Probing *in vivo* interactions between flavodiiron proteins and thioredoxins in *Synechocystis* sp. PCC 6803

Master's thesis in Molecular biosciences

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Synechocystis is a cyanobacterium that is capable of photosynthesis, and it can grow heterotrophically as well as phototrophically. Flavodiiron proteins are proteins that catalyze the Mehler-like reaction in photoreduction of oxygen into water. This works as an electron sink when light intensity suddenly increases or carbon assimilation is prohibited. *Synechocystis* has four different flavodiiron proteins that work as heterooligomers. Thioredoxins are enzymes that catalyze disulphide/dithiol exchange in order to adjust the activity of enzymes. Flavodiiron proteins have been found to have several conserved cysteine residues, which could work as targets for thioredoxin regulation. *Synechocystis* cells were transformed to produce fusion proteins of TrxA, Flv1, Flv2 and Flv3, where each protein was fused with one half of the yellow fluorescent protein Venus. By using bimolecular fluorescence complementation, it was possible to determine whether or not TrxA ever came close enough to one of the flavodiiron proteins for the Venus fragments to form a complete fluorescent protein. A successful completion of Venus would indicate that the two proteins in question interact with each other. TrxA was found to interact with Flv1 and Flv2, but not with Flv3. These interactions took place in the thylakoid membrane.

Keywords: Cyanobacteria, *Synechocystis* sp. PCC 6803, thioredoxin, TrxA, flavodiiron proteins, FDP

Content

1. Introduction

Life on Earth receives majority of the energy it needs from the Sun as light energy. Most of this energy is turned into heat energy, which cannot be properly utilized in cell metabolism. A small portion of the light energy is, however, captured as it is absorbed by molecules. Once absorbed, the energy can be bound into a chemical form by using the energy to form or break chemical bonds against the chemical equilibrium (Hohmann-Marriott and Blankenship 2011). In this form, energy can be readily utilized in cell metabolism. Absorption of light energy and its subsequent binding into biomolecules is referred to as photosynthesis.

Photosynthesis is a process where light energy is used to produce chemical energy by reducing inorganic carbon in the form of carbon dioxide into carbohydrates by taking electrons from an electron donor. The electron donor can for example be water or hydrogen sulphide, but oxygen is produced only if water works as the electron donor. The energy captured in this way is bound in adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), which can then be used in production of other biomolecules. Photosynthesis produces most of the energy used by the ecosystem, and it is responsible for the high concentration of oxygen in the atmosphere. The readily available oxygen has in turn enabled the evolution of large heterotrophic multicellular organisms like animals.

Photosynthetic organisms include cyanobacteria, algae, and plants. The photosynthetic groups of bacteria include cyanobacteria, purple bacteria, green sulphur bacteria, filamentous anoxygenic phototrophs, heliobacteria, and acidobacteria. Out of these, cyanobacteria, green sulphur bacteria, and most purple bacteria are photoautotrophs, meaning that they incorporate inorganic carbon in their metabolism with electrons acquired by light-driven reaction centers. Acidobacteria, heliobacteria, and some types of purple bacteria are photoheterotrophs, as they require organic carbon while they use

light energy for generating proton motive force, and for forming chemical bonds. (Hohmann-Marriott and Blankenship 2011).

2. Cyanobacteria

Cyanobacteria, also known as blue-green algae, are photosynthetic Gram-negative bacteria that are estimated to have appeared approximately 2,6 million years ago (Hedges et al. 2001), and they are believed to be the first organisms to produce oxygen through photosynthesis. Cyanobacteria can be found in a variety of different environments like freshwater, salt water, and terrestrial environments. They can be found across the world in surroundings with extreme temperatures from hot springs to the Antarctic, and are therefore considered ubiquitous in the world.

Cyanobacteria can be unicellular, filamentous, planktonic, or colonial. In filamentous growth the cells involved are divided into vegetative cells and heterocysts, where vegetative cells are responsible for photosynthesis while heterocysts fix N_2 from the surroundings (Kulasooriya and Magana-Arachchi 2016). Unicellular cyanobacteria can live on their own as free-living cells, or they can form colonies or planktonic aggregates together with other species in a symbiotic relationship (Sánchez-Baracaldo 2015).

Many cyanobacteria are capable of fixing nitrogen, however this reaction is sensitive to oxygen and must therefore be kept separate from photosynthetic reactions by either carrying out nitrogen fixing in its own cellular compartment, or by fixing nitrogen only in darkness. Aerobic nitrogen fixation in filamentous cell colonies is carried out by thick-walled heterocysts, but the details remain unclear on how it occurs in unicellular species. (Kulasooriya and Magana-Arachchi 2016).

In a process called primary endosymbiosis a non-photosynthetic eukaryotic organism has absorbed a photosynthetic cyanobacterium through phagocytosis. Instead of killing the cyanobacterium, the host cell established a symbiotic relationship with it, allowing the cyanobacteria to become chloroplasts in the cell. This event led to the creation of red

algae, glaucophyte algae and the “green lineage”, which includes all green algae and plants (Bhattacharya and Medlin 1995; Green 2011).

Cyanobacteria are photoautotrophic, which allows them to grow in conditions with little nutrients as long as there is water, light, and carbon dioxide available. This makes them easy to grow in laboratories, where the use of simple growth media enhances reproducibility of the exact growth conditions for the cells. In comparison to plants, cyanobacteria require less resources, space, and time to grow making them an excellent choice for research in photosynthesis. Cyanobacteria also have a fairly small genome with a size of 2-9 million base pairs, which makes modifying their genome easier.

Synechocystis sp. PCC 6803 (Stanier et al. 1971) is a unicellular strain of cyanobacteria that is capable of growing heterotrophically and phototrophically, allowing them to survive without fully functioning photosynthetic machinery. This in turn allows research on photosynthesis to be carried out with mutant strains that have parts of the photosynthetic machinery altered or completely removed. Unlike many other cyanobacteria, *Synechocystis* does not fix nitrogen from their surroundings. Instead, they uptake nitrogen in the form of nitrate, nitrite, ammonia and urea (Mills, McCormick, and Lea-Smith 2020). *Synechocystis* sp. PCC 6803 was the first cyanobacterial species to have its genome completely sequenced (Kaneko et al. 1996), and the genomes of many other strains of cyanobacteria have been thoroughly sequenced and analysed, which makes research on them efficient.

Cyanobacteria along with microalgae can be used for the production of renewable biofuels (Hu et al. 2008), food, feed (Smetana et al. 2017), cosmetics (Morone et al. 2020), and pharmaceutical compounds (Martins et al. 2008). As the need for fuel is constantly increasing and as the amount of available fossil fuels is constantly decreasing, the need for renewable fuels keeps growing. The use of fossil fuels also releases greenhouse gasses, which contribute to the global greenhouse effect. For these reasons, interest in sustainable and renewable energy sources is increasing. Cyanobacteria are an excellent candidate for producing carbon neutral biofuel due to their fast growth rate and ease of genetic engineering. Cyanobacteria can be used to produce products such as ethanol,

hydrogen, ethylene, fatty acids, and isoprenes by utilizing energy from light (Oliver et al. 2016; Touloupakis et al. 2016). Cyanobacteria can also be used for waste treatment and capturing harmful flue gasses (Uma et al. 2022).

In comparison to terrestrial feedstocks, cultivating cyanobacteria and algae have higher biomass productivity, and they can be grown on non-arable land around the year. As their cultivation is a moderately new innovation, technology for them still needs to be further developed to make it commercially attractive. The biorefineries used for extracting the high-value compounds need to be properly designed and optimised in order to maximize their commercial utilisation. Important steps for further enhancing the efficiency of biomass valorisation include research on cyanobacterial strains, streamlining the production before, during, and after cultivation, as well as development of fast and easy methods for valorising the products. (Uma et al. 2022).

3. Oxygenic photosynthesis

In oxygenic photosynthesis oxygen is produced by oxidizing water, which results in the production of oxygen and protons. The splitting of water demands a lot of energy to occur, and this energy is acquired from light. When a photon first arrives to the photosynthetic machinery in the thylakoid membrane of cyanobacteria or in chloroplasts, it gets absorbed by pigment molecules such as chlorophyll. In cyanobacteria these pigments are located in phycobilisomes at the surface of the thylakoid membrane in the cytosol, as well as in photosystems II (PSII) and I (PSI).

Phycobilisomes are large protein complexes that contain the phycobilin pigments phycoerythrin, phycocyanin, and allophycocyanin, along with the associated linker polypeptides. Phycoerythrin, phycocyanin, and allophycocyanin absorb light at 565 nm, 620 nm, and 650 nm, respectively. The absorption spectra of the phycobilisomes however changes depending on its structure and the surrounding proteins. Phycobilisomes change their size, composition and location depending on the quantity and quality of light received in order to optimize light absorption. Phycobilisomes can move across the

thylakoid membrane, and they are usually associated with PSII complexes. (Liberton et al. 2017; Singh et al. 2015).

Incoming photons are absorbed by the phycobilin, chlorophyll, and other pigments and passed on to PSII, which is a dimeric protein complex where in cyanobacteria each monomer is comprised of 21 protein subunits, pigment molecules, lipids, the oxygen-evolving complex, ions, and hemes among other biomolecules (Gisriel et al. 2022; Heinz et al. 2016). Inside PSII the energy is passed to the reaction center chlorophyll P_{680} by excited pigment molecules passing electrons to other molecules in redox reactions. The excited P_{680} reduces pheophytin, after which the oxidized P_{680} is reduced by the tyrosine residue Tyr161 in the D1 subunit of PSII. Tyr161 oxidizes the oxygen-evolving Mn_4CaO_5 cluster, and once four electrons have been taken from the oxygen-evolving complex two water molecules are split into four protons and one oxygen molecule (Shen 2015). Pheophytin reduces quinone Q_A, which passes the electron to quinone Q_B. Once Q_B has received two electrons it becomes plastoquinol PQH_2 and is released into the thylakoid membrane. PQH_2 binds to the Q_o binding site in the cytochrome b_6f complex which in turn reduces the soluble electron carrier protein plastocyanin.

One of the electrons coming from PQH_2 is passed to Q_B that is waiting in the Q_i binding site of the cytochrome b_6f. Once Q_B has received two electrons it protonates into PQH_2 and is released from the binding site. This process is called the Q cycle, and it causes protons to be moved from the stroma to the lumen when electrons are transferred from cytochrome b_6f to PSI. (Cramer, Hasan, and Yamashita 2011).

Plastocyanin passes the electron to PSI, where it is received by the reaction center chlorophyll P_{700}. PSI can work as a monomer, trimer or even a tetramer, where each monomer comprises of 11 protein subunits, carotenoids, chlorophyll a molecules, lipids, iron-sulphur clusters, phylloquinones, and calcium ions (Malavath et al. 2018; Netzer-El, Caspy, and Nelson 2019). PsaA and PsaB proteins form the reaction center of PSI. The electrons are then transported to the iron-sulphur cluster Fx, and further to iron-sulphur clusters FA and FB in the stromal peripheral subunit PsaC, where a docking site for oxidised ferredoxin (Fd) also resides in (Hippler and Nelson 2021; Lima-Melo et al. 2021).

