Doctoral Thesis in Biotechnology

Proteomic Investigation of Protein Interactions and Metabolic Regulation in Photosynthetic Organisms

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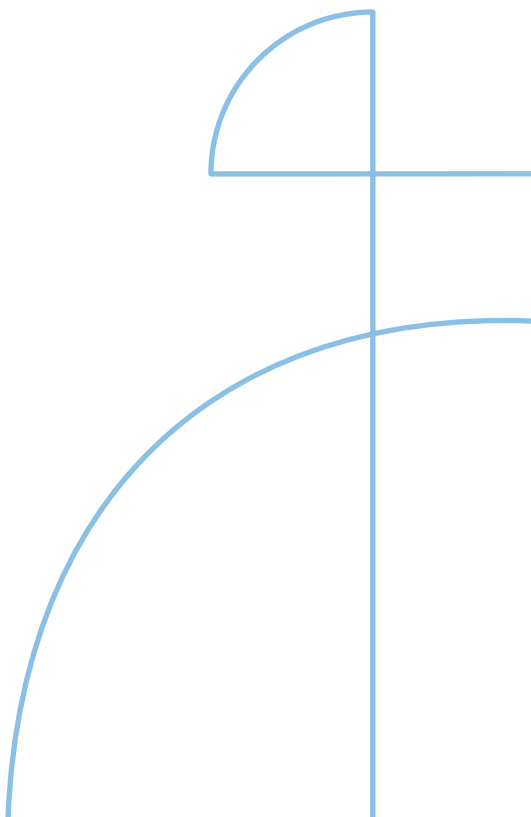
KTH ROYAL INSTITUTE OF TECHNOLOGY



Proteomic Investigation of Protein Interactions and Metabolic Regulation in Photosynthetic Organisms

ANNA KARLSSON

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“One must imagine Sisyphus happy.”

- *Albert Camus*

Abstract

The challenges we are facing as a society are many. Increasing CO_2 levels, destruction of habitats, and uncertainty about our ability to feed future generations are key issues that we have to solve. Photosynthetic organisms are sophisticated biological factories that convert sunlight and atmospheric carbon dioxide into useful products for humans, including food from plants and materials from forestry. Last but not least, the small photosynthetic cyanobacteria, which oxygenated the atmosphere billions of years ago and contributed to the development of plants, could enable the development of green cell factories for production of biofuels and chemicals. Despite the central role of photosynthetic organisms in society, many of the processes occurring inside photosynthetic cells remain not completely understood and could hold the key to improving their productivity or enabling their engineering. This thesis focuses on exploring regulation within photosynthetic cells and uses proteomics as a central tool to understand these regulatory processes.

In **Paper I** and **Paper III**, a high-throughput variant of thermal proteome profiling was used to investigate protein-metabolite interactions in cyanobacteria and plants. These studies examined regulatory mechanisms associated with nutrient limitation and interactions with ascorbic acid, providing insights into how metabolic regulation contributes to stress adaptation in photosynthetic organisms. In **Paper II**, an *in vivo* proximity-labeling proteomics approach was evaluated and applied to investigate protein-protein interactions in cyanobacteria. This approach was used to study enzymes involved in central carbon metabolism and to examine how their interactions change under different growth conditions. Finally, **Paper IV** investigated the regulation of a central carbon-fixation enzyme in plants and CO_2-fixing bacteria, focusing on metabolite regulation and oxidative sensitivity. This work also explored a bacterial enzyme variant in trees as an approach to improve plant growth.

Together, these studies provide a deeper understanding of the molecular regulation of photosynthetic organisms and demonstrate how proteomic approaches can be used to investigate interactions and regulatory mechanisms within these complex biological systems. The findings and methods used in this thesis could guide future studies and efforts to engineer plants and cyanobacteria for the sustainable production of biochemicals, food and materials.

Sammanfattning

Utmaningarna vårt samhälle står inför är många. Ökande halter av koldioxid, förstörelse av habitat och osäkerhet kring vår förmåga att försörja framtida generationer med mat är viktiga frågor som vi måste lösa. Fotosyntetiska organismer är sofistikerade biologiska fabriker som omvandlar solljus och atmosfärisk koldioxid till användbara produkter för människor, däribland mat från grödor och material från skogsbruket. Sist men inte minst kan de små fotosyntetiska cyanobakterierna, som syresatte atmosfären för miljarder år sedan och bidrog till växternas utveckling, vara en väg till att skapa gröna cellfabriker för produktion av biobränslen och kemikalier. Trots fotosyntetiska organismers centrala roll i samhället är många av de processer som sker inuti fotosyntetiska celler fortfarande dåligt förstådda och kan vara viktiga för att förbättra deras produktivitet. Denna avhandling fokuserar på att undersöka reglering i fotosyntetiska celler och använder proteomik som ett centralt verktyg för att förstå dessa regulatoriska processer.

I **Artikel I** och **Artikel III** användes en variant av thermal proteome profiling för att studera protein-metabolitinteraktioner i cyanobakterier och växter. Studierna fokuserade på regulatoriska mekanismer kopplade till stress vid näringsbrist samt interaktioner med vitamin C, och gav insikter i hur proteinreglering bidrar till stressanpassning hos fotosyntetiska organismer. I **Artikel II** undersöktes och tillämpades en proteomikmetod för att studera protein-proteininteraktioner i cyanobakterier. Metoden användes för att undersöka enzymer involverade i central metabolismen och hur deras interaktioner förändras under olika tillväxtförhållanden. Slutligen undersökte **Artikel IV** regleringen av ett centralt protein involverat i kolfixering i växter och CO_2-fixerande bakterier, med fokus på reglering och känslighet för oxidativ stress. Arbetet omfattade även en undersökning av en bakteriell enzymvariant i träd som en strategi för att förbättra växters tillväxt.

Sammantaget bidrar dessa studier till en djupare förståelse av den intracellulära regleringen av fotosyntetiska organismer och visar hur proteomiska metoder kan användas för att undersöka interaktioner och regulatoriska mekanismer i dessa komplexa biologiska system. De resultat och metoder som utvecklats inom denna avhandling kan vägleda framtida studier och försök att modifiera och utveckla växter och cyanobakterier för en hållbar produktion av biokemikalier, mat och material.

Acknowledgments

During my PhD, I have met many wonderful people I would like to express my gratitude to. Perhaps the real treasure was the friends I made along the way.

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List of publications and manuscripts

Paper I

Anna Karlsson, Rees Rillema, Emil Sporre, Elias Englund, Nikoleta Vogiatzi, Júlia Llavina Ramírez, Cenk O. Gurdap, Erdinc Sezgin, Fredrik Edfors, Cecilia Blikstad, Åsa Strand, Daniel C. Ducat, Elton P. Hudson. Identifying novel targets of the stringent response in plants and cyanobacteria using chemoproteomics. Biorxiv. 2026. DOI: 10.64898/2026.07.01.735797. *Under revision in Nature Communications.*

Respondent's contributions: Project design. Perfomed the majority of all experiment, including cell and plant cultures, proteomic screeing, data analysis, and validation of protein interactions. Wrote the full manuscript, with input from coauthors.

Paper II

Anna Karlsson and Marius Theune, Marko Böhm, Alva Lundmark Brånstad, Thanadol Sutantiwanichkul, Fredrik Edfors, Kirstin Gutekunst and Elton P. Hudson. Evaluation of BioID proximity labeling in cyanobacteria and proximity landscape of central carbon metabolism enzymes. *Manuscript draft*.

Respondent's contributions: Project design and major manuscript writing together with shared first co-author. Development of the proteomic method, performed majority of proteomic experiments including cell cultivation and main data processing.

Paper III

Dávid Tóth and Roland Tengölics, Fayezeh Aarabi, **Anna Karlsson,** André Vidal-Meireles, László Kovács, Soujanya Kuntam, Tímea Körmöczi, Alisdair R Fernie, Elton P Hudson, Balázs Papp, Szilvia Z Tóth. Chloroplastic ascorbate modifies plant metabolism and may act as a metabolite signal regardless of oxidative stress. Plant Physiology. 2024.

DOI: 10.1093/plphys/kiae409

Respondent's contributions: Cultivation of plants and extraction of chloroplasts. Proteomic metabolite-protein interaction experiments and data analysis. Expressed recombinant proteins and performed kinetic assays. Minor manuscript writing.

Paper IV

Bruno E. Rojas, **Anna Karlson** , Sara Luppachini, Leonor Ramirez, Åsa Strand, and Elton P. Hudson. A bifunctional F/SBPase from nonphotosynthetic bacteria enhances growth of transgenic poplar. *Manuscript draft*.

Respondent's contributions: Project design together with co-authors. Performed proteomic experiments and data analysis. Involved in design of enzyme mutants, purified all enzymes and design of activity assays. Minor manuscript writing.

Papers not included in the thesis

Ute A. Hoffmann and Axel F. Knave, Anna Z. Schuppe, Sebastià Capó-Bauçà, Pere Aguiló Nicolau, Karen Schriever, **Anna Karlsson**, Michele Russo, Emil Sporre, Mami Matsuda, Rui Miao, Tomohisa Hasunuma, Jeroni Galmés, Per-Olof Syrén, Elton P. Hudson. The fitness landscape of a form II rubisco in a photosynthetic bacterium guides engineering of oxygen tolerance. Science Advances. 2026. DOI: 10.1126/sciadv.aee9222

Hitesh Medipally, **Anna Karlsson**, Aman Dheer, Elton P. Hudson, Elias Englund. Expression landscape of metabolic engineering enzymes in cyanobacteria. Biorxiv. 2026. DOI: 10.64898/2026.04.08.717203. *Under revision in Nature Communications.*

Anna Karlsson, Emil Sporre, Linnéa Strandberg, Szilvia Tóth, Elton Hudson. Assessing Metabolite Interactions With Chloroplastic Proteins via the PISA Assay. BIO-PROTOCOL. 2025. DOI: 10.21769/bioprotoc.5298

Emil Sporre and Jan Karlsen, Karen Schriever, Johannes Asplund-Samuelsson, Markus Janasch, Linnéa Strandberg, **Anna Karlsson**, David Kotol, Luise Zeckey, Ilaria Piazza, Per-Olof Syrén, Fredrik Edfors, Elton P. Hudson. Metabolite interactions in the bacterial Calvin cycle and implications for flux regulation. Communications Biology. 2023.
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Johannes Yayo, Thomas Rydzak, Teun Kuil, **Anna Karlsson**, Dan J. Harding, Adam M. Guss, Antonius J. A. van Maris. The Roles of Nicotinamide Adenine Dinucleotide Phosphate Reoxidation and Ammonium Assimilation in the Secretion of Amino Acids as Byproducts of Clostridium thermocellum. Applied and Environmental Microbiology. 2023. DOI: 10.1128/aem.01753-22

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1 Why study protein regulation in photosynthetic organisms

As the global population continues to rise, humanity faces challenges of meeting growing demands for food, energy, and materials while reducing greenhouse gas emissions and protecting ecosystems. At the same time, atmospheric CO_2 concentrations have increased from approximately 280 ppm before industrialisation to more than 420 ppm today, and global temperatures have risen by around 1.5 °C above pre-industrial levels (1). These changes are already contributing to more frequent and severe extreme events (2), while rising sea levels are expected to increasingly threaten coastal populations, with up to 150 million people living in at-risk areas by 2050 (3). These challenges highlight the need for more sustainable approaches to resource production.

Our society is fundamentally dependent on photosynthesis and virtually all organic carbon in the world originates from photosynthetic activity. Through photosynthesis, plants, algae, and photosynthetic bacteria use sunlight to convert atmospheric carbon dioxide (CO_2) into organic matter, producing oxygen as a byproduct (Figure 1). While human efforts can efficiently mimic energy capture through solar cells, capturing CO_2 directly from the atmosphere remains challenging and energy intensive. Atmospheric CO_2 is present at relatively low concentrations, making its capture thermodynamically demanding and impossible without external energy input (4, 5). In contrast, photosynthetic organisms have evolved systems over billions of years that enable them to capture atmospheric CO_2 and convert it into organic matter using solar energy. Taken together with their already central role in food and material production, plants and photosynthetic bacteria are impressive systems to study.

As a biotechnologist, I want to use the complex systems nature has designed to solve problems. This thesis focuses on studying regulation of proteins in photosynthetic organisms. Understanding how cells are regulated, interact, and contribute to cellular processes can provide knowledge that may ultimately allow photosynthetic organisms to be more effectively adapted to human needs. The following sections explore how biotechnology can be used to harness the potential of photosynthetic organisms and why we should aim to know more about them.

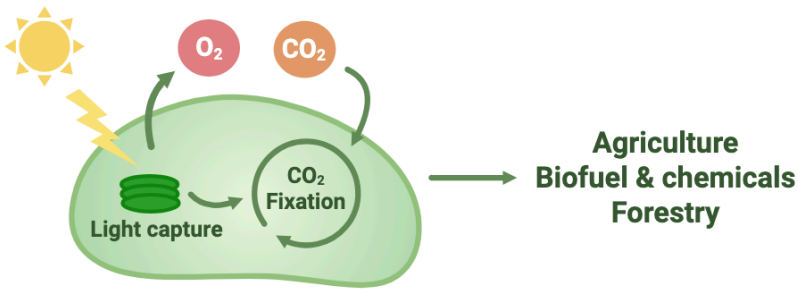

Figure 1: Schematic of a photosynthetic cell. Through photosynthesis, sunlight is harnessed as an energy source to capture carbon dioxide from the atmosphere and convert it into organic carbon, while releasing oxygen as a byproduct. Photosynthetic organisms are primary producers of food and materials, and also have the potential to serve as green cell factories for the production of biochemicals, such as biofuels.

