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II. Angeleri M, Zorina A, Aro EM and Battchikova N. (2018) Interplay of SpkG kinase and the Slr0151 protein in phosphorylation of Ferredoxin 5 in *Synechocystis* sp. Strain PCC 6803. *FEBS Lett*. 592, 411-421

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Other publications related to the topic:

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ABBREVIATIONS

3-PGA	3-phosphoglycerate
AET	alternative electron transfer
CCM	carbon concentration mechanism
CBB	Calvin-Benson-Bassham cycle
Chl-*a*	chlorophyll *a*
Ci	inorganic carbon
CET	cyclic electron transfer
Cu^{2+}	cupric ions
Cyt b_6f	cytochrome b_6f
Cyt c_6	cytochrome c_6
DDA	data dependent acquisition
FA	formic acid
Fd	ferredoxin
Fd5	ferredoxin 5
FNR	ferredoxin:NADP+ oxidoreductase
FRP	fluorescence recovery protein
GA3P	glyceraldehyde3-phosphate
GS	glucose-sensitive
GT	glucose-tolerant
HC	high carbon
HPLC	high-performance liquid chromatography
ICAT	isotope-coded affinity tag
ITRAQ	isobaric tags for relative and absolute quantitation
Km-R	kanamycin-resistance cassette
LC	low carbon
LC-MS/MS	liquid chromatography - tandem mass spectrometry
LET	linear electron transfer
MS	mass spectrometry
m/z	mass to charge ratio
nCE	normalized collision energy

ncRNAs	non-coding RNAs
NH_3	ammonia
Ni	inorganic nitrogen source
NPQ	non-photochemical quenching
OCP	orange carotenoid protein
OPP	oxidative pentose phosphate pathway
ORF	open reading frame
PAP	photosystem II assembly proteins
PBS	phycobilisomes
PC	plastocyanins
PQ	plastoquinones
PSI	photosystem I
PSII	photosystem II
PSM	peptide spectral match
PTM	post-translational modification
RBSs	ribosome binding sites
ROS	reactive oxygen species
RT	retention time
RTOs	respiratory terminal oxidases
RT-qPCR	real time quantitative polymerase chain reaction
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme
RuBP	ribulose 1,5-bisphosphate
SILAC	stable isotope labeling by/with amino acids in cell culture
SRM	selected reaction monitoring
TCEP	tris(2 carboxyethyl)phosphine hydrochloride
TM	thylakoid membrane
TMT	tandem mass tag
TPRs	tetratricopeptide repeats
TQQ	triple quadrupole
WT	wild type

ABSTRACT

Cyanobacteria are unique prokaryotic organisms performing oxygenic photosynthesis. Their ability to transform sunlight and CO_2 to sugar, together with the flexibility of metabolism, have made cyanobacteria interesting organisms for engineering the carbon and electrons fluxes toward exogenous pathways for the production of high value compounds. Understanding the complex mechanisms regulating photosynthesis and their dynamic interactions with cellular metabolism are crucial in order to improve the productivity of engineered strains.

In this Thesis, reversible O-type phosphorylation on S/T/Y amino acids, a ubiquitous posttranslational regulatory mechanism, was investigated in *Synechocystis* 6803 using proteomics approaches. A global discovery-driven investigation of the phosphoproteome revealed that S/T/Y phosphorylation is extensive in *Synechocystis* 6803, and phosphoproteins are involved in various metabolic pathways. A large share of the identified phosphoproteins participate in photosynthesis-related processes.

The quantitative targeted mass spectrometry method was set-up for (phospho)peptides of photosynthesis-related proteins. It was applied for screening a collection of S/T protein kinase mutants in *Synechocystis* 6803. The results revealed an intricate phosphoprotein-protein kinase network, including an interplay among several kinases and auxiliary proteins. In particular, the SpkG kinase was shown to phosphorylate the Fd5 protein, while Slr051 was revealed as an auxiliary protein regulating the balance between the phosphorylated and non-phosphorylated forms of Fd5. Moreover, the deletion of the SpkG kinase caused the induction of phosphorylation in several other peptides indicating a cross talk among the participants of the protein phosphorylation network.

Further, the data obtained in this Thesis showed that the induction of exogenous pathways in synthetic biology approaches might cause background proteome changes due to the acclimation of the cells to the concentration of metal ions. Particularly, the Cu^{2+} treatment, used to regulate the *petJ* promoter, irreversibly affected DNA replication, transcription and translation machineries, cell wall proteins, transporters, signaling proteins and enzymes involved in lipid biosynthesis. These changes might consequently affect the expression of a pathway or the recovery of a high value product.

In summary, the results presented in this Thesis provide a better understanding of *Synechocystis* 6803 regulatory mechanisms, which might support the optimization of engineered strains toward maximal productivity of high value compounds.

TIIVISTELMÄ

Syanobakteerit ovat ainoita happea tuottavaan fotosynteesiin kykeneviä esitumallisia organismeja, jotka auringon valoenergian avulla sitovat epäorgaanisen hiilen orgaanisiin molekyyleihin. Syanobakteerit ovat kiinnostava tutkimuskohde, sillä niiden aineenvaihduntareaktioita voidaan suhteellisen helposti muokata ja valjastaa arvokkaiden luonnonyhdisteiden tuottoon. Näiden yhdisteiden tuottaminen syanobakteereissa edellyttää syanobakteerien aineenvaihduntareaktioiden monimutkaisen säätelyverkoston tarkkaa tuntemusta.

Tässä tutkimuksessa selvitin syanobakteerin, *Synechocystis* 6803, proteiinien seriini-, treoniini-, tyrosiini -aminohappotähteiden O-fosforylaatiota proteomiikka-menetelmiä käyttäen, ja osoitin että kyseinen proteiinien translaation jälkeinen säätelymekanismi on hyvin yleinen tutkitussa syanobakteerilinjassa, ja useat tunnistetuista fosfoproteiineista toimivat fotosynteesireaktioissa. Kvantitatiivista massaspektrometriaa käyttäen seuloin seuraavaksi seriini- ja treoniinitähteiden fosforylaatiosta vastaavia proteiinikinaaseja. Tutkimuksessa löydettiin useista kinaaseista koostuva säätelyverkosto, jossa eri kinaasit toimivat synkronoidusti toistensa sekä muiden säätelyproteiinien kanssa. Osoitin että nk. SpkG-kinaasi vastaa Fd5-proteiinin fosforylaatiosta, ja Slr051-proteiini puolestaan säätelee Fd5-proteiinin fosforyloitujen ja fosforyloitumattomien muotojen suhdetta.

Lisäksi tutkimus osoitti, että synteettisen biologian menetelmin indusoidut metaboliareitit saattavat vaikuttaa *Synechocystis* 6803-solun koko proteomiin kohonneen metalli-ionikonsentraation johdosta. Erityisesti *petJ* promoottorin säätelyyn vaikuttava Cu^{2+}-käsittely muutti peruuttamattomasti DNA:n replikaatioon, transkriptioon ja translaatioon sekä soluseinäproteiineihin, kuljetusproteiineihin, signalointiproteiineihin ja lipidien biosynteesissä toimiviin proteiineihin. Nämä muutokset saattavat vaikuttaa metaboliareitin ilmenemiseen sekä halutun lopputuotteen saantoon.

Tässä väitöskirjassa esitetyt tulokset tuovat uutta tietoa *Synechocystis* 6803-solun säätelymekanismeista, ja näitä tuloksia on mahdollista soveltaa haluttujen arvokkaiden luonnonyhdisteiden tuotannon optimoinnissa

…It is according to the way
you look at things...

Walt Disney

-

1. INTRODUCTION

1.1. Cyanobacteria as a biotechnology platform

Cyanobacteria are the only prokaryotic organisms performing oxygenic photosynthesis, the fascinating process of trapping the energy of sunlight in sugars and other biomolecules using CO_2 as carbon source (for review see Drews, 2011). This ability makes them the most important primary producers on Earth (Bryant, 2003). In parallel, the release of O_2 as a bioproduct of photosynthesis contributes to the creation and maintaining of the current atmospheric conditions (Holland, 2006; DeRuyter and Fromme, 2008;). Photosynthesis has been thoroughly investigated in cyanobacteria, thus revealing the sophisticated structure and function of the photosynthetic machinery. However, research in this field is still going on in order to investigate the complex regulatory mechanisms and their dynamic interaction (Battchikova et al., 2015). An innovative biotechnological prospective for cyanobacteria as "green biofactories", has stimulated the development of new bioengineering tools (Wang et al., 2012; Berla et al., 2013; Gangl et al., 2015). Beside the economic advantage, the employment of autotrophic organism as biofactories has positive environmental impact since they consume CO_2, thus reducing "green house gases" pollution. Moreover, cyanobacteria have a short doubling time (Klemencic et al., 2017) and they can be cultivated in ponds, without competing for arable land (Rosgaard et al., 2012; Branco dos Santos et al., 2014). Cyanobacteria have been tested for production of low value compounds to use as biofuel or bulk chemicals (Klemencic et al., 2017), such as alcohol, alkanes/alkenes, lipid, and biohydrogen (Pacheco et al., 2014), as well as high value compounds like sugars, terpenoids, alkaloids, and phenylpropanoids for pharmaceutical application (Klemencic et al., 2017). Most of these compounds have been produced only on the lab-scale, but for some of them trials at pilot-scale facilities are going on to evaluate scale-up problems and economic competitiveness (Savakis and Hellingwerf., 2015; Wlodarczyk et al, 2016). Some species of cyanobacteria are considered safe for humans (WHO, 1999), and can thus be used for feed and food applications. Nowadays, an important direction in the development of "green biofactories" is the optimization of the engineered strains in order to balance the production of the compound of interest with the cells´ natural metabolism (Keasling, 2012). To this end, the highly advanced MS-based proteomics approaches are valuable tools for revealing novel regulatory mechanisms (Battchikova et al., 2018) and for evaluating proteome re-adjustments in mutant strains (Hagemann and Hess, 2018).

1.1.1. Synechocystis sp. PCC 6803 as a model species

Synechocystis sp. PCC 6803 (hereafter *Synechocystis* 6803) is a unicellular, non-toxic and non-diazotrophic freshwater β-cyanobacterium (Stanier et al., 1971; Ikeuchi and Tabata, 2001). The DNA content of *Synechocystis* 6803 consists of one multi-copy circular chromosome and 7 plasmids, encoding for a total of 3600 open reading frames (OFRs), of which about 3200 are in the main chromosome (3.6 Mbp) and additional 400 are in the plasmids (Mitschke et al., 2011). Currently, 4 different *Synechocystis* 6803 substrain genomes have been sequenced and annotated (Kaneko et al., 1196 A, B; Trautmann et al., 2012). The existence of a glucose-tolerant (GT) substrain, which is able to grow in the absence of light, allowed the study of heterotrophic and photoautotrophic metabolism via mutants defective in photosynthesis; such mutants are not viable in other substrains (Williams, 1988; Anderson and McIntosh, 1991; de Porcellinis et al., 2016). Thus, *Synechocystis* 6803 has been widely used as a model organism in studying photosynthesis and related processes. Beside photosynthesis, *Synechocystis* 6803 can obtain energy from the degradation of carbohydrates and from respiration, which are particularly important processes to provide the energy needed for cell metabolism in the dark (Vermaas et al., 2001). The photosynthetic machinery and some of the respiratory complexes are located in the thylakoid membrane (TM). *Synechocystis* 6803 is competent (Williams 1988, Barten and Lill, 1995) and can undertake natural transformation; additionally, a targeted mutagenesis protocol has been developed through the homologous recombination mechanism (Kufryk et al., 2002). All these features have promoted studies of *Synechocystis* 6803 as a model cyanobacterial organism, also in biotechnological approaches.

1.1.2. Photosynthetic apparatus: light and carbon reduction reactions

The energy from the sunlight is converted into chemical energy in the form of NADPH and ATP through a series of light-dependent reactions known as linear electron transfer (LET; Figure 1). Photosynthetic thylakoid-embedded protein complexes, namely Photosystem (PS) I and PSII and the Cytochrome b_6f complex (Cyt b_6f), interconnected by electron carriers (the membrane carrier plastoquinone, PQ, and the soluble carriers plastocyanin, PC; cytochrome c_6, Cyt c_6; ferredoxin, Fd and ferredoxin:NADPH oxidoreductase, FNR) are responsible for this process (see reviews by Nelson and Yocum, 2006; DeRuyter and Fromme, 2008). The soluble antenna system, phycobilisomes (PBS), function as efficient collector of solar energy. (Gantt and Conti, 1966; Wildman and Bowen, 1974). In *Synechocystis* 6803, PBSs are organized in specific hemidiscoidal structures (Yu and Glazer, 1982) that ensure a unidirectional transfer of energy toward P680 of PSII via terminal emitter proteins.

From P680, the excited electron is rapidly transferred to plastoquinone Q_A and then to a second plastoquinone Q_B. After receiving two electrons, Q_B is reduced to Q_B^{2-}, protonated to PQH_2 and released in the PQ pool. PQH_2 transfers electrons to Cyt b_6f which donates them to a lumen soluble carrier (PC or Cyt c_6) and at the same time releases the protons into the lumen. The soluble carrier transfers the electron to $P700^+$, a chlorophyll *a* (Chl-*a*) belonging to PSI that was excited by photons, similarly to P680. The electron released from the P700 in PSI is accepted by the cytosolic Fd. Finally, after receiving two electrons from Fd, FNR reduces $NADP^+$ to NADPH. In the entire process, two molecules of NADPH are generated per two water molecules split. At the same time, the active transfer of H^+ from the cytoplasm to lumen generates a proton gradient across the thylakoids, which in turns provides the energy for the photophosphorylation of ADP to ATP by the thylakoid-embedded ATP synthase complex. ATP synthase requires $12H^+$ to generate 3 ATPs. Thus, the ATP:NADPH ratio generated by LET is 3:2 (Behrenfeld et al., 2008).

The **cyclic electron transfer (CET) around PSI** generates ATP without net accumulation of reducing equivalents. In CET electrons are recycled from the PSI acceptor side to the PQ pool or Cyt b_6f, with concomitant transfer of protons across the thylakoid membranes, generating a proton gradient for the production of ATP (see review by Peltier, 2010; Yamori and Shikanai, 2016). The main pathway for CET in *Synechocystis* 6803 comprises the NDH-1_1 and NDH-1_2 complexes (Battchikova et al., 2011A; Strand et al., 2017), represented by violet arrows in Figure 1.