PsaC establishes the close contact needed between the iron-sulphur clusters of PSI and Fd, while PsaD and PsaE guide the Fd into the binding pocket of PSI (Lima-Melo et al. 2021). The electrons are then passed to Fd in the stroma, where Fd $NADP^+$ reductase (FNR) uses them in conversion of $NADP^+$ into NADPH (Knaff and Hirasawa 1991). NADPH can be used as an electron donor in various reactions in the cells, such as the reactions in the Calvin-Benson-Bassham (CBB) cycle.

During the electron transportation through the photosynthetic machinery a proton gradient is created over the thylakoid membrane. Protons are generated in the lumen by splitting of water by PSII, and they are also transported through the thylakoid membrane from stroma to lumen by the Q cycle in cytochrome b_6f. Other contributors to the formation of the proton gradient are NADPH dehydrogenase 1 complex (NDH-1) (Miller, Vaughn, and Burnap 2021) and possibly cytochrome *c* oxidases as well (Nikkanen et al. 2021). NDH-1 increases the proton motive force by transporting electrons from Fd or NADPH to quinone, which is then used in the photosynthetic electron flow. Cyanobacterial NDH-1 also has different versions that function in respiratory electron flow, CO_2 uptake, as well as cyclic electron flow (CEF) (Miller et al. 2021). The proton motive force caused by the proton gradient is used to power ATP synthesis by the ATP synthase.

ATP is synthesized from ADP and phosphate through photophosphorylation as protons travel from the lumen into the stroma. ATP synthase comprises of F_0 that permeates the thylakoid membrane, and F_1 that sticks out in the stroma. The movement of protons through F_0 causes the γ subunit of F_1 to spin. This causes conformational changes in the structure of the reaction center of F_1, driving synthesis of ATP out of ADP and P_i. (Junge and Nelson 2015).

In the cytosol protons are used in the production of NADPH by FNR (Knaff and Hirasawa 1991), and in the reduction of oxygen by cytochrome *c* oxidase, cytochrome *bd* quinol oxidase and flavodiiron proteins (FDPs) while they work as electron sinks for excess electrons (Ermakova et al. 2016; Nikkanen et al. 2021). Bidirectional hydrogenase

catalyzes $NAD(P)^+$-dependent H_2 uptake as well as NAD(P)H-dependent H_2 evolution in the cytosol (Mishra, Kaushik, and Tiwari 2018; Tamagnini et al. 2002).

3.1. Protection against photoinhibition

If photosynthetic cells receive too much light or carbon fixation is restricted, electrons begin accumulating in the photosynthetic machinery as there are more electrons in the photosynthetic electron transport chain (PETC) than what can be used in chemical reactions. PSI is particularly sensitive to photoinhibition due to the time it takes to repair a damaged PSI complex, which is a process that can take several days. This is due to the apparent absence of an efficient repair cycle like the one for PSII, and it could include the degradation and rebuilding of the entire PSI complex (Huang, Zhang, and Cao 2010; Kudoh and Sonoike 2002; Lima-Melo et al. 2021). PSI donates electrons to Fd, which provides electrons to be used in NADPH synthesis as well as Trx reduction, which are then used to regulate the activity of enzymes in processes such as the CBB cycle. The role of Trxs is discussed in further detail below. This central role makes it important to keep PSI working properly at all times. Fd works as an electron donor to several other processes as well. Fd donates electrons for the reduction of sulphite to sulphide, reduction of protons by hydrogenases, and reduction of nitrite to ammonia (Mondal and Bruce 2018). NDH-1 uses electrons donated by Fd in CEF in order to increase the proton motive force and reduce the plastoquinone pool (Miller et al. 2021; Sétif et al. 2020).

Failure to resume electron flow through the thylakoid membrane leads to excessive reduction of the components in the PETC. This causes PSI to become overly reduced, in which case electrons are donated to oxygen, which leads to the production of radical superoxide, which is disproportionated into hydrogen peroxide and oxygen (Asada, Kiso, and Yoshikawa 1974; Mehler 1951). Hydrogen peroxide can then react with the iron-sulphur clusters of PSI, causing hydroxyl radicals to be formed and electron transport through PSI to be halted. Another type of ROS that can be produced via photoinhibition of PSI is singlet oxygen (Takagi et al. 2016). Radical superoxide as well as singlet oxygen

can cause damage to proteins. PSI photoinhibition can occur in conditions with high light combined with low temperature, drought, or salinity, when assimilation of CO_2 is limited. PSI photoinhibition has also been shown to be intensified in red and blue light, which are more suitable for the excitation of PSII. (Lima-Melo et al. 2021).

In order to avoid the photosynthetic machinery becoming overly reduced and taking damage as a result, there exist alternative paths for the electrons to take. In paths relying on CEF electrons are returned to the PQ pool in the thylakoid membrane, thus supporting cytochrome b_6f in pumping protons into the lumen. The protons are used by ATP synthase in production of additional ATP, which is used to meet the needs of metabolic pathways like the CBB cycle and carbon concentration reactions. This alleviates the build-up of electrons in the PETC (Alboresi et al. 2019; Kramer and Evans 2011). In one of the major CEF pathways identified so far NDH-1 reduces PQ by using electrons acquired from Fd (Alboresi et al. 2019). NDH-1 also directly contributes to the upkeep of the proton gradient by pumping two protons to the lumen for every electron it receives (Miller et al. 2021). The water-water cycle transports electrons ultimately to oxygen, producing water in the process. One of these paths is the Mehler reaction (Mehler 1951).

Upon receiving electrons PSI reduces an electron acceptor, which in most cases is Fd. Other possible electron acceptors are the acceptor side of PSI, and plastosemiquinone in the PQ pool (Asada, Iho, and Yoshikawa 1974; Khorobrykh and Ivanov 2002; Kozuleva and Ivanov 2010). In Mehler reaction Fd reduces oxygen creating superoxide radicals, which converts into hydrogen peroxide. Peroxidases and peroxide reductases then reduce the hydrogen peroxide into water, thus completing the water-water cycle. This type of Mehler reaction is common in terrestrial plants. Being surrounded by air also makes dissipation of oxygen faster in comparison to an aquatic environment, decreasing accumulation of oxygen in cells. (Allahverdiyeva et al. 2011). In Mehler-like reaction oxygen is reduced to water without producing reactive oxygen species (ROS) by FDPs as explained in more detail below.

3.2. Thioredoxins

Thioredoxins (Trx) are small enzymes (12-14 kDa) with a highly conserved active site within a sequence motif that resides in a protrusion. Trxs catalyze disulphide/dithiol exchange, which is an important mechanism in regulating the activity of enzymes. Trxs are present in nearly all living organisms and they are divided into groups depending on what enzymes they regulate. The functions of the Trx regulated enzymes in *Synechocystis* include CO_2 fixation, glycolysis, and glycogen synthesis, among others. Trxs control or adjust the activity of enzymes by reducing one or several disulphide bonds into dithiols, or by causing the formation of disulphide bonds by oxidization of dithiol groups. Trx is then reduced by Fd-Trx-reductase, which in turn is reduced by Fd. The gene encoding the catalytic subunit of Fd-Trx-reductase has been found to be indispensable to the survival of *Synechocystis* sp. PCC 6803 cells. (Florencio et al. 2006).

Cyanobacteria have four distinct groups of Trxs, which are TrxA, TrxB, TrxQ and TrxC. Out of these four, TrxC is unique to cyanobacteria. The target proteomes of these Trxs have been shown to have little variation in *Synechocystis* sp. PCC 6803 (Florencio et al. 2006). TrxA is the only Trx to be essential for phototrophic and heterotrophic growth of *Synechocystis* sp. PCC 6803 cells (Florencio et al. 2006; Mallén-Ponce, Huertas, and Florencio 2022). TrxA also has the highest degree of conservation in its structure in comparison to TrxB, -Q, and -C. The most conserved areas are sections that bind and reduce the target proteins, as well as contributing to the reduction of TrxA by Trx-reductases. Some cyanobacteria have also been found to have another Trx that is evolutionarily distant from the other Trxs, and currently it is referred to as TrxE (*alr2205*). It is thought that TrxE reduces proteins in the antioxidant system. (Mallén-Ponce et al. 2022).

The expression of genes *trxA* and *trxB* as well as the gene encoding Fd-Trx-reductase is dependent on the photosynthetic growth conditions, whereas the expression of *trxQ* and *trxC* is independent of them. This is due to the dependence of *trxA/trxB* expression on the

photosynthetic electron transfer (Pérez-Pérez, Martín-Figueroa, and Florencio 2009). TrxA forms approximately 80-90 % of the total Trx pool in cyanobacteria. It regulates the activity of several protein complexes involved in photosynthesis, and its regulatory role is thought to be conserved along the evolution of oxygenic photosynthetic organisms . TrxA has a role in regulation of enzymes involved in the CBB cycle, oxidative pentose phosphate pathway, nitrogen fixation, glycogen metabolism, antioxidant defense, transcriptional regulation, and protein synthesis (Mallén-Ponce et al. 2022). TrxB might have a role in adaptation to high light, as demonstrated by the sensitivity to transition from low light to high light that cells deficient in TrxB possess (Pérez-Pérez et al. 2009). TrxB and TrxQ work as reducing substrates for the oxidative stress response, as demonstrated by the slight sensitivity the cells without them possess (Pérez-Pérez et al. 2009). The role of TrxC is not as well known. TrxC knockout mutants of *Synechocystis* have been shown to have differences in the pigment content in comparison to the wild types, as well as having an altered growth phenotype under low-carbon conditions (Mallén-Ponce et al. 2022).

In the study of Mallén-Ponce in 2021 a conditional *Synechocystis* sp. PCC 6803 mutant strain was created with an on-off promoter for controlling the level of transcription of *trxA*. Decreasing the amount of TrxA by 90 % did not cause major changes in photosynthetic activity, pigments and proteins related to photosynthesis, which allowed the cells to grow almost as well as the wild type. Lowering the amount of TrxA to 2 % of that of the wild type led to significant impairment of photosynthesis, CBB cycle and growth, as well as interference with the oxidative stress response, thus highlighting the necessity of TrxA in the cells. It also indicates that even reduced amounts of TrxA are sufficient to carry out all of the essential functions of TrxA. (Mallén-Ponce et al. 2021).

3.3. Flavodiiron proteins

FDPs are a large family of enzymes that catalyze the photoreduction of oxygen into water. A-type FDPs function in anaerobic organisms in detoxifying O_2 or NO by reduction. C-type FDPs are specific to organisms capable of oxygenic photosynthesis, and they possess a flavin-reductase-like domain. FDPs have a conserved metallo-β-lactamase-like domain with a diiron center as a catalytic site, and a conserved flavodoxin-like domain with a flavin mononucleotide (FMN). FDPs often work as oligomers in order to minimize the distance between these two domains by forming a head-to-tail formation. This allows the flavin of one FMN-binding domain to interact with the diiron center of another monomer. In addition to these domains, C-type FDPs also have an extra NAD(P)H:flavin oxidoreductase module, which suggests that they might be able to accept electrons from NADH/NADPH directly (Borges et al. 2019; Santana-Sanchez et al. 2019). FDPs carry out the Mehler-like reaction in the non-heme diiron center in the metallo-β-lactamase-like domain, where electrons are used to photoreduce oxygen into water (Santana-Sanchez et al. 2019). FDPs are most active in situations when the CBB cycle is repressed, working as a sort of safety valve for the electron flow. If the excess electrons are not removed, the PETC becomes overly reduced leading to damage to the photosystems.