1.1 Biotechnology in sustainable agriculture and forestry

Agriculture and forestry face a common challenge: increasing the production of renewable biological resources while limiting pressure on the ecosystem. Total food demand is estimated to increase approximately 35-56% between 2010 and 2050 (6). At the same time, climate change is expected to increase the frequency and severity of environmental stresses affecting agricultural production. Drought is expected to increasingly threaten agricultural production, particularly in vulnerable regions such as South America, Africa, Eastern Europe and Southeast Asia. By 2050, 62 countries are projected to experience maximum drought-related production losses exceeding 10%, while 24 countries may experience losses exceeding 20% (7). In a similar way, worldwide production from forestry has doubled since the 60s (8), and the demand for wood harvests is projected to increase by 54% from 2010 to 2050 (9).

Increasing production through land-use expansion can come with substantial environmental costs. Crop field expansion, for example, is estimated to account for almost 50% of global deforestation (10). Similarly, although forests are renewable resources with an important role in a sustainable bioeconomy, intensive forest management negatively affects biodiversity. Timber plantations and clear-cutting have been associated with approximately 40% and 20% lower local species richness than natural forests, respectively (11). Increasing crop and forest productivity can therefore help meet growing demand without further expanding production areas.

Conventional plant breeding has been a major contributor to increases in agricultural productivity. However, unlike conventional breeding, which relies on recombination and selection of existing genetic variation, genetic engineering can introduce genes from other organisms (transgenic) or directly modify existing genetic sequences (intragenic). This can enable traits that may be difficult or time-consuming to obtain through conventional breeding. A common way to modify plants is by *Agrobacterium*-mediated transformation, which uses a bacterial pathogen to insert genes at an unspecified location in the genome. More recent gene editing technologies such as CRISPR-Cas enable targeted modification of endogenous genes (12, 13).

Today, the major commercially cultivated GM crops are soybean, maize, cotton and canola, which together account for the vast majority of global GM crop area, with herbicide tolerance and pest resistance being the main targets (14). Genetic engineering has also been used in fruit crops to improve resistance to pests and diseases and modify traits such as ripening, browning and nutritional quality (12). Commercial examples include papaya engineered for resistance to papaya ringspot virus (PRSV) and virus-resistant squash via expression of transgenic viral coat-proteins. Other engineering targets, though less commercially established, include improving drought tolerance, modifying nutritional composition and extending shelf life (12).

Biotechnology is particularly relevant to forestry because trees are long-lived perennial organisms with much longer generation intervals than crops. Biotechnology can help accelerate selection and modify traits related to both productivity and wood properties. For example, genetically modified *Eucalyptus* with increased biomass production has been approved for commercial cultivation in Brazil. This variety expresses a transgenic enzyme from the plant *Arabidopsis thaliana* (thale cress) that loosens the cell wall and rapid expansion (15). In poplar, genetic modification of lignin biosynthesis has

produced trees with wood that can be more easily processed, requiring less chemical input during pulping (16). More recently, CRISPR-editing has enabled the simultaneous modification of multiple lignin-biosynthesis genes, allowing more systematic optimization of lignin composition and wood properties for efficient fiber production (17).

The extent to which genetic engineering of plants is used depends on regulation, which differs substantially between countries. The United States, Brazil, Argentina, India and Canada are among the largest producers of GM crops (14), while the European Union has historically applied stricter regulation. Under EU regulation adopted in June 2026 (Regulation 2026/1388), plants with genetic changes considered equivalent to those arising naturally or through conventional breeding can be exempt from EU GMO legislation. More complex modifications, including the introduction of transgenes, remain subject to more extensive regulatory requirements.

Overall, while measures such as reducing food waste, limiting material use, and increasing recycling are important for addressing the climate crisis and improving resource availability, biotechnology provides an attractive, complementary strategy for improvements in productivity that were previously limited by technological and legislational constraints.

1.2 Cyanobacteria as green cell factories

When discussing photosynthesis, plants are often the first organisms that come to mind. However, oxygenic photosynthesis evolved first in cyanobacteria, a diverse group of gram-negative bacteria, and are responsible for oxygenating Earth's atmosphere. Today they remain one of the main carbon and nitrogen fixators; Cyanobacteria account for half of all natural nitrogen fixation, and are responsible for >20% of marine primary production today (18, 19). Cyanobacteria are highly diverse and have adapted to a remarkable range of environmental conditions such as oceans, freshwater, hot springs, deserts, and in symbiosis with organisms, such as the case with lichen or nitrogen-fixing heterocysts in plant roots (20). One of the most important events in shaping life on Earth is the domestication of the cyanobacteria by eukaryotic cells, which created plants and algae. Approximately 1.5 billion years ago an eukaryotic cell engulfed a cyanobacteria that established an endosymbiotic relationship with its host (21). Modern chloroplasts, the organelle in eukaryotic cells where photosynthesis takes place, can therefore be seen as descendants of ancient cyanobacteria.

Sunlight provides an enormous and continuously renewable source of energy, and sunlight hitting the Earth for 1 hour is equivalent to a whole year's worth of energy consumption (22). Life across Earth uses photosynthetic organisms to store energy from the sun in more useful forms, such as the food we eat. Of course there are other things to imagine that are useful, such as fuel. Advances in genetic engineering have enabled the redirection of cyanobacterial metabolism towards the production of compounds of industrial interest, including biofuels, fatty acids, sugars, and terpenoids (23). Compared to established heterotrophic bacteria production of products, such as bio-ethanol, cyanobacteria have the advantage of directly using the solar energy and carbon dioxide, rather than having to rely on another carbon source. Cyanobacterial cultivation can also reduce direct competition with food production compared with conventional crop-based biofuel systems, as cyanobacteria do not require agricultural biomass as their primary carbon source and could be cultivated on non-arable land and using saline water or wastewater streams (24).

However, despite considerable progress, production titres generally remain below those achieved by established heterotrophic production organisms and are not economically viable (24). Improving carbon flux towards desired products, increasing product tolerance, and optimizing photosynthetic efficiency, therefore remain considerable challenges for industrial implementation of cyanobacteria.

Beyond their role as green cell factories, cyanobacteria are also valuable model systems for understanding and engineering plant photosynthesis. Their evolutionary relationship with chloroplasts, relatively short generation times, and well-established genetic tractability make organisms such as *Synechocystis* sp. PCC 6803 important experimental platforms for studying photosynthetic organisms. In some cases, cyanobacteria are even used for genetic inspiration and for transgenic engineering of plants (25, 26).

1.3 Regulation of metabolism and uses for biotechnology

As a crash-course in biology: DNA carries the genetic blueprints for creating proteins; Proteins builds structures and also function as machines, enzymes, that convert molecules from one molecule to another - called metabolites; The massive and complex networks of enzymes working together in the cell form what we call metabolism; These networks of enzymes are regulated in order for the cell to respond to environmental ques, which redirects metabolism towards for example growing or conserving energy.

Regulation of metabolism can be an issue when the goal of humans and cells do not overlap. In the case of cyanobacteria, producing a compound such as biofuel does not help the cell survive and proliferate, and instead causes a disadvantage by redirecting energy and carbon from other cellular processes into a product. To protect against unnecessary build-up of metabolites, enzymes are often inhibited by the end molecule in a pathway, called feedback regulation. This regulation can be removed in synthetic biology to allow for accumulation of products. By using feedback-resistant enzyme variants, cyanobacteria have been engineered to produce a wide range of aromatic amino acids and derivatives (27). Interestingly, plants have naturally evolved reduced feedback regulation of the aromatic amino acid biosynthetic pathway, allowing the accumulation of aromatic amino acids and their derivatives, such as lignin, which is an essential component of plant cell walls (28).

In some cases regulation can be hijacked to direct carbon flux towards a desired product (29). In cyanobacteria this is used when, for example, using metal stress-induced promoters for expression of transgenic genes (30), salt stress to increase sucrose production (31), or heat stress to increase terpenoid production (32). Together, regulation can either stand in the way or be used as a tool in synthetic biology. In Section 2 I review current knowledge of the regulation of photosynthetic organisms in greater depth, with a focus on protein-level regulation.

1.4 Aim of thesis

Beyond providing a fundamental understanding of the biology of photosynthetic organisms, knowledge of metabolic regulation is increasingly being used as a tool in synthetic biology. This thesis aims to increase our understanding of protein activity and regulation in plants and cyanobacteria, as part of addressing the broad challenge of sustainability. To do this, I have applied a systems biology proteomic approach to study protein interactions and enzymes:

In **Paper I** and **Paper III** I have used a high-throughput proteomic method to study protein-metabolite interactions in cyanobacteria and plant chloroplasts. Using this method, I study the stress-adaptive regulatory mechanism known as the stringent response across plants and bacteria. I also study interactions with ascorbic acid in plants.

In **Paper II** I have applied a proximity labeling proteomic technique to study protein-protein interactions *in vivo* in cyanobacteria. I apply it to several central carbon metabolism enzymes and evaluate protein interactions under different growth conditions, such as light and darkness.

Finally, **Paper IV** focuses on the comparison of a highly regulated enzyme used in carbon fixation between plants and CO_2-fixing bacteria, and study metabolite regulation and oxidative sensitivity. This is also combined with growth assessments of an bacterial transgenic enzyme variant in Poplar trees to improve growth.

2 Regulation of carbon metabolism in plants and cyanobacteria

Plants and cyanobacteria live in dynamic environments and must continuously adapt their metabolism to changing conditions. These include biotic stresses, such as pathogen infection, as well as abiotic stresses, including nutrient limitation, drought, high light, and changes in temperature (33–36). Photosynthetic organisms also face a unique challenge of the natural day-night cycle, requiring them to rewire their metabolism between light and darkness: During the day, they use sunlight to fix CO_2, whereas at night, they rely on stored carbon reserves for energy, shifting from photosynthetic to respiratory metabolism (37).

The flux through metabolic pathways can be regulated by changing the abundance of enzymes that catalyze individual reactions, primarily through transcriptional and translational control. However, altering protein abundance requires changes in gene expression and protein synthesis and is relatively slow and energy-demanding. A quicker and more flexible way to regulate metabolism is through direct modification of enzymes that are already present in the cell (Figure 2). Such protein-level regulation can rapidly alter enzyme activity without requiring the synthesis of new proteins, allowing metabolic flux to respond readily to changes in the cellular environment (38, 39).

Describing the protein regulation of photosynthetic organisms is nontrivial, partly because of the complexity and partly because we lack complete understanding. The proteome is a collective name for all proteins in a cell. In the highly studied model plant *Arabidopsis thaliana*, only 32% of the proteome has known annotation (biological process) supported by experimental evidence, 47% has evidence based on sequence or structural similarity with other organisms, and the remaining 21% consists of proteins with unknown function (TAIR database, 2023 data) (40). In the model organism *Synechocystis* PCC 6803, one of the most well-studied cyanobacteria, only about ~30% of protein-coding sequences had an assigned function in 2020 (41).

As with *A. thaliana*, many of these assignments are based on homologs in other organisms rather than experimental characterization in *Synechocystis* itself. Despite these challenges, considerable progress has been made in recent decades and several review studies provide comprehensive overviews of metabolic regulation in plants and cyanobacteria (33, 37, 42–44). In following sections, I discuss key concepts and regulatory mechanisms (Figure 2).

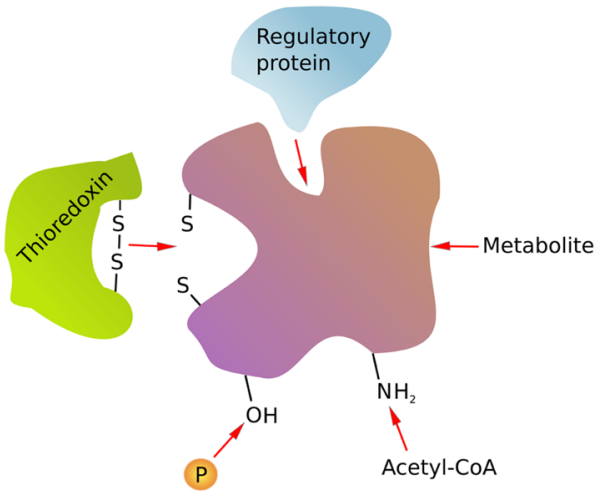

Figure 2: Examples of mechanisms for regulation of enzyme activity, including redox regulation, chemical modifications like phosphorylation and acetylation, metabolite-regulation and regulation by protein-protein interaction. Figure from Jan Karlsen 2021 (45).

2.1 Central carbon metabolism

All living organisms require the following things to survive: central building blocks such as carbon, nitrogen, and phosphorus; a way to gain energy to drive chemical reactions; a source of electrons to build molecules (reducing power); water as a solvent and for chemical reactions; and, to a lesser extent, trace elements and minerals that are needed, for example, for the activity of certain enzymes. In photosynthetic organisms, carbon building blocks are derived from CO_2, energy is derived from light, and water serves as a source of electrons. The main chemical pathways related to the conversion of carbon into other molecules are typically referred to as the central carbon metabolism.

Due to their shared evolutionary origin, plant chloroplasts and cyanobacteria retain many similarities in their central carbon metabolism (43). CO_2 is fixed through the Calvin-Benson-Bassham cycle (hereafter Calvin cycle), where the enzyme Rubisco catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP), ultimately generating triose phosphates that provide carbon skeletons for the synthesis of sugars and other metabolites (44). Cyanobacteria additionally concentrate inorganic carbon around Rubisco through organelles called carboxysomes, which increase the local CO_2 concentration and improve carbon fixation efficiency (46). Similar CO_2-concentrating mechanisms have also evolved in some plant species, for example through C_4 and crassulacean acid metabolism (CAM), which spatially and temporally concentrate CO_2 around Rubisco, respectively (47). The carbon fixed by Rubisco is used as building blocks for biomass and for carbon storage.