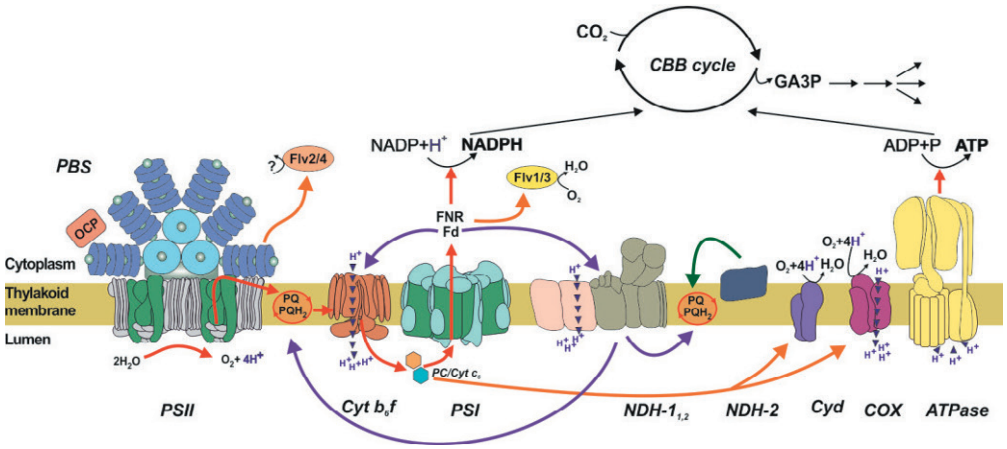

Figure 1: Schematic diagram of electron transport routes in the TM of *Synechocystis* 6803. Full arrows indicate electron transport: LET in red, CET in violet, alternative electron transfer in orange, and other electron inputs into the chain in green. Dotted violet arrows indicate proton translocation across the membrane.

Modified from Battchikova and Aro, 2014.

In the subsequent phase of photosynthesis, the Calvin-Benson-Bassham cycle (CBB), ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (Rubisco) uses ATP and NADPH to catalyze the fixation of CO_2 into ribulose 1,5-bisphosphate (RuBP), forming 3-phosphoglycerate (3-PGA). Cyanobacterial Rubisco has low affinity for CO_2 and low catalytic efficiency compared to the eukaryotic algae or higher plants enzyme. In order to increase the carbon fixation rate, cyanobacteria evolved active carbon uptake mechanisms, collectively known as the carbon concentration mechanism (CCM) (for review see Price et al., 2011) and a compartment called the carboxysome (see review by Badger and Price, 2003 and Montgomery et al., 2016). In this way, the first step of CCB takes place in an environment enriched in CO_2. 3-PGA is then exported from the carboxysome to the cytoplasm, where it is converted in glyceraldehyde3-phosphate (GA3P), that is used as a precursor for the synthesis of several compounds.

1.1.3. Acclimation mechanisms of the photosynthetic apparatus

The photosynthetic light-reactions should provide enough energy and reducing equivalent for maintaining the active CBB cycle and other biosynthetic pathways; on the other hand, excess of light causes over-reduction of LET that in turn may lead to production of toxic reactive oxygen species (ROS) damaging nucleic acids, proteins, lipids and pigments (Latifi et al., 2009). Avoiding excess of energy absorption is important and cyanobacteria evolved numerous regulatory mechanisms, which enable cells to acclimate rapidly to variable environmental conditions in water, especially in relation to the availability of light (Figure 1).

Distinct illumination conditions excite PSI and PSII differently, creating an imbalance in the excitation of PSI and PSII and altering the redox state of the PQ pool. The imbalance triggers the activation of **state transitions**, a process redistributing the energy absorbed by PBS between the two PSs functioning in LET (Bonaventura and Myers, 1969; Murata, 1969). N**on-photochemical quenching (NPQ) of Chl fluorescence** protects the photosynthetic machinery dissipating a part of the energy collected by PBS as heat (Wilson et al., 2008) thus reducing the amount of energy transfer to PSII. In *Synechocystis* 6803, NPQ is regulated by the interplay of two proteins, the orange carotenoid protein (OCP) that is activated by blue light or high white light, and the fluorescence recovery protein (FRP), converting OCP back to the inactive form (Kirilovsky and Kerfeld, 2016). In continuous stress condition, such as long exposure to high light, LET is protected from over-reduction by **PBS uncoupling and detachment** from the PSs (see review by Kirilovsky, 2015). Cyanobacteria also evolved **alternative electron transfer (AET)** routes (represented by the orange arrow in Figure 1) in order to deviate the excess of

electrons from LET. Flavodiiron proteins (Flv1-4; Zhang et al., 2012; Bersanini et al., 2014; Allahverdiyeva et al., 2013) and respiratory terminal oxidases (RTOs; Lea-Smith et al., 2013, Berry et al., 2002; Howitt and Vermaas, 1998) are the main alternative electron valves characterized in *Synechocystis*. Both Flv1/3 and RTOs donate electrons to O_2 reducing it to H_2O in the water-water cycle (Branco dos Santos et al., 2014) but their impact on LET changes according to the growth conditions.

Carbon acquisition, interconnected with photosynthesis, is affected by the availability of macronutrients. Carbon is essential for cell metabolism because it is present in nearly all biomolecules. Cyanobacteria utilize CO_2 and bicarbonate as the main sources of inorganic carbon (Ci) (Volokita et al., 1984). CO_2 passively diffuses into *Synechocystis* 6803 cells (Tchernov et al., 2001); this process is facilitated by porines (Kaldenhoff et al., 2014). Inside *Synechocystis* 6803 cells, CO_2 is converted to HCO_3^- by NDH-1_3 and NDH-1_4 complexes (Battchikova et al., 2011 A). HCO_3^- requires active transportation through membranes, which is performed by SbtA, BCT-1 and BicA (Price, 2011). For some mechanisms of Ci uptake the expression is strongly regulated by the availability of carbon sources; for example, NDH-1_3 and SbtA are strongly induced in low-CO_2 conditions (Price, 2011). The changes in Ci availability also affect the central carbon metabolism (Burnap et al., 2015): if an excess of GA3P is produced in the CBB cycle, the fixed carbon is stored by *Synechocystis* 6803 in the form of glycogen (Zilliges, 2014; Walkie et al., 2016; de Porcellinis et al, 2018) and/or polyhydroxybutyrate (Damrow et al., 2016). When organic carbon is needed for cell metabolism, the glycogen is degraded to glucose and further used by several catabolic pathways such as glycolysis and the oxidative pentose phosphate (OPP) pathway (Quintana et al., 2011; Xiong et al., 2017). Each pathway provides intermediate for several anabolic reactions and produces a different ratio of NADPH and ATP. Interestingly, the presence of several carbon anabolic/catabolic pathways confers to *Synechocystis* 6803 metabolic plasticity important for acclimation to various environmental conditions (Xiong et al., 2017; Wan et al., 2017).

Nitrogen is another essential macronutrient, needed for the synthesis of amino acids and intermediates for various pathways (Schwarz et al., 2014). The C:N ratio is important for the cells, and the PII protein regulates the Ci and Ni assimilation inside the cells (Garcia-Dominguez and Florencio, 1997; Tandeau de Marsac et al., 2001). Nitrogen-limiting conditions induce multiple Ni- transporters and Ni-assimilation enzymes (Schwarz et al., 2014) while up-regulating sugar catabolic pathways (Osanai et al., 2006). Further, in N-depleted conditions *Synechocystis* 6803 degrades PBSs that are used as a nitrogen source (Grossman et al., 1994) while reducing the absorption of excitation energy (Richaud et al., 2001).

Multiple acclimation processes are regulated at several levels of gene expression, including transcription and translation as well as post-transcriptional and post-translational processes (For review see Los et al., 2010; Zorina et al., 2011A). For example, the "low-carbon" induced expression of the CCM SbtA, CmpA and NDH-1_3 is transcriptionally regulated (McGinn et al., 2003), similarly to the expression of the *slr0217-0219* operon, encoding Flv2, Flv4 and the small Slr0218 protein (Zhang et al., 2009). Regulation at the post-transcriptional level is mediated by non-coding RNAs (ncRNAs) (Kopf and Hess., 2015) acting both by stabilizing mRNA from degradation, as described in the case of *psbA2* encoding D1 (Sakurai et al., 2012), or promoting mRNA degradation, like in the case of the *flv2-4* transcript (Eisenhut et al., 2012) and the *isiA* mRNA (Dühring et al., 2006) or even via ncRNA-protein interaction as suggested for glycogen accumulation (de Porcellinis et al., 2016). Post-translationally occurring phosphorylation of PBS linkers has been suggested to promote the degradation of PBS (Piven et al., 2005) described previously as a result of light/nutrient stress. Phosphorylation is also known to regulate the PII protein involved in maintaining the C:N balance (Tandeau de Marsac et al., 2001) and suggested to regulate the number of carboxysomes (Burnap et al., 2015). However, the knowledge of regulation at the post-translational level and of the physiological roles of these modifications is still limited in cyanobacteria

1.1.4. Exploitation of cyanobacteria flexibility in synthetic biology

The flexibility of cyanobacteria metabolism, especially regarding the regulation of photosynthetic activity and flexibility of the carbon metabolism in various growth conditions, have promoted the idea of engineering cyanobacterial metabolic pathways for the production of high value compounds. The productivity of cyanobacterial mutant strains has been improved by redirecting the Ci flux toward key intermediate metabolites (Angermayr et al., 2015) or by reducing carbon input into competing pathways, especially limiting the production of storage compounds (Savakis et al., 2015; Hagemann and Hess, 2018). Also, electrons can be redirected toward exogenous electron sinks. For instance, enhanced electron flux toward hydrogenase was achieved by the elimination of natural electron valves (Vignais et al., 2006). Another example is the transfer of electrons from PSI, via thioredoxin, to the exogenous P450 enzyme, a monooxygenase taking part in the synthesis of terpenoids (Wlodarczyk et al., 2015; Berepiki et al., 2016). Noteworthy, photosynthetic light-dependent activities and carbon metabolism strictly correlate and, consequently, the re-routing of electron and carbon fluxes are interdependent as well (Nogales et al., 2013). Therefore, the optimization of production of exogenous compounds is a challenge in synthetic biology (for review see Wang et al., 2012; Berla et al., 2013; Pacheco et al., 2014).

The expression of foreign genes in mutant strains can be customized by using various promoters, ribosome binding sites (RBSs) (Markley et al., 2015; Sakai et al., 2015) and terminators (Wang et al., 2012; Taton et al., 2014). Of these "building blocks", a promoter is one of the main components regulating gene expression. Several promoters have been characterized and compared for their efficiency in cyanobacteria. P_{psbA2} and P_{rbcL} are strong native promoters commonly used for constitutive gene expression (Englund et al., 2016). However, in designing production strains, an advantage can be gained by separation of the cell growth phase and the production phase (Keasling et al., 2012; Borowitzka et al., 2016; Giordano and Wang, 2018). This is usually achieved by the use inducible promoters which are often controlled by concentrations of specific metabolites or metal ions such as $Cu^{2+,}$ $Fe^{2+/3+}$, Ni^{2+}, $Zn^{2+}$and Co (Berla et al., 2013). Thus, IPTG-inducible promoters suitable for cyanobacteria have been recently developed (Klemencic et al., 2017) by improving *E. coli*-based ones like P_{tac}, P_{trc} or P_{lac} (Huang and Lindblad, 2013; Camsund et al., 2014) or native ones such as the P_{cpcB} in *Synechococcus* PCC7002 (Markley et al., 2015). The green light-regulated *cpcG2* promoter has also been recently used to control gene expression in *Synechocystis* sp. PCC 6803 (Miyake et al., 2014).

Inducible promoters controlled by metal ions are widely used in cyanobacteria. Two promoters, P_{petJ} and P_{petE}, are regulated by the concentration of cupric ions (Cu^{2+}) in the media. The Cyt c_6 protein encoded by *petJ* gene and the PC encoded by *petE* are soluble electron carriers involved in the transfer of electrons from the Cyt b_6f complex to PSI (Sandmann, 1986; Diaz et al., 1994). The expression of these genes and, consequently, accumulation of the two proteins differ according to the concentration of Cu^{2+} in the environment (Figure 2). In standard BG11 (containing 0,3 μM of $CuSO_4$), P_{petJ} is only partially activated, and the Cyt c_6 protein is synthetized in cells in low amounts. P_{petJ} is activated in Cu^{2+}-free BG11 and repressed in 1 μM of $CuSO_4$ (Zhang et al., 1992). Vice-versa, P_{petE} is repressed in absence of Cu^{2+} and induced by its presence (Zhang et al., 1992). Several examples can be found in literature, in which P_{petJ} has been used to switch the expression of proteins of interest on and off, both to investigate the function of the proteins itself (basic research, such as in Mitschke et al., 2011; Eisenhut et al., 2012; Savakis et al., 2012; Gandini et al., 2017), and to regulate the synthesis of biomolecules (Kuchmina et al., 2012) and chemicals (Dienst et al., 2014; Pade et al., 2017). Since PC and Cyt c_6 complement each other, both promoters can be used as tools in synthetic biology. The regulation by Cu^{2+} does not affect photosynthesis; however, other metabolic pathways may be

affected (Gudmundsson et al., 2018), resulting in a decrease in accumulation of the product of interest (Giordano and Wang, 2018).

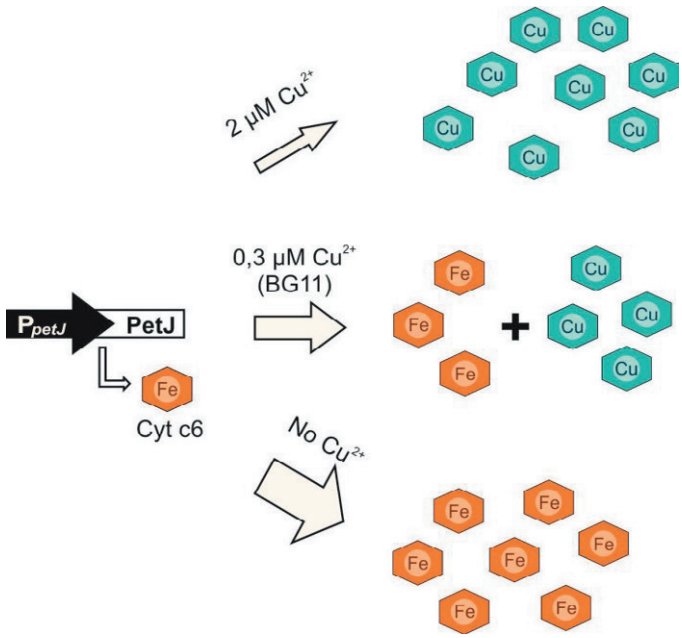

Figure 2: Cu^{2+} available in the media regulates the activation/repression of the *petJ* promoter. The Cyt c_6 protein encoded by the *petJ* gene is shown in orange and the PC proteins are shown in light-blue.

Understanding the metabolic rearrangements caused by various synthetic biology approaches is, in general, very important in order to optimized mutant strains (Keasling et al., 2012; Savakis et al., 2015). To this end, quantitative proteomics is a powerful tool to investigate the background protein changes caused by the exogenous pathways and to identify potential bottlenecks for production (Redding-Johanson et al., 2011; Hagemann and Hess, 2018).