Synechocystis sp. PCC 6803 possesses four C-type FDPs called Flv1, Flv2, Flv3, and Flv4, which are encoded by genes *sll1521, sll0219, sll0550 and sll0217*, respectively. Flv1 and Flv3 can be found in all cyanobacteria, but Flv2 and Flv4 are exclusive to β-cyanobacteria. The FDPs function as heterooligomers to catalyze the Mehler-like reaction in the photoreduction of oxygen without producing ROS in the process. Flv1 and Flv3 work together as a heterodimer in post-PSI electron transfer in fluctuating light conditions, which serves to keep P_{700} in its oxidized form (Allahverdiyeva et al. 2013). They prevent the production of ROS and damage to PSI after a sudden increase in light intensity, which could otherwise be lethal to the cell. The activity of Flv1/3 also depends on the CO_2 concentration, causing them to work as steady electron sinks in 3 % CO_2 growth conditions (HC) and as transient electron sinks in air-level CO_2 growth conditions (LC)

(Santana-Sanchez et al. 2019). Flv2 and Flv4 also work as heterodimers, but their expression is downregulated in HC. Instead, they work as a steady electron sink in LC (Santana-Sanchez et al. 2019). Flv3 has been found to also be able to function as a homodimer or as a homotetramer, however, in order to carry out Mehler-like reaction both Flv3 and Flv1 are needed. These Flv3 homooligomers do seem to have a role in alternative electron transfer pathways other than the Mehler-like reaction, but details on this remain unclear (Mustila et al. 2016).

According to recent research, the electron donor of Flv1/Flv3 is most likely Fd (Nikkanen et al. 2020; Sétif et al. 2020). Flv1/Flv3 have been found to work in unison with NDH-1 in maintaining the redox balance between carbon fixation and electron flow through the photosynthetic machinery when light intensity suddenly increases or carbon assimilation is obstructed (Nikkanen et al. 2020). Since NDH-1 also receives electrons from Fd, the activity of NDH-1 and Flv1/Flv3 could potentially be regulated together through Fd. The light-dependent Fd-Trx system could work as a regulator for them by competing for Fd and through light-dependent redox-sensitive cys residues identified in both FDPs and NDH-1 subunits (Guo et al. 2014; Nikkanen et al. 2020).

In fluctuating light conditions, the amount of light arriving to the cells can change rapidly. This necessitates a way to quickly regulate the activity of different FDPs. FDPs respond to changes in light intensity within seconds, indicating that their regulation is post-transcriptional. Upon increased light intensity FDPs activate after approximately 1 second, and remain active for the following 30 - 60 seconds working as a transient electron sink. Structural analyses have shown a possibility of the activity of FDP dimers to be regulated based on conformational changes. In the “open” conformation the FDP dimer would be capable of catazyling the reduction of oxygen into water, while in the “closed” conformation the dimer would be inactive. In the “closed” conformation the FDP monomers are in slightly different conformations increasing the distance between FMN and diiron center, thus inhibiting enzymatic activity. (Gerotto et al. 2016).

The details on the regulation of FDP activity remain unverified, but one of the theories suggests that they could be regulated by disulphide/dithiol exchange with the Trx system.

FDPs present in β cyanobacteria have been found to contain several conserved cys residues, which could imply post-translational regulation via disulphide/dithiol exchange. One of the proteins that could bind to said residues is TrxA. Because of the quickly changing light conditions there is a need for the ability to quickly adjust the activity of FDPs. The activity of Flv1 and Flv3 has been shown to react to increased light intensity as their thiols are oxidized or reduced depending on the light conditions. Flv1 also has two possible binding sites for TrxA (Guo et al. 2014).

Flv3 and Flv4 have been found to contain phosphopeptides which could be used as targets for post-translational modification in order to regulate their activity. Protein phosphorylation is a mechanism used for finely tuning and regulating the activity of proteins and phosphorylation is also often used in controlling the expression of genes. Protein phosphorylation is reversible and widespread in the regulation of metabolic processes, signal transduction and homeostasis, among others. *O*-phosphorylation is a type of protein phosphorylation that is based on post-translational modification at serine, threonine, and tyrosine residues. Many components in the photosynthetic machinery also contain phosphopeptides, which could be used in the regulation of photosynthesis. (Angeleri et al. 2016).

4. Aims of the research

The aim of the research was to decipher, whether or not TrxA interacts with FDPs 1, 2 and 3. This was to be done with bimolecular fluorescence complementation (BiFC) fusion proteins, which emit fluorescence if TrxA interacts with one of the FDPs. The results of this study could also shed some light on where in the cell this interaction might take place, and possibly provide insight on the details of the regulation of the activity of FDPs regarding the involvement of TrxA.

This knowledge can then be used to further optimize the use of photosynthetic organisms and production of compounds via photosynthesis in biotechnology. Understanding the regulation of different paths of electron flow in the photosynthetic machinery can create

opportunities to streamline electron flow to focus more on the production of the desired products, thus optimizing production efficiency.

5. Materials and methods

5.1. Building the vector plasmids

The entire workflow of building the vector plasmids is depicted in Figure 1. The figure shows all of the plasmids created along the way of creating the final pDF plasmids, and it also shows which restriction enzymes were used at what point. These steps will be described in more detail below.

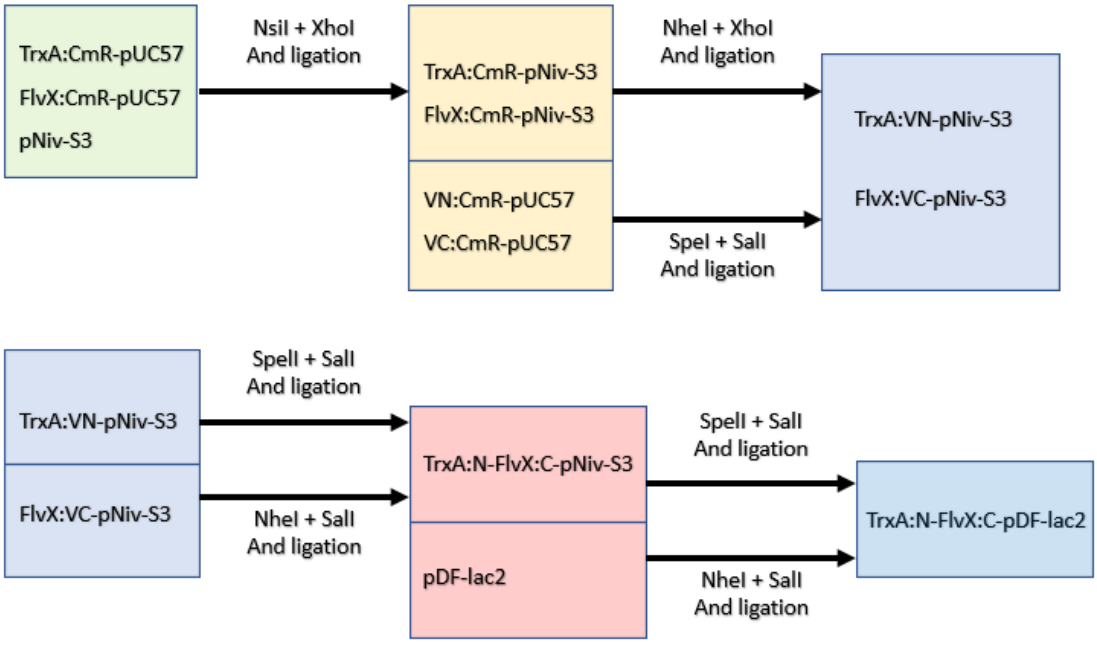

Figure 1. Chart depicting the workflow of building the pDF-lac2-TrxA:N-FlvX:C plasmids.

Escherichia coli DH5α cells (referred to as DH5α cells from now on) were transformed with TrxA:CmR_pUC57 (*slr0623)*, Flv1:CmR_pUC57 (*sll1521)*, Flv2:CmR_pUC57 (*sll0217)*,

Flv3:CmR_pUC57 (*sll0550)*, VN:CmR_pUC57 (VN155), VC:CmR_pUC57 (VC155), pNiv-S3, and pDF-lac2 -plasmids. The plasmids were synthetic DNA ordered from GenScript. VN155 and VC155 each encode for one half of the mutated version of the yellow fluorescent protein (YFP) Venus. The mutation decreases the chances of self-assembly, thus lowering the number of false positive results (Kodama and Hu 2010).

The transformation of the DH5α cells was done by adding 5 µl of DNA to 25 µl of E. coli cells, and the mixture was kept on ice for 30 minutes. Heat shock was applied by keeping the cells at 42 °C for 30 seconds, after which the cells were kept on ice for 2 minutes. After this, 170 µl of SOC-buffer (3,6 g/l dextrose; 0,19 g/l KCl; 4,8 g/l $MgSO_4$; 20 g/l tryptone; 5 g/l yeast extract) or liquid LB growth medium (10 g/l tryptone; 5 g/l yeast extract; 5 g/l NaCl) was added to the cells, and they were incubated at 37 °C for 1 hour. The cells were then plated on selection plates containing solid LB growth medium (liquid LB growth medium with 16 g/l agar), ampicillin (50 mg/ml, Amp50) and chloramphenicol (35 mg/ml, Cm35), and they were incubated at 37 °C overnight. Cells containing pDF-lac2 -plasmids were grown on selection plates containing spectinomycin (50 mg/ml, Sp50) instead of Amp50. Colonies were collected from the selection plates and they were grown in 4 ml of liquid LB growth medium with Amp50 and Cm35 overnight at 37 °C with constant shaking. As before, cells containing pDF-lac2 -plasmids were grown with Sp50 instead of Amp50. DNA was then collected from the growth culture with NucleoSpin Plasmid mini kit by Macherey-Nagel.

The plasmids also contained a resistance cassette, which provides resistance against ampicillin (Amp) and chloramphenicol (Cm). Amp inhibits synthesis of bacterial cell walls by irreversibly inhibiting transpeptidase from synthesising peptidoglycan (Edoo, Arthur, and Hugonnet 2017). Cm on the other hand inhibits protein synthesis by binding to the peptidyl transferase center of the 50S ribosomal subunit (Weisberger 1967). Bacterial cells can acquire resistance against Amp by producing β-lactamase that hydrolyzes the β-lactam ring of Amp (Livermore 1995). Resistance against Cm relies on the activity of chloramphenicol acetyltransferase, which acetylates Cm thus making it unable to bind to its target (Nakagawa, Nitahara, and Miyamura 1979). The pNiv-S3 contains the ribosome

binding site S3, where a ribosome binds and initiates translation of the genes into mRNA. Ribosome binding site S3 was chosen for the experiment due to the high translational efficiency it has when used for the expression of YFP (Thiel et al. 2018).

TrxA-, Flv-, and pNiv-S3 plasmids were digested with NsiI and XhoI, which opened the plasmids while leaving DNA overhangs of a few nucleotides without pairs as sticky ends. This allows the DNA fragments to be ligated in the correct orientation at the intended site. TrxA-, Flv-, and pNiv-S3 -plasmids were digested with by using 1 µl of NsiI and XhoI for 2 µg DNA. Samples were incubated at 37 °C for 2 – 4 hours, and the DNA fragments were separated by agarose gel electrophoresis by running the samples on a 1 % agarose gel with 80 V for 1 hour. The DNA fragments were then collected from the agarose gel.