The energy required for the reactions in carbon metabolism is generated by the photosystems, which capture light energy and convert it into chemical energy. Photosystem I (PSI) and photosystem II (PSII) are protein-pigment complexes embedded in the thylakoid membrane that capture sunlight and drive electron transfer (48). Notably, these pigments give many photosynthetic organisms their characteristic green color by absorbing light across the visible spectrum, with little absorption in the green region. In PSII, light energy is used to oxidize water, producing oxygen and releasing electrons and protons. The electrons are subsequently transferred through the photosynthetic electron transport chain to PSI, where a second photon of light provides additional energy, enabling the reduction of $NADP^+$ to NADPH (an electron carrier molecule). Electron transport also contributes to the formation of a proton gradient across the thylakoid membrane, which drives ATP synthase and generates ATP (an energy-dense molecule used to drive chemical reactions). Together, ATP and NADPH provide the energy and reducing power required for CO_2 fixation and other parts of the metabolism.

In plants, the reactions described above take place within the chloroplast. In contrast to cyanobacteria, the products of CO_2 fixation in plants are exported from the chloroplast to the rest of the cell, where they provide carbon precursors for the synthesis of proteins, fatty acids, nucleotides, and other metabolites. Both cyanobacteria and plants store a pool of carbon fixed during the day in the form of glycogen or starch, respectively, which is used as fuel during the night when photosystems are not active (37, 49).

2.2 Regulation through chemical modification

Protein-level regulation can occur in multiple ways to activate or reduce enzyme activity. A common form of chemical regulation in both cyanobacteria and plants is redox regulation, in which reversible oxidation and reduction of specific cysteine residues alters protein conformation and activity. In the light, photosynthetic electron chain generates reducing power, with electrons transferred to ferredoxin and ultimately used to reduce thioredoxin proteins. Reduced thioredoxin proteins can then reduce disulfide bonds in target proteins, converting cysteine residues from an oxidized disulfide state (-S-S-) to reduced thiol groups (-SH). This breaking or forming of bonds can induce conformational changes and activate the enzyme. This provides a direct link between light availability and metabolic activity.

Several Calvin cycle enzymes, including sedoheptulose-1,7-bisphosphatase (SBPase), fructose-1,6-bisphosphatase (FBPase), phosphoribulokinase (PRK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are activated through thioredoxin-dependent reduction in the light in plants (33). In cyanobacteria, these enzymes are likewise redox regulated, and additional enzymes, including phosphoglycerate kinase (PGK), phosphoglucomutase (PGM), and the bifunctional fructose-1,6-/sedoheptulose-1,7-bisphosphatase (F/SBPase, SBPase homolog), have also been identified as redox-regulated enzymes (44).

Although the thioredoxin-based redox regulatory system is broadly conserved, differences exist between plants and cyanobacteria. For example, phosphoglycerate kinase (PGK) is redox regulated in cyanobacteria and in the green algae *Chlamydomonas reinhardtii*, whereas this regulation does not appear to be conserved in land plants as it lacks the relevant cysteine residues (50).

Another well-studied modification of enzymes is phosphorylation, the attachment of phosphate groups on primarily serine and threonine residues (but also tyrosine, histidine, and aspartate) mediated by kinases. The removal of phosphate groups is catalyzed by phosphatases. Phosphorylation in cyanobacteria inhibits phosphoglucomutase (PGM) during nitrogen starvation, an important node for glycogen synthesis, as well as regulatory proteins such as PII (discussed in more detail in the following section), and the circadian clock (42, 51). Similarly, plants regulate starch metabolism and the circadian rhythm

via phosphorylation (33). However, the regulatory protein PII is not regulated by phosphorylation in plants (52).

Many other chemical modifications exist, such as acetylation, nitration, and carbonylation (33). Collectively, these modifications represent important mechanisms for regulating protein function and cellular metabolism. Redox regulation plays a prominent role in coordinating light- and dark-dependent metabolism, and phosphorylation contributes to the regulation of carbon and nitrogen metabolism, as well as components of the circadian clock. Understanding the chemical regulation of proteins, including the specific residues targeted by these modifications, provides opportunities for rational enzyme engineering. This knowledge is applied in a follow-up project to **Paper IV**, in which we engineer the plant Calvin cycle enzyme SBPase (Section 4.4).

2.3 Regulatory proteins

Regulatory proteins can modulate enzymes by binding to them, changing their activity, localization or interaction partners. Some of these proteins are highly conserved across plants and cyanobacteria, for example, the PII protein is a metabolic sensor that regulates target proteins according to cellular carbon and nitrogen status.

PII senses the cellular carbon/nitrogen balance primarily through the binding of 2-oxoglutarate (2-OG), while the binding of ATP or ADP provides information about the cellular energy state. During nitrogen limitation, 2-OG, which sits at the interface of nitrogen and carbon metabolism, accumulates because carbon skeletons are available but cannot be efficiently consumed through nitrogen assimilation. Increased 2-OG, together with the cellular ATP/ADP ratio, alters the conformation of PII and consequently its interactions with target proteins, contributing to the cellular response to nitrogen limitation. Conversely, when carbon or energy availability is low, the ATP/ADP ratio decreases as ATP levels decline relative to ADP, resulting in a different PII signaling state and altered interactions with its target proteins. Depending on these signals, PII interacts with different target proteins, including proteins in nitrogen uptake (AmtB, cyanobacteria-specific), arginine synthesis (NAGK, plants and cyanobacteria), and fatty acid synthesis (ACCase, plants and cyanobacteria) (53, 54).

In cyanobacteria, PII is also part of a cyanobacteria-specific PII-PipX-NtcA regulatory network that connects cellular carbon and nitrogen status to gene expression. PipX acts as an adaptor protein that can interact with either PII or the global nitrogen-responsive transcription factor NtcA. When nitrogen is

abundant and 2-OG levels are low, PII binds PipX and sequesters it, limiting its interaction with NtcA. During nitrogen limitation, 2-OG accumulates and promotes a change in the PII signaling state, resulting in the release of PipX. PipX can then associate with 2-OG-activated NtcA, promoting NtcA-dependent transcription of genes involved in nitrogen assimilation and adaptation to nitrogen limitation (55).

Other than the canonical PII protein, there are several structurally related proteins, such as SbtB found in cyanobacteria. SbtB interacts with adenine nucleotides (ATP, ADP, AMP, cAMP and c-di-AMP) and controls central carbon metabolism by regulating the bicarbonate transport and glycogen metabolism via interacting with SbtA and GlgB (53).

Recently discovered in cyanobacteria, the small protein PirC provides another example of protein-protein regulation linking carbon and nitrogen metabolism. PirC interacts with the 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (PGAM), which represents a branching point between the Calvin cycle and lower glycolysis. Binding of PirC inhibits PGAM activity, reducing the flow of fixed carbon from the CBB cycle into lower glycolysis. PirC itself is controlled by the PII protein. Under nitrogen-rich conditions, PII sequesters PirC, whereas during nitrogen limitation, accumulation of 2-oxoglutarate causes dissociation of the PII-PirC complex, making PirC available to inhibit PGAM (56). In plants, however, the PGAM is not associated with PII regulation (57). Recently, the PirC-PGAM regulation has been used in synthetic biology to channel carbon flux into products (58).

Some regulatory proteins are coupled to redox regulation, such as the small CP12 protein that has a well-conserved mechanism for controlling Calvin cycle activity in both cyanobacteria and plants. Under oxidizing conditions, such as darkness, cysteine residues in CP12 form disulfide bonds that convert the otherwise flexible protein into a structured state capable of binding phosphoribulokinase (PRK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). CP12 thereby causes the formation of a GAPDH-CP12-PRK complex in which both enzymes are inhibited, reducing Calvin cycle activity when photosynthetic energy production is low. In the light, reducing power generated by photosynthetic electron transport promotes reduction of CP12 disulfide bonds, leading to dissociation of the complex and reactivation of GAPDH and PRK (59).

Another redox-regulated regulatory protein is OpcA, a distinct cyanobacterial strategy for redox regulation of carbon metabolism. It regulates glucose-6-phosphate dehydrogenase (G6PDH/Zwf), the first and rate-limiting enzyme of the oxidative pentose phosphate pathway, which generates reducing power and metabolic intermediates. Notably, I use the Zwf-OpcA interaction as a control in **Paper II** (Section 4.3) for method optimization and evaluation.

Unlike plastidic plant glucose-6-phosphate dehydrogenase, cyanobacterial Zwf lacks the redox-sensitive cysteine residues that allow direct thioredoxin-mediated regulation. Instead, cyanobacteria use the auxiliary protein OpcA as a redox-sensitive regulator of Zwf. OpcA binds the Zwf tetramer and alters its activity according to its own redox state: oxidized OpcA promotes Zwf activity, whereas reduced OpcA inhibits the enzyme. During photosynthetic growth in the light, thioredoxin keeps OpcA predominantly reduced, which inhibits Zwf and minimizes carbon catabolism through the oxidative pentose phosphate pathway (60). OpcA also shows interplay with other metabolites, such as citrate, which can be used as an additional regulatory layer during prolonged nitrogen starvation (61).

Together, these examples illustrate the importance of protein-protein interactions in the regulation of photosynthetic metabolism. In an effort to map other protein-protein regulations, **Paper II** screens for further protein-protein interactions in the central carbon metabolism of cyanobacteria under different growth conditions.

2.4 Metabolite interactions and signaling molecules

Metabolites are more than simply substrates and products of biological processes; they can also function as signaling molecules and regulators of enzyme activity (62). Some metabolites regulate enzymes by competing with the substrate for binding to the enzyme's active site. Such molecules are referred to as competitive inhibitors. In contrast, allosteric effectors bind to regulatory sites distinct from the substrate-binding site and induce conformational changes in the enzyme that alter its activity. Allosteric effectors may either inhibit or activate enzyme activity, thereby providing an important mechanism for regulating metabolic flux. Feedback regulation discussed in Section 1.3 is a typical example of how metabolites can regulate pathways, commonly seen in amino acid metabolism (63). In the Calvin cycle, the bifunctional cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase (F/SBPase) provides an example of direct metabolite-mediated regulation, being regulated by both AMP

and glyceraldehyde-3-phosphate (GAP) (64, 65). More broadly, large-scale screening of metabolite-protein interactions suggests that metabolite-mediated regulation is widespread in cyanobacterial central carbon metabolism (65).

Metabolites can act as signals for energy status (ATP/ADP/AMP), redox status (NADPH/NADH), or nitrogen status (2-OG), either by direct interaction with enzymes, or by mediating regulation through regulatory proteins (as outlined in the previous Section). Apart from molecules that actively partake in metabolism as cofactors or intermediates, some molecules are created for the main purpose of regulating metabolism. Secondary messenger molecules are a collective term for signaling molecules such as cAMP, c-di-AMP and c-di-GMP that coordinate cellular processes by regulating multiple targets, including responses to nutrient limitation, carbon and nitrogen status, and biofilm formation (66, 67). In plants, cyclic nucleotides such as cAMP and cGMP similarly contribute to the regulation of ion transport, stress response and hormone signaling (68).

The signaling molecule guanosine tetraphosphate (ppGpp) is a central regulator for adaptation to nutrient limitation that is well-conserved across bacteria, and also plant chloroplasts. During nutrient limitation, ppGpp causes what is known as the stringent response, which redirects metabolism from growth into survival mode, by for example, inhibition of protein translation, redirecting of what genes are transcribed, and through direct interaction with key metabolic enzymes (69).

The stringent response was first characterized in heterotrophic bacteria, with *Escherichia coli* and *Bacillus subtilis* serving as major model organisms for understanding ppGpp-mediated regulation. In these organisms, ppGpp synthesis and degradation are controlled by RelA/SpoT homologs, which are activated by nutrient limitation. In *E. coli*, for example, RelA is activated at the ribosome by uncharged tRNAs during amino acid starvation, whereas SpoT integrates additional stress signals and provides the major ppGpp hydrolase activity. Once produced, ppGpp interacts directly with a range of cellular proteins, including RNA polymerase to redirect the transcription profile, and translation-associated factors, as well as metabolic enzymes involved in processes such as nucleotide and central metabolism (69, 70). As a central regulator of growth and stress acclimation, the stringent response is an attractive target for biotechnology. Though early in development, it is explored for antimicrobial development and has been used to channel carbon into products in bacteria (71–73).

In plants and cyanobacteria, the stringent response has been adapted to a photosynthetic lifestyle. By studies in cyanobacterial genera such as *Synechococcus* and nitrogen-fixing *Anabaena*, ppGpp has been seen to accumulate rapidly in darkness, and through synthetic inhibition of the photosynthetic electron transport chain, and is vital for survival in natural dark-light cycles (67, 74, 75). ppGpp also accumulates during heat stress and nitrogen starvation, and is needed for formation of nitrogen-fixing heterocysts (67, 76). In plants the evidence is similar, where ppGpp regulates adaptation to nitrogen limitation by downregulating photosynthetic activity, and also spikes at the onset of night (77).

The evolution of the stringent response in plants is not a straightforward linear transfer of the cyanobacterial system. Whereas cyanobacteria generally rely on a single bifunctional RelA/SpoT homolog with both ppGpp synthetase and hydrolase activities (67), plants possess several chloroplast-targeted RelA/SpoT homologs (RSH) with distinct biochemical functions, including RSH1, RSH2/3, and the calcium-regulated ppGpp synthetase CRSH. Moreover, phylogenetic analyses indicate that some plant RSH proteins are more closely related to RelA/SpoT proteins from the Deinococcus-Thermus lineage than to their cyanobacterial counterparts, suggesting that the plant system has a complex evolutionary history involving lateral gene transfer and diversification (70).