1.2. S/T/Y protein phosphorylation and its roles in metabolic regulation

Post-translational modifications (PTMs) regulate protein functions via changes of molecular properties, such as folding, conformation and cellular localization. In most of the cases, PTMs represent reversible additions of chemical groups to a specific amino acid (Mann and Jensen, 2003). The most common and most studied PTM both in prokaryotic and eukaryotic organisms is phosphorylation. This modification can occur on the Serine (S), Threonine (T) and Tyrosine (Y), also known as O-type phosphorylation, or on Histidine (H) and Aspartate (D). Different

classes of kinases and phosphatases are responsible for these two types of phosphorylation, thus their mechanism of action and regulation are also different (for detailed mechanisms of phosphorylation/dephosphorylation see 1993 Hubbard and Cohen, 1993; Hunter, 1995; Salazar and Höfer 2006; Casino et al., 2010). The O-type of phosphorylation was originally considered exclusive to eukaryotic organisms (Zhang et al., 2005), while phosphorylation on H and D occurs during signaling through two-component systems in prokaryotic organisms (Mann, 1994; Liu et al., 2006; Los et al., 2010). However, more recent studies have shown that O-type phosphorylation occurs also in prokaryotic organisms (Mann, 1994; Zhang et al., 2005; Zorina, 2013). This dynamic process is carried out by S/T and Y protein kinases and phosphatases, and it is a mechanism by which both intracellular and external signals are transmitted in order to activate and inactivate enzymes (Pawson, 2003). Protein phosphorylation regulates several important life processes (Liu et al., 2015; Kumar et al., 2018) such as protein–protein interaction, cell signaling, cytoskeleton remodeling, cell cycle events, and cell–cell interactions (Seet et al., 2006). In mammalian cells, about one third of the proteins are predicted to be phosphorylated; the process is sub-stoichiometric, and the level of phosphorylation ranges from 1% to 90% depending on proteins and conditions (Macek et al., 2009). Proteomics studies revealed that signaling usually involves a cascade of kinases, and altering these signaling pathways often results in abnormalities leading to various diseases (Nita-Lazar et al., 2008; Najam-ul-Haq et al., 2012), including cancer (Irish et al., 2004). Interestingly, a discernment between phosphorylation sites is important since most of the phosphoproteins harbor at least two of them, and they often regulate protein function differently allowing response to different stimuli (Thingholm et al., 2009; Sacco et al., 2017).

In plants, S/T/Y protein phosphorylation has been shown to be involved in acclimation to biotic and abiotic stresses and in the regulation of several cellular processes (Yin et al., 2018). Often the regulation is dependent on light availability through an interplay between redox signaling and phosphorylation (Chiarugi and Cirri, 2003; van Montfort et al., 2003), in which thioredoxin plays an important role (Seo et al., 2004). Studies of chloroplast phosphoproteomes has revealed that protein phosphorylation is an important mechanism for the regulation of chloroplast photosynthesis (Reiland et al., 2009; Baginsky et al., 2016). Current understandings of the phosphoprotein–protein kinase network in *Arabidopsis* chloroplasts suggests that the STN8 protein kinase and the PBCP phosphatase are responsible for the phosphorylation/dephosphorylation of the PSII core proteins, while the phosphorylation of the LHCII antenna is determined by the STN7 kinase

and the TAP38 phosphatase (Bonardi et al., 2005;Wunder et al., 2013; Tikkanen and Aro, 2014). The reversible phosphorylation of PSII-LHCII regulates the interaction of PSII and PSI complexes with LHCII trimers via a still elusive mechanism to balance the energy distribution between the PSs (Tikkanen and Aro, 2012; Tikkanen and Aro, 2014). Beside regulation of photosynthesis, phosphorylation cascades are involved in the regulation of chloroplast gene expression and in the interaction between starch, carbohydrate, and energy metabolism (Reiland et al., 2009). Phosphorylation is also involved in seed germination (Han et al., 2014), anther/leave development (Ye at al., 2014; Lv et al., 2014) and wood formation (Mauriat et al., 2015). Moreover, studies in crop plants such as rice, maize, wheat and soy beans have shown changes in the phosphorylation of proteins involved in signaling transduction, in response to flooding, drought, heat, radiation and metal toxicity (Yin et al., 2018; Kumar et al., 2018). For these reasons, engineering of phosphoproteomes has been regarded as a promising approach toward the production of crops with increased tolerance to stresses and improved productive yields (Kumar et al., 2018).

In the recent years, investigation of S/T/Y phosphorylation has expanded to bacteria. About 100 O-type phosphorylation sites were detected in model organisms such as *Bacillus subtilis* (Macek et al., 2007), *Escherichia coli* (Macek et al., 2008) and *Lactococcus lactis* (Soufi et al., 2008). Phosphoproteins in these bacteria are involved in various metabolic processes, especially in carbohydrate metabolism and protein biosynthesis. Interestingly, some phosphoproteins and phosphorylation sites are well conserved from archaea to humans (Macek et al., 2009). Recent results showed that bacteria have a complex phosphorylation network with promiscuous activities of protein kinases involved in several cellular function, similarly to eukaryotic organisms (Jers et al., 2008). Investigations of cyanobacterial PTMs have been intensified recently. Results showed that cyanobacterial proteins also undergo various PTMs. Thus, sophisticated sensing and signaling mechanisms based on PTMs may have contributed to the evolutionary and ecological success of these organisms (Xiong et al., 2016).

1.3. Application of proteomics in cyanobacterial research

Cyanobacterial proteomes (the term refers to all proteins present in a cell, Wilkins, et al. 1996) are very dynamic; they change during the acclimation of cells to various environmental factors and treatments including pathway engineering. Proteomics, due to its ability to identify and quantify proteins, is a powerful tool to

clarify biological processes in detail (Gilmore and Washburn, 2010). In contrast to genomics and transcriptomics, proteomics reflects the overall levels of protein regulation in the cell, thus overcoming the limitations faced by other "-omics" (Gygy et al., 1999; de Groot et al., 2007; Pandhal et al., 2008 A, B; Waldbauer et al., 2012). Moreover, proteomics allows the investigation of post-translational modifications (Mann and Jensen, 2003). Proteomics has played an important role in studies of cyanobacterial compartments, analyses of protein complexes and their assembly, and many other investigations of cyanobacterial proteins including revealing proteome changes during the adaptation of cells to environmental changes (for review, see Battchikova et al., 2015).

1.3.1. Proteomic approaches

In proteomics, complex samples, often representing the total protein content of a cell, are commonly investigated using the "bottom-up" approach (Zhang et al., 2013) in which proteins are enzymatically digested with a specific protease, usually trypsin, before injection into a mass spectrometer. Two protocols can be used: 1) the **in-gel digestion,** in which proteins are separated via gel electrophoresis (SDS-PAGE, isoelectrofocusing) and specific spots from the gel are excised and digested; and 2) the **shotgun approach** where the whole proteome is directly digested (in-liquid digestion) and then analyzed by MS. Nowadays, the shotgun approach is often selected because it has superior throughput and sensitivity compared to gel-based proteomics (Dowell et al., 2008). Mass spectrometer instruments are usually operated in the automatic data dependent analysis (DDA) mode, in which fixed number of precursor ions detected in a survey scan are subjected to a second MS analysis (MS/MS). Peptide identification and the subsequent protein analysis are performed by bioinformatic approaches using search engines that match the m/z of precursor and fragment ions with theoretical values in a protein database with specific scoring algorithms (Deutsch et al., 2007; Mueller et al., 2008).

1.3.2. Cyanobacterial studies aimed at protein identification

An overview of *Synechocystis* 6803 studies performed by various proteomic approaches has been presented by Gao and co-workers (2015). Great results have been achieved in numbers of identified proteins, improving from 15% of the predicted proteome with in gel-digestion (Fulda et al., 2006) to 50% of the theoretical *Synechocystis* 6803 proteome detected in a single shotgun LC-MS/MS experiment (Gao et al., 2014 A). Further improvements have been obtained by a combination of shotgun and gel-based approaches (Gao et al., 2015). Soluble proteins in the stroma and periplasm (Fulda et al., 2000) have been easily detected (Fulda et al., 2006; Barrios-Llerena et al., 2006; Ran et al., 2007), while studies of

thylakoid-embedded photosynthetic and respiratory complexes remained challenging due to their hydrophobic nature (for review see Gilmore and Washburn, 2010; Whitelegge, 2013; Gao et al., 2014 A). This problem was alleviated by the development of MS-compatible detergents. In combination with native gel electrophoresis (Wittig et al., 2006; Rantala et al., 2017) and cross-linking (Liu et al., 2013 A), LC-MS/MS gave an important contribution toward understanding the composition of photosynthetic complexes and their organization in super-complexes (Herranen et al., 2004; Pisareva et al., 2007). Proteomics helped to identify differential protein locations between the thylakoids (Srivastava et al., 2005), plasma membrane (Huang et al., 2002) and outer membrane (Huang et al., 2004) as well as extracellular secreted proteins (Sergeyenko and Los, 2000; Gao et al., 2014 B). Proteomics has also become a valuable tool for identification of novel subunits in complexes such as PSII (Kashino et al., 2002) and NDH-1 (Battchikova et al., 2011 B).

1.3.3. Quantitative proteomics provided an overview of acclimation of cyanobacterial proteomes to environmental changes

As was already mentioned in section 1.1.3, cyanobacterial acclimation to changes in light and nutrient availability requires an adjustment of the stoichiometry of protein complexes and the regulation of their activity. Extensive comparative proteomic investigations performed in cyanobacteria (for review see Ow and Wright, 2009; Battchikova et al., 2015) revealed global proteome changes occurring in acclimation to natural environmental stresses (for instance: Suzuki et al., 2006; Slabas et al., 2006; Huang et al., 2006; Fulda et al., 2006; Pandhal et al., 2007 Ramanan et al., 2012), identified novel proteins involved in these processes (for instance Ermakova et al., 2014), and disclosed consequences of expression of exogenous pathways, especially when the product is toxic for the host (for instance Qiao et al., 2012; Liu et al., 2012; Agrawal et al., 2014; Chen et al., 2014).

Potentially, any change in the proteome can be detected using shotgun LC-MS/MS in a **global, or discovery-driven,** approach. Several techniques, either using labelling or label-free methods, and respective bioinformatics tools have been developed to this purpose (Ow and Wright 2009; Battchikova et al., 2015). Alternatively, in **hypothesis-driven** experiments, a **targeted MS** approach has been developed, aiming to measure, with a high accuracy, changes in a specific subset of proteins, (Picotti et al., 2007 A). The latter approach was introduced into cyanobacterial research only recently (Vuorijoki et al., 2015).

Label-based quantitative proteomic strategies in discovery-driven cyanobacterial studies

Main label-based proteomic applications include 1) labeling of peptides with isobaric chemicals (iTRAQ, TMT) followed by relative quantitation of reporter ions specific for each sample, and 2) labeling of peptides with different isotopes, e.g. ICAT using chemicals with stable isotopes or SILAC where ^{13}C or ^{15}N are added to growth media (Mueller et al., 2008; Xie et al., 2011). A complete review can be found in Battchikova et al., 2015. For example, iTRAQ has been used in *Synechocystis* 6803 to study acclimation to high salt stress (Quiao et al., 2013), phosphate limiting conditions (Fuszard et al., 2012) and the development of chassis for hydrogen production (Pinto et al., 2012), while in *Nostoc* species nitrogen fixation and hydrogen production have been investigated using ITRAQ (Ekman et al., 2001; Stensjö et al., 2007; Ow et al., 2008) or ICAT (Sandt et al., 2015). Responses to heavy metal exposure has been investigated by ITRAQ in *Cyanothece* (Mota et al., 2015). ^{15}N labeling has been used to compare salt stress response in *Synechocystis* 6803 and in *Euhalothece* BAA001 (Pandhal et al., 2008 B) and in *Cyanothece* to follow protein dynamics after addition of labelled $^{13}C^{15}N$-L-leucine in the medium (Aryal et al., 2012).

The main advantage of quantitation with label-based techniques is the analysis of several samples in the same LC-MS/MS run which increases the accuracy of the relative quantitation; up to 11 samples can be compared using the modern TMT labels (Rauniyar and Yates, 2014). However, low- abundance proteins are usually underestimated in these approaches, due to the stochastic selection of peptides fragmented for MS/MS (Picotti et al., 2010).

Label-free quantitative proteomic investigations of cyanobacteria

In contrast to labels-based quantitation, in the label-free approach samples are analyzed in different LC-MS/MS runs, and therefore the quantitation is not restricted by the number of samples. This method also has a larger quantitation range and proteome coverage than label-based techniques (Neilson et al., 2011, Xie et al., 2011). However, label free quantitation approaches are strongly dependent on the performance of mass spectrometers. Due to the high speed, sensitivity, and robustness of highly advanced mass spectrometers, in the recent years label-free quantitation has been preferred over the labels approaches. Label-free quantitation is based on peptide spectral counting or comparison of precursor ion intensities (for review describing the methods, see Mueller et al., 2008, Neilson et al., 2011 and Xie et al., 2011). Briefly, the spectral count approach directly correlates the frequency of a peptide detection with its abundancy, thus being biased by physicochemical

properties of the peptide (Neilson et al., 2011). It has been shown that spectral counting produces reliable quantitation only for abundant proteins identified by at least 3 unique peptides (Mueller et al., 2008). In the other approach, quantitation via the intensity of **precursor ions** in the total ion chromatogram is based on the integrated peak areas (Mueller et al., 2008; Neilson et al., 2011). This method requires specific software for comparison and normalization of the results.

In cyanobacterial research, label-free quantitation with spectral counting has been applied, for example, in the characterization of *Synechocystis* 6803 mutant with reduced antenna size (Kwon et al., 2013), for study of changes in C and N metabolisms under several stresses (Wegener et al., 2010), in the study of day/night cycle in *Cyanothece* 51142 (Stöckel et al., 2011) and to evaluate acclimation to metal toxicity and macronutrient scarcity in *Synechococcus* WH8102 (Cox and Saito, 2013). The importance of phosphatidylglycerol in regulating *Synechocystis* 6803 metabolism (Talamates et al., 2014), and the role of NdbC (Huokko et al., 2017) were instead revealed using ion intensity.

SRM, the targeted proteomic approach

Selective Reaction Monitoring (SRM) is the recently developed method for targeted quantitation of proteins (for review see Picotti and Aebersold, 2012). In SRM, relative quantitation is based on the intensities of peptide **transitions**, i.e. pairs of precursor and fragment ions specific for a peptide that are acquired by a mass spectrometer along the LC gradient (Picotti et al., 2010). It is recommended, whenever possible, to select proteotypic peptides for protein quantitation by SRM (Picotti and Aebersold, 2012). In the method setup, the choice of optimal transitions is crucial for the experiment; previous knowledge of the target peptide fragmentation pattern is an advantage, but not a requirement. Preparation of the SRM assays is time-consuming; however, once established, they allow precise and highly accurate quantitation of targeted proteins (Hagemann and Hess, 2018). SRM has great advantages compared to the other label-free methods. Since only the selected transitions are analyzed, SRM permits quantitation of low abundant peptides along with highly abundant ones, with high sensitivity and specificity (Picotti et al., 2007 B). The number of proteins investigated in one run is one of the limits of using SRM; however, currently around 1000 transitions can be measured in a single experiment (Picotti and Aebersold, 2012). Despite its novelty, the method has already been used in *Synechocystis* 6803 to study the response to iron limitation (Vuorijoki et al., 2015; Vuorijoki et al., 2017 a; Vuorijoki et al., 2017b; Georg et al., 2017) and to investigate the role of uncharacterized proteins in cultures grown in different condition (Huokko et al., 2017).

