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# Development of robust cyanobacterial strains for biotechnological applications

*Stress tolerance and cell-specific expression in heterocyst-forming cyanobacteria*

XIN LI

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**Abstract**
Li, X. 2018. Development of robust cyanobacterial strains for biotechnological applications. Stress tolerance and cell-specific expression in heterocyst-forming cyanobacteria. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1641. 80 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0259-1.

Synthetic biology tools can be used to exploit potential applications of cyanobacteria, promising photosynthetic hosts for production of fuels and chemicals. Specific genetic tools are needed for the development of robust cyanobacterial strains for bioengineering. The key work presented in this thesis is a characterization and design of bioengineering tools for the heterocyst-forming cyanobacterium *Nostoc punctiforme* strain ATCC 29133. This multicellular cyanobacterium may express several oxidative stress-managing systems, including five Dps proteins. Two of these Dps proteins, NpDps2 and NpDps5, are involved in the tolerance against oxidative stress that induced by $H_2O_2$ or high light intensities. The capacity of NpDps2 and NpDps5 to further enhance oxidative stress tolerance, was confirmed by homologous overexpression and a constitutive strong promoter in *N. punctiforme*. The results show the potential of Dps proteins as tools to create robust cyanobacterial cells with improved stress tolerance. This work also establishes a Dps-mediated link among light tolerance, $H_2O_2$ scavenging, and iron homeostasis, and provides evidence on the non-redundant role of multiple Dps in multicellular cyanobacteria. To address the lack of well-defined promoters in cyanobacteria, a minimal synthetic promoter, SynDIF, was designed for heterocyst-specific expression. Promoters with 5'TCCGGA, the DIF motif, at the -35 region, have been identified to give heterocyst-specific transcription. To identify promoter elements, critical for cell-specificity, DIF promoter sequences from *Anabaena* PCC 7120 were used in a consensus sequence approach. The importance of the consensus regions for cell-specificity was investigated with promoter-e*yfp* reporters. This result provides new insights to the details of DIF promoters, which suggest that the DIF-motif, only together with the consensus or a native DIF promoter -10 region, are sufficient for heterocyst-specificity. Therefore, the DIF-35 region was (i) not independent of other promoter elements, and (ii) not sufficient for heterocyst-specific expression. Besides, the strength of the synthetic promoter was improved by including the upstream element from the native heterocyst specific promoter $P_{NsiR1}$. Moreover, the SynDIF promoter is the shortest promoter ever reported to provide heterocyst-specific expression, indicating the potential of introducing this promoter in future biotechnological applications.

*Keywords:* Cyanobacteria, Dps protein, heterocysts, Nostoc, oxidative stress, cell specific promoter, ROS tolerance, synthetic biology tools transcriptional regulation.

*Xin Li, Department of Chemistry - Ångström, Molecular Biomimetics, Box 523, Uppsala University, SE-75120 Uppsala, Sweden.*



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*To my dear parents,*
*To my unaccomplished dreams,*
*To the new life and to my past.*

# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Moparthi, V. K., Li, X., Vavitsas, K., Dzhygyr, I., Sandh, G., Magnuson, A., and Stensjö, K. (2016). The two Dps proteins, NpDps2 and NpDps5, are involved in light-induced oxidative stress tolerance in the $N_2$-fixing cyanobacterium *Nostoc punctiforme*. *Biochimica et Biophysica Acta - Bioenergetics*, 1857(11), 1766–1776.

II Li, X., Magnuson, A., Stensjö, K. (2018) Homologous overexpression of NpDps2 and NpDps5 increases the tolerance for hydrogen peroxide and light-induced oxidative stress in the filamentous cyanobacterium *Nostoc punctiforme*, Submitted.

III Li, X., Sandh, G., Nenninger, A., Muro-Pastor, A. M., and Stensjö, K. (2015). Differential transcriptional regulation of orthologous *dps* genes from two closely related heterocyst-forming cyanobacteria. *FEMS Microbiology Letters*, 362(6), 1–8.

IV Li, X., Wegelius, A., Turco, F., Stensjö, K. (2018) Design and characterization of a synthetic minimal promoter for heterocyst-specific expression in filamentous cyanobacteria, Manuscript.

## BE A LEGEND

WHEN I STOP SACRIFICING MYSELF TO ASSIMILATE THE DOMINANT;
WHEN I REJECT FEEDING THE COUNTRY TYRANNIZE THE WEAK;
#
WHEN I REFUSE TO LEARN HISTORY WHITEWASHED BY POWER;
WHEN I SAY NO TO A LAW WE NEVER HAVE CHANCE TO VOTE;
#
WHEN I SURRENDER MY PROPERTY, MY BODY, NOT MY SOUL;
WHEN I DECIDE TO CHOOSE THE PATH NO ONE EVER TRIED;
#
RISE ~ UP.

*『2016-04-10 in Uppsala Sweden.*
*Inspired by Rise Against Band』*

# Contents

# Abbreviations

| | |
|---|---|
| ATCC | American type culture collection |
| ATP | Adenosine triphosphate |
| APX | Ascorbate peroxidase |
| BPV | Biophotovoltaic |
| Bfr | Bacterioferritins |
| Chl-*a* | Chlorophyll a |
| Cyt | Cytochrome |
| Dps | DNA- binding proteins for starved cells |
| *E. coli* | *Escherichia coli* |
| EPR | Electron paramagnetic resonance |
| ETC | Electron transport chain |
| eYFP | Enhanced Yellow Fluorescent Protein |
| FNR | Ferredoxin: NADP+ oxidoreductase |
| GFP | Green fluorescent protein |
| PAGE | Polyacrylamide gel electrophoresis |
| PCC | Pasteur culture collection |
| PCR | Polymerase chain reaction |
| PEC | Photosynthesis electron chain |
| PQ | Plastoquinone |
| PRX | Peroxiredoxins |
| PSI | Photosystem I |
| PSII | Photosystem II |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| *N. punctiforme* | *Nostoc punctiforme* strain ATCC 29133 |
| RBS | Ribosome binding site |
| ROS | Reactive oxygen species |
| RNAP | RNA polymerase |
| SDS | Sodium dodecyl sulphate |
| SOD | Superoxide dismutase |
| TSS | Transcription start site |

# Author's request

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# Introduction

In this section the general concepts about the key aspects of this thesis are introduced, including: cyanobacteria, heterocyst differentiation, oxidative stress, Dps proteins and synthetic biology tools. The background knowledge provided in this introduction will facilitate the understanding of the research presented and discussed in this thesis.

## Cyanobacteria and their potential applications for biotechnology

Cyanobacteria are photoautotrophic prokaryotes. They are important microorganisms that contributed to the early oxygenation of the atmosphere and oceans on Earth [1]. This great oxidation event is probably the most important environmental and geochemical transition in the evolution of our planet. Different morphologies with unicellular and filamentous forms can be found among cyanobacteria species [2].

In terms of sustainable bioproduction, several aspects should be considered when choosing a system for synthetic biology applications. Firstly, the most promising energy source is the solar energy. Secondly, the candidate of the system should use water as the electron source for oxygenic photosynthesis. Furthermore, the organism should have a sequenced genome, be possible to genetically modify and grow fast within a wide range of environment niches. Therefore, cyanobacteria as oxygen-evolving photosynthetic prokaryotes are good candidates for being a suitable system for biological applications [3–5].

Heterotrophic organisms like *Escherichia coli* and yeasts, are great competitors of cyanobacteria on the scale of commercialized bioproduction applications [6]. However, compared to heterotrophic organisms, cyanobacteria have special metabolic features of autotrophic carbon and nitrogen assimilation and energy supply via photosynthesis, which presents an increased potential for the next generation of sustainable bioproduction [7,8]. Considering the ability to convert atmospheric $CO_2$ into biomass with solar light as the energy and $H_2O$ as electron sources, cyanobacteria uncover a great potential as a platform for development of a sustainable society and to reduce greenhouse emissions [7]. Due to a lower need of supply of organic compounds, using cyanobacteria as

hosts for production will increase the efficiency on energy turnover in large scale cultivation process [8,9]. With a higher growth rate and well-studied genetic apparatus for bio-engineering compared to algae and plants, cyanobacteria show a larger potential for biotechnological applications and bioproduction in comparison to other photosynthetic organisms [8,10–13].

Since the development of alternative energy sources became a new trend for the next generation of sustainable energy, cyanobacteria have stand out as one of the promising candidates and production hosts for bioproduction [9]. With synthetic biology and metabolic engineering approaches, genetically modified cyanobacteria may have more potential in future biotechnological applications as compare with algae and plants [6,11].

Novel applications of cyanobacteria-based biotechnology, such as production of bioactive compounds, bio-material and microbial fuel cells (MFCs), are also booming up at the research level. In the past decades cyanobacteria have been exploited as a valuable source of biological compounds with specific enzyme inhibition and immunosuppressive activities, which could be utilized for anticancer [14–16], antibacterial [16,17], antifungal [18], and antiviral purposes [19,20]. Several strains of cyanobacteria have been found to accumulate polyhydroxyalkanoates (PHAs) naturally in the cell [21,22]. PHAs are attractive biomaterial with biodegradability, biocompatibility and thermoplastic properties, and could be used as a substitution of traditional petrochemical-based plastics, which is not biodegradable [17,23]. Recent studies of microbial bio-photovoltaic (BPV) cells [24] showed the ability of cyanobacteria to use water as the electron source for conversion of light energy into electrical current [25].

In spite of the promising potentials, there are still numerous of challenges during the transition towards cyanobacteria-based biotech-applications [26,27]. One of the challenges is to improve the natural productivity. When the volumetric productivity is calculated by the cell biomass, the natural accumulation of bioproducts in cyanobacteria is desperately lower than in heterotrophic bacteria [8,18]. Thus, the natural metabolism such as nitrogen assimilation, carbon fixation, and photosynthesis, must be improved in cyanobacteria for higher productivity. For example, tuning the metabolic flux to direct the production of desirable secondary metabolic products and biofuels through metabolic engineered pathways [27].

# The cyanobacterium *Nostoc punctiforme* sp. ATCC 29133

Filamentous heterocyst-forming cyanobacteria are fascinating due to their multicellularity, with an interdependent metabolism of the cell-types within one filament. Furthermore, the ability of survival under harsh environmental conditions like high temperature or nutritional limitation [28], and being able to be cultivated even in wastewaters, as well as the capacity of producing good quantity of biomass, are remarkable [29]. All these interesting features make it likely that filamentous cyanobacteria will become good candidates for a wide range of industrial applications in the future.

*Nostoc punctiforme* is the main target cyanobacterium in this thesis and also the most important character in this thesis. It is a $N_2$-fixing filamentous cyanobacterium that can been found predominantly living in terrestrial environments either independently or as symbiosis with plants and fungi [30]. Different physiological properties and special cell types developmental alternatives are required for *N. punctiforme* to survive in a wide range of ecological niches such as nutritional limited, cold and desiccated environments [31]. With sufficient nitrogen sources, *N. punctiforme* filaments will grow photo-autotrophically and naturally form cell clumps with a cluster of filaments in liquid medium [30]. The cells within these filament are named as vegetative cells [30]. When exposed to environmental changes, the vegetative cells may differentiate into three different types as a response towards certain stress conditions [31]. One of the differentiation process is the formation of hormogonia, which are motile dispersal filaments functional as infection units for symbiotic association [32]. While in response to severe energy-limiting conditions, such as light and phosphorous depletion, the filaments will differentiate into dormant spore like cells named akinetes [33]. Under the morphology of akinetes, *N. punctiforme* may proceed cell metabolism by accumulating glycogen and cyanophycinase for carbon and nitrogen storage [33] until the environmental conditions are beneficial for growth again [34]. The host strain in this thesis, *Nostoc punctiforme* strain ATCC 29133 (from now one *N. punctiforme*), was originally isolated from coralloid roots of the cycad *Macrozamia* spp. [35].

In the absence of sources of combined nitrogen, filamentous cyanobacteria such as *N. punctiforme*, *Cylindrospermum stagnale*, and *Anabaena* strains. will develop specialized cells named heterocysts [28]. Every 10 to 20 of the vegetative cells along a filament will differentiate, to a nitrogen-fixing heterocyst [36].

## Heterocyst differentiation

Heterocyst differentiation occurs under nitrogen depleted condition when the culture transform into a $N_2$-fixing (diazotrophic) culture. The cells along a diazotrophic filaments have a clear functional division, in which the vegetative cells perform photosynthesis, and the micro-oxic heterocysts do $N_2$ fixation [36] Biological fixation of $N_2$ is a highly energy-consuming process, in which a large proportion of ATP generated by respiratory electron transport reactions and by cyclic electron transfer around PSI are required [37]. The nitrogenase which is the catalyst that reduce molecular nitrogen to ammonia is an oxygen sensitive enzyme [38], Therefore, diazotrophic cyanobacteria have evolved various strategies to protect the nitrogenase against oxidative damage and permit the $O_2$ level for energy regeneration simultaneously. The mechanism of nitrogenase protection has been studied since the early 1980s [39,40]. The heterocyst-forming cyanobacteria indeed provide multiple strategies to prevent $O_2$ access to the nitrogenase, such as forming permanently micro-oxic environment in heterocysts, and generating physical barriers around the heterocysts [41].

As a specialized cell for $N_2$ fixation, during the process of cell differentiation, the heterocyst undergoes major physiological and morphological changes [36]. Heterocyst-forming cyanobacteria contributes all these efforts to create a natural micro-oxic environment and a sufficient level of energy supply and reducing power for the nitrogenase to be functional [31,42]. After the molecular nitrogen has been reduced into ammonia by the nitrogenase, the nitrogen source will be transported into vegetative cells in the form of amino acids as bioavailable nitrogen source [31,43]. The vegetative cells in return provide a reduced carbon source to heterocysts for sustaining metabolism to produce ATP and NADPH [43,44]. Thus, a flux of carbon and nitrogen containing metabolites is established between vegetative cells and heterocysts along the filaments.

## Oxidative stress tolerance in heterocyst-forming cyanobacteria

In order to use cyanobacteria in general, and the heterocysts more specific for biotechnological applications and production of biofuels or other useful products, we need to engineer robust cyanobacteria strains to keep a high fitness in a variation of cultivation conditions. One of the important parts of cell robustness is tolerance to oxidative stress [45].

Cyanobacteria are the only prokaryotes that are capable of oxygenic photosynthesis [46]. They also inevitably encounter reactive oxygen species (ROS). ROS are naturally formed as byproducts during oxygenic photosynthesis in all photosynthetic microorganisms [47] The chemistry behind ROS formation has been well documented [48]. There are several different ROS, such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{-}\cdot$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH\cdot$) [47]. For example, under various environmental stress, such as UV, high light, nutrient limitations and heat exposure, intercellular ROS level will increase dramatically [49–51]. If the levels of ROS is not properly Controlled, and accumulated high levels of ROS leads to severe oxidative stress, that subsequently results in serious damage to biomolecules [47]. $H_2O_2$ will specifically oxidize protein containing Iron-Sulphur clusters, such as proteins within PSI complex, nitrogenase, and hydrogenases. These proteins considered as highly important in cyanobacteria for biotechnological purposes, and therefore ROS Control is certainly one of the important aspects to take care for bioproduction [52].

The major proportion of the ROS produced in cyanobacteria are generated from photosynthesis electron chain (PEC) [26]. In cyanobacteria, light is essential for photosynthesis. However, since the ROS are generated by photosynthetic electron flow inevitably, high intensity of light can also become a source of oxidative stress [47]. When the light intensity is rising, surplus excitation energy will be absorbed by photosystem II (PSII) and leads to excess photosynthetic electron flow [49]. The main function of PSII is to capture photons and transport electrons from water splitting to reduce plastoquinone to start all photosynthesis reactions [53]. In this scenario, the electron input driven by a high intensity of light will not be compatible with the electron consumption rate through $CO_2$ fixation [49]. During high light intensities charge recombination reactions in the photoinhibited PSII produce triplet chlorophyll that will react with oxygen to produce the very aggressive ROS, singlet oxygen ($^1O_2$) that, together with other ROS will inhibit the repair cycle of the D1 protein, could lead to photo-oxidative stress and loss of PSII activity [54]. At the donor side of photosystem I (PSI) other ROS are formed superoxide ($O_2^{-}$) due to that oxygen rather than ferredoxin will be used as an electron acceptor [55]. Hydrogen peroxide ($H_2O_2$) is then produced by dismutation of $O_2^{-}$ a reaction catalyzed by superoxide dismutase (SOD) [47,48], and finally, the $H_2O_2$ is reduced to $H_2O$ by different enzymes such as peroxidases. However if one electron instead is transferred from ferrous iron towards $H_2O_2$ by metal catalyzed Fenton Reaction (Equation 1.1), the highly toxic hydroxyl radical ($OH\cdot$) will be produced [56].

$$Fe^{2+} + H_2O_2 \leftrightharpoons Fe^{3+} + OH^{-} + OH\cdot \quad \text{Eq. (1.1)}$$

The formation of ROS at the donor side of PSI is called the Mehler reaction [55]. Interestingly in cyanobacteria there is another reaction present, in which flavodiiron proteins (Flvs) directly reduce $O_2$ to $H_2O$, and thus act in efficient photoprotection of the photosystems, without the production of any ROS. From research on Flvs in *Anabaena* sp. PCC 7120, this reaction was named "Mehler-like" reaction. The physiological importance of the Flvs in *Anabaena* has been dissected in detail and carefully reviewed [57].