Although it is evident that ppGpp plays a central role in coordinating metabolism during stress, the biochemical mechanisms through which it acts in photosynthetic organisms are less understood. To address this gap in knowledge, **Paper I** investigates the protein targets of ppGpp in plants and cyanobacteria.

3 Proteomic methods to study protein regulation

Regulation of metabolism is complex with several dependent and independent mechanisms, as reviewed in the previous section. The rise of systems biology techniques such as genomics, transcriptomics, proteomics and metabolomics, enabled by new technology, presents an opportunity to study the cell in a high-throughput cell-wide manner. Though single-focused, detailed biochemical studies are often needed for a full biological picture, system-wide technologies offer a holistic perspective.

Technological advances in the 2000s made it possible to quantify thousands of proteins simultaneously, establishing proteomics as a new powerful complement to genomics and transcriptomics. While genomics provides information about the genetic blueprint and transcriptomics measures what genes are actively transcribed, neither directly captures the abundance of proteins (78, 79), the primary workhorses of cellular processes. Proteomics is therefore an essential approach for understanding cells and is an integral part of this thesis, where it is used to investigate protein interactions.

Biotechnology is an interdisciplinary field that merges biological sciences with engineering, computer science, and physical sciences. As covered in the following sections, proteomics is a prominent example of this, where advanced physics and computational methods are used to quantify biology.

3.1 How the mass spectrometer works

At its core, a mass spectrometer measures the mass-to-charge ratio (m/z) of ions (80, 81). There are several technologies for measuring m/z, such as time-of-flight (TOF), which measures the time required for an ion to travel a defined distance after being accelerated by an electric field, or ion traps, which use oscillating electric fields to confine ions according to their m/z. By varying the frequency of the applied electric field, ions can be selectively destabilized and

ejected from the trap for detection. The m/z can then be calculated based on the frequency at which the ion is ejected (80).

Later invented, the Orbitrap measures the frequency of ions oscillating around a central electrode, where ions with different m/z oscillate at different frequencies. The movement of the ions produces an electrical signal that is recorded over time and converted into frequencies using Fourier transformation. The frequencies are then used to calculate the corresponding m/z values (80). The Orbitrap is particularly well suited for proteomics due to its high mass resolution and mass accuracy, which enable precise measurement and distinction of peptide ions.

In bottom-up proteomics, used in this thesis, proteins are enzymatically digested into smaller peptide fragments before mass spectrometric analysis. This facilitates protein identification in complex mixtures by generating peptides whose masses and fragmentation patterns provide sequence-specific information that can be used to identify the proteins from which they originated (81). The protease trypsin is commonly used because of its well-characterized cleavage specificity, producing peptides that are well suited for mass spectrometric identification. The peptides are then made into ions, which converts molecules in liquid into gas-phase ions so they can enter the mass spectrometer. Using the electrospray ionization method, the peptides are passed through a very fine needle at high voltage, producing a spray of small, charged droplets. As each droplet shrinks by evaporation, the electric field on its surface becomes intense enough to release individual molecules from the droplet, forming free ions (notably, the electrospray method gave John B. Fenn the Nobel Prize in Chemistry 2002)(82).

The mass spectrometer first measures the intensity and m/z of these charged peptide ions, generating an MS1 spectrum. Selected peptide ions are then further fragmented inside the mass spectrometer by collision with inert gas. This produces smaller fragment ions that are analyzed as an MS2 spectrum (81). The mass spectrometer used in this thesis also uses filtering of ions by a quadrupole, essentially an inverse-purpose ion trap, using an electric field to remove unwanted ions (83). As an additional layer of separation, mass spectrometers are usually coupled to reverse-phase liquid chromatography, which separates peptides based on their hydrophobic interactions with a chemical matrix. This separation means that peptides enter the mass spectrometer at different times rather than all at once, reducing the complexity of the sample being analyzed at

any given time. Retention time also provides an additional property of the peptide ions besides their m/z that can be used for protein identification.

The resulting data consist of MS1 and MS2 mass spectra (Figure 3), showing ion intensity over m/z. These spectra are acquired sequentially over time, with individual ion accumulation and detection events occurring on the millisecond timescale. By combining consecutive measurements across the liquid chromatography separation, ion intensity can be recorded as a function of retention time, typically based on the MS1 intensity.

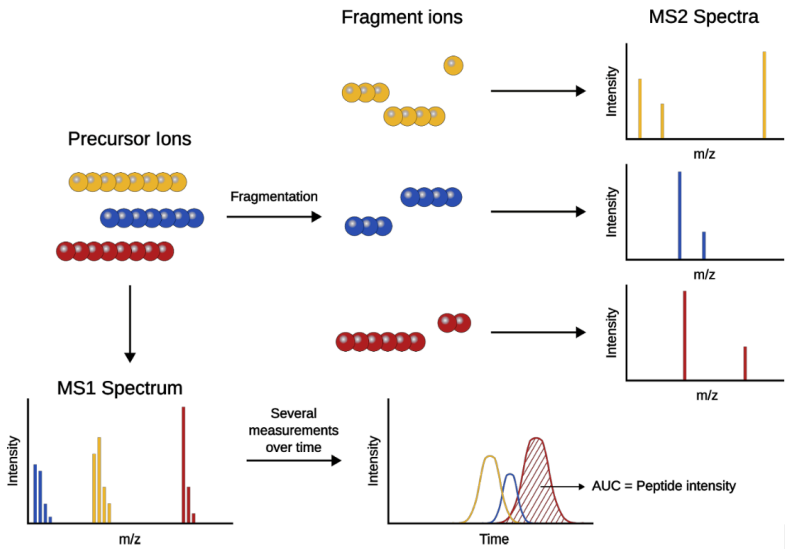

Figure 3: Overview of a typical DDA proteomics workflow. Proteins are digested into peptides and injected into the mass spectrometer. The m/z of the peptides are measured in an MS1 spectrum, which measured over time generates an intensity over time chromatogram. The area under the curve corresponds to the relative intensity. The peptides are further fragmented and measured again in MS2. This generates a fingerprint that can be used for identification of the peptide. Figure from Emil Sporre 2026 (84).

3.2 Turning mass spectrometry signal into protein data

Taken together, the mass spectrometer measures the intensity and m/z of ions, and retention time of peptide fragments. For each peptide, these measurements form a characteristic pattern ("fingerprint") of signals that can be used to identify peptides and map them together into the proteins they originated from. Although this process is too complex to perform manually, computational methods can compare the measured signals with experimentally derived peptide fingerprints (called a spectral library) to assign peptide identities. Spectral

libraries can also be predicted computationally, as in this thesis, by neural networks trained on experimental data (85, 86).

However, proteomes are large; the cyanobacteria proteome contains around 3500 proteins, while plants such as *Arabidopsis thaliana* approach 35 000 proteins (UniProt reference proteomes UP000001425 and UP000006548). When analyzing the peptides from proteomes, even after separation with a chromatography gradient, the resulting spectra are complicated. In addition, the events of MS1 and MS2 happen on the scale of milliseconds, producing an enormous amount of data.

To handle the complexity of the acquired MS2 spectra, different data-acquisition strategies can be used to determine which ions are used for MS2 fragmentation. Two approaches are data-dependent acquisition (DDA) and data-independent acquisition (DIA) (87, 88). In DDA, only a subset of the most intense precursor ions detected in MS1 are selected for fragmentation in MS2. DDA was historically predominant because the resulting MS/MS spectra were relatively simple to interpret. However, the selection of ions can result in missing peptides and variability between sample injections. In contrast, DIA fragments all isolated MS1 ions, generating less biased and more comprehensive, but computationally complex datasets that require advanced computational methods to assign fragment ions to peptides.

To limit false peptide identifications, the false discovery rate (FDR) is typically estimated using a target-decoy approach. In this approach, made-up decoy protein sequences are generated from the proteome, and the resulting decoy peptides are used alongside true peptides during the identification of peptides to estimate the rate of false identifications (89).

The resulting peptide and protein intensities are relative rather than absolute measures of abundance. Comparison is therefore most reliable when comparing the same peptide across samples, rather than comparing intensities between different peptides, which can differ in their ionization efficiency and detection. For the simple experimental designs used in this thesis, relative protein abundance is typically compared between conditions using t-tests. However, hypothesis testing across thousands of proteins increases the probability of false-positive findings. Therefore, p-values are adjusted for multiple testing, which increases the bar for what is considered statistically significant (90).

As discussed in the following sections, proteomics can be used for more than the comparison of cellular protein abundances. By coupling proteomics with assays and other complementary approaches, it can be used to investigate protein regulation.

3.3 Assay-based methods to study protein-metabolite interactions

Proteomics is today an established method to identify binding between proteins and small molecules. Though the principles between methods vary greatly, the final readout with mass spectrometry is peptide/protein detection and abundance.

Pulldown-based methods rely on the direct binding affinity between a protein and a molecule of interest (91). The molecule is immobilized on a solid surface, such as agarose or magnetic beads, typically through a chemical linker, and incubated with a cellular lysate. Proteins that bind the immobilized metabolite are captured, washed, and subsequently identified by mass spectrometry. To minimize false positives arising from nonspecific interactions with the bead surface or chemical linker rather than the metabolite itself, appropriate controls, such as competition with the free metabolite, are important for distinguishing specific interactors from nonspecific binders. However, chemical modification of the metabolite is typically nontrivial and may alter its binding properties or biological activity. In addition, washing steps may disrupt weak or transient interactions, potentially resulting in the loss of biologically relevant interactors (92–94). Pulldown methods are extensively used, for example, to capture ppGpp-binding proteins in non-photosynthetic organisms (95, 96), complementary to my approach in **Paper I**.

Another principle to detect protein-metabolite interactions is that interaction with molecules can alter the structure and properties of a protein, which does not require chemical modification of the molecule. For example, the method limited proteolysis is based on that a molecule that binds to a protein can cause changes in protein structure that block or expose protein residues to proteases (97). In limited proteolysis, cells are broken into lysates, lysates are incubated with and without the metabolite as a control. Then a quick, unspecific protease is added (Proteinase K) to the samples to cleave proteins at random sites for a limited amount of time. The proteins are digested completely by specific-cleaving trypsin and analyzed by mass spectrometry. Peptides that have been cleaved by proteinase K are not detected as trypsin-cleaved peptides and are

therefore not detected. Changes in the abundance of individual peptides reveal regions that have been altered upon metabolite exposure.

As used in **Paper I** and **Paper IV**, limited proteolysis can provide information on where the metabolite is interacting. However, because limited proteolysis compares changes in peptide abundances rather than protein-level abundances, it is particularly sensitive to missing values and variability at the peptide level, as protein-level abundances are usually averaged across several peptide abundances (98). It is not guaranteed that peptides affected by limited proteolysis directly interact with the metabolite, as binding can also cause conformational changes, and in practice, multiple peptides can be affected. Therefore, I would describe it as a method to be used complementarily with other approaches such as molecular docking. Nonetheless, when applied to the cyanobacterial fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase (F/SBPase), limited proteolysis identified distinct peptides affected by GAP and NADPH compared with AMP inhibition (65).

Binding of metabolites to proteins can also affect a protein's temperature stability, which is used in Thermal proteome profiling. In this method, metabolite targets are identified based on changes in protein temperature stability upon binding a molecule. Samples are heated across a temperature gradient in the presence and absence of the metabolite, which causes partial denaturation of the proteome. Denatured proteins are removed, for example, by ultracentrifugation, and the remaining soluble proteins are quantified by mass spectrometry. By measuring individual fractions of the heat gradient, a melting curve for each protein can be reconstructed and compared to the treatment without metabolite (99).

Central to **Paper I** (and **Paper III**), a more high-throughput version is Proteome Integral Solubility Alteration (PISA). PISA applies the same principle as thermal proteome profiling but instead pools samples across the temperature gradient and measures the total change in protein solubility (Figure 4). This substantially reduces the number of mass spectrometry measurements and bias in fitting a melting curve to a protein (100). Other methods use the same underlying principle as TPP and PISA, but employ alternative approaches to assess protein stability, such as resistance to chemical denaturation (101).

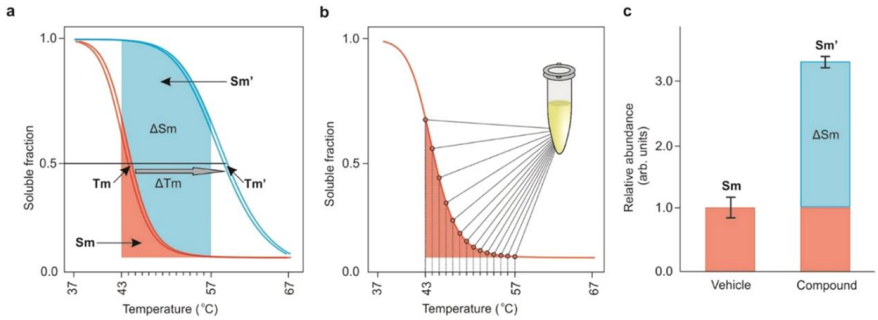

Figure 4. Schematic of the PISA method. A) Comparison of PISA concept of integral analysis with TPP concept of sigmoidal curve fitting: 1D PISA integral measurements of treated (blue area) and untreated (red area) sample for obtaining ΔSm; 1D TPP - temperature scanning for treated (blue) and untreated (red) samples, curve fitting, and derived ΔTm. B) PISA concept of pooling together individual samples corresponding to different temperature points and thus hardware integration of the melting curve without detailed determination of its shape. C) Measuring ΔSm as a difference between the integral abundances of the protein in the treated and untreated sample. Figure and description from Gaetani et al 2019 (100).