The DNA containing genes for TrxA and FDPs as well as resistance cassettes against Amp and Cm were separated and ligated into the opened pNiv-S3 plasmids. The ligation was done by using T4 ligase and incubating the DNA at 16 °C for 1 hour.

The TrxA-pNiv-S3 and Flv-pNiv-S3 plasmids were then used to transform DH5α cells in order to acquire more plasmids and verify the presence of the correct plasmids. Transformed cells were plated on LB Amp50 Cm35 selection plates and they were incubated at 37 °C overnight. Colonies were picked and grown in liquid LB Amp50 Cm35 as before, and the DNA was collected. The presence of the correct plasmids was verified by digesting them with NsiI and XhoI and separating the DNA fragments with gel electrophoresis as before to ensure that the plasmids were built correctly.

The TrxA-pNiv-S3 and Flv-pNiv-S3 plasmids were digested with NheI and XhoI, while VN:CmR-pUC57 and VC:CmR-pUC57 plasmids were digested with SpeI and SalI. DNA fragments were separated and collected as before. The pUC57 plasmids contained genes for a Cm resistance cassette and either the N-terminal (VN:CmR-pUC57) or C-terminal (VC:CmR-pUC57) half of the Venus protein. TrxA-pNiv-S3 was ligated with VN:CmR-pUC57, while each of the Flv-pNiv-S3 plasmid fragments was ligated with VC:CmR-pUC57, thus forming TrxA:N-pNiv-S3 and Flv:C-pNiv-S3. These plasmids were used to transform DH5α cells, which were grown on LB Amp50 Cm35 growth plates as before, and the DNA

was collected. During digestion the pNiv plasmids lose their CmR cassette, which is then replaced with the CmR cassette in the pUC57 plasmids. This ensures that only the bacteria with the complete plasmid can survive on the selection plates.

TrxA:VN-pNiv-S3 plasmid was digested with SpeI and SalI which separates a DNA fragment with genes for TrxA, N-terminal half of Venus, and Cm resistance cassette. Flv:VC-pNiv-S3 plasmids were digested with NheI and SalI, which removes the Cm resistance cassette. The DNA fragments were separated and purified as before. TrxA:VN fragments were then ligated to the Flv:VC fragments, producing TrxA:N-Flv:C-pNiv plasmids. These plasmids were used to transform DH5α cells as before, and the DNA was collected.

The TrxA:N-Flv:C-pNiv and pDF-lac2 plasmids were digested with SpeI and SalI, and the DNA fragments were separated and collected as before. TrxA:N-Flv:C fragments were then ligated with the opened pDF-lac2 plasmids, which allows the inserted genes to work under a *lac* operon. The *lac* repressor represses transcription of the genes in the *lac* operon when lactose is absent, and activates transcription when lactose is present. When *lac* repressor binds to the *lac* operator it partially covers the promoter, preventing ribosomes from binding to the site and starting the transcription. Isopropylthio-β-galactoside (IPTG) is also capable of binding to the *lac* repressor and make it detach from the *lac* operator, thus allowing gene transcription. Using IPTG in the induction of gene transcription allows more precise control over gene activity as IPTG binds to the repressor (Zhao et al. 2018). The pDF-lac2 plasmid also contains a Sp resistance cassette, providing resistance against Sp. Sp inhibits protein synthesis by binding to the ribosome (Bilgin et al. 1990), so resistance against it relies on modification of Sp to make it unable to bind to its target (Davies and Wright 1997). The TrxA:N-Flv:C-pDF plasmids were used to transform DH5α cells. The cells were grown on LB Sp50 Cm35 selection growth plates at 37 °C overnight, and colonies were picked to be grown in LB Sp50 Cm35 liquid growth medium at 37 °C overnight with continuous shaking. The DNA was collected as before.

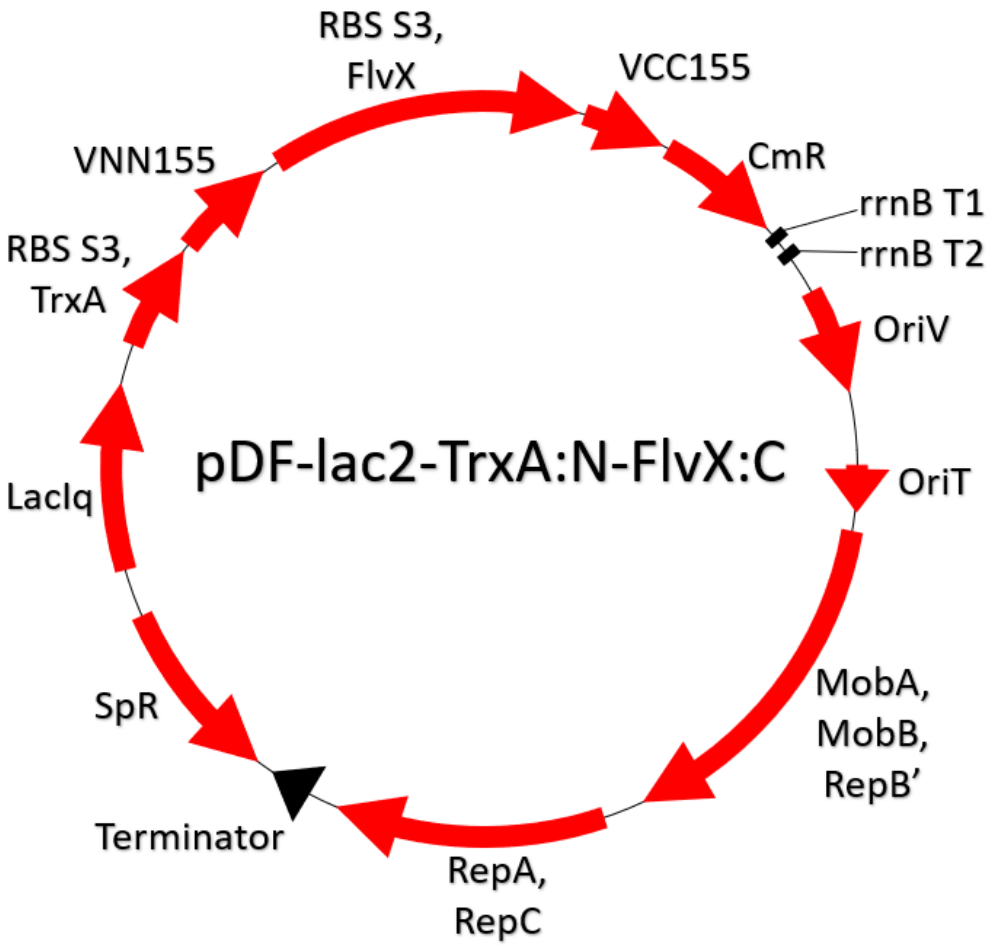

Figure 2. Genes present in a pDF-lac2-TrxA:N-FlvX:C plasmid, where FlvX stands for one of the FDPs Flv1-Flv3.

Figure 2 shows a schematic picture of the TrxA:N-Flv:C-pDF plasmids. Each plasmid contained resistance cassettes for Sp and Cm, as well as genes encoding RBS S3, VNN155 and VCC155, TrxA, and one of the FDPs. Along with antibiotic resistance cassettes (*SpR* and *CmR*) the plasmid contains several other genes necessary for replicating the plasmid and transferring it to other bacteria.

MobA and *MobB* encode proteins that assemble at the origin of transfer (*oriT*) to form a relaxosome. MobA is the most important part of the relaxosome and it locally disrupts the helical structure of double-stranded DNA (dsDNA). The C-terminal primase of MobA

forms primers at the origin of replication (*oriV*). MobA also has an N-terminal relaxase or nickase , which is an enzyme that nicks dsDNA. It cleaves one of the DNA strands at *oriT* while forming a covalent adduct with the 5' end of the DNA. The nicked DNA is then transferred to the host bacterium through bacterial conjugation in temporary cell-to-cell contact, after which MobA rejoins the two ends of DNA together and detaches. Complimentary DNA strands are then synthesized for the circular plasmids by polymerases. MobB stabilizes the formation of the relaxosome at *oriT* and stimulates the nicking of DNA by MobA, thus enhancing the frequency of mobilisation of the plasmid. (Meyer 1999; Xia and Robertus 2009).

Rep promoters work as enhancers of transcription, while replication proteins (Rep) inhibit their function by binding to them (Rozhon 2017). Rep proteins also possess nickase activity, and the nicking of DNA forms a free 3'-OH that works as a primer for leading strand synthesis in replication of the plasmid (Rozhon et al. 2011). RepB is able to bind to DNA and bend it, while RepA works as a primase (Chatterjee et al. 2007). RepC is able to nick DNA and then covalently bind to the 5' terminus of the DNA (Rasooly 1997).

rrnB T1 and *rrnB T2* along with *terminator* are terminator sequences, where *rrnB T1* and *rrnB T2* represent two regions of the complex *rrnB* gene (Orosz, Boros, and Venetianer 1991). Terminator sequences slow down or stop the transcription process by forming an RNA hairpin structure. Once stopped, the terminator causes the RNA polymerase to dissociate, thus terminating transcription. They can also protect upstream gene mRNA from hydrolysis enhancing upstream transcription (He et al. 2020).

5.2. Transformation and growth of *Synechocystis* cells

Once collected from the DH5α cells, the TrxA:N-Flv:C-pDF plasmids were used to transform *Synechocystis* sp. PCC 6803 cells which had been grown in 20 ml of liquid growth medium BG11 in continuous light (50 µmol photons·m^{-2}·s^{-1}) with air-level CO_2 conditions (LC) at 30 °C until the OD750 of the culture was in the range of 0,7-1,0. The cells were pelleted by centrifugation at 6000 rpm for 8 minutes, and the pellet was

resuspended in 400 µl of BG11. 1 µg of plasmid DNA was then added to 100 µl of cell suspension, and the cells were incubated in darkness overnight. The cells were then grown at 30 °C with shaking at LC conditions.

Synechocystis cells are slower to accept new DNA in comparison to DH5α cells, so transformed cells were plated on selection plates with Sp2,5 and Cm1,75, which is only 1/20 of the final antibiotic concentration. The plates were kept in darkness for one more day in order to allow the cells to recover, while the rate of metabolism is slowed down. This allows the cells more time to start efficiently using the newly acquired plasmids. The plates were then allowed to grow under continuous light for two weeks, after which colonies were picked and grown on BG11 plates with higher concentrations of Sp and Cm. As colonies appeared on the new plates, they were moved into ever increasing concentrations of Sp and Cm, until Sp50 and Cm35 were reached. Colonies were then picked from these plates to be grown in liquid BG11 with Sp and Cm. The *Synechocystis* growth cultures were adjusted to the same cell concentration before the addition of IPTG to begin the expression of the TrxA:N-Flv:C-pDF plasmids. After three days the cells were ready for protein extraction and bimolecular fluorescence complementation (BiFC).