1.3.4. Analyses of post-translational modifications

One of the advantages of proteomic studies compared to other -omics techniques is the possibility to investigate PTMs such as phosphorylation, methylation, acetylation etc. (for review on cyanobacterial PTMs in cyanobacteria, see Xiong et al., 2016). Investigation of various PTMs in cyanobacteria has been limited; however, a novel strategy applied on pools of *Synechococcus* PCC 7002 cultures grown in different conditions discovered 23 different PTMs and suggested the existence of PTM cross-talk (Xiong et al., 2016).

While the occurrence of O-type protein phosphorylation and its importance for the optimization of the photosynthetic machinery is well established in plants (see session 1.2), studies of S/T/Y phosphorylation and their biological functions in cyanobacteria have become focus of interest only recently. They have been facilitated by the development of the phosphoproteomic LC-MS/MS approach where phosphopeptides are enriched using affinity chromatography prior to mass spectrometry analysis (Mann et al., 2002; Macek et al., 2009; Engholm-Keller and Larsen, 2013). TiO_2 is the most commonly used resin; alternative materials used for this purpose can be found in Thingholm et al., 2008 and Najam-ul-Haq et al., 2012.

Recently, increased interest has been shown toward the quantitation of changes in PTM under various conditions. Several approaches have been used for the quantitation of changes in phosphorylation in plant and other eukaryotic organism (Kline-Jonakin et al., 2011; Nakagami et al., 2012). Between them, the SRM offers the advantage of distinguishing between different O-type phosphopeptide isoforms (Liu et al., 2013 B), thus allowing to quantify them separately. Noteworthy, the quantitation of changes in phosphopeptides is a direct way to reveal the role of these modifications, that are often still unknown.

2. AIMS OF THE STUDY

In cyanobacteria the study of the phosphoproteome has become an attractive topic in the recent years. In this Thesis the complexity of the phosphorylation network involving S/T/Y-phosphoproteins was investigated in the model organism *Synechocystis* 6803. A variety of novel LC-MS/MS based proteomics methods were used to reveal novel acclimation and regulatory mechanisms. In parallel to the natural regulation, this Thesis investigated proteomics changes involved in the use of genetic tools for biotechnological applications. The understanding of both these aspects of cyanobacterial (phospho)proteins regulation supports the development of cyanobacteria as a platform in the solar-driven bioindustry.

The key objectives of this Thesis were:

1. Elucidation of the importance of phosphorylation as a natural regulatory mechanism affecting the whole metabolism; focus was placed on phosphoproteins involved in photosynthesis and its regulation.

2. Identification of a new phosphorylation network for Ferredoxin5 involving the kinase SpkG and the Slr0151 auxiliary protein.

3. Investigation of the proteome changes in *Synechocystis* 6803 cells when using the *petJ* inducible promoter in bio-engineered mutant strains.

3. METHODOLOGY

3.1. Cyanobacterial strains and growth conditions

The *Synechocystis* 6803 strains used in this Thesis are described in the Table I.

Table 1: The strains used in the present Thesis

Strain	Description	Constructed in:	Used in:
WT	*Synechocystis* 6803 wild-type (WT), glucose-tolerant strain		I, II
Δ*spkG*	Knock-out of the SpkG kinase	Paper II	II
Δ*slr0151*	Knock-out of the Slr0151 protein	Paper II	II
WT/pVZ321	*Synechocystis* 6803 glucose-tolerant WT strain carrying the empty pVZ321 vector	Mitschke et al., 2011	III
WT(GS) and Δ*spkA-L(GT)*	The glucose-sensitive *Synechocystis* 6803 WT and the complete set of the *spk* knock-out mutants	Zorina et al., 2011 B	this Thesis

The Δ*spkG* and Δ*slr0151 Synechocystis* 6803 mutants were obtained via homologous recombination. The Δ*spkG* mutant was constructed by insertion of the kanamycin resistance cassette (KmR) into the native PstI site of the *slr0152* locus. For generation of Δ*slr0151,* the part of the s*lr0151* ORF encoding Asn15-Gln145 was replaced by KmR (Paper II).

Cells were cultivated in photoautotrophic conditions using constant illumination of 50 μmol photons m^{-2} s^{-1} at +30 °C. The cells were grown in Erlenmeyer flasks shaking at 120 rpm, in modified BG-11 medium (Rippka et al., 1979) buffered with HEPES-NaOH (10 mM, pH 7,5), and incubated in atmospheric air (this condition will be referred as standard growth). When high CO_2 conditions (HC) were necessary (Paper I), the medium was supplemented with Na_2CO_3 and buffered with TES-KOH (10 mM, pH 8.0), and cultures were incubated under 3% (v/v) CO_2. For selection and storage of the mutants (Paper II and III), the necessary antibiotics were added to media. However, antibiotics were omitted when the cells were grown to conduct experiments.

Synechocystis 6803 cells were grown from OD_{750} ≈ 0,2 to OD_{750} ≈ 1, and collected by centrifugation (Paper I-III). In the experiment described in Paper III, cultures were supplemented with $CuSO_4$ and subsequently shifted to Cu^{2+}-depleted BG11 (Figure 1 in Paper III).

3.2. Sample preparation for MS analyses

Cell pellets were re-suspended in a denaturing lysis buffer at pH 8, containing 8-9 M urea and protease inhibitors. EDTA, EGTA and phosphatase inhibitors (NaF, $Na_4P_2O_7$, β-glycerophosphate, and Na_3VO_4) were added in order to preserve the phosphorylation state of the proteins in Papers I and II. Re-suspended cells were broken mechanically, the details are provided in the corresponding Papers. Recovery of membrane proteins was enhanced by addition of MS-compatible detergents. Proteins were purified using methanol and chloroform (Wessel and Flügge, 1984) (Paper I), or by precipitation with 5 volumes of ice-cold acetone:ethanol (1:1) solution at -20 °C overnight. Reduction and alkylation of disulfide bonds were performed as described in Papers I-III.

3.2.1. Digestion of proteins to peptides

In Papers I and II, pellets of purified proteins were dissolved in a buffer containing urea and phosphatase inhibitors. In Paper III, RapiGest was added to improve the protein´s solubilization. The digestion of proteins with MS-grade trypsin (Thermo Scientific) was performed at 30°C overnight, and tryptic digests were acidified to pH ≈ 2 using formic acid (FA). Peptides were desalted using C18 cartridges (Sep-Pack, 50 mg, 3cc, Waters) according to the manufacturer's protocol; the eluted peptides were lyophilized in SpeedVac (Savant SPD1010, Thermo Scientific™) and stored at -80 °C. Purified peptides were reconstituted in 1% (v/v) FA, and their amounts were estimated using Nanodrop ND-1000 (Thermo Scientific) or the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

3.2.2. Peptide fractionation and enrichment of phosphopeptides

In Paper I, the desalted peptides were fractionated using C2-, C8- and C18-cartridges (Waters). Further, each fraction was enriched for phosphopeptides using home-made tip columns containing TiO_2 beads. Enriched phosphopeptides were desalted prior to injection into a mass spectrometer using solid phase extraction C18 disks according to Imanishi et al. 2007.

3.3. Protein identification and quantitation

The shotgun approach was used in the identification of the phosphopeptides and corresponding phosphoproteins (Papers I and III). Some of the phosphoproteins identified in Paper I were also quantified using the target SRM method (Paper I and II). In Paper III, proteins were quantified based on peptide ion intensities in a discovery-driven experiment.

3.3.1. Protein identification and label-free quantitation by LC-MS/MS

Mass spectrometry analysis

Peptides were analyzed using Q Exactive (Thermo Scientific) (Paper I) or Orbitrap Velos Pro (Thermo Scientific) (Paper III) mass spectrometers connected in line with a nanoflow HPLC system (EasyNanoLC 1000, Thermo Fisher Scientific). Purified peptides were loaded on a C18 pre-column and then separated on a C18 analytical nanocolumn with a 300 nl/min flow rate by reverse phase chromatography for 60 min (Paper III) or 120 min (Paper I). Eluted peptides were ionized by a nano-ESI source, and MS data were acquired in a positive ionization mode using the Thermo Xcalibur software (Thermo Scientific™). In the DDA mode, full scan spectra were registered with a resolution of 60000 in the range 300-2000 m/z. The ten most intensive ions with charge state +2, +3 or +4 were selected for MS/MS analysis; peptides were fragmented with normalized collision energy of 35%, and MS/MS ion scans were collected in a mass range of 100–2000 m/z with resolution of 17500.

Bioinformatic analysis

Data analysis was performed using the in-house Mascot 2.4 (Matrix Science, London, U.K.) server via Proteome Discoverer 1.4 (ThermoFisher Scientific). The search was carried out against the *Synechocystis* 6803 protein database (http://genome.microbedb.jp/cyanobase/Synechocystis), which included genome- and plasmid- encoded proteins and was supplemented with the list of common laboratory contaminants. The probability value for a primary Mascot search was 95%. The following search criteria were applied: one missed cleavage allowed for trypsin digestion, 10 ppm tolerance for precursor mass/charge (m/z) values, 0.02 Da tolerance for fragment m/z, carbamidomethylation of Cys was set as a fixed modification, whereas variable modifications included Met oxidation and acetylation for N-termini of proteins. In Paper I and II, identification of phosphopeptides was performed adding phosphorylation of S, T, and Y amino acid residues as a variable modification.

Progenesis software as the bioinformatic tool

In the Paper III, global label-free protein quantitation was performed using Progenesis QI for proteomics software v. 4.0 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Peak areas of peptide ions measured on the full scan surveys provided information about relative peptide amounts. After peptide identification in each run, the data were aligned and normalized, allowing relative comparisons. Proteins abundancies were calculated by summarizing normalized peak areas for unique peptides belonging to a protein. Three biological replicas were analyzed. The statistical significance threshold in Anova was set to p-value<0.05, and the practical significance threshold for differential expression was set to FC +/- 1.4.

3.3.2. Analysis of phosphopeptides with SRM

A group of phospho and unmodified peptides was quantified by Selected Reaction Monitoring in Paper I and II. The design of the SRM assays and the analysis of the experimental data were performed using Skyline software version 3.1. (MacLean et al., 2010). The targeted SRM data acquisition was performed using the triple quadrupole (QQQ) mass spectrometer TSQ Vantage (Thermo Scientific).

Generation of the SRM assay

The spectral library for selected peptides was created based on the results of LC-MS/MS DDA analyses described in section 3.3. Q1/Q3 transitions sets for individual peptides were designed in Skyline using the most abundant and selective fragment ions. The characteristic retention time (RT) values for each peptide were empirically determined by LC-MS/MS or by unscheduled SRM runs in which all the transitions were monitored throughout the entire LC gradient (Escher et al., 2012) using the native fractionated peptides. In some cases, synthetic peptides containing heavy isotopes in C-terminal Lys and Arg residues were applied to estimate or verify a position of a specific phospho-isoform. The synthetic peptides were analyzed separately and then spiked into the samples. Standard iRT peptides (Biognosys AG) were added to each sample to improve the precision of RT prediction.

Peptide quantitation by QQQ

Phospho and unmodified peptides, isolated and desalted as described in section 3.2 were separated in a C18 nanocolumn connected in-line with TSQ Vantage as described in the Papers I and II. The peptides were ionized by nano-electrospray before injection into the QQQ. The mass spectrometer was operated in the positive ion mode. Q1 and Q3 peak width (fwhm) parameters were set to 0.7. The selected transitions were measured using a cycle time of 2.5s; minimal dwell time was 30ms.

Application of Skyline software for SRM analysis

Raw files were imported into Skyline for peaks identification. Automatically assigned SRM peak groups were manually checked based on retention time information from DDA and dotp values. Quantitation of peptides was performed by summing the integrated peak areas of all the fragment ions. The 2-tailed *t*-test was used to confirm statistically significant differences (Paper II).

3.4. Bioinfomatic investigation of protein sequences and prediction of 3D structures

The multiple sequence alignments in Paper II were performed using Clustal Omega suit (https://www.ebi.ac.uk/Tools/msa/clustalo/) and BioEdit, version 7.2.527. Protein sequences from *Synechocystis* 6803 and other cyanobacteria were downloaded from Cyanobase (http://genome.microbedb.jp/cyanobase) and the corresponding accession numbers were used. The 3D models were created using the Expasy suit tool Swiss Model (https://swissmodel.expasy.org/), and graphics of the models were customized using PyMOL Molecular Graphics System, Version 2.0 (Schrödinger,LLC).

3.5. qRT PCR

Expression of the *slr0152* gene in the Δ*slr0151* strain was verified by Real Time Quantitative PCR (RT q-PCR) using primers 5´-GTGAGGACAGTGCCACAGAA-3´ and 5´-CCTTTGCACCCTTACCCTTT-3´ as described in Mustila et al., 2014.

4. OVERVIEW OF THE RESULTS

4.1. Phosphoproteins in *Synechocystis* 6803 are detected by LC-MS/MS

In Paper I, the occurrence of S,T,Y protein phosphorylation events in *Synechocystis* 6803 was investigated using the shotgun LC-MS/MS approach in DDA mode. In order to increase the number of identified phosphorylation events, cultures were grown in air (0.04% CO_2, LC) and in high CO_2 (3% CO_2, HC), in triplicate. Detection of phosphopeptides was facilitated by peptide fractionation and enrichment using TiO_2 before DDA analysis.

4.1.1. Phosphopeptides detected for Synechocystis 6803

Combined results obtained from 6 phosphoproteomic analyzes revealed about 430 phosphopeptides (Supplemental Table 1 and Figure S1 in Paper I). The list included peptides modified by methionine oxidation and the ones with trypsin miscleavage. Considering these events, the list was condensed to 300 unique peptide sequences where S/T/Y phosphorylation occurred. Some phosphopeptides (16 out of 428) were doubly or triply phosphorylated. Nevertheless, in this Thesis only singly phosphorylated phosphopeptides were investigated.

The localization of a phosphorylated residue in a peptide suggested by a Mascot search was further investigated using the PhosphoRS algorithm (Taus et al., 2011) that shows a probability for each putative position in every peptide spectrum match (PMS). Many phosphopeptides with more than one potential phosphorylation site were identified as isoforms, with a differently located phosphate group. In a majority of detected phosphopeptides, the location of a phosphorylated residue was identified with a high probability. For example, the Ac-TTTLQQR peptide at the N-terminal of the D1 protein (PsbA, Slr1311) was found to be phosphorylated on each of the three T residues in different isoforms (Figure 3 in Paper I). Based on the amount of PMSs, the pT2 was the most abundant isoform. Among the peptides where the position of the phosphate group was identified with a probability > 75%, the distribution of the phosphorylated amino acid residue was observed as 61% pS, 34%pT and 5%pY. However, in some

Table 2: Representation of the probability for phosphosite localization according to the PhosphoRS algorithm. Location is consider unknown when the probability is lower than 75%.