Despite its high throughput, PISA has several limitations. Changes in protein solubility are not necessarily caused by direct ligand binding, as indirect effects, such as co-melting or changes in protein-protein interactions, can also produce altered solubility profiles. Conversely, ligand binding does not necessarily result in a detectable change in protein stability, meaning that genuine interactions may be missed. In addition, the temperature range used must be carefully optimized for the organism and proteome under study to capture the melting behavior of as many proteins as possible. A temperature range that is too broad, however, can increase background variation and noise, reducing the ability to detect metabolite-induced changes (102).

3.4 Proximity labeling techniques to study protein-protein interactions

Proximity labeling approaches have emerged as powerful tools for studying protein-protein interactions in living cells. Classical proteomic approaches to study protein-protein interactions, such as affinity pulldown, rely on preserving protein complexes during cell lysis and purification (103). In a typical affinity pulldown, a protein of interest is affinity-tagged or immobilized and used to capture associated proteins from a cell lysate. Following incubation, the sample is washed to remove nonspecifically bound proteins, and the retained proteins are identified by mass spectrometry. However, interactions that are weak or

transient may be disrupted during cell lysis, purification, or washing and therefore escape detection.

Proximity labeling provides an alternative approach for studying protein-protein interactions (104, 105). These methods use enzymes that generate reactive molecules capable of modifying proteins near the protein of interest. After cell lysis, the modified proteins are purified using affinity purification. A major benefit of this approach is that protein interactions can be studied in the native cellular environment and under different growth conditions. As protein interactions and their regulation are often dependent on the growth environment, this provides an important advantage over classical pulldown approaches. Furthermore, because labeling occurs prior to cell lysis, nearby proteins can be identified even when their association is not stable *in vitro*.

In BioID-based approaches, the protein of interest is fused to a promiscuous biotin ligase, which catalyzes the formation of a reactive biotin intermediate that labels nearby proteins (106). Following addition of biotin to the cultivation medium, the biotinylated proteins are enriched using streptavidin-bead pulldown and identified by mass spectrometry (Figure 5). This approach enables the mapping of the local protein environment of a protein of interest directly *in vivo*, reducing artifacts associated with cell lysate-based methods.

Several BioID variants have been developed to improve labeling efficiency and adapt the method to different experimental systems. The original BioID enzyme was derived from *Escherichia coli* biotin ligase BirA, with greater promiscuity than the native enzyme (106). The engineered variants TurboID and miniTurbo were developed through directed evolution to achieve substantially faster labeling, enabling efficient proximity labeling within minutes rather than the several hours required by the original BioID (107). BioID2 is a biotin ligase derived from *Aquifex aeolicus* that is smaller than the previously mentioned variants to reduce steric hindrance (108). BioID2 is used in **Paper II**.

BioID-based proximity labeling has since been applied across diverse organisms, including photosynthetic systems. For example, TurboID has been used in *Arabidopsis thaliana* to investigate protein interactions and subcellular protein environments (109), as well as in the green algae *Chlamydomonas reinhardtii* to study chloroplast protein complexes and the HSP70 chaperone system (110). Recently, proximity labeling has also been applied in cyanobacteria to study protease activity (111).

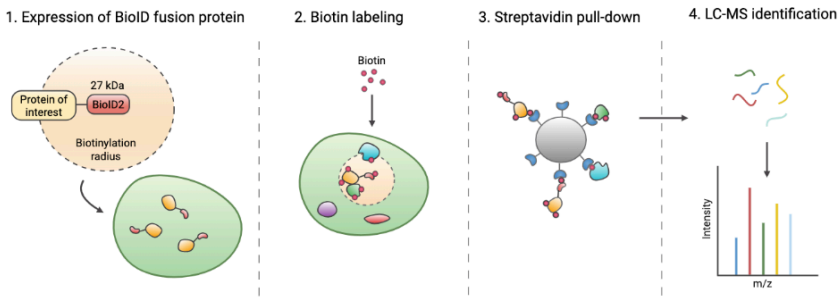

Figure 5. Overview of the BioID workflow. The BioID enzyme is expressed as a fusion with the protein of interest. Biotin is added to the cell culture, allowing BioID to biotinylate nearby proteins. Following cell lysis, biotinylated proteins are captured using streptavidin-coated beads. The beads are washed to remove nonspecifically bound proteins, and captured proteins are eluted from the beads by on-bead digestion and analyzed by mass spectrometry. Detected proteins represents potential interactions with the protein of interest.

An important consideration for proximity labeling is that it detects spatial proximity rather than direct physical interaction. Proteins within the labeling radius can therefore be identified even when they do not directly interact with the bait protein, and abundant or highly proximal proteins may contribute to background labeling. Yet, this can be advantageous when the aim is to characterize the local protein environment rather than identify only direct interaction partners, as shown in the previous paragraph.

However, proteins that interact with the bait but are located outside the effective labeling radius, typically around 10 nm (112), might not be detected, and the BioID tag can cause steric hindrances that inhibit interactions. In addition, BioID-based approaches rely on biotinylation of accessible lysine. Proteins with few or poorly accessible lysine residues may therefore be labeled less efficiently, potentially reducing their detection. Other proximity labeling approaches have been developed that use alternative labeling chemistry, including APEX, PUP-IT, EXCELL, and NEDDylation-based methods (104, 105).

3.5 Beyond a single method

As a final comment to conclude Section 3, chemoproteomic and other interaction-based methods all have inherent limitations, and complementary approaches can therefore provide different and sometimes only partially overlapping views of cellular regulation. For example, a recent study in *Synechococcus elongatus* combined global proteomics, limited proteolysis mass spectrometry, thermal proteome profiling, and redox proteomics to investigate

the response to changes in light availability (113). The four approaches identified largely distinct sets of proteins, with less than 10% of significantly affected proteins overlapping across all methods. At the same time, the methods converged on several biological processes, including photosynthesis, translation, and carbon metabolism.

Complementarity can also be extended across molecular layers through multi-omics approaches. For example, recent workflows have enabled the generation of DNA, RNA, and protein from a single starting sample, allowing genomic, transcriptomic, and proteomic measurements to be integrated (114). In PROMIS (PROtein-Metabolite Interactions using Size separation), proteomic and metabolomic measurements are combined following size-exclusion chromatography to globally assess protein-metabolite associations (115).

Lastly, distinguishing true protein interactions from false positives often requires considerable experimental effort. An example is provided by Zhang et al., where thermal proteome profiling of the FBP-metabolite interactome was followed by in vitro thermal stability measurements, phosphorylation analysis, mutational analysis, molecular docking and dynamics simulations, and enzymatic assays to investigate the mechanism of the FBP-PGAM1 interaction (116). Taken together, these examples illustrate how combining complementary approaches, from orthogonal measurements to multi-omics and targeted mechanistic validation can build a more complete picture of cell biology.

4 Present investigation

When I started my PhD, I became interested in applying high-throughput interaction proteomics to study protein regulation. Yet, many of the approaches that have been used in this thesis were originally developed for other purposes and organisms, typically mammalian cell cultures (100, 107, 108). As such, these methods had not previously been established in cyanobacteria, and their application in photosynthetic organisms in general was limited. A part of this thesis is therefore focused on method evaluation and optimization, starting with **Paper III**.

4.1 Paper III: Chloroplastic ascorbate modifies plant metabolism and may act as a metabolite signal regardless of oxidative stress

Ascorbate (vitamin C) is an abundant plant metabolite with well-established roles in antioxidant defense. In this paper, it is proposed that ascorbate may also modulate metabolism beyond its role as an antioxidant in the plant *Arabidopsis thaliana*. To investigate this, mutants with low ascorbate levels were examined, namely the ascorbate biosynthesis mutant *vtc2-4* and the chloroplastic ascorbate transporter mutants *pht4;4-3* and pht4;4-4. The plants were grown under short-day conditions at low-to-moderate light intensity, and photosynthetic performance was evaluated through measurements of Fv/Fm and non-photochemical quenching (NPQ). Given ascorbate's antioxidant role, oxidative stress was further assessed by measuring hydrogen peroxide levels inside the cells. Despite relatively minor effects on photosynthesis and no apparent oxidative damage under the normal-light conditions tested, the ascorbate-deficient mutants displayed widespread and largely overlapping changes in metabolite abundances, measured using metabolomics. This indicates that ascorbate availability has broader effects on plant metabolism than can be explained by oxidative stress alone.

As an exploratory approach, we wanted to investigate whether ascorbate could directly affect proteins, and therefore used the PISA assay to assess changes in protein thermal stability. The methodological work undertaken during this study also resulted in the publication of a protocol for the application of PISA in plants (Figure 6) (117). Since the lab group had primarily focused on cyanobacteria prior to my PhD studies, I first established plant cultivation using *A. thaliana* and isolated intact chloroplasts for interaction studies (intactness verified via oxygen evolution). As chloroplasts share a close evolutionary history with cyanobacteria, it aligned well with our group's interest in comparing these two systems.

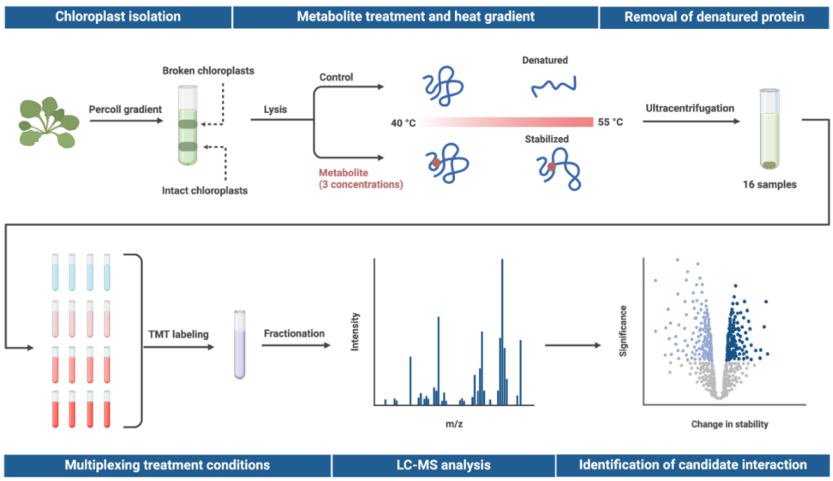

Figure 6. Proteome integral solubility alteration (PISA) assay in plant chloroplasts to screen for protein-metabolite interactions. Intact chloroplasts are isolated from leaves, lysed, and spiked with a metabolite of interest before undergoing temperature treatment. Soluble proteins are extracted via ultracentrifugation, multiplexed using tandem mass tags (TMT), and fractionated. Relative protein abundance is quantified through untargeted proteomics, and changes in protein abundance are compared to a heat-treated control sample without the metabolite. Proteins exhibiting altered temperature stability are considered potential candidates for interaction with the metabolite. To assess dose-response effects, the metabolite is tested at multiple concentrations in technical quadruplicates, with up to three concentrations analyzed using TMTpro 16plex in a single experiment. Figure from Karlsson et al 2025 (117).

For the temperature gradient to be used, I selected a range that covered the majority of the proteome's thermal stability based on the Meltome Atlas (118). I further removed steps that introduced additional protein losses, such as acetone precipitation, and established the method for mass spectrometric detection and data analysis. An important part of the PISA workflow turned out to be the use of Tandem Mass Tag (TMT) labeling (119). TMT is an isobaric labeling strategy

in which peptides from different samples are chemically labeled with tags that have the same overall mass. This allows peptides from multiple samples to be combined and analyzed in a single mass spectrometry run. During MS2 fragmentation, TMT-labeled peptides generate reporter ions with distinct masses depending on the tag, allowing the relative abundance of the same peptide across different samples to be quantified simultaneously.

The PISA assay measures changes in protein thermal stability. Because ligand-induced shifts in thermal stability can be relatively small, sometimes only a few degrees Celsius, the method requires high quantitative sensitivity. In our workflow, DIA label-free proteomics did not provide sufficient sensitivity and reproducibility to reliably detect these subtle changes. TMT labeling therefore provided an advantage by enabling multiplexed analysis and more precise relative quantification across temperature conditions. Since all samples are combined into one, which greatly reduces analysis time, it was also beneficial to combine the protocol with fractionation of the samples before mass spectrometry analysis to increase coverage (Figure 6). However, a limitation of TMT is ratio compression, where co-isolated peptides interfere with reporter ion measurements, causing differences in peptide abundance between samples to be underestimated (120). Finally, other studies have reported successful use of PISA with DIA-based proteomics (121), showing that my experience is not necessarily generalizable to other experimental setups and instruments.

Applying the optimized method, several proteins showed altered stability in the PISA assay across three concentrations of ascorbate, for example proteins in amino acid metabolism and photosynthetic electron transport (Figure 7).