5.3. Protein isolation

Transformed *Synechocystis* cells were grown in BG11 with Sp50 and Cm35 at 30 °C with 3 % CO_2 in continuous light while being constantly shaken. After 3 days the OD750 of each cell culture was adjusted to 0,5 and one day later 1 mM of IPTG was added to them. After 3 days 30 ml of liquid cell culture was collected by centrifugation at 6000 rpm for 8 minutes in 4 °C and the pellet was washed with 2 ml of washing buffer (50 mM Hepes · NaOH, pH 7,5; 30 mM $CaCl_2$). Samples were centrifuged at 6000 rpm for 8 minutes in 4 °C and the supernatant was discarded. For protein extraction the cells were broken with a bead beater by resuspending the pellets in 200 µl of resuspension buffer (50 mM Hepes · NaOH, pH 7,5; 30 mM $CaCl_2$; 800 mM sorbitol; 1 mM ε-amino-n-caproic-acid) and 200 mg of zirconium oxide grain (grain diameter 150 µm) was added and the samples were

blended with bead beater at full speed for 5 minutes. The bead beater vigorously shakes the sample causing the zirconium oxide beads to break the cells by colliding into them at high speeds. Zirconium oxide beads are particularly durable in comparison to other bead materials, such as silica and stainless steel, which makes them more effective at sample homogenization (Gibbons, Brangs, and Burden 2014).

The proteins present in the supernatant were carefully collected by centrifuging at 1500 rpm for 1 minute in 4 °C and the supernatants were collected. 100 µl of resuspension buffer was added to the remaining pellets, which were blended again as before, and the supernatant was once again carefully collected and centrifuged to get rid of as much of the remaining cell debris and zirconium beads as possible. The remaining sample went through another bead beater treatment, and the supernatants were centrifuged at 1500 rpm for 2 minutes in 4 °C and were collected into fresh tubes. The protein extraction and the subsequent protein analysis with was done in very dim lighting to avoid photodamage to the proteins. Protein concentrations were measured with the Lowry method using the Bio-Rad *DC* protein assay kit (ThermoFisher scientific).

The Lowry method for quantification of protein in samples is based on chemical reactions that take place when copper(II) ions react with peptide bonds under alkaline conditions, producing copper(I) ions as a result. Folin-Ciocalteu reagent, a mixture of phosphomolybdate and phosphotungstate, then reacts with copper(I) and produces molybdenum blue, which is then detected with a spectrophotometer. The intensity of the resulting blue color is proportional to the amount of protein present in the sample. (Lowry et al. 1951).

Protein samples were solubilized in sample buffer (40 % Laemmli buffer with 6 M urea; 4,5 % β-mercaptoethanol; 0,00023 % bromophenol blue) at 4 °C overnight. The proteins were then separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Before being loaded on the SDS PAGE gels, the protein samples were denatured with sample buffer containing β-mercaptoethanol, SDS and urea. SDS also provides a negative charge to the proteins, ensuring that they migrate to the same direction on the gels. 10 µg of each protein sample was run on 12 % SDS PAGE with 80 V

for 30 minutes and then with 120 V for 2 hours. The SDS PAGE gels were overlaid on nitrocellulose transfer membranes with blotting paper on top of the gels and below the membranes and transfer buffer (9,75 mM glycine; 12 mM Tris; 0,009375 % SDS; 20 % methanol) was added. Proteins were transferred to the membranes for one hour by using an electric current (mA) equivalent to the surface area of the membranes (cm^2) multiplied by 0,8. Binding the proteins on membranes keeps them in the same locations in comparison to one another, and makes it possible to visualize them with antibodies.

The membranes were then thoroughly washed with TBS (2,124 g/l tribase; 29,22 g/l NaCl; HCl to adjust pH to 7,5), and the membranes were blocked with 5 % milk for one hour in RT with shaking at 60 rpm in order to bind any free binding sites on the membrane, thus preventing nonspecific binding of the antibodies. The membranes were washed with TTBS (0,05 % Tween-20 in TBS) for 5 minutes twice, and they were incubated in the primary antibodies in 1 % milk in 4 °C on a swing shaker. Each of the primary antibodies bind specifically to Flv1, Flv2, Flv3, or α-YFP-N. The antibodies were allowed to bind overnight, after which the membranes were once again washed with TTBS for 5 minutes four times to wash off any unbound antibodies. After the wash secondary antibodies in 1 % milk were added on the membrane, where they bound to the primary antibodies by incubating the membranes in RT for 1 hour with shaking at 60 rpm. The secondary antibodies were anti-mouse α-YFP antibody and anti-rabbit IgG antibody. The anti-mouse α-YFP binds to the α-YFP-N antibody, while anti-rabbit IgG binds to the anti-Flv antibodies. The unbound antibodies were washed off with TTBS for 5 minutes three times and with TBS for 5 minutes twice.

Enhanced chemiluminescence (ECL) reagent was added to the membranes, and they were incubated on a swing shaker for 5 minutes. ECL works as a substrate to the horseradish peroxidase that is attached to the secondary antibody, which produces luminescence as the product of the reaction it catalyzes. This luminescence is used as a signal for determining the amount of bound secondary antibodies, which is proportional to the amount of the protein of interest present on the membranes. The luminescence was

detected with x-ray film with 5 minutes of exposure time, after which the films were developed.

5.4. Bimolecular fluorescence complementation tests

Synechocystis cells were grown and collected the same way as they were for the protein isolation. The cells were collected by centrifugation at 6000 rpm for 8 minutes and resuspended in 1 ml BG11 (20 mM Hepes · NaOH, pH 7,5; 17,6 mM $NaNO_3$; 0,3 mM $MgSO_4 \cdot 7\ H_2O$; 245 µM $CaCl_2 \cdot 2\ H_2O$; 31 µM citric acid; 2,7 µM EDTA-Na_2; 1,75 mM K_2HPO_4; 0,189 mM Na_2CO_3; 6 mg/l ferric ammonium citrate). The cells were detected with a Zeiss LSM880 confocal microscope while being excited with 488 nm and 543 nm light.

Protein complementation assay is a method used for investigating interactions between proteins by fusing truncated reporter proteins to the proteins of interest. Once in close proximity to one another, the truncated reporter proteins are able to fold and assemble properly and form functioning reporter proteins (Miller et al. 2015). BiFC is a type of protein complementation assay that utilizes a fluorescent protein as a reporter protein. BiFC is a method of probing interactions between proteins by covalently attaching one half of a fluorescent protein to the protein of interest, and then attach the second half to another protein. If the proteins of interest ever come close enough to each other for it to be considered interaction, the two halves of the fluorescent protein also come together and form a complete protein (Kodama and Hu 2010). The Venus protein is a YFP that is more robust against environmental changes than other fluorescent proteins. In this study, I152L mutant of the Venus protein VN155/VC155 was used due to its higher signal-to-noise ratio and lower chance of self-assembly in comparison to other fluorescent proteins (Kodama and Hu 2010).

Use of BiFC allows direct visualization of protein-protein interactions in living cells, while also revealing the subcellular location in which the interaction takes place. The signal emitted by the fluorescent proteins can be detected with fluorescent microscopy or flow cytometry without the need for additional treatments to the cells. BiFC provides a

sensitive protein-protein interaction assay with little background signal due to the specificity of the fluorescent protein interaction that produces the fluorescence signal. However, the interaction between truncated fluorescent proteins can occur even without the direct interaction between the proteins they are attached to, leading to false-positive results. Due to the irreversible nature of the completion of a functional fluorescent protein, the subcellular location where the signal is detected is not necessarily the location where the initial interaction took place. The irreversibility can however, allow the detection of fast protein-protein interactions that would otherwise be difficult to detect. Since BiFC assays are easy and inexpensive to carry out, they can be used in a larger scale for screening multiple protein-protein interactions in parallel. (Miller et al. 2015).

6. Results

6.1. Accumulation of BiFC-fusion proteins in *Synechocystis* cells

Figure 3A shows the proteins when exposed to a primary antibody that binds to YFP. Each tested strain had YFP present, however the sizes of the recognized protein complexes varied. TrxA:N-Flv1:C had the YFP in a protein that was approximately 35 kDa, while TrxA:N-Flv2:C had complexes that were roughly twice the size of that. It is possible that the TrxA:N-Flv2:C complexes had formed dimers without hindering the bonding of the anti-YFP antibody. One of the tested TrxA:N-Flv2:C strains (TrxA:N-Flv2:C 2.) showed two more distinct bands than the other (TrxA:N-Flv2:C 1.), with the largest protein complex being approximately 95 kDa in size and the smallest band being 55 kDa. This could also be due to the formation of oligomers. TrxA:N-Flv3:C showed many bands of proteins ranging in size from 34 kDa to 150 kDa. These bands were a rough match for the sizes of the monomer, homodimer, -trimer, and -tetramer of Flv3. Figure 3B shows the proteins that were exposed to antibodies that bind to each of the different FDPs used. TrxA:N-Flv1:C produced two bands at the sizes of roughly 25 kDa and 75 kDa. Figure 3C shows a different membrane with TrxA:N-Flv1:C detected with an anti-Flv1 antibody, where the Flv1:C with

or without YFP show up as 75 kDa and 55 kDa bands, respectively. TrxA:N-Flv2:C produced a barely visible band of protein with the size of 75 kDa. The poor visibility of the band could be due to Flv2 proteins forming oligomers and hindering the ability of the anti-Flv2 antibody to bind to its proper binding site. The band was most likely formed by Flv2:C with YFP attached. TrxA:N-Flv3:C once again produced multiple bands ranging in size from 20 kDa to 150 kDa. The bands at roughly 150 kDa and 110 kDa are most likely Flv3:C dimers with and without YFP, respectively.

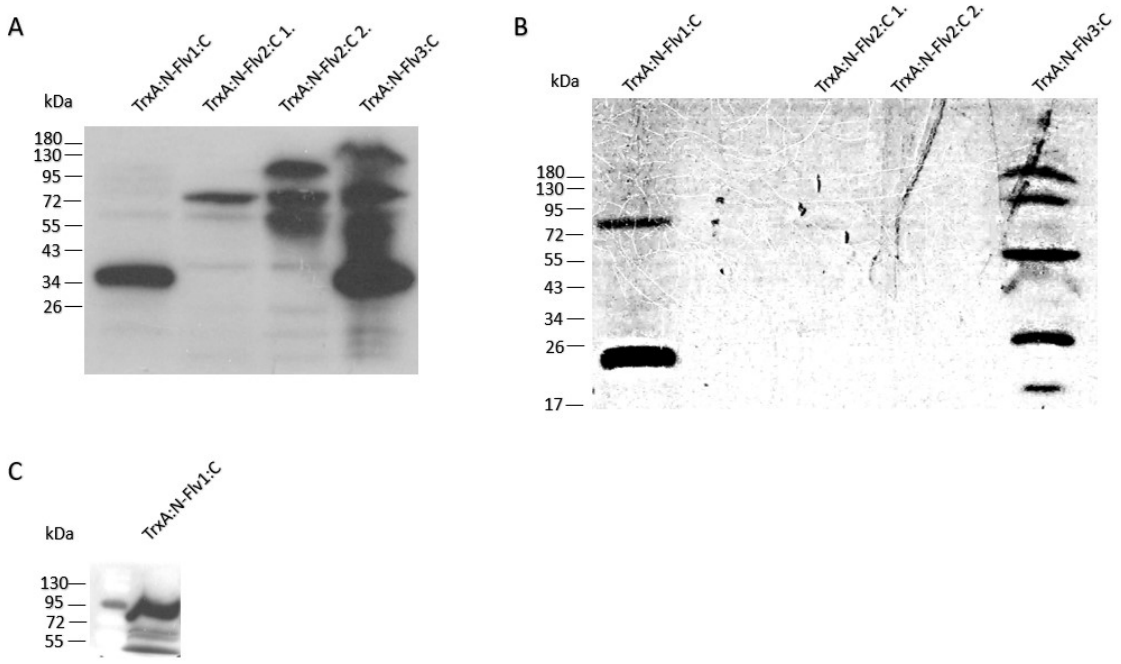

Figure 1. Immunodetection of BiFC-fusion proteins extracted from Synechocystis sp. PCC 6803 cells transformed with TrxA:N-Flv1:C-, TrxA:N-Flv2:C-, and TrxA:N-Flv3:C plasmids. The exposure time on film was 5 minutes. A) Proteins detected with an antibody that binds to the N-terminal fragment of YFP. B) Proteins detected with antibodies that bind to Flv1, Flv2, and Flv3 specifically. C) Picture of another membrane showing TrxA:N-Flv1:C detected with an anti-Flv1 antibody.