Probability for P-site localization	Number of P-peptides
<75%	64
75-99%	16
99-100%	287

peptides the phosphorylated residue(s) remained unknown (probability values <75%, Table 2), often due to low intensities of corresponding fragment ions.

4.1.2. Phosphoproteins are widespread in Synechocystis 6803

The detected phosphopeptides revealed the occurrence of 190 phosphoproteins in *Synechocystis* 6803 cells grown in air and in high CO_2 conditions. They are involved in a broad range of biological functions, as shown in Figure 2 in Paper I. The most abundant group included proteins taking part in photosynthesis related processes. It includes the multi-phosphorylated phycobilisome proteins, several subunits of both PSs, Cyt b_6f and ATP synthase, as well as Rubisco. Additionally, proteins involved in photoprotections were phosphorylated, such as flavodiiron proteins Flv3 and 4, the OCP and Slr0148 belonging to the PSII assembly proteins (PAP) operon. Many phosphoproteins were involved in nutrient metabolism (the 2nd most abundant group) including the regulatory PII protein and several proteins related to carbon and nitrogen metabolism. Other phosphoproteins were involved in DNA replication and repair, protein synthesis and folding and regulation of cell processes, namely histidine kinases, response regulators, O-type kinases and phosphatases (Table S1 in Paper I). Phosphoproteins annotated as unknown or hypothetical were also discovered.

4.2. Protein phosphorylation sites were verified by an SRM approach

Further investigation was focused on the proteins involved in light harvesting, photosynthetic electron transfer, carbon fixation and photoprotection (depicted by the red color in Figure 1 in Paper I). The phosphopeptides originating from these phosphoproteins were selected in the design of the SRM assays for verification/improvement of the DDA results and for phosphopeptide quantitation.

4.2.1. Location of phosphorylated amino acids in phosphopeptides of photosynthesis related proteins

For each target phosphopeptide and phosphopeptide isoform, an SRM assay was created in Skyline choosing the most abundant and selective fragment ions detected by DDA LC-MS/MS, as described in section 3.3.2.1 and represented in Figure 3. Fragment ions providing information about the localization of a phosphorylated amino acid residue were prioritized.

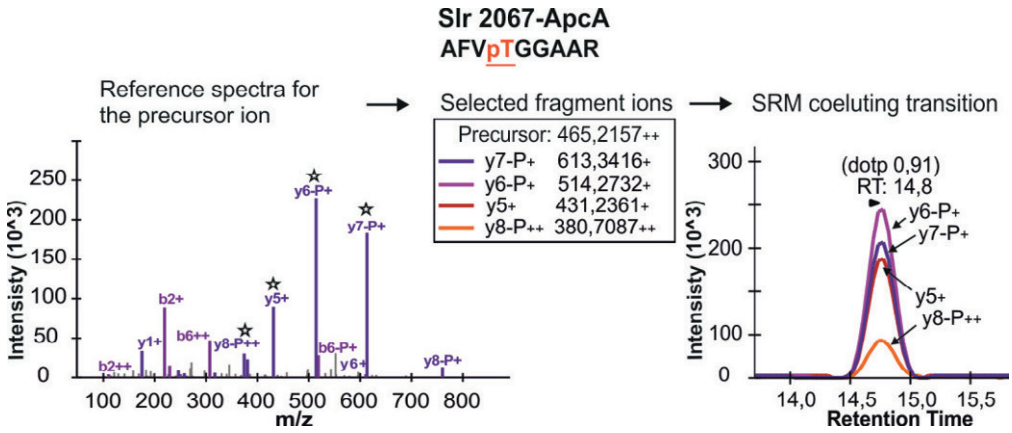

Figure 3: Preparation of the SRM assay for selected a phosphopeptide.
Left: DDA spectra of a selected phosphopeptide, the most intensive fragment ions are marked with stars; Center: the transition list, including a precursor ion and the fragments ions; Right: co-eluting transition in the SRM assay; Retention Time (RT) and dopt score are marked on the top of the peak.

The transitions selected in Skyline were verified experimentally using TiO_2-enriched fractionated native samples, since the separation of the complex peptide mixture to subfractions led to a substantial increase in phosphopeptide abundances. In parallel to verification of transitions, the individual retention time (iRT) values for peptide ions were determined. The transitions and iRT values were then finalized using TiO_2-enriched unfractionated *Synechocystis* 6803 samples.

4.2.2 Phosphopeptide isoforms in Synechocystis 6803

During the preparation of the SRM method, special attention was paid to correct assignment of phosphorylated residues in phosphopeptides containing several potential phosphorylation sites and present as various phospho-isoforms; in some cases, synthetic heavy peptides were used as a reference.

An example of well-resolved phospho isomers is the N-terminus of the D1 protein (Figure 4). Isomer specific fragment ions $y6^+/y6\text{-}P^+$, $y5^+/y5\text{-}P^+$ demonstrated that the phosphopeptides eluted in the following order: T3, pT1 and pT2.

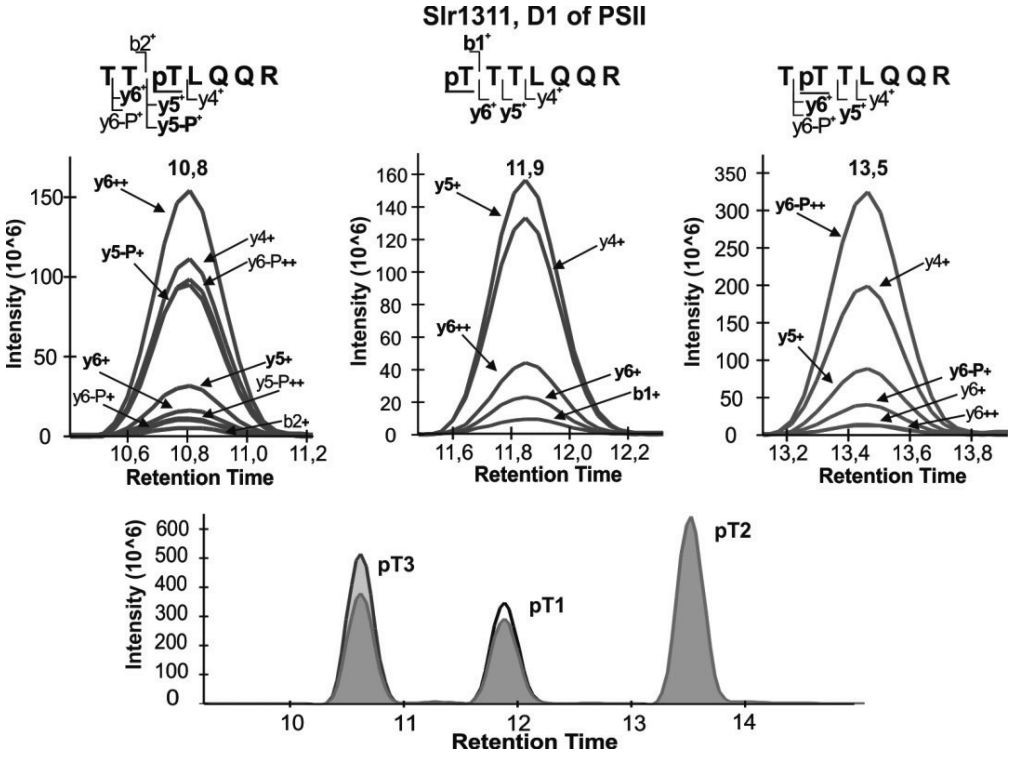

Figure 4: The N-terminal peptide from the D1 protein is phosphorylated in any of the three adjacent T in different singly phosphorylated peptides. The three phosphopeptide isoforms are distinguished by specific fragment ions marked in bold in the top part of the figure and reported in Table I in Paper I. The bottom part of the figure shows the distribution of the three isoforms along the gradient.

For the peptide TFDLSPSWYVEALK from CpcA (Sll1578), T1 was the only phosphorylated amino acid identified by DDA LC-MS/MS. However, the SRM analysis of the native samples revealed that the phosphorylation occurred also at S5. The two peptides, pTFDLSPSWYVEALK and TFDLpSPSWYVEALK, partially co-eluted and, therefore, were not fully resolved during HPLC, as shown in Figure 5 (and in Figure 5, Paper I). However, despite overlapping, the selection of distinct individual transitions allowed to assess these phospho-isomers separately, with $y9^+$, $y10^+$ and b3-P+ fragment ions characteristic for the pT1 form, and y9-P^+, y10-P^+ and $b3^+$ characteristic for pS5.

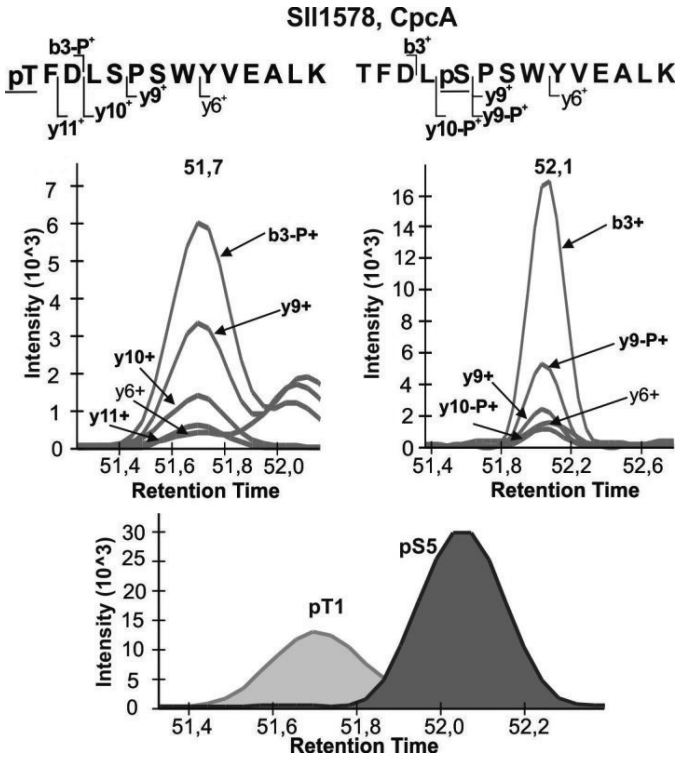

Figure 5: On top: SRM peaks for each of the two isoforms of the phosphopeptide TFDLSPSWYVEALK from CpcA, Sll1578; characteristic transitions allowing identification of each form are marked in bold. On the bottom: elution of the phosphor-isomers along the gradient showing partially overlapping of the two peaks.

Not all phospho-isomers could be resolved. The TSLVSAQR peptide originating from CpcC2 (Sll1579) was predicted by Mascot and pRS engines to be singly phosphorylated either on pT1, pS2 or pS5. However, the intensities of the characteristic $y7^+$/y7-P^+ fragment ions were too low to allow discrimination between pT1 and pS2 isoforms. Here, synthetic heavy peptides were used as controls. Results showed that the pS5 isoform was separated well from the other two variants, while the peaks corresponding to pTSLVSAQR and TpSLVSAQR were fully co-eluting. Thus, they cannot be assessed individually (Figure 6) in the native samples

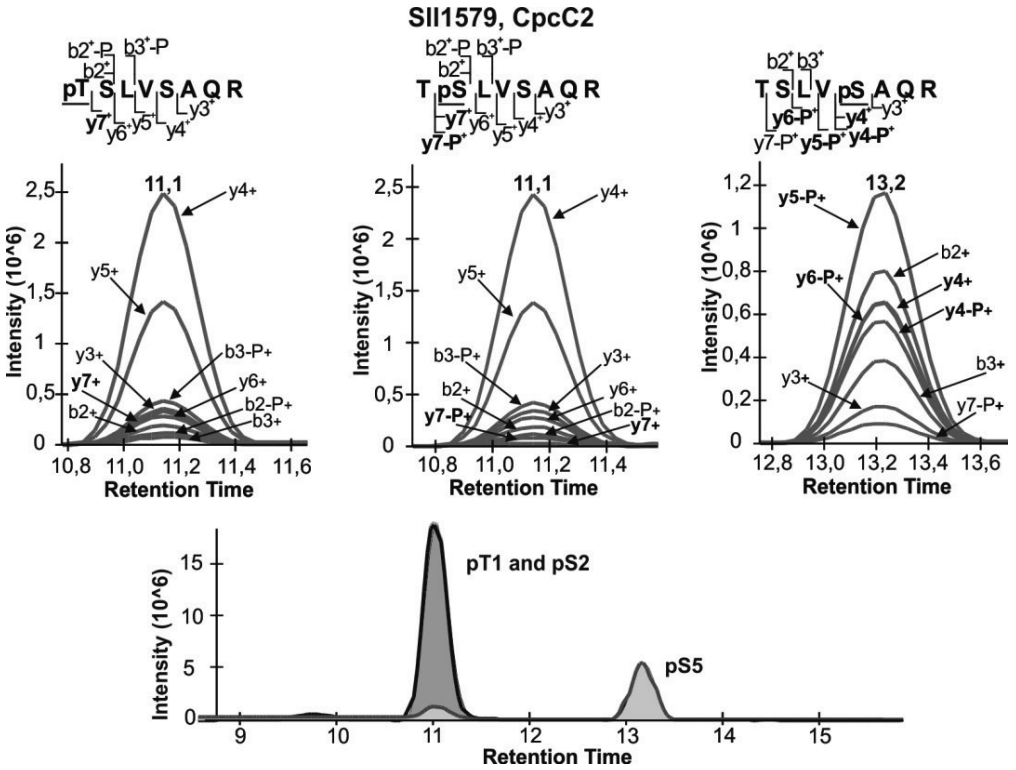

Figure 6: On top: characteristic transition peak for each of the three isoform of the phosphopeptide TSLVSAQR. Characteristic fragment ions are marked in bold and RT is on top of the peak. On the bottom: chromatogram showing the elution of the three isoform, pT1 and pS2 are coeluting at 11.1 min while pS5 is eluting at 13.2 min.

Finally, for each phosphopeptide precursor, the SRM assay comprised the iRT value and 4-to-6 transitions, including fragment ions which unambiguously specify the position of the phosphorylation event. The SRM library created in this Thesis included 46 phosphopeptides and phosphopeptides isomers (Table 1, Paper I), and the method was made publicly available on Panorama public (https://panoramaweb.org), Skyline file 2.

4.3. Revealing the phosphoprotein — protein kinase network in *Synechocystis* 6803

The SRM library designed for photosynthesis related phosphopeptides (described in session 4.2.1, and Paper I) was applied to investigate the S/T phosphoprotein – protein kinase network in *Synechocystis* 6803.