With 2.4 billion years' evolution [58], cyanobacteria have evolved in the presence of ROS on Earth [2]. Depending on different growth states of the cell and variations of environmental conditions, the discrepancy of ROS production and scavenging ratio may be different. An intercellular network of antioxidative processes has been buildup to control ROS levels and fight against oxidative damage in the cyanobacteria cells [2,47].

In plants, the ROS not only cause cellular damage, but also has a major role as agent of signaling to regulate metabolism [59]. However, the ROS-based signaling system in plants has not yet been explored in cyanobacteria [59,60]. In cyanobacteria, $H_2O_2$ can be scavenged by different antioxidants, enzymes as peroxidases, peroxiredoxins (PRX), catalases such as and ascorbate peroxidase (APX) [61,62] and also by non-protein antioxidants such as glutathione [47]. These antioxidants can perform as electron donors to convert $H_2O_2$ into water and oxygen. Besides, catalases [63], and PRX [64,65], DNA-binding proteins for starved cells (Dps) are also found in many prokaryotes, where they act as $H_2O_2$ detoxifiers [66].

## Dps related ROS protection

After it was characterized for the first time in *Escherichia coli* in 1992 [67], Dps proteins have been recognized with two main functions for bacteria. Many studies have now discovered that the Dps proteins rarely have the non-specific DNA-binding capacity [68], but mainly function in iron storage and $H_2O_2$ detoxification [69].

For most organisms, Iron is an essential element, but also problematic since free ferrous iron ($Fe^{2+}$) in the presence of $H_2O_2$ may produce the hydroxyl radical, $OH^{\cdot}$. Dps proteins are multifunctional proteins functioning in the defense against oxidative stress, possessing both $H_2O_2$ scavenging and regulation of free ferrous iron simultaneously. With a shell-like assembly and iron uptake capacity, the Dps proteins have been assigned to the ferritin superfamily, which is involved in bacterial iron homeostasis [66]. Under ROS stress conditions, Dps proteins function as dodecamers [68], that will reversibly oxidize $Fe^2$

$^{+}$ into $Fe^{3+}$ at the ferroxidase center (FOC) [69,70], thus preventing Fenton reaction [71]. Dps proteins prefer $H_2O_2$ rather than $O_2$ as oxidant for oxidation of ferrous irons, which makes Dps proteins 100 times more efficient in using $H_2O_2$ than ferritins [71].

Normally, a bacterial strain only encodes a single Dps protein. However, there are a few species that encodes more than one Dps, such as *Bacillus anthracis* [72], *Deinococcus radiodurans* [73], and *Mycobacterium smegmatis* [74] which all encode two Dps proteins. The coexistence of multiple Dps protein is generally more common in $N_2$-fixing filamentous cyanobacteria [70]. For example, in the host cyanobacteria of this study *N. punctiforme*, there are five unique ferritin-like proteins encoded in the genome, namely NpDps1 (*Npun_R3258*), NpDps2 (*Npun_F3730*), NpDps3 (*Npun_R5701*), NpDps4 (*Npun_R5799*) and NpDps5 (*Npun_F6212*) [34]. To understand the biological basis of the coexistence of multiple Dps proteins within one single organism, these five Dps proteins in *N. punctiforme* is a highly interesting system to study.

## Synthetic biology and genetic tools

The term synthetic biology defines the artificially design and construction of new biological parts, devices, and systems, and the re-design of existing, natural biological systems for desired application purposes [75]. Synthetic biology combines different scientific disciplines and technologies based on the deep understanding of complex natural biological systems. Biochemists uses bioengineering approaches to create complex artificial biological systems following engineering rules such as abstraction, standardization, and decoupling [76,77].

The essence of synthetic biology is the collection of standard biological parts. The function of the synthetic biology device should be well-defined and characterized by the functions encoded in these biological parts [77,78]. Therefore, the behavior of complex biological systems should be predictable from the biological parts used. For example, the standard system for gene cloning by the BioBrick foundation (http://parts.igem.org/Main_Page), uses defined prefix and suffix sequences, which contain specific restriction sites on each sides of an independent synthetic biology part to insert sequences of interest into a vector [79,80]. The standardization interfaces make biological parts reusable and makes the function after assembly process reliable.

The properties of individual biological parts, such as promoters, ribosome binding sites, terminators and functional gene sequences, might vary when

applied in different microorganisms [77]. There have been many attempts to use synthetic biology tools developed for *E. coli* to engineer cyanobacteria [3,5,81,82]. However, these attempts have often led to different problems, since these tools are not suitable or compatible with the cellular processes in cyanobacteria. If a promoter is developed for a specific purpose, for example cell specificity, the function and impacts of different promoter parts need to be well characterized for a specific desired function. This is the aim of the second part of the thesis.

Considering the variable function of a specific regulatory part, when used in different hosts, it is necessary to develop cyanobacterial specific genetic tools. For example, to achieve efficient $H_2$ production from heterocyst forming cyanobacteria, we need to develop suitable heterocyst specific promotors to regulate the expression of proteins of importance for this process.

## Transcriptional regulation and transcription factors

The promoter function is defined by the transcriptional context in terms of transcription factors and cis-acting promoter elements [83,84]. Transcriptional regulation including activation and repression, are Controlled through promoters and their contributing regulatory factors. Promoters play a fundamental role in the activation and suppression of gene expression. The strength of a promoter is normally defined by how strong the binding of the RNA polymerase (RNAP) is to the promoter. The binding sites for the RNAPs are located at -10 and -35 sites in relation to the transcription start site (TSS) of the gene of interest (GOI) (Figure 1). A typical promoter contains, from upstream to downstream: UP element, -35 element, , -10 element, and TSS [83]. In addition to this, the promoter often contains cis-acting elements that are conserved binding motifs, to which transcriptional regulators can bind and thereby interact with the RNA polymerase and activate or repress the expression of the GOI. The coordination of cis-acting elements regulate genes expression as a response to changes in, for example, the environment such as under stress condition or cell differentiation [84]. These promoter elements and their binding transcription factors, interact with the RNAP during transcription [83]. The essential -10 element has two base-specific interactions with sigma factors, which is a subunit of the RNAP complex. The sequence in between the -10 elements and TSS is important for the formation of the RNAP-promoter open complex [85]. After the RNAP recognition at the -10 elements, the transcriptional initiation starts, followed by the formation of a close complex RPc, the bent and wrapped close complex I1, the initial open complex I2, and, finally construction of a stable open complex RPo [86]. The RNAP then escapes the

promoter through the DNA scrunching mechanism [87] and starts the transcription elongation.

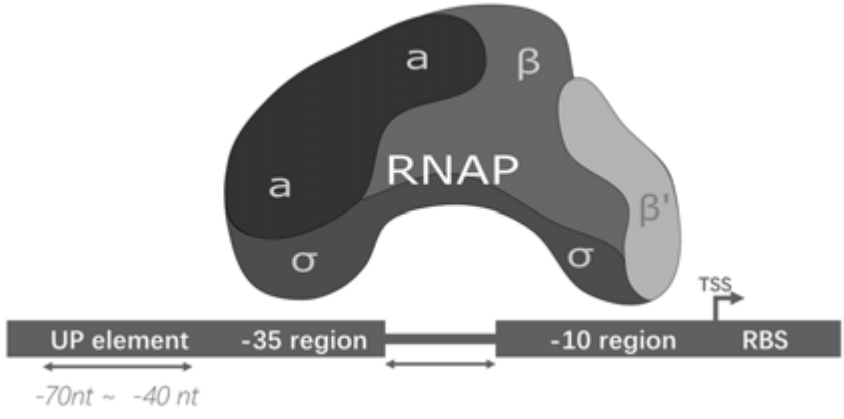


*Figure 1.* Schematic outline of a bacterial promoter. The model visualizes the RNA polymerase binding sites at -35 and -10 regions, the -35 upstream element (UP-element), the transcription start site (TSS) and the ribosome binding site (RBS) located in the 5'untranslated region (5'UTR).

Transcription factors can perform as activators and help recruits the RNAP binding in the initiation step of transcription. Their cognate binding site on the cis-acting elements which may located at the -35 region or at the UP-element region [88,89]. While another type of transcription factors work as repressors by binding to a cognate site close to the core promoter on the -10 region or between the -35 and -10 regions, which may create a steric hindrance to prevent the RNAPs binding and thus prevent transcriptional initiation [85].

σ factors are part of the RNAP and normally recognize both -10 and -35 regions of a promoter sequence. There are two main families of σ factors in prokaryotic cells, the σ70 family and the σ54 family. The σ70 family are responsible for most of transcription during growth of the organism, while the σ54 family, which is absent in cyanobacteria, is involved in regulation of transcription upon different environmental and stress conditions [90]. The σ70 family could be characterized into four groups. Group one is the fundamental for basic metabolic functions in cell survival and genes expression in the exponential growth. Group two is involved in tuning the metabolism upon nutrient stress but not essential for exponential growth. Group three is also not an essential factor. It regulates genes transcription during flagellum and sporulation synthesis as well as the heat shock response. Finally, the fourth group named as σ ECF (Extra Cytoplasmic Function), is non-essential for the survival of cells under normal growth conditions [91,92].

## Heterocyst-specific promoters and regulators

The differentiation of a vegetative cell into a heterocyst as a response to nitrogen deprivation has been thoroughly investigated [31,36]. Several genes have previously been reported with a transient or constant induction of expression during heterocyst differentiation. This has been shown by large transcriptomic data regarding the exposure of *Anabaena* sp. strain PCC 7120 to nitrogen deprivation [93–95]. Under diazotrophic growth, the specific proteomes of the different cell types have been studied, which provides better understanding of the specific metabolism in heterocysts [96–98]. Quantitative proteomics showed that 16% of the proteome, with a high number of proteins involved in oxidative stress tolerance and redox regulation, displays differently in abundance in heterocyst as compared to in vegetative cells, in a diazotrophic filament of *N. punctiforme* [97].

Particularly, the transcriptional regulation of the heterocyst differentiation processes has been studied for many years [31,42]. This regulation process depends mainly on two transcription factors: the global nitrogen regulator NtcA, and the heterocyst differentiation regulator HetR [43]. NtcA is involved in the early stages of heterocyst development regulating the activation or repression of genes related to nitrogen metabolism [99]. HetR is the main regulator of heterocyst differentiation that coordinates the transcriptional regulation of heterocyst development [43]. HetR has been shown to bind and regulate the promoters of *patS*, *hepA*, *hetR, pknE*, *hetP* and *hetZ*, which all encodes major players in the differentiation process [100–104].

In addition, specific biochemical pathways and related genetic interactions involved in the developmental process of heterocyst differentiation as well as in the spatial patterning of mature heterocyst along the filaments have been characterized [44,98,104]. The timeline of a heterocyst differentiation is displayed in Figure 2. An increase in the level of 2-oxoglutarate will be the cellular signal that response to a decreased N to C ratio. This signal will be subsequently sensed by NtcA that in turn indirectly will activate the regulator HetR, thus trigger heterocyst differentiation [105,106]. During the patterning phase, the expression of a nitrogen-responsive response regulator *nrrA* will be upregulated by the NtcA [93,107]. NrrA is required for the induction of *hetR* following the upregulation of *patS*, and *pknE* within the patterning phase of heterocyst differentiation [104]. Meanwhile, there are also several transcriptional factors reported to be interacting with HetR, including the repressors, PatS and HetN [108]. After the patterning phase, the upregulation of *hetP*, *hetC* and *hetZ* indicates the commitment phase of heterocyst differentiation [36,104]. During the morphogenesis phase, a heterocyst specific outer envelope is synthesized with

additional polysaccharide layers to the outer membrane, the activity of respiration as well as the oxidative pentose pathway are increased, the cyclic electron flow around PSI is enhanced and nitrogen fixation start [42]. Thus, the mature heterocyst is formed.

As discussed above, the mechanisms of transcriptional regulation during heterocyst differentiation are well defined in heterocyst-forming cyanobacteria [98,109]. However, the regulation of cell specificity is still not fully clear. Therefore, this study has also been focused on understanding more about the mechanisms behind cell specific gene expression and stress induced responses of the NpDps proteins in the heterocyst forming cyanobacterium *N. punctiforme*.

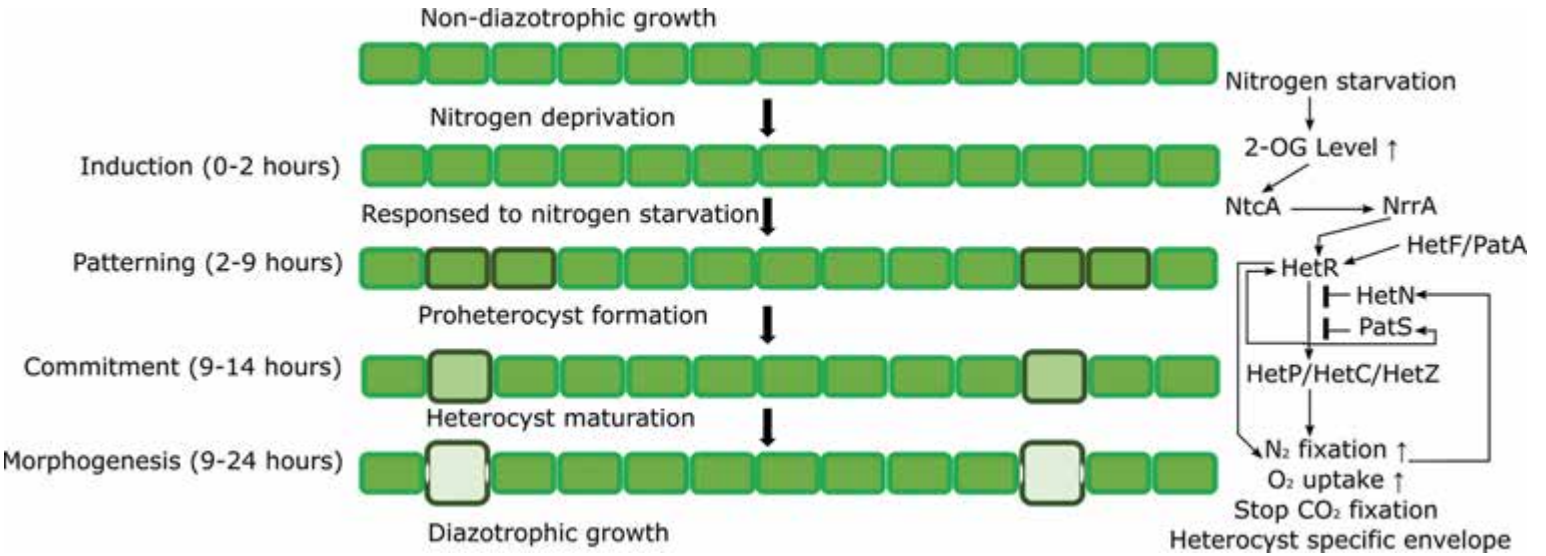


*Figure 2.* Schematic representation of various stages of heterocyst differentiation in a filamentous cyanobacterium. A schematic model depicting the time duration and the regulation factors involved in the developmental cascade of heterocyst differentiation. On the right side of the image, the interaction of regulation factors in each developmental phase and specific metabolism in heterocysts is indicated with arrows and bars. Arrows indicate positive interactions and up-regulation, while bars indicate negative interactions or down-regulation.

# Aim and significance of this study

Although Dps proteins have been reported with a function in detoxification of ROS, the mechanisms behind the cellular tolerance to high light and $H_2O_2$ stress in multicellular cyanobacteria are largely unknown. This thesis is part of a larger microbial chemistry project with a focus on enhancing the ability of cyanobacteria to acclimate to environmental variations. The overall objective of this thesis was to increase the cell tolerance of cyanobacteria against oxidative stress caused by ROS. This increased tolerance would make the cyanobacteria more robust and therefore useful in biotechnological applications for both commercial and academic purposes.

In the first part of the study, the biological functions of the five Dps proteins in *Nostoc punctiforme* strain ATCC 29133 have been identified and characterized. The aim was to resolve cell-activities of importance for the ability of cells to acclimate to different growth conditions to avoid oxidative stress. Additional to the physiological studies of the Dps proteins, their regulation on transcriptional level has also been investigate (Part I).