6.2. Bimolecular fluorescence complementation tests

Cells from the TrxA:N-Flv1:C, -Flv2:C, and -Flv3:C strains were examined with a confocal microscope to determine whether or not the YFP molecules were emitting light. Figure 4 shows the cells fluorescing when excited with different wavelengths of light. Column 1 of Figure 4 shows cells when exposed to 543 nm light that causes chlorophyll to fluoresce in the thylakoid membrane, thus showing all cells present. Column 2 shows cells exposed to 488 nm light that causes YFP to fluoresce if the studied proteins interact with each other. The emission was detected in the range of 518 nm to 621 nm. Column 1+2 shows columns 1 and 2 overlaid on each other. TrxA:N-Flv1:C strain shows that TrxA and Flv1 probably do interact with each other, and the same goes for one of the TrxA:N-Flv2:C strains (TrxA:N-Flv2:C 2.). The other TrxA:N-Flv2:C strain (TrxA:N-Flv2:C 1.) shows no YFP fluorescence. This leaves the results for TrxA:N-Flv2:C unclear, however since one of the TrxA:N-Flv2:C strains shows strong fluorescence from YFP it most likely means that TrxA does interact with Flv2. TrxA:N-Flv3:C shows barely any YFP fluorescence, meaning that TrxA probably does not interact with Flv3.

Because chlorophyll is situated in the thylakoid membrane, its fluorescence forms a ring along the inner edges of the cell leaving the cytosol as a dark center. The fluorescence of YFP is yellow, causing it to make the overall color of the overlaid pictures of the cells to be orange. The cells without YFP are shown as red. In mutant lines TrxA:n-Flv1:C and TrxA:n-Flv2:C 2. the inner edge of the cells appear orange, while the cytosol in the center remains dark. Based on this it is safe to assume that the interaction between TrxA and Flv1 or Flv2 takes place in the thylakoid membrane.

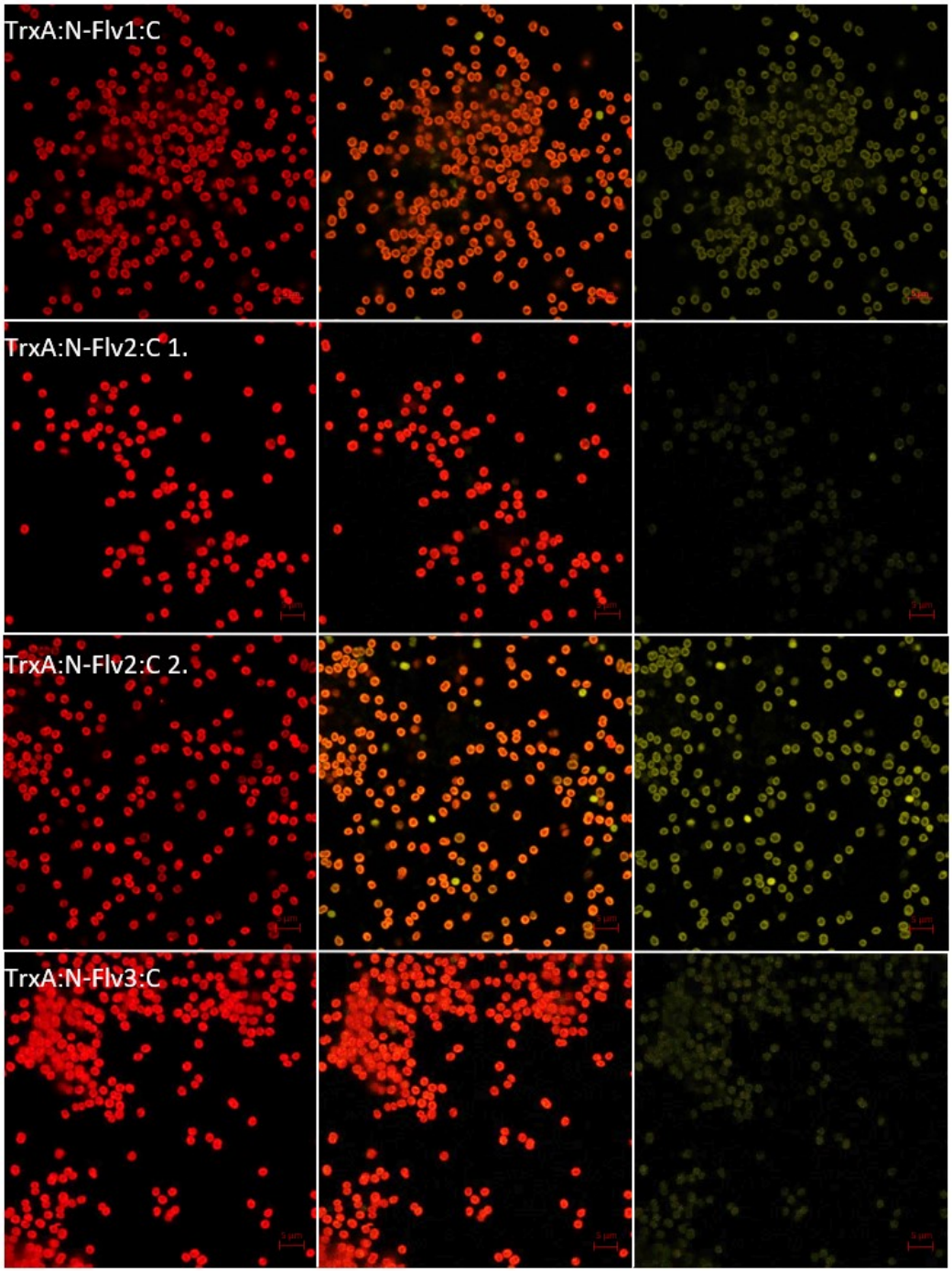
TrxA:N-Flv1:C
TrxA:N-Flv2:C 1.
TrxA:N-Flv2:C 2.
TrxA:N-Flv3:C
1
1+2
2

Figure 2. Results of BiFC with Synechocystis sp. PCC 6803 cells transformed with TrxA:N-Flv1:C-, TrxA:N-Flv2:C-, and TrxA:N-Flv3:C plasmids. Column 1 shows cells excited with 543 nm light that causes chlorophyll to fluoresce, and column 2 shows cells excited wit with 543 nm light that causes chlorophyll to fluoresce, and column 2 shows cells excited with 488 nm light that causes YFP to fluoresce. Column 1+2 shows columns 1 and 2 layered on top of each other.

7. Discussion

Cyanobacteria are photosynthetic bacteria that are the source of the chloroplasts found in plants today. Since they use light energy as the major energy source for their metabolism, they also need methods to protect themselves against excess light. One of these methods is the Mehler-like reaction, in which oxygen is converted into water in a reaction catalyzed by FDPs. One theory states that FDPs could be regulated by the Trx system, since FDPs have been found to contain several conserved cys residues (Guo et al. 2014). The conserved cys residues are Cys-185, Cys-207, Cys-393, and Cys-532 in Flv1 (uniprot.org), and in *Synechocystis* they are conserved in Flv1-4. These residues were also conserved in other cyanobacteria, such as *Nostoc* and *Thermosynechococcus vestitus*. In a folded protein these residues are generally in the same area along the surface of the protein, which makes them easy to access for possible interaction with TrxA.

Three strains of *Synechocystis* sp. PCC 6803 were created to produce TrxA with Flv1, Flv2, or Flv3 and each of these fusion proteins had one half of a Venus YFP attached to it. These strains were used to determine whether TrxA interacts with any of these three FDPs by measuring the fluorescence emitted by chlorophyll and the Venus YFP fragments with confocal microscopy from living cells. TrxA was found to interact with Flv1 and Flv2, but not with Flv3. These interactions were found to take place in the thylakoid membrane.

These results are partially consistent with past research demonstrating light-dependent thiol oxidation of Flv1 and Flv3 (Guo et al. 2014). Guo's study shows no significant light-dependent thiol oxidation of Flv2 at ambient CO_2 levels, which contradicts the results of the BiFC tests. This could be due to the role of Flv2 as a constant electron sink, rather than a temporary electron sink induced by the growth of light intensity. It is still possible that Flv2 is regulated by TrxA independent of the light conditions. Another possibility is that due to the similarities between Flv1 and Flv2, overproduction of Flv2 lead to interaction with TrxA even if they normally would not interact with each other. This could partially explain the different results acquired from the two TrxA:N-Flv2:C strains. The failure of Flv3 to interact with TrxA is interesting, because it has a similar function to Flv1, which is to work as a transient electron sink. It could be that when Flv3 forms a heterooligomer with Flv1 there is no need to regulate the activity of the oligomer with light-dependent thiol oxidation through all involved protein subunits. It is possible that regulation through Flv1 alone is enough to regulate the activity of the Flv1/Flv3 heterooligomer. Flv1 has been found to form functional oligomers only with Flv3, while Flv3 is able to form functional homodimers and homotetramers (Mustila et al. 2016). Perhaps the role of Flv1 in these heterooligomers is to function as a target for TrxA-regulation for the oligomer, since Flv3 by itself does not interact with TrxA.

In order to verify the results, the experiments should be done again with more strain replicates along with positive and negative controls to reduce ambiguity of interpretation.

8. References

Alboresi, Alessandro, Mattia Storti, Laura Cendron, and Tomas Morosinotto. 2019. "Role and Regulation of Class-C Flavodiiron Proteins in Photosynthetic Organisms." *Biochemical Journal* 476(17):2487–98.

Allahverdiyeva, Yagut, Maria Ermakova, Marion Eisenhut, Pengpeng Zhang, Pierre Richaud, Martin Hagemann, Laurent Cournac, and Eva Mari Aro. 2011. "Interplay between

Flavodiiron Proteins and Photorespiration in Synechocystis Sp. PCC 6803." *Journal of Biological Chemistry* 286(27):24007–14. doi: 10.1074/jbc.M111.223289.

Allahverdiyeva, Yagut, Henna Mustila, Maria Ermakova, Luca Bersanini, Pierre Richaud, Ghada Ajlani, Natalia Battchikova, Laurent Cournac, and Eva Mari Aro. 2013. "Flavodiiron Proteins Flv1 and Flv3 Enable Cyanobacterial Growth and Photosynthesis under Fluctuating Light." *Proceedings of the National Academy of Sciences of the United States of America* 110(10):4111–16. doi: 10.1073/pnas.1221194110.