4.3.1. Screening of the collection of the S/T protein kinase mutants

In a preliminary investigation, SRM was used to screen 13 *Synechocystis* 6803 strains, including glucose-sensitive WT (WT GS) and the full set of the 12 *Synechocystis* 6803 S/T protein kinase knock-out mutants, Δ*spkA*-Δ*spkL* (Zorina et al., 2011 B) looking for changes in the amounts of the phosphopeptides. Samples were normalized for the amounts of peptides used for injection into the mass spectrometer. Several phosphopeptides accumulated differentially in the mutants compared to WT GS. The most relevant examples for this Thesis are the phosphopeptides VAIEpTNDNLLSGLLGQDLR and TLEVIpTTHNR (Figure 7) which originate from the Fd5 (Slr0148) protein. The screening suggested that the SpkG kinase is responsible for phosphorylation of Fd5 on both sites. Another kinase, SpkA, could also participate in the phosphorylation of the VAIEpTNDNLLSGLLGQDLR peptide.

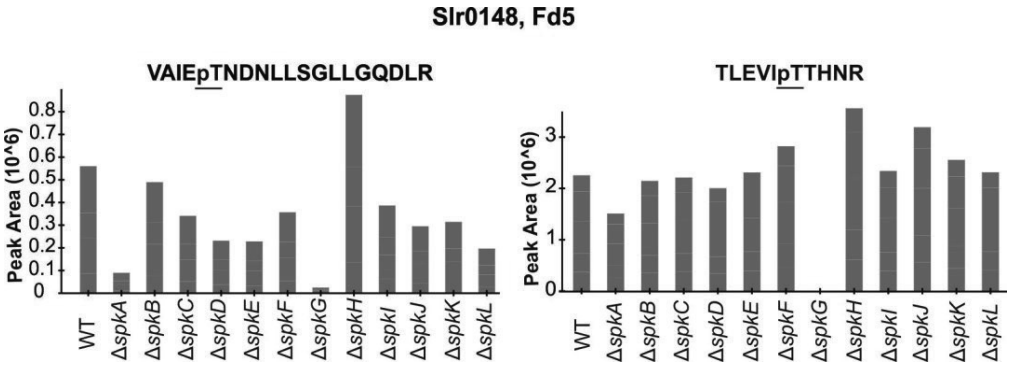

Figure 7: Screening of the set of knock-out kinases mutants for the 2 phosphopetides belonging to the Fd5 proteins. Both phosphopetides are affected in the ΔspkG mutant.

4.3.2. Construction of a novel spkG mutant for further investigation of the Fd5 phosphorylation network

To avoid possible involvement of the SpkA in the phosphorylation of Fd5, the *spkG* knock-out mutant was constructed (Paper II) on the background of the glucose-tolerant *Synechocystis* WT, where the SpkA kinase is not expressed due to the natural

frameshift in the *spkA* gene (Kamei et al., 2001; Trautmann et al., 2012). The Fd5 and SpkG kinase are encoded in the same gene cluster, *slr0144-slr0152*, described by Wegener et al (2008) as the PAP operon. However, mapping the transcription sites in *Synechocystis* 6803 (Mitschke et al., 2011) revealed that Fd5 and SpkG are transcribed in distinct operons, *slr0144-slr0150* and *slr0151-slr0152* (*spkG*), respectively. The *slr0151* gene encodes for a protein with unknown function, but is probably involved in protein–protein interaction via the tetratricopeptide repeats (TPRs) motif. To examine the potential influence of the Slr0151 protein on SpkG function, the knock-out *slr0151* mutant was also generated. Complete segregation of the Δ*spkG* and Δ*slr0151* mutants was demonstrated in Figure S1 in Paper II.

4.3.3. Fd5 phosphorylation in WT, ΔspkG and Δslr0151 mutants was quantified by SRM

SRM assays described in section 4.1.2 were used for relative quantitation of Fd5-derived phosphopeptides, VAIEpTNDNLLSGLLGQDLR and TLEVIpTTHNR, in WT, Δ*spkG* and Δ*slr0151* strains. Fd5 was one of the most abundant phosphoproteins identified in the global phosphoproteome (Paper I). Thus, SRM quantitation was performed on samples obtained without TiO_2 enrichment of the phosphopeptides providing an opportunity for parallel examination of several non-modified peptides (designed in Paper II) including the non-phosphorylated forms of the two Fd5 phosphopeptides. LDPIDLK, DGSILVEK and SMISQLDDQLQAAK, also from Fd5, were monitored to control the general level of this protein´s accumulation independently on the PTM occurrence. Additionally, the experiment comprised quantitation of the FQGDIQTSLGQQQQAIAANQENLTK and LYFDQGDLDSYEVAR peptides belonging to the Slr0151 protein. Peptides from the SpkG kinase were not possible to inspect due, most probably, to the low amount of the enzyme in *Synechocystis* cells. Therefore, the level of the *spkG* expression was monitored by qRT-PCR. Finally, two peptides and one phosphopeptide belonging to the phycobilisomes, ApcA (Slr2067) and CpcB (Sll1577) were used as indicators of the total amounts of peptides loaded in the experiment. The SRM assays specifically design to investigate the phosphorylation of Fd5 in the complex *Synechocystis* 6803 background proteome were submitted to Panorama Public, https://panoramaweb.org. The experiment was performed in triplicate, and averaged values were normalized to the amounts in WT (calculations are presented in Table S2 and S3, and final ratios in Table 1, in Paper II).

The amounts of the VAIEpTNDNLLSGLLGQDLR and TLEVIpTTHNR phosphopeptides were strongly decreased in Δ*spkG* compared to WT (Figure 8A and Figure 3 in Paper II), confirming the screening results described in 4.3.1. Concomitantly, the corresponding non-modified forms slightly increased their

abundance in this mutant (Figure 8A and Figure 4 in Paper II). The amounts of the other three Fd5-derived peptides were similar in both strains (Figure 8A and Figure 4 in Paper II). These results indicated that only phosphorylation of Fd5 was affected in the Δ*spkG* mutant, but not the total amount of the protein. Precisely, TLEVIpTTHNR was completely absent in the Δ*spkG* strain, while a small amount of VAIEpTNDNLLSGLLGQDLR was still detected.

Further, unexpectedly, the level of the Slr0151 protein accumulation strongly decreased (about 50%) in Δ*spkG* mutant (Figure 8B and Figure 4 in Paper II). In contrast, higher amounts of both VAIEpTNDNLLSGLLGQDLR and TLEVIpTTHNR, in parallel to a slight decrease of non-phosphorylated forms and a general decrease, around 15% of the total amount of Fd5, were observed in the Δ*slr0151* strain compared to WT (Figure 8A and Figures 3 and 4 in Paper II). The amounts of PBS peptides were similar in all the three studied strains (Figure 4 in Paper II).

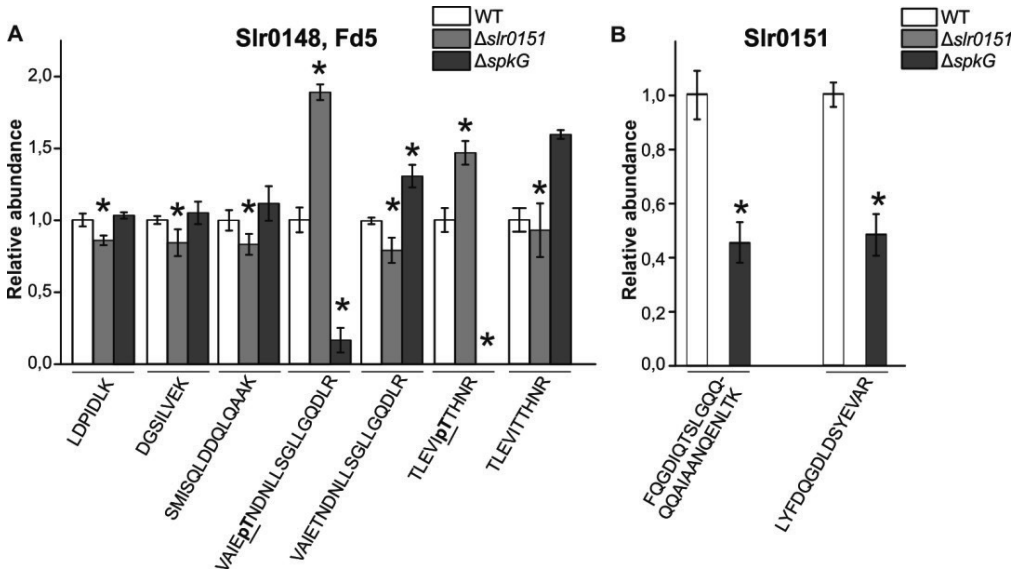

Figure 8: Relative quantification by SRM of (phospho)peptides originated from the Fd5 and Slr0151. **A**) Peptides representing Fd5: from the right three non-modified peptides, phosphorylated and non-phosphorylated form of VAIETNDNLLSGLLGQDLR and phosphorylated and non-phosphorylated form of TLEVITTHNR. **B**) Slr0151 is represented by FQGDIQTSLGQQQQAIAANQENLTK and LYFDQGDLDSYEVAR peptides. The graphs represent average values obtained for 3 biological replicates normalized to the amount of peptides present in WT. Modified from Publication II

4.3.4. SRM screening revealed a complex regulation of kinases activity

The results of the SRM screening of kinase mutants highlight other interesting features of the glucose-tolerant Δ*spkG* strain: some phosphopeptides were more abundant in the mutant compared to WT (GS), as shown in Figure 9 for the D1 protein belonging to the PSII complex and OCP (data not published).

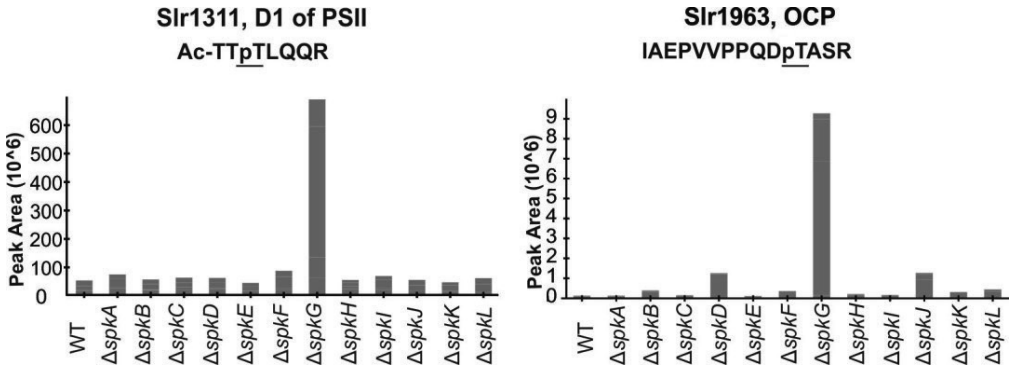

Figure 9: Screening of the knock-out kinases mutants for peptides belonging to the D1 protein (left) and OCP (right). Both phosphopeptides increase in amount in the Δ*spkG* mutant**.**

These findings require further investigation in order to reveal protein kinases involved in the phosphorylation of these peptides and to clarify mechanisms responsible for the remarkable increase in D1 and OCP phosphorylation in the absence of the SpkG kinase.

4.4. Proteomics-based insights into application of the P_{petJ} dependent over-expression of proteins as a biotechnological tool

In biotechnological applications, the P_{petJ}-dependent overexpression of targeted pathways is controlled by amounts of copper ions in growth media. The changes in the growth conditions utilized for controlling the promoter activity might cause rearrangement of intracellular metabolic pathways. In order to detect the background proteomics rearrangement, which occur in response to the addition and subsequent removal of Cu^{2+} ions, a label-free LC-MS/MS quantitative analysis was performed on a *Synechocystis* 6803 WT strain carrying an empty self-replicating vector (WT/pVZ321; Mitschke et al., 2011). These proteomics rearrangements are independent from the expression of foreign genes and might become a bottleneck in any biotechnological application.

4.4.1. The experimental setup used for the over-expression of proteins under control of the petJ promoter is based on changes in copper concentrations

Synechocystis 6803 WT/pVZ321 cells were subjected to changes in growth media, which mimicked an industrial application; the experimental setup used for this purpose is depicted in Figure 1 in Paper III. For protein quantitation, cells were collected at three points: in standard BG11 (S) containing 0.3 μM $CuSO_4$, after acclimation to 5 μM $CuSO_4$ (H), and after 48h-incubation in Cu^{2+} depleted media (D). The label-free LC-MS/MS method used provides relative quantitation, so the changes in protein expression were evaluated following experimental steps: from standard BG-11 to medium with 5 μM of Cu^{2} (H/S) and from the high copper medium to the depleted condition (D/H). Finally, indirect comparison of the depleted condition with the standard conditions (D/S) pointed out the proteins whose expression was affected at the end of the experiment.

4.4.2. Proteome responses in Synechocystis cells grown in standard conditions, in the excess of copper and during copper depletion

Totally, 1736 proteins were identified in at least one of the three experiments. Considering only the proteins represented by at least 2 unique peptides and with Anova p-value below 0.05, 812 proteins were quantified (Table S1 in Paper III). From them, 360 were differentially expressed in response to the changes of Cu^{2+} concentrations in the medium when using ±1,40 as threshold (Table 2 and S2 in Paper III). The numbers of proteins affected in each step of the experiment are showed in Table 1 in Paper III.

In this Thesis, the most intense changes in proteins with known functions were examined aiming to reveal the ones that might be relevant from an industrial perspective. Beside Cyt c_6 and PC whose expression was known to be regulated by copper (Zhang et al., 1992), many other functionally important proteins were affected by the changes in the media. Noteworthy, numerous hypothetical and unknown proteins were affected by the addition and subsequent depletion of Cu^{2+}, and several of them were still differentially regulated at the end of the experiment (Table S2 in Paper III). This group of proteins will not be discussed further since their function and consequently the implication of their changes remain unknown.

<u>Response of photosynthesis proteins</u>

The product of the *petJ* gene, the Cyt c_6 protein, was strongly reduced upon acclimation of cells from the standard conditions (S) to the media with high concentration of Cu^{2+} (H), and markedly up-regulated in copper-depleted conditions (D). Conversely, the amount of plastocyanin (PC) increased during the acclimation

to high copper concentration and then decreased after Cu^{2+}depletion. These results corroborated the previous knowledge on the regulation of *petJ/petE* promoters by Cu^{2+} (Zhang et al., 1992; Giner-Lamia et al., 2014; Giner-Lamia et al., 2016). Additionally, other proteins involved in photosynthesis were influenced by the Cu^{2+} treatment. The increase of Cu^{2+} affected proteins involved in the LET: Fed1 accumulated while PSI (represented by Ssr283, PsaE and Slr1655, PsaL) and Cyt b_6f (represented by PetB) diminished during this phase. However, Cu^{2+}had a stronger impact on proteins maintaining photosynthesis activity *via* photoprotection or assisting the assembly of the photosynthetic machinery. The flavodiiron proteins 2 and 4 (Slr0219 and Slr0217) functioning in the photoprotection of PSII (Bersanini et al., 2014) were down-regulated. Proteins involved in PSs biosynthesis/maintenance, especially under high light, like the products of the PAP operon (Slr0144, Slr0147, Slr0149, Slr0151; Wegener et al., 2008; Yang et at., 2014; Rast et al., 2016; Kubota et al., 2010), the membrane-associated rubredoxin A (Slr2033) and Slr1768 (Bryan et al.,2011), instead increased their levels. After the shift to Cu^{2+}-free medium, most of these proteins recovered their standard expression, with the exception of Slr0144 and Slr0147, which remained more abundant than in the standard conditions. The Cu^{2+} depletion also affected the photosynthetic linear electron transfer, since several proteins belonging to PBS and PsbE from PSII decreased while proteins from PSI and Cyt b_6f remained down-regulated.