The potential of cyanobacteria-based bioproduction also lead this thesis to investigate more about synthetic biology tools associated with regulation of the production of valuable biomolecules. In the second part of the thesis, the objective was to design synthetic promoters as tools to express target proteins involved in oxygen sensitive synthesis of valuable compounds in heterocyst-forming cyanobacteria. Promoter elements necessary, or unnecessary, for cell specific expression, in reported heterocyst specific promoters, have not been characterized in any detail. The aim was to generate (i) knowledge of benefit for the understanding of the regulatory function of promoter elements that confer heterocyst-specific expression, and (ii) tools that could be beneficial for future metabolic engineering approaches in cyanobacteria-based biotechnological applications (Part II).

# Part I Dps proteins and oxidative stress tolerance in $N_2$-fixing filamentous cyanobacteria (Paper I and II)

## Specific aims of Part I

1) To resolve the physiological functions of ferritin-like proteins (NpDps) in stress tolerance, in the filamentous heterocyst-forming cyanobacterium *Nostoc punctiforme.*
2) To understand the biochemical role of these NpDps proteins in interaction of photosynthesis and iron homeostasis during cellular response towards ROS stress.
3) To investigate whether NpDps proteins can be used as a novel biological tool for enhancing cell robustness in multicellular cyanobacteria.

## Results and discussion

To improve the tolerance against high light intensities, and thus oxidative stress, is one of the key aspects of importance to increase the production level in photosynthetic organisms. In the first part of this thesis, the results concerning the NpDps proteins and their possible roles in oxidative stress tolerance is described. The investigation of these ferritin-like proteins provides an understanding of their involvement in overall cellular response to environmental stress conditions. The focus is to investigate the role of Dps in iron homeostasis and in the regulation of ROS levels in filamentous cyanobacteria.

The cellular regulation, protein structure, as well as biochemical characterization of the single Dps protein in *E. coli* have already been well studied[110–115]. There are also experimental evidences that suggest that Dps proteins from cyanobacteria could be a key engineering target in synthetic biology approaches to enhance cellular tolerance against multiple oxidative stress [113,116].

Although this knowledge of Dps proteins exists, the understanding of multiple Dps proteins in one bacterium is still insufficient, and the cellular specificity of Dps in multicellular cyanobacteria even more so [34,117]. These earlier results on Dps proteins in *Nostoc* spp. lead me to hypothesize that NpDps can be utilized as a biological tool for ROS tolerance in cyanobacteria.

Before using NpDps proteins as biological tools in cyanobacteria, a more thorough understanding of the specific biological functions of these multifunctional proteins is required. To investigate this, NpDps deletion mutants of *N. punctiforme* were used in which the individual NpDps proteins were genetically inactivated [34]. This study also aimed to expand the repertoire of potential stress protection with homologous overexpression of NpDps in *N. punctiforme*. To test the outcome of this strategy, NpDps-over-expression strains (OENpDps) were constructed. The possible decrease or increase in stress tolerance of the deletion mutant strains, and the OENpDps strains were tested under conditions of high light illumination, or externally added $H_2O_2$ (Papers I and II).

Specifically, to test the feasibility of this ideas, two questions must be answered with this work to fill the knowledge gaps: 1) Why does *N. punctiforme* express five Dps proteins? 2) Are there any functional collaborative relations between different NpDps proteins in the process of increasing the robustness of the cells? Thus, a multi-dimensional characterization, with physiological, biochemical and genetic/bioinformatic strategies, of the five NpDps proteins in *N. punctiforme* were implemented.

The specific *in vivo* roles of individual NpDps proteins were studied by physiological characterization of the phenotypes of the *Npdps* deletion mutant strains, as well as NpDps overexpression strains. Additionally, to analyze the genetic organization and the possible co-expression of the five *Npdps* genes with other genes, the operons organization were studied with Reverse Transcription (RT) qPCR (Paper I). Besides, putative transcription start sites (TSS) were identified by 5'Rapid Amplification of cDNA Ends (5'RACE) (Paper I). To understand if the five NpDps proteins were co-regulated and to study their putative interdependence, the transcriptional regulation profile of all *Npdps* genes was investigated by RT-qPCR analysis on RNA isolated from the *Npdps* deletion strains (Paper I). By homologous overexpression of NpDps2 and NpDps5 in *N. punctiforme,* the study determined the sensitivity change under different $H_2O_2$ treatments and high light conditions (Paper II). The results indicated a role of NpDps2 and NpDps5 in $H_2O_2$ tolerance and light-induced oxidative stress (Papers I and II).

This study also investigated the Dps-mediated link between photosynthesis, $H_2O_2$ detoxification and iron homeostasis. The impact of the NpDps proteins on photosynthetic activity and on the photosystems, were investigated by the study of *Npdps* deletion and complemented strains, as well as NpDps overexpression strains, using immunoblot analysis, $O_2$ evolution analysis and Electron Paramagnetic Resonance (EPR) spectroscopy. The results revealed by these approaches provided a deep insight of the possible biological functions of the NpDps proteins in *N. punctiforme*.

## Construction of NpDps overexpression strains in *N. punctiforme*

The non-native promoter $P_{trc2O}$ (Huang *et al*., 2010), which is a strong constitutive promoter, was used. The RBS* (5'-TAGTGGAGGT-3') was used as ribosomal binding site (RBS) in the constructs, since it has been shown to determine a strong translation activity [82]. A terminator and a 3' strep-tag sequence were also assembled with the promoter and Npdps genes by overlap extension PCR [119] to form the final overexpression plasmid (Fig. 3). A Control strain carrying an empty vector pPMQAK1, in which the *ccdB* gene was removed, was used.

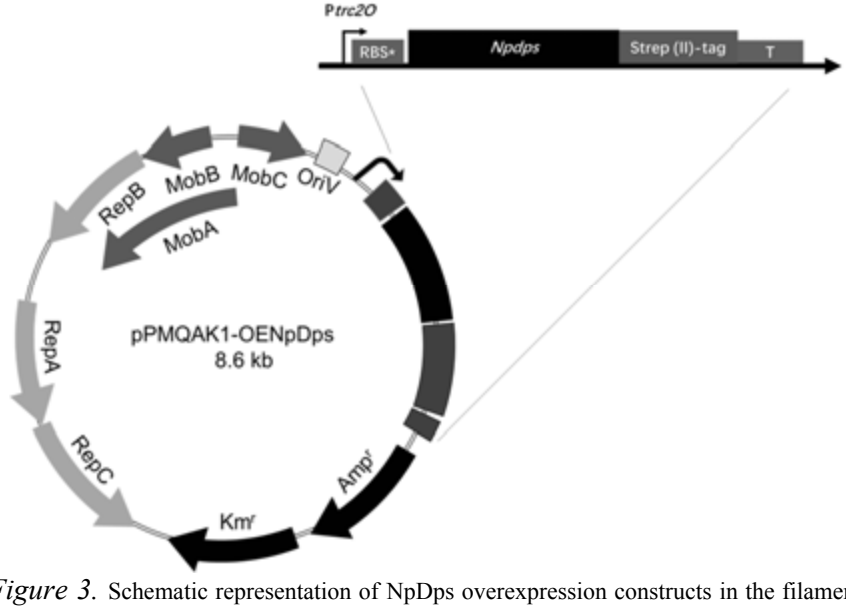


*Figure 3.* Schematic representation of NpDps overexpression constructs in the filamentous cyanobacterium *N. punctiforme*. Adapted from paper II.

*N. punctiforme* overexpression strains were constructed, namely OENpDps2 and OENpDps5. Two genes, *Npun_3730* (*Npdps2*) and *Npun_6212* (*Npdps5*), were individually inserted into the shuttle vector

pPMQAK1 [5,118], and the constructs (Fig. 3) were transformed into *N. punctiforme* for homologous overexpression. The overexpression and Control plasmids were successfully transformed into *N. punctiforme* by conjugation [120].

## Overexpression of NpDps2 and NpDps5 enhanced *N. punctiforme* tolerance to $H_2O_2$ stress

To investigate if the physiological function of NpDps2 and NpDps5 could be used to improve the tolerance to $H_2O_2$, the overexpressing strains OENpDps2 and OENpDps5 were investigated. The growth of the OENpDps2 and OENpDps5 strains as compared to the empty vector Control was examined under the addition of $H_2O_2$ with increasing concentrations (Paper II).

To confirm that the NpDps-strep(II)-tag overexpressions were successful, western blotting with strep(II)-tag antibody was used (Paper II). The results clearly showed that the target NpDps-strep(II)-tag fusion proteins were expressed in the correct sizes, corresponding to the clear bands at ~20 kDa for NpDps2 and ~18 kDa for NpDps5 in the light conditions used in this study. The two NpDps proteins were expressed at a similar level as compared to each other. The expression levels were also similar for the three different light regimes tested (Fig. 4) (Paper II).

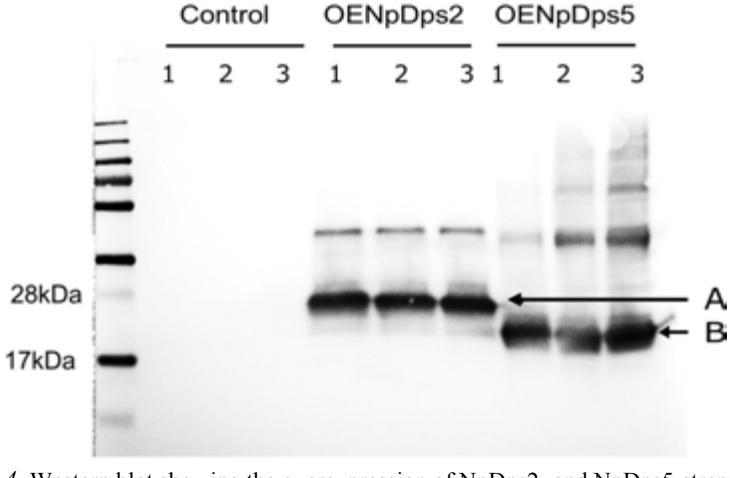


*Figure 4.* Western blot showing the overexpression of NpDps2- and NpDps5-strep(II)-tag fusion proteins from the filamentous cyanobacterium *Nostoc punctiforme*. From left to right: molecular weight ladder (PageRuler$^{TM}$ Prestained Protein Ladder), Control (empty vector), OENpDps2 and OENpDps5. Three light intensities were used: 40 µmol photons $m^{-2}s^{-1}$ (1), 60 µmol photons $m^{-2}s^{-1}$ (2), and 500 µmol photons $m^{-2}s^{-1}$ (3). Clear protein bands for both proteins can be seen at 20 kDa (A) and at 18 kDa (B). An antibody against the strep(II)-tag was used. Adapted from paper II.

Elevation of the intracellular $H_2O_2$ levels may accelerate the damage of DNA and enzymes and thus influence the growth rate [121]. WT and empty vector Control strain were studied with different concentrations of $H_2O_2$ to identify the $H_2O_2$ tolerance levels of the Control strains. The result showed that after addition of 1.5 mM $H_2O_2$, both WT and empty vector Control strain showed significantly decreased growth, after 4 days. Accordingly, to test the possible increased tolerance to $H_2O_2$ in the OENpDps strains, both $N_2$-fixing (Fig. 5A and B) and ammonium supplemented cultures (Fig. 6A and B) were treated with $H_2O_2$ concentrations from 0.5 mM to 5.0 mM. Non-treated cells without $H_2O_2$ were used for comparison.

The homologous overexpression of NpDps proteins indeed provided increased cell robustness of *N. punctiforme* compared to the Control strain under an increasing hydrogen peroxide concentration. Besides NpDps2, which has been suggested to be a $H_2O_2$ detoxifier. Surprisingly, the OENpDps5 strain also showed enhanced $H_2O_2$ tolerance under increasing level of $H_2O_2$ and performed even better than the OENpDps2 strain under high $H_2O_2$ concentrations (Fig. 5Bf and Fig. 6Bf). In both diazotrophic (Fig. 5B) and ammonium supplemented (Fig. 6B) growth conditions, the Control strain displayed a significant reduction in the specific growth rate when the $H_2O_2$ concentration was above 2.5 mM $H_2O_2$ (Paper II).

At 5.0 mM of externally added $H_2O_2$, the OENpDps5 strain displayed a higher growth rate than the OENpDps2 strain which showed dramatic decrease in growth (Fig. 5A). On the other hand, the OENpDps5 strain was still growing under 5.0 mM of $H_2O_2$. Besides, the minus value of specific growth rate indicates that $H_2O_2$ levels higher than 2.5 mM lead to complete stop of growth for the Control strain in diazotrophic condition (Fig. 5B), whereas in ammonium supplemented condition the growth rate decreased from 3.5 mM (Fig. 6B). From these results, a conclusion can be made that an increased $H_2O_2$ stress tolerance in *N. punctiforme* can be obtained by overexpression of NpDps2 and NpDps5 (Paper II).

The previous results of the physiological study on *Npdps* gene knock-out mutants, present a clear relation between NpDps2 (Npun_F3730) protein and $H_2O_2$ tolerance [34]. Combined with the current results in this thesis, the function of NpDps2 may be confirmed as a typical Dps protein which uses $H_2O_2$ for the oxidation of ferrous iron in the FOCs, and thus reduce the intercellular ROS levels.

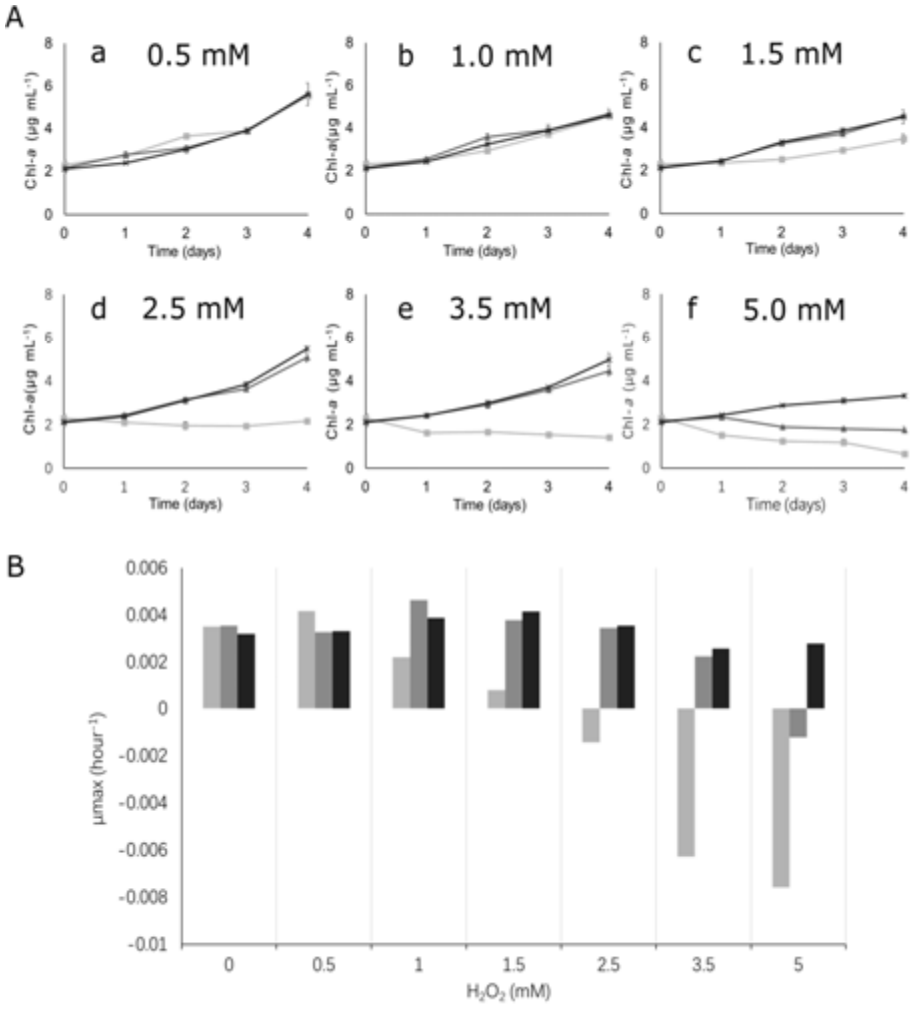


*Figure 5.* Growth of *N. punctiforme* strains: Control, OENpDps2 and OENpDps5 in diazotrophic growth conditions under different concentration of added $H_2O_2$. (A) Growth of Control (Light gray bars), OENpDps2 (Gray bars) and OENpDps5 (Black bars), under $H_2O_2$ concentrations of 0.5 (a), 1.0 (b), 1.5 (c), 2.5 (d), 3.5 (e) and 5.0 mM (f). (B) The specific growth rates of Control (Light gray), OENpDps2 (Gray) and OENpDps5 (Black) under 0.5, 1.0, 1.5, 2.5, 3.5 and 5.0 mM $H_2O_2$. Growth was determined by Chl-*a* concentration (µg $mL^{-1}$) during 5 days of culturing in 6-well culture plates. Each sample was measured in biological and technical triplicates and the error bars indicate standard deviations. Adapted from paper II.