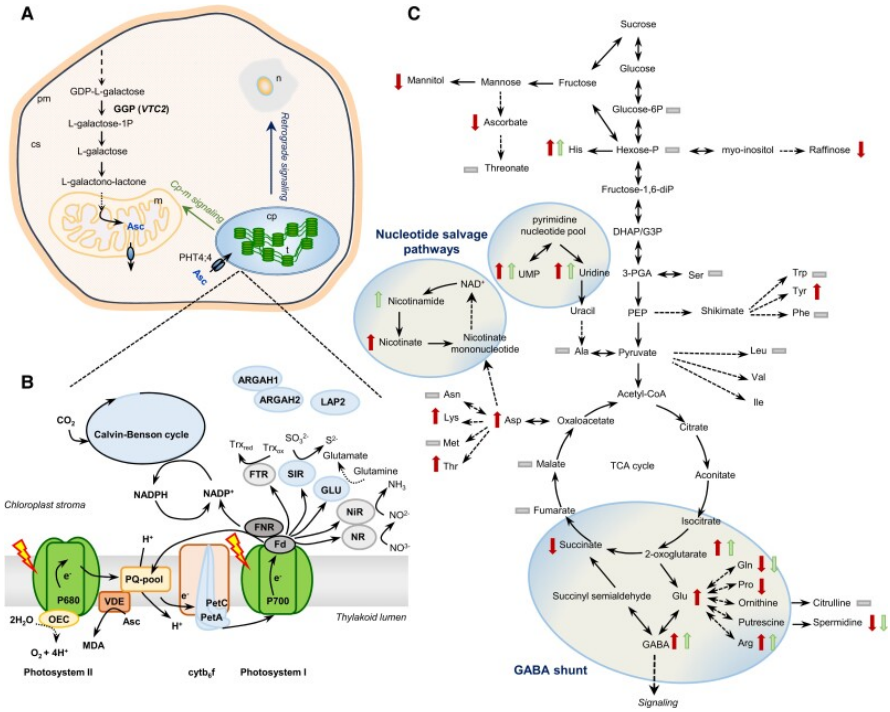

Figure 7. Chloroplastic ascorbate controls plant metabolism. A) ascorbate biosynthesis takes place in the cytosol, with the exception of the last step, occurring in mitochondria. vtc2 encodes GDP-L-galactose phosphorylase (GGP), catalyzing the first committed step of ascorbate biosynthesis. PHT4;4 transports ascorbate into the chloroplast. B) Photosynthetic electron transport components and stromal enzymes potentially interacting with chloroplastic ascorbate (indicated in blue, based on the PISA assay of this study). C) Chloroplastic and cellular ascorbate deficiencies lead to widespread changes in the metabolome, including the induction of the GABA shunt and nucleotide salvage pathways (circled). Dark red and light green arrows indicate significant changes in the respective metabolites identified at level "B" (MS2 level identification) in the vtc2-4 and the pht4;4-3 mutants compared to the WT (Col-0), respectively. Gray bars mean that the given metabolite was identified but no significant changes relative to the WT were detected. Figure from Tóth et al 2024 (122).

Among the candidate proteins identified by PISA was sedoheptulose-1,7-bisphosphatase (SBPase), an enzyme of the CO_2-fixing Calvin cycle, which is subjected to protein-level regulation as covered in Section 2. Measuring the activity of purified SBPase, we could observe an >50% inhibition of activity upon addition of 10 mM pH-corrected ascorbate, even in the presence of the reducing enzyme thioredoxin and the reducing agent dithiothreitol (DTT). At the same time, the plant SBPase is known to be particularly sensitive to a range of chemicals (as further explored in **Paper IV**), and its response therefore requires careful interpretation.

Importantly, while the experiments do not by themselves establish that a direct ascorbate-SBPase interaction is biologically responsible for regulation of the enzyme *in vivo*, they demonstrate that the PISA method can identify proteins that are directly affected by a ligand *in vitro* and present potential interactions. In hindsight, it would have been interesting to investigate the validation of other proteins identified in the PISA assay, for example, those involved in amino acid metabolism, notably arginine synthesis (Figure 7), which also corresponds to changes observed in the metabolome of the ascorbate-deficient mutants.

4.2 Paper I: Identifying novel targets of the stringent response in plants and cyanobacteria using chemoproteomics

Having optimized the PISA method in **Paper III** and demonstrated that it can be used to identify protein-ligand interactions, I wanted to apply it to my own research project. Initially, we set out to map protein targets for several signaling molecules, for example, c-di-AMP and cAMP. However, the amount of data quickly became too extensive to investigate all these molecules in sufficient depth and at consistent data quality. We therefore narrowed the focus to one signaling molecule with a particularly strong impact on cellular metabolism: the stringent-response molecule guanosine tetraphosphate (ppGpp). As a brief recap of the stringent response presented in Section 2.4, ppGpp is an alarmone that accumulates in response to nutrient limitation and helps reprogram cellular metabolism towards energy conservation and survival. This response is particularly relevant in cyanobacteria and plants under conditions such as darkness and nitrogen starvation.

In this project, I applied the PISA assay to four different organisms: the common heterotrophic model organism *E. coli*, *A. thaliana* chloroplasts, and two cyanobacteria, *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803. Both *Synechococcus* and *Synechocystis* are widely used model organisms in cyanobacterial research. In particular, *Synechococcus* has been used for characterization of the stringent response in cyanobacteria, such as during darkness (74–76). *A. thaliana* chloroplasts were investigated as a model organism for plants, and since ppGpp synthases and hydrolases are located in the chloroplasts rather than the cytosol (70). To benchmark our method, E. coli was included as a comparison.

As a dose-response analysis, we tested three ppGpp concentrations: 200 µM, 600 µM, and 1 mM in cell lysates of the four organisms. These concentrations were selected roughly based on previously reported intracellular ppGpp levels,

with 200 μM representing levels measured in cyanobacteria during darkness and 1 mM corresponding to E. coli under a fully induced stringent response (75, 76, 123). Across the four systems, the proteomic analysis quantified 2,143 proteins in *Synechocystis*, 1,594 in *Synechococcus*, 1,868 in E. coli, and 1,286 in isolated chloroplasts. Using 600 μM ppGpp, we identified significant changes in protein thermal stability for 183 proteins in *Synechocystis*, 63 in *Synechococcus*, 78 in E. coli, and 52 in chloroplasts. Thus, across all four systems, PISA detected a substantial number of proteins whose thermal stability was altered in the presence of ppGpp, providing candidate targets for further investigation.

We detected several classes of proteins affected by ppGpp (Figure 8), including multiple GTPases and translation factors. This is an expected outcome, as the stringent response is known to affect several proteins involved in translation in heterotrophic bacteria (123). Notably, the elongation factor Tuf, the signal recognition particle components Ffh/FtsY (important for targeting proteins to the thylakoid membrane), and the purine metabolism enzyme PurA were significantly affected across all organisms examined, suggesting that these proteins may represent conserved targets of ppGpp binding. The guanosine monophosphate synthase involved in GTP biosynthesis GuaA was found to be affected across both cyanobacteria. Interestingly, GuaA was recently found to be mutated in *Synechococcus* strains deficient in (p)ppGpp, with the authors proposing that these mutations reduce GTP biosynthesis and thereby partially alleviate the effects of (p)ppGpp deficiency (124). Together, these findings highlight both conserved and diverging components of the ppGpp response across the tested organisms.

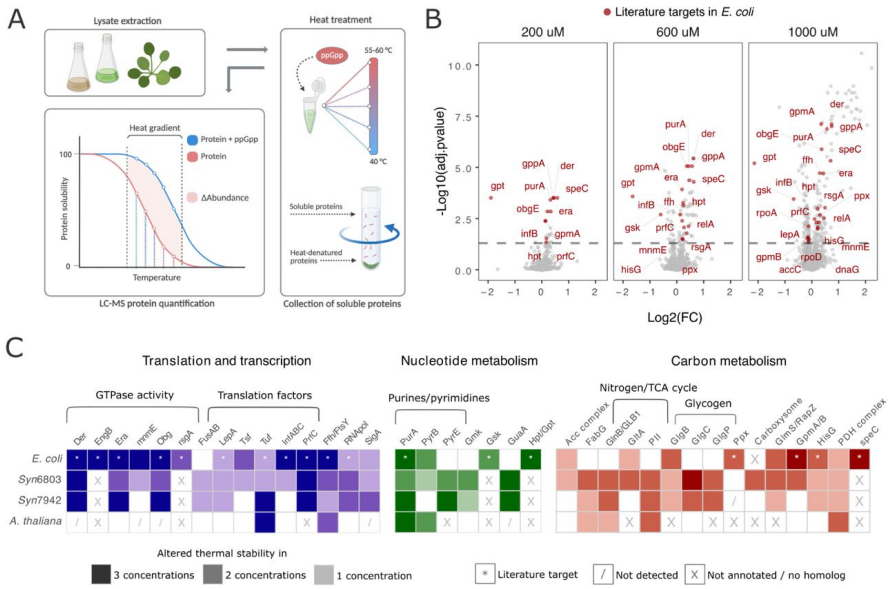

Figure 8. Screening of ppGpp-binding proteins using PISA. A) Overview of workflow. B) PISA method validation using ppGpp added to E. coli extracts. Previously reported ppGpp-binding targets in E. coli that showed an altered thermal stability in the presence of ppGpp are highlighted in red. Treatments were with 200, 600 and 1000 µM ppGpp with technical quadruplicates, compared against a control without ppGpp added. C) Proteins with significantly (adjusted p.value ≥ 0.05) affected thermal stability in PISA for the tested organisms. The tested concentrations of ppGpp were 200, 600 and 1000 µM with technical quadruplicates compared to a control without ppGpp.

To investigate whether ppGpp directly affects enzyme activity, we recombinantly expressed and purified candidate proteins and assayed their activities *in vitro* (Figure 9). Using this approach, we found that the plant pyrimidine biosynthesis enzyme aspartate carbamoyltransferase (PyrB) is inhibited by ppGpp. Molecular docking and limited proteolysis analyses further suggest that ppGpp may interact with the PyrB active site. Interestingly, the predicted binding mode overlaps with that of UMP, a plant-specific inhibitor of PyrB (125), raising the possibility that ppGpp inhibits PyrB through a similar mechanism. The evidence for PyrB regulation in cyanobacteria was weaker in our PISA screen, and I did not manage to recombinantly express *Synechocystis* PyrB as a comparison. Interestingly, another pyrimidine enzyme, orotate phosphoribosyltransferase (PyrE), was affected in both cyanobacterial species. Although we were unable to assess the enzymatic activity of PyrE in the presence of ppGpp due to time constraints, measurement of thermal stability *in vitro* suggests that ppGpp may bind PyrE and act as an alternative regulatory node.

A difference we observed between plants and cyanobacteria was the presence of glycogen synthesis enzymes affected in the PISA assay, where several glycogen-related enzymes are affected in the cyanobacteria species. Interestingly, both plants and cyanobacteria accumulate these carbon and energy reserves during nitrogen starvation, related to ppGpp signaling. Unlike cyanobacteria, where ppGpp overproduction mutants display glycogen accumulation, ppGpp overproduction in plants does not increase starch levels (76, 126). One protein of particular interest was the ADP-glucose pyrophosphorylase (GlgC/AGPase), which catalyzes the first committed step in glycogen biosynthesis. By assaying the activity of recombinant cyanobacterial GlgC and the plant homolog in lysates, we observed an activation of the cyanobacterial enzyme but not the plant enzyme. Consequently, activation of cyanobacterial GlgC by ppGpp may present a biological mechanism for glycogen accumulation.

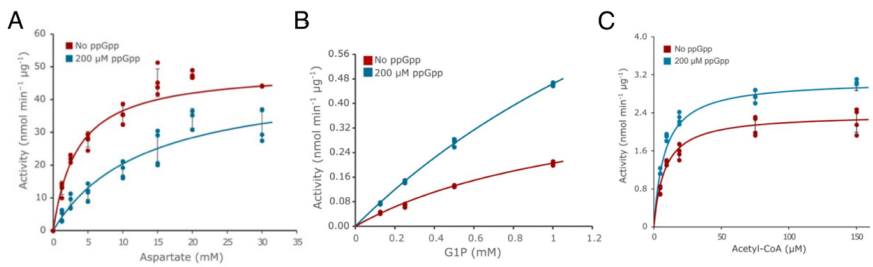

Figure 9. Kinetic analysis of proteins found in the PISA assay with an without ppGpp present. A) Inhibition of the plant pyrimidine enzyme PyrB. B) Activation of the glycogen enzyme GlgC in Synechocystis. C) Activation of the citric acid cycle enzyme GltA in Synechocystis.

Though the PISA can identify interaction partners, several proteins affected likely represent false positives and unspecific/indirect protein effects. For example, we saw citrate synthase (GltA) in *Synechocystis* with an affected thermal stability. Assessment of the stability *in vitro* using recombinant protein and NanoDSF showed that ppGpp strongly affected the stability without magnesium ions present, but the effect was diminished through addition of excess magnesium. Despite this, as an unexpected finding, we still observed an increase in enzyme activity in the presence of ppGpp, regardless of magnesium presence. We also observed that ppGpp can shift the oligomeric state from hexamers to dimers. As another example, the PII regulatory protein was seen affected across plants and cyanobacteria, but *in vitro* characterization did not reveal a direct effect of ppGpp. We also found the GTPase Der (EngA) to be affected by ppGpp, which, through the PipX regulatory network, is connected to the PII protein (127).

Lastly, we observed that several carboxysome proteins were affected by ppGpp in *Synechocystis*. The altered stability of one of the carboxysome proteins was retained *in vitro* when the protein was recombinantly expressed**.** Carboxysomes are carbon-concentrating organelles in cyanobacteria that increase the local CO_2 concentration around the carbon-fixing enzyme Rubisco. As an exploratory approach, we expressed the *B. subtilis* ppGpp synthase in *Synechocystis* and *Synechococcus* and examined carboxysome phenotypes using fluorescently tagged carboxysome-localised proteins. Interestingly, ppGpp overproduction caused carboxysomes to cluster into puncta, resembling the organization observed during prolonged darkness. A recent preprint indicates that polyphosphate can spatially organize carboxysomes into similar clusters we observed (128). Since polyphosphate accumulates upon ppGpp overexpression (75), this raises the possibility that polyphosphate provides a mechanistic link between ppGpp accumulation and carboxysome organization.