Angeleri, Martina, Dorota Muth-Pawlak, Eva Mari Aro, and Natalia Battchikova. 2016. "Study of O-Phosphorylation Sites in Proteins Involved in Photosynthesis-Related Processes in Synechocystis Sp. Strain PCC 6803: Application of the SRM Approach." *Journal of Proteome Research* 15(12):4638–52. doi: 10.1021/acs.jproteome.6b00732.

Asada, Kozi, Kuniaki Kiso, and Kyoko Yoshikawa. 1974. *Univalent Reduction of Molecular Oxygen by Spinach Chloroplasts on Illumination**. Vol. 249.

Bhattacharya, Debashish, and Linda Medlin. 1995. "The Phylogeny of Plastids: A Review Based on Comparisons of Small-Subunit Ribosomal RNA Coding Regions." *J. Physiol* 31:489–98.

Bilgin, N., A. A. Richter, M. Ehrenberg, A. E. Dahlberg, and C. G. Kurland. 1990. "Ribosomal RNA and Protein Mutants Resistant to Spectinomycin." *EMBO Journal* 9(3):735–39. doi: 10.1002/j.1460-2075.1990.tb08167.x.

Borges, Patrícia T., Célia v. Romão, Lígia M. Saraiva, Vera L. Gonçalves, Maria A. Carrondo, Miguel Teixeira, and Carlos Frazão. 2019. "Analysis of a New Flavodiiron Core Structural Arrangement in Flv1-ΔFlR Protein from Synechocystis Sp. PCC6803." *Journal of Structural Biology* 205(1):91–102. doi: 10.1016/j.jsb.2018.11.004.

Chatterjee, Sujoy, Arnab Basu, Abhijit Basu, and Sujoy K. das Gupta. 2007. "DNA Bending in the Mycobacterial Plasmid PAL5000 Origin-RepB Complex." *Journal of Bacteriology* 189(23):8584–92. doi: 10.1128/JB.01155-07.

Cramer, William A., S. Saif Hasan, and Eiki Yamashita. 2011. "The Q Cycle of Cytochrome Bc Complexes: A Structure Perspective." *Biochimica et Biophysica Acta - Bioenergetics* 1807(7):788–802.

Davies, Julian, and Gerard D. Wright. 1997. "Bacterial Resistance to Aminoglycoside Antibiotics." *Trends in Microbiology* 5(6):234–40. doi: 10.1016/S0966-842X(97)01033-0.

Edoo, Zainab, Michel Arthur, and Jean Emmanuel Hugonnet. 2017. "Reversible Inactivation of a Peptidoglycan Transpeptidase by a β-Lactam Antibiotic Mediated by β-Lactam-Ring Recyclization in the Enzyme Active Site." *Scientific Reports* 7(1). doi: 10.1038/s41598-017-09341-8.

Ermakova, Maria, Tuomas Huokko, Pierre Richaud, Luca Bersanini, Christopher J. Howe, David J. Lea-Smith, Gilles Peltier, and Yagut Allahverdiyeva. 2016. "Distinguishing the Roles of Thylakoid Respiratory Terminal Oxidases in the Cyanobacterium Synechocystis Sp. PCC 6803." *Plant Physiology* 171(2):1307–19. doi: 10.1104/pp.16.00479.

Florencio, Francisco J., María Esther Pérez-Pérez, Luis López-Maury, Alejandro Mata-Cabana, and Marika Lindahl. 2006. “The Diversity and Complexity of the Cyanobacterial Thioredoxin Systems.” *Photosynthesis Research* 89(2):157–71.

Gerotto, Caterina, Alessandro Alboresi, Andrea Meneghesso, Martina Jokel, Marjaana Suorsa, Eva Mari Aro, and Tomas Morosinotto. 2016. “Flavodiiron Proteins Act as Safety Valve for Electrons in Physcomitrella Patens.” *Proceedings of the National Academy of Sciences of the United States of America* 113(43):12322–27. doi: 10.1073/pnas.1606685113.

Gibbons, Lindsay E., Halley C. G. Brangs, and David W. Burden. 2014. *Bead Beating: A Primer*.

Gisriel, Christopher J., Jimin Wang, Jinchan Liu, David A. Flesher, Krystle M. Reiss, Hao-Li Huang, Ke R. Yang, William H. Armstrong, M. R. Gunner, Victor S. Batista, Richard J. Debus, and Gary W. Brudvig. 2022. “High-Resolution Cryo-Electron Microscopy Structure of Photosystem II from the Mesophilic Cyanobacterium, Synechocystis Sp. PCC 6803.” *Proceedings of the National Academy of Sciences of the United States of America* 119(1). doi: 10.1073/pnas.2116765118/-/DCSupplemental.

Green, Beverley R. 2011. “Chloroplast Genomes of Photosynthetic Eukaryotes.” *Plant Journal* 66(1):34–44. doi: 10.1111/j.1365-313X.2011.04541.x.

Guo, Jia, Amelia Y. Nguyen, Ziyu Dai, Dian Su, Matthew J. Gaffrey, Ronald J. Moore, Jon M. Jacobs, Matthew E. Monroe, Richard D. Smith, David W. Koppenaal, Himadri B. Pakrasi, and Wei Jun Qian. 2014. “Proteome-Wide Light/Dark Modulation of Thiol Oxidation in Cyanobacteria Revealed by Quantitative Site-Specific Redox Proteomics.” *Molecular and Cellular Proteomics* 13(12):3270–85. doi: 10.1074/mcp.M114.041160.

He, Zhiyun, Yanting Duan, Weiji Zhai, Xiaomei Zhang, Jinsong Shi, Xiaojuan Zhang, and Zhenghong Xu. 2020. “Evaluating Terminator Strength Based on Differentiating Effects on Transcription and Translation.” *ChemBioChem* 21(14):2067–72. doi: 10.1002/cbic.202000068.

Hedges, S. Blair, Hsiong Chen, Sudhir Kumar, Daniel Y. C. Wang, Amanda S. Thompson, and Hidemi Watanabe. 2001. *A Genomic Timescale for the Origin of Eukaryotes*.

Heinz, Steffen, Pasqual Liauw, Jörg Nickelsen, and Marc Nowaczyk. 2016. “Analysis of Photosystem II Biogenesis in Cyanobacteria.” *Biochimica et Biophysica Acta - Bioenergetics* 1857(3):274–87. doi: 10.1016/j.bbabio.2015.11.007.

Hippler, Michael, and Nathan Nelson. 2021. “The Plasticity of Photosystem I.” *Plant and Cell Physiology* 62(7):1073–81.

Hohmann-Marriott, Martin F., and Robert E. Blankenship. 2011. “Evolution of Photosynthesis.” *Annual Review of Plant Biology* 62:515–48. doi: 10.1146/annurev-arplant-042110-103811.

Hu, Qiang, Milton Sommerfeld, Eric Jarvis, Maria Ghirardi, Matthew Posewitz, Michael Seibert, and Al Darzins. 2008. “Microalgal Triacylglycerols as Feedstocks for Biofuel Production: Perspectives and Advances.” *Plant Journal* 54(4):621–39.

Huang, Wei, Shi Bao Zhang, and Kun Fang Cao. 2010. “The Different Effects of Chilling Stress under Moderate Light Intensity on Photosystem II Compared with Photosystem I and Subsequent Recovery in Tropical Tree Species.” *Photosynthesis Research* 103(3):175–82. doi: 10.1007/s11120-010-9539-7.

Junge, Wolfgang, and Nathan Nelson. 2015. “ATP Synthase.” *Annual Review of Biochemistry* 84:631–57.

Kaneko, Takakazu, Shusei Sato, Hirokazu Kotani, Ayako Tanaka, Erika Asamizu, Yasukazu Nakamura, Nobuyuki Miyajima, Makoto Hirosawa, Masahiro Sugiura, Shigemi Sasamoto, Takaharu Kimura, Tsutomu Hosouchi, Ai Matsuno, Akiko Muraki, Naomi Nakazaki, Kaoru Naruo, Satomi Okumura, Sayaka Shimpo, Chie Takeuchi, Tsuyuko Wada, Akiko Watanabe, Manabu Yamada, Miho Yasuda, and Satoshi Tabata. 1996. *Sequence Analysis of the Genome of the Unicellular Cyanobacterium Synechocystis Sp. Strain PCC6803. II. Sequence Determination of the Entire Genome and Assignment of Potential Protein-Coding Regions*. Vol. 3.

Khorobrykh, S. A., and B. N. Ivanov. 2002. *Oxygen Reduction in a Plastoquinone Pool of Isolated Pea Thylakoids*. Vol. 71.

Knaff, David B., and Masakazu Hirasawa. 1991. *Ferredoxin-Dependent Chloroplast Enzymes*. Vol. 1056.

Kodama, Yutaka, and Chang Deng Hu. 2010. “An Improved Bimolecular Fluorescence Complementation Assay with a High Signal-to-Noise Ratio.” *BioTechniques* 49(5):793–803. doi: 10.2144/000113519.

Kozuleva, Marina A., and Boris N. Ivanov. 2010. “Evaluation of the Participation of Ferredoxin in Oxygen Reduction in the Photosynthetic Electron Transport Chain of Isolated Pea Thylakoids.” *Photosynthesis Research* 105(1):51–61. doi: 10.1007/s11120-010-9565-5.

Kramer, David M., and John R. Evans. 2011. “The Importance of Energy Balance in Improving Photosynthetic Productivity.” *Plant Physiology* 155(1):70–78. doi: 10.1104/pp.110.166652.

Kudoh, Hideki, and Kintake Sonoike. 2002. “Irreversible Damage to Photosystem I by Chilling in the Light: Cause of the Degradation of Chlorophyll after Returning to Normal Growth Temperature.” *Planta* 215(4):541–48. doi: 10.1007/s00425-002-0790-9.

Kulasooriya, S. A., and D. N. Magana-Arachchi. 2016. “Nitrogen Fixing Cyanobacteria: Their Diversity, Ecology and Utilization with Special Reference to Rice Cultivation.” *Journal of the National Science Foundation of Sri Lanka* 44(2):111–28.

Liberton, Michelle, William B. Chrisler, Carrie D. Nicora, Ronald J. Moore, Richard D. Smith, David W. Koppenaal, Himadri B. Pakrasi, and Jon M. Jacobs. 2017. “Phycobilisome Truncation Causes Widespread Proteome Changes in Synechocystis Sp. PCC 6803.” *PLoS ONE* 12(3). doi: 10.1371/journal.pone.0173251.

Lima-Melo, Yugo, Mehmet Kılıç, Eva Mari Aro, and Peter J. Gollan. 2021. “Photosystem I Inhibition, Protection and Signalling: Knowns and Unknowns.” *Frontiers in Plant Science* 12.

Livermore, David M. 1995. *Lactamases in Laboratory and Clinical Resistance*. Vol. 8.

Lowry, Oliver H., Nira J. Rosebrough, A. Lewis Farr, and Rose J. Randall. 1951. *PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT**. Missouri.

Malavath, Tirupathi, Ido Caspy, Sigal Y. Netzer-El, Daniel Klaiman, and Nathan Nelson. 2018. "Structure and Function of Wild-Type and Subunit-Depleted Photosystem I in Synechocystis." *Biochimica et Biophysica Acta - Bioenergetics* 1859(9):645–54. doi: 10.1016/j.bbabio.2018.02.002.