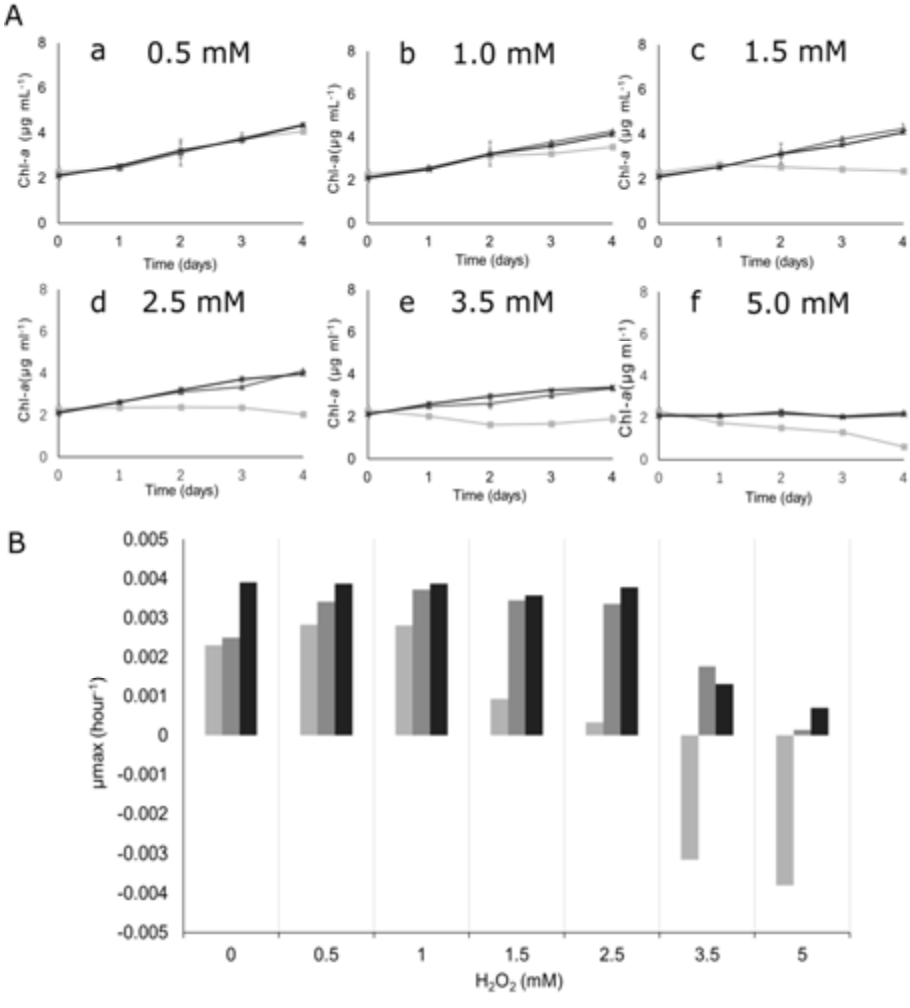


*Figure 6.* Growth of *N. punctiforme* strains: Control, OENpDps2 and OENpDps5 in ammonium supplemented conditions under different concentration of added $H_2O_2$. (A) Growth of Control (Light gray bars), OENpDps2 (Gray bars) and OENpDps5 (Black bars), under $H_2O_2$ concentrations of 0.5 (a), 1.0 (b), 1.5 (c), 2.5 (d), 3.5 (e) and 5.0 mM (f). (B) The specific growth rates of Control (Light gray), OENpDps2 (Gray) and OENpDps5 (Black) under 0.5, 1.0, 1.5, 2.5, 3.5 and 5.0 mM $H_2O_2$. The growth was determined by Chl-*a* concentration (µg $mL^{-1}$) during 5 days of culturing in 6-well culture plates. Each sample was measured in biological and technical triplicates and the error bars indicate standard deviations. Adapted from paper II.

## NpDps2 and NpDps5 proteins are involved in protection to light stress.

From the perspective of large scale bioproduction using cyanobacteria, photodamage induced by high light is still an issue [50]. An increased tolerance towards high light can provide a better intercellular balance between energy input (from light) and energy consumption mainly through cellular metabolism [122].

The *Npdps* deletion mutants were used to reveal if any of the NpDps proteins would be particularly relevant to cellular tolerance against light stress (Paper I). To explore the physiological roles of NpDps proteins and the possibility to use them for improvement of cellular robustness, the OENpDps2 and OENpDps5 were investigated under high light intensity treatments (Paper II).

In the first study, Δ*Npdps* strains were used for discovering the physiological function, and the impact of NpDps2 and NpDps5 on growth, and on photosynthetic activity under different light regimes (Fig. 7).

The Δ*Npdps2* strain showed reduced growth at both 70 and 200 µmol photons $m^{-2}$ $s^{-1}$ (Fig. 7). The Δ*Npdps5* strain showed a more severe reduced growth under 70 and 200 µmol photons $m^{-2}$ $s^{-1}$, and even at low light (20 µmol photons $m^{-2}$ $s^{-1}$) (Fig. 7). Those results indicate that NpDps2 and NpDps5 proteins play prominent roles in *N. punctiforme* for acclimation to different light intensities, but the mechanism of protection is probably different due to the phenotypic differences between the respective mutant strain (Paper I).

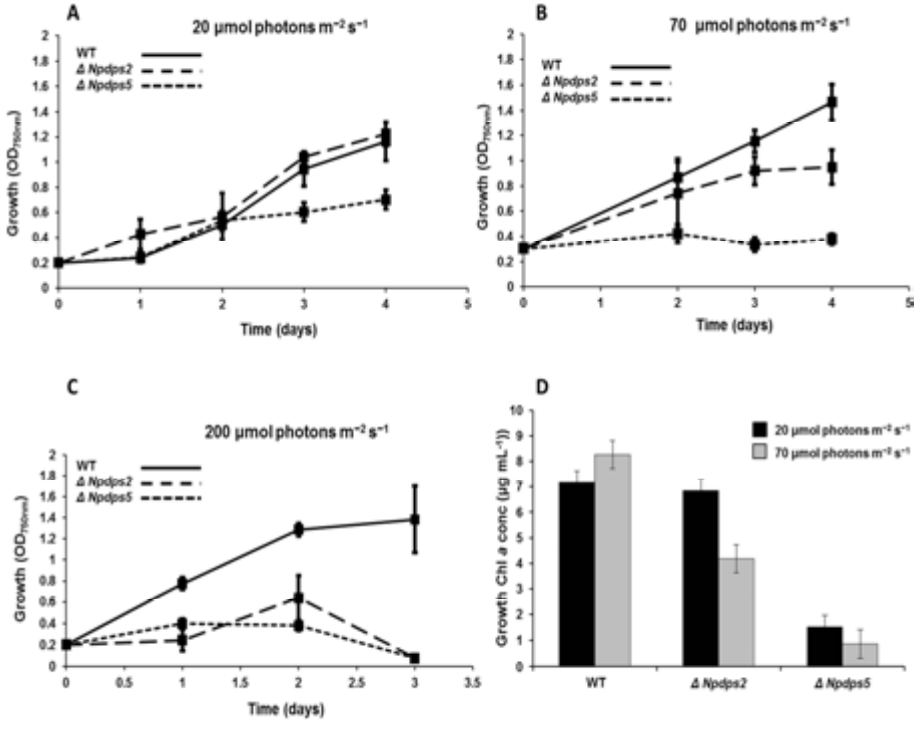


*Figure 7.* Growth properties of WT *N. punctiforme* and engineered strains Δ*Npdps2* and Δ*Npdps5* in diazotrophic conditions under different light intensities (Paper I). Cells grown at different light intensities 20, 70 and 200 µmol photons $m^{-2}$ $s^{-1}$ are shown in panels, A, B and C respectively. The strains are presented as wild type (WT) (continuous line), Δ*Npdps2* (dashed line) and Δ*Npdps5* (dotted line) Growth at 200 µmol photons $m^{-2}$ $s^{-1}$ were terminated after 3 days due to lack of growth. Panel D shows Chl-*a* concentration (µg $mL^{-1}$) of *N. punctiforme* cultures after 4 days of growth, at 20 µmol photons $m^{-2}$ $s^{-1}$ (black bars) and 70 µmol photons $m^{-2}$ $s^{-1}$ (grey bars) illumination. Adapted from paper I.

To investigate if an increased abundance of these proteins could further enhance the tolerance to high light, the growth (Chl-*a* concentration) of OENpDps2 and OENpDps5 strains were analyzed at both, moderate light (60 µmol photons m-² s -¹) and high light (500 µmol photons m-² s -¹) (Paper II). The OENpDps and the Control strains grow to a similar Chl-*a* level at medium light regimes in both growth conditions (Fig. 8A1 and B1). At high light and diazotrophic growth both NpDps5 and NpDps2 showed a lower level of Chl-*a* at day 4 as compared to Control (Fig. 8A2). The results indicated that the overexpression of NpDps proteins are a burden to the diazotrophic grown cells at high light intensities (Paper II).

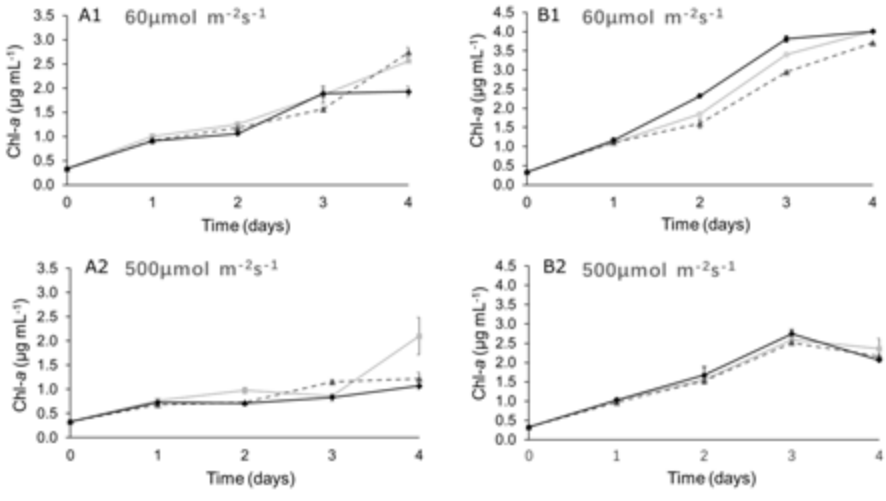


*Figure 8.* Growth of *N. punctiforme* overexpression strains under different light intensities. Growth curves of Control, OENpDps2 and OENpDps5 under diazotrophic growth (A1, A2) and $NH_4^+$-supplemented growth (B1, B2) for four days under 60 µmol photons $m^{-2}$ $s^{-1}$ (A1, B1) and 500 µmol photons $m^{-2}$ $s^{-1}$ (A2, B2). Each sample was measured in biological and technical triplicates and the error bars indicate standard deviation. The strains are represented as: Control (light gray line), OENpDps2 (dark gray dashed line) and OENpDps5 (black line) respectively. Adapted from paper II.

The photosynthetic fitness of both the NpDps overexpression and deletion mutants were determined by $O_2$ evolution activity (Berman-Frank *et al.*, 2003). After four days of growth under different light intensities, the Chl-*a* based $O_2$ evolution values of both overexpression and Δ*Npdps* strains were measured. Under moderate light condition (70 µmol photons $m^{-2}$ $s^{-1}$), the Δ*Npdps5* strain showed increased $O_2$ evolution activity per Chl-*a*, followed by Δ*Npdps2* strain, compared with WT, in diazotrophic conditions (Paper I). For the overexpression strains it was the opposite, OENpDps2 clearly showed higher $O_2$ evolution activity than OENpDps5 and Control, in both diazotrophic and $NH_4^+$ -supplemented conditions, under 60 µmol photons $m^{-2}$ $s^{-1}$ (see Fig. 5 in Paper II).

The amount of photosynthetic pigments per cell were measured as Chl-*a*/$OD_{750}$ ratio [125]. Under moderate light condition (70 µmol photons $m^{-2}$ $s^{-1}$), the Chl-*a*/$OD_{750}$ ratio of Δ*Npdps5* strain was 1.6 times lower than for the Δ*Npdps2* strain, while 2.5 times lower than the WT, after four days of growth in diazotrophic conditions (see Table 3 in Paper I). The Chl-*a*/$OD_{750}$ ratios of the OENpDps strains were less than 30% than of the Control under both moderate and high light, in $NH_4^+$ -supplemented growth condition (Table 1). However, when cultivated in diazotrophic conditions under high-light intensity (500 µmol photons $m^{-2}s^{-1}$) the OENpDps5 strain had a Chl-*a*/$OD_{750}$ ratio which

was 25% higher as compared to OENpDps2 and Control under the same conditions. This result indicated that OENpDps5 under high light and diazotrophic growth condition, has a better protection of photosynthetic proteins than OENpDps2 and Control.

Under 500 µmol photons $m^{-2}$ $s^{-1}$, the Chl-*a*/$OD_{750}$ ratio of both OENpDps strains cultivated in $NH_4^+$-supplemented growth were lower as compared to in diazotrophic condition (Table 1).

Table 1. Chl-*a*/$OD_{750}$ ratios of *N. punctiforme* OENpDps2, OENpDps5 and Control strains after the 4th day of growth under different light intensities. Adapted from Paper II

| | $NH_4^+$ supplemented | | Diazotrophic | |
|---|---|---|---|---|
| **µmol photons $m^{-2}$ $s^{-1}$** | 60 | 500 | 60 | 500 |
| **Control** | 3.9 | 2.3 | 4.6 | 2.4 |
| **OENpDps2** | 2.8 | 1.5 | 2.3 | 2.4 |
| **OENpDps5** | 2.3 | 1.8 | 3.3 | 3.0 |

By considering the higher $O_2$ evolution/Chl-*a* (see Fig. 5 in Paper II) in relation to the lower Chl-*a*/$OD_{750}$ (Table 1) in OENpDps2 strain, as compared to Control under 60 µmol photons $m^{-2}s^{-1}$ it seems that OENpDps2 could compensate the lower amount of chlorophylls per cell by increasing the photosynthetic efficiency (Table 2).

Table 2. $O_2$ evolution/$OD_{750}$ ratios of *N. punctiforme* OENpDps2, OENpDps5 and Control strains after the 4th day of growth under different light intensities. Adapted from Paper II

| | $NH_4^+$ supplemented | | Diazotrophic | |
|---|---|---|---|---|
| **µmol photons $m^{-2}$ $s^{-1}$** | 60 | 500 | 60 | 500 |
| **Control** | 726.57 | 415.771 | 678.04 | 652.08 |
| **OENpDps2** | 950.32 | 219.39 | 496.34 | 559.2 |
| **OENpDps5** | 414.92 | 425.214 | 509.85 | 648.3 |

Too high concentration of ammonia has been revealed as damaging to the manganese cluster in the PSII oxygen-evolving complex [126]. The toxic effect of ammonia can be triggered by high light intensity and lead to PSII photoinhibition, thus the PSII performance will decreases [127].

Under high light conditions (500 µmol photons $m^{-2}$ $s^{-1}$) Control and OENpDps2 strains displayed lower $O_2$ evolution activities under the $NH_4^+$-supplemented condition than in diazotrophic growth (see Fig. 5 in Paper II). This decrease may be due to increased intolerance to ammonium when the cells are under high light stress. However, OENpDps5 strain showed an enhanced $O_2$ evolution per Chl-*a* under high light, in ammonium-supplemented conditions, as compared to Control and OENpDps2. Suggesting that overexpression of NpDps5 indirectly improved the tolerance to ammonium toxicity

(see Fig.5 in Paper II). These results clearly suggest that in ammonium-supplemented growth under high-light intensity, PSII activity was affected negatively, but the presence of high levels of NpDps5 did reduce this effect. By considering that both Control and OENpDps strains were showing similar lower $O_2$ evolution/Chl-*a* ratio than OENpDps5 strain under high light, the conclusion of this result was that NpDps5 enhanced the tolerance to ammonium toxicity by enhanced stability of the PSII repair cycle.

To investigate the photosynthetic activity differences in the deletion strains more thoroughly, the PSII/PSI ratio was calculated by immunoblot analysis, quantifying the PsaB and PsbA proteins. Interestingly, the Δ*Npdps5* mutant presented a significant increase of PSII/PSI ratio with four times higher levels than in WT under moderate light (Table 3). This result could be explained as follow. Under normal growth conditions, the two photosynthetic reaction centers PSII and PSI have different abundancy in WT *N. punctiforme*. The PSI complexes are three to five times more abundant, than the PSII complex in the thylakoid membranes [128]. The number of chlorophylls involved in electron transfer reactions which act as terminal acceptors within the iron-sulphur (FeS) type reaction center of PSI is higher than in PSII [129]. The TyrD*EPR signal, indicating the number of PSII protein complexes, of the Δ*Npdps5* strain was only 20% higher than in the WT (see Fig. 5 in Paper I), Thus, in this study the increase of the PSII/PSI ratio in Δ*Npdps5* as compared to in WT indicates a decrease concentration of PSI in the cell, rather than an increase of PSII. (Table 3). In summary, these results indicated that NpDps5 protein is involved in PSI stability in heterocysts under light induced oxidative stress.