In line with the effect on proteins involved in photosynthesis, the amount of enzymes involved in the synthesis of chlorophyll and porphyrin was also affected. In 5 μM of Cu^{2+}, HemF (Sll1185), HemB (Sll1994) and Hem L (Sll0017), ChlP (Sll1091) and Hox1 (Sll1184) were down-regulated. From them, HemL remained at low levels also upon the Cu^{2+} depletion, while the others recovered their expression to levels observed in standard conditions.

Nitrogen metabolism was strongly affected by the copper concentrations

The 5 μM Cu^{2+} treatment at the first step of the experiment led to an accumulation of transporters for nitrate/nitrite and urea intake. Several subunits of the nitrate/nitrite transport system (Sll1450, NrtA; Sll1452, NrtC; Sll1453, NrtD) and the UrtA (Slr1256) and D (Sll0764) forming the urea transporter were up-regulated. NirA (Slr0898) and the urease (Slr1256, UreA and Sll1750, UreC) involved in degradation of nitrite and urea, respectively, to ammonia also increased. Similarly, the cyanate lyase (Slr0899) responsible for the hydrolysis of cyanate to ammonia and carbon dioxide was up-regulated. The glutamate-ammonia ligase (Slr0288, GlnN) was up-regulated, while both glutamine synthetase inactivating factor, IF7 and IF17 (Ssl1911 and Sll1515), were strongly reduced in amount, with IF7 being 16 times less abundant than in standard conditions. Interestingly, after shift to

Cu^{2+}depleted BG11, the majority of these proteins showed an accumulation similar to the standard condition, thus being not affected at the end of the experiment. The exceptions were GlnN, IF7 and IF17 whose amounts remained altered.

Response of cell wall proteins, transporters and signaling molecules

The S-layer protein Slr1704 (Huang et al., 2004) and the CccP (Slr1668) protein involved in the synthesis of cell surface structures and biofilm (Yoshimura et al., 2010) increased after the Cu^{2+} addition to the media. Conversely, proteins involved in the peptidoglycan biosynthesis (Slr0017, MurA and Sll0899, GlmU) were down-regulated; however, MurG (Slr1656), whose function is also related to peptidoglycan biosynthesis, was up-regulated. After two days in Cu^{2+}-free media, the amounts of the S-layer protein and MurG were further increased; the GlmU amount was still lower than in standard BG11, while cccP and murA were at a similar level as in standard growth conditions. Another protein involved in peptidoglycan biosynthesis, LpdX (Slr0776), accumulated after Cu^{2+} depletion. Several proteins at the cell surface were affected, such as the porins Slr1908 and Slr1841, SynToc75 (Slr1227, Reumann et al., 1999) and PilQ (Slr1277) which accumulated at 5 µM of Cu^{2+} and remained at a similar level, or further increased during depletion. Conversely, 2 transporters were negatively affected by the high copper concentration. Strong changes were measured for the lipopolysaccharide transporter (Sll0575, RfbB) and the zinc efflux pump (0798, ZiaA). The latter further diminished in amount after the shift of media to Cu^{2+}-free medium, while RfbB amount increased again, returning to levels observed at standard conditions. The Nat permease for neutral amino acids (Slr1881, NatE) was not affected by the high Cu^{2+} concentration, but its amount decreased after Cu^{2+} depletion. Further, the changes of the media composition strongly affected cell regulators such as two-component systems (Sll0094, Hik37; Sll1473, Hik32; Slr1982, Rre21), the anti-sigma factor (Slr1859) and chaperon proteins (Slr2075, GroES and Slr0093, DnaJ2).

Effect of copper on the protein synthesis machinery

Proteins involved in DNA replication, transcription and especially in translation were affected by the Cu^{2+} treatment. Some ribosome subunits were repressed during the acclimation to high Cu^{2+} but for the majority of them the stronger effect was observed after Cu^{2+} depletion. Indeed, several proteins forming both large and small ribosome subunits, translation initiation factors IF-1, -2, -3 (Ssl3441, Slr0744, Slr0974, respectively) and the elongation factor P (Slr0434) were down-regulated at the end of the experiment compared to standard conditions. Next,

the periplasmic processing protease YmxG (Slr1331) was up-regulated, while aminopeptidase P (Sll0136), which is involved in high light-stress protein degradation (Pojidaeva et al., 2013), was down-regulated during the high Cu^{2+} phase and further decreased its amount after the switch to Cu^{2+} depleted medium.

Taken altogether, it was demonstrated in Paper III that drastic changes of Cu^{2+} availability in the growth media, used for the control of P_{petJ} activity, cause significant background alterations in *Synechocystis* 6803 proteome. Figure 2 in Paper III represents the background changes still present at the end of the experiment.

Stepwise proteomics changes induced by the high copper treatment and upon the subsequent shift to Cu^{2+} depleted medium are shown in Paper III, Figures S1 and S2, respectively.

5. DISCUSSION

In this doctoral Thesis, some of the protein regulatory mechanisms in the cyanobacterium *Synechocystis* 6803 were investigated using modern mass-spectrometer approaches. The first part of the work revealed extensive S/T/Y protein phosphorylation in this organism, and further work investigated the phosphoprotein-protein kinase network. The second part aimed to reveal the background changes in the *Synechocystis* 6803 proteome that could be potential bottlenecks in synthetic biology applications.

Taking together, this Thesis improves our understanding of cyanobacterial physiology and provides novel insights into the use of cyanobacteria as biofactories.

5.1. S/T protein phosphorylation in *Synechocystis* 6803

5.1.1. Phosphoproteins are widespread in Synechocystis 6803

Signaling and regulatory mechanisms based on S,T,Y-phosphorylation are widespread in eukaryotic organisms (Mann et al., 2002; Macek et al., 2009). In *Synechocystis* 6803, the importance of protein phosphorylation has been shown for relatively few cellular processes (Mann, 1994; Zhang et al., 2005), such as PBS degradation (Piven et al., 2005), modification of PII protein activity (Hisbergues et al., 1999), cell mobility (Kamei et al., 2001; Kamei et al., 2003) or the circadian rhythm (Golden, 2003; Wiegard et al., 2013).

The results described in this Thesis (Paper I) demonstrated that phosphorylation extensively occurs in *Synechocystis* 6803. About 190 phosphoproteins which are involved in various metabolic pathways were detected. Photosynthesis processes are especially enriched in phosphoproteins in *Synechocystis* 6803 (Paper I): several light harvesting antenna proteins, proteins involved in photosynthetic light reactions, in carbon fixation, as well as proteins involved in photoprotection and regulation of photosynthesis appeared to be phosphoproteins. In line with this results, widespread phosphorylation of photosynthesis-related proteins has been observed in chloroplasts of green plants (Baginsky et al., 2016).

During the performing of the Thesis work, few other investigations of the *Synechocystis* 6803 phosphoproteome were published (Spät et al., 2015; Chen et al., 2015); similar work was carried out in *Synechococcus* PCC 7002 (Yang et al., 2013). Comparison of the results from this Thesis with other research (Spät et al., 2015; Chen et al., 2015) showed many common phosphopeptides. However, for the photosynthesis-related phosphoproteins, several novel phosphopeptides were

identified in this Thesis, and some differences were revealed for the location of the phosphorylated amino acids (Table S7). Observed differences in the phosphorylation pattern most probably reflect variations in the growth conditions, thus highlighting the flexibility of S/T/Y phosphorylation in *Synechocystis* 6803. In addition, some of the differences might be due to technical variations in sample preparations and MS data acquisition.

In plants, it has been shown that the phosphorylation of the subunits of PSs and LHCs plays important biological roles. It is involved in balancing the excitation energy between PSI and PSII, in the formation of super-complexes, and in maintaining thylakoids organization (Tikkanen and Aro, 2012; Tikkanen and Aro, 2014). In *Synechocystis* 6803, the phosphorylation of the beta subunit of phycocyanin (CpcB) has been shown to enhance the energy transfer toward PSII and, probably, to regulate state transitions (Chen et al., 2015). The involvement of S/T phosphorylation in response to N depletion was shown using global quantitation with iTRAQ labels (Spät et al., 2016). The biological function of a few cyanobacterial phosphosites was investigated via *in vitro* de-phosphorylation (Piven et al., 2005) and direct mutagenesis (Chen et al., 2015).

However, the physiological roles for an absolute majority of the phosphorylation events in cyanobacteria remain unknown, and it is also puzzling how only 12 S/T protein kinases manage the phosphorylation of hundreds of proteins in a controlled manner. Quantitation of protein phosphorylation in cyanobacterial cells grown in different environmental conditions and in various mutants would provide insights into the functional importance of phosphorylation events and clarify the responsible kinase/phosphatase network.

In this Thesis, the S/T protein phosphorylation was quantified using SRM, which is a targeted proteomic approach (Picotti et al., 2012). The focus was set on the investigation of phosphoproteins related to the photosynthetic apparatus, since the understanding of the role of protein phosphorylation and identification of respective kinases and phosphatases in the regulation of the cyanobacterial photosynthetic machinery is not only providing new insights into the complex mechanisms of photosynthesis regulation but is also needed for design of engineered strains.

5.1.2. SRM is a valuable tool for analysis of (phospho-) proteins in Synechocystis 6803

An SRM library specific for the quantitation of phosphopeptides in photosynthesis-related proteins was developed in this Thesis (Table 1, Paper I). This library, after a careful selection of the transition pairs in order to pinpoint specific phosphosites, specifically allowed the quantitation of different phosphopeptide isoforms.

Prior to quantitation, the library was used for the verification/improvement of assignments of the modified residues of phosphopeptide isomers. The majority of phospho-isomers could be resolved during HPLC and, therefore, individually assessed in quantitation. The analysis of the "heavy" analogs demonstrated that sometimes isomers co-eluted. In the latter cases, quantitation of peptide phosphorylation is still possible; however, it is important to keep in mind the uncertainty of the modified residue. Resolving of phospho-isomers was important as Trotta and colleagues (2016) showed recently in *Arabidopsis* that different phosphorylation events have a specific effect on proteins, even when the phosphorylated amino acids are close each other. The designed library will allow to investigate if such a specificity exists in cyanobacteria as well.

The photosynthesis-related proteins comprising the SRM-verified phosphorylation sites are shown in red in Figure 10. Due to the high sensitivity, the SRM assays verified the occurrence of 18 novel phosphorylation sites.

The designed SRM library was deposited in the Panorama public repository to be used by other research groups. Thus, results obtained in this Thesis provided reliable tools for the quantitation of protein phosphorylation in *Synechocystis* 6803 to a broad scientific community.

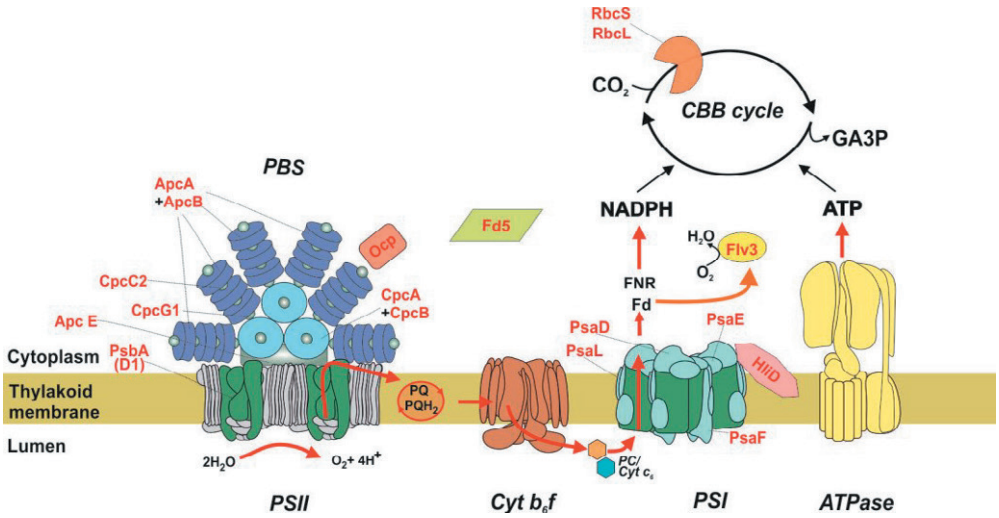

Figure 10: *Synechocystis* 6803 phosphoproteins of the photosynthetic apparatus. The proteins that contain phosphopeptides whose phosphosite(s) have been verified by SRM are marked in red. Multiprotein complexes involved in light harvesting, photosynthetic electron transfer, and CO_2 fixation are indicated by bold italic. Photosynthesis-driven electron flow is shown by red arrows.

Modified from Publication I

5.1.3. SRM revealed the phosphorylation network in Synechocystis 6803

Synechocystis 6803 genome encodes 12 S/T kinases; the activity of several of them has been confirmed in vitro (Kamei et al., 2001; Kamei et al., 2002; Kamei et al., 2003; Laurent et al., 2008; Liang et al., 2011; Zorina et al., 2011 B; Zorina, 2013), but their substrates in cyanobacterial cells have not been discovered yet. In order to investigate the phosphoprotein-protein kinase network in *Synechocystis* 6803, the SRM library created in this Thesis was applied in the screening of the full set of S/T protein kinase knock-out mutants, Δ*spkA*-Δ*spkL*. The mutants which were made based on glucose-sensitive WT (Zorina et al., 2011 B), did not show strong phenotypic differences when grown in standard conditions. However, several changes in the phosphorylation of the proteins were revealed. For instance, the phosphorylation of Fd5 phosphopeptides was abolished in the Δ*spkG* mutant (see Figure 7 in section 4.3.1), indicating that SpkG is involved in Fd5 phosphorylation. Moreover, the screening revealed that phosphorylation of several photosynthesis-related proteins, such as D1, PsaA, and OCP, strongly increases when SpkG is deleted and Fd5 is present in the non-phosphorylated form (see Figure 9 in session 4.3.1).

These results indicate a cross-talk between elements of the cyanobacterial S/T phosphorylation network which, however, remains elusive at present (Figure 11).