Mallén-Ponce, Manuel J., María José Huertas, and Francisco J. Florencio. 2022. "Exploring the Diversity of the Thioredoxin Systems in Cyanobacteria." *Antioxidants* 11(4):654. doi: 10.3390/antiox11040654.

Mallén-Ponce, Manuel J., María José Huertas, Ana María Sánchez-Riego, and Francisco J. Florencio. 2021. " Depletion of m -Type Thioredoxin Impairs Photosynthesis, Carbon Fixation, and Oxidative Stress in Cyanobacteria ." *Plant Physiology*. doi: 10.1093/plphys/kiab321.

Martins, Rosário F., Miguel F. Ramos, Lars Herfindal, José A. Sousa, Kaja Skaerven, and Vitor M. Vasconcelos. 2008. *Antimicrobial and Cytotoxic Assessment of Marine Cyanobacteria - Synechocystis and Synechococcus*. Vol. 6.

Mehler, Alan H. 1951. *Studies on Reactions of Illuminated Chloroplasts. I. Mechanism of the Reduction of Oxygen and Other Hill Reagents*. Chicago.

Meyer, Richard J. 1999. *Stabilization of the Relaxosome and Stimulation of Conjugal Transfer Are Genetically Distinct Functions of the R1162 Protein MobB*. Vol. 181.

Miller, Kristi E., Yeonsoo Kim, Won Ki Huh, and Hay Oak Park. 2015. "Bimolecular Fluorescence Complementation (BiFC) Analysis: Advances and Recent Applications for Genome-Wide Interaction Studies." *Journal of Molecular Biology* 427(11):2039–55.

Miller, Neil T., Michael D. Vaughn, and Robert L. Burnap. 2021. "Electron Flow through NDH-1 Complexes Is the Major Driver of Cyclic Electron Flow-Dependent Proton Pumping in Cyanobacteria." *Biochimica et Biophysica Acta - Bioenergetics* 1862(3). doi: 10.1016/j.bbabio.2020.148354.

Mills, Lauren A., Alistair J. McCormick, and David J. Lea-Smith. 2020. "Current Knowledge and Recent Advances in Understanding Metabolism of the Model Cyanobacterium Synechocystis Sp. PCC 6803." *Bioscience Reports* 40(4).

Mishra, Arun Kumar, Manish Singh Kaushik, and D. N. Tiwari. 2018. "Nitrogenase and Hydrogenase: Enzymes for Nitrogen Fixation and Hydrogen Production in Cyanobacteria." Pp. 173–91 in *Cyanobacteria: From Basic Science to Applications*. Elsevier.

Mondal, J., and B. D. Bruce. 2018. "Ferredoxin: The Central Hub Connecting Photosystem I to Cellular Metabolism." *Photosynthetica* 56(1):279–93.

Morone, Janaína, Graciliana Lopes, Marco Preto, Vítor Vasconcelos, and Rosário Martins. 2020. "Exploitation of Filamentous and Picoplanktonic Cyanobacteria for Cosmetic Applications:

Potential to Improve Skin Structure and Preserve Dermal Matrix Components." *Marine Drugs* 18(9). doi: 10.3390/MD18090486.

Mustila, Henna, Pasi Paananen, Natalia Battchikova, Anita Santana-Sánchez, Dorota Muth-Pawlak, Martin Hagemann, Eva Mari Aro, and Yagut Allahverdiyeva. 2016. "The Flavodiiron Protein Flv3 Functions as a Homo-Oligomer during Stress Acclimation and Is Distinct from the Flv1/Flv3 Hetero-Oligomer Specific to the O2 Photoreduction Pathway." *Plant and Cell Physiology* 57(7):1468–83. doi: 10.1093/pcp/pcw047.

Nakagawa, Yoji, Yoshiyuki Nitahara, and Sadao Miyamura. 1979. *Kinetic Studies on Enzymatic Acetylation of Chloramphenicol in Streptococcus Faecalis*. Vol. 16.

Netzer-El, Sigal Yoli, Ido Caspy, and Nathan Nelson. 2019. "Crystal Structure of Photosystem I Monomer from Synechocystis Sp. PCC 6803." *Frontiers in Plant Science* 9. doi: 10.3389/fpls.2018.01865.

Nikkanen, Lauri, Anita Santana Sánchez, Maria Ermakova, Matthias Rögner, Laurent Cournac, and Yagut Allahverdiyeva. 2020. "Functional Redundancy between Flavodiiron Proteins and NDH-1 in Synechocystis Sp. PCC 6803." *Plant Journal* 103(4):1460–76. doi: 10.1111/tpj.14812.

Nikkanen, Lauri, Daniel Solymosi, Martina Jokel, and Yagut Allahverdiyeva. 2021. "Regulatory Electron Transport Pathways of Photosynthesis in Cyanobacteria and Microalgae: Recent Advances and Biotechnological Prospects." *Physiologia Plantarum* 173(2):514–25. doi: 10.1111/ppl.13404.

Oliver, Neal J., Christine A. Rabinovitch-Deere, Austin L. Carroll, Nicole E. Nozzi, Anna E. Case, and Shota Atsumi. 2016. "Cyanobacterial Metabolic Engineering for Biofuel and Chemical Production." *Current Opinion in Chemical Biology* 35:43–50.

Orosz, Andras, Imre Boros, and Pal Venetianer. 1991. *Analysis of the Complex Transcription Termination Region of the Escherichia Coli RrnB Gene*. Vol. 201.

Pérez-Pérez, M. Esther, Eugenio Martín-Figueroa, and Francisco J. Florencio. 2009. "Photosynthetic Regulation of the Cyanobacterium Synechocystis Sp. PCC 6803 Thioredoxin System and Functional Analysis of TrxB (Trx x) and TrxQ (Trx y) Thioredoxins." *Molecular Plant* 2(2):270–83. doi: 10.1093/mp/ssn070.

Rasooly, Avraham. 1997. "Double-Stranded Origin Nicking and Replication Initiation Are Coupled in the Replication of a Rolling Circle Plasmid, PT181." *FEMS Microbiology Letters* 151(2):185–89. doi: 10.1111/j.1574-6968.1997.tb12568.x.

Rozhon, Wilfried. 2017. "The Replication Protein of PHW126 Auto-Controls Its Expression." *Plasmid* 90:38–43. doi: 10.1016/j.plasmid.2017.03.003.

Rozhon, Wilfried, Mamoona Khan, Elena Petutschnig, and Brigitte Poppenberger. 2011. "Identification of Cis- and Trans-Acting Elements in PHW126, a Representative of a Novel Group of Rolling Circle Plasmids." *Plasmid* 65(1):70–76. doi: 10.1016/j.plasmid.2010.09.002.

Sánchez-Baracaldo, Patricia. 2015. “Origin of Marine Planktonic Cyanobacteria.” *Scientific Reports* 5. doi: 10.1038/srep17418.

Santana-Sanchez, Anita, Daniel Solymosi, Henna Mustila, Luca Bersanini, Eva-Mari Aro, and Yagut Allahverdiyeva. 2019. “Flavodiiron Proteins 1-to-4 Function in Versatile Combinations in O 2 Photoreduction in Cyanobacteria.” doi: 10.7554/eLife.45766.001.

Sétif, Pierre, Ginga Shimakawa, Anja Krieger-Liszkay, and Chikahiro Miyake. 2020. “Identification of the Electron Donor to Flavodiiron Proteins in Synechocystis Sp. PCC 6803 by in Vivo Spectroscopy.” *Biochimica et Biophysica Acta - Bioenergetics* 1861(10). doi: 10.1016/j.bbabio.2020.148256.

Shen, Jian Ren. 2015. “The Structure of Photosystem II and the Mechanism of Water Oxidation in Photosynthesis.” *Annual Review of Plant Biology* 66:23–48. doi: 10.1146/annurev-arplant-050312-120129.

Singh, Niraj Kumar, Ravi Raghav Sonani, Rajesh Prasad Rastogi, and Datta Madamwar. 2015. “The Phycobilisomes: An Early Requisite for Efficient Photosynthesis in Cyanobacteria.” *EXCLI Journal* 14:268–89. doi: 10.17179/excli2014-723.

Smetana, Sergiy, Michael Sandmann, Sascha Rohn, Daniel Pleissner, and Volker Heinz. 2017. “Autotrophic and Heterotrophic Microalgae and Cyanobacteria Cultivation for Food and Feed: Life Cycle Assessment.” *Bioresource Technology* 245:162–70. doi: 10.1016/j.biortech.2017.08.113.

Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire. 1971. *Purification and Properties of Unicellular Blue-Green Algae (Order Chroococcales)*.

Takagi, Daisuke, Shigeo Takumi, Masaki Hashiguchi, Takehiro Sejima, and Chikahiro Miyake. 2016. “Superoxide and Singlet Oxygen Produced within the Thylakoid Membranes Both Cause Photosystem I Photoinhibition.” *Plant Physiology* 171(3):1626–34. doi: 10.1104/pp.16.00246.

Tamagnini, Paula, Rikard Axelsson, Pia Lindberg, Fredrik Oxelfelt, Röbbe Wünschiers, and Peter Lindblad. 2002. “Hydrogenases and Hydrogen Metabolism of Cyanobacteria.” *Microbiology and Molecular Biology Reviews* 66(1):1–20. doi: 10.1128/mmbr.66.1.1-20.2002.

Thiel, Kati, Edita Mulaku, Hariharan Dandapani, Csaba Nagy, Eva Mari Aro, and Pauli Kallio. 2018. “Translation Efficiency of Heterologous Proteins Is Significantly Affected by the Genetic Context of RBS Sequences in Engineered Cyanobacterium Synechocystis Sp. PCC 6803.” *Microbial Cell Factories* 17(1). doi: 10.1186/s12934-018-0882-2.

Touloupakis, Eleftherios, George Rontogiannis, Ana Margarita Silva Benavides, Bernardo Cicchi, Demetrios F. Ghanotakis, and Giuseppe Torzillo. 2016. “Hydrogen Production by Immobilized Synechocystis Sp. PCC 6803.” *International Journal of Hydrogen Energy* 41(34):15181–86. doi: 10.1016/j.ijhydene.2016.07.075.

Uma, V. S., Zeba Usmani, Minaxi Sharma, Deepti Diwan, Monika Sharma, Miao Guo, Maria G. Tuohy, Charalampos Makatsoris, Xiaobin Zhao, Vijay Kumar Thakur, and Vijai Kumar Gupta.

2022. “Valorisation of Algal Biomass to Value-Added Metabolites: Emerging Trends and Opportunities.” *Phytochemistry Reviews*.

Weisberger, Austin S. 1967. *Inhibition of Protein Synthesis by Chloramphenicol*.

Xia, Shuangluo, and Jon D. Robertus. 2009. “Effect of Divalent Ions on the Minimal Relaxase Domain of MobA.” *Archives of Biochemistry and Biophysics* 488(1):42–47. doi: 10.1016/j.abb.2009.06.004.

Zhao, Ming, Xin Yi Tao, Feng Qing Wang, Yu Hong Ren, and Dong Zhi Wei. 2018. “Establishment of a Low-Dosage-IPTG Inducible Expression System Construction Method in Escherichia Coli.” *Journal of Basic Microbiology* 58(9):806–10. doi: 10.1002/jobm.201800160.