Table 3. Comparison of PSII/PSI ratio by immunoblot analysis in *N. punctiforme.* Δ*Npdps2* strain and Δ*Npdps5* strain. Proteins were extracted after four days of cell growth at a light intensity of 70 μmol photons $m^{-2}$ $s^{-1}$. Adapted from Paper I

| Strain | PSII/PSI Ratio (mean± SD) |
|---|---|
| WT | 0.25±0.06 |
| Δ*Npdps2* | 0.29±0.07 |
| Δ*Npdps5* | 1.01±0.36 |

To test if the phenotypes seen were due to the lack of the specific protein and not any other secondary effects, the *Npdps2 and Npdps5* were expressed in trans in the respective deletion strain Δ*Npdps2* and Δ*Npdps5*. Under 150 μmol photons $m^{-2}$ $s^{-1}$, the Chl-*a* level of the complemented strains reached 80% of the Chl-*a* level in WT after 6 days of diazotrophic growth (Paper I). This result suggested that it is the absence of NpDps5 that lead to severe damage on the photosynthetic apparatus. The sensitivity of *Npdps* deletion strains towards high light can be restored by complementation of the NpDps protein by homologues expression of the *Npdps* on a self-replicating vector (Paper I).

## Co-regulation and interactions between the five NpDps proteins.

According to earlier quantitative proteomic studies, several nitrogen depletion related proteins were revealed to also be involved in redox responses [34,96,97,130]. One phylogenetic analysis of ferritin-like proteins showed the phylogenetic relations of the five NpDps proteins in *N. punctiforme*[34]. Four of them, NpDps1-4, encoded by *Npun_R3258*, *Npun_F3730*, *Npun_R5701* and *Npun_R5799*, were grouped in the Dps clade, while the NpDps5 (*Npun_F6212*) clusters with the bacterioferritins (Bfr) clade [34]. Even though NpDps1-4 all grouped into Dps clade they were members of four different Dps-subgroups. The results indicate that these proteins have different and specific functions.

Besides of the individual physiological roles of the NpDps in $H_2O_2$ or light-induced ROS stress, the study also focused on possible interactions among different *Npdps* genes on transcription level. The results of the deletion strains clearly showed that the NpDps proteins are not physiological redundant under the tested conditions. It was not known if there was some interaction of the transcriptional regulation of the *Npdps* genes, or with the *Npdps* genes and other genes. Therefore, the co-transcription and putative co-regulation of *Npdps* and other genes within the identified operons were studied by transcriptional analysis (Paper I).

The gene cluster organization and the transcriptional regulation of all five *Npdps* are shown in Table 4 and Figure 9. *Npdps1, Npdps2* and *Npdps5* are transcribed together with other genes in operons, while *Npdps3* and *Npdps4* are both single transcribed *Npdps* genes (Fig. 9).

Table 4. Functions of upstream and downstream genes of the the five *Npdps* genes. Transcription start site (TSS) of each *Npdps* gene is indicated in the 5'RACE results. The orthologous gene in *Anabaena* sp. PCC 7120 is listed

| ***Npdps* Genes** | **TSS location** (5'RACE) | **Upstream** | **Downstream** | **Orthologs** |
|---|---|---|---|---|
| ***Npdps1*** *(Npun_R3258)* | +25 nt | ***Npun_R3257*** Hypothetical protein and function as cation transport regulator | ***Npun_R3259*** Low temperature-induced protein | *all0458* |
| ***Npdps2*** *(Npun_F3730)* | not determined | ***Npun_F3729*** Nuclease Sulfiredoxin-like protein | ***Npun_F3731*** Co-chaperone GrpE and function group is Heat Shock Chaperone of Heat shock (GrpE) | *all4145* |
| ***Npdps3*** *(Npun_R5701)* | +25 nt | ***Npun_R5700*** Pyrin domain-containing protein | ***Npun_R5702*** Aldo/keto reductase | *all1173* |
| ***Npdps4*** *(Npun_R5799)* | +44 nt | ***Npun_R578*** Carbamoyl-phosphate synthase, large subunit | ***Npun_F5800*** Hypothetical protein | *alr3808* |
| ***Npdps5*** *(Npun_F6212)* | +132 nt | ***Npun_F6211*** Hypothetical protein | ***Npun_F6213*** Iron permease FTR1 | *all3940* |

The results from transcript abundance study revealed that when *Npdps2* has been knocked out, other *Npdps* genes in *N. punctiforme* showed a different transcript abundance level (Paper I). *Npdps1* transcript level was increased in the Δ*Npdps2* strain as compared to in WT. *Npdps1* and *Npdps2* are the closest homologous among the five *Npdps* [34], which is pointing towards the possibility that these proteins have similar functions. However, the transcriptional regulation could also be different in two homologous genes (Paper III). Our results, and results from others, indicate that this might be the case, since *Npdps1* was shown to be co-expressed with two membrane proteins, encoded by *Npun_R3257* and *Npun_R3259* which are annotated as low temperature and a desiccation protein respectively (Table 4). Furthermore the homologous gene of *Npdps1* in *Anabaena variabilis* strain M3 encodes a low-temperature and salinity induced protein Lit46.2 [131]. The results above points towards a more specific role of NpDps1, perhaps in protection against temperature and salinity stress.

The NpDps2 protein, is a typical Dps protein expressed in both heterocyst and vegetative cells but more abundance in vegetative cells in *N. punctiforme* [34]. Our results show that NpDps2 is a key player in $H_2O_2$ tolerance. *Npdps2* was co-expressed with a gene encoding a redox-dependent sulfiredoxin-like protein *Npun_F3729* and a heat-shock chaperone-like protein gene *Npun_F3731* (Fig. 9). Interestingly, the sulfiredoxin-like *Npun_F3729* protein is a homolog of SrxA protein, which has been reported as an activator of peroxiredoxins (Prxs) for the reduction of $H_2O_2$ in *Anabaena* sp. PCC 7120 [132]. In consideration of the similarity of genome organization in *Anabaena* sp. PCC 7120 and *N. punctiforme* [133], there is a possibility that SrxA and NpDps2 are co-regulated for a more efficient handling of oxidative stress. However, in Paper I, the results showed that by overexpressing *Npdps2* on a shuttle vector in the Δ*Npdps2*, the $H_2O_2$ sensitive phenotype of Δ*Npdps2* strain was recovered, which also signified the importance of the NpDps2 protein in $H_2O_2$ tolerance.

A promoter-GFP reporter study located the *Npdps5* (*Npun_F6212*) expression specifically to heterocysts[34]. The heterocyst specific *Npdps5* was co-expressed with *Npun_F6211* and *Npun*_F6213, annotated as an iron permease (Fig. 9). Considering the transcript abundance results on *Npdps2* in Δ*Npdps5* strain (Paper I), the absence of *Npdps5* had no effect on the *Npdps2* expression level, indicating that NpDps5 protein has a different function compared to NpDps2 in oxidative stress tolerance.

With the analyses of the genetic context of the five *Npdps* genes in *N. punctiforme,* some putative functions of the proteins encoded by the *Npdps* gene clusters have been revealed. I concluded that with an interdependent regulation, multiple NpDps proteins are possibly involved in response to multiple stress tolerance.

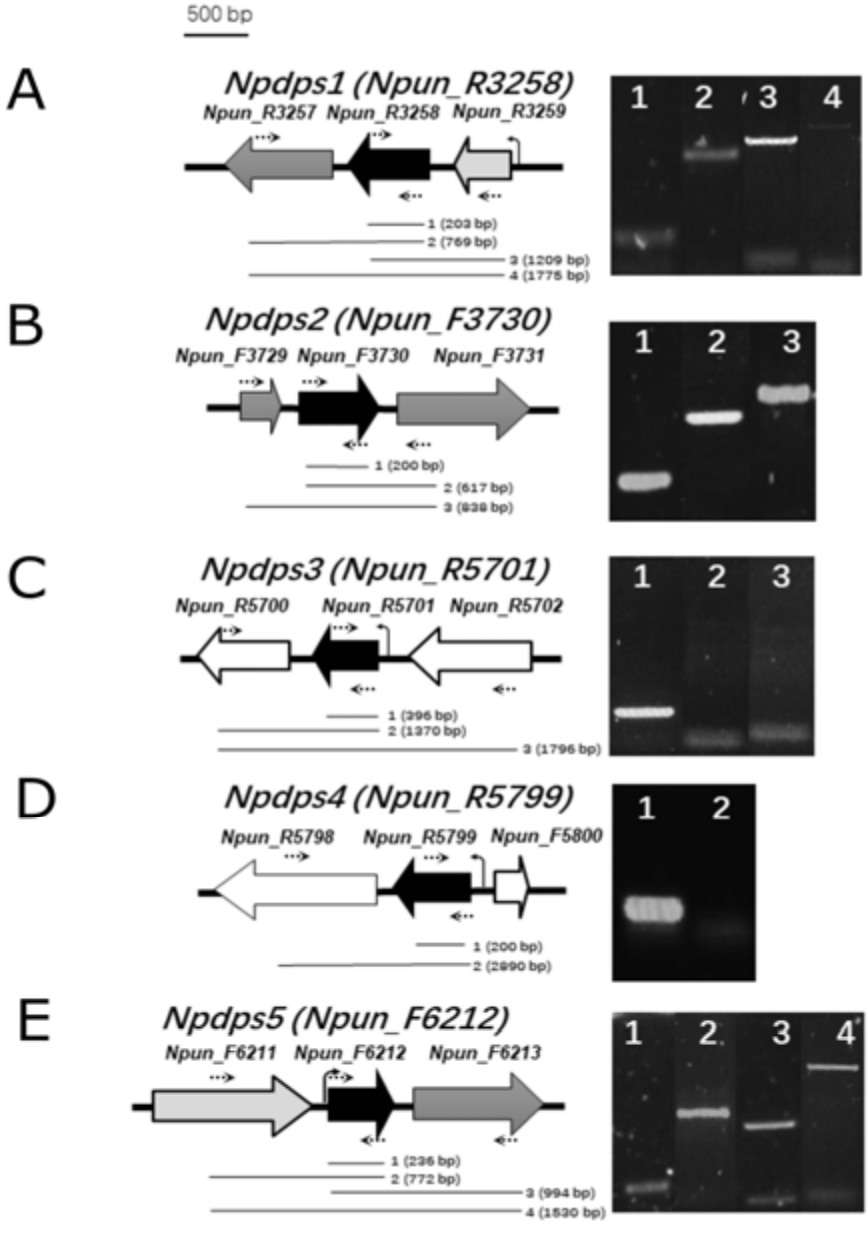


*Figure 9.* Organization of the genetic loci harboring the *Npdps* genes in *N. punctiforme*. *Npdps* genes are shown in black arrows. Upstream and downstream genes that are co-expressed with the *Npdps* genes are presented as grey arrows. The dotted black arrows represent the location of RT- PCR primers. The respective product sizes are indicated with black lines below the gene models. The location of the TSS are indicated by a black full line arrow. The amplified products are shown as cDNA bands on the agarose gels to the right. The numbers indicated on top of each band corresponds to the number of the RT-PCR products sizes below the gene models.

## The NpDps5 protein and the protection of photosynthesis

In the experiments of this study, both NpDps2 and NpDps5, were shown to have important physiological roles in the protection against light induced oxidative stress (paper I) and presented an increased capacity of protecting against $H_2O_2$ and to some extent to light induced oxidative stress (paper II). As a typical Dps protein, the function of NpDps2, as a ROS detoxifier, can be easier understood than the function of NpDps5. There are two types of ferritin-like proteins for iron-storage in bacteria, the ferritin (Ftn) and the heme-containing bacterioferritin (Bfr). Unlike other NpDps proteins in *N. punctiforme*, NpDps5 protein was in a phylogenetic study classified as a bacterioferritin (Bfr) [34]. If NpDps5 is a Bfr, as suggested, the function would mainly be in iron-storage. However, the interesting physiological results on NpDps5 implies a strong connection between NpDps proteins, iron-storage, $H_2O_2$ scavenging and protection of the photosystems.

The absence of NpDps5 could affect the level of free iron in the Δ*Npdps5* strain under diazotrophic growth. Insufficient iron availability in heterocysts will affect the function of the nitrogenase, which is an iron-rich metalloenzyme [42]. This will lead to a reduced function of nitrogen fixation [26], and to nitrogen starvation. Moreover, in the heterocysts, where the iron-rich PSI protein complex is highly active [128], the absence of NpDps5 protein might indirectly result in an increased damage of PSI. That is possibly one reason to the increased ratio of PSII/PSI ratio identified in the Δ*Npdps5* strain under diazotrophic growth. Another interpretation of the results concerning the function of NpDps5 might be that without NpDps5 the iron storage capacity of the cell is reduced and thus the level of free iron is increased. High levels of $Fe^{2+}$ is toxic to the cells and might produce hydroxyl radicals in the presence of $H_2O_2$ and cause severe damage too, for example the PSI protein complex.

The combined results of the studies from the Δ*Npdps5* and OENpDps5 strains, suggest that the NpDps5 protein can be indirectly involved in the repair system of photosynthetic apparatus (Paper I and II). The explanations are as follow: The availability of iron (Fe) plays a fundamental role in the photosynthetic electron transport chain, specifically, the PSI (12 iron atoms), PSII (3 iron atoms), Cyt b6f (6 iron atoms) and the phycobilisomes synthesized by iron-containing proteins [134–136]. Therefore iron limitation will influence the formation of those photosynthetic complexes and affect the electron transport chain [134].

A sufficient iron supply on the other hand will improve the efficiency of formation of both photosynthetic complexes. Hence the electron transport

chain may support more photon for the ATP synthesis [134]. To meet the cellular requirements for iron, ferritins such as Bfr and Dps, must present flexibility of iron flux [137]. With more NpDps5 in the cell, the capacity of capturing iron as an $Fe^{3+}$ mineral deposit will increase, and this will likely lead to a higher potential of releasing $Fe^{2+}$ as a response to the environmental deficiency [137,138]. As a consequence, the PSII complex can be repaired faster in the OENpDps5 than in the Control and OENpDps2 strains, and thus be less sensitive to to ammonium-induced photodamage (Paper II). The function of NpDps5 involved in $H_2O_2$ scavenging (Paper II) can also be explained as an improved efficiency of capturing the excess amount of iron. Thus, the amount of ferrous iron that could be oxidized by $H_2O_2$, in the Fenton Reaction, will decrease, and the production of toxic hydroxyl radical ($OH\cdot$) will be reduced [56].

# Summary, the role of NpDps2 and NpDps5 proteins in cell robustness

In this part of the thesis, the results indicate that multiple Dps proteins in *N. punctiforme* could have specific physiological functions, spatial distributions as well as interdependent regulation on both the transcript and protein levels. The physiological study with different $H_2O_2$ treatments and high light conditions revealed the specific *in vivo* roles of individual NpDps proteins, especially NpDps2 and NpDps5. The organization of genetic operons and the possible co-regulation of the five *Npdps* genes with other genes were investigated. All this study confirms the important role of NpDps2 protein in $H_2O_2$ induced oxidative stress tolerance as well as the role of NpDps5 protein in iron homeostasis and light acclimation. The Dps-mediated link between photosynthesis, $H_2O_2$ detoxification and iron homeostasis has also been revealed. The iron storage function of NpDps5 protein has been shown to indirectly help in protection of the photosystems, and in $H_2O_2$ tolerance, by Controlling the concentration of ferrous iron. The functions of NpDps2 and NpDps5 protein in cell robustness of *N. punctiforme* are summarized in Figure 10.

Additionally, the feasibility of using NpDps protein for enhancing cell robustness in multicellular cyanobacteria was evaluated. The positive results indicated the potential of NpDps2 and NpDps5 proteins as novel biological tools to improve cell robustness in *N. punctiforme*. The results of this thesis improved the knowledge, and identified novel players, of oxidative stress protection in multicellular cyanobacteria. It will be a foundation for resolving the oxidative tolerance problem during biotechnological applications in cyanobacteria.

