthesis on the level of both photosystems. In this way FDPs may have contributed to the very emergence of oxygenic photosynthesis.

5.2 Multiple routes to O_2 photoreduction?

Flv1/3 is a powerful system for the protection of PETC and under steady-state light it is plausible that this heterodimer is responsible for all photosynthesis-related O_2 photoreduction in cyanobacteria (Paper I, II). The absence of light-induced O_2 uptake in the Δ*flv1/3* mutant under ambient levels of CO_2 suggests

that O_2 photoreduction by other reactions is rather low and cannot be tracked by gas exchange analysis. However, under severe environmental stresses, O_2 photoreduction by various alternative pathways is initiated in the Δ*flv1/3* mutant in order to avoid the "true" Mehler reaction.

As demonstrated in Paper I, in the absence of Flv1/3, light-induced O_2 uptake by Rubisco might support the oxidation of PETC under severe C_i-deprived conditions. It has already been reported that, rather than being located exclusively in carboxysomes, some Rubisco molecules could be located in the cytoplasm of cyanobacteria, and thus be potential targets of oxygenation (Cossar et al. 1985, Cameron et al. 2013). Under FL, the Δ*flv1/3* mutant cells demonstrated the light-induced O_2 uptake carried out by RTO(s) (Paper II). Indeed, in the absence of Flv1/3, and consequently during the drastic over-reduction of PETC, RTOs might contribute to the alleviation of excess pressure by redirecting electrons from intersystem chain to O_2. However, the capacity of RTO(s) for O_2 photoreduction is likely limited and/or not adjustable to the light intensity. Thus, it is highly possible that the KCN-insensitive portion of light-induced O_2 uptake, up-regulated in Δ*flv1/3* during the high light phases of FL, belongs to the "true" Mehler reaction and, therefore, explains the oxidative stress and immediate damage of PS I (Paper II).

Despite the likely functioning of the "true" Mehler reaction in Δ*flv1/3*, the production of ROS under FL could also be partially assigned to RTOs. Plastid terminal oxidase (PTOX) of plants, although not related to Cyd, Cox and ARTO phylogenetically (McDonald and Vanlerberghe 2004), might be considered as a Cyd analog in chloroplasts. Both enzymes oxidize PQ and thus can act as a safety electron valve of the PQ pool (Berry et al. 2002, Heyno et al. 2009). However, it was demonstrated that PTOX produces superoxide in the case of limited substrate availability (Feilke et al. 2014) so it is possible that cyanobacterial RTOs, performing a four-electron reaction, also produce ROS under certain conditions (*e.g.* high light).

It is still questionable whether alternative O_2 photoreduction pathways co-exist under standard or stress conditions, or if they function only in the absence of Flv1 and Flv3 proteins. Helman et al. (2005) attempted to distinguish the impact of different processes in O_2 consumption under light, using triple isotopic O_2 fractionation. The application of this method led authors to suggest that, under the conditions studied, 40% of electrons extracted from water were directed to O_2 by Flv1/3 and 6% of the electrons were likely directed by RTOs.

Unfortunately, the experiments were conducted in the presence of 10 mM $NaHCO_3$ and, therefore, the impact of photorespiratory O_2 uptake was not addressed. Certainly, the high activity of Flv1/3 complicates the precise detection of light-induced O_2 uptake which may be carried out by other systems in WT cells. However, the co-existence of O_2 photoreduction by multiple systems might be expected, for example, in severe stress conditions.

5.3 Cell-specific O_2 photoreduction for the protection of both photosynthesis and N_2 fixation in *Anabaena*

The filaments of *Anabaena* consist of vegetative cells and heterocysts, with these two different cell types operating aerobically and anaerobically, respectively. O_2, produced during photosynthesis in vegetative cells, is likely able to diffuse into heterocysts along concentration gradients and, thus, is a constant threat to N_2 fixation. However, O_2 photoreduction mediated by FDPs appeared to be important for the protection of both photosynthesis and N_2 fixation. For this purpose there are "*A*" and "*B*" forms of the *flv1* and *flv3* genes in the genome of *Anabaena*. Flv1A and Flv3A are expressed in photosynthesizing vegetative cells and functionally match Flv1 and Flv3 from *Synechocystis*, while Flv1B and Flv3B are expressed upon combined N step-down exclusively in heterocysts (Paper III). Flv3B is directly involved in O_2 photoreduction in heterocysts and its absence severely compromises the N_2-fixing activity of nitrogenase in oxic, but not in microoxic conditions (Paper IV). Like nitrogenase, Flv3B is distributed throughout the cytoplasm of heterocysts (Paper III) therefore suggesting that FdxH, obtaining electrons from PS I and transferring them to nitrogenase, can also be an electron donor for Flv3B.