Together, this project identifies multiple potential ppGpp regulatory targets and fills an important gap in knowledge of the stringent response in photosynthetic organisms. Further validation is needed to establish these as bona fide ppGpp targets, for example, by measuring ppGpp binding affinity using isothermal titration calorimetry (ITC) and identifying ppGpp-insensitive mutants through targeted mutagenesis coupled to *in vivo* characterization. In retrospect, a more defined analysis pipeline of potential protein-ppGpp interacting proteins would have been beneficial, for example, by using ITC, although the instrument was not available at the time. Then I could have narrowed the biochemical analysis to fewer enzymes, such as PyrB and PyrE, and investigated them in greater depth and systematically compared purified proteins across organisms. Nevertheless, the broad scope allowed me to explore diverse pathways and methods, which has been highly valuable for my scientific development.

4.3 Paper II: Evaluation of BioID proximity labeling in cyanobacteria and proximity landscape of central carbon metabolism enzymes

Protein-protein interactions are important regulators of metabolism (Section 2.3). In this project, we aimed to use BioID-based proximity labeling to map protein-protein interactions. As previously described in Section 3.4, BioID is a promiscuous biotin ligase that biotinylates proteins in proximity to the enzyme, thereby enabling the identification of putative interaction partners. Essentially, the biotinylation of proteins near the BioID-tagged enzyme is induced by

supplying excess biotin to the cultivation media. Afterward, the cells are broken, and biotinylated proteins are captured using beads coated with streptavidin. The proteins attached to the beads are washed, and the remaining proteins are cleaved off using the protease Trypsin and measured by mass spectrometry. In contrast to the PISA assay used in **Paper I** and **Paper III**, which maps protein-metabolite interactions, BioID maps protein-protein interactions. An additional highlight of BioID is that it is performed *in vivo* and can therefore be used to study how interactions change depending on environmental conditions in a native context. Given these advantages, we set out to benchmark the method and study several proteins involved in central carbon metabolism.

This project was a collaboration with the University of Kassel, where we combined their cloning capacity with my expertise in proteomics. To avoid artifacts from overexpression of proteins, we inserted the BioID protein into the genome at the native gene location. Initially, we tested cloning several biotin ligases, including miniTurbo and TurboID, as well as the smaller BioID2. However, cloning of TurboID and miniTurbo strains was less successful. In addition, literature reports toxicity issues with TurboID (and to a lesser extent miniTurbo), and another issue is that TurboID is not generally inducible and is active even before the addition of excess biotin (129). We reasoned that this was not ideal, as it could biotinylate proteins under conditions that we did not want to study during, for example, darkness exposure during sampling. We therefore decided to move forward with the smaller biotin ligase BioID2.

To start, we wanted to test whether the BioID2 protein could capture known interactions. For this, we used glucose-6-phosphate dehydrogenase (G6PDH/Zwf) and the regulator OpcA, covered in Section 2.3. Briefly, OpcA is a redox-regulated protein that binds Zwf and regulates Zwf activity. As a constitutive, strong interaction, we considered it an optimal positive control. We also hypothesized that the BioID2 protein would have a significant unspecific background biotinylation, as well as proteins that are natively biotinylated (such as enzymes involved in fatty acid synthesis), in addition to proteins that can unspecifically bind to the beads. Therefore, we constructed a control strain with BioID2 fused to a “dummy protein” (in our case, the fluorescent protein mVenus) that was under the inducible rhamnose promoter to control the expression strength (= background biotinylation strength).

We optimized the BioID2 protocol based on the time required for biotinylation to occur after the addition of biotin and the strength of the background biotinylation control (mVenus-BioID2). In these experiments, we compared the

abundances of proteins from the streptavidin pull-down in Zwf-BioID2 to the mVenus-BioID2 control. We saw that low or no induction of the control led to a significantly larger number of identified candidate interacting proteins, presumably false positives, whereas the number of candidate interacting proteins became lower with higher induction of the mVenus-BioID2 control. Then, by varying the time that cells were grown with excess biotin in the media, we found that too short a biotinylation time did not capture the OpcA interactor, while too long a biotinylation time led to lower OpcA enrichment due to background labeling in the mVenus-BioID2 control. Importantly, we saw that without the addition of biotin to the media, no enrichment of the Zwf protein occurred, indicating low biotinylation activity of BioID2 without the addition of excess biotin and establishing the inducibility of the BioID2 protein. Based on these experiments, we selected the optimal conditions where we could 1) detect the OpcA-interacting protein and 2) limit the amount of false positives: 8 hours of biotinylation and 1 mM rhamnose induction strength.

Then, we applied the BioID method across an additional 15 enzymes involved in carbon metabolism (Figure 10). We grew them under regular autotrophic conditions (light), mixotrophic conditions (light, with the addition of glucose), and heterotrophic conditions (darkness, with the addition of glucose). These were selected based on the contrasting fluxes during autotrophy and heterotrophy, since in the light, carbon and energy are derived from photosynthesis and the Calvin cycle, while in darkness, carbon is derived from glycogen storage (and supplied glucose). We also included additional positive controls: OpcA-BioID2 (Zwf interactor), HoxF-BioID2 (subunit of the Hox complex involved in redox balancing), as well as Gap2-BioID2 and Prk-BioID2, which together form a complex with the CP12 protein in darkness covered in Section 2.3.

In general, enrichment of the BioID2-tagged protein was consistently and strongly enriched compared to the proteins captured by the mVenus-BioID control, showing that the BioID2 protein efficiently biotinylates the tagged protein. However, Gap2-BioID2 could not be statistically significantly enriched across experiments, likely as the background detection of Gap2 in the mVenus-BioID2 control was high.

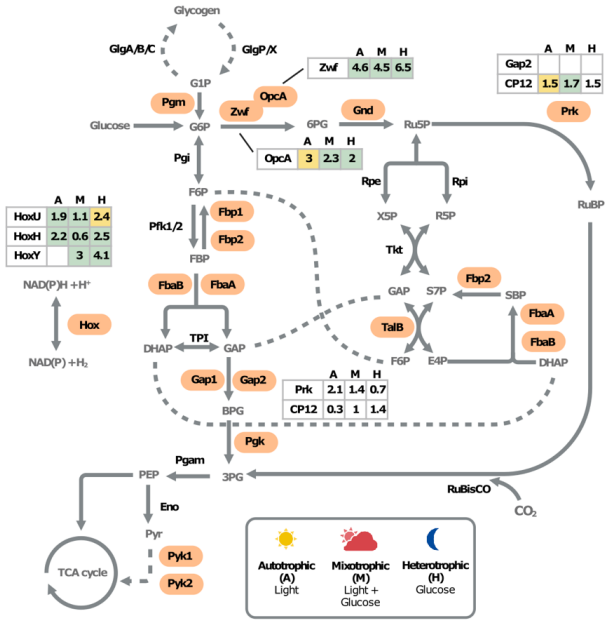

Figure 10. Central carbon metabolism with BioID2-tagged enzymes marked in orange. Positive log2 fold changes for known protein interactions are shown as tables next to the bait protein, where green indicates an adjusted p-value ≤ 0.05, yellow indicates an adjusted p-value ≤ 0.1, and white indicates adjusted p-value >0.1. A, M, and H stand for autotrophic, mixotrophic, and heterotrophic conditions.

We could consistently recover the Zwf-OpcA interaction across our tested conditions and partially the Hox complex, consisting of the HoxE, HoxF, HoxU, HoxY and HoxH subunits. Notably, the Hox complex has been found to form a variety of subcomplexes, although their biological roles remain uncertain (130). We did not detect the HoxE subunit, which has been reported to dissociate relatively easily from the Hox complex during protein extraction and preparation (130). We did not observe that the BioID2 tag itself interfered with the Hox complex formation. Interestingly, we also captured a small protein, Slr0598, with unknown function, which was enriched across all experiments in the HoxF-BioID2 mutants.

Our method could not capture the Prk-CP12-Gap2 complex well, which is expected to be primarily formed during darkness. Prk-BioID2 detected CP12 under autotrophic and mixotrophic conditions, but not significantly under heterotrophic conditions. A similar pattern was observed for Gap2-BioID2, which did not significantly enrich Prk or CP12. At least to some degree, this may be explained by high background biotinylation of Gap2 and CP12 by the

mVenus-BioID2 control. Therefore, the mVenus-BioID2 control may have been too strongly induced for the detection of some interactions, and it would have been beneficial to include additional induction levels in the optimization experiments.

As an alternative control, we also evaluated an *in vivo* TEV-protease cleavage system (Figure 11). The aim was to generate a background control in which the BioID2 tag was expressed at the same level as in the BioID2-tagged protein of interest, avoiding differences in expression between the bait and the background control. To achieve this, BioID2 was connected to the protein of interest through a flexible linker containing a TEV-protease cleavage site. Before cleavage, the construct therefore expressed the protein of interest with the BioID2 tag, while induction of TEV protease was intended to remove the tag from the protein of interest while leaving the protein itself unchanged. The resulting cleaved protein could then serve as a background control with a matched expression level. However, this approach did not provide a reliable alternative to the mVenus-BioID2 control. TEV activity varied between the tested strains, with unintended cleavage observed in some strains even in the absence of induction, while complete cleavage could not be achieved in others. Consequently, the system did not consistently produce a clear distinction between the BioID2-tagged and cleaved states, limiting its usefulness as a background control for BioID experiments.

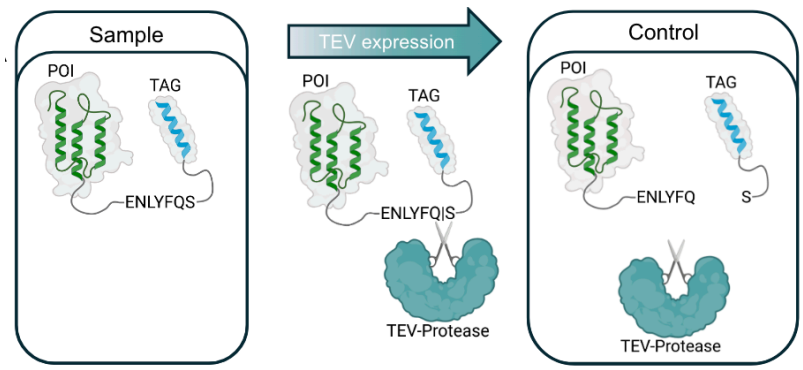

Figure 11. Concept of the TEV-based BioID2 control. BioID2 was fused to the bait protein through a flexible linker containing a TEV protease cleavage site. Upon induction of TEV protease with rhamnose, BioID2 was intended to be released from the bait and become freely diffusing, providing a matched background control for comparison with the non-cleaved BioID2-tagged bait.

Overall, we found limited evidence for additional strong constitutive protein-protein interactions. Although some associations between carbon metabolism enzymes were detected, these were not consistently observed. For instance, FbaB was detected in the Zwf- and Fbp2-BioID2 strains under heterotrophic conditions, while the glycogen phosphorylase GlgP (Sll1356) was detected in the Prk- and FbaA-BioID2 strains under autotrophic conditions. Likewise, the Pgm-BioID2 strain detected Fbp2 under autotrophic conditions. However, none of these associations were recovered reciprocally, making it difficult to distinguish genuine interactions from potential false-positive results. This is consistent with recent His-tag pulldown experiments in *Synechococcus*, in which no strong protein-protein interactions were identified for Pgk, TalB, or Fba (131).

Our experiments raise the question of whether many of the enzymes evaluated under the tested conditions genuinely lack strong constitutive protein-protein interactions, or whether the limited number of interactions detected instead reflects limitations of the method. Further refinement of the data analysis could potentially extract additional information from the experiments. For example, five additional BioID-tagged mutants were excluded from the main analysis because they did not show detectable expression of the BioID2 protein or biotinylation of the BioID2-tagged protein. However, these strains were still subjected to the same experimental procedures and could potentially be used as additional within-experiment negative controls, similar to approaches used by for example, the SAINT framework (131, 132).

Missing data is a well-recognized challenge in quantitative proteomics (133), and proximity-labeling workflows (and other pulldown approaches) may introduce further variability at several stages, such as peptide recovery from the pulldown, and availability of lysine residues to biotinylate. An additional complication arises from the modification of lysine residues by biotin, which interferes with trypsin cleavage and increases the frequency of missed cleavages (134). Combined, these effects may limit the detection and identification of peptides and lead to greater data missingness and experimental variability. In our dataset, several proteins were detected exclusively in BioID2 mutants and were not detected in the mVenus-BioID2 control. This presents a challenge, as it is difficult to distinguish between “biologically true” missing proteins (not biotinylated) and proteins missing by random or mass spectrometer instrument limitation. In other wording: Are bait-only detected proteins at (particularly at lower intensities) true interactions, or the result of random errors. We investigated approaches for imputing missing values, including the ProDA

method (135), but ultimately decided to keep the missing values rather than impute them to limit bias.

As a conclusion, this project demonstrates that the BioID proximity labeling method can identify known protein-protein interactions, while also highlighting the limitations of the method and the importance of careful control design. Per usual, the potential interactions identified in this study require further validation to determine whether they represent genuine protein-protein interactions, reflect proximity within the same local environment, or arise from experimental bias.

4.4 Paper IV: A bifunctional F/SBPase from nonphotosynthetic bacteria enhances growth of transgenic poplar

In this project, we focus on the Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase (SBPase) and compare it with bacterial homologs. In contrast to the plant SBPase, cyanobacteria possess a bifunctional fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase (F/SBPase) that, in addition to catalyzing the Calvin cycle reaction converting sedoheptulose-1,7-bisphosphate (SBP) to sedoheptulose-7-phosphate (S7P), can also catalyze the glycolytic reaction converting fructose-1,6-bisphosphate (FBP) to fructose-6-phosphate (F6P) (see Figure 10). We also evaluate the bifunctional F/SBPase from the CO_2-fixing bacteria *Cupriavidus necator*, capable of using hydrogen gas (H_2) as an energy source while fixing CO_2 through the Calvin cycle. Interestingly, though the plant SBPase and the cyanobacterial F/SBPases catalyze the same Calvin cycle reaction, they come from different evolutionary origins (supported by phylogenetic analysis presented in this paper) (136, 137).