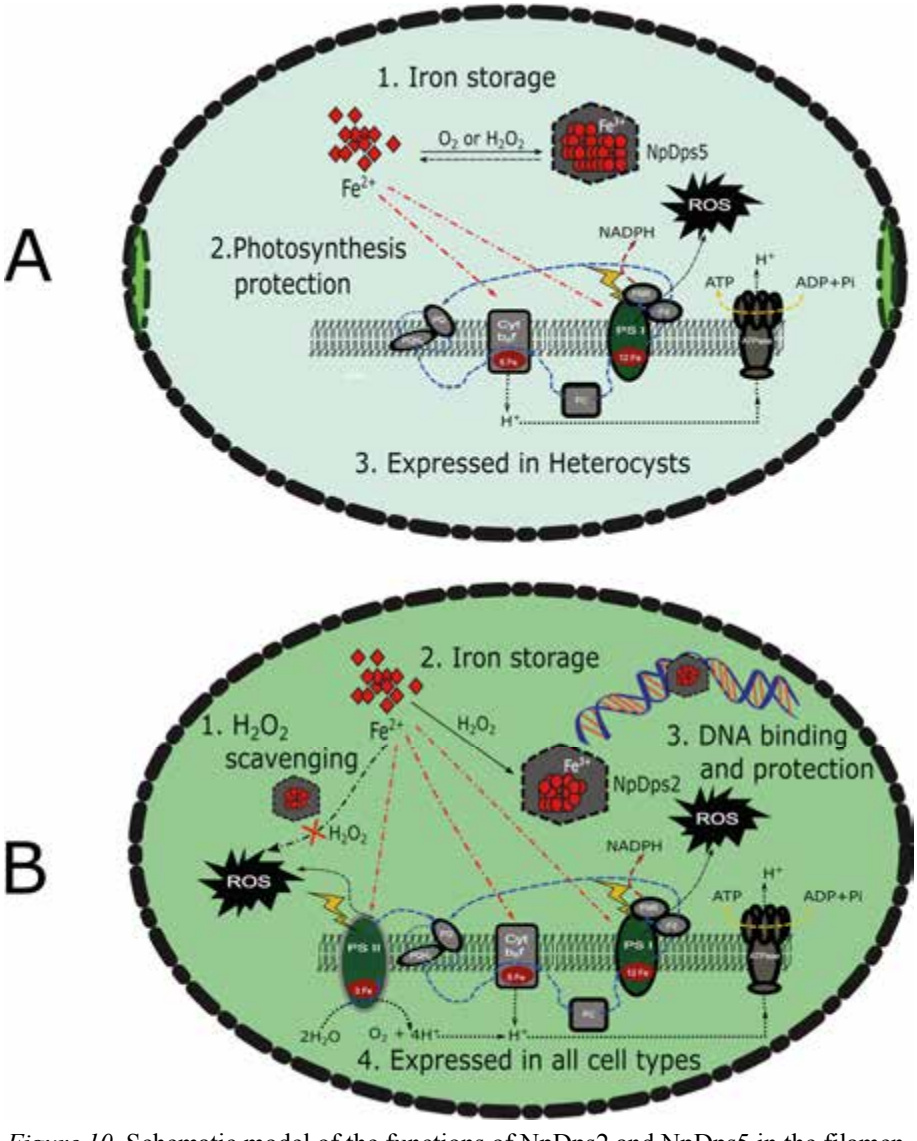


*Figure 10.* Schematic model of the functions of NpDps2 and NpDps5 in the filamentous cyanobacteria *N. punctiforme*. A summary of the functions of NpDps2 (B) and NpDps5 (A) based on results from *N. punctiforme* in this thesis.

# Part II Synthetic heterocyst specific promoters (Paper III and IV)

## Specific aims of part II

1) To design promoter parts as synthetic biology tools for biotechnological applications. The parts should: (i) be short enough to be easily assembled with other genetic parts, (ii) be well characterized with clear function, and (iii) and give heterocyst specific expression.
2) To expand the knowledge of early heterocyst differentiation, on transcriptional level, using the knowledge from these promoter tools.

## Results and discussion

One of the most important considerations in synthetic biology applications is the strategic choice of promoters. In conventional synthetic biology tool boxes, most of the well characterized promoters, whether constitutive or inducible, were isolated from native genomes and modified by truncations or randomized point mutations [139]. There are many means of increasing the speed of promoter design and engineering. For example High through-put approaches such as error-prone PCR mutagenesis have been successful [139]. I named this way of characterizing and engineering promoters from long native ones, without detailed knowledge about the function of each promoter element, as "top-down approach". Nonetheless, even though a desired promoter could be obtained by the "top-down approach", we still have a non-precise knowledge about the accurate function, or even the location, of each promoter element of importance. However, huge challenges still remains of improving and modifying promoters for synthetic biology requests, especially in cyanobacteria [82]. Meanwhile, the well-characterized, robust and orthogonal promoter libraries are now becoming highly desired for synthetic biology in high-precision laboratory applications, or larger commercial-scale production [3,140].

Sophisticated knowledge of different promoter elements is also required in computational and statistical analyses for *de novo* promoter design [139].

In parallel with the physiological function characterization of the NpDps proteins, this study also aimed to obtain more knowledge about the mechanisms that confer cell specificity and stress-specific induction of these Dps proteins in *N. punctiforme*. This part of the study was focused on the heterocyst differentiation and on promoters that confer cell-type specificity. Another target for the study was to design and characterize a synthetic promoter with heterocyst specificity for biotechnological applications in heterocyst-forming cyanobacteria (Paper IV).

Quantitative and qualitative approaches were employed to characterize the activities of the synthetic promoters. The designed and constructed promoters were verified with a standardized Promoter-eYFP reporter construct. The performance was examined *in vivo* by fluorescence using confocal microscopy and flow cytometry. With this method, it was possible to both locate the promoter activity and quantify the promotor strength at single cell level.

In this study, one of the aims was to explore how different promoter elements coordinates their regulation to shape the function of heterocyst specific expression (promoter elements characterization). This knowledge is of importance for us in our strive to reach the aim, which was to construct novel synthetic promoters for desired biotechnological applications in cyanobacteria For instance to introduce heterologous hydrogenases into the heterocysts for efficient bio-hydrogen production [141]. The strategy in the thesis was to use a "bottom-up approach", in which, based on a clear knowledge of the functions, different putative regulatory promoter elements were assembled with short randomized sequences as spacers in between.

## Heterocyst specific promoters and the DIF motif

The first promoters to be investigated were the promoters of *Npdps4* (*Npun_R5799*) and its orthologous gene, *alr3808*, in *Anabaena* sp. PCC 7120. Compared to the *alr3808* gene which has two transcriptional start sites (TSS1 and TSS2) in the upstream region [95], only one TSS was identified for *Npdps4* under both ammonium supplemented and $N_2$-fixing growth conditions. This TSS had the identical location to the previously identified TSS2 of *alr3808* (Fig. 11) (Paper III).

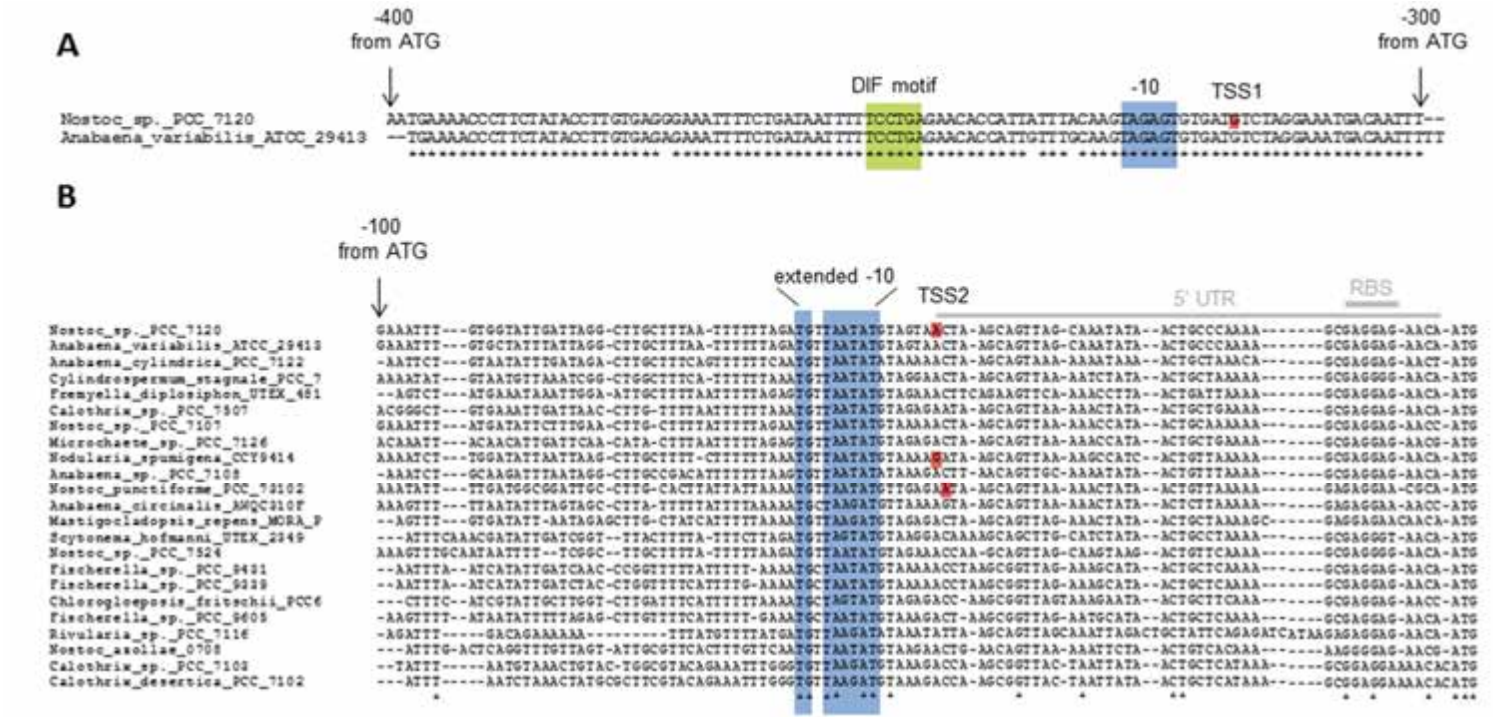


*Figure 11.* Sequence alignment of promoter regions of *dps* genes orthologous to *Npdps4* from heterocyst-forming cyanobacteria. (A) Alignment of the 5'-region (–400 nt to –300 nt with respect to the translation start site) from the two orthologous *dps* genes in *Anabaena* sp. PCC 7120 and *A. variabilis* ATCC 29413. The DIF motif [95] is depicted by a green box centered at –33.5 nt with respect to the distal TSS (TSS1) in *Anabaena* sp. PCC 7120. (B) Alignment of the 100 nt upstream region of *alr3808*, with respect to the translation start site, including the TSS2, with the corresponding regions of 24 orthologous *dps* genes. Adapted from paper III.

Even though *Npdps4* and *alr3808* are orthologs, their transcriptional regulations are clearly different. Sequence comparisons suggest that the promoter region containing the distal TSS (TSS1) of homologous genes to *Npdps4* is not conserved upstream of orthologous genes among heterocyst-forming cyanobacteria (Fig. 11B). Only the homolog in *Anabaena* sp. PCC 7120 contained a heterocyst specific transcript [95]. Furthermore, after removal of combined nitrogen, only the strain with the TSS1 promoter of *alr3808* (fragment –490 to –215nt with respect to the ATG of *alr3808*) Mitschke *et al.*[95] showed a very early heterocyst specific expression in immature heterocysts, with GFP fluorescence detected at 5.5 hours after nitrogen depletion (Fig. 12).

The confocal microscopy analyses of promoter GFP-reporter strains showed differences in cell-type specificity between the proximal (TSS2) and distal (TSS1) promoter regions of *alr3808* (Fig. 13). In contrast, the complete promoters of both *Npdps4* and *alr3808* were active in all cells. The regulatory properties of the promoters of the two close homologues (*Npdps4* and *alr3808*) are clearly very different, indicating that the *in vivo* function of the encoded proteins might also be different. This is one typical examples where it is shown that proteins encoded by homologous gene sequences do not necessarily have the same *in vivo* role.

The subsequent sequence analyses of two promoter sequences from orthologous *dps* genes in *Anabaena* sp. PCC 7120 and *A. variabilis* ATCC 29413 lead to the discovery of a conserved differentiation- (DIF) motif (5'-TCCGGA-3') in the upstream region of TSS1 (Fig. 11A). This motif has previously been associated with heterocyst differentiation in filamentous cyanobacteria [95]. Additionally, the sequence alignment of 24 *dps* orthologous promoter regions identified a Pribnow-box (-10 sequence) (5'-TAGAGT-3') that was highly conserved (Fig. 11B).

Several promoters have been reported as native promoters for heterologous heterocyst specific expression such as *nif, hupS* and *nsiR1* promotors [98]. The *nsiRI* promoter is a very complex native promoter, that consist of twelve tandem repeats in *Anabaena* sp. PCC 7120. *NsiRI* encodes for a heterocyst specific sRNA named Nitrogen Stress inducible RNA1 (NsiRI) [142]. This promoter has been reported as natively conferring heterocyst specific expression in both immature and mature heterocysts [95,143]. However, the promoter activity has not been characterized in detail yet.

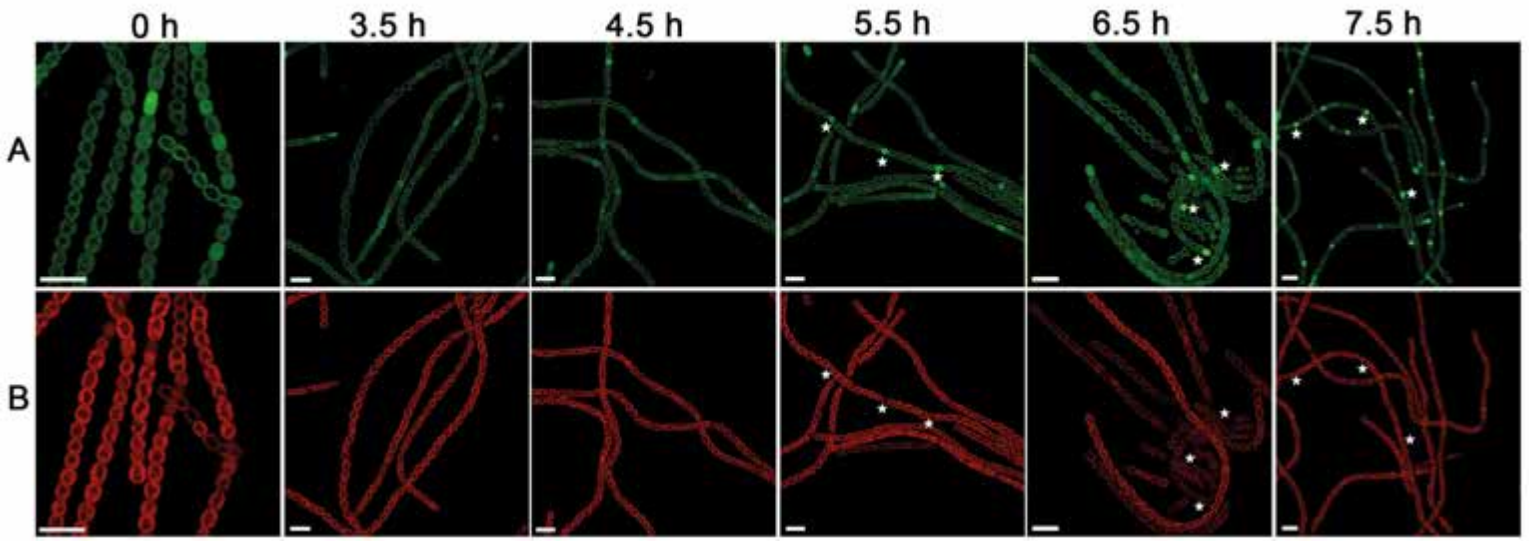


*Figure 12.* GFP fluorescence from the *alr3808* $P_{TSS2}$ *-gfp* construct in filaments of *N. punctiforme* strains following combined nitrogen deprivation. The images on the top and bottom represent GFP- (green color) and auto (red color) fluorescence, respectively. Six time-points during the early stages of heterocyst differentiation are depicted. Scale bars = 10 µm. Adapted from paper III.