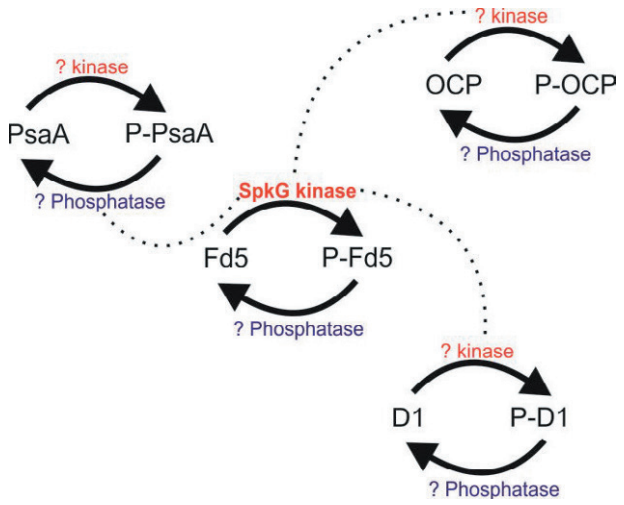

Figure 11: Schematic representation of the phosphorylation network around Fd5 in *Synechocystis* 6803. Phosphorylation of Fd5 by SpkG was verified. Unknown cross-talk signaling is affecting the phosphorylation of D1, PsaA and OCP.

Zorina and colleagues (2011 B) have shown that the phosphorylation of the GroES factor is dependent on three kinases, SpkC, F and K. The authors suggested that these kinases form a signal cascade similar to the one reported for eukaryotic organisms. Taken together, results of this Thesis and those of Zorina et al (2011 B), allow to suggest that the complexity of the S/T/Y phosphorylation network in cyanobacteria might be comparable with the one in eukaryotic species.

5.1.4. Interplay of proteins encoded by the slr0144-0152 gene cluster

The SpkG kinase- ferredoxin 5 pair discovered by the SRM screening was confirmed by the investigation of the independent Δ*spkG* kinase mutant constructed on the background of the glucose-tolerant WT. An expanded SRM library which contained SRM assays for several non-modified peptides, was used for this purpose (Paper II). Additionally, the investigation of the Δ*slr0151* strain, in parallel to Δ*spkG* (Δ*slr0152*), revealed a novel element in the *Synechocystis* protein phosphorylation network. Results are consistent with the theory that Slr0151 acts as the auxiliary protein regulating the balance between phospho- and dephospho-forms of Fd5 (Figure 12). Indeed, SpkG and Slr0151 influenced the status of Fd5 phosphorylation in an opposite way. It is possible to speculate that Slr0151 prevents the over-phosphorylation of Fd5 by interacting with SpkG, possibly binding to it through the TPR motif. How Slr0151 carries out this regulation, and it is direct or indirect, remains unknown. The results suggested an interaction between Slr0151 and SpkG, since the amount of the Slr0151 protein in the Δ*spkG* mutant was only 50% of that

in the WT strain. It is possible that cells limited the synthesis of Slr0151 when its function was not needed due to the lack of Fd5 phosphorylation in ΔSpkG. Alternatively, the lack of *slr0152* mRNA could destabilize the *slr0151-slr0152* bicistronic mRNA and negatively affect the Slr0151 protein accumulation.

Auxiliary proteins regulating kinase functions have been shown for His-kinases in *Synechocystis* and other cyanobacteria (Sakayori et al., 2009), but this Thesis showed for the first time the existence of a similar auxiliary protein regulating the S/T kinase.

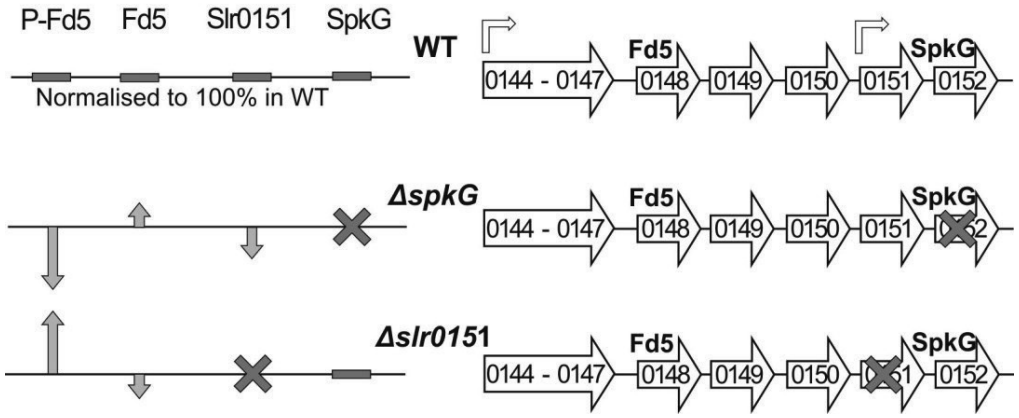

Figure 12: Schematic representation of changes in the amounts of (phospho)peptides detected by SRM in the Δ*spkG* and Δ*slr0151* mutants (left) and scheme of the gene organization (right). The arrows indicate the direction and intensity of the changes of (phopho)peptide amounts; a cross indicates knock-out gene; a line indicates proteins levels in WT and no changes detected in mutants (**spkG* in the Δ*slr0151* strain was measured by RT-qPCR since it cannot be detected by SRM)

The biological role of Fd5 proteins is still unknown, and thus it is difficult to assign the function of Fd5 phosphorylation. The alignment of the Fd5 sequence to the ones of plant-type ferredoxins in *Synechocystis* 6803 (Fd1-4, Figure 5 in Paper II) showed that both phosphorylated T residues in Fd5 are aligned with negatively charged amino acids (Glu or Asp). The addition of a phosphate group to the T in Fd5, resulting in an appearance of a negative charge that is conserved in other sequences in this position, might modify the Fd5 function in electron transfer. On the other side, the 3D model created for Fd5 showed that the two phosphorylation sites are located on the opposite sides of the protein (Figure 6 in Paper II), suggesting a function in protein-protein interaction. It can be speculated that specific conditions trigger phosphorylation and modify Fd5 affinity toward interaction partners. Interestingly, both T18 and T72 kinases are conserved within cyanobacteria species possessing a gene cluster similar to the *slr0144-0152* of *Synechocystis* (Figure S3 in Paper II)

indicating that the physiological role for Fd5 phosphorylation is conserved among cyanobacterial species.

Future studies are required to solve the intricate phosphoprotein-protein kinase network and the function of auxiliary proteins in *Synechocystis* 6803; the interaction with the phosphatase is also still mostly unknown. Furthermore, the biological functions of most of the phosphorylation events are unknown and require investigation as well. Noteworthy, revealing the biological role of protein phosphorylation in the regulation of photosynthesis may provide valuable hints for the application of cyanobacteria as biofactories.

5.2. Background proteome changes in engineered *Synechocystis 6803*

5.2.1 Regulation of the petJ promoter in Synechocystis 6803

In *Synechocystis* 6803, the *petJ* promoter is a good candidate for synthetic biology approaches, since 1) its activity is regulated by the concentration of Cu^{2+} in the medium and 2) its expression is not vital for the cell to survive. A sudden increase in the amount of Cu^{2+} in the medium was shown to be lethal (Zhang et al., 1992; Giner-Lamia et al., 2014), and therefore in this Thesis the $CuSO_4$ was added in a stepwise manner (Figure 1 in Paper III). The gradual addition of the metal ions is compatible with culture survival; indeed, during this treatment, cells continue growing, albeit more slowly than cells in standard conditions (data not shown). However, Cu^{2+} is a micronutrient whose homeostasis is strictly controlled in cyanobacteria (Tottey et al., 2001; Raimunda et al., 2010; Raimunda et al., 2011; Giner-Lamia et al., 2012; López-Maury et al., 2012; Giner-Lamia et al., 2016 a, b), thus changes in its concentration are expected to affect cell metabolism. The *Synechocystis* 6803 WT strain carrying an empty vector (WT/pVZ321) was tested in order to investigate a possibility to use this system in industrial application (Paper III). The changes measured in the proteome were direct effects of copper on the culture, without influence from the expression of exogenous genes, proteins or product.

5.2.2 Regulation of petJ promoter influenced the proteome of Synechocystis 6803

The proteomics investigation revealed profound metabolic rearrangements in *Synechocystis 6803* during the experiment. The increase of copper concentration induced acclimation changes in several pathways. Some proteins were affected only temporarily, and their expression was modified again after the shift to Cu^{2+} depleted media. In other cases instead, the acclimation to the high Cu^{2+}concentration was irreversible. Some proteins were affected by the lack of Cu^{2+} rather than by its excess.

Nitrogen metabolism is an example of a pathway that was strongly affected by the addition of Cu^{2+} to the BG11, but the cells restored normal expression of N-metabolism related proteins after Cu^{2+} removal from the media (Table 2, S1 and S2 in Paper III). The data suggested that upon increase of Cu^{2+} the cells require additional nitrogen, thus they increased the uptake and the assimilation of nitrogen-containing substrates such as nitrite, nitrate, urea and cyanate. All these metabolic adjustments may explain the retarded growth of the strains during the acclimation to high Cu^{2+}. However, after Cu^{2+} depletion, most of the proteins involved in N-metabolism were expressed at levels similar to ones in standard conditions (Paper III, column D/S in Table 2 and Figure 2). Thus, in a biotechnological application, the expression of the compound of interest should not be affected by the changes in proteins related to nitrogen metabolism.

On the other hand, the irreversible modifications in other pathways (Figure 13) may affect the over-expression of proteins of interest.

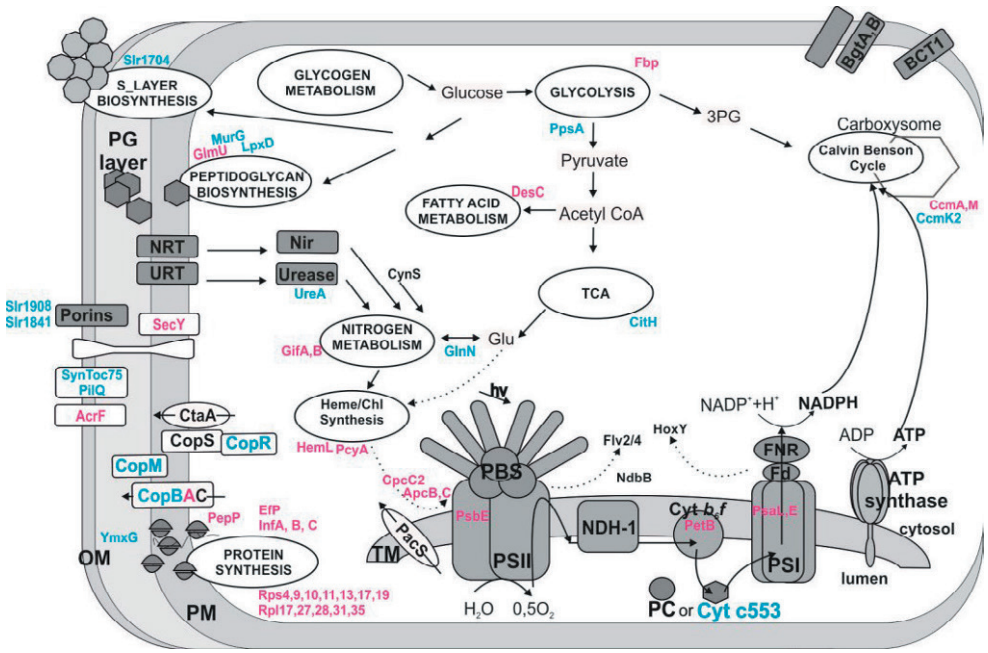

Figure 13: Schematic representation of the background changes occurring in the *Synechocystis* 6803 proteome under a subjective biotechnological application using the *petJ* promoter. Cells were first acclimated to 5 µM Cu^{2+} and then shifted to Cu^{2+} free medium. Proteins which were differentially regulated at the end of the experiment (D) compared to the standard conditions (S) are shown in blue (up-regulated) and magenta (down-regulated). The FC threshold value is ± 1.40.

For instance, to some extent, proteins involved in DNA replication and transcription to mRNA were negatively affected. Next, the protein synthesis

machinery was strongly down-regulated at the end of the experiment, and thus the synthesis of proteins is suggested to be impaired by the by lack of Cu^{2+}. This effect of Cu^{2+} on protein synthesis might have a negative impact in industrial applications if the product of interest is a protein, while it might result in a positive effect, keeping protein synthesis at minimum level, if the product of interest is not a protein. The results also suggested that the cell periphery, i.e. both the plasma and outer membranes, the peptidoglycan layer and the cell surface were remodeled as a consequence of Cu^{2+} treatments. These changes might affect the secretion of the exogenous proteins of interest, which is often a desirable feature of a cell factory. Further, the production of fatty acids and lipids might be negatively affected by the down-regulation of acyl-lipid desaturase DesC (Sll0541).

5.3. Deep knowledge of cyanobacterial metabolism is needed for synthetic biology applications

The model organism *Synechocystis* 6803 has been thoroughly studied for several tens of years (Kufryk et al., 2002; Knoop et al., 2010 Lopo et al., 2012; Branco dos Santos., 2014). Nevertheless, several regulatory mechanisms are still unknown or not fully understood. The functional roles of the extensive phosphorylation network shown in this thesis (Paper I and II) are one example. Overlooking this regulatory mechanism may affect the efficiency of the cyanobacterial strains used as cell factories.

In addition, synthetic biology tools and gene expression strategies might affect cellular metabolism (as shown in Paper III). Thus, analyses of the background proteome changes, which occur in response to genetic modifications or a treatment, should became routine steps in the optimization of production strains in order to improve the applicability of cyanobacteria to industrial scale production (Hagemann and Hess, 2018).

6. CONCLUDING REMARKS

The data presented in this doctoral Thesis demonstrated that:

1) S/T/Y phosphorylation events take place in many proteins from various pathways in *Synechocystis* 6803 (Paper I). A large group of phosphoproteins is involved in photosynthesis and photosynthesis-related processes.

2) The developed SRM library is a valuable tool to quantify the phosphorylation of photosynthesis-related proteins in *Synechocystis* 6803 and thus to provide insights into the biological roles of the phosphorylation events and into the corresponding kinase network (Paper I and II).

3) A complex S/T/Y phosphoprotein-protein kinase network exists in *Synechocystis* 6803. This network includes interconnections between kinases that regulate each other´s activities and auxiliary proteins regulating the function of the kinases (Paper II).

4) Gene expression strategies in synthetic biology affect cellular metabolism. Changes in Cu^{2+} concentrations in growth media, which were applied to control the *petJ* promoter, disturbed the proteome of *Synechocystis* 6803 (Paper III).

5) Proteins involved in gene expression, namely DNA replication, transcription to mRNA and further translation to protein, as well as in signaling, transport of compounds inside and outside the cell, and lipid biosynthesis were irreversibly affected by Cu^{2+} treatment. Thus, this Thesis demonstrated that it is important to evaluate which background proteome changes occur in response to synthetic biology approaches before designing production strains for industrial applications, (Paper III).

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