Throughout my work, I have observed that SBPase is generally sensitive to compounds associated with oxidative stress, for example, in PISA experiments using chlorophyll intermediates. SBPase is also sensitive to ascorbate, as described in **Paper III**. These observations led to the hypothesis that SBPase may be particularly sensitive to oxidative stress. To investigate this, we compared the sensitivity of F/SBPase enzymes to hydrogen peroxide (H_2O_2), including from the cyanobacterial enzyme from *Synechocystis* (*Syn*F/SBPase), the *C. necator* enzyme (*Cne*F/SBPase), and the *Arabidopsis thaliana* plant enzyme (*Ath*SBPase). Strikingly, *Ath*SBPase was substantially more sensitive to H_2O_2 than the bacterial enzymes, whereas *Cne*F/SBPase displayed a pronounced resistance to oxidative inactivation (Figure 12).

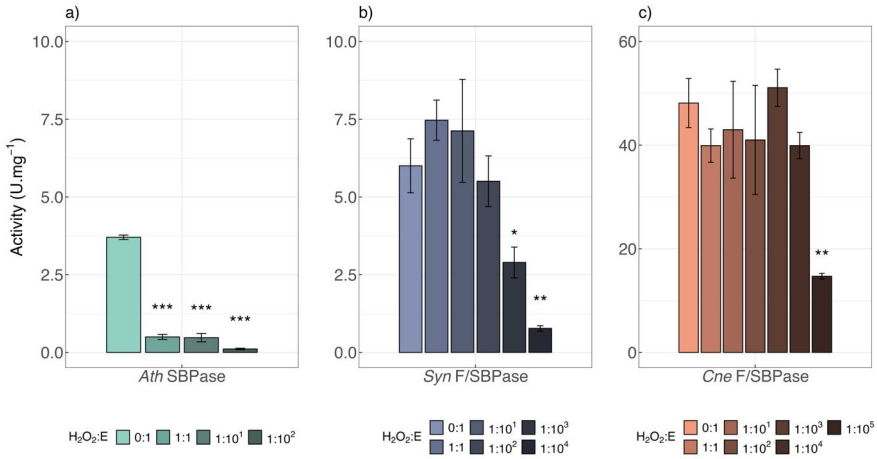

Figure 12. Enzyme activity under oxidative conditions. The Arabidopsis (AthSBPase), Synechocystis (SynF/SBPase), and Cupriavidus (CneF/SBPase) was incubated with increasing molecular ratios of H_2O_2 to enzyme. Assays were conducted as described in the Materials and Methods, using FBP as the substrate. Data show the mean ± SEM from at least four replicates. The H_2O_2 concentration range was 0-0.1 mM (AthSBPase), 0-6 mM (SynF/SBPase), and 0-30 mM (CneF/SBPase). Asterisks denote statistically significant differences from the control without H_2O_2, as determined by Student's t-test: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

In addition to this, we tested the effect of metabolites on enzyme activity (Figure 13). The *Syn*F/SBPase is inhibited by AMP (65) while *Ath*SBPase is inhibited by glycerate and 2-phosphoglycolate (2-PG) (138). 2-PG is produced directly by the oxygenation reaction catalyzed by Rubisco (a side reaction from unspecific incorporation of oxygen instead of CO_2) and is particularly high during heat stress and high light (139, 140). We found that AMP affected all the enzymes, in particular the plant enzyme. In addition, the *Cne*F/SBPase proved resistant to 2-PG regulation.

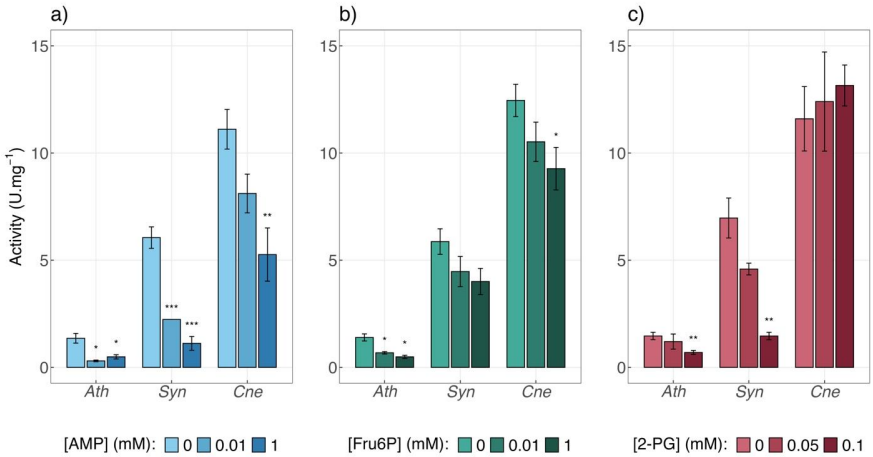

Figure 13. Metabolite regulation of Arabidopsis SBPase and bacterial F/SBPases. The metabolites AMP (a), Fru6P (b), and 2-PG (c) were tested on AthSBPase (Ath), SynF/SBPase (Syn), and CneF/SBPase (Cne). Bars represent the mean ± SEM from at least four replicates. Asterisks indicate statistically significant differences from the control sample without metabolite based on Student's t-test: $p < 0.05$ (*), < 0.01 (**), and $p < 0.001$ (***).

To investigate the interaction of 2-PG with *Ath*SBPase, we performed limited-proteolysis experiments. Comparison of the limited-proteolysis patterns revealed that FBP and 2-PG produced overlapping digestion profiles, with particularly pronounced changes in peptides containing the redox-regulatory cysteine residues. These results suggest that 2-PG induces conformational changes in *Ath*SBPase that are similar to those induced by the substrate FBP, potentially reflecting binding to the same or an overlapping regulatory region. Alternatively, 2-PG binding may modulate the redox sensitivity of the enzyme by altering the accessibility or reactivity of the regulatory cysteine residues (Figure 14).

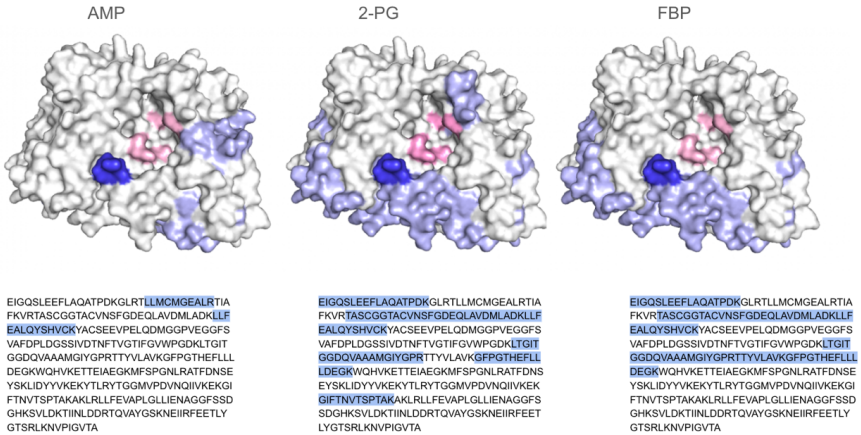

Figure 14. Dimeric AthSBPase residues affected by limited proteolysis in the presence of 5 mM AMP, 5 mM FBP, and 5 mM 2-PG when compared to a control without metabolite present. Consistently detected peptides across all conditions with an absolute log2 fold change >1 and an adjusted p-value of 0.05 were considered significantly affected. Peptides detected in less than n=3 replicates per condition or only in one condition were excluded from analysis. Affected peptides are highlighted in light blue. Regulatory cysteines and active site residues are highlighted in dark blue and pink respectively.

In summary, based on our kinetic analysis, *Cne*F/SBPase appeared to be more robust than the other enzymes with respect to both metabolite regulation and oxidative stress. In collaboration with SweTree Technologies, a company specializing in forest biotechnology and the development of increased value from trees and wood, we therefore investigated whether heterologous expression of *Cne*F/SBPase could enhance plant growth. Transgenic plants expressing *Cne*F/SBPase were generated and assessed by Umeå University. Though early in the evaluation, expression of *Cne*F/SBPase resulted in a 10% increase in stem height and a 9-23% increase in leaf size across the transgenic lines compared with wild-type plants grown in greenhouses (Figure 15). Further investigation under stress conditions would be of interest to determine whether these effects extend to other conditions. In addition to this, we observed an increase in sucrose production when expressing the *Cne*F/SBPase in sucrose-producing *Synechocysis*.

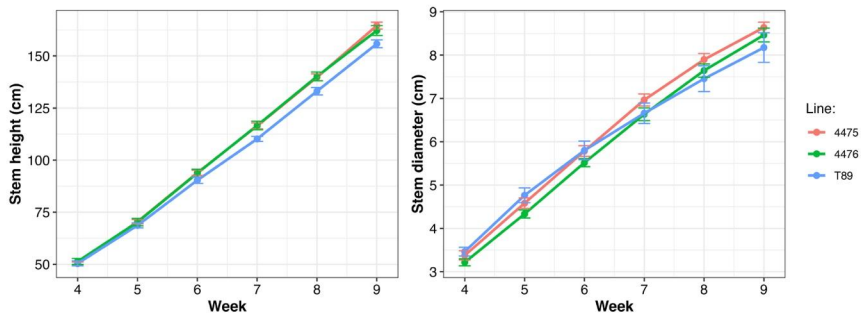

Figure 15. Stem height and diameter growth in transgenic Populus lines expressing CneFBPase. Stem height and diameter were measured from weeks 4 to 9 in wild-type (T89) plants and transgenic lines expressing CneFBPase under the control of the CaMV 35S promoter and fused to either the PtPSBS transit peptide (line 4475) or the AtRbcS transit peptide (line 4476). Values represent the mean ± SE of four independent lines per construct, with six biological replicates per line.

The work in this project is also being translated toward industrial application. Within EU regulations, transgenic genes will remain restricted for commercial use, as mentioned in Section 1.1. On the other hand, plants with genetic changes considered equivalent to those arising naturally or through conventional breeding can be exempt from EU GMO legislation. Consequently, in conjunction with this project, we have engineered mutants of the SBPase enzyme to be used as an alternative to the transgenic *Cne*F/SBPase. Mutants were designed partly based on limited proteolysis data mentioned here (Figure 14). The approach and mutant enzymes are now considered for a patent application and are therefore not discussed further. Interestingly, some variants seem less sensitive to 2-PG inhibition and exhibit increased catalytic efficiency.

5 Conclusions and future perspective

Plants and cyanobacteria are fascinating biological systems, and understanding and potentially engineering these organisms could help us address challenges such as climate change, food production, and the sustainable production of renewable materials. Plant engineering provides a promising tool alongside traditional plant breeding, while cyanobacteria hold potential as green cell factories for the production of chemicals and other valuable products. Knowledge is power, and developing a detailed understanding of cellular processes provides the foundation for elucidating and ultimately engineering complex biological systems.

This thesis has been dedicated to improving our understanding of the complex and finely tuned metabolism of photosynthetic organisms using proteomics. In **Paper I** and **Paper III**, I applied the PISA method, which measures changes in protein thermal stability, to identify proteins affected by metabolites. In **Paper I**, this approach was used to investigate how the nutrient-stress signaling molecule ppGpp interacts with proteins across multiple organisms. This revealed several previously unknown regulatory mechanisms involving pyrimidine synthesis, glycogen storage, the citric acid cycle, and carboxysomes. In **Paper III**, the method was used to study how the potential metabolite regulator ascorbate affects proteins in plants, and resulted in the development of a protocol paper. In **Paper II**, I evaluated the BioID proximity-labeling method in cyanobacteria, benchmarking the performance, and used it to map protein-protein interactions involving several enzymes in central carbon metabolism under light and dark conditions. Finally, **Paper IV** identified novel regulatory mechanisms of the Calvin-cycle enzyme SBPase and demonstrated that a bacterial variant of the enzyme exhibits increased resistance to oxidative stress. Heterologous expression of this enzyme also showed potential to enhance the growth of poplar trees, highlighting its potential for future applications in biotechnology and forestry, and collaborations with industry.

The rapid development of high-throughput technologies is fundamentally changing the scale at which biological systems can be studied. Proteomics, metabolomics, genetic screening, and other systems biology approaches can now generate measurements across thousands of features in a single experiment. For example, in cyanobacteria, genome-scale CRISPRi screening has enabled the systematic assessment of gene function across diverse environmental conditions, while proteome-wide approaches have revealed large-scale changes in the subcellular organization of metabolic enzymes, and protein-metabolite interactions (65, 141–143).

At the same time, as the scientific literature and the number of publicly available datasets continue to expand, only a fraction of the available data is typically explored in depth in the article in which it is presented, as is also evident from my own work. Furthermore, the format of supplementary datasets is generally not standardized. Consequently, I believe an important challenge for future research will be how to extract biological knowledge from the increasing volume and complexity of available data. Large datasets inevitably contain noise and method-specific biases, but they can also provide powerful information when measurements from independent, orthogonal approaches are integrated (144). Computational modeling and, increasingly, artificial intelligence could play an important role in this integration. Of course, this would not replace the needed work of detailed biochemical validation.

Overall, the work presented in this thesis forms one part of this larger scientific ecosystem: I have specialized in high-throughput proteomic methods to identify candidate relationships and generate further hypotheses, and while I validate some of them, further targeted biochemical experiments are essential for establishing mechanisms and biological relevance. I hope for my work presented here to expand our understanding of photosynthetic metabolism, broaden the toolbox of methods available for studying it, and eventually be applied in industrial settings to help address sustainability challenges.

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