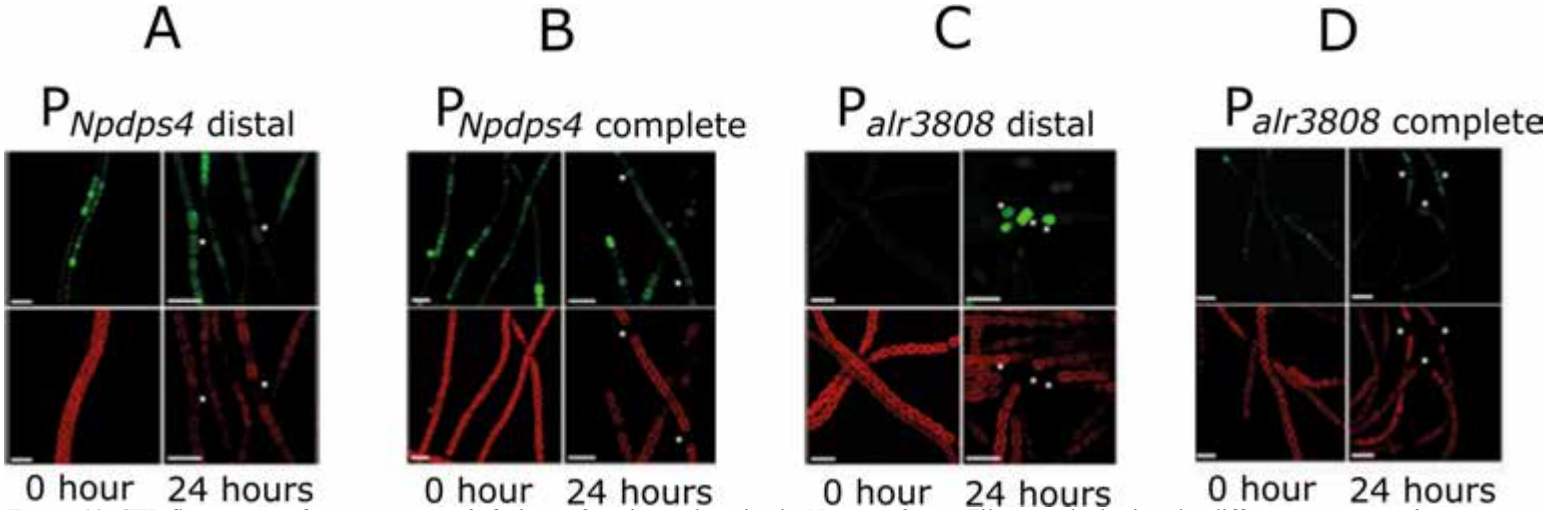


*Figure 13.* GFP fluorescence from promoter-*gfp* fusions of engineered strains in *N. punctiforme*. Filaments harboring the different promoter-*gfp* constructs grown with combined nitrogen (0 hour), or 24 hours after removal of combined nitrogen (24 hours). The Promoter-*gfp* constructs are indicated as (A) distal promoter regions of *Npdps4* (B) complete promoter regions of *Npdps4* or (C) distal promoter regions of *alr3808*, (D) complete promoter regions of *alr3808*, respectively. The distal promoter of *alr3808* includes TSS1. The GFP- and auto-fluorescence are presented as a green or red color, respectively. Heterocysts are distinguished by a reduced auto-fluorescence and indicated by stars. Scale bars = 10 µm. Adapted from paper III.

## Design a synthetic heterocyst specific promoter

The first step of the design of a well characterized synthetic promoter for heterocyst-specific expression was the promoter sequence comparison study based on the literature regarding the *NsiR1* promotor [142–144] in which the so called DIF motif was identified. In order to obtain an overview of the sequence structure of the DIF containing promotors, a sequence alignment for the DIF+ promotors reported by transcriptomic data [95] was done, and presented as a WebLogo (Fig. 14).

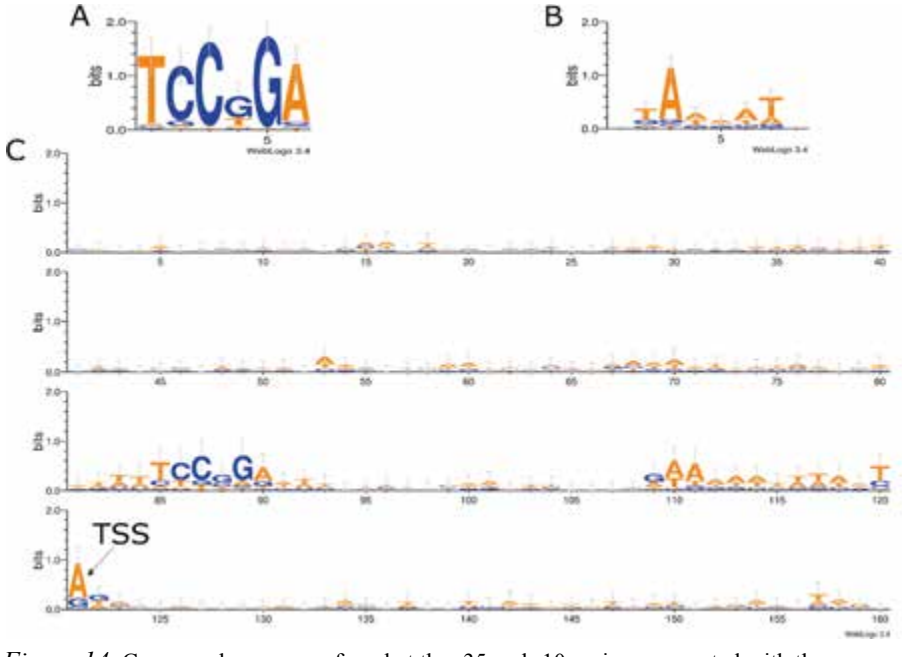


*Figure 14.* Conserved sequences found at the -35 and -10 regions presented with the weighted sequence logo (WebLogo). The size of the letters indicates the frequency of which a certain nucleotide is present at the certain position of the promoter sequence. Two conserved regions around -35 and between the -10 region and TSS were found. Image generated by Weblogo 3.0 [145]. The TSS is indicated in the figure. Adapted from paper IV.

The WebLogo (Fig. 14A and B) shows the sequence alignment result of the putative -35 region and extended -10 region, in all DIF+ promoters reported in the dRNA-seq data of *Anabaena* sp. PCC 7120 [95]. The promoter sequences from 40 nt downstream to 120 nt upstream of TSS of respective DIF+ promoter, which was reported in Mitschke *et al.*, [95] were evaluated. By aligning these promoters, the result showed that these sequences shared not only a conserved DIF motif at the -35 position upstream of the transcription start site, but also a consensus region situated directly upstream of TSS and

also including an extended -10 region (Fig. 14C), which is not similar to the consensus region at -10 reported in the dRNA-seq data [95] (Fig. 14B).

To investigate more details about the sequence specificity in the -10 region of *hetR* related promoters, this study combined transcriptomic data of *Anabaena* sp. PCC 7120 from Flaherty *et al*. [93] , and Mitschke *et al*., [94]. From this investigation 8 DIF+ promoters, out of the 73, were found to be stronger than the *NsiR1* promoter [94,95]. The comparison of those eight promoter sequences showed novel conserved parts in both the -10 region and in the upstream element (UE) of -35. No conserved sequences were found between the -10 and -35 regions. By considering the new findings from the transcriptomic and bioinformatics studies above, the design of synthetic promoter variants based on the consensus sequences from the alignments of Dif+ promoters (Fig. 15).

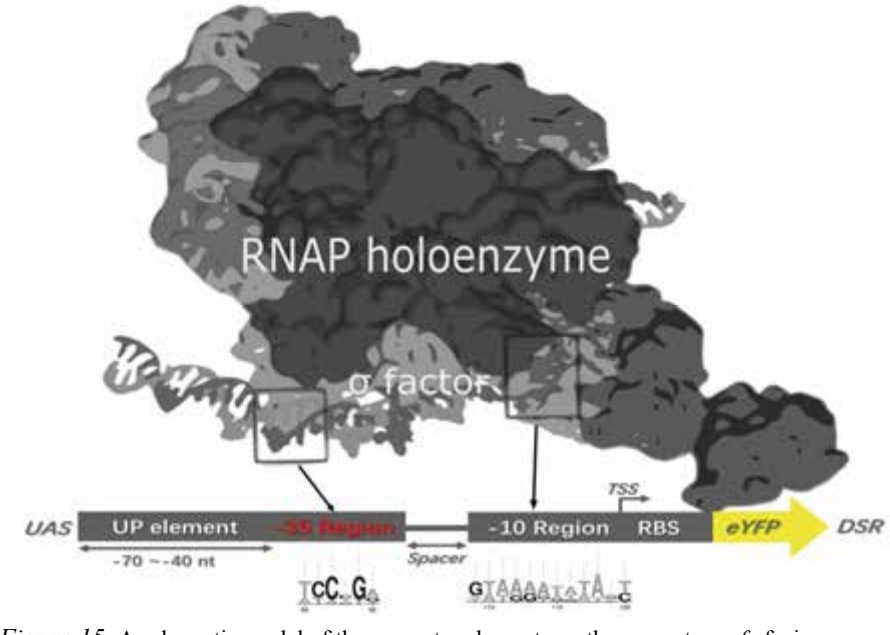


*Figure 15.* A schematic model of the promoter elements on the promoter-*eyfp* fusion construct and binding of the RNA polymerase. The promoter elements shown are: UP element, -35 region, 17nt spacer, -10 region, and transcription start site (TSS). The promoter binding sites at -35 and -10 by sigma factor on RNA polymerase holoenzyme are indicated with arrows. The conserved sequences of the -35 as well as of the -10 regions are depicted as WebLogo below the promoter construct. UAS stands for the upstream and DSR for the downstream part of the constructs, located in the host plasmid.

With deletions and substitutions of certain promoter parts, and by fusing different promoter elements with spacer regions of random sequences, seven promoter-variants were constructed, for a general schematic outline of the promoter elements, see (Fig. 15) The reason for the scrambling of the 17nt non-

conserved sequence between -35 and -10 regions, was to ensure that this sequence (spacer) had no influence on the transcriptional regulation of the promoters. The design of each construct with different promoter variants and eYFP reporter are listed in Figure 16, together with the cell specificity study results.

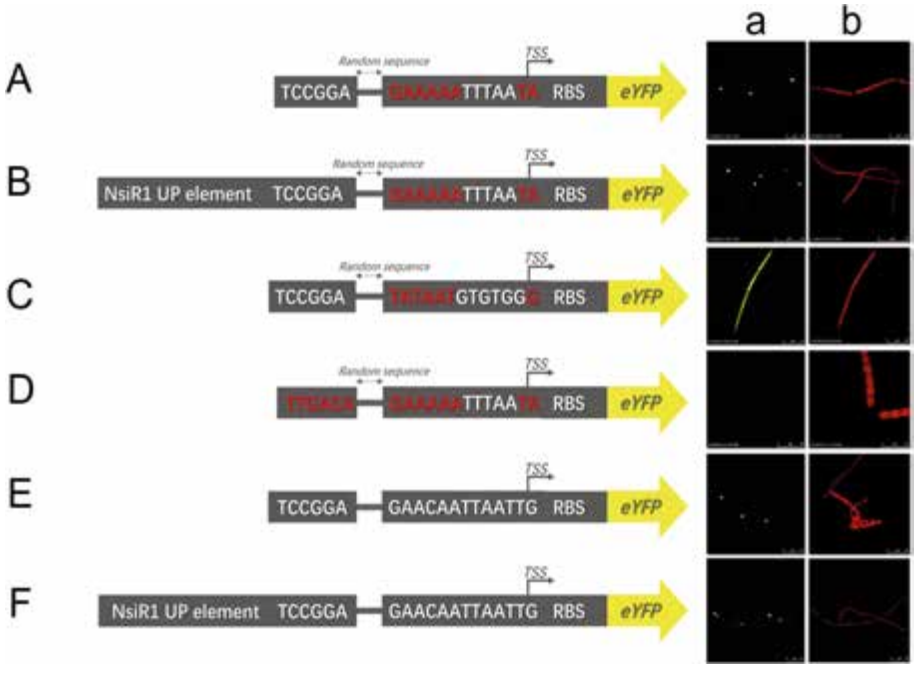


*Figure 16.* Design and the cell specificity study of different promoter-*eyfp* reporter constructs in *N. punctiforme*. The schematic images on the left represent the design of promoter variants with eYFP reporter. The different promoter constructs fused to *eyfp* are indicated as (A) SynDIF and (B) UESynDIF (UE stands for the UP-element) (C) Ptrc-10 change; (D) Ptrc-35 change; (E) NsiR1-core; and (F) NsiR1-long, respectively. The images with black background on the right present the results of confocal microscopy showing promoter activity as fluorescence in *N. punctiforme*. The eYFP fluorescence is shown in (a) and the auto-fluorescence is shown in (b). The heterocysts are distinguished by a reduced auto-fluorescence. Sample from *N. punctiforme* filaments harboring the different promoter-*eyfp* constructs were taken 12 hours after the removal of combined nitrogen. Scale bars = 10 µm. Adapted from paper IV.

The main targets of this investigation were the synthetic promoter versions SynDIF and UESynDIF (Fig. 16A and B). These two promoters were designed with -10 and -35 regions based on the consensus sequences of the promoter sequences alignment study, as shown by the WebLogo descriptions of Figure 14 and 15. Other promoter constructs feature the sequence of the synthetic short promoter (SynDIF) with a substitution at the -10 region (Fig. 16C) or at the -35 region (Fig. 16D). For these particular promoters, the synthetic -10 and -35 regions were substituted with these regions from a well-studied constitutive non-heterocyst-specific $P_{trc10}$ promoter from *E. coli* [118]. The native

promoters (Figs. 16E and F) are both versions of the consensus sequence of the twelve native *NsiR1*-promoter repeats of *Anabaena* sp. PCC 7120 [143]. The difference between the native-core and native-long promoter is that the long version of the promoter features an UP-element consisting of a 25bp AT-rich region further upstream of the -35 site (UE).

**Qualitative analysis of promoter expression**

The eYFP reporter gene was inserted downstream of the promoters as a fluorescent reporter to investigate the spatial distribution of the activity of the promoters in the filamentous cyanobacterium *N. punctiforme* (Paper IV). In order to get a deep insight of the activities of the promoters *in vivo*, confocal microscopy was used to visualize the location of eYFP expression in individual cells of the *N. punctiforme* strains carrying different constructs (Fig. 16). All the promoter-YFP reporter constructs were examined 12 hours after nitrogen depletion. Results from these experiments proved the successful construction of a heterocyst-specific synthetic promoter since the cell specificity expression could be observed in both the two synthetic constructs (Fig. 16A and B) as well as in the native promoter constructs (Fig. 16E and F). It is also shown that the -10 region of the promoter is significant for the cell specificity. The switch of the native -10 region to P*trc*-10 region indeed affected the behavior of the promoter specificity. The cell specificity was not detected at all for the P*trc*-10 change, instead the promoter showed a constitutive expression in both vegetative cells and in heterocysts (Fig. 16C).

Additionally, this study also discovered that the expression of the native *NsiRI* core promoters (Fig. 16E and F) and the UESynDIF promoter (containing the UP-element) (Fig. 16B) were less heterocyst specific as compared to the SynDIF promoter (Fig. 16A). During the early differentiation phase the fluorescence was localized to a selection of roughly four to six cells in a non-random pattern. Only approximately 12 hours after nitrogen depletion the heterocyst specific fluorescence from the reporter proteins were seen in the UP element containing promoter constructs (Fig. 16B and F).

**Quantitative analysis of promoter expression**

To investigate whether the identified fluorescence was merely due to accumulation of eYFP over time, or if it was a change in the promoter activity, the transcript levels of eYFP from the different promoters were determined by RT-qPCR. Furthermore, to provide a better resolution in quantifying promoter strength per single cell, flow cytometry was applied.

Flow cytometry has been shown to be useful when low emission levels of fluorescence protein are to be analyzed [147]. This technique employs the fluidics, optics, and electronic systems in a flow cytometer to preform single cell measurement for the scattered light and fluorescence. Therefore, cell-to-cell variations in a population can be efficiently analyzed [86]. In this study, a method was developed to quantify the intensity of YFP fluorescence in single heterocysts within a multicellular cyanobacterium, by use of imaging flow cytometry.

Cell cultures of engineered *N. punctiforme* strains carrying SynDIF-pSAW and UESynDIF-pSAW were cultivated under nitrogen depleted conditions and cells were collected by centrifugation during the logarithmic growth phase ($OD_{750}$ = 1) after approximately 12 hours. The cells of the filaments were separated by sonication. Then, the fluorescence intensity was analyzed by flow cytometry. A minimum number of 10,000 cells were acquired on an image-based flow cytometer (Merck Amnis FlowSight) and analyzed using analysis software IDEAS. Single cells were identified based on the first gating with area scatter and aspect ratio scatter parameters. Thereafter, the cells were sub-gated with intensity scatter on YFP fluorescence channel, at 488nm channel (for YFP, FITC, AF488, and GFP). In order to isolate the heterocysts, a third step gating was performed. Based on the 488nm channel data, the cells were sub-gated with the intensity scatter on 745-800nm channel, indicating the auto-fluorescence. The targeted single heterocyst cells were selected based on significantly decreased auto-fluorescence compared to in the vegetative cells (Fig. 17B).

The single cell measurement from the flow cytometry experiments showed that the promoter strength of UESynDIF-pSAW was increased twice as compared to SynDIF-pSAW (Fig. 17A). Together with the qualitative study of YFP fluorescence localization, this result indicated the UP-element was functional as a critical promoter part for enhancing the expression level.