As vegetative cell-specific and heterocyst-specific FDPs perform similar reactions, reducing O_2 using electrons provided on the reducing side of PS I, it is surprising that *flv1* and *flv3* differentiated into paralogues genes. However, differences in gene expression between vegetative cells and heterocysts have required the duplication of *flv1* and *flv3* genes for their independent regulation. While transcript amounts of *flv1A* and *flv3A* are increased upon the requirements of PETC in vegetative cells (Paper III), the *flv3B-flv1B* operon is likely under developmental control, as suggested for the genes expressed during heterocyst formation (Elhai and Wolk 1990, Valladares et al. 1997). However, our data (Paper IV) suggest that, in mature heterocysts, gene expression might be additionally regulated by the redox status of cells, which is indirectly dependent on the amount of O_2 inside heterocysts. In line with this, a recent study revealed

many potentially redox regulated proteins in heterocysts of *Nostoc punctiforme*, including homologues of Flv1B and Flv3B (Sandh et al. 2014).

Importantly, although Flv3B has a presumptive partner inside heterocysts (Flv1B), my thesis provided the first evidence showing that this cyanobacterial flavodiiron protein does not require the formation of a heterodimer to perform O_2 photoreduction. In this way Flv3B might be more closely related to FDPs from anaerobic species, since they form homodimers or homotetramers and function in microoxic environments, like heterocysts. The expression of the *flv3B* gene, together with nitrogenase genes, starts during the latest stage of heterocyst formation, when O_2 levels inside the cells has already decreased significantly (Flaherty et al. 2011). Therefore, it is conceivable that Flv3B, like FDPs from anaerobic species, might tolerate only low amounts of O_2, as compared to the FDPs from O_2-producing vegetative cells.

The function of Flv1B in heterocysts is not yet clear. Deletion of the *flv1B* gene effects the metabolism of heterocysts and whole filaments of *Anabaena*, with increased rates of dark respiration, lower activity of nitrogenase, and increased transcription of *nifH2*, together indicating a mild oxidative stress condition in Δ*flv1B* heterocysts. Despite Flv3B being capable of O_2 photoreduction alone, Flv1B might be required for the regulation of this reaction, or for the effective conversion of O_2 to water without the production of ROS. However, it is also possible that Flv1B has other, not yet elucidated functions related to electron transport inside heterocysts. Likewise, PS II in heterocysts lacks water-splitting activity, but is still capable of electron transport and might have a completely distinct function. Therefore, other novel functions of FDPs in cyanobacteria might still be expected.

6. CONCLUDING REMARKS AND FUTURE PLANS

The study of O_2 photoreduction by flavodiiron proteins Flv1 and Flv3 undertaken for this thesis resulted in a series of far-reaching discoveries, beyond the originally assumed role of FDPs in photosynthesis. The data presented here unambiguously demonstrate that the light-driven reduction of O_2 can be performed by multiple systems and is of crucial importance in cyanobacteria. Upon the oxygenation of the Earth's atmosphere, the photoreduction of O_2 became a potent strategy to prevent oxidative damage of photosynthetic components and nitrogenase. The elegant reduction of O_2 directly to water, as catalyzed by flavodiiron proteins, is gaining popularity in research due to the evidence for the important role of FDPs in protecting against oxidative stress. This protective role has been found to be significant, not only in the originally studied anaerobic prokaryotes, but also in aerobic prokaryotes and anaerobic and aerobic eukaryotes.

The complex regulation of photosynthesis and the numerous reactions shaping the environment of heterocysts still holds many puzzles. Therefore, the work started in this thesis will be further continued in our laboratory. A more detailed investigation of RTO(s) activity under light is a priority, since their impact in the regulation of PETC has not yet been well addressed. Besides this, it is important to clarify the mystery of the Flv1/3 heterodimer. Although the heterodimer is undoubtedly responsible for the Mehler-like reaction, closer examination suggests that Flv1 and Flv3 also have roles to play. The apparent difference between the "extra" functions of these proteins was noticed by Hackenberg et al. (2009) under particular conditions in *Synechocystis*. However, the difference between mutants lacking either Flv1A or Flv3A is more striking in *Anabaena* than in *Synechocystis* (unpublished results), and thus, studies of Flv1 and Flv3 analogs in *Anabaena* might provide novel insights into more diverse functions of cyanobacterial FDPs. The role of Flv1B in heterocysts also remains to be elucidated, thus deserving further investigation. Future discoveries resulting from these proposed investigations will, along with the data provided in my thesis, yield a better understanding of naturally occurring acclimation to environmental changes in cyanobacteria. Metabolic plasticity, convincingly, played a crucial role in the heyday of cyanobacteria and made possible the consequent evolution of eukaryotic phototrophs. Moreover, the knowledge gained from this thesis work, will be useful in employing synthetic biology for the creation of novel cyanobacterial strains with desirable features for important applications, such as the production of biofuels.

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Mama

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