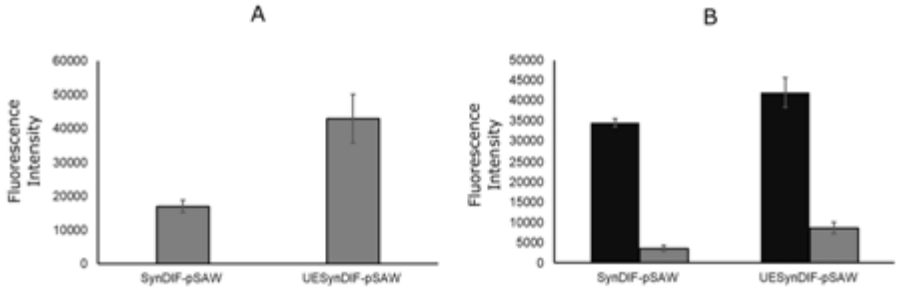


*Figure 17.* Quantitative study of heterocyst specific promoter activity in single cells of *N. punctiforme*. (A) Mean value of specific eYFP fluorescence intensity per heterocyst in *N. punctiforme* strains carrying SynDIF-pSAW and UESynDIF-pSAW constructs. (B) Mean value of specific auto-fluorescence intensity per vegetative cell (black) and heterocyst (gray) in *N. punctiforme* strains carrying SynDIF-pSAW and UESynDIF-pSAW constructs. Sample of the cells was measured after 12 hours diazotrophic cultivation. YFP fluorescence intensity data acquired from Merck Amnis FlowSight was analyzed using IDEAS software. The data represent mean ± SD of triple measurements of three independent cultivations. Adapted from paper IV.

# Summary, Synthetic heterocyst specific promoter

A synthetic heterocyst specific promoter was constructed with a DIF motif. The SynDIF strain with the SynDIF-pSAW plasmid showed clear eYFP fluorescence specific to heterocysts. This result confirmed that our synthetic heterocyst-specific minimal promoter with only 48nt was functional in *N. punctiforme*. This is the shortest promoter ever reported to provide heterocyst-specific expression of a reporter. To further improve the functionality of the SynDIF promoters, additional promoter cis-elements could be assembled on the promoter for additional functions, such as inducibility or enhancement of the promoter activity.

Moreover, comparing with the SynDIF-pSAW construct, the Ptrc-10 changed promoter did not have such specific expression property. the Ptrc-10 changed promoter showed an overall strong YFP fluorescence signal in all cells in both ammonium-supplemented filaments and in filaments after combined nitrogen depletion. This might be an interesting result for further study to understand the regulatory relationship between the DIF motif and the conserved -10 region of the SynDIF promoter. This result also highlights the significance of the -10 region for the heterocyst specificity in the DIF promoters. The results in this thesis have provided a new insight on the DIF promoters as compared with earlier studies on DIF promoters, which have suggested that solely the -35 position holding the DIF-motif is sufficient to form a functional promoter to render heterocyst specificity. A novel method to quantitatively

characterize the promoter activity in single heterocyst has been established with imagine flow cytometry (Flowsight). The data clearly showed that by including the UP-element from the $P_{NsiR1}$ promoter, the promoter activity of SynDIF was improved. This result indicated that the UP-region provides enhanced promoter activity.

Furthermore, the discoveries of the specific transcriptional regulator of the DIF promoters, and the regulatory mechanisms on molecular level, are of high interest. Both for fundamental understanding of cell specific transcriptional regulation, but also for the purpose to use this pair of transcriptional regulator/promoter part (DIF) as a tool for metabolic engineering in cyanobacteria. Considering that the TSS1 promoter of the gene *alr3808* performed heterocyst-specific expression in *N. punctiforme*, the specific sigma factor, or another transcriptional regulator that recognize the DIF motif (5'-TCCGGA-3') in *Anabaena* sp. PCC 7120 is most probable conserved in *N. punctiforme*. The significance of -10 and -35 regions on the synthetic promoter indicated that the transcription factor that regulate DIF promoters could possibly be an alternative sigma factor.

Alternative sigma factors such as SigG, SigC and SigE have been shown to be upregulated under nitrogen starvation [109]. The SigE and SigC have also been shown to regulate genes encoding proteins involved in formation of the polysaccharide envelope during heterocyst development [109]. However, the identification of, and the mechanisms for regulation of these alternative σ factors involved in heterocyst differentiation has not been revealed yet. This is something that deserves to be investigated in the future.

# Conclusion and future perspective

In this thesis, the biological functions of five Dps proteins in *N. punctiforme* have been identified and characterized. Two of the five Dps proteins, NpDps2 and NpDps5, are involved in the tolerance against oxidative stress induced by $H_2O_2$ or high light intensities. The capacity of NpDps2 and NpDps5 to enhance stress tolerance was confirmed by homologous overexpression of these two proteins in *N. punctiforme*. Furthermore, overexpression of NpDps5 gave a significant increase in tolerance of the cells to ammonium induced oxidative stress. The results provide information of how Dps proteins may be utilized in engineering of cyanobacteria for enhanced stress tolerance. This work also established a Dps-mediated link between light tolerance, $H_2O_2$ scavenging, and iron homeostasis, and provides further evidence on the non-redundant role of multiple Dps proteins in this multicellular cyanobacterium.

To resolve the collaborative roles of multiple Dps proteins' interaction involved in abiotic stress in cyanobacteria is one of the future challenges. Also a more detailed biochemical and biophysical characterization of NpDps proteins is still required for the fundamental understanding of the biochemistry of these proteins. The physiological functions of NpDps1, NpDps3 and NpDps4 have yet to be determined. The mechanism of $Fe^{2+}$ release from the iron core of NpDps5 is still unknown. To, in more detail, understand the role of NpDps5 in iron homeostasis, physiological experiments with both the deletion and overexpression strains under different iron concentrations should be developed.

Furthermore, to explore the potential of NpDps2 and NpDps5 proteins as novel biological tools, we need to characterize them more deeply. One strategy would be to design the genetic constructs to: lower the expression, be able to regulate the time of expression, and to determine in what cell types the NpDps proteins should be specifically expressed.

It would be interesting to understand the complete complex regulatory network of ROS scavenging and oxidative stress protection in heterocystous cyanobacteria. In order to investigate how the high number of proteins involved in ROS scavenging work together to protect the cells from different causes of oxidative stress, the changes in transcript or protein abundances on omics-level in the overexpression strains should be analyzed in future study.

Based on conservation of the DIF motif in heterocyst specific promoters, an artificial promoter was designed for expression of oxygen sensitive proteins in heterocysts. The results have provided new insights on the regulation of DIF promoters, which suggested that only together are the -35 region holding the DIF-motif and the -10 region sufficient to form a functional promoter to render heterocyst specificity. Besides, the promoter activity of the synthetic promoter SynDIF can be improved by including the UP-element from the native heterocyst specific promoter $P_{NsiR1}$. The SynDIF promoter is the shortest promoter ever reported to provide heterocyst-specific expression of a reporter, indicating the potential of introducing this minimal promoter in further biotechnological applications.

The cell specific function of NpDps in heterocysts can be investigated by using a cell specific promoter, for example the SynDIF promoter, in future studies. The NpDps5 protein expressed in heterocysts might have a large potential to increase the stability and activity of iron-containing enzymes of importance for more efficient heterocyst-based $H_2$-production.

As for the purposes of utilizing NpDps proteins, and the developed synthetic promoter as novel biological tools for biotechnological purposes, the work in the thesis has shown the possibilities, and made a path for future research. However, concerning the overall aim, improving cell robustness, the journey has just begun!

# Sammanfattning på svenska

## Cyanobakteriella cellfabriker

När vi pratar om lösningar på hantering av industriella föroreningar och avfall och hur vi ska skydda vår miljö och minska klimatpåverkan, så fokuserar vi sällan på hur dessa föreningar har producerats. Kemin är grundläggande för produktion av alla dessa föroreningar och avfall. Som mikrobiell kemist så anser jag att det är min plikt att bidra till mer hållbara produktionsvägar av de produkter/kemikalier som behövs i vårt samhälle. Mitt intresseområde är forskning kring principer för grön industri som baseras på fotosyntetiska mikroorganismer.

Låt oss tänka på de tre mest potenta resurserna vi har på jorden: 1) Koldioxid i atmosfären, som har ett orsakssamband med global uppvärmning, 2) solljuset, vilket är den ultimata energikällan vi har på jorden, och 3) den återvinningsbara källan till vatten. Strategin för den gröna industrin bygger på att vi strävar efter en övergång från ett fossilt samhälle mot ett grönt samhälle. I detta hållbara gröna samhälle bör koldioxid, solljus och vatten användas som substitut för fossila källor. Genom att tillämpa gröna biokemiska principer för produktion kan vi skapa ett samhälle med minskat avfall och utsläpp av koldioxid, och som kräver mindre från vår planets minskade resurser.

Att utnyttja cyanobakterier som produktionsvärdar har visat sig ha stor potential att bidra till en framtida grön bio-baserad ekonomi. Cyanobakterier är prokaryota organismer kapabla att utföra syregivande fotosyntes, en process där koldioxid ($CO_2$) omvandlas till mer komplexa organiska ämnen (biobränslen) med hjälp av solenergi. Cyanobakterierna, eller ”blågröna alger” som de felaktigt kallas i folkmun, står för en stor del av jordens totala koldioxidupptag och är bland de viktigaste primärproducenterna av organiskt kol med ett estimerat bidrag på 20-30% av den totala biomassan. I jämförelse med andra fotosyntetiserande organismer, såsom högre växter, utför cyanobakterier fotosyntes med upp till ca 10 gånger så hög effektivitet. Vidare är har cyanobakterier mycket högre tillväxt, alltså producerar mer biomassa än växter, och är enklare att modifiera genetisk än både växter och eukaryota alger. På grund av detta har en ingenjörsmässig omvandling av cyanobakterier till cellfabriker blivit en attraktiv möjlighet att utnyttja solenergi och återvinna koldioxid för att skapa framtidens bränsle och biomolekyler, och samtidigt reducera våra

miljö/klimatproblem. Genom att ”omprogrammera” cyanobakteriers metaboliska nätverk kan den fotosyntetiska metabolismen utnyttjas till att syntetisera värdefulla produkter från endast $CO_2$, solljus och vatten. Utan tvekan spelar flertalet faktorer en viktig roll för att man framgångsrikt, med genteknik, skall kunna skapa en robust och effektiv cyanobakterie som kan användas som värdorganism för produktion. Utmaningarna inför denna uppgift är stora! Vi behöver ytterligare grundläggande kunskap om; cellens genetiskt reglerade nätverk, hur celler känner av och anpassar sig till sin miljö. Den samt utveckla genetisk ingenjörskonst och syntetisk biologi för på cyanobakterier är fortfarande i sin linda.

Inom fältet mikrobiell bioteknik utnyttjas rationell design för att utveckla cellulära fabriker med önskad funktion, såsom exempelvis produktion av råmaterial för biobränslen, biokemikalier, eller bioplaster. Generellt finns tre viktiga hållpunkter för välutvecklade cellulära fabriker; cellen måste vara robust mot förändringar i närmiljön, cellen måste uppnå tillräcklig effektivitet vad gäller omvandling från solenergi till bioprodukt och cellen måste uppnå tillräcklig höga koncentrationer av den önskade produkten. De flesta studierna i ämnet har fokuserat på att introducera nya biosyntesvägar för specifika produkter, och på att förbättra effektiviteten av denna biosyntes. Metabolisk ingenjörskonst har även använts för att styra om kolflödet i cellen för att optimera produktionen av en önskad bioprodukt.

## Var robust, var specifik!

Forskningen i denna avhandling behandlar i huvudsak karaktärisering samt design av biologiska- och syntetiska verktyg anpassade för biotekniska applikationer i heterocystbildande cyanobakterier. Ett specifikt sådant verktyg relaterar till proteingrupper med uppgift att öka cyanobakteriers robusthet. En robust cell känner av och anpassar sin metabolism till förändringar i sin tillväxtmiljö, vilket leder till en balanserad metabolism och en stabil produktion. Saknas denna anpassningsförmåga så kan toxiska reaktiva syrearter (ROS) ackumuleras i cellen, vilket kan leda till oxidativ stress i cellerna, och en kollaps av produktionssystemet.

Cyanobakteriers utnyttjande av fotosyntes där solenergi omvandlas till kemisk energi, och syrgas bildas, är källan till detta. När solenergiupptaget genom fotosyntesen är för högt i förhållande till den energi som används i cellen så leder det till en obalans. Vid höga ljusnivåer så bildas ROS, exempelvis väteperoxid ($H_2O_2$), och om inte cellen har regleringssystem för att kontrollera ROS-nivåerna, så kan en oxidativ stress uppstå. Alla celler, framförallt syrgasutvecklande organismer, har välutvecklade processer för att hantera ROS. Exempelvis har en grupp proteiner, så kallade Dps-proteiner visats ha en viktig funktion i skyddet mot en mängd typer av oxidativ stress i både ”vanliga

bakterier" som *Escherichia coli* och i cyanobakterier. Intressant nog, återfinns fem olika Dps-proteiner hos den multicellulära cyanobakteriestammen Nostoc punctiforme. Dessa har visats vara ytterst viktiga för bakteriens toleransnivåer mot ROS, ett faktum ledde till hypotesen att Dps från Nostoc kan utnyttjas som biologiskt verktyg för att öka toleransen mot oxidativ stress, förorsakat av höga halter av $H_2O_2$ eller höga ljusintensiteter i cyanobakterier. Själva mekanismen bakom vad som styr den cellulära toleransen mot höga ljus- och $H_2O_2$-nivåer i multicellulära cyanobakterier är dock relativt outforskad.

Syftet med detta avhandlingsarbete var att förbättra cyanobakteriers förmåga att anpassa sig till förändringar i miljön, såsom växlingar i näringstillgång och ljus. Hypotesen att Dps proteiner kan fungera som verktyg för att öka toleransen mot ROS-inducerad stress undersöktes genom att Dps proteiner uttrycktes till höga nivåer i *Nostoc*, som sedan utsattes för starka ljusförhållanden och för höga halter av $H_2O_2$. Resultaten visade att överuttryck av två av Dps proteinerna, NpDps2 och NpDps5, individuellt förbättrar cellens förmåga att skydda sig mot stress inducerad av $H_2O_2$. Ingen ökad tolerans kunde däremot observeras vid stress genererad av starka ljusförhållanden, men en positiv effekt på aktiviteten av de viktiga enzymerna för fotosyntesen påvisades. Inom detta arbete etablerades även en Dps-förmedlad länk mellan ljustolerans, $H_2O_2$ skydd och järnbalansen i *Nostoc*. Det var också intressant att upptäcka att varje enskilt Dps-protein har en specifik roll, både vad gäller specifik funktion, och cellspecificitet i denna multicellulära cyanobakterie.

ROS-tolerans är ett olöst problem inom cyanobakteriebaserad produktion av biodrivmedel såsom vätgas, Höga ROS-nivåer kan potentiellt leda till skada på biomolekyler. Enzymer (bio-katalysatorer) som innehåller järnsvavelkluster.ex. hydrogenaser och nitrogenaser, är speciellt känsliga. Hydrogenaser; nyckel-enzymer i vätgasproduktion, är syrekänsliga vilket leder till stora tekniska svårigheter inom forskning och utveckling av fotosyntetiska, syrgasproducerande cyanobakterier för biovätgastillverkning. En möjlighet, för att lösa detta, är att utnyttja specifika cyanobakterier, *Nostoc*, vilka under kvävebrist bildar en speciell celltyp, kallad heterocyst, där atmosfärt kväve kan fixeras till ammonium. Den inre miljön i heterocysten är så gott som syrefri, vilket gör den till en ideal cellulär fabrik, där syrekänsliga katalysatorer, såsom hydrogenaser kan vara aktiva. Mitt bidrag till utvecklingen av effektiva vätgasproducerande cyanobakterier var att skapa en syntetisk promotor för heterocyst-specifikt genuttryck, med syfte att uttrycka proteiner, såsom hydrogenaser.

Utveckling av verktyg för syntetisk biologi i cyanobakterier är viktigt för att vidga möjligheterna till optimering av cellernas produktivitet. Antalet, väl karaktäriserade, standardiserade DNA-komponenter, "BioBricks", såsom promoterer, ribosombindande sekvenser och terminatorer, för cyanobakterier är i

dagsläget mycket begränsat. Det finns en stor brist av promotorer för biotekniska applikationer i cyanobakterier generellt, men för heterocystbildande cyanobakterier är läget än värre. Nyligen identifierades ett specifikt DNA-motiv, DIF, TCCGGA, i promotorer, som gav ett heterocystspecifikt proteinuttryck. Jag blev intresserad av ”DIF” då detta motiv fanns i en av de komplexa och långa promotorer som reglerar ett Dps-protein i *Nostoc*. Baserat på konserverade regioner i ett antal DIF-promotorer designades en väl karaktäriserad, minimal artificiell/syntetisk cellspecifik promotor med syfte att uttrycka syrekänsliga proteiner av intresse för heterocyst-baserad bioproduktion. Mina resultat har även bidragit med ny grundläggande förståelse av DIF-promotorers funktion. Exempelvis utvecklade, och testade, jag en ny metod för kvantitativ analys av promotoraktivitet i heterocyster med ”Flowsight” (avbildnings flödescytometri). Dessa cellspecifika kvantitativa resultat visar att uttrycksnivån från den minimala syntetiska DIF-promotorn ökade markant när en ytterligare DNA sekvens inkluderas i promotorn, uppströms DIF.

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